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Contribution of phase variation of Opa proteins to persistent carriage and immune evasion of *Neisseria meningitidis*

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Abstract

Neisseria meningitidis is one of the main causes of bacterial meningitis and septicaemia but colonizes the upper respiratory tract of humans asymptomatically as a normal commensal. Phase variation (PV) in the surface antigens is proposed as an effective mechanism to enable these bacteria to adapt and persist in the human host. Opa proteins are expressed on the outer surface of meningococcal cells playing an important role in the pathogenicity by mediating the adhesion to and invasion of human cells. These proteins are encoded by three/four loci and each locus is phase variable due to pentameric repeats within the coding region. The phase variability in Opa proteins was investigated in meningococcal isolates from 19 carriers and time points representing up to six months of asymptomatic carriage. Changes in repeat tracts were analyzed by GeneScan, and a high frequency of PV was observed in at least two loci with a rate of 0.06 mutations/gene/month during colonization. The expression state of Opa was confirmed by Western blotting indicating expression of a limited number of Opa variants. Around 70% of the isolates expressed only one Opa and none simultaneously expressed four Opa. Intergenic and intragenic recombination was detected in two carriers, leading to new *opa* alleles with functions differing from the previous alleles. These results revealed that persistent carriage was correlated with a high rate of variation and switching between different Opa variants with stable expression of one or more alleles that may maintain Opa-mediated adhesion. The study also highlights the role of Opa proteins in mediating *in vitro* escape of *N. meningitidis* strain MC58 from anti-Opa bactericidal antibodies while selection for populations expressing other Opa variants was also observed *in vivo* indicating the importance of switching between the different variants for immune evasion and maintaining the function of this protein. Four recombinant Opa proteins were generated in this study and used for developing bactericidal polyclonal antibodies that can be used for further investigations of *in vitro* and *in vivo* immune escape.

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Abbreviations

%	**Percent**
^{0}C	degree Centigrade
A	Adenine
aa	Anino acid
APS	Ammonium persulphate
BA	Blood agar
BHI	Brain Heart Infusion
BHIA	Brain Heart Infusion agar
bp	Base pairs
BSA	Bovine serum albumin

C	Cytosine
$CaCl_2$	Calcium chloride
CC	Clonal complex
CFU	Colony-forming units
CO2	Carbon dioxide
CSF	Cerebrospinal fluid
Dam	DNA adenine methyltransferase
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUS	DNA uptake sequence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FI	Fluorescence intensity
G	Guanine
g	Grams
H_2O	Water
HCl	Hydrogen chloride
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IMD	Invasive meningococcal disease
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase pairs
kDa	Kilodaltons
L	Litres

LOS	Lipooligosaccharide
M	Molar
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
mg	Milligrams
MgCl2	Magnesium chloride
ml	Millilitres
mM	Millimolar
MW	Molecular weight
NaCl	Sodium chloride
NIBSC	National Institute for Biological Standards and Control
OD	Optical density
OMP	Outer membrane protein
OMV	Outer membrane vesicles
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Decimal cologarithm of hydrogen
pI	Isoelectric point
PNACL	Protein Nucleic Acid Chemistry Laboratory, University of Leicester
PV	Phase variation
SBA	Serum bactericidal assay
SDS	Sodium dodecyl sulphate

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSM	Slipped strand mispairing
SSR	Simple sequence repeats
ST	Sequence type
T	Thymine
TEMED	Tetramethylethylenediamine
Tris.Cl	Trisaminomethane base adjusted with hydrogen chloride
V	Volts
µg	Micrograms
µl	Microliters

1 Introduction

1.1 *Neisseria meningitidis*

N. meningitidis is a pathogenic bacterium that is considered to be one of the leading causes of bacterial meningitis and septicaemia around the world (Christodoulides 2012). An infection with this organism usually correlates with a high rate of mortality and morbidity especially in children and young adults (Rouphael and Stephens 2012). It is an encapsulated or unencapsolated Gram –ve diplococcus (figure 1-1), belonging to the bacterial family Neisseriaceae and it is an obligate commensal of humans, which act as the only natural reservoir for these bacteria (Trotter and Maiden, 2016). It colonises the upper respiratory tract mucosa of humans as a member of the normal microbiota without causing disease in the carriage state (Yazdankhah and Caugant 2004b; Christodoulides 2012). The bacterium is not motile, but it utilises twitching motility mediated by type IV fimbria to move over the epithelial surfaces and cross the epithelial layer (Merz and So 2000). It is aerobic, fastidious bacteria and their optimal growth conditions are 35–37 °C with 5–10% (v/v) carbon dioxide (Rouphael and Stephens 2012). The bacterium transmits from person to person through aerosols or by respiratory or oral secretions such as saliva, and people who are in direct contact with meningitis patients are at high risk of getting an infection (Tzeng and Stephens 2000; Caugant and Maiden 2009). It can be isolated from the nasopharynx of carriers and from the cerebrospinal fluid or the blood of patients, and in some cases it can also be isolated from other parts of the body such as the genitourinary tract (Tzeng and Stephens 2000).

N. meningitidis are serogrouped according to variability in the structure of the capsular polysaccharide, which has been classified into 13 different serogroups including A, B, C, D, E (previously termed 29E), H, I, K, L, W, X, Y and Z, but only six of these serogroups (serogroup A, B, C, W, X, and Y) were reported to be responsible for the majority of invasive infections around the world (Lewis and Ram 2014). Identification of serogroup is achieved by slide agglutination or PCR assays.

Other methods have been used in typing meningococcal strains by utilizing the antigenic variability in the outer membrane structures including lipopolysaccharides (immunotype), outer membrane proteins such as PorB (serotype) and PorA (serosubtype) as well as FetA (Stephens 2007; Read 2014). Such meningococcal typing methods are performed using monoclonal antibodies (mAbs), PCR approaches, and DNA sequencing.

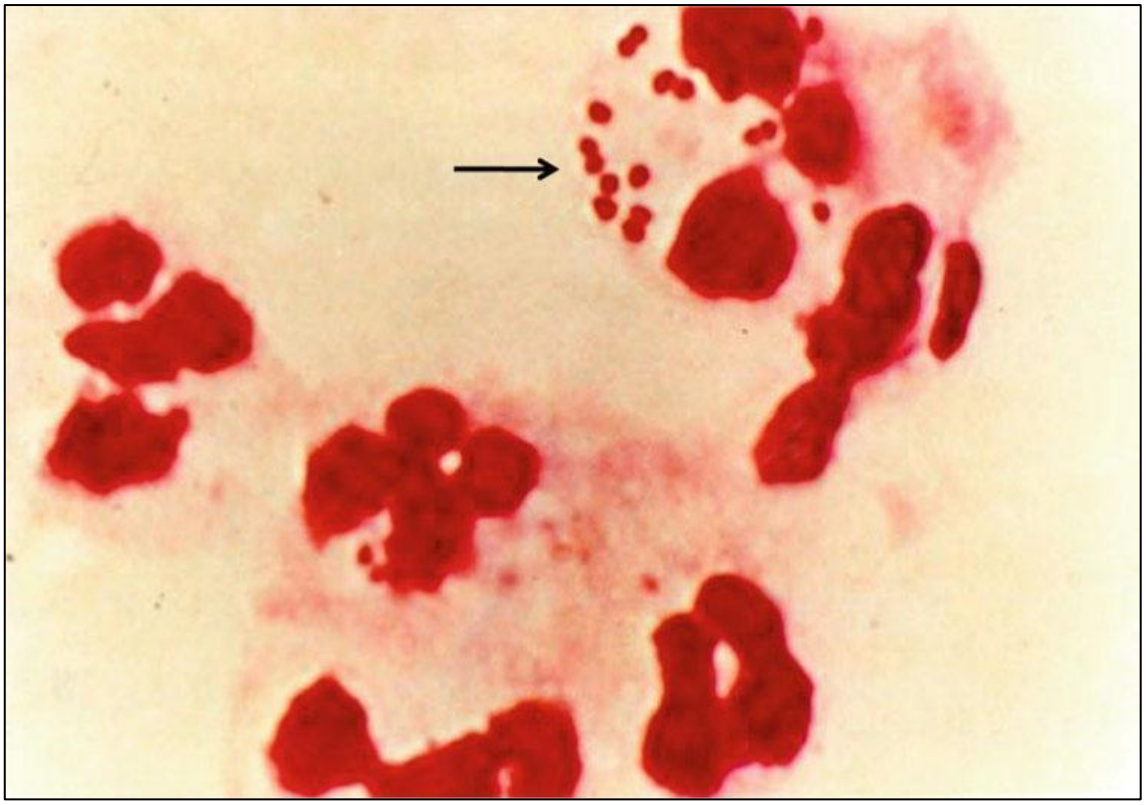


Figure 1-1: Gram staining of *N. meningitidis* diplococci with human leukocytes in CSF samples of meningitis patients. The arrow refers to the meningococcal cells that appear as diplococci**.** Adapted from (Rouphael and Stephens 2012)

Molecular typing methods are another approach for classifying and typing meningococcal isolates. These techniques tend to be more sensitive and reliable methods to characterise meningococcal strains making them more applicable for epidemiological studies and population biology of *N. meningitidis.* Usually these methods are used to identify meningococcal clonal complexes and characterise the strains that will potentially cause outbreaks. They are also used for screening and predicting the coverage of vaccines and to investigate the population genetic structure of the bacteria (Yakubu *et al.* 1999; Rouphael and Stephens 2012). Molecular typing involves using different techniques including pulsed-field gel electrophoresis (PFGE), multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST) and PCR (Yakubu *et al.* 1999). Multilocus sequence typing (MLST) is considered the best standard molecular method, which depends on utilizing differences in the sequences of seven housekeeping genes, and relying on sequence variation of 400-500 bp of the internal fragment of those housekeeping genes (Brehony *et al.* 2007). Such fragments can be sequenced on both strands and they provide enough variability in the nucleotide sequences at each locus giving large number of allelic profiles that can then be used to differentiate between the different isolates (Urwin and Maiden 2003). In such a manner, each unique allele

combination is given a specific Sequence Type (ST) number, and the closely related ST strains, which share at least four of the seven loci, are grouped into Clonal Complexes (CC) (Jolley et al., 2006).

1.2 The genome

Whole genome sequencing was carried out for a number of *N. meningitidis* strains representing different serogroups such as MC58 and H44/76 (serogroup B), FAM18 (serogroup C), and Z2491 (serogroup A) (Parkhill *et al.* 2000; Tettelin *et al.* 2000; Piet *et al.* 2011). According to the data from these genome sequences, *N. meningitidis* has a circular chromosome with a length of 2 – 2.2 megabases and a GC content of 52%, involving about 2000 open reading frames (Schoen *et al.* 2008). Around 70% of the chromosome encodes for metabolically essential proteins. There are also several genetic islands, which are predicted to encode for surface proteins and virulence factors (Caugant 2008; Christodoulides 2012). A large number of repetitive DNA sequences are present within different sites in the chromosome and some of these play important roles in genetic recombination events and antigenic variability in *N. meningitidis* (Davidsen and Tonjum 2006). These repetitive sequences include the Neisserial DNA uptake sequences (which play a role in import of DNA from the surrounding environment), the Correia repeat elements, insertion sequences (IS), and simple sequences repeats (SSRs, homopolymeric or heteropolymeric). The SSRs are present in around 50 loci and play a role in phase variation of expression of many of these 50 loci (Caugant 2008; Schoen *et al.* 2008). Some *N. meningitidis* strains have also been observed to contain plasmids that confer resistance to antibiotics such as β-lactams and tetracycline (Backman *et al.* 2000).

The molecular typing analysis revealed that there is a very high level of genetic diversity in the meningococcal population, and it is defined as non-clonal population in comparision to the majority of other bacterial species because of the high rate of genetic recombination resulting from the natural competency of these bacteria (Davidsen and Tonjum 2006; Maiden 2006). However, although their diversity is extensive, the meningococcal population is highly structured, and the MLST analysis was able to group the genetically related isolates into genetic lineage or clonal complexes (Bentley *et al.* 2007; Rouphael and Stephens 2012). Grouping and characterising meningococcal STs and clonal complexes found that the clonal structure was independent from the serogroups, and only a limited number of clonal complexes were correlated with the

majority of the disease cases worldwide rather than asymptomatic carriage, and so termed as hyperinvasive lineages (Yazdankhah *et al.* 2004; Rouphael and Stephens 2012).

The overall genetic variation of the genome of *N. meningitidis* was characterised by the molecular typing methods such as MLST, and the data generated by such approaches highlights the relationships between the isolates depending on their allelic profiles, which can be shared and exchanged between different academic and research institutes via an internet database (http://pubmlst.org/neisseria) (Watkins and Maiden 2012). Another important database is the pubmlst database, which was designed for storing and sharing both MLST data and Bacterial Isolate Genome Sequence Data (BIGSDB) (https://pubmlst.org/software/database/bigsdb/). These databases enable linking between phenotype and genotype data for an unlimited number of isolates. These databases also enable archiving and organising whole genome sequences data as well as the sequences of the partial genome fragments in a manner making them easy to be interrogated using different number of schemes involving different group of loci such as ribosomal multilocus sequence typing (rMLST) and antigen gene sequence typing (AGST), (Jolley and Maiden 2010; Jolley *et al.* 2012). The genome data available in this database can be used for both clinical and epidemiological analysis, and the database enables assembling of a large number of whole genome sequences, which facilitate the study of meningococcal isolates in terms of their evolution, epidemiology, and mechanisms of their pathogenicity (Maiden and Harrison 2016).

1.3 The carriage state

N. meningitidis is an obligate commensal for human hosts who are considered as the only reservoir for this bacteria in nature (Christodoulides 2012). The number of healthy individuals that carry *N. meningitidis* in their upper respiratory tracts ranges from 10 – 35 % at any time during non-epidemic periods, but this rate can increase to >50% in populations who have close or semi close contact such as university students in dormitories and military recruits as well as households who are in direct contact with meningococcal patients (Yazdankhah and Caugant 2004b; Read 2014). The carriage state is also age dependent, and it occurs at high frequency in adolescents and young adults (peaking in 20-24 years old) , while it tends to be quite rare in infants and less than 10 % in people older than 25 (Caugant *et al.* 2007; Caugant 2008). It is assumed that most people are colonised by *N. meningitidis* one or more times during their life (Caugant and Maiden 2009). The duration of the asymptomatic colonisation is quite variable, and it can

be transient (several days), sporadic or persistent lasting for several months (Yazdankhah and Caugant 2004b). Duration of carriage also depends on the colonizing strain, as not all the carriage strains have the same ability to persist for long periods in the host (Caugant *et al.* 2007).

The exposure to *N. meningitidis* strains during persistent carriage leads to elicitation of an antibody response such as mucosal IgA, which may act as a natural immunizing process conferring cross protection against challenges by other strains of *N. meningitidis* and helping to prevent invasion of the circulatory system. It also elicits serum bactericidal antibodies that have a cross reactivity with heterologous strains of *N. meningitidis* and are known to protect against infection (Jordens *et al.* 2004; Yazdankhah and Caugant 2004b).

Successful colonisation of the human nasopharynx by meningococci requires the attachment of the bacteria firstly to the mucosal membrane and then the resistance and the avoidance of the host defence mechanisms (Hill *et al.* 2010). The adhesion is achieved by expression of different surface adhesins (Figure 1-2), such as a Type IV pilus (TfP), which mediates the initial adhesion to the non-ciliated epithelial cells, which is considered the first step of colonisation. After that, retraction of the pilus brings the bacterial cells into close contact with the epithelial surface. This close contact allows for starting of intimate adhesion mediated by the binding of the outer membrane opacity proteins (Opa and Opc) to their receptors on the epithelial cells such as HSPG and CEACAMs (Hung and Christodoulides 2013). As the bacteria are usually encapsulated during transmission between hosts, the presence of the capsule hinders the intimate adhesion of the bacteria as it masks the major adhesins proteins and prevents them from interaction with their receptors (Hill *et al.* 2010). Therefore, after the initial adhesion, the expression of capsule may be down modulated via phase variation allowing the outer membrane proteins to interact with their host receptors (Deghmane *et al.* 2002). This will maintain a more stable adhesion that is necessary for long term of colonisation (Trivedi *et al.* 2011). The resistance of the bacteria to the mechanical forces such as the sheer force and the mucus flow is essential for such successful colonisation, and this can be achieved by the rapid and strong adhesion of the bacteria to the epithelial mucosa mediated by forming cortical plaques as well as biofilm formation (Yi *et al.* 2004; Mikaty *et al.* 2009; Hill *et al.* 2010).

Immune avoidance is another important strategy that is necessary for the colonisation to be successful. Immune avoidance is achieved by several mechanisms including secretion of IgA protease that deactivates the mucosal IgA, antigenic, and phase variation of the surface components involving the adhesins themselves (virji, 2009).

However, in a few cases during the asymptomatic colonisation, the bacteria may enter the mucosal membrane and reach the blood stream and cause disease (Yazdankhah and Caugant 2004b).

1.4 Pathogenesis of *N. meningitidis*

Although the rate of meningococcal asymptomatic colonization is quite high within human populations, switching from carriage to invasive disease happens at quite a low rate while the mechanisms leading to this change are not completely understood (Stephens 2007; Pace and Pollard 2012). After colonizing the human nasopharynx, the bacteria may cross the mucosal membrane either by transcytosis, direct invasion or via the phagocytic cells (Hung and Christodoulides 2013). When the bacteria reach the blood stream, they will encounter a large number of host defence mechanisms such as antibody/complement mediated bacteriolysis and phagocytosis (Hill *et al.* 2010). If the bacteria can overcome the immune defences in the blood stream, it will survive and multiply rapidly and spread to different parts of the body (Virji 2009). In such cases, the bacteria either cause septicaemia, or cross the vascular endothelium of the brain barrier to infect the meninges and the cerebrospinal fluid (Pizza and Rappuoli 2015b).

Expression of virulence factors is one of the key strategies for the meningococcus to survive and avoid the immune system in the blood. Capsule and LPS are the main virulence factors that enhance the survival of the bacteria during invasion into the bloodstream as they mediate resistance to antibody/complement-mediated killing (Geoffroy *et al.* 2003). Capsule also mediates bacterial protection by inhibiting the phagocytosis process (Uria *et al.* 2008; Pizza and Rappuoli 2015b). Binding of the bacteria to the negative regulators of complement pathway such as factor H, vitronectin and the major complement inhibitor C4b-binding protein (C4bp), is another strategy to increase their resistance to complement-mediated killing and then survive in blood (Hill *et al.* 2010). The meningococcal factor H binding protein (fHbp) can bind Factor H protein, a key regulator of the alternative complement pathway, which has been shown to increase meningococcal serum resistance (Madico *et al.* 2006). Vitronectin, which

inhibits the formation and penetration of the membrane attack complex (MAC) on the meningococcal cell membrane, can be recruited by binding with Opc protein, whereas PorA protein (the major outer membrane porine) binds and recruits the C4bp, both factors have been shown to enhance the bacterial resistance to human serum (Jarva *et al.* 2005; Sa *et al.* 2010). A further mechanism that enables the meningococcal cells to escape nonopsonic phagocytosis by PMN is their ability to utilize L-glutamate from the environment and convert it to glutathione, which is the key component that maintains the intracellular redox and protects the bacteria from the effects of the oxidative burst of neutrophils (Tala *et al.* 2011).

As mentioned above, when the bacteria successfully survive in the bloodstream, it will rapidly multiply, disseminate and cause bacteremia. The level of the bacteremia can affect the ability of the bacteria to invade the meninges, as it is correlated with the level of their interaction with the components of the brain-blood barrier (BBB) (Coureuil *et al.* 2012). When the bacteria reach the BBB, which is formed of specialized endothelial cells and tight intracellular junctions, it will adhere to the endothelial cells via pili, and then forming a firm adhesion that enables the bacteria to resist the high flow force of the blood stream (Miller *et al.* 2013). After the attachment, the bacteria may cross the endothelial layer via transcytosis, or via the disruption of the intracellular junction due to the intracellular signaling induced by pili and then passed the barrier (Coureuil *et al.* 2012). Once the bacteria breach the BBB, it will interact with cells lining the leptomeninges and consequently lead to an inflammatory reaction due to the release of pro-inflammatory cytokines which then results in meningitis (Hill *et al.* 2010).

Many other important factors are also supposed to participate in enabling the bacteria to invade the circulatory system and to successfully survive and multiply in the blood. Host susceptibility is one of the factors leading to meningococcal invasion and people can be susceptible due to a deficiency in their complement pathway or related immune system, co-infection by other pathogens such as viruses, smoking, other genetic polymorphisms or lack of specific immunity to the invading strain (Tzeng and Stephens 2000; Stephens 2007; Hill *et al.* 2010).

The rate of meningococcal diseases within human populations ranges from 1 to 1000 cases in each 100,000 individuals worldwide per year (Caugant and Maiden 2009). While

different kinds of diseases can result from infection by *N. meningitidis*, the most common diseases caused by these bacteria are meningitis and septicaemia (meningococcemia) (Christodoulides 2012). Further clinical implications of meningococcal infection involve septic arthritis, pneumonia, otitis, urethritis, purulent pericarditis and sinusitis. In spite of effective antibiotic treatment, mortality caused by meningococcal infection is still high, ranging from 10–20% in developed countries, while the survivors may suffer from permanent sequelae such as deafness, cognitive dysfunction and amputation (Tzeng and Stephens 2000; Christodoulides 2012).

1.5 Virulence factors

N. meningitidis express different virulence factors that play significant roles in the pathogenesis of these bacteria, the most important factors are discussed in the following sections.

1.5.1 Capsular polysaccharide

N. meningitidis, as with many Gram –ve bacteria, has a polysaccharide capsule that lies outside of the cell membrane. This structure is considered as the main virulence factor for this bacterial species as it enables these bacteria to evade the immune response by inhibiting opsonisation and phagocytosis as well as reducing complement-mediated bactericidal killing of the bacteria (Stephens 2007). The capsule may also play a role in preventing desiccation, which can enhance the ability of the bacteria to transmit between different hosts (Rosenstein *et al.* 2001). The invasive strains of *N. meningitidis* are always encapsulated, while the carriage isolates vary in their capsulation status (Christodoulides 2012). The structure of the capsule of some of the meningococcal serogroups, including B, C, Y and W, contain sialic acid that is also commonly present on human cell surfaces, and this provides those serogroups an ability to evade human immune response due to molecular mimicry mechanism. The most strong mimicry is for serogroup B which contains α(2-8)-linked polysialic acid in their capsule components that is structurally identical with the human neural cell-adhesion molecule (NCAM), with such identity making the serogroup B capsule poorly immunogenic (Finne *et al.* 1983; Christodoulides and Heckels 2017).

1.5.2 Lipo-oligosaccharide LOS

LOS are surface structures that mediate the adherence of *N. meningitidis* to human cells and also play an important role in the evasion of the innate immune system (Plant *et al.* 2006). It is an amphipathic glycolipid structure that mediates the interaction of *N.*

meningitidis with different host receptors and acts as virulence factor. LOS is the most abundant antigen of the *N. meningitidis* cell envelope. This structure is composed of two different components: the lipophilic portion, termed lipid A (which is highly toxic and acts as endotoxin), is composed of hydroxy fatty-acid chains and phosphoethanole amine; and a polysaccharide core, which is comprised of a variety of oligosaccharide units (may contain up to 40 subunits) with a conserved inner and variable outer core portions (Frosch and Maiden, 2006). The polysaccharide portion includes sialic acid and other modifications that mimic the outer surfaces of human cells, thus enabling the bacteria to avoid the immune response (Virji 2009). The LOS of these bacteria lacks the O-side chain antigens presents in the entire LPS of other Gram-negative bacteria (Kahler and Stephens 1998). Phase and antigenic variation of the LOS can lead to changes and variability in the oligosaccharide chain both between strains and within a clonal population. Differences in the antigenic structure of the LOS are determinants of the immunotype classification of meningococcal strains with strains varying between from L1 to L12 (Jennings *et al.* 1999). It has been noticed that the majority of carriage isolates of *N. meningitidis* expressed LOS with short chains, immunotypes L1 and L8, whereas the disease causing isolates tend to express LOS with long α-chains (Alonso *et al.* 2007). Such variability can result from phase variation as switching between immunotypes can occur due to phase variation of *lgt* genes including *lgtA, lgtB, lgtC, lgtD, lgtE*, and *lgtG*. Phase variation of LOS epitopes plays a role in immune avoidance of the bacteria to the serum bacteridcdal activity by mediating escape of epitope-specific LOS antibodies (Bayliss *et al.* 2008). In addition, the LOS share the same characteristics as the capsules in terms of enabling the bacterial cells to escape bactericidal activity and phagocytosis (Virji 2009).

1.5.3 Adhesins

Colonisation by *N. meningitidis* of the human host acts as the first step towards causing disease. Colonization involves adhesion of the bacteria to the epithelial cells, aggregation, formation of microcolonies and biofilms and then evasion of immune system. The adhesion step depends mainly on the contact and the interaction of particular surface structures of the bacteria called adhesins, with specific receptors on human cells (Hung and Christodoulides 2013). These surface structures are classified as major (Figure 1-2) and minor adhesins.

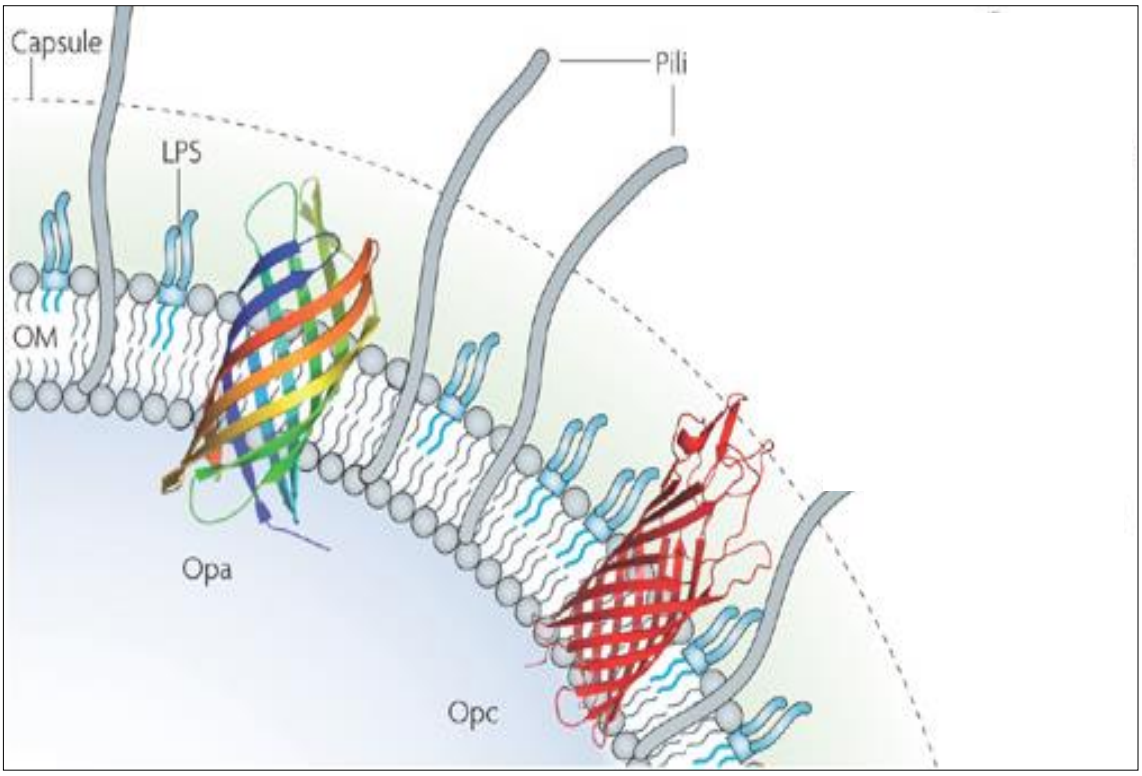


Figure 1-2: The main surface structures of meningococcal cells that play the central role in the colonisation and the invasiveness of these bacteria(Virji 2009). The figure presents the major adhsisns (Pili, Opc and Opa) of meningococcal as well as the capsular polysaccharide and LOS, and all of these structures act as virulence factors mediating a key role either in adhesion and ainvasion process of the meningococcal cells to human cells or in resistance to avoidance of human immune response as discussed in the text (adapted from (Virji 2009).

1.5.3.1 **Pili (type IV fimbria*)***

One of the major adhesins that mediates the initial adherence of the capsulated meningococcal cells to the human epithelial and endothelial cells is pili (Pinner *et al.* 1991). Pili are a homopolymeric fiber composed mainly of the major pilin subunits PilE (Kolappan *et al.* 2016) combined with other minor proteins including PilD , E, G , X , V and comP that contribute to the structure and the function of the pili. There are up to 15 different proteins that participate in the biogenesis of the meningococcal pili including PilC, PilD, PilE, PilF, PilG, PilH, PilI, PilJ, PilK, PilM, PilN, PilO, PilP, PilQ and PilW (Carbonnelle *et al.* 2005). Pilus receptors on human cells was not well documented, while a direct interaction of piliated gonococcal cells with human recombinant CD46 has been reported, other studies with gonococcal pili suggested that this surface structure binds to α1 and α2 integrins rather than CD46 (Helena *et al.* 2001; Edwards and Apicella 2005)Whereas, piliated meningococci showed to successfully colonise and then infected CD46-humanised transgenic mice suggesting the role of this human ligand in mediating binding of piliated meningococci to human cells (Johansson

et al. 2003). Pili also mediates the twitching motility of the bacteria, which plays a role in the passage of the bacterial cells through the epithelial layer, movement over epithelial surfaces, and formation of microcolonies (Merz and So 2000). This surface structure also mediates the uptake of foreign DNA from the environment, which facilitates the transformation and consequently the genetic variability via homologous recombination events (Fussenegger *et al.* 1997). *N. meningitidis* express two structurally different pilin families (class I and II), and class I family are subject to phase and antigenic variation particularly of the PilC and PilE proteins in contrast to class II which their pilE gene encodes for conserved pillin subunits (Wörmann *et al.* 2014). Such phase and antigenic variability in pili plays a role in immune evasion and avoidance of host defenses (Carbonnelle *et al.* 2009; Rouphael and Stephens 2012). Posttranscriptional modifications such as pilin glycosylation can also facilitate secretion of soluble pilin units, which can bind and then block anti-pili antibodies leading to protect the bacteria from the anti-pili antibodies (Rouphael and Stephens 2012).

1.5.3.2 **Opacity proteins**

Other important surface structures that participate in the virulence of the bacteria and mediate the adhesion of the bacteria to human cells are the opacity proteins of which there are two types, Opc and Opa. Both of these surface adhesins mediate the attachment to human cells that is essential in starting the colonization of human airways and also play an important role in invasion of human cells after intimate adherence (Virji 2009). The Opa proteins are discussed in section 1.8, so this section focuses on Opc.

In contrast to Opa proteins which are expressed by different neisserial species, Opc protein (27–31 kDa) is only expressed by *N. meningitidis*, and is encoded by a single gene called *opcA*. Opc has a β- barrel structure with 10 transmembrane domains forming 5 loops that stand out from the outer surface of the cell envelope (Prince *et al.* 2002). This proteins mediates adhesion and invasion of the meningococcal cells into human epithelial and endothelial cells (Virji *et al.* 1992). It was also observed that strains expressing Opc at high level tend to be highly invasive to human umbilical vein endothelial lid cells HUVECS (Virji *et al.* 1995). Opc has also implicated role in crossing of the blood-brain barrier (Unkmeir *et al.* 2002). The protein mediates the interaction with endothelial cells by binding to members of the integrin family proteins, which are considered as its main receptor on endothelial cells. Binding involves a sandwich mechanism in which Opc binds to vitronectin forming a tri-complex with the avβ3 and α5β1 integrin present on the

apical surface of endothelial cells (Virji *et al.* 1994). The binding of Opc to serum vitronectine can also enhance the resistance of Neisserial cells to human complement (Sa *et al.* 2010). Other receptors that can be recognised and bound by Opc are heparin like molecules and heparan sulphate proteoglycan (HSPG) present on human epithelial cells (de Vries *et al.* 1998).

1.5.3.3 *Minor adhesins*

Several minor adhesins have been identified and described for *N. meningitidis*. However, the functions and the roles of these adhesins in the pathogenesis of *N. meningitidis* is not well characterised. Some of these adhesins belong to the autotransporter protein family such as NadA (Neisserial adhesion A), which is a promising vaccine candidate. This protein interacts with human epithelial cells and mediates adhesion and invasion of meningococcal cells into Chang epithelial cells, however the receptors on this cell type are still unknown (Capecchi *et al.* 2005). Other minor adhesins are NhhA (Neisseria hia homologue A) and the auto-transporter MspA (meningococcal serine protease A), which were found to play a role in enhancing the bacterial adhesion to the human brain microvascular endothelial cells (HBMEC) (Serruto *et al.* 2003; Turner *et al.* 2006). The ACP (adhesion complex protein) is another adhesin that was shown to mediate the interaction of the meningococcal cells with human umbilical vein endothelial cells (HUVECS) (Hung *et al.* 2013). App (adhesion and Penetration Protein) is also an auto transporter protein that was suggested to play a role in the colonization of *N. meningitidis* as it mediates their adhesion to epithelial cells (Serruto *et al.* 2003).

Molecular mimicry and switching between different virulence phenotypes by phase and antigenic variation are additional molecular mechanisms that are thought to contribute to avoidance of innate and adaptive immune barriers (Tzeng and Stephens 2000).

1.6 Diagnosis and treatment of meningococcal diseases.

One of the main measures that should be taken to prevent lethal complications and long term-physical morbidity of meningococcal infection is the early diagnosis and treatment of the disease (Nadel 2016). Such early diagnosis is challenging as the specific meningococcal symptoms such as fever, headache, neck stiffness and photophobia are not clear at the early stage of the disease, and early clinical manifestations resemble the symptoms of other common infections such as viral infections (Rosenstein *et al.* 2001). This delay in diagnosis is always accompanied by a rapid progression in the infection,

which in many cases leads to death within few hours of hospital presentation (Slack 1982; Lala *et al.* 2007). The laboratory diagnosis of meningococcal infection involves using several classical bacteriological techniques relying on confirming the presence of the bacteria or their related products in blood and CSF (Sarfatti and Nadel 2015). Blood culture and Gram staining of the CSF are the main techniques used for such diagnosis (Dunbar *et al.* 1998; Brouwer *et al.* 2010), but the limitation with these techniques is that they are time consuming and have a low rate of specificity and sensitivity especially when samples were taken from patients after antibiotic administration (Wylie *et al.* 1997; Munoz-Almagro *et al.* 2009). Therefore, molecular methods including PCR and real time PCR were developed and proved to be more reliable for identification of meningococcal infections (Munoz-Almagro *et al.* 2009). These methods offer rapid diagnosis for the infection without requiring the presence of living organism in the blood or CSF samples and results with these approaches are of very high sensitivity (100 %) and specificity (around 88 %) as evaluated by multiple studies (Sarfatti and Nadel 2015).

Antibiotic therapy is the best treatment strategy for meningococcal diseases, as it tends to be very efficient if it is given appropriately and at a suitable time before or after diagnosis of the meningococcal infection (Sudarsanam *et al.* 2017). Due to the rapid progression of meningococcal infections, pre-hospital antibiotic treatment for a suspected meningococcal case is crucial and can be very effective to protect against mortality (Perea-Milla *et al.* 2009). Similarly for a confirmed case of meningococcal infection, antibiotic treatment should be given as early as possible (at least within 1 hour) after the confirmation of the case (Nadel 2016). Such early antibiotic administration can lead to immediate cessation of meningococcal replication in the blood and CSF, and complete sterilization of the CSF can be occur within 2-4 hrs of the antibiotic treatment (Kanegaye *et al.* 2001).

Parenteral administration of β-lactam antibiotics such as cephalosporins and penicillins is the best choice for meningococcal meningitis treatment. For those patients with confirmed invasive meningococcal disease (IMD), penicillin, cefotaxime and ceftriaxone is the most effective antibiotic treatment (Sarfatti and Nadel 2015). However, many meningococcal strains are showing resistance to penicillin and some other antimicrobial agents such as chloramphenicol, tetracycline and rifampin, and cases of penicillin treatment failure have been reported (Goldani 1998; Brouwer *et al.* 2010)Jorgensen, 2005 #327}. Therefore, for patients with an unconfirmed case of meningococcal disease,

empirical treatments with broader-spectrum cephalosporins (third generation) should be given until the positive diagnosis is confirmed, as there is still a potential infection either with a penicillin resistant strain, or with another bacterial species (Sarfatti and Nadel 2015).

1.7 Vaccination

Although intensive care and effective treatment of the meningococcal infections can reduce the rate of mortality, the survivors of meningococcus infections frequently suffer from permanent morbidity. Therefore, efforts had focused mainly on the prevention of meningococcal disease via vaccinations (Vipond *et al.* 2012; Batista *et al.* 2017). Three different kind of vaccines are available now for meningococcal disease including capsular polysaccharide-based vaccines, glycoconjugate vaccines and protein based vaccines. (McCarthy *et al.* 2018) Capsular polysaccharide-based vaccines were developed and used firstly in the 1970s against the five disease causing serogroups of *N. meningitidis* (A, C, W, X and Y). These vaccines depend on purifying the capsular polysaccharide of these serogroups (Girard *et al.* 2006). They are administered as bivalent vaccines (containing the capsule of serogroup A and C), trivalent (serogroups A, C and W capsules) and quadrivalent involving capsules of serogroups A, C, W and Y (Crum-Cianflone and Sullivan 2016). These vaccines were used to control endemic and outbreak disease cases, and they were shown to be safe and to provide good protection in adults and older children but were not immunogenic in infants or young children (under 2 years). In addition, polysaccharides are T-cell independent antigens and thus they elicit only short term immune memory (Shao *et al.* 2009) (McCarthy *et al.* 2018) (Assaf-Casals and Dbaibo 2016).

To overcome such limitations, glycoconjugate vaccines were developed in which the purified capsular polysaccharides are linked to a carrier protein that can trigger the B cells and T cell-dependent immunity and then elicit immune memory (Kelly *et al.* 2006). Such vaccines were effective in reducing carriage rates and break transmissibility among populations, and, most importantly, provide effective protection in infants and young children (Crum-Cianflone and Sullivan 2016). Currently three monovalent conjugate vaccines are licensed against MenC (Meningtec, Menjugate and NeisVac-C) (McCarthy *et al.* 2018). These vaccines show a high efficiency in infants and young children as well as other age groups (Gasparini and Panatto 2011), and when they were introduced, the incidence of disease caused by MenC strains decreased significantly in the UK and other

European countries. (Trotter *et al.* 2004; Gray *et al.* 2006) In addition, these vaccines reduced the rate of carriage due to the development of herd immunity (Maiden and Stuart 2002). A MenA monovalent vaccine (MenAfriVac) was developed for the meningococcal belt of sub-Saharan Africa and it was successful in making a dramatic reduction in MenA cases in African countries (Crum-Cianflone and Sullivan 2016). Tetravalent conjugated vaccines, such as MenVeo, involving the capsular polysaccharide of four serogroups of *N. meningitidis* (A, C, W and Y) were also licensed and showed broader coverage in terms of numbers of meningococcal serogroups and the age group of the vaccinees (McCarthy *et al.* 2018). while combined conjugated vaccines such as MenHibrix (Hib-MenCY-TT) provide protection against particular meningococcal serogroups and the other pathogenic bacteria *Haemophilus influenzae* serogroup b, as it involves the capsular antigens of a meningococcal serogroup and the key immunogenic component (polyribosylribitol phosphate) of the *H. influenzae* b capsule (Hale *et al.* 2013)

However, developing polysaccharide conjugate vaccines against serogroup B strains was not possible due to the poor immunogenicity of this capsule and the similarity of the polysaccharide structure with the one that is expressed by human brain cells making it a potential auto-antigen with a risk of causing an autoimmune response when is used as a vaccine (Finne *et al.* 1983; Bruge *et al.* 2004). Development of protein-based vaccines was the alternative strategy for providing protection against infection with this invasive serogroup. Detergent extracted outer membrane vesicles (OMVs) were developed as the best alternative vaccines for strain-specific outbreaks in many countries such as Cuba, Norway and New Zealand (Holst *et al.* 2013). The vesicles released naturally from the outer membranes of Gram –ve bacteria during growth and contain many surface components such as phospholipids, lipooligosaccharides, and outer membrane proteins in their native states (van der Pol *et al.* 2015). Although all of these components are immunogenic, the immunogenicity of this vaccine was mediated mainly by the strong antigenicity of PorA (Wedege *et al.* 2003). As PorA shows high variability between the different meningococcal strains, these vaccines provide only strain–specific protection, making them unsuitable for systematic immunization campaigns (Feavers and Maiden 2017b).

In order to improve these vaccines, other meningococcal proteins were added into the OMVs in an attempt to increase coverage of a wider range of protein antigens and hence wider protection against more meningococcal strains. Recently, two vaccines against

serogroup B were licensed which depend on such a strategy including 4cMenB (Bexsero) and MenB-FHbp (Trumenba). Bexsero is composed of OMV of the epidemic NZ98/254 strain (the MenB strain responsible for the outbreak in NewZealand) which provides immunodominant PorA variant (P1.7), and three other highly immunogenic recombinant proteins including factor H binding protein (fHbp), Neisserial adhesin A (NadA) and Neisseria heparin binding antigen (NHBA) (Gorringe and Pajón 2012). Whereas the bivalent Trumenba is composed of two dominant variants of lipidated fHBP (subfamilies A and B) without involving any OMVs (Donald *et al.* 2017).

In 2014, Bexsero had been used in the USA to prevent infections with MenB strains during outbreaks among university students. The vaccine successfully disrupted the outbreak caused by this strain and no further cases were recorded after one year of assessment (McNamara *et al.* 2015; Feavers and Maiden 2017a). Bexsero was also incorporated into the national immunization program for infants in the UK in 2015, and the effectiveness of the two-dose priming schedule was high and successful for preventing MenB disease in infants (Parikh *et al.* 2016). The effectiveness of Trumenba is mainly predicated on serological traits, which indicated that this vaccine provides protection against a panel of strains expressing different variants of fHbp and representing the most prevalence epidemiological strains in USA (Shirley and Dhillon 2015). However, the efficacy of both vaccines, the duration of their protection, the breadth of the strains that they cover as well as their impact on the carriage state still need to be investigated by assessing of their impacts using clinical and epidemiological analysis following implementation in a relatively large population (Feavers and Maiden 2017b; Toneatto *et al.* 2017)

1.8 Phase variation

Phase variation is an adaptive mechanism that is used by many pathogenic bacteria to adapt to fluctuations in environmental conditions and to survive and overcome adverse conditions. It involves reversible mutations in particular sites (hypermutable sequences) within the bacterial chromosome called contingency loci (Moxon *et al.* 1994). This mutation can lead to switching ON / OFF in the expression of particular proteins in a numbers of cells within the clonal bacterial population leading to generation of new heterogenic phenotypes, which under selective conditions will be selected to multiply and become the dominant phenotype in the new population (Figure 1-3) (Bayliss 2009)

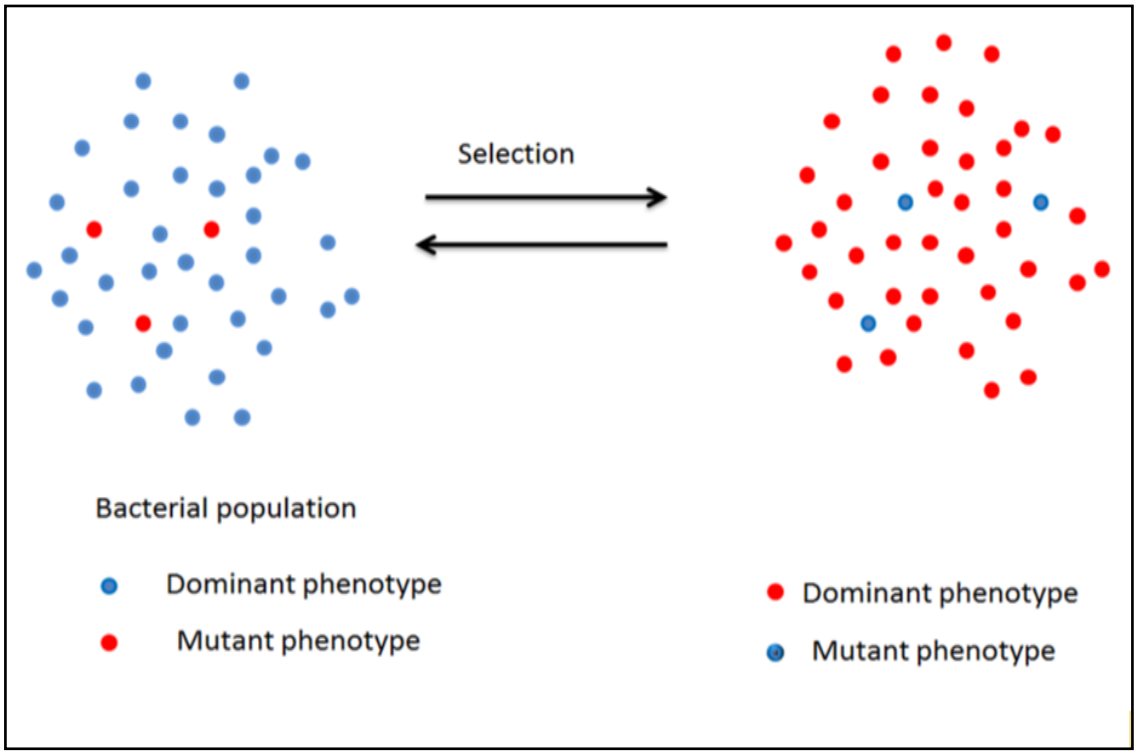


Figure 1-3: Phase variation mediates the switching between two different phenotypes in the bacterial population. High mutation rates occur in the contingency loci of the bacteria leading to generation of heterogeneous phenotypes within the bacterial populations, each one of these phenotypes can be selected under a particular selective condition to be the dominant phenotype that can successfully multiply and adapt to the new conditions. Therefore, such reversible mutations confer a flexibility to the bacteria to adapt and colonise different biological niches**.**

There are several mechanisms involved in phase variation including slipped strand mispairing (SSM), homologous recombination, site-specific recombination and epigenetic modification via DNA methylation.

1.8.1 Homologous recombination

Homologous recombination is one of the main mechanisms that mediates generation of genome diversity and genetic rearrangements in bacterial cells. This mechanism requires the presence of long homologous DNA sequences (>50 bp) and it utilise proteins of the DNA repair machinery and maintenance of the cell. Gene conversion can result from such mechanisms when unidirectional exchange of DNA happens between two alleles (van der Woude and Baumler 2004). The best-characterised example of this mechanism mediating gene conversion is in the type IV fimbria of *Neisseria gonorrhoea* where recombination happen between the *pilE* locus (encoding for PilE) and one or more of silent *pilS* loci that are distinct from *pilE* locus (Henderson *et al.* 1999). In such recombination events, DNA fragments of *pilS* will be transferred to the *pliE* locus by the RecA recombinase with lose of the *pilE* segment during the process (Haas and Meyer 1986). All *pilS* loci contain

variable sequences within conserved sequences of *pil* genes but lack promoter regions, therefore, gene conversion events lead to expression of *pilS* variable segments instead of *pilE* segments and consequently lead to antigenic variation in pili of N. *gonorrhoea (Hill and Davies 2009).*

In some cases, the recombination event leads to a deletion in the 5' end of *pilE,* or in another case, the sequences of one of the *pilS* segments contains a premature stop codon, and in such events gene conversion leads to reversible switching from On to Off in the expression of pili (Figure 1-4) (Swanson *et al.* 1986; Howell-Adams and Seifert 2000). A switch from piliated to non-pilated *N. gonorrhoea* can occur when recombination between *pilS* and *pilE* genes leads to insertion of multiple tandem copies of *pilS* into the expression region, which then leads to production of L-pilin subunits (Henderson *et al.* 1999). Such pilin subunits accumulate in the periplasmic space or outer membrane and cannot form pili structures. Deletion of the extra copies of *pilS* from the expression site leads to recovery of the piliated phenotype (Seifert 1996; Henderson *et al.* 1999).

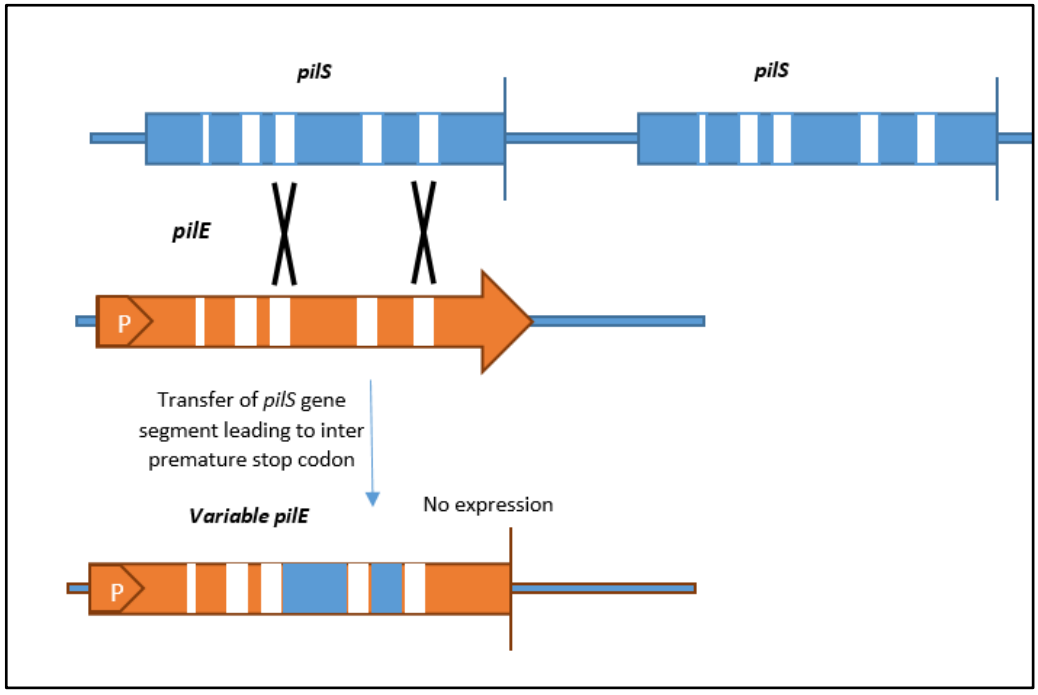


Figure 1-4 Phase and antigenic variation of gonococcal pili mediated by homologous recombination. Intra-genomic recombination events occur between *pilE* (encoding for the major pilin protein) and the silent gene *pilS* of N. *gonorrhoea* leading to transfer of a DNA segment of *pilS* (involving a variable sequence region of the *pilE* gene) into *pilE* . Recombination of this segment generates new variable *pilE* alleles, and in some cases, the recombined fragment may contain a premature stop codon or the recombination leads to deletion in the nucleotide sequences of *pilE* and then generation of a stop codon that consequently leads to switching the gene off. The letter P in the diagrams of the *pilE* gene refers to the promoter that is absent from the *pilS* silent genes.

1.8.2 Site-specific recombination

Site-specific recombination mediates inversion of DNA fragments that are flanked by short (30 bp), homologous inverted repeat sequences. Such DNA fragments can contain the promoter of a particular gene or gene regulatory element whose inversion leads to a change in the expression of the gene from ON to off or from Off to On (Hallet and Sherratt 1997; Van Der Woude and Bäumler 2004). This mechanism requires specific recombinase enzymes for mediating the excision and the inversion of the DNA fragment as well as one or more cofactors. This mechanism occurs independently from the homologous recombination pathway (van der Woude and Baumler 2004). Phase variation in the *fimA* gene, encoding for the major subunit of type 1 fimbria of *E. coli,* is a well characterised example of phase variation mediated by such a mechanism. The variation occurs by inverting a 315 bp DNA fragment located upstream of *fimA* and containing its promoter region. Changing the orientation of this fragment alters the orientation of the promoter of this gene and consequently leads to a change in expression of the gene from ON (the correct position) to Off (incorrect position)(Abraham *et al.* 1985). Site-specific recombination is controlled by the *fimB* and *fimE* genes which are located upstream of the promoter region of *fimA* and encode two site-specific recombinases (Klemm 1986). Another example of phase variation mediated by this mechanism is in the *S. typhimurium* flagella (Zieg *et al.* 1977; Silverman *et al.* 1979). This example is more complex than the one in *E. coli* as the inversion does not change the expression from ON to Off, but leads to a change between the expression of two antigenically different flagellin subunits, H1 and H2. The inverted repeat surrounds the promoter region of H2 encoding gene (*h2*) and other gene that encode for a repressor for flagella *h1*. Therefore, when the promoter of H2 is in the correct direction, the expression of *h2* will be in the On state, which in the same time lead to transcript and express the repressor (*rh1*) of the other flagella encoding gene (*h1)*, and consequently lead to repress the expression of H1 flagella. When the inversion happen, no expression will be for flagella *h2* as well as the repressor of flagella 1, which then will be transcribed and expressed (Figure 1-5) (Zieg *et al.* 1977).

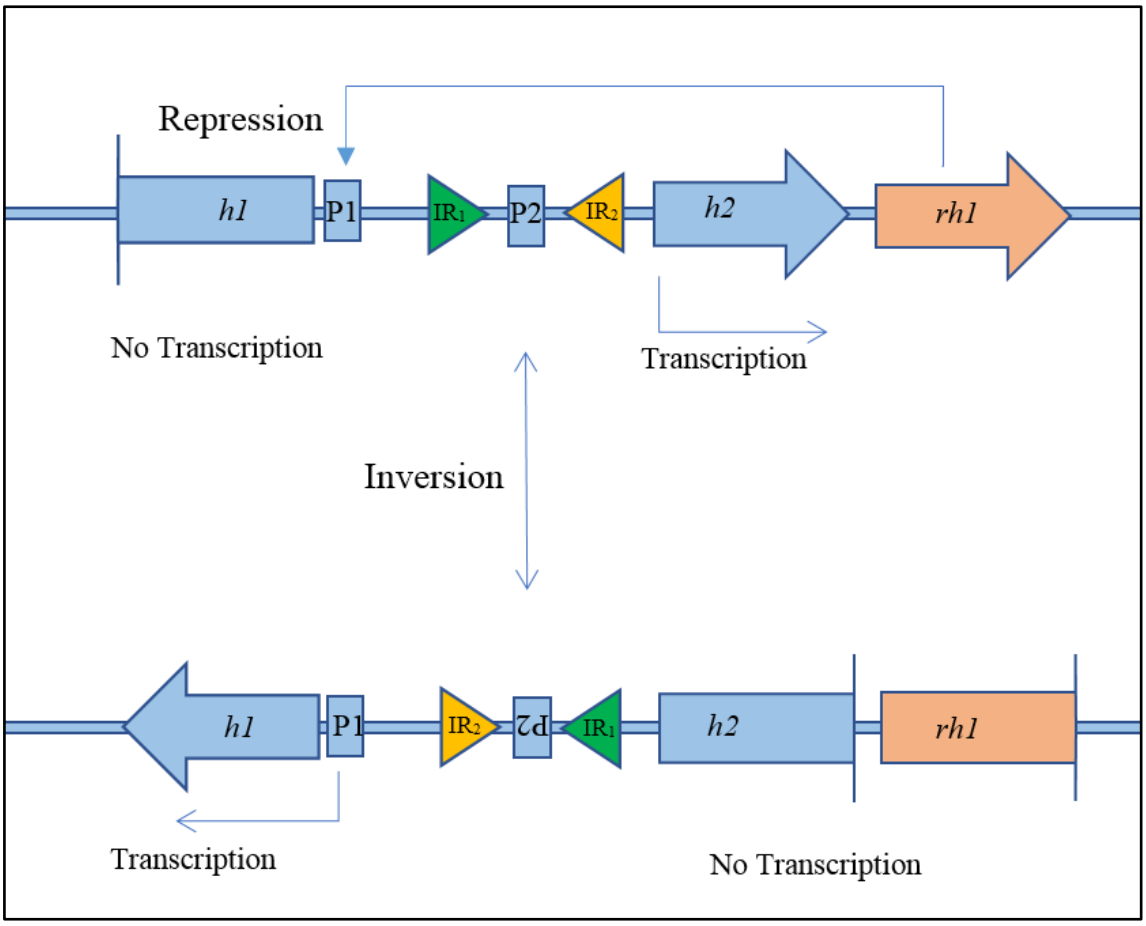


Figure 1-5 : Site-specific recombination mediating the phase variation between two antigenically different flagella of *S. typhimurium*. The promoter region of the gene encoding for the flagellin 2 (*h2*) also acts as a promoter for gene encodes for protein (*rh1*) that acts as repressor for flagellin1 encoding gene (*h1*). The promoter is flanked by inverted repeat that is target for recombinase mediated excision and flipping of these repeats. When the promoter is in the correct direction, the gene encoding for flagellin 2 (*h2*) will be expressed along with the flagellin 1 repressor gene, leading to repression of the expression of the flagellin 1. Whereas, when the flip happens, the expression of both flagellin2 and the repressor of flaggellin1 will be stopped, and this leads to expression of flagellin 1 (*h1*). IR, inverted repeats; P1, promoter 1; P2, promoter 2.

1.8.3 Epigenetic Regulation

This mechanism is different from the other mechanisms of phase variations as it does not involve a change in the DNA sequence (Van Der Woude and Bäumler 2004). The variation of this mechanism is mediated by inheritable and reversible changes in the methylation state of particular sites within the promoter region of the phase variable gene. Such methylation affects the binding of the regulatory factors to the promoter and consequently leads to a change in gene expression (Bayliss 2009). One of the best-characterised examples of phase variation mediated by epigenetic modification is reversible switching of Ag43 protein expression in *E. coli*. This protein is an outer membrane protein and plays a key role in biofilm formation and autoaggregation of the bacteria (Wallecha *et al.* 2002). The phase variation of the *ag43* gene requires the activity

of deoxyadenosine methyltransferase (Dam) and the DNA binding protein OxyR, which acts as a global regulator for preventing oxidative stress (Haagmans and van der Woude 2000). Both of those proteins compete for binding to the three GATC sequences present in the promoter region of *ag43*. When these sequences are fully methylated by Dam, the binding of OxyR will be blocked and at the same time this enhances the binding of RNA polymerase and consequently allowing transcription and the gene then be in the ON state. While in the hemi-methylated GATC, OxyR will bind to this region, prevent Dam from methylating the sequences, which consequently prevents the binding of RNA polymerase and blocking the transcription process (Haagmans and van der Woude 2000).

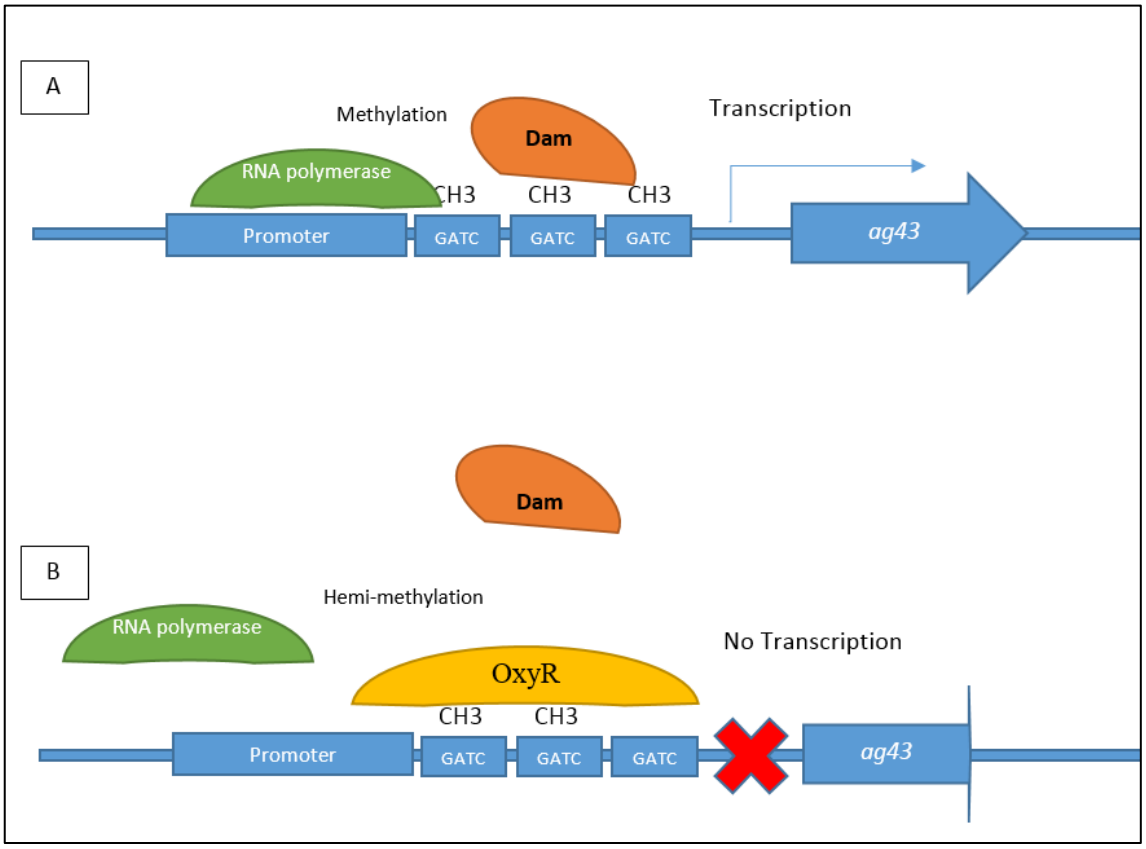


Figure 1-6 Phase variation of Ag43 protein mediated by epigenetic regulation. The promoter region of *ag43* gene that encodes for the outer membrane protein Ag43 in *E. coli* contains three sequence repeats (GATC), which are targets to be methylated by DAM methylate. When these sequences are fully methylated by Dam (figure A), RNA polymerase will bind to the promoter region allowing the transcription of the gene to be carried out. Whereas, when these sequences are not methylated, or are hemi-methylated (figure B), the DNA binding protein OxyR will bind to these sequences preventing Dam methylase from completing methylation of these sequences and consequently preventing the RNA polymerase from binding to the promoter, and the transcription process will be blocked.

1.8.4 Slipped strand Mispairing (SSM)

Slipped strand mispairing (SSM) is one of the most commonly occurring mechanisms that mediate phase variation in many surface proteins of bacteria. This mechanism involves reversible inherited mutation in the simple sequence repeats (SSR) such as a poly G tract due to a misalignment occurring between the template strand and the nascent strand during chromosomal replication (Figure 1-7) (van der Woude and Baumler 2004). This misalignment leads to a change in the number of the simple sequence repeats (insertion or deletion) which will affect protein expression either by leading to premature termination of the translation process if the simple sequence repeats is present in the coding region (frameshift mutation), or by blocking the transcriptional process if it lies within the regulatory region (promoter) (Bayliss 2009; Dale and Park 2010).

The SSR, which are also called repetitive DNA sequences, may consist of simple homopolymeric repeats such as poly G or poly C tracts or may be composed of multimeric repeat units that can be homogeneous or heterogeneous. Such multimeric repeat units also vary in the number of the nucleotides (i.e. it can be dimeric, trimeric or pentameric repeats etc.). Genome regions containing SSR tend to be a hot spot for mutation during DNA replication resulting in reversible changes in the expression of a particular gene (Henderson *et al.* 1999).

Many surface proteins of *N. meningitidis* are subject to phase variation due to the presence of SSR in their promoters or open reading frames. One of these proteins is the Opacity protein Opc, whose expression level is controlled at the transcription level due to the presence of polyC tracts in their promoter region, which vary in their number due to SSM (Sarkari *et al.* 1994). The change in the spacing between the -10 and -35, which for the binding of the RNA polymerase, has a strong effect on the transcription level of the particular gene even in one nucleotide (van der Woude and Baumler 2004). When the number of the repeat tract of *opc* is 12 to 13 the protein will be expressed at a high level, and when the number is changed to 11 or 14 , the expression will be at intermediate level. Whereas when the repeat tract number is reduced to equal or less than 10 repeats or increased to more than 15, the gene will be in the off state (Sarkari *et al.* 1994). The level of expression can also be affected when the repeat tract lies out of the promoter region, and the change of repeat number affects the binding of some regulatory proteins which then affect the promoter strength (van der Woude and Baumler 2004). An example of

such variability is in the *nadA* gene of *N. meningitidis*. The tetrameric repeat units (TAAA) of this gene lie upstream of the putative -35 region of the promoter and the variability in the number of these repeats lead to effects at the transcription level due to effects on the promoter strength (Martin *et al.* 2003b).

An example for phase variation that controls the gene expression at the translation level can be seen in *nalP* of *N. meningitidis.* This gene encodes for the autotransporter protease NalP that plays a role in enhancing the survival of *N. meningitidis* in human serum, as it cleaves C3 which is the central protein of the complement pathway (Del Tordello *et al.* 2014). The coding region of this gene contains a poly cytosine tract, and when the number of repeats are, 10 or 13 the gene will be in frame and the translation will be continue so that the protein will be expressed. While when the number of the poly C repeats is changed by addition or deletion of one nucleotide, the open reading frame will be altered and a premature stop codon will be brought into frame leading to generation of a truncated protein(Oldfield *et al.* 2013) (Alamro *et al.* 2014).

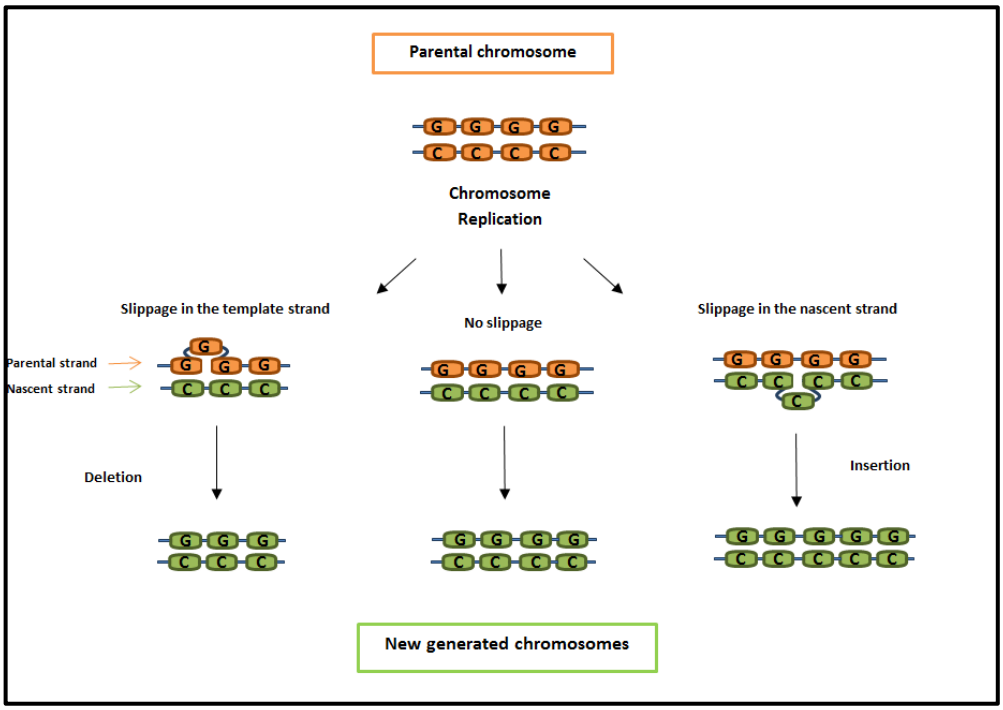


Figure 1-7: Insertion and deletion of nucleotide sequences in the Simple Sequence Repeat (SSR) region mediated by the Slipped-Strand Mispairing (SSM) mechanism. The change in the number of the repeats happens due to misalignment during chromosomal replication mediated by an SSM mechanism, and this leads to either insertion or deletion of one or multiple repeats in the newly generated chromosome. Three possibilities can happen during the DNA replication, 1- replication with no slippage, no insertion or deletion, 2- replication with slippage in the nascent strand, leading to an insertion and 3- replication with slippage in the template strand, leading to deletion of one or multiple repeats. Such changes in the number of the repeats leads to a change in the expression state of genes either by generation of a premature stop codon blocking translation or by affecting the transcription process if the repeats are present in the promoter region.

