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Declaration

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

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I would like to thank my supervisor's Dr. Helen Findlow, Professor Ray Borrow and Dr. May Azzawi for all their help and support throughout the duration of this study. I would also like to thank Dr. Xilian Bai, Dr. Jamie Findlow, all the staff at the Vaccine Evaluation Unit and the Meningococcal Reference Unit for their friendship, support and encouragement throughout the project duration.

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List of abbreviations

A	Absorbance
aP	Acellular pertussis
BB	Bactericidal Buffer
BHI	Brain heart infusion
BSA	Bovine serum albumin
C4BP	C4b binding protein
cap	Capsulation
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CI	Confidence interval
CSF	Cerebrospinal fluid
D	Diptheria
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administtration
FH	Factor H
FH-L1	Factor H-Like protein-1
GAVI	Global Alliance for Vaccines and Immunisation
GMC	Geometric mean concentration
GMT	Geometric mean titre
GSK	GlaxoSmithKline
HBS	Hanks balanced salts

Hi	*Haemophilus influenzae*
Hib	*Haemophilus influenzae* type b
HPA	Health Protection Agency
HRU	Haemophilus Reference Unit
hSBA	SBA assay with human complement
IgA	Immunoglobulin A
IgA1	Immunoglobulin A1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL2	Interleukin 2
IL4	Interleukin 4
IPV	Inactivated polio virus
LOS	Lipooligosaccharide
LPA	Latex particle agglutination
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MBL	Mannose-binding lectin
Men A	Meningococcal serogroup A
Men B	Meningococcal serogroup B
Men C	Meningococcal serogroup C
NAD	Nicotinamide adenine dinucleotide
NIBSC	National Institute of Biological Standards and Controls
NTHi	Non typeable *Haemophilus influenzae*
OD	Optical density

OMP	Outer membrane proteins
PCR	Polymerase chain reaction
PRP	polyribosyl-ribitol-phosphate
RABA	Radioantigen binding assay
RC	Rabbit complement
rSBA	SBA assay with rabbit complement
SBA	Serum bactericidal antibody
T	Tetanus
TI-2	Thymus-independent type 2
TT	Tetanus Toxoid
V factor	Nicotinamide adenine dinucleotide
VEU	Vaccine Evaluation Unit
WHO	World Health Organization
wP	Whole cell pertussis
X factor	Haemin

Abstract

Prior to routine immunisation, *Haemophilus influenzae* serotype b (Hib) was a major cause of serious bacterial infections, particularly in young children. In 1992, immunisation against Hib disease was introduced into the infant vaccination schedule along with a catch-up campaign in those aged 1-4 years which resulted in a rapid and sustained reduction in invasive Hib disease across all age groups. Since 1999, however, the number of reported invasive Hib infections began to rise. In 2006, the routine infant immunisation schedule was changed to include a booster immunisation to Hib in the second year of life. This had an immediate effect, resulting in a rapid and sustained reduction in invasive Hib disease across all age groups.

Evaluation of the immune response to Hib conjugate vaccines includes the measurement of serum antibodies to the Hib capsular polysaccharide (polyribosyl-ribitol-phosphate (PRP)) by ELISA, with accepted short term and long term levels of ≥0.15 μg/mL and ≥1.0 μg/mL, respectively. The relevance for protection in children who have been primed with glycoconjugate vaccines remains unclear, as these levels were derived by passive immunisation, or immunisation with pure polysaccharide. Therefore the aim of this project was to develop and optimise a serum bactericidal antibody (SBA) assay for the evaluation of Hib conjugate vaccines.

In order to assess the functional activity of Hib antibodies, we developed, optimised and evaluated a serum bactericidal antibody (SBA) assay. Validation of the Hib SBA assay was deemed acceptable in all assay parameters tested. In vaccinated adults, a strong correlation (r=0.81) between anti-PRP IgG concentrations and SBA titres were shown. The optimised Hib SBA assay was tested on sera from infants immunised under the current UK 2-3-4-12 month schedule. Good correlations between anti-PRP IgG concentration and SBA

titres were shown. (r=0.635, post primary, r=0.746, post booster). A predictive SBA titre of 8 was calculated using the established correlates of protection.

As Hib strains may contain multiple capsular loci, we also performed the SBA assay with strains expressing 1-5 copies of the *cap b* locus using convalescent sera from children with Hib vaccine failure. Geometric mean SBA titres for 1, 2, 3, 4 and 5 copy strains were 46, 24, 43, 55 and 20, respectively. There was no strong association between the number of *cap* b copies and SBA titres. Although an increased capsule may be an advantage, antibodies to other surface antigens may play a role in clearance.

1.0 Introduction

Haemophilus influenzae (Hi) is a non-spore- forming Gram-negative coccobacillus in the family *pasteurellaceae*. It requires two accessory factors for growth which are present in erythrocytes, specifically X factor (haemin) and V factor (nicotinamide adenine dinucleotide (NAD)). The requirement of both these factors characterise Hi from any other *Haemophilus* species such as *H. parainfluenzae* (Tebbutt, 1983). *Haemophilus influenzae* type b (Hib) is an important pathogen in children worldwide particularly those under the age of five years (Ladhani, 2012). Prior to 1992, when Hib conjugate vaccines were introduced into the UK, Hib was the leading cause of meningitis as well as a common cause of pneumonia, epiglottitis, pericarditis, bone and joint infections, and cellulitis (Todd and Bruhn, 1975). Hi was first identified as a pathogen in 1883 by Robert Koch. During the 1889 influenza pandemic, Richard Pfeiffer isolated Hi from post-mortem cultures (referred to as 'Pfeiffer's bacillus'), and it was mistakenly thought to be the causative organism of influenza (Heath and McVernon, 2002). During the influenza pandemic of 1918, it was discovered that Hi was not the causative organism of influenza and was recognised as a separate entity (Olitsky and Gates, 1921). In 1920, Winslow *et al*. (1920) renamed the organism *Haemophilus influenzae* to emphasise its requirement for blood derived factors (X and V) for growth (*Haemophilus* meaning blood loving) and to acknowledge its historical association with influenza.

1.1 Classification

Hi can be classified into encapsulated (typeable) and non encapsulated (non-typeable) strains (Pittman, 1931). Encapsulated strains produce a polysaccharide capsule which has been classified based on antigenic differences into 6 serotypes (a-f). Encapsulated strains possess the genes for capsule production within their capsulation

(*cap*) locus. The *cap* locus for all Hi serotypes consists of three functionally defined regions: 1, 2, and 3 (Satola *et al*., 2003). Region 2 genes are involved in capsular biosynthesis and are unique to each of the six capsule types (Satola *et al.*, 2007). Regions 1 and 3 are common to all six capsule types and contain genes necessary for the processing and transport of capsular material and surface expression (Satola *et al*., 2007). Non encapsulated strains lack a polysaccharide capsule due to the absence of the genes for capsule production (St. Geme and Falkow, 1991). Among the typeable strains, serotype b (Hib) has been responsible for most Hi meningitis cases (Watt *et al*., 2009). Hib has greater pathogenic potential than any other encapsulated Hi strains due to the capsule consisting of a repeating polymer of ribosyl and ribitol phosphate (polyribosyl-ribitol-phosphate (PRP)), which is known to be especially effective at enabling the organism to evade complement-mediated killing and avoiding splenic clearance (Swift *et al.*, 1991). The region of the Hib chromosome known as the *cap* b locus is involved in PRP capsule expression (Hoiseth *et al.*, 1986). Most Hib isolates contain a partial duplication that results in two complete copies of regions 2 and 3, one complete copy of region 1, and a truncated copy of region 1, with a 1.2- kb deletion within the *bexA* gene and IS*1016*, which is thought to stabilise capsule production (Satola *et al*., 2003). Although, most Hib isolates contain 2 copies for detectable capsular expression (Noel *et al.*, 1996), isolates with 3 or more copies of the *cap* b locus have been obtained from patients with invasive disease (Cerquetti *et al.*, 2005).

Hi can be classified further into eight biotypes (I-VIII) on the basis of indole production, urease activity, and ornithine decarboxylase reactions, to study the pattern of colonization, and to identify the strains of bacterium commonly known to be pathogenic (Munson and Doern, 2007). Biotype I has shown to be predominant amongst typeable strains in particular serotype b (Sharma *et al*., 2002). Biotypes II and III are commonly

associated with non typeable strains and upper respiratory tract infections (Jain *et al*., 2006).

1.2 Virulence factors

1.2.1 Hib Pili

The first step in the pathogenesis of invasive Hi infections is asymptomatic colonisation of the nasopharynx (McCrea *et al.*, 1998). Pathogenic bacteria are specifically adapted to bind to their host. Both encapsulated and non encapsulated strains express adhesive pili that mediate the binding of Hi to human erythrocytes and to specific human epithelial cells (Gilsdorf *et al.*, 1997). This allows colonisation of the human respiratory tract, thus promoting its ability to establish infection (Watson *et al.*, 1994). Pili expression in Hib was first identified in 1982 where a correlation between piliation and increased levels of attachment to human oropharyngeal epithelial cells and agglutination of human erythrocytes was reported (Pichichero *et al.*, 1982). By amino acid sequence analysis of strain Eagan, pili were found to contain 196 amino acids similar to structures of adhesive pili from other organisms including the *Escherichia coli* P pilus, type 1 pilus and F17 pilus subunits (Guerina *et al.*, 1985).

1.2.2 Lipopolysaccharide/Lipooligosaccharide

The lipopolysaccharide (LPS) is an essential characteristic surface component (Schweda *et al.*, 2007) and has been implicated to be a major contributory factor in the organism's ability to cause invasive disease (Humphries and High, 2002). LPS is common to gram negative bacteria and is one of the main constituents of the outer membrane. It has been demonstrated that LPS is required at several stages in the pathogenesis of invasive disease including colonisation of the upper respiratory tract (Hood *et al.*, 1996) and

systemic dissemination (Maskell *et al.*, 1992). LPS can also be referred to as the lipooligosaccharide (LOS) as the bacterium has been found to express short chain LPS, lacking O-specific polysaccharide chains (Humphries *et al*, 2002). LOS is subject to phase variation due to the spontaneous loss and gain of oligosaccharide structures situated in the outer core (Kimura *et al.*, 1986). The phase variable expression of LOS biosynthesis genes enables individual strains of Hib to express a wide variety of different LOS structures allowing the organism to adapt to diverse host environments (Humphries *et al.*, 2002; Van Alphen *et al.*, 1990). Sialylation of the LOS enables Hib to evade the lytic effects of human serum, playing an important role in the survival of this pathogen (Jones *et al*., 2002).

1.2.3 Outer membrane proteins

Bacterial lipoproteins form a group of structurally and functionally diverse proteins. They may be potent immunomodulators for both B cells and T cells (Yang *et al.*, 1997), act as adhesins in colonisation (Hultgren *et al*., 1993) as well as be involved in the cell wall nutrient transport system and sensory signal system (Tam and Saier, 1993). Outer membrane proteins have become of great interest as possible vaccine candidates especially for non typeable Hi (NTHi) as they play a major role in virulence and survival (Van Alphen *et al.*, 1990).

Outer membrane proteins (OMP) P1 and P2, have shown to bind complement component C3 during opsonisation of Hib. Anti-P1 and anti P2 antibodies have shown to be bactericidal, making these proteins promising vaccine candidates for NTHi (Hetherington *et al.*, 1993). Outer membrane protein P5 is a heat modifiable, antigenically variable protein. It has been reported to bind to the sialic acid residues on mucin and has

been implicated in colonisation and pathogenesis (Hill *et al.*, 2001). Outer membrane protein P4 is essential for the utilisation of V factor (NAD) and is therefore essential for growth of Hi, deletion of which, results in growth deficient serotypes (Kemmer *et al.*, 2001). Protein D has been of great interest in vaccine development as it is present in every Hi strain. It is antigenically invariable and has been an attractive candidate for a NTHi vaccine and as for use as a carrier protein in glycoconjugate vaccines (Hetherington *et al.*, 1993). A 10-valent pneumococcal glycoconjugate vaccine with protein D as the carrier protein (Synflorix, GSK) has been shown to be immunogenic and safe. The licensure of Synflorix has established the use of protein D as an alternative carrier protein (Lagos *et al*., 2011).

1.2.4 IgA1 proteases

Bacteria which produce IgA1 protease are able to cleave specific peptide bonds in the human immunoglobulin A1 (IgA1) hinge region (Mistry and Stockley, 2006). Proteolytic enzymes have been found in several species of pathogenic bacteria such as *Streptococcus pneumoniae, Neisseria meningitidis, Neisseria gonorrhoeae* and Hi (Mulks *et al*., 1980). IgA1 proteases are thought to be an important virulence factor as destroying the structure and function of human IgA1 is detrimental to host defence during infection.

1.2.5 Iron and Heme uptake

The requirement for iron is ever present for all living cells. In the human host iron is bound to the iron binding glycoproteins lactoferrin and transferrin. Intracellular, they are secreted as heme containing proteins such as haemoglobin and cytochromes (Lee, 1992). Therefore, free irons are not ready available and bacteria encounter a hostile, iron restricted environment. Hi has evolved to develop high affinity iron acquisition mechanisms which

are capable of sequestering the glycoprotein bound iron. These systems are comprised of low molecular mass iron chelators (siderophores) and their outer membrane receptors (Williams *et al.*, 1990).

1.3 Pathogenesis and disease

Hib succeeds as a commensal of the human respiratory tract, where it can spread from host to host occasionally causing disease in the immunocompromised and the very young. Hib is spread from person to person via respiratory droplets and through direct contact with respiratory secretions (Moxon, 2009). Hib enters the body via the respiratory tract and colonises the pharyngeal mucosa (Ulanova and Tsang, 2009). Colonisation in the nasopharynx can range from weeks to months and historically, before the introduction of glycoconjugate vaccines, persons would encounter and carry Hib in early life (Michaels and Norden, 1977). In the pre Hib vaccine era 3-5% of healthy preschool aged children were asymptomatic carriers of Hib (Michaels *et al*., 1976). Several factors have been associated with increased rates of carriage and disease. These include; age (McVernon *et al*., 2008), season of the year (Howard *et al*., 1988), presence of a respiratory virus infection (Long *et al*., 1983), family size, and overcrowding. (Turk, 1975; Ward *et al*., 1978). Most patients with Hib disease have not been in close contact with a person with invasive Hib disease and thus, asymptomatic carriers are the major source of infection (Ward *et al*., 1978).

Hib has the potential to cause fatal diseases, the most common being meningitis. The events that occur between initial mucosal colonisation and bacteraemia are unclear and remain under investigation. To enter the blood stream the organism must penetrate basement membrane and subepithelial tissue, where it can enter the endothelium or a blood vessel (Barbour, 1996). The organism may also be transported to regional lymph nodes

where it may enter the systemic circulation via the lymphatics (Rubin and Moxon, 1983). Possible mechanisms for entry to the circulation include the ingestion of Hib by polymorphonuclear leukocytes or macrophages with the Hib bacterium surviving internalised (Jacobs *et al*., 1982). Another theory is that an intense inflammatory response may cause disruption to the mucosal barrier allowing entry into the capillaries (Rubin and Moxon, 1983). Once Hib has maintained survival in the blood, it can disseminate to distant sites such as the meninges by penetration of the blood brain barrier and entry into the cerebrospinal fluid (CSF) (Filippidis and Fountas, 2009).

Hib meningitis shares the same symptoms as other kinds of bacterial meningitis. In older children and adults the early stages of disease present with fever, headache, and vomiting similar to that of other mild illnesses. A stiff neck and photophobia, which characterises meningitis, usually happens later with the patient becoming becoming confused or delirious and seizures may occur. In infants symptoms include a refusal to feed, irritability, with high pitched crying, a blotchy pale or bluish rash, and fever. A purpuric rash is not always seen with Hib meningitis that is typical of a meningococcal infection. Following bacterial meningitis patients can develop serious and permanent sequelae such as hearing loss, vision loss, seizures and cognitive delay (Ramakrishnan *et al*., 2009).

1.4 Laboratory Diagnosis and Identification

Any patient presenting with signs and symptoms of meningitis should have a lumbar puncture performed and CSF evaluated for cell count, protein and glucose concentration. Hib can be cultured from CSF, blood or other infected bodily fluids onto chocolate agar with a temperature of 35-37°C and a 3-5% CO_2 environment (Levine *et al*., 2000). To confirm *Haemophilus* species an X and V factor dependency test is performed.

Polymerase chain reaction (PCR) techniques are used to identify Hi species and as confirmatory tests on isolates from clinical specimens. Biotyping is performed using API test kits to establish species identification in the *Haemophilus* genus. From the API strip, Hi can be classified into distinct biotypes (Munson and Doern, 2007). To confirm the serotype i.e. type b, a latex particle agglutination (LPA) test is used. Latex particle agglutination tests use latex particles coated with specific antibody and a visible agglutination reaction appears if a homologous antigen is presented to the bound antibody. This is a reliable, sensitive technique (90-100%), which allows detection even when a patient has negative CSF cultures because of recent antimicrobial therapy (Welch and Hensel, 1982). LPA is extremely rapid, practical, relatively inexpensive (Ajello *et al*., 1987), and can detect 1 ng or less of PRP/mL in clinical specimens (Macone *et al*., 1985). Following laboratory identification, pure cultures are sent on a chocolate agar slope to the Haemophilus Reference Unit (HRU), Health Protection Agency (HPA) to confirm the type. The HRU offers identification, serological typing and capsular genotyping of invasive strains of Hi. The HRU requests submission of all Hi isolated from blood culture or other normally sterile sites as part of the surveillance of invasive disease due to Hi infections and Hib vaccine failures in children (http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HaemophilusInfluenzaeTypeB/InvasiveHaemophilusDiseaseAdviceForCliniciansAndMicrobiol/).

1.5 Treatment

Bacterial meningitis needs rapid treatment and fast admission to hospital. Usually patients with suspected meningitis will be given an extensive course of antibiotics before confirmation by laboratory testing as a clinician cannot wait a day or longer for definitive culture results. Hib produces beta-lactamases and has gained resistance to the penicillin

family of antibiotics by modification of its penicillin binding proteins (Semczuk *et al*., 2004). Ampicillin was the drug of choice for the treatment of Hib infection, however, due to the development of resistance, highly active third-generation cephalosporins (e.g. ceftriaxone) are used (Jain *et al.,* 2006). Ceftriaxone is the first drug of choice as it is very effective, and achieves good concentrations in the meninges and cerebral tissues (Jain *et al.,* 2006). Rifampicin has also been shown to be highly effective at eliminating carriage and preventing secondary infection. (Ladhani *et al*., 2009).

1.6 Epidemiology

A large number of epidemiology studies in the pre-vaccine era have been carried out worldwide. Although there have been wide variations in the annual incidence of disease especially among high risk groups, there have been consistent findings of increased risk of invasive disease in children less than 2 years of age correlating with the absence of anticapsular polysaccharide antibody (Anderson *et al*., 1995). A surveillance study carried out by Booy *et al*. (1993) in the Oxford region of the UK showed that over a seven year period (1985-1991) 416 cases of invasive Hib disease were ascertained in children < 10 years of age, 405 cases of these cases were in children < 5 years of age, and 295 of these cases were in children < 2 years of age. Disease incidence was found to be highest in children less than 1 year when the concentration of antibody to the PRP type b capsule is at its lowest (Booy *et al*., 1993). A prospective population based study carried out in Wales between 1980 and 1990 showed similar findings to the Oxford study (Howard *et al*., 1991). A total of 207 cases of invasive Hib disease occurred in Wales between 1988 and 1990. The most common clinical manifestation in children was meningitis. Again, like the previous study, most patients were under 5 years of age and the highest incidence of disease was in children < 1 years of age. The annual incidence of invasive Hib disease

(1988-1990) in children < 5 years was 34.6/ 100 000. The annual rate of infection was found to be 12-44% higher in four Welsh counties to that previously reported for the United Kingdom (Howard *et al*., 1991; Booy *et al*., 1993).

Similar levels of attack rates have been reported in other European countries. In all Scandinavian countries most invasive Hib disease occurs before the age of five years. The disease incidence of Hib meningitis before the introduction of Hib glycoconjugate vaccines was estimated to be 26-35/100 000 per year in Denmark, Norway, Finland and Sweden, however higher attack rates (43/100 000) have been reported for Iceland. The yearly incidence of all invasive Hib disease in the pre vaccine era was estimated to be 50-65/100 000 in Scandinavia, higher than reported for the UK (Claesson., 1993).

In the pre Hib vaccine era, Hib was the leading pathogen causing meningitis in the US, with reported rates of 60-100/100 000 in children less than 5 years of age (Millar *et al*., 2000). The highest burden of Hib disease has been reported in American Indian (Navajo and White Mountain Apache), Aboriginal children, and Alaskan natives, and these populations have a 5-10 % higher disease incidence than the general US population (Coulehan *et al*., 1984; Santosham *et al*., 1991; Santosham *et al*., 2007). Among Navajo children, the incidence of disease was 214/ 100 00 in children < 5 years of age (Coulehan *et al*., 1984). Whilst in Alaskan Eskimos the incidence was 491/100 000 in children < 5 years of age (Ward *et al*., 1981). Furthermore, a three year study carried out in the Northern territory of Australia estimated the annual incidence of invasive disease to be ~ 450/100 000 children < 5 years of age in Aboriginal children and ~88 /100 000 in non-Aboriginal children (Gilbert, 1991).

Hib still causes a large number of deaths among children in developing countries despite the availability of an effective vaccine. Studies carried out in Africa have shown attack rates twice as high of that of the UK (Bijlmer and Van Alphen., 1992; Cowgill *et al*.,

2006). Poor diagnostic facilities limit the awareness of disease burden and the high cost of this vaccine is a major obstacle in its implementation into routine immunisation schedules in these countries (Feikin *et al*., 2004). Currently the World Health Organization (WHO) estimates that Hib causes approximately 3 million cases of serious disease each year and approximately 386,000 deaths annually (WHO position paper, 2006). The global burden of Hib disease remains substantial, yet almost entirely vaccine preventable. Most deaths occur in developing countries in Africa and Asia, which account for an estimated 61% of childhood Hib deaths as shown in figure 1.1 (Watt *et al*., 2009).

Figure 1.1 Global disease burden of Hib disease

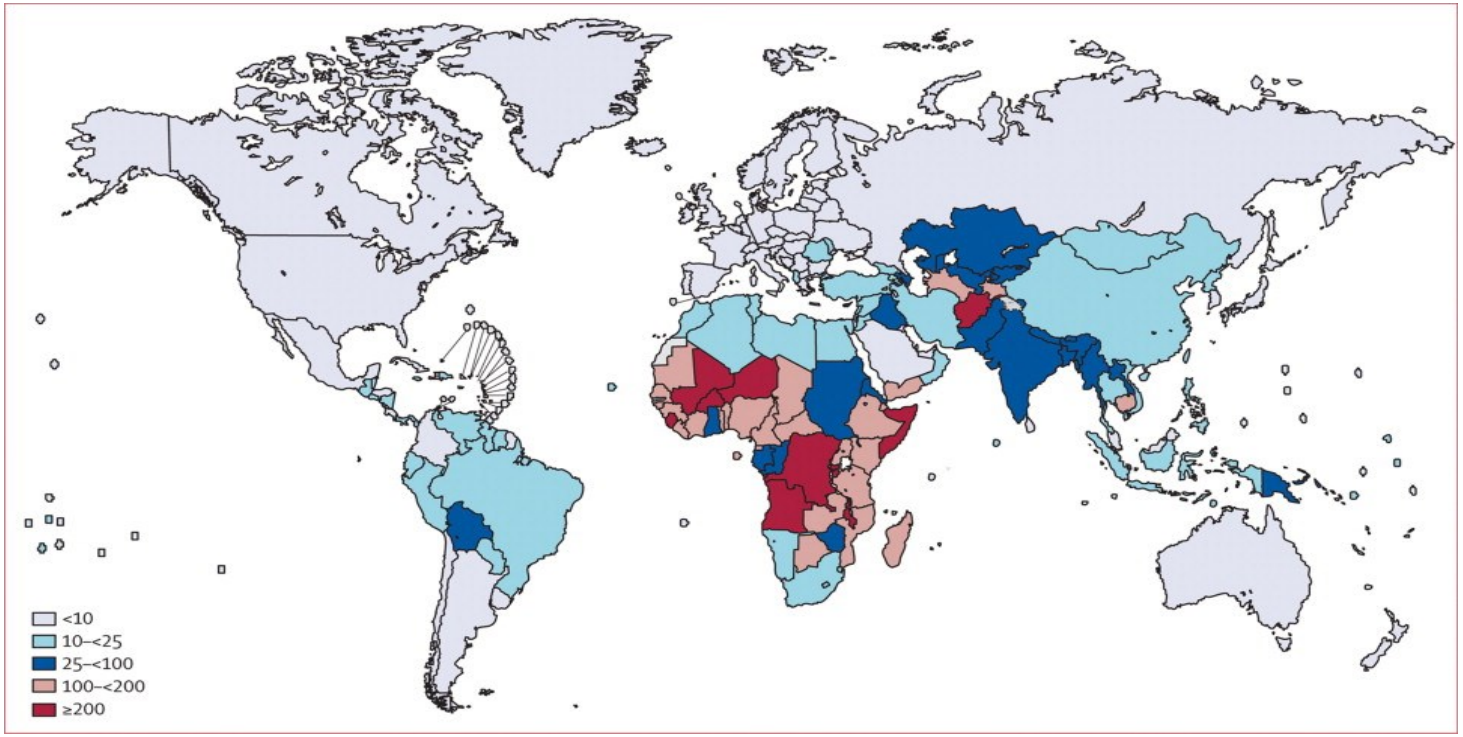


Figure 1.1 shows Hib deaths in children aged 1-59 months per 100,000 children (HIV negative Hib deaths only) in the year 2000. Actual estimates that year of 8.13 million serious illness and 371,000 deaths in children< 5 years (Watt *et al*., 2009).

1.7 Natural immunity

Serum antibodies against the capsular polysaccharide (PRP) play a major protective role against Hib infection. The detection of these antibodies in immunoassays such as the enzyme-linked immunosorbent assay (ELISA) have been used to estimate protective levels of immunity to Hib disease (Barra *et al*., 1988). Without vaccination, development of serum antibodies to the Hib polysaccharide usually begins from approximately 2 years of age via carriage of Hib and other bacteria that have cross-reacting polysaccharide structures (Fothergill and Wright, 1933; Leino *et al*., 2002).

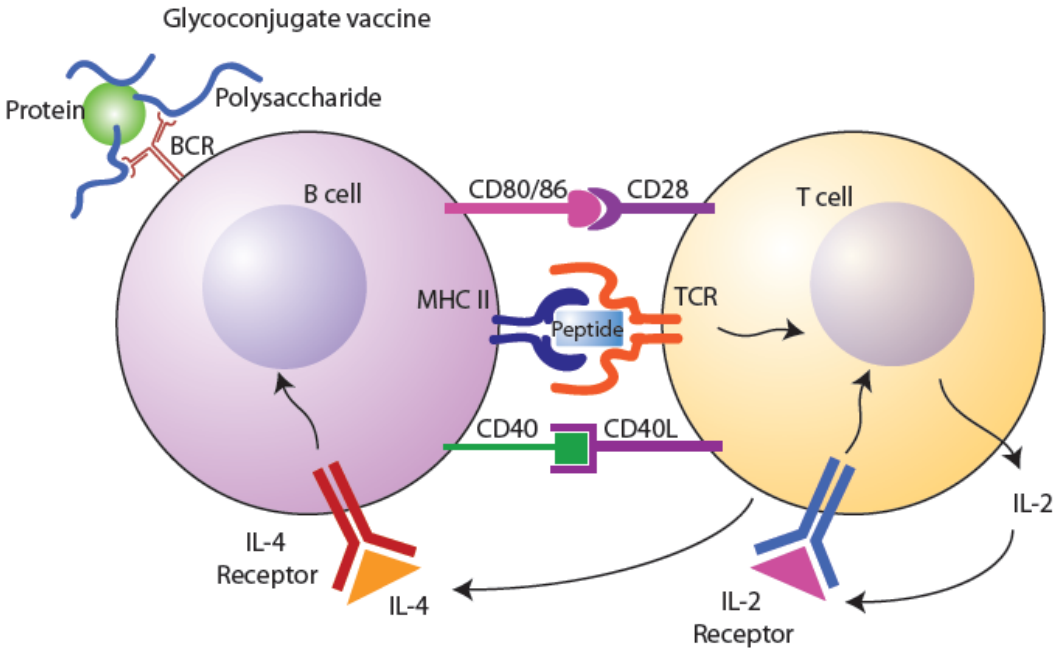
1.8 Polysaccharide vaccines

The first Hib vaccine to be tested in the field was a capsular polysaccharide vaccine (containing 25 μg of capsular polysaccharide per dose). A large field study in Finland, carried out in 1974, found the vaccine to have a 90% efficacy in children aged 18-71 months but was poorly immunogenic in the very young (Makela *et al*., 1977). Protection of infants is important and the polysaccharide vaccine only provided protective immunity in 45% of children < 18 months of age. This is in part due to the concentration of antibodies falling rapidly in children immunised before 24 months of age as well as the poor immunogenicity in this age group. Because of this, vaccination with polysaccharide was not recommended until children reach 24 months of age (Peltola *et al*., 1977). Bacterial polysaccharides are classified as Thymus-independent type 2 (TI-2) antigens which mostly stimulate antibodies of the IgM isotype and no induction of immunological memory and the antibodies are usually of short duration (Deauvieau *et al*., 2009).

1.9 Conjugate vaccines

Combining properties of the bacterial polysaccharide with a carrier protein (conjugation) enhances the immunogenicity of these vaccines, generating a protective response in the very young (Makela *et al*., 2003). Conjugating a polysaccharide to an immunogenic carrier protein converts the polysaccharide to a thymus dependent antigen (Lesinski and Westerink, 2001) leading to efficient B cell activation, isotype switching (IgM to IgG) and induction of memory (Deauvieau *et al*., 2009). An accepted theory is that the carrier protein in the glyconjugate vaccine is processed in the endosome of the B cell, from which, protein derived peptides are presented with major histocompatibility complex II (MHC II) on the surface of the Antigen presenting cell to the T cell. Cytokines are then produced yielding clonal expansion of polysaccharide specific B cells, producing IgG anti-polysaccharide antibodies and establishing memory B cells, illustrated in figure 1.2 (Lai and Schreiber, 2009; Avci *et al*., 2011).

Figure 1.2: T cell dependent B cell activation



Schematic representation of the processing and presentation of glycoconjugate vaccines. The B cell recognises the polysaccharide-protein conjugate specific to the B cell receptor (BCR) and binds. The protein portion is internalised in the endosome of the B cell to release peptides, leading to expression of preliminary antibodies (IgM). The B cell will 'present' the peptide on the cell surface attached to major histocompatibility complex II (MHC II). The CD4+ 'helper' T cells recognise the peptide bound to the MHCII leading to further co-stimulatory 'danger' signals (Interleukin 4 (IL4) and Interleukin 2 (IL2)) being sent via other receptors, leading to T cell activation, which secrete cytokines. Interactions between the B cell and the T cell in combination with the actions of cytokines, stimulate B cell maturation inducing isotype switching (IgM to polysaccharide specific IgG), and B cell memory (Avci *et al*., 2011).

Safe and effective Hib conjugate vaccines have been used since the late 1980s. Where introduced for routine use, virtual elimination of Hib disease has been achieved (Dagan *et al*., 1999; Ramsay *et al.,* 2003; Rossi *et al*., 2007; Ojo *et al*., 2010). Four conjugate vaccines for Hib have been developed, differing in the carrier proteins. They are: diphtheria toxoid (PRP-D), tetanus toxoid (PRP-T), a mutant diphtheria toxin, CRM_{197} (HbOC), and an outer membrane protein complex of the B_{11} strain of *Neisseria meningitidis* serogroup B (PRP-OMP) (Griffiths *et al*., 2012).

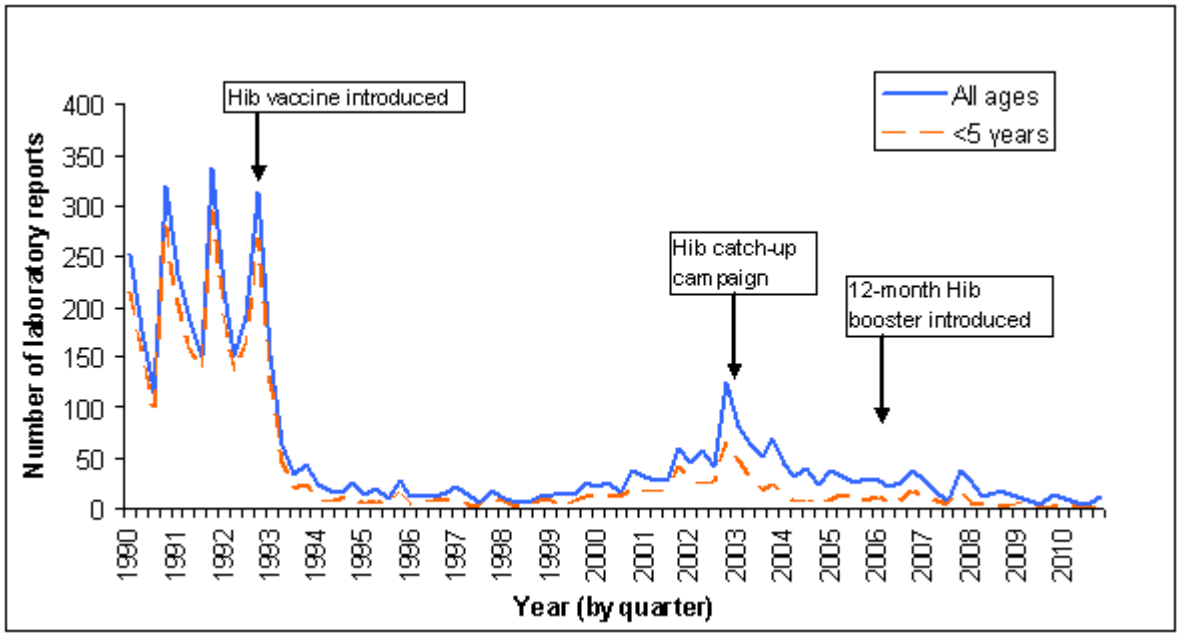
Hib conjugate vaccines were the first glycoconjugate vaccines to be included in the UK childhood routine immunisation schedule (Heath and McVernon, 2002). Initially the vaccine was given at 2, 3, and 4 months with no booster dose in the second year of life. At the same time a 12 month catch up campaign took place to vaccinate older children who were at risk of invasive Hib disease under four years of age. Three doses were given to infants between 4 and 12 months of age and one dose given to children aged 12-48 months (Ladhani *et al*., 2008). This vaccination schedule reduced invasive Hib disease incidence dramatically from 21-44/100,000 infants < 5 years of age in 1991 to 0.63/100,000 in 1998. A decline in Hib infection was also reported in older children and adults. This was probably due to a reduced asymptomatic pharyngeal carriage rate in vaccinated children, reducing transmission, leading to herd protection (Ladhani *et al*., 2008). No booster was included as earlier clinical trials confirmed that the vaccine was well tolerated and efficacious, producing antibody consistent with protection (≥0.15 μg/mL) in almost all children when given with a combined diphtheria, tetanus, whole cell pertussis (DTwP) and oral polio vaccines (Booy *et al*., 1992).

In 1999, the number of reported cases of invasive Hib disease began to rise in the UK. A year by year increase in disease in children < 5 years of age peaked at 120 cases in

2002. The reasons for the increase included a wearing-off of the initial catch up campaign, and a decline in vaccine effectiveness in those vaccinated in infancy (Trotter *et al*., 2003). Secondly, Hib conjugate vaccines reduce pharyngeal carriage therefore eliminating the chance of natural immune boosting to sustain antibody levels (Ladhani *et al*., 2010c). As well as this, a temporary change in the Hib vaccine combination from a whole cell pertussis (DTwP/Hib) to a 3 component acellular pertussis ($DTaP_3$/Hib (Infarix-Hib, GSK)) was also thought to play a role in reduced immunogenicity (McVernon *et al*., 2003). Due to the increase in disease, a Hib vaccination booster campaign was conducted between May and September 2003 which offered one dose to all children aged six months to four years. This had an immediate effect, quickly reducing the number of cases across all age groups (Ladhani *et al*., 2008).

