Natural Induced Antibodies Against Group B *Streptococcus* Surface Proteins and Capsular Polysaccharides.

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Dedication

This thesis is dedicated to my loving mother Mandisa B. Nkonjane, your kindness and unassailable wisdom has been my spear and shield to victory.

… *the best is yet to come*…

Publications and presentation

Publications in press

1. **Dzanibe S.,** Adrian P. V., Kimaro-Mlacha S.Z., and Madhi S.A., Natural acquired group B *Streptococcus* capsular polysaccharide and surface protein antibodies in HIV-infected and HIV-uninfected children, Vaccine 2016 Vol 34 (44), p5217-24.
2. **Dzanibe S.,** Adrian P. V., Kimaro-Mlacha S.Z., Dangor Z., Kwatra G., and Madhi S.A., Reduced transplacental transfer of group B *Streptococcus* surface protein antibodies in HIV infected mother-newborn dyads, J Infect Disease. 2017 Vol 215 (3), p415-19.

Publications in preparation

1. **Dzanibe S.,** Adrian P. V., Kwatra G., Kimaro-Mlacha S.Z., and Madhi S.A., Natural occurring antibodies against group B *Streptococcus* surface proteins in pregnant women
2. **Dzanibe S.,** Adrian P. V., Kimaro-Mlacha S.Z., Dangor Z., and Madhi S.A. Association of natural induced antibodies against group B *Streptococcus* surface proteins and invasive disease in neonates and early infancy.

Presentations

1. **Dzanibe S**., Adrian P. V., Kwatra G., Kimaro-Mlacha S.Z., Madhi S.A., Acquisition of Vaginal colonization with Group B *Streptococcus* in pregnant women induces higher antibody responses to protein antigens than rectal colonization, 9th Vaccine & ISV congress, 2015, Seoul, South Korea.

2. **Dzanibe S**., Adrian P. V., Kimaro-Mlacha S.Z., Madhi S.A., Natural antibodies against Group B *Strepotoccus* in HIV-infected and HIV-uninfected children of South Africa, 5th South African Immunology Society conference, 2016, Johannesburg, South Africa
3. **Dzanibe S**., Adrian P. V., Kwatra G., Kimaro-Mlacha S.Z., Madhi S.A., Acquisition of Vaginal colonization with Group B *Streptococcus* in pregnant women induces higher antibody responses to protein antigens than rectal colonization 5th South African Immunology Society conference, 2016, Johannesburg, South Africa (**poster presentation**).

Abstract

Background Vaccination of pregnant women with conserved group B *Streptococcus* (GBS) surface proteins has the potential to confer serotype independent protection against invasive GBS disease. Susceptibility to early onset (<7 days of age) GBS disease in infants is associated with maternal recto-vaginal colonisation, low circulating maternal antibodies and reduced trans-placental transfer of antibodies specific to GBS capsular polysaccharides (CPS). Additionally, invasive GBS disease beyond infancy has been reported in individuals with underlying conditions associated with immunosuppression including HIV infection. In this study we undertook retrospective analysis of serum IgG titres against select GBS surface proteins in relation to invasive GBS disease risk factors.

Methods Multiplex Luminex immunoassay was used to measure serum IgG titres against GBS capsular polysaccharides of serotype Ia, Ib, III and V and surface proteins Fibrinogen binding surface Antigen (FbsA), GBS Immunogenic Bacterial Adhesin (BibA), Surface immunogenic protein (Sip), gbs0393, gbs1356, gbs1539, gbs0392; and lipoproteins gbs0233, gbs2106 and Foldase PsrA. Furthermore, *in vitro* expression of Sip, gbs2106, gbs0393 and gbs1356 proteins on the surface of clinical GBS isolates was assessed by flow cytometry using protein specific polyclonal antibodies generated in rabbits.

Results Retrospective analyses of serum antibody titres against GBS surface proteins and CPS were measured in children between 4-7 years of age who were either HIV-infected (n=68) or HIV-uninfected (n=77). Lower geometric meant titres (GMT, U/mL) against Sip and gbs2106 were detected in HIV-infected children (77.03 and 53.10) compared to HIV-uninfected children (196.41 and 139.11, $p<0.001$) respectively.

Similar results were observed for antibodies against CPS, with HIV-infected children having lower IgG GMC for serotype Ib (p=0.012) and V (p=0.005).

Protein specific antibody titres were measured in pregnant women at 20-25 and ≥37 weeks of gestation age who were either non-colonised or colonised with GBS in the rectal and/or vaginal tract. Acquisition of GBS colonisation in the vagina was associated with higher antibody titres compared to colonisation in the rectum for proteins Sip (p=0.049), Foldase (p=0.0094), gbs0233 (p=0.0039), gbs0393 (p=0.027), gbs1539 (p=0.0004) and gbs1356 (p=0.039). The likelihood of acquiring GBS colonisation during pregnancy was lower in women having median IgG titres against gbs0233 ≥200 U/mL (adjusted OR=0.47 [95% CI: 0.25-0.89], p=0.021) and gbs1539 ≥85 U/mL (adjusted OR=0.44 [95% CI: 0.24-0.82], p=0.01).

The influence of maternal HIV infection on trans-placental transfer of antibodies against GBS surface proteins was evaluated by comparing IgG titres between 83 HIV-infected and 81 HIV-uninfected mother-newborn dyads. Maternal HIV infection was associated with reduced trans-placental transfer of antibodies, demonstrated by the difference in cord-maternal antibody ratios between HIV-infected and HIV-uninfected mother-newborn pairs for Sip (25.8%, p<0.001), Foldase (30.4%, p<0.001), gba0392 (36.5%, p=0.006), gbs0393 (32.9%, p<0.001), gbs1539 (39.2%, p<0.008), gbs2106 (35.7%, p<0.001) and BibA (19.4%, p=0.004).

A case control study was used to evaluate the association of protein specific IgG titres and invasive disease in neonates and young infants born to GBS colonised mothers, including 116 with healthy infants and 66 with invasive GBS disease at <90 days of age. Lower IgG GMT were detected in neonates who developed early-onset disease

compared to control infants for Sip (65.48 vs 145.43, p<0.001), Foldase (50.45 vs 109.87, p=0.005), gbs0393 (106.42 vs 221.81, p=0.006) and gbs1356 (45.34 vs 94.52, p=0.01). Similarly, young infants with late-onset disease had significantly lower IgG GMT (U/mL) compared to healthy controls for Sip (43.72 vs 79.69, p=0.04) and gbs2106 (30.46 vs 65.35, p=0.003). Infants born to women with Sip specific antibody titres ≥150 U/mL antibodies titres against Sip were 66% less likely to develop invasive disease.

Of the 82 GBS isolates collected from mothers who deliverd term babies (39 with invasive GBS disease and 43 healthy controls) that were assessed for *in vitro* expression of specific proteins, Sip, gbs2106 and gbs0393 were expressed in 70.7% 93.9% and 87.8% GBS isolates respectively. The expression of gbs2106 on the surface of GBS was more frequent in strains of women with infants who developed invasive disease compared to those with healthy infants (100% vs 88.4%, p=0.028). The distribution of Sip and gbs0393 expression between GBS isolates from women of infants with invasive GBS disease and healthy controls was similar, 71.8% vs 69.8% (p=0.84) and 89.7% vs 86.0% (p=0.61) respectively. Among women carrying GBS strains expressing gbs2106, having antibody titres ≥100 U/mL was associated with 90% lower likelihood for delivering infants that develop invasive GBS disease (OR=0.11 [95% CI: 0.02-0.54], p=0.002).

Conclusion Vulnerability to invasive GBS disease in HIV-infected immunocompromised individuals is possibly due to reduced antibody levels against CPS, Sip and gbs2106. Also, susceptibility to invasive GBS disease in infants may be exacerbated by maternal HIV-infection, which was associated with reduced trans-placental transfer of antibody against GBS surface proteins.

Higher maternal antibodies titres against Sip and gbs2106 proteins were associated with reduced odds of their infants developing invasive GBS disease. These data support consideration of Sip and gbs2106 proteins as possible vaccine candidates in pregnant women to protect their infants against invasive GBS disease. Furthermore, reduced GBS colonisation during pregnancy can be achieved by maternal immunisation of gbs0233 and gbs1539, which may subsequently result in lower rates of GBS transmission to newborns and thereby decreasing their risk for invasive GBS disease.

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Abbreviations

αCP	alpha C protein
ARV	anti-retroviral
CPS	capsular polysaccharide
CspA	cell-surface-associated protein
CRM	cross reactive material
BCR	B-cell receptor
βCP	beta C protein
BibA	bacterial adhesion protein A
ELISA	enzyme linked immunosorbent assay
EOD	early-onset disease
FbsA	fibrinogen binding protein
FI	Fluorescence intensity
FPLC	fast performance liquid chromatography
FSC	forward scatter
GBS	group B streptococcus
GMC	geometric mean concentration
GW	gestation weeks
HEU	HIV-exposed and uninfected
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HUU	HIV-uninfected unexposed

HvgA	hyper-virulence GBS protein
IgG	immunoglobulin G
IAP	intrapartum antibiotic prophylaxis
Lmb	laminin binding protein
LOD	late-onset disease
MHC	major histocompatibility complex
MLST	multilocus sequence typing
OPA	opsonophagocytic activity
PI	pilus Island
PMNL	polymorphonuclear leucocytes
rAlp	recombinant alpha like protein
ScpB	serine protease C5a peptidase
SI	sequence identity
Sip	surface immunogenic protein
SpI/II	signal peptidase I/II
SSC	side scatter
ST	sequence type
TD	thymus dependent
Td	tetanus diphtheria toxoid
TI	thymus independent
TLR	Toll-like receptor
TT	tetanus toxoid
UTI	urinary tract infection

Chapter One

Introduction

1.1 Study rationale

An estimated 6.3 million deaths occur in children ≤5 years of age, of which 44% of these deaths occur during the neonatal period (1). Group B streptococcus (GBS) is the most common infectious cause of neonatal morbidity and mortality with the highest incidence rate reported in sub-Saharan Africa (2). The strongest independent risk factor associated with development of invasive GBS disease in infants is maternal recto-vaginal colonisation during pregnancy (3). The prevalence of GBS colonisation in the recto-vaginal tract of pregnant women is 10-30%, resulting in 30-50% vertical acquisition of GBS *in utero* or at intrapartum in newborns, of whom 1-3% develop invasive disease (4).

Thus, administration of intrapartum antibiotic prophylaxis (IAP) in pregnant women identified to be at risk of transmitting GBS to their infants has been adopted in industrialised countries as an intervention strategy (5,6). Although this method of prevention has successfully reduced the incidence of GBS early onset disease in high income countries, cost and logistical problems has impeded the adoption of this strategy in low-income countries where the burden of invasive GBS disease is the highest (2,6,7). The majority (75-90%) of early onset disease in the absence of IAP occurs within 24 hours of birth; hence, limiting opportunity for an effective intervention once the baby is born (6,8).

The ascertainment that high maternally derived GBS capsular polysaccharide (CPS) antibodies in newborns are associated with reduced incidence of serotype-specific invasive GBS disease has prompted immunisation of pregnant women as an alternative prevention strategy (9–14). Current clinical studies have focussed on the development

of multivalent serotype-specific polysaccharide vaccines inclusive of a highly immunogenic protein conjugant (15–17). A limitation of this, however, includes geographic and temporal differences in serotypes associated with GBS colonisation and invasive disease.

Alternatively, a vaccine formulation strategy would be to use epitopes which are genetically conserved and shared among all GBS serotypes and strains as vaccine-targets. Protein antigens are highly conserved among GBS serotypes and therefore can confer serotype-independent protection. The prevalence of GBS-protein antibody titres in women and their respective neonates, and their association with invasive GBS disease in neonates has been shown for pilus island proteins, however, protective antibody threshold has not been established (18). Therefore, measuring antibody titres induced by surface proteins that are correlated with disease protection is warranted for identifying vaccine candidates that are capable of broad-serotype coverage.

Furthermore, maternal antibodies directed against GBS surface proteins induce antibody mediated opsonophagocytic killing associated with a reducing GBS colonisation in pregnant women (19). Therefore, consideration to investigate the potential role of GBS surface proteins in preventing and/or clearance of GBS colonisation is warranted. Identifying GBS proteins capable of eliminating GBS colonisation in pregnant women could reduce the risk of vertical acquisition of GBS in newborns.

Recently it has been reported that intrauterine HIV-exposure increases the risk of developing invasive GBS disease in HIV-exposed uninfected infants compared to HIV-unexposed uninfected infants (20,21). Reduced efficiency of placental transfer of protective antibodies against CPS epitopes are postulated to play a role in increased

susceptibility to invasive GBS disease in HIV-exposed uninfected infants. This suggests that increased GBS disease in infants may be mediated by the impaired ability to transfer maternal antibody in HIV infected mothers, and therefore further studies are required to investigate the effect of maternal HIV-infection on protein antibody titres and the trans-placental transfer thereof.

Moreover, invasive GBS disease has an unconventional age distribution that rarely occur in children >3 months of age when maternal derived antibodies have waned. About 6% of invasive GBS disease occur among 1 month to 17 year old children, with all cases having underlying comorbid conditions associated with immunosuppression such as diabetes mellitus, cancer and HIV infection (22). Therefore, immune-suppression is associated with an increased risk of invasive GBS disease in older children and adults (22,23); however, the particular mechanism that causes this increased risk is unclear.

1.2 Aims and Objectives

The aim of this study was to identify and produce highly immunogenic GBS surface proteins; and measure the natural induced antibody response specific to the selected GBS surface protein antigens in relation to maternal GBS colonisation, invasive GBS disease in infants, *in utero* HIV exposure in newborns and HIV infection in older children.

Objectives

1. Clone, express and purify GBS surface proteins identified *in silico* as highly immunogenic.

2. Measure the natural induced antibody response to GBS surface proteins and capsular polysaccharides in relation to:

   a. HIV status in children: compare antibody titres in HIV-uninfected and HIV-infected children against GBS surface proteins and capsular polysaccharides.

   b. GBS colonisation in pregnant women:

      i. Measure serum IgG titres in women at 25 weeks of gestation age and association with subsequent risk of GBS recto-vaginal acquisition during pregnancy.

      ii. Determine protein specific antibody titres associated with clearance of GBS colonisation by comparing antibody tires in women who are persistently colonised with GBS throughout pregnancy and those who clear GBS colonisation during pregnancy.

   c. *In utero* HIV exposure: Compare antibody titres in HIV-infected and HIV-uninfected mother-newborn dyads, to determine the effect of maternal HIV status on the efficiency of protein specific trans-placental antibody transfer during pregnancy.

   d. Invasive GBS disease in infants: Determine the association of maternal antibody titres specific to GBS surface proteins in relation to the odds of developing invasive GBS disease in infants born to GBS colonised women.

Chapter Two

Literature Review

2.1 *Streptococcus agalactiae*

Streptococcus agalactiae is a Gram-positive, β-haemolytic facultative anaerobe visible as short cocci chains and belonging to the Streptococcaceae family. *S. agalactiae* is also known as Lancefield group B streptococcus (GBS) according to the Lancefield classification based on the encapsulating polysaccharides (24). GBS is classified into ten serotypes (Ia, Ib, II through to IX) based on the chemistry of encapsulating polysaccharides (CPS), which are antigenically distinct (24–26).

Capsular serotyping is the classical GBS characterisation or typing technique, albeit additional molecular typing techniques for classifying GBS have been used, such as multi-locus enzyme electrophoresis, pulse-field gel electrophoresis, restriction endonuclease digestion pattern, restriction fragment length polymorphism and multi-locus sequence typing (MLST) (27). These techniques are used to distinguish GBS into clonally related strains and similar genetic structures in order to identify genotypes associated with invasive GBS disease. MLST is the least ambiguous typing technique utilising seven housekeeping gene sequences to define non-overlapping sequence types (ST) (28). The major sequence types identified are ST1, ST19, ST17, and ST23. ST-17 has been observed to be associated with hypervirulent clones, whilst ST1 and ST19 are associated with asymptomatic colonisation and ST23 is associated with both invasive and colonising clones (28).

GBS isolates can be further differentiated based on surface localised proteins: α-C, β-C, epsilon, alpha-like protein and Rib encoded by genes *acp*, *bac*, *epsl*, *apl* and *rib* respectively (29). The expression of these proteins occurs commonly in certain serotypes and thus in turn are associated with either colonising or invasive GBS isolates (29–32). Additionally, clinical isolates have been characterised based on pilus island

genes (PI) that express flagellin proteins that function as bacterial adhesions (33). The type or various combinations of PI genes expressed by a particular GBS isolate have been shown to be associated with either colonisation or invasiveness of the bacterial infection (34).

2.2 Clinical manifestation of group B streptococcus

GBS is the leading infectious cause of morbidity and mortality in neonates; causing pneumonia, meningitis and septicaemia (2,35). GBS disease among children clinically presents as a bimodal distribution in early-infancy, and hence classified as either early-onset disease (EOD, <7 days of age) or late-onset disease (LOD, 7-90 days of age). EOD manifests mainly as pneumonia or septicaemia and has a case fatality ratio of 2%-21% (2). EOD usually results from vertical acquisition of GBS, in which recto-vaginally colonised mothers infect the neonate either *in utero* or intrapartum. In industrialised countries, the introduction of intrapartum antibiotic prophylaxis (IAP) in GBS colonised women has resulted in >80% decrease in the incidence of EOD (36).

LOD mostly manifests as septicaemia or meningitis and has a case fatality ratio of 2-20% (2,37,38). Furthermore, 10-50% of infants who survive meningitis will develop permanent neurological sequelae such as mental retardation, blindness, deafness or severe disability (39). Although the mode of LOD infection is poorly understood, it is thought to be horizontally transmitted from mother to child, since the majority of cases have identical GBS isolates in the mother and infant (40). LOD has remained fairly constant in USA and elsewhere over the past decades, and is not prevented through IAP treatment during labour (2).

GBS infection also accounts for maternal morbidity among pregnant women causing bacteraemia, chorioamnonitis, endometritis and urinary tract infections (23,41,42). This subsequently results in pregnancy complications that are associated with prematurity, low-birth weight and also GBS-related stillbirths (43,44). Furthermore, a continued increase in the proportion of invasive GBS disease among the elderly especially in the population >65 years has been reported (23,45). Invasive GBS disease among the elderly commonly manifests as bacteraemia with no focus, pneumonia, urinary tract infections (UTI) and meningitis, in most cases occurring in patients with underlying conditions (45).

2.3 Epidemiology of invasive GBS disease

There is an estimate of 2.7 million deaths per year that occur during the neonatal period (1), with neonatal sepsis, pneumonia and meningitis accounting for approximately 20% of these cases, and GBS being a leading bacterial cause of death during the neonatal period (1,2,46). The burden of neonatal invasive GBS disease is highest in African countries with an estimated incidence of 1.21 cases per 1000 live births compared to 0.67 in the Americas, 0.57 Europe, 0.35 Eastern Mediterranean, 0.15 Western Pacific and 0.02 in South-East Asia (2).

Overall, the major invasive serotypes of GBS are type-Ia, Ib, II, III and V (2,37). Serotype Ia, and III account for 40% and 37% of EOD cases, while serotype III is the predominant (53%) cause of LOD (2). However, the prevalence and distribution of invasive GBS serotypes differ according to geographical region, where serotype V predominantly occurs in some industrialised countries, (47) and with type VI and VIII being frequent colonisers of pregnant women in South-East Asia (48). Additionally

non-typeable GBS strains that cause invasive disease in the neonatal period have been isolated in 3-15% of cases (2,49).

Molecular epidemiological classification of neonatal isolates showed that ST-17 belonging to serotype III was more frequent among LOD cases (75.3%) compared to 63.5% EOD cases (49). GBS isolates of ST-17 cause meningitis more frequently than sepsis, particularly for LOD cases (50). The hyper-virulence of ST-17 has been linked with an allelic variant of bacterial adhesion protein A (BibA) that mediates invasion of blood brain barrier and is expressed exclusively among ST-17 isolates (51). Furthermore ST-17 is associated with a specific combination of pilus island genes, such as PI-1 and PI-2b that frequently occur in LOD cases (52). ST-19 which is associated with maternal colonisation and accounts for 18-30% of GBS related EOD cases, almost exclusively expresses PI-1 and PI-2a combination (49,50,52,53).

Maternal morbidity due to GBS infection occurs in 0.04-0.12 cases per 1000 women-years (23,41). The distribution of invasive GBS serotypes among pregnant women is more heterogeneous than that observed in infants, with serotypes Ia to V occurring in similar percentages (9%-22%) (41). In non-pregnant adults, particularly the elderly, serotype V predominates among invasive isolates and accounts for 27.5% of cases (45).

The implementation of IAP has resulted in a significant reduction in EOD, effectively decreasing the number of cases from 1.8 to 0.26 cases per live births from 1990s to 2010 in the USA (6). However, in low income countries, particularly sub-Saharan African region, where cost and logistical problems limit implementation of IAP-like preventative strategies, the prevalence of EOD has remained high at 1.3 cases per 1000

live births (7). Furthermore, the incidence of LOD has remained unchanged in USA, despite successful adoption of IAP as a standard of care (6).

Given that the probability of introducing a multivalent GBS CPS serotype Ia, II, III and V vaccine could result in 50-90% neonatal invasive GBS disease case reduction (54), factors such as socio-economic status, geographic and temporal variation of invasive serotypes and the occurrence of non-typeable invasive isolates could result in residual cases. This could be partly overcome by the inclusion of GBS surface protein epitopes that are universally expressed and capable of conferring protection independent of serotype.

2.4 Risk factors associated with GBS colonisation and invasive disease

2.4.1 Maternal colonisation

The frequency and severity of GBS infection is age dependent (55). The greatest proportion of invasive GBS disease occurs in newborns (<7 days of age; 48.2%), with a lower percentage of cases occurring in the 7-90 day age group (38.02%) and rarely in infants >90 days (13.7%) (56). This onset of invasive GBS invasive disease early in life limits strategies that would alter the potential fatal progression in infants, hence the need for prevention being targeted during pregnancy or labour (8).

The strongest independent risk factor associated with invasive GBS disease in infants is maternal colonisation (3). Pregnant women are commonly colonised with GBS in the recto-vaginal tract, with higher prevalence reported from African countries, Table 2.1. Maternal colonisation status is in turn influenced by other factors such as maternal age, ethnicity, parity and previous GBS-related pregnancy complications (57,58). Women <20 years of age and/or of African ethnicity with previous spontaneous abortion are

observed to be frequently colonised with GBS (59–61), although these observations are restricted to specific study settings, Table 2.1.

Recto-vaginal colonisation of women during pregnancy has been reported to result in 30-70% GBS acquisition rate by their respective newborns', transmitted vertically *in utero* or intrapartum. About 2% of newborns' that acquire GBS colonisation will develop EOD, >90% of which occurs within 24 hours of life (4). Predisposing risk factors associated with neonatal invasive GBS disease include intrapartum fever, prolonged rapture of membranes (PROM) >18 hours, chorioamnonitis, prematurity and low birth weight (62–64). Premature infants (<37 weeks of gestation) and those born with a birth weight <2.5 kg are reported to be at an increased risk of invasive GBS disease, including being more likely to develop LOD (64,65). Maternal GBS colonisation has also been associated with stillbirth, with up to 12.1% stillbirth cases associated with GBS infection (44).

Table 2.1 Group B Streptococcus recto-vaginal colonisation in pregnant women between the years 2000-2015.

Region	Country	Prevalence (%)	Samples collected	Risk factors	Gestational week	Year	Ref
Africa	Zimbabwe	60.3	Lower vaginal and rectal swabs	Rural area setting	20-delivery	2003-2005	(66)
	Tanzania	23.0	High vaginal and rectal swabs	ND	≥37	2008-2009	(67)
	Malawi	21.0	Low vaginal and rectal swabs	ND	≥24	2008-2010	(68)
	Nigeria	11.3	Vaginal introitus	ND	35-40	2010	(69)
	South Africa	49.7	Lower vaginal and rectal swabs	Parity and gravidity	20-37+	2010-2011	(58)
	South Africa Kenya	10.0 14.3	Vaginal swabs	*C. albicans*, sexual intercourse, frequent sex worker, chlorehexidine vaginal washes and cervical ectopy	≥14	2010-2011	(70)
	DRC	20	Vaginal swabs	Low education, history of UTI , prematurity or	>24	2012-2013	(71)

				abortion and HIV-infection			
	Gambia	33.7	Recto-vaginal swabs and breast milk	Maternal haemoglobin < 10 g/dL, previous stillbirth, delivery assisted by midwife and delivering during the wet or hot dry season	Delivery	2014	(64)
	Egypt	25.3	Vaginal swabs	ND	35-40	2007-2008	(72)
Europe	Denmark	36	Vaginal and anorectal swab	ND	≥16	1999-2001	(73)
	Netherlands	21.0	Vaginal introitus and rectal (through anal sphincter) swabs	African origin	35-37	2000-2002	(74)
	Hungary	23.2	Distal vaginal and rectal swabs	ND	32-34	1995-2011	(75)
	Italy	20	Low vaginal and rectal swabs	ND	35-37	2008-2010	(76)
	UK	19.0	Lower vaginal, rectal and throat	ND	34-40	2011	(77)
America s	Brazil	17.0	Vaginal and rectal swabs	ND	35-37	2002-2004	(78)
	Chile	14.4	Vaginal and rectal swabs	ND	35-37	2010-2012	(79)

	USA	18.6	Vaginal-anal swab	ND	>36	2002	(80)
Asia	Taiwan	6.2	Upper vaginal swabs	ND	≥37	2005-2006	(81)
	Korea	8.3	Urine Vaginal and rectal swabs	Geographical region (hospital)	35-37	2006-2008	(82)
	India	16	Vaginal and rectal swabs	ND	35-37	2008	(83)
	Bangladesh	18.3	Vaginal and rectal swabs	Rapture of membrane ≥8h and PROM	≥30	2011	(84)
	Iran	25.4	Rectal and vaginal swabs	History of UTI	35-37	2013-2014	(85)
	China	7.1	Recto-vaginal swabs	ND	35-37	2011-2013	(86)

ND = not determined, DRC=Democratic Republic of Congo, UTI=urinary tract infection

2.4.2 Maternal GBS serotype specific capsular antibody levels

The risk of invasive GBS disease in neonates is associated with the level of maternal serum serotype specific capsular antibodies, where neonates born to mothers with higher serotype specific CPS antibody titre have reduced risk of developing invasive GBS disease. Newborns of mothers ≥34 weeks gestation age with serotype Ia anti-capsular specific IgG ≥5 µg/mL were less likely to develop EOD (11), whereas in other studies maternal IgG concentrations >2 µg/ml for serotype III was associated with protection against type-III EOD (9,10). Inter-study variation among the reported antibody threshold correlated with invasive GBS disease might be due to the lack of standardised immunological assays being used between studies. Furthermore, reference serum with high avidity compared to the test samples tend to underestimate antibody levels detected (87), and therefore the reported antibody threshold may vary depending on the study sample and reference serum used.

Lin *et al.* noted a 91% risk reduction in EOD incidence for infants born to mothers with ≥ 10 µg/mL IgG antibodies against CPS serotype-III (12). Recently, a risk reduction of 70-90% in GBS EOD cases has been reported in infants born to mothers with > 0.5 µg/mL IgG antibody concentration specific for GBS CPS serotype Ia, III and V (13). Furthermore, a study from South Africa reported a decrease in the risk of invasive GBS disease in infants with increasing maternal antibodies against CPS serotype Ia and III. The study demonstrated that antibody concentrations of ≥ 6 µg/mL and ≥ 3 µg/mL against GBS CPS serotype Ia and III respectively, result in < 10% risk for GBS invasive disease in infants (14). Therefore, increasing maternal GBS capsular antibodies by vaccination could reduce the incidence of invasive GBS disease in infants.

Currently, there are limited studies reporting changes in antibody levels against GBS surface proteins during pregnancy. The protective maternal antibody levels that have been determined are specific to GBS polysaccharide epitopes. β-C protein specific antibodies were detected in 99% of women evaluated, however, maternal antibody concentrations against β-C protein did not differ according to maternal GBS colonisation status, gestational age nor was associated with the risk of invasive GBS disease in their neonates (88). Moreover, no associations were observed between higher antibody concentration to surface immunogenic protein (Sip), bacterial adhesion protein A (BibA), fibrinogen binding protein (FbsA) and pilus island proteins for either neonatal invasive GBS disease or maternal vaginal colonisation (89,90). Studies by Larsson *et al*. and Fabbrini *et al.*, however, reported that low concentrations against α-C, Rib and pilus island proteins were associated with invasive GBS disease in neonates (18,91). This indicates that antibodies specific to some GBS surface proteins might be involved in protection against invasive disease and warrant consideration as possible vaccine candidates.

There is currently no established protective antibody threshold for GBS protein specific antibodies in pregnant women or their respective newborns (88–90,92), this is despite evidence of protein specific antibodies conferring protection in murine models and capable of inducing opsonophagocytic killing (93–97). These studies, however, either had a limited sample size or the quantification of antibody specific level was not correlated with invasive GBS disease from isolates expressing the target protein. Further studies evaluating the association between GBS protein antibodies with maternal GBS colonisation during pregnancy and invasive GBS disease in infants could identify a potential role of such proteins as vaccine candidates.

2.4.3 HIV infection and intrauterine exposure

The prevalence of HIV among pregnant women in South Africa is 30.2% (98). Improved management of HIV-infected pregnant women with antiretroviral treatment (ARV) has reduced the prevalence of HIV transmission from mother-to-child (99). This has resulted in a decrease of HIV-infected infants, however, a high proportion of children remain HIV-exposed, albeit uninfected with HIV (HEU). Maternal colonisation by GBS is similar between HIV-infected and HIV-uninfected pregnant women (100,101). HEU infants, however, have a higher incidence of EOD and LOD compared to infants born to HIV-uninfected mothers (20,21,102).

Increased morbidity and mortality among HEU infants may be due to severity of maternal HIV disease; increased exposure to opportunistic pathogens in early life, maternal antibody levels and reduced trans-placental transfer of maternal antibodies (102–104). Lower IgG antibodies against GBS CPS serotype Ia, Ib, II, III and V were reported in HIV-infected compared to HIV-uninfected pregnant women (105,106). These studies also reported significant reduction in trans-placental transfer of CPS-specific antibodies to newborns of HIV-infected women. Therefore, intrauterine HIV exposure may in part increase the risk of invasive GBS disease in HEU neonates by impairing the transfer of protective antibodies from mother to the foetus/newborn.

Antibodies induced by protein antigens are preferentially transported across the placenta (107,108), and are impaired in HIV infected women during pregnancy (109,110). Such an association has not been reported between maternal HIV infection and GBS surface protein specific antibodies. A study by Dangor *et al.* reported that HIV-infected pregnant women have low IgG antibodies against FbsA and pilus island proteins

compared to HIV-uninfected pregnant women, however, there was no significant reduction in the ratio of trans-placental antibody transfer from mother to newborn (106).

Further studies to identify GBS surface proteins that are associated with reduced transfer in HIV-infected compared to HIV-uninfected mother-newborn dyads, could identify GBS surface proteins that contribute to the increased risk of invasive GBS disease among HEU infants; which might also lead to the identification of potential GBS vaccine epitopes.

2.5 Group B *Streptococcus* virulence factors

GBS is a commensal bacterium that colonises the gastrointestinal and genitourinary tract of healthy adults (35). Development of invasive GBS diseases underlines the species evolution, enabling the utilization of extracellular matrix factors such as laminin, glycosaminoglycan's, fibronectin and fibrinogen to mediate pathogenesis by adhering and invading host epithelial and endothelial cells (111). Some of the surface virulence factors described have dual roles, enabling GBS invasion and evading immunological recognition and/or clearance.

2.5.1 Capsular polysaccharides

The encapsulating GBS polysaccharides identified by Lancefield to possess the ability to confer protection in murine immunisation models, have been subject of vaccine development (112). GBS CPS are key virulence factors, mediating GBS pathogenesis and immune-evasion (113). The chemical composition of CPS possesses sialic acid similar to that present on the surface of host epithelial cells. This molecular mimicry prevents deposition of C3 complement factors on the surface of GBS and therefore escapes opsonisation by phagocytic cells (114).

Serotype Ia and III share a similar oligosaccharide structure that only differ by the linkage of the galactose and sialic acid to the oligosaccharide backbone (115). This difference has been shown to have a similar anti-phagocytic capability but was quantitatively less for serotype Ia and hence CPS serotype-III predominantly occurs in both colonising and invasive clinical isolates (116).

2.5.2 Surface proteins

GBS surface proteins play an important role in mediating pathogenesis of invasive GBS disease. Proteins are involved in adhesion to epithelial cells, interaction with host extracellular matrix and also in evading immunological recognition (29). Figure 2.1 shows a pictorial summary of GBS surface proteins as virulence factors that enable GBS adherence and invasion of host epithelial cells to cause symptomatic infection.

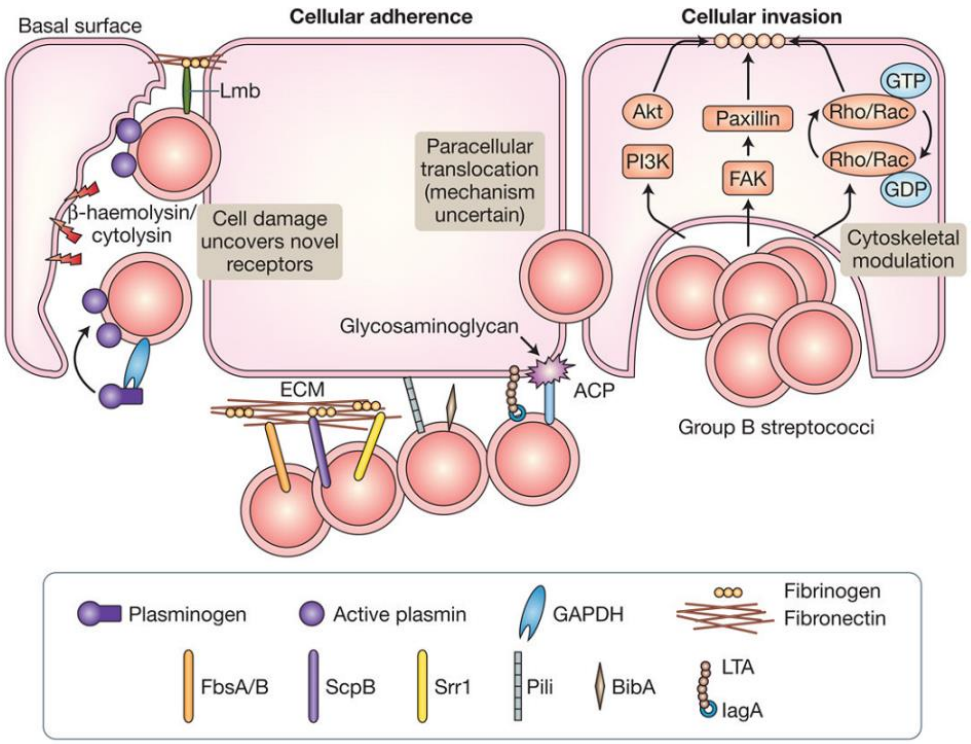

Figure 2.1 Mechanisms of group B Streptococcus pathogenesis using surface virulence factors to mediate cellular adherence and invasion by binding host extracellular matrix ligands/receptors (111).

β-haemolysin/cytolysin and *CAMP factor*

β-haemolysin is a pore-forming toxin secreted by GBS which has been shown to cause damage to lung epithelial cells and brain epithelium resulting in pulmonary infection and meningitis (117,118). Similarly, CAMP factor is another pore-forming toxin secreted by GBS to mediate damage to host epithelial cells (119). The potential role of antibodies to these toxins in protecting against GBS disease has not been evaluated, mainly due to the toxins being non-immunogenic and non-essential during pathogenesis (120,121).

C protein

Antibodies specific to C protein antigens that are identified on selected GBS strains were shown to be capable of conferring protection in immunised mice (112). The C protein antigen consists of two forms designated α- or β-C proteins based on trypsin digestion sensitivity. GBS strains that express the C protein are found to express either of the proteins or both except for isolates expressing CPS belonging to serotype-III (122). Further investigation on GBS serotype-III strains identified the Rib protein that was present in GBS strains not expressing the C protein (123). Molecular characterisation of αCP and Rib revealed that these proteins consist of highly identical tandem repeats and thus belong to a family of surface localised proteins denoted alpha like proteins (Alp) (124,125). The Alp family is comprised of αCP, Rib, R28 and Apl2 proteins which promote invasion by interacting with host cell's glycosaminoglycans (126). Alp are essential virulence factors and their ability to induce protective antibodies makes then suitable GBS vaccine candidates, however, these proteins are

heterogeneously distributed among GBS serotypes and therefore would require a multi-peptide vaccine to enhance coverage.

FbsA

Fibrinogen, present on the extracellular matrix, interferes with activation of complement mediated opsonophagocytosis and thus certain pathogens have developed mechanisms to accumulate fibrinogen on their surface to evade the immune system (127). Fibrinogen binding protein (FbsA) is a GBS surface anchored protein that consists of repeating units comprised of 16 amino acids that interact with fibrinogen during bacterial adhesion (128). FbsA interaction with fibrinogen also triggers polymer aggregation of fibrinogen on the surface of GBS that interferes with opsonophagocytosis by macrophages (129).

Another protein co-transcribed with FbsA, analogously designated FbsB, binds human fibrinogen in a dose dependant and saturable manner (130) and thus cumulatively increases the virulence of GBS. Interaction of FbsB and fibrinogen are, however, not essential during bacterial adhesion since deletion of the *fbsb* gene deletion does not influence adherence to epithelial cells. Furthermore, IgG antibody levels against FbsA were reported not to be associated with protection against invasive GBS disease during infancy (90).

Serine protease C5a peptidase (ScpB)

Cell-surface-associated protein ScpB is a serine protease that cleaves the complement factor C5a as a mechanism of immuno-evasion (131,132). C5a is a component of the complement system which functions as a chemoattractant of polymorphonuclear

leucocytes (PMNL). Cleavage of C5a by ScpB inactivates its ability to stimulate the influx of PMNL to the infection site hence contributing to GBS pathogenesis (132).

ScpB also interacts with fibrinogen and thus serves as an adhesin during GBS bacterial invasion (133,134). Other similar proteases localised on the surface of GBS have been described such as cell surface associated protein (CspA) that cleaves a panel of CXC chemokines essential for recruiting neutrophils to the site of infection (135). CspA is also capable of binding to human fibrinogen (136). These proteases are highly conserved among GBS strains and therefore are ideal as cross-protective vaccine candidates (131,137).

Lmb protein

Present on the extracellular matrix of mammalian cells are a fibrous network of high molecular weight glycoproteins called laminins (138). These proteins form the major part of the basement membrane of the epithelium layer and serve as structural organisation of tissue, cell differentiation and adhesion. Spellerberg et al. (1999) identified a GBS surface protein that is capable of binding to immobilised laminin, and as such designated as laminin binding protein (Lmb) (139).

Lmb protein is homologous to other *streptococcal* LraI (lipoprotein receptor antigen I) protein family that have been extensively characterised to mediate pathogenicity by adhering to laminin of damaged epithelial cells. The *lmb* gene encoding Lmb protein is situated immediately upstream of the *scpb* gene. Lmb protein is expressed by all GBS strains isolated from humans and binds to laminin on the surface of endothelial cells during colonisation and bacterial invasion. Deletion mutant studies of the lmb-scpb gene demonstrated that Lmb is also capable of binding to human brain endothelial cells

to promote the development of meningitis (140). Thus Lmb is an essential virulence factor of GBS pathogenesis expressed by most if not all GBS strains, making it a possible vaccine candidate.

BibA protein

GBS immunogenic bacterial adhesion protein A (BibA) is a cell wall anchored protein identified *in silico* and shown to possess epithelial cell adherence properties (141). BibA is also capable of immunological evasion by inhibition of C4bp (complement regulator) and thereby resisting opsonophagocytosis (141). Analysis of the hyper-virulence ST-17 clone identified an allelic *biba* gene that only occurs in ST-17 isolates (28,51). Further characterisation of the genetic diversity of the *biba* gene revealed that the protein is encoded by alleles occurring in three major phylogenetic clusters designated clusters A, B and C based on sequence types with shared *biba* gene homology (51). Cluster A and B are more related whilst cluster C was exclusively comprised of alleles belonging to ST-17, Figure 2.2. The BibA protein; belonging to cluster C, was shown to mediate specific adherence to intestinal epithelial cells and brain microvascular endothelial cells enabling migration through the blood brain barrier to cause meningitis and as such designated hypervirulent GBS adhesin (HvgA) (142).

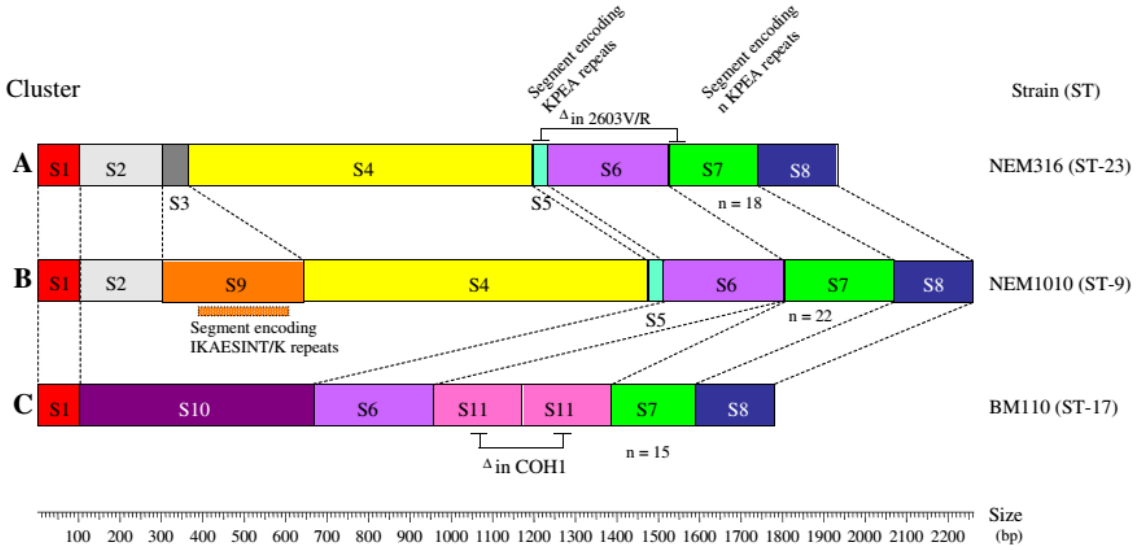

Figure 2.2 Structural depictions of the allelic forms of the biba genes in each phylogenetic cluster. S1-S11 represent identical gene segments present in more than one GBS strain.S1, S7 and S8 present in all clusters represent the flanking N-terminal signal peptide (S1), the KPEA amino acid repeats (S7) and the sorting signal with the LPxTG motif followed by the hydrophobic domain and the positively charged tail (S8) with > 90% identity (51).

Pilus island proteins

Analysis of the GBS genome sequences from different serotypes revealed the presence of genetic pilus island-like structures that encode pilus island proteins (PI) on the surface of GBS (143,144). Among the GBS strains, three structurally similar but phylogenetically distinct loci encode the PI proteins; the PI-1, PI-2a and PI-2b. Each PI encode for three different pili proteins; the backbone protein (BP) and two ancillary proteins (AP), and for two sortase proteins that enzymatically attach the pili to the peptidoglycan layer on the surface of GBS, Figure 2.3 (33,143,145).

Pili proteins are covalently polymerised proteins that form filamentous structures and extend on the surface of bacteria essential for motility (146). GBS pilus proteins play an essential role during bacterial adherence and invasion of lung and cervical epithelial cells (147). The pilus proteins also have the capacity to interact with brain microvascular endothelial cells to invade the blood brain barrier causing meningitis (148). Pilus backbone proteins of both PI-1 and PI-2a enable GBS paracellular trancytosis through the epithelial cells as demonstrated with cervical, pulmonary and intestinal cell lines (149).

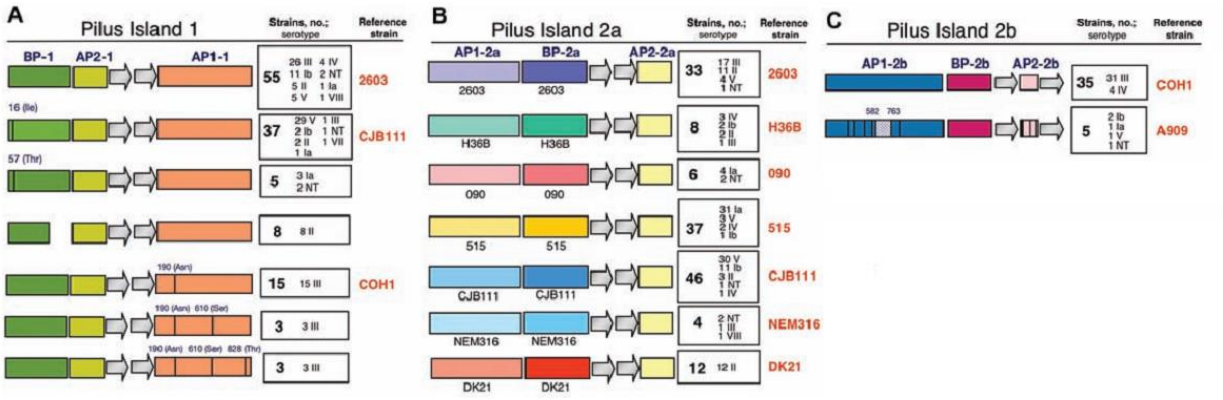

Figure 2.3 Schematic representation of the pilus island proteins assembly, inclusive of the backbone protein (BP) and the two ancillary proteins (AP) for PI-1, PI-2a and PI-2b. The sequence diversity of genes encoding pilus proteins are depicted with the sortase encoding region shown in grey (33).

2.6 Natural immune response to group B *Streptococcus*

2.6.1 Immunity to polysaccharides

The high density of CPS on the surface of GBS triggers a thymus independent (TI) immune response that mounts an immunological clearance of the pathogen independent of T lymphocytes (150). There are two types of TI immune responses that can be triggered depending on the nature of the antigen; the type I (TI-1) and the type II TI (TI-

2) that result in the activation of the B cell and subsequent secretion of pathogen neutralising antibodies (151). TI-1 immune response is induced by bacterial products mostly lipids that stimulate the B cell. The 1st signal is mediated by the interaction of the B cell receptors (BCR) with the antigen and the 2nd signal results from the Toll-like receptors (TLR) binding the antigen which then stimulates polyclonal activation of the B cell. TI-2 immune response is induced by large molecular weight polymers with repetitive epitope such as polysaccharides that encapsulate GBS (152). The stimulatory signals that result in B-cell activation are mediated by extensive cross-linking of surface antibodies due to a repeating epitope presented by the polysaccharide antigen, Figure 2.4. The GBS pathogen thus triggers a TI-2 immune response that requires mature B cells that are capable of extensive cross-linking of membrane-bound antibodies (153). Once activated, these B cells are incapable of mediating clonal expansion into memory B cells (152) and predominantly secrete IgG_2 (154).

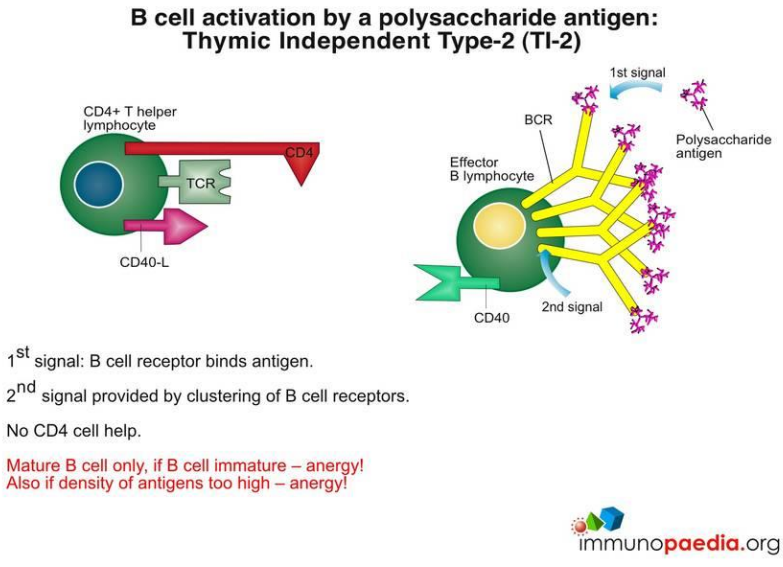

Figure 2.4 Schematic depiction of TI-2 immunological response that results in the activation of B lymphocytes from the extensive cross-linking of BCR by repeating epitopes of the antigen.

This particular type of immune response is not evident in neonates since they have a delayed ontogeny for TI immune response (155). The inability of neonates to recognise and mount an immune response against CPS (156), makes them highly dependent on the placental acquisition of maternal antibodies for protection against invasive GBS disease. Maternal transfer of antibodies to the foetus gradually increases and reaches a peak during the third trimester of gestation (108). This increases the vulnerability of preterm infants since they have low protective antibodies against GBS. Moreover amongst the IgG subclasses, IgG2 is less efficiently transported across the placenta and only found in trace amounts in cord blood even during the third trimester (107). Since IgG2 is the predominant antibody secreted by maternal B-cells against CPS antigens, this further increases the risk of susceptibility to invasive GBS disease in neonates (108). Thus a combination of these factors results in neonates exhibiting an immunodeficiency state towards GBS, due to a deficit in protective antibodies against the GBS CPS because of the delayed maturity in TI immune response and limited trans-placental transfer of maternal antibodies.