1.9 Opa proteins

Opacity-associated protein (Opa) is an outer membrane protein that mediates interactions of *N. meningitidis* with human cells and plays an important role in the colonization of human nasopharynx as well as in the pathogenicity of *N. meningitidis* (Callaghan *et al.* 2006). This protein binds to different types of receptors present on human epithelial and endothelial cells, mediating the intimate adhesion of the bacteria during the colonisation of the human nasopharynx that can be followed by the invasion of the blood stream (Malorny *et al.* 1998; Sadarangani *et al.* 2011). The expression of Opa proteins is encoded by 3-4 loci dispersed around the meningococcal chromosome termed as *opaA, opaB, opaD* and/or *opaJ* (Callaghan *et al.* 2006). All the four loci are phase variable in expression at the translational level due to the presence of pentanucleotide repeats (CTCTT) in the open reading frame within the sequences encoding for the signal peptide of each locus (Figure 1-8) (Stern *et al.* 1986).

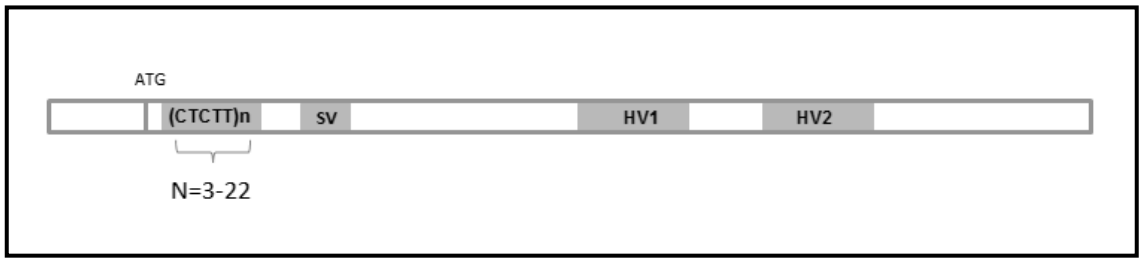


Figure 1-8: Map of an *opa* gene showing the location of the repeat tracts and the variable regions within the locus. *opa* genes are comprised of around 70% of conserved sequences (white color rectangles) that are interspersed with three variable regions apart from the repeat tract region (grey shaded rectangles). SV : semivariable region, HV1: hyper variable region 1 , HV2 : hypervariable region 2.

A change in the number of these repeats by the SSM mechanism leads to frame shift mutations and consequently switching ON or OFF in the expression of the proteins (Kawula et al., 1988). Therefore, different numbers and combinations of Opa variants can be expressed in meningococcal cells at any given time, generating a population with mixed phenotypes ranging in their Opa expressed repertoire from no Opa to one or multiple Opa variants (Hauck and Meyer 2003; Callaghan *et al.* 2011). As these Opa proteins vary in their functions and antigenicity, such heterogeneity in the meningococcal population provides flexibility to adapt, persist, and colonise different niches within the host along with immune avoidance (Callaghan *et al.* 2011). The frequency of the phase variation of *opa* was estimated to be between 10^{-3} - 10^{-4} per cell per generation, and it is affected by the length of the repeat tract as well as the promoter strength of the *opa* gene (Mayer 1982; Belland *et al.* 1997; Sadarangani *et al.* 2016).

The structure of Opa proteins is predicted to involve eight antiparellel β strands, representing eight domains spanning the outer membrane of the meningococcal cell to form four extracellular loops with a β- barrel structure (Malorny *et al.* 1998; de Jonge *et al.* 2002). The amino acid sequences of the second and the third loops are highly variable among Opa proteins and they are known as Hyper Variable Region 1 and 2 (HV1 &HV2), while the sequence in the first loop shows less variability among Opa proteins and is termed the semi-variable region (SV). So far, 345 different *opa* alleles have been characterised and contain 97 different HV1 and 127 different HV2 regions with 26 SV regions according to the database http://neisseria.org/nm/typing/opa/ (accessed at 16/4/2018). Several mechanisms participate in generating such high diversity in Opa proteins, which is then subject to selection and structuring by the high immune response imposed on the Opa repertoire by the human immune system, as a result the *opa* genes are constantly developing and new Opa alleles are still being generated (Hauck and Meyer 2003; Bilek *et al.* 2009).

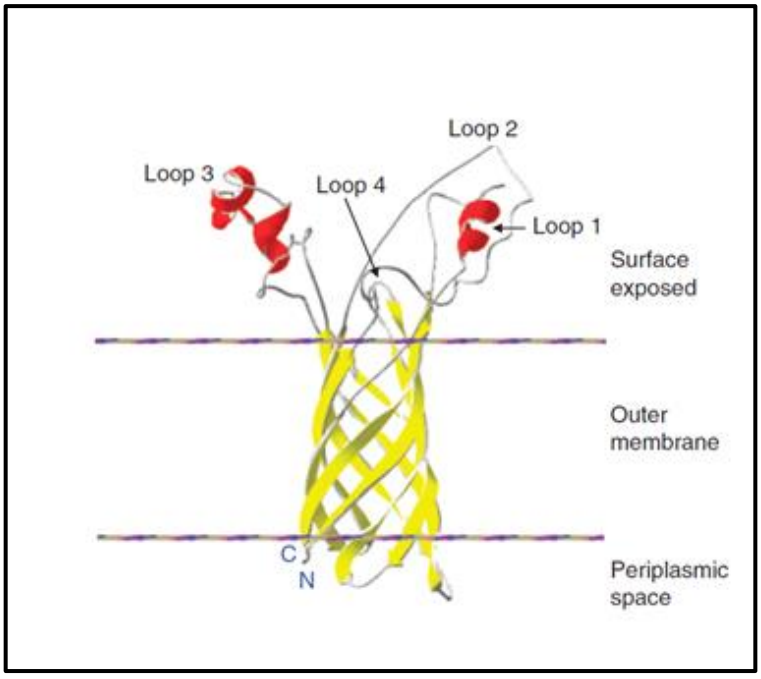


Figure 1-9: The three-dimensional predicted structure of the Opa protein. The β- Barrel structure consists of eight antiparallel strands spanning the outer membrane to form 4 extracellular loops, the three variable regions are localised on the first three loops (SV in loop1, HV1 in loop2 and HV2 in loop3) while the fourth loop as well as the intramembranous region of the protein are conserved among Opa variants (Sadarangani *et al.* 2011).

Intragenomic recombination is one of the main mechanisms that can diversify *opa* genes. These events occur between *opa* loci in the same genome and result in *opa* gene duplication or conversion and allelic mosaicism. Intergenic recombination can also happen by importing new *opa* sequences from the surrounding environments which leads

to either complete or partial replacement in *opa* loci (Bilek *et al.* 2009; Sadarangani *et al.* 2011). Point mutation in the HV1 or HV2 also has a role in generating the high diversity in *opa* alleles but it is less common in comparison to the other mechanisms (Hauck and Meyer 2003).

1.9.1 Interaction of Opa with human receptors.

1.9.1.1 *Opa-CEACAM interaction*

Generally, the majority of neisserial Opa proteins bind to human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (previously termed as CD66)(Dehio *et al.* 1998), which are a group of immunoglobulin-related glycoproteins that are expressed on a wide range of human cells and mediate different cellular processes such as cell – cell adhesion, cellular signalling, proliferation and cancer progression (Beauchemin and Arabzadeh 2013). Twelve different variants of CEACAM have been identified to be expressed on different human cells, and four of them including CEACAM1, CEACAM3, CEA (CEACAM5) and CEACAM6 act as target receptors for Neisseria Opa proteins (Popp *et al.* 1999; Kuespert *et al.* 2006). The extracellular part of these receptors is composed of a highly conserved N-terminal domain similar to the immunoglobulin variable domain (IgV-like domain) and up to six immunoglobulin constant-like domains (IgC- like domains) (Sadarangani *et al.* 2011). All neisserial Opa proteins that bind to human CEACAMs usually interact with the immunoglobulin like N-terminal domain of these receptors (Virji *et al.* 1996), and this interaction is mediated by the two hypervariable regions (HV1 and HV2) of each Opa variant (Malorny *et al.* 1998). Studies reveals that a particular conformational interaction between the 2nd and third loop of Opa (HV1 and HV2) is crucial for each Opa variant to bind to their receptors (De Jonge *et al.* 2003a). This also requires a specific combination of HV1 and HV2 to be present for each variant, as production of a chimeric Opa with HV1 and HV2 from different Opa variants (binding different CEACAMs) led to loss of the binding ability of the hybrid variant to any of the CEACAM receptors (Bos *et al.* 2002; De Jonge *et al.* 2003a). Presence of a conserved binding motif composed of Gly_{172}, Ile_{174} and Gly_{176} in the HV of the four Opa variants of H44/76 (that binds to different CEACAMs) has been confirmed, and this suggests that the binding sites for CEACAM are conserved among Opa variants inspite of their high sequence diversity, and similar motifs can be found in relatively all Opa variants that bind to CEACAMs (De Jonge *et al.* 2003a; Sadarangani *et al.* 2011). CEACAMs are expressed on a wide range of human cells including epithelial,

endothelial, neutrophils, T and B-lymphocytes (Kuespert *et al.* 2006). As different Opa variants can recognise and bind to one or multiple variants of CEACAMs, the distribution of these receptors within the different cells of the human body determines the cell tropism of the meningococcal cells that express particular variants of Opa (Bos *et al.* 1997).

The interaction of Opa with CEACAMs expressed on the epithelial cells mediates the intimate adhesion of the meningococci to the epithelial mucosa of the nasopharynx that follows the initial adhesion mediated by pili (Dehio *et al.* 1998; Hauck and Meyer 2003). Such interactions not only promote prolonged colonisation of the bacteria to the human mucosa, but also induces cell uptake and transcytosis of the meningococcal cells through the apical surface of epithelial cells and release from the basolateral surface of monolayers (Wang *et al.* 1998). In addition, due to the overexpression of the CEACAMs on the epithelial cells as consequences of inflamtory response, , Opa proteins can enhance binding of encapsulated meningococci to the CEACAM and thereby facilitates the engulfment and the translocation of the encapsulated neisserial cells into the blood stream (Virji *et al.* 1996; Sadarangani *et al.* 2011).

The immune suppression of lymphocytes is a further cellular effect that can be mediated by the interaction of Neisserial Opa with CEACAMs. It has been observed that binding of *Neisseria gonorrhoeae* expressing Opa variants to CEACAM1 expressed on the primary $CD4^+$ T-lymphocytes can supres their activation and proliferation (Boulton and Gray-Owen 2002). Similar observations were noticed when $CD4^+$ T-lymphocytes were exposed to OMV of meningococcal cells expressing Opa, and their activation and proliferation were inhibited in the presence of the activating stimuli (Lee *et al.* 2007). Whereas, the interaction of *N. gonorrhoeae* with B-lymphocytes mediated by Opa-CEACAM binding leads to inhibition of antibody production and also led to induced B-lymphocyte death (Pantelic *et al.* 2005). On the other hand, binding of Opa proteins to CEACAM3 expressed specifically on human neutrophils leads to bacterial engulfment via non-opsonic phagocytosis and triggering of a bactericidal oxidative burst (Gray-Owen 2003). This makes Opa proteins that bind to this variant of CEACAM (CEACAM3) disadvantageous to the expressing strain.

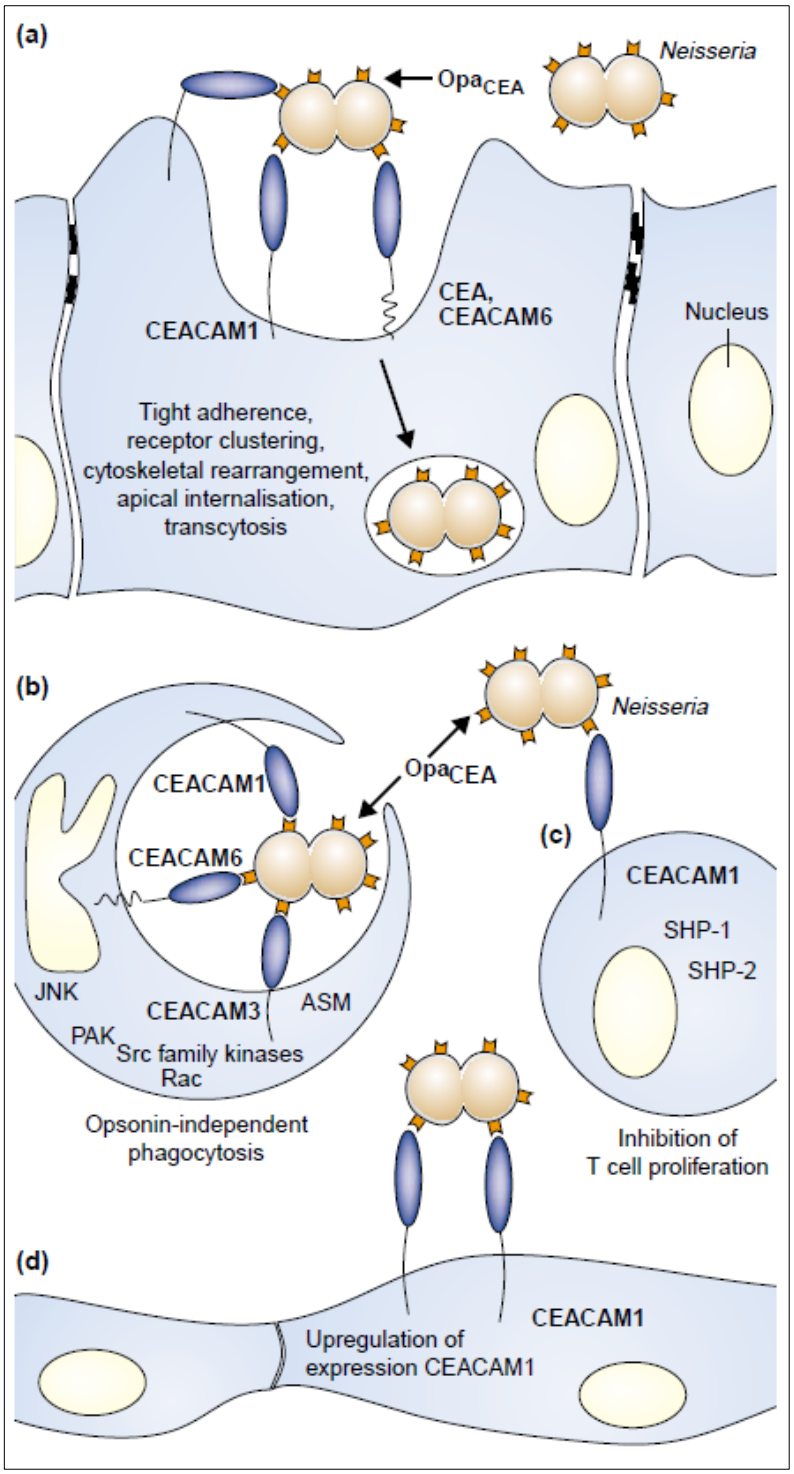


Figure 1-10 Opa-CEACAMs mediated interaction of Neisseria cells with different human cell types. A: Opa-CEACAM mediated adhesion of Neisserial cells to the epithelial cells followed by cytoskeleton changes and consequently the internalisation of the bacteria into the epithelial cells. B: adhesion of the bacteria to the polymorphonuclear granulocytes resulting in Opsonin-independent phagocytosis, C : Opa-CEACAM1 mediated binding of the bacteria to the T-cell resulting in inactivation of their proliferation and activation, D: stimulation of CEACAM1 expression on human endothelial cells due to bacterial induced factors resulting in enhancing the adhesion of Opa^{+} Neisserial cells (adapted from (Hauck and Meyer 2003)

1.9.1.2 *Interaction of Opa with HSPG and Extracellular matrix (ECM)*

A small number of Opa variants bind to heparin sulphate proteoglycan (HSPG) receptors (Chen *et al.* 1995) and other extracellular matrix (ECM) molecules including fibronectin and vitronectin (Gray-Owen 2003). HSPG is a membrane protein that is expressed on the majority of human cells and binds to various extracellular ligands mediating different cellular interactions such as cell-cell adhesion, signal transduction and cytoskeletal organisation. It is composed of a core protein which can be either GPI-anchor proteins (glypicans) or transmembrane proteins (syndecans), that are covalently linked with repeated units of heparansulphate glycosaminoglycan (HS-GAGs) (CAREY 1997; Dehio *et al.* 1998; Hauck and Meyer 2003). In contrast to Opa variants that bind to CEACAMs, binding of Opa to this receptor is mediated only by the HV1 of the first loop and no interaction between the two variable regions is needed to mediate such binding (Grant *et al.* 1999). Interaction of Opa with the HSPG and ECM receptors has been shown to enable the bacteria to penetrate and invade a number of cell lines *in vitro* (van Putten and Paul 1995; Gomez-Duarte *et al.* 1997). Such internalisation is mediated either by inducing cellular signaling mediated by binding of Opa proteins to HSPG, in which phosphatidylcholine-dependent phospholipase C (PC-PLC) and acidic sphingomyelinase (ASM) are activated and results in releasing of ceramide and diacylglycerol that leads to cytoskeletal reorganization and engulfment of the bacteria via a phagocytosis-like mechanism (Grassmé *et al.* 1997; Dehio *et al.* 1998). Another mechanism of cell uptake is mediated by binding of Opa proteins to the ECM including fibronectin and /or vitronectin inducing integrin- mediated engulfment of bacteria, this binding also requires the co-ligation of Opa with the HSPG and the integrin containing vitronectin or fibronectin (Hauck and Meyer 2003).

1.10 **Animal models to study meningococcal colonization and infection**

The specificity of *N. meningitidis* to colonize and infect humans (due in part to the specificity of the tissue tropism of their adhesins) has complicated utilizing or development of suitable animal models to study the key steps of their colonization and disease (Simonis and Schubert-Unkmeir 2016). The major information available regarding the pathogenicity and the interaction of this bacterium with human tissues were generated mainly with cell line and tissue cultures approaches as well as organ culture models (Trivedi *et al.* 2011).

The need for animal models that mimic the key steps of human nasal colonization and infection mediated by *N. meningitidis* is crucial to give a better understanding about the interactions of this pathogen with the human nasopharynx and the nature of immune responses correlated with this interaction. The development of humanized transgenic mice expressing human receptors has helped to fulfil such needs for meningococcal studies (Weyand 2017). CEACAM-humanized transgenic mice were developed by the Scott Gray-Own lab (University of Toronto-Canada). These mice express the human Opa-binding CEACAMs on their epithelial cells. The models allow the meningococcal Opa-CEACAM mediated adhesion and colonization of the epithelial mucosa, providing a promising approach to address both innate and adaptive immune response correlated with meningococcal colonization of humans (Johswich *et al.* 2015). Studies with this model showed that mice expressing CEACAM1 were successfully colonized by Opa expressing meningococcal strains, while animals expressing the other CEACAMs were not successfully colonized. This indicates that expression of CEACAM1 is essential to mediate the first step of colonization and infection, and highlights the central role of Opa-CEACAM1 interactions in the prolonged colonization (Johswich *et al.* 2013). This model was successfully used to show intranasal colonization for up to 10 days, and it was utilized to investigate the cross protection elicited by colonization with homologous and heterologous strains of *N. meningitidis*. In addition, it was a useful approach to highlight the role of the innate immune response in limiting the colonization, and how vaccination and the pre-exposure to meningococcal strains can confer sterilizing immunity against the incoming meningococcal strains (Johswich *et al.* 2015). However, this model is only suitable for investigations of the carriage state of *N. meningitidis* as the disease state is not observed after intra nasal inoculation.

Humanized transgenic mice expressing human CD46 have also been developed with the primary aim of investigation of the pathogenesis of the human measles virus (Mrkic *et al.* 1998). CD46 is a surface protein expressed on a wide range of human cells and acts as complement regulation factor and a target receptor for many pathogenic viruses and bacteria including meningococci and gonococci (Källström *et al.* 1997). Evidence from cell lines indicates that human CD46 is specifically recognized and bound by meningococcal pili, and expression of this receptor in transgenic mice enhances the interaction of piliated meningococci with the epithelial mucosa of the transgenic animals followed by dissemination of the bacteria and crossing the blood - brain barrier and

causing meningitis. The study recommended these transgenic mice as a suitable model for studying meningococcal pathogenicity as well as for vaccine development (Johansson *et al.* 2003). This model has also been utilized to investigate the route of meningococcal invasion and dissemination within the body during systemic infections. Siolinder and Jonsson (2010) found, using this model, that the bacteria can invade the meninges directly after crossing the nasopharynx without spreading in the blood stream using the olfactory nerve system as an alternative route.

One of the meningococcal iron acquisition receptors is specific for human transferrin, such specificity has affected utilization of animal models for studying the pathogenicity of this bacteria. Transgenic mice expressing human transferrin, were developed to investigate gene regulation in the brain and taste cells (Saleh *et al.* 2003; Lécureuil *et al.* 2004), but recently have been used to study meningococcal infections. The model provides transferrin as an iron source for meningococcal cells, which is essential for their virulence and growth during systemic infections (Zarantonelli *et al.* 2007). These transgenic mice were used to investigate meningococcal invasion after intranasal and intraperitoneal (IP) inoculation with meningococci, and the data showed that IP inoculation lead to crossing of the blood-brain barrier and causing meninges, while development of the disease occurred 7 days after the intranasal inoculation. These experiments were done without supplementing the animal with an exogenous iron source as required for systemic infections in Wild-type mice , again this study recommended this model as a useful tool to investigate the kinetics of meningococcal infection as well as for assessment of protection for new vaccine candidates(Yi *et al.* 2003; Zarantonelli *et al.* 2007).

1.11 **The aims and objectives of the project**

Studying the carriage state of *N. meningitidis* is essential to improve our understanding about the epidemiology and pathogenesis of these bacteria (Caugant and Maiden 2009), as well as the basic mechanisms that enable the bacteria to adapt to the human host and to evade the immune response.

As mentioned previously, phase variation and switching between different phenotypes are the main mechanisms that have been reported to enable meningococcal bacteria to persist in the human host (Tzeng and Stephens 2000). Such phase variability mostly happens in the surface antigens of the bacteria such as PorA, NadA, Opc and FetA.

Recently meningococcal carriage isolates have been reported to undergo significant reductions in expression of combinations of these surface proteins during persistent carriage due to phase variation (Alamro *et al.* 2014). The Alamro *et al.* (2014) study concluded that the reductions in the expression levels of these proteins was due to prolonged exposure to the host immune responses, which act as a selective pressure for the OFF state, and they suggested that phase variation in those proteins enabled the bacteria to avoid these immune defences and to persist in the human carriers.

To extend our understanding of the relevance of phase variation to the complete protein repertoire of *N. meningitidis* during prolonged colonisation, it was important to investigate phase variation in other surface proteins including the Opa proteins, which mediate a key role in the persistent colonisation as well as in the invasiveness of *N. meningtidis*. Although phase variation of these proteins had been investigated previously in terms of the frequency and mechanism, no data were available regarding the switching in the Opa repertoire in meningococcal carriage isolates during prolonged colonisation and how such switching may be correlated to immune avoidance and enhancing the fitness of the bacteria for both the asymptomatic colonisation and diseases cases. Therefore, phase variation in this protein was investigated in this project with the specific objectives including:

1. Characterisation of the numbers and alleles of the *opa* loci in meningococcal carriage isolates from persistent carriers.
2. Investigation of phase variation and changes in the repeat numbers of *opa* genes within these carriage isolates at different time points, and correlation of the variability with changes in the expression state of each *opa* gene, with the aim of evaluating the changes in the expression of the Opa repertoire of each strain during the longitudinal colonisation.
3. Study of the impact of phase variation of Opa proteins on escape of *N. meningitidis* from the bactericidal activity of anti-Opa antibodies *in vitro* using a modified *in vitro* serum bactericidal assay.

4. Cloning and expression of Opa proteins and generation of allele-specific mouse anti-Opa polyclonal antibodies as useful tools for further investigation of anti-Opa protein immune responses during persistent carriage.

2 Materials and methods

2.1 Bacterial strains and growth conditions

The meningococcal carriage isolates were isolated from student volunteers at the University of Nottingham during the period between 2008 and 2009 at different time points (Bidmos *et al.* 2011). The samples were taken by cotton swabs from the posterior pharynx of the volunteers. The swabs were then streaked directly on a selective chocolate GC agar plates (Oxoid) and incubated at 37ºC overnight in 5% CO_2. After incubation, 20 colonies were taken from each plate and subcultured again overnight on the same media and culture conditions and then subjected to DNA extraction. A glycerol stock was made for the isolates and stored at -80 ºC.

N. meningitidis disease isolates, MC58 and H44/76 were provided by Dr. Chris Bayliss (Laboratory 121, Department of Genetics, and University of Leicester, UK). The following growth conditions were applied to the all strains (37^0C, 5% CO2, overnight) on Brain Heart Infusion Agar (BHIA) plates (Oxoid) supplemented with Leventhals (20 ml of Levinthals were added to each 200 ml of BHIA). Carrying and handling the living meningococcal cells were always done by using class II microbiological safety cabinet. The living cells were also heat inactivated by killing them by incubation at 56 ^{O}C for 18 hours before using them in the assays that do not requires living cells such as whole cell lysates for western blot. Before use of whole cells in assays, meningococci were killed by incubation at 56 ^{O}C for 18 hours. Levinthals was prepared by adding 100 ml of defibrinated horse blood to 200 ml of Brain Heart Infusion broth (oxoid), and heated for 40 minutes at 95 ºC. The mixture was then left to cool below 50 ºC and centrifuged at 4000 rpm at 4 ºC for 25 minutes; the supernatant was then taken and stored at -20 ºC until used. *E. coli* strains BL21 (DE3) were obtained from laboratory stocks (Laboratory 121, Department of Genetics, University of Leicester). *E. coli* strains were grown in Luria broth (LB) (Oxoid) supplemented with ampicillin at 37 ^{0}C under aerobic conditions. *E. coli* cells were also cultured on Luria Agar (LA) (Oxoid). Selection for antibiotic resistance was achieved by supplementing media with ampicillin at final concentrations (100 µg/ml)

2.2 Genomic DNA extraction

For the carriage isolates, the genomic DNA was extracted by using a DNeasy purification kit (Qiagen) by following the manufacturer's instructions (Bidmos *et al.* 2011). For other experiments, a boiled lysate was prepared by harvesting the bacterial cells (suspended in 200 µl of PBS) by centrifugation at 13,000 rpm for 5 min, and then re-suspension of the pellet in 100 µl of sterilised distilled water followed by heating to 56 ºC for 30 min in a water bath (to kill the bacteria). After this, the suspension was boiled at 98 ºC for 5 minutes in the thermocycler and centrifuged at 13,000 rpm for 5 minutes in microcentrifuge before the supernatant was taken and stored at -20 ºC.

2.3 Polymerase chain reaction (PCR)

A polymerase chain reaction (PCR) was used to amplify the four *opa* loci of the meningococcal carriage isolates using different sets of primers as shown in Table 2-1. In each reaction, 1 µl of genomic DNA was used as a template and the total volume of the reaction mixture was set up to 10 µl including adding of 1 µl of each forward and reverse primers (2 µM stock), 1 µl of 10xPCR buffer, 0.4 µl of 25mM of $MgCl_2$, 0.25 µl (10mM) dNTPs, 0.1 µl (5U / µl) Taq DNA polymerase and 5.25 µl of sterilized dH_2O. The number of cycles varied depending on the target products, while reaction conditions involved a denaturation step at 95 °C for 30 seconds, elongation at 72°C with the elongation time depending on the target product size while the temperature of the annealing step was varied on the primer sets used in each reaction.

Table 2-1. List of primers

Primers used to amplify the repeat tract region of *opa* genes		
Primer name	Sequences	use
opa-for-1	CTGATATAGTCCGCTCCTGC	Forward primers (fluorescently labelled and unlabelled) anneal to the conserved region upstream the repeat tract region of each *opa* gene and used to amplify and sequence the repeat tract region.
opa-for-2	TTGAAACATCGCCCCAAACC	
locus1-174-rev.	CTATCCAGTTGCCAGCGTTA	Reverse primer anneals to the HV1 of *opaJ* of *N. meningitidis* CC174
locus2-174-rev.	CATGGGTGGAGGTAACAACCT	Reverse primer anneals to the HV2 of *opaD* of *N. meningitidis* CC174
locus3-174-rev.	GGTGCCGTTTTGGTGTTTA	Reverse primer anneals to the HV1 of *opaA* of *N. meningitidis* CC174
locus4-174-rev.	GTTGGTGCAAGGAAAGTAGTCG	Reverse primer anneals to the HV2 *opaB* in CC174, *opaB* of CC23 and *opaA* and *opaJ* of CC167
locus1-23-rev.	TATTGGTGGCGACGGTAACAAC	Reverse primer anneals to the HV2 of *opaJ* of *N. meningitidis* CC23
locus3-23-rev.	GCATTATCTCTATTTTCTGCATT	Reverse primer anneals to the HV2 of *opaB* of *N. meningitidis* CC23
locus1-167-rev.	CCTTGCGTTGGTTTAGAGGTAAC	Reverse primer anneals to the HV2 of *opaB* of *N. meningitidis* CC167
locus2-167-rev.	TTTCTGGTGCCTTGGTTTTC	Reverse primer anneals to the HV1 of *opaD* of *N. meningitidis* CC167
locus1-58-rev.	GACAAGCCGAGAGAAGAAAC	Reverse primer anneals to the HV1 of *opaA* of *N. meningitidis* MC58

locus2-58-rev.	ACCTCCCTGTGCAGCGCCTTGCG	Reverse primer anneals to the HV2 of *opaJ* of *N. meningitidis* MC58
locus3-58-rev.	CTTGCCAGATGCCACTGCTA	Reverse primer anneals to the HV2 of *opaD* of *N. meningitidis* MC58
locus4-58-rev.	TTTGCTACTCGGTCTCTCTGG	Reverse primer anneals to the HV2 of *opaB* of *N. meningitidis* MC58
locus2-60-rev.	TTGCCATTGCTTTTATGCAC	Reverse primer anneals to the HV1 of *opaJ* and *opaD* of *N. meningitidis* CC60
locus3-60-rev.	CCAGTTGTCCTGGGTTTCTT	Reverse primer anneals to the HV1 of *opaA* of *N. meningitidis* CC60
locus4-60-rev.	CTCATCGGTTTGTCTATGATC	Reverse primer anneals to the HV2 of *opaB* of *N. meningitidis* CC60
locus2-N88-rev.	TCTTGATGAGACAGGTCCTCC	Reverse primer anneals to the HV2 of *opaA* and *opaB* of *N. meningitidis* CC174 isolates of V88
locus3-N88.rev.	TTCTTGCCAGTTTTCAGTGC	Reverse primer anneals to the HV1 of *opaJ* of *N. meningitidis* CC174 isolates of V88
OpaJ-N459-Rev	CCACAGGAGTAACAGTAATAG	Reverse primer anneals to the HV2 of *opaJ* of *N. meningitidis* isolates V222 N391 and N459 (samples of 3rd and 4th time points)
N54-opaA-Rev	CCAGTTTTCAGTGCCAGTGC	Reverse primer anneals to the HV1 of *opaA* and *opaJ* of *N. meningitidis* isolates of V54
Primers used to amplify the whole *opa* genes.		
O87	GCA TGC CCA ATG AGA CTT CGT GGG	Revers primer anneal to the conserved region (end) downstream the HV2 (Callaghan *et al.* 2006)

NMB1466-Rev	TATCCCAATAATGCACGCCG	Revers primer anneals to the gene NMB1466 downstream *opaD*
opaD-rev-ppx	GTCTTTGGCGGTGATTTTGT	Revers primer anneals to the gene *ppx* (NMB1467) downstream *opaD*
NMB1464-For.	CATTGCGGTAAAAACGACGG	Forward primer anneals to the gene NMB1464 upstream *opaD*
opaA-Rev-0444	CAATAAACGAGCTGCTCCAGAT	Revers primer anneals to the gene NMB0444 downstream *opaA*
OpaA-Fw-0441	5'-CACAGCATGATTGTCGATCC	Forward primer anneals to the gene NMB0441 upstream *opaA* (modified from Sadarangani *et al.,* (2012)
opaB-fw-1624	CAACTATGCCGCCTTCCTCC	Forward primer anneals to the gene NMB1624 upstream *opaB* (CC174)
opaB-fw-1634	AACTTCTTCCGATCCCAACC	Forward primer anneals to the gene NMB1634 upstream *opaB* (modified from (Sadarangani *et al.* 2012))
opaB-rev-1637	AAGGCGAGGTAGGATTGC	Revers primer anneals to the gene NMB1637 downstream *opaB* (modified from (Sadarangani *et al.* 2012)
opaJ-rev -pip	ACCAATCCCTTTACCCGTTC	Revers primer anneals to the gene *pip* downstream *opaJ*
opaJ-fw-0925	TCCATCTGCGACATAATCCA	Forward primer anneals to the gene NMB0925 upstream *opaJ*
3- primers used for coning and expression of *opa* genes		
Opa-stop-T-expr	TATCCACCTTTACTGTCAGAAGCGGTAGCG CATGCCCAA	Reverse rimers used for amplify and cloning *opa* genes into the expression vector, bind to the end

Opa-stop-C -expr	TATCCACCTTTACTGTCAGAAGTGGTAGCGCATGCCCAA	of *opa* genes with extra nucleotide tags to enable insrtion of the gene to the vector via homologous recombination
Opa-signal-for -expr	TACTTCCAATCCATGGCAGCGCAGGCGGCAAGT	Forward primer used for amplify and cloning opa genes to the expression vector, binds to the region downstream the repeat tract region of opa genes to avoid amplification the sequences encoding for the signal peptide.

2.5 Gel electrophoresis

To detect the PCR products of each reaction, 1 % Agarose gels (prepared by dissolving 1 g of agarose in 100 ml of 1X TAE buffer) containing 5% ethidium bromide were loaded with 5 µl of PCR products (mixed with 1 µl of 6X loading dye) along with 5 µl of DNA hyper ladder I (size standard) and run at 100 v for 50 minutes. The gel was then visualized with UV light and images were taken for each PCR run.

2.6 A-tailing

This step was used before sending the PCR products for GeneScan as the Taq DNA polymerase adds non-templated adenine at the 3' end of the amplicon, which leads to extra peaks in the GeneScan. To ensure that all the PCR products have non-templated A on the 3' end, 4 µl of a mixture of 0.4 µl 10X PCR buffer, 0.4 µl of MgCl2, 0.01 µl of Taq DNA polymerase and 3.15 µl of dH_2O were added to 10 µl of the PCR products and incubated at 72°C for 45 min.

2.7 GeneScan

Capillary electrophoresis was used to determine the length of the PCR products of each *opa* loci. The assay involves amplifying the target gene using fluorescently labelled primers, then diluting the amplicon 1:5 and mixing 1 µl of the diluted amplicon with 0.25 µl of size standard (GS1200 LIZ) and 8.75 µl of formamide. Samples are transferred to the protein and nucleic acid analyses unit (PNACL) and run in DNA sequencer API 3730 (Applied Biosystem) .The received data (.fsa files) were analysed by using Peakscanner software v1.0.

2.8 Sanger sequencing

Sanger sequencing was used to determine the number of repeats in each *opa* gene to correlate them with the product size detected in the Genescan. The procedure involving mixing 0.5-2 µl Sanger sequencing was used to determine the number of the repeats in each *opa* loci to correlate them with the products size detected in the GeneScan. The procedure involved mixing 0.5-2 µl of PCR products with 4 µl of 1:8 diluted Big Dye (v3.1) and using 1 µl of sequencing primer (2µM stock) and adjusting the volume to 10 µl by adding sterilized dH_2O. The sequencing reaction was run for 29 cycles of 30 sec at 95°C (denaturation), 30 sec at 50°C (annealing) and 4 min at 60 °C (elongation). The sequencing mixtures were then cleaned up by adding 2 µl of 2% SDS and 8 µl of dH_2O followed by heating to 95°C for 5 minutes. To remove the incorporated ddNTPs, the mixtures were applied to gel filtration cartridge columns before being sent to PNACL for analysis in API 3730 sequencer (Applied Biosystem). The received data (.ab1 files) were analysed by using FinchTV software.

2.9 Protein analysis

2.9.1 Preparation of cell lysate

Meningococcal cells grown on BHI agar plates were suspended in PBS and then inactivated by incubating the suspension in a water bath at 56^0C overnight. The inactivated cells were washed three time in PBS and their concentrations were normalised to an optical density OD_{600} of ~0.4. After normalising the suspensions, four ml were taken and cells were pelleted by centrifugation at 15,000 rpm for 5 minute cell. The pellets were suspended in 100µl of 2x SDS loading buffer (4% SDS, 1M Tris pH 6.8, 0.1% Bromophenol blue, 20% glycerol, 200mM DTT), and heated at 98°C for 5 minutes in the PCR block to get the cell lysates.

2.9.2 SDS Polyacrylamide gel electrophoresis (SDS-PAGE) and staining

The stacking gel (6%) was prepared by mixing the following components (for 2 gels): 3.75 ml buffer B (250mM Tris.Cl pH6.8 and 0.2%SDS,), 1.5 ml UltraPure Protogel (30% (w/v) Acrylamide stock solution, Geneflow), 75 µl 10% APS, 3.75 µl TEMED (Sigma-Aldrich), and 2.175 ml distilled water. The separating gel (12 %) was composed of (content of 2 gels) 7.5 ml buffer A (0.75 M Tris.Cl pH 8.8, 0.2% SDS), 6 ml of UltraPure Protogel (Geneflow), 375 µl 10% APS, 7.5 TEMED (Sigma-Aldrich), and 1.05 dH2O. Bacterial whole cell lysates samples were loaded at equal volume into stacking gel along

with protein size standard (PageRuler , Thermo Scientific), and the electrophoresis was done in SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) at 100 V for 3 hrs. after that, gels were stained with Coomassie blue stain (0.25% w/v Brilliant Blue (Sigma-Aldrich), 10% glacial acetic acid and 45% methanol) for 45 minutes or overnight with shaking. Destaining was performed to remove the extra stain and identifying the proteins bands and their positions on the SDS-PAGE by using a de-staining solution (7.5% glacial acetic acid, 20% methanol).

2.9.3 Western blotting

After separating the proteins by SDS-PAGE, proteins were then transferred into polyvinylidene fluoride (PVDF) membranes. The membranes were activated by suspending in 100% methanol, followed by rinsing in water and suspension in transfer buffer for 10 minutes. The transfer was performed in cold transfer buffer (192 mM glycine, 25 mM Tris pH ~8.3, and 20% methanol) at a constant current of 100 V for 1 hour. The membranes were blocked overnight at 4°C with blocking buffer (0.5% Tween-20, 5% non-fat dry milk in PBS). The membrane was incubated with an appropriate concentration of primary antibodies in blocking buffer and then washed three times with PBS-T (0.05% Tween-20, PBS) for 5 minutes at room temperature with gentle shaking. Then membranes were incubated with suitable concentrations of peroxidise-conjugated secondary antibodies in the blocking buffer for 1 hour at room temperature. Membranes were washed three times (15 minutes each) with PBS-T to remove unbound secondary antibodies, and signals were developed using a Chemiluminescence detection kit (EZ-ECL) (Geneflow) and detected on X-ray film.

2.9.4 Immunodot blot

2.9.4.1 **Bacterial lysates**

Meningococcal isolates were grown on BHI agar plates overnight at 37ºC. Sweeps from bacterial growth were taken and bacterial suspensions were prepared in 1 ml of dH_20 contain protease inhibitor cocktail (Roche) (1 tablet/10 ml) to avoid the protein degradation. Whole cell lysates were prepared by three cycles of freezing (-80ºC) and thawing (37ºC). 20 µl of the cell laysates was further lysed in 980 µl (i.e. diluted 1:50) of alkali lysis buffer (1% SDS and 0.1 M NaOH) and the concentration of cells was estimated by measuring the OD at 260 nm on a NanoDrop spectrophotometer using a

glass cuvette with PBSB as a blank. Depending on the resultant readings, the following formula was applied to count the cell number:

OD260 of 1.8 = 1×10^9 cells, therefore:

Volume (ml) containing 1×10^9 cells = 1.8/OD260 (the measured reading) × dilution of sample (usually 50).

A suspension of 1×10^9 cells per ml was prepared in PBSB according to the OD measurements and the formula above, and then diluted serially to get the target cell numbers to be used and loaded in the assays.

2.9.4.2 **Immunodotblot**

The concentration of the bacteria was normalized at OD260 of 0.5 , and 50 µl of the suspension which contain around to (~$1x10^7$ bacterial cells were loaded on nitrocellulose membrane in duplicates, and for recombinant protein , samples were diluted in PBS for the suitable concentration and then loaded to the membrane. The membrane were then left for 10 minutes for air dry and then blocked with blocking buffer (Dulbecco's PBS , 0.05 Tween-20 and 3% BSA) for 1 hour at room temperature or overnight at 4ºC. The recombinant CEACAMs-Fc (0.1- 1 µg/ml) prepared in PBST with 1% BSA was added to the membrane and incubated for 1 hour at room temperature. The membranes were then washed by ELISA wash (0.9% NaCl, 0.05% Tween-20) (three times for 5 minutes each), and then the membrane incubated with anti-human Fc-alkaline phosphatase (AP)-conjugate for 1 hour at room temperature to detect the receptor binding. The membrane then washed by ELISA wash three times and signal was developed by adding 5-bromo-4-chloro-3-indolyl-phosphate-nitroblue tetrazolium solution (Perkin Elmer) to the membrane and incubated with shaking until getting the suitable color. The reaction was terminated by washing in dH_20 until desired color development had been obtained.

2.10 **Statistical analysis**

Two-tailed Fisher's exact test was used to analyse the statistically significant changes in the repeat tract length of *opa* loci between different time points by using Graphpad Prism 6 software. A Wilcoxon matched-pairs signed rank test was used to compare the means of the initial and final expression states of Opa proteins in the first and last time points of the longitudinal carriers using Graphpad Prism version 6.

2.11 Serum sensitivity and bactericidal assays

2.11.1 Preparation of a meningococcal inoculum

Meningococcal cells from an overnight culture on BHI agar plates were used ether directly for preparation of the inoculum or after inoculating blood agar plates and grown for a further four hours. Sweeps from bacterial cultures were re-suspended in PBSB (PBS + 0.5 mM MgCl2 and 0.9 mM CaCl2, pH 7.4), and the concentration of cells was then estimated by lysis of 20 µl of the cell suspension in 980 µl of alkali lysis buffer (1% SDS and 0.1 M NaOH) and then measuring the OD at 260 nm on a NanoDrop spectrophotometer in the same methods mentioned previously in section 1.9.4.1

2.11.2 Serum sensitivity and bactericidal assays

The assays were done in 96-well tissue culture plates and the following materials were added to each well: PBSB buffer to make the final volume of the assay 50 µl, 10 µl of bacterial suspension of $1x10^6$ cells (equal to $1x10^4$ cells), human pooled sera or baby rabbit sera (complement sources) to a final concentration of 5%, 10% and 20% (when testing the sensitivity to the complement source). While testing the sensitivity of the bacteria to the antibodies, the assay was done with fixing the final concentration of the complement source in each well at 5%, and adding the antibodies (pre-heated for 56 ^{0}C in water bath to inactivate the mouse complement) to the wells with a two-fold dilution ranging from 1:20 to 1:320. Three different controls were set up with each assay including: the meningococcal cells with human sera without antibodies, meningococcal cells with heat inactivated human sera (pre-heated for 56 ^{0}C in water bath) without antibodies and meningococcal cells with heat inactivated sera with antibodies. Each assay was performed in duplicate, and the plates were incubated at 37°C in 5% CO_2 for 1hr. Before the incubation, 10 µl from the control well was taken and plated in duplicate on BHI agar plates supplemented with Levinthals to get the CFU count at time zero (T0), and in the same manner samples from all other wells were plated after incubation to provide T60 counts, and get the CFUs. The mean of 4 counts (two counts for each duplicate well) was taken as the total CFU.

2.12 Escape assay:

The bacterial escape assay, as developed by Bayliss and co-workers (Bayliss et al., 2008), was applied in this study. Mouse polyclonal anti-Opa antibodies recognised OpaA and OpaD of strains MC58 and H44/76 were utilised in this study. The bacterial inoculum

was prepared either from a growth started from a glycerol stock or from a single colony identified to be a positive for the target antigen after testing by colony immunoblot. In both cases, the bacteria were grown overnight on BHI agar plates supplemented with Leventhals. A sweep of the bacterial growth was taken and re-suspended in 1 ml PBSB (PBS supplemented with MgCl2 (0.5 mM) and CaCl2 (0.9 mM) at pH 7.4). The cell suspension was diluted (1:50) in lysis buffer (1% SD, and 0.1 M NaOH,) and the concentration of cells was determined as described previously by taking the OD_{260} nm using the NanoDrop spectrophotometer (Thermoscientific) with using PBSB as a blank. A suspension of 1×10^9 cells per 1 ml (depending on the OD measures) was prepared and diluted serially in PBSB containing 0.1% glucose. Different inoculum sizes were prepared by transferring 10 µl from the different serial dilutions in to wells of a 24 well tissue culture plate and mixed with 30 µl of PBSB containing 0.1% glucose. To get the CFU counts of the input inoculum, appropriate volumes from the seral dilutions of the prepared suspensions were spreaded on BHI agar plates and incubated overnight at 37 ^{0}C with 5 % CO2.

The inoculum in each well was mixed with 50 µl of 10% human pooled serum prepared in PBSB-0.1% glucose. Suitable concentrations of antibody were added to all wells except the control wells, which were kept without antibodies, and 10 µl of only PBSB were added to those wells. The total volume of each culture was 100 µl, and the plate was incubated at 37°C in the presence of 5% CO2 for 60 minutes. After the incubation of the first passage, 50 µl of the culture was taken from each well, transferred to a new plate and mixed with 50 µl of fresh 10% human pooled serum (5% final concentration) and antibody, and incubated again in the same condition for the second passage. The cells that remained from the first passage were diluted serially and an appropriate volumes was plated overnight on BHI agar plates to get the CFU count. The 3rd passage was performed in the same manner as the second passage.

2.13 **Colony immunoblotting**

The meningococcal colonies from overnight cultures on agar plates were transferred into nitrocellulose membrane filters (Whatman) and left to dry at room temperature for 10 minutes. Membranes were blocked with PBS-T (PBS 0.05% Tween-20) containing 5% non-fat dry milk and 0.1% sodium azide for 1 hour at room temperature with gentle shaking. The membranes were washed three times with PBS-T and incubated with an appropriate concentration of primary antibodies prepared in blocking buffer (5 ml)

without sodium azide. The incubation was performed for 2-3 hrs at room temperature or overnight at 4 ^{0}C. Membranes were then washed three times with PBS-T and incubated for 1-2 hrs with suitable dilutions of alkaline phosphatase conjugated secondary antibodies prepared in blocking buffer followed by three washes with PBS-T to remove the unbound secondary antibodies. Signal was developed by adding a 5-bromo-4-chloro-3-indolyl-phosphate-nitroblue tetrazolium solution (Perkin Elmer) to membranes and incubated with shaking until getting the suitable colour. The reaction was stopped by washing the membrane with dH2O. The frequencies of phase variants were calculated by counting the number of –ve variants (differentiated depending on their colour) and dividing by the total number of colonies on the filter.

2.14 Generation of anti-Opa antisera

2.14.1 Cloning Expression, Purification, and Refolding of Opa.

The protocol for expression and purification depended mainly on those described by Fox *et al.* (2014) and de Jonge *et al.* (2003) with slight modifications. Some steps, such as washing of the inclusion bodies, were derived from protocol characterised in (Alamro *et al*., 2014), which was used for purification of recombinant FetA proteins.

2.14.2 Preparation of chemically competent cells

The chemically competent cells of *E. coli* BL21 were prepared by inoculating 100 ml of LB, supplemented with 10 mM MgSO4, with 1 ml of an overnight culture of the bacteria and incubated at 37 ^{0}C with shaking until the OD_{600} reach around 0.4. The culture was then divided into 2 cultures (in two tubes) and incubated on ice for 5 minutes. The cells were harvested by centrifugation at 4 ^{0}C and then washed with 20 ml of filter-sterilised TFB1 (30 mM KOAC, 50 mM $MnCl_2$.4H2O, 100 mM KCl, 10 mM CaCl2.6H2O, 15% v/v glycerol). The cells then were pelleted and suspended in 4 ml of filter-sterilised TFB2 (10mM Na-MOPS, (pH 7.0), 10mM KCl, 75 mM $CaCl_2.6H_2O$, 15% v/v glycerol) and then stored at -80 ^{0}C in aliquots.

2.14.3 PCR clean up

The PCR products of *opa* genes used for cloning were purified using E.Z.N.A. Cycle Pure Kit (Omega) following the manufacturer's instructions to remove any contaminants that might interfere in the cloning of the PCR amplicon into the expression vector.

2.14.4 Plasmid extraction

Plasmids were transformed into competent *E. coli* strains and extracted from cells using EZNA plasmid Mini Kit (Omega Bio-Tek) following the manufactor's instruction.

2.14.5 Heat-shock transformation

Purified plasmid DNA (5 µl) was mixed with 50 µl of *E coli* BL21 competent cells and the mixture was incubated on ice for 25 minutes, and then transferred immediately to a water bath and incubated at 37 °C for 5 minutes followed by immediate incubation on ice also for 5 minutes. After that, 900 µl of LB were added to the cells-DNA mixture, incubated at 37°C for 1 hr in a water bath, and then the bacterial suspensions were streaked on LA plates supplemented with ampicillin and incubated overnight at 37°C.

2.14.6 Expression of Opa

Amplification of four *opa* alleles, without the sequence region of the repeat tract which encodes for the signal peptides, was done from genomic DNA of four carriage strains:- N222.1, N459.1, N370.4 and N309.1. Each allele was amplified firstly from one of the flanking genes and then the amplicons were diluted and used as a template for a second PCR using the specific primers listed in table 2.2. The purified PCR products were sent to the Protein Expression Laboratory (PROTEX) to be cloned into the pLEICS-01 expression vector. Cloning was confirmed by sequencing the inserted *opa* allele sequences with part of the plasmid sequences. For protein expression, clones were transformed into *E. coli* expression strain BL21 by the standard heat shock technique. For the pilot protein expression and purification, 250 ml of LB media supplemented with ampicillin (100µg/ml) were inoculated with 10 ml of overnight culture of the transformant BL21 in LB, and the cultures incubated at 37 °C in a shaker incubator until the OD_{600} reached between 0.5 and 0.7. Then the cultures were divided into two tubes (two cultures prepared) and expression was induced by adding isopropyl-β-thio-D-galactoside (IPTG) to a final concentration of 1 mM to one of the cultures, and cultures then were incubated for a further 3 hrs. A 500 µl sample of the both cultures was taken after each one hour, the cells were harvested and lysed in 100 µl of 2x SDS loading buffer. The expression of Opa proteins was confirmed by analysing samples with SDS-PAGE and Western blotting using anti-Opa specific antibodies. For the large scale production, the volume of the culture was raised to 800 ml and the culture induced with IPTG to a final concentration of 1 mM, for 3 hrs. and then the cells were harvested and the purification of the recombinant protein applied according to the protocol below.

2.14.7 Purification of recombinant Opa

The purification of recombinant Opa proteins was done according to a protocol modified from (Fox *et al.* 2014). Bacterial cells were harvested from expression cultures by centrifugation at 2500 g for 20 minutes. The pellets were re-suspended in an appropriate volume (5 ml /1 g of wet pellet weight) of lysis buffer (50 mM Tris−HCl and 150 mM NaCl, 1 tablet of protease inhibitor (Roche) and a final working concentration of 350 µg/ml of Lysozyme), the suspension was mixed by vortexing and incubated at 37 °C for 30 minutes. Cells were then sonicated for 30 minutes (30 seconds on, 30 seconds off). Cell debris from the lysate was removed via centrifugation at 12000g for 30 min. The pellets containing inclusion bodies were washed 3 times with washing buffer (50 mM Tris.Cl pH 8, 100mM NaCl. 2M Urea, 0.5% Triton X-100). The insoluble fraction (containing inclusion bodies) were solubilised in solubilising buffer (50 mM Tris−HCl pH 8, 150 mM NaCl and 8 M Urea) overnight at room temperature with stirring. After incubation, the suspensions were centrifuged at 18,000 g for 30 minutes to remove any insoluble fractions, and the supernatant was taken. The soluble fraction was loaded into a Ni^{+2} immobilized metal affinity chromatography column His –Trap HP (GE healthcare) equilibrated with washing buffer (pH 7.8, 150 mM NaCl, 20 mM imidazole, 8 M urea) and washed with 10 CV of the washing buffer followed by elution with (20 mM sodium phosphate, pH 7.0, 150 mM NaCl, 250 mM imidazole, 8 M urea) in 7 fractions with 1ml of elution buffer for each fraction. From each step of purification, an appropriate volume of protein samples was collected and mixed with equal volume of 2x SDS loading buffer and run on 12% SDS-PAGE gels to check for the presence and the purity of the recombinant Opa. The proteins eluted under denaturing conditions and the refolding step was done separately by rapid dilution of the protein samples 1:20 in refolding buffer (20 mM Tris−HCl, pH 8.0, 500 mM NaCl, and 1% Zwittergent 3-14). The fractions of purified proteins (containing pure protein without aggregates) were pooled together and concentrated with a 10 kDa MWCO concentrator (Amicon) to reduce the volume to 2.5 ml, and then rapidly diluted 20-fold in the refolding buffer. After 5 days of incubation at room temperature, protein folding was assessed by a SDS−PAGE gel shift analysis. The samples were concentrated and dialyzed against 3 changes (6 h each) of 1 L of PBS, pH 7.5, using Slide-A-Lyzer G2 Dialysis Cassettes with a 10 KDa molecular weight cut off. The dialysed protein was concentrated and stored at -80 °C to be used in further applications. The concentration of the protein was estimated using a Bradford assay and a Bradford kit (Biorad Laboratories Inc.), following the manufacturer's instructions. The

identity of the proteins were confirmed by using mass spectrometry at the PNACL facility in the University of Leicester.

The refolding step was performed using another protocol (de Jonge *et al.* 2003b), and this involved diluting the concentrated protein samples (1 ml) in 100 ml of refolding buffer (PBS containing ethanol amine, pH12, and SP12) with stirring at 4 ^{0}C overnight. After the incubation, the pH was normalised to 7.5 by adding HCl, and 10 mM of Tris were added to buffer the solution. The proteins were concentrated and stored at 4 ^{0}C until use for further applications.

2.14.8 Mass spectra analysis of recombinant proteins

The recombinant protein samples were run on a 12% SDS-PAGE gel and the gel was stained with Coomassie blue stain and then de-stained using the de-staining solutions described before in section 2.9.2. After exhaustive de-staining, the gel was taken to the PNACL unit (proteins and nucleic acid analysis laboratory, Department of Biochemistry, University of Leicester), and the target bands were cut out and collected using micropipette tips. The spectra were analysed by PNACL and the identity of the proteins were determined as Opa depending on whether the score of all the bands in the spectra was significantly similar to the predicted spectra for Opa proteins.

2.14.9 Determination of protein concentration

The concentration of protein was measured by using the Bradford Assay Kit (Sigma Aldrich). Different concentrations of protein standard (bovine serum albumin BSA) were prepared in the same buffer used to store the proteins. The range of the different concentrations was between 0.1–1.4 mg/ml. 5 µl of the protein standards and recombinant protein samples were pipetted in to separated wells in a 96-well plate in duplicate, and mixed with 250 µl of the Bradford reagent with shaking for ~30 seconds and then incubated at room temperature for 5–45 minutes. Then the absorbance at 595 nm was measured using a plate reader (Biorad) and the OD_{595} of each standard concentration was plotted against the concentration and a standard curve was drown. The concentration of the unknown sample was estimated according to the standard curve.

2.14.10 Development of polyclonal anti-Opa antibodies from purified Opa antigens

Development of anti-Opa antisera was performed at the National Institute for Biological Standards and Control (NIBSC) by Dr Hannah Chan. The Opa protein was firstly

concentrated using centrifugal concentrators (Amicon) to the following final concentrations: OpaA 0.5 mg/ml, OpaB 0.9 mg/ml, OpaJ-N222 0.2 mg/ml and OpaJ-N459 0.1 mg/ml , all in Tris buffer (20 mM Tris+ 0.05 % Zwittergent 3-14 , pH 7.5) except OpaB which was in PBS buffer, pH 7.5. The polyclonal antisera was raised against concentrated antigen and five groups of mice (r=each group composed of 10 mice) were immunized with three doses of 200 ul of protein and Freunds adjuvant (5 µg of rOpa/ dose, with ratio of 4:6 of protein: Freunds). Group 1 was a control group which were immunised with the adjuvant only (Freunds), while the other groups were immunised with rOpas accompanied with adjuvant.

2.14.11 E L ISA with polyclonal antibodies

An ELISA protocol was used to detect the binding of polyclonal antisera with purified Opa protein. 10 ug/ml of purified rOpa were prepared in coating buffer (15mM Na2CO3, 35 mM $NaHCO_3$, pH 9.6) and 100 µl of the protein solution (1 µg final concentration) was coated in duplicates on flat-bottom 96-well plates and incubated overnight at 4°C. After the incubation, the supernatants were discarded and the protein antigens bound to the walls of wells were blocked with 150 µl of blocking buffer (PBS, 0.05% Tween-20, 1% BSA) for 1 hr at room temperature. After that, 100 µl of the appropriate dilutions of the primary antibodies (prepared in the blocking buffer) were added to the wells, except the control wells to which only blocking buffer were added (antigen only without antibodies) and incubated for 1 hr at room temperature. Three washes were performed with washing buffer (PBS, 0.05 Tween-20), then the anti-mouse alkaline phosphatase conjugated secondary antibodies prepared in blocking buffer (diluted 1:2000) were added and incubated for 1 hr at room temperature. No secondary antibodies controls were involved in the assay in which only blocking buffer were added without secondary antibodies. The wells were washed three times with 250 µl washing buffer to remove the unbound secondary antibodies and then the signal was developed by adding 100 µl of alkaline phosphatase substrate solution (PNP tablets, Sigma). The plate was incubated at room temperature for 30 min and the colour development was measured at OD_{405} nm on a plate reader.

3 Characterization of *opa* genes in meningococcus clonal complexes

3.1 Introduction

The expression of Opa proteins in *N. meningitidis* is controlled by phase variation at the translation level due to the variability generated by chromosomal replication in the number of the pentameric repeat tracts (CTCTT) present in the leader peptide-coding region of *opa* genes. The switching between different expression states in the four *opa* loci leads to generation of a highly diverse meningococcal population which might be utilized by this bacteria as an effective mechanism to adapt to and overcome the adverse conditions during colonisation of human host.

One of the problems that has been faced when meningococcal genomes were screened for Opa genes using Bigsdb in the Neisseria PubMLST database is the missing of one or more copies of the expected four *opa* genes. It is not clear whether absence of these genes is due to poor assembly or due to the deletion of Opa genes in the genome. This study aimed to detect the number of *opa* genes in a set of persistent carriage isolates and to develop a PCR approach for amplification of *opa* genes from a large number of other isolates.

3.2 Genome context of *opa* genes

Opa proteins in *N. meningitidis* are encoded in 4 loci dispersed around the meningococcal chromosome. The position and genome context of the four *opa* genes have been investigated in three references strains of *N. meningitdis*, MC58 (figure 3-1), FAM18 and Z2491. The four genes located in the same place within the genome in the different strains of *N. meningitdis* but they exhibit variations in the flanking genes.

Whole genome sequences are available for a number of carriage isolates that were investigated in this project. These sequences represent different clonal complexes including CC174, CC23, CC167 and CC60. For the first three clonal complexes as well as samples of V88 and V54 (CC174), the screening approach was done by performing a BLAST search with conserved sequences of the strain MC58 *opa* genes using both BIGSdb and the Artemis program. The blast search revealed that some of the isolates have four copies but others only had two or three *opa* loci and it was not clear whether the other loci were present in these strains or not (table 3-1). In addition, due to the

sequences being divided into short read contigs, sequences of a number of *opa* loci were present without the flanking region, which made it difficult to differentiate them as the typing scheme depends mainly on their location within the genome. In order to confirm the presence or the absence of the four loci and to allocate the others, *opa* genes had to be amplified from either the two flanking genes spanning the whole *opa* locus or from one of the conserved ends of *opa* towards one of the flanking genes. The first step for both strategies was to confirm and identify conserved flanking genes as the different clonal complexes may contain different *opa* genome contexts.

The genome context was first investigated in the genome sequences of carriage isolates representative of the four different clonal complexes. Examination of Artemis files and performance of BLAST searches using MC58 gene sequences identified homologs of *opa* flanking genes. From this information, a set of these flanking genes were selected to amplify of each *opa* locus as shown in table 3-2. Although these genes are conserved in each meningococcal genome analyzed, they showed variability in term of their location and distances regarding *opa* genes (figure 3-2), in which they might be separated from *opa* by one or multiple genes and they may present in other countigs without the presences of the *opa* gens. All of these make it difficult to predict the suitable conditions for amplifying *opa* genes in these strains.

Table 3-1: *opa* genes and their location within the genome of the meningococcal carriage isolates. The table summarizes the results of the BLAST search with conserved sequences *opa* genes using both BIGSdb and the Artemis program that has been done for the five clonal complexes. Apart from cc174, the missing of one copy of opa genes was the dominant in the other clonal complexes, and the majority of the *opa* genes were present in partial sequences within individual short contigs without the flanking genes, which make it difficult to differentiate them according to locus number.

CC	Contig number	*opa* gene number	Downstream flanking gene (in the same contig)	Upstream flanking gene (in the same contig)	*opa* locus name
CC174					
1	000026	NMB0663_1	------	NMB0925	*Opa J*
2	000067	NMB0663_2			*opaD*
3	000119	NMB0663_4		NMB0441	*opaA*
4	000142	NMB0663_5	NMB1624	NEIS1552_NMB1637	*opaB*
CC167					
	000208	NMB0663_2-		NIS1552_NMB1637	*opaB*
	000103	Partial (no gene number)			
	000066	Partial (no gene number)			
	000033	Partial (no gene number)			
	000047	Partial (no gene number)			
	000125	Partial (no gene number)			
CC23					
	000046	NMB0663_1	NIS1552_NMB1637	----------	*opaB*
	198	NMB0663_ 3	----------	NMB0925	*opaJ*
	242	NMB0663_4	----------		
	116	Partial (no gene number)	NMB0444		*opaA*
	150	Partial (no gene number)			
Cc60					
	227	NMB0663_3	NIS1552_NMB1637		*opaB*
	224	NMB0663_2	NEIS1720_NMB0441	NMB0444	*opaA*
	000052	Partial (no gene number)			
	0000190	Partial (no gene number)		NMB 1466	*opaD*
	122 (short)	Partial (no gene number)			
	113	Partial (no gene number)			
CC1157					
	000139	NMB0663_1		NEIS1720_NMB0441	*opaA*
	000172	NMB0663_2	NMB1466 (flanking contig)		
	144 (truncated)	Partial (no gene number)			

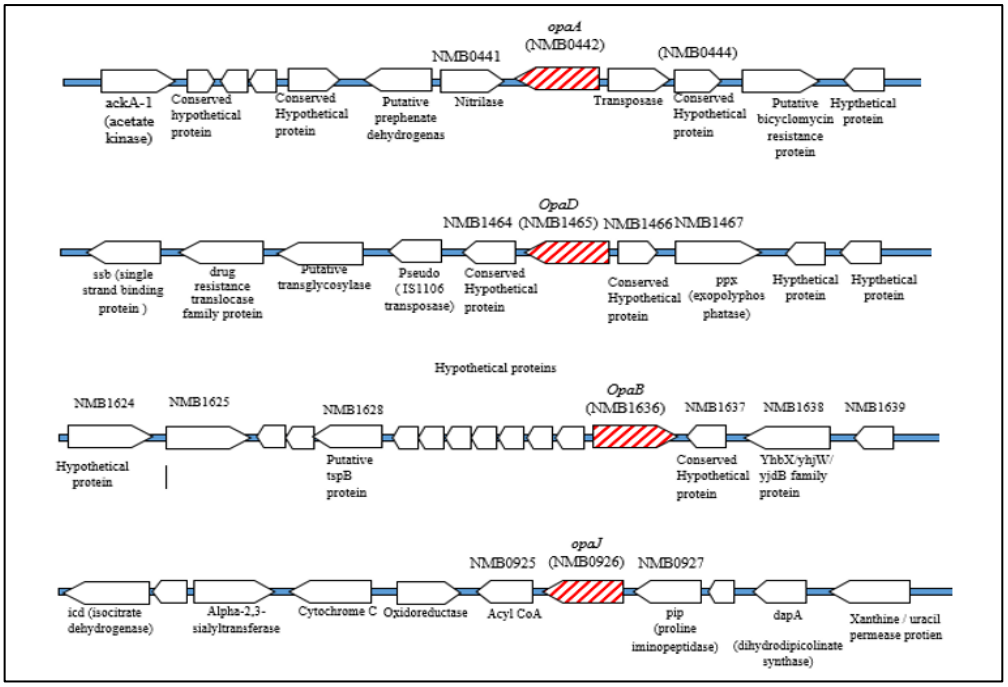


Figure 3-1: The genome contexts of the *opa* genes of meningococcal strain MC58. The genes were shown to be separated within the genome and to exhibit variability in their flanking genes. Each locus tends to have its own conserved flanking genes that can be found in the majority of the other meningococcal strains.

Table 3-2: Conserved upstream and downstream genes for design of primers to amplify *opa* genes from different meningococcal clonal complexes. The table shows the similarity of these flanking genes with the reference genes of *N. meningitidis* strain MC58.