In 2004, the recommended childhood immunisation schedule was changed from DTwP to a vaccine containing DTaP, inactivated polio and Hib ($DTaP_5$/Hib/IPV, Pediacel, Sanofi Pasteur). The acellular pertussis components used in this vaccine is different to the one used previously which contributed to the rise in disease. Pediacel contains five purified pertussis antigens, a formulation shown to produce satisfactory protective antibody levels under the UK 2, 3, 4 month primary immunisation schedule (Kitchin *et al*., 2007). In September 2006, the Department of Health introduced a booster dose of a combined Hib-Meningococcal serogroup C glycoconjugate vaccine (Menitorix, GSK) into the national immunisation programme at 12 months of age. A catch up campaign was also introduced for children too young for the 2003 booster campaign and too old to receive the scheduled 12 month booster dose (Ladhani *et al*., 2009). Invasive Hib disease in the UK is currently at an all time low as shown in Figure 1.3.

Figure 1.3 Laboratory confirmed cases of Hib from England and Wales, 1990-2010. (Health Protection Agency)



This figure shows the laboratory confirmed cases of Hib from England and Wales, 1990-2010. Hib disease fell rapidly after 1992 when Hib glycoconjugate vaccines were introduced. A steady increase in disease starts from 1999 peaking in 2003. After Hib vaccination catch up campaign, Hib infection rates declined to levels similar to that as just after the initial introduction of the Hib glycoconjugate vaccines. (Health Protection Agency http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HaemophilusInfluenzaeTypeB/EpidemiologicalData/HibGraph/23/07/12)

The WHO recommends that all countries should introduce Hib vaccine into their immunisation schedules. Hib conjugate vaccines have been proven safe and effective, and can almost eliminate disease in countries where they are routinely used. Hib causes around 3 million cases of serious disease and 386,000 deaths annually, them all being vaccine preventable, making Hib a target for global elimination (WHO factsheet December, 2005). Soon after licensure, Hib glycoconjugate vaccines were quickly introduced into North America and Western Europe, however, in developing countries the introduction of Hib vaccines has been slow. The high cost, limited vaccine supply and uncertainty/lack of data on disease burden have been factors affecting the introduction of the vaccine (Watt *et al*., 2009).

In 2005, the GAVI alliance (Global Alliance for Vaccines and Immunisation) created the Hib initiative to expedite and sustain evidence-informed decisions regarding the use of Hib vaccination in GAVI eligible countries, to prevent childhood meningitis and pneumonia. The GAVI alliance provides support to national governments through the GAVI fund with a result that countries with a Gross national income per capita below US $1,000 in 2003 qualify for support. Uptake for Hib containing vaccines has now increased dramatically from 2007. A total of 72 countries are eligible for vaccine support, and 68 countries qualify for the Hib conjugate vaccines. As of January 2009, 58 of these 68 countries had their applications approved (http://fr.gavialliance.org/performance/commitments/haemophilius/index.php). Almost all high income countries and GAVI eligible countries have introduced Hib conjugate vaccines or have been approved, yet only 45% of the world's children were fully vaccinated with the Hib vaccine at the end of 2009. This is largely due to the fact that a few countries with large birth cohorts such as India and China have not yet introduced the

vaccine. Therefore, additional efforts are still needed to increase vaccination in lower middle income countries (Ojo *et al*., 2010).

1.10 Immunological Evaluation

The immunological evaluation of responses to Hib vaccination usually includes the measurement of serum antibodies to the capsular polysaccharide (PRP) (Amir *et al*., 1990a). The short term protective level of ≥0.15 µg/mL of anti-PRP antibody and ≥1 µg/mL for long term protection has been established by antigen binding assays such as enzyme linked immunosorbent assay (ELISA) and radioantigen binding assay (RABA) as shown in Figure 1.4 (Käyhty *et al*., 1983; Santosham *et al*., 1987; Phipps *et al*., 1990). These values have been established through animal studies, studies of passive immunisation (Santoshma *et al*., 1987), analysis of natural immunity (Robbins *et al*., 1973), and through the early Hib vaccine efficacy trials with Hib polysaccharide vaccines (Peltola *et al*., 1977). Since these levels have been established through immunisation with pure polysaccharide or passive immunisation, the relevance to infants vaccinated with glycoconjugate vaccines remains uncertain (Goldblatt *et al*., 1998). In 1933, Fothergill and Wright described an inverse relationship between the occurrence of Hib meningitis and the presence of bactericidal activity against the pathogen in blood. They found that Hib meningitis was rarely seen in infants under 2 months when maternal antibodies are present or beyond six years of age when natural immunity has been induced. What is more, they found that children between two months and three years had practically no bactericidal activity where Hib disease was highest. Bactericidal activity rises above three years staying constant in adult life as shown in Figure 1.5 (Fothergill and Wright, 1933).

Although measurement of antibodies through RABA and ELISA provide a good measure of immunogenicity for vaccine evaluation, the results do not predict functional

and possibly protective activity of these antibodies. These assays provide a good assessment of the immune response but high/low avidity antibodies may go undetected. One limitation in determining protective antibody levels is that there is variability in the isotype distribution of antibody among individuals. A combination of IgM, IgG and IgA has been shown to be elicited by immunisation with Hib. IgG in the presence of complement has shown to have opsonic and bactericidal activity, IgM has shown only to be bactericidal, and IgA having neither activity (Amir *et al*., 1990a; Schreiber *et al*., 1986). In addition, antibodies of the same isotype may also result in different functional activity. A study by Amir *et al*. (1990b) found that IgG1 anti-PRP antibody was functionally more effective that IgG2 antibody although both subclasses were protective. Antibody function can be measured by a serum bactericidal antibody (SBA) assay which measures the titre of antibodies that bind to the specific target strain and fix complement onto the bacterial surface, initiating complement mediated lysis. The SBA assay is reliable and reproducible when evaluating the humoral response following vaccination (Schlesinger *et al.,*1992).

Figure 1.4 The protective level of serum antibodies to the capsular polysaccharide of Hib.

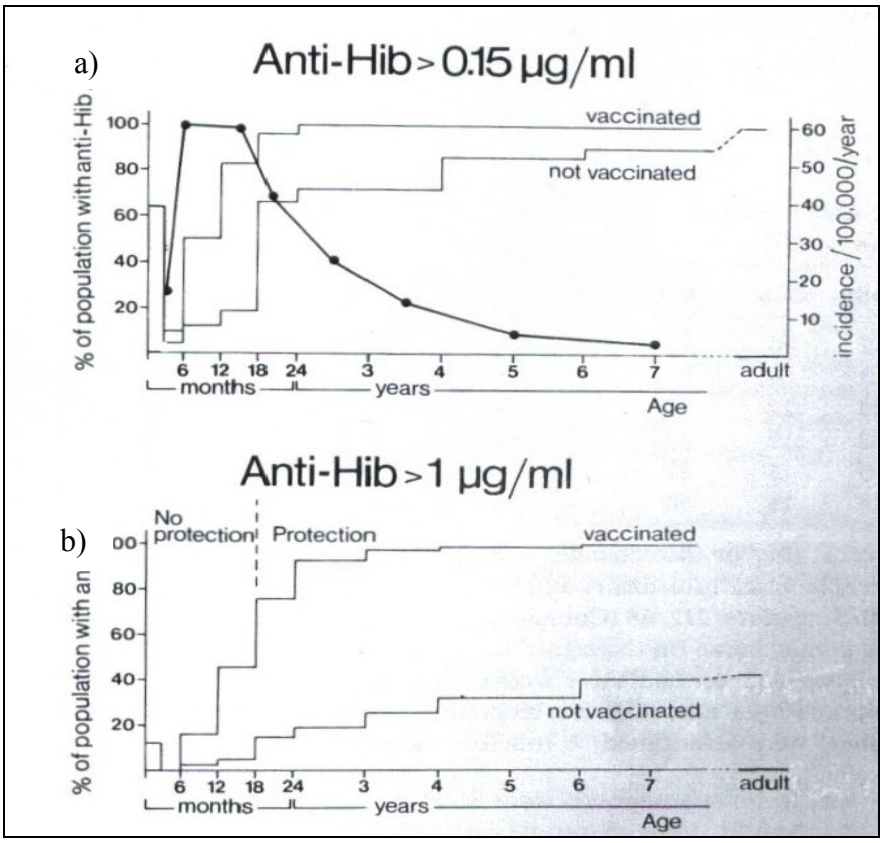


Figure 1.4 (a) shows the incidence of meningitis due to Hib (●——●). Both graphs show the protective effect of the Hib polysaccharide vaccine compared with the percentage of the population at each specified age having anti-Hib polysaccharide (anti-Hib) antibody levels exceeding 0.15 µg/mL (a) and 1.0 µg/mL (b) before and after immunisation. In the non vaccinated population, a good inverse correlation is shown between the anti-Hib level of 0.15 µg/mL and disease incidence, suggesting that this concentration of antibody is sufficient for protection against bacterial infection. However, in the vaccinated population the correlation is less good with 80% of infants aged 12-17 months reaching this level after vaccination, without being protected from Hib disease. A concentration of 1.0 µg/mL gives a better correlation with protection (Käyhty *et al*., 1983).

Figure 1.5 The inverse relationship between Hib disease and bactericidal activity

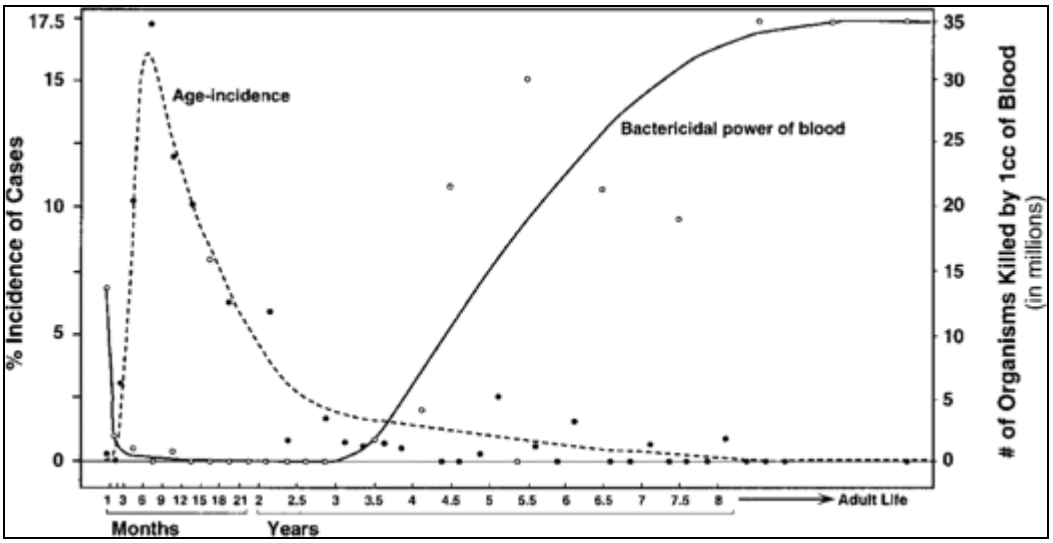


Figure 1.5 shows the inverse relationship between Hib disease and bactericidal activity of blood. The dash line shows cases of Hib disease from infancy to adult life. Solid line shows the bactericidal activity of blood which is able to kill Hib in the presence of phagocytes (Fothergill and Wright, 1933).

1.11 The complement system

The complement system is the first line of defence and an important part of the innate immune system. Activation of the complement system by one of three pathways leads to a cascade of protein activation and deposition on the surface of the pathogen, resulting in the formation of the membrane attack complex (MAC) (Zipfel *et al*., 2007; Trouw *et al*., 2003). Invading pathogens activate the complement system either spontaneously due to foreign envelope or membrane composition (Alternative pathway and Lectin pathway) or through antibody binding (Classical pathway) (Wurzner, 1999). All three pathways follow the same terminal pathway leading to lysis of the pathogen as shown in the Figure 1.6.

For pathogens to establish disease in the human host, they must be able to avoid, resist and neutralise host defences including the complement system. The complement system is tightly regulated to avoid extensive host tissue damage. Important regulators of the complement system are Factor H (FH), Factor H-Like protein-1 (FHL-1) (Alternative pathway), C4b binding protein (C4BP) (classical/Lectin) and Vitronectin (Terminal pathway) (Alexander and Quigg, 2007). Factor H is a 150 kDa fluid phase protein which regulates the alternative pathway. A glycoprotein in plasma, it is able to regulate both in fluid phase and on cell surfaces by inactivating C3b (Hallstrom *et al*., 2008). Binding of human Factor H via bacterial surface proteins leads to the down regulation of the alternative complement system therefore enhancing serum resistance of the pathogen. Binding of this protein is an important mechanism for survival in the human host (Jiang *et al*., 2010). Encapsulated Hi strains have been shown to bind FH and FHL-1 (a product of alternative splicing of the *FH* gene). A study by Hallstrom *et al*. (2008) showed that Hib

strains with low Factor H binding were more sensitive to killing by human serum, than those strains which had strong binding. This suggests that binding Factor H is an important survival strategy for this pathogen to evade complement-mediated attack (Hallstrom *et al*., 2008). Factor H has been a protein of much interest, as Factor H binding protein on the surface of *Neisseria meningitidis* has shown to be a promising vaccine candidate for group B strains (Lucidarme *et al*., 2011). In addition to Factor H, Hib has also been shown to bind vitronectin. It has been suggested that binding vitronectin would play a protective role from bacteriolysis following opsonisation due to interactions with the complement C5b-9 complex (Eberhard and Ullberg, 2002).

Figure 1.6 The complement system

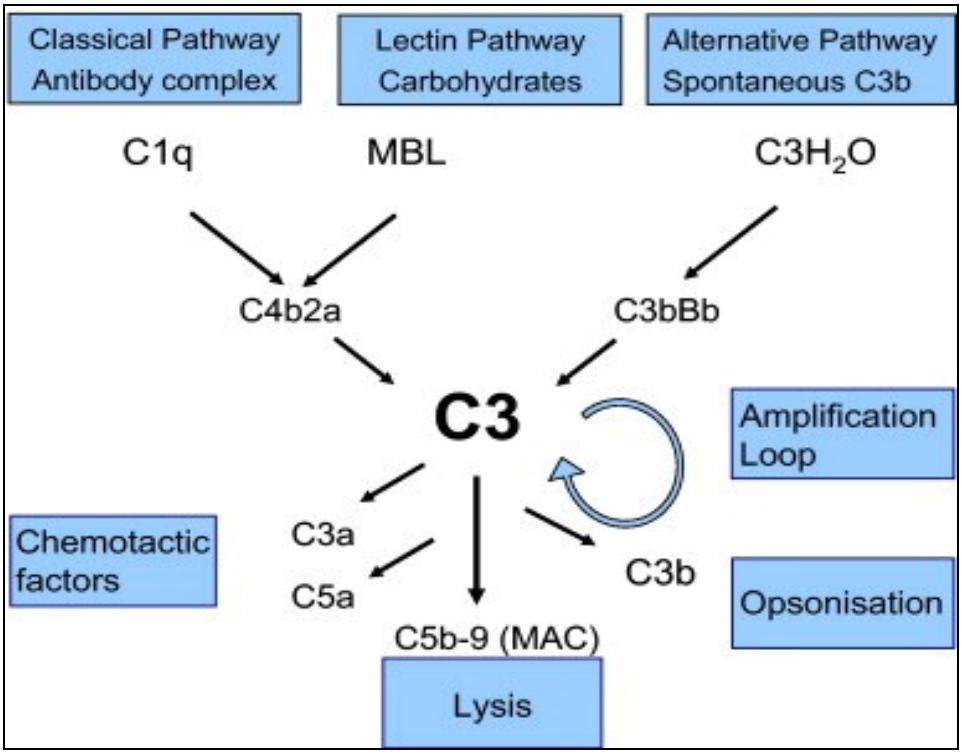


Figure 1.6 shows the complement system. All 3 pathways lead to the formation of the C3 convertases, cleaving C3 to 3a and 3b. C5 convertases are formed, leading all three pathways into the same terminal pathway resulting in cytolytic MAC. The complement system is tightly regulated to avoid extensive host tissue damage (Trouw *et al*., 2003).

1.12 Aims and objectives

Evaluation of the immune response to Hib conjugate vaccines includes the measurement of serum antibodies to the Hib capsular polysaccharide (PRP) by ELISA with accepted short term and long term levels of ≥0.15 μg/mL and ≥1.0 μg/mL, respectively. Their relevance for protection in children who have been primed for memory response remains unclear, as these levels were derived by passive immunisation or immunisation with pure polysaccharide and so, the search of adequate markers of protection in primed persons continues (Goldblatt *et al*., 1998). Previous SBA methodologies have been published for Hib which have shown good correlation between anti-PRP antibody concentrations with SBA titres; however no studies have been performed comparing SBA to anti-PRP IgG concentrations in serum collected from infants under the UK immunisation schedule. (Romero-Steiner *et al*., 2001; Schlesinger *et al*., 1992; Kim *et al*., 2008).

The first aim was to develop, optimise and evaluate the Hib SBA assay in the Vaccine Evaluation Unit (VEU) using previous knowledge and understanding of the SBA with *N. meningitidis* and compare the assay to a method used by the Centres for Disease Control and Prevention (CDC) (Romero-Steiner *et al*., 2004). The second aim was to apply the assay to test samples that have shown to have antibody responses to Hib, and compare SBA titres to known anti-PRP IgG concentrations. The optimised assay will then undergo assay validation to produce a validation report. Following assay validation, the Hib SBA assay will be used to test serum samples taken from infants under the UK immunisation schedule to measure Hib SBA titres and compare to known anti-PRP IgG concentrations. As Hib strains may contain multiple capsular loci, the Hib SBA assay will be used to test convalescent sera from children with Hib vaccine failure against strains

expressing 1-5 copies of the *cap* b locus, to determine whether multiple *cap* b loci is functionally important or not.

2.0 Methods

2.1 Replication of CDC method

The methodology for a SBA assay for Hib has been previously published by the CDC, Atlanta, USA (Romero-Steiner *et al*., 2001). When establishing the Hib SBA assay, the first step was to replicate this method.

2.2 Strains, Media and serum samples

The target strain used to replicate this method was M07 240381 (Hib strain Eagan) which was provided by the CDC. The mother culture was stored between -70 and -95°C in Microbank cryovials (ProLab Diagnostics, South Wirral, U.K.). For colony isolation, a single bead from the cryovial was removed and streaked onto a chocolate agar plate (Columbia agar with chocolated horse blood, Oxoid, Hampshire, U.K.) followed by overnight culture (16 hours) at 37°C (±2) with 5% (±2) CO_2.

Serum samples from a phase IV, single group study to evaluate the immunogenicity and safety of UK laboratory workers of a licensed Hib and meningococcal C conjugate combined vaccine (Menitorix, GSK, Middlesex, U.K.) were used to replicate the CDC method. Subjects enrolled received a single dose of Menitorix. Blood was collected prior to vaccination and 4-6 weeks following vaccination. The study group consisted of 30 laboratory staff enrolled from Health Protection Agency, Manchester, UK. Appropriate ethical approval was obtained from Northern and Yorkshire Research Ethics Committee, REC reference number: 07/MRE03/6. European Union Drug Regulating Authorities, clinical trials (EudraCT) number: 2006-004302-74, (appendix I). A positive control serum was included in the assay, (National Institute of Biological Standards and Controls (NIBSC), Hertfordshire, UK, Code 96/536). This standard serum was produced from a pool of sera from adults containing antibodies specific for the polysaccharide (PRP) from Hib. The lyophilised standard is supplied in glass DIN ampoules each containing the

freeze-dried powder from 0.5 mL of serum. The freeze dried powder was reconstituted with 0.5 mL of injectable water (Hameln Pharmaceuticals Limited, Gloucester, U.K.) and appropriate volumes aliquoted into microtubes (Alpha laboratories, Eastleigh, Hampshire, UK) and stored at -65°C to -95°C.

2.3 Bactericidal buffer

Bactericidal Buffer (BB), used as a dilution buffer in the assay, was prepared by aliquoting aseptically 20 mL Hanks balanced salts (HBS) with Ca^{++} and Mg^{++} (Gibco, Paisley, U.K.) and 2% Fildes extract (Oxoid, UK) into 25 mL Universal tubes (Sarstedt, Leicester, U.K), which were then stored at +4°C (±2).

2.4 Bacterial preparation

The Hib strain M07 240381 was inoculated on a chocolate agar plate as described in section 2.2 and incubated overnight for 16 hours at 37°C (±2), 5% (±2) CO_2 . Working in a class 1 safety cabinet (TriMAT model, Medical Air Technology, Lancashire, U.K.), ten isolated bacterial colonies were transferred to 10 mL of brain heart infusion (BHI) broth (Oxoid) with 2% Fildes extract (Oxoid) and incubated at 37°C (±2), 5% (±2) CO_2 . For bacteria to enter exponential phase of growth the broth required a change from yellow to amber whilst incubated. Thus, the colour was checked every half an hour. The broth changed to amber after four hours and removed from the incubator and placed in the safety cabinet. After four hours, the optical density at 600nm (OD_{600}) was 0.4. A volume of 1.5 mL (15%) of sterile glycerol (Sigma, Dorset, U.K.) was added to the bacterial culture and mixed well. After mixing, 0.5 mL of the bacterial culture was dispensed into microtubes (Alpha laboratories), labelled with a working stock batch number and stored at -65°C to -95°C.

2.5 Optimisation of working stock

From the frozen vials, the dilution necessary to gain approximately 1000 colony forming units (cfu) per 20 µL of working stock was determined. Six tubes were prepared with 0.9 mL of BB. An aliquot of frozen bacterial culture (section 2.4) was thawed. A volume of 100 µL of bacterial culture was added to 1 mL of BB, from which, six 10 fold serial dilutions were made by transferring 100 µL into 0.9 mL. From each serial dilution, a volume of 10 µL was plated out onto chocolate agar (Oxoid) in triplicate (30 µL) for each serial dilution. The plates were incubated overnight at 37°C (±2) with 5% (±2) CO_2 for 16 hours and the colonies counted the following day using an automated colony counter (Perceptive Instruments, Suffolk, U.K.). The average count from each dilution was determined and a dilution that would yield ~1000 cfu per 20 µL was chosen.

2.6 Replication of CDC Hib SBA assay

To columns 1 to 11 of a U-bottomed microtitre plate (Sterilin, Staffordshire, U.K.) 10 µL of BB (section 2.3) was added. A volume of 10 µL of heat inactivated (56°C (±2) for 30 minutes) test/control serum was added to column 1. An aliquot of 5 µL BB and 5 µL heat inactivated (56°C (±2) for 30 minutes) test/control serum was added to column 12. The diluted test/control sera in column 1 (10 µL) was double diluted across the microtitre plate to column 9 (discarding the 10 µL at column 9 after mixing). An aliquot of bacteria as described in section 2.5, was thawed and diluted in BB to prepare 1000 CFU per 20 µL of BB. An aliquot of 20 µL of bacterial suspension was added to each well of the microtitre plate. The microtitre plates were gently tapped and sealed with plate sealers (Thermoquest, Hants, U.K.), and incubated at 37°C (±2) with 5% (±2) CO_2 for 15 minutes. After incubation, 25 µL of complement preserved baby rabbit complement (RC) (Pel-freez Biologicals, Arkansas, USA) was added to columns 1-10. Heat- inactivated RC was added to columns 11 and 12. Following the addition of RC, 25 µL of BB was added to each well.

Microtitre plates were gently tapped and sealed with plate sealers and incubated at 37°C (±2) with 5% (±2) CO_2 for 60 minutes. Figure 2.1 shows the microtitre plate layout for the CDC SBA assay. Following incubation, a volume of 5 µL from the wells of the first six columns of the first row/ sample was plated out using a multichannel pipette (Biohit, Devon, U.K.) onto a chocolate agar plate (Oxoid) using the tilt method. The tilt method allows the reaction mixture to slide down the chocolate plate without running into each other. The procedure was repeated for the wells of the second six columns of the first row/sample containing the higher dilutions and the control wells. Figure 2.2 shows an example of an operator completing the tilt method. Once all samples/controls from the microtitre plate had been plated out using the tilt method, the chocolate agar plates were incubated overnight at 37°C (±2) with 5% (±2) CO_2 for 16 hours. The following morning, colonies were counted using a colony counter with Sorcerer software (Perceptive Instruments) and the SBA titre expressed as the reciprocal of the final dilution giving ≥50% killing as compared to the number of cfu in column 11 at 60 minutes (T60).

A panel of adult sera (section 2.2) were assayed using the CDC method with a total of 19 post Hib vaccination serum samples chosen to test the reproducibility of the assay. Each sample was assayed on three separate runs/days. Reproducibility was deemed acceptable if >85% of the test samples produced SBA titres, which fell within ±1 titres of each other (Bioanalytical method validation, FDA, May 2001 available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf)

Figure 2.1 Microtitre plate layout for the CDC method.

ASSAY	1	2	3	4	5	6	7	8	9	10	11	12
BB (µL)	10	10	10	10	10	10	10	10	10	10	10	See Below
PATIENT SERUM (µL)	9 TWOFOLD SERIAL SERUM DILUTIONS Transfer 10 µL from column ≠ 1 -- 9, mix 6 times and discard 10 µL from column # 9									0	0	
COMPLEMENT (µL)	25	25	25	25	25	25	25	25	25	25	25*	
CELLS (µL)	20	20	20	20	20	20	20	20	20	20	20	
BB (µL)	25	25	25	25	25	25	25	25	25	25	25	
FINAL VOL. (µL)	80	80	80	80	80	80	80	80	80	80	80	
RECIPROCAL FINAL SERUM DILUTION	1/16	1/ 32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	n/a	n/a	

This figure shows the microtitre plate layout for the replication of the CDC method. Column 12 contains 30 µL of BB, 5 µL of heat inactivated serum, 25 µL of heat inactivated complement, and 20 µL of working solution of organisms. *column 11: heat inactivated complement

Figure 2.2 Example of an operator completing the tilt method

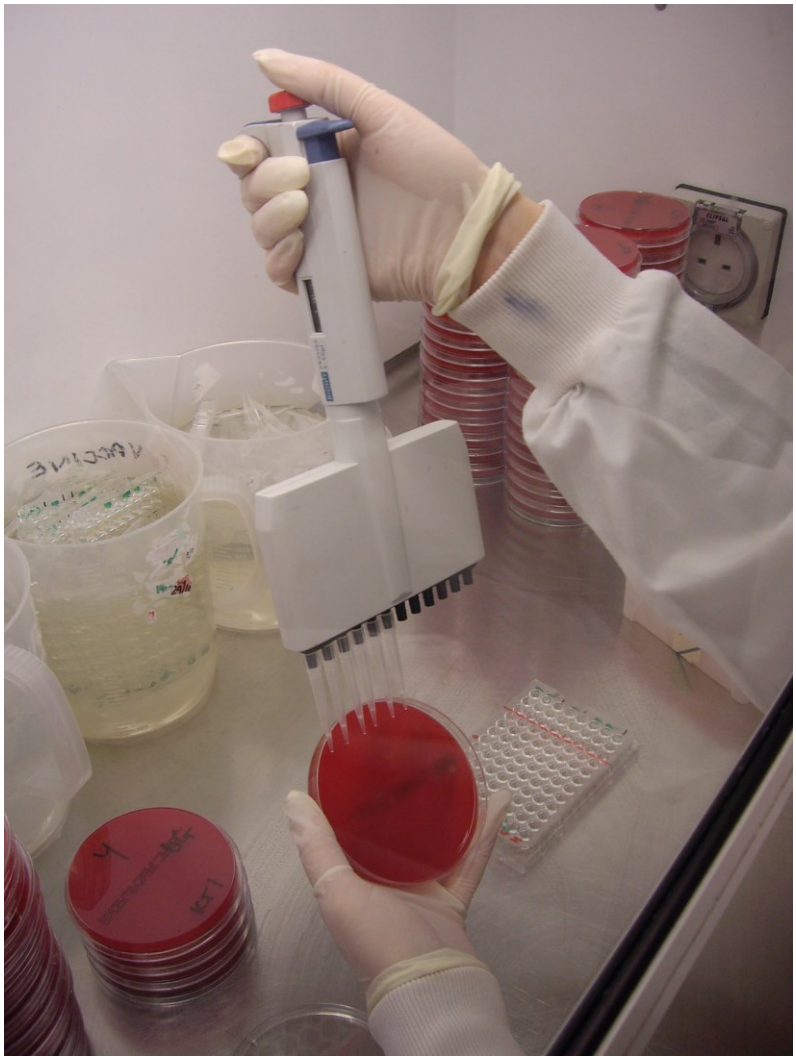


Figure 2.2 shows an operator completing the tilt method. A multichannel pipette is used to plate out the first six columns of the first row/sample onto an agar plate. The agar plate is angled to allow the reaction mixture to slide down the agar plate without running into each other. The procedure is completed for the wells of the second six columns of the first row/sample containing higher assay dilutions and control wells. The procedure is repeated for the remainder of the rows (test samples).

2.7 Rabbit complement

To investigate whether the rabbit complement may affect the SBA assay, all batches of rabbit complement were assayed for intrinsic bactericidal activity at the same complement concentration used in the assay against appropriate target strains. Any changes to the assay throughout the development stage, which altered the complement concentration, were tested for intrinsic activity again. Batches of RC were validated by assaying the complement source in duplicate at the appropriate complement concentration used in the assay. For CDC method, the target strain and buffers were prepared as detailed in section 2.3 and 2.5. A volume of 10 µL of BB was added to the wells of columns 1, 2, 3 and 4 of each row of a microtitre plate (8 assays). Working in a class 1 safety cabinet, 20 µL of the target strain was added to each well of the relevant rows. A volume of 25 µL of heat inactivated RC was added to column 3 and 4. A volume of 25 µL of the relevant RC was added to the rows of columns 1 and 2. Following the addition of RC, 25 µL of BB was added to each well. The microtitre plate was sealed and incubated for 60 minutes at 37°C (±2) with 5% (±2) CO_2. The tilt method was performed to plate out the four columns of the first row onto a chocolate agar plate. The procedure was repeated for the remainder of the rows. Plates were incubated as detailed in section 2.6. The following morning, colonies were counted using the complement validation template in the Sorcerer software (Perceptive instruments). The averages of the two control wells (columns 3 and 4) were automatically calculated. The percentage of survival of bacteria in each of the complement source dilutions (columns 1 and 2) were automatically calculated using the average of the two control wells. The acceptance criteria of >85% survival was automatically applied.

2.8 Development of the Hib SBA assay by adaptation of the CDC method

The initial Hib SBA assay was adapted from the method currently used in the VEU for *N. meningitidis* and the CDC Hib method. The first step in adapting the method was to change the preparation of the target strain to be used in the assay.

2.8.1 Working stock

Hib working stock was made by streaking one bead from a microbank cryovial (ProLab diagnostics) of the mother culture (M07 240381) onto chocolate agar (Oxoid) for heavy growth and incubated overnight at 37°C (±2), 5% (±2) CO_2 (16-24 hours). Following incubation all of the growth on the plate was transferred into 9 mL of BHI broth (Oxoid) using a sterile swab, to this 15% sterile glycerol (Sigma) and 2% Fildes extract (Oxoid) was added. A volume of 0.5 mL of broth was transferred into individual microtubes and labelled with a batch number.

2.8.2 Optimisation of working suspension

A single dilution which produced a working suspension of 60-250 cfu was determined. Samples were prepared as in the CDC method (section 2.6). The target strain preparation was altered by retrieving a single tube of working stock as prepared in section 2.8.1, defrosting and streaking onto chocolate agar for single colony isolation and incubating overnight at 37°C (±2), 5% (±2) CO_2. The following morning ~ 50 colonies were streaked for confluent growth onto a fresh chocolate agar plate (Oxoid) and incubated for 4 hours (±15 minutes) at 37°C (±2), 5% (±2) CO_2 (for bacteria to enter exponential phase of growth). Following incubation (working in a class 1 safety cabinet), a sterile swab was used to suspend bacteria into 5 mL of BB. Approximately 1 mL of the Hib suspension was placed into a semi micro-cuvette (Sigma) and the absorbance read on a spectrophotometer (UV1100 WPA spectrophotometer, Jencons, West Sussex, U.K.) at a

wavelength of 650 nm (spectrophotometer was blanked with 1x BB). The suspension was adjusted to A_{650} = 0.1. Working suspensions with differing numbers of cfu were gained by preparing 1/10 and 1/20 dilutions of the OD_{650} = 0.1 bacterial suspension, followed by 1/100 dilution in BB. The equivalent to column 11 (35 µL, BB, 20 µL target strain, 25 µL complement) for both dilutions were plated out at T60. The dilution that produced between 60-250 cfu was chosen.

2.8.3 Procedure

A single tube of working stock (section 2.8.1) was defrosted and streaked onto a chocolate agar plate (Oxoid). Samples were prepared as per CDC (section 2.6). After 4 hours incubation grown on chocolate agar (section 2.8.2), the Hib suspension was adjusted to A_{650} = 0.1 (section 2.8.2) followed by a 1/10 and a 1/100 dilution. After the addition of 20 µL of the target strain to every well the method continued to follow the CDC method as per section 2.6, with the exception of 15 minute incubation after the addition of bacteria. Secondly, the volume of reaction mixture which is plated out after the addition of target strain, buffer and complement was increased from 5 µL to 7.5 µL. A total of seven pre and post Hib vaccination sera (section 2.2) were tested using this method and titres compared to those generated following the CDC method.

2.9 Further optimisation of the Hib SBA assay

Keeping the current adapted method (section 2.8) many variables were tested to optimise the assay.

2.9.1 Comparison of the complement concentration

The concentration of complement is crucial in the assay. A concentration that is activating enough using less volume of complement as possible is useful in the assay due to the high cost of baby rabbit complement. A total of seven known positive serum samples

(section 2.2) were tested at various concentrations in the assay and compared to the titres obtained from the CDC method.

2.9.2 Comparison of the incubation period of serum/target strain/complement/buffer.

Due to the observation of a inhibited cfu growth in column 12 (non complement mediated lysis control) and enhanced cfu growth in column 11 with positive adult sera at T60, varying incubation times of 30, 40, 50 and 60 minute were compared using a complement concentration of 33.3% as determined by section 2.9.1. The method followed that described in section 2.8.

2.9.3 Comparison of the incubation period with 5% CO_2 and without 5% CO_2

The procedure in the CDC method (section 2.6) incubates microtitre plates after the addition of bacterial suspension, complement and buffer in a 37°C incubator in a 5% CO_2 environment. The microtitre plates are sealed and so the CO_2 environment might not be essential in this incubation. The assay was run as described in section 2.8 using a complement concentration of 33.3%. Seven post Hib vaccination samples (section 2.2) were assayed with and without a 5% CO_2 environment at T50 and T60 time points.

2.9.4 Comparison of the media used in the Hib SBA assay

The best media for optimal growth is essential in the assay. The agar is used for numerous procedures in the SBA assay. A comparison of chocolate agar plates used in the assay was performed method described in section 2.8. The two types of chocolate agar plates compared were, chocolate agar + PolyViteX (Biomérieux, Marcy l'Etoile, France) and Columbia agar with chocolated horse blood (Oxoid). Nineteen known positive serum samples (section 2.2) were tested using both media and the titres compared.

2.9.5 Comparison of the use of bovine serum albumin in BB.

Bovine serum albumin (BSA) can be used in immunoassays to address the issue of non specific binding and stability. To address the issue of non specific killing in the assay

i.e. non complement mediated lysis, known positive samples (section 2.2) were assayed using bactericidal buffer with BSA (Sigma) and without BSA and the SBA titres compared.

2.9.6 Validation of the specific optical density and its correlation to inoculum count.

Due to the changes made to the assay during optimisation it was important to validate the suspension of Hib at an OD of 0.1 at a wavelength of 650nm and find a suitable dilution which produced an inoculum count between 60-250 cfu. Following the method detailed in section 2.8 at a 33.3% complement concentration, the Hib suspension was adjusted to OD of 0.1 at 650nm. The adjusted suspension was diluted in BB in repeats of five at 1/30, 1/40 and 1/50 followed by 1/100 and a further 1/6 on the microtitre plate. The mean cfu count was taken from the five results gained at each dilution and the number of cfu/mL was calculated.