2.6.2 Immunity to proteins

Protein antigens trigger a thymus dependent (TD) immune response that requires activating signals from T lymphocytes (152). This particular type of immune response is more advantageous in neonates and their mothers than the TI immune response; since neonates are capable of producing antibodies towards these antigens and TD immune response result in inducing memory B cells. Moreover, the predominating subclass secreted is the IgG1, (157) that has an increased efficiency of crossing the placenta-barrier than IgG2 isotype (107). This results in an increased ability of neutralising the

invading pathogen in neonates, thus presenting an ideal class of potential vaccine antigens against GBS.

Peptides are either endogenously processed, presented complexed with human leukocyte antigen (HLA) class I, or exogenously recognised (presented by HLA class II) as epitope by B lymphocytes. Class II antigens comprise mainly of surface exposed peptides and are presented to $CD4^+$ T lymphocytes, which then aid in stimulating antibody production by B lymphocytes (158). HIV gp120 (viral-envelope glycoprotein) recognises the CD4 molecule as a receptor during infection into T lymphocytes. This results in these lymphocytes having defects in soluble antigen processing and presentation (159–161). Contrary to these studies Woc-Colburn (162) maintains that antigen presentation is preserved in HLA class II restricted T cells and that HIV infection marginally affects the presentation of antigens. Nevertheless, the particular type of immune response induced by GBS surface protein in HIV infected individuals has not been fully elucidated. Thus, by determining the antibody dynamics toward GBS protein antigens and how it is affected by underlying HIV infection could help identify potential vaccine epitopes against invasive GBS disease. Moreover pregnant women exhibit an immuno-attenuated state due to the interaction between the endocrine and the immune system, where a shift towards humoral immunity away from cell mediated immunity is observed at the maternofoetal interface (163). Therefore, the design and development of an efficacious vaccine against invasive GBS disease has to incorporate the plasticity of the immune system during pregnancy, the maternofoetal relationship together with the influence of HIV infection.

2.7 GBS vaccines

Currently, the recommended GBS prevention strategy is the use of IAP in pregnant women who are identified to be at high risk of transmitting GBS to their infants. The implementation of the IAP method has successfully reduced the incidence of EOD in high-income countries, however, due to cost and logistical problems its adoption in low-income countries has not been considered (6). IAP also does not protect against LOD, GBS-related prematurity and stillbirth. Moreover, the use of IAP could result in the emergence of antibiotic-resistant bacteria, which may necessitate alternative strategies (164).

The use of vaccines as a prevention strategy against invasive GBS disease has been a subject of extensive research. This has been primarily based on the findings that the probability of invasive GBS disease decreases with increasing maternal serotype specific capsular antibodies in the women and newborns (9–11). Hence, maternal immunisation could provide immunity to the physiologically immunocompromised infants that might persist during infancy. Therefore, development of a GBS vaccine is an alternative approach to IAP for the prevention of invasive GBS disease in infants and possibly reducing GBS colonisation and associated morbidity in pregnant women. The characteristics of an ideal GBS vaccine candidate is that the antigen: (i) must be immunogenic, (ii) the antigen specific antibodies must efficiently cross the placenta, (iii) able to confer protection to both the mother and the infant, (iv) antigen specific antibodies must persist into infancy and (iv) possess a broad-spectrum target against the various strains of the pathogen (89).

2.7.1 Polysaccharide antigens

GBS encapsulating polysaccharides play a major role as virulence factors capable of eliciting an immune response that triggers the production of protective antibodies (25). The antigenicity of the CPS serotypes has been exploited to synthesise vaccines that provide immunity against GBS. CPS-based vaccines have been shown to provide a significant level of protection against other encapsulated bacteria such as *H. influenzae* type b and *S. pneumoniae* (165).

The major problem encountered in GBS vaccine development is the antigenic variation amongst the serotypes (166), and with the prevalence and distribution of the serotypes varying according to geographical region (48). Kotloff *et al.* (16) evaluated a tetravalent CPS (Ia, Ib, II and III) vaccine potential to confer broad-spectrum serotype protection. The vaccine was modestly immunogenic and well tolerated among recipients, but none of the recipients had responses to all four antigens and responders had lower IgG GMC than those who were immunised with the monovalent CPS. Furthermore, to induce a ≥ 4-fold IgG GMC post-vaccination, vaccine recipients had to have CPS specific IgG prior immunisation, with higher dosing concentrations unable to enhance the vaccine immunogenicity.

To date, several clinical trials have investigated GBS CPS vaccines candidates to assess their immunogenic potential. Table 2.2 provides a detailed summary of GBS vaccine clinical trials that have been conducted. The majority of the clinical trials have primarily focused on the safety and tolerability of the multivalent polysaccharide vaccine in non-pregnant and pregnant women. Given that CPS are poor immunogens, most potential vaccine constructs have incorporated a highly immunogenic peptide such as tetanus

toxoids (TT) or cross-reactive material (diphtheria mutant protein; CRM_{197}) that stimulates a TD immune response, and enhances the immunogenicity of CPS.

Overall, the limitations of CPS-based vaccines include (i) limited serotype cross-reactivity, (ii) poor immunogenicity on its own, which requires protein conjugation process to increases its immunogenicity and (iii) inefficient materno-foetal transfer of predominantly IgG2 antibodies produced.

Recently the use of GBS molecular characterisation techniques such as MLST and pulse-field gel electrophoresis has revealed that within the hyper-virulent clonal complex there is capsular switching (167). The switch involved the dominant disease causing serotype-III switching to serotype-IV, which could impact on vaccine design (56).

Table 2.2 Clinical studies that reported the safety and immunogenicity of group B Streptococcus capsular polysaccharides based vaccines.

Antigen	Population	Objective	Study phase and setting	Finding	Ref
Tetravalent CPS (Ia, Ib. II and III)	Healthy adults with pre-existing antibodies prior vaccination.	Assess the safety and immunogenicity of the vaccine.	Phase Ia, USA	Vaccine was well tolerated among participants. Immune response did not vary with increased dosage. Seroconversion (≥4 fold) was observed in 17-70% participants. No participant had detectable antibodies to all four CPS serotype.	(16)
CPS serotype-III conjugated to TT	Healthy non-pregnant adults (n=100) between 18-40 years.	Evaluation of the safety and immunogenicity of vaccine	Randomised, placebo controlled phase, USA	Dose dependent immune response was observed with CPS type-III specific antibodies being highest when immunised with III-TT (50.1-fold increase) compared to uncoupled CPS type-III (8.8-fold increase) at 8-weeks post-immunisation.	(168)
CPS serotype-Ia and -Ib conjugated to TT	Healthy non-pregnant women (n=105 for type-Ia and n=85 for type-Ib) between 18-40 years.	Determine safety, immunogenicity and optimal dose.	Randomised, placebo controlled phase 1 or 2, USA	GBS CPS specific IgG increased post-vaccination in a dose dependent manner and was higher in participants immunised with TT conjugated vaccine formulations that resulted in higher OPA killing.	(15)

CPS serotype-III conjugated to TT with or without alum as adjuvant	60 Non-pregnant adults randomised to receive four doses of CPS with or without aluminium hydroxide and 36 adults who previously (> 21 months) received CPS-type III-TT vaccine.	Evaluate the effects of aluminium hydroxide adsorption and inclusion of a booster dose on the immunogenicity of CPS.	Randomised, blinded (alum adjuvant effect) and open label (booster dose response) phase I, USA	Similar CPS-III specific IgG concentrations at 4 weeks post-vaccination between participants immunised with or without alum adjuvant. IgG decreased 21 months post initial vaccine dose. Booster effect was achieved in 22% participants with IgG increasing 3-fold higher compared to 2-months post initial dose.	(169)
CPS serotype-III conjugated to TT	Pregnant women between 30-32 weeks of gestation who delivered ≥37 weeks of gestation. Their respective infants were followed up for 6-months.	Evaluate the safety and immunogenicity of vaccine, efficiency of placental transport and durability in infancy. Correlate antibody level to OPA killing.	Randomised, double-blind, placebo controlled phase I, USA	No vaccine-associated AE were noted. A >50-fold increase in type-III specific IgG GMC persisted 2 months postpartum in infants.	(170)

Bivalent CPS (serotype-II and –III) conjugated to TT	Healthy adults (n=75) between 18-45 years.	Compare immune elicited by the bivalent vaccine formulation to that elicited by either of the monovalent vaccines.	Randomised, double blind phase II, USA	Bivalent vaccine formulation induced ≥4-fold increase of both CPS type-II and –III specific antibodies 4-8 weeks post-immunisation with no statistical difference to the monovalent vaccines. However, OPA killing was not observed in serum of recipients of the monovalent CPS type-III vaccine as compared to the recipient of the bivalent vaccine.	(171)
CPS serotype-V conjugated to TT	Healthy adults between 65-85 years old.	Assess the reactogenicity and immunogenicity and also determine the durability and functional activity of the CPS type-V specific antibodies induced.	Randomised, double blind, Td controlled phase I, USA	Vaccine was well tolerated with no AE reported. Vaccine induced ≥4-fold increase in CPS type-V specific IgG, IgM and IgA in 68%, 76% and 59% of recipients 8-weeks post-immunisation which persisted for 52-weeks with increasing avidity. Higher OPA was achieved in recipients who had ≥1 µg/mL type-V specific antibodies prior-immunisation.	(172)
CPS serotype-V conjugated to either TT or CRM_{197}	35 Nonpregant adults receiving type V-TT, V-CMR or placebo.	Evaluate immunogenicity and OPA activity	Randomised, double blinded placebo controlled phase I, USA	A ≥4-fold increase in CPS-V specific IgG concentration in 93-100% participants 26 weeks post-immunisation which persisted in 85-93% 2 years post vaccination. IgA and	(173)

				IgM also increased post-immunisation, peaking at 8-weeks and persisted until 26-weeks post-immunisation.	
CPS serotype-V conjugated to TT	Healthy non-pregnant adults	Determine the dose of vaccine with optimum immunogenicity and functional activity.	Phase I open label, USA	Unconjugated vaccine modestly increased type-V specific GMC but was significantly lower than TT conjugated vaccine. Significantly less OPA killing was observed for the unconjugated compared to the TT glycoconjugate.	(174)
CPS serotype-Ia, III and V conjugated to TT or CRM_{197}	33 Healthy adults who had low antibody concentrations pre-immunisation.	To determine the persistence of functional activity of GBS CPS specific antibodies.	Phase I and II, USA	Significant increase in IgG post-immunisation that declined by 40-50% at 104 weeks post-immunisation. OPA activity persisted 104 weeks post-immunisation although lower compared to 4 weeks post-immunisation.	(175)
Trivalent CPS (serotype-Ia, -Ib and -III) conjugated to CRM_{197}	Pregnant women between 24-35 weeks of gestations with or without HIV infection and their respective infants.	Assess placental transfer of antibodies specific to CPS in infants relative to HIV *in utero*-exposure. Compare vaccine safety and immunogenicity in pregnant women with or without HIV	Non-randomised open-label phase II, Malawi and South Africa	CPS type specific antibody GMC increased following vaccination and was significantly higher in HIV-uninfected mothers compared to HIV-infected mothers. Placental antibody transfer was similar between the HIV-uninfected and HIV-infected mother-infant dyads. Antibody GMC in infants decreased	(176)

		infection.		to 28-58% atfter 42 days compared to those recorded at birth.	
Trivalent CPS (Ia, Ib and III) conjugated to CRM_{197} with or without aluminium hydroxide or MF59 as adjuvant	340 Non-pregnant women followed for 2 years (longevity) and 338 pregnant followed for 61 days (immunogenicity).	Determine optimal dosage, vaccine formulation and injection schedule.	Randomised, observer blind phase Ib, Belgium	Higher antibody GMC in all vaccine formulations compared to the placebo group but no additional benefit from higher dosage, adjuvanted vaccine or injection of second dose.	(177)
Trivalent CPS (Ia, Ib and III) conjugated to CRM_{197} with aluminium hydroxide as adjuvant	Healthy non-pregnant and pregnant women (32-gestation week) were followed post-vaccination together with their respective newborn infants.	Asses safety and immunogenicity in pregnant and non-pregnant women together with antibody trans-placental transfer to newborns	Randomised, observer blind placebo controlled phase Ib/II, South Africa	Specific IgG against all GBS CPS serotype was significantly high 1year post vaccination compared to the non-pregnant women who received the placebo. Similarly, higher IgG specific to CPS serotypes were observed in immunised pregnant women resulting in 49-79% antibody transfer to their newborns.	(17)

2.7.2 Protein antigens

Protein antigens have several advantages to polysaccharide antigens as vaccine candidates; including eliciting a TD response that results in immunological memory, being immunogenic without conjugation to other molecules and can also be produced inexpensively in large quantities by employing DNA recombination techniques (166). Several GBS surface proteins have been identified as potential vaccine epitopes against invasive GBS disease, based on their virulence potential and antibody reactivity, Table 2.3. Investigation to elucidate the immunogenicity of these proteins has, however, been limited to murine models with only few studies in humans.

Table 2.3 Virulence and immunogenic properties of GBS surface proteins considered as potential vaccine candidates.

Protein name	Virulence	Cohort and sample size	Immunogenic potential	Ref
Sip	Unknown	Pregnant women (n=644) between 35-37 weeks of gestation who were either GBS colonised or non-colonised and their newborns (n=176)	GBS colonised women were detected to have higher IgG specific to Sip compared to non-colonised women. IgG levels in newborns were correlated to that of maternal levels.	(89)
BibA	Complement inhibitor and adhesion protein, occurs in three allelic variants, with HvgA associated with hypervirulence	Studies limited to murine models and uncategorised human sera	BibA specific IgG is capable of conferring up to 69% protection against GBS disease in pups born to mice dams immunised with BibA protein. Recombinant BibA protein N-terminal is recognised by human IgG and IgA.	(51,95,141)
FbsA	Induces fibrinogen aggregation, masking GBS from opsonophagocytosis	Cervical and ano-rectcal culture confirmed colonisation in pregnant women.	FbsA is highly immunogenic and recognised by IgA obtained from cervical secretions	(96,129)
ScpB	Cleaves complement component C5a to attenuate recruitment of polymophornuclear leukocytes to site of infection. Binds to fibronectin as an adhesin during infection.	Pooled sera form asymptomatic adults	Natural occurring IgG inhibits activity of soluble C5a-ase, however GBS surface anchored C5a-ase is masked by the encapsulating polysaccharide from neutralisation by antibodies	(133,178)

Rib	Unknown	Culture confirmed neonates (1week post-partum) and their respective maternal serum (26-48 gestation weeks) Neonates and their mothers with blood negative cultures were used as case controls	Colonisation with Rib expressing GBS elicits an antibody response associated with maternal gestational age. Low IgG levels against Rib were associated with invasive GBS disease and there were correlation between maternal IgG levels and neonate IgG levels	(91,166)
Alpha C protein (αCP)	Binds to integrin of fibronectin on epithelial cells to mediate GBS invasion.	Sera of pregnant women collected at delivery together with neonatal cord blood (n=570). Colonised women were culture confirmed with vaginal and rectal swabs (n=156).	α-C specific IgG titre was similar in both non- and colonised women, however, women presenting with peripartum GBS bacteraemia had elevated IgG titre together with their respective healthy neonates. Neonates with invasive disease had half the IgG level required for effective opsonophagocytic killing.	(19,179)
Beta C protein (βCP)		Sera from 570 pregnant women was collected of which 156 were colonised with GBS and 16 had βCP expressing strains.	IgA and IgG GMCs were similar between colonised and non-colonised pregnant women, however, bacteremic women (n=2) had significantly elevated IgA and IgG GMCs.	(92)
Pilus Island	GBS adhesion and invasion in epithelial cells, with serotype Ia and III showing greater	Maternal sera was collected for 984 colonised women who delivered healthy infants, 153	Maternal IgG specific to the three pilus proteins BP-1, AP1-2a and BP-2b were	(18,147,148)

proteins	invasion capability.	from those who delivered infants with invasive GBS disease and 473 who were non-colonised together with their respective infants.	significantly higher in women colonised with GBS expressing the respective protein compared to those non-colonised or colonised by strains not expressing the respective PI protein. Significantly lower maternal IgG titres to PI-1 and 2a were reported for women who delivered EOD infants.	
R protein	Trypsin resistant protein sub-classified into 4 distinct genetic types with R4 most prevalent in isolates recovered from humans	Sera of 40 GBS II/III colonised mothers and 26 non-colonised mothers were assayed for presence of antibodies to R4. Furthermore cord blood of 14 mother-infant pairs were assayed.	R4 specific antibodies were present in 92.5% of colonised mothers compared with 54% of non-colonised mothers. A correlation of 0.68 antibody concentration was observed between mothers and their newborns suggesting materno-fetal transfer of antibodies across the placenta.	(180)
Laminin binding protein (Lmb)	Adhesion protein that binds to human laminin to mediate bacterial attachment during infection. Lmb plays an important role in transcytosis of brain microvascular endothelial cells.	Lack of relevant data on the immunogenicity of the protein, which is further enunciated by the protein's inability to stimulate neutrophil to secrete cytokines.		(139,140)
Luecine rich	LrrG is homologous to PsaA a choline binding	Mice immunisation study was performed by	LrrG immunisation provided 91%	(181)

repeat protein (LrrG)	protein of *S. pneumoniae* in which its C-terminal region adheres to epithelial cells.	injecting s.c the recombinant mixture with alum adjuvant and a booster dose after 28 days to a group of 12 mice of 6-7 weeks old. Challenge lethal dose was administered 3 weeks post-booster dose.	protection and a substantial increase of $IgG_1 > IgG_2$ consistent with administration of protein antigens in immunisation models. No further progress has been made regarding LrrG immunogenicity.	
Ornithine carbamoyltransferase (OTK) and Phosphoglycerate kinase (PGK)	Outer membrane localised GBS proteins with unknown virulence properties.	Anti-sera collected from rabbits immunised with recombinant protein were mixed with GBS challenge dose that was subsequently injected i.p to 24 h old mice. Sample size was 48-54.	Anti-sera provided 46% and 35% protection in neonatal mice for OTK and PGK relative to 64% protection conferred by whole cell anti-sera.	(182)

Other factors which could influence the immunogenicity of proteins e.g., surface accessibility and/or masking effect of CPS and their genetic variability, have not been fully studied. These factors may limit the potential of a GBS protein antigen from serving as a vaccine candidate capable of conferring protection against invasive disease (183). Fluegge *et al.* noted a pronounced genetic variability in the distribution of virulence conferring surface proteins among invasive and non-invasive strains isolated from neonates (49). Figure 2.5 highlights that 21% of the invasive strains did not contain genes encoding any of the known virulence surface proteins. Therefore, factors such as the physiological and physiochemical properties that play complex roles in the immunogenicity of GBS should be considered during the selection of peptides as vaccine antigens for the prevention of invasive GBS disease.

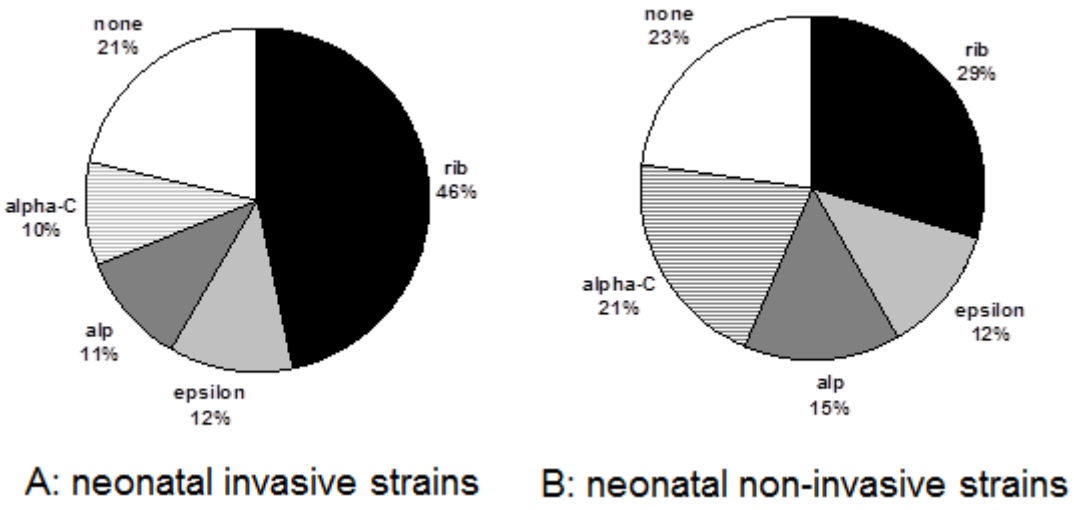

Figure 2.5 Distribution of group B Streptococcus surface protein genes in A, neonatal invasive strains and B, neonatal non-invasive strains(49).

2.7.3 Polysaccharide-protein glycol-conjugate

Proteins are considered to be more immunogenic than polysaccharides, although the polysaccharide capsule is the major virulence factor of GBS, making it the ideal target for the immune system. The immunogenicity of CPS conjugated to tetanus toxoid (TT) has been extensively evaluated in several clinical trials. The conjugated vaccines, inclusive of the major invasive serotypes (Ia, Ib, II-III and V), were well tolerated and elicited immunological responses with functional antibodies that mediated opsonophagocytic killing (15,168,174,184). The immunogenicity of each vaccine in pregnant and non-pregnant adults was dose dependent, with the highest dose resulting in ≥ 4-fold increase in CPS specific antibody geometric mean concentration (GMC) that persisted for almost a year post immunisation. A recent clinical trial by Leroux-Roels *et al.*, however, reported that there was no additional benefit when increase vaccine dose >5 µg/mL (177). The predominant antibody isotype elicited by the conjugate vaccines was IgG, except for type V conjugants in which IgM was the predominantly secreted isotype. For CPS III-TT and the CPS-III unconjugated vaccines; IgG_2 was higher, with insignificant levels of the other IgG subclasses observed in immunised sera (168). The unconjugated vaccines were less immunogenic, eliciting antibody concentrations that peaked 2 weeks post-immunisation and declined after 8-26 weeks, which highlights the role of TT in activating cell mediated immunity that subsequently serves as immune modulators into polarising clonal expansion of plasma B cell to memory cells.

Only five serotypes account for 95% of invasive GBS disease isolates globally, hence a pentavalent conjugate vaccine could possibly result in substantial disease reduction. Evaluation of the immunogenicity of a bivalent type II and III-TT conjugate showed that in 80-90% individuals, ≥ 4-fold increase in IgG GMC was retained 8-weeks post-

immunisation, which persisted for 26 weeks (171). Although there is some cross-reactivity between serotype II and III, this cross-reactivity was not associated with ≥ 4-fold GMC increase inter-serotype specific IgG; and insignificant level of OPA killing occurred with the heterologous GBS serotype. Furthermore, the functional activity of the antibodies elicited by the bivalent conjugate vaccine mediated similar OPA killing as the monovalent conjugate of either type II or III resulting in 1.6-1.8 $\log_{10}$ reduction of colony forming units. Substituting the conjugate protein by a highly immunogenic GBS protein, such as in Table 2.4, could increase the vaccine's potential as a universal GBS vaccine, and possibly mitigate against replacement disease by non-vaccine serotypes.

Table 2.4 Studies investigating group B Streptococcus capsular polysaccharide protein glyco-conjugates

Conjugate protein	Serotype	Finding	Ref
TT	IV and VII	Despite the CPS type-IV and -VII having a similar structure each vaccine induced CPS-type specific antibody response with no cross-reactivity. CPS type-IV and –VII specific antibodies conferred protection to 93% and 100% of pups challenged with a homotypic GBS strains respectively.	(185)
C5a peptidase	III	Immunisation with C5a-III provided greater bacterial clearance compared to TT-III conjugate due to independent serotype protection.	(137)
Cα		Administration of 20 µg conjugate to mouse dams resulted in 95% and 60% protection of pups when challenged with type-III GBS or Cα positive GBS, results comparable to TT-III vaccine.	(186)
Cβ		Cβ-III immunisation triggers serotype independent opsonophagocytic killing in rabbit sera. Pups from mouse dams immunised with conjugate vaccine resulted in 93% and 76% survival when challenged with either type-III or Ia GBS lethal dose.	(187)

Recombinant alpha like protein (rAlp)		Vaccine formulation induced dose dependent antibodies titres against serotype-III and rAlp. Protection was conferred to 94% of pups born to immunised dams when challenged with a lethal dose of GBS expressing CPS type-III. However, survival was reduced to 23% when pups were challenged with GBS expressing Alp3 but not the CPS type-III.	(188)
Duck hepatitis B core antigen		Serotype-III specific IgG GMC increased to 22.6 µg/ml on mouse dams administered the conjugate vaccine which conferred protection to 97% of neonates when challenged with a lethal dose of GBS.	(189)
Diphtheria toxin		CPS type-III specific IgG GMC increased from baseline level (0 µg/mL) to 42.4 µg/mL on mouse dams administered the conjugate vaccine which conferred protection to 98% of neonates when challenged with a lethal dose of GBS.	(189)
Cross-reactive material (diphtheria mutant protein)	V	Conjugate vaccine triggered a ≥ 4-fold increase of type-V specific IgG concentration that persisted 26 weeks post vaccination with 95% of recipients having ≥ 1µg/ml type-V specific IgG concentrations. Other Ig subtypes were higher than IgG post-immunisation.	(173)

Pilus protein (PI-1)	II	Conjugate vaccine induced both type-II and PI-1 specific antibodies capable of OPA killing in immunised dams. Antibodies were trans-placental transferred to their respective pups which conferred protection when challenged with lethal dose of GBS expressing either type-II CPS or PI-1 proteins.	(190)

2.8 Reverse vaccinology

The use of vaccines as a strategy for disease prevention has been successfully used for over two centuries to eradicate or cause near elimination of several invasive pathogens such as variola-virus, rubella virus, poliovirus, *C. diphtheriae etc.* The conventional methods used in vaccine development involved *in vitro* cultivation of the pathogen which is then inactivated by killing or attenuated and with the whole-organism used as a vaccine. Alternatively, subunits of the cultivated pathogen could be isolated one at a time to be analysed for their immunogenic properties, Figure 2.6a (191). This method of vaccine development involves a laborious task which is time consuming and proves ineffective when the pathogens cannot be cultured *in vitro.* Additionally most pathogens occur in variant strains that have mutated their virulence factor to avoid immunological clearance, hence, increasing the complexity of designing and developing a vaccine for all possible variable strains (192). Other detrimental effects are also observed when the whole organism is used as a vaccine; either it may re-activate upon immunisation or consist of toxic chemicals. Consequently, there is a need for more reliable and feasible methods in developing vaccines.

Reverse vaccinology, a term coined by Rappuoli (191), refers to a technique that use the high-throughput genomic data available to select for potential antigenic molecules with potential to be vaccine targets. Computational algorithms are used to analyse and access for conserved sequences and/or motifs within the pathogen's genome to select for peptides that are potentially immunogenic, Figure 2.6b. The *in silico* identified antigens are then cloned and expressed in *E. coli* or a suitable host and assayed for their immunogenic capabilities both *in vivo* and *ex vivo*.

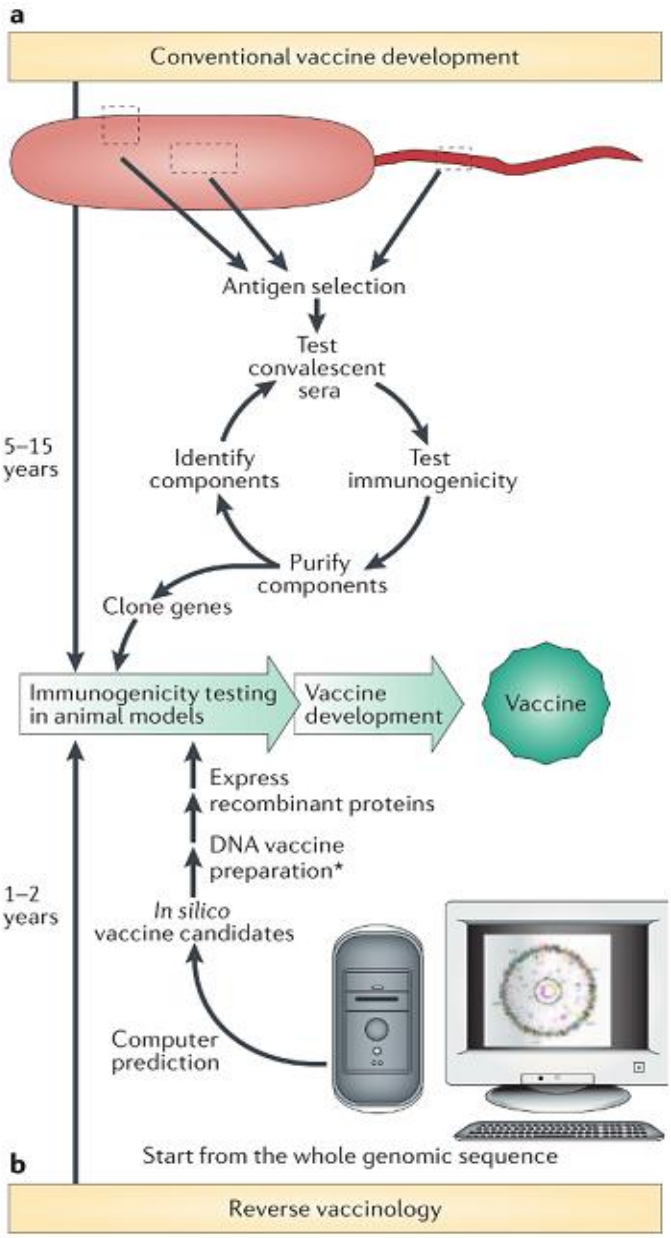

Figure 2.6 Schematic representation of approaches used to develop vaccines, A, conventional methods and ,B, reverse vaccinology (164).

The process of reverse vaccinology in vaccine development has been used successfully in identifying essential virulence factors of *N. meningitidis* and *S. agalactiae* which are conserved amongst several serotypes thus making them possible universal vaccines (193,194). A limitation of reverse vaccinology is that it can only be used to identify

protein antigens and unable to identify other essential virulence factors such as polysaccharides and glycolipids.

Proteins anchored on the surface of the bacteria are easily accessible and are targeted by the antigen presenting cells, which subsequently activate an immune response to clear the evading pathogen. The primary structure of proteins contains conserved intragenic sequences that determine subcellular localisation of the particular protein. These conserved sequences are used to construct computational algorithms that can be used to predict subcellular localisation of hypothetical proteins of related bacterial genomes (195). Available GBS genomes of strains isolated from humans are listed in table 2.5, in which reverse vaccinology to identify immunogenic GBS peptides may be executed.

Table 2.5 List of group B Streptococcus strains with complete genome sequences. Only GBS strains isolated from human subjects are included.

Strain	Serotype	Size (Mb)	Genes	Proteins	Ref
2603V/R	V	2.2	2279	2127	(196)
A909	Ia	2.1	2150	2026	(197)
COH1	III	2.1	2067	1929	
CNCTC 10/84	V	2.0	2031	1903	(198)
GBS6	II	2.2	2222	2079	
GBS2-NM	II	2.2	2198	2057	(199)
GBS1-NY	II	2.2	2223	2083	(199)
SS1	V	2.1	2100	1977	(200)
H002	III	2.1	2140	2008	(201)
GBS85147		2.0	1982	1876	
SG-M1	III	2.1	2169	2037	(202)
NGBS128	III	2.1	2077	1914	(203)

NEM316	III	2.2	2217	2103	(204)
515	Ia	2.09	2110	1886	(197)
18RS21	II	2.19	2238	2146	(197)
H36B	Ib	2.2	2382	2376	(197)
CJB111	V	2.11	2114	1917	(197)

S. agalactiae NEM316 strain isolated from a fatal case of septicaemia (204) is a representative genome of the major invasive serotype-III and colonising ST-23. The genome is comprised of 14 putative pathogenic islands of which one is triplicated in the genome and saturated by open reading frames for protein coding. Comparative genomics reveals that the GBS pan-genome consist of approximately 1806 core genome and 33 strain specific genes (197). Almost one third of the core genome is comprised of hypothetical proteins and the functions of the vast majority of the dispensable genes are unknown, Figure 2.7, which enunciates the necessity of reverse vaccinology in further elucidating the virulence and immunogenic proteins with vaccine potential.

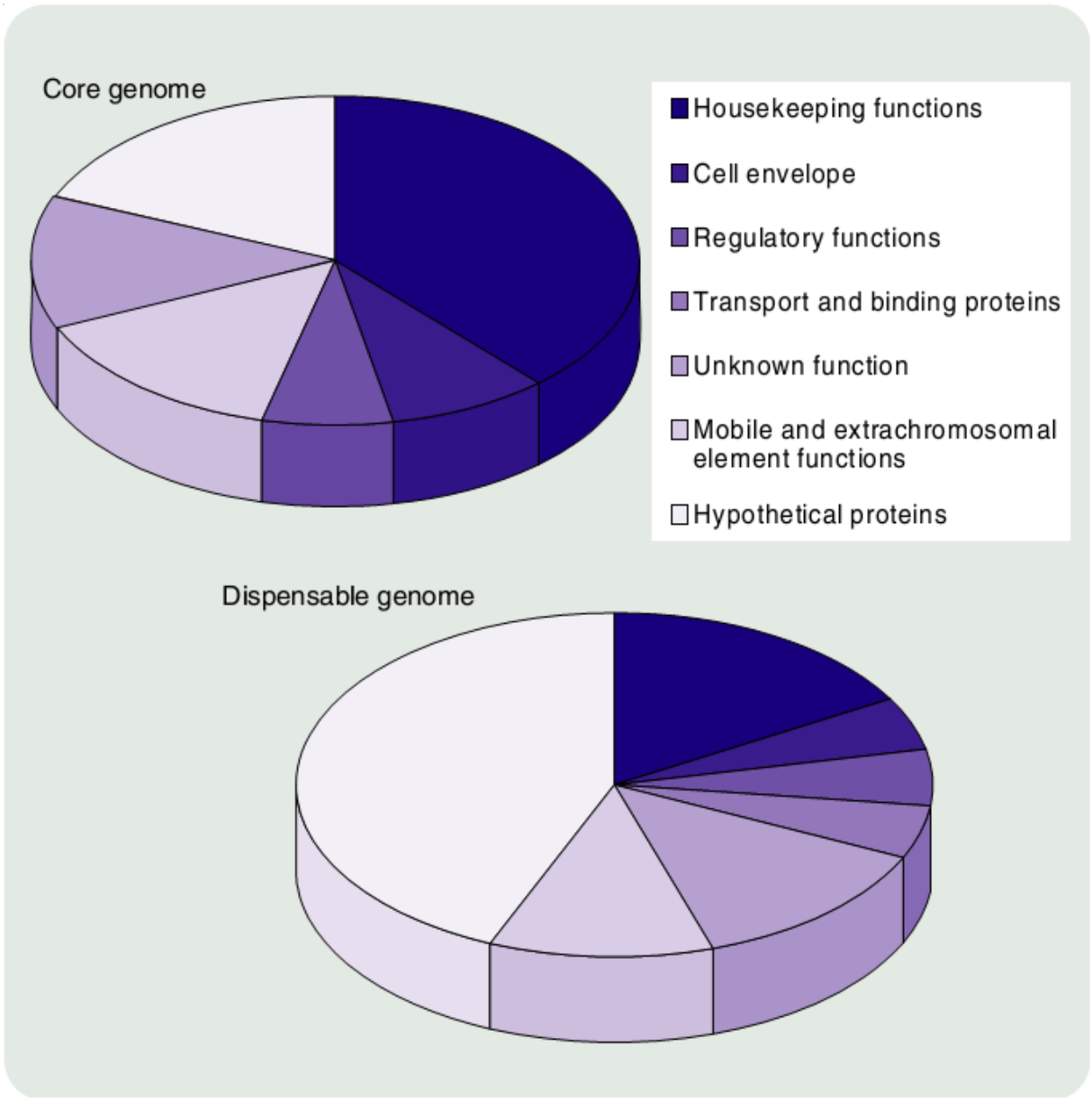

Figure 2.7 Schematic representation of group B Streptococcus core genes and dispensable genes of the eight GBS strains isolated from humans (192).

Therefore, using *in silico* identified GBS surface proteins together with those with known virulence capacity; the objectives of my study were:

i. Determine the association between natural occurring antibodies and prevention of GBS acquisition and/or clearance of colonisation during late pregnancy;

(ii) Evaluate the effect of maternal HIV infection on trans-placental transfer of IgG specific to GBS surface proteins from mother to newborn;

iii. Evaluate the association of GBS surface protein specific IgG titres with risk of disease against invasive GBS disease in infants;

iv. Explore the possible immunological characteristics associated with the increased likelihood of invasive GBS in older children by comparing IgG titres in HIV-infected and HIV-uninfected children against CPS with that induced by GBS surface proteins.

Chapter Three

Methods

3.1 *In silico* surface protein selection

Surface proteins were predicted using an adaptation of the Giombini et al. protocol designed to automatically identify surface localised proteins using query genomic sequences (205). A systemic cycle-process was adopted rather than the filtration system to analyse all proteins for potential cell surface features. A cut-off ≤ 3 membrane helices per protein was permitted since proteins with more than 3 membrane spanning regions tend to be insoluble (194). Proteins supplied by Valneva Austria GmbH were removed prior to selection of immunogenic proteins. Additional bioinformatics tools: SignalP and TargetP, were used to decrease the level of false positives, Figure 3.1. Proteins that were identified as extracellular and/or cell wall surface anchored proteins were screened for B-cell epitpope regions using BepiPred and an average threshold score of 0.4 was considered significant for immunogenic proteins (206). Ideal immunogenic proteins were further selected based on the presence of repeating proline residues (peptidase resistance), repeating serine residues (family of adhesins in Gram positive bacteria) and LysM domain (surface anchor motif).

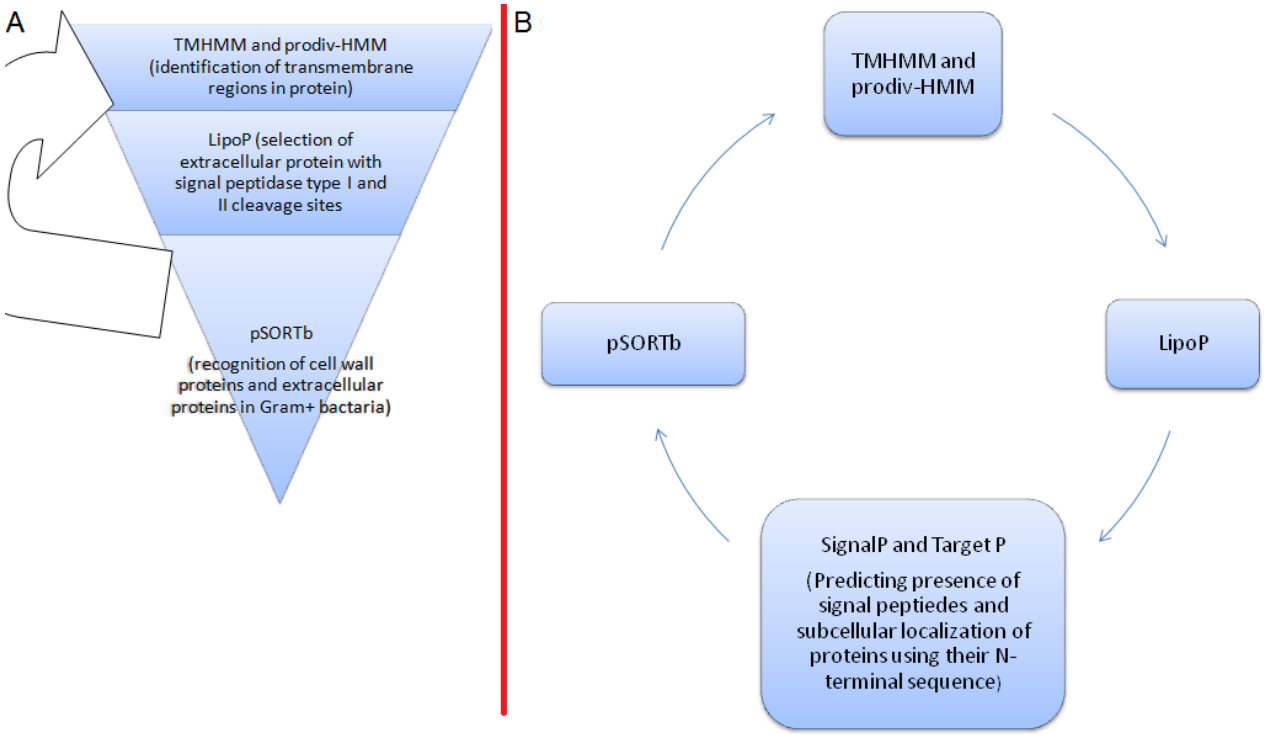

Figure 3.1 Extracellular surface protein selection methods, A: Giombini et al filtration protocol, B: systemic cycle-process that analyse each protein with all the available bioinformatics tools.

3.2 Protein structure and immunogenic domain prediction

Protein structure of the *in silico* identified protein was predicted using Swiss Model database available at http://swissmodel.expasy.org which uses known protein structures that are homologous to the query protein sequence. The modelled structure was then accessed for distinguishable domains using domain linker prediction database (DLP) (128). The database identifies domains based on the presence of a consensus sequence of residues that are highly associated with linking protein domains. The domain structures were superimposed onto the complete protein structure using Superimpose CERPROXY software and the respective immunogenicity of each domain accessed with DISCOTOPE (129).

3.3 Gene cloning, expression and protein purification

GBS serogroup III (CDC) was grown overnight on 5% sheep blood agar and subsequently cultured in Todd Hewitt broth (Merck, South Africa) at 37^{o}C with shaking. The genomic DNA was extracted and purified with the QIAgenDNAamp kit according to manufactures instruction, and DNA concentration and purity determined spectophotemtrically with the $A_{260/280}$ ratio. Genes encoding putatively immunogenic proteins were amplified with manually designed and optimised primers, Table 3.1, with the PCR mixture inclusive of 1.0 µM of each primer (Inqaba Biotec, South Africa), 25-50 ng gDNA and Phusion Flash Enzyme mix (2.5 U DNA polymerase, DMSO, 50 µM MgCl, 400 µM per dNTPs) (Thermo Scientific) and the reaction cycles were: an initial denaturation cycle at 98^{o}C for 2 min, 30 cycles of 98^{o}C for 5 sec, 50-55^{o}C for 30 sec, 72^{o}C for 15 sec/kb and a final extension cycle at 72^{o}C for 5 min. The PCR product was then purified with QIAgen PCR purification kit and cloned into the pETite C-His vector (Lucigen, USA) and propagated in HI *E. Coli* competent 10G cells (Lucigen, USA) according to manufactures instructions.

Table 3.1 Primer sequences (5'-3') flanked by att-L/R homologous oligonucleotides

	Forward primer	Reverse primer
Foldase protein*	**GAAGGAGATATACATATG**GTTGTTA CAATGAAGGGCGACAC	**GTGATGGTGGTGATGATG**TTGTGAT AAGATGCCTGCAAATGC
Sip	**GAAGGAGATATACATATG**GCACAA GAAACAGATACGACGTGG	**GTGATGGTGGTGATGATG**TGATACG TGAACGTGGTCATAGTG

gbs0392	**GAAGGAGATATACATATG**GTCATCC CAGTAGACCCAGCAGTACC	**GTGATGGTGGTGATGATG**TGGGAGC GTCTTTTCAGCACCTG
gbs0393	**GAAGGAGATATACATATG**GCTGTTA CAACCACACCTGCAGTTAATC	**GTGATGGTGGTGATGATG**TGGCAGT CCTTTAGCTGGAGC
gbs1356	**GAAGGAGATATACATATG**GTAGGG AGAACTGTTGCGACAAGTG	**GTGATGGTGGTGATGATG**ACCGTCC GCATTTTCATTGTCCTT
cell wall protein	**GAAGGAGATATACATATG**GCTGATA CAAGTGATAAGAATACTGAC ACG	**GTGATGGTGGTGATGATG**TCCCGTA GATGCTATTACAGTATCTG
immunogenic secreted proteins	**GAAGGAGATATACATATG**GAAACT GCTTCACCTTCAGCCG	**GTGATGGTGGTGATGATG**TTGTGCA GCTTGTGCTGAACT
gbs2106	**GAAGGAGATATACATATG**GCAGAT AAAGTTCGCGTAGCC	**GTGATGGTGGTGATGATG**CCAAGCT GATAAACCTTGAGCACG
LysM domain protein	**GAAGGAGATATACATATG**ACCGTG AAATCAGGTGATACCTTATCA GC	**GTGATGGTGGTGATGATG**AAATGAT AGCGCTGCCGACC
pEtite vector	TAATACGACTCACTATAGGG	CTCAAGACCCGTTTAGAGGC

Positive recombinant colonies were identified by colony PCR, which were then cultured in YT broth Kan^+ for ~6hr at 37ºC with shaking (220rpm). The culture recombinant-clones were diluted to a final concentration of 30% glycerol and stored in 1 ml aliquots at -70ºC. Plasmids isolated from the recombinant-clones were sequenced to confirm correct ORF orientation prior to protein expression.

Sequence confirmed plasmids were subsequently transformed into *E. coli* BL21(DE3) competent cells (Lucigen, USA) by heat shock at 42^{o}C in a water bath (Scientific, South Africa) and cultured overnight in YT agar plates supplemented with 30 µg/ml kanamycin and 0.5% glucose at 37^{o}C. The overnight culture was diluted 1/100 to 100 ml YT broth and cultured for ~ 3 h at 37^{o}C after which protein expression was induced by adding IPTG (Sigma Aldrich) to a final concentration of 1 mM. After induction of protein expression, the culture was allowed to continue to grow at 37^{o}C with shaking (220 rpm), and 1 ml samples were collected every half an hour for 3 h. The collected samples were centrifuged and the pellet re-suspended in 100 µl SDS sample loading buffer (250 µM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercaptalethanol and 0.02% bromophenol blue) (Biorad), incubated for 5 min at 95^{o}C and stored at -20^{o}C for SDS-PAGE analysis. The remaining culture was used to access the solubility of the protein. The pellet was suspended in 4 ml of PBS, sonicated at 6-10 pulses of 10 s. The lysed cells were centrifuged at 18514g for 30 min at 4^{o}C and the supernatant mixed with SDS sample buffer in a 1:1 ratio. The pellet was re-suspended in 4 ml of 8 M urea with vigorous vortexing, and subsequently centrifuged at 12857g for 30 min and the supernatant mixed with the sample buffer in a 1:1 ratio, similarly with the PBS re-suspended pellet. The collected samples were visualised by SDS-PAGE analysis.

Proteins were purified by fast performance liquid chromatography (FPLC) that utilised an immobilised metal ion affinity column. Overnight cultures were diluted 1/100 in 1 L YT broth Kan^{+}, grown to an OD_{600} nm 0.5 and protein expression induced with 0.1 mM IPTG (Sigma, Germany) and further cultured for an additional 3 h. The culture was centrifuged at 2000g for 10 min and the pellet re-suspended in either 20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 20 mM imidazole (soluble proteins) or 20 mM phosphate

buffer pH 7.4, 0.5 M NaCl, 20 mM imidazole, 8 M urea (insoluble proteins) and were lysed by a cycle of freezing (-20^{o}C) and thawing. The cells were further lysed by sonication and the cell lysate purified by centrifugation at 18514g for 30 min at 4^{o}C. The histidine tag on the C-terminal of the recombinant proteins was allowed to bind to immobilised Ni^{2+} cations of the resin column. A step gradient elution was performed to remove protein with low column binding affinity with 50 mM imidazole and with a subsequent elution of His-tagged protein by 500 mM imidazole. Purity of eluted proteins was chromatographically analysed at 254 nm and visualised by SDS-PAGE analysis.

3.4 Multiplex immunological assay

3.4.1 Conjugation of recombinant proteins to microsphere beads

Purified proteins were desalted by gel filtration using PD-10 desalting columns (GE healthcare, Germany) with an exclusion limit of 5 KDa and eluted with 3.5 ml PBS pH 7.2 in 1 ml aliquots. Desalted proteins were immobilised to carboxylated beads using a 2-step carbodiimide reaction, with each protein being conjugated to a unique bead region (207). Beads were homogenised by vortexing for 1 min and transferred to 1.5 ml eppendorf tube where they were centrifuged for 5 min at 15000g. The supernatant was discarded and the bead pellet was washed with PBS pH 6.1 and re-centrifuged. Binding site of the beads was activated by adding PBS pH 6.1, 20 mg 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl and 20 mg *N*-Hydroxysulfosuccinimide which was incubated at room temperature with shaking at 100 rpm for 20 min in the dark. Afterwards the beads were washed twice with PBS pH 7.2 and the beads suspended in 50 µg/ml proteins at room temperature with shaking at 100 rpm overnight.

Quantification of optimal protein conjugation concentration to the beads is described in appendix 10.5.1.