***opa* loci**	**MC58 *opa* flanking genes**	**Percentages of similarity of the flanking genes of the strains with MC58 genes.**						
		CC174	CC60	CC23	CC167	FAM18	Z2491	V185
NMB0442 (*opaA*)	NMB0444	99	99	99	99	99	99	99
	NMB0441	96	97	97	97	96	96	100
NMB1636 (*opaB*)	NMB1634	79	79	79	79	99	80	98
	NMB1637	98	98	98	99	99	99	98
	NMB1624	99	89	99	99	99	97	95
NMB 1465 (*opaD*)	NMB1466	98	96	96	96	97	97	96
	NMB1464	99	99	99	98	99	99	100
NMB 0926 (*opaJ*)	NMB0925	97	98	98	98	99	98	98
	NMB0927	97	98	98	97	99	97	98

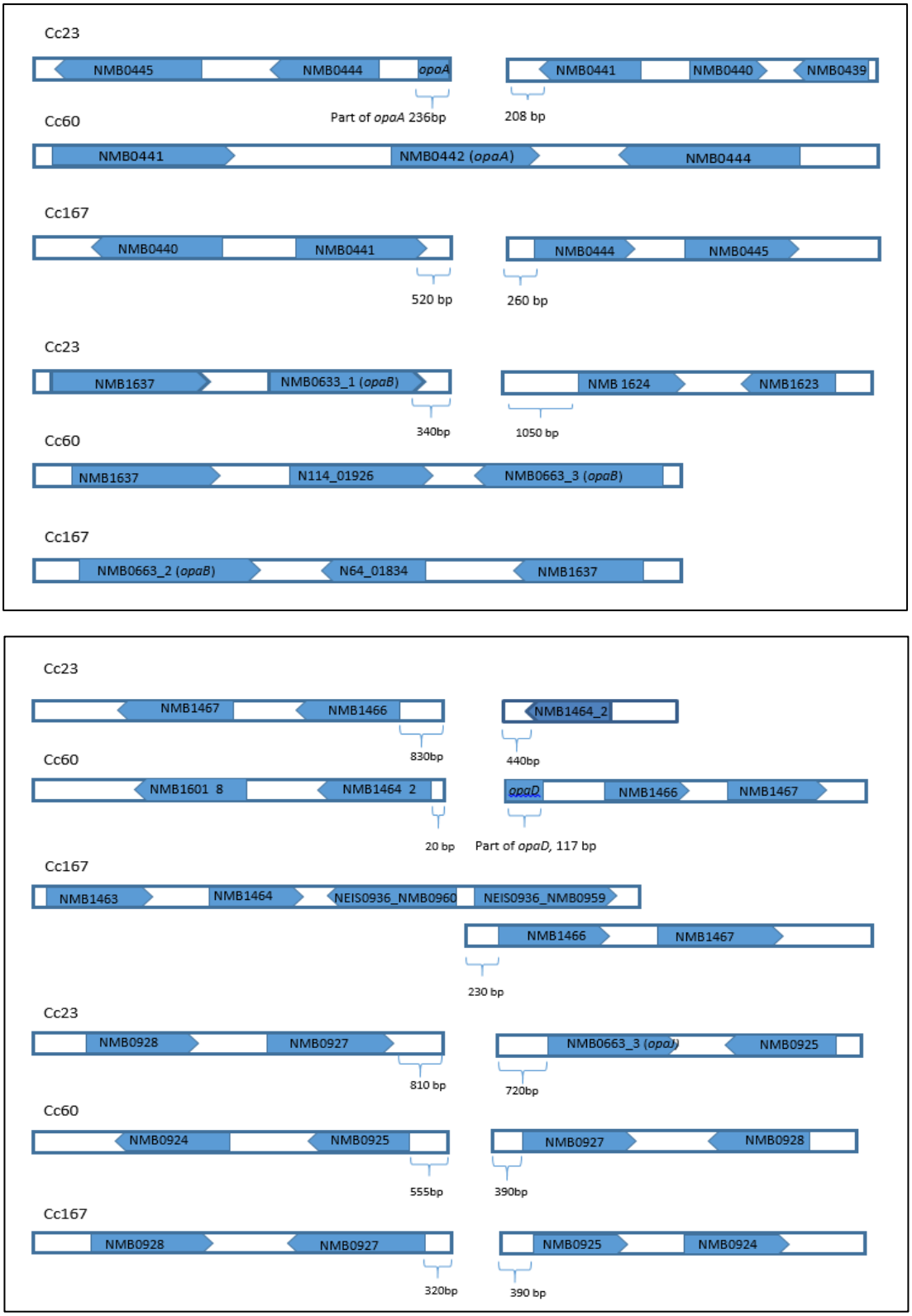


Figure 3-2 : **Genome context of *opa* genes within the genome of the meningococcal carriage isolates.** The figure presents the location of the conserved flanking genes and how they are dispersed within different contigs and the presence or the absence of the *opa* genes alongside those genes within the same contigs. Some of the *opa* genes are present in partial and small individual contigs without any flanking genes (table 3-1) and they were not described in the figure.

3.3 Designing PCR assays to detect all the potential *opa* genes in a range of meningococcal isolates

A PCR assay was required to detect the missing copies of *opa* genes in the different carriage isolates. The approach was to design primers that bind to the conserved flanking genes and to the variable regions of *opa* as well as to their conserved ends. Amplifying *opa* genes from one flanking gene to another or from the conserved N-terminal end toward the downstream flanking gene was not successful in most cases. Conversely amplifying the loci from their C-terminal conserved end or from one of the hypervariable regions toward the upstream flanking gene was successful in most of the analyzed genes (Figure 3.2). This may reflect higher conservation of the region upstream of *opa* genes whereas the downstream region contains a number of inserted genes, as determined when comparing *opa* flanking regions in a number of meningococcal strains in the database.

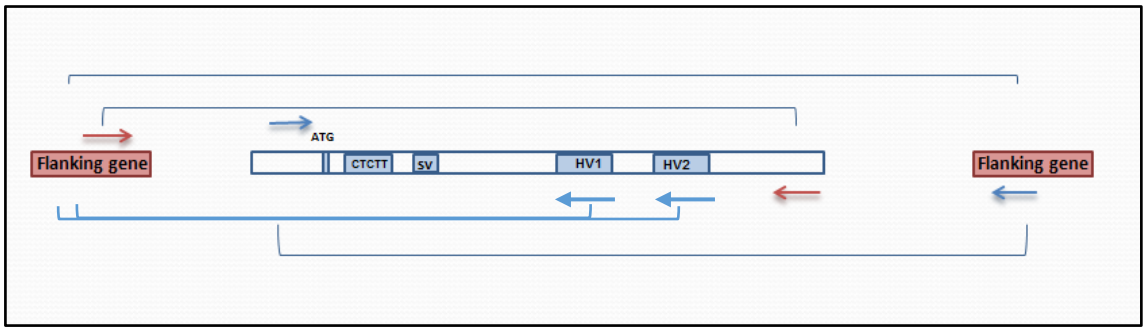


Figure 3-3 : **The sites of primers that were designed to detect and amplify *opa* genes in the carriage isolates.** Different sets of primers were designed in order to detect and allocate the different *opa* genes in the carriage isolates, these sets either general primers can be used to the different *opa* genes (bind to the conserved ends of *opa*, and to the flanking genes) or locus, allele specific primers which bind to the one pf the variable regions for each *opa* allele.

Overall, four loci were confirmed to be present in the genome of each clonal complex that was investigated in this study. Sequence analyses of putatively duplicated loci were found to be identical in their variable regions with another locus for the same clonal complex Figure 3-5. This was observed for all missing loci that were detected in the isolates of the four clonal complexes as summarized in Table 3-3. In contrast, *opa* loci of each clonal complex are different in their sequences from *opa* loci of other clonal complexes, i.e. the four *opa* loci of CC60 are different from the loci of CC174 and other clonal complexes which also differ in their loci between each other. In total, 16 different *opa* alleles were analysed in this study sharing different rates of similarities in their sequences (Appendix 1). Thus, only isolates of clonal complex CC174 (except V88 and V54) contained four different *opa* allele whereas two or three different alleles were

present in the four *opa* loci within the other three clonal complexes (cc167, cc23 and cc60) analysed in this study.

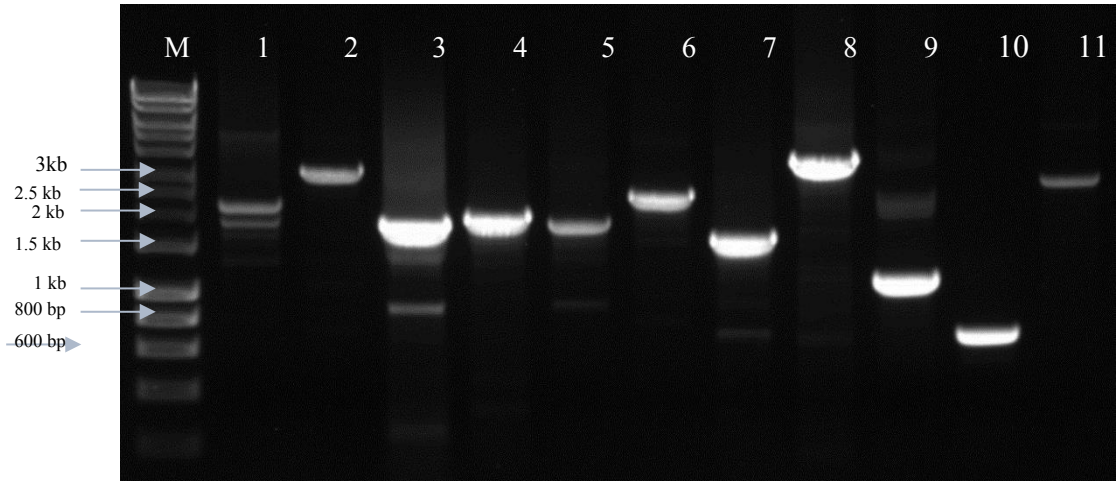


Figure 3-4 : **Agarose gel electrophoresis of PCR products of the detected *opa* loci with their identical loci.** The different loci were amplified from their variable regions using the same reverse primers (in each pair) toward the flanking genes of each locus. M: DNA marker (Hyperladder I), 1 , 2 : *opaJ* and *opaD* of CC60 (N113.1), 3,4 *opaA* and *opaJ* of CC167 (N117.2), 5,6 *opaA* and *opaB* V88, CC174, ST1466 (N449.1), 7,8 *opaA* and *opaJ* V54 ,CC174, ST 8510 (N54.4) , 9,10 *opaD* and *opaA* of CC23, 11 *opaA* CC23 from the flanking genes (N459.5). N: refers to the isolate number.

Table 3-3: **The detected and allocated *opa* loci in *N. meningitidis* carriage strains and their identical loci within the same strains**

N. meningitidis **clonal complexes**	**loci with identical** ***opa*** **alleles**
CC167, ST 767	*opaJ, opaA*
CC167, ST 767 (V124)	*opaA*, *opaB* and *opaJ*
CC23, ST 1655	*opaD, opaA*
CC60, ST 1383 (4 carriers)	*opaJ, opaD*
CC174, ST1466 (V88)	*opaB, opaA*
CC60, ST 1430 (V185)	*opaJ, opaD;* *opaB, opaA*

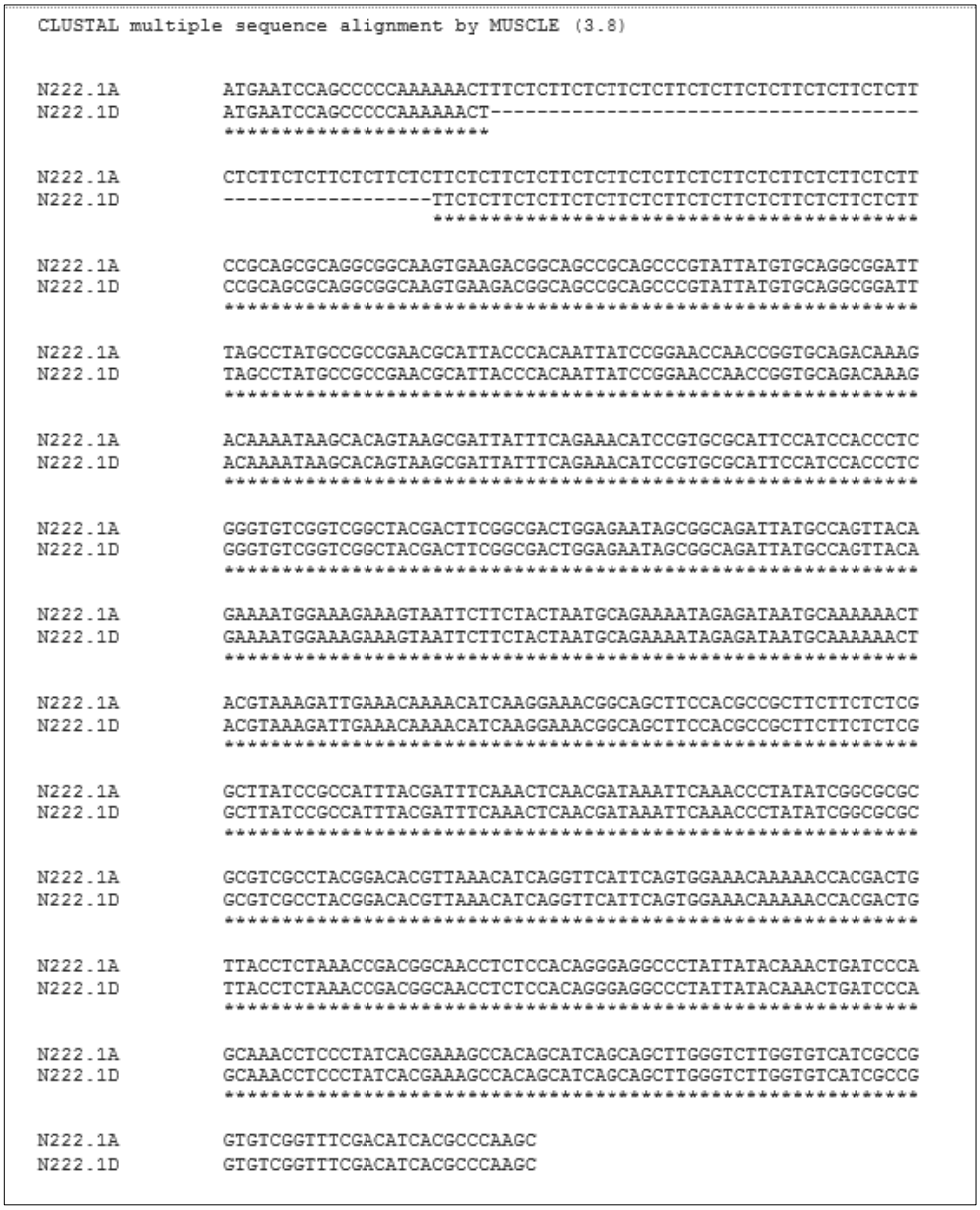


Figure 3-5: **Multiple sequence alignment of *opaA* and *opaD* of CC23 (V222) of the isolate N222.1.** The alignment showed that these two loci are identical in the sequences of their variable regions and only differ in the repeat tract region sequence. Both loci were amplified with the same reverse primer which was designed to specifically bind to the conserved end of *opa* genes and the amplification covered the whole genes from the starting codon to the stop codon.

3.4 Typing and suggested nomenclature system

In order to type all of the *opa* alleles investigated in this study, the three variable regions of each allele were blasted against the sequences of 345 *opa* allele *opa* allele's available in the *opa* database http://neisseria.org/nm/typing/opa/ (accessed at 25/7/2015). The nomenclature system suggested by (Callaghan et al. 2006) was used, which involves giving an allele number for the gene and a number for the variable regions, which refers

to the family of these regions. For example locus A of CC23 isolates contain *opa* allele: 34, SV:4-3, HV1:18-3, HV2:14-1. This mean that the number of this allele is 34 and it sequences involve the 3rd variants of semi-variable region subfamily number 4, and the third variant of the subfamily number 18 of HV1 and variant 1 of subfamily 14 of HV2 . From the 17 alleles investigated, only five were identical with previously detected alleles, while the others differed from all other *opa* alleles available in the database and hence can be considered as new alleles with a suggested number according to the Callaghan system (**Table** 3-4)

Table 3-4 : *opa* alleles and suggested nomenclature

Clonal complex	*opa* genes	Homologous allele					Homologous variable regions with similarity (%)			Suggested nomenclature of new alleles			
		Allele	SV	HV1	HV2	Similarity	SV	HV1	HV2	Allele	SV	HV1	HV2
Cc23	*opaA*	34	4-3	18-3	14-1	172/172 (100%)							
	opaB	208	2-2	3-2	8A-3	173/175 (98%)	2-2 (90%)	3-2 (100%)	8A-3 (100%)	346	2-11	3-2	8A-3
	opaJ	158	4-7	15-2	1-7	146/172 (84%)	1-1 (100%)	17-1 (100%)	1-7 (95%)	347	1-1	17-1	1-12
	opaA (N445)	15	4-2	19-8	14-1	158/174 (90%)	4-3 (100)	17-1(100)	14-1 (100%)	348	4-3	17-1	14-1
	opaJ (N459)	72	4-2	19-3	14-1	130/172 (75%)	4-3 (84%)	19-5 (92%)	21-1 (71%)	349	4-9	19-11	21-2
CC60	*opaA*	130	3-2	12-1	20-1	159/168 (94%)	2-1 (100%)	14-2 (100%)	20-1 (100%)	350	2-1	14-2	20-1
	opaB	27	4-2	7-1	5-2	168/176 (95%)	4-2 (100%)	7-3 (100%)	5-3 (88%)	351	4-2	7-3	5-4
	opaD, opaJ	157	2-7	2-2	1-5	171/172 (99%)	2-7 (100%)	2-2 (100%)	1-5 (100%)				
CC60 (V185)	*opaA, opaB*	182	4-2	14-2	4A-1	172/173 (99%)	4-2 (100%)	14-2 (100%)	4A-1 (98%)	352	4-2	14-2	4A-7
CC167	*opaA, opaJ*	206	2-1	1-1	8A-4	172/176 (97%)	2-1 (100%)	1-1 (96%)	8A-4 (96%)	353	2-1	1-7	8A-7
	opaB	228	2-2	19-10	11-3	173/173 (100%)							
	opaD	158	4-7	15-2	1-7	164/172 (95%)	4-7 (100%)	16-2 (100%)	1-7 (95%)	354	4-7	16-2	1-13
CC174	*opaA*	210	2-2	17-1	17-1	164/165 (99%)	2-2 (100%)	17-1 (100%)	17-1 (97%)	355	2-2	17-1	17-9
	opaB	206	2-1	1-1	8A-4	170/178 (95%)	2-1 (100%)	53 (96%)	8A-4 (100%)	356	2-1	5-9	8A-4
	opaD	163	2-2	11-2	1-6	170/170 (100%)							
	opaJ	150	2-1	1-1	5-6	166/176 (94%)	2-1 (100%)	1-5 (93%)	5-1 (94%)	357	2-1	1-8	5-5
Cc174 (V88)	*opaA, opaB*	182	4-2	14-2	4A-1	173/173 (100%)							
	opaJ	291	2-1	5-2	18-1	177/177 (100%)							

3.5 Intergenic and intragenic recombination

Some of the loci analysed in this study displayed variability within samples of the same strain not only in their repeat tract length, but also in the sequences of their variable

regions. This was noticed in the samples of V222, in which *opaJ* from the isolates of the 3rd and 4th time points was not amplified by the same primers used to amplify this locus from the isolates of the first and second time point. Analysis of the sequences indicated that the Opa allele in this locus was different from allele in the same locus for samples of the 1st and 2nd time points (Figure 3-6) In addition, the locus of the later time points was switched ON in all colonies analysed in the two time points while the previous one was switched OFF in the all samples of the first and second time points. Blasting the sequences of the new locus against the sequences of other carriage isolates available in BIGSdb database (http://pubmlst.org/neisseria/) as well as against the sequences of the 345 known *opa* alleles (available at the website http://neisseria.org/nm/typing/opa/, accessed at 25/7/2015) did not show any identical allele or allele with strong similarity even in one of the variable regions. This indicates that allelic mosaicism has occurred at this locus via homologous recombination either with an Opa located in another locus in the same genome or with an *opa* imported from another strain and that this event led to generation a new *opa* allele within the samples of the later period

Intragenic recombination has also been noticed in samples of the V96 , as the isolates from the 3rd time points and one isolate from the 2nd time point showed to have different *opa* allele (*opaA*) that is differ from the one exist in the samples from the first time point, as well as the other isolates from the 2nd time points. The analysis of the sequences showed that the new allele has sequence come from the two alleles of the same strain which are *opaJ* and *opaA* of the first time point isolates, and it showed that the new allele has the HV1 of *opaJ* while the rest of the sequences is the same as in the old *opaA* (Figure 3-7) This mean that a recombination happened between the two alleles which led to replace the HV regions and generate new *opa* allele.

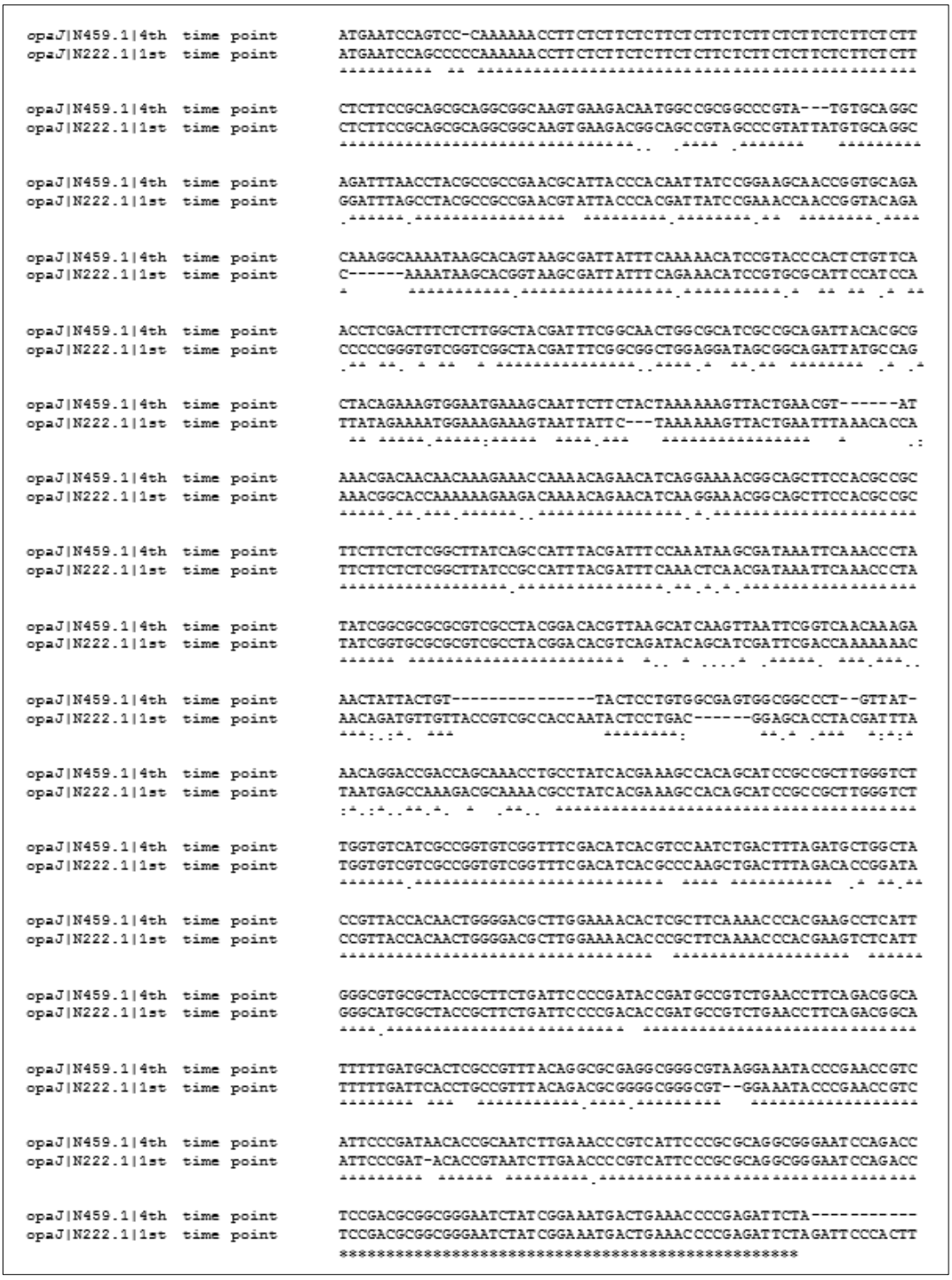


Figure 3-6 Multiple sequence alignment showing the differences between nucleotide sequences of two *opaJ* genes of the same strain but from different time points. Samples from the third and fourth time points of V222 (N391 AND N459) showed to have *opaJ* that differ from *opaJ* of samples of the first and second time points (N222 and N309) which were isolated from the same carrier. The comparison of the sequences revealed the later isolates have new *opa* allele that generated via homlogous recombination

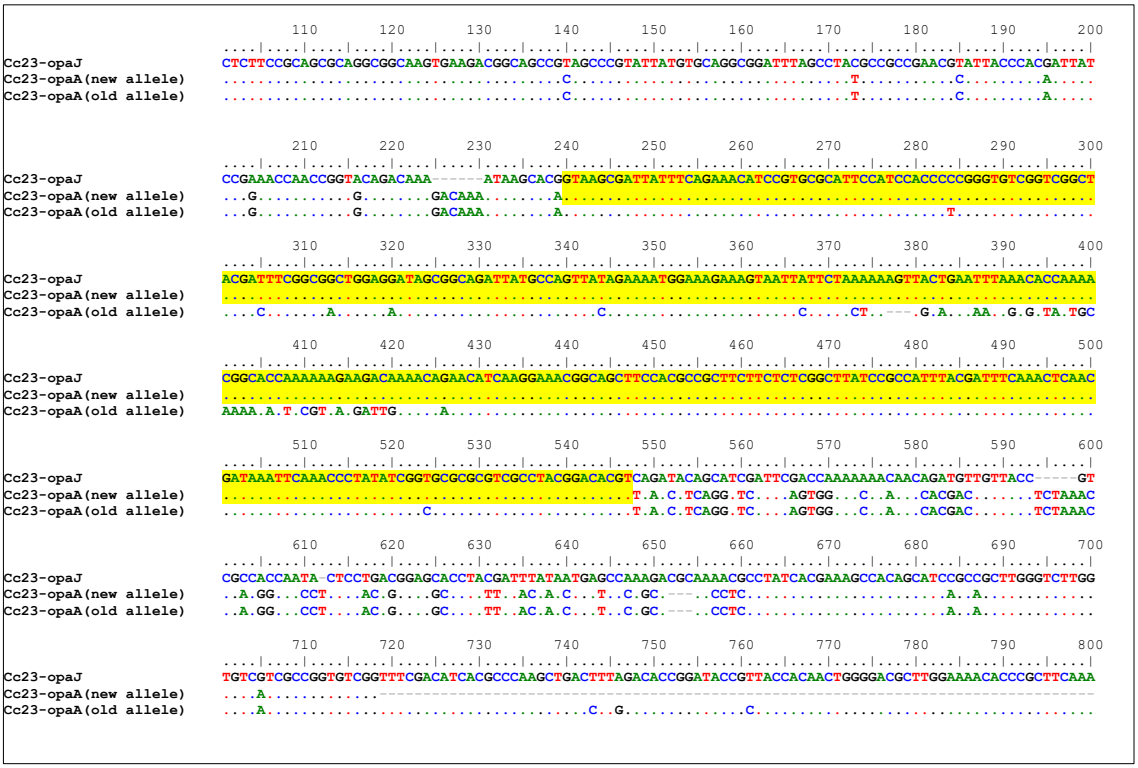


Figure 3-7: **DNA Sequence alignment of three** *opa* alleles of meningococcal isolates isolated at different time points from the same carrier (V96). The alignments show that homologous intragenomic recombination between *opaA* and *opaJ* has led to replacing a sequence fragment (yellow shading) resulting in generating a new *opaA* allele detected in the samples isolated at later time points (N445) from the same carrier V96.

3.6 Discussion

The whole genome sequence of several carriage isolates, which are available in the Neisseria PubMLST database, were utilised in this study to design strain specific primers to amplify *opa* loci in the isolates of different clonal complexes. The sequences of these isolates were obtained by assembly of paired-end Illumina reads, and they lacked the expected number of *opa* loci in most clonal complexes. Using a PCR-based approach, four *opa* loci were confirmed as being present in each clonal complex. Analysis of these sequences showed that the missing loci were identical in sequence with other loci in the same genome. This duplication of sequences may be the reason why these loci were not present in the whole genome sequences, as during assembling and annotating the genome, the two identical loci might be interpreted as a single gene due to their sequence similarity. This is supported by the presence of partial sequences of *opa* truncated at the middle of the repeat tract region, which is always variable among the loci, and in absence of the sequence downstream of the repeat tract which were proved to be identical between the detected genes.

The presence of identical alleles in the majority of the isolates analysed (72%) suggests that duplication of *opa* alleles was favourable in the carriage isolates. This mechanism might be utilised by *N. meningitidis* to reduce the antigenic diversity of their Opa variants to enable the bacteria to evade the immune response by decreasing the probability of exposure to an immune response elicited by a previous colonisation by other strains displaying one or more similar Opa variants (Callaghan *et al.* 2008b). This pattern was also noticed in a previous study carried out by Callaghan et al. (2008) where the presence of identical *opa* alleles within different loci was observed in 41 % of *N. meningitidis* carriage isolates from the Czech Republic. One suggested explanation is that a strong immune pressure imposed on the bacteria during circulation among different hosts might lead to selection of isolates with lower diversity in their Opa repertoire (Callaghan *et al.* 2008b; Sadarangani *et al.* 2011).

Two identical alleles were noticed in the isolates of CC174 (ST 1466) of the carrier V88, which showed differences in three of the *opa* loci from other CC174 (ST 1466) isolates of other carriers. Only one allele (*opaD*) in this isolate was the same as one in the isolates of other CC174 carriers. Blasting the sequence of the different alleles of V88 against the sequences of other carriage isolates (isolated in the same study) showed that *opaA*/*opaB* were identical (100% similarity) with the sequences of *opaA* of isolates of V185 (CC60, ST1430), while the sequences of *opaJ* was identical (100%) with the sequences of

opaJ/*opaA* of isolates of V54 (CC174, ST 8510). This suggests that natural transformation and recombination with different strains has occurred in isolates of this carrier leading to import of such different alleles, but it is not clear whether this has happened at an early stage of colonising of this carrier (V88), which might be previously colonised by the mentioned strains, or it has happened over the long term of circulating within different hosts colonised by strains having such *opa* alleles. Such events may confer fitness to different isolates of the same strain during transmission and colonisation of carriers with differences in their immunological reactivity for these proteins.

Homologous recombination is one of the main mechanisms utilised by *N. meningitidis* to raise the diversity of their Opa repertoire in response to continuous exposure to the human immune response as an obligate commensal (Sadarangani *et al.* 2011). The recombination happens either between two *opa* loci within the same strain or from importing another locus from co-infecting strains and it leads either to duplication of an existing allele on another locus, or results in allelic mosaicism (Bilek *et al.* 2009).

Homologous recombination has been noticed in *opa* loci in a number of samples analysed. In V222, the *opaJ genes* of the samples from the third and fourth time points differed in sequence from *opaJ* of samples of the first and second time points whereas all isolates were of the same strain and clonal complex (i.e. CC23). The total or partial sequences of the new locus did not show any identity with any other *opa* alleles , which indicates that allelic mosaicism has occurred and led to development of a new *opa* allele at this locus. This allele was not similar or shared similarity with other alleles of the same strain as well as with alleles of other strains (according to the blast search against all *opa* sequences available in *opa* database, see section 3.5). Therefore, this allele is more likely to come from another strain via intergenic recombination. The expression state of the previous allele was OFF in most of the colonies of the 1st and 2nd time point while the expression state of the new allele was ON in all colonies analyzed in the third and fourth time points. This is an indication that there was immune selection against expression of the initial allele, which was lifted by generation of the new allele.

Another event has been noticed in the samples of V96, where the isolates from the 3rd time point and one of the isolates of the 2nd time points showed to have new *opaA* with variable regions come from *opaJ* and *opaA* of the same strain but from the isolates of the first and 2nd time points. It could be suggested here that the bacteria utilized this

mechanism to change the *opa* allele to avoid the immune response imposed on the previous allele.

These findings suggest that phase variation is not the only mechanism used by *N. meningitidis* to adapt and persist within the host and generating new *opa* alleles is another mechanism used by this bacteria during the carriage state either as an alternative way to avoid the immune response or to gain new functions within their Opa repertoire.

4 Phase variation of Opa proteins in Neisseria *meningitidis* during persistent carriage

4.1 Introduction

Phase variation is one of the major mechanisms that enable commensal and pathogenic bacteria to adapt and survive in humans. This mechanism confers fitness to the bacteria to transmit between different hosts, colonise particular human tissues, and evade immune defences (Martin *et al.* 2003a; Bayliss 2009). It involves stochastic, reversible mutations in particular sites (hypermutable sequences) within the bacterial chromosome called contingency loci that lead to alterations in surface exposure molecules as a response to particular selective conditions (Bayliss 2009). The expression of many of these contingency loci are controlled by simple sequence repeats (SSR) which lie either in the promoter or in the coding region of these genes, and act as a hot spot for reversible, *rec*-independent mutations mediated by slipped-strand mispairing mechanism (SSM) during DNA replication (Bayliss *et al.* 2001).

N. meningitidis is an obligate commensal of humans and is considered as a leading cause for bacterial meningitis and septicaemia. The bacteria can colonise and persist in the human nasopharynx for several months without causing disease in the carriage state (Yazdankhah and Caugant 2004b). A large number of loci (~ 40 genes) of these bacteria are subject to phase variation due to the presence of SSRs in their promoters or ORFs (Snyder *et al.* 2001; Martin *et al.* 2003a). The majority of these genes encode surface antigens that mediate different functions such as adhesion / invasion, iron acquisition or encoding for enzymes that participate in the biosynthesis and modification of surface molecules such as lipopolysaccharides (Saunders *et al.* 2000).

The opacity-associated outer membrane proteins (Opa) of *N. meningitidis* are encoded by four loci dispersed within the meningococcal chromosome. Each locus is phase variable independently due to the pentameric SSR (CTCTT) present in the leader peptide-coding region of these genes and that control the expression of Opa proteins at the translational level (Kawula *et al.* 1988). These proteins mediate the adhesion to and invasion of the bacteria into human cells via binding to a member of the CEACAM family of human proteins. Opa play an important role in asymptomatic colonisation as well as in meningococcal disease (Sadarangani *et al.* 2011). As each *opa* gene encodes for different Opa variants with the same function, the switches in expression of these genes leads to

generation of a heterologous meningococcal population with different antigenic variants whilst maintaining the essential functions (Bayliss *et al.* 2001). This mechanism is supposed to be an effective strategy to evade the human immune defences and enable the bacteria to adapt to different niches within the host as well as enhancing the switching from the carriage to disease state.

The frequency of phase variation of Opa was estimated to be around 10^{-3} and it is influenced by the promoter strength and the length of the repeat tract of the locus. Bacterial transformation has been shown to increase the PV rate in meningococcal *opa* genes 180 fold (Mayer 1982; Belland *et al.* 1997; Sadarangani *et al.* 2016). A more recent study investigated the expression of Opa proteins in the UK meningococcal disease isolates in the MRF MGL database. This study indicated that the majority (68.0%) of Opa encoding genes were in the on state which reflects the important role mediated by Opa during the meningococcal disease (Sadarangani *et al.* 2016). This study referred to several limitations regarding the assessment of Opa expression in disease isolates such as the poor coverage of the whole genome sequence in the database for the *opa* genes and the unknown conditions of treating the meningococcal isolates between sampling and sequencing, which may vary between the isolates with a possible *in vitro* selection for Opa^+ variants. Contrastingly, the PV of Opa proteins, and its possible implications, during meningococcal colonisation of humans has not been investigated.

Due to the potentially significant role of PV of Opa proteins in enhancing the fitness of these bacteria to persist and evade the immune response in the human nasopharynx, this study aimed to investigate the phase variability of these proteins during prolonged carriage state of *N. meningitidis* in human carriers. The alteration of the repeat tracts and its effects on the expression state of Opa were characterised in sequential meningococcal samples isolated from different healthy carriers at different time points. These samples were obtained from first year students volunteers in the University of Nottingham during a carriage study carried out between 2008 and 2009 (Bidmos *et al.* 2011)

4.2 Investigation the repeat tract length of *opa* genes in the meningococcal isolates

The number of the repeat tracts of each locus has been investigated in carriage isolates obtained from a total number of 19 carriers for different time points during the period between 2008 and 2009 in the carriage study carried out by (Bidmos *et al.* 2011). The isolates were isolated from student volunteers at the University of Nottingham, and the ages of those volunteers were ranged between 17 and 23 years old and 46 % of them were females. No data were available regarding the vaccination records of the volunteers but none of them were vaccinated during the course of the study and the majority of them were not expected to take any other meningococcal vaccinations as the study was carried out before introducing the routine MenACWY and MenB vaccines. However, the majority of the volunteers are expected to have infant MenC immunization. (Bayliss, personal communication). The meningococcal carriage isolates from each volunteer were confirmed to be of the same strain by whole genome sequencing of one isolate from the intial and one isolate from the final time points. Further molecular typings were done for one isolate per time point using sequencing of the PCR products of the variable regions of *porA* or *fetA* or sequencing of the seven house keeping genes for MLST typing (Alamro 2014).

Another confirmation by allele specific PCR for PorA and FetA type was performed for all isolates at the different time points (Alamro *et al.* 2014). The carriage isolates from the 19 carriers belonged to four different clonal complexes of *N. meningitidis* including CC174 (7 carriers), CC60 (5 carriers), CC23 (4 carriers) and CC167 (3 carriers). Isolates were taken from the each carrier at up to 4 time points separated by 1 month (1^{st} - 2^{nd} time points), two months (2^{nd} - 3^{rd} time points), and three months (3^{rd} - 4^{th} time point), and 1-20 colony isolates were analysed for each time point. Samples from some carriers were taken only in two or three time points as the carriers either cleared during the period of study or showed clonal replacement during one of the time points. The regions spanning the repeat tracts were amplified using locus specific reverse primers that bind to one of the variable regions of each locus with fluorescently labelled forward primers annealing to the conserved region upstream of the repeat tract (Figure 4.1). The size of each PCR product was determined by GeneScan for a precise measurement. The GeneScan data were then analysed by peak scanner software which presents the size of the PCR amplicons as peaks against the signal strength (Figure 4.2. B).

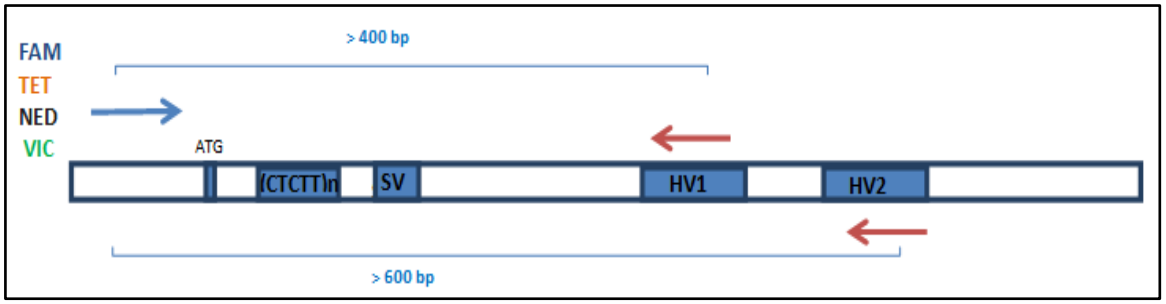


Figure 4-1: **Map of the *opa* gene showing the positions of the primers used in this study to amplify the different *opa* loci and the expected size of PCR products**. The forward primer binds to the conserved region upstream of the repeat tracts of *opa* and this primer is fluorescently labelled with different fluorescent dyes (FAM, TET, NED and VIC), while the reverse primers are locus specific and bind specifically to one of the variable regions of each locus.

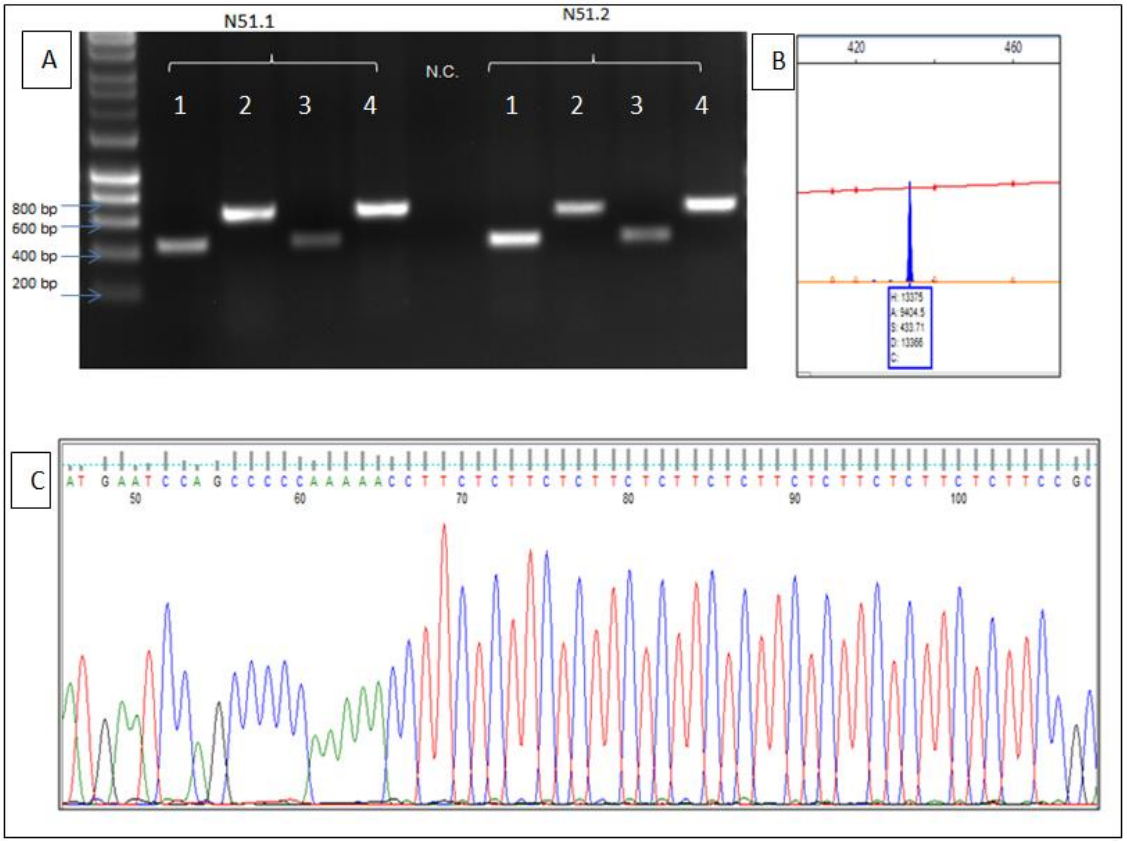


Figure 4-2: The three steps used to determine the repeat tract length in each *opa* gene. Each locus were firstly amplified with fluorescently labelled primers and the product size then determined by GeneScan (to give the precise size of the PCR amplicons). Products with a particular size were then sequenced in a number of isolates to determine the repeat numbers. A: agarose gel electrophoreses of PCR products of 4 opa loci in two colony isolates of the carriar V51 (CC174), N51.1 and N51.2. The numbers above the bands refer to *opa* loci amplified, 1: *opaJ*, 2: *opaD*, 3: *opaA*, 4: *opaB*, N.C: negative control. B: the peak of the PCR product of *opaJ* (band 1) of N51.1 obtained by GeneScan analyses as shown in PeakScanner v1.0 software which indicates that the size of this product is 438.71 bp. C: the sequences of the *opaJ* (band 1)of N51.1 displaying the pentameric repeat tracts of this locus (CTCTT) showing that there was 7 repeats in this locus of this isolate.

The four *opa* loci were sequenced in a number of isolates from each clonal complex and the number of the repeat tracts of each gene was correlated with the size of the PCR products as determined in the GeneScan assay. These sequenced products were considered as a standard for the further analysis of the GeneScan data from other isolates (Figure 4.2. C). In the case where there were identical *opa* loci, each locus was firstly amplified from one of its variable regions toward the flanking conserved upstream gene, and then the resultant PCR products were diluted and used as a template for a second PCR to amplify the repeat tract region from the conserved upstream end of *opa* toward the variable region. This was to differentiate between the identical loci and also to make the product size within the range of the size standard used in GeneScan which has maximum size of 1200 bp.

The product size of each locus varied among the different strains due to differences in the number of the repeats and also in the sites where the reverse primers annealed, as the variable regions of *opa* genes are different between the different clonal complexes as well as within the same clonal complex. The product size and the correlated number of the repeat tracts of *opa* loci are summarized in table 4-1.

4.3 Testing the effect of in vitro passage on the repeat tract length in opa genes.

In order to test whether subculturing of *N. meningitidis* isolates, which is usually involved in bacterial sampling methods, leads to a change in the repeat tract length of *opa* loci, a sample of *N. meningitidis* MC58 strain was subjected to serial passage accompanied with analysis of the repeat length of *opa* loci over different time points. The procedure simply involved subculturing the isolate on BHIA plates over six time points and determining the repeat tracts of *opa* loci in six single colonies (named A-F) obtained in each time point. The size of the products was detected by GeneScan while the repeat tract number was determined by sequencing the four loci in two colonies one from the 1st time point (colony A0) and the second from the 6th time point (colony A5), and the repeat tract length was then correlated with the product size .

The results of GeneScan analysis indicated that the product size was constant in each locus over the six time points and no changes have been detected in all the colonies analysed. This indicates that the repeat tract length did not change (which was confirmed by sequencing) during the serial passage of the bacteria. This suggests that subculturing itself is not enough to generate a change in the number of the repeat tracts in *opa* loci, and

the presence of other factors might be necessary to induce such reversible mutations in the pentameric repeat region of *opa* genes.

Table 4-1: The size of PCR products of *opa* loci determined by GeneScan and its correlated number of repeat tracts in different strains of *N. meningitidis* analysed in this study

N. meningitidis strains	*opa* loci	Primer 's annealing site	Product size (detected by GeneScan) bp	Correlated number of repeat tracts (CTCTT)
CC174	*opaA*	HV1	450	12
	opaB	HV2	655	10
	opaD	HV2	640	8
	opaJ	HV1	433	7
CC60	*opaA*	HV1	458	13
	opaB	HV2	700	11
	opaD	HV1	464	14
	opaJ	HV1	413	4
CC167	*opaA*	HV2	616	4
	opaB	HV2	651	12
	opaD	HV1	420	4
	opaJ	HV2	616	4
CC23	*opaA*	HV1	471	17
	opaB	HV2	641	9
	opaD	HV1	426	8
	opaJ	HV2	626	8
CC174 , V88	*opaA*	HV2	672	9
	opaB	HV2	677	10
	opaD	HV2	671	14
	opaJ	HV1	441	7
CC23, V222 (3rd , 4th time point)	*opaJ*	HV2	639	8
CC23, V96 (3rd , 4th time point)	*opaA*	HV2	728	16

4.4 Analysis of the variability in the number of the repeats during persistent carriage

GeneScan analyses indicated that considerable switching in the numbers of the repeats occurred between different time points in the four *opa* loci. The numbers tend to vary among the samples of different carriers of the same clonal complex as well as of different clonal complexes. The changes in the repeat tracts in the all loci are summarised in Figure 4.3. Generally, *opaA* was the most variable locus among the four loci. The range of the repeat numbers for this locus was between 4 and 22 CTCTT for all CCs, while samples of each clonal complex had specific ranges for this locus. In the samples of CC174, the

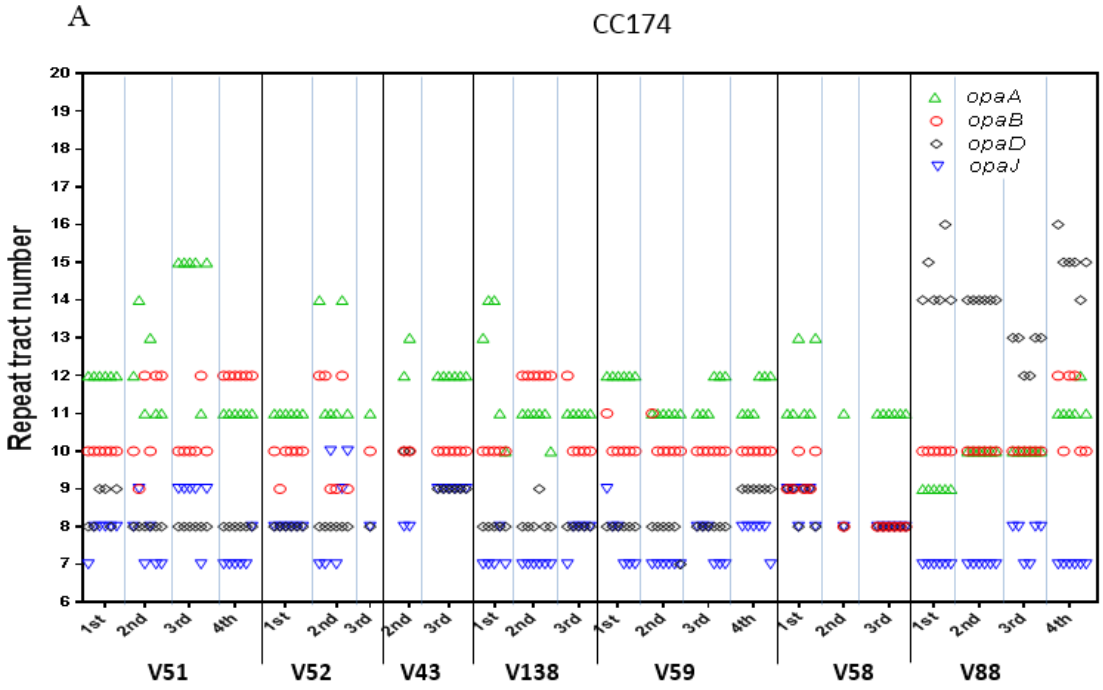
repeat length of this locus was fluctuating between 9 and 15 repeats, while in the samples of CC23 the range was between 15 and 22 repeats. Changes in the number of the repeats of *opaA* were noticed in all carriage samples analysed over the different time points except in the samples of V64 and V124 (carriers of CC167) where the repeat tract length was fixed in this locus at 4 CTCTT in all samples.

On the other hand, the overall range of the repeats of locus B was between 5 and 18 repeats. The changes in repeat number of this tract ranged from a high switching frequency in some carriers such as V117 (CC167), to rare switching in samples of other volunteers such as V59 (CC174) in which only two isolates out of 24 showed a change in the repeat length of this locus. Surprisingly, no changes were detected in the samples from carriers V43 (CC174), V113 (CC60) and V124 (CC167), where the repeat length of *opaB* remained stable at 10, 11 and 12 CTCTT over the time points in the samples of those carriers respectively.

However, different patterns were noticed in *opaD* which showed less variability in their repeat tracts within the samples of a number of carriers. The repeat length of this locus was stable over all-time points in the samples of CC23 either at 7 CTCTT in V93, or 8 in V222 and V69 (except one colony which showed 9 repeats) and at 4 CTCTT in the samples of CC167 as well as in the samples of carrier V52 of CC174 at 8 repeats. Significant fluctuations in the repeat length of this locus were noticed in the samples of carrier V88 (CC174) as well as in the carriers of the CC60 strain where the repeat number showed switching between 8 CTCTT and 16 CTCTT.

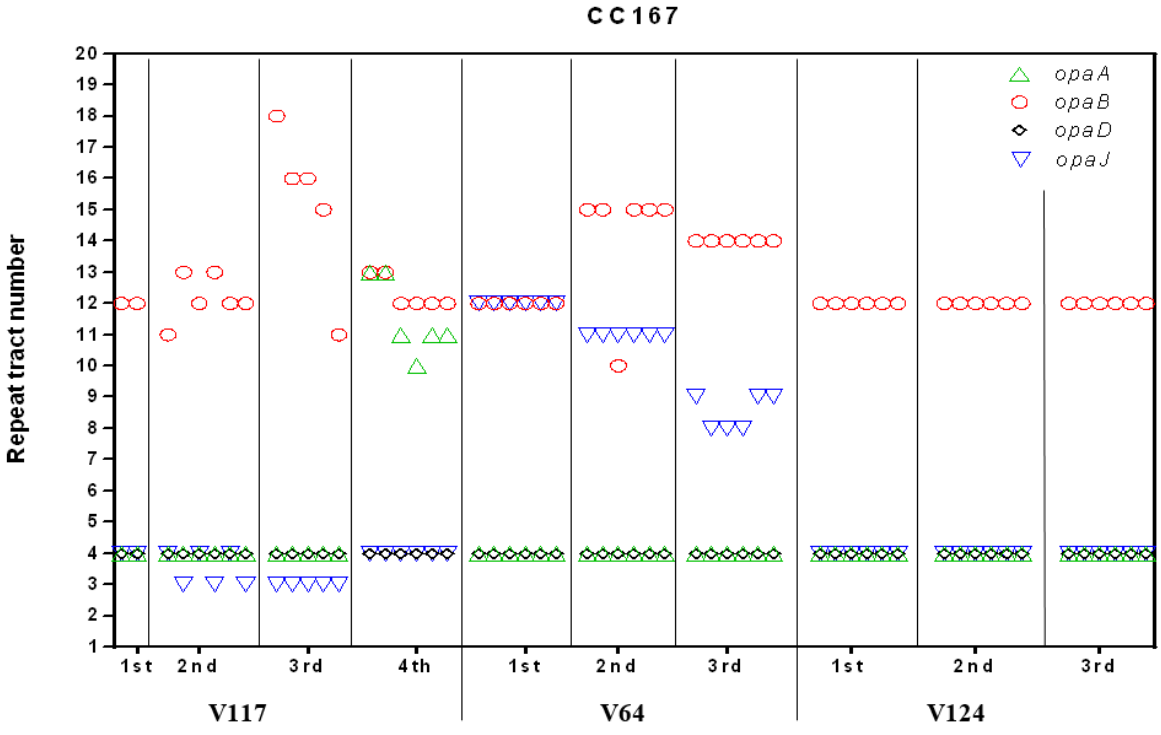
Finally in *opaJ*, the change in the repeat tracts was within an overall range of 3 CTCTT (which represents the lowest limit for repeat length detected in this study) to 12 CTCTT., In the samples of CC60, this locus did not show any change in repeat number, which remained stable at 4 CTCTT over the different time points. A similar pattern was also noticed in the samples of CC23, as the repeat length of *opaJ* remained constant either at 7 repeats in V93 or at 8 repeats in V69 and V222 with an exception of one colony of V222 which switched the repeat length of this locus to 9 repeats in the 2nd time point. Whereas the highest variability in this locus was noticed in the samples of CC174 where the switching was between a range of 7 to 12 repeats.

A



Carriers with time points

B



Carriers with time points

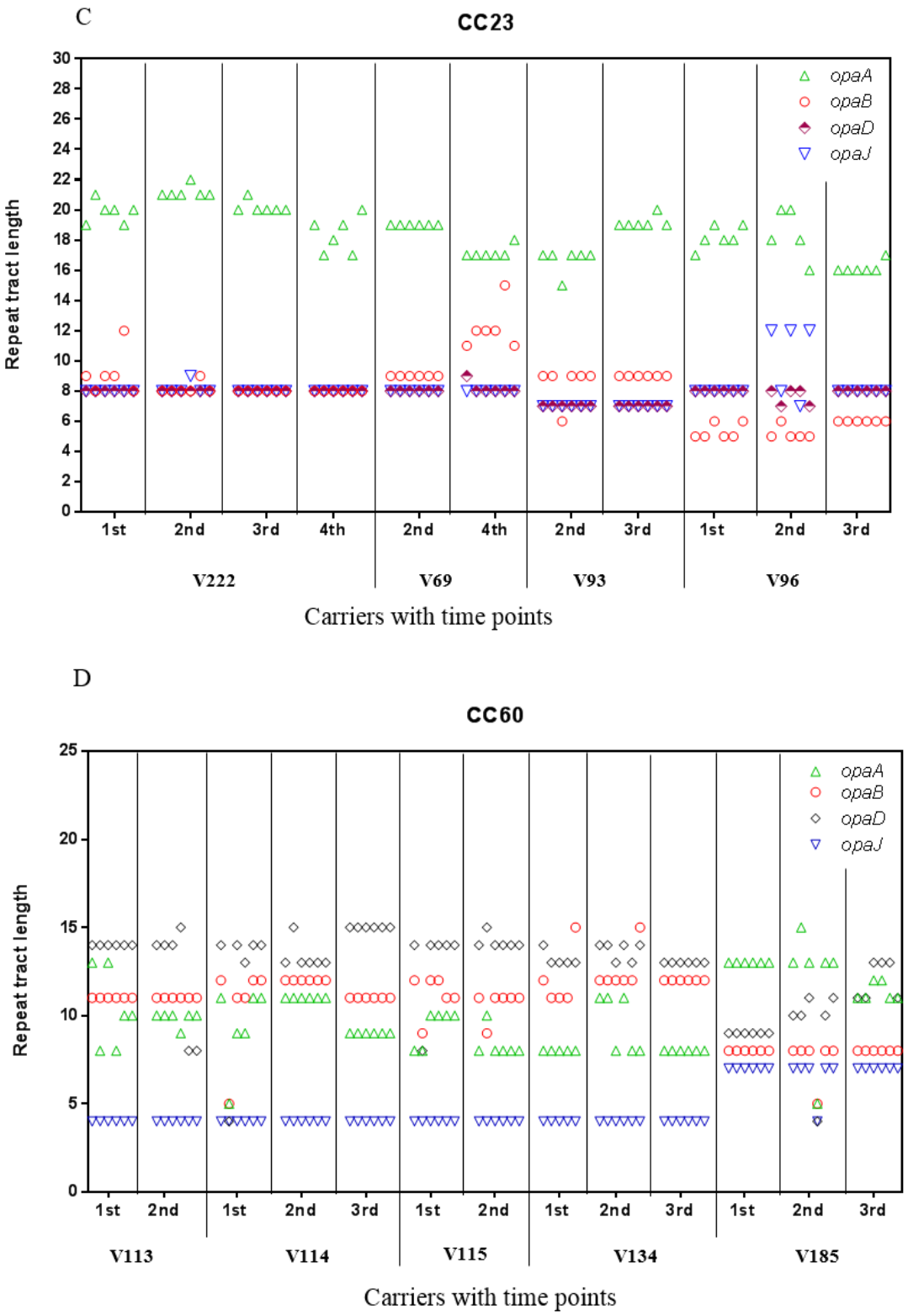


Figure 4-3 **The changes in the SSR length of the four *opa* loci in meningococcal carriage isolates over different time points.** The repeat length was analysed in samples from19 different carriers representing 4 different complexes and up to six colonies were analysed in each time point. Graph A: samples of cc174, B: samples of cc167, C: samples of cc23, and D: samples of cc60.

4.5 Correlation of the switching rate of the repeat tract with the length of colonisation

In order to determine whether the length of colonisation plays a role in increasing the rate of the P.V of *opa* genes, the variability in the repeat tract length of each locus in each carrier was firstly compared between each pair of time points and examined using two tailed Fisher`s exact test to determine whether these changes were statistically significant or not (significance was defined as a p value of >0.05). The number of carriers analysed for each pair of time points were 14 in the 1^{st}-2^{nd} , 14 in the 2^{nd} -3^{rd} , 6 in the 3^{rd} -4^{th} and 9 in the $1^{st\ or}$ 2^{nd} -4^{th} time points. The percentage of carriers showing a significant difference between time points was determined for each Opa locus and for each of the four time periods (Figure 4-4). The graph shows that the rate of changes increased with the length of colonisation and that the highest rate of change was observed in the period between the 3^{rd} and 4^{th} time points (3 months of colonisation) in three genes, followed by a decline in the 4^{th} pair of time points (1^{st} or 2^{nd} to 4^{th}) which represents 5 to 6 months of persistent carriage. This decline was partly due to a number of samples switching back to the repeat number as found in the early time points. In general, the highest frequency of changes in the repeat number was in *opaA* in three pairs of time points with a peak in the third pair of time points where around 65 % of the samples showed statistically significant changes in the repeat tract of this gene. Similarly *opaB* showed the second highest level of variability with a gradual increase in the variability over the length of carriage starting from 20% after one month to around 45% after 5-6 months of colonisation. Although *opaD* and *opaJ* were less variable than the other two genes, *opaD* showed a gradual increase in the variability during the first three pairs of time points and then declined in the 4^{th} pair, while variability in *opaJ* fluctuated and also peaked after 3 months of carriage at 33% of the carriers (Figure 4.4)

However, it is known that the rate of switching tends to be affected by the length of the repeat tracts. The data was examined to determine if the percentages of statistically significant changes increased with increasing length of the repeat tracts. The highest percentage of switching was correlated with the range of repeat length between 13 and 22 CTCTT repeats. The data revealed that 33 % of *opa* loci showed significant switching either to or from repeat numbers within this range (Figure 4.5). Contrastingly, no significant changes were observed from or to repeat lengths below 7 CTCTT, and, as mentioned

before, genes in this range tend to be stable at a particular repeat length over the whole period of carriage.

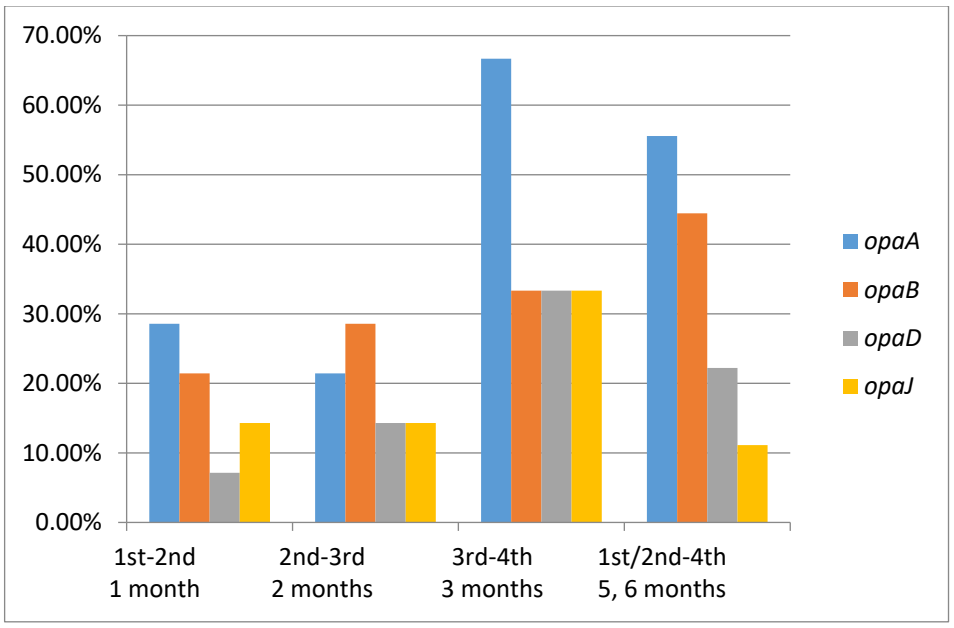


Figure 4-4: Percentage of carrier samples showing statistically significant changes in the repeat number between different time points. For each *opa* gene, the repeat number was compared between two different time points in each carrier using a two-tailed Fisher exact test to determine if there was a significant change ($P< 0.05$). The number of carriers analysed in each pair of time points are: 14 (1^{st}-2^{nd}), 14 (2^{nd} -3^{rd}), 6 (3^{rd} -4^{th}) and 9 (1^{st}, 2^{nd} -4^{th}).

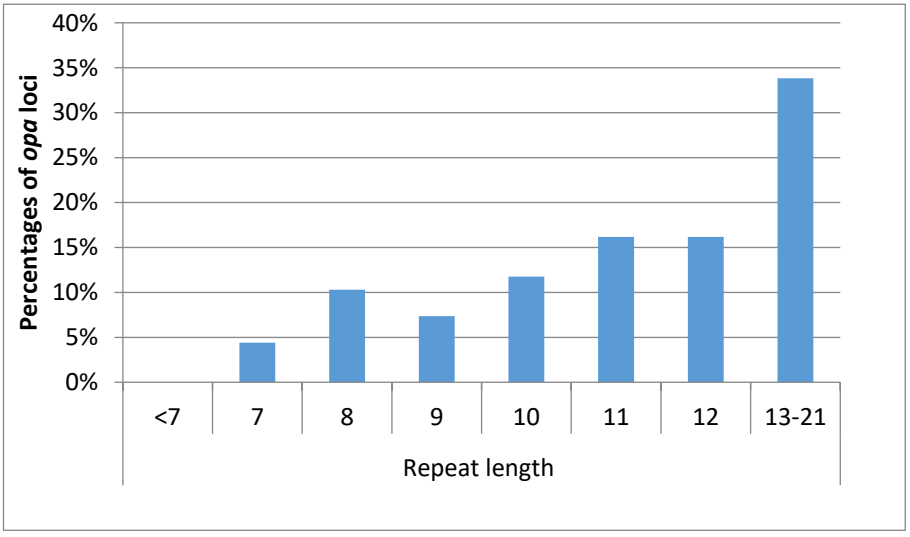


Figure 4-5: The proportion of *opa* loci displaying statistically significant switching from or to a given number of repeat tracts.

Depending on the analysis above, the mutation rate was estimated in *opa* loci according to the number of significant switching events between the 1st or 2nd time points and the 4th time points. This was achieved by dividing the rate of switches for all Opa loci (number of loci showing statistically significant switches divided by the total number of events analyzed) by the average of the number of months (5 and 6 months). This calculation indicated that the rate of switching was **0.06** mutations/gene/month, and this was according to the equation:

Mutation rate = (number of genes showing significant changes (12) / number of total events (genes) analyzed (4 loci per carrier) 36) / average period (5.5 months) = (12/36)/5.5= 0.0606

4.6 Correlation of the expression state of Opa proteins with the repeat tract number

The expression state of *opa* loci is dependent on the number of the repeats in each locus and can be predicted by analysing the open reading frames of each locus with different repeat lengths to see which repeat number gives an ON or OFF state. This analysis was necessary in order to take into account the size of the poly C and poly A tracts that are present upstream of the CTCTT region in *opa* genes as shown in Figure 4-6 . These tracts are not phase variable but vary in repeat number among the different *opa* genes, with these differences giving rise to alternative expression states for a specific number of CTCTT tracts.

```
opaD/cc174/N241.1    ATGAATCCAGCCCCCAAAAAACCTTCTCTTCTCTTCTCTTCTCTT 45
OpaJ/CC174/N241.1    ATGAATCCAGCCCCCAAAAA-CCTTCTCTTCTCTTCTCTTCTCTT 44
opaD/CC60/N114.1     ATGAATCCAGCCCCAAAAAA-CCTTCTCTTCTCTTCTCTTCTCTT 44
opaJ/cc23/N459.1     ATGAATCCAGTCCCAAAAAA-CCTTCTCTTCTCTTCTCTTCTCTT 44
                     ********** ***.***** ************************
```

Figure 4-6: Multiple sequence alignment of the sequences upstream the pentameric repeat tract region of different *opa* loci. The interstrain and intrastrain alignment of *opa* genes showed variability in the sequences of the genes in the poly C and poly A tracts region upstream of the pentameric repeat region. The alignment shows the differences in this region among different loci due to the deletion in the poly C or polyA tracts.

The most common pattern noticed in these tracts within the samples of this study was 5 Cs with 6As upstream of the CTCTT region. Only *opaJ* and *opaD* in strains of cc60 showed the pattern of 4 Cs and 6 As. while having 5 Cs and 5 As was only present in *opaJ* of CC174 strains. In this case, the number of repeats that correlates with the

expression state in this locus is similar to the pattern in *opaD* and *opaJ* of CC60 (4Cs and 6As). In the case of *opaJ* in CC23, one of the cytosine repeats was replaced by a thymine (Figure 4.6), but no deletion happened in this region and the same prediction for the expression state was found as for 4Cs and 6As. All possible combinations of the numbers of the repeat tracts that were detected in these samples and the correlation with the ON state of *opa* expression are summarized in table 4.2.

Table 4-2: The combinations of the number of the C, A and CTCTT tracts that are predicted to give an ON state of *opa* expression in the samples analyzed in this study.

C tract	A tract	CTCTT tract	C tract	A tract	CTCTT tract	C tract	A tract	CTCTT tract
5	**6**	**3**	**5**	**5**	**5**	**4**	**6**	**5**
5	**6**	**6**	**5**	**5**	**8**	**4**	**6**	**8**
5	**6**	**9**	**5**	**5**	**11**	**4**	**6**	**11**
5	**6**	**12**	**5**	**5**	**14**	**4**	**6**	**14**
5	**6**	**15**	**5**	**5**	**17**	**4**	**6**	**17**
5	**6**	**18**	**5**	**5**	**20**	**4**	**6**	**20**
5	**6**	**21**	**5**	**5**	**23**	**4**	**6**	**23**

The expression states of *opa* with different repeat tracts was then confirmed by western blotting. Samples from different carriers representing the four different clonal complexes expressing different Opa variants with different repeat tracts were probed with rabbit anti-Opa polyclonal antibodies that recognize and bind to the fourth loop of the protein which is conserved among the all Opa variants. The results matched the predicted expression state as shown in (Figure 4.7 & Figure 4.8), and more clarification for the data presented in the blots and how they correlate to the repeat tract length is summerised in table 4-3. Two proteins, PorA (different variants) and fHbp variant 1, were utilized as a loading control in the strains analyzed and have been probed with mouse monoclonal anti-PorA antibodies (P1.16, P1.2 and P1.5 depending on the strain analyzed) and mouse polyclonal anti-fHbp antibodies variant 1.