2.9.7 Comparison of the incubation length of the target strain prior to use in the Hib SBA assay

As the incubation length of the target strain can affect the expression of surface antigens on the bacteria, the target strain (M07 240381) was incubated overnight for 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours prior to use in the assay. Pre and post Hib vaccination serum samples (section 2.2) were assayed in the optimised Hib SBA assay as described below (section 2.10). Titres were compared between 16-24 hours incubation.

2.10 Optimised Hib SBA assay

Pre and post Hib vaccination samples (section 2.2) were assayed in the optimised Hib SBA assay to select for validation.

2.10.1 Target strain

The target strain chosen for the optimised Hib SBA assay was M07 240381. Mother cultures were stored in at least 2 microbank cryovials (Pro-lab) at -80°C (-65 to -

95°C). Working cultures were made by streaking a single bead from the mother culture to produce a heavy growth on a chocolate agar plate (Biomérieux) and incubated over night for 16-24 hrs at 37°C (±2), 5% (±2) CO_2. Using a sterile swab (Medical Wire and Equipment co, (Bath) Ltd, Wiltshire, England) the cultured Hib was used to inoculate 9 mL of BHI (Oxoid). To the BHI, 2% Fildes extract (Oxoid) and 15% sterile glycerol (Sigma) was added. The bacteria were mixed gently to produce the working culture. A volume of 0.5 mL of the working culture was dispensed into microtubes and labelled with a batch number and expiry date and stored at -80 ºC (-65°C to -95°C).

2.10.2 Bactericidal buffer

BB is used as a serum and bacteria diluent in the Hib SBA assay. Initially a 10x solution containing 20% (w/v) of BSA (Sigma) dissolved in HBS (Gibco) was prepared and filtered through a 0.22 µm filter (Millipore, U.K.). A volume of 2 mL of 10x filtered solution was dissolved in 18 mL HBS (Gibco) to obtain a 1 x 2% BSA (Sigma) (w/v) bactericidal buffer solution. To which, 2 % Fildes (Oxoid) extract was added. This buffer is used throughout the optimised SBA method.

2.10.3 Pre assay

A frozen tube of working stock as prepared in section 2.10.1 was retrieved from the -80°C (-65°C to -95°C) freezer and placed within the safety cabinet. Once the culture had equilibrated to room temperature the culture was streaked for single colony isolation onto a chocolate agar plate (Biomérieux) and incubated overnight for 16-24 hours at 37°C (±2) 5% CO_2 (±2).

2.10.4 Preparing the target strain for exponential phase of growth

After 16-24 hours incubation the overnight agar plate (section 2.10.3) was placed into a safety cabinet and using a fresh sterile loop (Scientific Laboratory Supplies, Wilford, Nottingham, U.K.) approximately 50 colonies were transferred from a section on the

chocolate agar plate which allows enumeration and streaked onto a fresh chocolate agar for confluent growth. The freshly inoculated agar plate was placed in a 37°C (±2), 5% CO_2 (±2) incubator for 4 hours (±15 minutes).

2.10.5 Sample preparation

A volume of 15 µL of BB was added to columns 1-11 of a U-bottomed 96 well microtitre plate (Sterilin) and 7.5 µL added to column 12. A volume of 15 µL of heat inactivated (56°C (±2) for 30 minutes (±5)) test/control sera was added to column 1 and 7.5 µL added to column 12. The test/control sera (15 µL) was double diluted across the microtitre plate from column 1 to 9 (15 µL discarded at column 9). Leaving each well with a total volume of 15 µL with a range of doubling dilutions from 1/8 to 1/1024 (the initial starting dilution was adjusted for more positive test and control sera). Figure 2.3 shows the layout of the microtitre plate of the optimised Hib SBA assay. The positive control serum (NIBSC 96/536) as described in section 2.2 was included in each assay.

2.10.6 Procedure

Following a 4 hour (±15 minutes) incubation (section 2.10.4) a sterile swab was used to sweep across the centre of the bacterial growth on the chocolate agar plate (Biomérieux). The inoculated swab was suspended in 5 mL of 1x BB in a sterile universal tube. A volume of 1 mL was removed and placed into a semi micro-cuvette (Sigma) and the absorbance read on a spectrophotometer at a wavelength of 650 nm (spectrophotometer was blanked with 1x BB). The suspension was adjusted to A_{650} = 0.1. The adjusted suspension was diluted 1/30 with BB followed by 1/100 to prepare the working suspension. An aliquot of 10 µL of the working suspension was added to every well of the microtitre plate. A volume of 20 µL of heat inactivated (56°C (±2) for 30 (±5) min) complement was added to all wells of columns 11 and 12. A volume of 20 µL of complement, (thawed and equilibrated to room temperature), was added to columns 1 to 10

and gently tapped to mix plates. A volume of 15 µL of 1x BB was added to every well. The microtitre plates were sealed and incubated at 37°C (±2) for 50 minutes. Prior to incubation, 10 µL was removed from column 11 for T0 count. Following 50 minutes incubation the plates were transferred back to the safety cabinet. Using the tilt method described in section 2.6, 10 µL of reaction mixture from the wells of the first six columns of the first row/sample was plated out onto chocolate agar (Biomérieux). The tilt procedure was repeated for the second six columns of the first row/sample containing the higher assay dilutions and the control wells. Once all the samples and controls were plated out from the microtitre plates the chocolate agar plates were left to dry for ten minutes before being transferred into a 37°C (±2), 5% (±2) CO_2 incubator for 16-24 hr. The following morning colonies were counted using a colony counter (Sorcerer software, perceptive instruments) and SBA titres expressed as the reciprocal of the final dilution giving ≥50% killing as compared to the number of cfu in column 11 at 50 minutes (T50). Titres were only assigned to test sera if target strains were not complement sensitive (column 10) and any killing of the target strain was due to non-complement mediated lysis (column 12).

Figure 2.3 microtitre plate layout for optimised Hib SBA assay

ASSAY	1	2	3	4	5	6	7	8	9	10	11	12
BB (µL)	15	15	15	15	15	15	15	15	15	15	15	See below
PATIENT SERUM (µL)	9 TWOFOLD SERIAL SERUM DILUTIONS Transfer 15 µL from column ≠ 1 -- 9, mix 6 times and discard 15 µL from column # 9									0	0	
COMPLEMENT (µL)	20	20	20	20	20	20	20	20	20	20	20*	
CELLS (µL)	10	10	10	10	10	10	10	10	10	10	10	
BB (µL)	15	15	15	15	15	15	15	15	15	15	15	
FINAL VOL. (µL)	60	60	60	60	60	60	60	60	60	60	60	
RECIPROCAL FINAL SERUM DILUTION	1/8	1/ 16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	n/a	n/a	

This figure shows the microtitre plate layout for the optimised Hib SBA assay. Column 12 contains 22.5 µL of BB, 7.5 µL of heat inactivated serum, 20 µL of heat inactivated complement, and 10 µL of working solution of organisms. * Column 11: heat inactivated complement.

2.11 Validation of the optimised Hib SBA assay

Validation was completed for the optimised Hib SBA assay (section 2.10). The assay was validated by the evaluation of the following parameters of assay performance: specificity, accuracy, assay precision/reproducibility and linearity. FDA guidelines for bioanalytical validation was followed (Bioanalytical method validation, FDA, May 2001 available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf). A full range of samples were chosen including negative sera. Acceptable variability was defined as at least ≥85% of test samples meeting the stated requirements. Hib strain M07 240381 was used to validate the SBA assay. This strain was previously found to be suitable for use in the SBA assay.

2.11.1 Serum samples

Approximately 44 samples (section 2.2) were screened and selected for use in the validation. Sample screening would ideally involve the repeated testing of the samples to eliminate those with presence of low avidity antibodies causing variability in the titres obtained. Due to insufficient sample volume this could not be completed. Ideally infant sera would be preferable; however, these samples are often limited in sample volume.

2.11.2 Specificity

Specificity was evaluated by inhibition of Hib with another appropriate antigen (*N. meningitidis* group C) and spiking of negative sample with positive sera.

2.11.3 Inhibition

Specificity of the Hib SBA assay was determined by inhibition with homologous (Hib type b polysaccharide NIBSC code 02/208) and heterologous (*N. meningitidis* serogroup C polysaccharide NIBSC code 07/318) antigens. For inhibition, polysaccharide was used at 200 μg/mL, which has shown to be the appropriate concentration for inhibition

in *N. meningitidis* serogroup C and A, SBA assays (Maslanka *et al*., 1997). A total of 19 positive samples with an SBA titre of ≥128 were assayed in this parameter. Individual samples were assayed neat (without inhibitors) and with both inhibitors on the same run. Samples run with an inhibitor were incubated with the appropriate inhibitor for one hour at room temperature, vortexing every 15 minutes. The difference in SBA titre (+/-) between inhibited result and neat result was calculated. Specificity was considered acceptable if ≥85% of samples undergoing homologous inhibition resulted in a reduction of >2 SBA titres. In addition ≥85% of samples undergoing heterologous inhibition should not result in a reduction of > 2 SBA titres.

2.11.4 Spiking

Spiking was completed by the testing of SBA negative samples which have been shown to have high antibody concentrations to other antigens. Negative samples were then spiked with an SBA positive sample in three different ratios. A total of 5 negative samples which had no quantifiable SBA activity were selected for use. These samples were determined as having positive antibody to various meningococcal serogroups. One positive sample (017B) was selected to spike into negative samples. This sample had an expected SBA titre of 8192- 37268 for Hib strain M07 240381. For each of the 5 negative samples, the positive sample was spiked into the 5 negative samples in three different ratios (1:2, 1:4, 1:8). The positive and negative samples were assayed neat on the same run. A single result for neat positive, neat negative and three spiked samples was achieved for each sample. The spiked negative results (x dilution factor) were compared to the positive sample result. Specificity was considered acceptable if for ≥85% of samples, the neat negative sample should give a negative (<8) and the spiked negative samples (multiplied

by the dilution factor) should be within ±1 SBA titre of the neat positive sample result. The spiking analysis was repeated five times to measure accuracy.

2.11.5 Assay precision/reproducibility

The validation of assay precision/ reproducibility was split into intra-assay, and inter assay over a range of negative and positive samples.

2.11.5.1 Intra assay precision/reproducibilty

Intra assay precision/reproducibility describes the closeness of individual measures of a given analyte within the same day/run of the assay completed by a single operator. Reproducibility was determined over 5 replicate measurements. Thirty six samples were assayed (positive and negative samples). Each of the 36 samples was assayed 5 times on the same day/run. Preparation of the samples was completed individually. Five results (replicates) for each sample were achieved on the same day/run for the 36 samples. If any replicates failed assay acceptance criteria, the complete analysis for that sample was undertaken. The median was calculated between the 5 replicates of the 36 samples tested. Intra assay precision was considered acceptable if for ≥85% of results, the SBA titre should be within ±1 SBA titre of the median for that sample. Samples with SBA titres <8 were excluded from this analysis.

2.11.5.2 Inter assay precision/reproducibility

Inter assay precision/reproducibility describes the closeness of individual measures of titres against a target strain across multiple days/runs completed by a single operator. A total of 39 samples covering the range of expected titres were chosen high (>128) medium (32-128) and negative samples were chosen. Each of the samples were assayed 5 times (replicates) on a different run. Five results (replicates) for each sample from the separate days/runs were achieved for all the 39 samples. If any replicate failed assay acceptance

criteria, the sample was repeated. The median was calculated between the five replicates for each of the samples. Inter assay precision was considered acceptable if for ≥85% of results, the SBA titre should be within ±1 titre of the median for that sample. Following validation, titres obtained from inter assay precision analysis were correlated with previously determined anti-PRP IgG concentrations.

2.11.6 Linearity/recovery

Linearity, also known as recovery of an antibody in an assay, is the detector response obtained from an amount of the SBA antibody added to and extracted from the sample, compared to the detector response obtained for the true concentration of the pure authentic standard. After diluting a known positive sample with commercially available IgG deficient sera (Sigma) at 1:4, the antibody titre of that positive serum should be recovered by multiplying by the dilution factor in this case 4 (1:4). Eighteen samples covering a range of positive concentrations were chosen. Samples were diluted 1:4 in IgG deficient sera. The neat (undiluted) and diluted samples were assayed on the same run. The diluted sample result (x4) was compared top the neat sample. Linearity was considered acceptable if for ≥85% of samples, the diluted sample should be within (+/-) 1 SBA titre of neat sample result.

2.12 Infant post primary and post booster responses

2.12.1 Sample size

Samples from a Phase IV, randomised study to evaluate the immune response of UK infants receiving licensed DTaP/IPV/Hib-TT, meningococcal C conjugate and pneumococcal conjugate vaccines was used in the optimised Hib assay. Favourable ethics opinions were gained from the Public Health Laboratory Service ethics committee, the East and North Hertfordshire Local Research Ethics Committee (LREC), and the Gloucestershire LREC EudraCT number: 2004-001049-14, see appendix II (Southern *et*

al., 2009; Borrow *et al*., 2010). For primary vaccination, three doses, 0.5 mL each, of Pediacel (Sanofi Pasteur) were given at 2, 3 and 4 months as per UK routine vaccination schedule. A booster dose of combined meningococcal serogroup C (MenC) and Hib (Menitorix) was given in the second year of life. A total of 280 samples taken 1 month post primary vaccination schedule were analysed in the Hib SBA assay (section 2.10). A total of 206 serum samples taken 1 month post booster were also assayed in the optimised Hib SBA assay.

2.12.2 Statistical Analysis

Geometric mean titres (GMT) with 95% confidence intervals (CI) were calculated for post primary and post booster responses. Secondly, SBA titres were compared to previously determined anti-PRP IgG concentrations determined by a Hib Bioplex assay (Standard Operating Procedure (SOP) 1646, HPA, Manchester) for correlation. The Pearson`s correlation coefficients (*r* value) for SBA titres and previously determined anti-PRP IgG antibody concentrations were calculated for both visits. The power function equation displayed on the top of the chart $y=a\ x^b$ (y=log transformed IgG concentration, log a=y intercept, x= log transformed SBA titre, b= slope) was used to calculate the predictive protective SBA titre that corresponds to the established long term protective antibody concentration of 1.0 µg/mL and short term protection of 0.15 µg/mL.

The optimised Hib assay (section 2.10) was altered to include the number of viable cfu per sample at T0 as well as T50. T0 time point was used as a control well in the optimised assay and to observe growth in column 11 and 12 as this was the first time infant sera was used in the assay. Statistical analysis was carried out for both time points. Following analysis, the assay was changed from having a 50 minute incubation period (T50) to a 60 minute incubation period (T60). This was changed to adapt the assay to ones currently used in the VEU after showing no difference in SBA titre between T50 to T60.

2.13 Vaccine failure samples assayed against strains with increasing copy numbers of the *cap* b locus

2.13.1 Strains and sera

Hib strains with various numbers of copies of the capsulation (*cap*) b locus have been isolated from patients with invasive disease. Hib strains containing increasing numbers of copies are shown to express more capsule and thought to play a role in some cases of Hib conjugate vaccine failure in children (Cerquetti *et al*., 2006). A total of 164 serum samples were previously collected from children who developed invasive Hib disease despite having being vaccinated against Hib (vaccine failures) The recruitment of these samples has been described elsewhere (Ladhani *et al*., 2010a). Hib vaccine failure was defined as invasive Hib disease occurring anytime after three vaccine doses given in the first year of life, or >1 week after two doses in the first year, or > 2 weeks after one dose given after 12 months of age. Briefly, cases diagnoses between October 1992 and December 2005 were identified through routine laboratory reporting, enhanced surveillance and clinical reporting schemes. Upon permission from their general practitioner, the parents of children with Hib vaccine failure allowed a blood sample to be obtained from the child. Appropiate ethics were gained from the Thames Valley Multi-centre Research Ethics Commtee (REC reference number: 05/MRE12/50) Listed in Appendix III. All vaccine failure samples were assayed in the optimised Hib SBA assay (section 2.10, with a change from T50 to T60 time point) against 5 strains of Hib containing either 1, 2 3, 4 or 5 copies of the cap b locus. Table 2.1 shows the strains used in the Hib SBA assay along with the number of copies of the *cap* b locus.

2.13.2 Statistical analysis

GMTs with 95% CI were calculated for samples assayed against each strain at T0 and T60 time points. The SBA titres for each strain were correlated against known anti-PRP IgG concentrations measured by a Hib bioplex assay (HPA, Manchester). To measure whether an increasing expression of capsule would have any importance functionally, the percentage of subjects achieving an SBA titre ≥8 were calculated for each different strain.

Table 2.1 Hib strains used to test vaccine failure samples

Assigned number	No of copies (*cap* b locus)
M07 240825	**1**
M07 240381	**2**
M07 240814	**3**
M07 240810	**4**
M07 240811	**4**
M07 240809	**4**
M07 240805	**5**

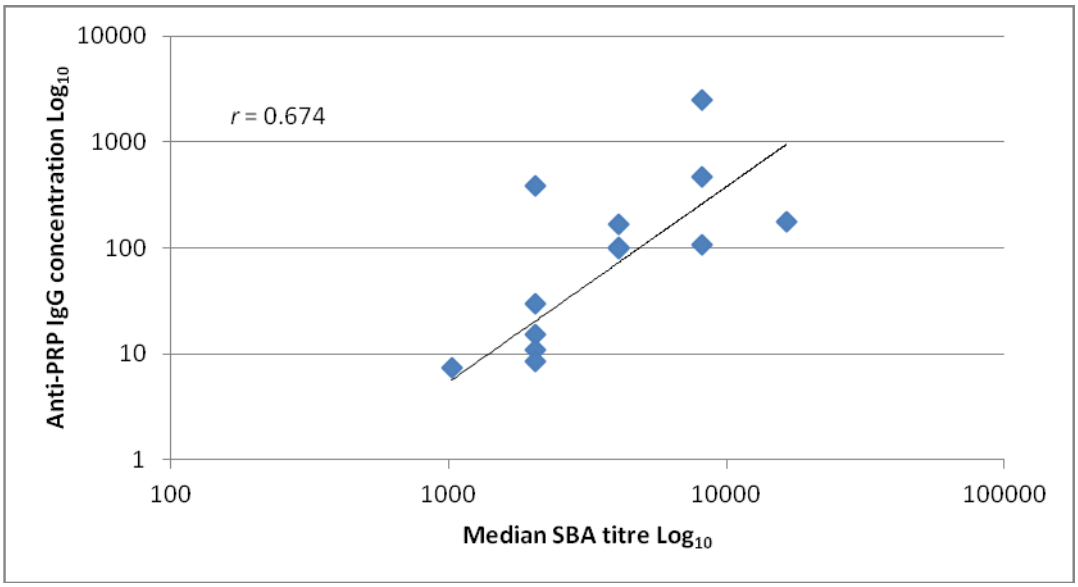
This table shows the Hib strains used to test vaccine failure samples. Three strains with four copies of the *cap* b locus were used, as strain M07 240811 became complement sensitive despite passing RC validation. Strains M07240810 and strain M07 240809 were used to re test the samples that shown higher titres than the other copy strains.

3.0 Results

3.1 Replication of CDC method

The first step to develop a Hib SBA assay was to replicate a previously published method from the CDC (Romero-Steiner *et al*., 2001) as detailed in section 2.8. A panel of 19 post Hib vaccination serum samples (detailed in section 2.2) and standard control serum (NIBSC code 96/536) were chosen to test the assays reproducibility and expected SBA titre ranges. Each serum sample was tested on three separate days/runs. SBA titre results were compared to previously determined anti-PRP IgG concentrations. Reproducibility was deemed acceptable if >85% of the test samples were within three SBA titre steps as calculated at T60. From the first run, five samples failed to meet assay acceptance criteria due to either producing multiple SBA titres or needed to be assayed at a higher starting dilution, and therefore removed from calculations. The remaining samples produced acceptable SBA titres from all three runs. From the three runs, 12/14 (86%) were within three T60 SBA titre steps of each other. Of the 14 serum samples, nine (64%) were within two T60 SBA titre steps. Two (14%) were within one T60 SBA titre step. As >85% of samples were within three T60 SBA titre steps the assay was deemed reproducible. The median of the three SBA titres generated in this method was compared to known anti-PRP IgG concentrations measured by a Hib bioplex assay. A good correlation was shown (r=0.674) as seen in figure 3.1.

Figure 3.1 Correlation of anti-PRP IgG concentrations and median SBA titres



This figure shows the good correlation (r = 0.674) between the median SBA titre generated by the CDC method to previously determined anti-PRP IgG concentrations.

3.2 Development of a Hib SBA assay by adaptation of the CDC method

The first step in the adaptation of the CDC method to mirror the meningococcal SBA assays currently used in the VEU (HPA, Manchester) was to change the procedure for growing bacteria to log phase, from broth-grown to agar plate-grown. A total of seven post-vaccination serum samples were assayed. The SBA titres of Hib grown to log phase on chocolate agar plates were compared to SBA titres of Hib grown to log phase in broth. Of the seven samples, one sample produced a titre greater than the highest dilution and was removed from calculations. Of the six samples to compare 5/6 (83%) were within three T60 SBA titre steps of each other. Five (83%) were within two T60 SBA titre steps of each other. Two (33%) were within one T60 SBA titre step. From these results the preparation of bacterial working stock and 4 hour growth conditions for exponential phase were changed to bacteria grown on agar as opposed to broth as 83% of titres were within two T60 SBA titre steps.

3.3 Further optimisation of the Hib SBA assay

The previous CDC Hib SBA method was adapted to mirror the meningococcal SBA assays used in the VEU (Borrow *et al*., 2005). Various assay parameters were investigated to optimise and produce a reproducible assay.

3.3.1 Comparison of complement concentration

Baby rabbit complement (RC) is widely used in SBA assays for the licensure of meningococcal polysaccharide and glycoconjugate vaccines. It is commercially available in large batches and avoids the difficulties obtaining complement from human donors (Gill *et al*., 2011). The previously published CDC method has a final complement concentration of 31.3% (total well volume 80 µL) but uses 25 µL of RC per well. A volume that required

less complement per well but was still in excess of that required to give full complement-mediated bactericidal activity was investigated. The ratio of serum/buffer/target strain/complement concentration was altered to produce complement concentrations of 29.6% (total well volume 67.5 µL), 33.3% (total well volume 60 µL) and 37.5% (total well volume 40 µL). A complement concentration of 25% (total well volume 80 µL) was also investigated but was shown not to be sufficient enough producing inconsistent SBA titres. A total of seven adult post Hib vaccination samples were assayed at all three various concentrations. SBA titres were compared to titres produced from that of the CDC method.

Of the seven samples, one sample failed to meet assay acceptance criteria as it was greater than the top dilution and so was removed from the calculations. Of the remaining samples 5/6 (83%) were within 3 T60 SBA titre steps of each other. One (17%) was within 2 T60 SBA titre steps. All three complement concentrations produced titres consistent with CDC method and therefore a complement concentration of 33.3% was chosen. At 33.3% the total well volume is reduced to 60 µL. This uses less complement per well than the CDC method and thus sera can be assayed at a lower initial starting dilution.

3.3.2 Comparing the incubation time of serum/buffer/target strain/complement reaction

During replication of CDC method and development of the initial Hib SBA, non complement mediated lysis was observed in the control well (column 12) at T60. A total of eight samples were assayed. The total viable cells (column 11) was plated out for each sample at T0 and T60 to investigate whether this was true non complement mediated lysis or a reduced growth rate compared to column 11. From the eight samples assayed there

was an 8-33% increase in cfu from T0 – T60 in column 11. The average increase was 20%. Data are shown in Table 3.1. The assay acceptance criteria are set that the number of cfu in complement control wells (column 10, column 12) should lie within -30% of the number of cfu in column 11. From the eight samples, four failed for column 12 as they were less that 30% of column 11 at T60.When calculated from T0, the cfu in column 12 was not less than 30% of column 11. The column 12 fails were not treated as true non complement mediated lysis after the above observation and the fact that all samples had been assayed with beta lactamase which should inhibit any antibiotics in the sera which would cause non complement mediated lysis. The >30% reduction in column 12 was in part due to the average 20% increase in cfu from T0-T60 in column11.

From this observation incubation of serum/buffer/target strain/complement was investigated to find an incubation time that produced reproducible SBA titres and did not cause an increased cfu growth in column 11. A total of eight post Hib vaccination samples were assayed at a complement concentration of 33.3% and incubated for 30, 40, 50, and 60 minutes after the addition of sera/buffer/target strain/complement. Titres, column 12 cfu, and column 11 cfu were compared for all four incubation times.

Figure 3.2 shows the comparison of the SBA titres when assayed with incubation times of T30, T40, T50 and T60. Of the samples tested, 7/8 had increased titres from T30 to T60. The greatest increase from T30-T60 was 4 titres shown in samples 009B and 013B. One sample, 014B, produced the same titre from T30-T60. All titres were within 3 SBA titre steps between T50-T60. The assay at T30 was producing positive titres but the killing curves produced for each sample were not as consistent as they were at T50 and T60.

Table 3.2 shows the average column 11 and column 12 cfu growth from T30-T60. The average column 11 cfu at T30 and T60 were 104 and 135, respectively. The cfu in column 11 increased by 30% from T30 to T60. The average cfu in column 12 at T30 and

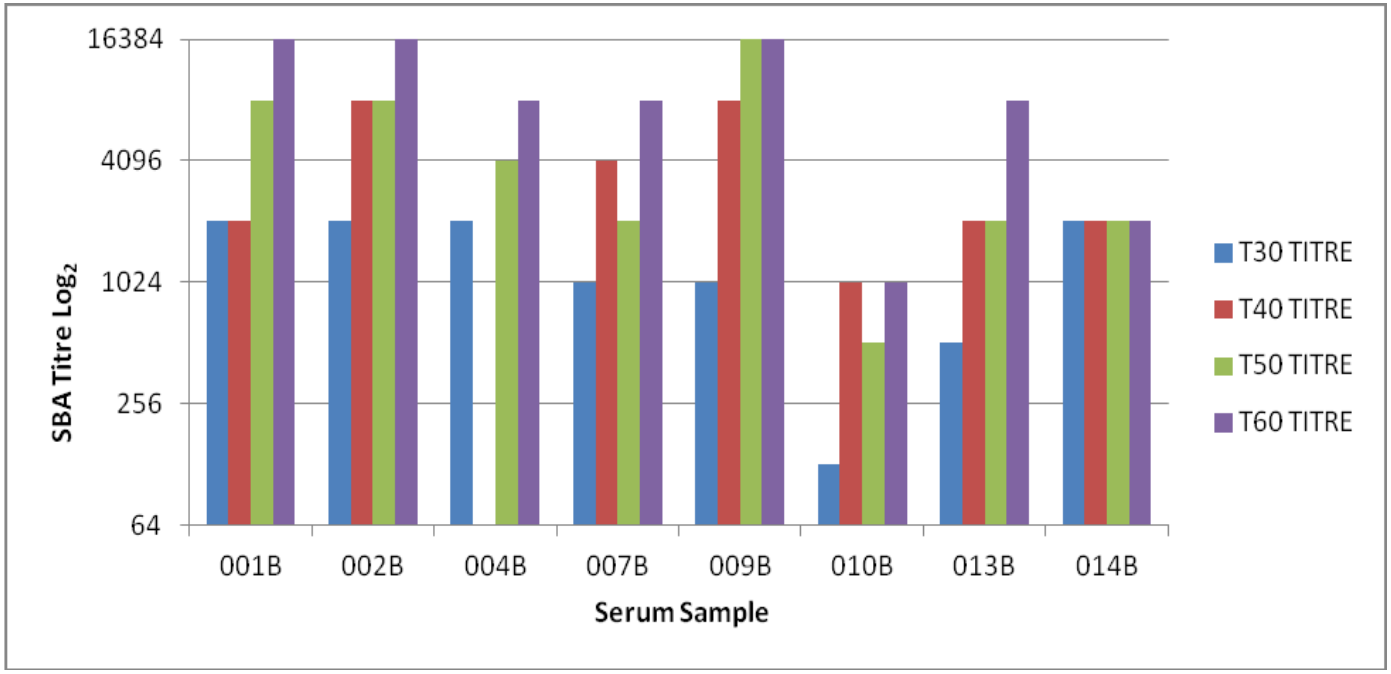
T60 was 88 and 87, respectively. There was a 1% decrease in cfu in column 12 from T30 toT60. Because of the average 30% increase in cfu in column 11 at T60, 6/8 samples failed for column 12 at T60. Secondly, 4/8 samples failed for column 12 at T50. One sample failed for column 12 at both T30 and T40. Therefore the cfu in column 12 did not increase at the rate as cfu in column 11 did from T30 to T60. The cfu in column 12 stays relatively the same. This seemed to be causing column 12 to fail the acceptance criteria at T60. The average percentage decrease from cfu in columns 11 to column 12 for T30, T40, T50 and T60 were 15%, 20%, 34% and 36%, respectively. Only the average T30 and T40 column 12 were found to lie within -30% of its respected column 11. At T50 the average % decrease from column 11 to column 12 was 34%. Although at T50 the decrease was >30%, the assay was changed from a T60 incubation to T50 incubation as all samples produced titres within three SBA titre steps of each other, and less column 12 fails than T60. Preliminary data of running the assay at T40 showed samples often failing acceptance criteria due to producing inconsistent titres. This incubation period was not activating enough complement to produce the maximum titre for each sample consistently (data not shown).

Table 3.1 Viable cfu (column 11) percentage increase from T0 to T60

SAMPLE	T0 COLUMN 11	T60 COLUMN 11	% INCREASE
001B	105	116	10
002B	92	111	21
004B	96	123	28
007B	110	130	18
009B	101	134	33
010B	112	135	21
013B	109	118	8
014B	98	119	21
Average	103	123	20

This table shows the viable cfu (column 11) percentage increase from T0 to T60 from eight samples assayed on the same run. The average increase was 20%, ranging from 8% to 33%.

Figure 3.2 SBA titres generated with varying incubation times (T30, T40, T50 and T60)



This figure shows the difference in SBA titres for each sample when calculated from T30, T40, T50 and T60 time points. Due to 004B failing assay acceptance criteria at T40 no data are shown.

Table 3.2 The average column 11 and 12 cfu at T30, T40, T50, and T60

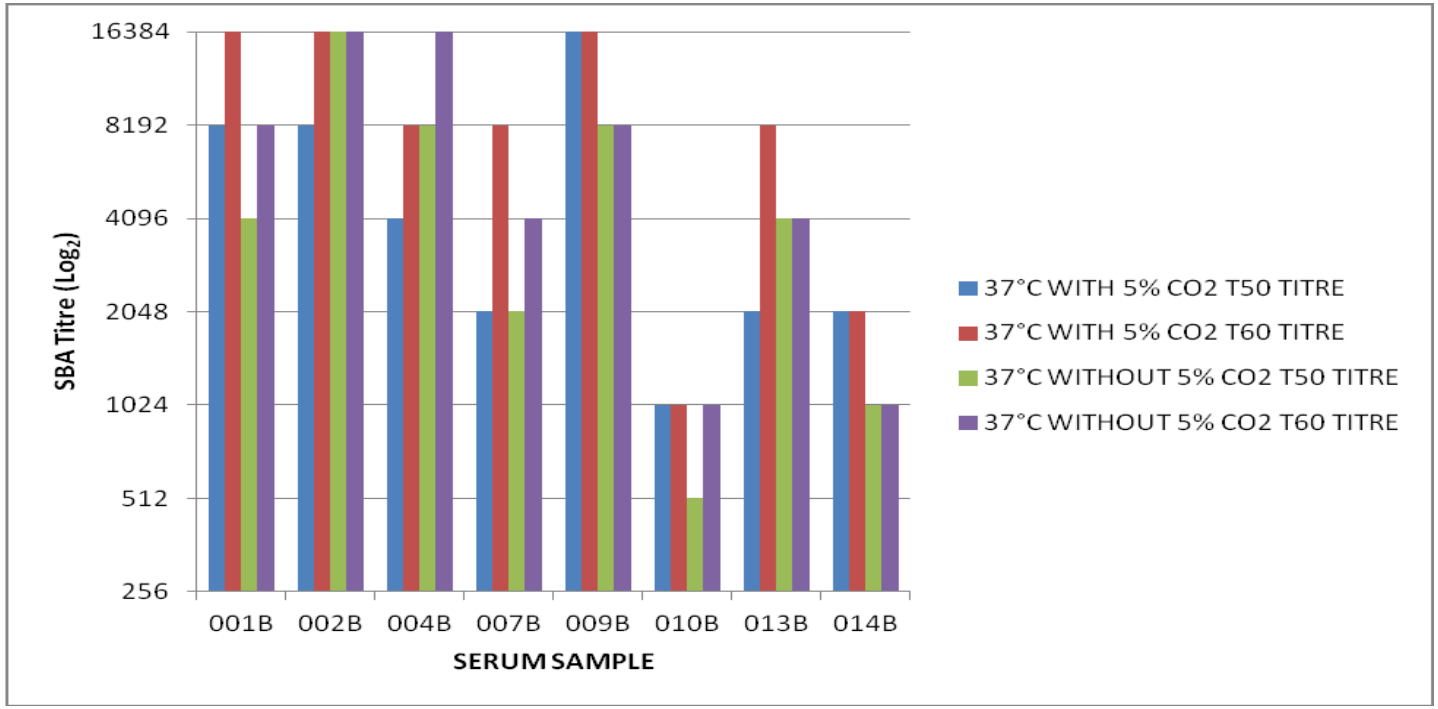
SAMPLE	T30 COL 11	T40 COL 11	T50 COL 11	T60 COL 11
001B	111	108	121	130
002B	93	129	126	144
004B	97	102	105	123
007B	116	125	109	142
009B	96	125	149	132
010B	104	116	123	131
013B	107	113	118	134
014B	108	120	139	141
AVERAGE	**104**	**117**	**124**	**135**
SAMPLE	T30 COL 12	T40 COL 12	T50 COL 12	T60 COL 12
001B	91	85	85	91
002B	77	86	78	94
004B	93	103	80	105
007B	80	88	72	74
009B	83	92	89	64
010B	102	97	89	90
013B	90	99	67	86
014B	91	99	96	94
AVERAGE	**88**	**94**	**82**	**87**
AVERAGE % DECREASE FROM COLUMN 11 TO COLUMN 12	**15%**	**20%**	**34%**	**36%**

Table 3.2 Shows the percentage difference between column 11 and 12 at T30, T40, T50 and T60 time points. Only T30 and T40 time points have cfu in column 12 which lie within - 30% of column 11. A 30% increase is shown in the average cfu in column 11 over the 60 minute incubation period. The cfu in column 12 stayed relatively the constant.

3.3.3 Comparison of incubation period with 5% CO_2 and without 5% CO_2

The CDC Hib SBA assay incubates microtitre plates in a 37°C, 5% CO_2 environment for 60 minutes after the addition of serum/buffer/target strain and complement (Section 2.8). As the microtitre plates are sealed during this incubation step, a 5% CO_2 environment may not be necessary. A set of seven post Hib vaccination samples were assayed as detailed in section 2.10 at a 33.3% complement concentration for 50 or 60 minutes with and without 5% CO_2 and SBA titres were compared (figure 3.3). All T50 and T60 titres assayed with and without 5% CO_2 were within 1 SBA titre step of each other. Moreover, all T50 and T60 titres under the same conditions were within 2 SBA titre steps of each other. From these results the assay was changed to a 50 minute incubation period without a 5% CO_2 environment as it did not seem to have an effect on the titres produced. In addition to this no significant difference in titres are seen between T50 and T60 for this environment.

Figure 3.3 SBA titres achieved with incubation titres of T50 and T60 with and without a 5% CO_2 environment



This figure shows that similar SBA titres were gained with or without a 5% CO_2 environment.