3.4.2 Reference serum, controls and sample analysis

Purified immunoglobulin G pooled from human adults (Polygam, National Bioproducts, South Africa) was used as a reference serum to measure IgG titres. Polygam was diluted 1:50 in PBS, 10% fetal bovine serum, 0.05% NaN_3 (PBSFN) and serially diluted 4-fold to obtain 7 standards. The lowest dilution standard (1:50) was arbitrarily assigned a value of 100 U/mL IgG titre. IgG titres to the Polygam standards were measured in true duplicates by adding 50 µL of each standard to 50 µL of protein-bead mixture (3500 beads per unique bead region) and incubated at room temperature for 1 h with 300 rpm to allow for antibody-antigen coupling. The samples were washed 3× using PBS, 0.05% Tween-20, 0.02% NaN_3 (PBSTN). Bound antibodies were quantified by adding 50 µL goat anti-human IgG conjugated to R-phycoerythrin (Jackson ImmunoResearch, USA) diluted 1:100 in PBSFN. The sample mixture was incubated for 30 min at room temperature with 300 rpm followed by washing 3×with PBSTN. IgG titres were measured using Bioplex 200 (Bio-Rad) instrument. Linearity, specificity and correlation between the singleplex and multiplex assay for Polygam are described in appendix 10.5.2.

In house controls were prepared by pooling sera from pregnant women with high IgG titres against the proteins (high control) and sera from those that had low IgG titres (low control). The pooled sera were diluted 1:100 in PBSFN, and IgG titre to the controls was measured similar to Polygam standards. Levey-Jennings plots of the controls were used to assess inter-assay reproducibility (appendix 10.4.2).

Collected serum samples were diluted 1:100 in PBSFN and IgG level determined similar to Polygam standards. Antibody titres were extrapolated using the reference serum and reported as U/mL. Serum samples with IgG higher than the upper limits of detection of the reference serum were re-analysed at 1:200 dilutions. Those below the lower limit of detection (LLD) were arbitrary assigned a value 4× lower than LLD for the respective antigen. All duplicate samples with coefficient of variation ≥30% were re-analysed. Use of all archived human serum samples was approved by the Human Research Ethics Committee of the University of Witwatersrand (approval letters included in appendix 10.1).

3.5 Generation of specific GBS surface protein antibodies

Specific polyclonal antibodies were generated in 6 weeks old New Zealand white female rabbits. Purified cell wall anchored proteins Sip, gbs0393, gbs1356 and lipoprotein gbs2106 were dialyzed in sterile PBS solution (pH 7.2) at 4^{o}C with the PBS solution changed three times every 2 h, and then overnight. Prior immunisation, 5 mL blood was collected from the lateral ear artery of each rabbit and was used as control. After which, 1 mL of the recombinant proteins (250 µg/mL) were emulsified in complete Freud's adjutant at a 2:1 ratio and administered subcutaneously (s.c) at the dorsal thoracic area of the rabbit.

Two booster doses of 250 µg/ml antigen mixed with incomplete Freud's adjuvant (2:1 ratio) were administered s.c at days 28 and 42 post-immunisation. To see if the rabbits were responding to the antigen, 5 mL blood was collected from the lateral ear artery a week later after each booster dose. Finally, at day 56 post-immunisation 20 mL blood

was collected via cardiac puncture on fully anaesthetised animals which were subsequently euthanized using sodium pentobarbital administered intravenously at a dose of 2 mL/kg. Table 3.2 gives a summary of the rabbit immunisation schedule performed in the study. Animal use for generating polyclonal antibodies was approved by the Witwatersrand University animal ethics screening committee (AESC number: 2015/05/20/B, approval letter included in appendix 10.1).

Table 3.2 Overview of the immunisation schedule used to generate protein specific polyclonal-antibodies using New Zealand white rabbits.

Day	Event	Procedure	Specifications
1	Collection of control serum	5 ml of blood from the lateral ear artery	Pre-immunisation bleed
1	Primary Immunisation	Subcutaneous injection at 4 sites at the back of the neck (250 µl/injection site)	Immunised with 250 µg/ml antigen in of CFA
28	1st Booster Immunisation		
35	Collection of test sera	5 ml from lateral ear artery	Test bleed for ELISA analysis
42	2nd Booster Immunisation	Subcutaneous injection at 4 sites (250 µl/injection site)	Immunise with 250 µg/ml antigen in IFA
56	Exsanguination	Heart puncture and withdrawal of blood (~20 ml)	Anesthetise with ketamine-xylazine mixture and then euthanize with sodium pentobarbital after blood collection

CFA= complete Freund's adjuvant, IFA=incomplete Freund's adjuvant

All blood samples collected were allowed to clot at room temperature for ≤1 h and sera was separated from blood cells by centrifuging at 2000g for 10 min. The serum was stored at -70^{o}C. GBS protein specific antibodies in post-immunisation sera were assessed using ELISA (see appendix 10.5) and compared with pre-immune sera to determine increase in antibodies specific to the respective GBS protein antigen.

3.6 Bacterial surface staining and FACS

GBS isolates were cultured on colorex strep B agar plates (MediaMage, South Africa) at 37^{o}C with 5% CO_2. A single GBS positive colony was inoculated in 10 mL Todd Hewitt medium (Davies Diagnostics, South Africa) and allowed to grow overnight at 37^{o}C with shaking (230 rpm). The overnight cultures of GBS isolates were then grown in fresh Todd Hewitt broth at 37^{o}C until an OD_{600nm} of 0.5. The fresh GBS culture was washed with PBS and then fixed using PBS, 0.1% paraformaldehyde solution at 37^{o}C for 1 h and at 4^{o}C for ~16 h. The fixed bacterial cells were washed with PBS and 50 µL of the bacterial cells (OD_{600nm} of 0.1) were incubated with 50 µL of PBS, 20% fetal calf serum and 0.1% bovine serum albumin (blocking solution) for 20 min at room temperature. Bacterial suspensions were then mixed with 100 µL of either pre-immunisation or post-immunisation rabbit serum diluted 1:200 in blocking solution. Protein specific antibodies were allowed to bind on bacterial surface for 1 h at 4^{o}C. After which the cells were centrifuged at 4000g and washed with 200 µL PBS, 0.1% bovine serum albumin (washing solution). Bound antibodies were detected by adding 50 µL of anti-rabbit IgG conjugated to R-phycoerythrin diluted 1:200 in blocking solution and incubated for 1 h at 4^{o}C. Unbound anti-rabbit IgG was washed with 200 µL of washing solution and bacterial cells re-suspended in 200 µL PBS. The bacterial cells were kept in the dark at 4^{o}C. Unstained and stained bacterial cells were analyzed using FACS (fluorescence activated cell sorting) assay.

The FACS Calibur instrument (BectonDickinson Bioscience, South Africa) was used to evaluate the accessibility of the proteins on the surface of GBS by proteins specific

antibodies. Unstained GBS bacterial cells were used to set the voltage and amp gain parameters for detection of GBS cells. The logarithmic scale was used for forward scatter (FSC), side scatter (SCC, threshold of 280) and R-phycoerythrin (R-PE) channels. Sample acquisition was performed at a rate of 300-600 events per second until 10000 events were acquired. Analysis was performed using FlowJo software (version 10.1r5, Tree Star Inc., CA). GBS clinical isolates were assessed for surface expression of Sip, gbs2106, gbs0393 and gbs1356 on the surface and the results are presented in chapter 8.

Chapter Four

Characterisation of group B Streptococcus surface proteins

4.1 Introduction

GBS surface proteins play a major role during bacterial pathogenesis, aiding in both host invasion and immune evasion (29). Efforts to identify highly conserved immunogenic GBS surface proteins as potential candidate vaccines are on-going and have been accelerated by the increasing number of available GBS genome sequences (191,208). Comparisons among the GBS genome sequences revealed that GBS strains consist of a core-genome that consist of genes present in all strains (197). Of the genes encoded by the core-genome, 396 are surface localised proteins and thus subject of interest as potential universal GBS vaccines (197).

Therefore, using the genome sequence of GBS serotype III of the NEM316 strain, *in silico* analysis was performed to identify immunogenic GBS proteins. This chapter discusses the selection and characterisation of GBS surface proteins that are used in subsequent immunological assays. This work has been published in the journal *Vaccine;* Appendix 10.7.

4.2 Results

4.2.1 Identification of surface localised proteins

Using the genome sequence of GBS NEM316 strain, open reading frames (ORF) with characteristics of surface localisation were identified using SignalP, THMM/prodiv, pSORT and LipoP as described in the methods section. The complete set of predicted ORFs of the GBS genome were screened for protein features that are associated with extracellular localisation. Of the 2094 predicted proteins, 32 proteins were identified as surface anchored proteins and 25 as lipoproteins, Table 4.1 and appendix 10.2. The accuracy for selecting GBS surface proteins was verified by comparing the predicted

subcellular localisation of the protein with that assigned in both the curated and uncurated Uniprot/SwissProt proteins database of GBS NEM316. The Uniprot/SwissProt database had 2000/2094 predicted ORF and only 268 were assigned subcellular localisation. A prediction accuracy of 98% was observed using the cycle-process to determine the subcellular localisation of the proteins.

Table 4.1 Group B Streptococcus surface protein features, complete table included in appendix 10.2, signal peptidase I (SpI) recognises the LPxTG motif of cell wall anchored proteins and signal peptidase II (SpII) recognises cleavage site LxxC for lipoproteins.

Protein name	Length (aa)	SignalP	TMHMM	pSORT	LipoP
Sip (gbs0031)	434	Yes	No	Extracellular	SpI
gbs0255	235	No	Yes	Extracellular	SpI
gbs0392	240	Yes	Yes	Cell wall	SpI
gbs0393	933	Yes	Yes	Cell wall	SpI
gbs0479	253	Yes	Yes	Cell wall	SpI
gbs0628	554	Yes	Yes	Cell wall	SpI
gbs0722	240	Yes	Yes	Cell wall	SpI
gbs0723	933	Yes	Yes	Cell wall	SpI
gbs0986	933	Yes	Yes	Cell wall	SpI
gbs0987	240	Yes	Yes	Cell wall	SpI
gbs1143	932	Yes	Yes	Cell wall	SpI
gbs1144	236	Yes	Yes	Cell wall	SpI
streptococcal C5a peptidase (gbs1308)	1150	Yes	Yes	Cell wall	SpI
gbs1356	1634	Yes	Yes	Cell wall	SpI
gbs1539	192	Yes	Yes	Cell wall	SpI
immunogenic secreted protein (gbs1727)	512	Yes	Yes	Cell wall	SpI

gbs2106	225	Yes	Yes	Membrane	SpII
LysM domain protein (gbs2107)	179	Yes	Yes	Cell wall	SpI

4.2.2 Prediction of B cell epitopes

The predicted surface proteins were screened for the presence of immunogenic regions using BepiPred, a bioinformatics tool that determines linear epitope regions within the protein sequence using hidden Markov models in combination with hydrophilicity propensity scale (206). There were 17 proteins that had an average score greater than the cut-off value of 0.4, Figure. 4.1. These included known virulence proteins such as streptococcal C5a peptidase, laminin binding protein (Lmb) and surface immunogenic protein (Sip). Immunogenic proteins were further narrowed to those that contain specific repeats of either proline or serine residues and those that had cell surface anchor motifs or domains, Table 4.2. The final list comprised of 10 proteins to be cloned and expressed for immunogenicity testing. Amongst the identified proteins; Foldase PrsA (gbs0827) protein which is a lipoprotein was included as a negative control for immunogenicity assays with the assumption that lipoproteins are mostly found embedded in the peptidoglycan layer and thus not easily accessible by antibodies.

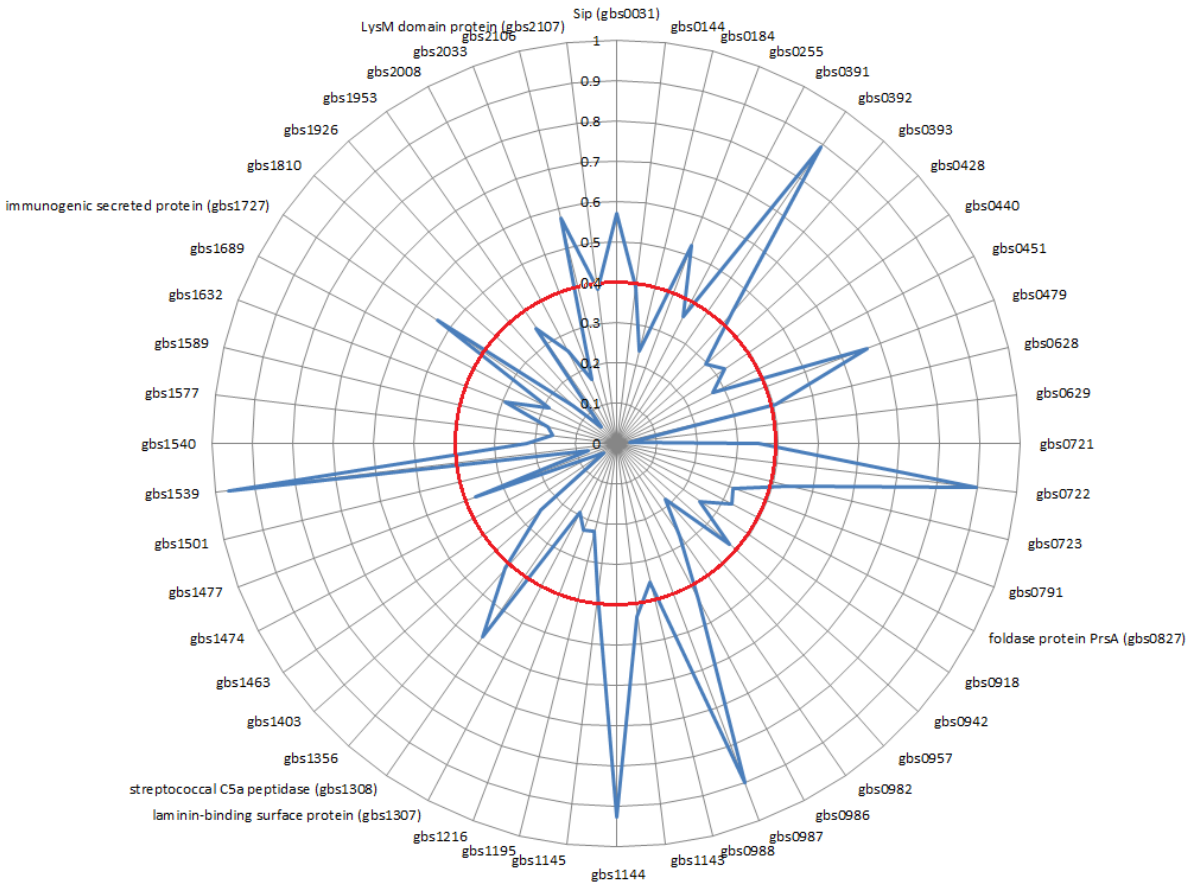

Figure 4.1 Average B cell epitope immunogenicity score for GBS cell surface proteins as predicted using BepiPred software, an arbitrary threshold of 0.4 (displayed in red) was used to select for highly immunogenic proteins.

Table 4.2 Group B Streptococcus immunogenic protein containing repetitive proline/serine, LPXTG motif or LysM domains which were considered for subsequent cloning and purification.

Protein name	**Proline**	**Serine rich**	**LPxTG**	**LysM**
Sip	Yes	Yes	No	Yes
gbs0255	No	No	No	-
gbs0392/0722/0987	Yes	No	Yes	-
gbs0393/0723/0986	No	No	Yes	-
streptococcal C5a peptidase	Yes	No	No	-
gbs1356	No	No	Yes	-
Gbs1539	No	Yes	Yes	-
immunogenic secreted protein	No	Yes	No	-
gbs2106	No	Yes	No	-
LysM domain protein (gbs2107)	No	Yes	No	Yes

Valneva GmbH (Austria) kindly provided recombinant proteins FbsA, BibA and gbs0233 that were selected using the ANTIgenome technique (209). Two BibA variants from strain CHO1 (ST-17) and strain NEM316 (ST-23) were provided. Amino acid sequence comparisons of the BibA variants revealed that the internal sequence fragment of BibA-NEM316 shared 16% similarity with BibA-COH1 peptide sequence,

Appendix 10.5.2. Therefore, for subsequent analysis the internal peptide fragment of BibA from GBS NEM316 strains was included together with the whole recombinant BibA peptide from COH1 strain.

4.2.3 Peptide sequence conservation

Conserved proteins among the genomes are determined as those that have ≥40% identity and ≥80% similarity of their sequences with a P-value ≤ 0.005. Homology of the selected proteins was quantified by performing a BLAST comparison of GBS sequences from strains A909 (Ia), 2603V/R (V), CJB111 (V), COH1 (III), 515 (Ia), H36B (Ib) and 18RS21 (II) that were used to define the pan-genome of GBS (197). A conservation index formula, $\frac{\sum_{i\geq 40\%}^{n-1} SIi}{n-1}$, where SI is sequence identity greater than 0.4 between the query sequence and the referent (NEM316 strain), n is available GBS sequences. Sip and gbs0233 proteins are highly conserved with sequence identity ≥98% among the all GBS strains. Gbs0392 is the leased conserved protein with no homologous proteins in other GBS strains isolated from human samples, Figure 4.2.

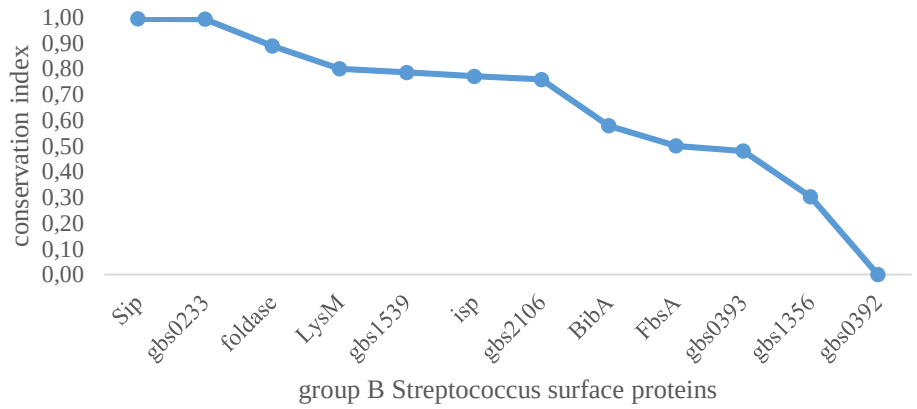

Figure 4.2 Representation of surface proteins conservation in GBS relative to the NEM316 strain as determined by $\frac{\sum_{i>0.4}^{n-1} SIi}{n}$.

4.2.4 GBS protein gene cloning

GBS genes encoding selected peptides were cloned, into *E. Coli* cells. Lipoprotein gbs0255 was omitted from cloning since an optimal primer set required to amplify the gene target were either too long or compromised the integrity of the expected recombinant protein. The peptide sequence of Gbs1356 was too large to be successfully amplified. Therefore, further characterisation of the protein using structural prediction revealed that gbs1356 consists of two domains separated by a linker peptide of residue 632-732 (appendix 10.2). Figure 4.3A shows the two domains of gbs1356 denoted gbs1356-D1 and gbs1356-0D2. Analysis using BepiPred showed that D1 had an average score of 0.548 compared to 0.413 of the complete protein (Figure 4.3D) and therefore was amplified and the recombinant clone transformed into *E. coli*, Figure 4.3B. Structural superimposition of gbs1356 and D1 shows that D1 exclusively forms the D1 domain with a root mean square deviation of 2.421, Figure 4.3C.

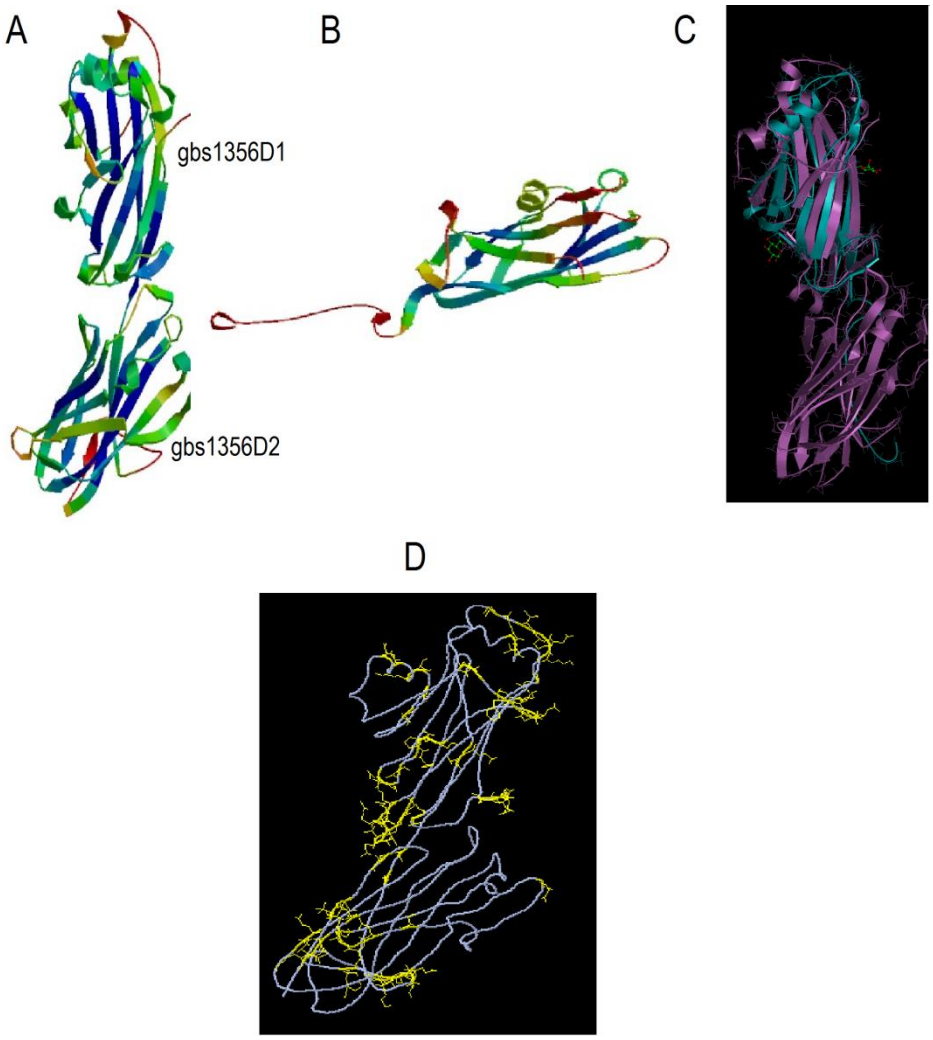

Figure 4.3 Secondary structure prediction of gbs1356, A, complete 3D structure of gbs1356 showing the two domains (D1 and D2) QMEAN score of 0.71. B, secondary structure displaying the recombinant clone of the D1 sequence. C, 3D-structure superimposition of gbs1356 and D1 showing D1 being exclusively gbs1356D1. D, B cell epitope distribution on gbs1356 highlighted in yellow.

The recombinant *E. coli* clones were further sequenced to confirm correct in-frame sequence in the pET C-His vector. A total of 8 of 9 of the cloned genes were correctly inserted into the vector inclusive of a start codon and a his-tag coding residues on the 3'-end of ORF. Recombinant immunogenic secreted protein (gbs1727) had multiple point mutations inclusive of premature termination site and therefore omitted from subsequent production.

The sequencing results for the successfully cloned GBS proteins were aligned with BLAST nr/nt database and are included in Appendix 10.3.

4.2.5 Protein expression and purification

Protein expression of the cloned GBS genes was induced and the solubility of the various proteins accessed for optimal purification conditions. Proteins that formed inclusion bodies were denatured with phosphate buffer containing 8 M urea to re-solubilise them and were subsequently desalted to their native conformation after purification. Chromatographic analysis of the purified proteins was performed at 254 nm wavelength and visualised by SDS-PAGE analysis, Figure 4.4 (corresponding data for the remaining proteins is shown in Appendix 10.4, Figure 10.3). High molecular weight proteins gbs0393 and gbs1356 were partially degraded during purification, Figure 4.4C. Table 4.3 summarises the extracellular localisation and immunogenicity features of the recombinant proteins produced for immunological testing. The immunological assay comprised of three lipoproteins; Foldases-PrsA, gbs0233 and gbs2106, and cell-wall anchored proteins FbsA, BibA, gbs0393, gbs1356, gbs1539, gbs0392 together with extracellular secreted protein Sip.

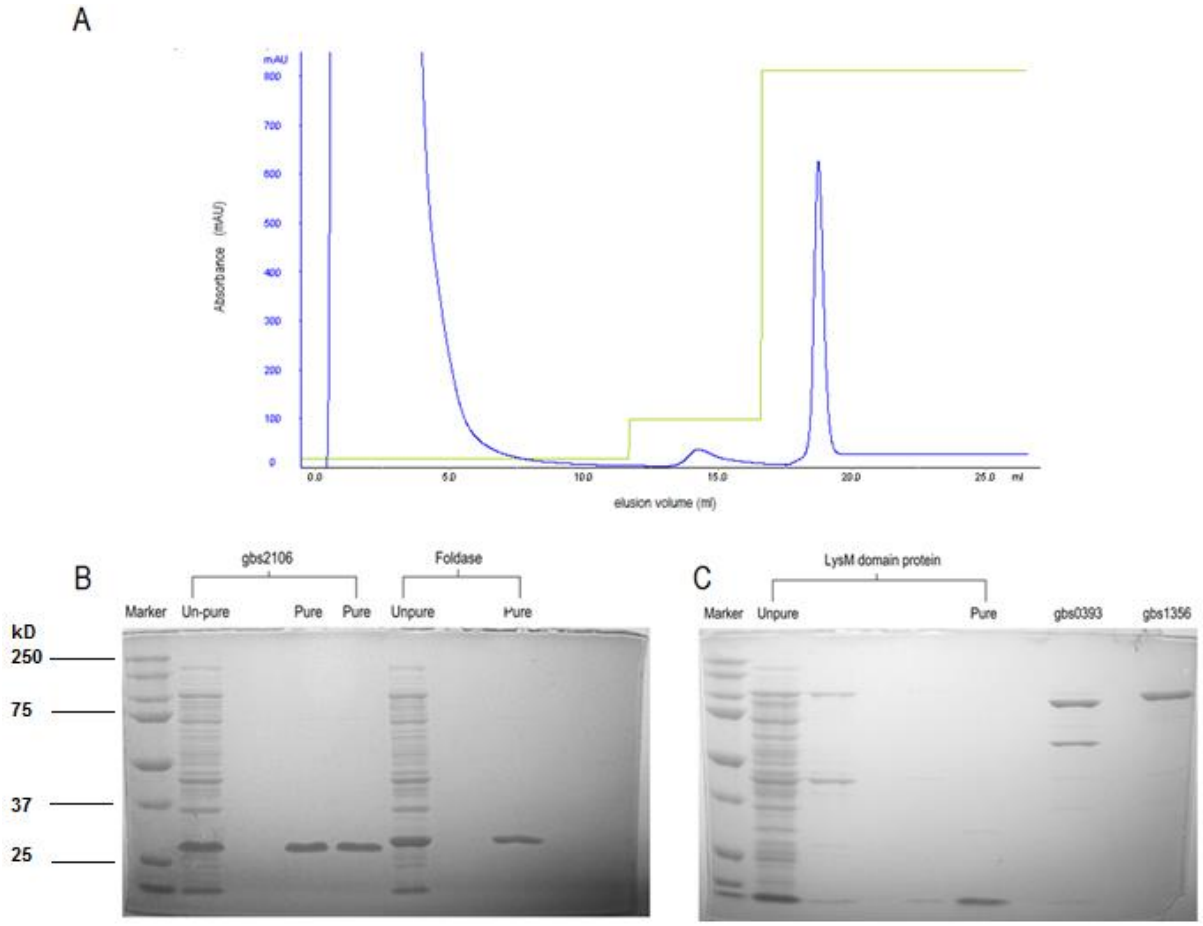

Figure 4.4 Purified recombinant group B Streptococcus proteins; A, chromatographic analysis of eluted gbs2106 protein, with blue line showing UV absorption at 254 nm and green line showing concentration of imidazole; B, SDS-PAGE visualisation of gbs2106 and foldase PrsA protein; C, LysM protein, gbs0393 and gbs1356 SDS-PAGE visualisation.

Table 4.3 Subcellular localisation characteristics and putative functions of group B Streptococcus surface proteins.

		Protein surface features							Putative function/pathogenicity
	Protein name	**Recombinant peptide Length (aa)**	**SignalP**	**TargetP**	**TMHMM**	**pSORT**	**LipoP**	**BepiPred score**	
Lipoproteins	Foldase PsrA protein (gbs0827)	272	Yes	Secreted	0	-	LXXC	0.33	Peptidyl-prolyl cis-trans isomerase activity
	Gbs0233	308	No	Secreted	0	-	LXXC	-0.03	Lipoprotein, ABC transporter
	Gbs2106	189	Yes	Secreted	1	-	LXXC	0.57	Unknown function, Hypothetical lipoprotein
Cell wall anchored proteins	FbsA (gbs1087)	340	Yes	Secreted	0	LPXTG	-	0.55	Induces fibrinogen aggregation, Adhesn
	BibA-COH1 (SAN2207)	460	Yes	Cytoplasmic membrane	1	LPXTG	-	0.45	Complement inhibitor, Adhesion
	BibA-1(gbs2018)	300	Yes	Cytoplasmic membrane	1	LPXTG	-	0.45	Complement inhibitor, Adhesion

Gbs0393	834	Yes	Secreted	2	LPXTG	-	0.44	Adhesin, surface antigen protein
Gbs1356	846	Yes	Cytoplasmic membrane	2	LPXTG	-	0.40	Adhesin, Agglutinin receptor
Gbs1539	156	Yes	Secreted	2	LPXTG	-	0.97	Unknown function
Gbs0392	119	Yes	Secreted	1	LPXTG	-	0.89	Unknown funtion
Sip (gbs0031)	414	Yes	Secreted	0	LysM	-	0.57	Immunogenic protein

4.3 Discussion

The pan-genome of GBS has a minimum of 1806 genes encoding proteins (197). Maione et al. identified 589 genes encoding surface proteins present in 8 GBS strains of serotype Ia, Ib, II, II and V of which 396 were conserved (194). Using a representative genome sequence of GBS serotype III NEM316 strain and a combination of available bioinformatics tools, putatively immunogenic surface proteins were selected and produced using recombinant techniques. The recombinant proteins included cell wall anchored proteins Sip, gbs0392, gbs0393, gbs1356 and gbs1539 and lipoproteins Foldase and gbs2106. The selected proteins were supplemented with recombinant proteins FbsA, BibA and gbs0233 provided by Valneva Gmb (Austria).

Sip is a highly conserved protein that is extracellular localised on the surface of GBS using the LysM surface anchor motif (94,210). *In vitro* expression of Sip is detectable at all growth stages suggesting that Sip is expressed throughout the life cycle of the bacteria. The immunogenicity of Sip has been extensively characterised and has been shown to be capable of conferring protection in mice pups born to immunised dams (94,211). Immunisation with Sip protein induces both systemic and mucosal immunity in the vaginal and rectal tract (212).

The genome sequence of GBS NEM316 strains consist of 14 genetic islands of which four are true pathogenicity islands, comprised of genes encoding virulence surface proteins (213). Gbs0392 and gbs0393 are syntenic genes localised in genetic island that has been triplicated within the GBS NEM316 genome and annotated as chromosomal integrated plasmid (204,213). Gbs1356 is dimeric protein located in a true pathogenicity island that includes genes that encode known virulence proteins such as Lmb and C5a

peptidase (213). Subtractive DNA hybridisation revealed that gbs0393 and gbs1356 homologous to a family of adhesion proteins (214). Furthermore gbs0393 and gbs1356 were identified as highly immunogenic peptides when using cervical secretion and serum samples from pregnant women screened against bacteriophage displaying recombinant GBS proteins (96).

Gbs1539 is a serine rich protein with repeating units flanked by either lysine or glutamine residues. Serine rich GBS proteins have been reported to mediate virulence in GBS strains (215). Moreover, analysis of differential transcription of GBS gene at different growth phases revealed that transcription of gbs1539 increases by >4-fold during transition from mid-log to stationary phase indicative of virulence capacity (216). Gbs0233 and Gbs2106 are highly conserved lipoprotein consisting of the LxxC motif for surface localisation. The proteins were among the most immuno-reactive peptides identified by Meinke et al., with gbs0233 confer protection against GBS to 40% of immunised mice (96).

FbsA was identified as a cell wall anchored protein comprised of 16 amino acid repeating units that interact with host fibrinogen essential for bacterial adhesion (128,217). *In silico* analysis of GBS genomes identified BibA protein, which is a surface exposed adhesion protein capable of binging human C4bp (complement 4 binding protein), thus inhibiting opsonophagocytosis (141). Three chimeric forms of BibA proteins have been characterised with strains belonging to the hyper-virulence ST-17 expressing a phylogenetically distant BibA protein (51). Heterogeneous peptide fragments of BibA from GBS strain COH1 (ST-17) and strain NEM316 (ST-23) were included in this study analysis.

Highly immunogenic GBS surface proteins were selected using robust computational algorithms. The proteins were varied according to extracellular localisation and heterogeneity among GBS strains. Using serological assays, the subsequent chapters discusses the immunogenicity of the recombinant peptides to determine their role possible GBS vaccine candidates.

Chapter Five

Natural acquired Group B *Streptococcus* capsular polysaccharide and surface protein antibodies in HIV-infected and HIV-uninfected children

5.1. Introduction

Cases of invasive GBS disease occurring in children > 3 months of age have been reported. The majority of these cases were reported during the pre-IAP era, where the prevalence of invasive GBS disease was 4% amongst children between 1-17 years of age (22). Table 5.1 list studies that reported the occurrence of invasive GBS disease beyond infancy. These cases occur comorbid with underlying conditions associated with immune-suppression including HIV infection. The commonest clinical manifestations were pneumonia, bacteraemia with no focus and UTI.

The prominent immuno-pathological progression of HIV infection is a continuous depletion and dysfunction of $CD4^+$ T lymphocytes. Additionally, HIV infection causes hyper activation of B cells, hypergammaglobulinaemia and loss of memory B lymphocytes, hence resulting in a dysfunctional humoral immune response (218). Collectively immuno-pathologies associated with HIV infection increases susceptibility to bacterial infections such as *S. pneumoniae, Haemophilus influenzae* type B and GBS (219). There is, however, a paucity of data regarding the pathogenesis of GBS and the immune-suppressive role of HIV-infection that result in susceptibility to invasive GBS disease beyond infancy.

Table 5.1 Clinical manifestations of invasive group B Streptococcus disease cases occurring in children > 3 months of age with underlying conditions.

Cases	Age	Clinical manifestation	Underlying conditions	Year	Country	Ref
3 infants who progressed to AIDS had positive GBS growth cultures from blood and CSF	3.5-5 month	Pneumonia, Frontal CSF space enlargement and seizures	HIV infection	1983-1985	USA	(220)
18 children with GBS isolates from sterile sites	15 weeks-18 years	Bacteraemia, meningitis, endocarditis and central venous catheter infection	Prematurity, hypergammaglobulinaemia, diabetes mellitus, HIV and congenital heart disease	1986-1993	USA	(221)
GBS isolated from a normally sterile sites (CSF and blood) were detected in 11 children	3-31 months	Respiratory distress and bacteraemia	Severe malnutrition and HIV infection	1997-1999	South Africa	(222)
24 children, a median age of 8.5 years with GBS recovered from infectious sites	15 weeks-12 years	UTI, acute tonsillitis, sepsis and soft tissue infection	Diabetes mellitus, vesicoureteral reflux and autoimmune disease	1991-2002	Taiwan	(223)
223 cases of children presenting with invasive GBS disease	3 months-14 years	Bacteraemia, meningitis, pneumonia, septic arthritis and peritonitis	Neurologic disorders. Immunosuppressive conditions, asthma, malignancy and renal disease	1999-2005	USA	(23)

Given that the GBS surface constitutes primarily of two types of antigens; polysaccharide encapsulating the bacteria that trigger a T lymphocyte independent immune response, and proteins embedded within the CPS that require secondary activating signals from $CD4^+$ T lymphocytes for production of antibodies by B cells. The aim of this investigation was to determine whether differences in IgG titres to GBS CPS and surface protein between HIV-uninfected and HIV-infected children may possibly contribute to increased susceptibility to invasive GBS disease. This study has been published in the journal *Vaccine;* Appendix section 10.7.

5.2. Study population

The immunogenicity of the selected GBS surface proteins together with that of CPS of serotype Ia, Ib, III and V was assessed in sera collected from 77 HIV-uninfected and 68 HIV-infected children between the age 4-7 years. These were archived samples from children who were enrolled in a previous study that was aimed at assessing the persistence of the pneumococcal conjugate vaccine (PCV) induced antibodies (224). Permission to use these samples without further parental consent was granted by the Human Research Ethics Committee of the University of Witwatersrand (Approval number: M121019).

5.3. Statistical analysis

The data was analysed using STATA software (version 13.0 Stata-Corp, Tx USA) and GraphPad Prism (version 5.03 GraphPad Software Inc, California USA). The IgG titres obtained were non-symmetric and therefore were $\log_{10}$ transformed for statistical comparisons. Statistical differences in IgG geometric mean concentration (GMC, µg/mL) for CPS or IgG geometric mean titres (GMT, U/mL) for proteins between HIV-

uninfected and HIV-infected children were measured using two tailed *Student's* t-test. Among HIV-infected children differences in IgG titres was compared between those who had $CD4^+$ lymphocyte count <500 cells/µL and those who had $CD4^+$ lymphocyte count ≥500 cells/µL using Mann-Whitney *U* test. A more conservative p value ≤ 0.01 was considered significant to allow for multiple comparisons in lieu of undertaking any correction for multiple comparisons.

5.4. Results

Demographic information

The demographic profiles of the HIV-infected and HIV-uninfected children were similar for gender and age (3.9 - 7.1 years), Table 5.2. This comprised of 55.9% males of which 41 were HIV-uninfected (median age=5.1 years) and 40 were HIV-infected (median age=5.2 years), and 44.1% females; 36 HIV-uninfected (median age=5 years) and 28 HIV-infected (median age 5.9 years). $CD4^+$ lymphocyte count data was available for 57 of the HIV-infected children with a median of 566 cells/µl (range 0-1897 cells/µl), including 30 (52%) with CD4+ count ≥ 500 cells/µl and 27 (47%) <500 cells/µl. Of the HIV infected children 12 (21%, 6 males and 6 females) were on antiretroviral (ARV) treatment, that comprised of two nucleoside reverse transcriptase inhibitors and one non-nucleoside reverse transcriptase inhibitor. The median $CD4^+$ count of the HIV-infected children receiving ARV treatment was 442 cells/µL (range 255-1094 cells/µL).

Table 5.2 Demographic features of HIV-infected and HIV-uninfected children between the age of 4-7 years evaluated for the prevalence of IgG anti-GBS surface antigens.

Demographic feature	**HIV-infected (n=68)**	**HIV-uninfected (n= 77)**
age years, median (range)	5.7 (3.9-7.1)	5.2 (3.9-6.3)
male	40 (58.8%)	41 (53.2%)
CD4 count cell/µL, median (range)	566 (0-1897)	
<500 cells/ µL, median (range)	351 (0-497)	
≥500 cells/ µL, median (range)	794 (505-1897)	
HIV viral load copies/mL, median (range)	37500 (25-460000)	
HAART-	56 (82.4%)	
HAART+*	12 (17.6%)	

*CD4 cell count information for four of the children receiving ARV treatment was not available

IgG antibodies against GBS antigens

The differences in antibody concentrations/titres between HIV-infected and HIV-uninfected children were epitope dependent. IgG GMC (µg/ml) against CPS were lower in HIV-infected compared to HIV-uninfected children particularly for serotype Ib (0.047 vs 0.076, p=0.01) and V (1.95 vs 2.97, p = 0.005), Table 5.3. In contrast, GMTs were higher against the majority of the protein epitopes in HIV-infected compared to

HIV-uninfected children, including the lipoprotein Foldase PsrA, and the cell wall anchored proteins FbsA, Gbs1356, Gbs0393, Gbs1539 and Gbs0392 ($p < 0.005$), Figure 5.1. Titres against the lipoprotein Gbs2106, and the lysM domain cell-wall anchored protein Sip were, however, significantly higher in HIV-uninfected compared to HIV-infected children ($p < 0.001$; Figure 5.2).

Table 5.3 IgG geometric mean concentration (GMC, µg/ml) of group B Streptococcus capsular polysaccharides in HIV-uninfected and HIV-infected children between the age of 4-7 years.

	Antigen	HIV-uninfected GMC (95% CI)	HIV-infected GMC (95% CI)	p value
Capsular polysaccharides	Ia	0.078 (0.066-0.093)	0.061 (0.047-0.079)	0.1048
	Ib	0.755 (0.62-0.92)	0.472 (0.34-0.65)	0.0119
	III	0.439 (0.38-0.51)	0.375 (0.32-0.44)	0.1541
	V	2.971 (2.49-3.55)	1.952 (1.54-2.47)	0.0045

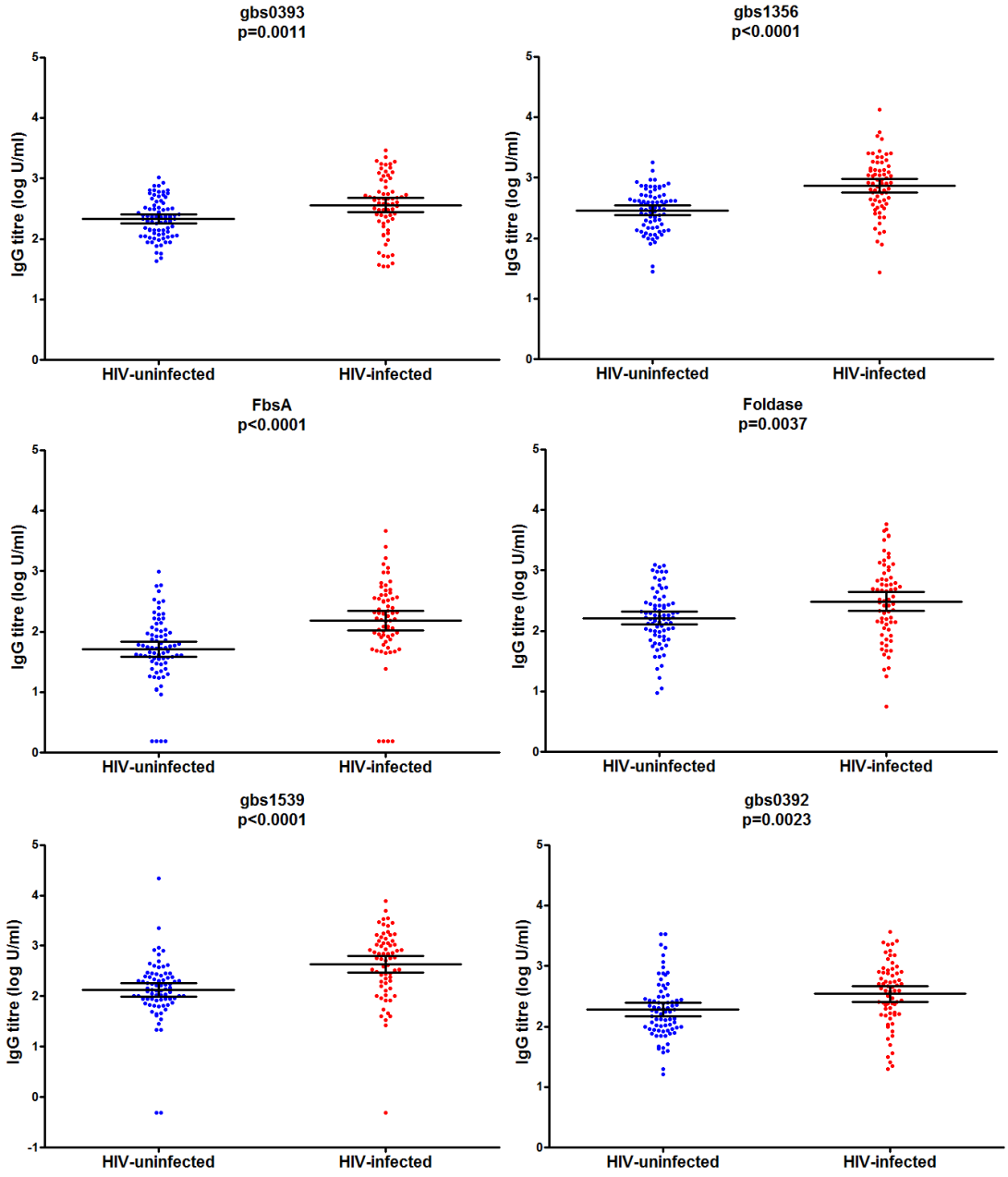

Figure 5.1 IgG geometric mean titre (log U/ml) of group B Streptococcus of GBS surface protein surface proteins between HIV-uninfected and HIV-infected children between the age of 4-7 years.

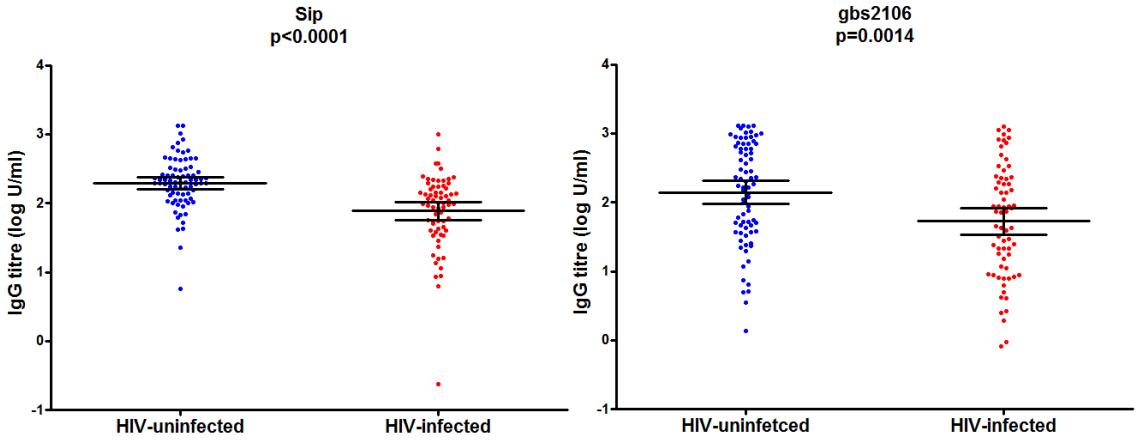

Figure 5.2 IgG geometric mean titre (log U/ml) of group B Streptococcus of GBS surface protein surface immunogenic protein (Sip) and gbs21016 between HIV-uninfected and HIV-infected children between the ages of 4-7 years.

Multivariate regression of IgG GMC specific to GBS CPS with IgG GMT specific to surface proteins was performed. Statistically significant positive correlation was observed between IgG levels of CPS and that of surface proteins with the exception of Sip, FbsA and gbs1356, Table 5.4. Further evaluation of the IgG level in HIV-infected children revealed that IgG titres were higher in children who had $CD4^+$ lymphocyte count <500 cell/µL compared to those with $CD4^+$ lymphocyte count ≥500 cell/µL for proteins Foldase, gbs1356, gbs1539, gbs0233 and gbs0393, Figure 5.3.

Table 5.4 Multiple linear regression of the geometric mean concentration (GMC, µg/ml) of group B Streptococcus capsular polysaccharides and the geometric mean titres (GMT, U/ml) of surface proteins among HIV-uninfected and HIV-infected children between the age of 4-7 years.