The blot showed that the different OpaA variants were with different molecular weights (figure 4.7) which was consistent with the sequences variability between these variants

in term of the length of the gene and the number of amino acids encoded. In addition samples that predicted to be negatives for all *opa* genes including N138.1, N272.1 and N253.1 were also confirmed in the blot, as they did not show any band at the expected size of Opa. The blot also presents sample that express three Opa variants simultaneously (N59.3, which express three loci), and it was consistent with the predicted expression state of this sample according to the repeat tract length.

Having confirmed the correlations between repeat number and expression state, the next step was to examine the changes in expression of the Opa for each carrier. This data is shown in Figure 4-9 . Generally, the switches in the expression state of *opa* indicated that phase variation in these genes has occurred at high rates within the carriage samples, and in at least one locus in each sample over the different time points. Only the samples of V124 showed no switches in expression as isolates from this carrier were of the same phasotype at the three time points and no switching were noticed in the repeat tract in any of the *opa* loci.

Locus A, in general, displayed strong phase variability as it was subjected to phase variation at a high rate in the samples of 9 carriers with clear fluctuation in its expression over the different time points. On the other hand, no phase variability has been detected in this locus in the samples of three carriers of CC174 (V58, V138 and V52), in the three carriers of CC167 (V117, V64 V124), and two carriers of CC60 (V134 and V115) and its state was OFF over the whole period of the colonisation in those samples.

Contrastingly, phase variation in *opaB* occurred in samples of 14 carriers wherein this locus was selected to be expressed in only one or two time points, except in V117 where the ON state in this locus was observed in all four time points in at least 2 colonies. No switching in the expression of this locus was noticed in the other 5 carriers, and it remained either in an on state such as in samples of V124 (cc167) and V93 (cc23) or in OFF state as in V113, V185 (cc60) and V59 (CC174) throughout the total period of colonisation.

Phase variation of *opaD* was detected in samples of 11 carriers, and this locus was expressed in particular time points rather than others in most cases. While no expression for this locus was detected in the samples of the other 8 carriers such as the 4 carriers of CC23 (except in one colony) and this locus is occupied by the same allele of *opaA* in

samples of this clonal complex. Similarly, *opaJ* was subjected to phase variation in the samples of 11 carriers, while in the other carriers this gene was in the OFF state in all samples of the CC60 carriers (note that this locus is identical with *opaD* that was expressed at a high rate in those carriers) and in two carriers of CC23 (V69 and V93) and V124 (CC167).

However, not all the switching events in the repeat tracts led to changes in the expression state of Opa, and hence the genes stayed in the same expression state so that switching was from the off to off or on to on state but with different repeat numbers such as in *opaA* of V138 and V58. In addition, there was a tendency to express a particular locus at a higher rate than others in the samples analysed, and this varied from one clonal complex to another. 29.7% of cc174 isolates expressed OpaA as a dominant locus while 8.8% of the isolates switched all the genes into the off state. OpaB was expressed dominantly in cc23 samples (i.e. in 51.4 % of the isolates) while ~3 % of the isolates switched all the genes off. OpaB was also dominantly expressed in the other clonal complexes, it was On in 61.2% of the cc167 isolates and in 29.1% of samples of cc60, whereas 11.2 % of cc167 and 20.1% of cc60 isolates had all the *opa* genes in the off state as shown in Figure 4.10. In the case of the identical loci, the expression of only one of them was detected over all the time points with switching from one to another being very rare and noticed only in a few colonies in V88 (cc174) and in one colony in V69 (cc23) (Figure 4.10)

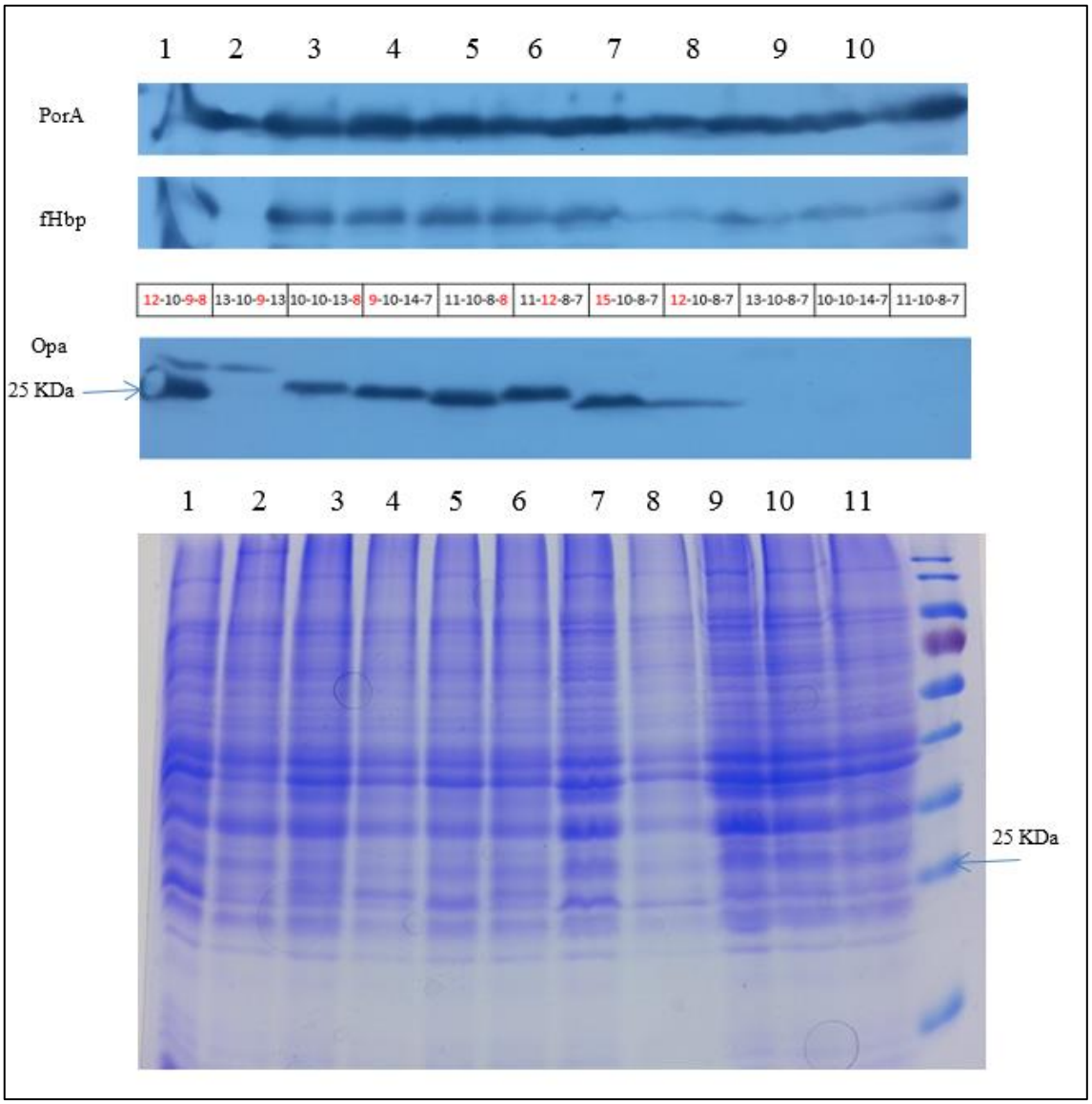


Figure 4-7 **Western blotting analysis of Opa expression**. Proteins from whole cell lysates of carriage isolates of cc174 expressing different Opa variants with different repeat tract length were separated by 12% SDS-PAGE and transferred to PVDF membrane and blotted with primary antibodies as following : rabbit polyclonal anti-Opa antibodies, mouse monoclonal anti-PorA antibodies (P1.16), and mouse polyclonal anti-fHbp V1 antibodies. Samples were loaded as the following from 1-11 : N59.3, N54.1, N369.1, N88.1, N352.2, N424.1, N354.1, N51.1 N138.1, N272.1 and N253.1. Numbers on the Opa blots refer to the repeat tract number of the four *opa* genes in each sample in the order ***opaA-opaB-opaD-opaJ***, the red colour refers to the genes in the phase **ON**, and the black colour refers to the genes in the phase **OFF**. PorA and fHbp protein were utilised in the experiments as a loading control.

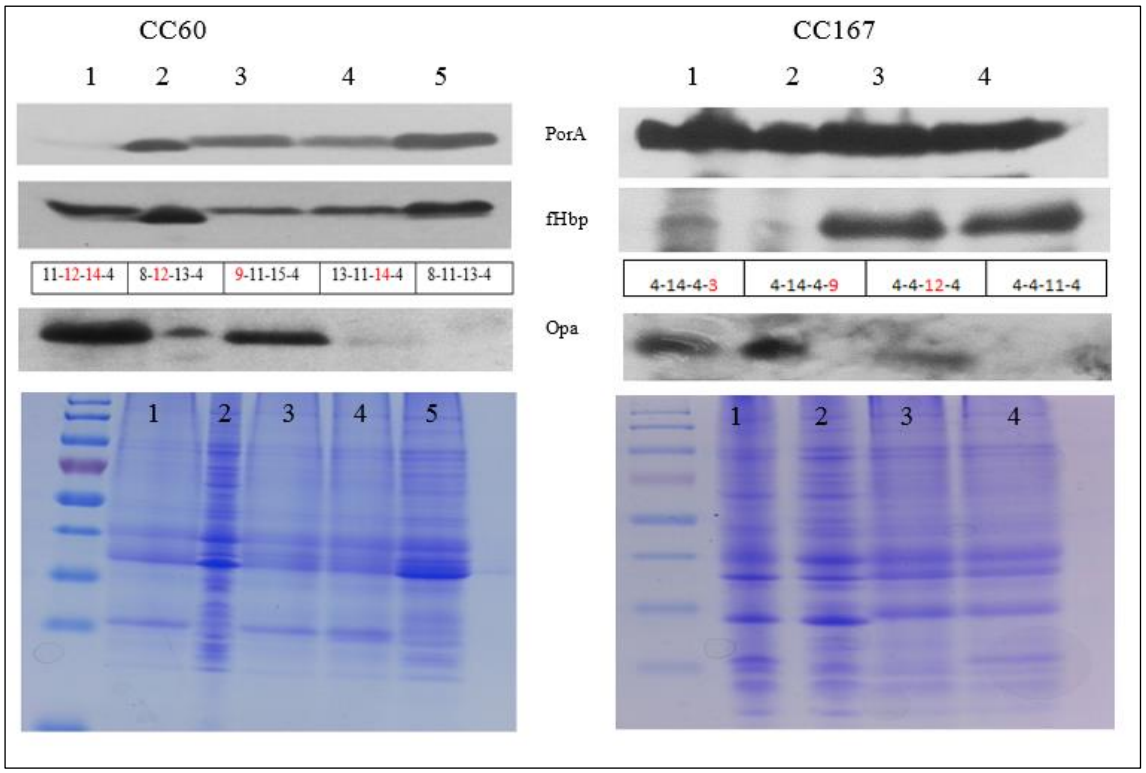


Figure 4-8 Western blotting analysis of Opa protein expression carriage samples of cc60 and cc167. Samples of these clonal complexes expressing different Opa variants with different repeat tract length were analysed by western blotting to confirm and correlate the expression state of different Opa variants with the repeat tract lengths. The details of the western blot are the same as the previous figure except the anti-PorA variants are different in this experiment in which anti-PorA variant P1.2 were used for samples of cc60, and P1.5 were used for cc167 samples. Samples were loaded as following: cc60 (1-5) including: N114.1, N333.1, N330.1, N113.1 and 134.2, while the cc167 samples are (from 1-4): N332.2, N348.1, 117.1 and N284.1.

4.7 The multiplex expression state of Opa proteins in meningococcal isolates

Switching between expression states of the four *opa* loci can result in a heterogeneous population with sixteen possible phenotypes expressing either no Opa, one or multiple Opa variants (Hauck and Meyer 2003). To assess such heterogeneity in the carriage isolates, the expression states of the four *opa* genes were combined together in each isolate which is then termed as a "phasotype" and given four digit numbers as a code that refers to the expression state of each locus, 0 refers to the gene in the OFF state and 1 refers to the gene in the ON state. Generally, the total number of the different phasotypes that have been detected in the whole data set was 13 out of 16 potential phasotypes, however samples from different carriers and different clonal complexes varied in the observed number of phasotypes. The highest heterogeneity was noticed in the CC174

isolates which, in total, displayed 11 different phasotypes, with samples from V88 and V59 (CC174) showing the highest diversity as they switched between 8 and 7 different phasotypes respectively .

Table 4-3: the predicted expression state of Opa variants in samples analysed by western blot according to the repeat tract length as well as the data from western blotting. The phase variable pentameric repeats in the coding region of each *opa* gene are preceeded by poly C and poly A tracts which are not phase variable but they vary in their number between different loci and different strains of *N. meningitidis* and their number in combinations with the CTCTT tracts number determines whether the gene is in frame or not**.**

Meningococcal samples (according to the clonal complex)	*opa* gene	Repeat tract number			Predicted expression state (ON/OFF)	Expression state confirmed by western blot
		No of C tracts (fixed)	No of A tracts (fixed)	No of CTCTT Tracts (variable)		
CC174	*opaA*	5	6	9	ON	ON
				10	OFF	OFF
				11	OFF	OFF
				12	ON	ON
				13	OFF	OFF
				15	ON	ON
	opaB	5	6	10	OFF	OFF
				12	ON	ON
	opaD	5	6	8	OFF	OFF
				9	ON	ON
				13	OFF	OFF
				14	OFF	OFF
	opaJ	5	5	7	OFF	OFF
				8	ON	ON
				13	OFF	OFF
CC60	*opaA*	5	6	8	OFF	OFF
				9	ON	ON
				13	OFF	OFF
	opaB	5	6	11	OFF	OFF
				12	ON	ON
	opaD	4	6	13	OFF	OFF
				14	ON	ON
				15	OFF	OFF
	opaJ	4	6	4	OFF	OFF
CC167	*opaA*	5	6	4	OFF	OFF
	opaB	5	6	4	OFF	OFF
				14	OFF	OFF
	opaD	5	6	4	OFF	OFF
				11	OFF	OFF
				12	ON	ON
	opaJ	5	6	3	ON	ON
				4	OFF	OFF
				9	ON	ON

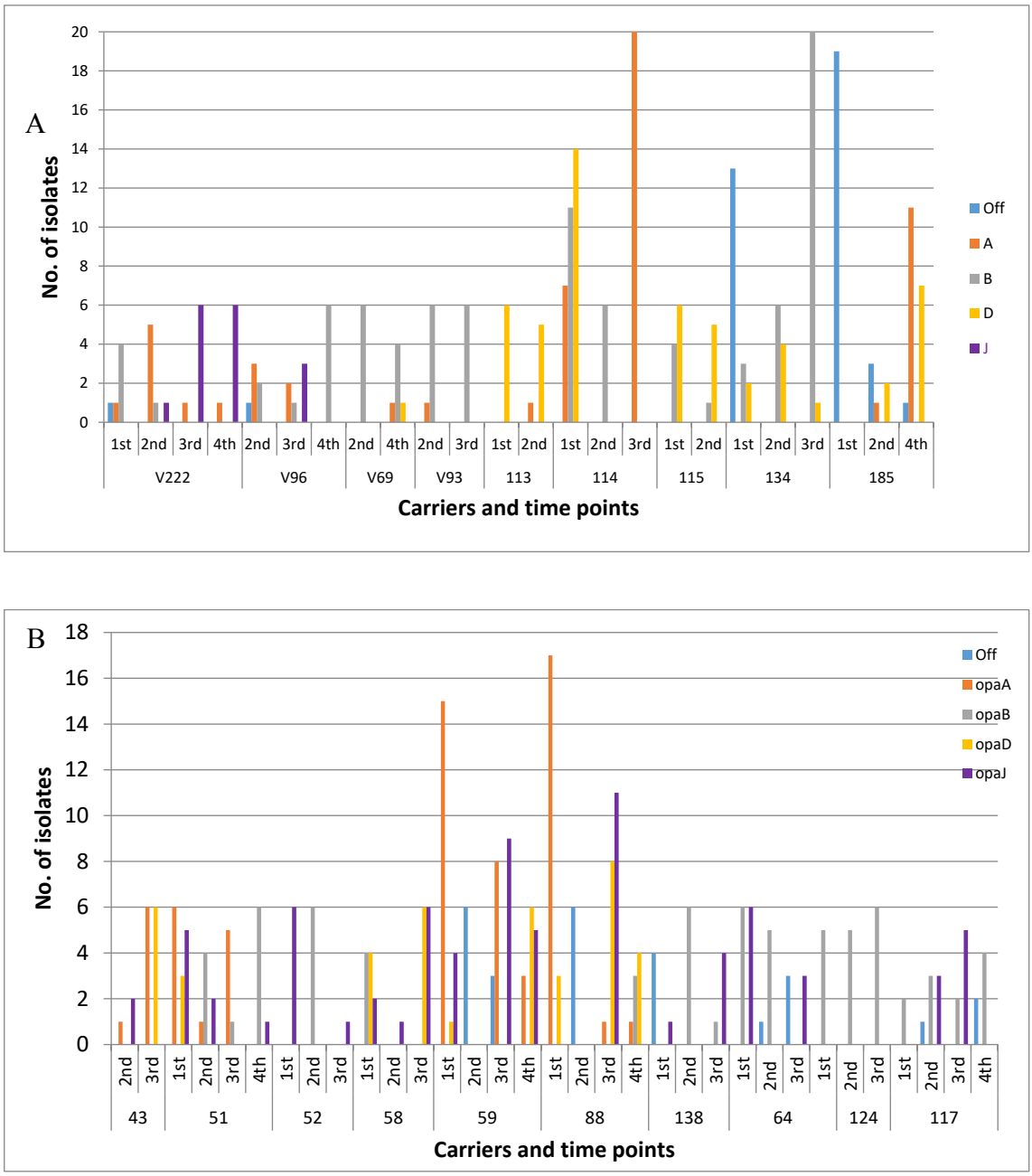


Figure 4-9: The expression of *opa* genes in the meningococcal carriage isolates at different time points. The expression of *opa* genes were correlated with the repeat tract length in each locus in the carriage isolates in the different time points. The graph shows the number of isolates with an On repeat number for each Opa and isolates where all the Opa were switched off. A: samples of carriers off cc23 (V222-V93) and cc60 (V113-V185), B samples of carriers of cc174 (V43-V138) and cc167 (v64-v117), the number of isolates analysed in each time point ranged from 1 -20isolates.

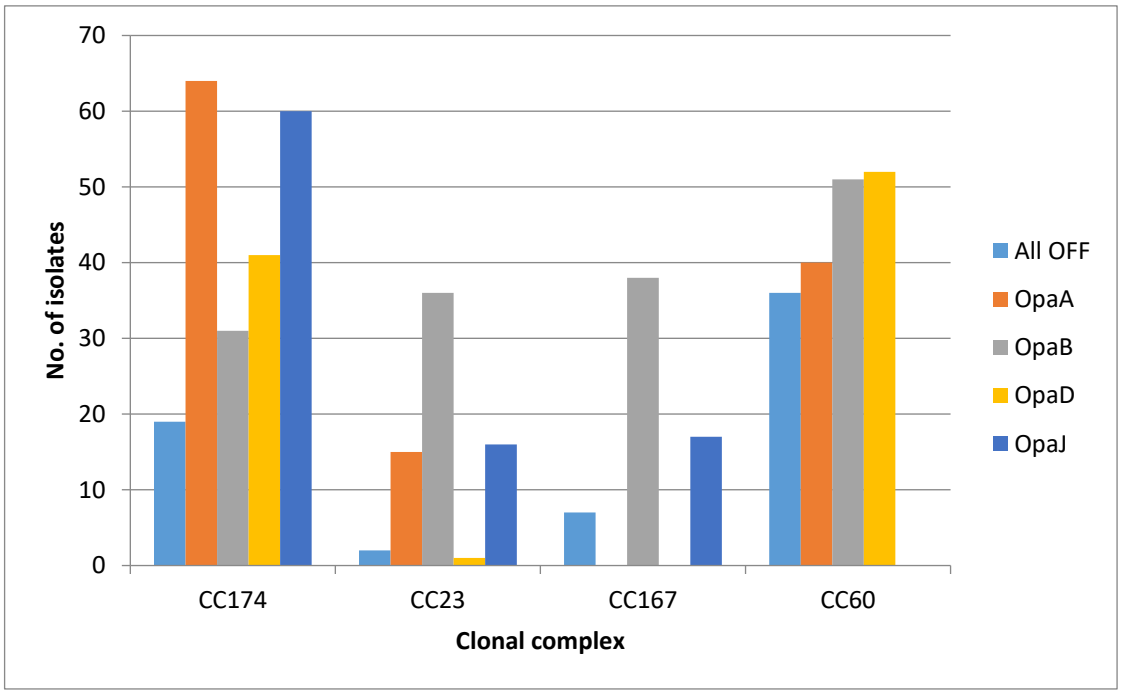
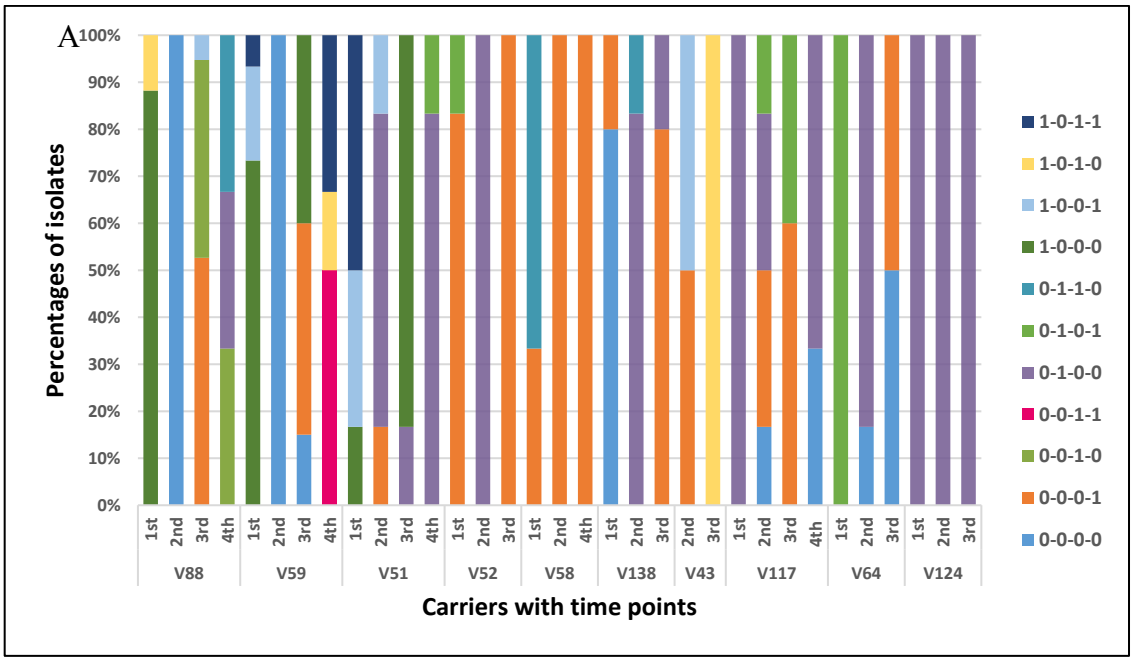


Figure 4-10 **The overall expression states of different *opa* loci in the samples of each clonal complex.** The expression of the four *opa* loci varied in samples from different clonal complex, and some loci expressed at a high rate while others were not expressed in any samples either due to the presence of identical alleles or due to the short repeat tracts which stabilize the expression of the gene at a particular expression state. Data in the graph indicate the rate of expression of each gene, which can be either individually or in a combination with other genes, in the total samples of each clonal complex.The total number of isolates analysed in each clonal complex are : CC174 :169 isolates , CC60: 152 isolates, CC23: 65 isolates and CC167: 53 isolates.

Whereas, the lowest diversity was noticed in samples of CC167 especially the carrier V124 as all isolates from this carrier were of the same phasotype, while isolates of the other two carriers were switching between only 4 different phasotypes (Figure 4.11A&B). Such variability may be related to the differences in the number of the isolates from each carrier and each clonal complex. It may also relate to the diversity of *opa* alleles within the strain, as the presence of identical alleles led to expression of only one of them as the previous data showed that (see section 4.5), and this might be the reason for the low diversity of V124 samples as these strains have only 2 different *opa* alleles (section 3,Table 3-3).



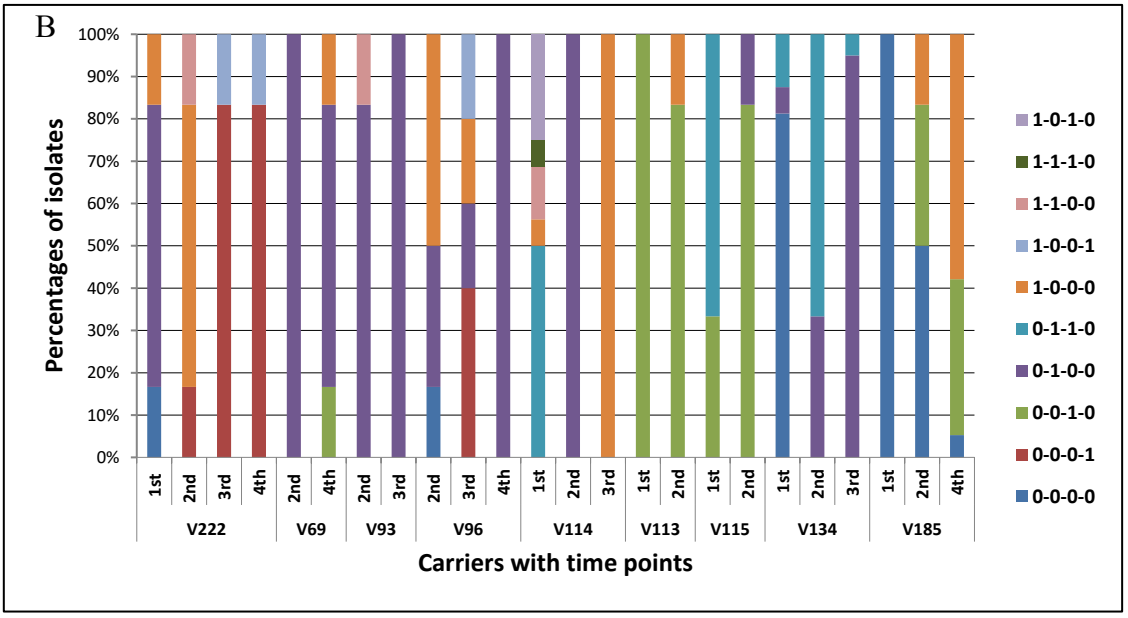


Figure 4-11 the switching of the carriage isolates between different phasotypes during persistent carriage. A total of 12 phasotypes expressing different number and combinations of Opa proteins were detected in the carriage samples from the 19 carriers. The figure A presents the data for samples of 9 carriers of cc23 (V222-V96) and cc60 (V114-V185) strains with 10 different phasotypes, whereas figure B presents the data for the carriers of CC174 (V88-V43) and CC167 (V117-V124). (e.g. 0-0-0-0 indicates that all four *opa* loci are in the OFF state while 1-1-1-0 indicates that *opaA*, *opaB* and *opaD* are ON but *opaJ* is OFF).

4.8 The expression of Opa repertoire at the population level during the persistent carriage

The data on Opa expression was analysed with all samples combined together in order to assess the expression state of the Opa repertoire at the population level during persistent carriage. Generally, expression of a limited number of Opa variants was dominant, as around 70% of the isolates expressed only one locus at any given time point, whereas about 14% of the isolates were in the phase OFF for all Opa proteins. Furthermore expression of more than two loci simultaneously within an individual isolates was very rare (~ 1% of the total samples) while no isolates were found to simultaneously express all the four Opa proteins (Figure 4.12).

To determine the trends in expression of *opa* loci during persistent carriage, *opa* genes were firstly divided into two groups depending on their expression rate in the initial time points. The first group involves the *opa* genes that were switched ON in lower than 65% of the isolates in the first time-point while the second group represents *opa* that were switched ON in ≥65 % of the isolates in the first time point.

The mean expression score was then calculated for each gene in each carrier from the individual expression scores (ranged from 0-1) for up to twenty isolates per time point (Figure 4.13). The initial time point was compared to the final observed time point, which represents one month (1^{st} -2^{nd}), three months (1^{st} -3^{rd}), five months (2^{nd} -4^{th}), and six months (1^{st}-4^{th}) of carriage. The data from many carriers presented a clear switching between different Opa variants over the two time-points, and in many cases, genes that started with a high frequency ON state tend to be switched OFF or expressed at a lower rate at the later time points and vice versa for genes that start in the OFF state. In cc174 carriers, for example (see Fig. 4-12), *opaD* was off in the samples of 6 carriers (expression score 0), while in the later time points this gene was in an On state in samples of three of these six carriers with expression scores of 1, 0.6 and 1 respectively. Whereas, *opaA* was on in samples from the initial time points of three cc174 carriers with an expression score of 1 (i.e. all isolates from this time point had the gene switched on), while in the last time points the expression score of this gene was reduced in these carriers to 0 in two of them and 0.1 in the other carrier.

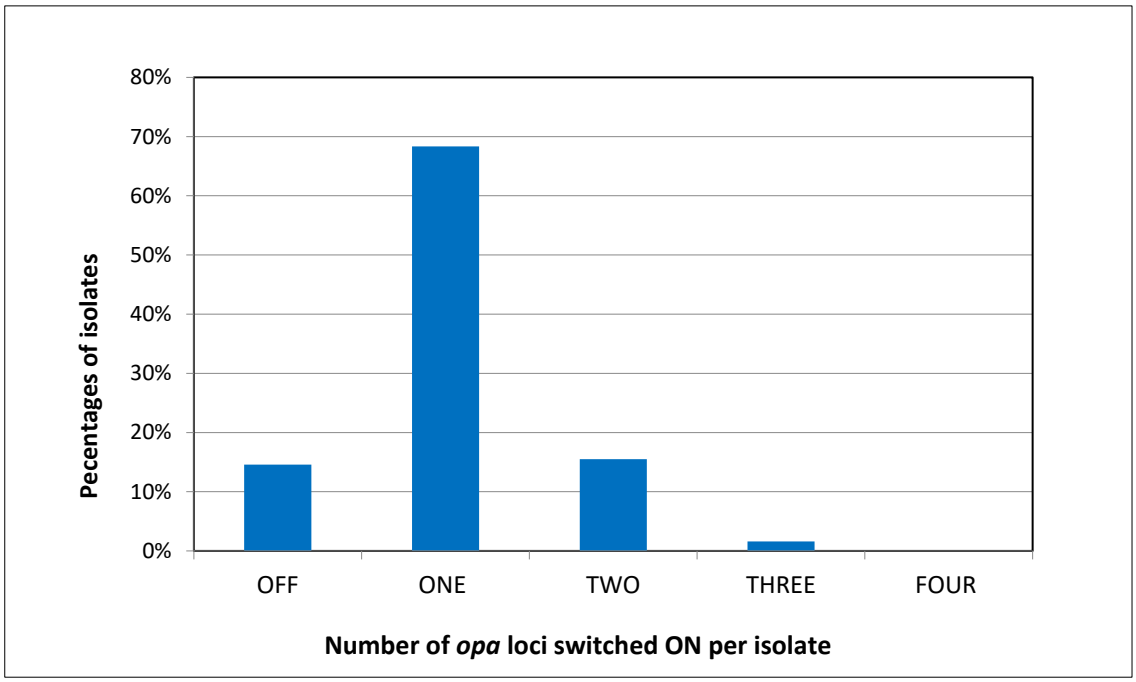


Figure 4-12 Percentages of the carriage isolates according to the number of *opa* loci switched ON. The phase variability of the four *opa* genes can result in each isolate expressing a different number of Opa variants ranging from no Opa to one or multiple Opa variants. This figure shows the overall percentages for the combined data of every isolate obtained from 19 carriers (n= 439).

The data for each group were combined together and examined by Wilcoxon matched-pairs signed rank test for the significant changes, and the analysis indicated that statistically significant changes occurred in mean expression between the initial and the final time points for Opa genes. However, the overall expression state of the Opa repertoire were assessed by comparing the mean expression of the four *opa* genes (scored 0-4) for the whole data set between the initial and final time points as shown in Figure 4.14. The data indicated that apart from the switching between different variants, a constant expression for one or more *opa* genes during prolonged colonisation.

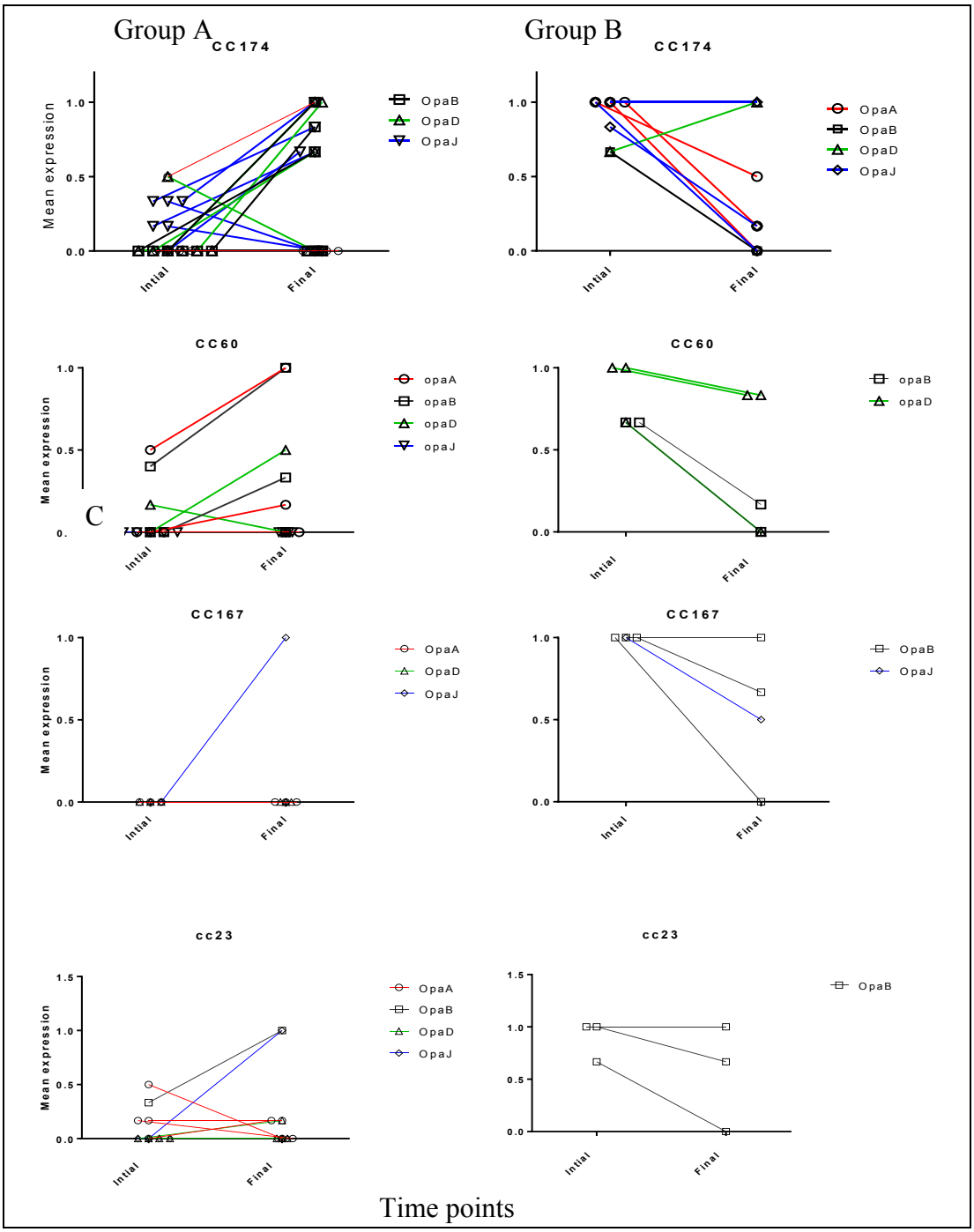


Figure 4-13 : **Changes in the mean expression of each Opa variant in samples of different carriers** . The mean was calculated for each Opa protein from the expression scores of up to twenty isolates per time point. The Opa genes were divided into two groups depending on their expression rate at the initial time point, group A :genes that were OFF or switched ON in lower than 65 % of the isolates in the initial time point, group B:Opa genes that switched ON at ≥ 65 % of the isolates.

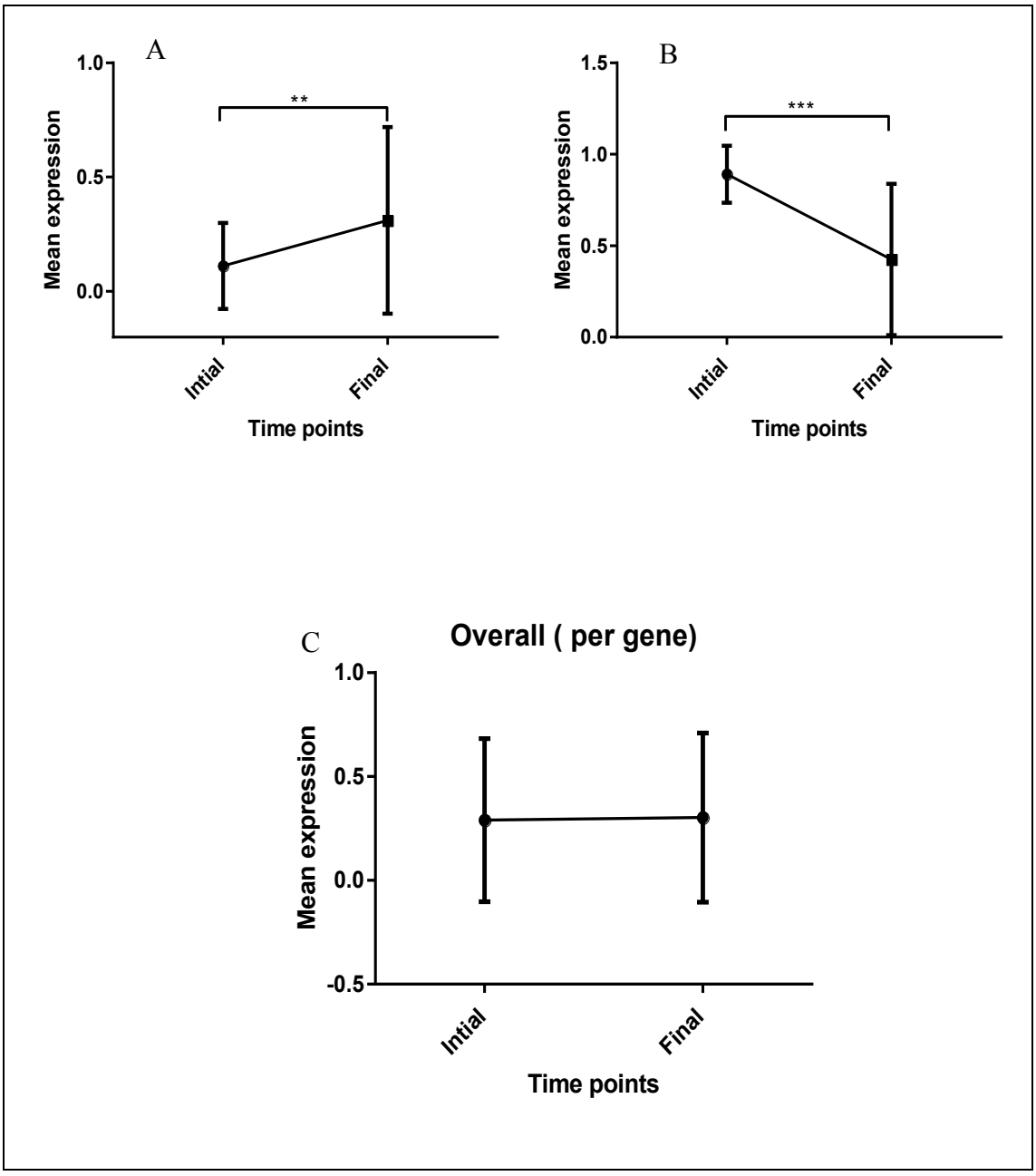


Figure 4-14: **The overall trend of the mean expression of Opa repertoire during the persistent carriage.** A mean expression score was calculated for each Opa protein from the expression scores of up to twenty isolates per time point. Graph A&B: the overall trend of the mean expression of *opa* genes (scored 0-1) of the two groups for all the carriage samples, graph C: the mean expression of the four-opa genes (scored 0-4) for all the samples analysed. Data in graph C&D were analysed by Wilcoxon matched-pairs signed rank test for the significant changes (** P= 0.0078, *** P=0.0001). The data presented as the mean with SD.

4.9 Receptor overlay dot blotting using soluble chimeric CEACAMs and meningococcal whole-cell lysates.

The specificity of the different Opa variants characterised in this study for binding CEACAMs receptors were investigated in order to determine whether the switching between different variants was related to their different functions, as well as to investigate whether homologous recombination led to changes in the specificity or the functionality of Opa variants. The assay involved blotting the whole cell lysates of the meningococcal strains expressing one of the Opa variants with the different purified human CEACAMs receptors including CEACAM1, 3 and 6, while mutated CEACAM1 (deleted N domain) was used as a negative control in the assay.The expression of the different Opa variants in different isolates were confirmed firstly by western blot and then whole cell lysates of 16 isolates expressing different Opa variants (each isolate expressed only one Opa variant) were prepared by freeze and thaw. Samples were normalised to an OD_{260} of 0.5 and 50 microliters (equivalent to 10^7 cells) were loaded into nitrocellulose strips, air dried and then blocked for 1 hr using blocking buffer containing 3% bovine serum albumin. This membrane was overlaid with CEACAM-Fc fusion receptors (0.25 µg/ml) in 5 ml of blocking buffer for 1 hr at room temperature. The binding was detected using anti-human Fc/alkaline phosphatase secondary antibodies. The lysates were also blotted against the secondary antibody only as a further negative control. The data showed that 16 variants out of 18 Opa variants bind to CEACAM1 but not CEACAM3 and CEACAM6 (Figure 4-15). In addition, the two new Opa variants of CC23 isolates generated via homologous recombination were found to have functions that differ from the old variants, thus OpaA of N370.5 does not bind to CEACAM1 while the old OpaA of samples of cc23 binds to the CEACAM1. Similarly, the OpaJ of N459.1 gained the ability to bind to CEACAM1 (but with lower affinity in comparison to the other variants), whereas the old OpaJ of the first and second time points of the same carrier (the same variant of N370.4) does not bind to CEACAM1. Finally, no binding was detected to the other CEACAM receptors by any of the variants tested (figure 4-15). However, the lack of +ve control for binding to the CECAM3and CEACAM6 was a big limitation for this assay, although Opa variants that binds to CEACAM3 and 6 tend to be rare, but it was crucial to involve such control in the assay to confirm the binding or not of these Opa variants to one of these receptors

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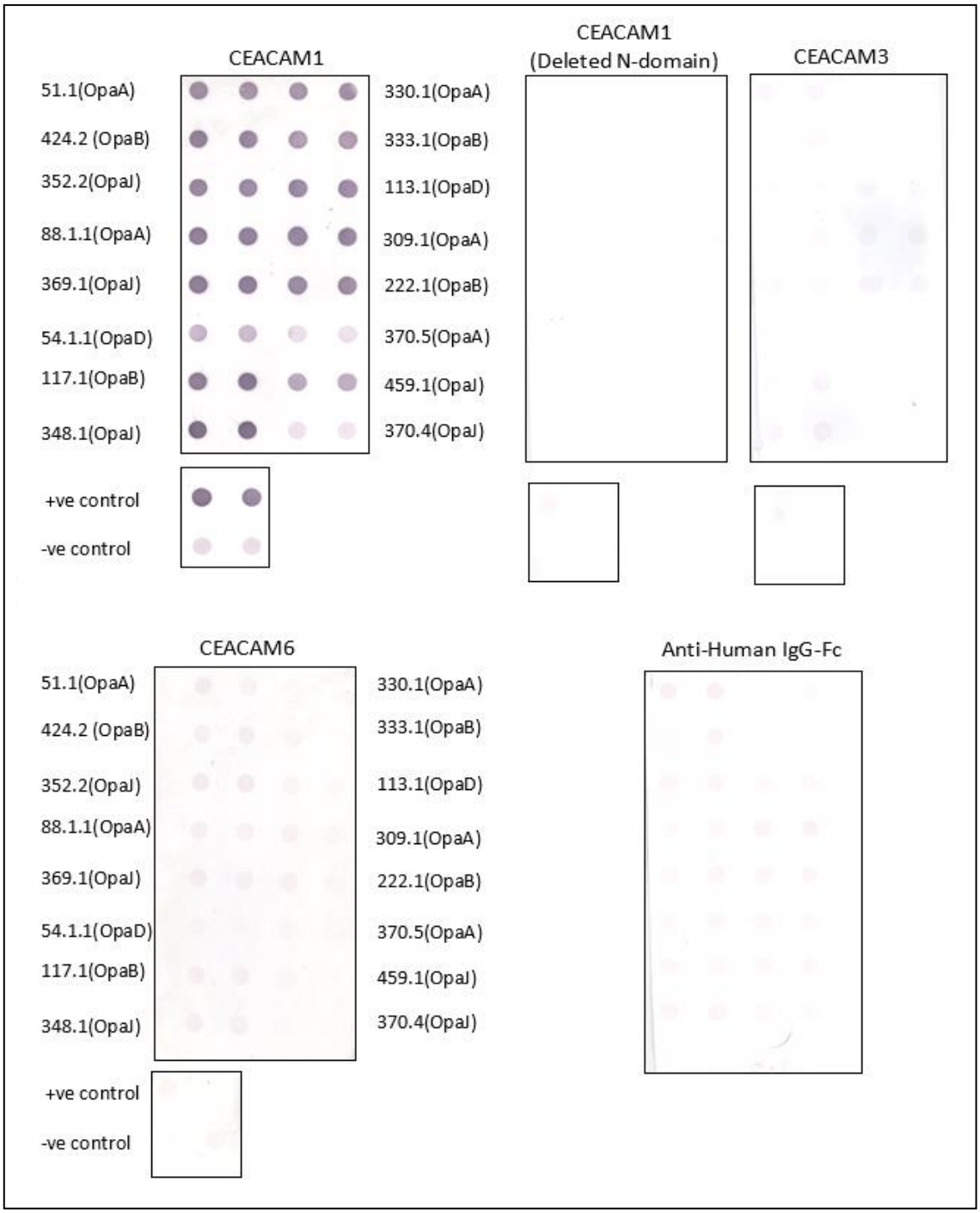


Figure 4-15: **Receptor overlay dot blotting using soluble chimeric CEACAMs and meningococcal whole-cell lysates.** Whole cell lysates were prepared by freeze and thaw from 16 different carriage isolates expressing single different Opa variants. Cell lysates were then normalised to OD_{260} to 0.5 in PBS and 50 µl (around 10^7 cells) were loaded on to nitrocellulose strips. The membrane was then air dried and blocked for 1 hr using blocking buffer (PBS containing 3% bovine serum albumin and 0.05% Tween-20). Then the membrane was overlaid with CEACAMs-Fc receptors (0.25 µg/ml) in 5 ml of blocking buffer for 1 hr at room temperature. The binding was detected using anti-human Fc/alkaline phosphatase conjugate (1:1000) and the signal developed by using nitro blue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate as substrates.

4.10 Discussion

Studying the carriage state of *N. meningitidis* is essential to improve our understanding about the epidemiology and pathogenesis of these bacteria (Caugant and Maiden 2009), as well as the basic mechanisms that enable the bacteria to adapt to the human host and to evade the immune response.

Phase variation mediated by changes in the SSR is one of the mechanisms that have been reported to enable the bacteria to persist in the human host (Tzeng and Stephens 2000). Many surface antigens of *Neisseria meningitidis* are subject to PV due to the presence of SSR in their promoter or coding region (Martin *et al.* 2003a). The latest study of the PV of eight of these antigens indicated a high mutation rate in the SSR region with a trend towards a reduction in the expression level of one or combinations of these antigens during the persistent carriage (Alamro *et al.* 2014). However, little is known about the PV in Opa protein of *Neisseria meningitidis* and its impacts on the expression state of the repertoire during prolonged colonisation. In the current study, PV in the Opa coding genes and its effects on expression were investigated in sequential meningococcal samples isolated from different carriers at time points spanning up to six months of carriage state. The data from the GeneScan analysis indicated that high rates of mutation occurred in the repeat tracts of *opa* loci in samples from 19 carriers, and that mutations happened in at least two loci within samples of individual carriers between each pair of time points. The rate of alteration in the SSR was estimated to occur at 0.06 mutations /gene/ month during the carriage state. This rate was consistent with the rate of PV of the other eight genes investigated in the meningococcal isolates which was also estimated to occur in those genes as combination at a rate of 0.06 mutations /gene/ month (Alamro *et al.* 2014).

However, the different *opa* loci showed different rates of switching in their repeat length within samples of the same strains and of different strains. The mutation rate was calculated for each locus as following: *opaA* 0.1, *opaB* 0.08, *opaD* 0.04 and *opaJ* 0.02 mutations /gene/ month. Such variability can be mainly correlated with the length of the repeat tracts of each locus, as genes with longer repeat length are subject to phase variation at higher rate than those with short repeat (Power and Moxon 2006; Sadarangani *et al.* 2016). *opaA* was correlated with long repeats in most of the samples analysed whereas *opaJ* was mainly with short repeats in the majority of the samples. The effect of the repeat tract length on the switching rate was obvious with the highest fraction of switching events (33%) occurring in loci with repeat lengths within a range of 13-21

CTCTT. Contrastingly changes in repeat length within loci with short repeat (lower than 7 CTCTT) were very rare and in most cases these tracts remained stable over the total period of colonisation. A critical point is that a reduction in the repeat length to a short number (<5 repeats) led to stabilisation of the gene at this length and a constant expression state over different time points. Conversely, some loci with high numbers of repeats such as *opaB* in V59 and V88 (10 CTCTT), and *opaB* of V124 (12 CTCTT) remained relatively stable at a constant repeat length over the different time points. This may be related to the expression state of this locus, which might be selected under particular conditions to be on or off resulting in stabilising the repeat length in this locus over the period of colonization, or these were protected by expression of other surface structure such as capsule or LPS. There was also an association between the length of colonisation and mutation rate, as a high frequency of alteration in the SSR happened in samples between the 2nd and 3rd (after 3 month of colonisation), which reflects the effects of the long term of carriage on the mutability rate of the SSR of the surface antigens.

Investigation of the expression state of Opa indicated that the alteration of the SSR resulted in a highly diverse meningococcal population with mixed phasotypes ranging from switching all the loci off to expression of one or multiple loci. Samples from different carriers varied in their diversity and some of them were less diverse especially carriers of cc167 in comparison with samples of other clonal complexes. This can be mainly related to the length of the repeat tracts as well as the presence of identical alleles in the different loci of those strains. Three loci of isolates of V117 and V124 (no phase variability were noticed in the any samples of latter carrier) and two loci of the isolates of V64 had repeat lengths between 3- 4 CTCTT, and loci at this repeat number tend to be stable with no change in number. In addition, three loci of V124 samples were occupied by the same allele, which in this case switching of only one of them "on" is the dominant pattern rather than switching between them. This can be supported by the high diversity noticed in the isolates of cc174, whose Opa loci were occupied with four different alleles with a long repeats in general. Another factor that may mediate such variability in the diversity of samples from different carriers is the immune response, which can play a role in shaping the antigenic repertoire of the meningococcal isolates during persistent carriage (Bidmos *et al.* 2011). This can depend on the immunological background of each carrier mainly in terms of their susceptibility and previous colonisation by one or more strains of *N. meningitdis* or other commensal *Neisseria.*The previous exposure to

different strains of *N. meningitidis* confers cross protection against the in-coming strain by eliciting bactericidal antibodies targeting the surface antigens including Opa proteins (Yazdankhah and Caugant 2004b). Therefore, the expression of a particular allele will be affected when the carriers were previously exposed to a similar allele, and Opa expression states will be disadvantageous leading to selection for expression of other Opa variants. This will be in contrast to when the carrier is naive to colonisation by a similar strain and so an in-coming strain might express one or more Opa variants.The effect of the age of individuals is obvious regarding the rate of meningococcal carriage state, and different age groups show a clear variability in the rate of carrying of *N. meningitids* (Christensen et al. 2010), whereas there is no strong variability in the carriage rate between males and females (Yazdankhah and Caugant 2004a). The age of carriers had also shown to correlate with the level of salivary IgA towards meningococcal OMVs. This was tested with distinct age groups involving infants, toddlers (3-4 years) children (7 years) and adults (>18). The study showed that the level of IgA against a range of meningococcal OMP is increased sucessivly with the age and current carriage and it is higher in the adults group in comparison to the other groups. (Horton *et al.* 2005). The study also suggested that transient colonization with meningococcal strains leads to acquisition of mucosal immunological memory towards *N. meningitidis.* This is expected to be happened with the carriers of this study according to their age, and they are expected to be previously exposed to different meningococcal strains. Therefore, such potential immune response in the carriers can mediate the high rate of Opa PV noticed in the carriage isolates analysed in this study, which then enable the bacteria to avoid such immune response towards this protein. The possible preimmunisation of carriers with meningococcal vaccine may not have a specific effects on the data obtained about the PV of Opa. As vaccines (such as MenC) depends mainly on capsular polysaccarides immunogenicity and the presence of an Opa variant as a part of meningococcal vaccine can be in the OMV involved in Bexero vaccine which was not introduced during the carriage study when these isolates sampled from those carriers. Populations with diverse antigenic variants expressing different numbers and combinations of Opa proteins confers functional flexibility to the bacteria in terms of enhancing their transmission among different hosts or to persist and evade immune response within individual carriers (Power and Moxon 2006; Callaghan *et al.* 2008b).

A trend to express a particular locus at higher rates than other loci have been noticed in samples of different clonal complexes such as expression of OpaB at a high frequency in samples of CC167 and CC23 and OpaA in CC174. This trend may also relate to the functionality of the Opa variants along with the possible effect of immune response in shaping the antigenic repertoire as mentioned before. The different Opa variants were proved to display different roles in the attachment to and invasion of human epithelial cells and their functionality has ranged from no effect, intermediate to a significant effect on the both processes (Virji *et al.* 1993). Thus, *opa* loci with an essential function might be expressed more frequently than others with a less important role. This also can be correlated with expression of only one of the identical alleles, as the two identical alleles must have the same function and expression of one of them might be enough for the cells to gain function.

At the population level, one of the key findings is that a limited number of Opa variants were expressed in each carriage isolate and the majority of the samples analysed (~70%) switched ON only one locus with a 14 % of the total number of samples switching all the loci OFF. Whereas, phenotypes expressing more than two loci were quite rare and no isolates were detected to switch the four *opa* genes ON. Such patterns can be correlated with the high immunogenicity of the Opa proteins and the role of the immune response in selecting phenotypes displaying lower numbers of Opa variants on their surface membrane. On the other hand, there was a trend for switching between different Opa variants in the earlier and later time points. A significant reduction in the expression of Opa variants was observed when a high proportion of isolates were in the ON state at the initial time point. This switch may be due to extended exposure to an antibody response elicited against those specific variants that resulted in selection for OFF variants. Simultaneously, there was selection for isolates switched other variants ON that were switched OFF or expressed at low rates in the early stages of colonisation. Such selection is potentially mediated by the functionality of Opa proteins to keep their role in the intimate adhesion to the epithelial mucosa and then enable the bacteria to persist for a long period of colonization. This was also consistent with the constant expression of at least one locus during long periods of colonization (apart from the switching between different variants), which may maintain the Opa-mediated adhesion to the human epithelial cells.

Testing the specificity of the different Opa variants to their CEACAMs receptors showed that the majority of the variants tested bind to CEACAM1 rather than CEACAM3 and CEACAM6. Although these results were consistent with the many studies that have revealed that the majority of the Opa variants bind to CEACAM1, and only a few variants bind to the other CEACAMs receptors as well as the HSPG receptor (Sadarangani *et al.* 2011), it was important to involve a positive control for binding to the CEACAM3, 6 with the assay to confirm the specificity of Opa variants to CEACAMs. However, these data indicate that switching between different Opa variants during long periods of colonisation was with maintaining the specificity of the expressed Opa for the CEACAM1 receptor enabling the bacteria to keep the same function during switching between different variants.

One of the key findings of the receptor specificity experiments is that the new *opa* alleles (generated by intergenic and intragenic recombination as shown in chapter 1) have different functions from the older parental alleles. OpaJ of the isolates of the 3rd and 4th time points of V222 was shown to bind to CEACAM1, while the parental OpaJ variant did not bind to this receptor. In addition, OpaA of the isolates of the later time points of V96, which were generated from intragenic recombination between *opaJ* and *opaA* of the isolates of the first time points of this carrier, does not bind to CEACAM1. This new allele was composed of the HV1 of *opaJ* and the HV2 of the older *opaA*,(see chapter 1 Figure , 1-5) this exchange and replacement of the HV regions may have led to the loss of the function of this new allele. Previous work has shown that hybrid Opa variants, generated by combining HV-1 and HV-2 regions derived from other alleles have a reduced binding to CEACAM1 and CEA in comparison to the parental alleles. This published work indicated that particular combinations of HV-1 and HV-2 with particular motifs are required for each Opa to bind to each type of CEACAMs receptor (De Jonge *et al.* 2003a). However, it is likely that intergenic and intragenic recombination events also led to generation of new opa alleles with altered antigenic structures, which can also enable the bacteria to evade the immune response.

In summary, this study indicated that the carriage state was correlated with a high rate of variation and switching between different Opa variants, driven by high repeat numbers, the presence of identical alleles, and the length of persistence within the host. A selection of isolates expressing a limited number of Opas and a significant reduction in the Opa variants switched ON at a high rate in the early colonisation time points was one of the

key findings of this study. The synchronized selection for other variants that were OFF or expressed at low rates in the early stages suggested the potential role of PV of this protein to evade the immune response during persistent carriage, which reflects a role for immunity in shaping the Opa repertoire of meningococcal populations. Finally, the stable expression of at least one locus during persistent colonisation provides a further confirmation for the essentiality of these proteins for the meningococcal cells to colonies the human nasopharynx.

5 Phase variation of Opa proteins mediates the escape of bactericidal activity of polyclonal antibodies

5.1 Introduction

N. meningitidis is one of the major causes of severe sepsis and meningitis around the world. This bacterium is an obligate commensal and prolonged colonization of the human host results in continuous exposure to the immune system (Lo *et al.* 2009). In order to colonize as well as to survive in the bloodstream and transmit from one host to another, the bacteria have several different mechanisms to evade the immune system and to survive in different environments (Pizza and Rappuoli 2015a). The alteration of surface structures by phase and antigenic variation are proposed to be key mechanisms for this bacterium to evade the immune defenses as well as to persist and colonize the host (van der Woude and Baumler 2004).

Complement-mediated bactericidal activity plays a significant role in the protection against meningococcal diseases. One of the well-known mechanisms that is used by this bacterium to evade complement mediated killing is the expression of capsular polysaccharide and lipooligosaccharide (Lewis and Ram 2014). However, phase variation mediating the change in a surface structure of the meningococcus cells has also been proved to mediate the escape of the bacteria from the complement-mediated bactericidal activity. Two *in vitro* studies have proved that successive incubation of the bacteria with specific bactericidal monoclonal antibodies targeting particular surface antigens (PorA, and LPS) in the presence of human complement led to a rapid development of meningococcal populations that are resistant to the bactericidal activity. This resistance was associated with the switching in the repeat tract length in the promoter or in the open reading frame of the genes encoding for those surface proteins which led to changes in the expression state either from a high level to low level (PorA) or from an On state to Off state (LPS) (Bayliss *et al.* 2008; Tauseef *et al.* 2013).

The expression of Opa proteins, which mediate the adhesion and invasion of the meningococcal cells into human tissues, are controlled at the translation level by phase variation mediated by the alterations in repeat number (CTCTT) for a tract present in the open reading frame during chromosomal replication. Changes in the repeat tracts of the multiple copies of the genes encoding for Opa variants can lead to development of heterologous meningococcal population expressing different numbers and combinations

of Opa variants which confers fitness to the bacteria to evade the immune response as well as to colonise different niches within the human host (Sadarangani *et al.* 2011). Apart from their biological role, Opa proteins have gained importance as a potential vaccine. Injection of mice with recombinant Opa proteins that represent the highly prevalent variants elicited the production of serum bactericidal antibodies, and those highly immunogenic variants can be utilised as a potential vaccine against hyperinvasive lineages (Callaghan *et al.* 2011). Phase variation of Opa proteins and their role in mediating the evasion of the bactericidal activity of anti-Opa antibodies has not been studied before. Such a potential role has been investigated in this study using the modified serum bactericidal assay described before by Bayliss et al (2008). The assay involved incubation of a meningococcal population of strain MC58 for multiple cycles with mouse poly clonal anti-Opa antibodies that specifically target the OpaD variant of strains H44/76 and MC58. The findings of this study highlight the biological significance of the P.V. of Opa proteins regarding their role in immune evasion and have implications for involving Opa proteins in meningococcal vaccines.

5.2 Testing the complement sensitivity of meningococcal strains

As the escape assay involved incubation of the bacteria with the polyclonal antibodies accompanied with human serum as a complement source, it was necessary to test whether the meningococcal strains that will be used in the assay are sensitive to the complement source in the absence of the antibodies. This is to avoid any nonspecific killing mediated by the complement source and to ensure that any bactericidal activity will be mediated by only the antibodies. Therefore, the sensitivity of meningococcus strain H44/76 was tested by incubating the bacteria with different concentrations of human serum for one hr at 37 ^{0}C and then the cfu counts were determined (see section 2.11). This assay showed that this bacterial stock was very sensitive to the complement source even with a low concentration of 5 % in comparison to the heat inactivated serum as a control (figure 5-1). The bacteria were also sensitive to the baby rabbit sera which were used as an alternative source of complement. To solve this problem, another strain was tested in the assay which is the reference strain *N. meningitidis* MC58. This strain showed a strong resistance to the human sera at two different concentrations 5% and 10% (figure 5-2). Therefore, this strain could exhibit escape in this assay rather than H44/76, as it is identical with H44/76 in three Opa variants (OpaA, OpaB, and OpaJ) and the antibodies generated against H44/76 Opa variants can be used with this strain.

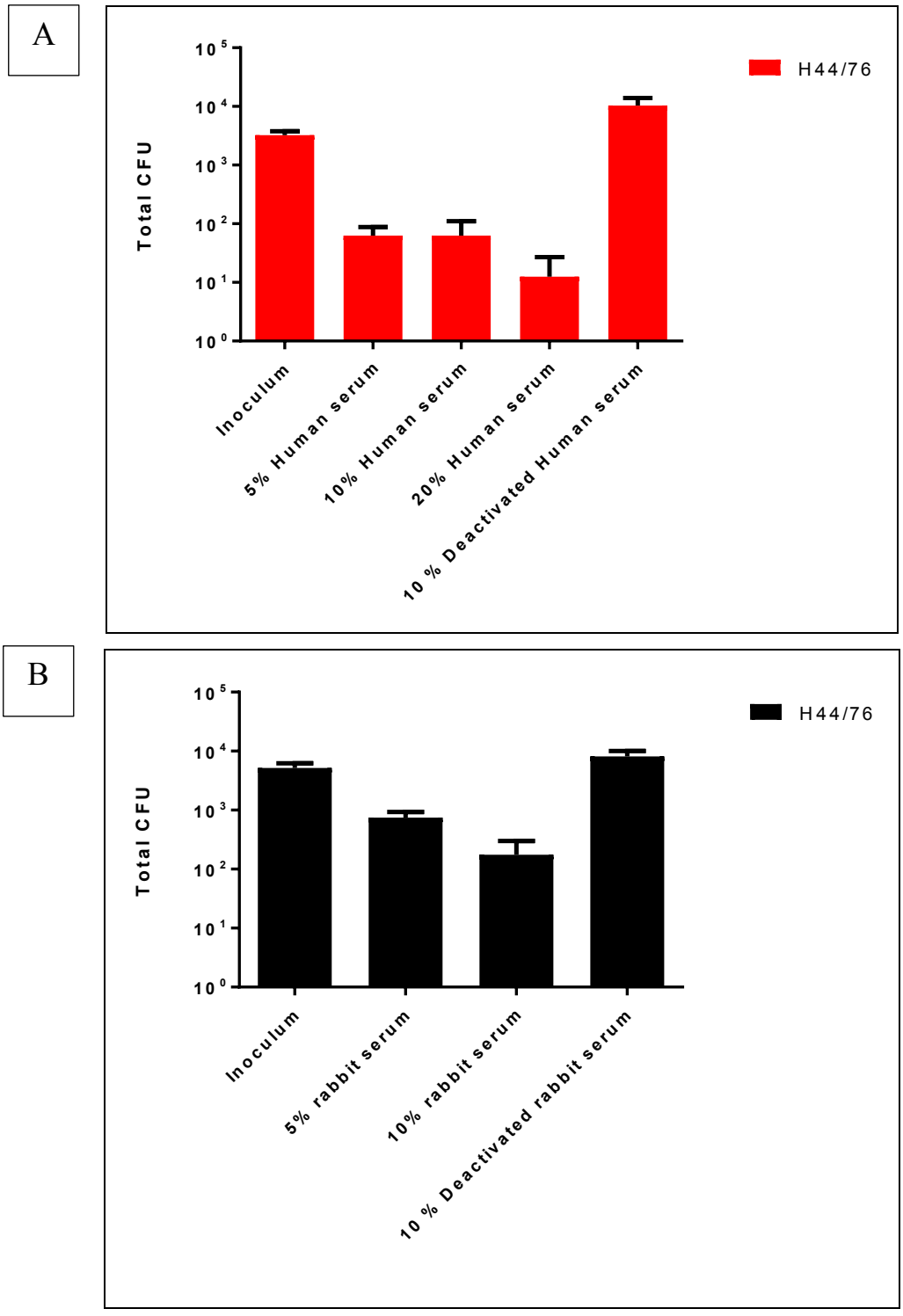


Figure 5-1: **The sensitivity of meningococcal strain H44/76 to different complement sources.** Around 10^4 of bacterial cells were prepared and the suspension were incubated with human pooled sera (graph A) and with baby rabbit sera (graph B) individually with different concentration (in duplicates) and incubated at 37 ^{0}C for 1 hr 5% CO2. 10 % of heat-inactivated sera was used as negative control with each assay. 10 µl of each mix were taken and spread on BHI plates in duplicate and incubated at 37 ^{0}C overnight with 5% CO2 to get the cfu count. The mean of 4 CFU counts was taken and plotted and the error bars represent the standard error of the 4 counts.

5.3 Serum bactericidal assay of anti Opa antisera

The bactericidal activity of anti-OpaD polyclonal antibodies (kindly provided by Dr. Manish Sadarnaghani, University of Oxford) were tested before use in the escape assay. The wild type strain MC58 was shown to express OpaD (confirmed by colony immunoblot) and was used in the assay. The bacteria were incubated for 1 hr with different concentrations of antibodies ranging from 1:20-1:320 in the presence of human sera at a concentration of 5%. Anti-PorA antibodies were also used in the assay as a control along with the anti-Opa. Heat inactivated sera and antibodies without sera were also used as negative controls. The anti-OpaD antibodies showed very strong bactericidal activity at all concentrations with a greater than 50% reduction in the cfu count in the highest dilution (1:320) of anti-OpaD antisera in comparison to the inoculum and the control (figure 5-3). This test confirmed that the antibodies are valid for use in the escape assay.