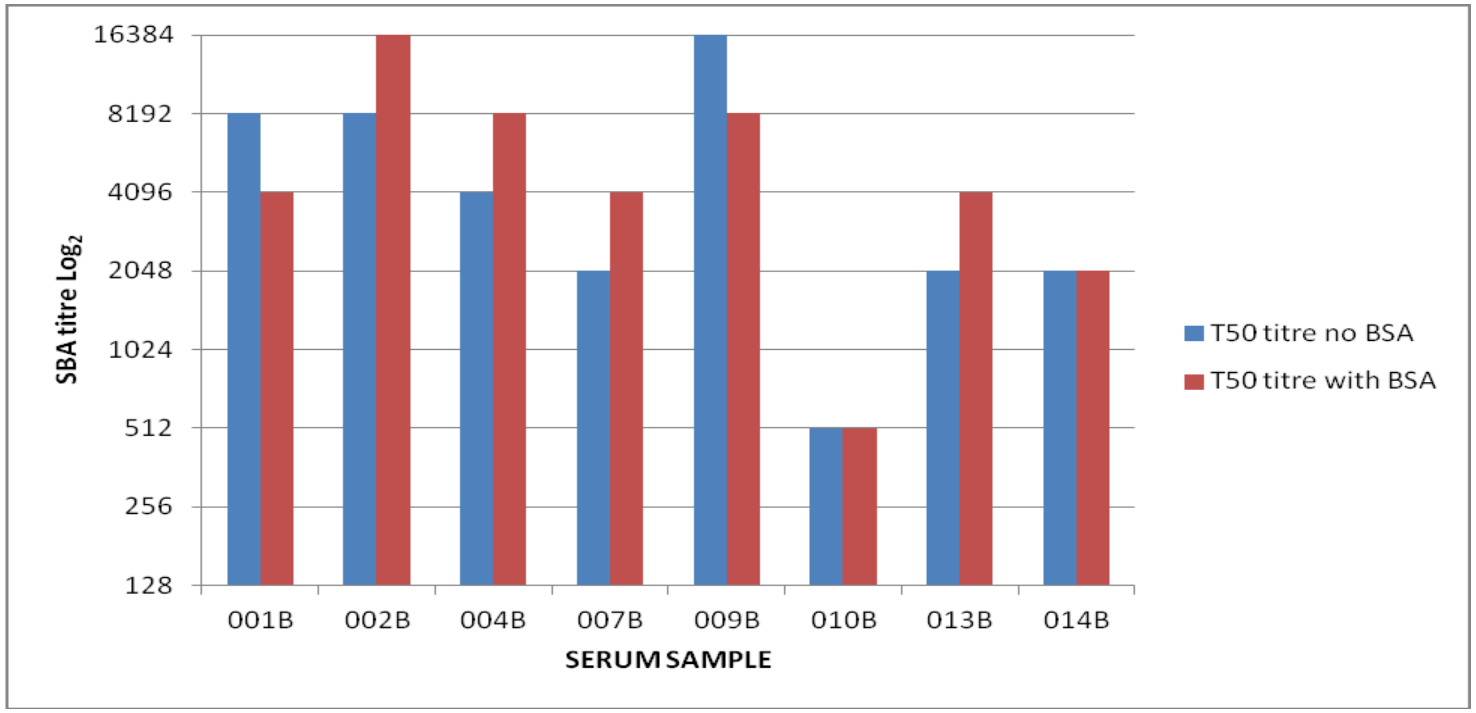
3.3.4 Comparison of the media used in the Hib SBA assay

Due to the observation of inhibited growth in column 12, two different manufacturers of chocolate agar plates (Oxoid and Biomérieux) were investigated for use in the assay. To investigate which growth medium would produce maximum colonies, nineteen samples were assayed. Although no titre differences were observed, Biomérieux agar supported bacterial growth better, producing more colonies and was selected for use in further optimisation.

3.3.5 Comparison of the use of BSA in bactericidal buffer

As BSA has been used in immunoassays to protect bacteria from non specific killing (Maslanka *et al*., 1997), 21 pre and post-vaccination serum samples were assayed on two separate days with BB containing a 2% BSA (w/v) concentration to observe whether this would protect against non specific killing and promote growth in column 12. Of the 21 samples assayed no column 12 fails were seen when using BB with BSA on both days. Secondly, eight of the known positive serum samples were compared to previously determined titres gained from assaying at T50 without BSA to see if the BSA would affect titres. All eight samples were within one T50 SBA titre step from each other. What is more, 4/8 samples had column 12 fails using BB without BSA. No column 12 fails were observed in the eight samples using BB containing BSA. As the BSA was found to support bacterial growth in column 12 and no significant difference in titres, BB with BSA was selected for further optimisation. Data are shown in figure 3.4.

Figure 3.4 Comparison of the SBA titre with and without use of BSA in the bactericidal buffer



This figure shows the titre comparison of samples assayed with BSA and without BSA. All samples were within 1 SBA titre step of each other.

Neither produced consitiently higher titres than the other. BB containing BSA was selected for further optimisation.

3.3.6 Validation of the specific optical density and its correlation to inoculum count

The assay was changed to increase the volume of reaction mixture in the tilt method from the CDC method of 7.5 µL to 10 µL. As the assay had now changed during optimisation, it was important to validate the suspension of Hib at an optical density (OD) of 0.1. An inoculum count between 150-200 cfu per well has shown to make counting inaccurate (Martin *et al*., 2005). A heavy inoculum count can make it harder to distinguish separate colonies and may have an effect on titres. Likewise, small errors in dilution technique or contaminants can have a large effects on counts of <60 cfu per well. The WHO recommends a target cell number of 100 cfu per well for meningococcal SBA assays (WHO, 1976). A count between 100-150 cfu per well was investigated and validated for the suspension at the OD of 0.1 at 650 nm. Colony forming unit counts are shown in table 3.3. As a 1/30 dilution produced cfu of 100-150 it was chosen for use in the assay. Calculating from the dilution factors, the liquid Hib culture suspension at A_{650} =0.1 contains approximately 2.5 x 10^8 cfu/mL.

Table 3.3 Number of cfu of Hib at dilutions of 1/30, 1/40 and 1/50 (OD of 0.1 read at 650nm) for both T0 and T60

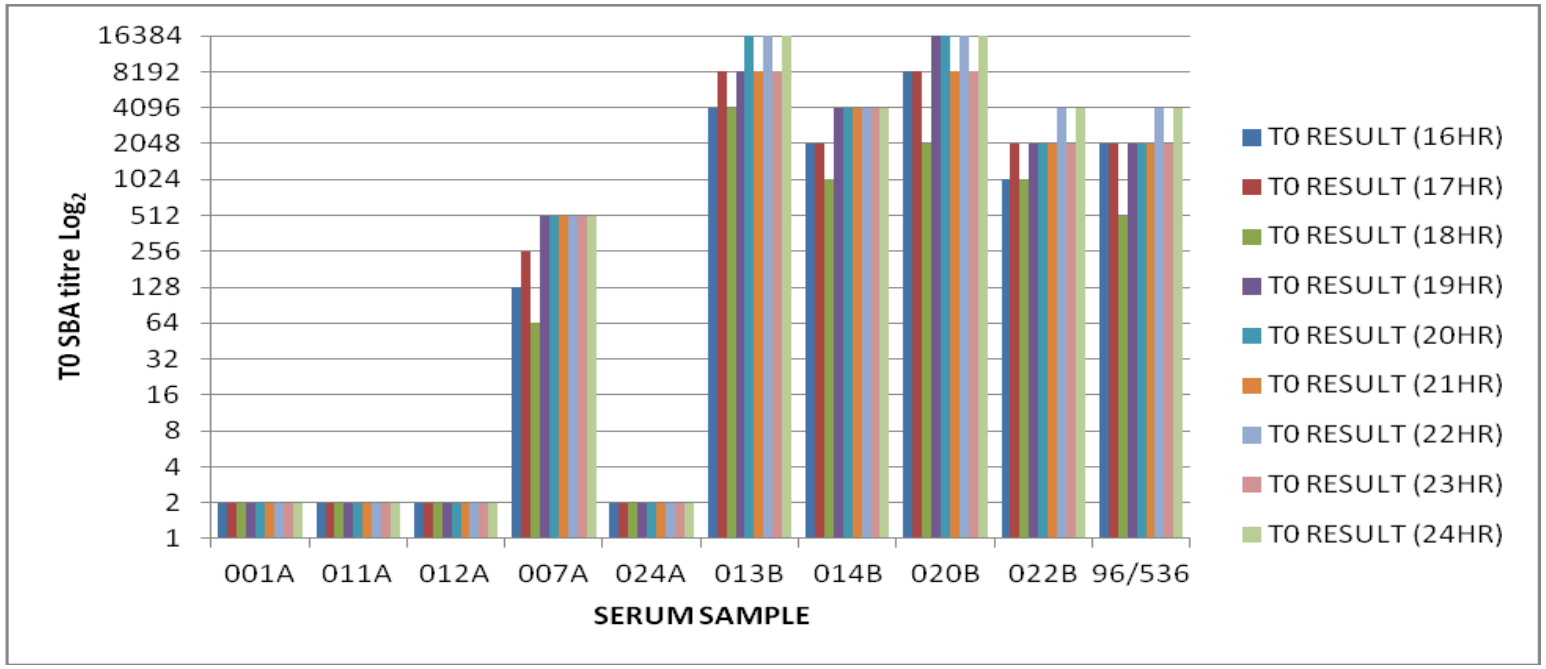
TIMEPOINT	DILUTION	COUNT	MEAN COUNT
T0	1/30	111	**126**
		128	
		129	
		132	
		128	
	1/40	93	**90**
		94	
		91	
		82	
		88	
	1/50	99	**99**
		110	
		98	
		100	
		89	
T60	1/30	141	**140**
		139	
		139	
		146	
		136	
	1/40	101	**102**
		96	
		100	
		99	
		114	
	1/50	116	**112**
		118	
		118	
		120	
		87	

This table shows the cfu counts at dilutions of 1/30, 1/40 and 1/50. As a 1/30 dilution produced cfu of 100-150 it was chosen for use in the assay.

3.3.7 Comparison of the incubation length of the target strain prior to use in the Hib SBA assay

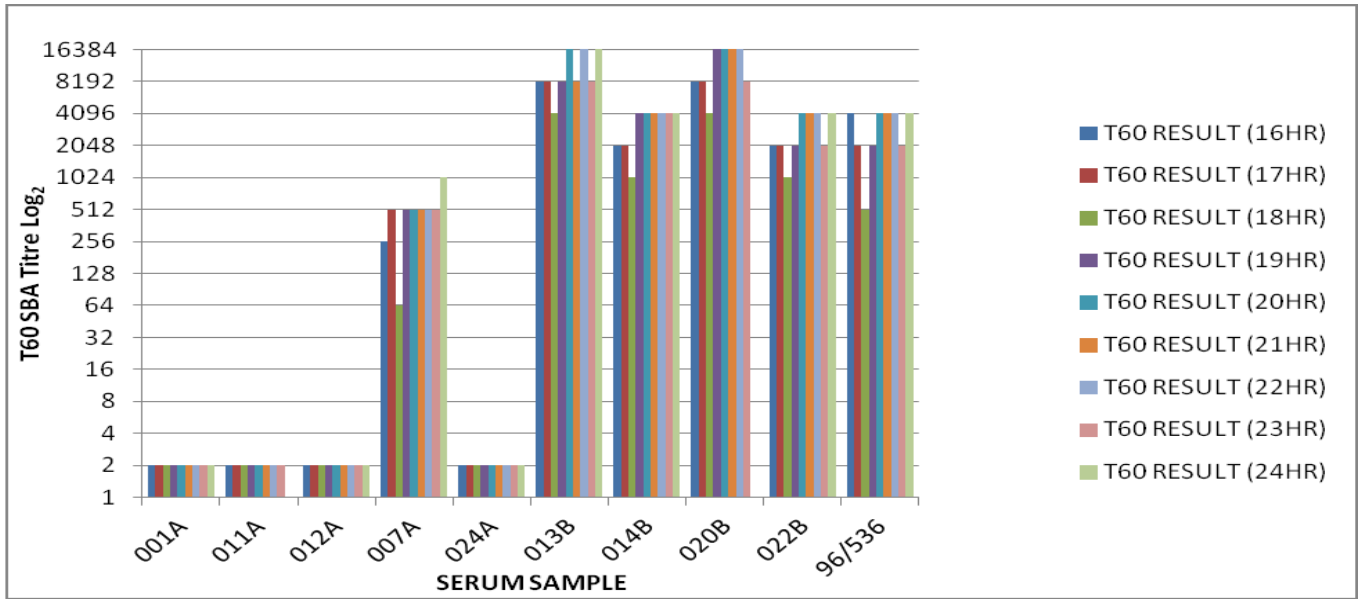
In the Hib SBA assay, the target strain is incubated for a minimum of 16 hours for bacterial growth prior to use in the assay. As the incubation time of the target strain can have an effect on bacterial surface antigen expression and growth, a suitable incubation period that would not have an effect on the SBA titre was investigated. A total of eleven pre and post Hib vaccination serum samples were assayed in the optimised Hib SBA to compare SBA titres when using the Hib target strain incubated for either 16, 17, 18, 19, 20, 21, 22, 23 or 24 hours prior to use in the assay. One sample was removed from calculations as it failed assay acceptance criteria for being greater than the top dilution at both time points. Two samples also failed to get a result at 24 hours at T60, one due to inconsistent titres produced and one sample exceeding the top dilution. As most samples were producing results within similar titre steps of each other, a time of 16 to 24 hour growth of the target strain prior to use in the assay was used. Data for T0 are presented in figure 3.5 and for T60 are presented in figure 3.6.

Figure 3.5 Comparison of SBA titres calculated at T0 for each serum sample, when using the target strain M07 240381 which had been incubated at 16 to 24 hours



A total of 7/10 samples produced SBA titre steps that are were within three SBA titres of each other when incubated for 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours. Therefore a 16-24 hour incubation time was acceptable for growth prior to use in the Hib SBA assay.

Figure 3.6 Comparison of SBA titres calculated at T60 for each serum sample, when using the target strain M07 240381 which had been incubated at 16 to 24 hours



A total of 8/10 samples produced SBA titre steps that are were within three SBA titres of each other when incubated for 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours. Therefore a 16-24 hour incubation time was acceptable for growth prior to use in the Hib SBA assay. No data are shown for samples 011A and 020B at 24 hours incubation due to inconsistent titres produced and exceeding the top dilution, respectively.

3.4 Validation of the Hib SBA assay

The Hib SBA assay was validated by evaluating the assay parameters of specificity, accuracy, assay precision/reproducibility and linearity.

3.4.1 Specificity, inhibition

Results for inhibition are shown in table 3.4. Nineteen post Hib vaccination samples were inhibited with 200 μg/mL of Hib polysaccharide (homologous) and Men C polysaccharide (heterologous). A hundred percent (19/19) of homologous (Hib polysaccharide) inhibited results had a reduction of >2 SBA titres. Whilst 95% (18/19) of heterologous (Men C polysaccharide) inhibited titres were ≤2 titres of the neat titre. Both homologous and heterologous inhibition exceed the acceptance criteria of ≥85%. Therefore Hib strain M07 240381 passed this validation acceptance criterion.

Table 3.4 Hib SBA assay validation-inhibition analysis with homologous Hib polysaccharide and heterologous Men C polysaccharide

	Hib			Men C	
Sample	**Neat titre result (no inhibitor)**	**Inhibited titre**	**Titre difference**	**Inhibited titre**	**Titre difference**
001B	4096	<8	-10	2048	-1
002B	4096	<8	-10	8192	1
004B	2048	<8	-9	4096	1
007B	4096	<8	-10	1024	-2
009B	16384	<8	-12	16384	0
020B	2048	<8	-9	2048	0
05B	512	<8	-7	512	0
013B	4096	<8	-10	2048	-1
031B	4096	<8	-10	4096	0
017B	16384	<8	-12	16384	0
027B	4096	<8	-10	4096	0
014B	4096	<8	-10	2048	-1
018B	256	<8	-6	256	0
022B	1024	<8	-8	1024	0
023B	2048	<8	-9	1024	-1
024B	16384	<8	-12	8192	-1
025B	4096	<8	-10	4096	0
026B	2048	<8	-9	2048	0
035B	2048	<8	-9	128	-3
Total number of results			19	-	19
No. of results with reduction >2 titres			19	-	1
No. of results with reduction of < 2 titres			0	-	18
Percentage of sample results within acceptance criteria			100%	-	95%

Both homologous and heterologous inhibition exceeds the acceptance criteria of ≥85%. Specificity was therefore deemed acceptable.

3.4.2 Specificity, spiking

Known Hib SBA negative samples were spiked with a known Hib SBA positive sample in three different ratios. The spiked negative results (x dilution factor) were compared to the SBA positive sample result. Results for spiking are presented in table 3.5. Ninety three percent of the results (x the dilution factor) were within the acceptance criteria of ±1 SBA titre of the neat positive sample result. Samples with no detectable SBA titre were expressed as <8. Specificity was deemed acceptable as Hib strain M07 240381 exceeded the acceptance criteria of ≥85%.

Table 3.5 Hib SBA assay validation- Spiking a known negative SBA sample with a known positive SBA sample

Observed Titres						**Spiked result x dilution factor**			**Difference in SBA titre between spiked result (x dilution factor) and neat +ve result**		
Spiked samples						**1:2**	**1:4**	**1:8**			
Negative Sample	Neat –ve titre	Neat +ve titre (017B)	1:2	1:4	1:8	(x2)	(x4)	(x8)	**1:2**	**1:4**	**1:8**
001A	<8	8192	8192	4096	1024	16384	16384	8192	+1	+1	0
002A	<8	8192	4096	2048	4096	8192	8192	32768	0	0	+2
004A	<8	32768	16384	8192	8192	32768	32768	65536	0	0	+1
009A	<8	8192	8192	2048	16384	16384	8192	131072	+1	0	+4
020A	<8	32768	16384	16384	4096	32768	65536	32768	0	1	0
Total no of results									15		
No of results (x dilution factor) within +/- 1 SBA titre of +ve sample									13		
Percentage of results within the acceptance criteria (results (x dilution factor) within ± 1 SBA titre of neat +ve titre)									86.6%		

This table shows that validation passed the acceptance criteria as ≥85% of results (x dilution factor) were within ±1 SBA titre of the neat positive sample titre.

3.4.3 Accuracy

Spiking analysis (section 3.4.2) was repeated five times to measure accuracy. Of the results 91%, (x dilution factor) were within ±1 SBA titre of the neat positive titre. This exceeds the acceptance criteria of ≥85%. All negative samples (100%) had a titre of <8. In addition to this 89% of results were within the acceptance criteria of ±1 SBA titre of the median (for that negative sample for that spiking ratio). Hib strain M07 240381 exceeded the acceptance criteria of ≥85% and therefore passed validation. The results are presented in table 3.6

3.4.4 Intra assay/inter assay precision

Intra and inter assay reproducibility are used to measure assay reproducibility and operator reproducibility. For intra assay reproducibility, 36 samples were assayed 5 times on the sameday/run. Of the 36 samples assayed, 98% of the results were within the acceptance criteria of ≥85% (table 3.7). Therefore Hib strain M07 240381 passed the intra assay validation acceptance criteria.

For inter assay reproducibility, 39 samples were assayed to gain 5 results on different runs/days . Of the results, 92% were within the acceptance criteria of within ± 1 SBA titre of the median (table 3.8). This exceeds the acceptance criteria of >85%. Hib strain M07 240381 passed the validation acceptance criteria for this parameter.

Table 3.6 Hib SBA assay validation- Accuracy analysis

Negative sample	Replicate	Observed Titres					Spiked result x dilution factor			Difference in SBA titre between spiked result (x dilution factor) and neat +ve result		
		Neat (-ve) titre	Neat (+ve) titre	Spiked samples			1:2	1:4	1:8	1:2	1:4	1:8
				1:2	1:4	1:8	(x 2)	(x 4)	(x 8)			
002A	1	<8	16384	8192	4096	1024	16384	16384	8192	0	0	-1
	2	<8	16384	4096	4096	1024	8192	16384	8192	-1	0	-1
	3	<8	16384	4096	4096	2048	8192	16384	16384	-1	0	0
	4	<8	8192	4096	1024	1024	8192	4096	8192	0	-1	0
	5	<8	16384	4096	4096	4096	8192	16384	32768	-1	0	+1
	Median SBA titre						8192	16384	8192	n/a		
009A	1	<8	16384	16384	8192	4096	32768	32768	32768	+1	+1	+1
	2	<8	32768	16384	8192	4096	32768	32768	32768	0	0	0
	3	<8	16384	8192	4096	4096	16384	16384	32768	0	0	+1
	4	<8	16384	8192	2048	512	16384	8192	4096	0	-1	-2
	5	<8	16384	4096	4096	1024	8192	16384	8192	-1	0	-1
	Median SBA titre						16384	16384	32768	n/a		
011A	1	<8	16384	16384	8192	4096	32768	32768	32768	+1	+1	+1
	2	<8	32768	8192	8192	4096	16384	32768	32768	-1	0	0
	3	<8	8192	4096	2048	4096	8192	8192	32768	0	0	+2
	4	<8	16384	8192	4096	2048	16384	16384	16384	0	0	0
	5	<8	16384	16384	8192	2048	32768	32768	16384	+1	+1	0
	Median SBA titre						16384	32768	32768	n/a		
012A	1	<8	16384	8192	2048	1024	16384	8192	8192	0	-1	-1

Table 3.6 Hib SBA assay validation- Accuracy analysis (continued)

012A	2	<8	32768	16384	16384	16384	32768	65536	131072	0	+1	+2
	3	<8	16384	4096	4096	4096	8182	16384	32768	-1	0	+1
	4	<8	16384	4096	4096	1024	8192	16384	8192	-1	0	-1
	5	<8	8192	4096	2048	2048	8192	8192	16384	0	0	+1
	Median SBA titre						8192	16384	16384	n/a		
001A	1	<8	16384	4096	2048	512	8192	8192	2048	-1	-1	-3
	2	<8	16384	4096	2048	1024	8192	8192	4096	-1	-1	-2
	3	<8	8192	4096	2048	1024	8192	8192	4096	0	0	-1
	4	<8	16384	8192	2048	1024	16384	8192	4096	0	-1	-2
	5	<8	4096	4096	4096	2048	8192	16384	8192	+1	+2	+1
	Median SBA titre						8192	8192	4096	n/a		
Total no. of results (spiked result x dilution factor)							75			n/a		
No. of results (spiked result x dilution factor) within ± 1 SBA titre of the median							67			n/a		
Percentage of results within the acceptance criteria (± 1 SBA titre of the median)							89%			n/a		
Total no. of results (Difference in SBA titre between spiked and neat result)							n/a			75		
No. of results (x dilution factor) within ±1 SBA titre of neat +ve sample titre							n/a			68		
Percentage of results within the acceptance criteria (results (x dilution factor) within ±1SBA titre of the neat +ve titre)							n/a			91%		

Validation passed as acceptance criteria was > 85 % of results (spiked result x dilution factor) within ± 1 SBA titre of the median AND > 85 % of results (x dilution factor) within ±1 SBA titre of the neat +ve sample titre

Table 3.7 Hib SBA assay validation- Intra assay precision

	Replicate							
Sample	1	2	3	4	5	Median	No. of samples within ±1 titre from median including negative results	No. of samples within ±1 titre from median excluding negative results
001A	<8	<8	<8	<8	<8	<8	5/5	n/a
002A	<8	<8	<8	<8	<8	<8	5/5	n/a
004A	<8	<8	<8	<8	<8	<8	5/5	n/a
007A	256	256	256	256	256	256	5/5	5/5
009A	<8	<8	<8	<8	<8	<8	5/5	n/a
011A	<8	<8	<8	<8	<8	<8	5/5	n/a
013A	<8	<8	<8	<8	<8	<8	5/5	n/a
014A	512	512	1024	1024	512	512	5/5	5/5
017A	<8	<8	<8	<8	<8	<8	5/5	n/a
018A	512	128	512	512	512	512	4/5	4/5
020A	<8	<8	<8	<8	<8	<8	5/5	n/a
022A	<8	<8	<8	<8	<8	<8	5/5	n/a
024A	<8	<8	<8	<8	<8	<8	5/5	n/a
026A	<8	<8	<8	<8	<8	<8	5/5	n/a
027A	<8	<8	<8	<8	<8	<8	5/5	n/a
028A	<8	<8	<8	<8	<8	<8	5/5	n/a
032A	<8	<8	<8	<8	<8	<8	5/5	n/a
002B	2048	4096	4096	4096	4096	4096	5/5	5/5
004B	16384	16384	16384	8192	4096	16384	5/5	5/5
007B	1024	2048	4096	2048	2048	2048	5/5	5/5
009B	8192	16384	16384	32768	32768	16384	5/5	5/5

Table 3.7 Hib SBA assay validation- Intra assay precision (continued)

010B	512	1024	1024	512	512	512	5/5	5/5
013B	4096	4096	4096	4096	4096	4096	5/5	5/5
014B	4096	4096	4096	2048	4096	4096	5/5	5/5
017B	32768	65536	65536	32768	32768	32768	5/5	5/5
018B	1024	1024	2048	2048	2048	2048	5/5	5/5
020B	4096	4096	4096	8192	8192	4096	5/5	5/5
022B	1024	512	1024	1024	1024	1024	5/5	5/5
023B	2048	4096	4096	4096	4096	4096	5/5	5/5
024B	16384	8192	8192	8192	8192	8192	5/5	5/5
025B	2048	2048	2048	1024	2048	2048	5/5	5/5
026B	2048	4096	4096	4096	4096	4096	5/5	5/5
027B	16384	16384	16384	32768	16384	16384	5/5	5/5
028B	4096	1024	4096	8192	8192	4096	4/5	4/5
05B	1024	1024	2048	1024	1024	1024	5/5	5/5
035B	2048	2048	2048	1024	512	2048	4/5	4/5
Total number of results							180	110
No. of results within the acceptance criteria (within ±1 SBA titre of the median)							177	107
percentage of results within the acceptance criteria							98%	97%

Validation passed as acceptance criteria was ≥85% of results (excluding negative results) within ±1 SBA titre of the median. Where negative samples were excluded from results not applicable (N/A) was inserted in to the table.

Table 3.8 Hib SBA assay validation- Inter assay precision

	Replicate							
Sample	1	2	3	4	5	Median	No. of samples within ±1 titre from median including negative results	No. of samples within ±1 titre from median excluding negative results
001A	<8	<8	<8	<8	<8	<8	5/5	n/a
002A	<8	<8	<8	<8	<8	<8	5/5	n/a
003A	<8	<8	<8	<8	<8	<8	5/5	n/a
004A	<8	<8	<8	<8	<8	<8	5/5	n/a
007A	<8	<8	<8	<8	<8	<8	5/5	n/a
009A	<8	<8	<8	<8	<8	<8	5/5	n/a
011A	<8	<8	<8	<8	<8	<8	5/5	n/a
012A	<8	<8	<8	<8	<8	<8	5/5	n/a
013A	<8	<8	<8	<8	<8	<8	5/5	n/a
016A	<8	<8	<8	<8	<8	<8	5/5	n/a
017A	<8	<8	<8	<8	<8	<8	5/5	n/a
020A	<8	<8	<8	<8	<8	<8	5/5	n/a
021A	<8	<8	<8	<8	<8	<8	5/5	n/a
022A	<8	<8	<8	<8	<8	<8	5/5	n/a
024A	<8	<8	<8	<8	<8	<8	5/5	n/a
026A	<8	<8	<8	<8	<8	<8	5/5	n/a
027A	<8	<8	<8	<8	<8	<8	5/5	n/a
028A	<8	<8	<8	<8	<8	<8	5/5	n/a
032A	<8	<8	<8	<8	<8	<8	5/5	n/a
001B	8192	8192	4096	4096	2048	4096	5/5	5/5
002B	16384	8192	8192	4096	4096	8192	5/5	5/5

Table 3.8 Hib SBA assay validation- Inter assay precision (continued)

004B	2048	8192	1024	16384	8192	8192	3/5	3/5
007B	2048	2048	8192	8192	4096	4096	5/5	5/5
013B	4096	4096	4096	8192	1024	4096	4/5	4/5
014B	2048	1024	2048	2048	1024	2048	5/5	5/5
017B	32768	32768	16384	16384	8192	16384	5/5	5/5
018B	512	1024	512	1024	2048	1024	5/5	5/5
020B	4096	4096	4096	4096	2048	4096	5/5	5/5
022B	2048	1024	2048	2048	1024	2048	5/5	5/5
023B	2048	2048	2048	2048	2048	2048	5/5	5/5
024B	8192	8192	8192	8192	1024	8192	4/5	4/5
025B	2048	2048	2048	2048	4096	2048	5/5	5/5
026B	2048	16384	4096	2048	2048	2048	4/5	4/5
027B	4096	16384	65536	32768	32768	32768	4/5	4/5
028B	8192	4096	2048	2048	16384	4096	4/5	4/5
030B	128	128	128	512	128	128	5/5	5/5
031B	4096	8192	4096	8192	2048	4096	5/5	5/5
05B	1024	512	1024	2048	2048	1024	5/5	5/5
035B	2048	2048	4096	2048	512	2048	4/5	4/5
Total number of results							195	100
No. of results within the acceptance criteria (within ±1 SBA titre of the median)							187	92
percentage of results within the acceptance criteria							96.0%	92%

Validation passed as acceptance criteria was ≥85% of results (excluding negative results) within ±1 SBA titre of the median

3.4.5 Linearity/recovery analysis

Eighty nine percent of results were within the acceptance criteria of within ±1 SBA titre of the median. As this is greater than the acceptance criteria of ≥85%, Hib strain M07 240381 passed the validation (table 3.9). SBA titres for each sample (diluted result x dilution factor and undiluted) were plotted in a scatter graph to show the correlation between the neat and the recovery of the titre. A strong correlation was shown (r= 0.89) as shown in figure 3.7.

Table 3.9 Hib SBA assay validation- linearity

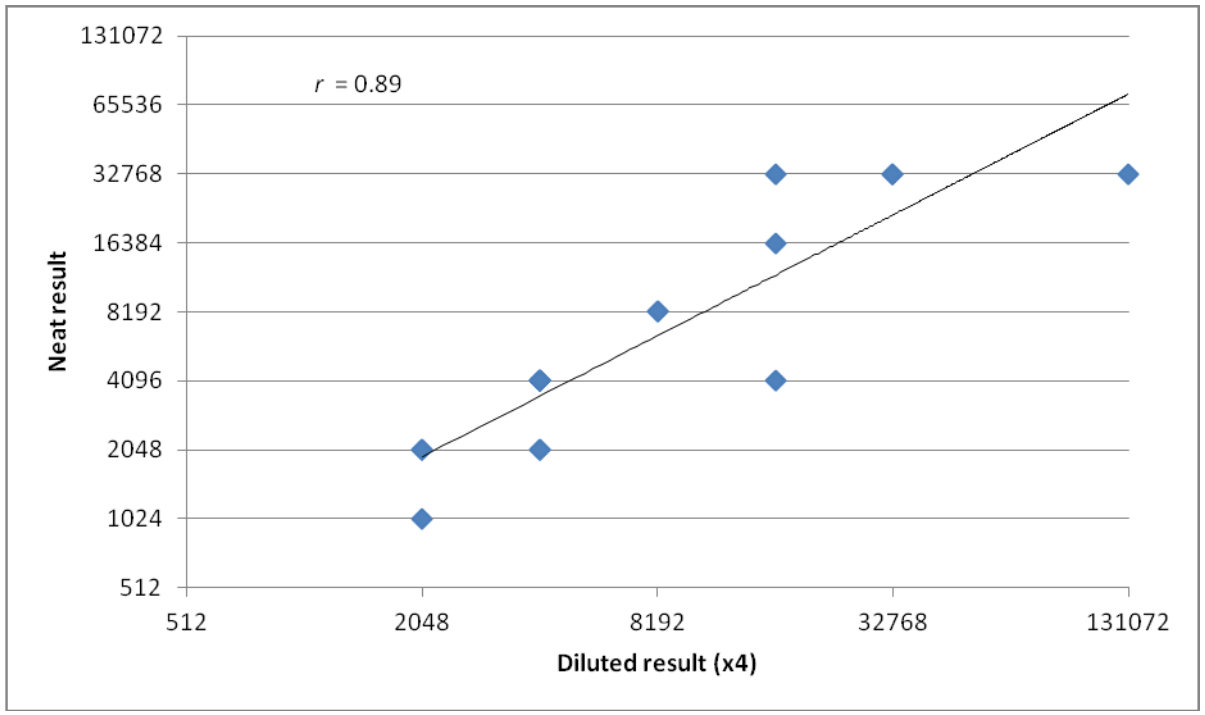
Sample	Observed SBA		Diluted results x dilution factor	Difference in SBA titre between diluted result (x dilution factor) and neat results, including negative results	Difference in SBA titre between diluted result (x dilution factor) and neat results, excluding negative results
	Neat titre	1:4 dilution	1:4 (x 4)		
001B	16384	1024	4096	-2	-2
002B	32768	8192	32768	0	0
004B	16384	4096	16384	0	0
007B	4096	1024	4096	0	0
009B	16384	8192	32768	+1	+1
013B	8192	2048	8192	0	0
014B	4096	1024	4096	0	0
017B	131072	8192	32768	-2	-2
020B	4096	1024	4096	0	0
022B	2048	512	2048	0	0
023B	4096	512	2048	-1	-1
024B	8192	2048	8192	0	0
025B	2048	512	2048	0	0
026B	2048	256	1024	-1	-1
027B	32768	8192	32768	0	0
028B	4096	1024	4096	0	0
031B	4096	1024	4096	0	0
05B	4096	512	2048	-1	-1
Total No. of results				18	18
No. of results within the acceptance criteria (within $\pm$ 1 SBA titre of the median)				16	16

Table 3.9 Hib SBA assay assay validation- Linearity (continued)

Percentage of results within the acceptance criteria	89	89

This table shows validation passed the acceptance criteria as > 85% of results (excluding negative results) were within $\pm$ 1 SBA titre of the median.

Figure 3.7 Linearity analysis

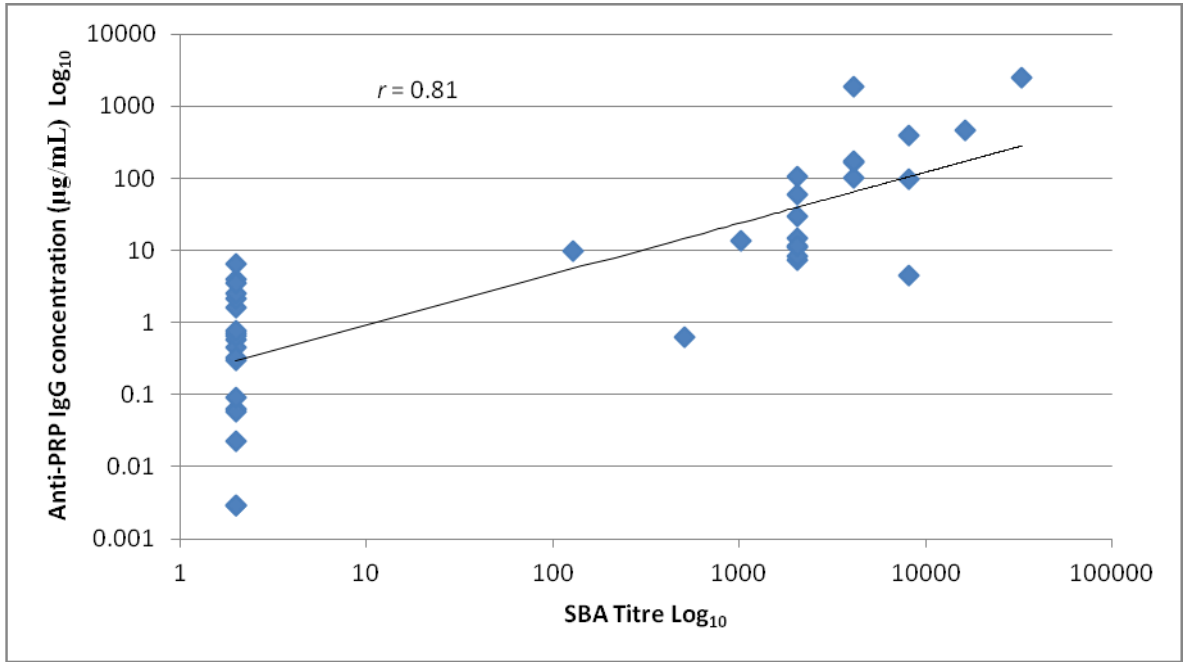


A strong correlation (r=0.89) was observed between the neat positive SBA result and the diluted (x4) SBA result indicating that samples can be diluted and still give comparative results to an undiluted sample.

3.4.6 Validated Hib SBA assay correlation with previously determined anti-PRP IgG concentrations

Following validation the assay was deemed acceptable in all assay parameters tested. The next step was to correlate the pre and post Hib vaccination samples used for validation with previously determined anti-PRP IgG concentrations. The Hib SBA data show a strong correlation (r= 0.81) with the anti-PRP IgG concentrations (figure 3.8).