	Ia		Ib		III		V	
	HIV-Uninfected	HIV-Infected	HIV-Uninfected	HIV-Infected	HIV-Uninfected	HIV-Infected	HIV-Uninfected	HIV-Infected
	Coeff (p value)		Coeff (p value)		Coeff (p value)		Coeff (p value)	
FbsA	0.16 (0.423)	0.10 (0.604)	0.08 (0.644)	0.12 (0.410)	0.23 (0.307)	0.50 (0.076)	0.20 (0.307)	0.14 (0.465)
gbs0233	0.14 (0.399)	0.35 (0.006)	0.22 (0.120)	0.26 (0.011)	0.05 (0.796)	0.67 (0.001)	0.23 (0.151)	0.41 (0.003)
BibACOH1	0.13 (0.329)	0.30 (0.005)	0.09 (0.431)	0.18 (0.043)	0.02 (0.878)	0.46 (0.006)	0.04 (0.784)	0.36 (0.002)
BibANEM	0.51 (0.009)	0.44 (0.001)	0.11 (0.542)	0.30 (0.004)	-0.03 (0.891)	0.79 (<0.001)	0.19 (0.340)	0.55 (<0.001)
gbs2106	0.44 (0.082)	0.64 (0.003)	0.34 (0.133)	0.48 (0.004)	0.27 (0.351)	1.21 (<0.001)	0.13 (0.607)	0.81 (<0.001)
gbs0393	0.09 (0.443)	0.21 (0.101)	0.17 (0.083)	0.19 (0.066)	0.17 (0.180)	0.49 (0.014)	0.19 (0.093)	0.34 (0.016)
Sip	0.10 (0.475)	0.17 (0.262)	0.20 (0.094)	0.11 (0.344)	0.05 (0.758)	0.33 (0.144)	0.13 (0.338)	0.13 (0.423)
Foldase	0.25 (0.132)	0.29 (0.090)	0.17 (0.246)	0.32 (0.018)	0.18 (0.334)	0.72 (0.006)	0.31 (0.057)	0.49 (0.007)
Gbs1539	0.18 (0.390)	0.24 (0.183)	0.14 (0.441)	0.19 (0.193)	0.10 (0.692)	0.61 (0.032)	0.18 (0.389)	0.35 (0.073)
gbs1356	0.29 (0.015)	0.21 (0.104)	0.28 (0.007)	0.15 (0.142)	0.27 (0.054)	0.32 (0.103)	0.29 (0.014)	0.23 (0.096)
gbs0392	0.36 (0.026)	0.44 (0.001)	0.24 (0.091)	0.33 (0.002)	0.43 (0.021)	0.78 (<0.001)	0.30 (0.066)	0.54 (<0.001)

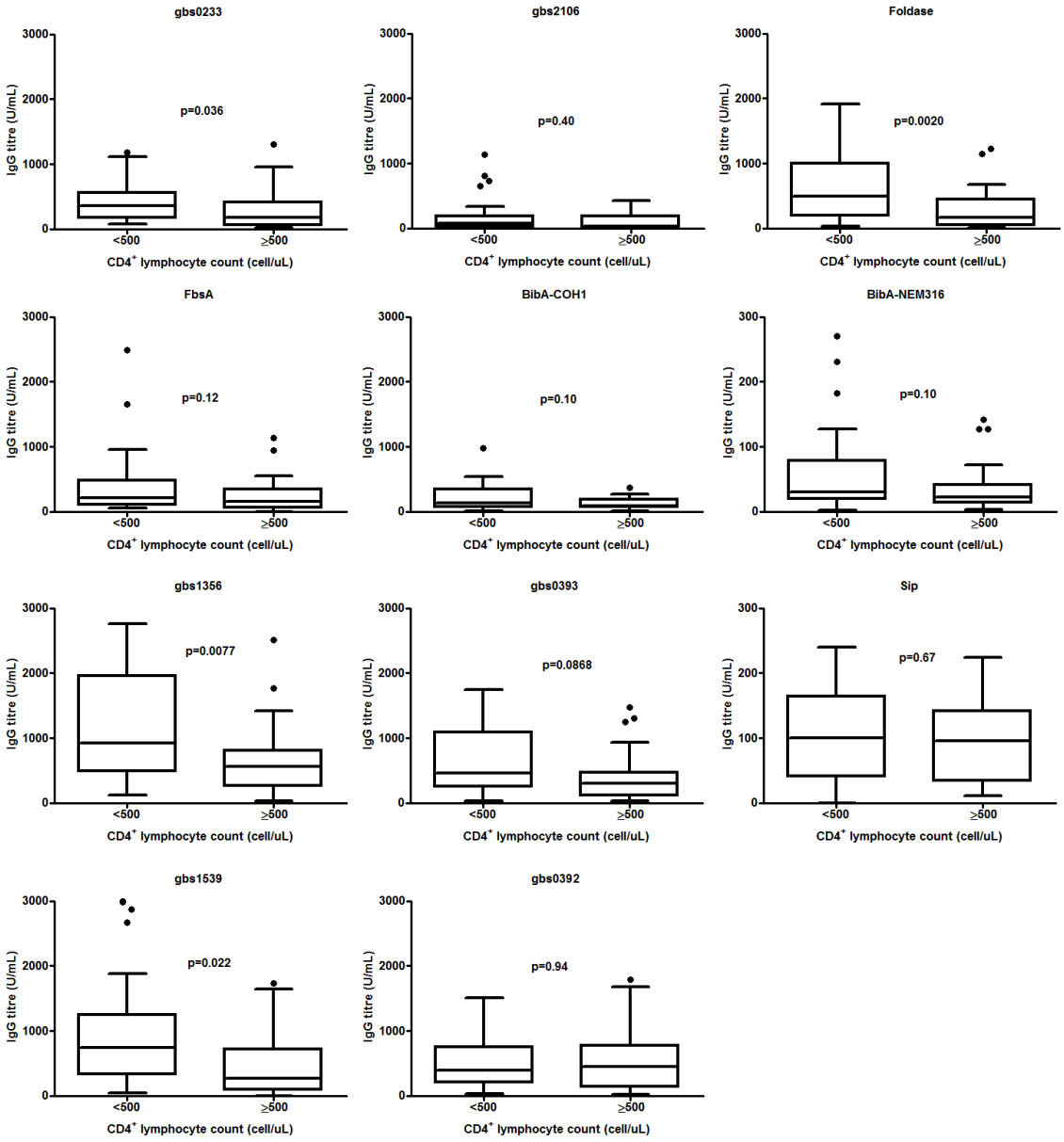

Figure 5.3 IgG antibody titre in HIV-infected children, comparing the difference between those with $CD4^+$ lymphocyte count < 500 cells/µL and those with $CD4^+$ lymphocyte count ≥ 500 cell/µl.

5.5. Discussion

Low levels of circulating antibodies to GBS polysaccharides has been associated with increased risk of developing invasive GBS disease in infants (9,11,12). Increased bacterial infection, inclusive of GBS, occurs in HIV-infected individuals due to immunopathologies associated with HIV infection (225).The increased susceptibility to invasive GBS disease in HIV-infected children is suggested to be a consequence of inadequate humoral immune responses to capsular polysaccharide (105,106). This was particularly evident for serotypes Ia and V, in which their respective IgG concentrations were shown to be lower among HIV-infected children. Also, we identified lower IgG titres to Sip and gbs2106 proteins. Sip has been identified as virulence factors in animal model studies (94), but for which an association to invasive disease in humans has not been established. Nonetheless, IgG titres against GBS surface antigens in both HIV-infected and HIV-uninfected children shows that humoral immune response occurred to these epitopes, likely due to GBS colonisation during childhood (226).

HIV infection causes immuno-pathogenesis associated with dysregulated and dysfunctional $CD4^+$ lymphocytes, in particular, the hyper-activation of B cells and hypergammaglobulinaemia (218). The proper immunological response to individual antigens may be affected by HIV infection depending on the mechanism and pathway of antigen recognition by the immune system. Since protein antigens require activating signals from T lymphocytes to activate B cells (152), the higher protein antibody titres in HIV-infected compared to HIV-uninfected children to most surface proteins may be a result of an aberrant response, due to hypergammaglobulinaemia that commonly occurs with HIV infection and frequently results in high antibody titres of lower functionality (227,228). This is further corroborated by the inverse relationship between

antibody titres to protein antigens and $CD4^+$ lymphocyte counts in HIV-infected children. Similar to these findings, serum IgG against pneumococcal surface proteins in HIV-infected adults were also found to be elevated compared to HIV-uninfected adults (229).

The frequency of GBS colonisation remains high beyond infancy, although often occurs asymptomatically, however similar serotypes are isolated in both invasive cases and in healthy colonised individuals (221,226,230). The frequent colonising serotypes in this population has been reported to be Ia, Ib, II, III and V (106,221). Factors influencing GBS acquisition and/or transition to invasive disease are poorly understood. GBS colonisation information among the study participants was not available, which presented a limitation in our analysis, albeit increased invasive disease was reported amongst HIV-infected and HIV-exposed uninfected infants (21,101) and also in immunocompromised children (220,221,223,225).

Responses to the evaluated GBS proteins differed based on the localisation of the protein on the surface of the bacteria (lipoprotein or cell wall anchored), and also according to their function as indicated by shared homology of proteins with known function. There was no clear relationship to HIV-status between the presumed function of the protein or in relation to whether it was mapped to an extra cellular location. This was in contrast to that observed for pneumococcal protein antigens in the same study population, in which a selection of membrane and cell-wall associated pneumococcal antigens, had a distinct dichotomy with respect to HIV status, where the HIV-infected group had high titres associated with cell membrane anchored proteins and low titres to cell-wall anchored proteins and vice versa (231). The author's hypothesised that these differences could have been due to some cross reactive epitopes from other bacteria in

HIV infected individuals due to increased gut microbial translocation in this population. Similarly, increased cross-reactive epitopes in HIV-infected children may also be responsible for the higher GMTs observed in this study despite the lack of an apparent dichotomy.

The study limitations included the use of Polygam as a reference serum, which is pooled IgG from human adults and thus may result in an overestimation of IgG titres with cross-reactive antibodies induced by other species, especially for antigens that had low specificity. However, we conceived that since the assay was performed consistently throughout, any cross-reactive antibodies detected would result in a proportional increase and therefore would not greatly affect the difference between the study groups compared. Furthermore, some of the study participants received PCV vaccine and/or were colonised with *S. pneumoniae,* albeit no cross-reactivity was evaluated between GBS surface antigens and pneumococcal specific antibodies. However, it is noteworthy that GBS specific IgG levels were not associated with PCV vaccination status or colonisation with *S. pneumoniae* in the respective children.

The process of reverse vaccinology in vaccine design and development has been used successfully in identifying essential virulence factors of *N. meningitidis* and *S. agalactiae* which are conserved amongst several serotypes thus making them possible universal vaccines (193,194). Using reverse vaccinology, we were able to identify novel immunogenic proteins localised on the surface of GBS. This study revealed that natural antibody responses to GBS surface antigens are produced post-infancy, irrespective of HIV status. However, differences in the antigen specific titres should be investigated as a possible cause of a dysfunctional immune response or increased GBS colonisation in HIV infected children. Furthermore, characterisation of antibody avidity and

functionality should be evaluated to determine if the risk of invasive GBS diseases beyond infancy is a factor of immunosuppression resulting from a dysfunctional and dysregulated immune system. Should the attenuated response to these epitopes, particularly serotype-specific CPS and select GBS surface proteins, be responsible for the increased risk in susceptibility to GBS in severely immunocompromised HIV infected individuals, these epitopes may have potential as future vaccine candidates (16,174,176,177).

Chapter Six

Natural occurring antibodies against group B *Streptococcus* surface proteins in pregnant women

6.1 Introduction

Colonisation of the recto-vaginal tract of pregnant women with GBS occurs in an intermittent, transient and persistent manner during pregnancy (58,73). Asymptomatic colonisation of the gastrointestinal site has been shown to be a requisite for GBS colonisation in the genitourinary tract (232). GBS colonisation in the genitourinary of women is a predisposition to acute symptomatic infection including chorioamnionitis, endometritis and urinary tract infections (42). GBS colonisation of the genitourinary tract during pregnancy is also a key predisposing factor for developing EOD, with a high case fatality ratio 9.6% (2). Moreover, GBS colonisation during pregnancy is associated with prematurity, late miscarriages and stillbirths (233).

Maternal immunisation during pregnancy has the potential to reduce GBS-related stillbirths or prematurity and also EOD and LOD in their young infants (234). Natural induced maternal α-C protein antibodies are capable of opsonophagocytic killing (19), which is associated with a lower prevalence of GBS colonisation among pregnant women. Therefore, a vaccine formulation which includes highly immunogenic GBS surface proteins capable of preventing GBS recto-vaginal colonisation, could contribute to reducing the risk of GBS acquisition in women, and consequently reduce vertical transmission of GBS to their foetus and newborns. This would ultimately reduce the risk of GBS associated morbidity and mortality, including that affecting the mother, foetus and the young infants.

This chapter investigated natural immune responses to 11 GBS surface proteins and the association thereof to GBS recto-vaginal acquisition and/or clearance of colonisation.

6.2 Study population

We retrospectively investigated serum samples from pregnant women who were previously enrolled in a study that determined the association between serotype-specific capsular antibody and GBS recto-vaginal colonisation in pregnant women (58). Briefly, the study cohort consisted of 661 asymptomatic pregnant women attending routine antenatal clinic visits in Soweto, South Africa during the period of August 2010 to August 2011 who were enrolled at 20 to 37+ weeks of gestational age. The women were confirmed to be HIV-uninfected and not on antibiotic treatment for at least two weeks prior to enrolment. The study population was documented to have a GBS acquisition rate of 27.9% from 20 to 37+ weeks of gestational age, with 49.6% of the women being colonised at least on one occasion during the study period. (58).

GBS colonisation was determined at 20-25, 26-30, 31-35, and at 37+ week of gestation by culturing lower vaginal and rectal swabs in chromagenic agar and subsequenctly evaluated for the CAMP factor, catalase activity, hydrolysis of esculin and group B antigen (58). A participant with GBS detected at either the vaginal and/or rectal swab was considered as colonised; and non-colonised if no growth was detected from either swab. In addition, sera were available from the 20-25 weeks and 37+ weeks visits and was stored at -70^{o}C until assayed for the quantification of naturally induced IgG titres specific to GBS surface proteins.

Paticipants were categorised according to previously determined colonisation status between visits as follows: women whom GBS could not be detected throughout the study visits were grouped as non-carriers, women who were not colonised at visit 1 but colonised at subsequent visits were grouped as “new-acquisition” cases. Furthermore,

women who were colonised with a specific GBS serotype at all study visits were denoted as “persistently colonised” and women who tested positive for GBS at visit 1 (20-25 weeks of gestation age) but had negative culture results at any stage up until the 37+ weeks gestation age visit were grouped as “transiently colonised”. Pregnant women that were only colonised at either the 2nd or 3rd visit were grouped as “intermittently colonised”.

6.3 Statistical analysis

Antibody titres remained skewed after $\log_{10}$ transformation and therefore median IgG antibody titres are reported. Statistical analysis was performed using STATA software (version 13.0 Stata-Corp, Tx USA) and GraphPad Prism (version 5.03 GraphPad Software Inc, California USA). Difference in IgG titres between groups was determined using Mann Whitney *U* test. Spearman’s rank test regression was performed to determine correlation of protein specific IgG titres with maternal age, gestational age, parity and gravidity. Association between colonising serotype and protein specific IgG titres was assessed using Kruskal-Wallis test. Multivariate quantile regression was used to correct for confounding variables with p value <0.2 when analysed as univariates. χ^2 and Fisher’s exact test were used to determine association between groups. Differences were considered significant for p-value <0.05.

6.4 Results

Association between IgG titres and concurrent GBS colonisation

Among the 661 pregnant women enrolled in the study, 505 (76.1%) pregnant women attended all four study visits and were included in the analysis. The main reason for non-inclusion of the other women were relocation, withdrawal of consent or lost to

follow up. The demographic characteristics of the study cohort are described elsewhere (58). Overall, GBS colonisation was detected in 161/505 (31.9%) women at 20-25 weeks and in 140/505 (27.7%) of gestation as detailed in Table 6.1. The predominant colonising serotypes were type-Ia and III, Table 6.1 (58).

The maternal age of the women was positively correlated with IgG titres to gbs0233 (p=0.033), foldase (p=0.02), gbs0393 (p=0.037) and Sip (p=0.03) detected in sera collected at 20-25 weeks of gestation age. In sera collected at 20-25 weeks of gestation age, gestational age was inversely correlated with IgG titres specific to FbsA (p=0.041) and BibA-NEM316 (p=0.02), whilst at ≥37 weeks of gestation age, an inverse association was detected between gestational age and IgG titres against FbsA (p=0.033). The women's parity was positively correlated with antibodies against Foldase (p=0.045), gbs0393 (p=0.034) and gbs0392 (p=0.048) at 20-25 weeks of gestation age, and in sera collected at ≥37 weeks of gestation, maternal parity was correlated to FbsA specific IgG titres (p=0.039). The women's number of pregnancies (gravidity) was also positively correlated with IgG titres against gbs0393 (p=0.009) and Sip (p=0.039) detected in sera collected at 20-25 weeks of gestation age. Protein specific IgG titres were not dependent on the colonising serotype at both 20-25 and ≥37 weeks of gestation age.

Table 6.1 Demographic characteristics of pregnant women who were either non-colonised or colonised with group B Streptococcus at 20-25 and ≥37 weeks of gestation.

Demographic	20-25 week of gestation		≥37 week of gestation	
	Non-colonised (n=344)	Colonised (n=161)	Non-colonised (n=365)	Colonised (n=140)
Median age, year (IQR)	25.4 (21.7-29.4)	25.5 (22.2-29.7)	25.2 (21.7-29.3)	25.6 (22.2-29.6)
Median parity (range)	0 (0-5)	1 (0-4)	0 (0-5)	1 (0-4)
Median gravidity (range)	2 (1-8)	2 (1-6)	2 (1-8)	2 (1-6)
Miscarriage(s)				
0	293 (85.2%)	128 (79.5%)	304 (82.3%)	117 (83.6%)
1	40 (11.6%)	30 (18.6%)	50 (13.7%)	20 (14.3%)
≥2	11 (3.2%)	3 (1.9%)	11 (3.0%)	3 (2.1%)
Colonisation site				
Rectal[†]		48 (29.8%)		46 (32.9%)
Vaginal[‡]		44 (27.3%)		29 (20.7%)
Recto-vaginal		69 (42.9%)		65 (46.4%)
Serotype				
Ia		71 (44.1%)		50 (35.7%)
Ib		7 (4.3%)		7 (5.0%)

II	13 (8.1%)	11 (7.9%)
III	52 (32.3%)	49 (35.0%)
IV	4 (2.5%)	2 (1.4%)
V	13 (8.1%)	17 (12.1%)
IX	1 (0.6%)	4 (2.9%)

† rectal colonisation without concomitant vaginal colonisation, ‡ vaginal colonisation without concomitant rectal colonisation.

Cross-sectional analysis at 20-25 weeks of gestation showed that median IgG titres (U/mL) for BibA protein of GBS NEM316 strain were higher in colonised (398.11) compared to non-colonised women; (295.12; p=0.03). A similar trend was observed at ≥37 weeks of gestation age with elevated median IgG titres (U/mL) in colonised compared to non-colonised women for BibA-COH1 (309.03 vs 245.47, p=0.01), BibA-NEM316 (380.19 vs 234.42, p=0.03), and Sip (223.87 vs 186.21, p=0.01), Table 6.2. These differences were, however, statistically significant only for BibA-NEM316 (p=0.001 and Sip (p=0.001) after adjusting for cofactor variables.

Table 6.2 Cross-sectional comparison of median IgG antibody titres (U/ml) between non-colonised and colonised pregnant women at 20-25 and ≥37 weeks of gestation age.

		20-25 weeks				37-40 weeks			
		Median IgG (IQR)				Median IgG titre (IQR)			
	Protein	Non-colonised (n=344)	Colonised (n=161)	p-value	Adj p-value[α]	Non-colonised (n=365)	Colonised (n=140)	p-value	Adj p-value[α]
Lipoprotein	Gbs0233	169.82 (91.20-389.05)	208.93 (104.71-371.54)	0.21	0.10	131.83 (81.28-309.03)	138.04 (81.28-234.42)	0.90	0.90
	Gbs2106	263.03 (128.82-524.81)	288.40 (131.83-478.63)	0.92	0.90	204.17 (91.20-398.11)	251.19 (147.91-363.08)	0.17	0.17
	Foldase PsrA	213.80	218.78	0.84	0.66	173.78	194.98	0.72	0.25

		(104.71-489.78)	(117.49-457.09)			(87.10-363.08)	(100.00-380.19)		
Cell wall	FbsA	104.71 (33.88-416.87)	93.33 (34.67-389.05)	0.81	0.80	83.18 (36.31-295.12)	85.11 (38.02-346.74)	0.77	0.97
	BibA-COH1	288.40 (138.04-524.81)	331.13 (169.82-575.44)	0.05	0.57	245.47 (125.89-446.68)	309.03 (165.96-602.56)	0.01	0.24
	BibA-NEM316	295.12 (120.23-630.96)	398.11 (190.55-691.83)	0.03	0.21	234.42 (104.71-478.63)	380.19 (173.78-616.60)	0.03	0.001
	Gbs0393	316.23 (158.49-645.65)	354.81 (194.98-616.60)	0.35	0.62	281.84 (141.25-562.34)	245.47 (128.82-489.78)	0.19	0.10
	Sip	239.88 (120.23-426.58)	257.04 (134.90-407.38)	0.68	0.53	186.21 (87.10-316.23)	223.87 (123.03-354.81)	0.01	0.001

Gbs1539	181.97 (75.86-446.68)	213.80 (89.13-467.74)	0.45	0.37	147.91 (69.18-363.08)	151.36 (75.86-380.19)	0.84	0.77
Gbs1356	269.15 (144.54-478.63)	295.12 (173.78-478.63)	0.55	0.80	213.80 (109.65-389.05)	218.78 (95.50-501.19)	0.66	0.77
Gbs0392	245.47 (107.15-537.03)	269.15 (141.25-501.19)	0.40	0.73	186.21 (95.50-436.52)	213.80 (95.50-380.19)	0.96	0.96

α, p value was adjusted for colonising serotype, maternal age, gestational age, parity and gravity

IgG titres among the pregnant women were further stratified according to the site of GBS colonisation. At 20-25 weeks of gestation age, median IgG titres specific to BibA-NEM316 were significantly higher in women who were colonised in the rectum only (489,78 U/mL) compared to those who were non-colonised (169,82 U/mL, p=0.04 adjusted for cofactor variables), Figure 6.1. Women who had concurrent GBS colonisation in the rectal and vaginal tract at 20-25 weeks of getation age had higher media Sip antibody titres (U/mL) compared to women who were colonised exclusively in the rectum (309,03 vs 204.17, p=0.019), Figure 6.1. IgG titres for the remaining GBS proteins were similar for non-colonised women and those who were GBS colonised at the rectal and/or vaginal tract, Figure 6.1.

At ≥37 weeks of gestation age, median IgG titres were higher in women who were colonised in the vaginal tract only compared to women were non-colonised for proteins FbsA (169.82 vs 83.18, p=0.015), BibA-NEM316 (478.63 vs 234.42, p=0.001), Foldase (269.15 vs 173.78, p=0.034), Sip (275.42 vs 186.21, p=0.023) and gbs1539 (281.84 vs 147.91, p=0.014). After adjusting for cofactor variables these results remained statistically significant for proteins BibA-NEM316 (p=0.006) and gbs1539 (p=0.031), Figure 6.2.

Multivairate quantile regression analysis adjusted for cofactor variables showed that GBS colonisation in vaginal tract alone was associated with higher IgG titres (U/mL) compared to women with only rectal colonisation for proteins Foldase (269.15 vs 91.20, p=0.013), gbs0233(177.83 vs 123.03, p=0.031) and gbs1539 (281.84 vs 114.82, p=0.001), Figure 6.2. Moreover, women with only vaginal colonisation had higher median IgG tires compared to women who were concurrently colonised in the rectal and

vaginal tract for proteins FbsA (169,82 vs 69.18, p=0.021) and BibA-NEM316 (478,63 vs 316,23, p=0.029) after adjusting for cofactor variables, Figure 6.2.

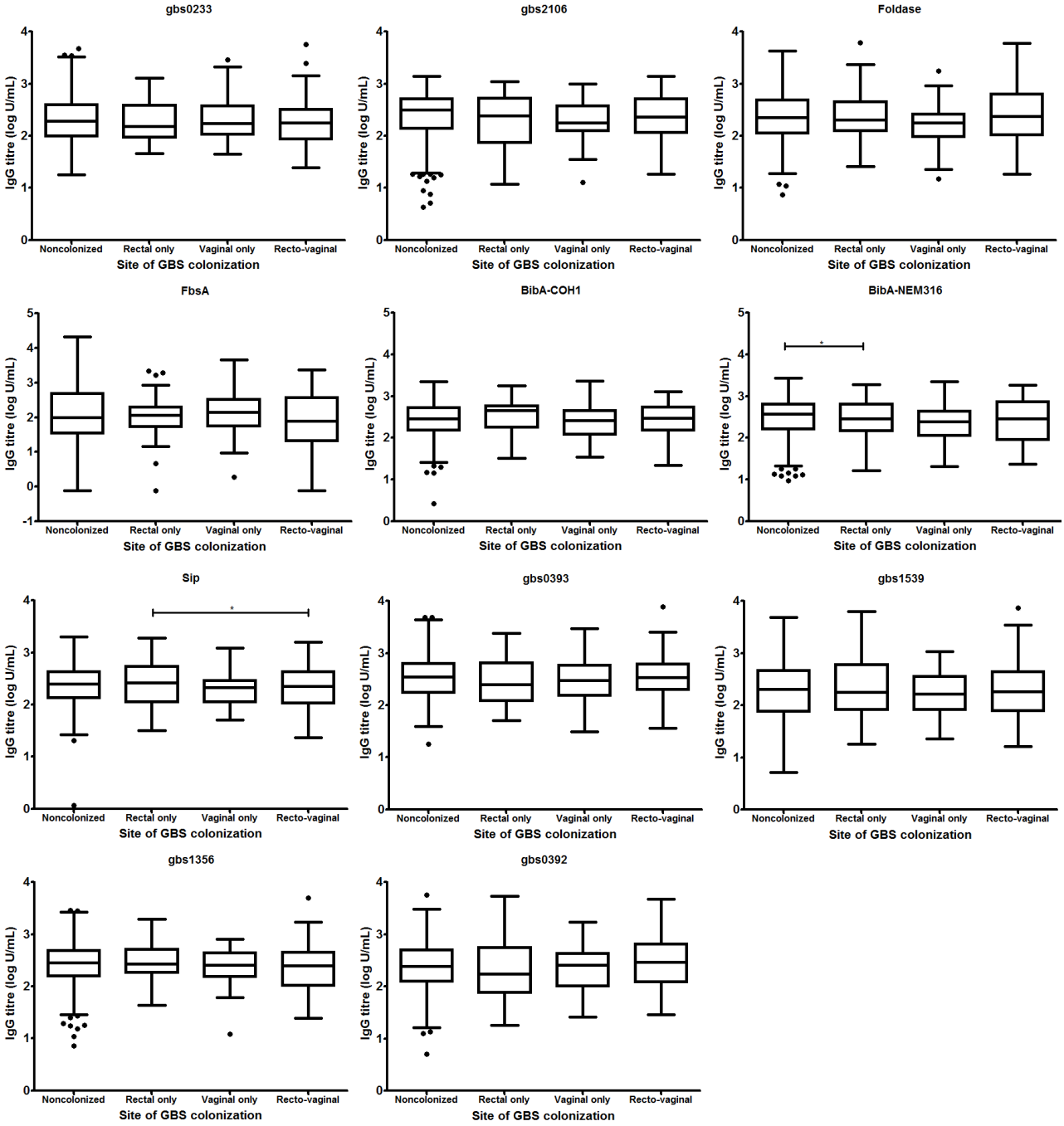

Figure 6.1 IgG titres (log U/mL) against group B Streptococcus surface protein present in pregnant women who were either not colonised or colonised at the rectal and/or vaginal tract at 20-25 weeks of gestation age. Statistical comparison between groups using quantile regression, * $p<0.05$ and** $p<0.01$ adjusted for cofactor variables. Turkey box-and-whisker plots representing the median (line within the box), 25th and 75th percentiles (box), the 1.5 times interquartile distances from the 25th and 75th centiles (whiskers) and outliers (solid dots)

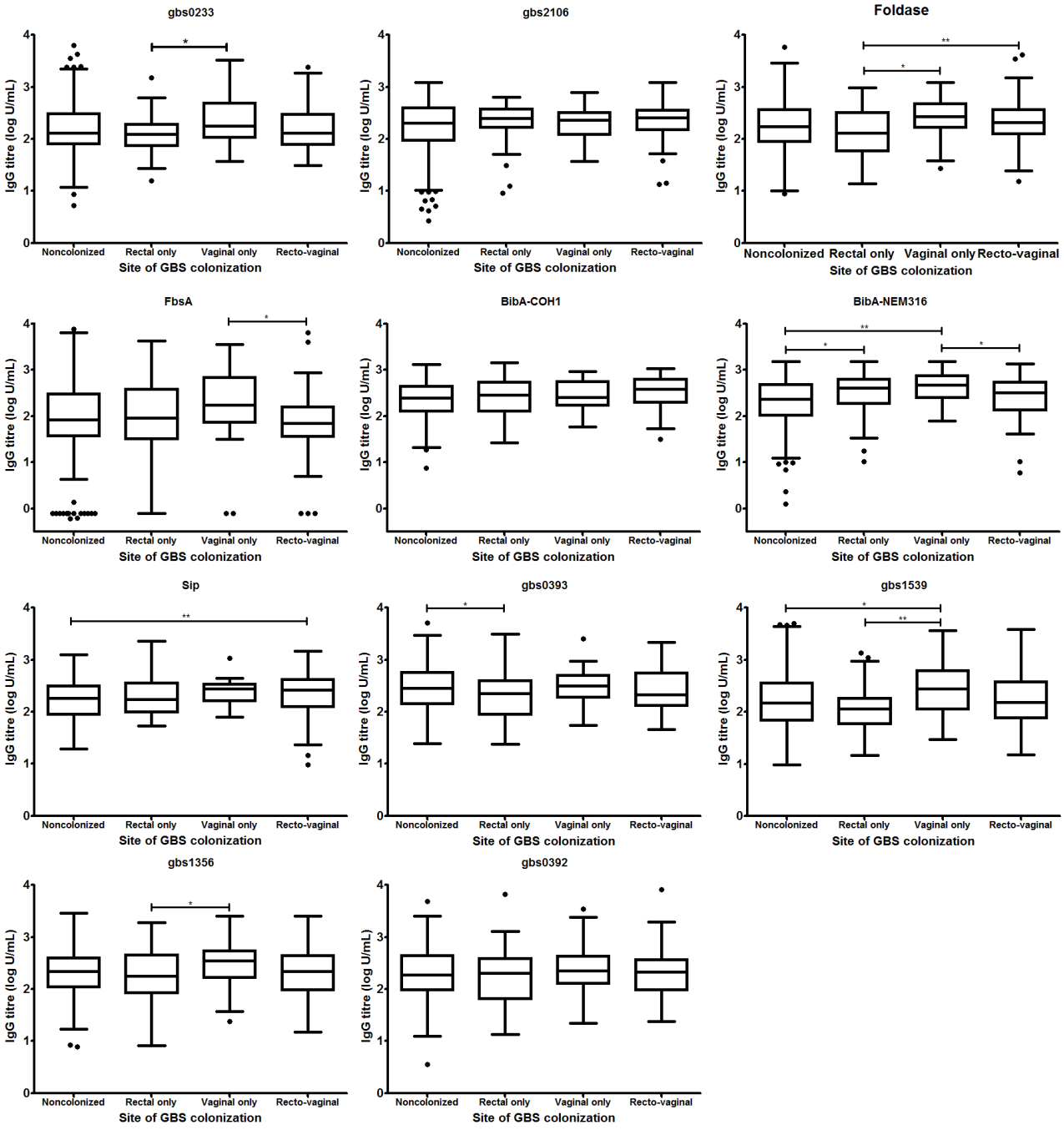

Figure 6.2 IgG titres (log U/mL) against group B Streptococcus surface protein present in pregnant women who were either not colonised or colonised at the rectal and/or vaginal tract at ≥37 weeks of gestation age. Statistical comparison between groups using quantile regression, * $p<0.05$ and** $p<0.01$ adjusted for cofactor variables. Turkey box-and-whisker plots representing the median (line within the box), 25th and 75th percentiles (box), the 1.5 times interquartile distances from the 25th and 75th centiles (whiskers) and outliers (solid dots).

Association of protein specific IgG antibody and acquisition of GBS colonisation

To determine the association of naturally occurring GBS surface protein IgG antibodies and the risk of recto-vaginal GBS acquisition during pregnancy, antibody titres were compared between women who were persistent non-carries (255/505, 50.4%) and those who were negative for recto-vaginal colonisation at enrolment and subsequently acquired GBS colonisation during the pregnancy (54/505, 10.7%).

Median IgG titres against all the GBS surface proteins at 20-25 weeks of gestation age were not statistically different between the two groups, Table 6.3 and Figure 6.3. The reverse cumulative plots, however, show that approximately 60% and 55% of pregnant women who were classified as non-carriers had predominant IgG titres against proteins gbs0233 and gbs1539 compared to women who subsequently acquired GBS colonisation following 20-25 weeks of gestation study visit, Figure 6.3.

Table 6.3 Median IgG titre (U/mL) against group B Streptococcus (GBS) surface proteins at 20-25 weeks of gestation age in pregnant women who were GBS non-carriers and those who newly acquired GBS recto and/or vaginal colonisation during pregnancy.

	Protein	Non-carrier (n=255) Median IgG (IQR)	New acquisition (n=54) Median IgG (IQR)	p
Lipoprotein	Gbs0233	177.83 (93.33-407.38)	147.91 (79.43-229.09)	0.09
	Gbs2106	269.15 (134.90-524.81)	239.88 (123.03-537.03)	0.79
	Foldase PsrA	223.87 (104.71-512.86)	186.21 (95.50-416.87)	0.37
Cell wall anchored proteins	FbsA	104.71 (33.88 380.19)	97.72 (33.88-630.96)	0.47
	BibA-COH1	295.12 (147.91-512.86)	302.00 (147.91-602.56)	0.78
	BibA-NEM316	281.84 (123.03-616.60)	371.54 (141.25-724.44)	0.44
	Gbs0393	331.13 (165.96-645.65)	295.12 (147.91-630.96)	0.75
	Sip	239.88 (123.03-426.58)	263.03 (162.18-501.19)	0.40
	Gbs1539	194.98 (87.10 436.52)	91.20 (57.54 389.05)	0.07
	Gbs1356	269.15	251.19	0.94

	(147.91-457.09)	(147.91-562.34)	
Gbs0392	239.88 (107.15-524.81)	190.55 (109.65-467.74)	0.69

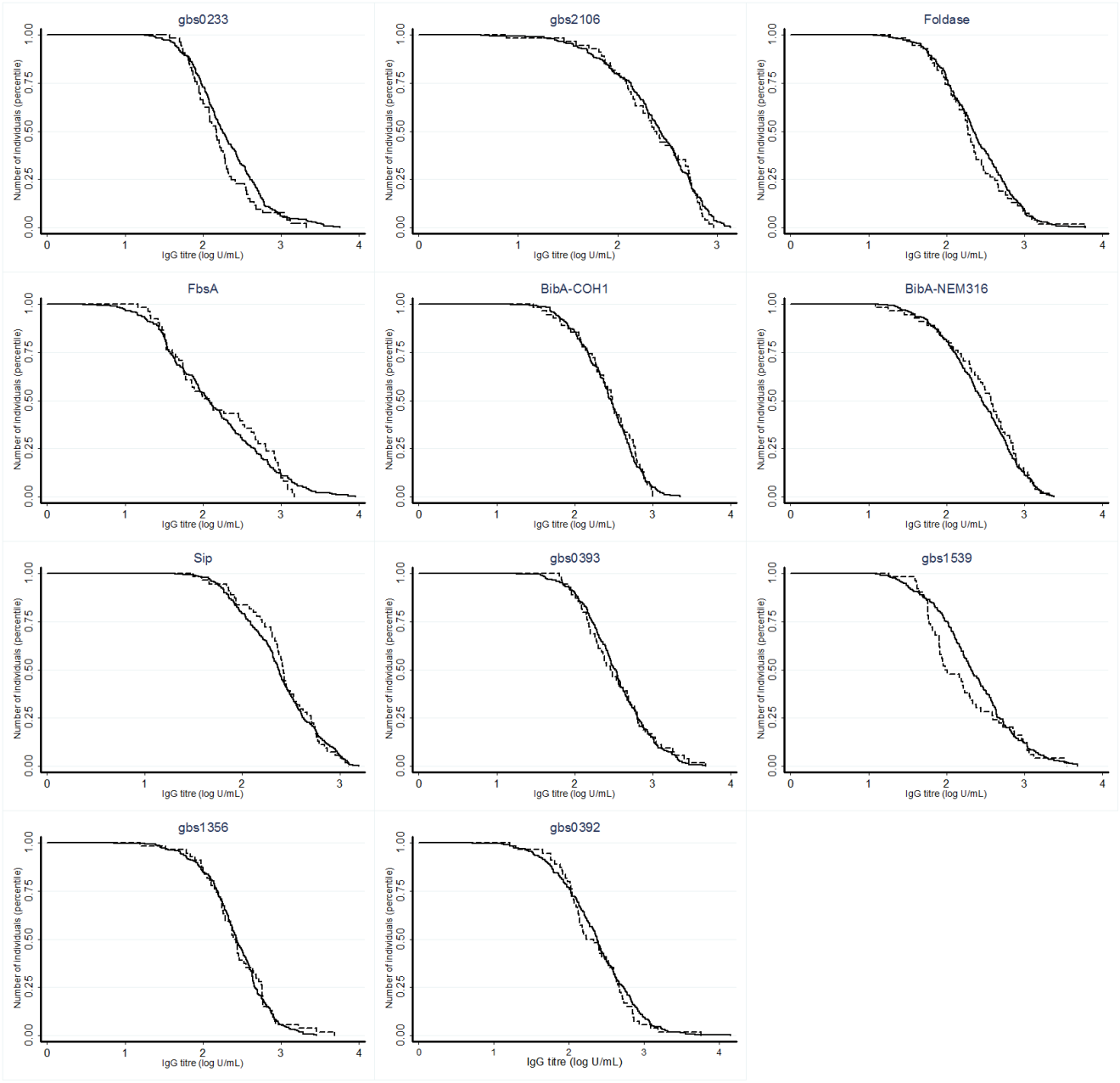

Figure 6.3 Reverse cumulative plots comparing IgG titres against group B Streptococcus (GBS) surface proteins between women who remained non-colonised throughout pregnancy (solid line) and those who were non-colonised at 20-25 weeks of gestation age but subsequently acquired GBS colonisation (dotted line).

Logistic regression analysis was used to determine the IgG antibody titre threshold against gbs0233 and gbs1539 (estimated from the reverse cumulative plot, Figure 6.3) required to prevent GBS colonisation during pregnancy. Of the women who remained non-carriers throughout pregnancy, 46.7% (119/255) had IgG titres ≥200 U/mL against surface protein gbs0233 at baseline compared to 29.6% (16/54) women who newly acquired GBS colonisation later in pregnancy. Thus, having gbs0233 specific IgG tires ≥200 U/mL was associated with reduced odds of acquiring GBS colonisation during pregnancy (adjusted OR=0.47 [95%CI: 0.25-0.89], p=0.021), Table 6.4. This strength of association remained similar at higher threshold, albeit not significant.

Similarly, for gbs1539, women who remained free of GBS recto-vaginal colonisation (i.e. non-carriers) were 56% more likely to have IgG titres ≥85 U/mL at baseline compared to women who had new-acquisition of GBS (adjusted OR=0.44 [95% CI: 0.24-0.82], p=0.01). This strength of association between higher gbs1539 antibodies and risk of GBS acquisition persisted at higher thresholds of up to ≥300, albeit not increasing or being significant thereafter, Table 6.4. No other such association was found for the other GBS surface proteins.

Table 6.4 IgG titres against group B Streptococcus (GBS) surface proteins gbs0233 and gbs1539 associated with prevention of acquisition of GBS colonisation.

IgG levels (U/mL)	Non-colonised (n=255)	New acquisition (n=54)	OR (95% CI)	P value	OR (95% CI)[α]	P value[α]
Gbs0233						
≥100	185 (72.5%)	34 (63.0%)	0.64 (0.35-1.19)	0.16	0.60 (0.32-1.14)	0.12
≥200	119 (46.7%)	16 (29.6%)	0.48 (0.26-0.91)	0.022	0.47 (0.25-0.89)	0.021
≥300	85 (33.3%)	12 (22.2%)	0.57 (0.29-1.14)	0.11	0.59 (0.29-1.19)	0.14
≥400	66 (25.9%)	8 (14.8%)	0.50 (0.22-1.11)	0.083	0.53 (0.23-1.18)	0.12
≥500	51 (20.0%)	5 (9.3%)	0.41 (0.15-1.08)	0.063	0.43 (0.16-1.15)	0.092
Gbs1539						
≥85	192 (75.3%)	30 (55.5%)	0.41 (0.22-0.75)	0.003	0.44 (0.24-0.82)	0.01

≥100	181 (71.0%)	26 (48.1%)	0.38 (0.21-0.69)	0.001	0.41 (0.22-0.75)	0.004
≥200	124 (48.6%)	18 (33.3%)	0.53 (0.29-0.98)	0.04	0.54 (0.29-1.00)	0.051
≥300	97 (38.0%)	15 (27.8%)	0.63 (0.33-1.200	0.15	0.64 (0.33-1.22)	0.18

α, adjusted for maternal age, gestational age, parity and gravidity

Effect of GBS acquisition on protein specific antibody response

Further evaluation of serum antibodies against GBS surface proteins revealed that IgG titres were dependent on the site where acquisition of GBS colonisation occured. Figure 6.4 shows the number of women with newly acquired GBS colonisation in the rectal and/or vaginal tract by study visits. Of the women grouped as "newly acquired", 25.9% (14/54) acquired GBS colonisation in the rectal tract only, 24.1% (13/54) in vagina tract only and 50.0% (27/54) in recto-vaginal tract. A higher number of women newly acquired GBS colonisation in the vaginal tract only at ≥37 weeks of gestation age (11 of 13, 84.6%) compared to those who newly acquired GBS colonisation in the rectal tract only (7 of 14, 50.0%), although this difference was not statistically different (p=0.32).

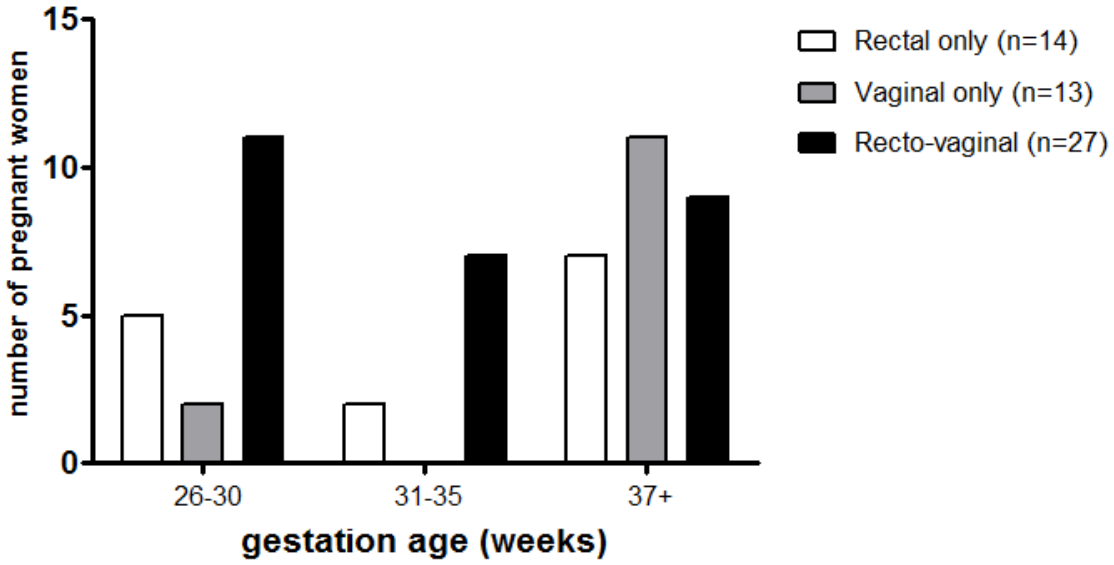

Figure 6.4 Pregnant women who newly acquired group B streptococcus colonisation after 20-25 weeks gestation age study visit in the rectal tract only (white bars), vaginal tract only (grey bars) and in the rectal and vaginal tract (black bars).

At ≥37 weeks of gestation age, pregnant women who acquired GBS colonisation in the vaginal tract only had higher median IgG titres (U/mL) compared to women who were non-carriers for GBS surface proteins BibA-NEM316 (478.63 vs 234.42, p=0.012), Foldase (269.15 vs173.78, p=0.036), Sip (275.42 vs 186.21, p=0.030) and gbs 1539 (281.84 vs 147.91, p=0.049), Figure 6.5. In contrast, lower IgG titres (U/mL) were observed in women who newly acquired GBS colonisation at the rectal tract only at ≥37 weeks gestational age compared to those who remained non-colonised throughout, inlcuding for Foldase (128.82 vs 173.78, p=0.043), gbs0233 (123.03 vs 131.83, p=0.010), gbs1539 (114.82 vs 147.91, p=0.002) and gbs1356 (177.83 vs 213.80, p=0.019), Figure 6.5.

Moreover, women who only acquired GBS in the vagina had higher median IgG titres compared to those who acquired GBS colonisation only in the rectum for proteins Sip (p=0.049), Foldase (p=0.0094), gbs0233 (p=0.0039), gbs0393 (p=0.027), gbs1539 (p=0.0004) and gbs1356 (p=0.039); Figure 6.4. BibA-COH1 specific median IgG titres (380.19 U/mL) where higher in women who newly acquired GBS colonisation in the recto-vaginal tract compared to those who were persistent non-carriesr (257.04, p=0.041). The was no significant difference observed for the other proteins when comparing protein specific IgG titres in women who acquired GBS colonisation at the recto-vaginal tract and those who acquired GBS colonisation either in the rectal or vaginal tract only, Figure 6.5.

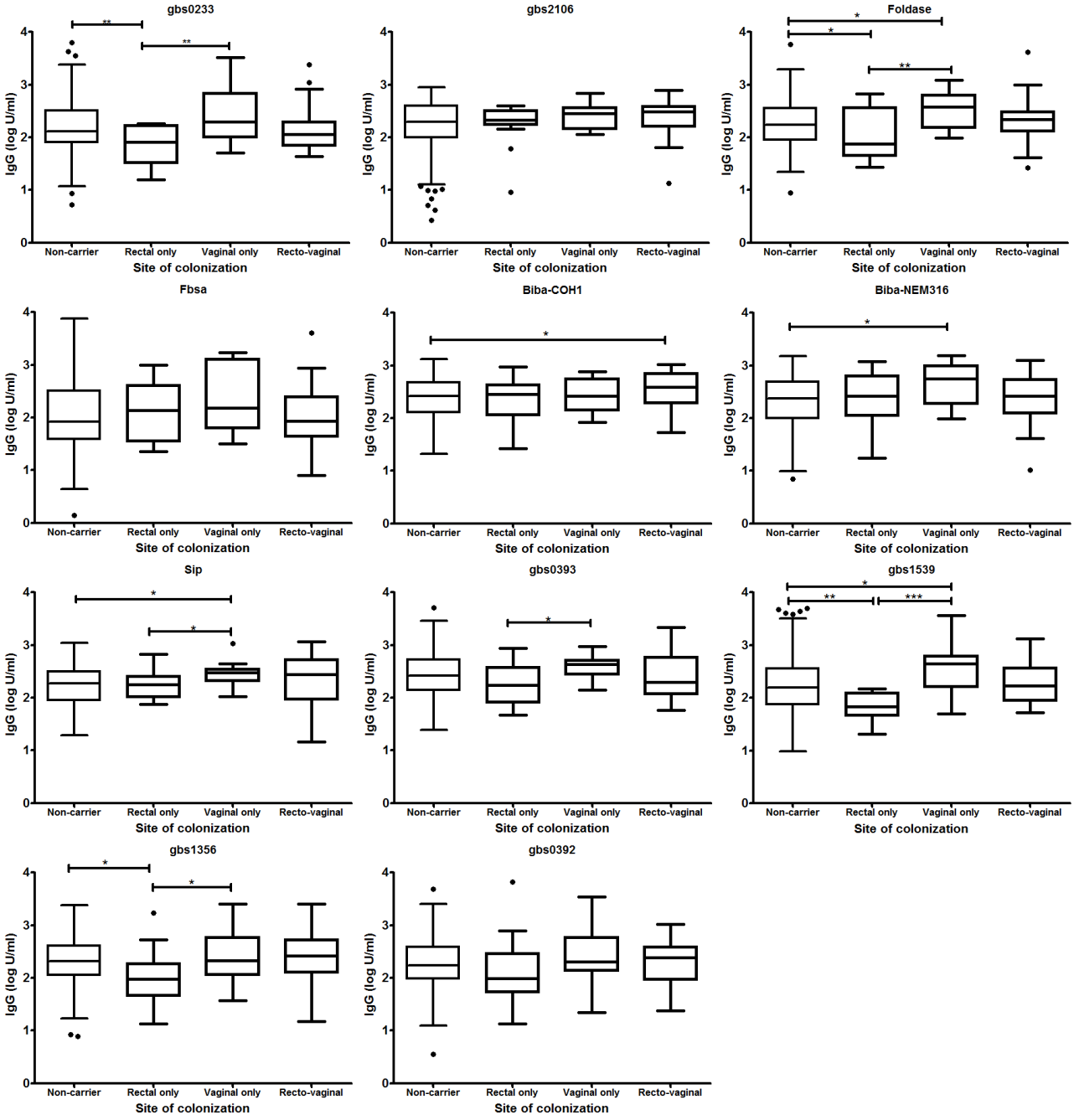

Figure 6.5 Comparison of median IgG titres to group B Streptococcus (GBS) proteins between pregnant women who were non-carriers and those who newly acquired GBS colonisation either at the rectal and/or vaginal tract at ≥37 weeks of gestation age. * $p < 0.05$, ** $p < 0.01$ and ** $p<0.001$. Turkey box-and-whisker plots representing the median (line within the box), 25th and 75th percentiles (box), the 1.5 times interquartile distances from the 25th and 75th centiles (whiskers) and outliers (solid dots).