5.4 The escape of meningococcal strain MC58 from the bactericidal activity of anti-Opa antibodies.

To investigate the escape of meningococcal cells from the bactericidal activity mediated by anti-Opa antisera, the bacteria were incubated with anti-OpaD antibodies for two cycles. In each cycle, fresh antibodies and human complement were added to the bacterial suspension. The bacterial inoculum was prepared from a single colony that was positive for OpaD expression. The bacteria grown overnight on a BHI plate and used to prepare the inoculum. The bacterial inoculum of ~ $1X10^5$ cfu were incubated with anti-OpaD antibodies at two different dilutions: 1:20, and 1:160 for two cycles in the presence of 5 % of human serum. After the incubation of the first cycles for 1 hr, half of the bacterial suspension were taken and incubated again with fresh antisera and human complement for 1hr. After each cycle, a suitable dilution of the bacterial suspension were prepared and spread on BHI plates to get the cfu count.

A strong bactericidal activity was noticed in the first cycle for both dilutions. The 1:20 dilution showed a significant reduction in the inoculum size which is around 9 fold, while the high dilution of the antibodies (1:160) showed a reduction to about half of the inoculum. There was also a slight reduction in the cfu count of the control population which is incubated only with human complement (figure 5-4).

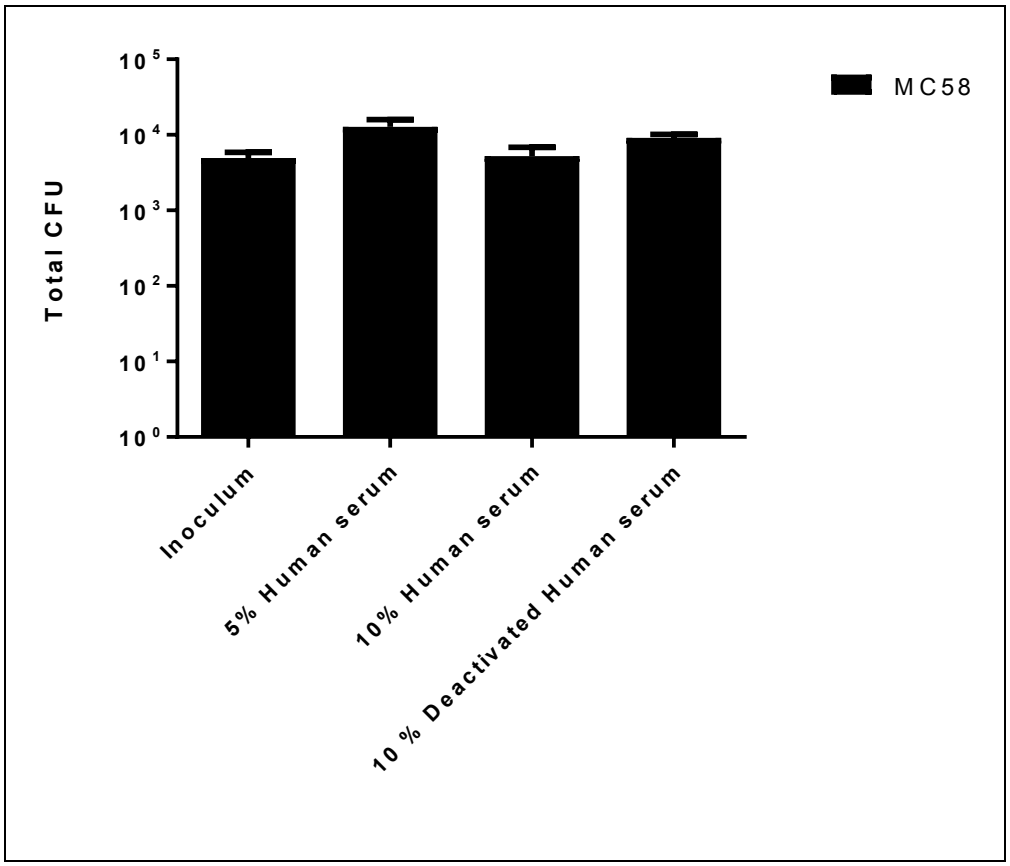


Figure 5-2 . **The sensitivity of meningococcal strain MC58 to human complement source**. A suspension of around 10^4 cells of MC58 were prepared and incubated with human pooled sera with different concentration (in duplicate) and incubated at 37 ^{0}C for 1 hr with 5% CO2. 10 % of heat inactivated sera was used as negative control with each assay. 10 µl of each mix were taken and spread on BHI plates in duplicate and incubated at 37 ^{0}C overnight with 5% CO_2 to get the cfu count. The figure showed the mean of 4 cfu counts with standard error.

In the second cycle, and after adding fresh antibodies and human complement, the bacterial population showed a recovery in the number of the cfu in both cultures of both dilutions, which indicated that the populations had started to escape the bactericidal activity of the antibodies. A colony immunoblot was done on selected plates from passage 1 and 2 for both dilutions as well as for the initial inoculum and the control at passage 2 (figure 5-5). The blot showed that in the case of a high concentration of antibodies (1:20 dilution), there was a clear accumulation of OpaD negative colonies in comparison to the inoculum and the control, with the increasing percentages of the negative variants in the second passage to 82 % of the total colonies tested. With the lower concentration of antibodies, a strong accumulation of negative variants was not detected and the percentage of negative variants was relatively similar in both cycles (21% and 20 % respectively).

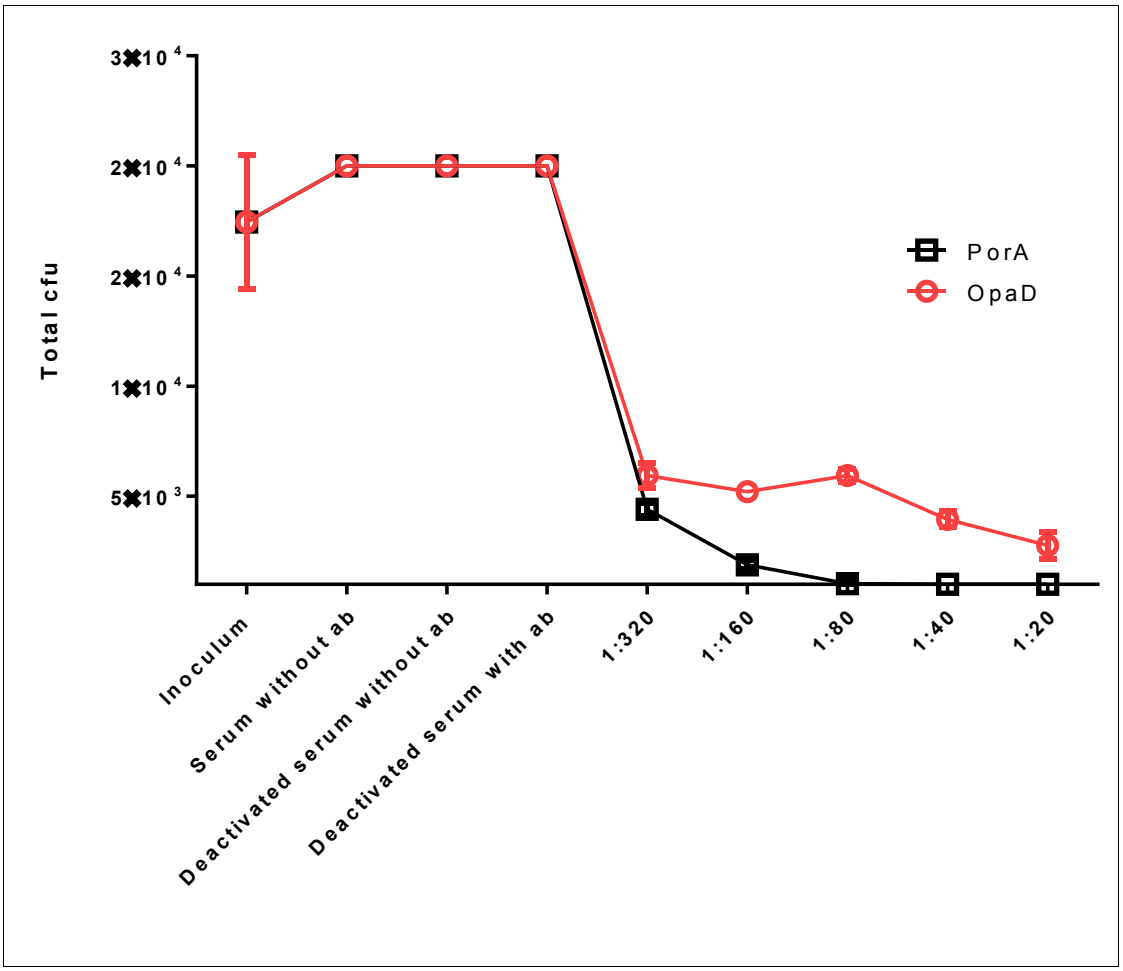


Figure 5-3 **: Serum bactericidal assay of mouse anti-OpaD antisera and PorA P1.7 mAb**. A suspension of around 10^4 cells of MC58 were prepared and incubated with human pooled sera as a complement source at a final concentration of 5% along with different dilutions of anti-OpaD antibodies or with anti-PorA P1.7 Mab in duplicates. Each mix was incubated for 60 minutes at 37 ^{0}C with 5% CO_2. Three different controls were involved including the bacteria with complement only, the bacterial cells with heat inactivated with antibodies and the bacterial cells with heat inactivated serum only. cfu counts were taken by plating 10µl of each assay on BHI plates (in duplicate) and incubating the plates overnight at 37^{0}C. The mean and SEM of up to 4 cfu counts were taken and plotted above.

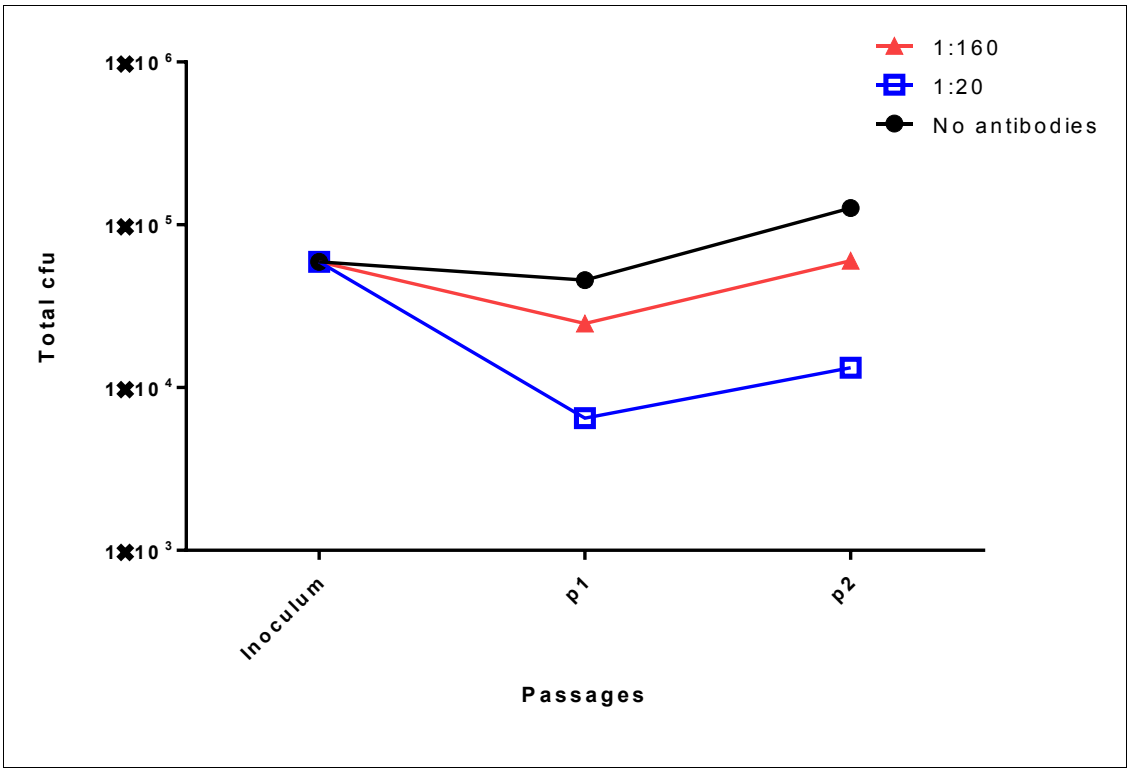
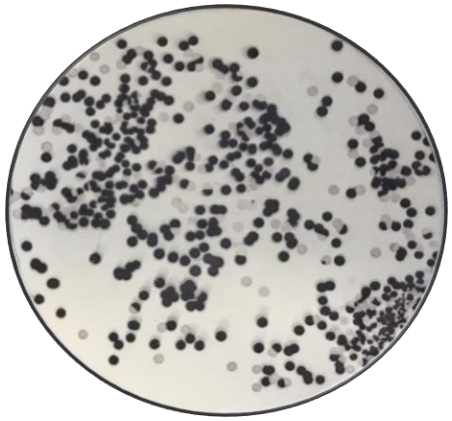


Figure 5-4: **Escape of meningococcal strain MC58 from the bactericidal activity of anti-Opa antisera**. An inoculum of around 10^5 cells was prepared from an overnight culture and incubated with two different dilutions of mouse anti-OpaD antisera in the presence of human complement at a final concentration of 5%, the mixture was diluted in PBSB supplemented with 0.1 % glucose at a final volume of 200µl. The experiment consisted of two passages, the first passage involves incubation of the mixture for 1 hr at 37 0C , and then taking the half of the mixture (100µl) and mixing it with 100µl of PBSB + 0.1% glucose containing 5% human sera and anti-OpaD antibodies (diluted 1:20 and 1:160) and incubate it again at 37 0C for 1hr . The input inoculum, 100µl from passage 1, and the total volume of passage 2 were serially diluted and plated on BHI plates and incubated overnight for taking the cfu counts. A control experiment was also set up by incubating the bacteria with human complement only without the antibodies. The graphs above represent the mean of four cfu counts.

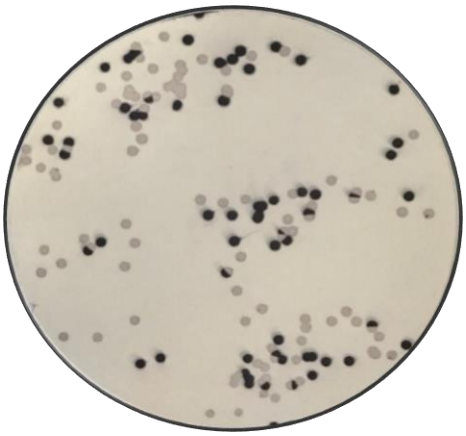
5.5 Genescan analysis of the escape population

The repeat tract length of the *opa* genes were analysed to see whether the non-reactivity of the negative colonies with anti-OpaD antisera was due to switching in the *opaD* gene and to investigate how exposure to the anti-Opa antisera will affect and shape the Opa repertoire of the meningococcal cells. A number of single colonies were picked up (16 colonies) from each passage as well as from the inoculum plates and from the control plate, DNA extraction was done followed by genescan of the four *opa* genes to check the repeat number of the pentanucleotide repeat tract.

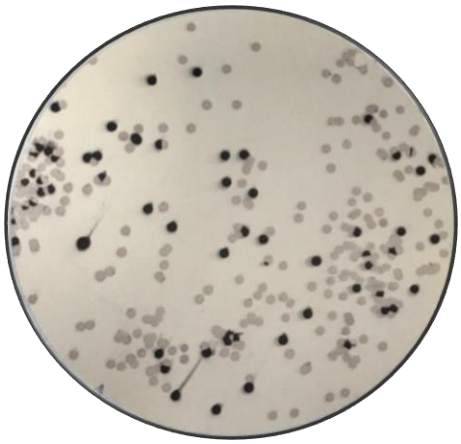
Inoculum: 12 %-ves



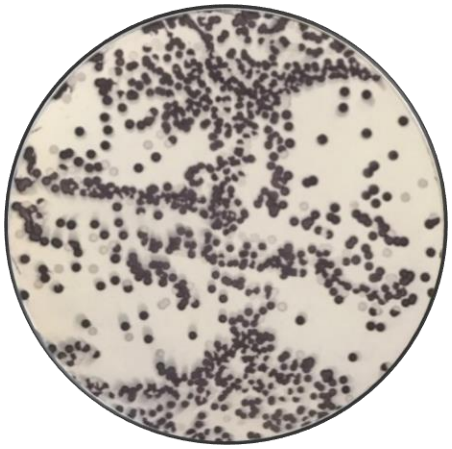
Control P2, no antibodies (17% -ves)



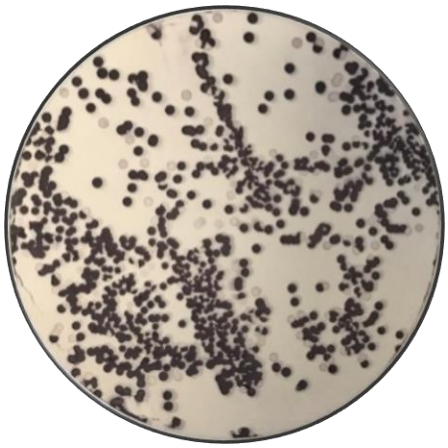
P1, 1:20, 61.4 % -ves



P2, 1:20, 82 % -ves



P1 1:160, 21%-ves



P2 1:160 , 20.6 %-ves

Figure 5-5: **Colony immunoblots of inoculum and passaged populations of *N. meningitidis* strain MC58 probed with pooled mouse polyclonal anti-OpaD antibodies**. An overnight growth of the inoculum and the passaged populations (serially diluted) were transferred to nitrocellulose filters and probed with anti-OpaD antibodies (1:1000), the reactivity was detected by using anti-mouse AP-conjugated secondary antibodies (1:2000). The blot were developed by incubating the membrane with AP substrate developer kit until getting the suitable colour. The percentages of –ve variants were counted by dividing the number of the –ve colonies by the total number of the blotted colonies. P1, passage 1 and p2 represents passage 2.

The Genescan data showed that the majority of the colonies (15 out of 16) from the inoculum have three *opa* genes in the ON state with 12 repeats in the *opaD* gene. This pattern was also observed for the control colonies (figure 5-6). However, after exposure to the antibodies during passage 1 and passage 2, there was switching in the *opaD* gene from 12 repeats (on state) to 13 repeats (off state) in 100% of the colonies showing switch in the repeats of this gene.

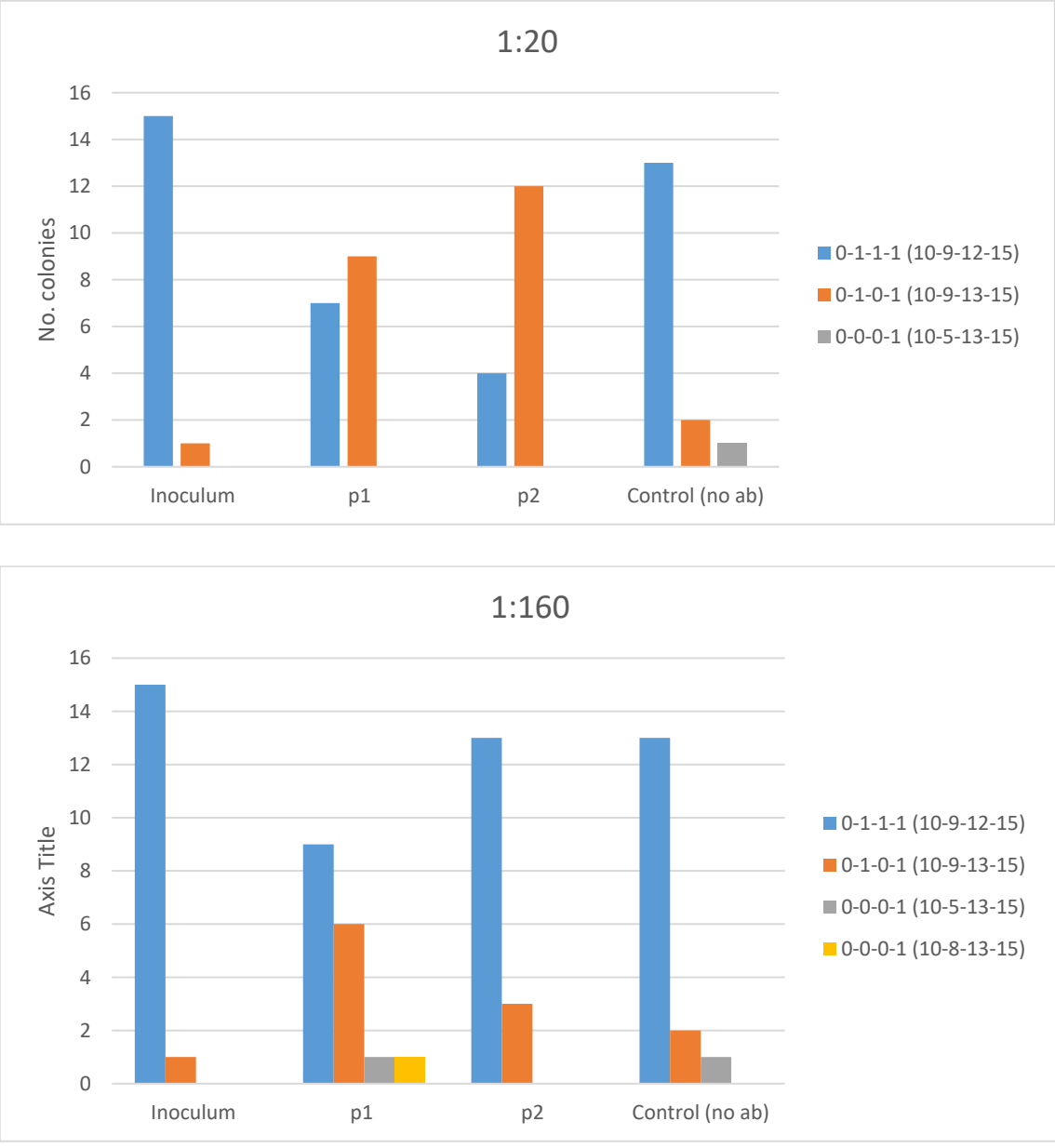


Figure 5-6 : **Phase variation state and repeat tract length of the four *opa* genes of unpassaged and passaged populations of strain MC58.** A total of 16 colonies from each population were picked up randomly followed by extraction of the DNA and then genescanned in order to get the repeat tract length and the phase variation state of the *opa* genes. The repeat numbers were converted to expression state with digital code: zero, for Off state and 1, for ON, while the repeat numbers of each locus were put in brackets as the *opa* genes organised (*opaA-opaB-opaD-opaJ*).

This pattern was the same in the exposure to high and low concentrations of antibodies. At the same time, there was no changes in the repeat tracts of the other *opa* genes except in a small number of colonies (2 colonies in p1 of 1:160 dilution). The new dominant phasotype (0-1-0-1) in the passaged population (treated with 1:20 dilution of antibodies) was present in one colony analysed in the inoculum population, which was then selected after the exposure to the bactericidal antibodies to overcome the bactericidal killing. This also indicates that exposure to anti-Opa antisera led to a specific selection for bacterial cells that switched off only the target Opa variant and there is no effect on the other Opa variants.

5.6 The effects of inoculum size on the meningococcal evasion of serum bactericidal activity

The previous preliminary experiment showed that exposure to the anti-Opa antisera led to a selection for variants that were negative for the target Opa and then escape the bactericidal activity of the anti-Opa antisera. In order to check the minimum population that can escape the bactericidal activity of anti-Opa antibodies, the escape assay was done with different inoculum sizes of *N. meningitidis* MC58 ($3x10^5$, $3x10^4$ and $3x10^3$ cells). The assay was done with three cycles of 1 hr of incubation for each cycle with a high concentration of antibodies (1:20 dilution). The results showed that all three population were able to escape from the bactericidal activity of the antisera (figure 5-7). However, there was a strong reduction in the population size in the first cycle of the two smallest inoculum sizes (intermediate and low inoculum) while the high inoculum showed a lower reduction than the other inoculum sizes (~ 3 fold of the initial population). However, in the second cycles, the higher inoculum stayed to be reduced due to exposure to the antibodies, while the intermediate and the low inoculum started to recover in the 2^{nd} cycle. The recovery in the population continued in the third cycles in the three inoculums (figure 5-7).

Colony immunoblots were done for antibody-treated cultures as well as for the inoculum and the negative control. The blots showed that the antibody-treated populations consisted mainly of –ve OpaD variants (more than 90% of the total colonies) in comparison to the initial inoculum, which was, composed mainly of OpaD +ve variants (figure 5-8). One of the limitations of this experiment is that the initial inoculum contained a high rate of –ve variants (25 %), which facilitates the population to escape from the bactericidal activity of the antibodies. This was due to the population being prepared from a glycerol stock of

the bacteria that had a high level of negative variants in the population. Another problem is that there was a reduction in the control population without antibodies, which might be due to inoculum having some sensitivity to the complement source in the absence of the added Opa antibodies.

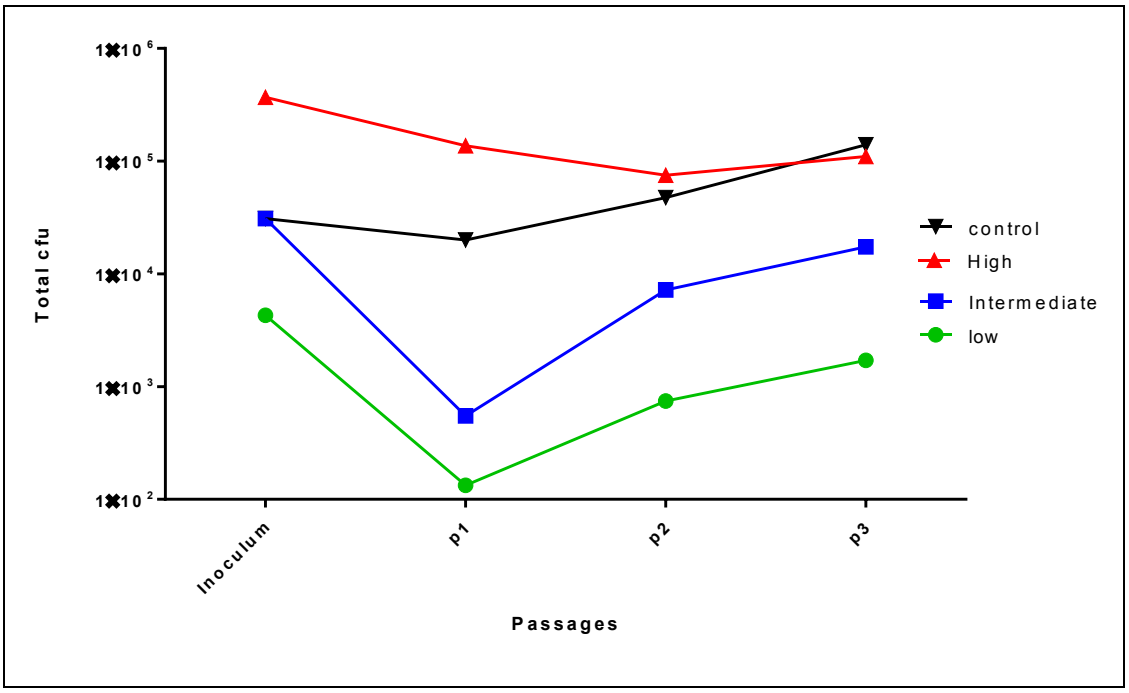


Figure 5-7 : **The effects of the size of inoculum on the escape of the meningococcal strain MC58 from the bactericidal activity of anti-Opa antisera**. Three different sizes of inoculum including $3x10^5$ cells (high inoculum), $3x10^4$ cells (intermediate inoculum), and $3x10^3$ cells (low inoculum) were passaged in the presence of anti-OpaD antibodies (diluted 1:20) and human complement (final concentration of 5%) for three cycles. The total cfu counts were taken from the inoculum as well as from the passaged populations after each passage. A control experiment was set up by passaging the bacteria with human complement without antibodies. The graph represents the mean of 4 cfu counts.

Genescan was performed on the passaged population and up to 16 colonies were picked up from each passage (figure 5-9). The analysis showed that the switching was mainly in opaD, which occurred in 97 % of the passaged colonies that showed changes in the repeats of *opa* genes in comparison to the dominant phasotype present in the inoculum (0-1-1-1 with 10, 9, 12, and 15 repeats respectively). The switching was mainly from 12 (on state) to 13 (off state) with a rare switch from 12 to 11 repeats in this locus (only 8 colonies out of 118 passaged colonies had this switch in *opaD* repeats). Whereas, the switching in the other *opa* genes was very rare (*opaB* 0 % and *opaJ* 4.9 % out of the total passaged colonies showed changes in *opa* loci in comparison to the dominant phenotype present in the inoculum).

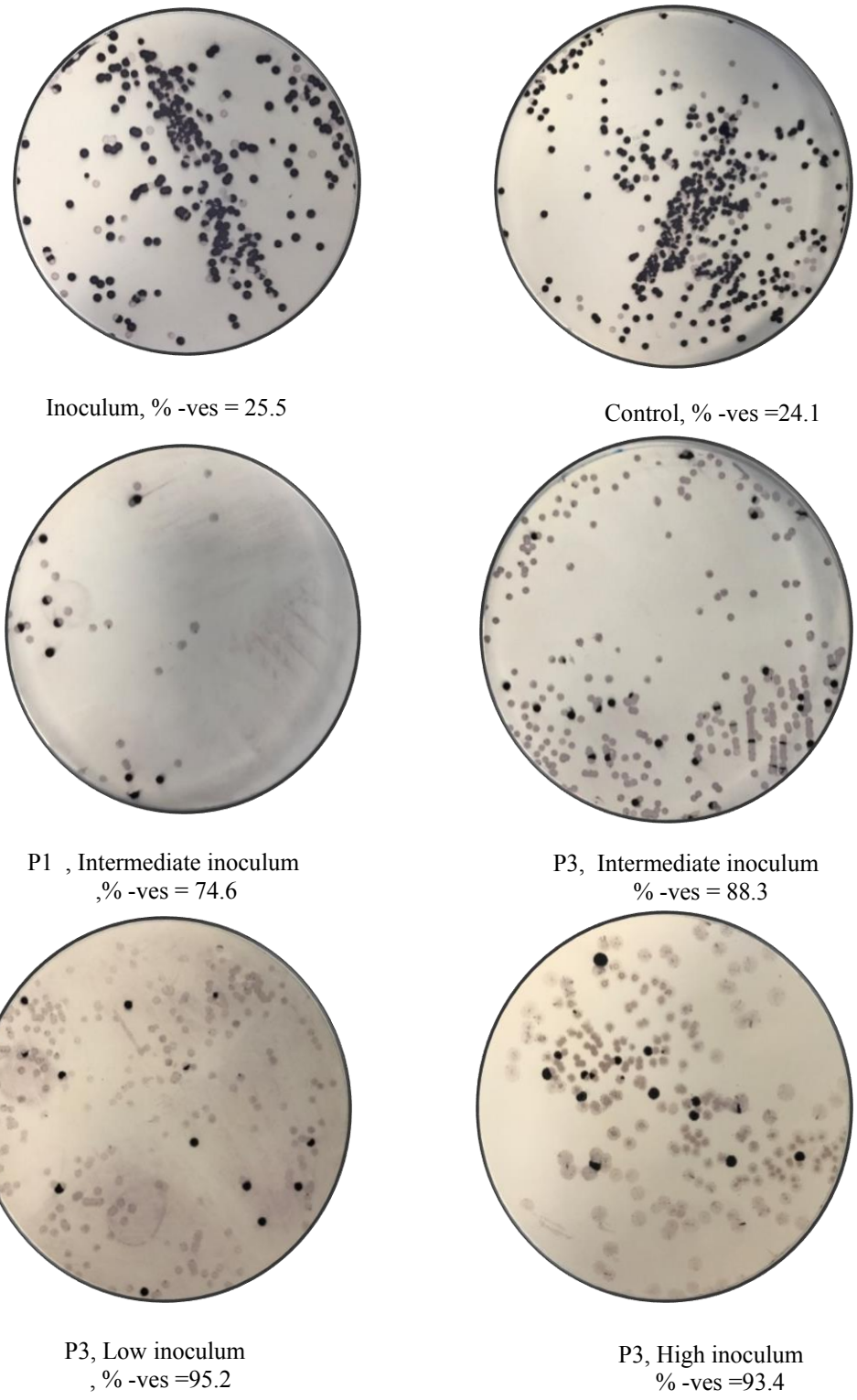


Figure 5-8 **Colony immunoblots of N. meningitidis strain MC58 before and after passage probed with anti-OpaD antibodies.** Colonies from overnight growth of the inoculum and the passaged populations were transferred to nitrocellulose filters and probed with anti-OpaD antibodies and then detected by using anti-mouse AP conjugated secondary antibodies. The percentages of –ves were counted by dividing the number of the –ve colonies by the total number of the blotted colonies.

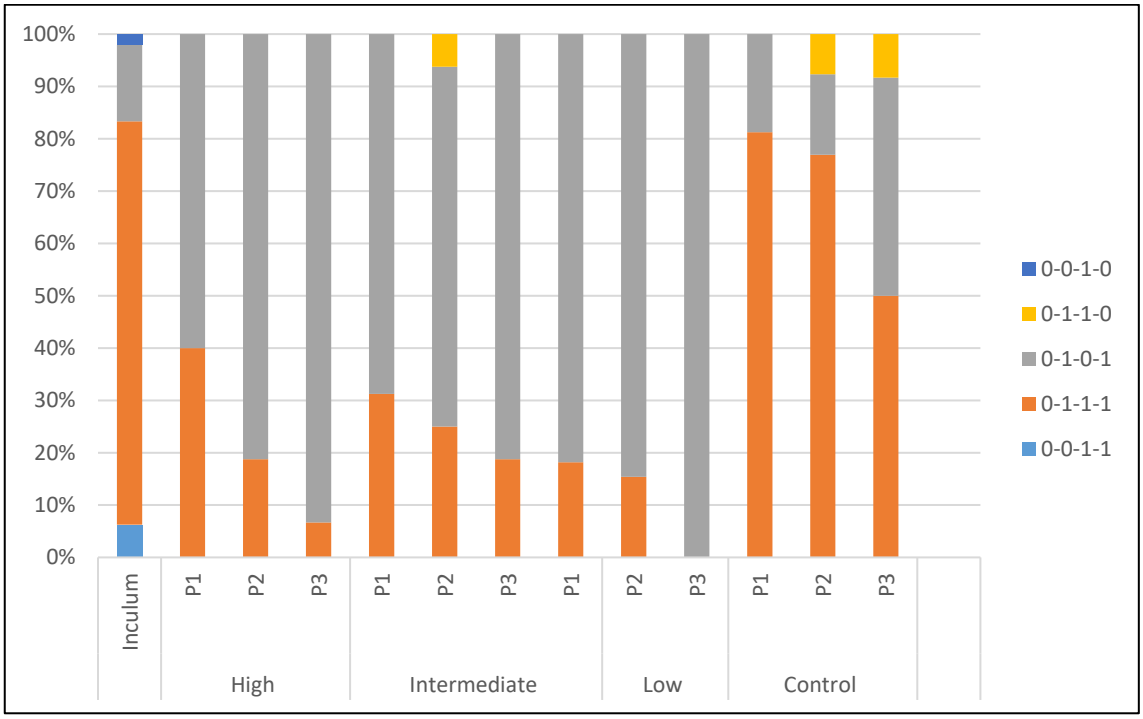


Figure 5-9: **Phase variation state of *opa* genes of unpassaged and passaged populations of the strain MC58.** In total, 224 colonies were picked up, 48 from the inoculum population and up to 16 colonies from each passaged population and were analysed by genescan to get the phase variation state of the four *opa* genes. The majority of the switching was in opaD with a minor switching noticed in the other *opa* loci (opaB and opaJ), while *opaA* was in a constant off state in all colonies analysed.

Thus, the selected variants had two *opa* genes in the ON state, which were *opaJ* and *opaB*, while *opaA* and *opaD* were in the off state, while the majority of the inoculum colonies had three *opa* genes in their ON state, including *opaB*, *opaD* and *opaJ*.

5.7 The effects of inoculum size of clonal populations on the meningococcal evasion of bactericidal activity

Due to the limitation in the previous assay with the presence of a high rate of opaD –ve variants in the inoculum, and in order to analyse whether the a clonal population started from a single colony can escape the bactericidal activity of the anti-Opa antibodies, the assay was repeated with an inoculum derived from a single colony that is positive for OpaD. A colony immunoblot was performed on multiple single colonies with anti-OpaD antibodies and a single positive colony was picked up and streaked onto a BHI plate. After overnight incubation, the bacterial growth on this plate was used to prepare the inoculum for the escape assay. Three different inoculum sizes (2 x $10^{6,}$ 2 x 10^5 and 2 x 10^4) were used and the assay was done with a 1:20 dilution of anti-OpaD antibodies for three cycles of 1 hr of incubation. The data showed that there was a very strong reduction in the inoculum size in the first cycle which lead to eradication of the whole population

of the low inoculum, and only few cells (i.e. cfus) escaped in the case of the intermediate population (Figure 5-10). However, there were also a strong reduction in the control population without antibodies which is probably due to the sensitivity of the cells to the complement source. Similar to the previous assay, the populations of the intermediate and the high inoculums showed a clear recovery during the 2nd and 3rd cycles indicating that the populations had become resistant to the bactericidal activity of the antibodies.

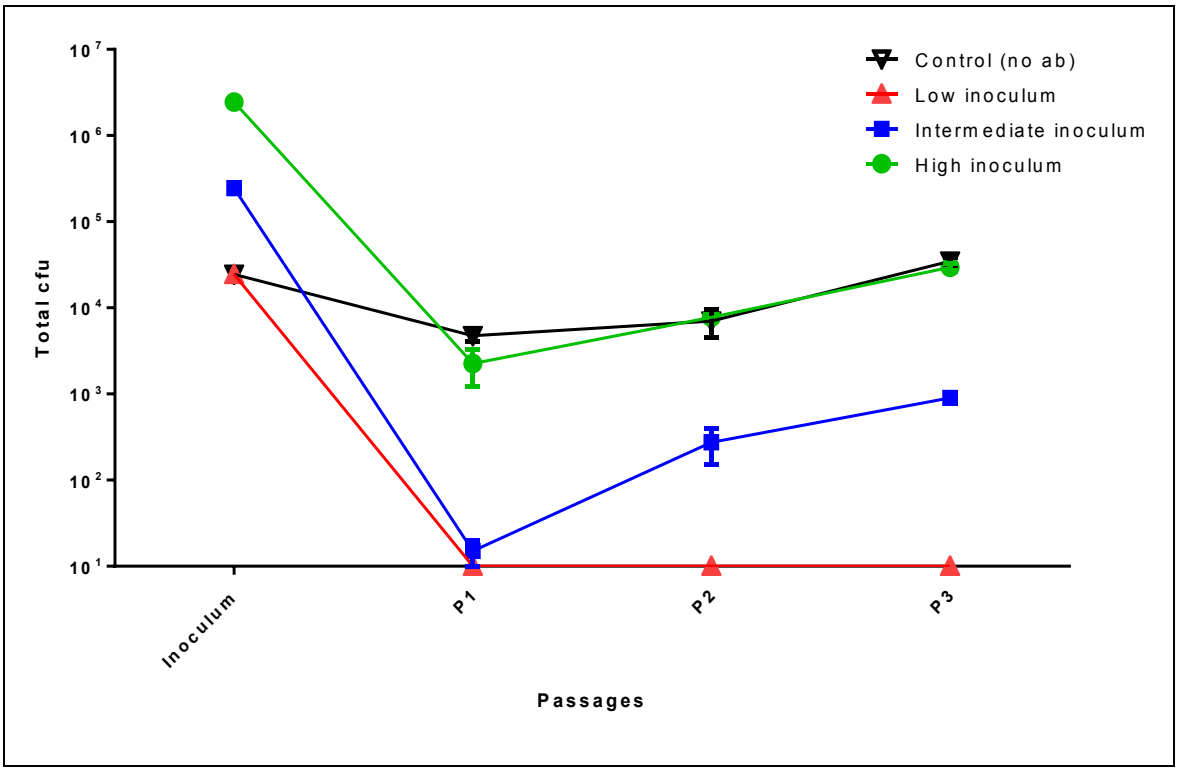


Figure 5-10 : **Eescape of meningococcal strain MC58 clonal populations from the bactericidal activity of anti-Opa antisera**. Three different sizes of inoculum including $5x10^6$, $5x10^5$and $5x10^4$ cells were prepared from a single colony to get clonal populations and passaged in the presence of anti-OpaD antibodies (diluted 1:20) and 5% of human complement for three cycles. The total cfu counts were taken as previously described in figure 6-7. Each point represents the mean of 4 cfu counts with standard error.

The colony immunoblot analysis showed that the percentage of the –ve variants in the inoculum was around 1% and relatively the same ratio was detected in the control population (figure 5-11), whereas the passaged populations showed 100 % -ves even in the p2 of the high inoculum. Genescan was performed to confirm the phase variation state of the escaped population and the data showed that all colonies analysed (up to 16 colonies from each passage) had *opaD* in an off state (figure 5-12), and in contrast to the other assays, the switching in the repeat length of *opaD* were to a variable number of repeats ranged between 11, 13, 14 and 16 repeats.

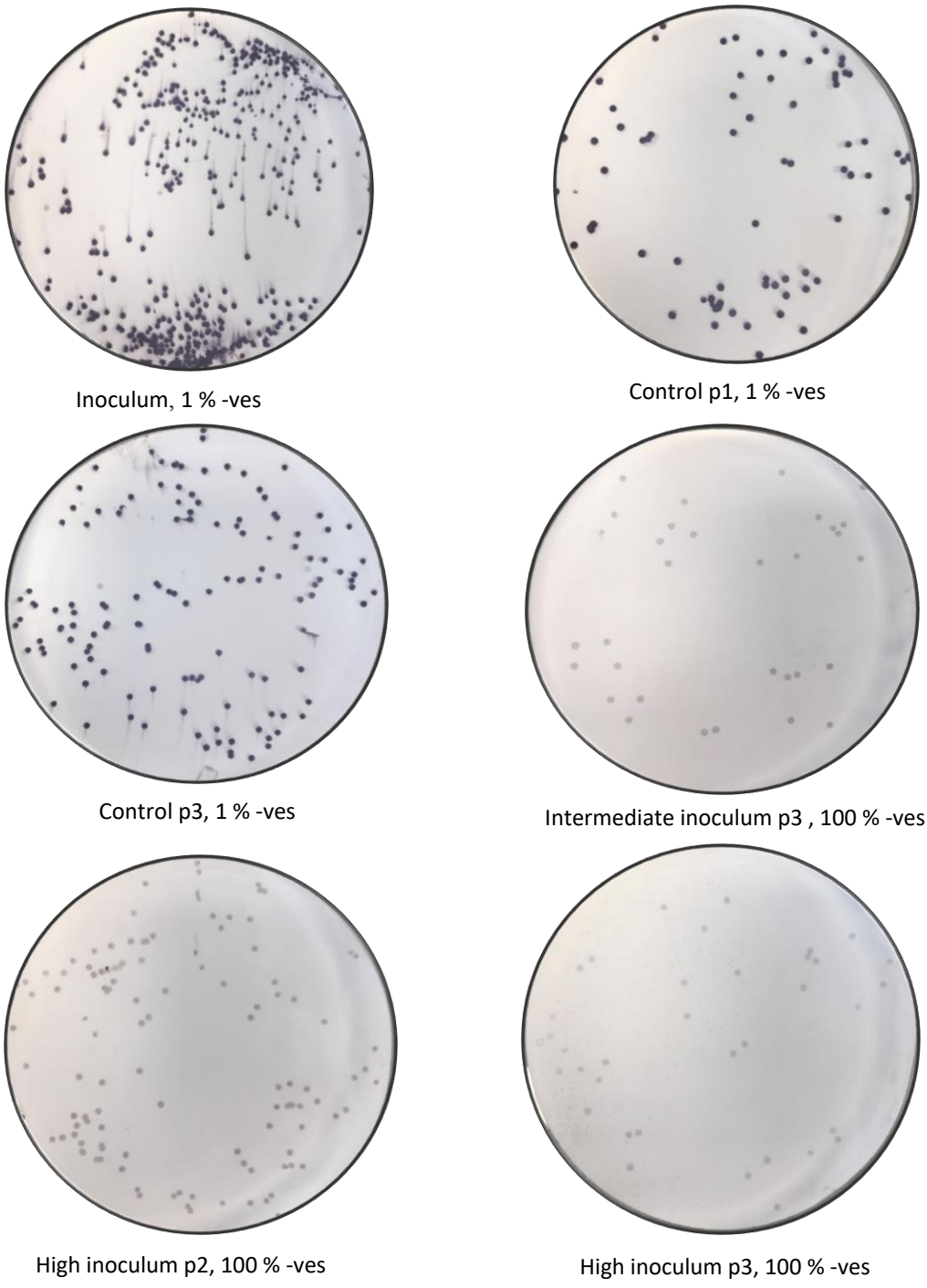


Figure 5-11 : **Colony immunoblots of *N. meningitidis* strain MC58 before and after passage probed with anti-OpaD antibodies**. Colonies from overnight growth of the inoculum and the passaged populations were transferred to nitrocellulose filters and probed with anti-OpaD antibodies and then detected by using anti-mouse AP-conjugated secondary antibodies. The percentages of –ve variants were counted by dividing the number of the –ve colonies by the total number of the blotted colonies.

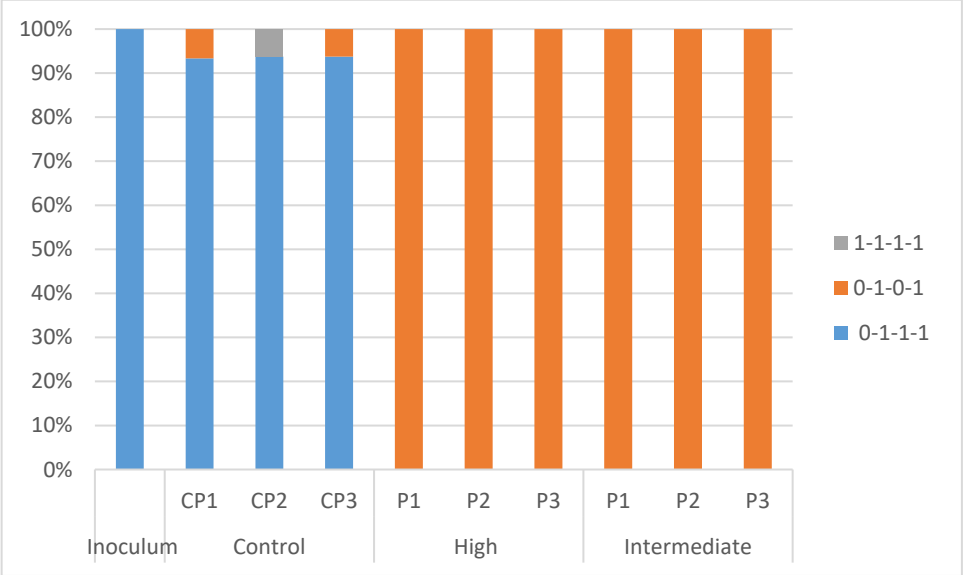


Figure 5-12 the genescan analysis and the phase variation state of *opaD* genes of the populations of the strain MC58. In total, 137 colonies were picked up randomly from the different passaged and un-passaged populations (up to 16 colonies from each population) and were analysed by genescan and the phase variation state of the four *opa* genes were confirmed by checking the repeat tract number of each gene. The figure showed the different phasotypes detected in the whole samples in percentages of colonies analysed in each population.

5.8 Testing the effect of different concentrations of antibodies on the escape of the population

The previous experiment proved that escape was affected by the size of the population and that a high number of meningococcal cells is required for escape of the bactericidal activity. In this experiment, the effect of the concentration of the antibodies was tested, and the assay was done with three different dilutions of the antibodies including 1:20, 1: 40 and 1:80 of anti-OpaD antibodies. The inoculum was prepared from a single colony in the same manner as in the previous experiment and the experiment was also done in three cycles of 1 hr of incubation. The results were different from the previous experiment. There was a lower selection for the –ve variants when the bacteria were incubated with the high concentration of antibodies (1:20 dilution), and the ratio was 57 % -ves in the output population. However, the other two dilutions showed very low selection and the output populations contained a low rate of negative variants involving 24.5 % in 1:40 dilution and 1.5 % in the case of 1:80 dilution (figure 5-13). The control population showed the same problem of sensitivity to the complement and there was a reduction in the cfu in the first cycle followed by a recovery in the second and third cycle.

To avoid the problem of the sensitivity of the bacteria to the complement, the assay was repeated using an inoculum started from a single colony derived from a population of the

no antibody control that has been treated with human complement only. The results showed that there was no reduction in the control population in the first cycle indicating that bacteria were resistant to the complement and there was no nonspecific killing (Figure 5-14). However, there were lower bactericidal activities in the inoculums treated with antibodies in comparison to the previous experiment with lower selection of the –ve variants in the output populations of the three different concentrations of the antibodies.

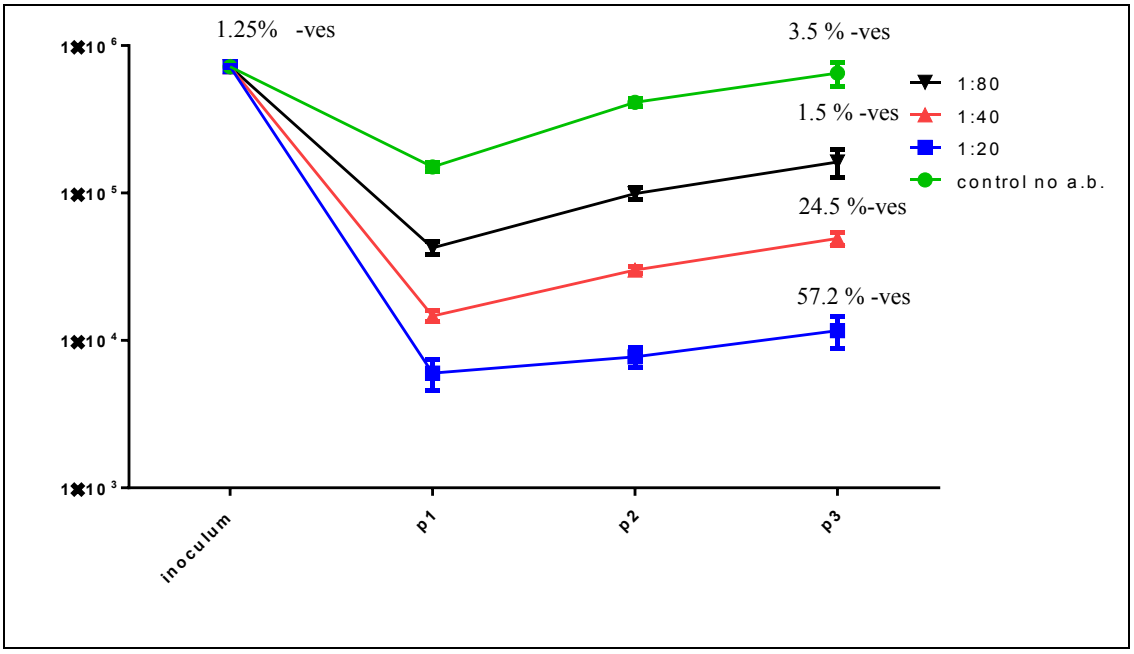


Figure 5-13: **The effects of the concentration of the antibodies on the escape of the meningococcal strain MC58 from the bactericidal activity of anti-Opa antisera.** An inoculum of MC58 (~ $1x10^6$ cells) prepared from a single colony were passaged in the presence of anti-OpaD antibodies with three different dilutions (1:20, 1:40 and 1:80) and human sera as complement source (final concentration of 5%) for three cycles. Control experiment were involved incubating the bacteria with human complement without antibodies. The mean of 4 cfu counts were blotted above and the error bar represent the standard error.

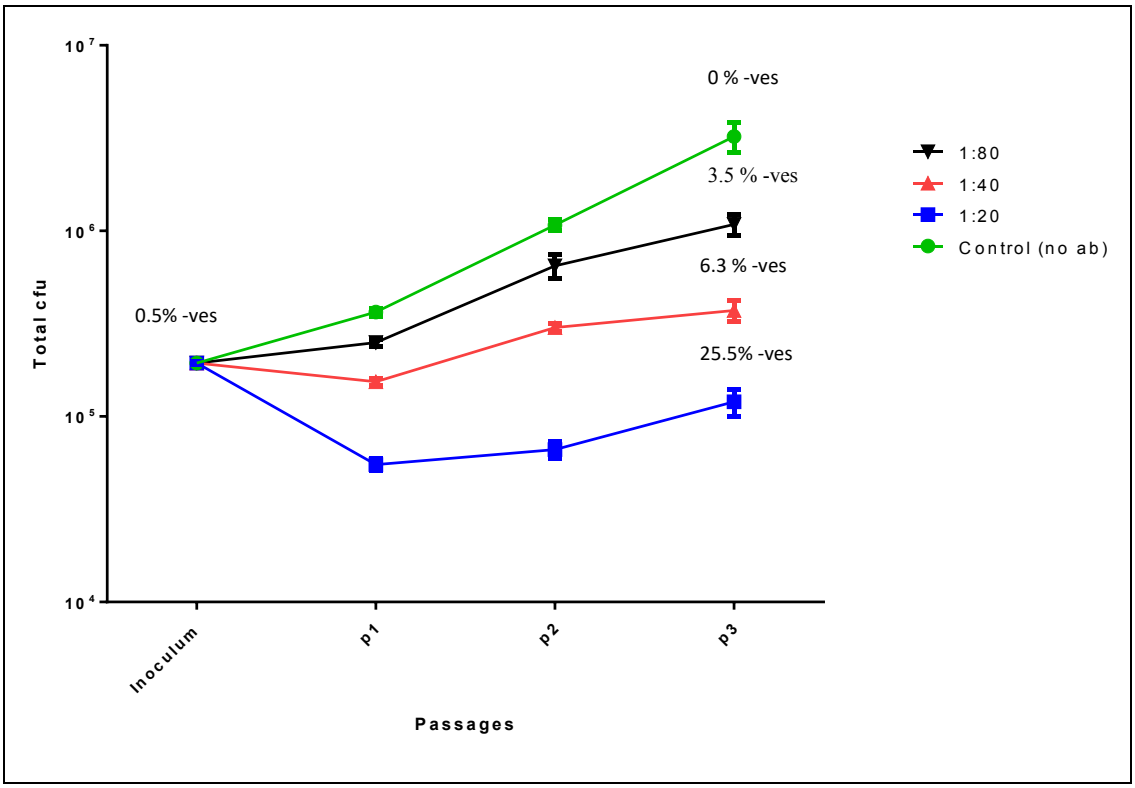


Figure 5-14: **The influence of the different concentrations of anti-Opa antibodies on the escape of the meningococcal strain MC58 after passaging the cells with human complement only.** An inoculum of the strain MC58 were prepared and incubated with human complement only for 60 minutes and then the suspension serially diluted and plated on BHI plate overnight. Colony immunoblots were done for the grown colonies using anti-OpaD antibodies, and a single positive colony was taken and streaked on BHI plate, and then used to prepare the inoculum for this assay. The inoculum (~4 x10^5 cells) was passaged in the presence of anti-OpaD antibodies with three different dilutions (1:20, 1:40 and 1:80) or without antibodies (control) in the presence of human sera as complement source (final concentration of 5%) for three cycles. The blot represents the mean of 4 cfu counts and the bar represents the standard error.

5.9 Discussion

Phase variation in Opa proteins and their role in mediating the evasion of the bactericidal activity of anti-Opa antibodies has not been studied before. In this study, the potential role of PV of Opa proteins on the escape of meningococcal population from their bactericidal antibodies was investigated using a modified *in vitro* serum bactericidal assay. Mouse polyclonal anti-Opa antibodies used in the assays were generated against Opa variant (OpaD) of the meningococcal strain H44/76 (Sadarangani *et al.* 2012), and the assay involved using human pooled serum as an exogenous source of complement. The H44/76 strain available in our lab was shown to be sensitive to the human sera as well as rabbit sera. Therefore it was replaced with the meningococcal strain MC58 which shared the same OpaD variant of H44 /76 and showed more resistance to human sera. Such variability in the killing effect of human natural serum on different meningococcal strains can be related to the possibility of the presence of circulating meningococcal specific antibodies or other antibodies that might cross react with meningococcal strains and trigger the complement pathways. This is one of the common problems faced with using human serum in the bactericidal assay as it is difficult to find individual donors who's sera does not contain such circulating antibodies (McIntosh *et al.* 2015). In the same time, the specifity of the meningococcal surface antigens to human complement inhibitors such as fHbp, which binds to Factor H protein that inhibit the alternative complement pathway, makes the meningococcal strains sensitive to the animal complement sources such as rabbit complement. Thereforeserum bactericidal antibody (SBA) assays using such animal complement source measures higher titres than when using human complement (Granoff *et al.* 2009; McIntosh *et al.* 2015). This might be the reason why we still see killing backround in the test of the H44/76 with baby rabbit sera, which does not expect to have such circulating antibodies that might specifically bind or cross-react with the strain.

The bactericidal activity of anti-OpaD polyclonal antibodies were investigated using meningococcal strain MC58, and the antibodies showed strong bactericidal activity at a high titre of 1:320 which killed more than 50 % of the meningococcal population.

The successive incubation of the meningococcal strain MC58 (OpaD +ve) with a high concentration of anti-OpaD antibodies (1:20 dilution) led to development of an Opa-antibody resistant population. The majority of the killing happened in the first cycle of the assay, while in the second cycle there were lower bactericidal activities and the

population recovered to a high number of cells. The colony blot revealed that after two hrs of incubation with the antibodies, the population showed a rapid turnover from mainly OpaD +ves, to mainly OpaD –ves. Analysing the repeat tract length by genescan showed that the input population was composed mainly of cells expressing three Opa variants (OpaB, OpaD and OpaJ) and the switching happened in the repeat tract length of *opaD* going from 12 repeats (On state) to 13 repeats (Off state) with no switching in the genes encoding for the other variants. Notably the majority of the output population had two Opa variants in the On state. This indicates that the antibodies target only the specific Opa antigen, and there is no effect on the other Opa variants in terms of selection, so that the population still express two Opa variants which is enough to mediate their biological functions.

The assay was extended to investigate the lowest limit of cell number to escape a high concentration of anti-Opa antisera. Three different inoculum sizes were incubated with the antibodies for three cycles and all the populations, even the lowest one ($3x10^3$) were able to escape the antibodies from the second cycle of the incubation (Figure 5-7). As shown by the output populations containing > 90% opaD -ves. One of the limitations in this assay was the high rate of OpaD –ves in the input population (the inoculum) which was around 25 % (which equal to around 750 cells negative for OpaD), and this might make it easy for the low size population to escape from the bactericidal activity. In addition, nonspecific killing was noticed in the control population in the first passage resulted in reducing the population by approximately 50 % (around 20,000 cfus), and this may have led to reduction in the expected cell number that should be seen in the first cycle of the treated populations. However, the assay was done with an input population started from a single OpaD positive colony with three different inoculum sizes but higher than the previous assay by one log (Figure 5-10). The data showed that the low inoculum size (2×10^4) couldn't escape the antibodies and was completely eradicated in the first cycle of the incubation. Conversely, the other populations successfully evaded the bactericidal activity and were completely converted to OpaD -ve at the 2^{nd} and 3^{rd} cycle of the incubation. From the colony immunoblot, the rate of –ves was calculated in the inoculum of this assay and it was around 1 %, which mean that around 200 cells that are off for OpaD were present in the low inoculum population but it was still cannot escape. This might be due to the nonspecific killing mediated by the human complement as shown in the control population, which led to reduction of about 5 fold of the control population

(around 20,000 cfu) in the first cycle of the assay. Similar patterns can be seen in the other populations, in which the number of the surviving cells in the first cycles did not match the expected number of the negative cells that should already be present in the inoculum and should escape the bactericidal antibodies. It was very difficult to determine the lowest limit of the population size to escape the bactericidal activity due to such nonspecific killing mediated by the complement source rather than the antibodies.

The PV frequency of *opa* was predicted to be between 10^{-3} and 10^{-4} per cell per generation, and PV of the same *opaD* gene in another strain (H44/76) with 14 repeats was estimated to be 6.9×10^{-3} per cell per generation (Sadarangani *et al.* 2016). However, the rate of PV of this locus was higher in our assay than expected. This might due to sub - culturing of the single colony before using it to prepare the inoculum, which might consequently lead to an increase in the number of –ve variants in the inoculum and raising the ratio to 1%.

The rate of PV was reported as an important determinant of the bacterial escape of the bactericidal activity. Studies indicated that deactivating the mismatch repair system (MMR) by constructing a mutation in the *mutS* gene into strain 8047 has increased the frequency of PV *lgtG* and *porA* 1000-fold in comparison to the wild type strain. Consequently, the *mutS* mutant strain showed a very rapid escape of the bactericidal antibodies against the both proteins (individually) in comparison to the wild type strain 8047 (Bayliss *et al.* 2008; Tauseef *et al.* 2013). The MMR deactivation has affected the PV rate of genes with homopolymeric tracts rather than tetrameric repeats, indicating that tetrameric repeats are reciprocal to MMR (Martin *et al.* 2004). Such pattern can be similar for the pentameric repeats of the *opa* genes , even though their PV rate was shown to be increased 180 fold during the natural transformation, but it was not clear whether that due to inactivation of MMR genes during the transformation or not (Sadarangani *et al.* 2016). In other words, the direct effect of MMR inactivation of *opa* pentameric repeats was not investigated, but it is more likely to behave similar to the tetrameric repeats in term of their resistance to the MMR system and then have a slippage rate higher than genes with monomeric repeats. Such high rate of PV has mediated a rapid escape of meningococcal population of the bactericidal antibodies highlighting the importance of PV of this protein in immune avoidance.

The genescan analysis revealed that the majority of the colonies analysed showed switching in *opaD* repeats which encode the target antigen of the antibodies used with a

very rare switching in the other *opa* loci. The switching was mainly from 12 repeats (on state) to 13 repeats (off state) (addbing of single pentanuclotide repeat), with rare switching mediated by deletion of one repeat (from 12 to 11 repeats) or by insertion of 2 or 4 repeats (switching from 12 to 14 or 16 repeats respectively).

The effect of the antibody concentration has also been investigated, and the data showed that the bacteria escaped the killing by lower concentrations of antibodies (1:40 and 1:80 dilution) without a clear selection of the negative variants, and only the highly concentrated antibodies (1:20 dilution) mediated selection for negative variants which might enable the bacteria to escape the killing. In all dilutions, the main bactericidal activity happened in the first cycle of the assay and then reduced in the later cycles as the population escaped both the specific and non-specific killing. This might be related to the sensitivity of the populations to the complement, as the control population also showed a reduction in the first cycle of the assay and then started to resist the complement in the later cycles. This might be due to selection of variants that are more resistant to complement and consequently lead to reduce the bactericidal activity of the antibodies. It has been noticed that expression of an L8 variant of LPS led to an increase in the sensitivity of meningococci to complement, such that anti-PorA antibodies killed the bacteria with titres 4 to 8 times higher than the variant that was not expressing an L8 LPS variant (Moran *et al.* 1994). This means that expressing a particular antigen can affect the bactericidal activity of antibodies that are targeting other antigens. This might have happened in our assay where the population developed resistance to complement, as observed in the 2nd and 3rd cycles in the control population. Similarly, the bactericidal activity of the anti-Opa antibodies became lower than in the first cycle (Figure 5-13) and the population escaped the antibodies without a selection to negative phase variants. This can be supported by the findings from the later assay (Figure 5-14), as the inoculum prepared from a single colony that was passaged through the human complement showed a level of the bactericidal sensitivity that was lower than what was observed in the previous experiments.

This might be also because the low ratio of the antibodies to the input bacterial inoculum. The total volume of the assay was 100 microliter and the inoculum size was around 10^6 cells, and the amount of antibodies (diluted 1:40 and 1:80) may not enough to mediate a strong killing that can reduced the cell number over the three cycles of the incubations. It may not mediate killing the majority of the bacterial population and then the rate of

doubling cells was more than the rate of killed cells and then the population showed recovery in cell number especially where the population start to show more resistance to the human complement.

In summary, these experiments provided evidence that PV of Opa proteins can mediate the escape of meningococcal cells from Opa-specific immune responses. This indicates that having four different Opa variants that can be expressed at any given time (individually or in combination) provides advantages to the bacteria for evasion of the anti-Opa antibodies by switching between those different variants with maintain their function (see section 1.9, Opa proteins). In addition, these experiments showed that exposure to a low concentration of antibodies enabled the population to escape the killing without a strong turning over to negative variants, which can be related to development of a resistant population to human complement. This most likely occurred via expression of other antigens that increase the resistance to the complement or even mask or protect Opa proteins from the bactericidal antibodies. Thus, exposure to a high concentration of antibodies requires Opa –ve variants to escape the bactericidal activity, while in the case of exposure to lower concentration of antibodies, the population can escape the killing without switching to –ve variants.

6 Evaluation of ligand binding and bactericidal response of Opa proteins

6.1 Introduction

Protein- polysaccharide conjugate vaccines based on the capsular antigen are available against *N. meningitidis* strains with capsular groups A, C, W and Y, but there was, up until recently, no widely protective vaccine against *N. meningitidis* serogroup B (Pillai *et al.* 2005). This is due to the polysaccharide structure of the capsule of this serogroup being poorly immunogenic and highly similar to the human sialylated glycoproteins which could lead to cross-reactivity of anti-capsular antibodies with glycoproteins in human tissues (Ala'Aldeen and Cartwright 1996). Therefore, alternative vaccines have been developed including outer membrane vesicles (OMVs) which have been shown to be successful and safe and provide protection against capsular group B meningococcal disease; however, this protection is only against the target strain.

Further attempts were carried out to develop vaccines based on outer membrane proteins (OMPs), and one of those proteins that is a candidate to be involved is the Opa protein (Callaghan *et al.* 2011; Christodoulides and Heckels 2017). A problem with using Opa proteins is ther high antigenic variability. However, it has been shown by molecular studies that meningococcal antigenic diversity is structured, and that particular combinations of Opa proteins are correlated with the hyperinvasive lineages of serogroup B and C strains which are considered the leading cause of meningococcal disease in European countries (Callaghan *et al.* 2008a; Callaghan *et al.* 2011).

Opa proteins have proved to be highly immunogenic and to generate bactericidal antibodies in mice (de Jonge *et al.* 2004; Callaghan *et al.* 2011), but there was no assessment for human immunity against Opa proteins and their role in shaping the Opa repertoire within meningococcal strains. In addition, the analysis of the PV state of Opa within longitudinal carriage isolates revealed that the majority of the isolates analysed expressed only one locus of the four opa loci (70 %) and a considerable number of the isolates had switched all the loci OFF (see chapter 4). There was also a reduction in the expression of Opa variants at later stage of colonisation when a high proportion of isolates were in the ON state at the initial time point. Simultaneously, there was selection for isolates expressing other variants that were switched OFF or expressed at low rates in the

early stages of colonisation. This behaviour may be correlated with the evasion of a specific immune response targeting the Opa antigens during the carriage state. Therefore, evaluating the immune response against the Opa proteins was crucial to interpret the observations of the PV analysis of the carriage state. Therefore, this study aimed to clone, express and purify rOpa proteins in order to be used for evaluating the immune response against the Opa proteins in sera taken from human carriers during the carriage state. The study also aimed to produce polyclonal anti-Opa antibodies which can be used for more analysis of the evasion of a specific immune response mediated by PV of Opa. Extending the assays of immune escapes with using different isolates as well as covering the four Opa variants of the same strain will highlight how the exposure to an immune response will shape the Opa repertoire of *N. meningitidis* and how the PV of these proteins can mediate the escape of the bacteria from the anti-Opa specific antibodies. Generating of new antisera can enable this study as well as overcoming the limitations that were in the previous study due to the depletion and the limited amount of Opa antisera. In addition, the generated antibodies can be used for testing the role of Opa proteins in adhesion and invasion of human cells, by which the role of Opa in the infection among other antigens can be evaluated. In this case, the antisera would be used for blocking the attachment of the Opa to their receptors.