Figure 3.8 Correlation of SBA titres of Validation panel sera with previously determined anti-PRP IgG concentrations



All SBA titres and IgG concentrations were plotted on log axis. Although a strong correlation (r=0.81) was shown between the two assays, the presence of high anti-PRP concentrations and low SBA titres were observed in some sera.

3.5 Infant post primary and post booster Hib vaccination responses

The optimised Hib SBA assay was used to test infant post primary and post booster Hib vaccination responses. The data were used to correlate with previously determined anti-PRP IgG concentrations.

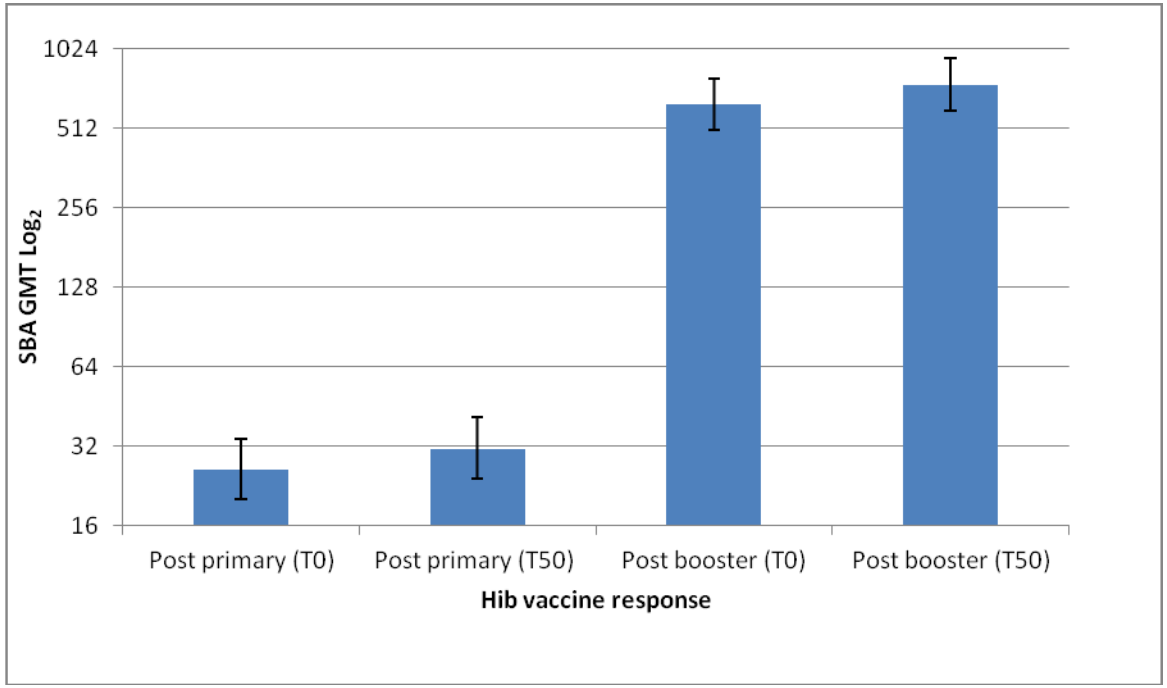
3.5.1 Sample size

A total of 486 infant serum samples were assayed in the validated Hib SBA assay. Of these, 280 serum samples were from infants who had been primed with 3 doses of Pediacel at 2, 3, and 4 months of age. The remaining 206 samples were from infants who had been boosted in the second year of life with Menitorix. Blood samples were taken one month after the three dose primary series and one month after the booster.

3.5.2 Responses to Hib vaccine

All SBA titres were log-transformed and the GMTs with 95% CI were calculated. The GMTs for T0 and T50 time points are presented in figure 3.9. The SBA GMTs for primary response (Pedicel) at T0 and T50 time points were 26 (95% CI, 20-34) and 31 (95% CI, 24-41), respectively. The SBA GMTs for booster response (Menitorix) at T0 and T50 time points were 631 (95% CI, 503-791) and 746 (95% CI, 594-938), respectively. The SBA GMT was lower following the three dose primary series than after the booster response.

Figure 3.9 SBA GMT comparison of Hib post primary response and Hib post booster response (T0 and T50 time points)



The SBA GMT rose significantly after receiving the booster dose when calculated from both T0 and T50 timepoints. Error bars show 95% CI.

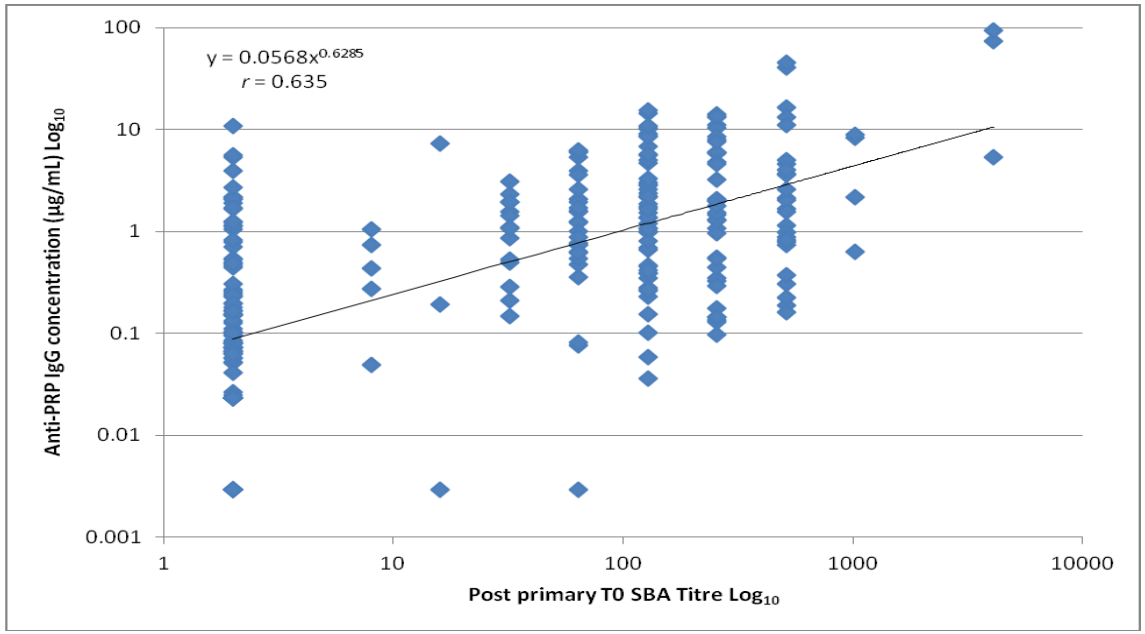
3.5.3 Comparison of the Hib SBA to IgG measured by a Hib bioplex assay

SBA titres measured by the validated Hib SBA assay were compared to previously determined anti-PRP IgG concentrations measured by a Hib Bioplex assay. Correlation analysis was performed for post primary responses and post booster responses. Analysis was performed for both T0 and T50 SBA assay time points. A moderate correlation was shown for post primary responses shown in figures 3.10 and 3.11. Both T0 and T50 time points gave a good correlation coefficient (r) of 0.635. A stronger correlation was shown for post booster responses (r= 0.729 at T0, r= 0.746 at T50) shown in figures 3.12 and 3.13.

3.5.4 Calculating a predictive SBA titre

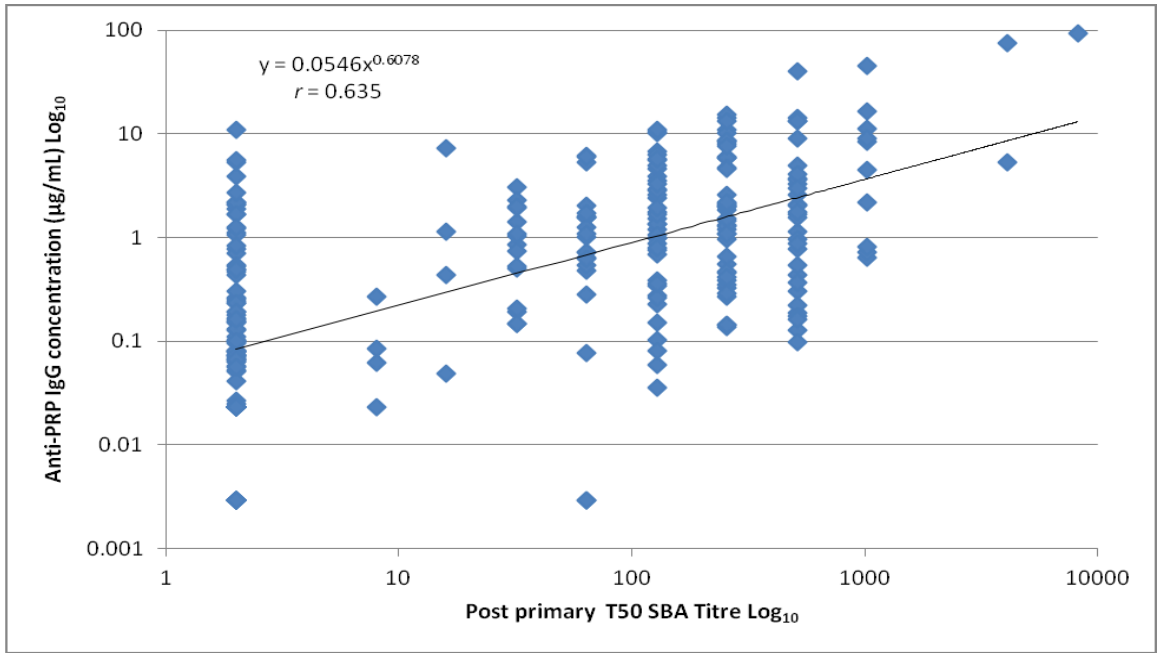
The predictive protective SBA titre that corresponds to the established long term protective antibody concentration of 1.0 µg/mL was calculated. As a good correlation was seen in post booster responses, only these data were used. The predictive protected SBA titre was calculated to be 8 at T50 and 6 at T0. When calculating the SBA titre using the short term correlate of protection of 0.15 µg/mL, the SBA titre was not determinable as it gave a value lower than the lowest starting dilution.

Figure 3.10 Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) post primary at T0. The correlation coefficient (*r* value) and the power function equation are displayed at the top of the chart.



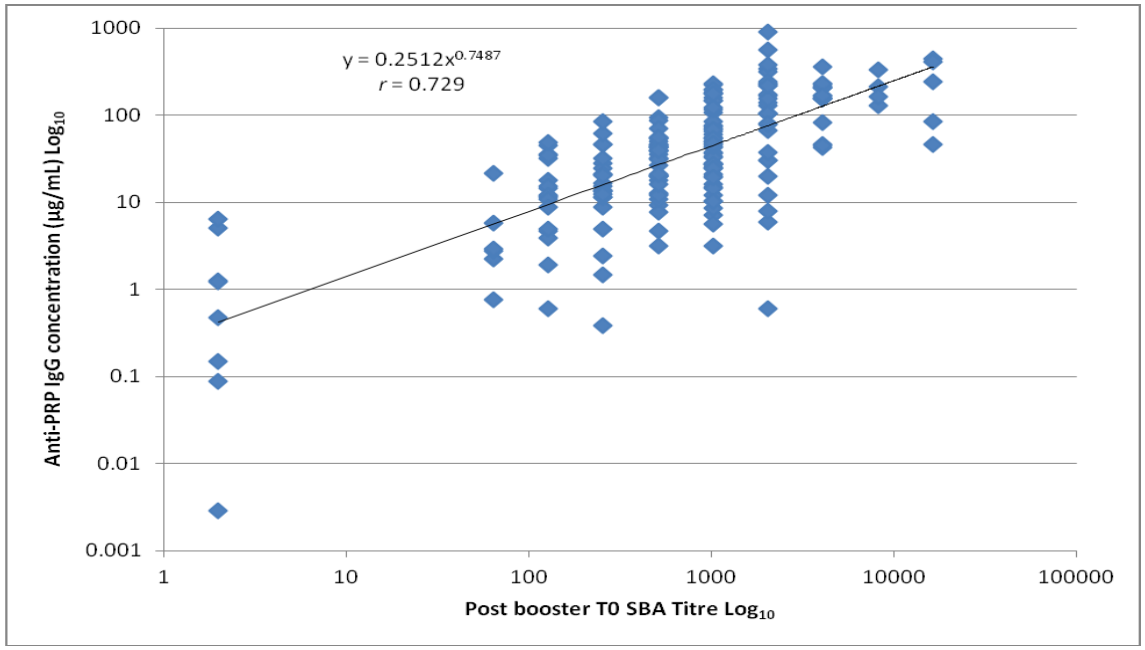
A moderate correlation (*r* =0.635) is shown between anti-PRP IgG concentrations and T0 SBA titres for post primary response serum samples. Sera with low SBA titres and high anti-PRP IgG concentrations were observed. Conversely sera with high SBA titres and low anti-PRP IgG concentrations were observed.

Figure 3.11 Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) post primary at T50. The correlation coefficient (*r* value) and the power function equation are displayed at the top of the chart.



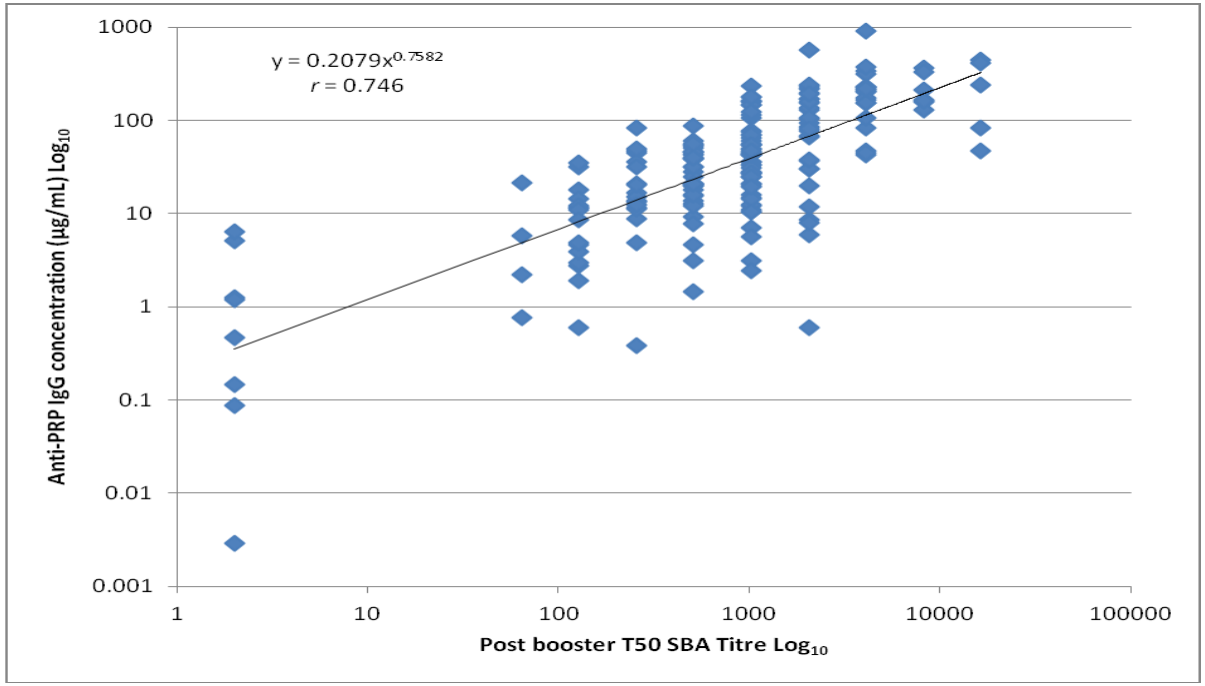
A moderate correlation (*r* =0.635) is shown between anti-PRP IgG concentrations and T50 SBA titres for post primary response serum samples. Sera with low SBA titres and high anti-PRP IgG concentrations were observed. Conversely sera with high SBA titres and low anti-PRP IgG concentrations were observed

Figure 3.12 Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) post booster at T0. The correlation coefficient (*r* value) and the power function equation are displayed at the top of the chart.



A stronger correlation (*r* =0.729) is shown between anti-PRP IgG concentrations and T0 SBA titres for post primary response serum samples. Sera with low SBA titres and high anti-PRP IgG concentrations were observed. Conversely sera with high SBA titres and low anti-PRP IgG concentrations were observed.

Figure 3.13 Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) post booster at T50. The correlation coefficient (*r* value) and the power function equation are displayed at the top of the chart.



The strongest correlation (*r* = 0.746) is shown between anti-PRP IgG concentrations and T50 SBA titres for post primary response serum samples. Sera with low SBA titres and high anti-PRP IgG concentrations were observed. Conversely sera with high SBA titres and low anti-PRP IgG concentrations were observed.

3.6 Vaccine failure samples assayed against strains with different copy numbers of the *cap* b locus

3.6.1 Sample size

A total of 164 serum samples collected from children who developed Hib disease despite receiving a Hib containing vaccine (vaccine failures) were assayed in the optimised Hib SBA. Five Hib strains containing 1, 2, 3, 4, and 5 copies of the *cap* b locus were assayed against each sample. Due to insufficient sample volume, only 127 samples were included in the analysis, having obtained a result for each strain.

3.6.2 Comparison of GMT`s for each strain

The comparison of GMTs for each strain calculated from T0 time point are presented in figure 3.14. The comparison of GMTs for each strain calculated from T60 time point are presented in figure 3.15. The GMTs at T0 for 1, 2, 3, 4, and 5 copy strains were 40 (95% CI, 26-63), 22 (95% CI, 14-34), 40 (95% CI, 25-64), 51 (95% CI, 32-83), and 18 (95% CI, 12-28), respectively. The GMTs at T60 for 1, 2, 3, 4, and 5 copy strains were 46 (95% CI, 29-73), 24 (95% CI, 15-38), 43 (95% CI, 27-69), 55 (95% CI, 33-90), and 20 (95% CI, 13-31), respectively. At both time points the 5 copy strain produced the lowest GMT. The 2 copy strain produced the second lowest. Surprisingly the 4 copy strain had the highest GMT, however it wasn't much higher than the GMT`s for the 1 copy and 3 copy strains. What is more, the 4 copy GMT still fell between the upper and lower 95% CI for the 1 copy and 3 copy strains.

Figure 3.14 Comparison of SBA GMTs of strains containing 1, 2, 3, 4 and 5 copies of the *cap* b locus calculated at T0

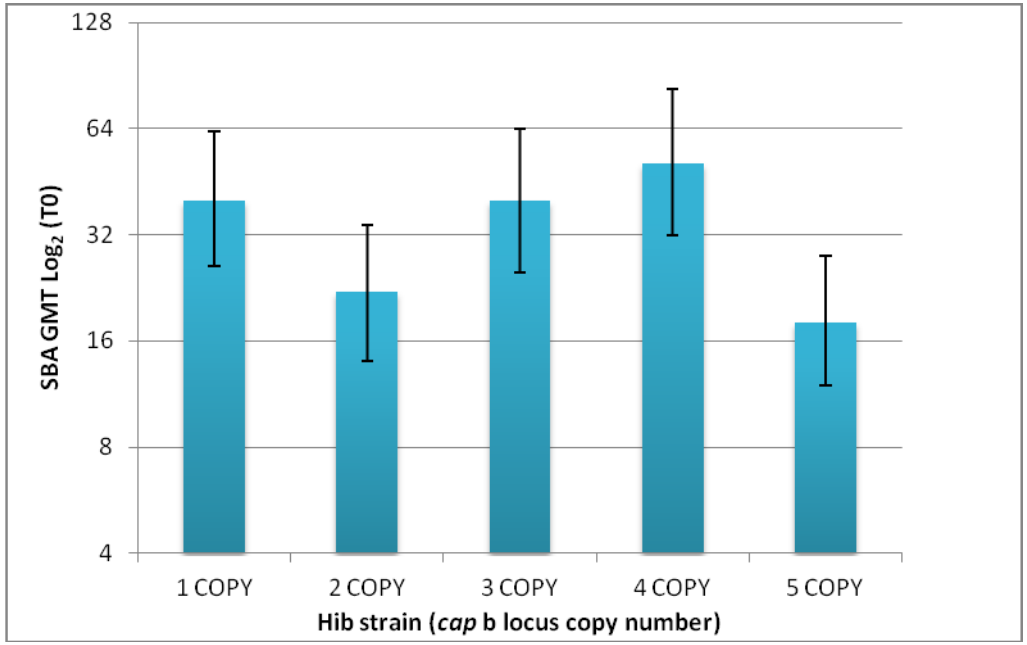
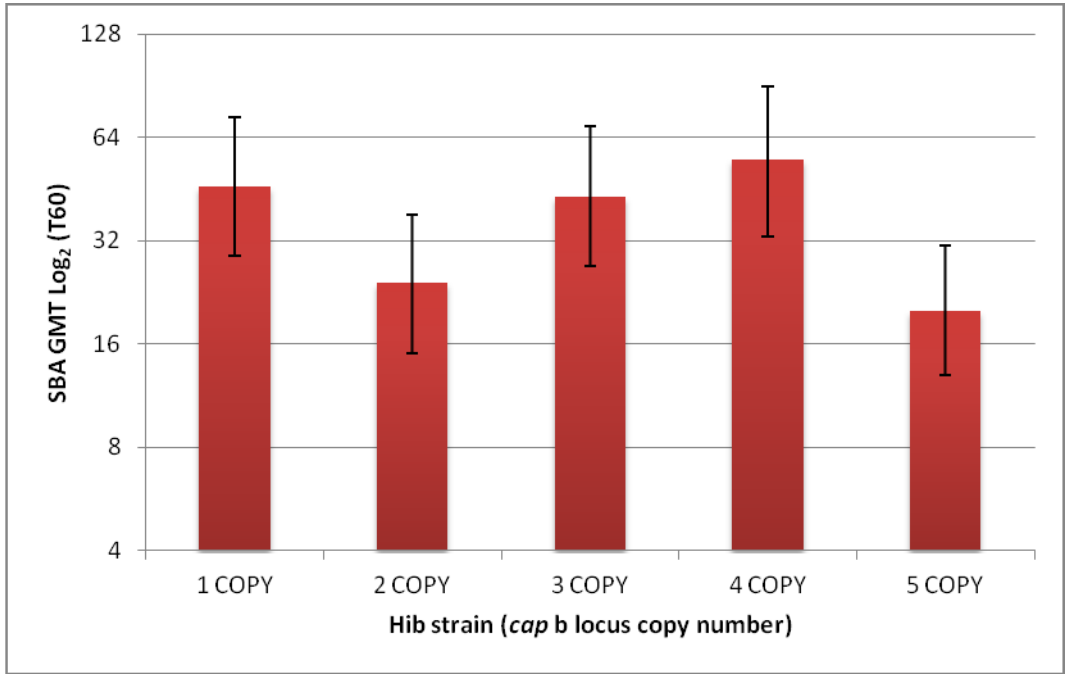


Figure 3.14 Shows the comparison of SBA GMTs at T0 (n=127). The 5 copy strain had the lowest SBA GMT. The 4 copy strain had the highest SBA GMT. The 1 and 3 copy produced the same SBA GMT. Error bars show 95% CI.

Figure 3.15 Comparison of SBA GMTs of strains containing 1, 2, 3, 4 and 5 copies of the *cap* b locus calculated at T60



Figure? Shows the comparison of the SBA GMTs at T60 (n=127). The 5 copy strain had the lowest SBA GMT. The 4 copy strain produced the highest SBA GMT. The 1 copy and 3 copy strain had similar SBA GMTs. Error bars show 95% CI.

3.6.3 Percentage of subjects achieving an SBA titre of ≥8

Figure 3.16 shows the percentage of subjects achieving an SBA titre of ≥8. The strain containing 1 copy of the *cap* b locus had the highest percentage at T60, with 62 % of samples achieving an SBA titre ≥ 8. The 1 copy and 4 copy strain both shared the highest percentage at T0 with 61% of samples achieving an SBA titre of ≥ 8. The 5 copy strain had the lowest % of samples achieving an SBA titre ≥8 at both T0 and T60 time points.

3.6.4 Correlation between SBA titre and previously determined ant-PRP IgG concentration

SBA titres were correlated with previously determined anti-PRP IgG concentrations by a Hib Bioplex assay. A weak to moderate correlation was shown for all five copy number strains. The strongest correlation was shown at T0 for the strain expressing 5 copies of the *cap* b locus (r= 0.608). The weakest correlation was seen at T60 for the strain expressing two copies of the *cap* b locus (r= 0.457). Data are presented in table 3.10. The scatter plots showing the correlation at T0 and T60 time points for each strain are listed in Appendix IV.

Figure 3.16 Percentage of samples achieving an SBA titre ≥8 for strains containing 1, 2, 3, 4 and 5 copies of the *cap* b locus

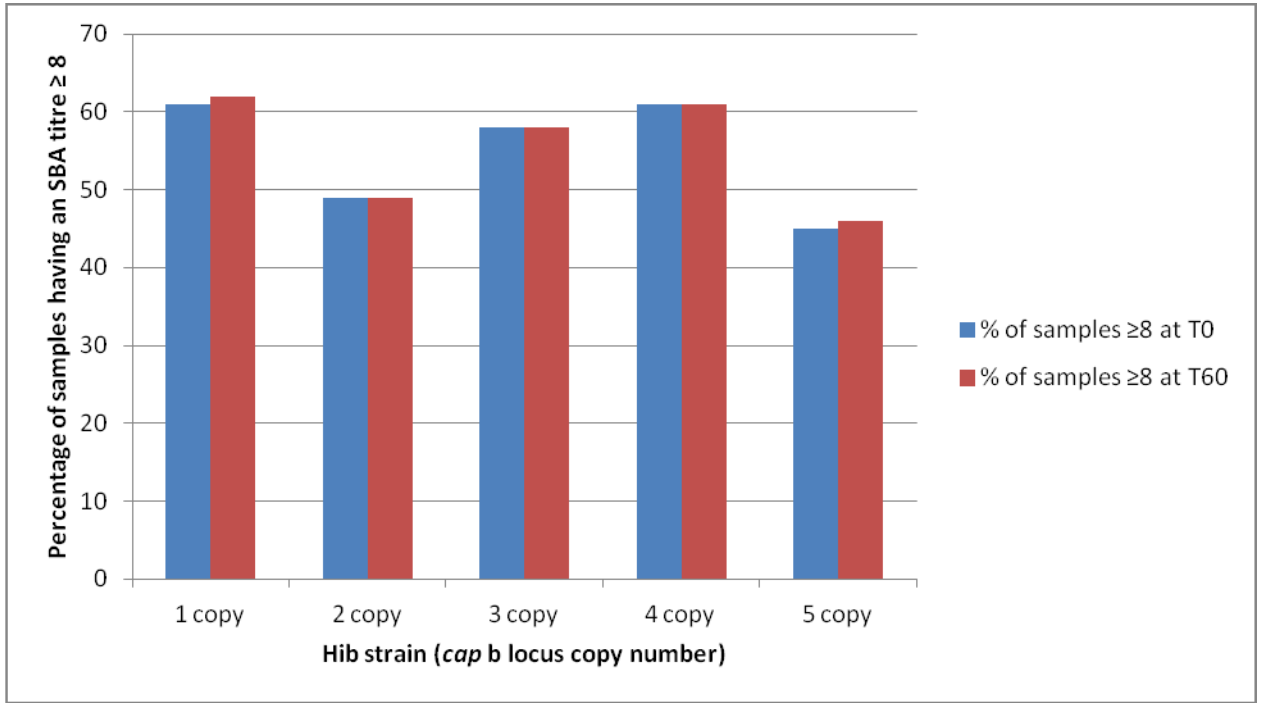


Figure 3.16 Shows the percentage of subjects achieving an SBA titre ≥8 (n= 127). The 5 copy strain had the lowest percentage of subjects achieving an SBA titre ≥8. The 1 copy strain had the highest percentage of subjects achieving an SBA titre ≥8 at T0 and T60.

Table 3.10 Correlation data (*r* value) calculated between SBA titres and anti-PRP IgG concentrations for strains containing 1-5 copies of the *cap* b locus

***cap* b copy number strain**	***r* value (T0)**	***r* value (T60)**
1 copy	0.540	0.535
2 copy	0.463	0.457
3 copy	0.506	0.509
4 copy	0.550	0.549
5 copy	0.608	0.595

This table shows that a weak to moderate correlation was shown for all copy number strains. The strongest correlation was shown at T0 for the strain expressing 5 copies of the *cap* b locus (r= 0.608). The weakest correlation was seen at T60 for the strain expressing two copies of the *cap* b locus (r= 0.457).

4.0 Discussion

Although the established correlates of protection (≥ 0.15 µg/mL short term, ≥ 1.0 µg/mL long term) against Hib disease have been determined through ELISA and RABA, their relevance to infants vaccinated with glycoconjugates remains uncertain (Goldblatt *et al*., 1998). The disadvantage in using ELISA and RABA is that they do not measure the functional and possible protective activity of these antibodies. The data of Fothergill and Wright in 1933 demonstrated the importance of bactericidal activity for the protection against Hib disease (Fothergill and Wright, 1933). It is accepted that functional activity is equally important or more so than just the measurement of IgG concentration alone and researchers have developed various assays to measure the functional activity of vaccine induced antibodies to Hib (Amir *et al*., 1990a; Schlesinger *et al*., 1992; Romero-Steiner *et al*., 2001). In other gram negative encapsulated bacteria such as *N. meningitidis*, bactericidal activity is the accepted correlate of protection (Borrow *et al*., 2001).

4.1 Replication of CDC method

This study set out with the initial aim of replicating a previously published SBA assay method by the CDC (Romero-Steiner *et al*., 2001), which is a modification of a method described by Schlesinger *et al.* (1992). A total of 19 post-Hib vaccination samples were chosen to assess whether the assay could produce reproducible SBA titres which correlated to previously determined anti-PRP IgG concentrations. Five of the samples failed to produce titres due to either being greater than the top dilution or, producing inconsistent titres. The reason the samples produced inconsistent titres is not known but most possibly due to the presence of low avidity antibodies in the sera. The assay was shown to be reproducible as > 85% of samples were within three SBA titre steps of each other. A full validation was not performed as the Food and Drug Administration (FDA)

guidelines (for bioanalytical method validation) state that a partial validation will suffice for a methodological transfer between laboratories.

A good correlation (r=0.674) was shown between the SBA titres produced in the CDC method and anti-PRP IgG concentrations. The CDC SBA assay has been previously used to measure functional antibody activity in infant sera following immunisation with fractional doses of a Hib conjugate vaccine (Romero-Steiner *et al*., 2001). Interestingly, the researchers did not correlate the SBA titres to IgG antibody concentrations measured by ELISA, stating the reasons being that the SBA assay does not distinguish between IgG and IgM antibodies, and antibodies of the IgM class have shown to be very potent modulators of complement. Although this method has not been used to correlate Hib SBA titres and IgG concentrations, it has been used to correlate to other Hib SBA assays using different methodologies. SBA assays with a colorimetric and fluorometric endpoint in the presence of AlamarBlue were developed by Romero-Steiner *et al.* (2004) and correlated to the standard Hib SBA assay. AlamarBlue is a metabolic indicator which can quantitatively measure the proliferation of bacteria, a variety of animal and human cells, mycobacteria and fungi. In response to metabolic activity, it yields a colorimetric redox change and a fluorescent signal via an indicator (Romero-Steiner *et al*., 2004). The colorimetric and fluorometric SBA assays both allow same day titre determinations using a spectrophotometer and a fluorometer, respectively. Both the colorimetric and fluorometric SBA assays highly correlated with the standardised Hib SBA (r = 0.87 and r =0.95, respectively). What is more, they showed to be faster, more reproducible, and easier to perform than the current standardised Hib SBA assay. However, they did show a higher sensitivity to buffer conditions and close monitoring was needed for long incubation times (Romero-Steiner *et al*., 2004). Moreover, there are safety issues regarding these assays as there will still be live broth cultures in the final step and therefore equipment would have

to be placed in the safety cabinet. The question of whether AlamarBlue will be triggered by human cells, present in samples tested in the SBA assay, must also be raised.

4.2 Development of Hib SBA by adaptation of the CDC method

The next step in this research project was to adapt the CDC method to standardised SBA assay methods currently used in the VEU and elsewhere for *N. meningitidis* (Borrow *et al*., 2005; Maslanka *et al*., 1997). Many factors have shown to influence the results of measurement of meningococcal SBA activity, including choice of the target strain, growth conditions of the bacteria, complement concentration, assay buffer, and target cell number (Maslanka *et al*., 1997). Therefore, various assay parameters were evaluated and optimised to produce a reproducible Hib SBA assay. The CDC reports a different method of how to grow bacterial cells prior to use in the assay. Bacterial cells that are grown to log phase are ideal as they are in a defined metabolic state for reproducibility and antigen expression (Anderson *et al*., 1976; Maslanka *et al*., 1997). The effect on the SBA titre of Hib strains grown for 4 hours on a chocolate agar plate were compared with the CDC method of log phase growth in broth. No major differences between the titres were observed, as 83% of the samples were within three SBA titre steps of each other. The assay was changed to include bacteria grown to log phase on chocolate agar, as it brought the assay in line with ones currently being used in the VEU. This finding supports the research by Maslanka *et al*. (1997) who reported no difference in MenC and MenA SBA titres when using bacteria grown to log phase in broth or agar. It is also in agreement with Rodriguez *et al.* (2002) who also reported no difference in titres in a Men B SBA assay.

4.2.1 Comparison of serum/buffer/target strain/complement concentration

Baby rabbit complement (RC) is widely used as a complement source against meningococcal strains A, C, Y and W and is specified in the WHO recommended procedure (WHO, 1976). It has not been used in meningococcal serogroup B (Men B)

assays as the use of rabbit complement has shown to give falsely high SBA titres due to the presence of low-avidity anti-MenB capsular IgM antibody in the test sera (Zollinger and Mandrell, 1983; Findlow *et al*., 2007). Researchers have reported differences in SBA titres produced when comparing rabbit complement (rSBA) and human complement (hSBA) in SBA assays against all meningococcal strains (Santos *et al*., 2001; Gill *et al*., 2011; Findlow *et al*., 2009). In all reports, RC yielded higher SBA titres than human complement, and persons who were considered protective in the rSBA assay were not shown to be protected in the hSBA. This was mainly when individuals had an rSBA titre between 8 and 64. For meningococcal serogroup C rSBA assays, Borrow *et al*. (2001) proposed a fourfold titre or greater rise between pre and post-vaccination sera as a correlate of protection.

Ideally a human complement source would be desirable but there are many difficulties in collecting large volumes of agammaglobulinemic sera or to screen healthy adults which lack intrinsic bactericidal activity in serum. RC which lacks intrinsic bactericidal activity is readily available and helps further standardisation between laboratories. As SBA titres assayed in the CDC method with RC were shown to correlate with anti-PRP IgG concentrations, it was chosen as a suitable complement source to develop the Hib SBA assay in the VEU.

The complement concentration in meningococcal SBA assays has been shown to work optimally at 25% (Maslanka *et al*., 1997). A complement concentration of 31.3% is used in the CDC method. A 25% complement concentration was investigated in the Hib SBA but the concentration was not activating enough to produce consistent titres. This is surprising as earlier studies using a complement concentration of 20% in a Hib SBA, showed to be activating enough, however this was using human complement. (Amir *et al*.,

1990; Schlesinger *et al*., 1992). This also accords with a study by Barra *et al*. (1993) who found a 25% complement concentration to be sufficient when using precolostral calf serum (serum taken from a neonate calf before the ingestion of immunoglobulin rich colostrum from the mother) as the complement source. However, it must be noted that the final well volume in this assay was 400 µL and was not performed on a microtitre plate. The total well volume in the studies performed by Amir *et al*. (1990a) and Schlesinger *et al*. (1992) also had a high total well volume of 120 µL. The total well volume, along with the complement source, may both be a factor affecting activation. Future work using precolostral calf serum and human complement would be help to establish this.

The serum dilution scheme is important in the SBA assay and a lower starting dilution is beneficial in the assay to determine true seronegative titres. By adjusting the ratio of serum, buffer, target strain, and complement, the complement concentration was adjusted from 31.3% to 33.3%. This enabled the assay to be started at a 1:8 dilution as opposed to a 1:16 dilution in the CDC method. In addition, the assay then required less volume of complement per well which was favourable due to the high cost of RC (£566/100 mL, Pel-freeze, 2012).