Association of serum IgG and clearance of GBS recto-vaginal colonisation

Of the 505 women included in the analysis, 69 (13.8%) had positive GBS cultured at all study visits (persistently-colonised), 75 (14.8%) were transiently-colonised and 52 (10.3%) were intermittently-colonised. The pattern of GBS colonisation in women who were persistently-colonised differed from women who were transiently-colonised with GBS acccording to the site of colonisation and serotype, albeit not statistically significant, Table 6.5.

To determine IgG antibody titres against GBS surface proteins associated with clearance of GBS colonisation during pregnancy; women who were persistently-colonised with GBS were compared to those who were transiently colonised. Median IgG titres (U/mL) among women who were persistently-colonised were higer than those occuring in transiently colonised women at 20-25 and ≥37 weeks of gestation age for the majority of proteins, Table 6.6 and Table 6.7. Multivariate analysis adjusting for maternal colonising serotype, maternal age, gestational age, parity and gravidity showed that median IgG titres were higher among persistently-colonised compared to transiently-colonised women for BibA-COH1 (p=0.03), Sip (p=0.03) and gbs1356 (p=0.04) at 20-25 weeks gestation, Table 6.6. The serum IgG titres against all the GBS surface proteins were not statistically different between persistently-colonised compared to transiently-colonised women at ≥37 weeks gestation age after adjusting for confounding variables, Table 6.7.

Table 6.5 Heat map display of serotype distribution in pregnant women who were either persistently or transiently colonised at the vaginal and/or rectal tract.

	Vaginal								Rectal								Recto-vaginal							
	Persistent (n)				Transient (n)				Persistent (n)				Transient (n)				Persistent (n)				Transient (n)			
Serotype	V1	V2	V3	V4	V1	V2	V3	V4	V1	V2	V3	V4	V1	V2	V3	V4	V1	V2	V3	V4	V1	V2	V3	V4
Ia	5	4	7	8	17	8	6	0	6	7	8	8	15	7	1	0	13	13	11	11	9	3	7	0
Ib	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	3	3	2	1	3	0	0	0
II	2	1	0	1	2	0	1	0	1	1	3	3	1	0	0	0	3	4	3	2	1	1	1	0
III	3	5	5	4	4	5	2	0	10	5	7	8	6	1	2	0	17	17	15	14	6	4	2	0
IV	1	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	2	0	0	0
V	1	1	0	1	3	1	2	0	2	1	0	1	2	3	1	0	1	3	3	2	4	2	2	0
IX	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	2	2	0	0	0	0
Total	12	12	14	15	26	15	11	0	20	15	19	21	24	12	4	0	37	42	36	33	25	10	12	0
Overall[α]	13.2 (19.2%)				17.3 (23.1%)				18.8 (27.2%)				13.3 (17.3%)				37.0 (53.6%)				15.7 (20.9%)			

α, rate of GBS colonisation

V, study visit during pregnancy

Table 6.6 Comparison of median IgG titre to group B Streptococcus (GBS) surface proteins between pregnant women who were persistently colonised and those who were transiently colonised at 20-25 weeks of gestation age.

	Protein	Transiently-colonised (n=75)	Persistently-colonised (n=69)	p value	Adj p value[α]
Lipoprotein	Gbs0233	199.53 (97.72-371.54)	245.47 (117.49-389.05)	0.15	0.59
	Gbs2106	234.42 (100.00-467.74)	346.74 (144.54-537.03)	0.05	0.11
	Foldase PsrA	190.55 (100.00-478.63)	234.42 (117.49-457.09)	0.73	0.23
Cell wall anchored proteins	FbsA	109.65 (33.88-338.84)	91.20 (45.71 501.19)	0.67	0.69
	BibA-COH1	288.40 (158.49-501.19)	436.52 (199.53-660.69)	0.03	0.03
	BibA-NEM316	354.81 (141.25-741.31)	467.74 (213.80-691.83)	0.35	0.29
	Gbs0393	354.81 (194.98-676.08)	407.38 (251.19-602.56)	0.60	0.17
	Sip	213.80 (109.65-389.05)	295.12 (177.83-426.58)	0.04	0.03
	Gbs1539	177.83	213.80	0.38	0.55

	(75.86-436.52)	(114.82-512.86)		
Gbs1356	263.03 (134.90-389.05)	354.81 (173.78-575.44)	0.02	0.04
Gbs0392	275.42 (141.25-575.44)	281.84 (177.83-436.52)	0.80	0.75

α, adjusted for colonising serotype, gestational age, maternal age, parity and gravidity

Table 6.7 Comparison of median IgG titre to group B Streptococcus (GBS) surface proteins between pregnant women who were persistently colonised and those who were transiently colonised at ≥37 weeks of gestation age.

	Protein	Transient	Persistent	p value	p value[α]
Lipoprotein	Gbs0233	141.25 (75.86-275.42)	158.49 (97.72-323.59)	0.20	0.71
	Gbs2106	218.78 (93.33-407.38)	245.47 (123.03-346.74)	0.73	0.56
	Foldase PsrA	158.49 (75.86-398.11)	194.98 (77.62-389.05)	0.79	0.63
Cell wall anchored proteins	FbsA	79.43 (36.31-251.19)	85.11 (42.66-398.11)	0.63	0.46
	BibA-COH1	239.88 (154.88-446.68)	338.84 (173.78-602.56)	0.08	0.57
	BibA-NEM316	208.93 (134.90-524.81)	398.11 (204.17-630.96)	0.02	0.24
	Gbs0393	302.00 (144.54-707.95)	269.15 (154.88-512.86)	0.37	0.38
	Sip	199.53 (93.33-338.84)	223.87 (123.03-354.81)	0.23	0.62
	Gbs1539	125.89 (61.66-363.08)	177.83 (69.18-478.63)	0.38	0.06

Gbs1356	223.87 (89.13-389.05)	245.47 (93.33-501.19)	0.46	0.61
Gbs0392	245.47 (97.72-575.44)	229.09 (104.71-398.11)	0.65	0.56

α, adjusted for colonising serotype, gestational age, maternal age, parity and gravidity

6.5 Discussion

We observed that antibodies against a selection of GBS surface proteins were present throughout pregnancy irrespective of the women's colonization status and the colonising serotype. This observation is consistent with the high GBS acquisition rate of 27.9% that occurred from ≥20 weeks of gestation age onward during the study period (58). Also, high prevalence of GBS colonisation during pregnancy has been reported in other studies, being greatest in the African region (235). The protein antibody levels detected were influenced by GBS colonisation status, the site of acquisition of GBS colonisation and gestational period.

Humoral immunity in pregnant women with GBS colonisation has been extensively described for capsular polysaccharides, with serotype specific antibodies significantly increased in women colonised with homotypic CPS serotype (18,60,236). Similarly, antibody titres against Sip and pilus island proteins have been reported to be higher among colonised pregnant women (89,237). In our study, cross-sectional analysis at 20-25 and ≥37 weeks of gestation revealed that IgG titres for BibA and Sip proteins were significantly increased in colonised compared to non-colonised women. Considering that this was not evaluated specifically for GBS isolates expressing the specific protein, this might be an under estimate of the protein's capacity to induce natural antibody response.

Changes in sera IgG titres against GBS surface proteins with advancing gestation were dependent on the site where GBS colonisation was detected. IgG titres against select GBS proteins were significantly lower in pregnant women colonised at the rectal tract compared to those who were only colonised at the vaginal tract at ≥37 weeks of

gestation. This is in contrast to Hordnes et al. (1996) who reported increased serotype-specific capsular IgA and IgG in cervical secretion with no corresponding increase in serum IgG in women who had GBS colonisation at either the rectum or vaginal tract at 17 weeks of gestation (238). Shen et al. (2000), however reported that vaginal immunisation with GBS serotype III CPS conjugated to cholera toxin induced a marked increase in sera IgG together with mucosal IgG and IgA (239). Given that the dominant IgG present in cervivcal secretions and vaginal washes tends to have been derived from both the local and circulating plasma cells (240). This in part could explain the occurance of higher serum IgG titres among women who were colonised at the vaginal tract.

The function of naturally induced antibodies was inferred by comparing pregnant women who remained non-colonised throughout the study visits to those that newly acquired GBS after 20-25 weeks of gestation age. Women with IgG titres ≥ 200 U/mL against gbs0233 were 53% less likely to acquire GBS colonisation. Similarly, having IgG titres ≥85 U/mL against gbs1539 reduced the odds of being colonised by 66%. These results suggest that increasing maternal antibodies to proteins by vaccination could reduce GBS acquisition and subsequently lower vertical transmission to their respective foetus/newborns. Similar observations were reported for antibodies against CPS of serotype Ia and III, with increasing IgG concentrations associated with reduced odds of acquiring GBS colonisation (241).

Acquisition of GBS at the vaginal tract induced elevated serum IgG titres against select GBS surface proteins. This resulted in significantly lower serum IgG titres against select GBS surface proteins were observed in women who newly acquired GBS colonisation at the rectal tract compared to those that acquired GBS colonisation at the vaginal tract.

An increase in systemic antibody production has been reported in women where the vaginal tract was used as the route for immunisation (239,242). We hypothesized that differential protein expression may occur between the two colonisation site resulting in an up-regulation of specific proteins and subsequently inducing a higher immune response. Alternatively, the lack of serum antibody stimulation observed following colonisation of the rectal tract may be due to either local immune dependent suppression of colonisation or immunological naivety in the rectum that allows for persistent GBS colonisation.

Pregnant women who were persistent GBS carriers had, overall, higher median serum IgG titres compared to those who cleared GBS colonisation in subsequent visits post 20-25 weeks of gestation. Similar results were observed for IgG concentration against CPS of serotype Ia, III and V, however, significantly high OPA killing was reported amongst women who lost GBS colonisation (243). Therefore, further experiments are required to determine the functional activity of protein specific antibodies produced in women who are persistently colonised and compared to those that are transient carriers.

Immunisation against GBS colonisation during pregnancy has the potential to reduce vertical transmission of the bacterium from mother to foetus/newborns, hence decreasing invasive GBS disease among neonates. Prevention and/or subsequent clearance of GBS colonisation requires a vaccine candidate capable of inducing neutralising antibodies at both the mucosal and systemic components (242). Identifying and targeting antigens that are preferentially expressed for mediating pathogenesis: those having dual importance in colonisation and invasive disease, may increase the efficacy of the vaccine against invasive disease. Moreover, these antigens may be highly expressed and more favourable for immune recognition for prevention of GBS

colonisation. Grifantini et al. identified 5 genes upregulated during *Neisseria meningitidis* adhesion, two of which were putative proteins, using DNA-microarray that were capable of inducing antibodies with opsonophagocytic activity in mice (244). Using both known virulence proteins and *in silico* identified surface proteins, this study reports on immunogenic GBS peptides that seem to be involved during bacterial infection and thus further experiments are required to determine their pathogenicity. The peptide antigens described are capable of inducing an antibody response and exhibit serological potential in preventing GBS acquisition. Current evidence suggest that these GBS surface proteins are not involved in clearance of GBS colonisation, further experiments are required to determine functionality of antibodies induced.

Chapter Seven

Reduced trans-placental transfer of antibodies against GBS surface proteins in HIV-infected mother-newborn dyads

7.1 Introduction

Risk factors associated with neonatal invasive GBS disease include intrapartum fever, premature rapture of membrane, preterm-delivery and low birth weight (245). Recently maternal HIV infection has been shown to increase the risk of invasive GBS disease in infants who have been exposed *in utero* (20,21). Moreover, maternal HIV infection is also associated with preterm delivery and low birth weight (246), and therefore further increasing the burden of invasive GBS disease in HIV-exposed uninfected (HEU) infants.

The increased susceptibility to bacterial and viral infections such as *Streptococcus pneumoniae, Streptococcus pyogenes, Haemophilus influenzae* type b, *Clostridium tetani, Bordetella pertussis* and Varicella-zoster virus and measles in HEU infants is partly attributable to reduced maternally derived antibodies (102–104). Similarly, reduced trans-placental transfer of antibodies against GBS capsular polysaccharide has been reported, resulting in low antibody levels that are capable of binding and neutralising GBS in infants (105,106). This may also contribute to the heightened susceptibility to invasive GBS disease amongst HEU infants (20,21).

The aim of this study was to evaluate the effect of maternal HIV infection on trans-placental transfer of antibodies against GBS surface proteins including BibA, Sip, Foldase, gbs2106, gbs0393, gbs1356, gbs1539 and gbs0392. Work included here has been published in *The Journal of Infectious Diseases*; Appendix 10.7.

7.2 Study population

To determine the influence of maternal HIV-infection on placental transfer of antibodies against GBS surface proteins, a retrospective analysis was performed in sera collected

from cord and maternal blood of mother-newborn pairs. The study participants were previously enrolled in a similar study that measured antibody concentrations specific to GBS CPS and proteins PI, FbsA and BibA (from hypervirulent ST-17) which are not included in this study analysis (106).

Pregnant women with confirmed HIV status delivering at ≥36 weeks of gestation age and whose babies were ≥2.5 kg birth weight were included in the study. As part of routine antenatal care, $CD4^+$ lymphocyte counts were determined for HIV-infected pregnant women and were used to determine ARV regimen given to each women. Those with $CD4^+$ lymphocyte count ≤350 cells/μL were given triple ARV, whereas those who had $CD4^+$ lymphocyte count >350 cells/μL were treated with zidovudine until delivery. HIV RNA-viral load was measured in blood of HIV-infected pregnant women using polymerase chain reaction which had a detection limit of 40 copies/mL. GBS colonisation was determined for the pregnant women by swabbing the lower vaginal and rectal tracts and inoculating selective medium. Positive GBS cultures were serotyped using latex agglutination. Use of the archived serum samples for this study was approved by the Human Research Ethics Committee of the University of Witwatersrand (Approval number: M140902)

7.3 Statistical analysis

Statistical analysis was performed using STATA software (version 13.0 Stata-Corp, Tx USA) and GraphPad Prism (version 5.03 GraphPad Software Inc, California USA). Antibody titres were $\log_{10}$ transformed for parametric statistical comparisons. Statistical differences or association between groups was measured using two tailed *Student's* t-test or χ^2 and Fisher's exact test. Confounding variables were assessed in a univariate

analysis and were corrected for using multivariate regression and a p value < 0.05 was considered statistically significant.

Cord-maternal antibody ratio (CMR) were calculated for each mother-newborn pair and median CMR were compared between HIV-uninfected and HIV-infected mother-newborn dyads using quantile regression. Relative efficiency of trans-placental transfer of antibodies between the two groups was measured as the difference in CMR of HIV-uninfected and HIV-infected mother-newborn dyads and reported as a percentile. The association between maternal $CD4^+$ lymphocyte count and HIV viral load with antibody titres and CMR was assessed using univariate quantile regression.

7.4 Results

Demographic characteristics

A total of 164 mother-newborn dyads were analysed in this study. Detailed demographic information about the study participants is shown in Table 7.1 (106). Briefly, of the 164 mothers, 81 were HIV-uninfected and 83 were HIV-infected. CD4+ lymphocyte counts were available for 71 (85.5%) of the 83 HIV- infected women, with a median of 423 cells/µL (range 46-1268 cells/µL). HIV-1 RNA viral loads were detected in 79 HIV-infected women with a median of 96 copies/mL (range 20 to 146 055 copies/mL). Using vaginal and rectal swabs, positive GBS growth at delivery were detected in 29.9% (49 of 164) women, including among 27.2% (22 of 81) HIV-uninfected and 32.5% (27 of 83; p=0.45) HIV-infected women, supplementary Table S1. Serotype distribution did not differ by HIV status with the frequently colonising serotypes being Ia (40.8%), III (28.6%), V (18.4%),II (8.2%) and Ib (4.1%) (106).

Table 7.1 Demographic characteristics of HIV-uninfected and HIV-infected pregnant women at delivery together with their respective HIV-unexposed and HIV-uninfected exposed infants.

	HIV-uninfected (n=81)	HIV-infected (n=83)	P value
age, years; median (range)	26.0 (18.1-42.0)	30.7 (18.7-42.7)	0.0057
Parity, median (range)	1 (0-5)	1 (0-4)	0.079
$CD4^+$ lymphocyte count, cells/μL; median (range)[ǂ]		423 (46-1268)	
<250		14 (16.8%)	
250-350		12 (14.5%)	
350-500		21 (25.3%)	
>500		24 (28.9%)	
HIV viral load, copies/mL; median (range)[ϕ]		96 (20-146055)	
<40		31 (37.3%)	
40-1000		21 (25.3%)	
1000-10000		14 (16.9%)	

>10000		13 (15.7%)	
GBS Colonised	22 (27.2%)	27 (32.5%)	0.45
Serotype			0.18
Ia	12 [54.6%]	8 [29.6%]	0.47
Ib	0 [0.0%]	2 [7.4%]	0.24
II	2 [9.1%]	2 [7.4%]	1.00
III	3 [13.6%]	11 [40.7%]	0.035
V	5 [22.7%]	4 [14.8%]	0.81
Infants			
Gender, male	45 (55.6%)	45 (54.2%)	0.86
Gestational age, weeks; median (range)	40 (36.1-44.0)	40 (36.4-43.5)	1.00
Birth weight, grams; median (range)	3130 (2510-4415)	3034 (2500-3910)	0.19

ǂ $CD4^+$ lymphocyte count was not recorded in 12 of the HIV-infected women.

ϕ HIV RNA viral load was not recorded in 4 of the HIV-infected women.

Maternal antibodies to GBS surface proteins

All maternal sera had detectable antibody titres for the analysed GBS surface proteins. IgG GMT against the GBS surface proteins analysed did not differ by GBS colonisation status among the women, Table 7.2. HIV-infected women had lower antibody GMT (U/ml) compared to HIV-uninfected women, including after adjusting for cofactor variables, for proteins Sip (132.42 vs 186.72, p=0.002), Foldase (162.06 vs 220.82, p=0.035) and other putative proteins including gbs2106 (153.62 vs 235.11, p=0.002), gbs0393 (258.97 vs 381.35, p=0.001), gbs1356 (156.36 vs 215.49, p=0.035), Figure 7.1. Similar trends were observed for gbs0392 and gbs1539 with antibody GMT being higher amongst HIV-uninfected compared to HIV-infected women, albeit not statistically significant. No significant correlation was observed between $CD4^+$ lymphocyte count and HIV-1 RNA viral load with maternal antibody titres for all the GBS surface proteins, Table 7.3 and 7.4.

Table 7.2 Comparison of IgG antibody geometric mean titres (GMT) against group B Streptococcus surface protein between non-colonised and colonised pregnant women who were either HIV-uninfected or HIV-infected mothers.

	HIV-uninfected			**HIV-infected**		
Surface proteins	**Non-colonised GMT (95%CI)**	**Colonised GMT (95%CI)**	**P value**	**Non-colonised GMT (95%CI)**	**Colonised GMT (95%CI)**	**P value**
Sip	191.70 (164.56-223.33)	173.97 (148.82-203.38)	0.47	128.22 (107.83-152.48)	141.58 (105.17-190.60)	0.54
BibA	200.13 (157.21-254.78)	235.12 (149.38-370.07)	0.51	180.36 (143.26-227.06)	251.09 (171.18-368.30)	0.13
Foldase	220.10 (177.61-272.75)	222.78 (145.24-341.71)	0.96	151.62 (114.95-199.98)	186.07 (138.05-250.80)	0.37

Gbs2106	227.51 (179.16-288.90)	256.78 (197.31-334.17)	0.57	138.82 (109.36-176.22)	189.53 (133.88-268.31)	0.14
Gbs1356	359.02 (297.55-433.20)	448.34 (293.64-684.54)	0.62	231.62 (182.28-294.33)	326.42 (250.58-425.21)	0.45
Gbs0393	222.30 (175.36-281.80)	198.26 (135.94-289.15)	0.27	146.65 (106.51-201.92)	178.58 (126.08-252.94)	0.08
Gbs1539	351.81 (286.38-432.18)	391.51 (250.58-611.71)	0.62	268.61 (209.61-344.20)	369.63 (265.00-515.57)	0.14
Gbs0392	324.67 (268.49-392.60)	329.44 (208.39-520.81)	0.94	250.64 (191.59-327.90)	397.10 (275.55-572.28)	0.05

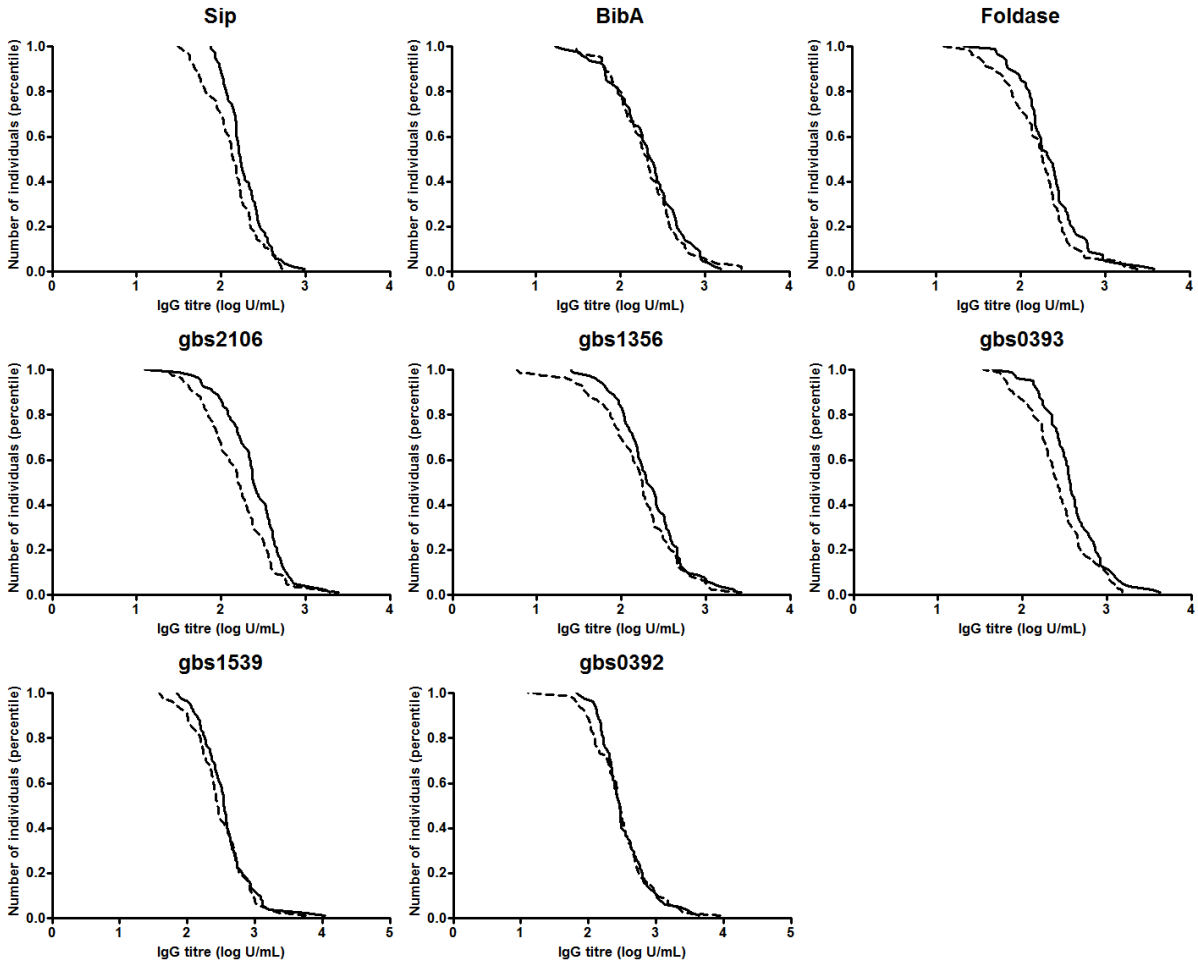

Figure 7.1 Reverse cumulative distributions of antibody titres against group B Streptococcus surface protein antigens in HIV-uninfected (solid line) and HIV-infected (dotted line) women at delivery.

Table 7.3 Association between group B Streptococcus specific IgG antibodies and maternal $CD4^+$ lymphocyte count in HIV-infected pregnant women.

	$CD4^+$ cells/mL				p value
	<250 (n=14)	**250-350 (n=12)**	**350-500 (n=21)**	**>500 (n=24)**	
Sip	160.47 (113.08-222.89)	116.43 (61.36-214.19)	149.96 (107.42-213.24)	136.76 (72.47-211.64)	0.62
BibA	166.75 (104.54-504.33)	221.41 (116.58-370.43)	271.16 (95.27-330.32)	177.51 (117.89-264.23)	0.72
Foldase	251.76 (178.47-310.21)	141.57 (51.46-208.51)	165.15 (76.33-283.74)	178.36 (87.12-240.64)	0.14
gbs2106	200.48 (124.31-361.34)	187.74 (61.4-382.76)	94.03 (61.4-382.76)	154.78 (91.76-213.84)	0.37
Gbs1356	271.59 (180.67-541.64)	82.42 (38.08-257.96)	173.89 (38.05-257.96)	170.5 (78.48-205.03)	0.54
Gbs0393	401.36 (192.64-1031.36)	187.43 (149.35-256.83)	230.03 (149.35-256.83)	283 (104.00-492.24)	0.77
Gbs1539	442.58 (144.13-853.10)	254.09 (97.92-290.14)	293.36 (97.92-290.14)	240.89 (163.96-518.56)	0.66
Gbs0392	314.42 (197.59-433.15)	273.32 (152.08-521.05)	275.11 (152.08-521.05)	282.85 (108.37-582.91)	0.64

Table 7.4 Association between group B Streptococcus specific IgG antibodies and maternal HIV-1 viral load in HIV-infected pregnant women.

	HIV-1 viral load copies/mL				
	<40 (n=31)	40-1000 (n=21)	1000-10000 (n=14)	>10000 (n=13)	P value
Sip	149.73 (108.35-218.53)	160.61 (107.42-216.32)	83.82 (56.87-116.36)	170.35 (130.29-259.70)	0.63
BibA	222.51 (139.44-364.64)	145.32 (101.45-228.02)	165.93 (60.19-330.32)	276.77 (122.02-377.95)	0.90
Foldase	184.86 (79.72-276.62)	195.66 (136.83-283.95)	98.76 (46.69-180.39)	207.6 (136.99-282.78)	0.90
gbs2106	215.07 (94.03-361.34)	146.92 (95.04-209.38)	88.66 (46.11-157.93)	234.18 (92.42-359.49)	0.17
Gbs1356	173.89 (93.02-346.58)	179.57 (85.01-237.71)	103.27 (35.78-248.64)	178.83 (150.57-305.47)	0.94
Gbs0393	293.01 (187.53-533.49)	283.15 (135.19-460.53)	172.52 (66.17-200.70)	297.53 (235.19-455.24)	0.60
Gbs1539	292.6	247.58	266.77	480.69	0.18

	(185.07-581.09)	(104.95-513.25)	(102.50-398.00)	(230.56-697.13)	
Gbs0392	350.59 (195.11-664.54)	312.54 (125.79-501.28)	251.56 (122.15-302.35)	259.41 (184.23-420.39)	0.20

Newborn antibodies to GBS surface proteins

Of the 164 cord sera analysed, 18 samples had antibody titres below the detection limit for gbs1539, two had no detectable titres to gbs1539 and gbs0392, one each for gbs1539, gbs0393, Sip proteins and gbs1356. Antibody GMT in HEU newborns (n=83) were significantly lower compared to HIV-unexposed newborns (n=81) for all proteins after multivariate regression controlling for cofactor variables; including for Sip (80.37 vs 153.70, $p<0.0001$), Foldase (71.07 vs 142.27, $p<0.0001$), BibA (127.46 vs180.42, p=0.026), gbs2106 (82.91 vs 205.83, $p<0.0001$), gbs0393 (113.50 vs 241.33, $p<0.0001$), gbs1539 (73.12 vs 138.38, $p=0.016$), gbs1356 (55.05 vs 104.06, $p=0.001$), gbs0392 (81.70 vs 130.2, $p<0.0001$), Figure 7.2.

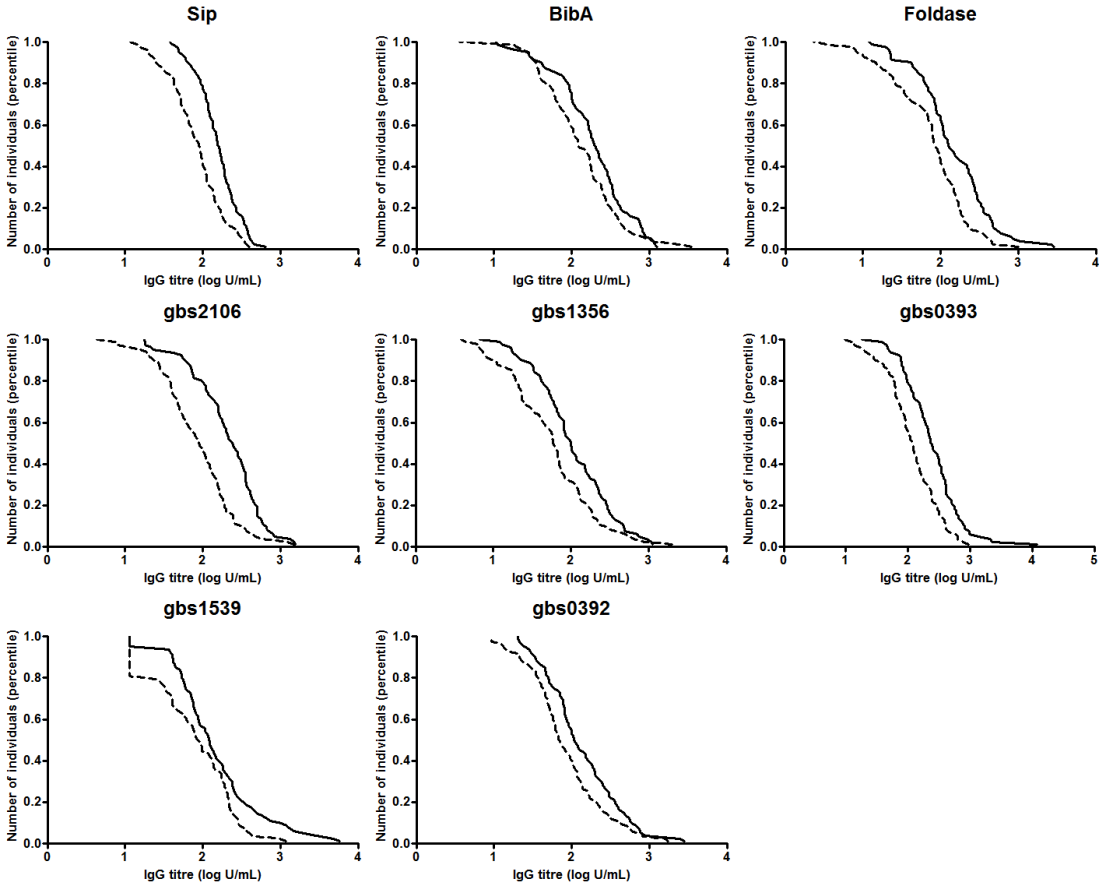

Figure 7.2 Reverse cumulative distributions of antibody titres against group B Streptococcus surface protein antigens in HIV-unexposed (solid line) and HIV-exposed uninfected (dotted line) newborn infants.

Effects of HIV infection on trans-placental transfer of antibodies

Maternal antibody titres correlated positively with cord antibody titres for all GBS surface proteins, $R^2 \geq 0.5$ and $p<0.0001$. Cord-maternal antibody ratios were compared between HIV-infected and HIV-uninfected mother-newborn dyads using quantile regression and adjusted for cofactor variables. Maternal HIV infection was associated with lower ratios of trans-placental transfer of antibodies for Sip (25.8%, $p<0.001$), Foldase (30.4%, $p<0.001$), gba0392 (36.5%, $p=0.006$), gbs0393 (32.9%, $p<0.001$), gbs1539 (39.2%, $p<0.008$), gbs2106 (35.7%, $p<0.001$) and BibA (19.4%, $p=0.004$),

Table 7.5. Antibodies to proteins gbs1539 and gbs0392 had ≤0.40 median cord-maternal antibody ratios irrespective of maternal HIV infection. No correlation was observed between cord-maternal antibody ratios with maternal $CD4^+$ lymphocyte count and HIV-1 RNA viral load, supplementary Table 7.6 and 7.7.

Table 7.5 Cord-maternal ratio (CMR) of antibody titres against group B Streptococcus surface proteins in HIV-uninfected and HIV-infected mother-newborn dyads.

Surface proteins	HIV-uninfected Mother-newborn dyad median CMR (IQR) n=81	HIV-infected Mother-newborn dyad median CMR (IQR) n=83	Reduction %[α]	P value[β]
Sip	0.819 (0.701-1.027)	0.608 (0.462-0.794)	25.8	<0.0001
Gbs2106	0.909 (0.733-1.110)	0.584 (0.421-0.809)	35.7	<0.0001
Gbs0393	0.641 (0.515-0.851)	0.430 (0.273-0.627)	32.9	<0.0001
Gbs1539	0.390 (0.227-0.555)	0.237 (0.138-0.415)	39.2	0.008
Gbs1356	0.507 (0.401-0.752)	0.319 (0.229-0.507)	37.1	<0.0001
Gbs0392	0.397 (0.267-0.630)	0.252 (0.163-0.425)	36.5	0.006
Foldase	0.654 (0.508-0.862)	0.455 (0.322-0.638)	30.4	<0.0001
BibA	0.877 (0.713-1.144)	0.707 (0.500-0.850)	19.4	0.004

α, CMR reduction calculated as (1-CMRHIV+/CMRHIV-)×100%

β, quantile regression p value adjusted for overall colonisation colonising serotype, maternal age, gestational age and parity.

Table 7.6 Association between group B Streptococcus specific cord-maternal IgG antibody ratios and maternal $CD4^+$ lymphocyte count in HIV-infected pregnant women.

	$CD4^+$ lymphocyte count (cells/µL)				
Median (IQR)	**<250 (n=14)**	**250-350 (n=12)**	**350-500 (n=21)**	**>500 (n=24)**	**P value**[α]
Sip	0.60 (0.44-0.90)	0.74 (0.57-0.88)	0.54 (0.46-0.67)	0.64 (0.44-0.84)	0.77
BibA	0.67 (0.54-0.81)	0.75 (0.44-0.84)	0.66 (0.56-0.83)	0.67 (0.47-0.91)	0.85
Foldase	0.44 (0.38-0.55)	0.44 (0.32-0.69)	0.46 (0.33-0.64)	0.51 (0.33-0.71)	0.20
gbs2106	0.66 (0.33-0.83)	0.67 (0.54-0.83)	0.49 (0.41-0.58)	0.59 (0.35-0.78)	0.83
Gbs1356	0.39 (0.27-0.66)	0.40 (0.23-0.52)	0.29 (0.24-0.57)	0.32 (0.25-0.50)	0.75
Gbs0393	0.42 (0.25-0.59)	0.42 (0.31-0.61)	0.45 (0.37-0.63)	0.46 (0.29-0.67)	0.64
Gbs1539	0.25 (0.19-0.42)	0.17 (0.13-0.32)	0.27 (0.16-0.44)	0.24 (0.11-0.39)	0.90
Gbs0392	0.28 (0.17-0.41)	0.29 (0.19-0.62)	0.29 (0.16-0.42)	0.25 (0.13-0.38)	0.63

α univariate quantile regression

Table 7.7 Association between group B Streptococcus specific cord-maternal IgG antibody ratios and maternal HIV-1 viral load in HIV-infected pregnant women.

	HIV-1 viral load copies/mL				
Median (IQR)	**<40 (n=31)**	**40-1000 (n=21)**	**1000-10000 (n=14)**	**>10000 (n=13)**	**p value[α]**
Sip	0.66 (0.54-0.84)	0.54 (0.44-0.74)	0.64 (0.42-0.85)	0.63 (0.46-0.77)	0.47
BibA	0.72 (0.54-0.93)	0.59 (0.45-0.76)	0.74 (0.48-0.84)	0.59 (0.52-0.83)	0.16
Foldase	0.43 (0.33-0.64)	0.46 (0.33-0.6)	0.49 (0.28-0.85)	0.43 (0.29-0.52)	0.74
gbs2106	0.62 (0.46-0.84)	0.54 (0.35-0.77)	0.60 (0.43-0.88)	0.44 (0.33-0.61)	0.31
Gbs1356	0.41 (0.25-0.51)	0.31 (0.19-0.42)	0.41 (0.20-0.75)	0.28 (0.21-0.45)	0.64
Gbs0393	0.49 (0.36-0.68)	0.40 (0.24-0.48)	0.38 (0.32-0.71)	0.35 (0.23-0.49)	0.36
Gbs1539	0.24 (0.14-0.4)	0.16 (0.11-0.34)	0.33 (0.18-0.68)	0.32 (0.14-0.42)	0.66
Gbs0392	0.29 (0.17-0.59)	0.30 (0.13-0.42)	0.22 (0.17-0.42)	0.22 (0.18-0.36)	0.79

α univariate quantile regression

7.5. Discussion

We observed 19% to 39% reduction in the concentration of trans-placental transfer GBS surface proteins antibodies in HIV-infected compared to HIV-uninfected mother-newborn dyads. Low antibody GMT in HIV-infected mothers together with reduced efficiency of trans-placental transfer of antibodies resulted in lower antibody GMT in HEU infants compared to HIV-unexposed infants. The results of this study are similar to that reported for antibody against *H. influenzae* type b, *B. pertussis,* tetanus and *S. pneumoniae* in HIV-infected compared to HIV-uninfected mother-newborn dyads (104).

A reduction in trans-placental transfer of antibodies against GBS capsular polysaccharide for the predominantly invasive serotype Ia, II, III and V has been noted previously (106). Maternally derived serotype specific capsular antibodies against these invasive serotypes play an important role in providing protection to neonates (176). It is therefore likely that the impediment of materno-foetal antibody transfer against GBS surface proteins together with antibodies against GBS CPS during intrauterine HIV exposure may in part contribute to the increased risk of developing invasive GBS disease in infants.

GBS surface proteins Sip and BibA, have been previously reported to confer protection in newborn pups challenged with a lethal dose of GBS, when born to mice dams immunised with the respective protein (51,211). The virulence and immunological role of the other evaluated GBS proteins are unknown as these proteins were identified computationally; however, these proteins have emerged in other studies as potential GBS virulence factors. Using subtractive DNA hybridisation, gbs1356 and gbs0393

were shown to be homologous to a family of adhesion proteins that play a role in binding of GBS to host epithelial cells (214).

IgG_1, primarily induced by protein antigens, is efficiently transported across the placenta compared to IgG_2 induced by CPS. Therefore, maternal immunisation using a protein-based vaccine might result in higher trans-placental transfer of antibody concentrations to provide protection against invasive GBS disease in newborns (107). Considerations, however, should be taken in selecting highly immunogenic proteins antigens that are capable of crossing the placenta and provide protection to both the mother and the infant. Antibodies to the highly conserved proteins such as Sip, expressed by all GBS isolates, BibA, existing in three different allelic forms (51), and gbs2106 protein were efficiently transferred across the placenta in the absence of maternal HIV infection.

The prevalence of GBS colonisation in HIV-infected women is similar to HIV-uninfected women, including for countries where there is a higher burden of HIV infection (235), including observed in our study population. Antibody GMT did not differ by colonisation status or colonising serotype amongst HIV-uninfected and HIV-infected mothers for the investigated GBS surface protein antigens. Acquisition of GBS colonisation occurs frequently during pregnancy (235), and therefore difference in IgG level that may result from GBS colonisation cannot be detected using a cross-sectional study (92). Other limitations of the study include use of Polygam as reference serum which does not allow quantification of absolute antibody concentrations comparable to other study populations.

Given that HEU infants are at high risk of invasive GBS due to reduced maternally derived antibodies, increasing maternal antibodies by vaccination has the potential to reduce the risk of invasive GBS disease. HIV-infected women, however, were reported to have lower antibody concentrations specific to CPS following immunisation with a polysaccharide-protein glycoconjugate based vaccine (176). The poor immunogenic response in HIV-infected women may be circumvented by adapting an alternate dosing schedule, dosing strength. Since it appears that the antibody response induced by proteins Sip, gbs2106, gbs1356, gbs0393 and Foldase are attenuated by HIV-infection, this might contribute to the increased susceptibility to invasive GBS disease in HEU infants. Further investigations are required to assess the association of antibody to these proteins in relation to risk for invasive GBS disease in neonates.

Chapter Eight

Association of Natural Induced Antibodies against Group B Streptococcus Surface Proteins and Invasive Disease in Neonates and Early Infancy

8.1 Introduction

There were approximately 6.3 million deaths reported in children ≤ 5 years of age in 2013, with 44% of the cases occurring during the neonatal period (1). GBS is the leading cause of neonatal pneumonia, septicaemia and meningitis, with a case fatality ratio between 2-20% (1,2,37,38). The use of intrapartum antibiotic prophylaxis (IAP) following universal or risk based screening of GBS in pregnant women has proven effective in reducing EOD by 70-90% in high income countries (6,247). Such a strategy, however, is unlikely to be feasible in low-income countries due to cost and logistical constrains.

Alternatively, maternal vaccination for prevention of invasive GBS disease is supported by studies reporting reduced risk of neonates developing invasive GBS disease when born to mothers with high antibody concentration against GBS capsular polysaccharides (CPS) (9–14). Phase I/II clinical trials have shown that such a vaccine is safe and immunogenic when administered to pregnant women (15–17). The immunogenicity of these CPS vaccines were enhanced by conjugating them to immunogenic protein carriers. Given that serotype Ia, Ib, II III and V account for >80% of neonatal GBS disease, substituting the conjugate protein with a conserved immunogenic GBS protein may further increase serotype coverage and thus confer broad-spectrum, including serotype-independent protection.

In this chapter we investigated the association of maternal antibody titres to GBS surface proteins BibA, Sip, Foldase, gbs2106, gbs1356 gbs0393, gbs1539 and gbs0392 and invasive GBS disease in neonates born to GBS colonised women, irrespective of serotype.

8.2 Study population

We retrospectively analysed serum samples from mothers and young infants (<90 days age) previously enrolled in a case-control study investigating the association of capsular antibody to serotype-specific invasive GBS disease (14). Briefly, infants <90 days of age with GBS cultured from either blood, cerebrospinal fluid or other normally sterile sites were enrolled into the study, and their mothers. Healthy young infants without invasive GBS disease were enrolled as the control group, matched for maternal HIV status, maternal age (±2.5 years), gestational age (≥37 weeks or ± 2 weeks for preterm), and age of disease onset in the cases (<6 days for EOD or ±14 days for LOD). Lower vaginal and rectal swabs were collected form the mothers at enrolment to determine GBS colonisation and the colonising serotypes.

8.3 Statistical analysis

The data was analysed using STATA software (version 13.0 Stata-Corp, Tx USA) and GraphPad Prism (version 5.03 GraphPad Software Inc, California USA). IgG antibody titres occurring in both mothers and infants were $\log_{10}$ transformed for parametric statistical comparisons and reported as geometric mean titres (GMT, U/mL). Student's t-test was used to compare the differences in IgG antibody titres between colonised women of healthy infants (controls) and those with invasive GBS disease (cases); and similarly so in the infants. Confounding variables were adjusted using multivariate regression. χ^2, Fischer's exact test and logistic regression were used to determine maternal antibody titres associated with protection against invasive GBS disease in infants. P-value < 0.05 was considered statistically significant.

8.4 Results

We included 182 women colonised with GBS in the recto-vaginal tract. Of those, 66 were mothers to infants who developed invasive GBS disease (cases: 36 EOD and 30 LOD) and 116 mothers of healthy infants (matched controls: 52 EOD and 64 LOD), Table 8.1. The distribution of colonising GBS serotypes differed between case and control mothers, with the predominant serotypes being Ia (40.9% vs 37.9%) and III (39.4% vs 28.4%), p=0.042, Table 8.1. The frequency of premature rupture of amniotic membrane (PROM; ≥18h) was higher among women of cases (16.7%) compared to controls (6.0%, p<0.001), Table 8.1. Other risk factor characteristics including maternal HIV status, premature delivery and birth weight were similar between cases and controls.

Table 8.1 Demographic characteristics of group B Streptococcus colonised mothers of asymptomatic infants (controls) and infants with invasive GBS disease (cases).

	Cases (n=66)	Controls (n=116)	P value
Maternal			
Median age. years (range)	27.5 (16.2-39.7)	27.3 (16.1-39.7)	0.98
Median parity (range)	1 (0-5)	1 (0-6)	0.32
Median gravidity (range)	2 (1-5)	2 (1-7)	0.16
PROM≥18 h	11 (16.7%)	7 (6.0%)	<0.001
Colonising serotype			0.042
Ia	27 (40.9%)	44 (37.9%)	
Ib	3 (4.5%)	4 (3.4%)	
II	1 (1.5%)	16 (13.8%)	
III	26 (39.4%)	33 (28.4%)	
IV	1 (1.5%)	0	
V	7 (10.6%)	14 (12.1%)	
Co-colonised[ǂ]	1 (1.5%)	5 (4.3%)	
HIV-infected. n	35 (53.0%)	51 (44.0%)	0.24

HIV-infected Median CD4. cells/µL (IQR)	n=33 387 (280-559)	n=50 384 (265-519)	0.81
Infant			
Gestational age (weeks)	39.1 (28.0-40.2)	38.6 (36.3-40.2)	0.82
preterm	22 (33.3%)	33 (28.4%)	
term	44 (66.6%)	83 (71.6%)	
Birth weight grams (IQR)	2887.5 (2140.0-3200.0)	3002.5 (2645.0-3400.0)	0.053
<2500	19 (28.8%)	23 (19.8%)	
≥2500	47 (71.2%)	93 (80.2%)	
Invasive GBS disease onset			0.21
EOD	36 (54.5%)	52 (44.8%)	
LOD	30 (45.5%)	64 (55.2%)	

PROM = premature rupture of membrane

ǂ colonised with more than one GBS serotype,

Antibodies against GBS surface proteins

Significantly lower IgG GMT against Sip protein were detected in women of cases (164.94 U/mL) compared to those of controls (218.90 U/mL, p=0.019), Table 8.2. Maternal antibody titres against the remaining GBS surface proteins were similar between mothers of cases and controls, Table 8.2. IgG GMT (U/mL) measured in

infants' sera were lower in cases compared to controls for proteins Sip (54.49 vs 104.36, p<0.001), Foldase (51.16 vs 79.78, p=0.025), gbs2106 (54.35 vs 85.76, p=0.022) and gbs0393 (94.68 vs 140.43, p=0.029), Table 8.3.

Table 8.2 IgG antibodies geometric mean titres (GMT, U/mL) against group B Streptococcus surface proteins in colonised women of infants with invasive disease (cases) or healthy controls.

Protein	Case (n=66) IgG GMT (95% CI)	Control (n=116) IgG GMT (95% CI)	P value[α]
BibA	257.43 (187.03-354.33)	222.55 (184.00-269.17)	0.41
Sip	164.94 (132.03-206.04)	218.90 (192.57-248.82)	0.019
Foldase	199.72 (149.95-266.02)	240.86 (202.90-285.92)	0.23
gbs2106	160.90 (133.30-194.21)	184.96 (156.12-219.12)	0.31
gbs0393	436.38 (337.76-563.78)	436.84 (375.31-508.45)	0.97
gbs1356	218.82 (168.58-284.02)	236.22 (201.39-277.06)	0.53
gbs1539	406.18 (317.72-519.27)	432.55 (361.08-518.18)	0.54
gbs0392	373.31 (310.90-448.25)	390.59 (332.13-459.34)	0.61

α, adjusted for colonising serotype and PROM≥18h

Table 8.3 IgG antibodies geometric mean titres (U/mL) against group B Streptococcus surface proteins in infants with invasive group B streptococcus disease (cases) or healthy infants delivered by colonised mothers (controls).

Protein	Case (n=66) IgG GMT (95% CI)	Control (n=116) IgG GMT (95% CI)	P value[α]
BibA	79,14 (55,56-112,74)	94,28 (74,55-119,22)	0.51
Sip	54,49 (40,76-72,86)	104,36 (88,19-123,49)	<0.001
Foldase	51,16 (37,24-70,29)	79,78 (64,64-98,45)	0.025
gbs2106	54,35 (40,68-72,62)	85,76 (68,73-107,00)	0.022
gbs0393	94,68 (72,05-124,41)	140,43 (115,71-170,44)	0.029
gbs1356	46,13 (33,08-64,32)	65,06 (52,55-80,56)	0.088
gbs1539	70,42 (50,03-99,14)	97,59 (77,60-122,71)	0.11
gbs0392	77,13 (59,63-99,75)	87,40 (71,75-106,46)	0.45

α, adjusted for colonising serotype and PROM≥18 h.

Antibody titres associated with EOD and LOD

Antibody titres in mothers and infants were further stratified based on EOD and LOD cases and their respective controls groups. No statistcal difference was observed for IgG GMT between mothers of EOD case and controls, Figure 8.1. Mothers of infants who developed LOD had significantly lower IgG GMT (U/mL) against protein Sip (170.35 [95% CI: 120.93-239.97]) compared to those of controls (255.64 [95% CI: 218.36-299.29], p=0.029), Figure 8.2. Similarly, IgG GMT against gbs2106 was lower in mothers of infants with LOD (133.42 [95% CI: 100.54-177.06]) compared to those of controls (205.25 [95% CI:165.81-254.08], p=0.018), Figure 8.2.