6.2 Cloning of *opa* genes

In order to produce recombinant Opa proteins to be used for developing anti-Opa antibodies as well as for the further liquichip assay applications, four *opa* alleles of the carriage isolates of *N. meningitidis* cc23 were chosen to be cloned into the expression vector pLEICS-01. Those alleles involved the three *opa* alleles of the isolate N222.1 (*opaA*, *opaB* and *opaJ*) and *opaJ* allele of the isolate N459.1(from the 4th time points of the carrier V222 that has an *opaJ* with multiple differences from the *opaJ* of the isolates of the 1st time point of the same carrier). OpaD was excluded as it is identical with OpaA of this strain. A set of primers were designed for amplifying and cloning *opa* alleles (section 2, Table 2-1), and these primers were specific to amplify the sequences that encodes for the mature Opa protein and exclude the repeat tract region that encodes the signal peptide. These primers also contained extra nucleotide sequences homologous to sequences in the expression vector in order to enable the insertion of the *opa* genes within the plasmid via homologous recombination. Since these primers bind to the conserved regions of *opa*, it cannot be used to amplify the 4 copies of *opa* genes directly from the

genome DNA. Therefore, the four alleles were firstly amplified from one of their flanking genes to differentiate them and then the resultant PCR products were used as a template for a second PCR using the primers designed specifically for cloning purposes.

The PCR products of the *opa* alleles were cloned into the expression vector pLEICS-01 in a cloning site that leads to fusion of the *opa* gene with sequences encoding for a 6x-histidine tag in the N-terminus, which facilitates the subsequent purification of the recombinant proteins using Nickel affinity chromatography technique. The vector preparation and the cloning process were done by the Protein expression facility (PROTEX) in the Department of Biochemistry in the University of Leicester. The expression plasmid was transferred into *E. coli* DH5α (which was received from the PROTEX facility). The cloning process was successful and the insertion of the *opa* alleles in the received clones were confirmed by sequencing and colony PCR.

6.3 Pilot expression of OpaB-N222

The four clones were transformed into *E. coli strain* BL21, and the transformation was confirmed by colony PCR using *opa* specific primers. The expression of the recombinant Opa was done by using this expression system with induction by IPTG. In the pilot expression experiment, *E. coli* BL21 transformed with pLEICS-01-OpaB were grown overnight in LB medium supplemented with ampicillin (100 µg/ml), and this culture was used to inoculate fresh media and incubated at 37 ^{0}C in a shaker incubator. When the culture reached OD_{600} between 0.6-0.7, it was divided into two cultures, and IPTG was added into one of them at a final concentration of 1mM to induce the expression while the other culture was left without adding IPTG and utilised as a control for the experiment. Both cultures were incubated again in the shaker incubator at 37 ^{0}C for 3 hrs, and after each hour, 500 µl of the samples were removed from both cultures and lysed in 100 ul of SDS loading buffer after harvesting the cells by centrifugation. Following that, 10 µl from each cell lysate was run on SDS-PAGE to check the expression of rOpa. The results showed that the optimum expression of rOpa was obtained after 3 hrs of induction with IPTG as a strong band was noticed in the whole cell lysates of BL21 from the induced cultures at the expected molecular weight of Opa (26 KDa) (Figure 6-1)and the intensity of this band was higher than the intensity of the other bands noticed in the samples of 1 hr and 2 hrs. No expression was detected in the 0 hr cultures as well as in the samples of the non-induced cultures (without IPTG). The presence of the OpaB variant in these

whole cell lysates was confirmed by western blotting using anti-Opa specific antibodies as shown in (Figure 6-2)

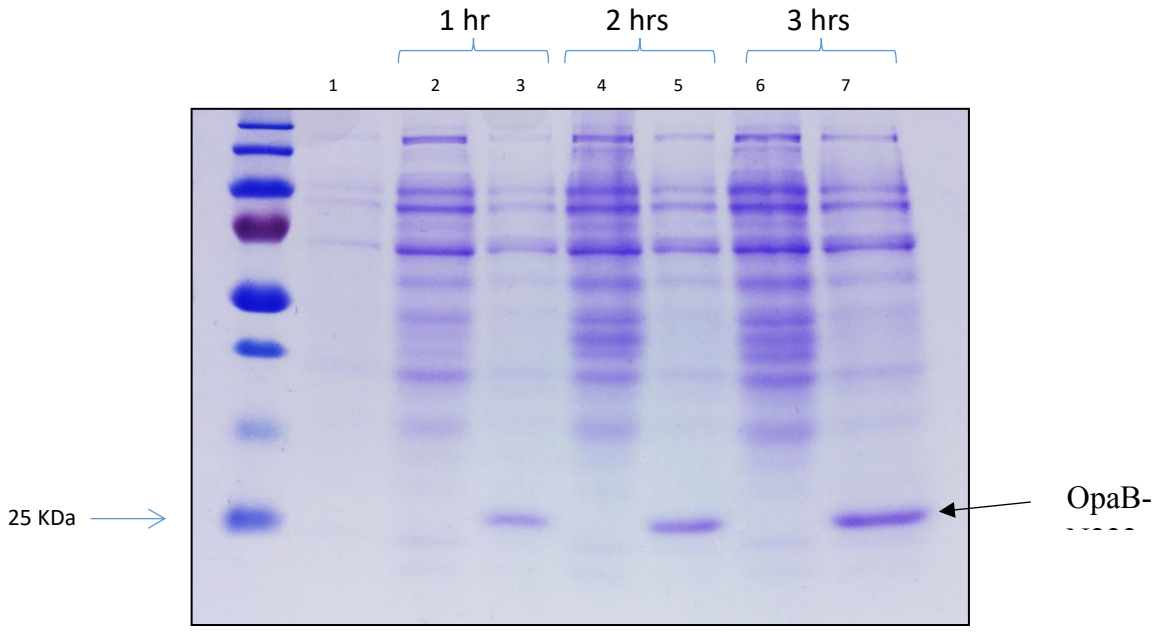


Figure 6-1 : **Expression of rOpaB protein in E. coli BL21 in the presence and absence of 1 mM of IPTG.** Cultures of E. coli BL21 containing expression plasmids of rOpaB were incubated for three hrs with and without 1mM of IPTG, and whole cell lysates were prepared from samples taken at 0, 1, 2, 3 hrs from both the induced and un-induced culture and 10 µl from each lysate were run on 12 % SDS-PAGE. Line 1, the zero time point of induction; lines 2, 4, 6, WCL of un-induced culture; lines 3,5,7, WCL of induced culture with 1 mM IPTG.

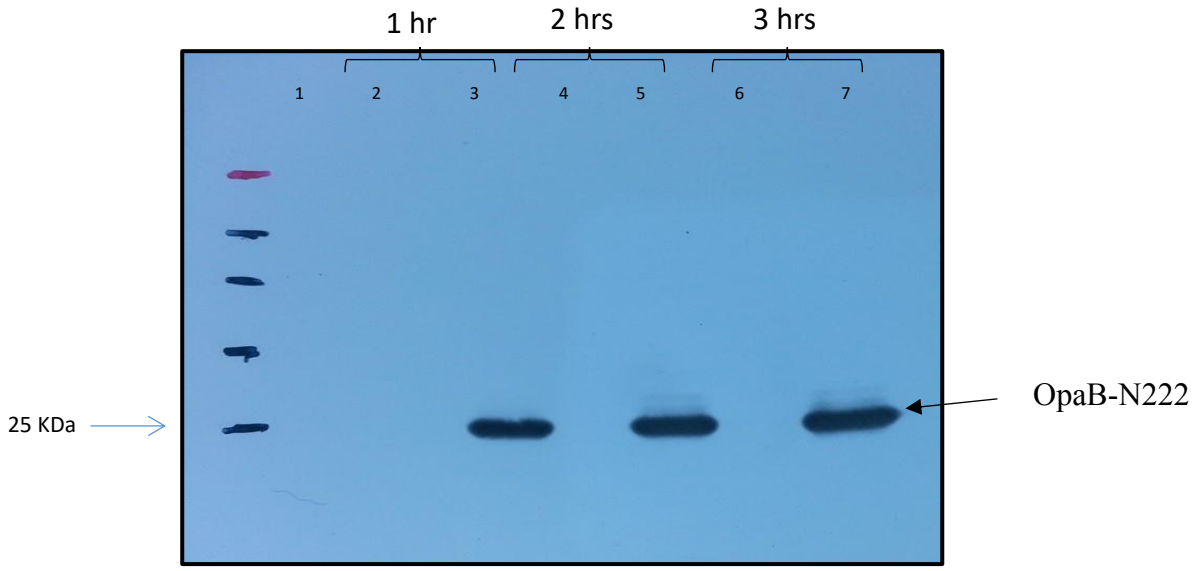


Figure 6-2 **: Western blotting analysis of the hourly expression of rOpa protein expressed in E. coli BL2 in the presence and absence of IPTG** .Samples analysed in this western blot are the same samples run on SDS-PAGE described in the previous figure (6.1). 10 µl of WCL from induced and un-induced cultures of BL21 were run on 12% of SDS-PAGE and then the proteins were transferred to PVDF membrane and probed with 1:1000 of anti-Opa antibodies. Then the membrane probed with peroxidase conjugated anti-rabbit IgG secondary antibodies and then the signal developed using ECL developer kit. Line 1, the zero time point of induction; lines 2, 4, 6, WCL of un-induced culture; lines 3,5,7, WCL of induced culture with 1 mM IPTG.

6.4 Pilot purification of Opa proteins

The recombinant OpaB was purified from *E. coli* BL21 according to the detailed protocol, which is presented in the Material and Methods section. The protein is produced as inclusion bodies within the bacterial cells and it was purified under denaturing conditions. The pilot purification experiment was performed with OpaB-N222, and it was started by extraction of the inclusion bodies from recombinant *E. coli* BL21, and this was done using a centrifugal step after sonicating the bacterial cells (see the detailed conditions in section 2.14.7). After that the inclusion bodies were solubilised in solubilising buffer (50 mM Tris−HCl pH 8 , 150 mM NaCl and 8 M Urea) overnight at room temperature with stirrer. The His-Tagged rOpaB protein then was separated and purified from the other proteins of *E. coli* BL21 using affinity chromatography by passing the soluble fractions through the Nickel affinity column (His-Trap). After several washing steps with washing buffer containing low concentrations of imidazole to remove the other proteins that may show low affinity to the Ni^{++} column, the rOpaB protein was eluted under denaturing t conditions using elution buffer (50 mM tris-HCl, pH8) containing 8 M Urea. The elution step was performed with several volumes of the elution buffer containing different concentrations of imidazole ranging from 150 mM to 500 mM to optimize the best concentration for the elution step. From each purification step, samples were taken and analysed on SDS-PAGE (as shown in Figure 6-3) to determine the best concentration of imidazole as well as the purity level of the eluted recombinant protein. According to the SDS-PAGE analysis, the highest concentration of the protein started to be eluted with 250 and 300 mM of imidazole, and no elution was detected in the lower concentration of imidazole. In addition, no elution was noticed in 400 or 500 mM imidazole, which means that the optimum elution of the protein occurred at 250 and 300 mM of imidazole. There was a considerable amount of recombinant protein eluted in the flow through and in the wash samples, which might be due to the high concentration of the recombinant protein within the samples that were passed through the column and might have led to column saturation.

Due to the presence of a smear of protein bands along with the Opa bands, it is not clear whether impurities were present, mass spectrometry was performed to confirm the identity of the Opa protein as well as the other small bands present in the elution fractions. Three bands were chosen to be cut out and analysed as shown in figure 6-3 and labelled as 1, 2 and 3. The results revealed that the main band and the other bands analysed showed

significant similarity with the sequences of OpaB-N222 (submitted to the database) with scores of (167, 78 and 159) as shown in Figure 4-6 and appendix (Figure 8-1, A). Therefore, the other bands (noticed as a smear) above the main band of Opa protein can be considered as an aggregation of Opa protein, while the band below the main band can be considered as degradation products of the same variant. Overall, these data revealed that the rOpa protein was eluted with a high purity without a considerable or detectable contaminant proteins coming from the proteins of the host.

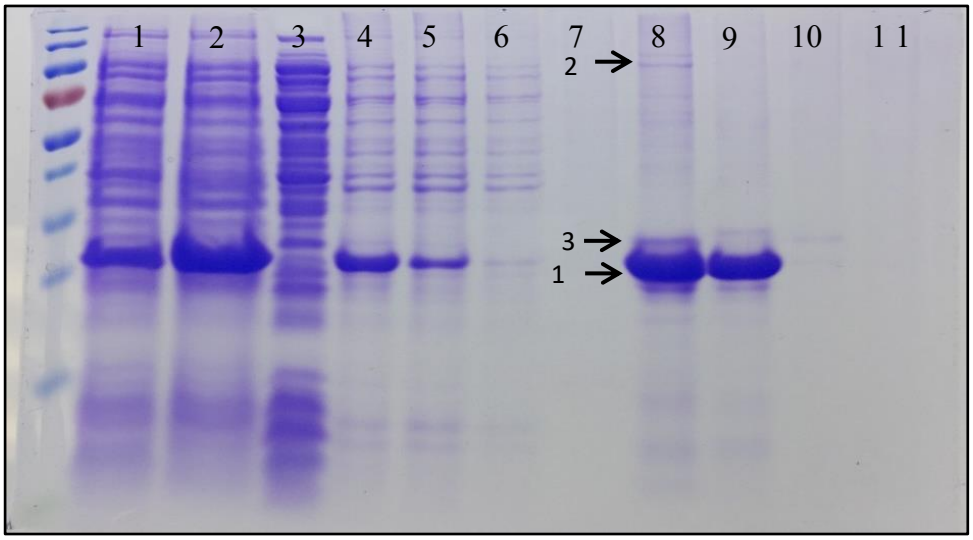
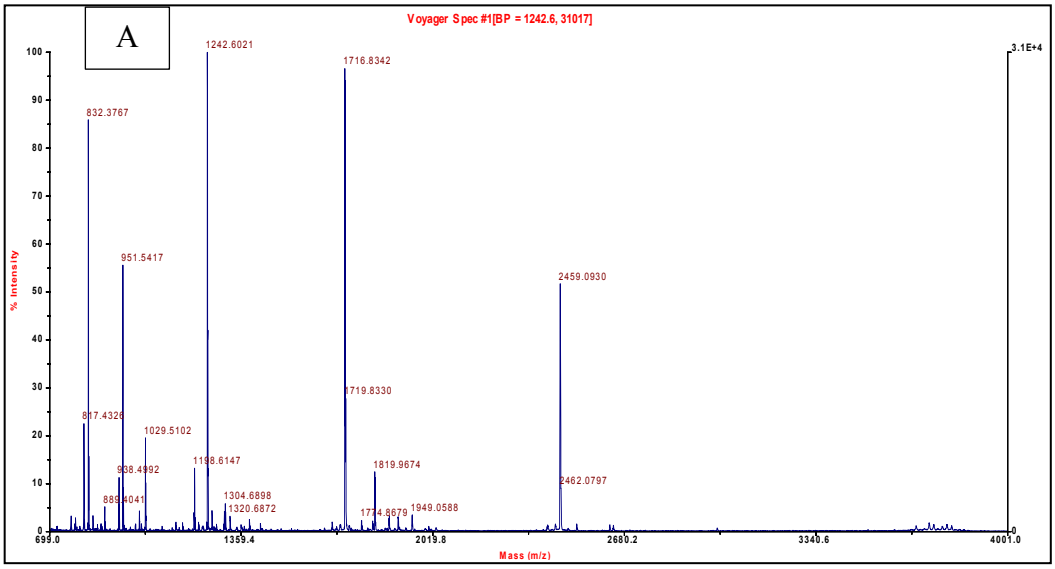


Figure 6-3. **Pilot purification of rOpaB expressed in *E. coli* BL21.** The protein expressed as inclusion bodies in the host which were released from the cells and solubilised in 8 M urea and the protein then purified by applying the sample to Nickel affinity column, which then eluted with gradient concentrations of Imidazole. From each step of purification, samples were taken and mixed with 2x SDS loading buffer and run on 12 % SDS-PAGE. Lane 1, BL21 WCL; Lane 2, Inclusion bodies ; Lane 3, supernatant (after separation the inclusion bodies) ; Lane 4, the IB solubilised in 8 M Urea ; Lane 5, Flow through; Lane 6, Unbound proteins (washed with washing buffer); line 7-11, eluted protein with 100 mM, 200 mM, 300 mM, 400 mM, and 500 mM of imidazole respectively.

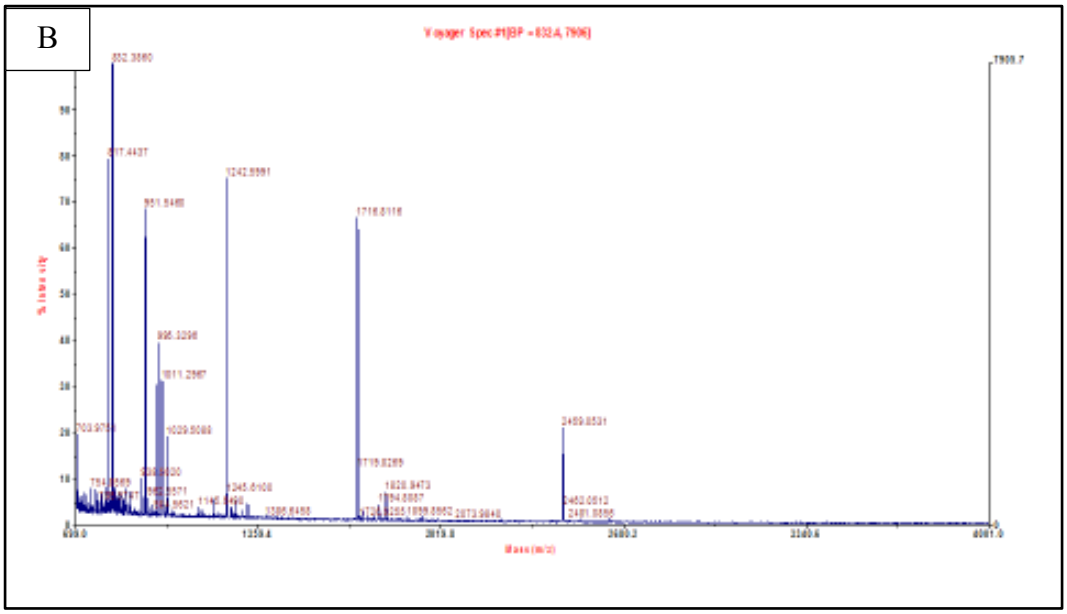
6.5 Large scale expression and purification of rOpa variants

The large scale production of the rOpas was performed after optimizing the key steps of the protocol depending on the previous pilot experiments. The volume of the transformant *E. coli* BL21 culture was raised to 800 ml and some modifications for the expression and purification steps were done. To avoid any possible contaminant in the final purified proteins, a washing step was added before solubilizing the inclusion bodies including washing the IB three times with 50 mM Tris buffer containing 2 M urea and 100 mM NaCl, in addition, the concentration of the NaCl in the washing and elution buffer was increased to 500 mM to prevent the aggregation of the Opa proteins during the binding to the column and during the elution. The proteins eluted with elution buffer containing

300 mM Imidazole and under denaturant conditions. As in the previous experiment, from each purification step, samples were taken and run on an SDS-PAGE gel as shown in figure 6-5 to confirm the elution of the protein as well as to evaluate their level of purity.



P00122 **Mass:** 30039 **Score:** 176 **Expect:** 8e-014 **Queries matched:** 13
EMBOSS_001_1 protein - provided by Ali Al rubaiawi 24/03/2016



	Accession	Mass	Score	Description
1.	P00122	30039	159	EMBOSS_001_1 protein - provided by Ali Al rubaiawi 24/03/2016

Figure 6-4: Mass spectrometry of samples of rOpaB protein. The samples of the purified rOpaB eluted with 250mM of imidazole were chosen to be analysed by Mass spectrometry in order to confirm the identity of the target protein band as well as the other bands presents in the sample when run on SDS-PAGE. The protein of three bands named as 1, 2, and 3 on the SDS-PAGE figure (6-3) were extracted from the gel and digested and applied for mass spectrometry. BSA protein was utilised as standard protein in the assay, and the data revealed that the proteins from the three bands matches the sequences of the submitted OpaB amino acids sequences with high scores as shown in the figure. Figure A. refer to spectra of band number 1, while figure B is the spectra of band number 3 as numbered on the gel in figure 6-3.

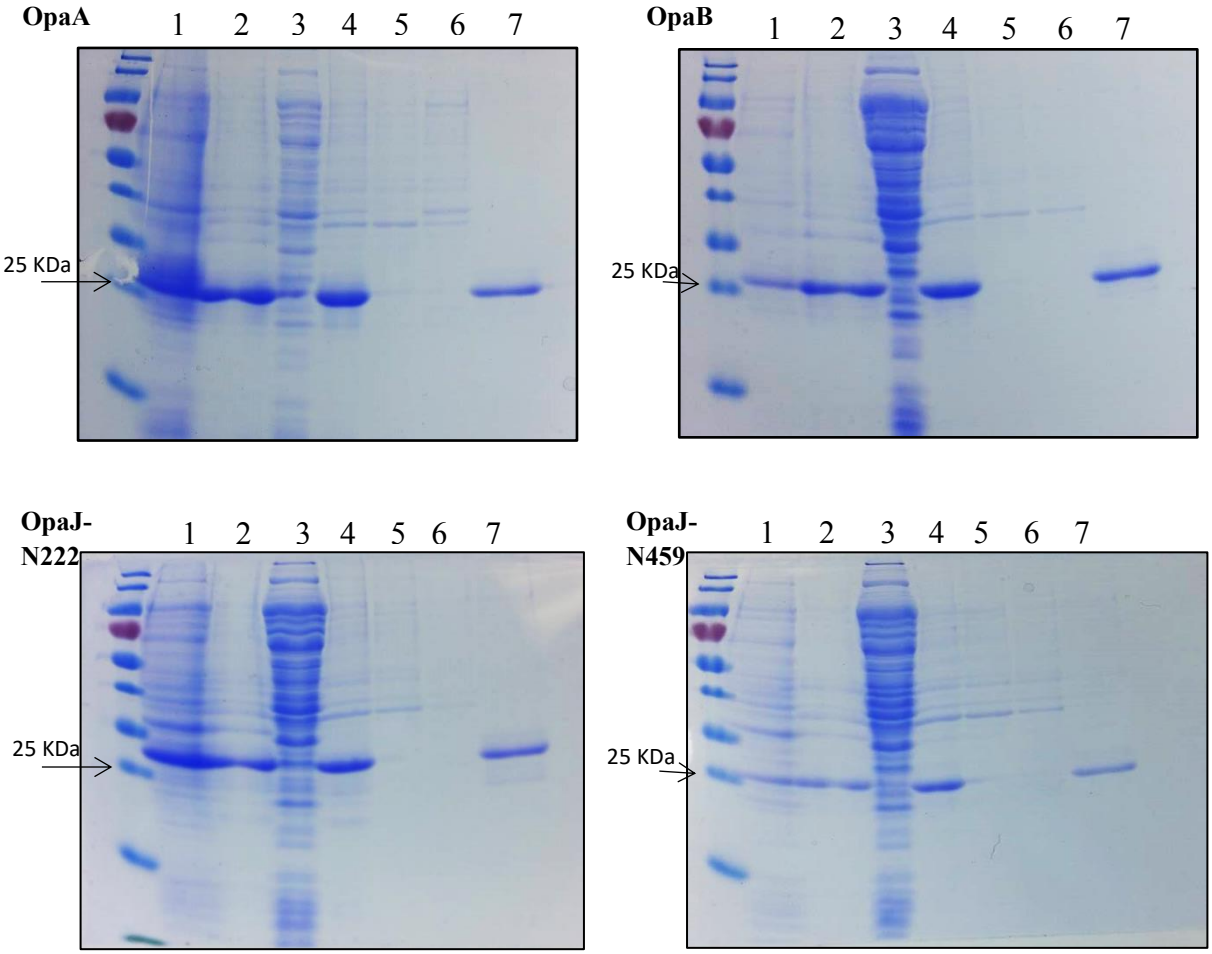


Figure 6-5: **Expression and purification the of rOpa proteins**. The four rOpa variants were expressed at a large scale in BL21 after optimising the key steps of expression and purification protocol. The proteins expressed as inclusion bodies within the host were extracted and solubilised in 8 M Urea, and then purified under denaturant conditions using Nickle affinity column. From each step of purification, samples were taken and mixed with 2x SDS loading buffer and run on 12 % SDS-PAGE. Samples were loaded in each gel as following: Lane 1, BL21 WCL; Lane 2, Inclusion bodies ; Lane 3, supernatant (after separation the inclusion bodies) ; Lane 4, the IB solubilised in 8 M Urea ; Lane 5, Flow through; Lane 6, Unbound proteins (washed with washing buffer); line 7, eluted proteins with 300 mM, of imidazole .

Mass spectrometry was done for the four variants after extracting them from the SDS-PAGE gel. The amino acid sequences of each recombinant protein were submitted to the database, and the spectra then searched about the similarity of proteins in these database. The analysis revealed that spectra of all the four recombinant variants showed significant similarity for the Opa sequences submitted to the database (OpaA-N222, OpaB-N222, OpaJ-N222 and OpaJ-N459) with scores of : 192, 176, 198 and 82 respectively (data in the Appendix figure 8.1).

Analysis of the SDS-PAGE gel showed that the eluted proteins were of a high purity without any extra bands except a faint band which are considered as an aggregation of

the same proteins, as seen above, as well as bands below the expected Opa band which are likely to be a degradation of the Opa proteins.

6.6 Refolding of the purified Opa variants

The refolding step of the purified rOpas involved applying two different protocols. Both of them were dependent on the rapid dilution of the purified proteins either 1:100 or 1:20 in the refolding buffers.

For the first protocol (de Jonge *et al.* 2002), the protein (in the elution buffer) was firstly concentrated using centrifugal filter-tubes to reduce the volume of the solution to 2 ml and then diluted rapidly in 198 ml PBS containing 328 mM of ethanolamine (pH 12), 0.5% SB12. The dilute protein was incubated for 24 hrs at 4 ^{o}C with stirring. After that, the pH of the refolding solution was neutralized to 7.5 by adding HCl and 10 mM of Tris to buffer the solution. The proteins were then concentrated again and the volume reduced from 200 ml to 5 ml. However, a problem that was encountered with using this protocol is that after concentrating and reducing the volume of the refolding mix the proteins aggregated and precipitated. To avoid this problem, the experiment was repeated with modifications of the protocol with addition of 300 mM NaCl to the refolding buffer, as the ionic strength of the NaCl was expected to prevent the aggregation of the proteins. However the same problem occurred and the proteins completely precipitated after concentrating the refolding mix. Therefore another protocol was used (Fox *et al.* 2014) which also depends on a rapid dilution of the denatured protein 20 folds in the refolding buffer (20 mM Tris−HCl, pH 8.0, 500 mM NaCl and 1% Z3-14). The soluble proteins in the elution buffer containing 8 M Urea was concentrated and the volume reduced to 5 ml before rapidly diluting in 100 ml of the refolding buffer. The protein was then incubated at room temperature for five days and concentrated again to reduce the volume to 5 ml. There was only a slight aggregation and precipitation of the proteins in comparison to the previous protocol. Another problem that was encountered after the refolding step is that the detergent Z3-14 (which is used in the refolding buffer with a concentration of 1%) cannot be removed by dialysis and it is concentrated with the proteins when washed or concentrated with concentrator filters. This detergent can interfere with the proteins and affect the downstream applications. Therefore, the refolded proteins were re-bound to the nickel column and washed five times with Tris buffer containing 0.05 % of Z3-14 and eluted with elution buffer also containing 0.05 % of Z3-14. To remove the Imidazole from the protein samples in the elution buffer, the proteins were dialysed against PBS buffer

containing 0.05 % Z3-14 using Slide-A-Lyzer G2 Dialysis Cassettes. The proteins were then concentrated and their final concentrations were determined by Bradford assay. The concentrations were as follows: OpaA 0.5 mg/ml, OpaB 0.9 mg/ml, OpaJ-N222 0.2 mg/ml and OpaJ-N459 0.1 mg/ml . The proteins were aliquoted and stored at -80.

The successful refolding of the Opa proteins was checked by semi-native SDS-PAGE as the native Opa protein migrates faster than the denatured protein in the semi-native PAGE (de Jonge *et al.* 2002; Otzen and Andersen 2013). From each variant, two samples were prepared , one of them was diluted 1:2 in 2x SDS loading buffer and heated at 95 ^{0}C for 5 minutes (denatured samples) , while the other sample was diluted 1:2 in 0.1% SDS loading buffer (folded samples) without heat treatment , and both of the samples were run alongside on the SDS-PAGE.

The results showed that all the folded samples migrated divergently from the denatured samples of each variant with a molecular weight below 25 KDa , while all the heat denatured samples gave bands above 25 KDa as shown in figure 6-6. The absence of an extra band in the folded samples at the same molecular weight as the denatured samples means that the entire protein sample was homogenous and there were no intermediate or denatured molecules in folded samples.

6.7 Immunodotblotting to analyse the functionality of the refolded Opa proteins

In order to confirm that the proteins were refolded properly, the functionality of the refolded Opa variants and their ability to react and bind to their native receptor on human cells (CEACAM1) were tested by using an immunodotblotting technique. For each variant, folded and denatured samples were prepared with five different concentrations by diluting them in PBS. For the denatured samples, the proteins were diluted in PBS containing 2% SDS and denatured by heating at 98 ^{0}C for 10 minutes. The folded and the denatured samples were loaded together on nitrocellulose membranes in identical amounts, and then the membrane was left to dry for 10 minutes. The samples were overlaid with human CEACAM1-Fc receptor (5 ml of 0.25 µg/ml) in blocking buffer (PBS + 3% BSA) and incubated for 1 hour at room temperature with shaking. After that, the samples were incubated with peroxidase-conjugated mouse anti-Human IgG antibodies (1:2000) for 1 hour followed by detection of peroxidase with the ECL developer kit. The WCL of *N. meningitidis* strain N117.1 (expressing OpaB and proven to bind to CEACAM1; see section 4.9, Figure 4-15) was used as positive control and the

WCL of isolate N370.4 (expressing OpaJ-N222 and proven to lack CEACAM1 binding) as a negative control. Whereas incubating the protein samples with only the secondary antibodies was also done as a further control.

The binding of Opa proteins to CEACAM1 is conformation-dependent and the protein in a denatured state should not react with human CEACAMs (de Jonge *et al.* 2002). Therefore, binding of the rOpa to this receptor reflects their refolding state. The results of the dot blot showed that folded samples of OpaA and OpaB showed a good reactivity with human CEACAM1 while no reactivity was noticed in the denatured samples, (Figure 6-7 A) which means that those two variants were folded properly. Relatively similar amounts of both proteins were loaded in the last three dots (see Figure 6-7, dots with lower amounts of proteins from the right to the left), but OpaA showed higher reactivity. This might relate to the quality and the folding state of this variant which might be better folded than OpaB. Whereas OpaJ-n459 did not show any reactivity to the receptor, the native protein in cell lysates of this variant showed reactivity with CEACAM1 (see chapter 4, figure 4.15, isolate number N459.1). However, the fourth variant, OpaJ-N222, did not react to the human CEACAM1 in either its native state as purified protein or in the WCL of isolate N 370.4 which express this variant and was used as negative control in these experiments (figure 6-7 B). Note that the WCLs exhibited some cross-reactivity with the secondary antibody but isolate N370.4 does not have a level of reactivity above background.

6.8 Generating of mouse anti-rOpa antibodies

Five groups of 10 mice were immunized with 5 μg /dose (three doses) of each purified rOpa variant. Group 1 were a control group which were immunised with the adjuvant only (Freunds), while the other groups were immunised with rOpas as following: group 2 immunised with rOpaA, group 3 with rOpaB, group 4 with OpaJ-N222 and group 5 were immunised with rOpaJ-N459. The immunisation and bleeding of the mice were done at the National Institute of Biological Standards and Control (NIBSC). The sera from each group were pooled together, and in order to test the specificity and the quantity of the generated antibodies to the Opa variants, ELISA tests were performed for each antisera against the target rOpa variant and the other variants of rOpa to check whether the antisera will cross react with the different Opa variants. The antisera of the control group (adjuvant only) were also probed against each different variant of Opa in each assay

as a control for non-specific binding, whereas PorA was used as an unrelated and non-homologous antigen. The sera were diluted 1:500 and probed against 1µg of each rOpa variant. The control wells, which involved the antigens without primary antibodies, were used as a blank and these values were subtracted from the mean of the value obtained for the other wells to get the final value of the fluorescence intensity.

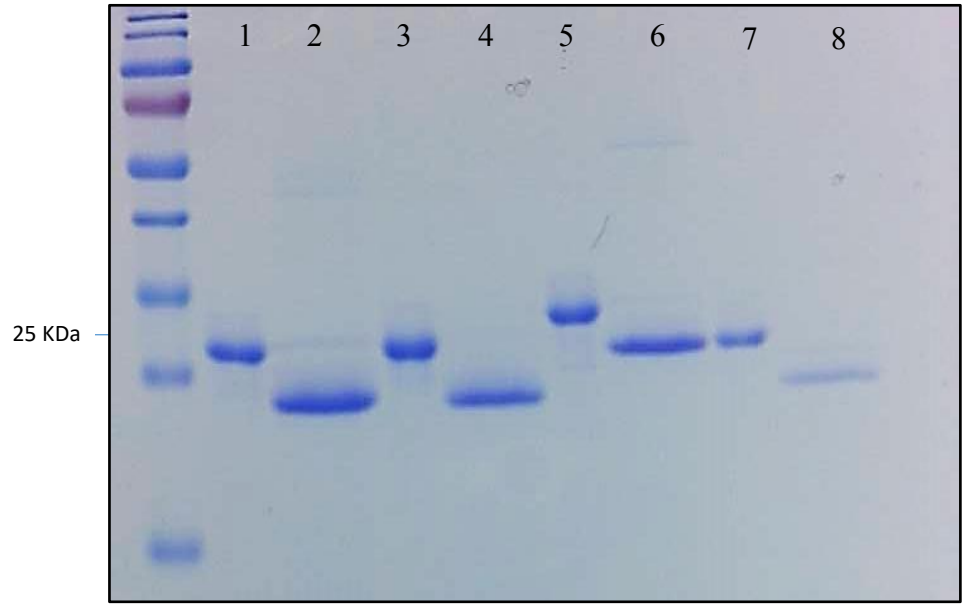


Figure 6-6: **SDS−PAGE gel shift analysis of the refolding state of the purified rOpa variants.** For each purified rOpa variants, two samples were prepared and run on 12 % SDS-PAGE to the refolding state of the folded samples which should move faster than the denatured samples on the gel. The denatured samples were diluted I 1:2 in 2x SDS loading buffer and heated at 95 ^{0}C for 5 minutes, while folded samples were diluted 1:2 in 0.1% SDS loading buffer without heat treatment. Samples were loaded on the gel as following: : Lane 1, denatures OpaA; Lane 2, folded OpaA ; Lane 3, denatured OpaB ; Lane 4, folded OpaB ; Lane 5, denatured OpaJ-N222; Lane 6, folded OpaJ-N222; Lane 7, denatured OpaJ-N459; and Lane 8, folded OpaJ-N459.

The results showed that each antisera displayed high reactivity to their target rOpa antigens, but differing degrees of cross reactivity with the other Opa variants. Anti-OpaB showed the highest cross reactivity with the different Opa variants followed by anti-OpaA (figure 6-8), while the antisera against two variant OpaJs showed a high specificity for their target antigens (figure 6-9).

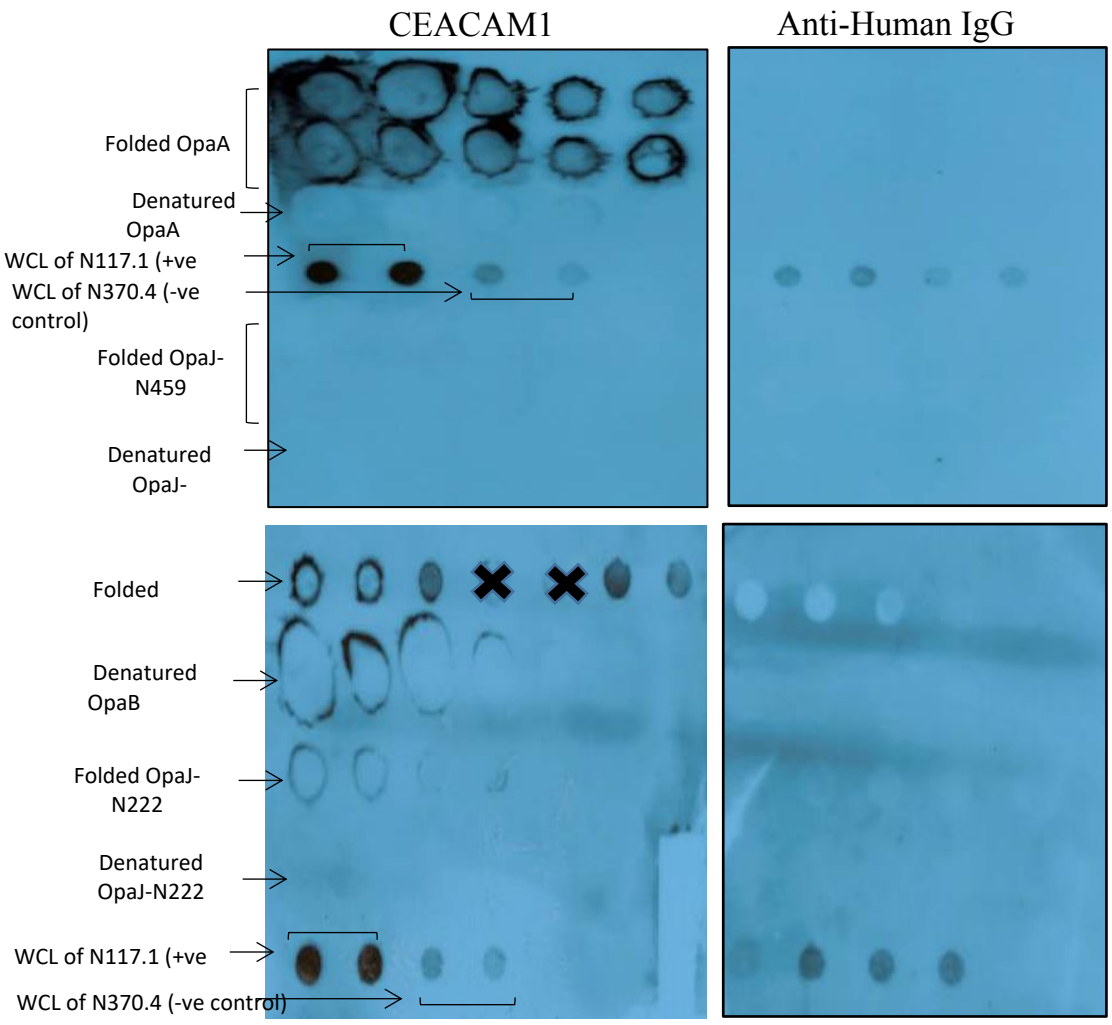


Figure 6-7 **Receptor overlay immunodotblot to analyse the functionality of the purified rOpa**. For each Opa variant, folded and denatured (samples were prepared with five different concentration and loaded together on nitrocellulose membrane in the same amount, and the membrane was then overlaid with human CEACAM1-FC receptor (5 ml of 0.25 µg/ml) and also with only the secondary antibodies (mouse antihuman IgG as a control.) and incubated for 1 hr. The reactivity to the CEACAM1 were checked using secondary antibodies mouse anti-Human IgG antibodies (1:2000). The signal then developed by using the ECL developer kit. The amount of protein loaded as following: rOpaA (the five dots from left to right): 25 µg, 20 µg, 10 µg, 5 µg and 2.5 µg; rOpaJ-N459: 20 µg, 10 µg, 5 µg, 2 µg and 1 µg. rOpaB : 50 µg, 40 µg 20 µg, 10 µg and 5 µg,; rOpaJ-n222: 25 µg, 20 µg , 10 µg, 5 µg and 2.5 µg . The WCL were loaded with 50 microliters of 0.5 of OD_{260} of cells (alkali lysed) which are equal to around 10^7 cells in each well in duplicate.

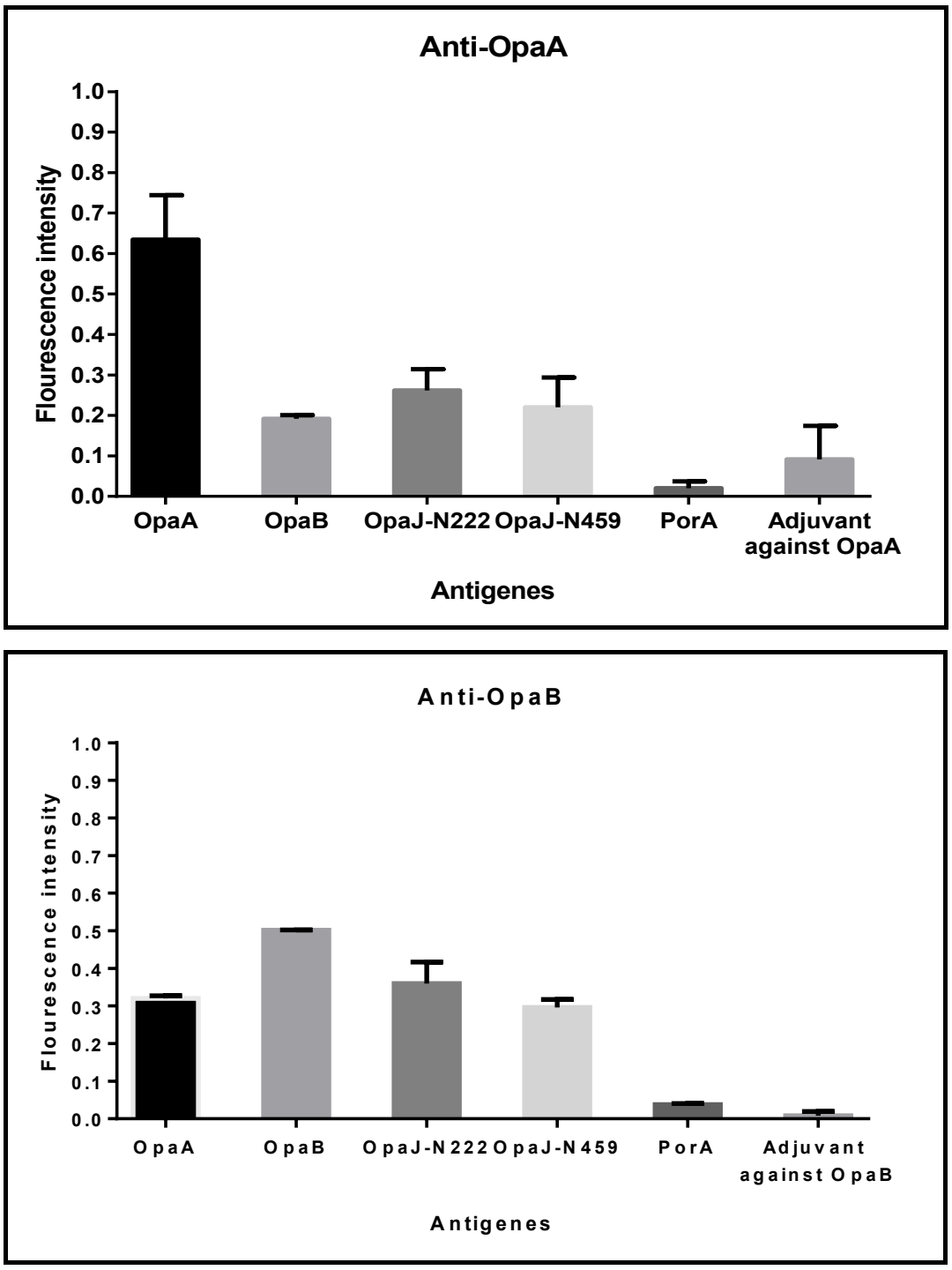


Figure 6-8 **ELISA reactivity and cross reactivity of the generated anti-opaA, and anti-OpaB antisera against their target antigens and other different variants and unrelated antigens**. 1 μg of each Opa variants were probed with anti-Opa antisera diluted to a 1:500 concentration. The final value of the fluorescence intensity was adjusted by subtraction of a background reading (the control wells without primary antibody) and blotted above as a mean of two reads with standard error

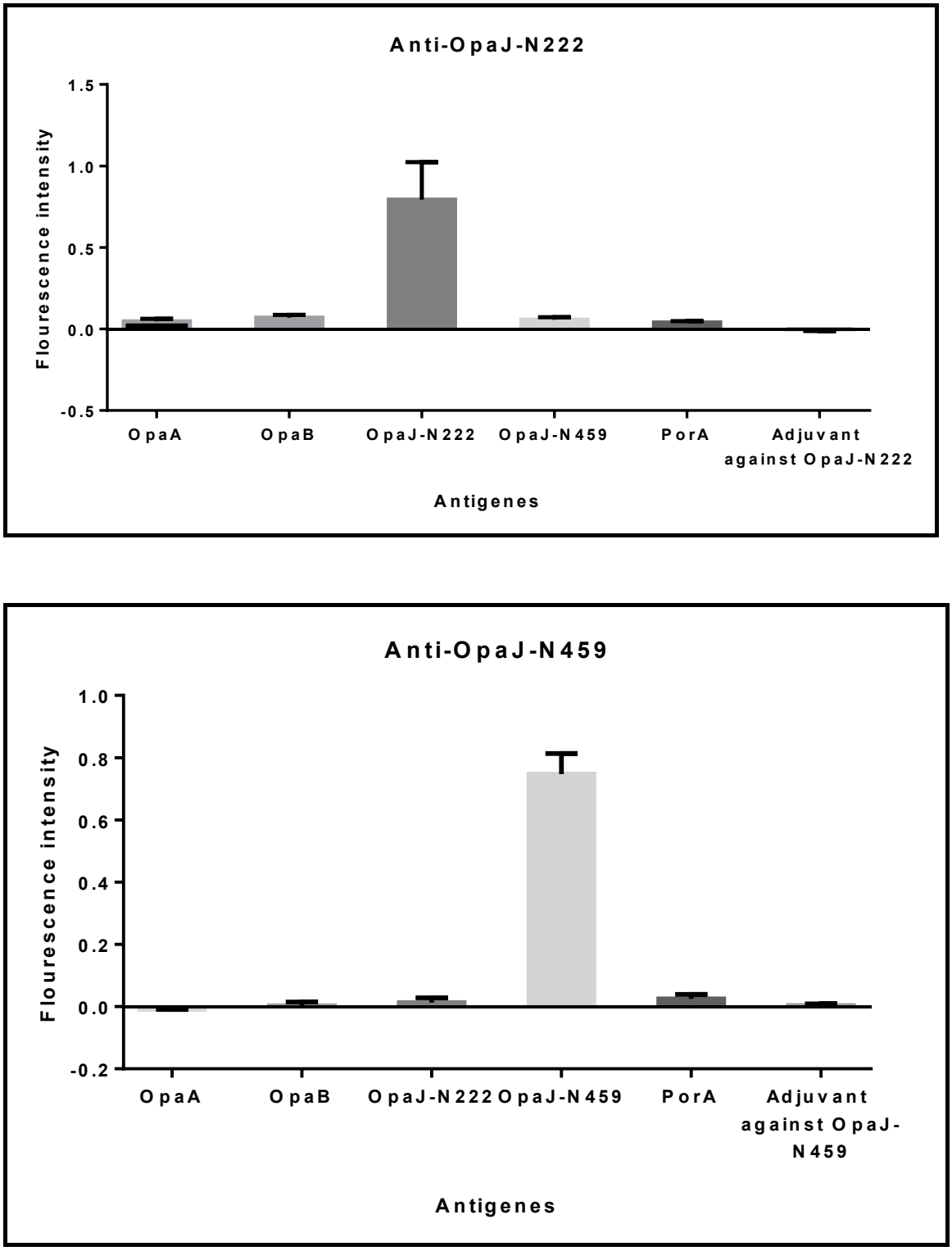


Figure 6-9 **ELISA reactivity and cross reactivity of the generated anti-opaJ-N222, and anti-OpaJ-N459 antisera against different rOpa variants and unrelated antigen**. 1 µg of each Opa variants were probed with anti-Opa antisera diluted to a 1:500 concentration. The final value of the fluorescence intensity was adjusted by subtraction of a background reading (the control wells without primary antibody) and blotted above as a mean of two reads with standard error.

6.9 Western blotting analysis of the generating antisera against the target and another Opa antigens

As a further confirmation for the specificity of the antisera in targeting their specific antigens, western blotting was performed for each antisera against all the Opa variants as shown in figure (6-10). Each anti-sera was diluted 1:1000 and used to probe the 4 Opa variants in the same membrane. The four Opa variants were also probed with the general anti-Opa antibodies (which binds to the conserved domain of Opa) as a control for the experiment. The results were consistent with the data gained from ELISA tests. Anti-OpaB showed the highest cross reactivity with the other Opa variants (figure 6-10). The anti-OpaA sera also showed a lower level of cross reactivity and was more specific to rOpaA than the other variants. Contrastingly the anti-OpaJs antisera Were highly specific for their target antigens as shown in figure (6-10 E&F) with very low reactivity with the other variants. These results can be related to the refolding state of the proteins when used to immunise mice.

6.10 Evaluating the serum bactericidal activity of the generated anti-Opa antisera

6.10.1 Sensitivity of meningococcal cc23 strains to human complement

The sensitivity of the meningococcal strains to human complement was a major limitation in the bactericidal assays performed in previous experiments (see chapter 5, section5.2). The wild type strain H44/76 was very sensitive to the complement source in the absence of the antibodies, while the wild type strain MC58 , even though it survives in 5% human complement, it showed variability in resistance in later assays when populations were prepared from different single colonies. In order to test the bactericidal activity of the newly generated antisera, it was necessary to test the resistance of the cc23 isolates expressing these Opa antigens to human complement. For this assay, a modification to the protocol used in the previous assays (chapter 5, sections5.2 and 5.3) was applied, whereby bacteria were firstly grown overnight on BHI plates and then single colonies were taken, streaked again on blood agar plates and incubated for 4 hrs at 37 ^{0}C in the presence of 5% CO_2. After that, the bacteria were harvested and used to prepare the inoculum for the assay. This protocol was used in a preliminary test with isolate N222.1 and the isolate was shown to survive better than using the previous protocol (i.e. bacteria grown overnight on BHI plates only without additional incubation on blood agar plates for 4 hrs).

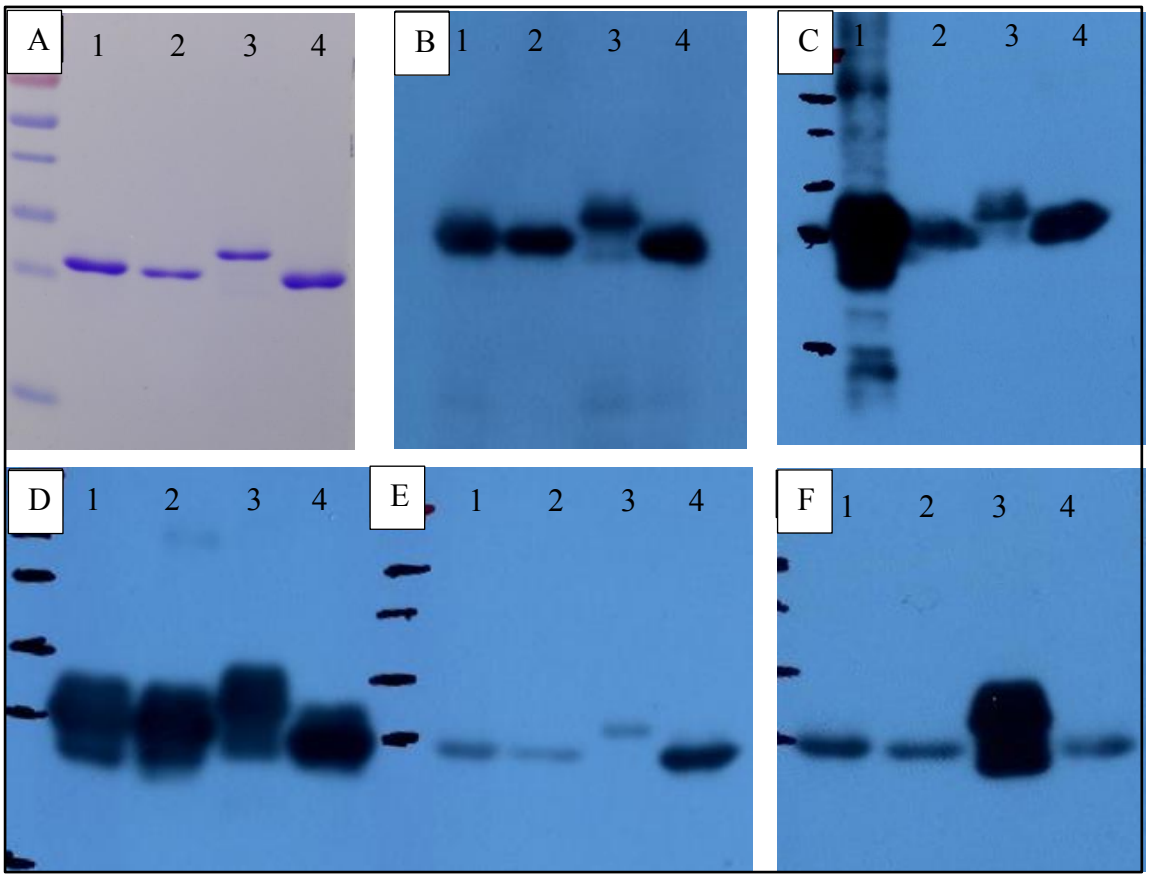


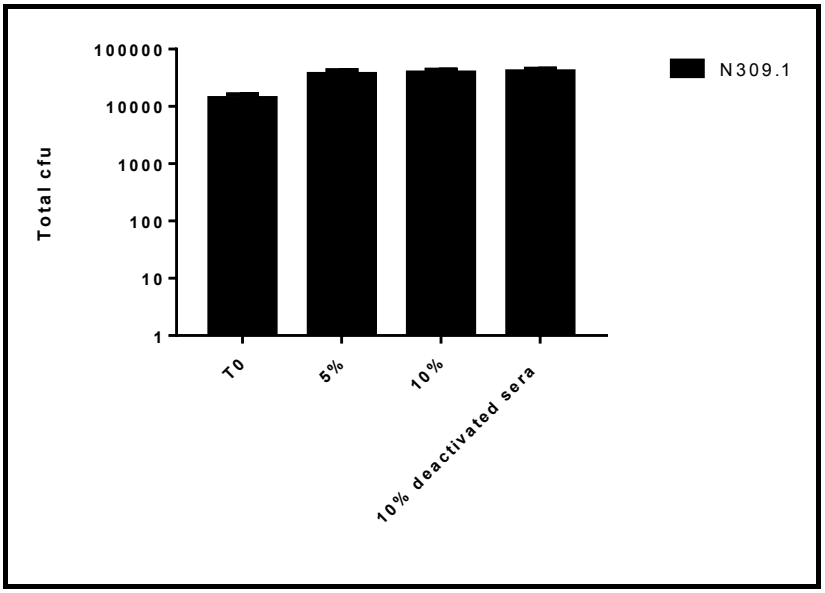
Figure 6-10 **western blot analysis of the reactivity and cross reactivity of the generated anti-Opa antisera against their target antigens and the other Opa variants**. Samples of denatured rOpa variants were prepared by mixing 5 microliter of protein samples (in 8 M Urea) with 5 microliters of 2x SDS loading and run of 12 % SDS-PAGE. The proteins then transferred to PVDF membrane and probed with different ant-Opa anti-sera diluted to 1:1000, and the reactivity then detected by using HRP conjugated anti-mouse IgG antibodies. Figures : A SDS-PAGE; B , Opa variants probed with general anti-Opa antisera; C, Opa variants probed with anti-OpaA antisera ; D Opa variants probed with anti-OpaB antisera; E, Opa variants probed with anti-OpaJ-N459 antisera; F Opa variants probed with anti-OpaJ-N222 antisera. Samples loaded in the same order iun the all blots as in the SDS-PAGE and including: 1, OpaA; 2 OpaB; 3, OpaJ-N222; and 4, OpaJ-N459.

Therefore, this protocol was used with other three carriage isolates of CC23 isolated from the SAME volunteer V222 but expressing different Opa variants including: N309.1 expressing OpaA, N370.4 expressing OpaJ-n222 and N459.1 expressing OpaJ-n459. An inoculum of around 10^4 cells of each isolate were prepared and incubated with two different concentrations of the complement (5% and 10%) for 1 hr at 37C, the bacteria were also incubated with 10% of heat-inactivated sera as a control. After the incubation, 10 microliter from each well were streaked directly on BHI plates and incubated overnight to get the CFU counts. The results showed that all the three isolates were resistant and able to survive in the both concentrations of the human sera and no reduction in the cfu counts were observed (Figure 6-11and Figure 6-12).

6.10.2 Serum bactericidal assays of the generated anti-Opa antibodies

The bactericidal activity of the generated polyclonal anti-Opa antibodies were tested against the target strains that express the matching Opa variants (individually) used to immunise the mice. The assays involved testing the pooled anti-Opa antisera with different concentrations ranging from 1:20 – 1:320 along with 10 % of human pooled sera as a complement source. The bactericidal activity of anti-PorA (P1.5) were also tested with the same range of concentrations against the target strains in the same assays as a control to show sensitivity of the isolates to the antibody- mediated bactericidal activity in the presence of the complement. The results showed that both anti-OpaB, and anti-OpaJ-n459 showed very significant bactericidal activity, and more than 50% of the bacterial population were killed when incubated with all the concentrations of the antisera including the 1:320 dilution (Figure 6-13).

However, the concentration of the human complement was reduced for the test of the anti-OpaA to 5% to see whether this would alter the bactericidal activity. The data showed that this antisera was also bactericidal, and killing of more than 50 % occurred with the first three dilutions (1:20, 1:40 and 1:80) while in the higher dilutions the antibodies did not mediate any reduction in the population as shown in figure (6-14), while the anti-PorA showed a strong reduction (more than 50%) of the population in the all dilutions.



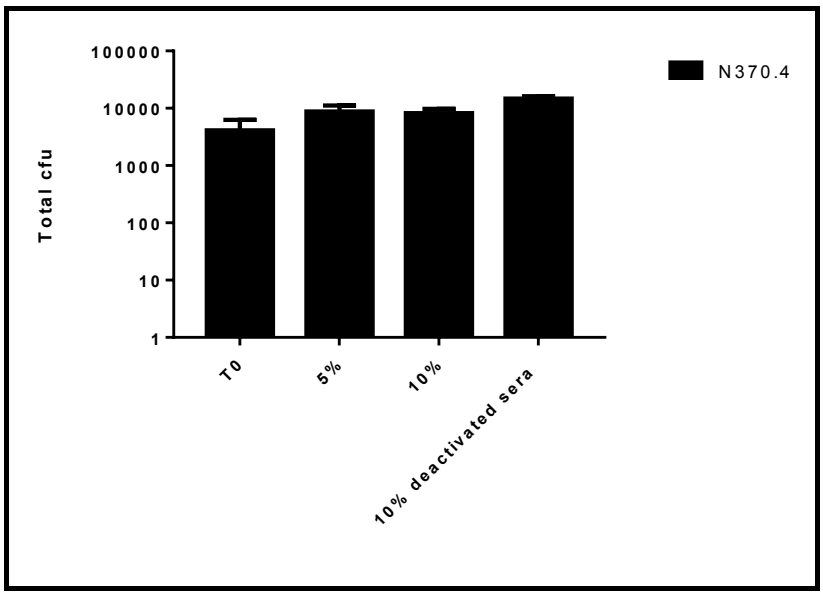


Figure 6-11 . **The sensitivity of the meningococcus strains N309.1 and N370.4 to human complement source.** A suspension of around 10^4 cells of each isolate were prepared and incubated with human pooled sera with different concentration (in duplicates) and incubated at 37 ^{0}C for 1 hr with 5% CO2. 10 % of heat inactivated sera was used as negative control with each assay. 10 µl of each mix were taken and spread on BHI plates in duplicate and incubated at 37 ^{0}C overnight with 5% CO2 to get the cfu count. The mean of 4 CFU counts was taken and plotted above, and the error bar represent the standard error of the 4 counts.

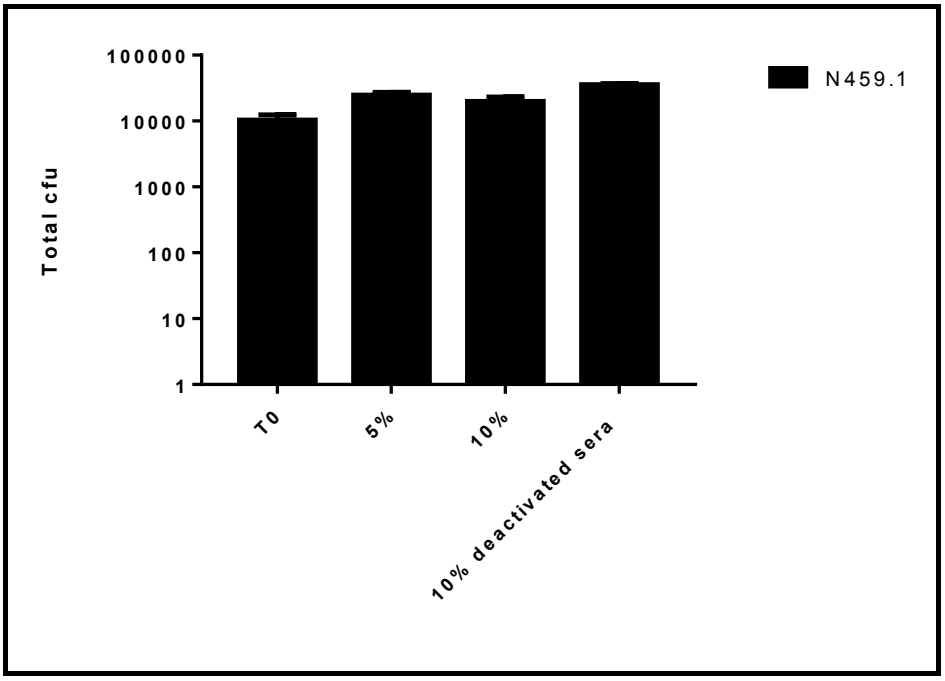


Figure 6-12: **The sensitivity of the meningococcus strain N459.1 to the human complement**. An inoculum of around 10^4 of bacterial cells were prepared and incubated with human pooled sera with different concentration (in duplicates) and incubated at 37 ^{0}C for 1 hr 5% CO2. 10 % of heat inactivated sera was used as negative control with the assay. 10 µl of each mix were taken and spread on BHI plates in duplicate and incubated at 37 ^{0}C overnight with 5% CO2 to get the cfu count. The graph showed the mean of 4 cfu counts with standard error.

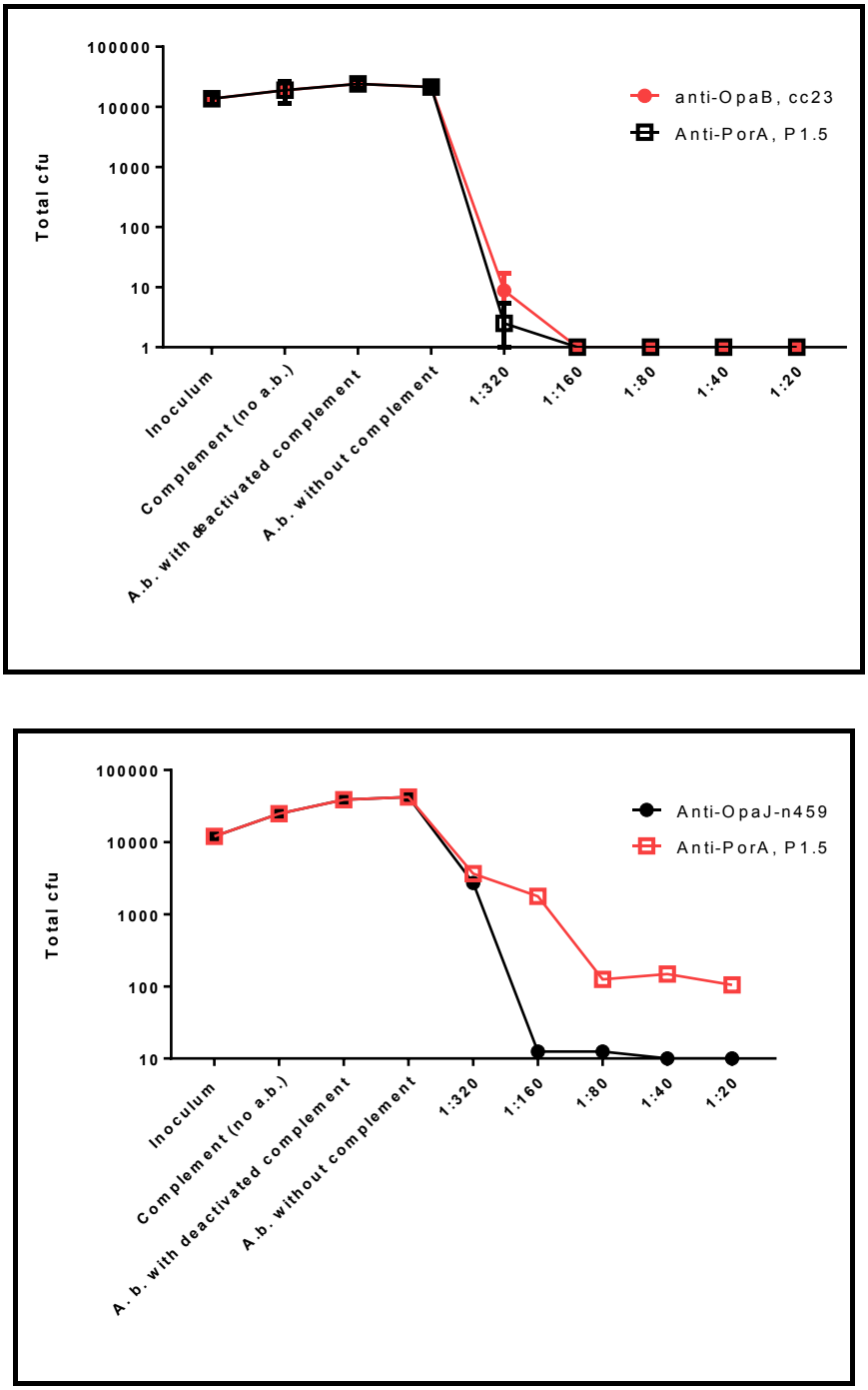


Figure 6-13 : **Serum bactericidal assay of the generated anti-Opa antisera and PorA P1.5 mAb**. A suspension of around 10^4 cells of *N. meningitidis* N222.1 (expressing OpaB variants) and N459.1 (expressing OpaJ) were prepared and incubated with different dilutions of anti-Opa antibodies or with anti-PorA P1.5 (as a control) in, in the presence of 10 % of human sera as a complement source. Each mix was incubated for 60 minutes at 37 0C with 5% CO_2. Three controls were set up including the bacteria with complement only, the bacterial with heat inactivated with antibodies and the bacterial cells with the antibodies only. cfu counts were taken by plating 10µl of each assay on BHI plates (in duplicates) and incubating overnight at 370C. The mean and SEM of up to 4 cfu counts were taken and blotted above.