4.2.2 Comparing the incubation length of the serum/buffer/target strain/complement

The recommended procedure by the WHO (1976) suggests an incubation length of 30 minutes for Men A and Men C SBA assays. However, an incubation time which allows maximum titre generation and a minimum reduction in control wells should be investigated (Maslanka *et al*., 1997), and many laboratories use an incubation length of 60 minutes (Maslanka *et al*., 1997). It was noticed during the replication of the CDC method, that quite often, the control well for non complement mediated lysis (column 12) failed to be within -30% of column 11 in post-vaccination sera. Acceptance criteria states that the cfu

in column 12 should be within -30% of column 11 or the sample will be classed as a fail. Investigations showed an increase of up to 30% of viable cfu from T30 to T60 (column 11). This finding has also been observed by Schelsinger *et al*. (1992) who reported a 200% increase in viable cfu between T0 and T60. It appeared that the column 12 fails at T60 were due to an inhibited growth as there was no reduction in cfu in column 12 over the T30-T60 time period. It seemed the sera containing high amount of antibody in column 12 would not allow the bacterial cells to grow at the rate in which bacteria in column 11 did. This has also been reported in meningococcal serogroup A and C assays (Maslanka *et al*., 1997). Therefore, it seems more likely that the column 12 fails are because of an inhibited growth rather than cell death. Evidence to support this theory is also the observation that there were no column 12 fails when running pre vaccination sera which lacked functional antibodies to Hib. The column 12 in pre vaccination serum samples were able to grow within -30% of column 11. Secondly, all post vaccination sera were all treated with beta lactamase to inhibit any antibiotics in the test sera.

Because of the inhibited growth observed in column 12 over the T0-T60 time period, the incubation was changed from T60 to T50. Activation begins at T30 however the clear and consistent titres were not being produced until T50. Although T40 produced fewer columns 12 fails, one sample (004B) failed to produce an SBA titre at this time point due to the sample producing inconsistent cfu. It seemed T40 time point would not be a good incubation time to use at it could cause a large number of samples to fail because of inconsistent cfu. A reduced incubation period is consistent with other published Hib SBA assays. Barra *et al*. (1993) and Denoel *et al*. (2007) used incubation periods of 30 and 45 minutes, respectively. However, they both used precolostral calf serum as the complement source. Schlesinger *et al.* (1992) also used a reduced incubation of 30 minutes in a Hib SBA assay with human complement.

4.2.3 Comparison of incubation period with 5% CO_2 and without 5% CO_2

Carbon dioxide enhances growth in Hib strains (Bolmstrom and Karlsson, 2002), however, a CO_2 environment was thought not to be necessary during the reaction mixture incubation when plates are incubated with plate sealers on. Due to safety requirements, incubations without plate sealers on, which would allow the CO_2 to diffuse into the reaction mixture could not be performed. The method was changed to an environment without CO_2 after observing no difference between titres when compared with and without CO_2. An incubation period without CO_2 is in agreement with SBA assay methods for *N. meningitidis* strains published by other laboratories (Borrow *et al*., 2005; Liu *et al*., 2004; Martin *et al*., 2005; Maslanka *et al*., 1997). Further investigations without plate sealers during incubation of the reaction mixture would demonstrate the true effect of CO_2 during this time.

4.2.4 Comparison of the media used in the Hib SBA assay

The chocolate agar plates used in the replication were different to that used by the CDC. Initially, the manufacturer (Oxoid) which supplied Columbia horse blood agar for meningococcal SBAs to the HPA VEU was chosen. The Oxoid agar was not supporting bacterial growth sufficiently in column 12 so other agar, supplied by Biomerieux, was investigated. The Oxoid chocolate plates were found to be inferior to chocolate plates supplied by Biomerieux. The composition of both agar differ and for reasons unknown the Biomerieux plates supported the Hib bacterial colonies better and was chosen for optimisation. A possible explanation is that Biomerieux plates contain PolyViteX (not present in Oxoid plates), which is reported to enrich the medium with X (hemin) and V (NAD) factors which are required growth factors of Hib.

4.2.5 Comparison of the use of BSA in bactericidal buffer

BSA has been widely used in bactericidal buffers in meningococcal SBA assays (Frasch and Robbins 1978; Maslanka *et al*., 1997; Martin *et al*., 2005). BSA can coat bacterial cells which may help avoid non specific killing in the assay. Due to the earlier observations of reduced bacterial growth in column 12, BSA was added to the BB as no differences were observed between titres. Maslanka *et al.* (1997) also reported no difference in SBA titres in Men A and Men C SBA assays when comparing buffer with and without BSA. However, they did report that buffer containing BSA had an increased pH after 2-3 weeks of storage which has shown to produce different titres in one laboratory which used different lots of BSA (Maslanka *et al.,* 1997). The BSA BB in the Hib SBA assay was given a two week expiry date after an observation of reduced control serum SBA titres during assay development. It is possible that the change in pH during storage could have had an effect on the SBA titres.

4.2.6 Validation of the optical density and its correlation to inoculum count

After increasing the volume of reaction mixture in the tilt method, an inoculum count which produced 100-150 cfu per well was established to improve the quality of counting. A heavy innoculum count would make it harder to distinguish separate colonies and therefore may have an effect on the titre. The innoculum count chosen was optimal for the colony counting method. Improved methods for colony counting have been described. A method that stains bacterial colonies which enables them to be counted with an imaging system has shown to be advantageous to current counting methods as it can detect up to 300 colony forming units (Liu *et al*., 2004). Other methods include assays with colorimetric and fluorometric end points as discussed earlier in section 4.1 (Romero-Steiner *et al*., 2004).

4.2.7 Comparison of the incubation length of the target strain prior to use in the Hib SBA assay

The handling of the target strain is an important aspect of the SBA assay. An interlaboratory standardisation study of a Men B SBA assay showed that different methods of colony isolation between different laboratories resulted in marked differences when measuring SBA titres between the laboratories (Borrow *et al*., 2005). Meningococcal SBA assays carried out in the HPA VEU require bacteria to be incubated 16-24 hours prior to use in the assay to introduce the bacteria into log phase. It has been demonstrated that Hib can adapt to changes in the environment by expressing different surface components based upon its current growth conditions (Clark *et al*., 2012) therefore a suitable incubation period that would not have an effect on the SBA titre was investigated. An incubation period of 16-24 hours was deemed acceptable as 7/10 samples fell within 3 SBA titre steps of each other. Also, all (10/10) samples fell within 4 SBA titre steps. Although there was not much variability in SBA titre over time, it was noted that 24 hour incubation did generate the highest titre for each sample. Incubation periods <16 hours and >24 hours were not investigated as this was not achievable over two consecutive working days.

4.3 Validation of the optimised Hib SBA assay

Assay validation is an essential requirement for any bioassay. It is important that the results produced are accurate, reliable, and reproducible. The specificity of the assay was determined by performing competitive inhibition with Hib and Men C polysaccharide. The SBA demonstrated high specificity as shown by complete inhibition in the presence of homologous polysaccharide, and no to low inhibition in the presence of heterologous polysaccharide, compared to the titre without the added competitor. One sample (035B) had a three titre reduction when inhibited with Men C polysaccharide. This was the only outlier as all other samples inhibited with Men C polysaccharide were all within 2 SBA

titre steps of the neat (no polysaccharide) result. A possible explanation for this outlier could be that the inhibition could have been diluted out when mixing. Specificity was investigated further by spiking known SBA negative samples with SBA positive samples in three different ratios. When multiplied by the dilution factor (1:8) sample 009A produced a titre which was 4 SBA titres higher than the neat positive result. It has been reported that circulating IgA which does not activate the classical pathway can block IgM and IgG bactericidal activity by both competitive inhibition and non competitive inhibition (Griffiss and Goroff, 1983). Maslanka *et al*. (1997) reported that increased titres may be due to a reduced inhibition by IgA, if the bacterial cell number increases, making more antigenic epitopes available. Nevertheless, the results demonstrate that the SBA assay is specific to Hib. Accuracy was determined by repeated spiking analysis. The SBA assay demonstrated high levels of accuracy and no outliers were observed.

The reproducibility of the assay was investigated in terms of inter and intra assay precision. Both inter and intra assay shown higher reproducibility when negative SBA results were included in the analysis. This is due to the larger sample size and every negative sample passing acceptance criteria. The SBA demonstrated excellent linearity and a strong correlation (r=0.89) was observed between neat SBA titres and `recovered` SBA titres (diluted result x 4). Samples can be diluted and still give comparative results to undiluted. Overall the Hib SBA assay was deemed highly specific, reproducible and suitable for conducting research in the HPA VEU which highly correlated to anti-PRP IgG concentrations (r=0.81). Although the validated assay demonstrated a strong correlation with anti-PRP IgG correlations, there were samples which had a low SBA titre and a high anti-PRP IgG concentration. The possible explanations for this observation are discussed in the next section (section 4.4).

4.4 Infant post primary and post booster responses

In England and Wales infants receive Pediacel ($DTaP_5$/IPV/Hib-TT) at 2, 3, and 4 months of age; Men C conjugate vaccination at 3 and 4 months of age and a pneumococcal conjugate vaccine (PCV) at 2 and 4 months of age. Infants are boosted with a combined Hib/Men C-TT (Menitorix) vaccine and PCV whilst receiving the first dose of MMR (Measles, Mumps and Rubella) vaccine in the second year of life. In this study the SBA GMTs following primary vaccination at T0 and T50 time points were 26 (95% CI, 20-34) and 31 (95% CI, 24-41), respectively. The SBA GMTs following booster vaccination at T0 and T50 time points were 631 (95% CI, 503-791) and 746 (95% CI, 594-938), respectively. As expected, the SBA GMTs were found to be lower in the primary response compared to the booster response.

The enhanced immune response following Hib booster vaccination has been demonstrated elsewhere. A study by Borrow *et al*. (2010) calculated the anti-PRP IgG GMCs before and one month after the administration of Menitorix under the current UK immunisation schedule. The percentage of subjects with anti-PRP IgG concentrations ≥0.15 μg/mL and ≥1.0 μg/mL before and after the administration of Menitorix were also calculated. In this study, the anti-PRP IgG GMCs were 0.58 (95% CI, 0.49-0.69) and 43.47 (95% CI, 36.56-51.70) for before and after the administration of Menitorix, respectively. What is more the percentage of subjects achieving an anti-PRP IgG concentration of ≥1.0 μg/mL were 33% (95% CI, 27-40) and 98% (95% CI, 96-100) before and after the administration of Menitorix, respectively. This enhanced immune response supports the GMTs for primary and booster responses calculated in this study. Conversely, the percentage of subjects achieving an anti-PRP IgG concentration calculating from ≥0.15 μg/mL (82% (95% CI, 76-87) and 99% (95% CI, 97-100) before and after Menitorix,

respectively) do not demonstrate the same enhancement. This is probably due to the level of ≥1.0 μg/mL being more representative of protection. (Käyhty *et al*., 1983).

The protective antibody concentration which is able to confer protection remains controversial (Amir *et* al., 1990a; Granoff and Holmes, 1991; Schlesinger *et al*., 1992). Although the accepted levels of short term (≥0.15 μg/mL) and long term (≥1.0 μg/mL) protection have been established, the isotype, avidity and functionality of these antibodies can vary among individuals (Käyhty, 1994). This is the first study to correlate Hib SBA titres to anti-PRP IgG concentrations in infants under the UK immunisation schedule. The results of this study showed a good correlation between SBA titres and anti-PRP IgG concentrations in the primary response for both T0 and T50 time points (r= 0.635). A stronger correlation was shown in the booster response at T0 and T50 time points (r = 0.729 at T0, r= 0.746 at T50). Antibody avidity is the strength of which an antibody binds to an antigen. Goldblatt *et al*. (1998) demonstrated that antibody avidity is relatively low following primary immunisation and significantly higher following boosting. The Hib SBA assay measures high avidity antibodies, which could explain the stronger correlation seen in the booster responses.

The observation of high anti-PRP IgG concentrations with low SBA titres were seen in both primary and booster response scatter plots. Discrepancies were expected as the SBA assay measures functional antibodies, regardless of isotype, to the whole organism, whereas anti-PRP IgG is simply a measure of total IgG just to the polysaccharide capsule. Even so, the discrepancy between high IgG concentration and low SBA could be explained by the presence of low avidity antibodies. Although a high IgG antibody concentration is produced it does not represent the functionality of these antibodies. This also accords with Findlow *et al*. (2009) who reported moderate correlations between IgG concentrations measured by ELISA, and SBA titres against Men A. They suggested the presence of low

avidity antibodies cannot be distinguished in an ELISA which measures both functional and non functional antibodies. Schlesinger *et al*. (1992) reported differences in antibody avidity upon vaccination with different Hib conjugate vaccines. They showed high avidity antibodies to be more potent than low avidity antibodies, as antibodies of low avidity were less active in the assay of complement mediated bactericidal activity. For future work, it would be interesting to see if an improved correlation could be seen comparing the Hib SBA titres against IgG concentrations measured by an ELISA adapted to select for high avidity antibodies. The production of high avidity antibodies is strongly associated with memory response and has been considered as a surrogate for successful priming. A person primed for memory response will be able to produce specific IgG of high avidity on encounter with the relevant antigen and so the measure of antibody avidity may help differentiate those successfully primed in infancy with high avidity to those with low avidity who are not (Goldblatt *et al*., 1998). Therefore the Hib SBA assay developed in this project could help distinguish those who are primed for memory and those who are not.

A second observation was the presence of sera with low anti-PRP IgG concentrations with high SBA titres. There are several possible explanations for this observation. Firstly, the established correlates of protection are a measure of IgG alone. The SBA assay is a measure of functional antibodies which can activate the complement system which will result in lysis of the bacterial cell (IgG, IgM). The high SBA titre and low IgG concentration can be explained by the presence of IgM which has shown to be a potent activator of complement. Barra *et al*. (1993) characterised the serum antibody response induced by a Hib conjugate vaccine in infants. They found that the vaccine induced the IgG, IgM and IgA classes. What is more they found that the concentrations of IgG and IgM rose significantly after 3 doses of the vaccination. Therefore, IgM not quantified in the current study, may contribute to SBA activity and affect the correlation

between the assays. Further work measuring total IgM in those with high SBA titres and low IgG concentrations, would help to establish this. A second possible explanation for the discrepancy is the due to the fact that the bioplex assay only measures IgG antibodies directed against the PRP polysaccharide of Hib. Although vaccine induced antibodies are usually directed to the polysaccharide, other surface antigens such as outer membrane proteins, have shown to be immunogenic and have been studied as promising vaccine candidates for non-typeable Hi (Chang *et al*., 2011; Winter and Barenkamp, 2006). Antibodies elicited against the outer membrane proteins of Hib could explain the high SBA titre and low anti-PRP IgG concentration if they were bactericidal in the SBA assay.

A 'predictive' SBA titre was calculated using the established correlates of protection. The predictive SBA titre, using the long term protective anti-PRP IgG concentration of 1.0 µg/mL, was calculated to be 8 and 6 at T50 and T0 time points, respectively. It is possible that an SBA titre of 8 would predict protection of Hib disease given the good correlation between the two assays; however, these data must be interpreted with caution. The scatter plots these data were calculated from use correlation coefficients of $r = 0.746$ and $r = 0.729$ for T50 and T0 time points, respectively. Although these values suggest a good correlation, they are not strong enough to predict a true protective SBA titre especially when they are a measure of two different assay outcomes (quantitative vs functional). This corroborates the ideas of Jódar *et al*. (2003) who reported an antibody concentration ≥0.20 µg/mL corresponds to the threshold opsonic antibody titre of 8, for protection against pneumococcal infection. The authors in this paper raised potential problems related to the relationship between the ELISA IgG concentrations and opsonophagocytic titres. They reported the accuracy of the ELISA may be influenced by substances in the sera, reagents and materials. The outcome of the opsonophagocytic assays may be affected by the types of phagococytes, bacteria and complement.

The overall findings from the evaluation of infant post primary and post booster Hib vaccination responses are noteworthy, as they show subjects achieving IgG levels considered to be protective of Hib disease, lacked the capacity to kill Hib in the presence of complement. Goldblatt *et al*. (1998) developed an assay for the measurement of anti-PRP antibody avidity. They found that infants with anti-PRP IgG concentrations < 1.0 µg/mL following primary immunisation, antibody avidity was low, indicating an absence of priming. Therefore, the data in this study supports the question of whether the current established correlates of protection of 0.15 µg/mL and 1.0 µg/mL generated through ELISA and RABA, remain relevant to infants primed for memory response when they have shown to lack functional capability in some subjects.

4.5 Vaccine failure samples assayed against strains with different copy numbers of the *cap* b locus

In the UK, Hib vaccine failure is rare, with an estimated vaccine failure rate of 2.2 per 100,000 vaccinees (Ladhani *et al*., 2010b). A six year study by Heath *et al*. (2000) found that 44% of children with Hib vaccine failure had clinical risk factors such as prematurity and/or immunological deficiencies (IgA, IgG, IgM). Persons with antibody concentrations <1.0 µg/mL were also found to be at risk of Hib vaccine failure. Most strains isolated from patients with invasive Hib disease generally posses a duplication of the *cap* b locus, however further amplification has been detected (three, four, five, six copies) and has thought to play a role in in Hib vaccine failure (Cerquetti *et al*., 2006). The Hib SBA assay was used to test convalescent sera from children with Hib vaccine failure against strains expressing 1-5 copies of the *cap b* locus, to determine whether this is functionally important or not.

The results of this study show that increasing copies of the *cap* b locus may have some importance functionally, as the strain containing five copies of the *cap* b locus gave the lowest GMTs calculated at T0 and T60 time points, 18 and 20, respectively. However, this is unlikely, as the Hib strain containing 4 copies of the *cap* b locus produced the highest SBA GMT, which also fell between the upper and lower 95% CI for the 1 copy and 3 copy strains. The strain containing two copies of the *cap* b locus gave the second lowest GMT. This was surprising as it was hypothesised that as the GMT would decrease with each increasing copy strain. The reasons for this are not clear, but a possible explanation is that most invasive Hib strains contain a duplication of the *cap* b locus. This duplicate arrangement has shown to serve as a template for further amplification (Cerquetti *et al*., 2005). Therefore Hib strains in this duplicate arrangement may be a highly virulent state. Studies have also shown that isolates can contain a mixed population of six, five, four, three and two copy arrangements of the locus, suggesting that a partial progressive shift from a six copy arrangement to a two copy arrangement can occur (Cerquetti *et al*., 2005, Cerquetti *et al*., 2006). What is more, a study by Cerquetti *et al*. (2005) showed that clinical presentation of Hib meningitis was more frequently associated with 1 or 2 copy strains than with multiple copy strains in children with true vaccine failure and unvaccinated children.

Another important finding was that the Hib strain containing 5 copies of the *cap* b locus had the lowest percentage of subjects achieving an SBA titre of ≥8. What is more the strain containing 1 copy had the highest percentage of subjects achieving an SBA titre of ≥8 demonstrating that an amplified state may play a role functionally. It is encouraging to compare these results to the work of Noel *et al*. (1996) who found that amplification of the *cap* b locus is associated with decreased susceptibility to complement mediated lysis. However, the researchers in this study reported that organisms containing 4 copies of the *cap* locus were more resistant to complement killing than organisms containing 2 copies.

The present study showed that the Hib strains containing 2 copies were more resistant to complement killing than the Hib strain containing 4 copies. Again reasons for this are unknown. It seems unlikely that it was due to the strain, as vaccine failure samples were re tested with different strains containing four copies of the cap b locus. Although an increased capsule production may be advantageous in evading complement killing, other surface components may be a factor affecting SBA titres. Antibodies raised against Hib surface proteins (anti P1, P2, P4, P6 and protein D) have been shown to be bactericidal and may play an important role in clearance in this study (Hetherington *et al.*, 1993; Gilsdorf, 1998; Kemmer *et al.*, 2001). It is also noteworthy that most invasive Hib disease are due to strains containing 2 copies, so this duplicate state must have some advantage (Cerquetti *et al*., 2006).

SBA titres were correlated to previously determined anti-PRP IgG concentrations. The strongest correlation (r= 0.608) was shown in correlations against the Hib strain containing 5 copies of the *cap* b locus. Cerquetti *et al*. (2005) reported a greater proportion of strains in true Hib vaccine failures contained multiple copies compared with strains from unvaccinated control children. This could explain a greater correlation with the strain containing the highest number of copies and the lowest correlation seen in the strain containing two copies. The weak correlations were due to the presence of high SBA titres with low IgG concentrations and low SBA titres with high IgG concentrations. Explanations for these observations have been described earlier in section 4.4.

The present study showed no strong association between increased *cap* b copies and SBA titres. This is in contrast to the research of Uria *et al.* (2008). They identified three meningococcal serogroup C isolates that resisted killing by bactericidal antibodies induced by the Men C conjugate vaccine. The resistance was due to the presence of an

insertion sequence, IS*1301*, which led to an increase in the transcript levels of surrounding genes increasing the amount of capsule expressed. The increasing amount of capsule was associated with the down regulation of the alternative pathway. The authors suggested that although the insertion may mark as a survival advantage, there are several potential reasons why this change is not more widespread. They suggested that the strains may be effectively cleared by other immune mechanisms such as opsonophagocytosis, or it may be detrimental at other stages of its lifecycle. This could be applied to why multiple *cap* b copies are not an advantage in this study. In future investigations it might be interesting to assay sera from children immunised under the UK primary schedule to see which amplified state plays an important role functionally and may pose the most threat to children who may develop disease following vaccination.

The development of invasive Hib disease in vaccinated individuals is rare. Following the introduction of routine Hib immunisation, NTHi now accounts for the majority of invasive Hi infections and is a common cause of pneumonia and upper respiratory tract infections (e.g otitis media) in children and adolescents (Ladhani *et al*., 2010c). Since NTHi lacks a polysaccharide capsule, a number of NTHi outer membrane proteins/surface antigens are under development as potential vaccine candidates (Gu *et al*., 2003). The recent protein D- based pneumococcal vaccine (PHiD-CV) has shown to offer protection aganst otits media suggesting this vaccine may offer some protection against Hi strains (Prymula *et al*.,2006). Development of an assay against the outer membrane proteins of NTHi/Hib strains would be important for future work, with the development of new NTHi vaccines.

4.6 Conclusion

The Hib SBA assay was deemed highly specific and reproducible, which highly correlated to anti-PRP IgG concentrations. It is interesting to note that the correlation (*r* values) between anti-PRP IgG concentrations and SBA titres were lower in the vaccine failure population than in samples taken from children immunised under the UK immunisation schedule. It has been shown that children with Hib vaccine failure often have an immunoglobulin deficiency (Ladhani *et al*., 2010a). In the vaccine failure population, there were more samples that had a low SBA titre and a high anti-PRP IgG concentration which weakened the correlation. It has been discussed that samples with a low SBA titre and a high IgG concentration could be due to the presence of low avidity antibodies. If this is true, this data would suggest that children with vaccine failure do not make a strong antibody reponse with high avidity upon vaccination, and therefore explain the weaker correlation seen in this population. Further work using an ELISA selected for high avidity antibodies would confirm this.

The SBA is reliable and reproducible when evaluating the humoral response following vaccination. The predictive SBA titre of 8 must be interpreted with caution as it was calculated from a correlation coefficient of r=0.746. There was no strong association between the number of *cap* b copies and SBA titres. Although an increased capsule may be an advantage, antibodies to other surface antigens may play a role in clearance.

References

Ajello, G.W., Bolan, G.A., Hayes, P.S., Lehmann, D., Montgomery, J., Feeley, J.C., Perlino, C.A. and Broome, C.V. (1987) 'Commercial latex agglutination tests for detection of *Haemophilus influenzae* type b and *Streptococcus pneumoniae* antigens in patients with bacteremic pneumonia.' *J Clin Microbiol,* 25(8) pp. 1388-1391.

Alexander, J.J. and Quigg, R.J. (2007) 'The simple design of complement factor H: Looks can be deceiving.' *Mol Immunol,* 44(1-3) pp. 123-132.

Amir, J., Liang, X. and Granoff, D.M. (1990a) 'Variability in the functional activity of vaccine-induced antibody to *Haemophilus influenzae* type b.' *Pediatr Res,* 27(4) pp. 358-364.

Amir, J., Scott, M. G., Nahm, M.H. and Granoff, D.M. (1990b) 'Bactericidal and opsonic activity of IgG1 and IgG2 anticapsular antibodies to *Haemophilus influenzae* type b.' *J Infect Dis,* 162(1) pp**.** 163-171.

Anderson, E.C., Begg, N.T., Crawshaw, S.C., Hargreaves, R.M., Howard, A.J. and Slack, M. P. (1995) 'Epidemiology of invasive *Haemophilus influenzae* infections in England and Wales in the pre-vaccination era (1990-2).' *Epidemiol Infect,* 115(1) pp. 89-100.

Anderson, P., Pitt, J. and Smith, D.H. (1976) 'Synthesis and release of polyribophosphate by Haemophilus influenzae type b in vitro.' *Infect Immun,* 13(2) pp. 581-589.

Avci, F.Y., Li, X., Tsuji, M. and Kasper, D.L. (2011) 'A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design.' *Nat Med,* 17(12) pp. 1602-1609.

Barbour, M.L. (1996) 'Conjugate vaccines and the carriage of *Haemophilus influenzae* type b.' *Emerg Infect Dis,* 2(3) pp. 176-182.

Barra, A., Dagan, R., Preud'Homme, J. L., Bajart, A., Danve, B. and Fritzell, B. (1993) 'Characterization of the serum antibody response induced by *Haemophilus influenzae* type b tetanus protein-conjugate vaccine in infants receiving a DTP-combined vaccine from 2 months of age.' *Vaccine,* 11(10) pp. 1003-1006.

Barra, A., Schulz, D., Aucouturier, P. and Preud'homme, J.L. (1988) 'Measurement of anti-*Haemophilus influenzae* type b capsular polysaccharide antibodies by ELISA.' *J Immunol Methods,* 115(1) pp. 111-117.

Bijlmer, H.A. and Van Alphen, L. (1992) 'A prospective, population-based study of *Haemophilus influenzae* type b meningitis in The Gambia and the possible consequences.' *J Infect Dis,* 165 Suppl 1**,** S29-S32.

Bolmstrom, A. and Karlsson, A. (2002) 'Influence of CO(2) incubation on quinolone activity against *Streptococcus pneumoniae* and *Haemophilus influenzae*.' *Diagn Microbiol Infect Dis,* 42(1) pp. 65-69.

Booy, R., Hodgson, S.A., Slack, M.P., Anderson, E.C., Mayon-White, R.T. and Moxon, E.R. (1993) 'Invasive *Haemophilus influenzae* type b disease in the Oxford region (1985-91).' *Arch Dis Child,* 69(2) pp. 225-258.

Booy, R., Taylor, S.A., Dobson, S.R., Isaacs, D., Sleight, G., Aitken, S., Griffiths, H., Chapel, H., Mayon-White, R. T., Macfarlane, J.A. and Moxon, E.R. (1992) 'Immunogenicity and safety of PRP-T conjugate vaccine given according to the British accelerated immunisation schedule.' *Arch Dis Child,* 67(4) pp. 475-478.

Borrow, R., Aaberge, I.S., Santos, G.F., Eudey, T.L., Oster, P., Glennie, A., Findlow, J., Hoiby, E.A., Rosenqvist, E., Balmer, P. and Martin, D. (2005) 'Interlaboratory standardization of the measurement of serum bactericidal activity by using human complement against meningococcal serogroup b, strain 44/76-SL, before and after vaccination with the Norwegian MenBvac outer membrane vesicle vaccine.' *Clin Diagn Lab Immunol,* 12(8) pp. 970-976.

Borrow, R., Andrews, N., Findlow, H., Waight, P., Southern, J., Crowley-Luke, A., Stapley, L., England, A., Findlow, J. and Miller, E. (2010) 'Kinetics of antibody persistence following administration of a combination meningococcal serogroup C and *haemophilus influenzae* type b conjugate vaccine in healthy infants in the United Kingdom primed with a monovalent meningococcal serogroup C vaccine.' *Clin Vaccine Immunol,* 17(1) pp. 154-159.

Borrow, R., Andrews, N., Goldblatt, D. and Miller, E. (2001) 'Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: reevaluation of correlates of protection.' *Infect Immun,* 69(3) pp. 1568-1573.

Cerquetti, M., Cardines, R., Ciofi Degli Atti, M.L., Giufre, M., Bella, A., Sofia, T., Mastrantonio, P. and Slack, M. (2005) 'Presence of multiple copies of the capsulation b locus in invasive *Haemophilus influenzae* type b (Hib) strains isolated from children with Hib conjugate vaccine failure.' *J Infect Dis,* 192 pp. 819-823.

Cerquetti, M., Cardines, R., Giufre, M., Castella, A., Rebora, M., Mastrantonio, P. and Ciofi Degli Atti, M. L. (2006) 'Detection of six copies of the capsulation b locus in a *Haemophilus influenzae* type b strain isolated from a splenectomized patient with fulminant septic shock.' *J Clin Microbiol,* 44(2) pp. 640-642.

Chang, A., Kaur, R., Michel, L.V., Casey, J.R. and Pichichero, M. (2011) '*Haemophilus influenzae* vaccine candidate outer membrane protein P6 is not conserved in all strains.' *Hum Vaccin,* 7(1) pp. 102-105.

Claesson, B.A. (1993) 'Epidemiology of invasive *Haemophilus influenzae* type b disease in Scandinavia.' *Vaccine,* 11 Suppl 1**,** S30-S33.

Clark, S.E., Snow, J., Li, J., Zola, T.A. and Weiser, J.N. (2012) 'Phosphorylcholine allows for evasion of bactericidal antibody by *Haemophilus influenzae*.' *PLoS Pathog,* 8(3) pp. e1002521.

Coulehan, J.L., Michaels, R.H., Hallowell, C., Schults, R., Welty, T.K. and Kuo, J.S. (1984) 'Epidemiology of *Haemophilus influenzae* type B disease among Navajo Indians.' *Public Health Rep,* 99(4) pp. 404-409.

Cowgill, K.D., Ndiritu, M., Nyiro, J., Slack, M.P., Chiphatsi, S., Ismail, A., Kamau, T., Mwangi, I., English, M., Newton, C. R., Feikin, D.R. and Scott, J.A. (2006) 'Effectiveness of *Haemophilus influenzae* type b conjugate vaccine introduction into routine childhood immunization in Kenya.' *Jama,* 296 pp. 671-678.

Dagan, R., Fraser, D., Roitman, M., Slater, P., Anis, E., Ashkenazi, S., Kassis, I., Miron, D. and Leventhal, A. (1999) 'Effectiveness of a nationwide infant immunization program against *Haemophilus influenzae* b. The Israeli Pediatric Bacteremia and Meningitis Group.' *Vaccine,* 17(2) pp. 134-141.

Deauvieau, F., Dussurgey, S., Rossignol, D., De Montfort, A., Burdin, N. and Guy, B. (2009) 'Memory B and T cell responses induced by serotype 4 *Streptococcus pneumoniae* vaccines: longitudinal analysis comparing responses elicited by free polysaccharide, conjugate and carrier.' *Vaccine,* 28(2) pp. 576-582.

Denoel, P.A., Goldblatt, D., De Vleeschauwer, I., Jacquet, J.M., Pichichero, M.E. and Poolman, J.T. (2007) 'Quality of the *Haemophilus influenzae* type b (Hib) antibody response induced by diphtheria-tetanus-acellular pertussis/Hib combination vaccines.' *Clin Vaccine Immunol,* 14(10) pp. 1362-1369.

Eberhard, T. and Ullberg, M. (2002) 'Interaction of vitronectin with *Haemophilus influenzae.*' *FEMS Immunol Med Microbiol,* 34(3) pp. 215-219.

FDA (2001) *Bioanalytical method validation* [online] [Accessed on 5th June 2011] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf)

Feikin, D.R., Nelson, C.B., Watt, J.P., Mohsni, E., Wenger, J.D. and Levine, O.S. (2004) 'Rapid assessment tool for *Haemophilus influenzae* type b disease in developing countries.' *Emerg Infect Dis,* 10(7) pp. 1270-1276.

Filippidis, A. and Fountas, K.N. (2009) 'Nasal lymphatics as a novel invasion and dissemination route of bacterial meningitis' *Med Hypotheses*, 72(6) pp. 694-697.

Findlow, H., Plikaytis, B.D., Aase, A., Bash, M.C., Chadha, H., Elie, C., Laher, G., Martinez, J., Herstad, T., Newton, E., Viviani, S., Papaspyridis, C., Kulkarni, P., Wilding, M., Preziosi, M.P., Marchetti, E., Hassan-King, M., La Force, F.M., Carlone, G. and Borrow,R. (2009) 'Investigation of different group A immunoassays following one dose of meningococcal group A conjugate vaccine or A/C polysaccharide vaccine in adults.' *Clin Vaccine Immunol,* 16(7) pp. 969-977.

Findlow, J., Holland, A., Martin, D., Oster, P., Balmer, P. and Borrow, R. (2007) 'Inadequacy of colominic acid as an absorbent intended to facilitate use of complement-preserved baby rabbit serum in the *Neisseria meningitidis* serogroup B serum bactericidal antibody assay.' *Clin Vaccine Immunol,* 14(5) pp. 556-561.

Fothergill, L.D. and Wright, J. (1933) 'Influenzal Meningitis: The relation of age incidence to the bactericidal power of blood against the causal organism.' *J Immunol*, 24 pp. 273-284.

Frasch, C.E. and Robbins, J.D. (1978) 'Protection against group B meningococcal disease. III. Immunogenicity of serotype 2 vaccines and specificity of protection in a guinea pig model.' *J Exp Med,* 147(3) pp. 629-644.

GAVI Alliance *Haemophilus influenzae type b vaccine* [online] [Accessed 24th July 2012] http://fr.gavialliance.org/performance/commitments/haemophilius/index.php

Gilbert, G.L. (1991) 'Epidemiology of *Haemophilus influenzae* type b disease in Australia and New Zealand.' *Vaccine,* 9 Suppl**,** S10-3; discussion S25.

Gill, C. J., Ram, S., Welsch, J.A., Detora, L. and Anemona, A. (2011) 'Correlation between serum bactericidal activity against *Neisseria meningitidis* serogroups A, C, W-135 and Y measured using human versus rabbit serum as the complement source.' *Vaccine,* 30(1) pp. 29-34.

Gilsdorf, J.R. (1998) 'Antigenic diversity and gene polymorphisms in *Haemophilus influenzae*.' *Infect Immun,* 66(11) pp. 5053-5059.

Gilsdorf, J.R., McCrea, K.W. and Marrs, C.F. (1997) 'Role of pili in *Haemophilus influenzae* adherence and colonization.' *Infect Immun,* 65 pp. 2997-3002.

Goldblatt, D., Vaz, A.R. and Miller, E. (1998) 'Antibody avidity as a surrogate marker of successful priming by *Haemophilus influenzae* type b conjugate vaccines following infant immunization.' *J Infect Dis,* 177(4) pp. 1112-1115.

Granoff, D.M. and Holmes, S.J. (1991) 'Comparative immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugate vaccines.' *Vaccine,* 9 Suppl**,** S30-S34; discussion S42-S43.

Griffiss, J.M. and Goroff, D.K. (1983) 'IgA blocks IgM and IgG-initiated immune lysis by separate molecular mechanisms.' *J Immunol,* 130(6) pp. 2882-2885.

Griffiths, U.K., Clark, A., Gessner, B., Miners, A., Sanderson, C., Sedyaningsih, E.R. and Mulholland, K.E. (2012) 'Dose-specific efficacy of *Haemophilus influenzae* type b conjugate vaccines: a systematic review and meta-analysis of controlled clinical trials.' *Epidemiol Infect,* 140(8) pp. 1343-1355.

Gu, X. X., Rudy, S.F., Chu, C., Mccullagh, L., Kim, H.N., Chen, J., Li, J., Robbins, J.B., Van Waes, C. and Battey, J.F. (2003) 'Phase I study of a lipooligosaccharide-based conjugate vaccine against nontypeable *Haemophilus influenzae*.' *Vaccine,* 21(17-18) pp. 2107-2114.

Guerina, N.G., Langermann, S., Schoolnik, G.K., Kessler, T.W. and Goldmann, D.A. (1985) 'Purification and characterization of *Haemophilus influenzae* pili, and their structural and serological relatedness to Escherichia coli P and mannose-sensitive pili.' *J Exp Med,* 161 pp. 145-159.