Infants who developed EOD had significantly lower IgG GMT (U/mL) compared to control infants for Sip (65.48 vs 145.43, p<0.001), Foldase (50.45 vs 109.87, p=0.005), gbs0393 (106.29 vs 221.81, p=0.006) and gbs1356 (45.34 vs 94.52, p=0.01), Figure 8.2. Similarly, infanst with LOD had lower IgG GMT (U/mL) compared to control infants for Sip (43.72 vs 79.69, p=0.040) and gbs2106 (30.46 vs 65.35, p=0.003), Figure 8.2.

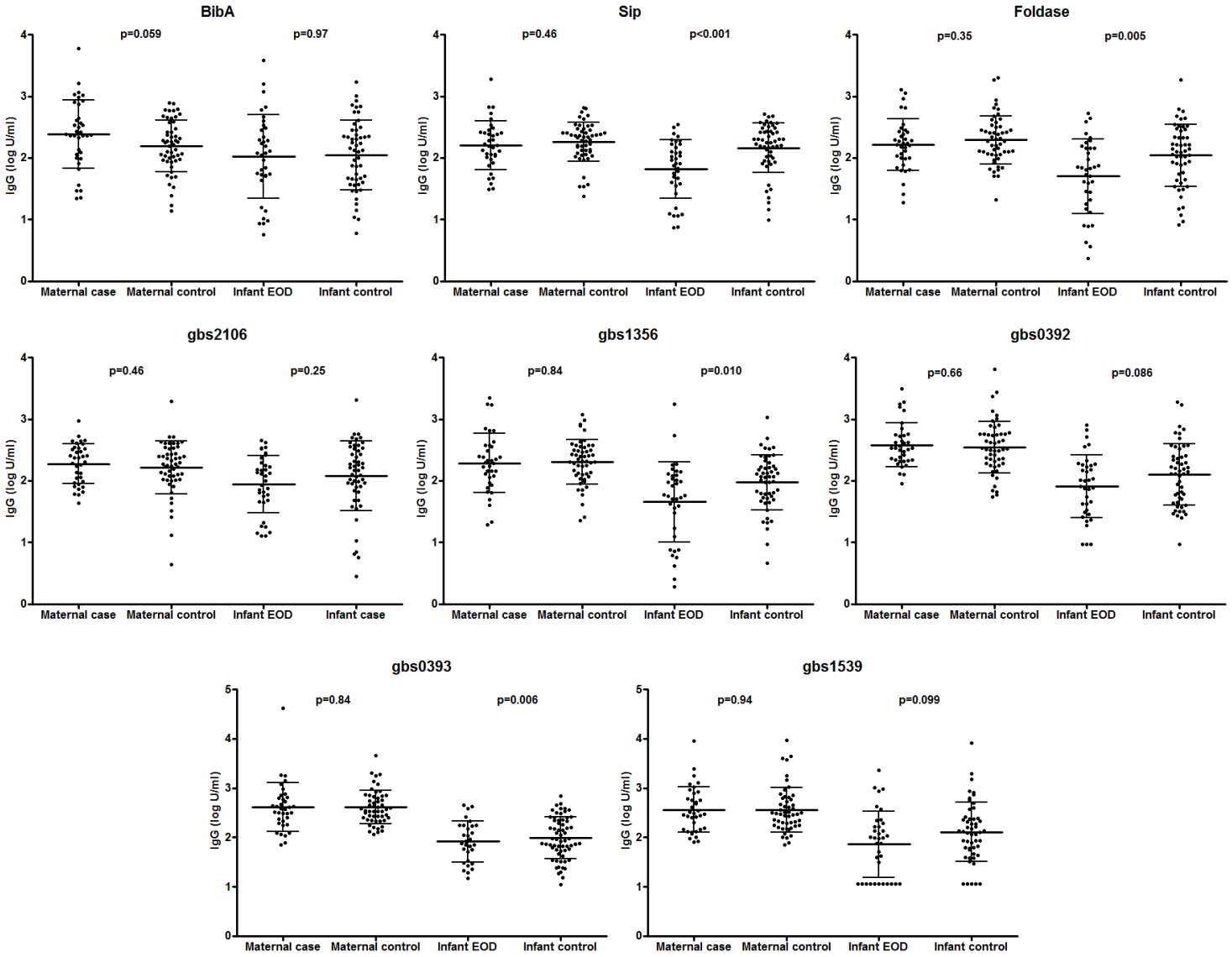

Figure 8.1 IgG antibody titres against group B Streptococcus surface protein in colonised mothers and their respective infants who either remained healthy or who developed early-onset GBS disease (EOD), p-value was adjusted for maternal colonising serotype and premature rupture of membrane >18 h.

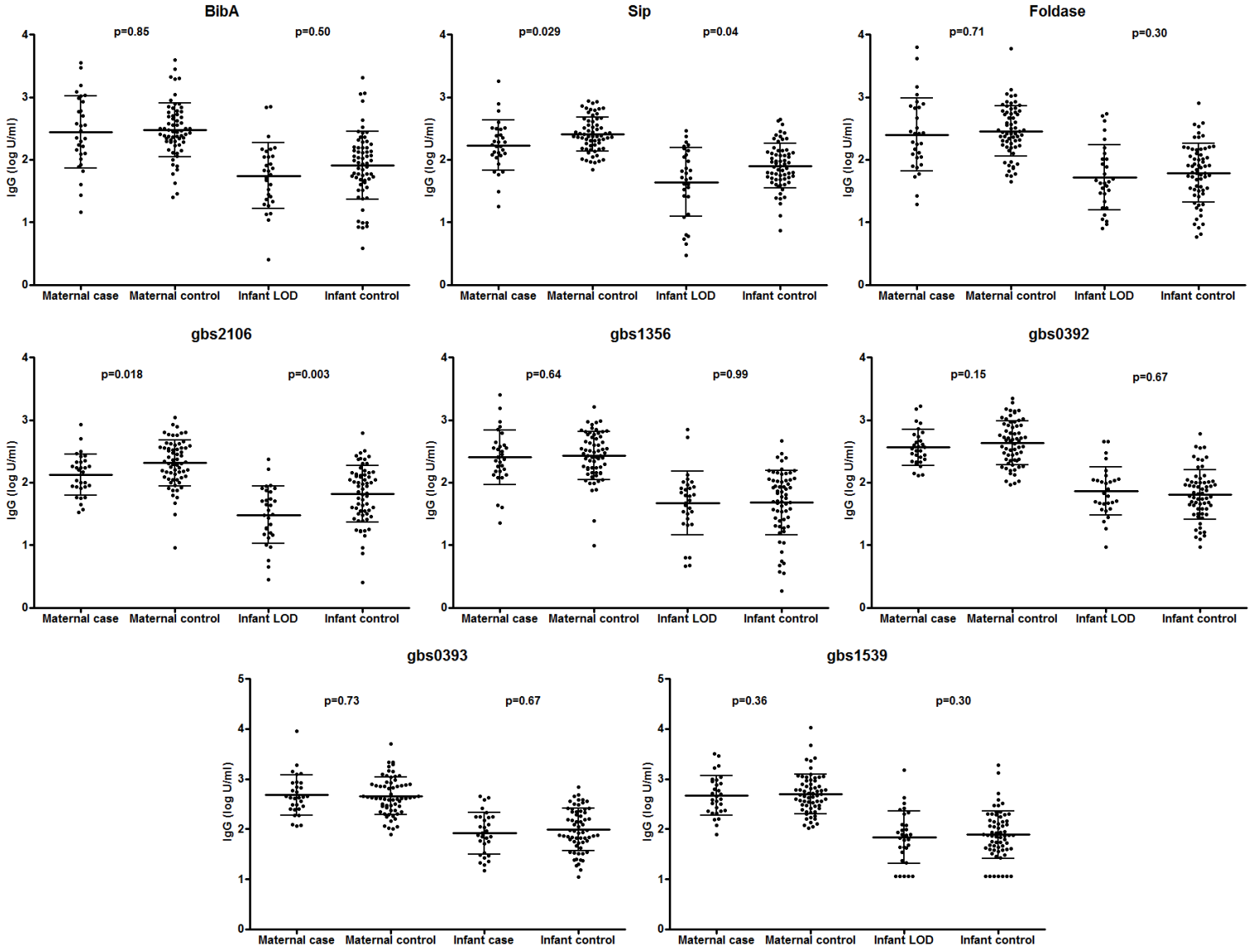

Figure 8.2 IgG antibody titres against group B Streptococcus surface protein in colonised mothers and infants and their respective infants who either remain healthy or who developed late onset GBS disease (LOD), p-value was adjusted for maternal colonising serotype and premature rupture of membrane >18 h.

Maternal antibody levels associated with protection against invasive GBS disease

Given that antibody titres against GBS surface proteins Sip, Foldase, gbs2106, gbs1356 and gbs0393 differed depending on invasive GBS disease status, antibody levels in mothers associated with reduced odds of delivering an infant that develop invasive GBS disease were determined for these proteins. Using the reverse cumulative distribution, the lowest maternal antibody titre against Sip associated with reduced likelihood of having an infant that develops invasive GBS disease was 150 U/mL (OR = 0.44, p=0.01). Increasing maternal antibodies against Sip was associated with decreasing odds of their infants developing invasive GBS disease, Figure 8.4 and Table 8.4.

IgG GMT ≥200 U/mL for Foldase protein were observed to occur in 42.4% (28/66) of mothers with infants that developed invasive GBS disease, significantly less compared to 59.5% (69/116) maternal control counter parts. Increasing maternal antibodies against Foldase, however, was not associated with reduced odds of invasive GBS disease in infants, Figure 8.3 and Table 8.4. Similarly, 68.2% (45/66) women with infants that developed invasive disease had antibody titres ≥99 U/mL for gbs2106 protein, significantly less compared to 83.6% (97/116) mother of controls (OR=0.42, p=0.016), Figure 8.3 and Table 8.4. No association was observed for maternal antibodies against gbs1356 and gbs0393 with reduced odds of delivering infants who develop invasive GBS disease, Figure 8.3 and Table 8.4.

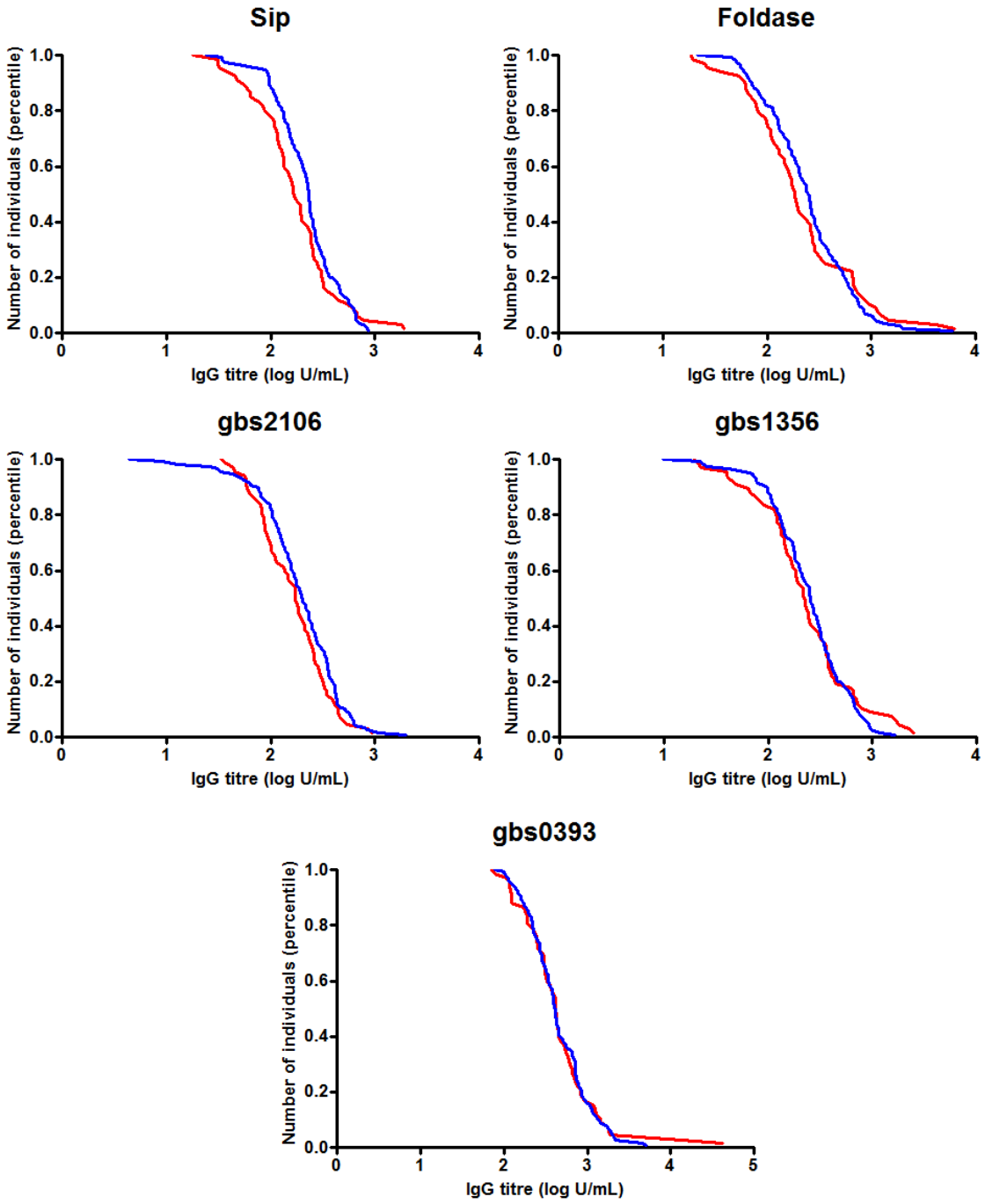

Figure 8.3 Reverse cumulative plots of IgG titre (U/mL) against group B Streptococcus (GBS) surface protein antigens in mothers who delivered either infants who remained healthy (blue) or those who developed invasive GBS disease (red).

Table 8.4 IgG antibody titre threshold against group B Streptococcus surface proteins in colonised mothers who delivered infant who were either healthy or developed invasive GBS disease.

IgG levels (U/mL)	Case (n=66)	Control (n=116)	OR (95% CI)	P value[α]
Sip				
≥100	51 (77.3%)	102 (87.9%)	0.47 (0.21-1.04)	0.059
≥150	36 (54.5%)	85 (73.3%)	0.44 (0.23-0.83)	0.01
≥200	25 (37.9%)	70 (60.3%)	0.40 (0.22-0.75)	0.004
Foldase				
≥100	49 (74.2%)	94 (81.0%)	0.67 (0.33-1.39)	0.28
≥200	28 (42.4%)	69 (59.5%)	0.50 (0.27-0.93)	0.027
≥300	18 (27.3%)	42 (36.2%)	0.66 (0.34-1.28)	0.22
gbs2106				
≥99	45 (68.2%)	97 (83.6%)	0.42 (0.21-0.86)	0.016
≥100	45 (68.2%)	94 (81.0%)	0.50 (0.25-1.01)	0.050
≥200	14 (21.2%)	38 (32.8%)	0.55 (0.27-1.12)	0.097
Gbs0393				
≥100	64 (97.0%)	115 (99.1%)	0.28 (0.025-3.13)	0.27
≥200	52 (78.8%)	96 (82.8%)	0.77 (0.36-1.66)	0.51
≥300	45 (68.2%)	76 (655%)	1.13 (0.59-2.15)	0.71
Gbs1356				
≥70	57 (86.4%)	110 (94.8%)	0.35 (0.12-1.02)	0.04
≥100	54 (81.8%)	101 (87.1%)	0.67 (0.29-1.53)	0.34
≥300	24 (36.4%)	46 (39.7%)	0.87 (0.47-1.62)	0.66

α, p value was determined using either χ^2 or Fisher's exact test.

In vitro expression of proteins on the surface of GBS

The expression of Sip, gbs2106, gbs1356 and gbs0393 on the surface of GBS were assessed by flow cytometry. Using the GBS strains from which the protein genes were cloned, Sip, gbs2106 and gbs0396 were shown to be expressed on the surface of GBS, with ≥6-fold higher antibody binding in antigen specific sera compared to pre-immunisation sera (control), Figure 8.4. Control sera in rabbits immunised with either Sip or gbs1356 had a high binding signal relative to unstained bacterial cells. This reduced the resolution power to infer expression of antigens on the surface of GBS (appendix 10.6). Antibody binding on the surface of GBS was 6-fold higher when using Sip specific post-immunisation sera despite the high binding signal of the relative control sera, and therefore was used to assess expression of Sip on GBS clinical isolates together with gbs2106 and gbs0393.

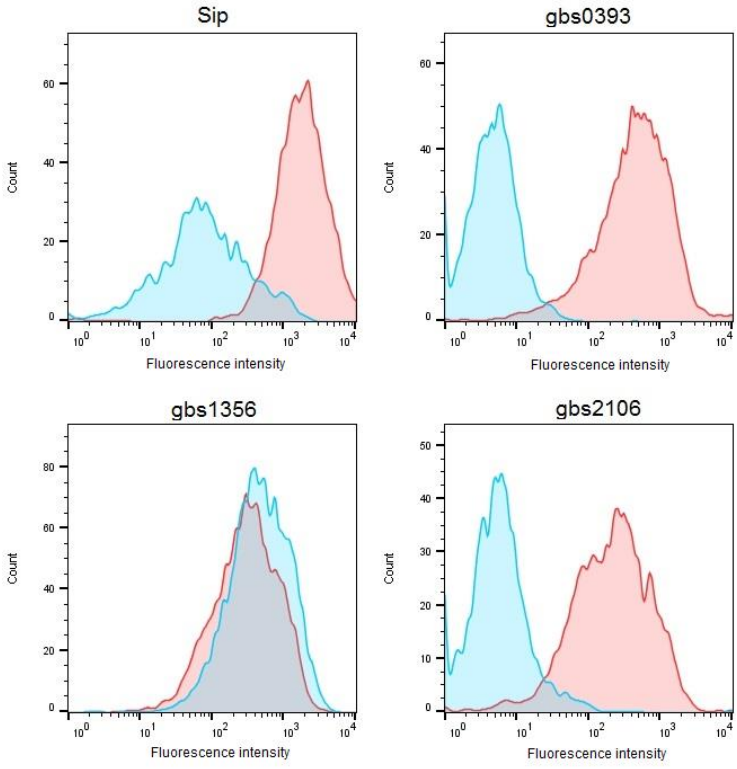

Figure 8.4 Expression of proteins on the surface of group B Streptococcus (serotype III NEM316 strain). Binding of antibodies using antigen specific rabbit sera (red) compared to the corresponding pre-immunisation sera (blue) was detected by flow cytometry to access expression of each protein on the surface of GBS.

GBS isolates from mothers who delivered infants ≥37 weeks of gestation age were assessed for surface expression of proteins Sip, gbs2106 and gbs0393. The demographic characteristics were similar between mothers of cases and controls whose colonising isolates were analysed, Table 8.5. Of the 82 GBS isolates screened (39 from cases and 43 from control), Sip was expressed in 77.3%, 64.7% and 69.8%% GBS strains isolated from mothers of EOD, LOD and control infants respectively, Figure 8.5. Gbs2106 protein was expressed by all GBS strains isolated from mothers of EOD and LOD infants. Gbs0393 protein was expressed in 95.5%, 82.4% and 86.0% in GBS strains isolated from mothers of EOD, LOD and control infants, Figure 8.5.

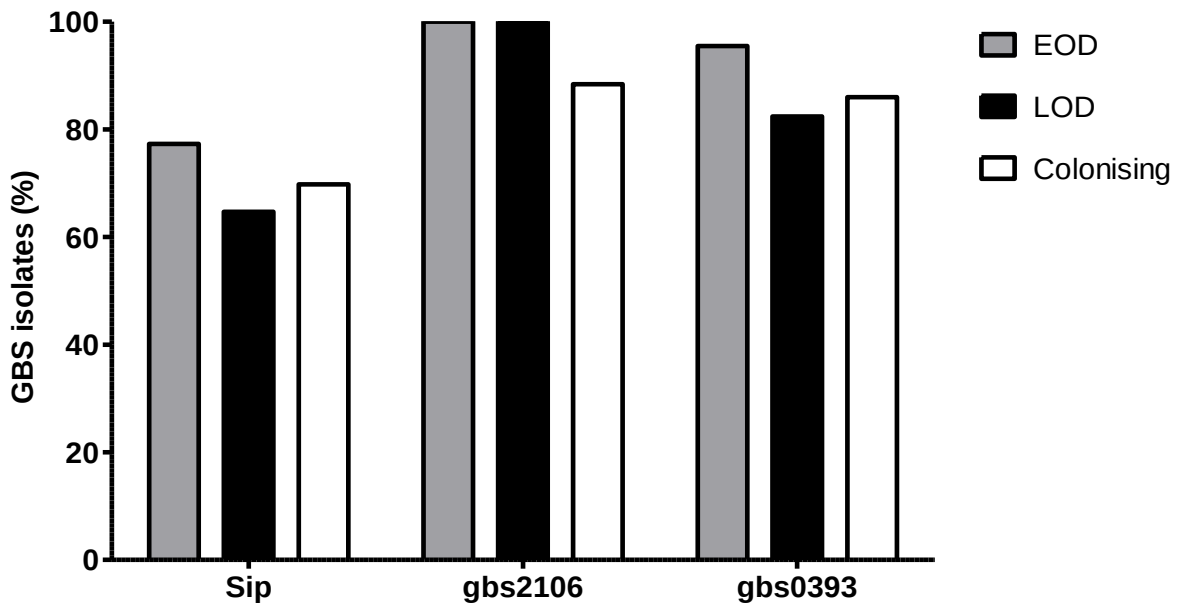

Figure 8.5 Distribution of surface proteins on group B Streptococcus isolates from mothers of infants who remained healthy (white) or those that developed EOD (grey) and LOD (black).

Table 8.5 Demographic characteristics for mothers colonised with group B Streptococcus isolates expressing either Sip, gbs2106 or gbs0393 on the surface.

	Sip		**gbs2106**		**gbs0393**	
	Cases (n=28)	**Controls (n=30)**	**Cases (n=39)**	**Controls (n=38)**	**Cases (n=35)**	**Controls (n=37)**
Median Age (range)	25.8 (19.4-36.8)	25.1 (18.3-35.8)	27.5 (19.4-36.8)	25.3 (18.3-33.5)	26.8 (19.4-36.8)	25.9 (18.3-35.8)
Median Parity (range)	1 (0-3)	0 (0-3)	1 (0-3)	0 (0-3)	1 (0-3)	0 (0-3)
Median Gravidity (range)	2 (1-5)	1.5 (1-5)	2 (1-5)	2 (1-5)	2 (1-5)	2 (1-5)
PROM≥18 h	5 (17.9%)	1 (3.3%)	5 (12.8)	2 (5.3%)	5 (14.3%)	2 (5.4%)
serotype						
Ia	14 (50.0%)	13 (43.3%)	17 (43.6%)	14 (36.8%)	17 (48.6%)	17 (45.9%)
Ib	2 (7.1%)	1 (3.3%)	2 (5.1%)	2 (5.3%)	2 (5.7%)	2 (5.4%)
II	1 (3.6%)	0	1 (2.6%)	1 (2.6%)	1 (2.9%)	1 (2.7%)
III	6 (21.4%)	11 (36.7%)	14 (35.9%)	16 (42.1%)	10 (28.6%)	12 (32.4%)
V	5 (17.9%)	5 (16.7%)	5 (12.8%)	5 (13.2%)	5 (14.3%)	5 (13.5%)
HIV-infected	13 (46.4%)	10 (33.3%)	20 (51.3%)	12 (31.6%)	19 (54.3%)	12 (32.4%)
Median Gestation age (IQR)	40.0 (38.5-40.6)	39.3 (38.5-40.5)	40.0 (39.0-40.5)	40.0 (39.0-40.4)	40.0 (38.6-40.6)	39.5 (39.0-40.3)
Median Birth weight, kg (IQR)	2.96 (2.80-3.24)	3.08 (2.86-3.45)	3.04 (2.84-3.38)	3.11 (2.92-3.50)	3.01 (2.80-3.39)	3.11 (2.93-3.45)

In vitro protein expression and protective antibody titres

Antibody levels against Sip, gbs2106 and gbs0393 were compared between women of cases and controls colonised with GBS expressing the respective protein. IgG GMT against gbs2106 were lower in case mothers (160.89 [95%CI: 125.02-207.05]) compared to control mothers (248.65 [95% CI: 197.41-313.19], p=0.019), Figure 8.6. The odds of invasive GBS disease in the infant was lower for mothers carrying GBS strain expressing gbs2106 with antibody levels ≥100 (OR=0.11 [95% CI: 0.02-0.54], p=0.002). No statistical difference was observed when comparing IgG GMT between case and control women colonised with GBS strains expressing Sip (202.26 [95% CI:138.64-295.07] vs 268.05 [95% CI: 205.02-350.47], p=0.099) and gbs393 (490.58 [95% CI: 327.28-735.36] vs 370.46 [95% CI: 289.25-474.47], p=0.30), however, antibody titres among mothers of controls was higher than those of cases for Sip, Figure 8.6. Antibody titres against Sip and gbs2106 in infants with EOD or LOD were significantly lower compared to their respective controls, Figure 8.7.

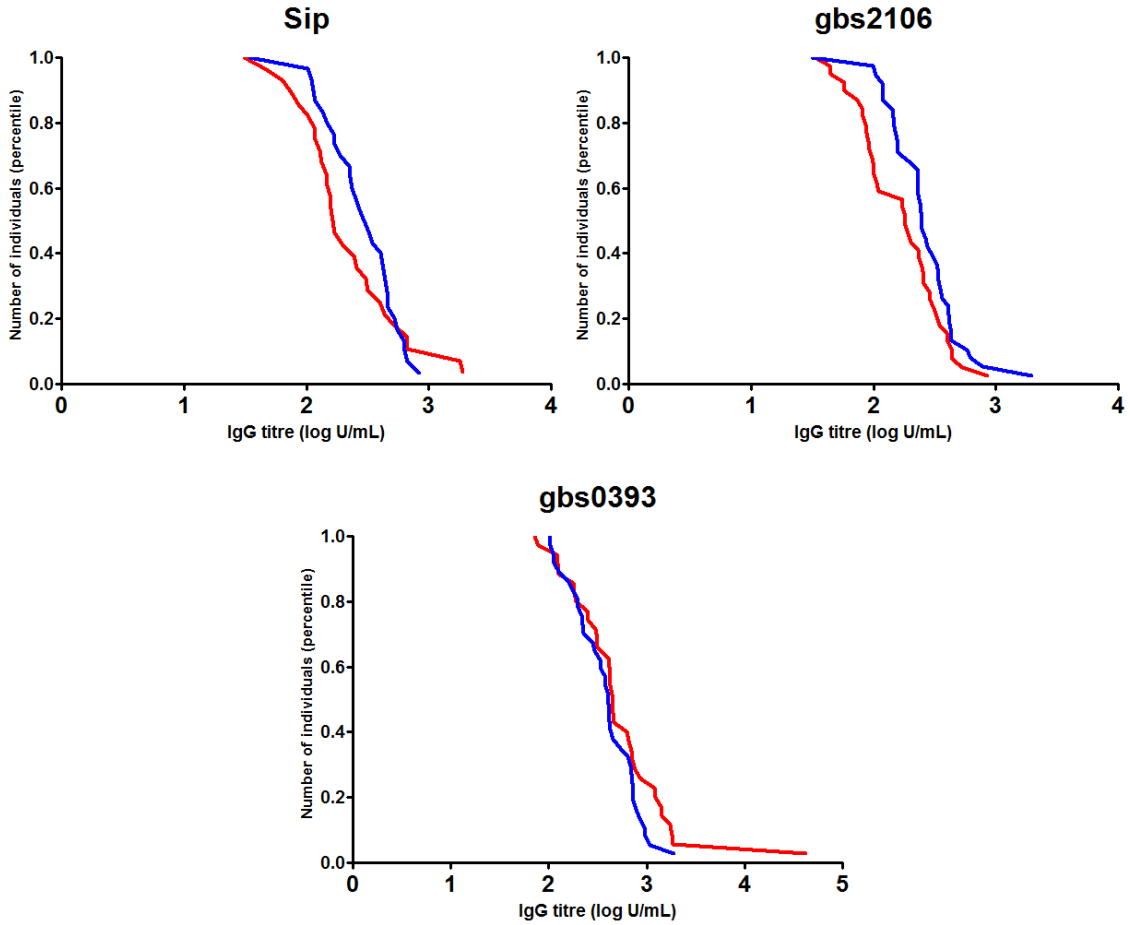

Figure 8.6 Reverse cumulative distribution of antibody titres against Sip, gbs2106 and gbs0393 proteins in group B Streptococcus (GBS) colonised women who had infant (<90 days of age) that either remained healthy (blue) or developed invasive GBS disease (red).

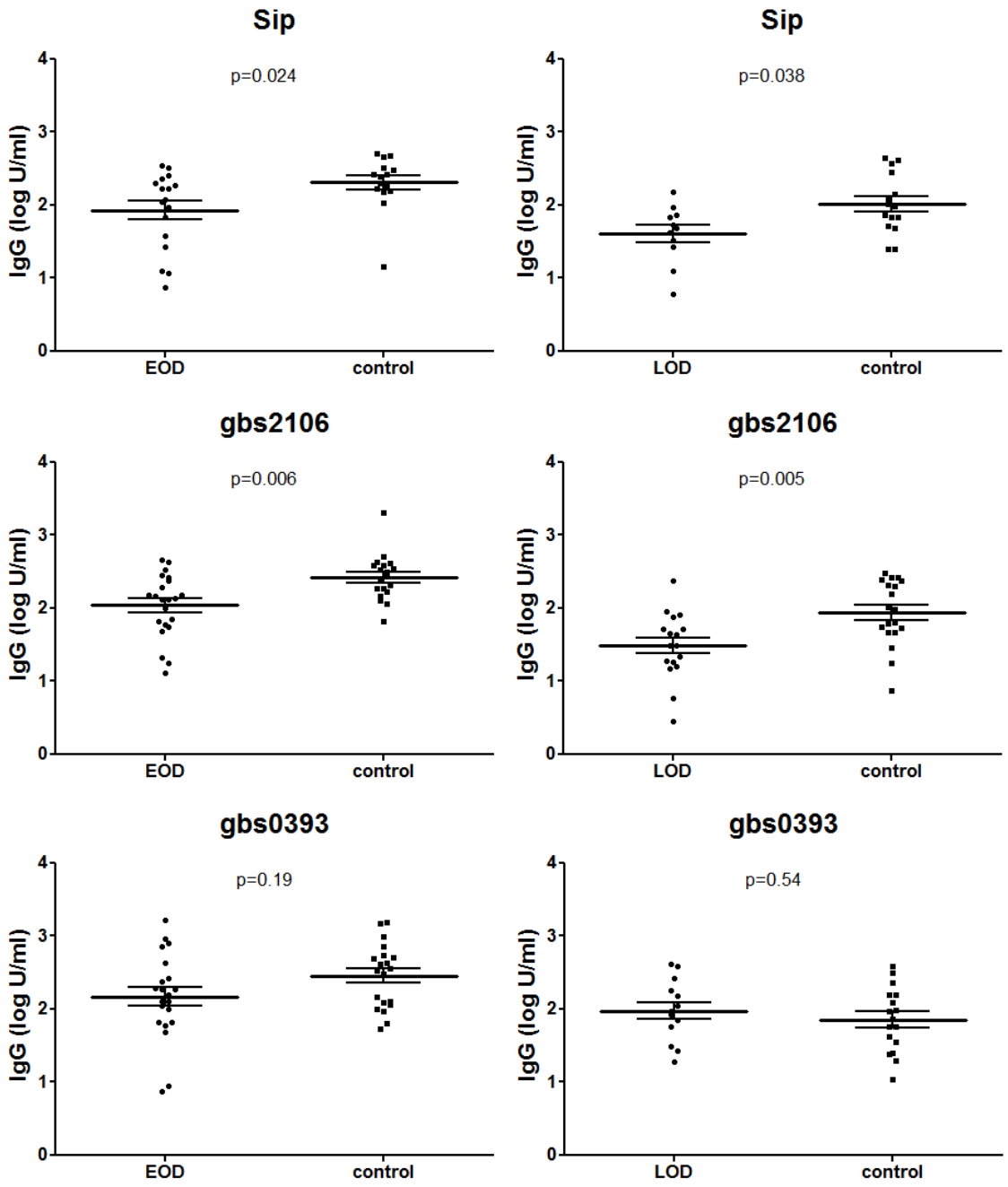

Figure 8.7 Comparison of IgG against GBS surface in infants born to mothers colonised with GBS stain expressing the respective protein between those who developed EOD or LOD and their corresponding controls.

8.5 **Discussion**

GBS continues to be the leading infectious cause of neonatal morbidity and mortality (2). The low incidence of GBS disease requires a large sample populations to prove efficacy of a vaccine and ultimately impeding vaccine licensure (248). An alternative pathway sought to expedite vaccine licensure is to establish maternal antibody levels associated with reduced risk of invasive GBS disease in infants. In this study we show that increasing maternal antibodies against GBS surface proteins Sip and gbs2106 result in reduced odds of developing invasive GBS disease in infants. These results suggest a possible contribution of these antigens in protecting against invasive GBS disease.

Infants born to mothers with low circulating antibodies against GBS capsular polysaccharides have an increased risk of developing invasive GBS disease. Protective antibody thresholds specific for CPS of serotype Ia, III, V and VIII have been reported, with variation among the studies due to lack of standardisation of the assay methods used (11–14,18,249).

We report here that maternal antibodies ≥150 U/ml for GBS surface proteins Sip reduces the odds of delivering infants with invasive GBS by 66%. Infants who developed EOD or LOD in turn had lower antibody titres against Sip compared to controls. Sip has been previously shown to confer protection to mice pups born to dams immunised with Sip (94,212). Therefore, increasing maternal antibodies by vaccination may protect against both EOD and LOD. *In vitro* expression of Sip on the surface of GBS was detected in 70.7% of GBS isolates analysed, however, it has been reported that Sip is a highly conserved protein expressed in all GBS proteins (250). The lower rate of detection of Sip in our isolates may be due to the low sensitivity of our assay

caused by the high background exhibited in the pre-immunisation sera. This may have resulted in underestimation of antibody protection conferred by Sip proteins.

Women carrying GBS strains expressing gbs2106, with antibody titres ≥100U/ml, reduced the odds of their infants developing invasive GBS disease by 90%. Infants who developed EOD and LOD had lower antibody titres against gbs2106. Furthermore, gbs2106 was expressed in 93.9% of the GBS isolates analysed and occurred in all of EOD and LOD cases, thus highlights the antigen's role during GBS pathogenesis.

The characteristics of an ideal GBS vaccine candidate is that the antigen (i) must be highly immunogenic, (ii) antigen specific antibodies must efficiently cross the placenta, (iii) confer protection to both the mother and infant, (iv) antibodies must persist throughout infancy and (iv) possess a broad-spectrum target against disease causing GBS serotypes. Using the ANTIgenome technique, Sip and gbs2106 were shown to be highly immunogenic GBS proteins (96). Furthermore, in the absence of maternal HIV-infection, both proteins were efficiently transferred across the placental as discussed in the previous chapter. Manning et al. reported that antibodies against Sip in infants persist throughout infancy with a halve life of 43.9 days being detected up until 6 months of age (89). In this study, antibodies specific to Sip and gbs2106 were capable of binding to the surface of 70% and 89% GBS isolates irrespective of serotype. Moreover, increasing maternal antibodies were associated with reduced odds of delivering infants that develop invasive GBS disease. Therefore, Sip and gbs2106 fulfil most if not all criteria for an ideal GBS vaccine warranting inclusion as future vaccine candidates. A standardised assay, however, is required for evaluating serological surrogate markers correlated with protection against invasive GBS disease that would be comparable to other study populations.

Chapter Nine

Concluding Remarks

9.1 Conclusion

In this study we used reverse vaccinology techniques to identify GBS surface proteins that could possibly be considered as vaccine candidates. Using robust computational algorithms, 7 immunogenic proteins predicted to be localised on the surface of GBS were recombinantly produced and subjected to serological testing together with virulence proteins, BibA, FbsA and gbs0233 provided by Vulneva Gmb Austria.

Increased susceptibility to invasive GBS disease beyond infancy was explored by comparing serum IgG titres between HIV-infected and HIV-uninfected children. Our findings were that HIV-infection attenuated antibody response against capsular polysaccharides and surface proteins Sip and gbs2106, which could play a role in susceptibility to GBS disease. Other factors such as immune-exhaustion as a result of chronic HIV infection may also contribute to GBS susceptibility as demonstrated by the inverse relationship observed between $CD4^+$ lymphocyte count and antibody levels amongst HIV infected children, indicative of hypergammaglobulinemia.

Recto-vaginal GBS colonisation in pregnant women is a strong risk factor of invasive GBS disease in infants. A high GBS acquisition rate of 27.9% was reported to occur during the last trimester of pregnancy in our study setting. Here, we show that GBS protein specific antibody titres might be used a relative measure of immunity against GBS acquisition. High antibody titres against gbs0233 and gbs1539 were associated with reduced odds of acquisition of GBS colonisation. Moreover, a longitudinal study evaluating GBS colonisation during pregnancy reported decreasing vaginal carriage overtime. Our data reveal heightened antibody responses against GBS surface proteins following acquisition of GBS colonisation in the vagina, whilst an attenuated antibody

response was observed when colonised at the rectum. Thus, we hypothesised that colonisation at the anorectal tract likely results in immunological tolerance or ignorance of GBS which may favour persistent colonisation.

Maternal HIV infection increases the risk of infants developing invasive GBS disease. The mechanism for this is partly attributable to reduced efficiency to transfer antibodies against GBS surface proteins across the placenta as reported here. Twenty-nine percent of pregnant women in our study population were reported to be HIV infected. The incidence of their infants developing invasive GBS disease was 2-fold greater than infants born to HIV-uninfected women, highlighting an urgent requirement for intervention.

Sero-epidemiological data against GBS capsular polysaccharides suggest that immunisation of women during pregnancy with the major serotypes (Ia, Ib, II, III and V) could reduce 50-90% of invasive GBS cases. Similarly, in this study we show that increasing maternal antibodies against Sip and gbs2106 will reduce 66-90% of invasive GBS disease cases in infants. Sip and gbs216 proteins are highly conserved proteins and have the potential to confer serotype-independent protection. Moreover, proteins can be inexpensively produced in large amounts using recombinant cloning techniques and are immunogenic without being conjugated to other molecules. Altogether this information warrants further considerations of these proteins as GBS vaccine candidates.

9.2 Future prospects

The *in silico* method used to select putatively immunogenic peptides in this study was a bioinformatics tool that incorporates hidden Markov models and propensity scale methods (hydrophilicity scores) to identify linear B cell epitopes. Since B cell activation

and subsequent antibody production requires secondary stimulatory signals from T cells to initiate B cell clonal expansion producing both memory and active B cells. The presence of T cell epitopes in protein antigens is essential for mounting an effective immunological response. Hence, *in silico* selection of proteins inclusive of epitopes with high affinity to HLA class II should be incorporated in reverse vaccinology. This will circumvent limitations that arise when using a running window propensity scale to identify B cell epitopes which is biased against peptides with large molecular weight containing discontinuous epitopes.

GBS asymptomatically colonises epithelial cells of the lower gastrointestinal and genitourinary tract of women. Transition to pathogenic infection entails utilisation of virulence factors that either interact with components of the host extracellular matrix (invasion) or evade immunological recognition, Figure 9.1. In this study we observed that acquisition of GBS colonisation at the vagina induced a heightened antibody response compared to acquisition of colonisation in the rectum. Given that colonisation of the anorectal tract is perceived as the requisite for colonisation in the vagina, these data suggest that differential protein expression may occur when colonising the vaginal tract. Thus, future studies should investigate the transcriptomic profile of GBS depending on the site where colonisation occurs. This information may highlight antigens involved at different pathogenic stages that are essential vaccine targets.

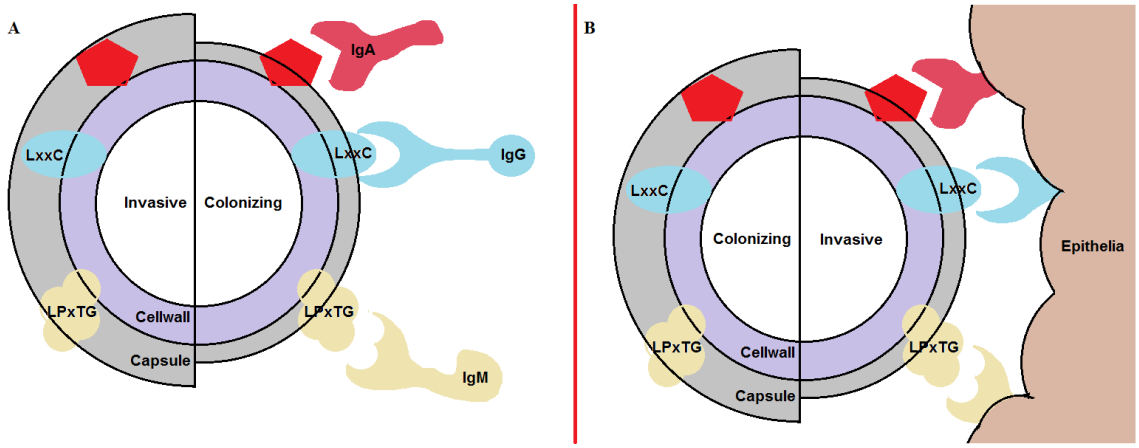

Figure 9.1 Pictorial depiction of group B Streptococcus postulated mechanisms for host invasion and immune evasion. A, thick capsular polysaccharide masks surface proteins to avoid opsonisation and B, capsular polysaccharide synthesis decreases during adhesion and invasion by binding to of host epithelial cells surface markers.

In this study, women who were colonised with GBS throughout pregnancy had high antibodies against GBS surface proteins compared to women who cleared GBS colonisation. Recto-vaginal colonisation of GBS during pregnancy occurs either in a persistent, transient or intermittent manner and mechanisms for this are poorly understood. Adherence of GBS strains to either the epithelial cells of the genitourinary tract or the gastrointestinal tract are dependent on both the colonising serotype and GBS proteins expressed on the surface (251). GBS strains of serotype III have an adherence capacity higher than that of serotype Ia, and with those expressing *alp* genes encoding alp2 and Rib proteins having a higher propensity to adhere to epithelial cells of the lower gastrointestinal tract (251). Moreover, persistence of GBS in the genital tract is mediated by strains with a higher affinity for cervical epithelial cells compared to the ones in the vaginal cells (252). Therefore, establishment of persistent GBS colonisation is dependent on an intricate interaction among innate immunity, adaptive immunity, site of colonisation, and the colonising serotype together with surface proteins expressed on

GBS strains. Therefore, complementary functional assays that measure killing activity of antibodies induced by proteins are essential in establishing immunity associated with clearance of GBS colonisation.

Chapter Ten

Appendices

10.1 Ethics approval letters

This thesis entails retrospective use of archived human serum samples to measure antibody titres specific to GBS surface proteins and capsular polysaccharides. Prior to enrolment on the original studies, signed informed consent was obtained from all study participants and from legal guardians in cases where minors were to be enrolled. Table 10.1 lists the study cohorts from which serum samples were taken. Use of the sera for this research thesis was approved by the Human Research Ethics Committee and the approval letters are included below.

To evaluate *in vitro* surface expression of GBS proteins, white New Zealand female rabbits were immunised with purified recombinant peptides to generate protein specific antibodies. The Animal Ethics Screening Committee (AESC) of the University of Witwatersrand approved the use of animals for generating polyclonal antibodies against GBS surface proteins Sip, gb2106, gbs0393 and gbs1356. The approval letter is attached below with AESC approval number:2015/05/20/B.

Table 10.1 Description of study cohorts used in fulfilment of study objects approved by the Human Research Ethics Committee (HREC).

HIV-uninfected and HIV–infected children (HREC number: M121019)

Original study	Age	Sample size	Description
Durability of antibody responses to an investigational 9-valent PCV (HREC #M970916)	4-7 years	145	HIV-uninfected and HIV-infected children who were participants of a phase 3 PCV efficacy trial.
GBS non-colonised and colonised pregnant women (HREC number: M12019)			
GBS correlates of protection trial (HREC #M090937)	16-42 years	661	Pregnant women between 20-37+ weeks of gestation age who were enrolled in a study to determine antibodies against capsular polysaccharides correlated to prevention and/or clearance of GBS colonisation.

HIV-uninfected and HIV-infected mother newborn dyads (HREC number: M140902			
Effects of maternal HIV infection on trans-placental transfer of GBS antibodies (HREC #M120905)	Infants: <24h Mothers: 18-42 yeas	164	Maternal and cord blood taken in HIV-uninfected and HIV-infected pregnant women ≥37 week of gestation age.
Infants GBS disease status (HREC number: M140902)			
Clinical and Immunological epidemiology of invasive GBS in South Africa: A case control study (HREC #M120963)	Infants: ≤ 90 days Mother: 16-40 years	183	GBS colonised pregnant women who delivered infants that subsequently developed invasive GBS disease (cases) or infants that remained health

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Sonwabile Dzanibe

CLEARANCE CERTIFICATE	**M121019**
PROJECT	Natural Immune Responses to Streptococcal Surface Protein Antigens
INVESTIGATORS	Mr Sonwabile Dzanibe.
DEPARTMENT	Microbiology & Infectious Disease/Pathology
DATE CONSIDERED	26/10/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 26/10/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof S Madhi

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

R14/49 Mr Sonwabile Dzanibe

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140902

NAME: (Principal Investigator) Mr Sonwabile Dzanibe

DEPARTMENT: School of Pathology
Respiratory and Meningeal Pathogens Research Unit

PROJECT TITLE: Natural Induced Antibodies against Group B Sreptococcus Surface Proteins and Association with Colonization in in Women and Invasive Disease in Newborns

DATE CONSIDERED: 03/10/2014

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Dr PV Adrian and Prof S Madhi

APPROVED BY: _____
Professor Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 22/11/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

_____ _____
Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/05/20/B

APPLICANT: Mr S S Dzanibe

SCHOOL: School of Pathology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Generation of anti-GBS surface protein antibodies in rabbits to measure the degree of surface exposure of these antigens on the cell wall

Number and Species

4 White New Zealand Female Rabbits

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/05/26. This approval remains valid until 2017/06/14.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: D.A. Gray (Chairperson, AESC) Date: 30th June 2015

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: (Registered Veterinarian) Date: 30 June 2015

cc: Supervisor: Dr P V Adrian
Director: CAS

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10.2 *In silico* predicted GBS surface proteins

Table 10.2 provides the full list of GBS proteins in NEM316 strain that were predicted to be either lipoproteins or cell wall anchored proteins.

Table 10.2 Extracellular localisation of group B streptococcus NEM316 proteins classified according to their predicted sub-cellular localisation.

Lipo**p**roteins	Cell wall Proteins
hypothetical protein gbs0086	hypothetical protein gbs0391
hypothetical protein gbs0113	hypothetical protein gbs0392
hypothetical protein gbs0144	hypothetical protein gbs0393
hypothetical protein gbs0184	cell wall surface anchor family protein
lipoprotein	hypothetical protein gbs0451
hypothetical protein gbs0233	hypothetical protein gbs0470
hypothetical protein gbs0397	hypothetical protein gbs0479
hypothetical protein gbs0419	hypothetical protein gbs0619
hypothetical protein gbs0440	cell wall surface anchor family protein
hypothetical protein gbs0473	hypothetical protein gbs0629
hypothetical protein gbs0727	hypothetical protein gbs0632
hypothetical protein gbs0778	hypothetical protein gbs0721
hypothetical protein gbs0796	hypothetical protein gbs0722
foldase protein PrsA	hypothetical protein gbs0723
hypothetical protein gbs0918	cell wall surface anchor family protein
hypothetical protein gbs0937	hypothetical protein gbs0966
hypothetical protein gbs0942	hypothetical protein gbs0986
hypothetical protein gbs0957	hypothetical protein gbs0987
hypothetical protein gbs0982	hypothetical protein gbs0988

phosphate ABC transporter phosphate-binding protein	hypothetical protein gbs1087
hypothetical protein gbs1057	hypothetical protein gbs1143
hypothetical protein gbs1216	hypothetical protein gbs1144
laminin-binding surface protein	hypothetical protein gbs1145
hypothetical protein gbs1463	hypothetical protein gbs1288
hypothetical protein gbs1489	streptococcal C5a peptidase
hypothetical protein gbs1501	hypothetical protein gbs1356
hypothetical protein gbs1577	hypothetical protein gbs1403
peptidyl-prolyl cis-trans isomerase, cyclophilin-type	hypothetical protein gbs1474
hypothetical protein gbs1589	hypothetical protein gbs1477
hypothetical protein gbs1632	cell wall surface anchor family protein
hypothetical protein gbs1659	cell wall surface anchor family protein
hypothetical protein gbs1689	amidase family protein
hypothetical protein gbs1810	hypothetical protein gbs1919
hypothetical protein gbs1908	bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase precursor protein
hypothetical protein gbs1926	hypothetical protein gbs2008
hypothetical protein gbs1953	hypothetical protein gbs2022
hypothetical protein gbs2033	
hypothetical protein gbs2106	

Protein domain prediction

Cloning of large proteins is a laborious task with a low success rate. Recognising domains within a protein that are able to adopt a conformation that is similar to the one they fold into when they are part of the complete protein would be ideal in truncating

proteins and cloning their constituents. The isolated domain peptides are easier to clone based on their smaller molecular weight and would subsequently fold into macromolecules that resemble a part of the parent peptide. Native gbs1356 has a molecular weight of 181 KDa, which is processed to 171 KDa by cleaving off the signal peptide and C-terminal residues posterior to LPXTG motif during cell wall anchoring. Cloning and expression of such a large protein was infeasible and therefore distinguishable domains were identified using DLP (domain linker peptide) with a linker peptide predicted between residues 579-679. The smooth value score was 3.472 for the linker peptide; significantly above the 0.0 threshold, highlighting the presence of two domains denoted as gbs1356D1 and gbs1356D2. The prediction of a second linker peptide was rationalised as a false positive since it occurred within 40 residues from the C-terminal (253).