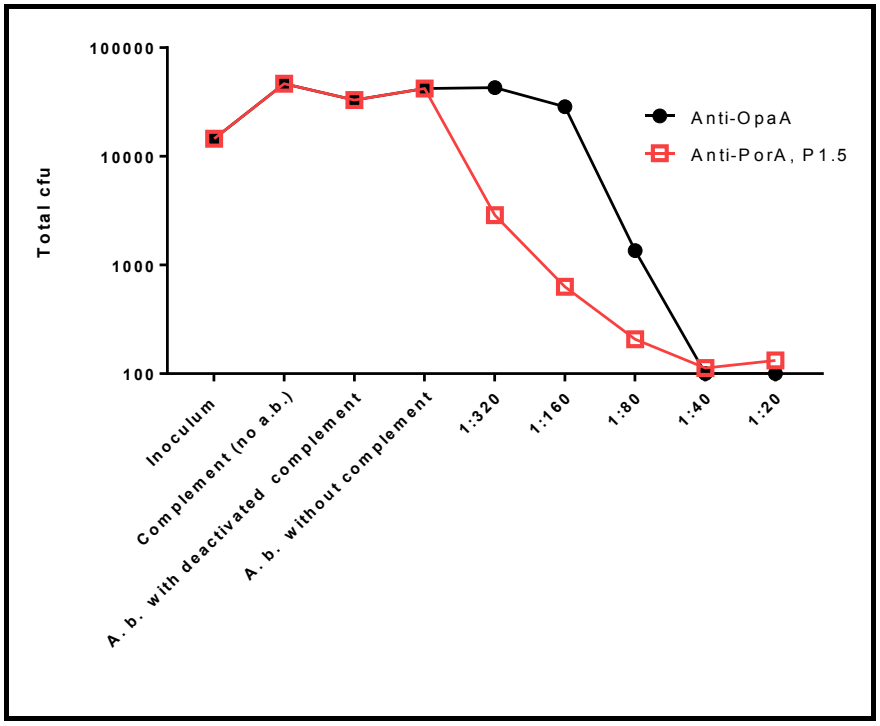


Figure 6-14: **Serum bactericidal assay of anti-OpaA polyclonal antisera and PorA P1.5mAb**. A suspension of around 10^4 cells of *N. meningitidis* N309.1 (expressing OpaA) were prepared and incubated with human pooled sera as a complement source at a final concentration of 5% with along with different dilutions of anti-OpaD antibodies or with anti-PorA P1.7 Mab in duplicates. Each mix was incubated for 60 minutes at 37 0C with 5% CO_2. Three different controls were involved including the bacteria with complement only, the bacterial cells with heat inactivated with antibodies and the bacterial cells with antibodies only. cfu counts were taken by plating 10µl of each assay on BHI plates (in duplicates) and incubating the plates overnight at 370C. The mean and SEM of up to 4 cfu counts were taken and blotted above.

6.11 **Discussion**

The aim of this part of my project was to produce recombinant Opa proteins of cc23 carriage strains in order to use these recombinant proteins in assessments of the level of immune response in human carrier's sera. The study also aimed to produce bactericidal polyclonal antibodies against Opa proteins that can be used for investigation of the role of PV of these proteins in evasion of the immune response as well as for blocking assay in testing the reactivity of the Opa with human antibodies (liquichip assay) or in evaluating the role of different Opa variants in invasion and adhesion processes.

Cloning of the Opa alleles was done successfully into the expression vector pLEICS-01, which was transformed into *E. coli* BL21 and used to express the recombinant proteins. The expression was done with induction by 1 mM IPTG, and the proteins were shown to be expressed at a high level using this system. The previous works on production and purification of rOpa (De Jonge *et al.* 2003a; Callaghan *et al.* 2011) depended on purifying

Opa with multiple steps of purification with different kinds of chromatographic techniques such as ion exchange and gel filtration. In our protocol, the proteins were purified under denaturing conditions with a single step of purification using Nickel affinity chromatography, while the refolding step was done after eluting the proteins in a denatured state. The fusion of the Histidine tag to Opa and purification using a Nickel affinity chromatography yielded protein with a high purity with a single step purification. The proteins were eluted with a smear or extra bands above and below the main band of Opa. Mass spectrophotometry confirmed that the extra bands were also Opa proteins suggesting that aggregation of the Opa proteins was occurring and that these proteins might bind to other proteins from the expression host. However, adding a washing step to the purification protocols and raising the concentration of NaCl in the elution buffer to 500 mM acted to reduce aggregation and decreased the extra bands significantly resulting in elution of Opa proteins with nearly single bands as shown in Figure 6-6.

The production of functional recombinant membrane protein is a complicated process because the hydrophobic nature of membrane proteins often causes expression as insoluble proteins (inclusion bodies) which then need to be denatured and renatured. Renaturing of membrane proteins relies on providing synthetic membrane mimics to stabilize the protein fold and maintain function (Dewald *et al.* 2011). Providing *in vitro* conditions that mimic the cellular system and the structure of the outer membrane with LPS and phospholipids is quite complicated. However, different conditions and models were applied by providing a hydrophobic environment that enables the recombinant proteins to be inserted in a membrane and folded. An example is Unilamellar vesicles, which are composed of a symmetric phospholipid bilayer; another is detergent micelles, which also provide conditions that are suitable for refolding of OMPs. Such different systems were applied to fold Opa proteins, with the aim to get fully and perfectly folded recombinant Opa suitable for NMR analysis (Otzen and Andersen 2013) . However, such conditions for refolding of Opa proteins were investigated and it has been shown that Opa proteins are not correctly folded using conditions that were successfully applied to folding of other OMPs (Dewald *et al.* 2011). Whereas, the best conditions for Opa folding has been reported to involve diluting the denatured proteins 1:20 in Tris buffer (pH8) in the presence of n-dodecylphosphocholine (DPC) (Dewald *et al.* 2011; Fox *et al.* 2014).

The refolding step of rOpa proteins was done in this study after purification of the proteins under denaturant conditions. This step was complicated due to the tendency of Opa proteins to aggregate and precipitate when diluted into the refolding buffer. This problem occurred when the proteins were concentrated after refolding in PBS buffer (pH12) supplemented with 0.5 SB12 (detergent). Therefore, the protocol that was developed by (Fox *et al.* 2014) was used for the refolding step in this project, which involved a long step of incubation for 5 days at room temperature in Tris buffer (pH 8) in the presence of detergent micelles of Z3-14 (zwitterioninc detergent) and 500 mM of NaCl. The micelles of the zwitterionic detergent (Z3-14) were used as a replacement of the dodecylphosphocholine (DPC) which is a lipid bilayer used for refolding of Opa in the original protocol. This detergent at a concentration of 1% along with the other buffer conditions was successful for refolding of Opa at room temperature. The only limitations with using this detergent is that the micelles have a molecular weight of 30 kDa which is higher than the MW of Opa proteins, and this led to complications with removal of this detergent from the final solutions of the refolded proteins by dialysis or by washing the protein with the concentrator. Therefore, a further purification step was needed to remove the extra amounts of detergent, and this was achieved by re-binding the folded proteins to the Nickel column and then washing and eluting it with Tris buffer and a lower concentration of detergent (0.05 %), followed by a dialysis step to remove the imidazole from the solution.

Using these conditions, folded and functional rOpa proteins were obtained as assessed by SDS- PAGE shift analysis and receptor overlay dot blot. The majority of Opa proteins detected and characterised were shown to bind and react with CEACAM1 receptors rather than other CEACAMs or other receptors (Heparanesulphate proteoglycan HPSG) (Hauck and Meyer 2003). Regarding the purified rOpas in our work, the OpaA and OpaB proteins from V222 were shown to react with CEACAM1 as were the native proteins of these variants present in cell lysates, which confirms that functional recombinant proteins had been obtained after purification and refolding. Conversely, no reactivity was noticed with the other two variants (OpaJ-N459, and OpaJ-N222). The native protein of OpaJ-N459 (from cell lysates) was shown to react with CEACAM1 (chapter 4) but with a lower reactivity in comparison to the other variants. This indicates that the recombinant OpaJ-N459 was either not folded properly (misfolded) or was denatured during the experiment. Another possible reason is that the native protein did not react with CEACAM1, and that

the reactivity in the WCL was due to isolate N459.1 being mixed with other variants that express another Opa which can bind to CEACAM1. This result in the WCL having a lower intensity in the experiment in comparison to other isolates expressing CEACAM1-reactive variants. Because the native protein of OpaJ-N222 did not show any reactivity with human CEACAM1, the refolding state and functionality of the OpaJ-N222 cannot be determined.

Immunisation of mice with these rOpas (the four variants) was successful for generation of polyclonal antibodies. This was confirmed by an ELISA test as well as western blot. The tests showed that the developed antisera were able to recognize and bind to their target purified antigens (used for the immunization), and were very specific towards the target antigens in the antisera developed against OpaJ-N222 and OpaJ-N459, as they showed high specificity to their antigens rather than the other variants of Opa and the unrelated antigen (PorA). These sera must be dominated by antibodies specific for unique variable regions. In contrast, the antisera developed against OpaA and OpaB showed less specificity to their antigens and showed strong reactivity with the other Opa variants but not to PorA. This was confirmed by the ELISA test as well as by western blot analysis which gave consistent data with each other. This can be related to the quality of the purified rOpa that was used to immunise the mice, as an old batch of rOpaB was used to immunize the mice in which the protein was in a poor quality and there were multiple bands in the SDS-PAGE due to denaturation and degradation of the proteins and this might lead to generation of antibodies against cross reactive epitopes that are normally not exposed on the surface of the protein.

Before evaluating the bactericidal activity of the generated antisera against the target meningococcal strains (expressing the target Opa variants), the sensitivity of these strains toward human complement was tested to determine and avoid any non-specific killing mediated by the human complement only. The protocol for this test was modified from the protocol used for the same test with strain MC58 (chapter 5). The modification involved harvesting the bacteria at their mid-log phase and using them to prepare the inoculum. The bacteria were streaked and grown on BHI plates overnight and then colonies were picked up and re-streaked on blood agar plates for 4 hrs prior to preparation of the inoculum. The results showed that all 4 isolates were resistant to human complement, whereas using the previous protocol these isolates did not survive in the complement only. This might be due to the bacteria being in mid-log phase where they

are healthier, and hence more resistant to complement. Alternatively blood agar may provide a factor that enhances complement resistance.

The bactericidal activity of the developed antisera were then evaluated against the isolates expressing the target Opa variant. Three of the developed antisera were tested, and the anti-OpaB and anti-OpaJ showed very strong bactericidal activity and killed more than 50% of the population with a high titer (1:320). The activity of these antisera was tested using 10% human sera as complement source, while anti-OpaA was tested with using 5 % human sera and was bactericidal (killing more than 50% of the populations) in the first three dilutions (1:20- 1:80). This variability might reflect the effect of the concentration of the complement used in each assay as it was reduced to 5% in the anti-OpaA assay, while in the other assays which showed very strong bactericidal, the antibodies were mixed with 10% of the human sera. However, further investigation is required in order to evaluate the bactericidal activity of the generated antibodies, this could involve testing the antisera against strains expressing the other variants of Opa that cross react with the antisera to see whether it was also bactericidal and testing with higher titres to determine the best dilutions for use in escape of the bactericidal activity of the anti-Opa antisera.

To sum up, in this part of the project, functional recombinant Opa proteins were successfully cloned and expressed from strain N222.1 and N459.1, which are both variants of one CC23 strain. The expression, purification and the refolding conditions were optimised to get functional rOpa for use in downstream applications. The study produced bactericidal anti-bodies with varying levels of specificity to their target antigens. The level of immune response in human sera against Opa proteins needs to be assessed as a correlate for the PV data of the Opa proteins during longitudinal colonisation by carriage isolates, both the purified rOpa and the generated antisera can be used for this purpose

7 General conclusion and future work

N. mningitidis is an obligate commensal for humans that asymptomatically colonises the nasopharynx of about 10 % of healthy individuals. This bacterium is also responsible for a high rate of lethal bacterial meningitis and septicaemia worldwide, while the survivors of infection always suffer from permanent sequelae (Zhang *et al.* 2016). The successful colonisation by this bacteria of the human epithelial mucosa is the first step for causing disease, and it depends on the ability of the bacteria to persist and avoid the immune defences in the mucosal surface. Such abilities can be achieved by expression of particular virulence factors that mediate the firm attachment of these bacteria to the epithelial cells in addition to the switching between different variants of these factors via phase and antigenic variation mechanisms as a means for immune evasion (Corbett *et al.* 2004). The role of the phase variable Opa proteins in the colonisation and in the disease is well documented as this protein mediates the intimate adhesion and the invasion of these bacteria to the human epithelia (Sadarangani *et al.* 2011). These proteins were also suggested as vaccine candidates in spite of their high diversity, as they showed to be highly structured among the hyper-invasive lineages of *N. meningitidis* (Callaghan *et al.* 2011). The intensive investigation of the phase variability and the expression pattern of this important virulence factor of *N. meningitiids* during the persistent carriage is crucial to give better insight about how these pathogenic bacteria interact with their host, which then has implications in developing suitable preventive or treatment strategies against this pathogen.

The study highlighted the phase variation of the Opa repertoire of *N. meningitidis* during long-term of colonisation of different human carriers. The project firstly focussed on characterising the different *opa* alleles in multiple isolates of 19 carriers in terms of the number and sequence variability in each isolate. A trend was revealed that the long term of colonisation was correlated with a lowering of the diversity of *opa* encoding genes rather than diversifying these genes. The presence of identical alleles in different loci and silencing of one of them (by locking the gene off due to a low number of repeats) was the dominant pattern in the carriage isolates analysed. This duplication and silencing of *opa* genes can result in lowering the diversity of the Opa repertoire at both the genetic level and the phenotypic level (due to phase variation). Such a pattern is advantageous for the meningococcal isolates, which require circulating between different human carriers as

lowering the diversity of these proteins increase the fitness of the meningococcal isolates by decreasing the probability of encountering anti-body immune responses elicited against similar Opa variants of other strains that had colonised these carriers previously (Callaghan *et al.* 2008b). Homologous recombination is one of the main mechanisms that mediates duplication and diversifying *opa* alleles of *N. meningitidis* (Bilek *et al.* 2009). However, from our data, this mechanism was less common for generating or importing new *opa* alleles (only two events were noticed). Such recombination events potentially participated in conferring fitness to the meningococcal population by importing and generating new alleles that enabled the bacteria to escape the bactericidal antibodies elicited against the previous allele, and potentially by gaining alleles with new function.

The project also analysed the phase variability of the Opa repertoires of the carriage isolates from the 19 different carriers at different time points representing of up to six month of colonisation. The changes in the repeat numbers that mediated changes in the expression states of Opa were analysed in those isolates, and the data indicated a high rate of switching in the repeats, which correlated with switching between expression of different Opa variants between different time point. The trend was detected towards reductions in the expression of the variants that were expressed at a high rate in the early time points of colonisation accompanied with a selection for on variants of loci that were off or expressed at lower rates in the early stages of carriage. The data were relatively consistent with the findings of (Alamro *et al.* 2014), who reported that the long term of colonisation of human nasopharynx was correlated with reductions in the expression levels of five OMPs of N. *meningitidis* as a possible consequence of the strong immune selection imposed on the meningococcal OMPs repertoire during long term of colonisation. The overall trend of Opa expression was also correlated with a constant expression of at least one variant during the term of colonisation. This can be correlated to the importance of Opa function that might be needed to be maintained, as these proteins have been reported as being essential to mediate the colonisation of nasal mucosa of CEACAM1- humanised transgenic mice, where challenging these transgenic animals with Opa –ve meningococcal populations led to recovery of Opa +ve cells after colonisation (Johswich *et al.* 2013).

Limiting the number of Opa variants expressed per isolate was another key finding reported in this study with the majority of the isolates analysed (70 %) expressing only

one *opa* gene, with no expression for more than two variants. Such trends also inform about the advantages of reducing the diversity of Opa repertoire at the phenotypic level as a means of immune avoidance. This strategy is clearer in the other isolates that were negative for the all Opa variants (16% of the total isolates), as it has been reported as one of the main mechanisms to avoid the bactericidal antibodies elicited against Opa during colonisation (Sadarangani *et al.* 2011).

The potential role of immune response in shaping the Opa repertoire and mediating switching and selection of a limited number of Opa variants was also evaluated in this project. Imposing such immune pressure on meningococcal population expressing three Opa variants (by a successive incubation with bactericidal anti-Opa antibodies) has led to a rapid shifting to populations expressing lower numbers of Opa proteins. The switching was toward expression only two variants, and the selection was for population that switched off only the Opa antigen that is targeted by the antibodies. Data from the total colonies analysed in the escape assays showed that the main switch was in the repeat tracts of *opaD*, which were 12 repeats, whereas the switching in *opaJ* was very rare even though this gene had 15 repeats and was in an on state in the input inoculum. This reflects the specificity of the selection imposed on the population by the bactericidal antibodies that may lead to select only OpaD-ve phenotypes, and may correlate with another factor (such as the promoter strength) which can increase the rate of mutation in *opa* genes independently from or synergistically with the repeat tract (Belland *et al.* 1997). Shifts in the population from one phenotype to another has mediated a rapid escape from the bactericidal activity of the Opa antibodies highlighting the important role of PV of the Opa repertoire in immune evasion along with maintaining the function of this major adhesin of *N. meningitidis* by switching between Opa variants of different allele types. Findings from this work are consistent with previous work that also reported the role of PV in *porA* and *lgtG* in mediating the escape of meningococcal population from bactericidal antibodies (Bayliss *et al.* 2008; Tauseef *et al.* 2013). These previous studies also reported that the rate of escape depends on the frequency of the PV as the *mutS* mutant strain increases the frequency of PV of genes by 1000-fold as compared to the wild type strain resulting in a rapid escape of the bactericidal activity. Such patterns can explain the rapid escape of the strain MC58 from anti-Opa antibodies, as the PV frequency of Opa is 10^{-3}, which is much higher than in many other phase variable genes including *porA* and *lgtG*. Such high frequencies might relate to the type of the repeat

tracts, mutation rates of tetrameric repeats were not affected by deactivating the mismatch correction systems (MMR) (such as in *mutS* strain of *N. meningitidis* and *H. influenzae*), which reflects that slippage in this kind of repeats is resistance to the mismatch correction systems (Martin *et al.* 2004; Bayliss 2009). This pattern can be similar in the pentameric repeat tracts of *opa* genes, which showed high rates of slippage in comparison to the genes with homopolymeric tracts such as *porA* and *lgtG* .

Although the *in vitro* assay showed that immune selection mediated the PV and the accumulation of variants with lower expression levels for PorA, such a positive correlation was not present during the long term of colonisation of human carriers (Bidmos, 2013). The study suggested that the *in vivo* selection was polygenic and the selection was by targeting other OMPs rather than PorA. Such patterns can be similar in the case of Opa proteins, and the reported overall trends of Opa expression during the persistent carriage might be driven by selection for other antigens, or even by functional selection rather than the immune response. Therefore, evaluating the level of immune response elicited against Opa proteins in human carriers and correlates that with the PV state of the meningococcal populations is crucial for a better understanding of the factors that mediate the PV of Opa *in vivo* and consequently affect the interaction of this bacteria with human mucosal surfaces. For this purpose, four recombinant Opa variants of the clonal complex CC23 were produced in this project and used as antigens for generating mouse polyclonal antibodies with the aim of using them for further evaluation of the level of anti-Opa IgG antibodies in the sera of successive human carriars by using liquichip asssay.

In summary, the phase variability and the trends in the expression states of the meningococcal Opa repertoire during the persistent carriage were investigated intensively in this project, giving a further understanding about the nature of the meningococcal interaction with the human nasopharynx during persistent carriage. Limiting the number of expressed Opa variants and switching between different variants between the early and the later stages of colonisation were key points characterised in this study. These findings along with the findings of *in vitro* immune escape analysis suggested the importance of PV of these proteins in mediating the immune avoidance as well as maintaining their essential function during the carriage state. However, the highly diverse population resulting from generating different phenotypes by PV of Opa, and the rapid turnover of

such populations from expressing one variant to another under selection pressures may weaken the candidacy of Opa proteins to be used as vaccine antigens. Therefore, extending the in vitro immune escape assay with using anti-Opa antibodies that targeting multiple opa variants individually and in combinations may give better insight about the ability of the bacteria to escape immune response against this antigen and give more evaluation about the possibility of using this protein as a vaccine antigen.

8 Appendix

Table 8-1 Data of the genescan analysis of *opa* genes in the carriage isolates correlated with the expression state (on/off). The table showed the length of the pentameric repeats (CTCTT) along with the C tracts and A tracts preceding these tracts which their length in combination with the penta-repeat length control the expression stat of the Opa proteins

Carriers with time points	**isolate**	***opa* genes**									
		opaA					***opaB***				
cc23		**Size**	**C tracts**	**A tracts**	**CTCTT length**	**ON/ OFF**	**Size**	**C tracts**	**A tracts**	**CTCTT length**	**ON/OFF**
V222/ 1st	222.1	482.5	5	6	19	OFF	641.5	5	6	9	ON
	222.2	492.6	5	6	21	ON	636.6	5	6	8	OFF
	222.3	487.1	5	6	20	OFF	641.0	5	6	9	ON
	222.4	487.2	5	6	20	OFF	640.9	5	6	9	ON
	222.5	482.1	5	6	19	OFF	654.7	5	6	12	ON
	222.6	487.1	5	6	20	OFF	636.0	5	6	8	OFF
V222/2nd	309.1	492.6	5	6	21	ON	636.5	5	6	8	OFF
	309.2	492.7	5	6	21	ON	636.6	5	6	8	OFF
	309.3	492.6	5	6	21	ON	636.5	5	6	8	OFF
	309.4	497.8	5	6	22	OFF	636.5	5	6	8	OFF
	309.5	492.3	5	6	21	ON	640.9	5	6	9	ON
	309.6	492.7	5	6	21	ON	636.6	5	6	8	OFF
V222/3rd	391.1	487.6	5	6	20	OFF	636.2	5	6	8	OFF
	391.2	492.7	5	6	21	ON	636.6	5	6	8	OFF
	391.3	487.6	5	6	20	OFF	636.5	5	6	8	OFF
	391.4	487.5	5	6	20	OFF	636.4	5	6	8	OFF
	391.5	487.5	5	6	20	OFF	636.4	5	6	8	OFF
	391.6	487.5	5	6	20	OFF	636.4	5	6	8	OFF
V222/4th	459.1	482.4	5	6	19	OFF	636.4	5	6	8	OFF
	459.2	472.3	5	6	17	OFF	636.8	5	6	8	OFF
	459.4	476.9	5	6	18	ON	636.0	5	6	8	OFF
	459.4	482.0	5	6	19	OFF	636.1	5	6	8	OFF
	459.5	471.7	5	6	17	OFF	636.0	5	6	8	OFF
	459.6	487.3	5	6	20	OFF	636.9	5	6	8	OFF
V96/1ST	259.1	472.0	5	6	17	OFF	620.7	5	6	5	OFF
	259.2	477.1	5	6	18	ON	620.7	5	6	5	OFF
	259.3	482.1	5	6	19	OFF	626.6	5	6	6	ON
	259.4	477.1	5	6	18	ON	621.1	5	6	5	OFF

	259.5	477.2	5	6	18	ON	621.1	5	6	5	OFF
	259.6	482.1	5	6	19	OFF	626.3	5	6	6	ON
V96/2nd	370.1										
	370.2	738.6	5	6	18	ON	621.4	5	6	5	OFF
	370.3	487.8	5	6	20	OFF	626.7	5	6	6	ON
	370.4	748.4	5	6	20	OFF	621.4	5	6	5	OFF
	370.5	738.6	5	6	18	ON	621.5	5	6	5	OFF
	370.6	728.5	5	6	16	OFF	621.5	5	6	5	OFF
V96/3rd	445.1	728.5	5	6	16	OFF	625.8	5	6	6	ON
	445.2	728.6	5	6	16	OFF	625.6	5	6	6	ON
	445.3	728.5	5	6	16	OFF	626.0	5	6	6	ON
	445.4	728.5	5	6	16	OFF	626.1	5	6	6	ON
	445.5	728.5	5	6	16	OFF	626.2	5	6	6	ON
	445.6	733.4	5	6	17	OFF	625.8	5	6	6	ON
V69/1st	258.1	482.0	5	6	19	OFF	641.7	5	6	9	ON
	258.2	482.1	5	6	19	OFF	641.2	5	6	9	ON
	258.3	482.1	5	6	19	OFF	641.4	5	6	9	ON
	258.4	482.6	5	6	19	OFF	641.8	5	6	9	ON
	258.5	482.6	5	6	19	OFF	641.5	5	6	9	ON
	258.6	482.0	5	6	19	OFF	641.6	5	6	9	ON
V69/2nd	431.1	472.2	5	6	17	OFF	652.1	5	6	11	OFF
	431.2	472.1	5	6	17	OFF	657.2	5	6	12	ON
	431.3	472.4	5	6	17	OFF	657.1	5	6	12	ON
	431.4	472.4	5	6	17	OFF	656.7	5	6	12	ON
	431.5	472.4	5	6	17	OFF	672.1	5	6	15	ON
	431.6	477.4	5	6	18	ON	652.1	5	6	11	OFF
V93/	264.1	472.5	5	6	17	OFF	641.6	5	6	9	ON
	264.2	472.4	5	6	17	OFF	641.4	5	6	9	ON
	264.3	461.9	5	6	15	ON	626.8	5	6	6	ON
	264.4	472.4	5	6	17	OFF	641.9	5	6	9	ON
	264.5	472.3	5	6	17	OFF	641.0	5	6	9	ON
	264.6	471.8	5	6	17	OFF	641.0	5	6	9	ON
V93/	359.1	482.7	5	6	19	OFF	642.0	5	6	9	ON
	359.2	482.7	5	6	19	OFF	641.6	5	6	9	ON
	359.3	482.4	5	6	19	OFF	642.1	5	6	9	ON
	359.4	482.6	5	6	19	OFF	641.2	5	6	9	ON
	359.5	487.7	5	6	20	OFF	641.6	5	6	9	ON
	359.6	482.3	5	6	19	OFF	641.0	5	6	9	ON

CC167											
V117/1st	117.1	616.5	5	6	4	OFF	652.8	5	6	12	ON
	117.2	616.7	5	6	4	OFF	652.8	5	6	12	ON
V117/2nd	284.1	617.0	5	6	4	OFF	647.9	5	6	11	OFF
	284.2	617.1	5	6	4	OFF	658.3	5	6	13	OFF
	284.3	617.1	5	6	4	OFF	653.1	5	6	12	ON
	284.4	617.0	5	6	4	OFF	658.1	5	6	13	OFF
	284.5	616.9	5	6	4	OFF	652.9	5	6	12	ON
	284.6	617.0	5	6	4	OFF	652.8	5	6	12	ON
V117/3rd	332.1	616.4	5	6	4	OFF	683.2	5	6	18	ON
	332.2	616.6	5	6	4	OFF	673.2	5	6	16	OFF
	332.3	616.8	5	6	4	OFF	673.1	5	6	16	OFF
	332.4	616.8	5	6	4	OFF	668.0	5	6	15	ON
	332.5										
	332.6	616.5	5	6	4	OFF	647.0	5	6	11	OFF
V117/4th	417.1	661.0	5	6	13	OFF	656.9	5	6	13	OFF
	417.2	661.8	5	6	13	OFF	657.6	5	6	13	OFF
	417.3	652.3	5	6	11	OFF	653.1	5	6	12	ON
	417.4	645.7	5	6	10	OFF	651.3	5	6	12	ON
	417.5	652.2	5	6	11	OFF	653.1	5	6	12	ON
	417.6	652.2	5	6	11	OFF	653.1	5	6	12	ON
V64/1st	64.1	616.6	5	6	4	OFF	652.8	5	6	12	ON
	64.2	616.7	5	6	4	OFF	652.7	5	6	12	ON
	64.3	616.8	5	6	4	OFF	652.7	5	6	12	ON
	64.4	616.8	5	6	4	OFF	652.8	5	6	12	ON
	64.5	616.4	5	6	4	OFF	651.7	5	6	12	ON
	64.6	616.6	5	6	4	OFF	652.7	5	6	12	ON
V64/2nd	257.1	616.6	5	6	4	OFF	667.7	5	6	15	ON
	257.2	617.0	5	6	4	OFF	668.3	5	6	15	ON
	257.3	617.1	5	6	4	OFF	643.0	5	6	10	OFF
	257.4	617.1	5	6	4	OFF	668.3	5	6	15	ON
	257.5	617.0	5	6	4	OFF	668.3	5	6	15	ON
	257.6	616.9	5	6	4	OFF	668.2	5	6	15	ON
V64/3rd	348.1	616.5	5	6	4	OFF	662.7	5	6	14	OFF
	348.2	616.7	5	6	4	OFF	662.8	5	6	14	OFF
	348.3	616.5	5	6	4	OFF	662.8	5	6	14	OFF
	348.4	616.7	5	6	4	OFF	662.7	5	6	14	OFF

	348.5	616.5	5	6	4	OFF	662.7	5	6	14	OFF
	348.6	616.6	5	6	4	OFF	662.0	5	6	14	OFF
V124/1st	124.1	617.0	5	6	4	OFF	657.1	5	6	12	ON
	124.2	617.0	5	6	4	OFF	657.3	5	6	12	ON
	124.3	616.3	5	6	4	OFF	656.0	5	6	12	ON
	124.4	616.3	5	6	4	OFF	655.8	5	6	12	ON
	124.5	616.3	5	6	4	OFF	656.0	5	6	12	ON
V124/2nd	290.1	616.4	5	6	4	OFF	656.8	5	6	12	ON
	290.3	616.9	5	6	4	OFF	657.3	5	6	12	ON
	290.4	616.8	5	6	4	OFF	657.1	5	6	12	ON
	290.5	616.5	5	6	4	OFF	656.8	5	6	12	ON
	290.6	616.4	5	6	4	OFF	656.8	5	6	12	ON
V124/3rd	336.1	616.6	5	6	4	OFF	657.0	5	6	12	ON
	336.2	616.7	5	6	4	OFF	657.1	5	6	12	ON
	336.3	617.0	5	6	4	OFF	657.2	5	6	12	ON
	336.4	616.8	5	6	4	OFF	657.1	5	6	12	ON
	336.5	616.7	5	6	4	OFF	656.9	5	6	12	ON
	336.6	616.6	5	6	4	OFF	656.8	5	6	12	ON
cc60											
V113/1st	113.1	458.6	5	6	13	OFF	700.5	5	6	11	OFF
	113.2	433.2	5	6	8	OFF	700.6	5	6	11	OFF
	113.3	458.7	5	6	13	OFF	700.7	5	6	11	OFF
	113.4	433.2	5	6	8	OFF	700.5	5	6	11	OFF
	113.5	443.3	5	6	10	OFF	700.7	5	6	11	OFF
	113.6	443.2	5	6	10	OFF	700.5	5	6	11	OFF
V113/2nd	281.1	443.6	5	6	10	OFF	700.9	5	6	11	OFF
	282.2	443.6	5	6	10	OFF	701.0	5	6	11	OFF
	282.3	443.7	5	6	10	OFF	701.0	5	6	11	OFF
	282.4	438.6	5	6	9	ON	701.0	5	6	11	OFF
	282.5	443.6	5	6	10	OFF	700.9	5	6	11	OFF
	282.6	443.6	5	6	10	OFF	700.9	5	6	11	OFF
V114/1st	114.1	448.4	5	6	11	OFF	705.8	5	6	12	ON
	114.2	438.3	5	6	9	ON	690.7	5	6	9	ON
	114.3	438.3	5	6	9	ON	700.6	5	6	11	OFF
	114.4	438.3	5	6	9	ON	700.8	5	6	11	OFF
	114.5	448.4	5	6	11	OFF	705.6	5	6	12	ON
	114.6	448.4	5	6	11	OFF	705.6	5	6	12	ON
	114.7	438.4	9	6	11	OFF	705.8	5	6	12	ON

	114.8	443.4	10	6	11	OFF	706.0	5	6	12	ON
	114.9	443.4	10	6	11	OFF	705.8	5	6	12	ON
	114,10	443.4	10	6	11	OFF	705.8	5	6	12	ON
	114.11	438.3	9	6	11	OFF	700.6	5	6	11	OFF
	114.12	438.4	9	6	11	OFF	700.8	5	6	11	OFF
	114.13	438.3	9	6	11	OFF	705.6	5	6	12	ON
	114.14	438.3	9	6	11	OFF	700.6	5	6	11	OFF
	114.16	443.7	10	6	11	OFF	706.1	5	6	12	ON
	114.18	443.8	10	6	11	OFF	706.1	5	6	12	ON
V114/2nd	283.1	448.3	5	6	11	OFF	705.4	5	6	12	ON
	283.2	448.8	5	6	11	OFF	706.2	5	6	12	ON
	283.3	448.8	5	6	11	OFF	706.1	5	6	12	ON
	283.4	448.4	5	6	11	OFF	705.6	5	6	12	ON
	283.5	448.7	5	6	11	OFF	706.0	5	6	12	ON
	283.6	448.8	5	6	11	OFF	706.0	5	6	12	ON
V114/3rd	330.1	438.1	5	6	9	ON	700.5	5	6	11	OFF
	330.2	438.2	5	6	9	ON	700.4	5	6	11	OFF
	330.3	438.6	5	6	9	ON	700.8	5	6	11	OFF
	330.4	438.5	5	6	9	ON	700.7	5	6	11	OFF
	330.5	438.3	5	6	9	ON	700.5	5	6	11	OFF
	330.6	438.1	5	6	9	ON	700.5	5	6	11	OFF
	330.7	438.3	5	6	9	ON	700.7	5	6	11	OFF
	330.8	438.4	5	6	9	ON	700.8	5	6	11	OFF
	330.9	438.4	5	6	9	ON	700.6	5	6	11	OFF
	330,10	438.4	5	6	9	ON	695.7	5	6	10	OFF
	330.11	438.4	5	6	9	ON	700.8	5	6	11	OFF
	330.12	438.3	5	6	9	ON	700.7	5	6	11	OFF
	330.15	438.7	5	6	9	ON	701.0	5	6	11	OFF
	330.16	438.7	5	6	9	ON	701.1	5	6	11	OFF
	330.17	438.7	5	6	9	ON	701.1	5	6	11	OFF
	330.18	438.8	5	6	9	ON	701.0	5	6	11	OFF
	330.19	438.7	5	6	9	ON	701.1	5	6	11	OFF
	330,20	438.7	5	6	9	ON	701.1	5	6	11	OFF
V115/1st	115.1	433.2	5	6	8	OFF	705.6	5	6	12	ON
	115.2	433.2	5	6	8	OFF	690.6	5	6	9	ON
	115.3	443.3	5	6	10	OFF	705.7	5	6	12	ON
	115.4	443.4	5	6	10	OFF	705.6	5	6	12	ON
	115.5	443.3	5	6	10	OFF	700.6	5	6	11	OFF
	115.6	443.3	5	6	10	OFF	700.6	5	6	11	OFF
V115/2nd	282.1	433.3	5	6	8	OFF	700.5	5	6	11	OFF

	282.2	443.7	5	6	10	OFF	690.9	5	6	9	ON
	282.3	433.6	5	6	8	OFF	701.0	5	6	11	OFF
	282.4	433.6	5	6	8	OFF	701.1	5	6	11	OFF
	282.5	433.6	5	6	8	OFF	701.0	5	6	11	OFF
	282.6	433.6	5	6	8	OFF	701.0	5	6	11	OFF
V134/1st											
	134.1	433.1	5	6	8	OFF	705.5	5	6	12	ON
	134.2	433.2	5	6	8	OFF	700.6	5	6	11	OFF
	134.3	433.2	5	6	8	OFF	700.6	5	6	11	OFF
	134.4	433.2	5	6	8	OFF	700.6	5	6	11	OFF
	134.5										
	134.6	433.2	5	6	8	OFF	720.8	5	6	15	ON
	134.7	433.2	5	6	8	OFF	700.7	5	6	11	OFF
	134.8	433.3	5	6	8	OFF	700.7	5	6	11	OFF
	134.9	433.3	5	6	8	OFF	700.7	5	6	11	OFF
	134,10	433.4	5	6	8	OFF	700.7	5	6	11	OFF
	134.11	433.3	5	6	8	OFF	700.7	5	6	11	OFF
	134.12	433.3	5	6	8	OFF	700.6	5	6	11	OFF
	134.13	433.3	5	6	8	OFF	700.7	5	6	11	OFF
	134.14	433.1	5	6	8	OFF	700.5	5	6	11	OFF
	134.16	433.6	5	6	8	OFF	701.2	5	6	11	OFF
	134.17	433.6	5	6	8	OFF	701.1	5	6	11	OFF
	134.18	443.8	5	6	10	OFF	706.1	5	6	12	ON
V134/2nd	295.1	448.7	5	6	11	OFF	705.6	5	6	12	ON
	295.2	448.8	5	6	11	OFF	706.0	5	6	12	ON
	295.3	433.3	5	6	8	OFF	705.6	5	6	12	ON
	295.4	448.4	5	6	11	OFF	705.5	5	6	12	ON
	295.5	432.9	5	6	8	OFF	705.0	5	6	12	ON
	295.6	433.6	5	6	8	OFF	720.6	5	6	15	ON
V134/3rd	333.1	433.1	5	6	8	OFF	705.5	5	6	12	ON
	333.2	433.1	5	6	8	OFF	705.5	5	6	12	ON
	333.3	433.6	5	6	8	OFF	706.0	5	6	12	ON
	333.4	433.5	5	6	8	OFF	706.0	5	6	12	ON
	333.5	433.1	5	6	8	OFF	705.4	5	6	12	ON
	333.6	433.0	5	6	8	OFF	705.4	5	6	12	ON
	333.7	433.3	5	6	8	OFF	705.8	5	6	12	ON
	333.8	433.4	5	6	8	OFF	705.9	5	6	12	ON
	333.9	433.5	5	6	8	OFF	705.9	5	6	12	ON
	333,10	433.5	5	6	8	OFF	705.9	5	6	12	ON
	333.11	433.4	5	6	8	OFF	705.8	5	6	12	ON
	333.12	433.4	5	6	8	OFF	705.8	5	6	12	ON
	333.13	433.4	5	6	8	OFF	705.7	5	6	12	ON

	333.14	433.2	5	6	8	OFF	705.6	5	6	12	ON
	333.15	433.6	5	6	8	OFF	706.2	5	6	12	ON
	333.16	433.7	5	6	8	OFF	706.2	5	6	12	ON
	333.17	433.7	5	6	8	OFF	706.1	5	6	12	ON
	333.18	433.7	5	6	8	OFF	706.1	5	6	12	ON
	333.19	433.7	5	6	8	OFF	706.2	5	6	12	ON
	333,20	433.7	5	6	8	OFF	706.2	5	6	12	ON
V185/1st	185.1	463.6	5	6	13	OFF	438.2	5	6	8	OFF
	185.2	463.7	5	6	13	OFF	438.2	5	6	8	OFF
	185.3	463.6	5	6	13	OFF	438.2	5	6	8	OFF
	185.4	463.7	5	6	13	OFF	438.2	5	6	8	OFF
	185.5	463.6	5	6	13	OFF	438.2	5	6	8	OFF
	185.6	464.1	5	6	13	OFF	438.7	5	6	8	OFF
	185.8	463.7	5	6	13	OFF	438.2	5	6	8	OFF
	185.9	463.7	5	6	13	OFF	438.2	5	6	8	OFF
	185,10	463.8	5	6	13	OFF	438.4	5	6	8	OFF
	185.11	463.7	5	6	13	OFF	438.3	5	6	8	OFF
	185.12	463.8	5	6	13	OFF	438.3	5	6	8	OFF
	185.13	463.7	5	6	13	OFF	438.3	5	6	8	OFF
	185.14	463.7	5	6	13	OFF	438.2	5	6	8	OFF
	185.15	463.6	5	6	13	OFF	438.1	5	6	8	OFF
	185.16	464.0	5	6	13	OFF	438.6	5	6	8	OFF
	185.17	464.1	5	6	13	OFF	438.7	5	6	8	OFF
	185.18	464.1	5	6	13	OFF	438.7	5	6	8	OFF
	185.19	464.2	5	6	13	OFF	438.7	5	6	8	OFF
	185,20	464.1	5	6	13	OFF	438.7	5	6	8	OFF
V185/2nd	306.1	463.5	5	6	13	OFF	438.1	5	6	8	OFF
	306.2	473.8	5	6	15	ON	438.2	5	6	8	OFF
	306.3	463.7	5	6	13	OFF	438.2	5	6	8	OFF
	306.4	463.7	5	6	13	OFF	438.2	5	6	8	OFF
	306.5	463.6	5	6	13	OFF	438.1	5	6	8	OFF
	306.6	463.6	5	6	13	OFF	438.2	5	6	8	OFF
V185/4th	456.1	453.6	5	6	11	OFF	438.4	5	6	8	OFF
	456.2	453.7	5	6	11	OFF	438.5	5	6	8	OFF
	456.3	458.8	5	6	12	ON	438.5	5	6	8	OFF
	456.4	458.9	5	6	12	ON	438.5	5	6	8	OFF
	456.5	453.7	5	6	11	OFF	438.5	5	6	8	OFF
	456.6	453.7	5	6	11	OFF	438.4	5	6	8	OFF
	456.7	453.9	5	6	11	OFF	438.3	5	6	8	OFF
	456.8	458.4	5	6	12	ON	438.1	5	6	8	OFF

	456.9	458.5	5	6	12	ON	438.2	5	6	8	OFF
	456,10	458.5	5	6	12	ON	438.2	5	6	8	OFF
	456.11	453.5	5	6	11	OFF	438.2	5	6	8	OFF
	456.12	458.5	5	6	12	ON	438.2	5	6	8	OFF
	456.13	453.5	5	6	11	OFF	438.2	5	6	8	OFF
	456.14	458.4	5	6	12	ON	438.1	5	6	8	OFF
	456.16	458.9	5	6	12	ON	438.5	5	6	8	OFF
	456.17	458.9	5	6	12	ON	438.6	5	6	8	OFF
	456.18	453.9	5	6	11	OFF	438.6	5	6	8	OFF
	456.19	458.9	5	6	12	ON	438.6	5	6	8	OFF
	456,20	458.9	5	6	12	ON	438.6	5	6	8	OFF
cc174											
V51/1ST											
	51.1	450.7	5	6	12	ON	655.7	5	6	10	OFF
	51.2	450.7	5	6	12	ON	655.7	5	6	10	OFF
	51.3	450.8	5	6	12	ON	655.9	5	6	10	OFF
	51.4	450.8	5	6	12	ON	655.7	5	6	10	OFF
	51.5	450.7	5	6	12	ON	655.8	5	6	10	OFF
	51.6	450.8	5	6	12	ON	655.9	5	6	10	OFF
V51/2nd											
	236.1	450.6	5	6	12	ON	655.7	5	6	10	OFF
	236.2	460.9	5	6	14	OFF	650.5	5	6	9	ON
	236.3	445.6	5	6	11	OFF	665.9	5	6	12	ON
	236.4	455.8	5	6	13	OFF	655.8	5	6	10	OFF
	236.5	445.6	5	6	11	OFF	665.9	5	6	12	ON
	236.6	445.7	5	6	11	OFF	665.9	5	6	12	ON
V51/3rd	354.1	465.7	5	6	15	ON	655.5	5	6	10	OFF
	354.2	465.8	5	6	15	ON	655.4	5	6	10	OFF
	354.3	465.7	5	6	15	ON	655.3	5	6	10	OFF
	254.4	465.7	5	6	15	ON	655.6	5	6	10	OFF
	254.5	445.3	5	6	11	OFF	665.5	5	6	12	ON
	254.6	465.7	5	6	15	ON	655.5	5	6	10	OFF
V51/4th	424.1	445.6	5	6	11	OFF	665.8	5	6	12	ON
	424.2	445.5	5	6	11	OFF	665.4	5	6	12	ON
	424.3	445.6	5	6	11	OFF	665.6	5	6	12	ON
	424.4	445.5	5	6	11	OFF	665.6	5	6	12	ON
	424.5	445.5	5	6	11	OFF	665.7	5	6	12	ON
	424.6	445.5	5	6	11	OFF	665.6	5	6	12	ON
V52/1st											

	52.1	445.8	5	6	11	OFF	656.1	5	6	10	OFF
	52.2	445.8	5	6	11	OFF	651.0	5	6	9	ON
	52.3	445.8	5	6	11	OFF	656.0	5	6	10	OFF
	52.4	445.8	5	6	11	OFF	656.0	5	6	10	OFF
	52.5	445.7	5	6	11	OFF	655.8	5	6	10	OFF
	52.6	445.7	5	6	11	OFF	655.9	5	6	10	OFF
V52/2nd											
	238.1	460.5	5	6	14	OFF	665.0	5	6	12	ON
	238.2	445.8	5	6	11	OFF	666.1	5	6	12	ON
	238.3	445.8	5	6	11	OFF	650.9	5	6	9	ON
	238.4	445.8	5	6	11	OFF	651.0	5	6	9	ON
	238.5	460.4	5	6	14	OFF	665.2	5	6	12	ON
	238.6	445.8	5	6	11	OFF	650.9	5	6	9	ON
V52/3rd	342.1	445.7	5	6	11	OFF	655.8	5	6	10	OFF
V58/1st	58.1	445.8	5	6	11	OFF	650.9	5	6	9	ON
	58.2	445.8	5	6	11	OFF	650.8	5	6	9	ON
	58.3	455.9	5	6	13	OFF	655.9	5	6	10	OFF
	58.4	445.8	5	6	11	OFF	650.8	5	6	9	ON
	58.5	445.8	5	6	11	OFF	650.9	5	6	9	ON
	58.6	456.0	5	6	13	OFF	656.1	5	6	10	OFF
V58/2nd	240.1	445.1	5	6	11	OFF	644.7	5	6	8	OFF
V58/3rd	429.1	445.8	5	6	11	OFF	645.9	5	6	8	OFF
	492.2	445.8	5	6	11	OFF	645.9	5	6	8	OFF
	429.3	445.8	5	6	11	OFF	645.8	5	6	8	OFF
	429.4	445.8	5	6	11	OFF	645.6	5	6	8	OFF
	429.5	445.7	5	6	11	OFF	645.6	5	6	8	OFF
	429.6	445.8	5	6	11	OFF	645.7	5	6	8	OFF
V59/1st	59.1	450.5	5	6	12	ON	655.4	5	6	10	OFF
	59.2	450.6	5	6	12	ON	655.5	5	6	10	OFF
	59.3	450.6	5	6	12	ON	655.5	5	6	10	OFF
	59.4	450.6	5	6	12	ON	660.6	5	6	11	OFF
	59.5	450.0	5	6	12	ON	655.5	5	6	10	OFF
	59.6	450.5	5	6	12	ON	655.4	5	6	10	OFF
	59.11	451.1	5	6	12	ON	655.8	5	6	10	OFF
	59.12	451.2	5	6	12	ON	656.1	5	6	10	OFF
	59.13	451.1	5	6	12	ON	656.1	5	6	10	OFF

	59.14	451.1	5	6	12	ON	656.0	5	6	10	OFF
	59.15	451.1	5	6	12	ON	656.0	5	6	10	OFF
	59.16	451.1	5	6	12	ON	655.9	5	6	10	OFF
	59.17	451.1	5	6	12	ON	655.9	5	6	10	OFF
	59.18	451.0	5	6	12	ON	655.9	5	6	10	OFF
	59,20	451.4	5	6	12	ON	656.5	5	6	10	OFF
V59/2nd	253.1	445.4	5	6	11	OFF	655.3	5	6	10	OFF
	253.2	445.4	5	6	11	OFF	655.4	5	6	10	OFF
	253.3	445.4	5	6	11	OFF	655.4	5	6	10	OFF
	253.4	445.4	5	6	11	OFF	655.4	5	6	10	OFF
	253.5	445.5	5	6	11	OFF	660.6	5	6	11	OFF
	253.6	445.4	5	6	11	OFF	655.4	5	6	10	OFF
V59/3rd	352.1	445.3	5	6	11	OFF	655.1	5	6	10	OFF
	352.2	445.3	5	6	11	OFF	655.1	5	6	10	OFF
	352.3	450.4	5	6	12	ON	655.2	5	6	10	OFF
	352.4	445.3	5	6	11	OFF	655.1	5	6	10	OFF
	352.5	450.3	5	6	12	ON	655.1	5	6	10	OFF
	352.6	450.4	5	6	12	ON	655.1	5	6	10	OFF
	352.7	446.0	5	6	11	OFF	656.0	5	6	10	OFF
	352.8	451.1	5	6	12	ON	656.1	5	6	10	OFF
	352.9	446.0	5	6	11	OFF	655.9	5	6	10	OFF
	352,10	446.0	5	6	11	OFF	661.2	5	6	11	OFF
	352.11	451.1	5	6	12	ON	655.9	5	6	10	OFF
	352.12	446.0	5	6	11	OFF	656.0	5	6	10	OFF
	352.13	445.9	5	6	11	OFF	655.9	5	6	10	OFF
	352.14	440.8	5	6	10	OFF	655.8	5	6	10	OFF
	352.15	451.3	5	6	12	ON	656.3	5	6	10	OFF
	352.16	456.5	5	6	13	OFF	656.3	5	6	10	OFF
	352.17	446.4	5	6	11	OFF	656.4	5	6	10	OFF
	352.18	446.4	5	6	11	OFF	656.5	5	6	10	OFF
	352.19	451.4	5	6	12	ON	656.4	5	6	10	OFF
	352,20	451.4	5	6	12	ON	656.4	5	6	10	OFF
V59/4th	438.1	445.3	5	6	11	OFF	655.3	5	6	10	OFF
	438.2	445.3	5	6	11	OFF	655.3	5	6	10	OFF
	438.3	450.4	5	6	12	ON	655.2	5	6	10	OFF
	438.4	450.4	5	6	12	ON	655.1	5	6	10	OFF
	438.5	445.2	5	6	11	OFF	655.1	5	6	10	OFF
	438.6	450.7	5	6	12	ON	655.5	5	6	10	OFF
V138/1st	138.1	455.6	5	6	13	OFF	654.7	5	6	10	OFF

	138.2	460.6	5	6	14	off	653.7	5	6	10	OFF
	138.3	double peak									
	138.4	460.6	5	6	14	OFF	655.1	5	6	10	OFF
	138.5	445.3	5	6	11	OFF	654.1	5	6	10	OFF
	138.6	440.7	5	6	10	OFF	656.0	5	6	10	OFF
V138/2nd	288.1	445.7	5	6	11	OFF	666.1	5	6	12	ON
	288.2	445.8	5	6	11	OFF	666.1	5	6	12	ON
	288.3	445.7	5	6	11	OFF	666.0	5	6	12	ON
	288.4	445.7	5	6	11	OFF	666.0	5	6	12	ON
	288.5	445.6	5	6	11	OFF	665.9	5	6	12	ON
	288.6	440.6	5	6	10	OFF	666.0	5	6	12	ON
V88/1st	N88.1.1	672.6	5	6	9	ON	677.7	5	6	10	OFF
	N88.1.2	672.6	5	6	9	ON	677.6	5	6	10	OFF
	N88.1.3	672.6	5	6	9	ON	677.7	5	6	10	OFF
	N88.1.4	672.6	5	6	9	ON	677.7	5	6	10	OFF
	N88.1.5	672.5	5	6	9	ON	677.6	5	6	10	OFF
	N88.1.6	672.6	5	6	9	ON	677.7	5	6	10	OFF
	88.1.7	673.6	5	6	9	ON	678.5	5	6	10	OFF
	88.1.8	673.6	5	6	9	ON	678.6	5	6	10	OFF
	88.1.9	673.7	5	6	9	ON	678.8	5	6	10	OFF
	88.1.10	673.5	5	6	9	ON	678.5	5	6	10	OFF
	88.1	673.2	5	6	9	ON	678.3	5	6	10	OFF
	88.2	673.2	5	6	9	ON	678.4	5	6	10	OFF
	88.3	673.2	5	6	9	ON	678.3	5	6	10	OFF
	88.4	673.2	5	6	9	ON	678.3	5	6	10	OFF
	88.5	673.1	5	6	9	ON	678.3	5	6	10	OFF
	88.6	673.1	5	6	9	ON	678.2	5	6	10	OFF
	88.8	673.2	5	6	9	ON	678.3	5	6	10	OFF
V88/2nd	N272.1	678.0	5	6	10	OFF	678.0	5	6	10	OFF
	N272.2	678.0	5	6	10	OFF	678.0	5	6	10	OFF
	N272.3	678.0	5	6	10	OFF	678.0	5	6	10	OFF
	N272.4	678.0	5	6	10	OFF	678.0	5	6	10	OFF
	N272.5	678.0	5	6	10	OFF	678.0	5	6	10	OFF
	N272.6	676.7	5	6	10	OFF	676.7	5	6	10	OFF
V88/3rd	N369.1	677.6	5	6	10	OFF	677.6	5	6	10	OFF
	N369.2	677.5	5	6	10	OFF	677.5	5	6	10	OFF
	N369.3	677.6	5	6	10	OFF	677.6	5	6	10	OFF
	N369.3	677.7	5	6	10	OFF	677.7	5	6	10	OFF
	N369.5	677.5	5	6	10	OFF	677.5	5	6	10	OFF

	N369.6	677.4	5	6	10	OFF	677.4	5	6	10	OFF
	369.7	678.4	5	6	10	OFF	678.4	5	6	10	OFF
	369.8	678.4	5	6	10	OFF	678.4	5	6	10	OFF
	369.9	678.6	5	6	10	OFF	678.6	5	6	10	OFF
	369,10	678.5	5	6	10	OFF	678.5	5	6	10	OFF
	369.11	678.3	5	6	10	OFF	678.3	5	6	10	OFF
	369.12	678.4	5	6	10	OFF	678.4	5	6	10	OFF
	369.14	678.3	5	6	10	OFF	678.3	5	6	10	OFF
	369.15	678.3	5	6	10	OFF	678.3	5	6	10	OFF
	369.16	678.7	5	6	10	OFF	678.7	5	6	10	OFF
	369.17	673.7	5	6	9	ON	668.7	5	6	8	OFF
	369.18	678.7	5	6	10	OFF	678.7	5	6	10	OFF
	369.19	678.8	5	6	10	OFF	678.8	5	6	10	OFF
	369,20	678.6	5	6	10	OFF	678.6	5	6	10	OFF
V88/4th	N449.1	682.9	5	6	11	OFF	687.7	5	6	12	ON
	449.2	677.8	5	6	10	OFF	682.9	5	6	11	OFF
	449.3	683.1	5	6	11	OFF	688.0	5	6	12	ON
	449.4	683.1	5	6	11	OFF	688.0	5	6	12	ON
	449.5	678.0	5	6	10	OFF	688.1	5	6	12	ON
	449.6	677.9	5	6	10	OFF	682.9	5	6	11	OFF
V43/2nd	241.1	450.9	5	6	12	ON	655.9	5	6	10	OFF
	241.2	456.0	5	6	13	OFF	655.9	5	6	10	OFF
V43/3rd	349.1	450.8	5	6	12	ON	655.8	5	6	10	OFF
	349.2	450.9	5	6	12	ON	655.9	5	6	10	OFF
	349.3	450.9	5	6	12	ON	656.1	5	6	10	OFF
	349.4	450.9	5	6	12	ON	656.0	5	6	10	OFF
	349.5	451.0	5	6	12	ON	656.0	5	6	10	OFF
	349.6	450.9	5	6	12	ON	656.1	5	6	10	OFF

Carrier with time points	isolate	*opa* genes									
		opaD						*opaJ*			
cc23		Size	C tracts	A tracts	CTCT T length	ON/ OFF	Size	C tracts	A tracts	CTCTT length	ON/ OFF
V222/ 1st	222.1	426.7	5	6	8	OFF	627.0	5	6	8	OFF
	222.2	426.7	5	6	8	OFF	627.0	5	6	8	OFF
	222.3	426.1	5	6	8	OFF	626.4	5	6	8	OFF
	222.4	426.1	5	6	8	OFF	626.6	5	6	8	OFF
	222.5	426.1	5	6	8	OFF	625.5	5	6	8	OFF
	222.6	426.2	5	6	8	OFF	626.5	5	6	8	OFF

V222/2nd	309.1	426.5	5	6	8	OFF	627.0	5	6	8	OFF
	309.2	426.7	5	6	8	OFF	627.1	5	6	8	OFF
	309.3	426.6	5	6	8	OFF	627.1	5	6	8	OFF
	309.4	426.7	5	6	8	OFF	632.2	5	6	9	ON
	309.5	426.1	5	6	8	OFF	626.5	5	6	8	OFF
	309.6	426.6	5	6	8	OFF	627.1	5	6	8	OFF
V222/3rd	391.1	426.5	5	6	8	OFF	639.5	3	6	8	ON
	391.2	426.5	5	6	8	OFF	639.2	3	6	8	ON
	391.3	426.8	5	6	8	OFF	639.5	3	6	8	ON
	391.4	426.6	5	6	8	OFF	639.6	3	6	8	ON
	391.5	426.5	5	6	8	OFF	639.4	3	6	8	ON
	391.6	426.6	5	6	8	OFF	640.2	3	6	8	ON
V222/4th	459.1	426.6	5	6	8	OFF	639.4	3	6	8	ON
	459.2	426.5	5	6	8	OFF	639.4	3	6	8	ON
	459.4	426.1	5	6	8	OFF	640.0	3	6	8	ON
	459.4	426.1	5	6	8	OFF	639.6	3	6	8	ON
	459.5	426.1	5	6	8	OFF	639.5	3	6	8	ON
	459.6	426.2	5	6	8	OFF	640.3	3	6	8	ON
V96/1ST	259.1	426.4	5	6	8	OFF	626.4	5	6	8	OFF
	259.2	426.3	5	6	8	OFF	626.4	5	6	8	OFF
	259.3	426.4	5	6	8	OFF	626.7	5	6	8	OFF
	259.4	426.4	5	6	8	OFF	626.8	5	6	8	OFF
	259.5	426.4	5	6	8	OFF	626.8	5	6	8	OFF
	259.6	426.3	5	6	8	OFF	627.1	5	6	8	OFF
V96/2nd	370.1										
	370.2	426.8	5	6	8	OFF	647.4	5	6	12	ON
	370.3	421.9	5	6	7	OFF	627.5	5	6	8	OFF
	370.4	426.8	5	6	8	OFF	647.4	5	6	12	ON
	370.5	426.8	5	6	8	OFF	622.4	5	6	7	OFF
	370.6	421.7	5	6	7	OFF	647.5	5	6	12	ON
V96/3rd	445.1	426.2	5	6	8	OFF	626.8	5	6	8	OFF
	445.2	426.2	5	6	8	OFF	626.3	5	6	8	OFF
	445.3	426.3	5	6	8	OFF	626.9	5	6	8	OFF
	445.4	426.3	5	6	8	OFF	626.8	5	6	8	OFF
	445.5	426.2	5	6	8	OFF	627.1	5	6	8	OFF
	445.6	426.3	5	6	8	OFF	626.7	5	6	8	OFF
V69/1st	258.1	426.3	5	6	8	OFF	626.6	5	6	8	OFF

	258.2	426.4	5	6	8	OFF	626.9	5	6	8	OFF
	258.3	426.3	5	6	8	OFF	626.7	5	6	8	OFF
	258.4	426.8	5	6	8	OFF	627.3	5	6	8	OFF
	258.5	426.7	5	6	8	OFF	627.2	5	6	8	OFF
	258.6	426.2	5	6	8	OFF	626.6	5	6	8	OFF
V69/2nd	431.1	431.6	5	6	9	ON	626.8	5	6	8	OFF
	431.2	426.5	5	6	8	OFF	626.9	5	6	8	OFF
	431.3	426.6	5	6	8	OFF	626.8	5	6	8	OFF
	431.4	426.8	5	6	8	OFF	627.1	5	6	8	OFF
	431.5	426.8	5	6	8	OFF	627.0	5	6	8	OFF
	431.6	426.8	5	6	8	OFF	627.1	5	6	8	OFF
V93/	264.1	421.7	5	6	7	OFF	622.0	5	6	7	OFF
	264.2	421.7	5	6	7	OFF	622.1	5	6	7	OFF
	264.3	421.3	5	6	7	OFF	621.7	5	6	7	OFF
	264.4	421.6	5	6	7	OFF	622.0	5	6	7	OFF
	264.5	421.6	5	6	7	OFF	621.7	5	6	7	OFF
	264.6	421.1	5	6	7	OFF	621.5	5	6	7	OFF
V93/	359.1	421.9	5	6	7	OFF	622.2	5	6	7	OFF
	359.2	421.8	5	6	7	OFF	622.3	5	6	7	OFF
	359.3	421.5	5	6	7	OFF	621.9	5	6	7	OFF
	359.4	421.7	5	6	7	OFF	622.3	5	6	7	OFF
	359.5	421.8	5	6	7	OFF	622.2	5	6	7	OFF
	359.6	421.4	5	6	7	OFF	621.7	5	6	7	OFF
CC167											
V117/1st	117.1	420.6	5	6	4	OFF	616.5	5	6	4	OFF
	117.2	420.5	5	6	4	OF	616.7	5	6	4	OFF
V117/2nd	284.1	421.0	5	6	4	OFF	617.0	5	6	4	OFF
	284.2	421.1	5	6	4	OFF	612.0	5	6	3	ON
	284.3	421.1	5	6	4	OFF	617.1	5	6	4	OFF
	284.4	421.0	5	6	4	OFF	611.8	5	6	3	ON
	284.5	421.0	5	6	4	OFF	616.9	5	6	4	OFF
	284.6	421.0	5	6	4	OFF	611.8	5	6	3	ON
V117/3rd	332.1	420.6	5	6	4	OFF	611.5	5	6	3	ON
	332.2	420.7	5	6	4	OFF	611.7	5	6	3	ON
	332.3	420.7	5	6	4	OFF	611.7	5	6	3	ON
	332.4	420.7	5	6	4	OFF	611.6	5	6	3	ON
	332.5										
	332.6	420.6	5	6	4	OFF	611.6	5	6	3	ON

V117/4th	417.1	420.4	5	6	4	OFF	616.4	5	6	4	OFF
	417.2	421.1	5	6	4	OFF	617.1	5	6	4	OFF
	417.3	421.1	5	6	4	OFF	617.2	5	6	4	OFF
	417.4	420.4	5	6	4	OFF	616.2	5	6	4	OFF
	417.5	421.2	5	6	4	OFF	617.0	5	6	4	OFF
	417.6	421.1	5	6	4	OFF	617.1	5	6	4	OFF
V64/1st	64.1	420.6	5	6	4	OFF	657.1	5	6	12	ON
	64.2	420.6	5	6	4	OFF	657.1	5	6	12	ON
	64.3	420.7	5	6	4	OFF	657.0	5	6	12	ON
	64.4	420.5	5	6	4	OFF	657.2	5	6	12	ON
	64.5	420.5	5	6	4	OFF	656.9	5	6	12	ON
	64.6	420.6	5	6	4	OFF	657.0	5	6	12	ON
V64/2nd	257.1	420.5	5	6	4	OFF	651.8	5	6	11	OFF
	257.2	421.2	5	6	4	OFF	652.3	5	6	11	OFF
	257.3	421.2	5	6	4	OFF	652.2	5	6	11	OFF
	257.4	421.1	5	6	4	OFF	652.4	5	6	11	OFF
	257.5	421.1	5	6	4	OFF	652.2	5	6	11	OFF
	257.6	421.2	5	6	4	OFF	652.2	5	6	11	OFF
V64/3rd	348.1	420.5	5	6	4	OFF	641.7	5	6	9	ON
	348.2	420.6	5	6	4	OFF	636.8	5	6	8	OFF
	348.3	420.5	5	6	4	OFF	636.8	5	6	8	OFF
	348.4	420.6	5	6	4	OFF	636.7	5	6	8	OFF
	348.5	420.5	5	6	4	OFF	641.6	5	6	9	ON
	348.6	420.6	5	6	4	OFF	641.4	5	6	9	ON
V124/1st	124.1	421.0	5	6	4	OFF	617.0	5	6	4	OFF
	124.2	421.1	5	6	4	OFF	617.0	5	6	4	OFF
	124.3	420.5	5	6	4	OFF	616.3	5	6	4	OFF
	124.4	420.5	5	6	4	OFF	616.3	5	6	4	OFF
	124.5	420.5	5	6	4	OFF	616.3	5	6	4	OFF
V124/2nd	290.1	420.5	5	6	4	OFF	616.4	5	6	4	OFF
	290.3	421.0	5	6	4	OFF	616.9	5	6	4	OFF
	290.4	420.9	5	6	4	OFF	616.8	5	6	4	OFF
	290.5	420.5	5	6	4	OFF	616.5	5	6	4	OFF
	290.6	420.4	5	6	4	OFF	616.4	5	6	4	OFF
V124/3rd	336.1	420.5	5	6	4	OFF	616.6	5	6	4	OFF
	336.2	420.7	5	6	4	OFF	616.7	5	6	4	OFF
	336.3	421.0	5	6	4	OFF	617.0	5	6	4	OFF

	336.4	420.8	5	6	4	OFF	616.8	5	6	4	OFF
	336.5	420.6	5	6	4	OFF	616.7	5	6	4	OFF
	336.6	420.5	5	6	4	OFF	616.6	5	6	4	OFF
cc60											
V113/1st	113.1	464.3	4	6	14	ON	413.4	4	6	4	OFF
	113.2	464.4	4	6	14	ON	413.5	4	6	4	OFF
	113.3	464.5	4	6	14	ON	413.5	4	6	4	OFF
	113.4	464.3	4	6	14	ON	413.4	4	6	4	OFF
	113.5	464.4	4	6	14	ON	413.4	4	6	4	OFF
	113.6	464.3	4	6	14	ON	413.5	4	6	4	OFF
V113/2nd	281.1	464.7	4	6	14	ON	413.8	4	6	4	OFF
	281.2	464.7	4	6	14	ON	413.9	4	6	4	OFF
	281.3	464.8	4	6	14	ON	413.9	4	6	4	OFF
	281.4	469.8	4	6	15	OFF	413.8	4	6	4	OFF
	281.5	434.3	4	6	8	ON	413.8	4	6	4	OFF
	281.6	434.3	4	6	8	ON	413.9	4	6	4	OFF
V114/1st	114.1	464.4	4	6	14	ON	413.5	4	6	4	OFF
	114.2	469.5	4	6	15	OFF	413.5	4	6	4	OFF
	114.3	464.5	4	6	14	ON	413.6	4	6	4	OFF
	114.4	459.4	4	6	13	OFF	413.5	4	6	4	OFF
	114.5	464.4	4	6	14	ON	413.6	4	6	4	OFF
	114.6	464.5	4	6	14	ON	413.5	4	6	4	OFF
	114.7	464.4	4	6	14	ON	413.7	4	6	4	OFF
	114.8	464.5	4	6	14	ON	413.5	4	6	4	OFF
	114.9	464.5	4	6	14	ON	413.5	4	6	4	OFF
	114,10	464.5	4	6	14	ON	413.6	4	6	4	OFF
	114.11	464.5	4	6	14	ON	413.6	4	6	4	OFF
	114.12	464.6	4	6	14	ON	413.6	4	6	4	OFF
	114.13	474.6	4	6	16	OFF	413.5	4	6	4	OFF
	114.14	464.4	4	6	14	ON	413.5	4	6	4	OFF
	114.16	464.8	4	6	14	ON	414.4	4	6	4	OFF
	114.18	464.9	4	6	14	ON	414.3	4	6	4	OFF
V114/2nd	283.1	459.3	4	6	13	OFF	413.4	4	6	4	OFF
	283.2	469.9	4	6	15	OFF	414.1	4	6	4	OFF
	283.3	459.7	4	6	13	OFF	414.0	4	6	4	OFF
	283.4	459.3	4	6	13	OFF	413.1	4	6	4	OFF
	283.5	459.6	4	6	13	OFF	413.9	4	6	4	OFF
	283.6	459.7	4	6	13	OFF	414.0	4	6	4	OFF
V114/3rd	330.1	469.3	4	6	15	OFF	413.5	4	6	4	OFF