Hallstrom, T., Zipfel, P.F., Blom, A.M., Lauer, N., Forsgren, A. and Riesbeck, K. (2008) '*Haemophilus influenzae* interacts with the human complement inhibitor factor H.' *J Immunol,* 181(1) pp. 537-545.

Health Protection Agency. (2011) *Graph showing Haemophilus influenzae type b laboratory reports: England and Wales, 1999-2010* [online] [Accessed on 23rd July 2012] http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HaemophilusInfluenzaeTypeB/EpidemiologicalData/HibGraph/23/07/12)

Health Protection Agency. *Invasive Haemophilus disease advice for clinicians and microbiologists* [online] [Accessed on 25th September 2012] http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/HaemophilusInfluenzaeTypeB/InvasiveHaemophilusDiseaseAdviceForCliniciansAndMicrobiol/

Heath, P.T. and McVernon, J. (2002) 'The UK Hib vaccine experience.' *Arch Dis Child*, 86 pp. 396-399.

Heath, P.T., Booy, R., Griffiths, H., Clutterbuck, E., Azzopardi, H.J., Slack, M., Fogarty, J., Moloney, A.C and Moxon, E.R. (2000) 'Clinical and immunological risk factors associated with *Haemophilus influenzae* type b conjugate vaccine failure in childhood' *Clin Infect Dis*, 31(4) pp. 973-980.

Hetherington, S.V., Patrick, C.C. and Hansen, E.J. (1993) 'Outer membrane protein binding sites of complement component 3 during opsonization of *Haemophilus influenzae*.' *Infect Immun,* 61(12)**,** 5157-5163.

Hill, D.J., Toleman, M.A., Evans, D.J., Villullas, S., Van Alphen, L. and Virji, M. (2001) 'The variable P5 proteins of typeable and non-typeable *Haemophilus influenzae* target human CEACAM1.' *Mol Microbiol,* 39(4)**,** 850-862.

Hoiseth, S.K., Moxon, E.R. and Silver, R.P. (1986) 'Genes involved in *Haemophilus influenzae* type b capsule expression are part of an 18-kilobase tandem duplication.' *Proc Natl Acad Sci U S A,* 83 pp. 1106-1110.

Hood, D.W., Deadman, M.E., Allen, T., Masoud, H., Martin, A., Brisson, J.R., Fleischmann, R., Venter, J. C., Richards, J.C. and Moxon, E.R. (1996) 'Use of the complete genome sequence information of *Haemophilus influenzae* strain Rd to investigate lipopolysaccharide biosynthesis.' *Mol Microbiol,* 22 pp. 951-965.

Howard, A.J., Dunkin, K.T. and Millar, G.W. (1988) 'Nasopharyngeal carriage and antibiotic resistance of *Haemophilus influenzae* in healthy children.'*Epidemiol Infect,* 100 pp. 193-203.

Howard, A.J., Dunkin, K.T., Musser, J.M. and Palmer, S.R. (1991) 'Epidemiology of *Haemophilus influenzae* type b invasive disease in Wales.' *BMJ,* 303(6800) pp. 441-445.

Hultgren, S.J., Abraham, S., Caparon, M., Falk, P., St Geme, J.W., 3rd and Normark, S. (1993) 'Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition.' *Cell,* 73 (5) pp. 887-901.

Humphries, H.E. and High, N.J. (2002) 'The role of licA phase variation in the pathogenesis of invasive disease by *Haemophilus influenzae* type b.' *FEMS Immunol Med Microbiol,* 34 pp. 221-230.

Jacobs, R.F., Wilson, C.B., Laxton, J.G., Haas, J.E. and Smith, A.L. (1982) 'Cellular uptake and intracellular activity of antibiotics against *Haemophilus influenzae* type b.' *J Infect Dis,* 145 (2) pp. 152-159.

Jain, A., Kumar, P. and Awasthi, S. (2006) 'High ampicillin resistance in different biotypes and serotypes of *Haemophilus influenzae* colonizing the nasopharynx of healthy school-going Indian children.' *J Med Microbiol,* 55 pp. 133-137.

Jiang, H.Q., Hoiseth, S.K., Harris, S.L., McNeil, L.K., Zhu, D., Tan, C., Scott, A. A., Alexander, K., Mason, K., Miller, L., Dasilva, I., Mack, M., Zhao, X. J., Pride, M.W., Andrew, L., Murphy, E., Hagen, M., French, R., Arora, A., Jones, T.R., Jansen, K.U., Zlotnick, G.W. and Anderson, A.S. (2010) 'Broad vaccine coverage predicted for a bivalent recombinant factor H binding protein based vaccine to prevent serogroup B meningococcal disease.' *Vaccine,* 28(37) pp. 6086-6093.

Jodar, L., Butler, J., Carlone, G., Dagan, R., Goldblatt, D., Kayhty, H., Klugman, K., Plikaytis, B., Siber, G., Kohberger, R., Chang, I. and Cherian, T. (2003) 'Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants.' *Vaccine,* 21(23) pp. 3265-3272.

Jones, P.A., Samuels, N.M., Phillips, N.J., Munson, R.S., Jr., Bozue, J.A., Arseneau, J. A., Nichols, W.A., Zaleski, A., Gibson, B.W. and Apicella, M.A. (2002) '*Haemophilus influenzae type* b strain A2 has multiple sialyltransferases involved in lipooligosaccharide sialylation.' *J Biol Chem,* 277(17) pp. 14598-14611.

Käyhty, H. (1994) 'Difficulties in establishing a serological correlate of protection after immunization with *Haemophilus influenzae* conjugate vaccines.' *Biologicals,* 22(4) pp. 397-402.

Käyhty, H., Peltola, H., Karanko, V. and Makela, P.H. (1983) 'The protective level of serum antibodies to the capsular polysaccharide of *Haemophilus influenzae* type b.' *J Infect Dis,* 147(6). 1100.

Kemmer, G., Reilly, T.J., Schmidt-Brauns, J., Zlotnik, G.W., Green, B.A., Fiske, M.J., Herbert, M., Kraiss, A., Schlor, S., Smith, A. and Reidl, J. (2001) 'NadN and *e* (P4) are essential for utilization of NAD and nicotinamide mononucleotide but not nicotinamide riboside in *Haemophilus influenzae*.' *J Bacteriol,* 183(13)**,** 3974-3981.

Kim, K.H., Lee, H., Chung, E.H., Kang, J.H., Kim, J. H., Kim, J.S., Lee, H.J., Oh, S.H., Park, E.A. and Park, S.E. (2008) 'Immunogenicity and safety of two different *Haemophilus influenzae* type b conjugate vaccines in Korean infants.' *J Korean Med Sci,* 23(6) pp. 929-936.

Kimura, A. and Hansen, E.J. (1986) 'Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence.' *Infect Immun,* 51(1) pp. 69-79.

Kitchin, N.R., Southern, J., Morris, R., Hemme, F., Thomas, S., Watson, M. W., Cartwright, K. and Miller, E. (2007) 'Evaluation of a diphtheria-tetanus-acellular pertussis-inactivated poliovirus-*Haemophilus influenzae* type b vaccine given concurrently with meningococcal group C conjugate vaccine at 2, 3 and 4 months of age.' *Arch Dis Child,* 92(1) pp. 11-16.

Ladhani, S. (2012) 'Two decades of experience with the *Haemophilus influenzae* serotype b conjugate vaccine in the United Kingdom.' *Clin Ther*, 34(2) pp. 385-399.

Ladhani, S., Borrow, R., Heath, P.T., Ramsay, M.E. and Booy, R. (2010a) 'Low serum serotype-specific pneumococcal antibody concentrations in young children with *Haemophilus influenzae* serotype b (Hib) vaccine failure.' *Vaccine,* 28(28) pp. 4440-4444.

Ladhani, S., Heath, P.T., Aibara, R.J., Ramsay, M.E., Slack, M.P., Hibberd, M.L., Pollard, A. J., Moxon, E.R. and Booy, R. (2010b) 'Long-term complications and risk of other serious infections following invasive *Haemophilus influenzae* serotype b disease in vaccinated children.' *Vaccine,* 28(10) pp. 2195-2200.

Ladhani, S., Neely, F., Heath, P.T., Nazareth, B., Roberts, R., Slack, M. P., McVernon, J. and Ramsay, M.E. (2009) 'Recommendations for the prevention of secondary *Haemophilus influenzae type* b (Hib) disease.' *J Infect,* 58(1)**,** 3-14.

Ladhani, S., Slack, M.P., Heath, P.T., Von Gottberg, A., Chandra, M. and Ramsay, M.E. (2010c) ' Invasive *Haemophilus influenzae* Disease, Europe, 1996-2006.' *Emerg Infect Dis,* 16(3) pp. 455-463.

Ladhani, S., Slack, M.P., Heys, M., White, J. and Ramsay, M.E. (2008) 'Fall in *Haemophilus influenzae* serotype b (Hib) disease following implementation of a booster campaign.' *Arch Dis Child,* 93(8) pp. 665-669.

Lagos, R.E., Munoz, A.E., Levine, M.M., Lepetic, A., Francois, N., Yarzabal, J.P. and Schuerman, L. (2011) 'Safety and immunogenicity of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV) in Chilean children.' *Hum Vaccin,* 7(5) pp. 511-522.

Lai, Z. and Schreiber, JR. (2009) 'Antigen processing of glycoconjugate vaccines; the polysaccharide portion of the pneumococcal CRM(197) conjugate vaccine co-localizes with MHC II on the antigen processing cell surface.' *Vaccine,* 27(24) pp. 3137-3144.
Lee, B.C. (1992) 'Isolation of haemin-binding proteins of *Neisseria gonorrhoeae*.' *J Med Microbiol,* 36 (2) pp. 121-127.

Leino, T., Auranen, K., Makela, P. H., Kayhty, H., Ramsay, M., Slack, M. and Takala, A.K. (2002) '*Haemophilus influenzae* type b and cross-reactive antigens in natural Hib infection dynamics; modelling in two populations.' *Epidemiol Infect,* 129(1) pp. 73-83.

Lesinski, G.B. and Westerink, M.A. (2001) 'Vaccines against polysaccharide antigens.' *Curr Drug Targets Infect Disord,* 1(3) pp. 325-334.

Levine, O.S., Liu, G., Garman, R.L., Dowell, S.F., Yu, S. and Yang, Y. H. (2000) '*Haemophilus influenzae* type b and *Streptococcus pneumoniae* as causes of pneumonia among children in Beijing, China.' *Emerg Infect Dis,* 6(2) pp. 165-170.

Liu, X., Wang, S., Sendi, L. and Caulfield, M.J. (2004) 'High-throughput imaging of bacterial colonies grown on filter plates with application to serum bactericidal assays.' *J Immunol Methods,* 292(1-2) pp. 187-193.

Long, S.S., Henretig, F.M., Teter, M.J. and Mcgowan, K.L. (1983) 'Nasopharyngeal flora and acute otitis media.' *Infect Immun,* 41 (3) pp. 987-991.

Lucidarme, J., Newbold, L.S., Findlow, J., Gilchrist, S., Gray, S. J., Carr, A.D., Hewitt, J., Kaczmarski, E.B. and Borrow, R. (2011) 'Molecular targets in meningococci: efficient routine characterization and optimal outbreak investigation in conjunction with routine surveillance of the meningococcal group B vaccine candidate, fHBP.' *Clin Vaccine Immunol,* 18(2) pp. 194-202.

Macone, A. B., Arakere, G., Letourneau, J.M. and Goldmann, D.A. (1985) 'Comparison of a new, rapid enzyme-linked immunosorbent assay with latex particle agglutination for the detection of *Haemophilus influenzae* type b infections.' *J Clin Microbiol,* 21(5) pp. 711-714.

Makela, P. H., Peltola, H., Kayhty, H., Jousimies, H., Pettay, O., Ruoslahti, E., Sivonen, A. and Renkonen, O.V. (1977) 'Polysaccharide vaccines of group A *Neisseria meningtitidis* and *Haemophilus influenzae* type b: a field trial in Finland.' *J Infect Dis,* 136 Suppl**,** S43-S50.

Makela, P.H., Kayhty, H., Leino, T., Auranen, K., Peltola, H., Ekstrom, N. and Eskola, J. (2003) 'Long-term persistence of immunity after immunisation with *Haemophilus influenzae* type b conjugate vaccine.' *Vaccine,* 22(2) pp. 287-292.

Martin, D., McCallum, L., Glennie, A., Ruijne, N., Blatchford, P., O'Hallahan, J. and Oster, P. (2005) 'Validation of the serum bactericidal assay for measurement of functional antibodies against group B meningococci associated with vaccine trials.' *Vaccine,* 23(17-18) pp. 2218-2221.

Maskell, D.J., Szabo, M.J., Butler, P.D., Williams, A.E. and Moxon, E.R. (1992) 'Molecular biology of phase-variable lipopolysaccharide biosynthesis by *Haemophilus influenzae*.' *J Infect Dis,* 165 Suppl 1**,** S90-S92.

Maslanka, S.E., Gheesling, L. L., Libutti, D.E., Donaldson, K.B., Harakeh, H.S., Dykes, J. K., Arhin, F. F., Devi, S.J., Frasch, C.E., Huang, J.C., Kriz-Kuzemenska, P., Lemmon, R.D., Lorange, M., Peeters, C.C., Quataert, S., Tai, J. Y. and Carlone, G.M. (1997)

‘Standardization and a multilaboratory comparison of *Neisseria meningitidis* serogroup A and C serum bactericidal assays. The Multilaboratory Study Group.’ *Clin Diagn Lab Immunol,* 4(2) pp. 156-167.

McCrea, K.W., Sauver, J.L., Marrs, C.F., Clemans, D. and Gilsdorf, J.R. (1998) ‘Immunologic and structural relationships of the minor pilus subunits among *Haemophilus influenzae* isolates.’ *Infect Immun,* 66 pp. 4788-4796.

McVernon, J., Andrews, N., Slack, M., Moxon, R. and Ramsay, M. (2008) ‘Host and environmental factors associated with Hib in England, 1998-2002.’ *Arch Dis Child,* 93(8) pp. 670-675.

McVernon, J., Andrews, N., Slack, M.P. and Ramsay, M.E. (2003) ‘Risk of vaccine failure after *Haemophilus influenzae* type b (Hib) combination vaccines with acellular pertussis.’ *Lancet,* 361(9368) pp. 1521-1523.

Michaels, R.H. and Norden, C.W. (1977) ‘Pharyngeal colonization *with Haemophilus influenzae* type b: a longitudinal study of families with a child with meningitis or epiglottitis due to H. influenzae type b.’ *J Infect Dis,* 136 pp. 222-228.

Michaels, R.H., Poziviak, C.S., Stonebraker, F.E. and Norden, C.W. (1976) ‘Factors affecting pharyngeal *Haemophilus influenzae* type b colonization rates in children.’ *J Clin Microbiol,* 4 pp. 413-417.

Millar, E.V., O'Brien, K.L., Levine, O.S., Kvamme, S., Reid, R. and Santosham, M. (2000) ‘Toward elimination of *Haemophilus influenzae* type B carriage and disease among high-risk American Indian children.’ *Am J Public Health,* 90(10) pp. 1550-1554.

Mistry, D. and Stockley, R.A. (2006) ‘IgA1 protease.’ *Int J Biochem Cell Biol,* 38(8) pp. 1244-1248.

Moxon, E.R. (2009) ‘Bacterial variation, virulence and vaccines.’ *Microbiology,* 155 pp. 997-1003.

Mulks, M.H., Kornfeld, S.J. and Plaut, A.G. (1980) ‘Specific proteolysis of human IgA by *Streptococcus pneumoniae* and *Haemophilus influenzae*.’ *J Infect Dis,* 141(4) pp. 450-456.

Munson, E.L. and Doern, G.V. (2007) ‘Comparison of three commercial test systems for biotyping *Haemophilus influenzae* and *Haemophilus parainfluenzae*.’ *J Clin Microbiol,* 45(12) pp. 4051-4053.

Noel, G.J., Brittingham, A., Granato, A.A. and Mosser, D.M. (1996) ‘Effect of amplification of the Cap b locus on complement-mediated bacteriolysis and opsonization of type b *Haemophilus influenzae*.’ *Infect Immun,* 64(11) pp. 4769-4775.

Ojo, L.R., O'Loughlin, R.E., Cohen, A. L., Loo, J.D., Edmond, K.M., Shetty, S.S., Bear, A. P., Privor-Dumm, L., Griffiths, U. K. and Hajjeh, R. (2010) ‘Global use of *Haemophilus influenzae* type b conjugate vaccine.’ *Vaccine,* 28(43) pp. 7117-7122.

Olitsky, P.K. and Gates, F.L. (1921) 'Experimental studies of the nasopharyngeal secretions from influenza patients: V. Bacterium pneumosintes and concurrent infections.' *J Exp Med*, 34(1) pp. 1-9.

Peltola, H., Kayhty, H., Sivonen, A. and Makela, H. (1977) '*Haemophilus influenzae* type b capsular polysaccharide vaccine in children: a double-blind field study of 100,000 vaccinees 3 months to 5 years of age in Finland.' *Pediatrics,* 60(5) pp. 730-737.

Phipps, D.C., West, J., Eby, R., Koster, M., Madore, D.V. and Quataert, S.A. (1990) 'An ELISA employing a *Haemophilus influenzae* type b oligosaccharide-human serum albumin conjugate correlates with the radioantigen binding assay.' *J Immunol Methods,* 135 (1-2) pp. 121-128.

Pichichero, M.E., Loeb, M., Anderson. and Smith, D.H. (1982) 'Do pili play a role in pathogenicity of *Haemophilus influenzae* type B?' *Lancet,* 2 pp. 960-962.

Pittman, M. (1931) 'Variation and Type Specificity in the Bacterial Species *Hemophilus Influenzae*.' *J Exp Med,* 53 pp. 471-492.

Prymula, R., Peeters, P., Chrobok, V., Kriz, P., Novakova, E., Kaliskova, E., Kohl, I., Lommel, P., Poolman, J., Prieels, J. P. and Schuerman, L. (2006) 'Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both *Streptococcus pneumoniae* and non-typable *Haemophilus influenzae*: a randomised double-blind efficacy study.' *Lancet,* 367(9512) pp. 740-748.

Ramakrishnan, M., Ulland, A.J., Steinhardt, L.C., Moisi, J.C., Were, F. and Levine, O.S. (2009) 'Sequelae due to bacterial meningitis among African children: a systematic literature review.' *BMC Med,* 14 pp. 7- 47.

Ramsay, M.E., McVernon, J., Andrews, N.J., Heath, P.T. and Slack, M.P. (2003) 'Estimating *Haemophilus influenzae* type b vaccine effectiveness in England and Wales by use of the screening method.' *J Infect Dis,* 188(4) pp. 481-485.

Robbins, J.B., Parke, J.C., Jr., Schneerson, R. and Whisnant, J.K. (1973) 'Quantitative measurement of "natural" and immunization-induced *Haemophilus influenzae* type b capsular polysaccharide antibodies.' *Pediatr Res,* 7(3) pp. 103-110.

Rodriguez, T., Lastre, M., Cedre, B., Del Campo, J., Bracho, G., Zayas, C., Taboada, C., Diaz, M., Sierra, G. and Perez, O. (2002) 'Standardization of *Neisseria meningitidis* serogroup B colorimetric serum bactericidal assay.' *Clin Diagn Lab Immunol,* 9(1) pp. 109-114.

Romero-Steiner, S., Fernandez, J., Biltoft, C., Wohl, M.E., Sanchez, J., Feris, J., Balter, S., Levine, O.S. and Carlone, G.M. (2001) 'Functional antibody activity elicited by fractional doses of *Haemophilus influenzae* type b conjugate vaccine (polyribosylribitol phosphate-tetanus toxoid conjugate).' *Clin Diagn Lab Immunol,* 8(6) pp. 1115-1119.

Romero-Steiner, S., Spear, W., Brown, N., Holder, P., Hennessy, T., Gomez de Leon, P. and Carlone, G. M. (2004) 'Measurement of serum bactericidal activity specific for

Haemophilus influenzae type b by using a chromogenic and fluorescent metabolic indicator.' *Clin Diagn Lab Immunol,* 11(1) pp. 89-93.

Rossi, I.A., Zuber, P.L., Dumolard, L., Walker, D.G. and Watt, J. (2007) 'Introduction of Hib vaccine into national immunization programmes: a descriptive analysis of global trends.' *Vaccine,* 25(41) pp. 7075-7080.

Rubin, L.G. and Moxon, E.R. (1983) 'Pathogenesis of bloodstream invasion with *Haemophilus influenzae* type b.' *Infect Immun,* 41(1) pp. 280-284.

Santos, G.F., Deck, R.R., Donnelly, J., Blackwelder, W. and Granoff, D.M. (2001) 'Importance of complement source in measuring meningococcal bactericidal titers.' *Clin Diagn Lab Immunol,* 8(3) pp. 616-623.

Santosham, M., Reid, R., Ambrosino, D.M., Wolff, M.C., Almeido-Hill, J., Priehs, C., Aspery, K. M., Garrett, S., Croll, L., Foster, S. and et al. (1987) 'Prevention of *Haemophilus influenzae* type b infections in high-risk infants treated with bacterial polysaccharide immune globulin.' *N Engl J Med,* 317(15) pp. 923-929.

Santosham, M., Reid, R., Chandran, A., Millar, E.V., Watt, J. P., Weatherholtz, R., Donaldson, C., Croll, J., Moulton, L. H., Thompson, C.M., Siber, G.R. and O'Brien, K.L. (2007) 'Contributions of Native Americans to the global control of infectious diseases.' *Vaccine,* 25(13) pp. 2366-2374.

Santosham, M., Wolff, M., Reid, R., Hohenboken, M., Bateman, M., Goepp, J., Cortese, M., Sack, D., Hill, J. and Newcomer, W. (1991) 'The efficacy in Navajo infants of a conjugate vaccine consisting of *Haemophilus influenzae* type b polysaccharide and *Neisseria meningitidis* outer-membrane protein complex.' *N Engl J Med,* 324(25) pp. 1767-1772.

Satola, S.W., Collins, J.T., Napier, R. and Farley, M.M. (2007) 'Capsule gene analysis of invasive *Haemophilus influenzae*: accuracy of serotyping and prevalence of IS1016 among nontypeable isolates.' *J Clin Microbiol,* 45 pp. 3230-3238.

Satola, S.W., Schirmer, P.L. and Farley, M.M. (2003) 'Complete sequence of the cap locus of *Haemophilus influenzae* serotype b and nonencapsulated b capsule-negative variants.' *Infect Immun,* 71 pp. 3639-3644.

Schlesinger, Y. and Granoff, D.M. and The Vaccine Study Group (1992) 'Avidity and bactericidal activity of antibody elicited by different Haemophilus influenzae type b conjugate vaccines' *JAMA,* 267(11) pp. 1489-1494.

Schreiber, J.R., Barrus, V., Cates, K.L. and Siber, G.R. (1986) 'Functional characterization of human IgG, IgM, and IgA antibody directed to the capsule of *Haemophilus influenzae* type b.' *J Infect Dis,* 153(1) pp. 8-16.

Schweda, E. K., Richards, J.C., Hood, D.W. and Moxon, E.R. (2007) 'Expression and structural diversity of the lipopolysaccharide of *Haemophilus influenzae:* implication in virulence.' *Int J Med Microbiol,* 297 pp. 297-306.

Semczuk, K., Dzierzanowska-Fangrat, K., Lopaciuk, U., Gabinska, E., Jozwiak, P. and Dzierzanowska, D. (2004) 'Antimicrobial resistance of *Streptococcus pneumoniae* and *Haemophilus influenzae* isolated from children with community-acquired respiratory tract infections in Central Poland.' *Int J Antimicrob Agents,* 23(1) pp. 39-43.

Sharma, A., Kaur, R., Ganguly, N.K., Singh, P.D., Chakraborti, A. (2002) 'Subtype distribution of *Haemophilus influenzae* isolates from north India.' *J Med Microbiol,* 51 pp. 399-404.

Southern, J., Borrow, R., Andrews, N., Morris, R., Waight, P., Hudson, M., Balmer, P., Findlow, H., Findlow, J. and Miller, E. (2009) 'Immunogenicity of a reduced schedule of meningococcal group C conjugate vaccine given concomitantly with the Prevenar and Pediacel vaccines in healthy infants in the United Kingdom.' *Clin Vaccine Immunol,* 16(2) pp. 194-199.

St. Geme III, J.W. and Falkow, S. (1991) 'Loss of capsule expression by *Haemophilus influenzae* type b results in enhanced adherence to and invasion of human cells.' ' *Infect Immun*, 59(4) pp. 1325-1333.

Swift, A.J., Moxon, E.R., Zwahlen, A. and Winkelstein, J.A. (1991) 'Complement-mediated serum activities against genetically defined capsular transformants of *Haemophilus influenzae*.' *Microb Pathog,* 10 pp. 261-269.

Tam, R. and Saier, M.H., Jr. (1993) 'Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria.' *Microbiol Rev,* 57(2) pp. 320-346.

Tebutt, G.M. (1983) 'Evaluation of some methods for the laboratory identification of *Haemophilus influenzae.*' *J Clin Pathol*, 36(9) pp. 991-995.

Todd, J.K. and Bruhn, F.W. (1975) 'Severe *Haemophilus influenzae* infections.' *Am J Dis Child*, 129(5) pp. 607-611.

Trotter, C.L., Ramsay, M.E. and Slack, M.P. (2003) 'Rising incidence of *Haemophilus influenzae* type b disease in England and Wales indicates a need for a second catch-up vaccination campaign.' *Commun Dis Public Health,* 6(1) pp. 55-58.

Trouw, L.A., Seelen, M.A. and Daha, M.R. (2003) 'Complement and renal disease.' *Mol Immunol,* 40(2-4) pp. 125-134.

Turk, D.C. (1975) 'An investigation of the family background of acute Haemophilus infections of children.' *J Hyg (Lond),* 75 (2) pp. 315-332.

Ulanova, M. and Tsang, R.S. (2009) 'Invasive *Haemophilus influenzae* disease: changing epidemiology and host-parasite interactions in the 21st century.' *Infect Genet Evol,* 9 pp. 594-605.

Uria, M.J., Zhang, Q., Li,Y., Chan, A., Exley, R.M., Gollan, B., Chan, H., Feavers, I., Yarwood, A., Abad, R., Borrow, R., Fleck, R.A., Mulloy, B., Vazquez, J.A. and Tang,

C.M. (2008) ‘A generic mechanism in *Neisseria meningitidis* for enhanced resistance against bactericidal antibodies.’ *J Exp Med,* 205(6) pp. 1423-1434.

Van Alphen, L., Levene, C., Geelen-Van Den Broek, L., Poole, J., Bennett, M. and Dankert, J. (1990) ‘Combined inheritance of epithelial and erythrocyte receptors for *Haemophilus influenzae.’ Infect Immun,* 58(11) pp. 3807-3809.

Ward, J. I., Margolis, H.S., Lum, M.K., Fraser, D.W., Bender, T.R. and Anderson, P. (1981) ‘*Haemophilus influenzae* disease in Alaskan Eskimos: characteristics of a population with an unusual incidence of invasive disease.’ *Lancet,* 1(8233) pp. 1281-1285.

Ward, J. I., Gorman, G., Phillips, C. and Fraser, D.W. (1978) ‘*Hemophilus influenzae* type b disease in a day-care center. Report of an outbreak.’ *J Pediatr,* 92(5) pp. 713-717.

Watson, W.J., Gilsdorf, J.R., Tucci, M.A., McCrea, K.W., Forney, L.J. and Marrs, C.F. (1994) ‘Identification of a gene essential for piliation in *Haemophilus influenzae* type b with homology to the pilus assembly platform genes of gram-negative bacteria.’ *Infect Immun,* 62 pp. 468-475.

Watt, J.P., Wolfson, L.J., O`Brien, K.L., Henkle, E., Deloria-Knoll, M., McCall, N., Lee, E., Levine, O.S., Hajjeh, R., Mulholland, K. and Cherian, T. (2009) ‘Burden of disease caused by *Haemophilus influenzae* type b in children younger than 5 years: global estimates.’ *Lancet,* 374 pp. 903-911.

Welch, D.F. and Hensel, D. (1982) ‘Evaluation of Bactogen and Phadebact for detection of *Haemophilus influenzae* type b antigen in cerebrospinal fluid.’ *J Clin Microbiol,* 16(5) pp. 905-908.

World Health Organization (2005) *Haemophilus influenzae type B (Hib) Fact sheet N°294* [online] [Accessed on 23rd July 2012] http://www.who.int/mediacentre/factsheets/fs294/en/index.html

World Health Organization (2006) WHO position paper on *Haemophilus influenzae* type b conjugate vaccines. (Replaces WHO position paper on Hib vaccines previously published in the Weekly Epidemiological Record. *Wkly Epidemiol Rec,* 81**,** 445-52.

Williams, P., Morton, D.J., Towner, K.J., Stevenson, P. and Griffiths, E. (1990) ‘Utilization of enterobactin and other exogenous iron sources by *Haemophilus influenzae, H. parainfluenzae* and *H. paraphrophilus*.’ *J Gen Microbiol,* 136(12) pp. 2343-2350.

Winslow, C.E., Broadhurst, J., Buchanan, R.E., Krumwiede, C., Rogers, L.A. and Smith, G.H. (1920) ‘The Families and Genera of the Bacteria: Final Report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types.’ *J Bacteriol,* 5 pp**.** 191-229.

Winter, L.E. and Barenkamp, S.J. (2006) ‘Antibodies specific for the high-molecular-weight adhesion proteins of nontypeable *Haemophilus influenzae* are opsonophagocytic for both homologous and heterologous strains.’ *Clin Vaccine Immunol,* 13(12) pp. 1333-1342.

World Health Organization. 1976. ‘Requirements for meningococcal polysaccharide vaccine (requirements for biological substances no. 23).’ W. H. O. Tech. Rep. Ser. 594 pp**.** 72–73.

Wurzner, R. (1999) ‘Evasion of pathogens by avoiding recognition or eradication by complement, in part via molecular mimicry.’ *Mol Immunol,* 36(4-5) pp. 249-260.

Yang, Y.P., Munson, R.S., Jr., Grass, S., Chong, P., Harkness, R.E., Gisonni, L., James, O., Kwok, Y. and Klein, M.H. (1997) ‘Effect of lipid modification on the physicochemical, structural, antigenic and immunoprotective properties of *Haemophilus influenzae* outer membrane protein P6.’ *Vaccine,* 15(9) pp. 976-87.

Zipfel, P.F., Wurzner, R. and Skerka, C. (2007) ‘Complement evasion of pathogens: common strategies are shared by diverse organisms.’ *Mol Immunol,* 44(16) pp. 3850-3857.

Zollinger, W.D. and Mandrell, R. E. (1983) ‘Importance of complement source in bactericidal activity of human antibody and murine monoclonal antibody to meningococcal group B polysaccharide.’ *Infect Immun,* 40(1) pp. 257-264.

Appendix I Ethical approval (REC reference number 07/MRE03/6)

NHS

Northern and Yorkshire Research Ethics Committee

Room 215
TEDCO Business Centre
Viking Industrial Park
Jarrow
Tyne & Wear NE32 3DT

Telephone 0191 4283545/4283438 Fax 0191 4283303

Bill Hackett (Co-ordinator) e-mail: bill.hackett@suntpct.nhs.uk
Helen Wilson (Asst Co-ordinator) e-mail: helen.wilson@suntpct.nhs.uk

06 March 2007

Dr Edward Kaczmarski
Consultant Medical Microbiologist
Health Protection Agency
North West Regional H.P.A. Laboratory, MMMP,
2nd floor, Clinical Sciences Building II, PO Box 209,
Manchester Royal Infirmary,
Oxford Road,
Manchester,
M13 9WZ

Dear Dr Kaczmarski

Full title of study:	**A Phase IV, single group study to evaluate the immunogenicity and safety in UK laboratory workers of a licensed Hib and meningococcal C conjugate combined vaccine (Menitorix).**
REC reference number:	**07/MRE03/6**
Protocol number:	**Version 3**
EudraCT number:	**2006-004302-74**

Thank you for your letter of 27 February 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by myself under Chair's Action.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Appendix I (continued)

Approved documents

The final list of documents reviewed by the Committee is as follows:

Document	*Version*	*Date*
Application	5.2	13 December 2006
Investigator CV	Edward B Kaczmarski	12 December 2006
Protocol	2	15 November 2006
Protocol	Version 3	30 January 2007
Covering Letter		27 February 2007
Covering Letter		13 December 2006
Letter from Sponsor	from Dr P Mortimer	24 November 2006
Advertisement	2	15 November 2006
GP/Consultant Information Sheets	2	15 November 2006
Participant Information Sheet	2	15 November 2006
Participant Consent Form	2	15 November 2006
Response to Request for Further Information		
Signature page from sponsor	Signature page from sponsor	
Answers to provisional opinion	Answers to provisional opinion	
Letter from funder		21 August 2006
MHRA request for authorisation		13 December 2006

R&D approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final approval from the R&D office for the relevant NHS care organisation.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

07/MRE03/6 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Professor Peter Heasman
Chair

Email: bill.hackett@suntpct.nhs.uk

Appendix I (continued)

Enclosures:	*Standard approval conditions* *Site approval form*
Copy to:	Dr Philip Mortimer Health Protection Agency Clinical Goverenance 61 Colindale Avenue Colindale London NW9 5EQ Clinical Trials Unit, MHRA

Northern & Yorkshire REC

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	07/MRE03/6	**Issue number:**	0	**Date of issue:**	06 March 2007
Chief Investigator:	Dr Edward Kaczmarski				
Full title of study:	A Phase IV, single group study to evaluate the immunogenicity and safety in UK laboratory workers of a licensed Hib and meningococcal C conjugate combined vaccine (Menitorix).				

This study was given a favourable ethical opinion by Northern & Yorkshire REC on 07 March 2007. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* (1)
Dr Edward Kaczmarski	Consultant Medical Microbiologist	Health Protection Agency North West (part of the NHS)	Central Manchester Research Ethics Committee	06/03/2007	

Approved by the Chair on behalf of the REC:

..... (Signature of Chair/Co-ordinator)
(delete as applicable)

BILL HACKETT. (Name)

Appendix I (continued)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

Appendix II Ethical approval (REC reference number 04/MRE5/44)

SL14 Favourable opinion following consideration of further information
Version 2, October 2004

Dr S Evans- Chairman **Dr M Wilkinson –Vice Chairman**
Eastern Multi-Research Ethics Committee
The REGUS Centre
1010 Cambourne Business Park
Cambourne
Cambridge
CB3 6DP
Tel: 01223 598467
Fax: 01223 598118
Email: Admin@easternmrec.com

Our ref: SL1404mre544Favopfurtherinfonov04

19th November 2004

Prof. E Miller
Immunisation Dept
Health Protection Agency
61 Colindale Avenue
London, NW9 5EQ

Dear Prof. Miller

Full title of study: A phase IV, randomised study to evaluate the immune response of UK infants receiving licensed DtaP/Hib/IPV, Meningococcal C conjugate and pneumococcal conjugate vaccines
REC reference number: 04/mre5/44
Protocol number: version 2 dated 7/10/04
EudraCT number: 2004-001049-14

Thank you for your letter of 3/11/2004 responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chairman and Vice Chairman.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Studies requiring SSA with at least one site approved

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed that they have no objection.

Conditions of approval
The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

23 NOV 2004

Appendix II (continued)

SL14 Favourable opinion following consideration of further information
Version 2, October 2004

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

List documents with version numbers and dates:
Letters dated 3/11/2004 and 7/10/2004
Letter from applicant dated 24/8/04
Letter from applicant dated 31/8/04-further info
Application form
Additional information to parts A, B and C-
automatically generated.
Parent information leaflet SCHED1 Protocol,
v 2 dated 7/10/2004
Reply slip SCHED1 protocol, v2 dated 7/10/2004
Information letter-5 months of age, SCHED1 Protocol,
v 2 dated 7/10/2004
Parental consent form SCHED1 Protocol, v 2
dated 7/10/2004
Patient Diary- SCHED 1 Health Diary version-14.9.04
Flowchart SCHED 1 study process v1 dated 28/8/04
Letter from study vaccine nurse SCHED1 Protocol, v 2
dated 7/10/2004
GP information leaflet SCHED1 Protocol, v1
dated 15/5/04
Participation card v 1 dated 7/10/2004
Letter from statistician dated 25/8/04
Letter 4/9/02 Re: Grant Allocation
Scientific report- consortium meeting minutes
dated 18/6/04 (2 pages)
CV Prof. E Miller
Research Protocol SCHED1v 2 dated 7/10/2004
(includes copies of letters and forms)
Summary of product characteristics-Pediacel
Summary of product characteristics-Menjugate
Summary of product characteristics-Meningitec
Summary of product characteristics- Neis Vac-C
Annex 1-Summary of product characteristics

Management approval

Studies requiring SSA:

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Notification of other bodies

The Committee Administrator will notify the research sponsor and for CTIMPs, the Medicines and Health-care products Regulatory Agency that the study has a favourable ethical opinion.