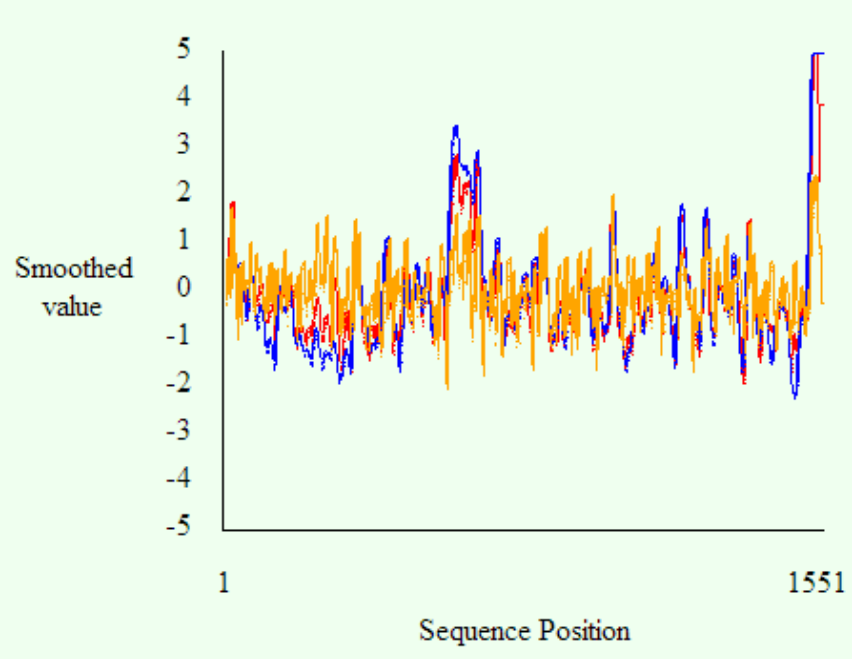

Figure 10.1 *Domain linker prediction for gbs1356 showing linker peptide at between residues 632-732 with a smooth value of 2.879. The second predicted linker peptide is 40 residues from the C-terminal and therefore invalid.*

10.3 Recombinant genes cloning

The genes encoding the selected GBS surface proteins were amplified using the PCR conditions detailed in Methods chapter 3.2. The PCR conditions were optimised for each gene encoding target protein. Table 10.3 describes the optimal temperature and time that was used to clone each respective GBS protein. The 5' and 3' end of the primer set to amplify specific genes were flanked with DNA base pairs homologous to cloning vector regions that enable homologous recombination. The annealing conditions of the PCR reaction did not incorporate the flanking regions required for homologous recombination.

Table 10.3 Polymerase chain reaction conditions for amplifying genes encoding group B Streptococcus surface proteins.

Protein name	Length (bp)*	Annealing temperature (^{o}C)	Elongation Time (min) (1min/1Kb)
Foldase protein	830	50.3	1
Surface immunogenic protein	1221	51.5	1.5
gbs0392	588	54.9	0.5
gbs0393	2517	53.3	2.5
gbs1356-D1	2553	53.1	2.5
Gbs1539	484	53.1	0.5
Immunogenic secreted protein	1368	51.7	1.5
gbs2106	582	52.2	0.5
LysM domain protein	444	53.2	0.5

* gene length excludes the signal peptide region and cell wall anchoring motif

The amplified DNA genes were cloned into pETite plasmid vector (Lucigen, USA) encoding C-terminal His-tag and kanamycin resistant genes, Figure 10.2. The specific primer set for each GBS protein was designed to exclude the signal peptide region together with the surface anchoring motif (LxxC or LPxTG). Amplified genes were inserted into the vector using homologous flanking regions as according to the manufactures instructions.

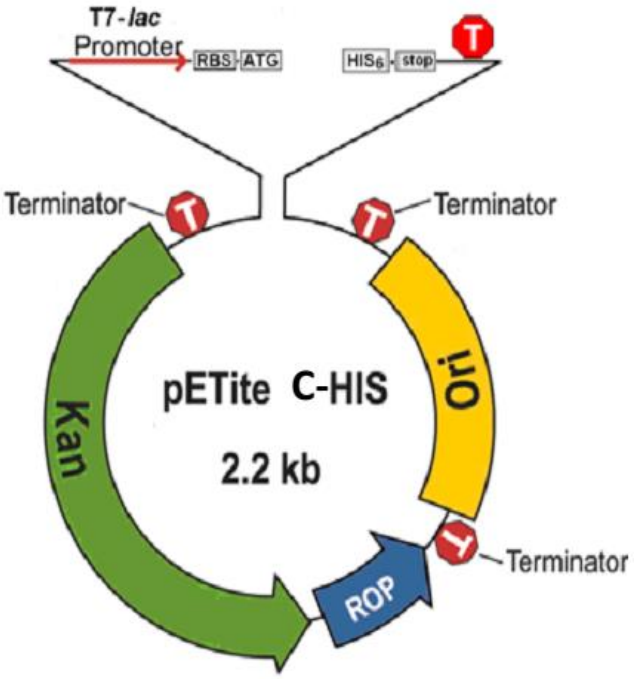

Figure 10.2 Schematic representation of the pETite vector displaying the origin of replication (Ori), repressor of primer (ROP), kanamycin resistant gene (Kan) the ribosomal binding site (RBS) and the 6 histidine tag (HIS_6).

Selected GBS surface protein gene amplicons are listed in Table 10.4. The gene length included the homologous flanking regions and the peptide length includes the recombinant peptide together with the 6×Histidine tag on the C-terminal.

Table 10.4 Group B Streptococcus surface protein amplified gene properties.

Protein name	**Gene length (bp)**	**Vector size**	**Pepetide length (Da)**
Foldase protein (gbs0827)	1010	3063	30,388.41
Sip (gbs0031)	1401	3454	45,419.37
gbs0392	770	2823	20,399.70
gbs0393	2699	4752	91,639.05
gbs1356-D1	2735	4788	94,820.20
Cell wall protein	666	2179	16,921.68
gbs2106	764	2817	20,645.82
LysM domain protein (gbs2107)	626	2679	16,091.58

Protein encoding genes cloned into the pETite vector were sequenced using forward and revers primers to determine correct and in-frame insertion of recombinant gene. The sequencing result of the recombinant GBS genes (query) was aligned with GBS NEM316 strain gene sequences from NCBI nucleotide BLAST database to show correct recombinant gene inserts as shown below (reverse sequence not included).

Gbs0392

```
Score =   994 bits (538),  Expect = 0.0
 Identities = 545/552 (99%), Gaps = 0/552 (0%)
 Strand=Plus/Minus

Query  1      GTCATCCCMGTAGAMCMAGCAGTACCATCTACTGAAGTGGTCACACCACCAAACYCWKTA  60
              |||||||| ||||| | |||||||||||||||||||||||||||||||||| |  ||
Sbjct  81061  GTCATCCCAGTAGACCCAGCAGTACCATCTACTGAAGTGGTCACACCACCAAACTCAGTA
81002

Query  61     GATCCTAGTTTACCAACTGATACAACAGATCCTAGCACCCCAGTAGTACCTGAGAAACTA
120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81001  GATCCTAGTTTACCAACTGATACAACAGATCCTAGCACCCCAGTAGTACCTGAGAAACTA
80942

Query  121    CCAGACACCTCAAAACCAGCAGAGCCAGAAACACCAAAAGAAGAGCTACCAGCGCCAGTG
180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80941  CCAGACACCTCAAAACCAGCAGAGCCAGAAACACCAAAAGAAGAGCTACCAGCGCCAGTG
80882

Query  181    GATCCGACAACTCCATCAGCTGGCAAAGAAGACAAACAAGAAACTGTTCCAGGTCAGGCT
240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80881  GATCCGACAACTCCATCAGCTGGCAAAGAAGACAAACAAGAAACTGTTCCAGGTCAGGCT
80822

Query  241    GAAACGCCTaaagaagaagtaaaacctgaaaaccctaagtctagtgaagacaagaaaaaa
300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80821  GAAACGCCTAAAGAAGAAGTAAAACCTGAAAACCCTAAGTCTAGTGAAGACAAGAAAAAA
80762

Query  301    gaagaaCTTGTCCCAGATAATGTTTCAAAAGACATTATTGACAAGGTGGATACCACTACA
360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80761  GAAGAACTTGTCCCAGATAATGTTTCAAAAGACATTATTGACAAGGTGGATACCACTACA
80702

Query  361    GGTGAAGTGGTCTTTAAACCGATTCCAGTCACACCAGACAAAACAGTTATCGGTACTAAG
420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80701  GGTGAAGTGGTCTTTAAACCGATTCCAGTCACACCAGACAAAACAGTTATCGGTACTAAG
80642

Query  421    GACAGTCAGGTGATTATTCGTGACCAWGCAGGCCGTACACAAGTCGTTACCCCTGAGAGC
480
              |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  80641  GACAGTCAGGTGATTATTCGTGACCAAGCAGGCCGTACACAAGTCGTTACCCCTGAGAGC
80582

Query  481    CTTGGTGGCCACGTTAATCCTAATGGAACCGTTACACTTAAAGATGCTACAGGTGCTGAA
540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80581  CTTGGTGGCCACGTTAATCCTAATGGAACCGTTACACTTAAAGATGCTACAGGTGCTGAA
80522

Query  541    AAGACGCTCCCA  552
              ||||||||||||
Sbjct  80521  AAGACGCTCCCA  80510
```

Gbs0393

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Score =  4567 bits (2473),  Expect = 0.0
 Identities = 2477/2481 (99%), Gaps = 0/2481 (0%)
 Strand=Plus/Minus

Query  56     GCTGTTACMMCCACACCTGCAGTTAATCATGCGACAACTACTGATGCACAAGCTGATTTA
115
              ||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80140  GCTGTTACAACCACACCTGCAGTTAATCATGCGACAACTACTGATGCACAAGCTGATTTA
80081

Query  116    GCTAATCAAACACAAGCTGTTAAAGATGTTACTGCAAAAGCACAAGCTAATACACAAGCT
175
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80080  GCTAATCAAACACAAGCTGTTAAAGATGTTACTGCAAAAGCACAAGCTAATACACAAGCT
80021

Query  176    ATTAAAGATGCTACTGCTGAAAATGCAAAAATTGATGCTGAAAACAAAGCAGAGGCAGAG
235
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80020  ATTAAAGATGCTACTGCTGAAAATGCAAAAATTGATGCTGAAAACAAAGCAGAGGCAGAG
79961

Query  236    CGTGTTGCAAAAGAAAACAAGGAAGGTCAAGCAGCCGTAGATGCACGTAACAAAGCAGGT
295
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79960  CGTGTTGCAAAAGAAAACAAGGAAGGTCAAGCAGCCGTAGATGCACGTAACAAAGCAGGT
79901

Query  296    CAAGCAGCCGTAGATGCACGTAATAAAGCGAAACAGCAAGCGCAAGACGATCAAAAAGCA
355
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79900  CAAGCAGCCGTAGATGCACGTAATAAAGCGAAACAGCAAGCGCAAGACGATCAAAAAGCA
79841

Query  356    AAAATTGATGCTGAAAACAAAGCAGAGTCTCAACGTGTAAGTCAGTTAAATGCACAAAAT
415
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79840  AAAATTGATGCTGAAAACAAAGCAGAGTCTCAACGTGTAAGTCAGTTAAATGCACAAAAT
79781

Query  416    AAAGCAAAAATTGACGCAGAAAATAAAGATGCGCAAGCTAAAGCAAATGCGACTAATGCA
475
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79780  AAAGCAAAAATTGACGCAGAAAATAAAGATGCGCAAGCTAAAGCAAATGCGACTAATGCA
79721

Query  476    CAATTACAAAAAGATTATCAAGCTAAATTAGCGGAAATCAAATCAGTTGAAGCTTATAAT
535
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79720  CAATTACAAAAAGATTATCAAGCTAAATTAGCGGAAATCAAATCAGTTGAAGCTTATAAT
79661

Query  536    GCAGGTGTACGTCAACGTAATAAAGATGCACAAGCTAAAGCAGATGCGACTAACGCACAG
595
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79660  GCAGGTGTACGTCAACGTAATAAAGATGCACAAGCTAAAGCAGATGCGACTAACGCACAG
79601

Query  596    TTACAAAAAGACTATCAAGCTAAATTAGCACTTTATAATCAAGCTCTAAAAGCTAAAGCA
655
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Sbjct  79600  TTACAAAAAGACTATCAAGCTAAATTAGCACTTTATAATCAAGCTCTAAAAGCTAAAGCA
79541

Query  656    GAAGCAGATAAACAGTCTATTAATAATGTTGCTTTTGATATCAAAGCTCAAGCTAAAGGT
715
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79540  GAAGCAGATAAACAGTCTATTAATAATGTTGCTTTTGATATCAAAGCTCAAGCTAAAGGT
79481

Query  716    GTTGATAACGCTGAATATGGAAACTCAATCATGACTGCAAAAACTAAACCTGACGGAAGT
775
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79480  GTTGATAACGCTGAATATGGAAACTCAATCATGACTGCAAAAACTAAACCTGACGGAAGT
79421

Query  776    TTCGAGTTTAACCATGATATGATCGATGGTGTGAAGACAATCGGCTATGGGAAATTGACA
835
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79420  TTCGAGTTTAACCATGATATGATCGATGGTGTGAAGACAATCGGCTATGGGAAATTGACA
79361

Query  836    GGTAAAGTTAATCATCATTATGTTGCTAACAAGGATGGCTCTGTGACAGCATTTGTTGAT
895
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79360  GGTAAAGTTAATCATCATTATGTTGCTAACAAGGATGGCTCTGTGACAGCATTTGTTGAT
79301

Query  896    TCTGTCACTCTTTACAAGTACGAGTATCGTAATGTTGCTCAAAATGCTGCTGTTAACCAA
955
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79300  TCTGTCACTCTTTACAAGTACGAGTATCGTAATGTTGCTCAAAATGCTGCTGTTAACCAA
79241

Query  956    AATATTGTATTTAGAGTTTTAACAAAAGATGGTCGTCCTATTTTTGAAAAAGCTCATAAT
1015
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79240  AATATTGTATTTAGAGTTTTAACAAAAGATGGTCGTCCTATTTTTGAAAAAGCTCATAAT
79181

Query  1016   GGTAACAAAACTTTTGCAGAAACTTTAAACAAAACTTTACAACTCAATCTTAAATATGAG
1075
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79180  GGTAACAAAACTTTTGCAGAAACTTTAAACAAAACTTTACAACTCAATCTTAAATATGAG
79121

Query  1076   CTTAAACCACATGCTTCCAGCGGTAACGTCGAAGTCTTTAAGATTCATGATGACTGGGTA
1135
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79120  CTTAAACCACATGCTTCCAGCGGTAACGTCGAAGTCTTTAAGATTCATGATGACTGGGTA
79061

Query  1136   CATGACACACATGGGTCTGCTTTAGTGTCTTATGTTAATAATAATGATGCTGTTCCTAAT
1195
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Sbjct  79060  CATGACACACATGGGTCTGCTTTAGTGTCTTATGTTAATAATAATGATGCTGTTCCTAAT
79001

Query  1196   GTGGTCATCCCAGAACGGCCAACTCCACCAAAGCCAGTGAAAGTTACACCTGAAGCAGAA
1255
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Sbjct  79000  GTGGTCATCCCAGAACGGCCAACTCCACCAAAGCCAGTGAAAGTTACACCTGAAGCAGAA
78941

Query  1256   AAACCAGTACCTGAAAAGCCAGTTGAGCCTAAATTGGTAACGCCTACATTAAAAACTTAT
1315
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Sbjct  78940    AAACCAGTACCTGAAAAGCCAGTTGAGCCTAAATTGGTAACGCCTACATTAAAAACTTAT
78881

Query  1316     ACTCCAGTCAAATTTATTCCGCGAGAATACAAACCAGAACCAATCACCCCTGAGACGTTT
1375
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78880    ACTCCAGTCAAATTTATTCCGCGAGAATACAAACCAGAACCAATCACCCCTGAGACGTTT
78821

Query  1376     ACCCCTGAGAAATTTACTCCAGCTCAACCAAAAGTGAAACCACATGTGTCTATTCCTGAA
1435
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78820    ACCCCTGAGAAATTTACTCCAGCTCAACCAAAAGTGAAACCACATGTGTCTATTCCTGAA
78761

Query  1436     AAGATTAACTACTCAGTTAGTGTTCATCCTGTTTTAGTTCCAGCTGCTAATCCTTCAAAA
1495
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Sbjct  78760    AAGATTAACTACTCAGTTAGTGTTCATCCTGTTTTAGTTCCAGCTGCTAATCCTTCAAAA
78701

Query  1496     GCTGTCATTGATGAAGCAGGTCAATCTGTTAATGGTAAAACCGTATTACCAAATGCAACA
1555
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78700    GCTGTCATTGATGAAGCAGGTCAATCTGTTAATGGTAAAACCGTATTACCAAATGCAACA
78641

Query  1556     TTAGACTATGTTGCTAAACAAAACTTTAGTCAATACAAAGGTATTAAAGCTTCTGCAGAA
1615
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78640    TTAGACTATGTTGCTAAACAAAACTTTAGTCAATACAAAGGTATTAAAGCTTCTGCAGAA
78581

Query  1616     GCGATCGCAAAAGGTTTTGCATTTGTAGATCAACCAAATGAAGCGTTAGCTGAATTGACT
1675
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78580    GCGATCGCAAAAGGTTTTGCATTTGTAGATCAACCAAATGAAGCGTTAGCTGAATTGACT
78521

Query  1676     GTTAAGTCTATCAAAGCATCTAATGGTGATGATGTATCAAGCTTGTTAGAAATGCGTCAT
1735
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78520    GTTAAGTCTATCAAAGCATCTAATGGTGATGATGTATCAAGCTTGTTAGAAATGCGTCAT
78461

Query  1736     GTTTTATCAAAAGATACTTTAGACCAAAAACTTCAATCTCTTATTAAAGAGGCAGGAATT
1795
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78460    GTTTTATCAAAAGATACTTTAGACCAAAAACTTCAATCTCTTATTAAAGAGGCAGGAATT
78401

Query  1796     AGTCCAGTTGGTGAGTTTTACATGTGGACTGCAAAAGATCCACAAGCTTTTTATAAAGCT
1855
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78400    AGTCCAGTTGGTGAGTTTTACATGTGGACTGCAAAAGATCCACAAGCTTTTTATAAAGCT
78341

Query  1856     TATGTTCAAAAAGGACTAGATATCACTTATAATCTATCCTTTAAAATCAAAGCTAACTTT
1915
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78340    TATGTTCAAAAAGGACTAGATATCACTTATAATCTATCCTTTAAAATCAAAGCTAACTTT
78281
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Query  1916   ACTAAAGGTCAAATCAAAAATGGTGTTGCACAGATTGATTTTGGGAATGGATATACAGGT
1975

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Sbjct  78280  ACTAAAGGTCAAATCAAAAATGGTGTTGCACAGATTGATTTTGGGAATGGATATACAGGT
78221

Query  1976   AATATTGTAGTCAATGATGTTACTGTTCCAGAAGTACATAAGGATATACTTGATAAAGAG
2035

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Sbjct  78220  AATATTGTAGTCAATGATGTTACTGTTCCAGAAGTACATAAGGATATACTTGATAAAGAG
78161

Query  2036   GATGGTAAATCAATTAATAATAGTACGGTTAAGTTAGGTGATGAAGTGACCTACAAACTT
2095

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Sbjct  78160  GATGGTAAATCAATTAATAATAGTACGGTTAAGTTAGGTGATGAAGTGACCTACAAACTT
78101

Query  2096   GAAGGATGGGTTGTACCAGCAAACCGTGGTTACGATCTTTTTGAATACAAATTTGTGGAT
2155

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78100  GAAGGATGGGTTGTACCAGCAAACCGTGGTTACGATCTTTTTGAATACAAATTTGTGGAT
78041

Query  2156   CAATTACAACACACACATGATCTTTACTTACGTGATAAAGTGGTCGCTAAAGTTGATGTG
2215

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78040  CAATTACAACACACACATGATCTTTACTTACGTGATAAAGTGGTCGCTAAAGTTGATGTG
77981

Query  2216   ACATTAAAAGATGGTACAGTCATTAAAAAAGGGACTAATTTAGGAGAGTACACAGAAACA
2275

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77980  ACATTAAAAGATGGTACAGTCATTAAAAAAGGGACTAATTTAGGAGAGTACACAGAAACA
77921

Query  2276   GTTTACAACAAAACGACAGGTCATTATGAGCTTGCCTTTAAAAAAGAGTTTTTAGCTAAG
2335

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77920  GTTTACAACAAAACGACAGGTCATTATGAGCTTGCCTTTAAAAAAGAGTTTTTAGCTAAG
77861

Query  2336   GTTTCTCGTGAATCAGAATTTGGTGCAGATGATTTTATTGTAGTTAAACGTATTAAGGCA
2395

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77860  GTTTCTCGTGAATCAGAATTTGGTGCAGATGATTTTATTGTAGTTAAACGTATTAAGGCA
77801

Query  2396   GGTGATGTTTACAATACTGCTGATTTATATGTTAATGGATATAAAGTTAAGTCAGAAGCA
2455

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77800  GGTGATGTTTACAATACTGCTGATTTATATGTTAATGGATATAAAGTTAAGTCAGAAGCA
77741

Query  2456   GTTGTGACTCATACTACTGAGAAATCAAAACCAGTTGAACCACAAAAAGCAACTCCAAAA
2515

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77740  GTTGTGACTCATACTACTGAGAAATCAAAACCAGTTGAACCACAAAAAGCAACTCCAAAA
77681

Query  2516   RCTCCRGCTAAAGGACTGCCA  2536
                ||||  ||||||||||||||
Sbjct  77680  GCTCCAGCTAAAGGACTGCCA  77660
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Gbs1356-D1

```
Score =  4645 bits (2515),  Expect = 0.0
 Identities = 2516/2517 (99%), Gaps = 0/2517 (0%)
 Strand=Plus/Minus

Query  40     GTAGGGAGAACTGTTGCGACAAGTGTACAGACGGAAACGAATCCTGCAACAAATTTAAAA  99
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95303  GTAGGGAGAACTGTTGCGACAAGTGTACAGACGGAAACGAATCCTGCAACAAATTTAAAA
95244

Query  100    GAAAATCAGCCAAGTCCGATTGCGGAACAAAAAGATAGTCTTGCAGCAACCGGTMAATCA
159
              |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  95243  GAAAATCAGCCAAGTCCGATTGCGGAACAAAAAGATAGTCTTGCAGCAACCGGTCAATCA
95184

Query  160    ACTGGAACGGTGACAGTCACTGTTCCACATGATAAGGTGACACAAGCTGTCGATAAGGCA
219
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95183  ACTGGAACGGTGACAGTCACTGTTCCACATGATAAGGTGACACAAGCTGTCGATAAGGCA
95124

Query  220    AAGACCGAAGGAATAAAAGCAGTACAAGACAAGCCGATGGACTTAGGCAATACAGTATCC
279
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95123  AAGACCGAAGGAATAAAAGCAGTACAAGACAAGCCGATGGACTTAGGCAATACAGTATCC
95064

Query  280    GCAGCTGAAACCAGCCAACAACTCAAAAAGGCAGAAGAAGATGCCACAAACCAAACAACA
339
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95063  GCAGCTGAAACCAGCCAACAACTCAAAAAGGCAGAAGAAGATGCCACAAACCAAACAACA
95004

Query  340    ACTATTTCTAAAACTGTTGAAATCTACAAGTCTGATAAAGCAACTTATGAAGCTGAAAAG
399
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95003  ACTATTTCTAAAACTGTTGAAATCTACAAGTCTGATAAAGCAACTTATGAAGCTGAAAAG
94944

Query  400    AAGTGGGTAGAGAAGCGTAATGAAGAGTTAACTGCTGCTTATGATAAGGCAGAACAAACA
459
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94943  AAGTGGGTAGAGAAGCGTAATGAAGAGTTAACTGCTGCTTATGATAAGGCAGAACAAACA
94884

Query  460    GGGACTGGCTTAAACCATTCGGTTGATACGACTGTTTCAGAATTGAAGTCGCAAGACCAA
519
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94883  GGGACTGGCTTAAACCATTCGGTTGATACGACTGTTTCAGAATTGAAGTCGCAAGACCAA
94824

Query  520    AACGCTCATGTGACCGTGAACACACAAACAGTAAAATCAGGAGATGGGACGAGTGTTTCA
579
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Sbjct  94823  AACGCTCATGTGACCGTGAACACACAAACAGTAAAATCAGGAGATGGGACGAGTGTTTCA
94764

Query  580    GGCTATCAGGAGTACGTCAAGTCTGTTGCGGCCATTGATAAGAAGAATAAAGCGAACTTA
639
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94763  GGCTATCAGGAGTACGTCAAGTCTGTTGCGGCCATTGATAAGAAGAATAAAGCGAACTTA
94704
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Query  640    GCTGATTATCGGACTAAGAAACAAGCCGCAGACGCTGTTGTGGCTAAGAATCAACTCATT
699

Sbjct  94703  GCTGATTATCGGACTAAGAAACAAGCCGCAGACGCTGTTGTGGCTAAGAATCAACTCATT
94644

Query  700    CAAAAAGAGAATGAAGCTGGGCTTGCTAAGGCAAAAGCAGAAAATGAAGCGATTGACAGA
759

Sbjct  94643  CAAAAAGAGAATGAAGCTGGGCTTGCTAAGGCAAAAGCAGAAAATGAAGCGATTGACAGA
94584

Query  760    CGGAATAAAGAAGGGCAAAAAGCTGTTGATGAAGCAAATAAGGCTGGGCAAGCCGCAGTA
819

Sbjct  94583  CGGAATAAAGAAGGGCAAAAAGCTGTTGATGAAGCAAATAAGGCTGGGCAAGCCGCAGTA
94524

Query  820    GAGCAAGCGAACCAAGAAAAACAAAAACAGGCCGCAAACCGTGCTTTTGAAATTGCGACA
879

Sbjct  94523  GAGCAAGCGAACCAAGAAAAACAAAAACAGGCCGCAAACCGTGCTTTTGAAATTGCGACA
94464

Query  880    ATTACCAAACGGAATAAAGAAAGAGAAGAAGTCGCAAAGAAAGAAAATGCAGCGATTGAT
939

Sbjct  94463  ATTACCAAACGGAATAAAGAAAGAGAAGAAGTCGCAAAGAAAGAAAATGCAGCGATTGAT
94404

Query  940    GCTTATAATGCGAAAGAATGGATCCGCTATAAGCGGGATTTAGCAAACATCTCAAAAGGG
999

Sbjct  94403  GCTTATAATGCGAAAGAATGGATCCGCTATAAGCGGGATTTAGCAAACATCTCAAAAGGG
94344

Query  1000   GAGGAAGGCTACATTTCAGAAGCCCTTGCGCAGGCTCTAGATTTAAATCATGGCGAACCG
1059

Sbjct  94343  GAGGAAGGCTACATTTCAGAAGCCCTTGCGCAGGCTCTAGATTTAAATCATGGCGAACCG
94284

Query  1060   CAGGTCAAACATGGTGCAGGTACTCGAAATCCAGATCGAATCATTTCAAAGGGAGATGCC
1119

Sbjct  94283  CAGGTCAAACATGGTGCAGGTACTCGAAATCCAGATCGAATCATTTCAAAGGGAGATGCC
94224

Query  1120   ATGTTGGGTGGCTATTCTAACATTCTTGATTCAACGGGTTTCTTTGTCTACAATCACTTT
1179

Sbjct  94223  ATGTTGGGTGGCTATTCTAACATTCTTGATTCAACGGGTTTCTTTGTCTACAATCACTTT
94164

Query  1180   AAAACAGGTGAAACGCTGAACTTTACCTATCAAAATCTGAAGCATGCACGTTTTGATGGG
1239

Sbjct  94163  AAAACAGGTGAAACGCTGAACTTTACCTATCAAAATCTGAAGCATGCACGTTTTGATGGG
94104

Query  1240   AAGAAAATTACAGCCATAACTTATGATATTACCAATCTGGTTTCACCAACTGGAACCAAT
1299
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Sbjct  94103  AAGAAAATTACAGCCATAACTTATGATATTACCAATCTGGTTTCACCAACTGGAACCAAT
94044

Query  1300   GCGGTGCAGTTAGTTGTTCCAAACGACCCAACAGAAGGCTTTATTGCTTATCGCAATGAT
1359
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94043  GCGGTGCAGTTAGTTGTTCCAAACGACCCAACAGAAGGCTTTATTGCTTATCGCAATGAT
93984

Query  1360   GGCGCTGGAAATTGGCGGACAGATAAGATGGAGTTCCGTGTCAAAGCTCGGTATTTCTTA
1419
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93983  GGCGCTGGAAATTGGCGGACAGATAAGATGGAGTTCCGTGTCAAAGCTCGGTATTTCTTA
93924

Query  1420   GAAGACGGTTCACAAGTGACCTTTACTAAAGAAAAACCAGGTGTCTTTACCCACTCGTCA
1479
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93923  GAAGACGGTTCACAAGTGACCTTTACTAAAGAAAAACCAGGTGTCTTTACCCACTCGTCA
93864

Query  1480   CTCAATCATAATGATATTGGGCTTGAATATGTTAAAGACTCATCAGGTAAATTTGTTCCT
1539
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93863  CTCAATCATAATGATATTGGGCTTGAATATGTTAAAGACTCATCAGGTAAATTTGTTCCT
93804

Query  1540   ATTCATGGTTCAAGTGTGCAAGTAACCAATGAGGGCTTAGCCCGTTCGTTAGGTTCAAAC
1599
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93803  ATTCATGGTTCAAGTGTGCAAGTAACCAATGAGGGCTTAGCCCGTTCGTTAGGTTCAAAC
93744

Query  1600   CGAGCGAGTGACTTGAAGTTGCCAGAAGAATGGGATACGACTTCTAGTCGCTATGCTTAT
1659
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93743  CGAGCGAGTGACTTGAAGTTGCCAGAAGAATGGGATACGACTTCTAGTCGCTATGCTTAT
93684

Query  1660   AAAGGAGCGATTGTATCAACAGTCACATCGGGGAATATTTATACCGTAACCTTTGGCCAA
1719
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93683  AAAGGAGCGATTGTATCAACAGTCACATCGGGGAATATTTATACCGTAACCTTTGGCCAA
93624

Query  1720   GGAGATATGCCAACCCAAGTGGGAGGGAAGACCTATTGGTTTGCTTTAAATACTTTGCCA
1779
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93623  GGAGATATGCCAACCCAAGTGGGAGGGAAGACCTATTGGTTTGCTTTAAATACTTTGCCA
93564

Query  1780   GTCGCAAAAACAGTGACTCCCTACAATCCAAAGACTCATGTAAGACCACAGCTGGATCCA
1839
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93563  GTCGCAAAAACAGTGACTCCCTACAATCCAAAGACTCATGTAAGACCACAGCTGGATCCA
93504

Query  1840   GTTCCTGAACCGATAAAAGTTACACCAGAAACTTATACTCCTAAAATCTTTACTCCTGAA
1899
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93503  GTTCCTGAACCGATAAAAGTTACACCAGAAACTTATACTCCTAAAATCTTTACTCCTGAA
93444

Query  1900   AAACCAGTAACCTTTACTCCAAAATCAGTAGAAAAAGTGCCCCAACCTAGTTTGACCTTA
1959
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                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93443  AAACCAGTAACCTTTACTCCAAAATCAGTAGAAAAAGTGCCCCAACCTAGTTTGACCTTA
93384

Query  1960   ACAAAAGTCACACTACCAACAAATCTGAAGCTAGAACCATTACCCAAAGCTCCACAAAAG
2019
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93383  ACAAAAGTCACACTACCAACAAATCTGAAGCTAGAACCATTACCCAAAGCTCCACAAAAG
93324

Query  2020   CCAACCGTTCATTACCACGATTACCTCTTAACCACAACACCCGCTATCGCAAAAGAAGTG
2079
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93323  CCAACCGTTCATTACCACGATTACCTCTTAACCACAACACCCGCTATCGCAAAAGAAGTG
93264

Query  2080   ATGAATGTTGACAAAGTTAATCTTCATGGTAAACAGGTGGCTAAGGATTCCACTGTTATT
2139
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93263  ATGAATGTTGACAAAGTTAATCTTCATGGTAAACAGGTGGCTAAGGATTCCACTGTTATT
93204

Query  2140   TATCCCTTGACAGTAGATGTTTTATCTCCAAATCGTTCCAAGATAACCAGTCTTATCTTT
2199
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93203  TATCCCTTGACAGTAGATGTTTTATCTCCAAATCGTTCCAAGATAACCAGTCTTATCTTT
93144

Query  2200   GAAGATTATCTGCCTGCTGGTTATGCGTTTGATATGACGAAGACACAAGCGGAGAATAGC
2259
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93143  GAAGATTATCTGCCTGCTGGTTATGCGTTTGATATGACGAAGACACAAGCGGAGAATAGC
93084

Query  2260   GACTATGACTTAACCTTTGATAAAAATAAGAACTTTGTGACCTTGAAAGCGAAAGATAGC
2319
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93083  GACTATGACTTAACCTTTGATAAAAATAAGAACTTTGTGACCTTGAAAGCGAAAGATAGC
93024

Query  2320   TTACTTCAAACGTTGAATAAAGAGTTAAACAAGTCTTATCAACTGTCTGCTCCAAAACTT
2379
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  93023  TTACTTCAAACGTTGAATAAAGAGTTAAACAAGTCTTATCAACTGTCTGCTCCAAAACTT
92964

Query  2380   TATGGTTCAGTTCAAAATGATGGGGCGACTTATTCTAATAGCTATAAACTCCTTATTAAC
2439
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92963  TATGGTTCAGTTCAAAATGATGGGGCGACTTATTCTAATAGCTATAAACTCCTTATTAAC
92904

Query  2440   AAGGACACCCCAAACACCTATACGGTTATTTCAAACGTAGTTAGAATTCGGACTCCAGGA
2499
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92903  AAGGACACCCCAAACACCTATACGGTTATTTCAAACGTAGTTAGAATTCGGACTCCAGGA
92844

Query  2500   GACGGTGAAACGACCAGCCGAATCCGGCCTAAAAAGGACAATGAAAATGCGGACGGT  2556
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92843  GACGGTGAAACGACCAGCCGAATCCGGCCTAAAAAGGACAATGAAAATGCGGACGGT  92787
```

Gbs1539

```
Score =   826 bits (447),  Expect = 0.0
 Identities = 447/447 (100%), Gaps = 0/447 (0%)
 Strand=Plus/Minus

Query  1      GCTGATACAAGTGATAAGAATACTGACACGAGTGTCGTGACTACGACCTTATCTGAGGAG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92787  GCTGATACAAGTGATAAGAATACTGACACGAGTGTCGTGACTACGACCTTATCTGAGGAG
92728

Query  61     AAAAGATCAGATGAACTAGACCAGTCTAGTACTGGTTCTTCTTCTGAAAATGAATCGAGT
120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92727  AAAAGATCAGATGAACTAGACCAGTCTAGTACTGGTTCTTCTTCTGAAAATGAATCGAGT
92668

Query  121    TCATCAAGTGAACCAGAAACAAATCCGTCAACTAATCCACCTACAACAGAACCATCGCAA
180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92667  TCATCAAGTGAACCAGAAACAAATCCGTCAACTAATCCACCTACAACAGAACCATCGCAA
92608

Query  181    CCCTCACCTAGTGAAGAGAACAAGCCTGATGGTAGAACGAAGACAGAAATTGGCAATAAT
240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92607  CCCTCACCTAGTGAAGAGAACAAGCCTGATGGTAGAACGAAGACAGAAATTGGCAATAAT
92548

Query  241    AAGGATATTTCTAGTGGAACAAAAGTATTAATTTCAGAAGATAGTATTAAGAATTTTAGT
300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92547  AAGGATATTTCTAGTGGAACAAAAGTATTAATTTCAGAAGATAGTATTAAGAATTTTAGT
92488

Query  301    AAAGCAAGTAGTGATCAAGAAGAAGTGGATCGCGATGAATCATCATCTTCAAAAGCAAAT
360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92487  AAAGCAAGTAGTGATCAAGAAGAAGTGGATCGCGATGAATCATCATCTTCAAAAGCAAAT
92428

Query  361    GATGGGAAAAAAGGCCACAGTAAGCCTAAAAAGGAACTTCCTAAAACAGGAGATAGCCAC
420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92427  GATGGGAAAAAAGGCCACAGTAAGCCTAAAAAGGAACTTCCTAAAACAGGAGATAGCCAC
92368

Query  421    TCAGATACTGTAATAGCATCTACGGGA  447
              |||||||||||||||||||||||||||
Sbjct  92367  TCAGATACTGTAATAGCATCTACGGGA  92341
```

Gbs2106

```
Score =   998 bits (540),  Expect = 0.0
 Identities = 543/546 (99%), Gaps = 0/546 (0%)
 Strand=Plus/Minus

Query  1      GCAGATAAAGTTCGCGTAGCCaaaaaatcaaaaaTGACTAAGGCGACATCTAAAYCWWAA  60
              |||||||||||||||||||||||||||||||||||||||||||||||||||| |  ||
Sbjct  73484  GCAGATAAAGTTCGCGTAGCCAAAAAATCAAAAATGACTAAGGCGACATCTAAATCAAAA
73425

Query  61     GTAGAAGATGTAAAACAGGCTCCAAAACCTTCTCAGGCATCTAATGAAGCCCCAAAATCA
120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73424  GTAGAAGATGTAAAACAGGCTCCAAAACCTTCTCAGGCATCTAATGAAGCCCCAAAATCA
73365

Query  121    AGTTCTCAATCTACAGAAGCTAATTCTCAGCAACAAGTTACTGCGAGTGAAGAGGCAGCT
180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73364  AGTTCTCAATCTACAGAAGCTAATTCTCAGCAACAAGTTACTGCGAGTGAAGAGGCAGCT
73305

Query  181    GTAGAACAAGCAGTTGTAACAGAAAACACCCCTGCTACCAGTCAGGCACAACAAGCTTAT
240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73304  GTAGAACAAGCAGTTGTAACAGAAAACACCCCTGCTACCAGTCAGGCACAACAAGCTTAT
73245

Query  241    GCTGTTACTGAGACAACTTATAGACCTGCTCAACACCAGACGAGTGGCCAAGTATTGAGT
300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73244  GCTGTTACTGAGACAACTTATAGACCTGCTCAACACCAGACGAGTGGCCAAGTATTGAGT
73185

Query  301    AATGGAAATACTGCAGGGGCTATTGGCTCAGCAGCTGCAGCACAAATGGCTGCTGCAACA
360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73184  AATGGAAATACTGCAGGGGCTATTGGCTCAGCAGCTGCAGCACAAATGGCTGCTGCAACA
73125

Query  361    GGAGTCCCTCAGTCTACTTGGGAACATATTATTGCCCGTGAATCAAATGGTAATCCTAAT
420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73124  GGAGTCCCTCAGTCTACTTGGGAACATATTATTGCCCGTGAATCAAATGGTAATCCTAAT
73065

Query  421    GTTGCTAATGCCTCAGGAGCTTCAGGACTTTTCCAAACGATGCCAGGTTGGGGTTCAACA
480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73064  GTTGCTAATGCCTCAGGAGCTTCAGGACTTTTCCAAACGATGCCAGGTTGGGGTTCAACA
73005

Query  481    GCTACAGTTCAGGATCAAGTTAATTCAGCTATTAAAGCTTATCGTGCTCAAGGTTTATCA
540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73004  GCTACAGTTCAGGATCAAGTTAATTCAGCTATTAAAGCTTATCGTGCTCAAGGTTTATCA
72945

Query  541    GCTTGG  546
              ||||||
Sbjct  72944  GCTTGG  72939
```

LysM domain protein (gbs2107)

```
Score =   750 bits (406),  Expect = 0.0
 Identities = 407/408 (99%), Gaps = 0/408 (0%)
 Strand=Plus/Minus

Query  1      ACCGTGAAATCAGGTGATACCTTATCAGCTATTGCTAAAAATCATAAAACTACGGTACAA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74171  ACCGTGAAATCAGGTGATACCTTATCAGCTATTGCTAAAAATCATAAAACTACGGTACAA
74112

Query  61     GAGTTAGTGTCTCTCAATAGTATCAGTAACGCTGATGTCATCAGTATAGGTGATGTTTTA
120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74111  GAGTTAGTGTCTCTCAATAGTATCAGTAACGCTGATGTCATCAGTATAGGTGATGTTTTA
74052

Query  121    AAATTGGATAATTCTAAAGCTAGTCAAGCAGAAGCAAAATCTCAACCAACAATTGAAAAT
180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74051  AAATTGGATAATTCTAAAGCTAGTCAAGCAGAAGCAAAATCTCAACCAACAATTGAAAAT
73992

Query  181    TCAATGAATTCTTCATCAAATTTGAGTTCAAGTGATTCAGCCGCMAAAGAAGAAATAGCT
240
              |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  73991  TCAATGAATTCTTCATCAAATTTGAGTTCAAGTGATTCAGCCGCAAAAGAAGAAATAGCT
73932

Query  241    CGTCGTGAATCAAATGGTAGTTATACTGCACAGAATGGACAATATTATGGAAGATATCAA
300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73931  CGTCGTGAATCAAATGGTAGTTATACTGCACAGAATGGACAATATTATGGAAGATATCAA
73872

Query  301    CTGTCTCAATCTTACCTAAATGGCGACTTATCTCCTGAAAATCAAGAAAAAGTAGCGGAC
360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73871  CTGTCTCAATCTTACCTAAATGGCGACTTATCTCCTGAAAATCAAGAAAAAGTAGCGGAC
73812

Query  361    AATTATGTGGTTTCTCGTTACGGATCTTGGTCGGCAGCGCTATCATTT  408
              |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73811  AATTATGTGGTTTCTCGTTACGGATCTTGGTCGGCAGCGCTATCATTT  73764
```

Foldase PrsA protein (gbs0827)

```
Score =  1465 bits (793),  Expect = 0.0
 Identities = 794/795 (99%), Gaps = 0/795 (0%)
 Strand=Plus/Plus

Query  1      GTTGTTACMATGAAGGGCGACACTATCACAGTCTCTGATTTTTATGATCAAGTAAAAACA  60
              |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66812  GTTGTTACAATGAAGGGCGACACTATCACAGTCTCTGATTTTTATGATCAAGTAAAAACA
66871

Query  61     TCAAAAGCTGCACAACAATCAATGCTTACATTGATCCTCTCACGTGTTTTTGATACACAG
120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66872  TCAAAAGCTGCACAACAATCAATGCTTACATTGATCCTCTCACGTGTTTTTGATACACAG
66931

Query  121    TATGGTGATAAAGTTTCAGATAAAAAAGTATCAGAAGCTTATAATAAGACAGCTAAAGGC
180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66932  TATGGTGATAAAGTTTCAGATAAAAAAGTATCAGAAGCTTATAATAAGACAGCTAAAGGC
66991

Query  181    TATGGTAATTCATTTTCAAGCGCACTTTCACAAGCAGGTTTGACTCCAGAAGGTTACAAA
240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  66992  TATGGTAATTCATTTTCAAGCGCACTTTCACAAGCAGGTTTGACTCCAGAAGGTTACAAA
67051

Query  241    CAACAAATTCGCACAACTATGCTTGTGGAATATGCTGTAAAAGAAGCAGCTAAGAAAGAA
300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67052  CAACAAATTCGCACAACTATGCTTGTGGAATATGCTGTAAAAGAAGCAGCTAAGAAAGAA
67111

Query  301    TTAACAGAAGCAAACTATAAAGAAGCATATAAAAACTATACTCCTGAAACTTCTGTACAA
360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67112  TTAACAGAAGCAAACTATAAAGAAGCATATAAAAACTATACTCCTGAAACTTCTGTACAA
67171

Query  361    GTAATCAAATTGGATGCAGAAGATAAAGCTAAATCTGTCCTTAAAGATGTAAAGGCTGAT
420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67172  GTAATCAAATTGGATGCAGAAGATAAAGCTAAATCTGTCCTTAAAGATGTAAAGGCTGAT
67231

Query  421    GGAGCTGATTTTGCAAAGATTGCAAAAGAAAAAACAACAGCTACTGATAAAAAAGTTGAG
480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67232  GGAGCTGATTTTGCAAAGATTGCAAAAGAAAAAACAACAGCTACTGATAAAAAAGTTGAG
67291

Query  481    TATAAATTTGATTCTGCAGGGACAAGCCTCCCTAAAGAAGTTATGTCAGCAGCCTTTAAG
540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67292  TATAAATTTGATTCTGCAGGGACAAGCCTCCCTAAAGAAGTTATGTCAGCAGCCTTTAAG
67351

Query  541    CTAGATAAAAATGGTGTTTCAGATGTGGTTTCAACGGTTGATTCAACAACTTATAAAACA
600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67352  CTAGATAAAAATGGTGTTTCAGATGTGGTTTCAACGGTTGATTCAACAACTTATAAAACA
67411
```

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Query  601    AGTTACTACATCATTAAAGTAACTGATAAGACAGAGAAAAAATCTGATTGGAAATCTTAC
660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67412  AGTTACTACATCATTAAAGTAACTGATAAGACAGAGAAAAAATCTGATTGGAAATCTTAC
67471

Query  661    AAAAATCGCTTAAAAGAAGTTATTCTTAAGGATAAAACAAGCGATAGATCCTTCCAAAAT
720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67472  AAAAATCGCTTAAAAGAAGTTATTCTTAAGGATAAAACAAGCGATAGATCCTTCCAAAAT
67531

Query  721    AAAGTGATTTCAAAAGCCTTAGAAAAAGCTAACGTTAAGATTAAAGATAAAGCATTTGCA
780
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67532  AAAGTGATTTCAAAAGCCTTAGAAAAAGCTAACGTTAAGATTAAAGATAAAGCATTTGCA
67591

Query  781    GGCATCTTATCACAA  795
              |||||||||||||||
Sbjct  67592  GGCATCTTATCACAA  67606
```

Surface immunogenic protein (gbs0031)

```
Score =  2255 bits (1221),  Expect = 0.0
 Identities = 1221/1221 (100%), Gaps = 0/1221 (0%)
 Strand=Plus/Plus

Query  41     GCACAAGAAACAGATACGACGTGGACAGCACGTACTGTTTCAGAGGTAAAGGCTGATTTG
100
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47593  GCACAAGAAACAGATACGACGTGGACAGCACGTACTGTTTCAGAGGTAAAGGCTGATTTG
47652

Query  101    GTAAAGCAAGACAATAAATCATCATATACTGTGAAATATGGTGATACACTAAGCGTTATT
160
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47653  GTAAAGCAAGACAATAAATCATCATATACTGTGAAATATGGTGATACACTAAGCGTTATT
47712

Query  161    TCAGAAGCAATGTCAATTGATATGAATGTCTTAGCAAAAATAAATAACATTGCAGATATC
220
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47713  TCAGAAGCAATGTCAATTGATATGAATGTCTTAGCAAAAATAAATAACATTGCAGATATC
47772

Query  221    AATCTTATTTATCCTGAGACAACACTGACAGTAACTTACGATCAGAAGAGTCATACTGCC
280
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47773  AATCTTATTTATCCTGAGACAACACTGACAGTAACTTACGATCAGAAGAGTCATACTGCC
47832

Query  281    ACTTCAATGAAAATAGAAACACCAGCAACAAATGCTGCTGGTCAAACAACAGCTACTGTG
340
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47833  ACTTCAATGAAAATAGAAACACCAGCAACAAATGCTGCTGGTCAAACAACAGCTACTGTG
47892

Query  341    GATTTGAAAACCAATCAAGTTTCTGTTGCAGACCAAAAAGTTTCTCTCAATACAATTTCG
400
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47893  GATTTGAAAACCAATCAAGTTTCTGTTGCAGACCAAAAAGTTTCTCTCAATACAATTTCG
47952

Query  401    GAAGGTATGACACCAGAAGCAGCAACAACGATTGTTTCGCCAATGAAGACATATTCTTCT
460
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  47953  GAAGGTATGACACCAGAAGCAGCAACAACGATTGTTTCGCCAATGAAGACATATTCTTCT
48012

Query  461    GCGCCAGCTTTGAAATCAAAAGAAGTATTAGCACAAGAGCAAGCTGTTAGTCAAGCAGCA
520
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  48013  GCGCCAGCTTTGAAATCAAAAGAAGTATTAGCACAAGAGCAAGCTGTTAGTCAAGCAGCA
48072

Query  521    GCTAATGAACAGGTATCACCAGCTCCTGTGAAGTCGATTACTTCAGAAGTTCCAGCAGCT
580
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  48073  GCTAATGAACAGGTATCACCAGCTCCTGTGAAGTCGATTACTTCAGAAGTTCCAGCAGCT
48132

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48192

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48252

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48312

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Sbjct  48373  TTACAAGCGACTGAAGTTAAGAGCGTTCCGGTAGCACAAAAAGCTCCAACAGCAACACCG
48432

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48492

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Sbjct  48553  AGTACATACCGTGCGGGAGATCCAGGTGATCATGGTAAAGGTTTAGCAGTTGACTTTATT
48612

Query  1061   GTAGGTACTAATCAAGCACTTGGTAATAAAGTTGCACAGTACTCTACACAAAATATGGCA
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48672

Query  1121   GCAAATAACATTTCATATGTTATCTGGCAACAAAAGTTTTACTCAAATACAAACAGTATT
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48732

Query  1181   TATGGACCTGCTAATACTTGGAATGCAATGCCAGATCGTGGTGGCGTTACTGCCAACCAC
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Sbjct  48793  TATGACCACGTTCACGTATCA  48813
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10.4 Recombinant protein purification

Overexpressed GBS proteins were purified using Fast Perfomance Liquid Chromatography (ATKA FPLC, GE-Healthcare) and visualised by SDS-PAGE analysis. Figure 10.3 depict the un-purified lysate and the pure protein eluted from the Ni^{2+} affinity column. All proteins were successfully purified, however, gbs0393 and Tgbs1356 partially degraded. Proteins BibA-COH1, BibA-NEM316, FbsA and gbs0233 were kindly provided by Valneva Austria GmbH.