	330.2	469.3	4	6	15	OFF	413.3	4	6	4	OFF
	330.3	469.7	4	6	15	OFF	413.8	4	6	4	OFF
	330.4	469.6	4	6	15	OFF	413.6	4	6	4	OFF
	330.5	469.4	4	6	15	OFF	413.5	4	6	4	OFF
	330.6	469.4	4	6	15	OFF	413.3	4	6	4	OFF
	330.7	469.6	4	6	15	OFF	413.5	4	6	4	OFF
	330.8	469.7	4	6	15	OFF	413.6	4	6	4	OFF
	330.9	469.6	4	6	15	OFF	413.5	4	6	4	OFF
	330,10	469.6	4	6	15	OFF	413.7	4	6	4	OFF
	330.11	469.5	4	6	15	OFF	413.6	4	6	4	OFF
	330.12	469.5	4	6	15	OFF	413.6	4	6	4	OFF
	330.15	469.9	4	6	15	OFF	414.1	4	6	4	OFF
	330.16	470.0	4	6	15	OFF	414.2	4	6	4	OFF
	330.17	470.0	4	6	15	OFF	414.2	4	6	4	OFF
	330.18	469.9	4	6	15	OFF	414.2	4	6	4	OFF
	330.19	470.0	4	6	15	OFF	414.1	4	6	4	OFF
	330,20	470.0	4	6	15	OFF	414.1	4	6	4	OFF
V115/1st	115.1	464.4	4	6	14	ON	413.5	4	6	4	OFF
	115.2	433.9	4	6	8	ON	413.5	4	6	4	OFF
	115.3	464.5	4	6	14	ON	413.5	4	6	4	OFF
	115.4	464.4	4	6	14	ON	413.6	4	6	4	OFF
	115.5	464.3	4	6	14	ON	413.6	4	6	4	OFF
	115.6	464.3	4	6	14	ON	413.5	4	6	4	OFF
V115/2nd	282.1	464.4	4	6	14	ON	413.4	4	6	4	OFF
	282.2	469.9	4	6	15	OFF	414.1	4	6	4	OFF
	282.3	464.8	4	6	14	ON	414.0	4	6	4	OFF
	282.4	464.8	4	6	14	ON	414.1	4	6	4	OFF
	282.5	464.8	4	6	14	ON	414.0	4	6	4	OFF
	282.6	464.9	4	6	14	ON	414.0	4	6	4	OFF
V134/1st											
	134.1	464.3	4	6	14	ON	413.4	4	6	4	OFF
	134.2	459.3	4	6	13	OFF	413.5	4	6	4	OFF
	134.3	459.2	4	6	13	OFF	413.4	4	6	4	OFF
	134.4	459.3	4	6	13	OFF	413.5	4	6	4	OFF
	134.5										
	134.6	459.2	4	6	13	OFF	413.5	4	6	4	OFF
	134.7	459.4	4	6	13	OFF	413.6	4	6	4	OFF
	134.8	459.4	4	6	13	OFF	413.5	4	6	4	OFF
	134.9	459.4	4	6	13	OFF	413.6	4	6	4	OFF
	134,10	459.4	4	6	13	OFF	413.6	4	6	4	OFF
	134.11	459.4	4	6	13	OFF	413.5	4	6	4	OFF
	134.12	459.3	4	6	13	OFF	413.5	4	6	4	OFF

	134.13	459.3	4	6	13	OFF	413.3	4	6	4	OFF
	134.14	459.3	4	6	13	OFF	413.5	4	6	4	OFF
	134.16	459.7	4	6	13	OFF	414.2	4	6	4	OFF
	134.17	459.8	4	6	13	OFF	414.2	4	6	4	OFF
	134.18	464.9	4	6	14	OFF	414.3	4	6	4	OFF
V134/2nd	295.1	464.3	4	6	14	ON	413.5	4	6	4	OFF
	295.2	464.8	4	6	14	ON	414.0	4	6	4	OFF
	295.3	459.3	4	6	13	OFF	413.5	4	6	4	OFF
	295.4	464.4	4	6	14	ON	413.5	4	6	4	OFF
	295.5	459.2	4	6	13	OFF	413.6	4	6	4	OFF
	295.6	464.7	4	6	14	ON	413.8	4	6	4	OFF
V134/3rd	333.1	459.3	4	6	13	OFF	413.4	4	6	4	OFF
	333.2	459.3	4	6	13	OFF	413.3	4	6	4	OFF
	333.3	459.7	4	6	13	OFF	413.8	4	6	4	OFF
	333.4	459.5	4	6	13	OFF	413.7	4	6	4	OFF
	333.5	459.3	4	6	13	OFF	413.4	4	6	4	OFF
	333.6	459.2	4	6	13	OFF	413.4	4	6	4	OFF
	333.7	459.4	4	6	13	OFF	413.7	4	6	4	OFF
	333.8	459.4	4	6	13	OFF	413.6	4	6	4	OFF
	333.9	459.5	4	6	13	OFF	413.7	4	6	4	OFF
	333,10	459.6	4	6	13	OFF	413.7	4	6	4	OFF
	333.11	459.6	4	6	13	OFF	413.6	4	6	4	OFF
	333.12	459.4	4	6	13	OFF	413.7	4	6	4	OFF
	333.13	464.5	4	6	14	OFF	413.6	4	6	4	OFF
	333.14	459.4	4	6	13	OFF	413.5	4	6	4	OFF
	333.15	459.7	4	6	13	OFF	414.1	4	6	4	OFF
	333.16	459.8	4	6	13	OFF	414.2	4	6	4	OFF
	333.17	459.9	4	6	13	OFF	414.2	4	6	4	OFF
	333.18	459.9	4	6	13	OFF	414.2	4	6	4	OFF
	333.19	459.8	4	6	13	OFF	414.1	4	6	4	OFF
	333,20	459.7	4	6	13	OFF	414.1	4	6	4	OFF
V185/1st	185.1	438.8	4	6	9	OFF	428.8	4	6	7	OFF
	185.2	438.9	4	6	9	OFF	429.0	4	6	7	OFF
	185.3	438.9	4	6	9	OFF	428.9	4	6	7	OFF
	185.4	439.0	4	6	9	OFF	429.0	4	6	7	OFF
	185.5	439.0	4	6	9	OFF	428.9	4	6	7	OFF
	185.6	438.9	4	6	9	OFF	428.9	4	6	7	OFF
	185.8	438.9	4	6	9	OFF	428.9	4	6	7	OFF
	185.9	439.2	4	6	9	OFF	429.1	4	6	7	OFF
	185,10	439.1	4	6	9	OFF	428.9	4	6	7	OFF
	185.11	439.0	4	6	9	OFF	428.8	4	6	7	OFF

	185.12	439.0	4	6	9	OFF	429.0	4	6	7	OFF
	185.13	438.9	4	6	9	OFF	428.9	4	6	7	OFF
	185.14	438.9	4	6	9	OFF	429.0	4	6	7	OFF
	185.15	438.8	4	6	9	OFF	428.6	4	6	7	OFF
	185.16	439.3	4	6	9	OFF	429.3	4	6	7	OFF
	185.17	439.3	4	6	9	OFF	429.3	4	6	7	OFF
	185.18	439.4	4	6	9	OFF	429.3	4	6	7	OFF
	185.19	439.4	4	6	9	OFF	429.3	4	6	7	OFF
	185,20	439.4	4	6	9	OFF	429.4	4	6	7	OFF
V185/2nd	306.1	443.9	4	6	10	OFF	428.9	4	6	7	OFF
	306.2	443.9	4	6	10	OFF	428.8	4	6	7	OFF
	306.3	449.1	4	6	11	ON	428.8	4	6	7	OFF
	306.4	444.0	4	6	10	OFF	428.9	4	6	7	OFF
	306.5	443.9	4	6	10	OFF	428.8	4	6	7	OFF
	306.6	449.0	4	6	11	ON	428.8	4	6	7	OFF
V185/4th	456.1	448.9	4	6	11	ON	428.7	4	6	7	OFF
	456.2	448.9	4	6	11	ON	428.7	4	6	7	OFF
	456.3	459.2	4	6	13	OFF	429.0	4	6	7	OFF
	456.4	459.2	4	6	13	OFF	428.7	4	6	7	OFF
	456.5	459.2	4	6	13	OFF	428.8	4	6	7	OFF
	456.6	449.0	4	6	11	ON	428.7	4	6	7	OFF
	456.7	449.2	4	6	11	ON	429.5	4	6	7	OFF
	456.8	459.2	4	6	13	OFF	428.7	4	6	7	OFF
	456.9	459.3	4	6	13	OFF	428.6	4	6	7	OFF
	456,10	459.2	4	6	13	OFF	428.8	4	6	7	OFF
	456.11	449.1	4	6	11	ON	428.9	4	6	7	OFF
	456.12	459.2	4	6	13	OFF	428.9	4	6	7	OFF
	456.13	449.1	4	6	11	ON	428.7	4	6	7	OFF
	456.14	459.2	4	6	13	OFF	428.9	4	6	7	OFF
	456.16	459.5	4	6	13	OFF	428.9	4	6	7	OFF
	456.17	459.6	4	6	13	OFF	429.0	4	6	7	OFF
	456.18	449.5	4	6	11	ON	429.1	4	6	7	OFF
	456.19	459.6	4	6	13	OFF	429.3	4	6	7	OFF
	456,20	459.7	4	6	13	OFF	429.3	4	6	7	OFF
cc174											
V51/1ST											
	51.1	640.8	5	6	8	OFF	433.7	5	5	7	OFF
	51.2	641.1	5	6	8	OFF	438.7	5	5	8	ON
	51.3	646.0	5	6	9	ON	438.9	5	5	8	ON
	51.4	646.1	5	6	9	ON	438.7	5	5	8	ON
	51.5	640.9	5	6	8	OFF	438.7	5	5	8	ON

	51.6	646.0	5	6	9	ON	438.6	5	5	8	ON
V51/2nd											
	236.1	641.0	5	6	8	OFF	438.7	5	5	8	ON
	236.2	641.0	5	6	8	OFF	443.8	5	5	9	OFF
	236.3	641.1	5	6	8	OFF	433.8	5	5	7	OFF
	236.4	641.1	5	6	8	OFF	438.7	5	5	8	ON
	236.5	641.1	5	6	8	OFF	433.7	5	5	7	OFF
	236.6	641.1	5	6	8	OFF	433.7	5	5	7	OFF
V51/3rd	354.1	640.8	5	6	8	OFF	443.6	5	5	9	OFF
	354.2	641.0	5	6	8	OFF	443.6	5	5	9	OFF
	354.3	640.9	5	6	8	OFF	443.6	5	5	9	OFF
	254.4	641.0	5	6	8	OFF	443.7	5	5	9	OFF
	254.5	641.0	5	6	8	OFF	433.6	5	5	7	OFF
	254.6	640.9	5	6	8	OFF	443.6	5	5	9	OFF
V51/4th	424.1	640.9	5	6	8	OFF	433.6	5	5	7	OFF
	424.2	640.9	5	6	8	OFF	433.5	5	5	7	OFF
	424.3	640.9	5	6	8	OFF	433.6	5	5	7	OFF
	424.4	640.9	5	6	8	OFF	433.4	5	5	7	OFF
	424.5	640.9	5	6	8	OFF	433.6	5	5	7	OFF
	424.6	640.8	5	6	8	OFF	438.5	5	5	8	ON
V52/1st											
	52.1	641.3	5	6	8	OFF	439.0	5	5	8	ON
	52.2	641.1	5	6	8	OFF	438.9	5	5	8	ON
	52.3	641.2	5	6	8	OFF	439.0	5	5	8	ON
	52.4	641.2	5	6	8	OFF	439.0	5	5	8	ON
	52.5	641.1	5	6	8	OFF	438.8	5	5	8	ON
	52.6	641.1	5	6	8	OFF	438.9	5	5	8	ON
V52/2nd											
	238.1	640.2	5	6	8	OFF	432.9	5	5	7	OFF
	238.2	641.2	5	6	8	OFF	433.9	5	5	7	OFF
	238.3	641.2	5	6	8	OFF	449.2	5	5	10	OFF
	238.4	641.3	5	6	8	OFF	434.0	5	5	7	OFF
	238.5	640.0	5	6	8	OFF	443.2	5	5	9	off
	238.6	641.3	5	6	8	OFF	449.1	5	5	10	OFF
V52/3rd	342.1	641.1	5	6	8	OFF	438.8	5	5	8	ON
V58/1st	58.1	646.3	5	6	9	ON	444.0	5	5	9	OFF
	58.2	646.2	5	6	9	ON	444.0	5	5	9	OFF

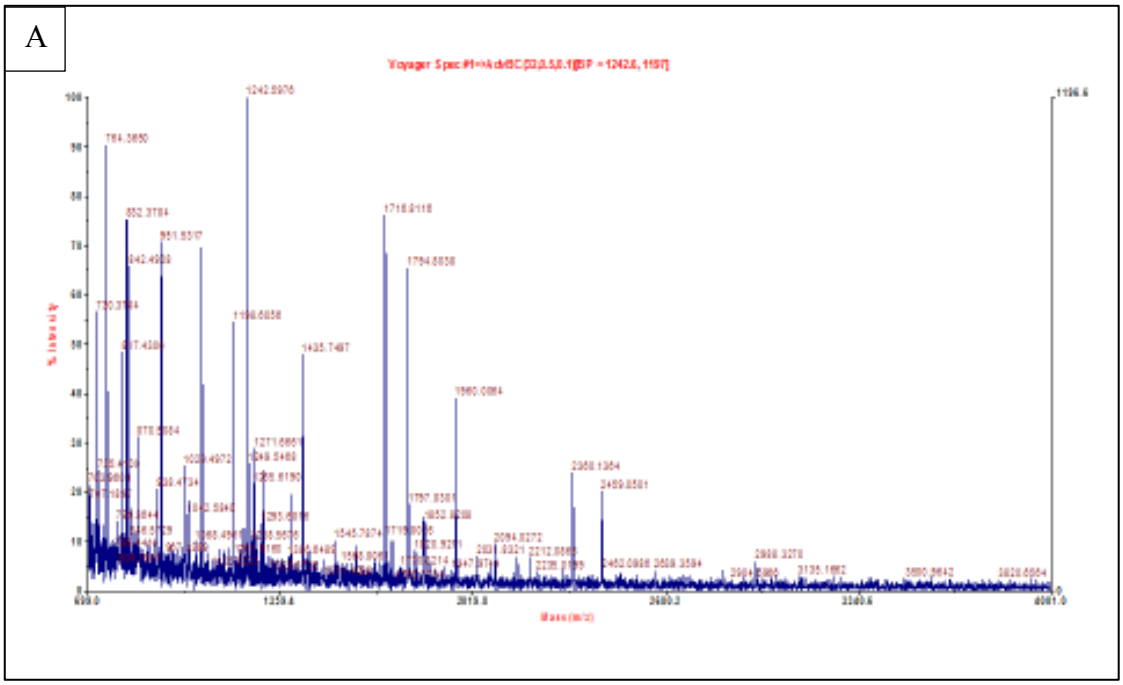
	58.3	641.2	5	6	8	OFF	438.8	5	5	8	ON
	58.4	646.2	5	6	9	ON	443.8	5	5	9	OFF
	58.5	645.6	5	6	9	ON	443.0	5	5	9	OFF
	58.6	641.3	5	6	8	OFF	439.0	5	5	8	ON
V58/2nd	240.1	640.3	5	6	8	OFF	437.9	5	5	8	ON
V58/3rd	429.1	641.2	5	6	8	OFF	438.9	5	5	8	ON
	492.2	641.1	5	6	8	OFF	438.9	5	5	8	ON
	429.3	641.2	5	6	8	OFF	438.9	5	5	8	ON
	429.4	641.1	5	6	8	OFF	438.8	5	5	8	ON
	429.5	641.1	5	6	8	OFF	438.8	5	5	8	ON
	429.6	641.1	5	6	8	OFF	438.8	5	5	8	ON
V59/1st	59.1	640.7	5	6	8	OFF	433.5	5	5	7	OFF
	59.2	640.7	5	6	8	OFF	438.5	5	5	8	ON
	59.3	640.8	5	6	8	OFF	443.5	5	5	9	OFF
	59.4	640.7	5	6	8	OFF	438.4	5	5	8	ON
	59.5	640.5	5	6	8	OFF	433.2	5	5	7	OFF
	59.6	640.6	5	6	8	OFF	433.3	5	5	7	OFF
	59.11	640.6	5	6	8	OFF	433.2	5	5	7	OFF
	59.12	640.8	5	6	8	OFF	433.4	5	5	7	OFF
	59.13	640.8	5	6	8	OFF	433.2	5	5	7	OFF
	59.14	640.8	5	6	8	OFF	433.3	5	5	7	OFF
	59.15	640.7	5	6	8	OFF	433.3	5	5	7	OFF
	59.16	645.7	5	6	9	OFF	438.2	5	5	8	ON
	59.17	640.6	5	6	8	OFF	433.1	5	5	7	OFF
	59.18	640.5	5	6	8	OFF	433.1	5	5	7	OFF
	59,20	641.2	5	6	8	OFF	438.7	5	5	8	ON
V59/2nd	253.1	640.7	5	6	8	OFF	433.4	5	5	7	OFF
	253.2	640.7	5	6	8	OFF	433.4	5	5	7	OFF
	253.3	635.7	5	6	7	OFF	433.5	5	5	7	OFF
	253.4	640.8	5	6	8	OFF	433.4	5	5	7	OFF
	253.5	640.8	5	6	8	OFF	433.4	5	5	7	OFF
	253.6	640.7	5	6	8	OFF	433.3	5	5	7	OFF
V59/3rd	352.1	640.6	5	6	8	OFF	438.2	5	5	8	ON
	352.2	640.6	5	6	8	OFF	438.3	5	5	8	ON
	352.3	640.6	5	6	8	OFF	433.2	5	5	7	OFF
	352.4	640.6	5	6	8	OFF	438.2	5	5	8	ON
	352.5	640.7	5	6	8	OFF	433.3	5	5	7	OFF
	352.6	640.6	5	6	8	OFF	433.1	5	5	7	OFF
	352.7	640.5	5	6	8	OFF	438.2	5	5	8	ON

	352.8	640.6	5	6	8	OFF	433.3	5	5	7	OFF
	352.9	640.7	5	6	8	OFF	433.4	5	5	7	OFF
	352,10	640.7	5	6	8	OFF	438.2	5	5	8	ON
	352.11	640.6	5	6	8	OFF	433.2	5	5	7	OFF
	352.12	640.5	5	6	8	OFF	438.2	5	5	8	ON
	352.13	640.6	5	6	8	OFF	438.1	5	5	8	ON
	352.14	640.6	5	6	8	OFF	438.0	5	5	8	ON
	352.15	641.0	5	6	8	OFF	433.6	5	5	7	OFF
	352.16	641.1	5	6	8	OFF	433.6	5	5	7	OFF
	352.17	641.1	5	6	8	OFF	433.6	5	5	7	OFF
	352.18	641.2	5	6	8	OFF	438.7	5	5	8	ON
	352.19	641.2	5	6	8	OFF	433.6	5	5	7	OFF
	352,20	641.1	5	6	8	OFF	433.6	5	5	7	OFF
V59/4th	438.1	645.8	5	6	9	ON	438.4	5	5	8	ON
	438.2	645.6	5	6	9	ON	438.2	5	5	8	ON
	438.3	645.6	5	6	9	ON	438.3	5	5	8	ON
	438.4	645.7	5	6	9	ON	433.3	5	5	7	OFF
	438.5	645.6	5	6	9	ON	438.3	5	5	8	ON
	438.6	645.5	5	6	9	ON	437.9	5	5	8	ON
V138/1st	138.1	640.3	5	6	8	OFF	432.8	5	5	7	OFF
	138.2	640.5	5	6	8	OFF	433.0	5	5	7	OFF
	138.3										
	138.4	640.3	5	6	8	OFF	432.9	5	5	7	OFF
	138.5	640.4	5	6	8	OFF	437.8	5	5	8	ON
	138.6	641.3	5	6	8	OFF	433.8	5	5	7	OFF
V138/2nd	288.1	641.1	5	6	8	OFF	433.8	5	5	7	OFF
	288.2	641.1	5	6	8	OFF	433.7	5	5	7	OFF
	288.3	641.0	5	6	8	OFF	433.7	5	5	7	OFF
	288.4	645.3	5	6	9	ON	433.7	5	5	7	OFF
	288.5	641.0	5	6	8	OFF	433.6	5	5	7	OFF
	288.6	640.9	5	6	8	OFF	433.6	5	5	7	OFF
V88/1st	N88.1.1	671.1	5	6	14	OFF	441.3	5	5	7	OFF
	N88.1.2	676.4	5	6	15	ON	441.4	5	5	7	OFF
	N88.1.3	671.2	5	6	14	OFF	441.5	5	5	7	OFF
	N88.1.4	671.1	5	6	14	OFF	441.4	5	5	7	OFF
	N88.1.5	681.2	5	6	16	OFF	441.4	5	5	7	OFF
	N88.1.6	671.1	5	6	14	OFF	441.3	5	5	7	OFF
	88.1.7	671.6	5	6	14	OFF	441.4	5	5	7	OFF
	88.1.8	671.7	5	6	14	OFF	441.5	5	5	7	OFF

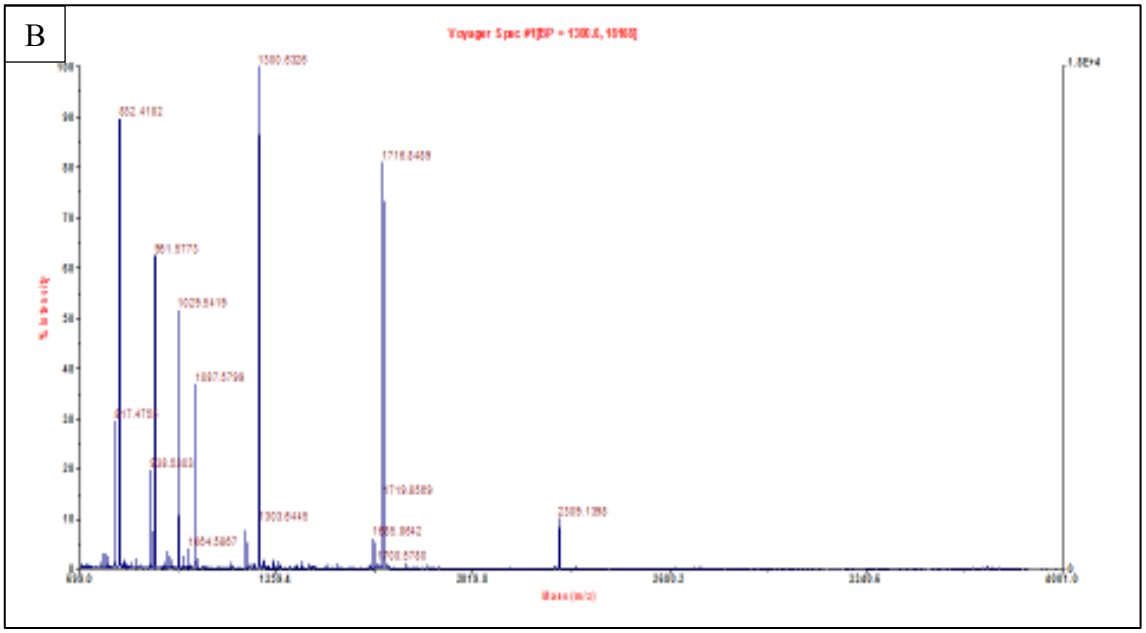
	88.1.9	671.8	5	6	14	OFF	441.4	5	5	7	OFF
	88.1.10	676.6	5	6	15	ON	441.3	5	5	7	OFF
	88.1	671.2	5	6	14	OFF	441.0	5	5	7	OFF
	88.2	671.2	5	6	14	OFF	441.0	5	5	7	OFF
	88.3	671.3	5	6	14	OFF	441.0	5	5	7	OFF
	88.4	671.3	5	6	14	OFF	441.0	5	5	7	OFF
	88.5	671.1	5	6	14	OFF	440.9	5	5	7	OFF
	88.6	671.1	5	6	14	OFF	441.0	5	5	7	OFF
	88.8	671.1	5	6	14	OFF	440.7	5	5	7	OFF
V88/2nd	N272.1	671.5	5	6	14	OFF	441.0	5	5	7	OFF
	N272.2	671.6	5	6	14	OFF	441.0	5	5	7	OFF
	N272.3	671.6	5	6	14	OFF	441.0	5	5	7	OFF
	N272.4	671.5	5	6	14	OFF	440.9	5	5	7	OFF
	N272.5	671.5	5	6	14	OFF	440.9	5	5	7	OFF
	N272.6	671.5	5	6	14	OFF	441.0	5	5	7	OFF
V88/3rd	N369.1	665.9	5	6	13	OFF	446.3	5	5	8	ON
	N369.2	665.9	5	6	13	OFF	446.4	5	5	8	ON
	N369.3	661.1	5	6	12	ON	441.4	5	5	7	OFF
	N369.3	660.8	5	6	12	ON	441.3	5	5	7	OFF
	N369.5	665.9	5	6	13	OFF	446.4	5	5	8	ON
	N369.6	665.9	5	6	13	OFF	446.3	5	5	8	ON
	369.7	661.2	5	6	12	ON	441.1	5	5	7	OFF
	369.8	666.2	5	6	13	OFF	446.2	5	5	8	ON
	369.9	661.2	5	6	12	OFF	441.2	5	5	7	OFF
	369,10	666.4	5	6	13	OFF	446.2	5	5	8	ON
	369.11	666.2	5	6	13	OFF	446.1	5	5	8	ON
	369.12	671.3	5	6	14	OFF	446.1	5	5	8	ON
	369.14	666.2	5	6	13	OFF	446.1	5	5	8	ON
	369.15	661.1	5	6	12	ON	440.9	5	5	7	OFF
	369.16	666.5	5	6	13	OFF	446.5	5	5	8	ON
	369.17	681.9	5	6	16	OFF	446.6	5	5	8	ON
	369.18	661.6	5	6	12	ON	441.5	5	5	7	OFF
	369.19	661.5	5	6	12	ON	441.6	5	5	7	OFF
	369,20	661.4	5	6	12	ON	441.5	5	5	7	OFF
V88/4th	N449.1	681.2	5	6	16	OFF	440.4	5	5	7	OFF
	449.2	676.4	5	6	15	ON	440.4	5	5	7	OFF
	449.3	676.6	5	6	15	ON	440.5	5	5	7	OFF
	449.4	676.4	5	6	15	ON	440.5	5	5	7	OFF
	449.5	671.3	5	6	14	OFF	440.7	5	5	7	OFF
	449.6	676.5	5	6	15	ON	440.6	5	5	7	OFF

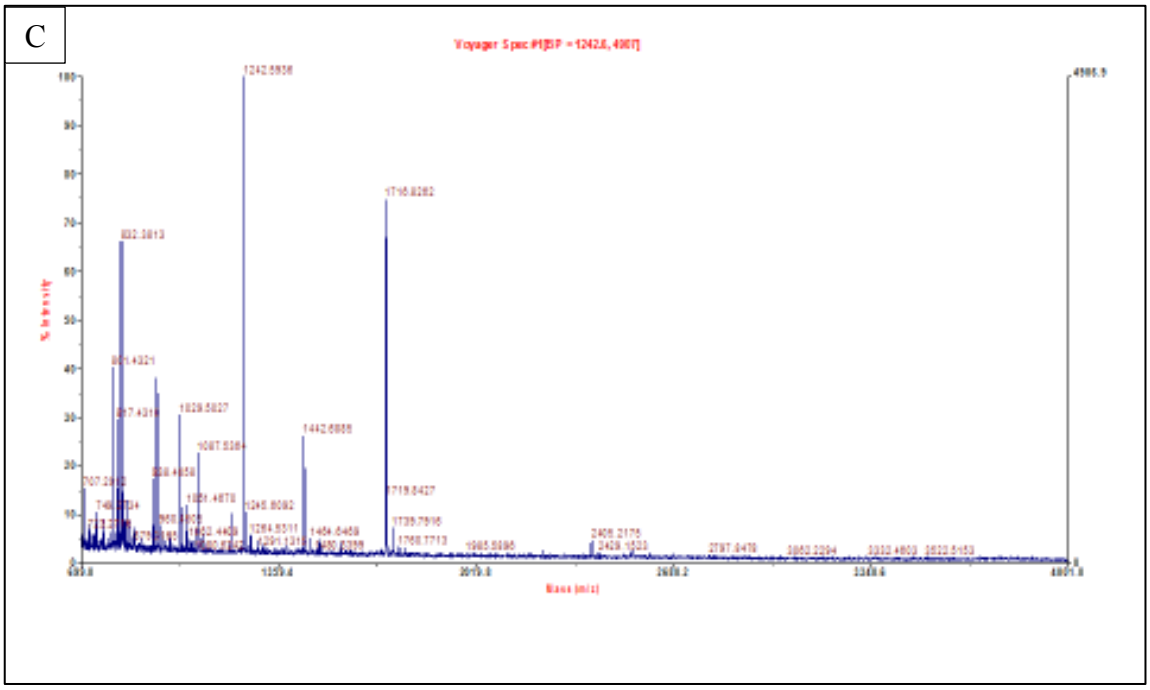
V43/2nd	241.1	651.3	5	6	10	OFF	438.9	5	5	8	ON
	241.2	651.3	5	6	10	OFF	438.9	5	5	8	ON
V43/3rd	349.1	646.1	5	6	9	ON	443.9	5	5	9	OFF
	349.2	646.1	5	6	9	ON	444.0	5	5	9	OFF
	349.3	646.4	5	6	9	ON	444.0	5	5	9	OFF
	349.4	646.4	5	6	9	ON	444.1	5	5	9	OFF
	349.5	646.3	5	6	9	ON	444.0	5	5	9	OFF
	349.6	646.3	5	6	9	ON	444.0	5	5	9	OFF

A



B





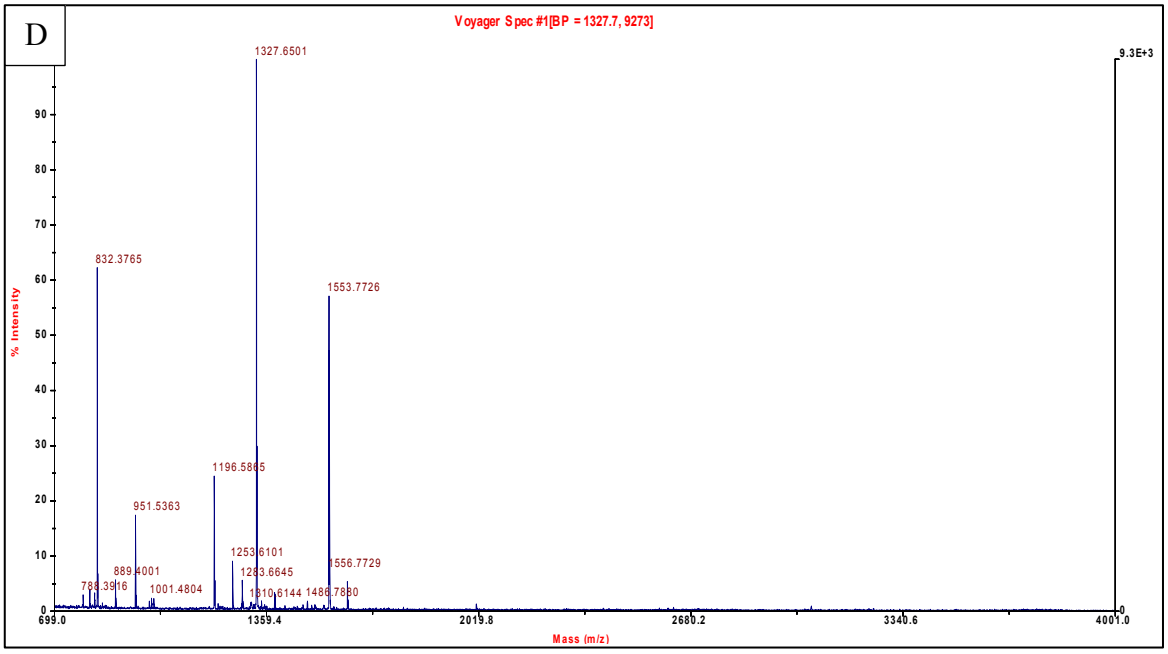


Figure 8-1 : Mass spectrometry of the purified recombinant Opa protein samples. Opa proteins were purified using nickle affinity chronmatography column and samples from each variant were run on 12% SDS- PAGE, after gel staining target bands were cut out , digested with trypsin and analysed for mass spectra (by PNACLE staff). Figure A: mass spectra of protein samples shown along with OpaB protein band in SDS-PAGE (band number 2 on SDS-PAGE in Figure 6-3), figure B: spectra of OpaA, figure C: spectra of OpaJ-N222, and figure D: spectra of OpaJ-N459.

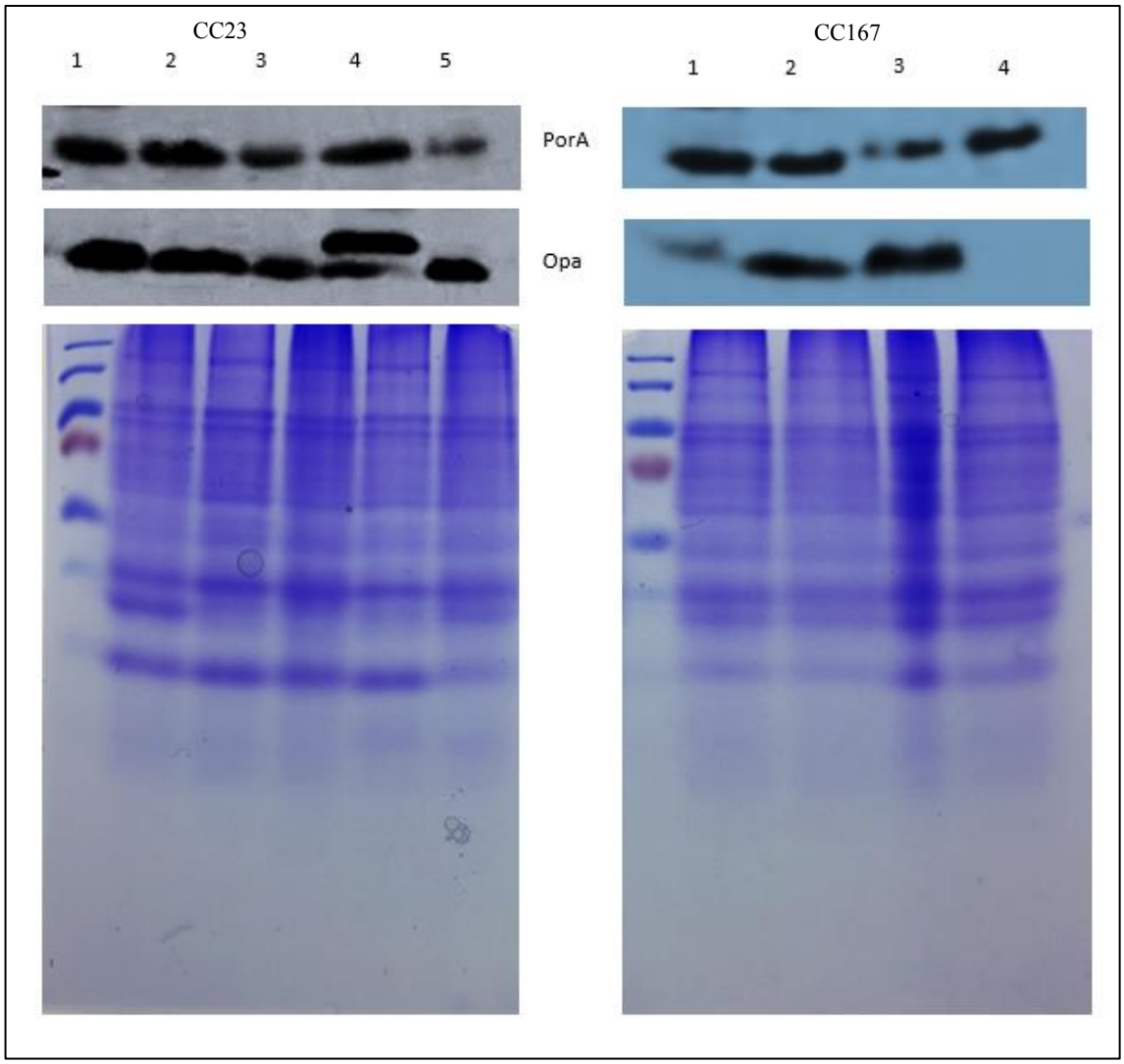


Figure 8-2: Western blot analysis of the expression of Opa proteins in different carriage isolates of CC23 and CC167. whole cell lysates of samples from different isolates expressing different Opa variants with variable repeat tract length loaded on 12% SDS-PAGE, separated and probed with anti-Opa mouse polyclonal antibodies and anti-PorA monoclonal antibodies (variant P1.2) as Loading control. Samples were loaded as following: cc23 (1-5): 309.1, 222.1, 459.6 370.4 and 370.5; while cc167 samples (1-4): N332.2, N348.1, 117.1 and N284.1.

Table 8-2: the predicted expression state of Opa variants in samples of CC23 analysed by western blott according to the repeat tract length as well as the data from western blotting. The number of the not phase variables poly C and poly A tracts preceeding the pentameric repeats region of each opa gene in combinations with the number of CTCTT tracts determines whether the gene is in frame or not**.** Note that the data of the CC167 samples are the same as presented in table 4-3 in chpter 4.

Meningococcal samples (CC23	*opa* gene Repeat tract number			Predicted expression state (ON/OFF)	Expression state confirmed by western blot
	No of C tracts (fixed)	No of A tracts (fixed)	No of CTCTT Tracts (variable)		
opaA	5	6	18	ON	ON
			19	OFF	OFF
			20	OFF	OFF
			21	ON	ON
opaB	5	6	5	OFF	OFF
			8	OFF	OFF
			9	ON	ON
opaD	5	6	8	OFF	OFF
opaJ	5	6	7	OFF	OFF
			8	OFF	OFF
			12	ON	ON
OpaJ (n459.6)	3 (+T SNP)	6	8	ON	ON

```
opaA,                    GCAGCGCAGGCGGCAAGTGAAGACGGCAGCCGCAGCCCGTATTATGTGCAGGCGGATTTA
16990|N222.1|363598|43   GCAGCGCAGGCGGCAAGTGAAGACGGCAGCCGCAGCCCGTATTATGTGCAGGCGGATTTA
                         ************************************************************

opaA,                    GCCTATGCCGCCGAACGCATTACCCACAATTATCCGGAACCAACCGGTGCAGACAAAGAC
16990|N222.1|363598|43   GCCTATGCCGCCGAACGCATTACCCACAATTATCCGGAACCAACCGGTGCAGACAAAGAC
                         ************************************************************

opaA,                    AAAATAAGCACAGTAAGCGATTATTTCAGAAACATCCGTGCGCATTCCATCCACCCTCGG
16990|N222.1|363598|43   AAAATAAGCACAGTAAGCGATTATTTCAGAAACATCCGTGCGCATTCCATCCACCCTCGG
                         ************************************************************

opaA,                    GTGTCGGTCGGCTACGACTTCGGCGACTGGAGAATAGCGGCAGATTATGCCAGTTACAGA
16990|N222.1|363598|43   GTGTCGGTCGGCTACGACTTCGGCGACTGGAGAATAGCGGCAGATTATGCCAGTTACAGA
                         ************************************************************

opaA,                    AAATGGAAAGAAAGTAATTCTTCTACTAATGCAGAAAATAGAGATAATGCAAAAAACTAC
16990|N222.1|363598|43   AAATGGAAAGAAAGTAATTCTTCTACTAATGCAGAAAATAGAGATAATGCAAAAAACTAC
                         ************************************************************

opaA,                    GTAAAGATTGAAACAAAACATCAAGGAAACGGCAGCTTCCACGCCGCTTCTTCTCTCGGC
16990|N222.1|363598|43   GTAAAGATTGAAACAAAACATCAAGGAAACGGCAGCTTCCACGCCGCTTCTTCTCTCGGC
                         ************************************************************

opaA,                    TTATCCGCCATTTACGATTTCAAACTCAACGATAAATTCAAACCCTATATCGGCGCGCGC
16990|N222.1|363598|43   TTATCCGCCATTTACGATTTCAAACTCAACGATAAATTCAAACCCTATATCGGCGCGCGC
                         ************************************************************

opaA,                    GTCGCCTACGGACACGTTAAACATCAGGTTCATTCAGTGGAAACAAAAACCACGACTGTT
16990|N222.1|363598|43   GTCGCCTACGGACACGTTAAACATCAGGTTCATTCAGTGGAAACAAAAACCACGACTGTT
                         ************************************************************

opaA,                    ACCTCTAAACCGACGGCAACCTCTCCACAGGGAGGCCCTATTATACAAACTGATCCCAGC
16990|N222.1|363598|43   ACCTCTAAACCGACGGCAACCTCTCCACAGGGAGGCCCTATTATACAAACTGATCCCAGC
                         ************************************************************

opaA,                    AAACCTCCCTATCACGAAAGCCACAGCATCAGCAGCTTGGGTCTTGGTGTCATCGCCGGT
16990|N222.1|363598|43   AAACCTCCCTATCACGAAAGCCACAGCATCAGCAGCTTGGGTCTTGGTGTCATCGCCGGT
                         ************************************************************

opaA,                    GTCGGTTTCGACATCACGCCCAAGCTGACCTTGGACACCGGATACCGCTACCACAACTGG
16990|N222.1|363598|43   GTCGGTTTCGACATCACGCCCAAGCTGACCTTGGACACCGGATACCGCTACCACAACTGG
                         ************************************************************

opaA,                    GGACGCTTGGAAAACACCCGCTTCA
16990|N222.1|363598|43   GGACGCTTGGAAAACACCCGCTTCA
                         *************************
```

Figure 8-3 : Sequence alignment of the sequenced opaA gene of CC23, N222.1 that was cloned to the Pleics-1 plasmid vector with the sequence of this gene available in whole genome sequence of this isolate available in bigsdb database.

```
opaJ,           GCAGCGCAGGCGGCAAGTGAAGACGGCAGCCGTAGCCCGTATTATGTGCAGGCGGATTTA
opaj,n22,       GCAGCGCAGGCGGCAAGTGAAGACGGCAGCCGTAGCCCGTATTATGTGCAGGCGGATTTA
                ************************************************************

opaJ,           GCCTACGCCGCCGAACGTATTACCCACGATTATCCGAAACCAACCGGTACAGACAAAATA
opaj,n22,       GCCTACGCCGCCGAACGTATTACCCACGATTATCCGAAACCAACCGGTACAGACAAAATA
                ************************************************************

opaJ,           AGCACGGTAAGCGATTATTTCAGAAACATCCGTGCGCATTCCATCCACCCCCGGGTGTCG
opaj,n22,       AGCACGGTAAGCGATTATTTCAGAAACATCCGTGCGCATTCCATCCACCCCCGGGTGTCG
                ************************************************************

opaJ,           GTCGGCTACGATTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCAGTTATAGAAAATGG
opaj,n22,       GTCGGCTACGATTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCAGTTATAGAAAATGG
                ************************************************************

opaJ,           AAAGAAAGTAATTATTCTAAAAAAGTTACTGAATTTAAACACCAAAACGGCACCAAAAAA
opaj,n22,       AAAGAAAGTAATTATTCTAAAAAAGTTACTGAATTTAAACACCAAAACGGCACCAAAAAA
                ************************************************************

opaJ,           GAAGACAAAACAGAACATCAAGGAAACGGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTA
opaj,n22,       GAAGACAAAACAGAACATCAAGGAAACGGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTA
                ************************************************************

opaJ,           TCCGCCATTTACGATTTCAAACTCAACGATAAATTCAAACCCTATATCGGTGCGCGCGTC
opaj,n22,       TCCGCCATTTACGATTTCAAACTCAACGATAAATTCAAACCCTATATCGGTGCGCGCGTC
                ************************************************************

opaJ,           GCCTACGGACACGTCAGATACAGCATCGATTCGACCAAAAAAACAACAGATGTTGTTACC
opaj,n22,       GCCTACGGACACGTCAGATACAGCATCGATTCGACCAAAAAAACAACAGATGTTGTTACC
                ************************************************************

opaJ,           GTCGCCACCAATACTCCTGACGGAGCACCTACGATTTATAATGAGCCAAAGACGCAAAAC
opaj,n22,       GTCGCCACCAATACTCCTGACGGAGCACCTACGATTTATAATGAGCCAAAGACGCAAAAC
                ************************************************************

opaJ,           GCCTATCACGAAAGCCACAGCATCCGCCGCTTGGGTCTTGGTGTCGTCGCCGGTGTCGGT
opaj,n22,       GCCTATCACGAAAGCCACAGCATCCGCCGCTTGGGTCTTGGTGTCGTCGCCGGTGTCGGT
                ************************************************************

opaJ,           TTCGACATCACGCCCAAGCTGACTTTAGACACCGGATACCGTTACCACAACTGGGGACGC
opaj,n22,       TTCGACATCACGCCCAAGCTGACTTTAGACACCGGATACCGTTACCACAACTGGGGACGC
                ************************************************************

opaJ,           TTGGAAAACACCCGCTTCAAAACCCACGAAGTCTCATTGGGCATGCGCTACCGCTTCTGA
opaj,n22,       TTGGAAAACACCCGCTTCAAAACCCACGAAGTCTCATTGGGCATGCGCTACCGCTTCTGA
                ************************************************************
```

Figure 8-4 Sequence alignment of the sequenced opaJ gene of CC23, N222.1 that was cloned to the Pleics-1 plasmid vector with the sequence of this gene available in whole genome sequence of this isolate available in bigsdb database.

```
n459-opaJ-constract     GCAGCGCAGGCGGCAAGTGAAGACAATGGCCGCGGCCCGTATGTGCAGGCAGATTTAACC
n459-opaJ-Bigsdb        GCAGCGCAGGCGGCAAGTGAAGACAATGGCCGCGGCCCGTATGTGCAGGCAGATTTAACC
                        ************************************************************

n459-opaJ-constract     TACGCCGCCGAACGCATTACCCACAATTATCCGGAAGCAACCGGTGCAGACAAAGGCAAA
n459-opaJ-Bigsdb        TACGCCGCCGAACGCATTACCCACAATTATCCGGAAGCAACCGGTGCAGACAAAGGCAAA
                        ************************************************************

n459-opaJ-constract     ATAAGCACAGTAAGCGATTATTTCAAAAACATCCGTACCCACTCTGTTCAACCTCGACTT
n459-opaJ-Bigsdb        ATAAGCACAGTAAGCGATTATTTCAAAAACATCCGTACCCACTCTGTTCAACCTCGACTT
                        ************************************************************

n459-opaJ-constract     TCTCTTGGCTACGATTTCGGCAACTGGCGCATCGCCGCAGATTACACGCGCTACAGAAAG
n459-opaJ-Bigsdb        TCTCTTGGCTACGATTTCGGCAACTGGCGCATCGCCGCAGATTACACGCGCTACAGAAAG
                        ************************************************************

n459-opaJ-constract     TGGAATGAAAGCAATTCTTCTACTAAAAAAGTTACTGAACGTATAAACGACAACAACAAA
n459-opaJ-Bigsdb        TGGAATGAAAGCAATTCTTCTACTAAAAAAGTTACTGAACGTATAAACGACAACAACAAA
                        ************************************************************

n459-opaJ-constract     GAAACCAAAACAGAACATCAGGAAAACGGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTA
n459-opaJ-Bigsdb        GAAACCAAAACAGAACATCAGGAAAACGGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTA
                        ************************************************************

n459-opaJ-constract     TCAGCCATTTACGATTTCCAAATAAGCGATAAATTCAAACCCTATATCGGCGCGCGCGTC
n459-opaJ-Bigsdb        TCAGCCATTTACGATTTCCAAATAAGCGATAAATTCAAACCCTATATCGGCGCGCGCGTC
                        ************************************************************

n459-opaJ-constract     GCCTACGGACACGTTAAGCATCAAGTTAATTCGGTCAACAAAGAAACTATTACTGTTACT
n459-opaJ-Bigsdb        GCCTACGGACACGTTAAGCATCAAGTTAATTCGGTCAACAAAGAAACTATTACTGTTACT
                        ************************************************************

n459-opaJ-constract     CCTGTGGCGAGTGGCGGCCCTGTTATAACAGGACCGACCAGCAAACCTGCCTATCACGAA
n459-opaJ-Bigsdb        CCTGTGGCGAGTGGCGGCCCTGTTATAACAGGACCGACCAGCAAACCTGCCTATCACGAA
                        ************************************************************

n459-opaJ-constract     AGCCACAGCATCCGCCGCTTGGGTCTTGGTGTCATCGCCGGTGTCGGTTTCGACATCACG
n459-opaJ-Bigsdb        AGCCACAGCATCCGCCGCTTGGGTCTTGGTGTCATCGCCGGTGTCGGTTTCGACATCACG
                        ************************************************************

n459-opaJ-constract     TCCAATCTGACTTTAGATGCTGGCTACCGTTACCACAACTGGGGACGCTTGGAAAACACT
n459-opaJ-Bigsdb        TCCAATCTGACTTTAGATGCTGGCTACCGTTACCACAACTGGGGACGCTTGGAAAACACT
                        ************************************************************

n459-opaJ-constract     CGCTTCAAAACCCACGAAGCCTCATTGGGCATGCGCTACCGCTTCTGA
n459-opaJ-Bigsdb        CGCTTCAAAACCCACGAAGCCTCATTGGGCGTGCGCTACCGCTTCTGA
                        ****************************** ****************
```

Figure 8-5 : Sequence alignment of the sequenced opaJ gene of CC23, N459.1 that was cloned to the Pleics-1 plasmid vector with the sequence of this gene available in whole genome sequence of this isolate available in bigsdb database.

```
opaB,           GCAGCGCAGGCGGCAAGTGAAGACAGCAGCCGCAGCCCGTATTATGTGCAGGCGGATTTA
opaB,           GCAGCGCAGGCGGCAAGTGAAGACAGCAGCCGCAGCCCGTATTATGTGCAGGCGGATTTA
                ************************************************************

opaB,           GCTTATGCCGCCGAACGCATTACCCACGATTATCCGCAAGCAACCGGTGCAAACAACACA
opaB,           GCTTATGCCGCCGAACGCATTACCCACGATTATCCGCAAGCAACCGGTGCAAACAACACA
                ************************************************************

opaB,           AGCACAGTAAGCGATTATTTCAGAAACATCCGTGCGCATTCCATCCACCCCCGGGTGTCG
opaB,           AGCACAGTAAGCGATTATTTCAGAAACATCCGTGCGCATTCCATCCACCCCCGGGTGTCG
                ************************************************************

opaB,           GTCGGCTACGATTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCAGTTACAGAAAATGG
opaB,           GTCGGCTACGATTTCGGCGGCTGGAGGATAGCGGCAGATTATGCCAGTTACAGAAAATGG
                ************************************************************

opaB,           AACAACAATAAATATTCCGTCAACACAAAAGAGGTGCTAAGAAACAATAATGGCAACAGG
opaB,           AACAACAATAAATATTCCGTCAACACAAAAGAGGTGCTAAGAAACAATAATGGCAACAGG
                ************************************************************

opaB,           AAAGAACTGAAGACGGAAAATCAGGAAAACGGTACATTCCACGCAGTCTCATCGCTCGGC
opaB,           AAAGAACTGAAGACGGAAAATCAGGAAAACGGTACATTCCACGCAGTCTCATCGCTCGGC
                ************************************************************

opaB,           TTATCCGCCATTTACGATTTCAAACTCAACGATAAATTCGATAAATTCAAACCCTATATC
opaB,           TTATCCGCCATTTACGATTTCAAACTCAACGATAAATTCGATAAATTCAAACCCTATATC
                ************************************************************

opaB,           GGTGCGCGCGTCGCCTACGGACACGTTAAACATCAGGTTCATTCAGTGAAAAAAGAAACC
opaB,           GGTGCGCGCGTCGCCTACGGACACGTTAAACATCAGGTTCATTCAGTGAAAAAAGAAACC
                ************************************************************

opaB,           ACGACTACTTTCCTTGCACCAACGCAAGGCGCTACAGTGCCAGGCAAGATCGTAGAAGGT
opaB,           ACGACTACTTTCCTTGCACCAACGCAAGGCGCTACAGTGCCAGGCAAGATCGTAGAAGGT
                ************************************************************

opaB,           CCGTTCAGCAAACCTGCCTATCACGAAAGCCACAGCATCAGCAGCTTGGGTCTTGGTGTC
opaB,           CCGTTCAGCAAACCTGCCTATCACGAAAGCCACAGCATCAGCAGCTTGGGTCTTGGTGTC
                ************************************************************

opaB,           ATCGCCGGTGTCGGTTTCGACATCACGCCCAAGCTGACTTTAGACACCGGATACCGCTAC
opaB,           ATCGCCGGTGTCGGTTTCGACATCACGCCCAAGCTGACTTTAGACACCGGATACCGCTAC
                ************************************************************

opaB,           CACAACTGGGGACGCTTGGAAAACACCCGCTTCAAAACCCACGAAGTCTCATTGGGCATG
opaB,           CACAACTGGGGACGCTTGGAAAACACCCGCTTCAAAACCCACGAAGTCTCATTGGGCATG
                ************************************************************

opaB,           CGCTACCACTTCTGA
opaB,           CGCTACCACTTCTGA
                ***************
```

Figure 8-6 Sequence alignment of the sequenced opaB gene of CC23, N222.1 that was cloned to the Pleics-1 plasmid vector with the sequence of this gene available in whole genome sequence of this isolate available in bigsdb database.

```
N113.1-opaJ      ATGAATCCAGCCCCAAAAAAC----------------------------------------
N113.1-opaD      ATGAATCCAGCCCCAAAAAAACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTC
                 ********************

N113.1-opaJ      -----------CTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGAGGGCAAGTGAAGACGGC
N113.1-opaD      TCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGAGGGCAAGTGAAGACGGC
                           **********************************************

N113.1-opaJ      AGCCGCAGCCCGTATTATGTGCAGGCGGATTTAGCCTACGCCGCCGAACGCATTACCCAC
N113.1-opaD      AGCCGNAGCCCGTATTATGTGCAGGCGGATTTAGCCTACGCCGCCGAACGCATTACCCAC
                 ***** **************************************************

N113.1-opaJ      GATTATCCGGAAGCAACCGGTGCAAACAACACAAGCACAGTAAGCGATTATTTCAGAAAC
N113.1-opaD      GATTATCCGGAAGCAACCGGTGCAAACAACACAAGCACAGTAAGCGATTATTTCAGAAAC
                 ************************************************************

N113.1-opaJ      ATCCGTACGCATTCCATCCACCCCCGGGTGTCGGTCGGCTACGACTTCGGCGGCTGGAGG
N113.1-opaD      ATCCGTACGCATTCCATCCACCCCCGGGTGTCGGTCGGCTACGACTTCGGCGGCTGGAGG
                 ************************************************************

N113.1-opaJ      ATAGCGGCAGATTATGCCCGTTACAGAAAATGGAACAACAATAAATATTCCGTAAACACA
N113.1-opaD      ATAGCGGCAGATTATGCCCGTTACAGAAAATGGAACAACAATAAATATTCCGTAAACACA
                 ************************************************************

N113.1-opaJ      AAAAATGTGCAGGTGCATAAA
N113.1-opaD      AAAAATGTGCAGGTGCA----
                 *****************
```

Figure 8-7 Sequence alignment of *opaJ* and *opaD* of cc60 , N113.1 .

```
117.2-opaA      CTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACAGCGGGCGCAGCCCGT
N124-opaB       CTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACAGCGGGCGCAGCCCGT
117.2-opaJ      CTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACAGCGGGCGCAGCCCGT
                ************************************************************

117.2-opaA      ATTATGTGCAGGCGGATTTAGCCTATGCCGCCGAACGCATTACCCACGATTATCCGCAAG
N124-opaB       ATTATGTGCAGGCGGATTTAGCCTATGCCGCCGAACGCATTACCCACGATTATCCGCAAG
117.2-opaJ      ATTATGTGCAGGCGGATTTAGCCTATGCCGCCGAACGCATTACCCACGATTATCCGCAAG
                ************************************************************

117.2-opaA      CAACCGGTGCAAACAACACAAGCACAGTAAGCGATTATTTCAGAAACATCCGTGCGCATT
N124-opaB       CAACCGGTGCAAACAACACAAGCACAGTAAGCGATTATTTCAGAAACATCCGTGCGCATT
117.2-opaJ      CAACCGGTGCAAACAACACAAGCACAGTAAGCGATTATTTCAGAAACATCCGTGCGCATT
                ************************************************************

117.2-opaA      CCATCCACCCCCGGGTGTCGGTCGGCTATGATTTCGGCGGCTGGAGGATAGCGGCAGATT
N124-opaB       CCATCCACCCCCGGGTGTCGGTCGGCTATGATTTCGGCGGCTGGAGGATAGCGGCAGATT
117.2-opaJ      CCATCCACCCCCGGGTGTCGGTCGGCTATGATTTCGGCGGCTGGAGGATAGCGGCAGATT
                ************************************************************

117.2-opaA      ATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCAACACAAAAGAGGTGGAAA
N124-opaB       ATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCAACACAAAAGAGGTGGAAA
117.2-opaJ      ATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCAACACAAAAGAGGTGGAAA
                ************************************************************

117.2-opaA      GAAACAATATCAGTGGCAACTGGAAAGAACTGAAGACGGAAAATCAGGAAAACGGCAGCT
N124-opaB       GAAACAATATCAGTGGCAACTGGAAAGAACTGAAGACGGAAAATCAGGAAAACGGCAGCT
117.2-opaJ      GAAACAATATCAGTGGCAACTGGAAAGAACTGAAGACGGAAAATCAGGAAAACGGCAGCT
                ************************************************************

117.2-opaA      TCCACGCCGCTTCTTCTCTCGGCTTATCCGCCATTTACGATTTCAAACTCAACGATAAAT
N124-opaB       TCCACGCCGCTTCTTCTCTCGGCTTATCCGCCATTTACGATTTCAAACTCAACGATAAAT
117.2-opaJ      TCCACGCCGCTTCTTCTCTCGGCTTATCCGCCATTTACGATTTCAAACTCAACGATAAAT
                ************************************************************

117.2-opaA      TCGATAAATTCAAACCCTATATCGGTGTGCGCGTCGCCTACGGACACGTTAAACATCAGG
N124-opaB       TCGATAAATTCAAACCCTATATCGGTGTGCGCGTCGCCTACGGACACGTTAAACATCAGG
117.2-opaJ      TCGATAAATTCAAACCCTATATCGGTGTGCGCGTCGCCTACGGACACGTTAAACATCAGG
                ************************************************************

117.2-opaA      TTCATTCAGTGAAAAAAGAAACCACGACTA------------------------------
N124-opaB       TTCATTCAGTGAAAAAAGAAACCACGACTACTTTCCTTGCACCAACGGGAGACGCTAAAG
117.2-opaJ      TTCATTCAGTGAAAAAAGAAACCACGACTACTTTCCTTGCACCAAC--------------
                ****************************

117.2-opaA      ------------------------------------------------------------
N124-opaB       TGCCAGGCAAGATCGTAGAAGGTCCGTTCAGCAAACCTGCCTATCACGAAAGCCACAGCA
117.2-opaJ      ------------------------------------------------------------


117.2-opaA      ------------------------------------------------------
N124-opaB       TCCGCCGCTTGGGTCTTGGTGTCGTCGCCGGTGTCGGTTTCGACATCACGCCCAAG
117.2-opaJ      ------------------------------------------------------
```

Figure 8-8 : Sequence alignment of *opaA*, *opaJ* of N117.1 (CC167) and *opaB* of N124.1 (CC167).

```
opaA/J, CC167    --CTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACAGCGGGCGC
opaB,N124        CTCTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACAGCGGGCGC
                 ************************************************************

opaA/J, CC167    AGCCCGTATTATGTGCAGGCGGATTTAGCCTATGCCGCCGAACGCATTACCCACGATTAT
opaB,N124        AGCCCGTATTATGTGCAGGCGGATTTAGCCTATGCCGCCGAACGCATTACCCACGATTAT
                 ************************************************************

opaA/J, CC167    CCGCAAGCAACCGGTGCAAACAACACAAGCACAGTAAGCGATTATTTCAGAAACATCCGT
opaB,N124        CCGCAAGCAACCGGTGCAAACAACACAAGCACAGTAAGCGATTATTTCAGAAACATCCGT
                 ************************************************************

opaA/J, CC167    GCGCATTCCATCCACCCCCGGGTGTCGGTCGGCTATGATTTCGGCGGCTGGAGGATAGCG
opaB,N124        GCGCATTCCATCCACCCCCGGGTGTCGGTCGGCTATGATTTCGGCGGCTGGAGGATAGCG
                 ************************************************************

opaA/J, CC167    GCAGATTATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCAACACAAAAGAG
opaB,N124        GCAGATTATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCAACACAAAAGAG
                 ************************************************************

opaA/J, CC167    GTGGAAAGAAACAATATCAGTGGCAACTGGAAAGAACTGAAGACGGAAAATCAGGAAAAC
opaB,N124        GTGGAAAGAAACAATATCAGTGGCAACTGGAAAGAACTGAAGACGGAAAATCAGGAAAAC
                 ************************************************************

opaA/J, CC167    GGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTATCCGCCATTTACGATTTCAAACTCAAC
opaB,N124        GGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTATCCGCCATTTACGATTTCAAACTCAAC
                 ************************************************************

opaA/J, CC167    GATAAATTCGATAAATTCAAACCCTATATCGGTGTGCGCGTCGCCTACGGACACGTTAAA
opaB,N124        GATAAATTCGATAAATTCAAACCCTATATCGGTGTGCGCGTCGCCTACGGACACGTTAAA
                 ************************************************************

opaA/J, CC167    CATCAGGTTCATTCAGTGAAAAAAGAAACCACGACTACTTTCCTTGCACCAACGGGAGAC
opaB,N124        CATCAGGTTCATTCAGTGAAAAAAGAAACCACGACTACTTTCCTTGCACCAACGGGAGAC
                 ************************************************************

opaA/J, CC167    GCTAAAGTGCCAGGCAAGATCGTAGAAGGTCCGTTCAGCAAACCTGCCTATCACGAAAGC
opaB,N124        GCTAAAGTGCCAGGCAAGATCGTAGAAGGTCCGTTCAGCAAACCTGCCTATCACGAAAGC
                 ************************************************************

opaA/J, CC167    CACAGCATCCGCCGCTTGGGTCTTGGTGTCGTCGCCGGTGTCGGTTTCGACATCACGCCC
opaB,N124        CACAGCATCCGCCGCTTGGGTCTNGGTGTCGTCGCCGGTGTCG-----------------
                 **********************.******************

opaA/J, CC167    AAGCTGACTTT
opaB,N124        -----------
```

Figure 8-9 sequence alignment opaA/J of cc167 and opaB of N124 (cc167).

```
opaB            ATGAATCCAGCCCCCAAAAAACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTT 60
opaA            ATGAATCCAGCCCCCAAAAAACCTTCTCT-----TCTCTTCTCTTCTCTTCTCTTCTCTT 55
                ****************************     ************************

opaB            CTCTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACAGCAGCCGC 120
opaA            CTCTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACAGCAGCCGC 115
                ************************************************************

opaB            AGCCCGTATTATGTGCAGGCGGATTTAGCCTATGCCGCCGAACGCATTACCCACGATTAT 180
opaA            AGCCCGTATTATGTGCAGGCGGATTTAGCCTATGCCGCCGAACGCATTACCCACGATTAT 175
                ************************************************************

opaB            CCGAAACCAACCGGTACAGACAAAGACAAAATAAGCACAGTAAGCGATTATTTCAGAAAC 240
opaA            CCGAAACCAACCGGTACAGACAAAGACAAAATAAGCACAGTAAGCGATTATTTCAGAAAC 235
                ************************************************************

opaB            ATCCGTGCGCATTCCATCCACCCTCGGGTGTCGGTCGGCTACGATTTCGGCGGCTGGAGG 300
opaA            ATCCGTGCGCATTCCATCCACCCTCGGGTGTCGGTCGGCTACGATTTCGGCGGCTGGAGG 295
                ************************************************************

opaB            ATAGCGGCAGATTATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCAACACA 360
opaA            ATAGCGGCAGATTATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCAACACA 355
                ************************************************************

opaB            AAAGTGTTGAAAGAAACCCAGGACAACTGGCAAGAACTGAAGACGGAAAATCAGGAAAAC 420
opaA            AAAGTGTTGAAAGAAACCCAGGACAACTGGCAAGAACTGAAGACGGAAAATCAGGAAAAC 415
                ************************************************************

opaB            GGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTATCCGCCATTTACGATTTCAAACTCAAC 480
opaA            GGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTATCCGCCATTTACGATTTCAAACTCAAC 475
                ************************************************************

opaB            GATAAATTCAAACCCTATATCGGTGCGCGCGTCGCCTACGGACACGTTAAACATCAGGTT 540
opaA            GATAAATTCAAACCCTATATCGGTGCGCGCGTCGCCTACGGACACGTTAAACATCAGGTT 535
                ************************************************************

opaB            CATTCGGTGGAAACAAAAACCACGACTGTTACCTCTAAACCAAACGGAAAAAATCTAAAG 600
opaA            CATTCGGTGGAAACAAAAACCACGACTGTTACCTCTAAACCAAACGGAAAAAATCTAAAG 595
                ************************************************************

opaB            GGAGG 605
opaA            GGAGG 600
                *****
```

Figure 8-10 sequence alignment of opaA and opaB of cc174 N88.1.

```
opaA-N185       ATGAATCCAGCCCCCAAAAAACCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTT
N185.3-opaB     ATGAATCCAGCCCCCAAAAAAC-----------------------CTTCTCTTCTCTT
                **********************                       *************

opaA-N185       CTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACGGCA
N185.3-opaB     CTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCCGCAGCGCAGGCGGCAAGTGAAGACGGCA
                ************************************************************

opaA-N185       GCCGCAGCCCGTATTATGTGCAGGCGGATTTAGCCTACGCCGCCGAACGTATTACCCACG
N185.3-opaB     GCCGCAGCCCGTATTATGTGCAGGCGGATTTAGCCTACGCCGCCGAACGTATTACCCACG
                ************************************************************

opaA-N185       ATTATCCGAAACCAACCGGTACAGACAAAGACAAAATAAGCACAGTAAGCGATTATTTCA
N185.3-opaB     ATTATCCGAAACCAACCGGTACAGACAAAGACAAAATAAGCACAGTAAGCGATTATTTCA
                ************************************************************

opaA-N185       GAAACATCCGTGCGCATTCCATCCACCCTCGGGTGTCGGTCGGCTACGATTTCGGCGGCT
N185.3-opaB     GAAACATCCGTGCGCATTCCATCCACCCTCGGGTGTCGGTCGGCTACGATTTCGGCGGCT
                ************************************************************

opaA-N185       GGAGGATAGCGGCAGATTATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCA
N185.3-opaB     GGAGGATAGCGGCAGATTATGCCAGTTACAGAAAATGGAACAACAATAAATATTCCGTCA
                ************************************************************

opaA-N185       ACACAAAAGTGTTGAAAGAAACCCAGGACAACTGGCAAGAACTGAAGACGGAAAATCAGG
N185.3-opaB     ACACAAAAGTGTTGAAAGAA----------------------------------------
                ********************

opaA-N185       AAAACGGCAGCTTCCACGCCGCTTCTTCTCTCGGCTTATCCGCCATTTACGATTTCAAAC
N185.3-opaB     ------------------------------------------------------------

opaA-N185       TCAACGATAAATTCAAACCCTATATCGGTGCGCGCGTCGCCTACGGACACGTTAAACATC
N185.3-opaB     ------------------------------------------------------------

opaA-N185       AGGTTCATTCGGTGGAAACAAAAACCACGACTGTTACCTCTAAACCAAACGGAAAAAATC
N185.3-opaB     ------------------------------------------------------------

opaA-N185       TAAAGGGAGGACCTGTCTCATCAAGAGAGCCCAGCAAACCTCCCTATCACGAAAGCCACA
N185.3-opaB     ------------------------------------------------------------

opaA-N185       GCACCAGCAGCTTGGGTCTTGGTGTCATCGCCGGTGTCGGTTTCGACATCACGCCCAAGC
N185.3-opaB     ------------------------------------------------------------
```

Figure **8**-**11**: sequence alignment of opaB and opaA of N185 (CC60

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