Statement of compliance

CTIMPs

Appendix II (continued)

SL14 Favourable opinion following consideration of further information
Version 2, October 2004

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

All studies

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference number :04/mre5/44 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely

Anne M Burnley
For Dr S Evans
E-mail: Admin@easternmrec.com

Enclosures List of names and of members who were present at the meeting and those who submitted written comments below.

Standard approval conditions

Site approval form (SF1)

List of members present at meeting dated 23/09/2004
Dr S Evans- Lay Chairman
Dr M Wilkinson- Clinical Vice Chairman
Dr S Ariyanayagam- Clinician
Mr A S Baker- Clinician
Mrs A Colvill- Lay Member
Mr R Driver- Lay Member
Mr L Gelling- Clinician
Mr M Sydes- Statistician

Appendix III Ethical approval (REC reference number 05/MRE12/50)

NHS

Thames Valley Multi-centre Research Ethics Committee

13 September 2005

Prospect Park Hospital
Orchid Suite
Honey End Lane
Tilehurst
Reading RG30 4EJ

Tel: 0118 960 5191/5192
Fax: 0118 960 5368
www.corec.org.uk

Prof Robert Booy
Head, Centre for Child Health
Centre for Child Health
Queen Mary's School of Medicine and Dentistry
Barts and the London, Royal London Hospital
38 New Road, Whitechapel, London
E1 2AX

Dear Prof Booy

Full title of study: **Long-term follow-up of childen who developed invasive Haemophilus Influenzae serotype b (Hib) disease despite vaccination against Hib and characterisation of vaccine failures using large scale immunogenetics.**

REC reference number: **05/MRE12/50**

Thank you for your letter of 01 September 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

With regard to Dr Ladhani's query in his email to us dated 21 June 2005, we would prefer if the genetic information could **not** be linked back to the patient.

The Committee has designated this study as having "no local investigators". There is no requirement for Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		20 May 2005
Application - Part B: Section 4 - Use of Existing Stored		20 August 2005

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The Central Office for Research Ethics Committees is responsible for the operational management of Multi-centre Research Ethics Committees

Appendix III (continued)

Samples, signed by Prof M Levin		
Investigator CV		20 May 2005
Protocol	1	30 March 2005
Covering Letter		22 May 2005
Summary/Synopsis - Background of Research Proposal	4	20 May 2005
Letter from Sponsor - Joint Director of R&D, Queen Mary, University of London School of Medicine and Dentistry		08 April 2005
Compensation Arrangements - Letter of provisional indemnity from Barts and The London NHS Trust		08 April 2005
Copy of Questionnaire - GP Questionnaire	13	20 May 2005
Copy of Questionnaire - Parent Questionnaire	3	26 March 2005
Copy of Questionnaire - Paediatrician questionnaire	3	15 May 2005
Letters of Invitation to Participants - Parent Invitation Letter	3	05 August 2005
GP/Consultant Information Sheets - Letter to GP	4	20 May 2005
GP/Consultant Information Sheets - Reminder for request to complete a questionnaire	2	20 May 2005
GP/Consultant Information Sheets - GP reply slip	4	20 May 2005
GP/Consultant Information Sheets - Reminder to return the reply slip	2	20 May 2005
GP/Consultant Information Sheets - Request to complete a questionnaire	3	20 May 2005
Participant Information Sheet - Parent reply slip	1	15 March 2005
Participant Information Sheet - Reminder to take part in a clinical study	2	20 May 2005
Information sheet and assent form for children	1	08 August 2005
Participant Information Sheet - Information sheet for Parents	15	08 August 2005
Participant Consent Form - Parent Consent Form	4	15 March 2005
Response to Request for Further Information		01 September 2005
Letter to R&D re: Participation of local paediatrician in a MREC approved study	1	20 May 2005
Registration form for health advocacy service of clinical trials	signed on 04/04/2005	07 January 2003
Approval from St Mary's LREC for the use of Control Samples from a previous study		13 February 1996
Letter from Dr Mary Ramsay, Immunisation Department, Health Protection Agency		09 August 2005
Letter from Dr Martin Hibberd of Genome Institute Singapore		05 August 2005
List of Appendices	5	20 May 2005
Letter to paediatrician: Reminder for request to complete a questionnaire	1	20 May 2005
Letter to paediatrician: Request to complete a questionnaire	2	20 May 2005

Management approval

You should arrange for all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research must obtain management approval from the relevant care organisation before commencing any

Appendix III (continued)

Samples, signed by Prof M Levin		
Investigator CV		20 May 2005
Protocol	1	30 March 2005
Covering Letter		22 May 2005
Summary/Synopsis - Background of Research Proposal	4	20 May 2005
Letter from Sponsor - Joint Director of R&D, Queen Mary, University of London School of Medicine and Dentistry		08 April 2005
Compensation Arrangements - Letter of provisional indemnity from Barts and The London NHS Trust		08 April 2005
Copy of Questionnaire - GP Questionnaire	13	20 May 2005
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Copy of Questionnaire - Paediatrician questionnaire	3	15 May 2005
Letters of Invitation to Participants - Parent Invitation Letter	3	05 August 2005
GP/Consultant Information Sheets - Letter to GP	4	20 May 2005
GP/Consultant Information Sheets - Reminder for request to complete a questionnaire	2	20 May 2005
GP/Consultant Information Sheets - GP reply slip	4	20 May 2005
GP/Consultant Information Sheets - Reminder to return the reply slip	2	20 May 2005
GP/Consultant Information Sheets - Request to complete a questionnaire	3	20 May 2005
Participant Information Sheet - Parent reply slip	1	15 March 2005
Participant Information Sheet - Reminder to take part in a clinical study	2	20 May 2005
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Registration form for health advocacy service of clinical trials	signed on 04/04/2005	07 January 2003
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Letter from Dr Mary Ramsay, Immunisation Department, Health Protection Agency		09 August 2005
Letter from Dr Martin Hibberd of Genome Institute Singapore		05 August 2005
List of Appendices	5	20 May 2005
Letter to paediatrician: Reminder for request to complete a questionnaire	1	20 May 2005
Letter to paediatrician: Request to complete a questionnaire	2	20 May 2005

Management approval

You should arrange for all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research must obtain management approval from the relevant care organisation before commencing any

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Appendix III (continued)

research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Notification of other bodies

The Committee Co-ordinator will notify the research sponsor that the study has a favourable ethical opinion.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference: 05/MRE12/50 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely

pp

Peter Tausig
Chair

Email: lavenda.lee@berkshire.nhs.uk

Enclosure: Standard approval conditions

Appendix III (continued)

Thames Valley MREC

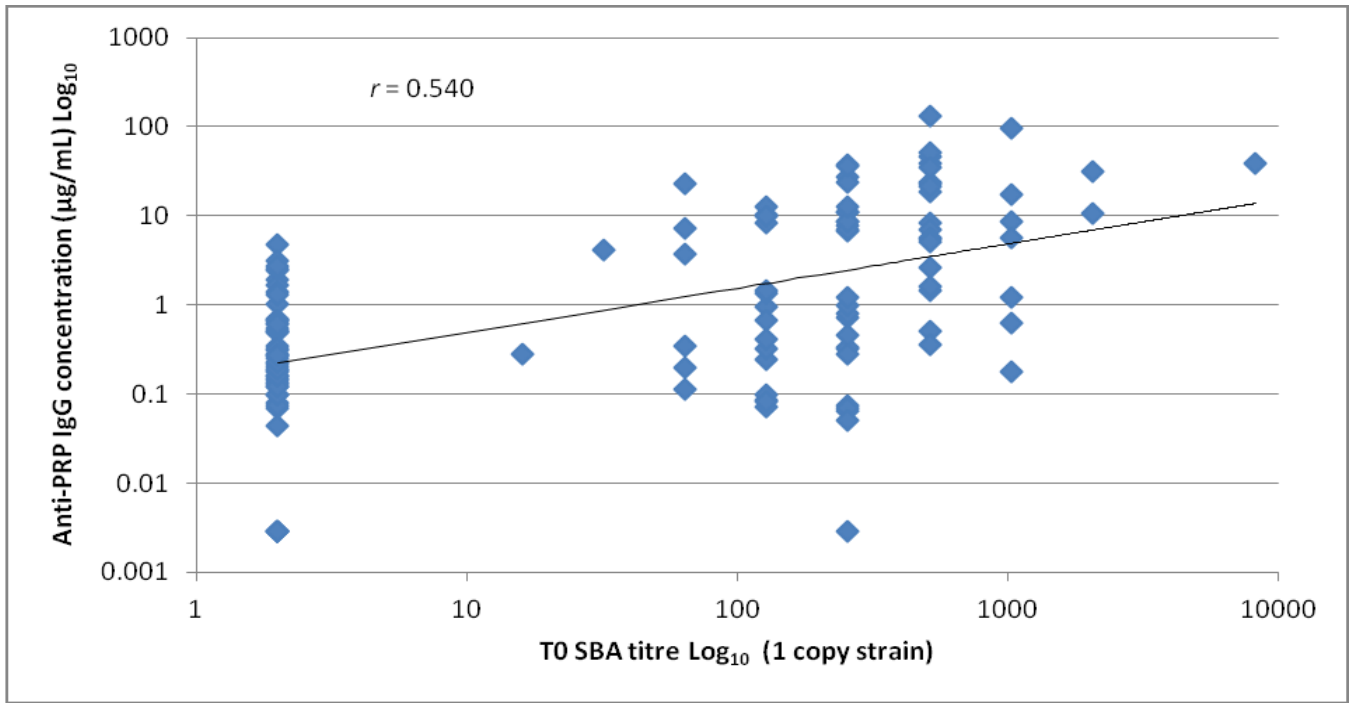
Attendance at Committee meeting on 21 June 2005

Committee Members:

Name	*Profession*	*Present?*	*Notes*
Mr P Tausig	(Chair, Lay Member)	Present	
Dr C Cheetham	(Consultant Paediatrician)	Present	
Mr P Allen	(Oral and Dental Surgeon)	Present	
Mr A Gillian	(Pharmacist)	Present	
Mr K Reel	(Occupational Therapy Lecturer)	Present	
Mr G Vallance	(Lay Member)	Present	
Dr D Parker	(GP)	Present	
Mr J Hughes	(Medical Statistician)	Present	

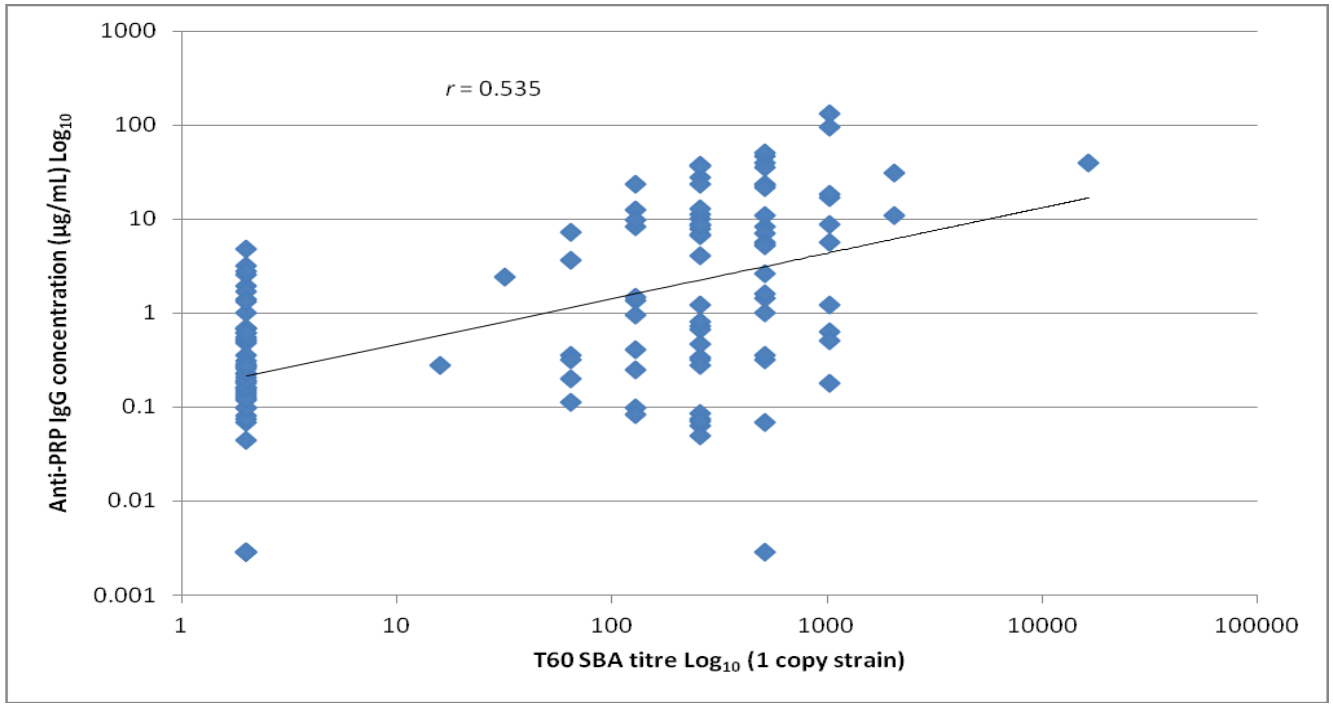
Appendix IV Scatter plots showing the correlation between anti-PRP IgG concentrations and SBA titres calculated at T0 and T60 time points, for strains expressing 1-5 copies of the *cap* b locus

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T0 for 1 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



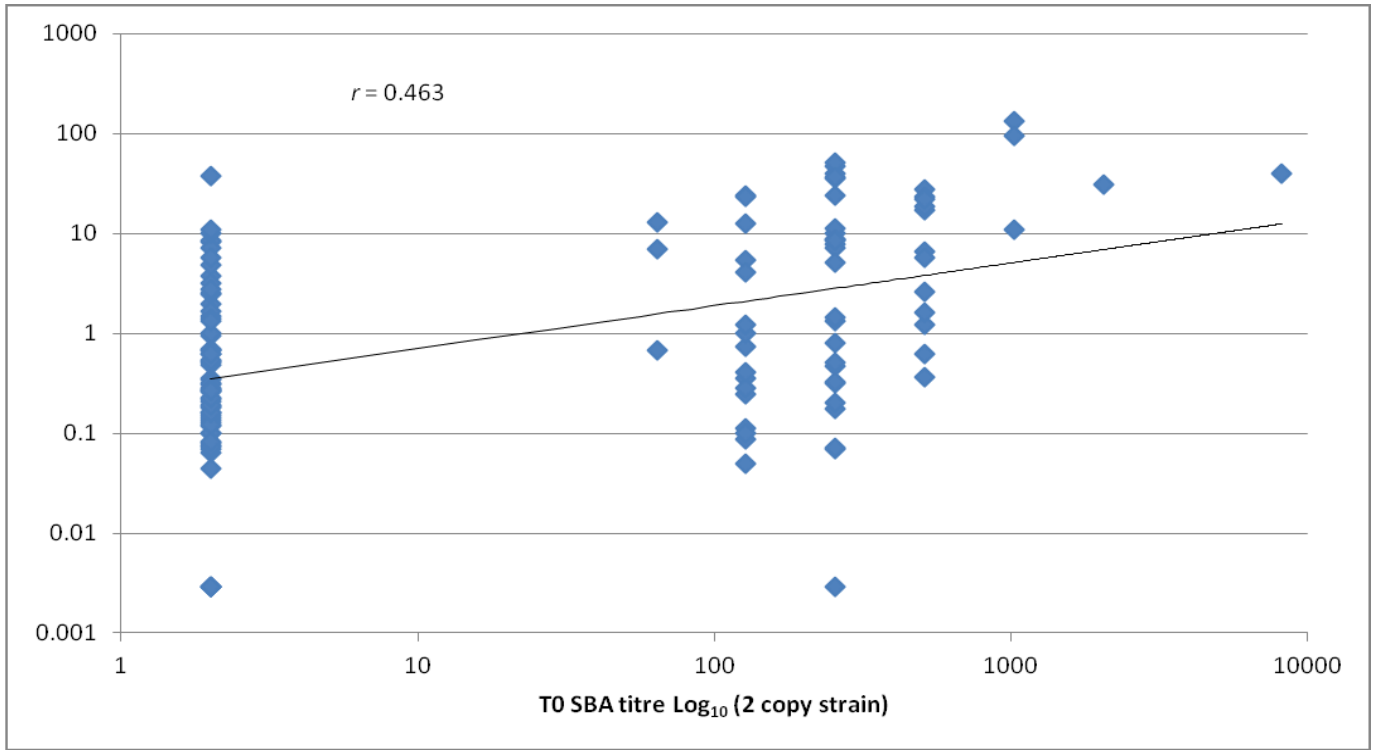
Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T60 for 1 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



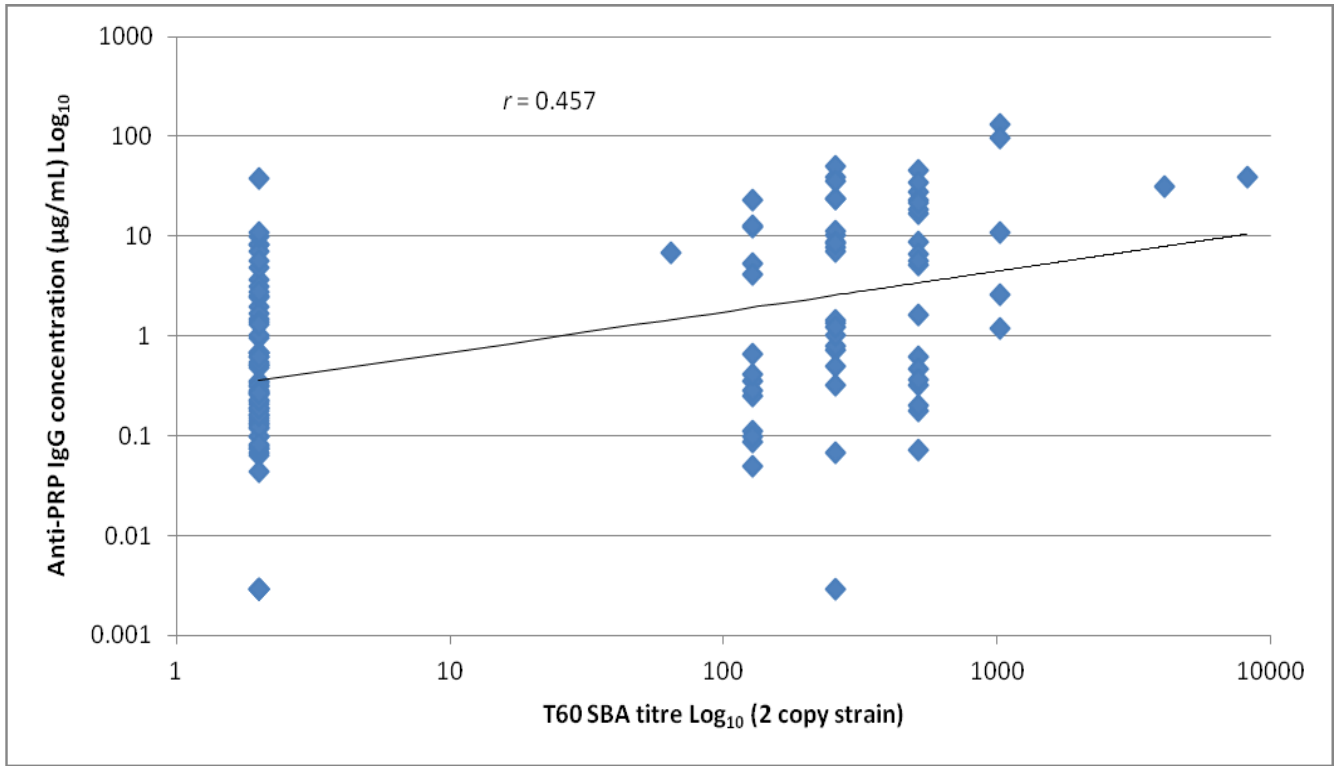
Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T0 for 2 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



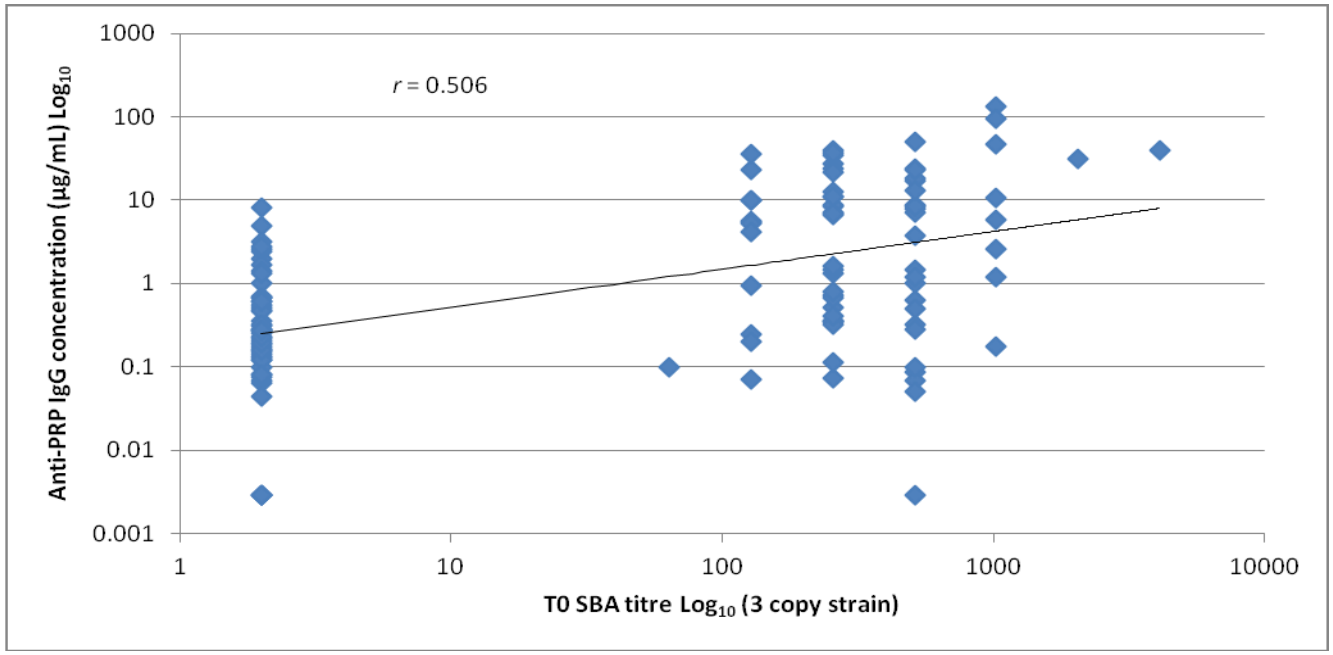
Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T60 for 2 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



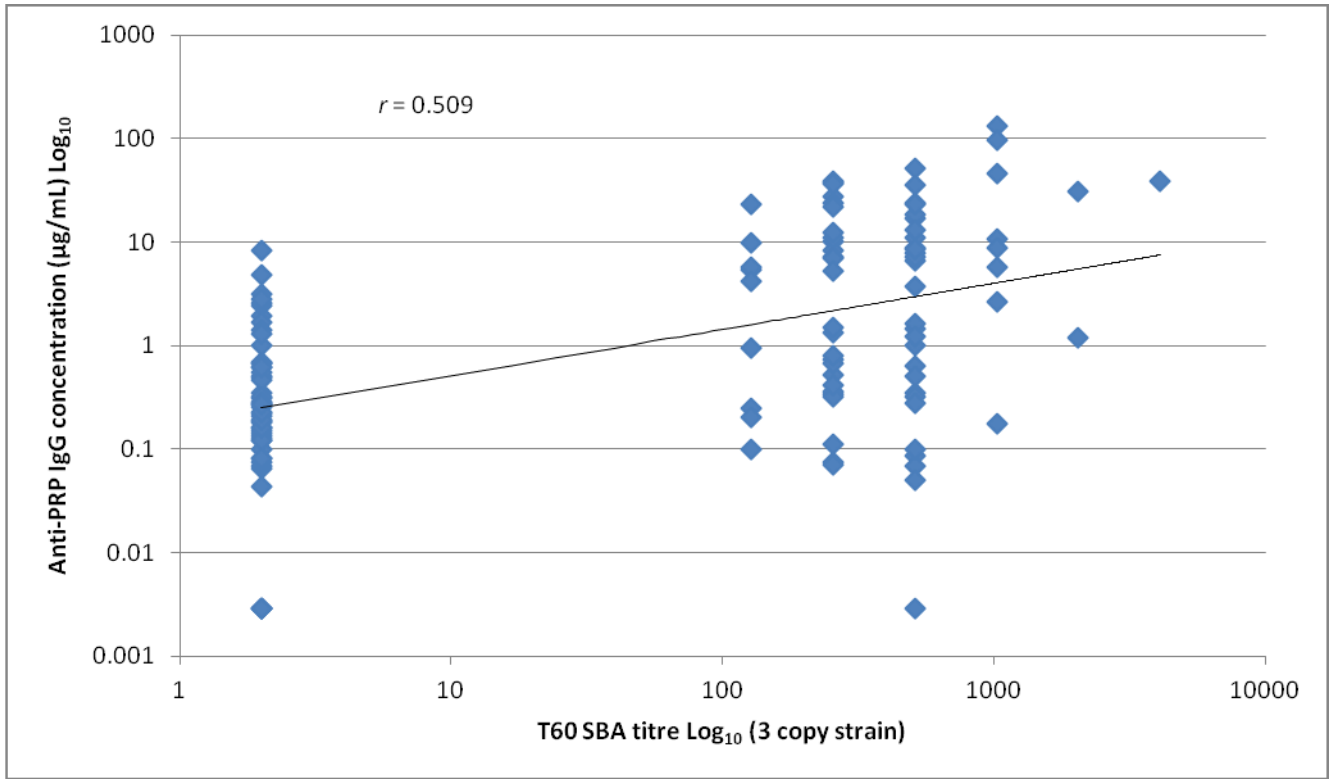
Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T0 for 3 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



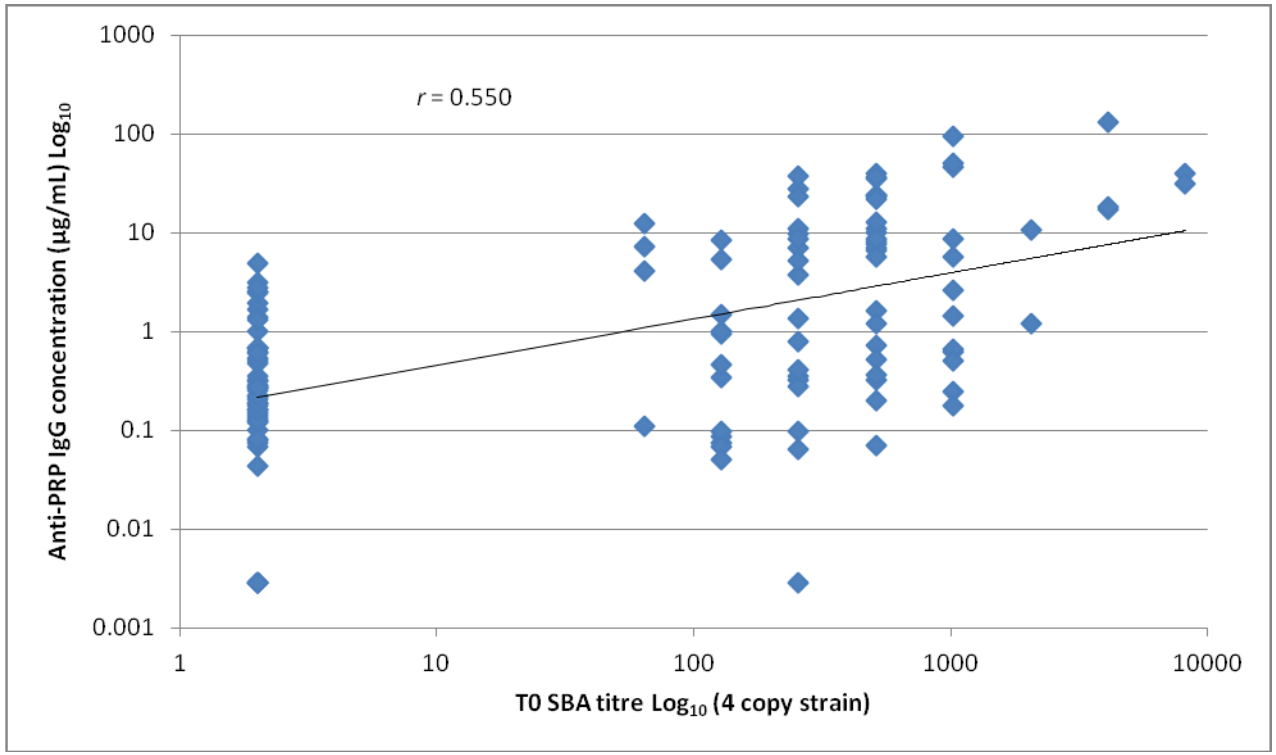
Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T60 for 3 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



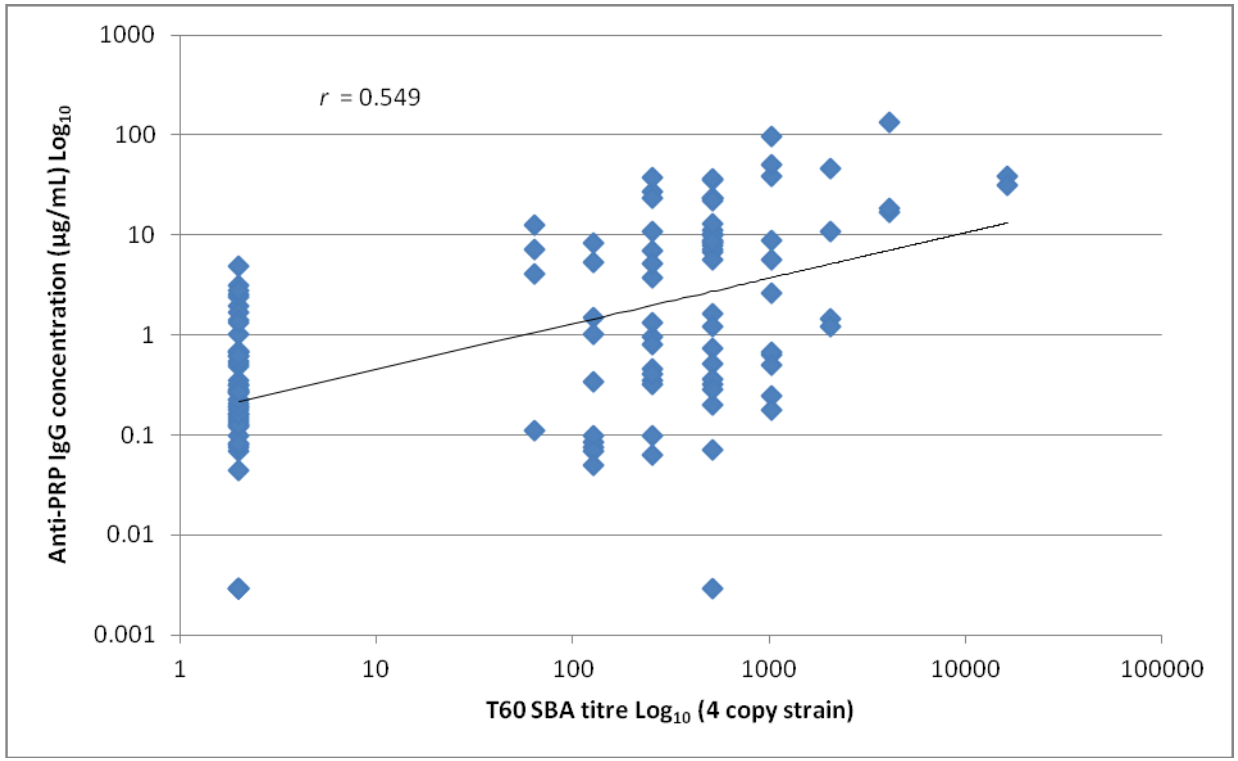
Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T0 for 4 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



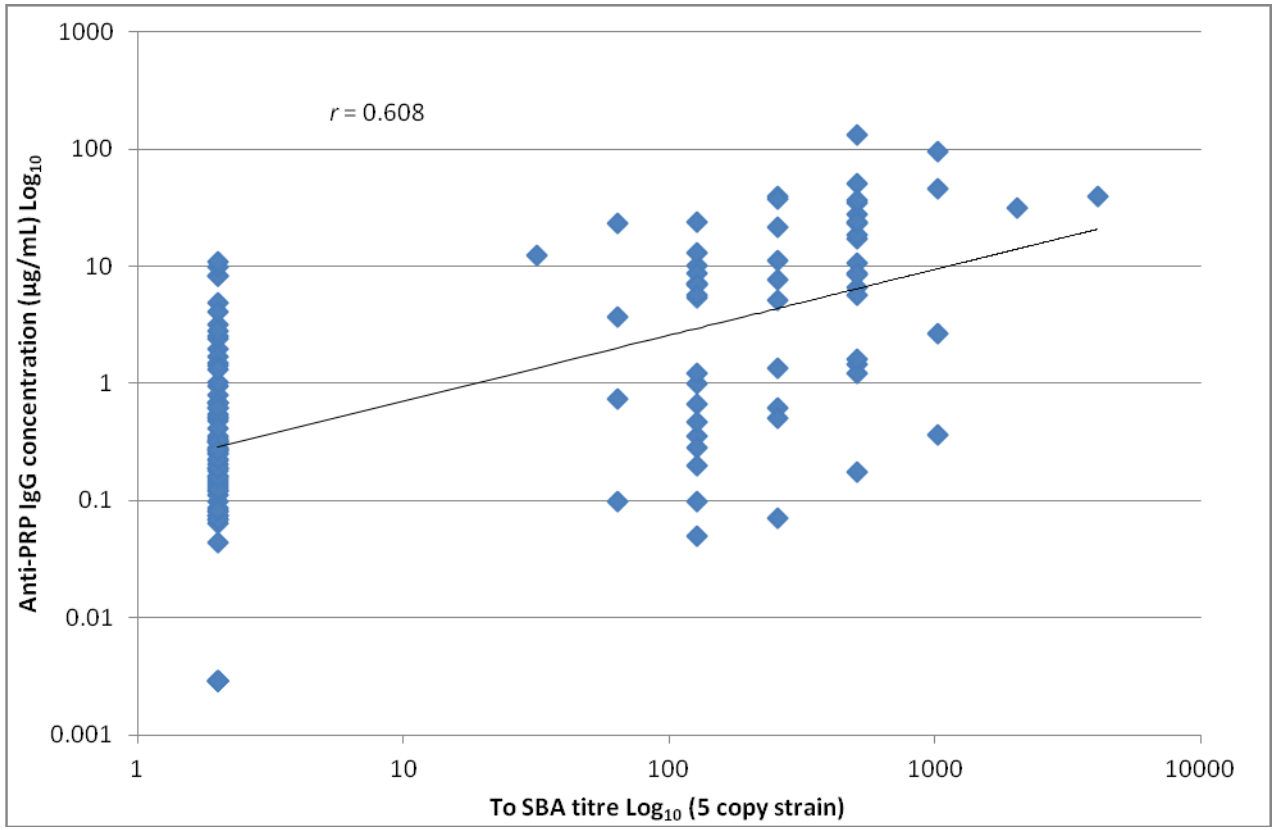
Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T60 for 4 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T0 for 5 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.



Appendix IV (Continued)

Correlation of anti-PRP IgG concentrations and SBA titres (Log_{10} values) at T60 for 5 copy strain. The correlation coefficient (*r* value) is displayed at the top of the chart.