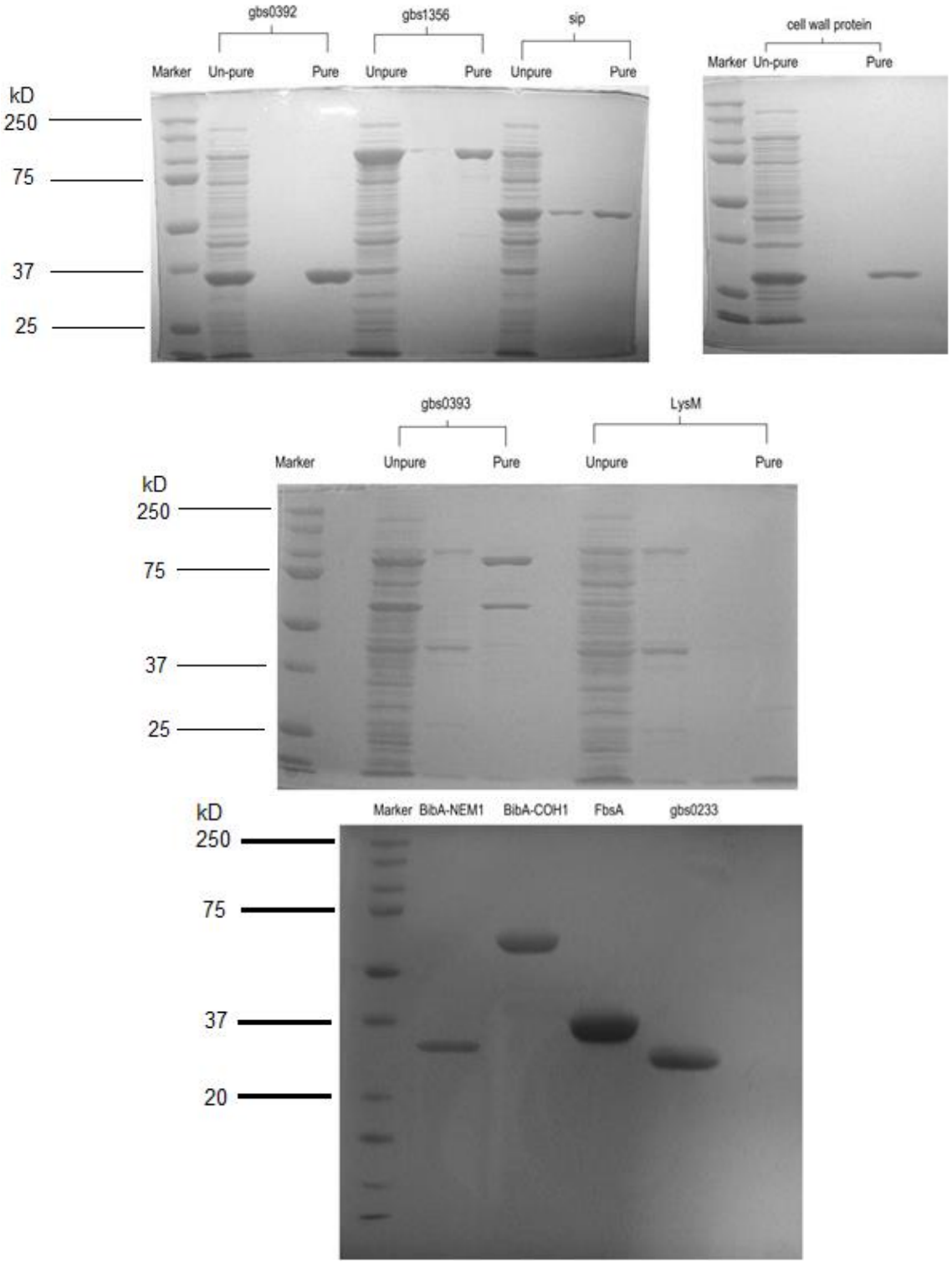


Figure 10.3 SDS-PAGE visualisation of purified group B Streptococcus recombinant proteins

10.5 Multiplex Luminex assay validation and standardisation

Protein specific IgG titres in serum collected from study participants were measured using the Bio-plex 200 Luminex system (BioRad, USA). The method is comparable to an ELISA immunological assay, however, the Luminex system is capable of simultaneous quantification of antibodies targeting different antigens. This is achieved by using a dual laser, flow cytometric principle that identifies fluorescent micro-beads conjugated with the antigen and simultaneously quantifying bound antibodies. Quantification of the bound antibodies is based on an indirect antibody capture immunoassay where a secondary antibody (anti-human IgG) tagged with a fluorophore is used as a signal reporter. Data is measured as the fluorescent intensity relative to antigen-antibody concentration.

This section describes the validation of the multiplex Luminex assay for the quantification of IgG titres specific to GBS surface proteins Sip, BibA-COH1, BibA-NEM316, Foldase, gbs0233 gbs0392, gbs0393, gbs1356, gbs1539 and 2106. The optimal protein concentration for conjugating to the microbeads and the specificity of the reference serum (Polygam) were established for each protein antigen included in the assay. Intra-assay reproducibility was monitored using pooled sera of participants with high IgG titres (high titres control) and those that had low IgG titres (low titres control).

10.5.1 Quantification of optimal protein concentration

To determine the optimal protein concentration for conjugating to the microbeads, antibodies against proteins BibA-COH1, FbsA, Foldase, gbs0233, gbs0392 and gbs0393 were measured using Polygam. The concentrations of the purified recombinant proteins were determined using Pierce® BCA Protein Assay Kit (ThermoScientific, USA)

according to manufacturer's instructions. Proteins were diluted in PBS solution into 5 dilutions of 125 µg/mL, 100 µg/mL, 75 µg/mL, 50 µg/mL and 25 µg/mL protein concentrations. Proteins were coupled to unique micro-beads as described in the methods section 3.4.1. A singleplex assay was performed for each protein dilution using 1:100 Polygam dilutions and fluorescence intensity (FI) was measured, Figure 10.4. Protein concentration of 50 µg/mL was established as the optimal conjugating concentration and used in subsequent Luminex immunoassays.

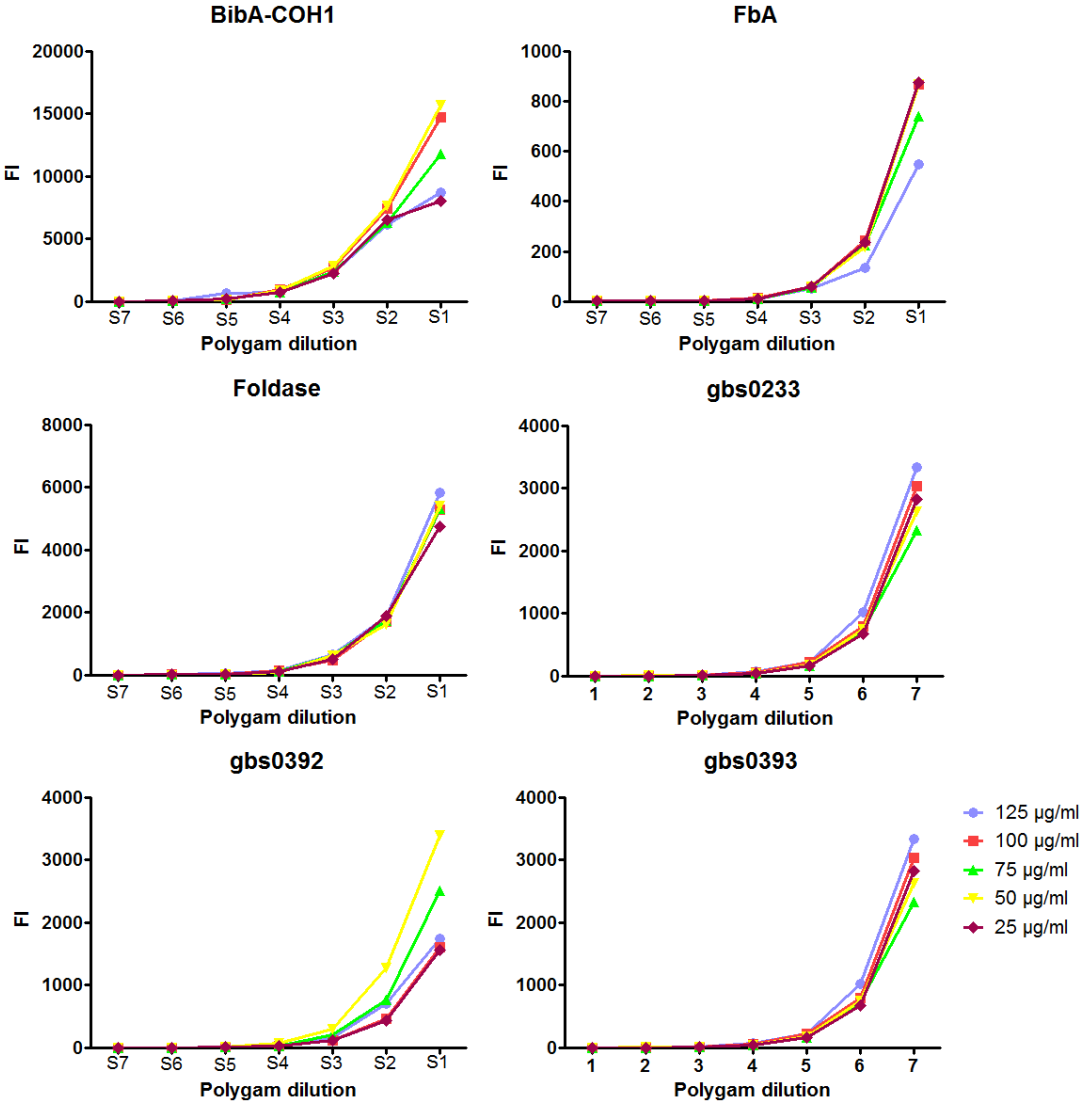


Figure 10.4 Quantification of optimal protein concentration for conjugating to micro-beads.

10.5.2 Cross-reactivity and specificity of reference serum

Cross-reactivity of BibA-NEM316 and BibA-COH1

GBS recombinant proteins supplied by Vulneva Gmb Austria of gbs2018 (BibA)-NEM316 a variant of BibA of strain COH1 had a sequence identity of 43%. Sequence comparisons of BibA protein from NEM316 and COH1 strains have highly identical flanking sequences. Heterogeneity occurs within internal regions of the two proteins that may have a significant role in pathogenicity and immunogenicity (51). To include both BibA variants in a multiplex assay; fragment peptide of BibA-NEM316 inclusive of the variable region that showed least identity with BibA-COH1 was selected based on multiple sequence alignment as depicted below.

```
(gbs2018COH1:gbs2018-1) Aligned. Score: 16
(gbs2018COH1:gbs2018-A) Aligned. Score: 22.5806
(gbs2018COH1:gbs2018-C) Aligned. Score: 88.6792

gbs2018COH1     MDSPDTLKVEKLGKLKDVKSVHELTPISIPNELKGAKEQALSSIISHPNITNSEVDKLAS
gbs2018-C_      ------------------------------------------------------------
gbs2018-A       ------------------------------------------------------------
gbs2018-1       ------------------------------------------------------------


gbs2018COH1     DYSFRINTSNDVNDVKRLLNEFYNAVARKQLDTNSADYRSKIDNISTTGLAIALEAKEIY
gbs2018-C_      ------------------------------------------------------------
gbs2018-A       ------------------------------------------------------------
gbs2018-1       ----------------------MATHAKVSDQELGKQSRRSQDIIKSLGFLSSDQKDILV


gbs2018COH1     EANKSILPHRYKDSVGTYVNSFEERRSPGKFNIWNGQEGFNAAQKLLEDVKKLLLELQNL
gbs2018-C_      ------------------------------------------------------------
gbs2018-A       ------------------------------------------------------------
gbs2018-1       KSISSSKDSQLILKFVTQAT---------QLNNAESTKAKQMAQNDVALIKNISPEVLEE


gbs2018COH1     TKNNKPNIQVPKQAPTEAAKPALSPEALTRLTTWYNQAKDLLKDDQVKDKYVDILSVQKA
gbs2018-C_      -MEAKPNIQVPKQAPTEAAKPALSPEALTRLTTWYNQAKDLLKDDQVKDKYVDILAVQKA
gbs2018-A       ---------MDTSSGISASIPHKKQVNLGAVTLKNLISKYRGND--------------KA
gbs2018-1       YKEKIQRASTKSQVDEFVAEAKKVVNSNKETLVNQANGKKQEIAKLENLSNDEMLRYNTA
..   .: .           .*              .*

gbs2018COH1     VDQAYDHVEEGKFITTDQANQLANKLRDALQSLELKDKKVAKPVAKGTYDVKYVDTEGKE
gbs2018-C_      VDQAYDHVEEGKFITTDQANQLANKLRDALQSLELKDKKVAKPEAK--------------
gbs2018-A       IAILLSRVNDFNRASQDTLPQLINSTEAEIRNILYQG-----------------------
gbs2018-1       IDNVVKQYNEGKLNITAAMNALNSIKQAAQEVAQKNLQKQYAKKIER-------------
:   .: :: :      *. . .   :

gbs2018COH1     VAKSRHFEGEEGAAFVTSAKEVAGYKLVRTEGAVSNVFTAGAQVRTYVYEKVKPEVKPDV
gbs2018-C_      ----------------------------------------------PEAKPEA
gbs2018-A       ------------------------------------------------------------
gbs2018-1       ----------------------------------------------------ISSK


gbs2018COH1     KPEAKPEAKPEVKPDVKPEAKPEAKPEVKSDVKPEAKPEAKPEAKPEVKPDVKPEAKPEA
gbs2018-C_      KPEAKPEAKPEAKPEAKPEAKPDVKPEAKPDVKPEAKPEAKPEAKSEAKPEAKLEAKPEA
gbs2018-A       ------------------------------------------------------------
gbs2018-1       GLALSKKAKEIYEKHKSILPTPGYYADSVGTYLNRFRDKQTFGNRSVWTGQSGLDEAKKM
```

```
gbs2018COH1     KPATKKSVNTSGNLVAKKAIENKKYSKKLPSTLEHHHHHH
gbs2018-C_      KPATKKSVNTSGNLAAKKAIENKKYSKKLPSTLEHHHHHH
gbs2018-A       ----------QIGKQNKPSVTLEHHHHHH
gbs2018-1       LDEVKKLLKELQDLTRGTKEDKKPDVKPEAKPLEHHHHHH
```

An ELISA competitive inhibition assay was performed to determine the degree of cross-reactivity between BibA from COH1 strain and the internal fragment BibA peptide form NEM316. Firstly, optimal coating concentration for each antigen was measured to determine the sensitivity of the assay. A 2-fold serial dilution of 20 µg/mL of protein antigen (20 µg/mL-0.002 µg/mL) in 100 µL PBS, 0.05% NaN_3 was absorbed on to Nunc maxisorb 96 well microtitre flat bottomed plates (ThermoScientific, Denmark) at 37ºC for 5 h and then overnight at 4ºC. Unbound protein antigen was washed 4 times with PBS, 0.05% Tween20 (washing buffer) and the binding sites were blocked by adding 300 µL/well of PBS, 10% fetal bovine serum (blocking buffer) and incubated at 37ºC for 1 h. After blocking, the plate was washed 4× with washing buffer. Antigen specific antibodies in Polygam (pooled IgG) sera diluted 1:100, 1:200, 1:400 and 1:1800 in blocking buffer was added 100 µL/well and allowed to bind to the respective antigen at 37ºC for 2 h. Unbound antibodies were washed 4× with washing buffer and bound IgG was detected by adding 100 µL/well of goat anti-human IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch, USA) diluted 1:2000 in blocking buffer at 37ºC for 2 h. Unbound antibodies was removed by washing 4× with washing buffer and 2× with distilled water. Signal was developed by adding 100 µL/well of 1 mg/mL of p-nitrophenol phosphate disodium (Sigma-Aldrich), with signal allowed to develop for 1 h at room temperature. Absorbance was measured at 405_{nm} using MultiSkan II plus plate reader (LabSystems). Optimal plate coating concentration was determined to be 2.5 µg/mL and 5µg/mL for BibA-COH1 and BibA-NEM316 respectively, Figure 10.5

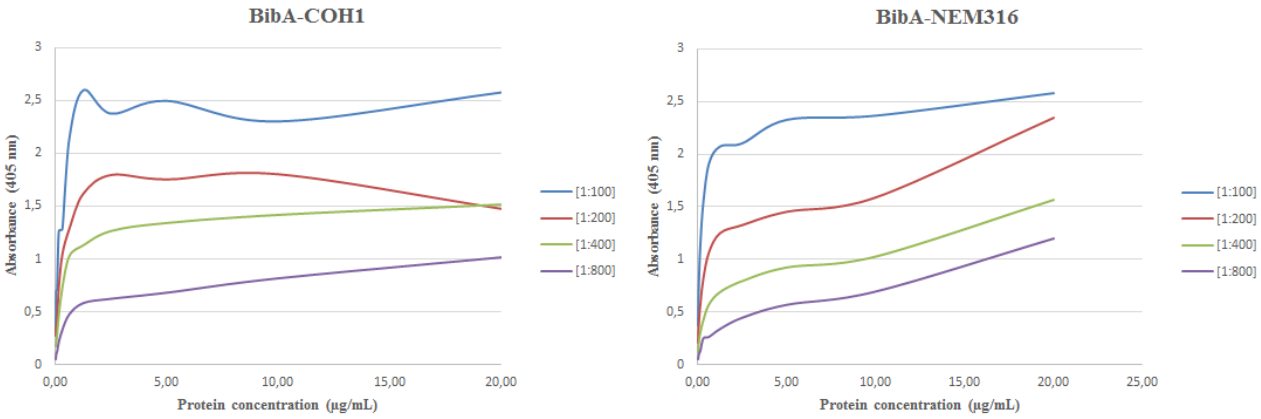


Figure 10.5 Sensitivity ELISA assay to determine optimal protein concentration for absorption of BibA-COH1 and BibA-NEM316 to microtitre plate.

The Competitive inhibition ELISA assay for BibA-COH1 and BibA-NEM1 was performed similar to the sensitivity ELISA assay with the exception that 2-fold diluted Polygam (1:100-1:64000) was mixed with 20 µg/mL of either BibA-COH1 or BibA-NEM316 prior adding to bound antigen on the plate. Absorbance signal detected from homologous and heterologous inhibition was compared to uninhibited Polygam. There was no significant cross-reactivity observed between the two peptides and therefore no quantitation inference was expected during multiplex immunoassay of the two antigens, Figure 10.6.

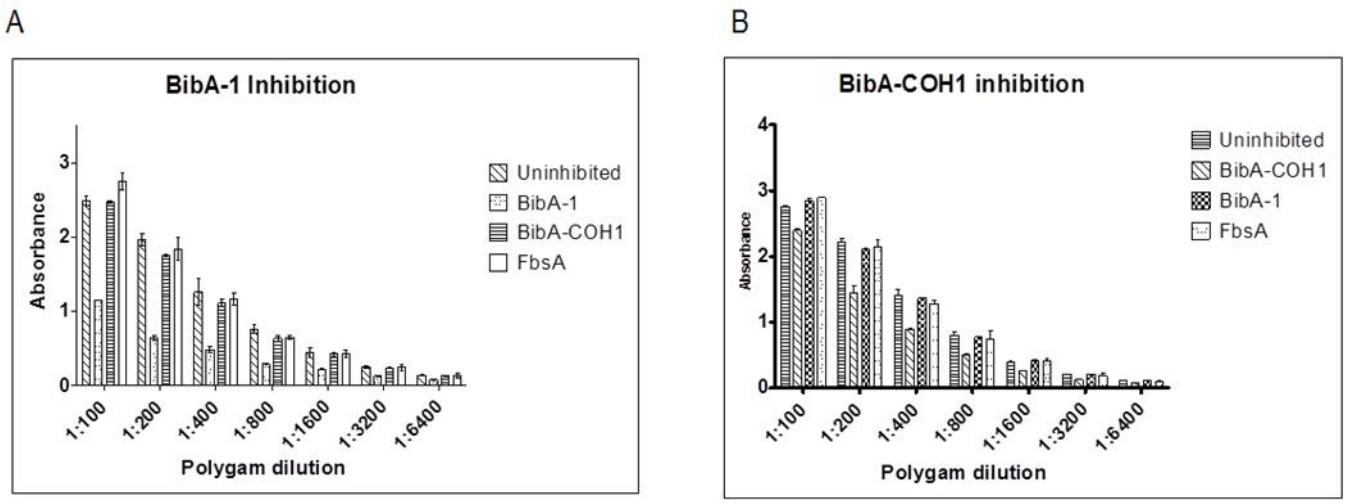


Figure 10.6 ELISA competitive inhibition assay, A, homologous inhibition of BibA-1 peptide and B, homologous inhibition of BibA-COH1 protein, error bars are indicative of standard error of the mean.

Cross-reactivity of GBS surface protein in multiplex assay

Comparisons of a singleplex and multiplex assays were conducted for each GBS surface protein to determine variation of IgG binding signal in the presence of multiple analytes. A strong positive correlation (R^2>0.9) was detected for all evaluated GBS proteins between the singleplex and multiplex assay, Figure 10.7.

Specificity of the reference serum (Polygam) to GBS surface proteins was determined by performing a competitive inhibition assay. A multiplex Luminex was conducted as described in the methods section, with the exception that 5 µg of GBS protein antigen was incubated with 1:100 Polygam dilution at RT for 1 h prior being probed in a multiplex assay. The specificity of polygamy to each protein was determined by comparing mean fluorescent intensity (MFI) detected for each protein in the presence and absence of inhibiting protein. Homologous and heterologous percentage inhibition was calculated as $\left(\frac{\mathrm{MFI}-\mathrm{MFI_{inh}}}{\mathrm{MFI}}\right)\times 100\%$, where $\mathrm{MFI_{inh}}$ is the binding intensity of

Polygamy in the presence of inhibiting protein. Table 10.5 includes the specificity for each protein included in the multiplex.

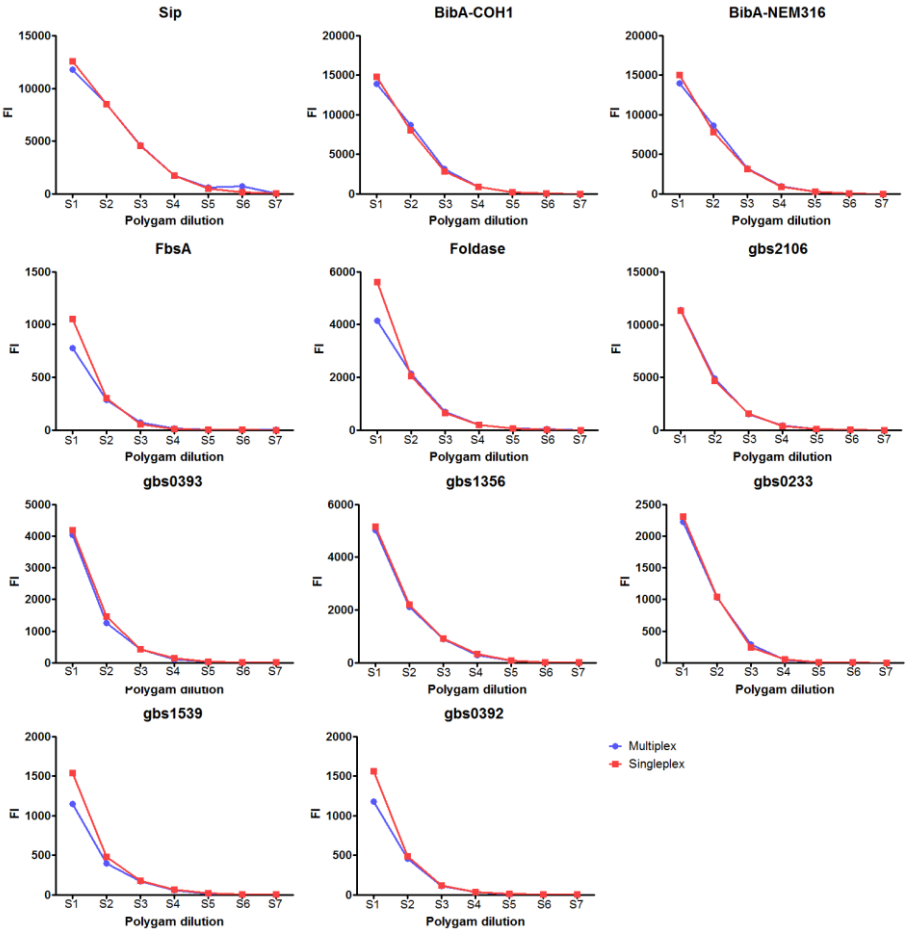


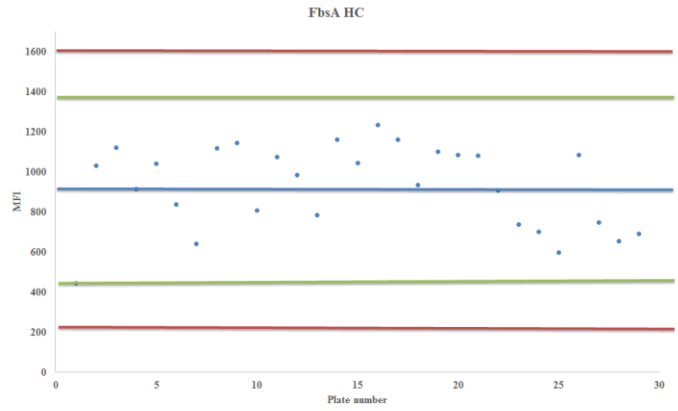
Figure 10.7 Comparisons of IgG binding signal by multiplex (blue) and singleplex (red) immunoassay.

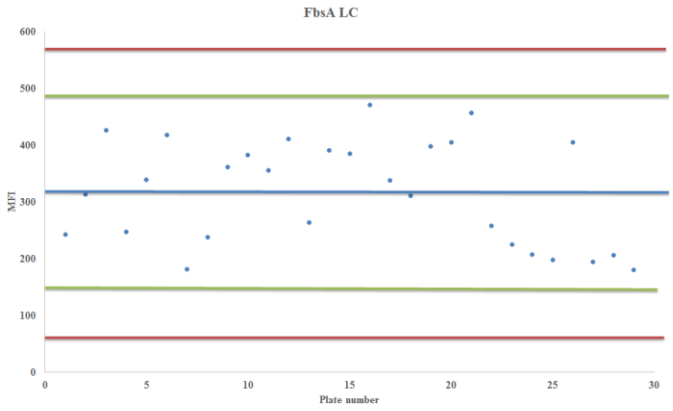
Table 10.5 Specificity absorption assay for Group B Streptococcus surface proteins, homologous inhibition is indicated in light grey, and heterologous cross-reactivity ≥ 20% is marked as dark-grey.

	Foldase	Gbs0392	FbsA	Gbs1539	Gbs0393	Gbs2106	BibA-NEM316	BibA-COH1	Gbs1356	sip	Gbs0233
Foldase	76.3	5.8	11.6	-60.5	13.7	2.9	-8.3	-2.4	16.9	3.4	-29.1
Gbs0392	5.1	80.7	-2.9	-21.3	20.3	-10.8	-7.3	-7.1	0.3	-0.7	-2.9
FbsA	-19	-20.2	99.9	-10.9	-14	-5.1	-6.9	1.9	-31.4	-6.6	9.6
Gbs1539	-20.4	9.7	-10.4	80.3	-36	-18.1	-10.2	16.7	3.2	-18.4	-8.4
Gbs0393	18.3	24.1	13.5	-20.1	99.9	11.3	1.5	9.5	54	11.4	18.2
Gbs2106	17.1	1.5	2.4	9.	6.7	97.8	-9.6	-5.6	2	-9	16.4
BibA-NEM316	19.1	-13.9	4.3	28.6	18.9	9.1	93.2	28.2	19.9	11	-19.8
BibA-COH1	12	-23.8	-1.6	8.9	12.1	0.7	0	95.2	3.5	-1.2	-39.7
Gbs1356	39.4	-12.4	0.3	53.7	33.5	9.7	6.6	6.8	95	10.5	-24
sip	30.9	-12.1	-8.1	8.3	18.6	-4.9	-1.9	-3.4	39	86.8	-40.3
Gbs0233	0.1	-30.3	-6.4	16.1	-.2.3	-5.4	13.4	-5.5	-18.4	-13.2	31.9

Quality control of the multiplex Luminex assay

In-house reference sera was prepared by screening IgG titres against GBS proteins in serum from pregnant women with or without GBS colonisation. Sera with high IgG titres against the GBS surface proteins were pooled together and used as high-titred control (HC). Similarly, sera with low IgG titres against the proteins were pooled together and used as low-titred control (LC). Inter-asssay reproducibility of the assay was determined by constructing Levey-Jenning plots using HC and LC mean fluorescence intensity (MFI) for each assay run, Figure 10.8. Assay runs that had HC and LC values outside ±2SD limits were repeated.


FbsA HC
MFI
Plate number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MFI
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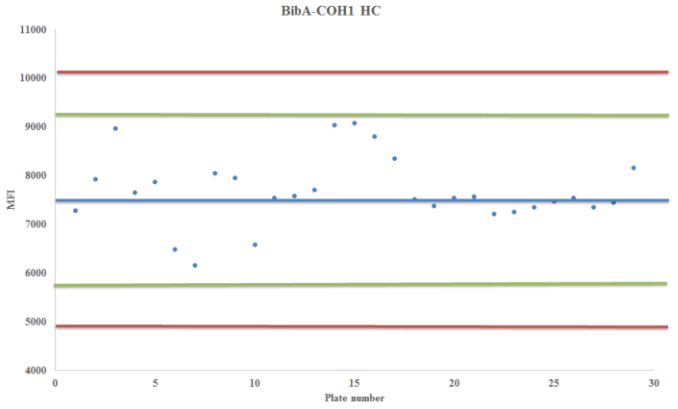
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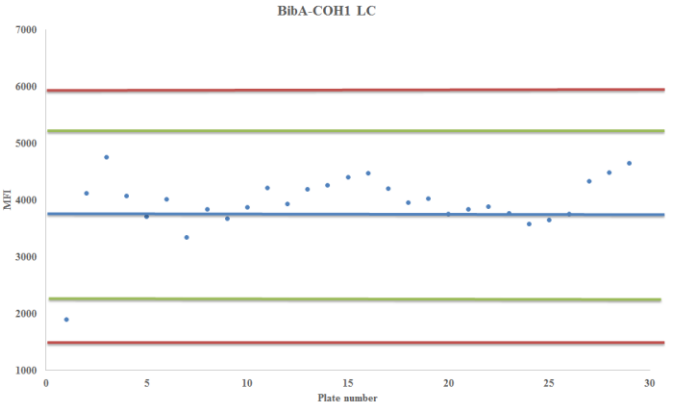
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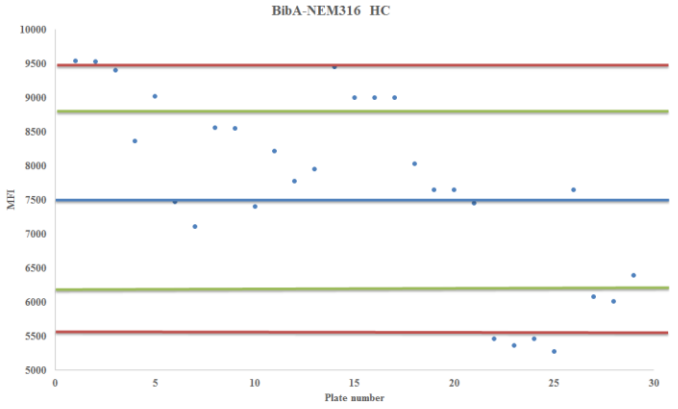
MFI
Plate number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MFI
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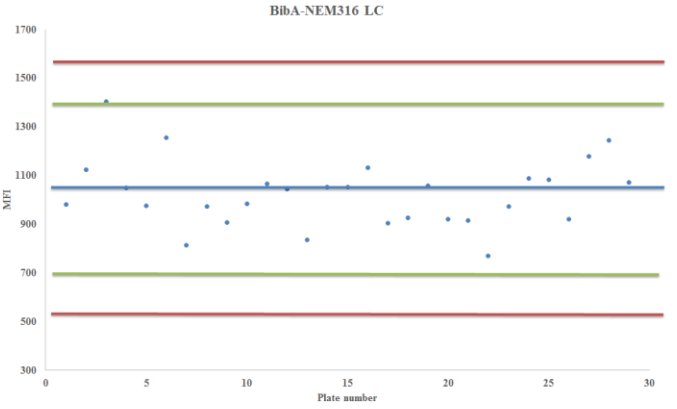
BibA-NEM316 LC
MFI
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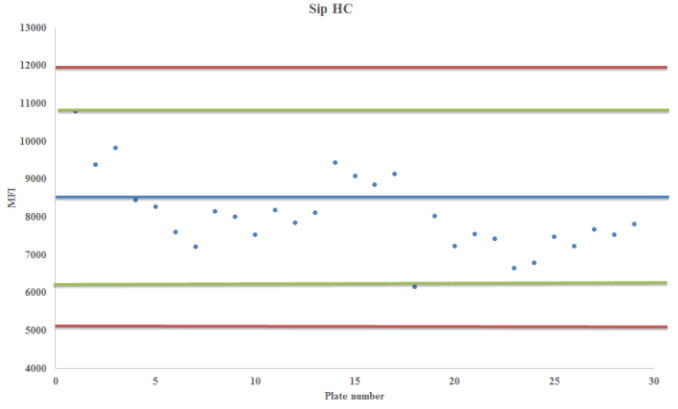
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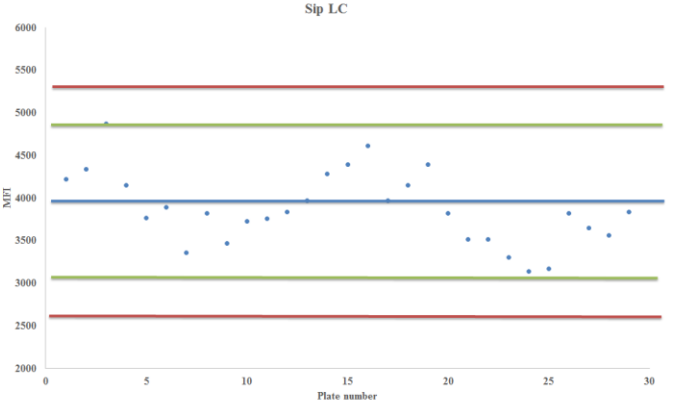
Sip LC
MFI
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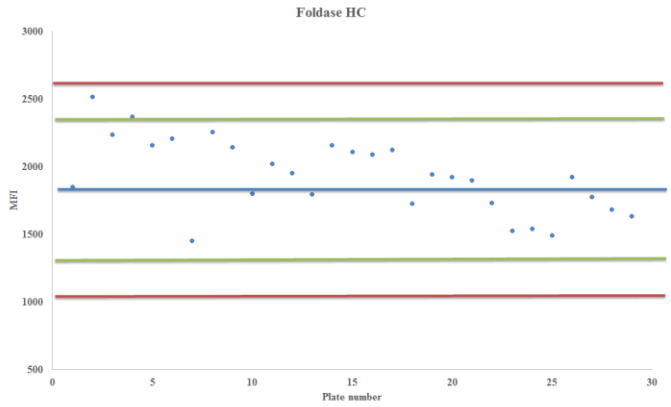
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MFI
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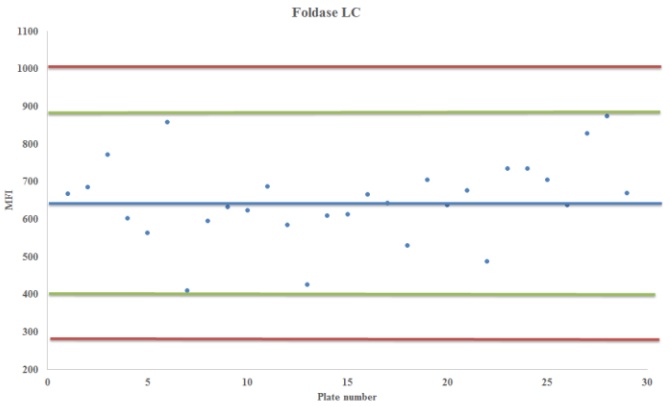
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MFI
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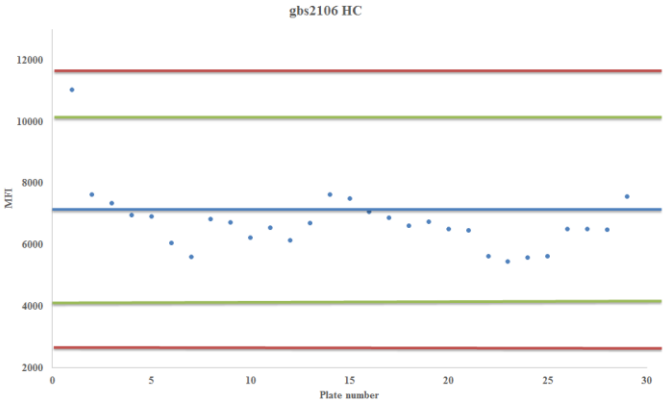
gbs2106 HC
MFI
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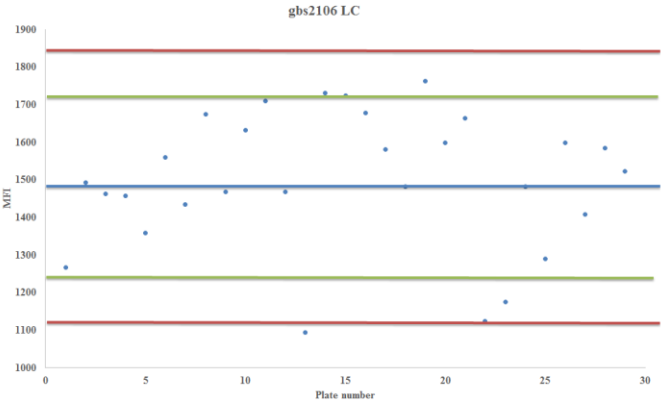
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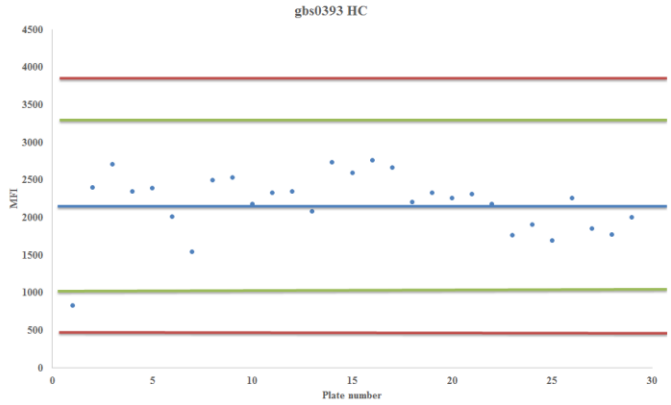
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MFI
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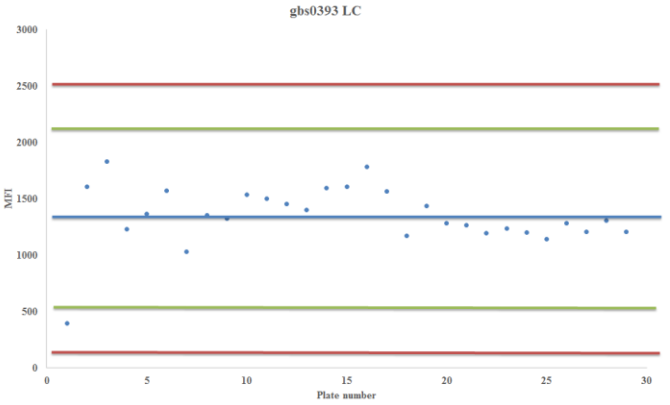
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MFI
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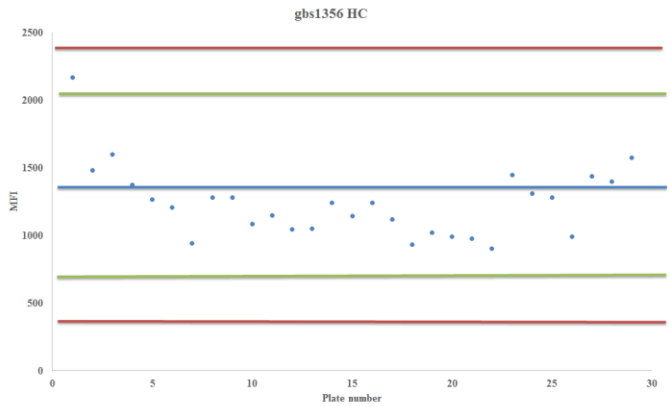
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MFI
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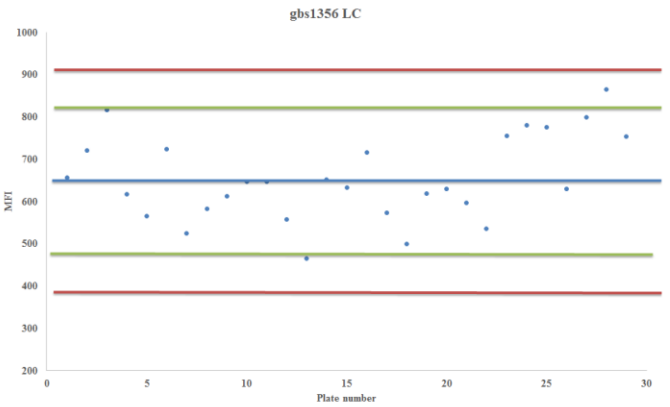
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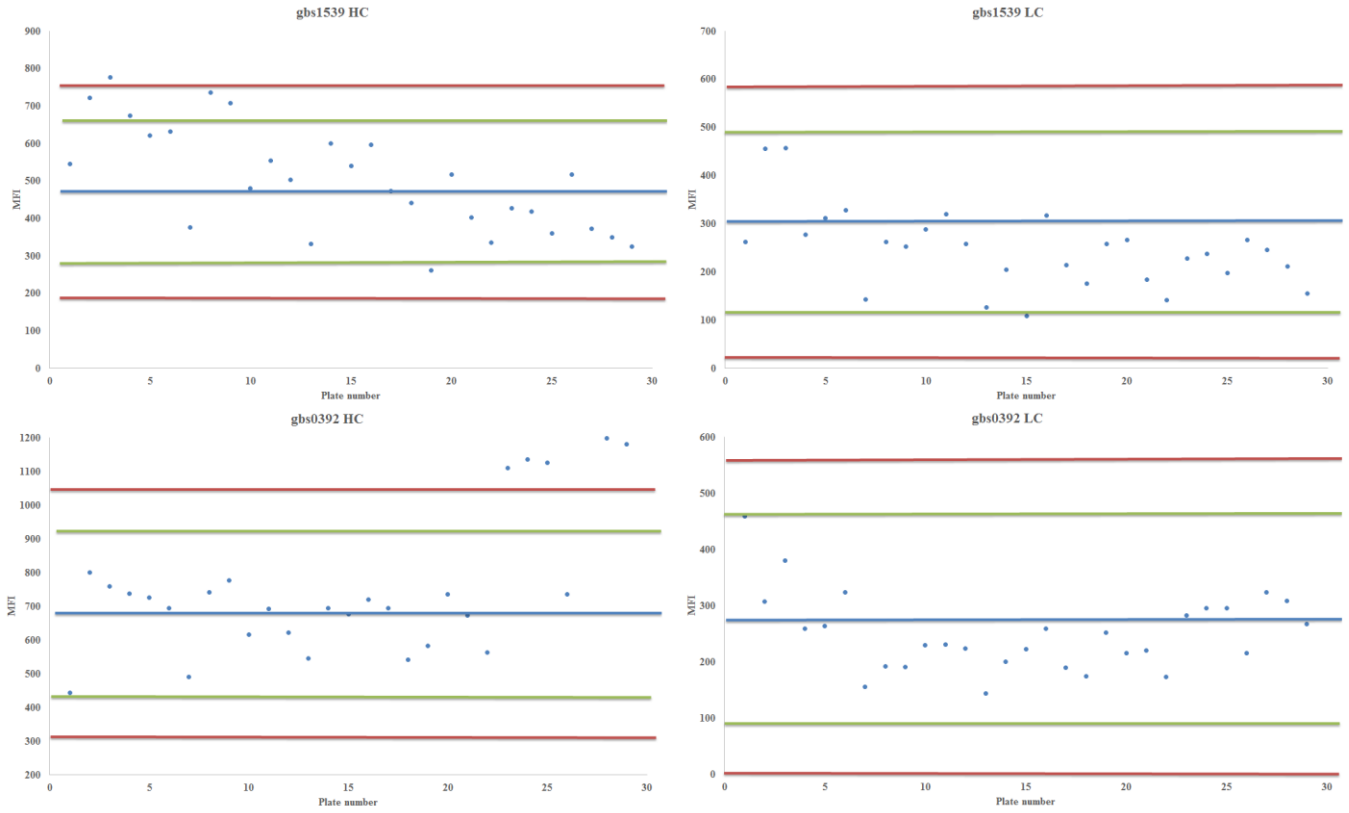

Figure 10.8 Levey-Jennings plot of high and low titre sera against group B Streptococcus surface proteins included in multiplex Luminex assay. The lines represent the mean (blue), ±2SD (green) and ±3SD (red).

10.6 Generation of specific antibodies to GBS recombinant proteins

Polyclonal antibodies specific to GBS surface proteins Sip, gbs2106, gbs0393 and gbs1356 were generated using rabbits. Purified recombinant protein was emulsified in either complete or incomplete Freund's adjuvant and administered subcutaneously in three doses as described in the methods sections. Immunisation of the Sip and gbs1356 recombinant proteins differed from the proposed immunisation schedule due to development of inflammation or consolidated granuloma on the injection site as a result of using complete Freund's adjuvant.

Rabbit immunised with Sip recombinant protein developed a consolidated granuloma after primary immunisation and therefore administration of the 1st booster dose was delayed by 14 days to allow the rabbit to recover after consulting with a veterinarian. Similarly, rabbit immunised with gbs1356 had inflammation at the injection site after primary immunisation and administration of the 1st booster dose was delayed by 7 days. Table 10.6 describes the amended immunisation schedule for the two respective protein antigens.

Table 10.6 Amended immunisation schedule for rabbits that had adverse reaction following administration of antigen emulsified in complete Freund's adjuvant.

Sip		**gbs1356**	
Day	**Event**	**Day**	**Event**
1	Collection of control serum	**1**	Collection of control serum
1	Primary Immunisation	**1**	Primary Immunisation
28	Collection of test sera	**28**	Collection of test sera
42	1st Booster Immunisation	**35**	1st Booster Immunisation
49	Collection of test sera	**42**	Collection of test sera
56	2nd Booster Immunisation	**49**	2nd Booster Immunisation
70	Exsanguination	**63**	Exsanguination

IgG antibodies against the GBS surface proteins were measured following immunisation of rabbits using ELISA assays. The optimal coating concentration for each antigen was measured to determine the sensitivity of the assay (described in section 10.5). A 5-fold serial dilution of 5 µg/mL of protein antigen (5 µg/mL, 1 µg/mL, 0.2 µg/mL, 0.04 µg/mL and 0.008 µg/mL) was absorbed on to microtitre plates. Protein specific antibodies in pre-immunisation and post-immunisation sera (diluted 1:200, 1:600 and 1:1800) were measured using goat anti-rabbit IgG conjugated with alkaline phosphatase (Jackson Immunology, USA) and signal was developed by adding p-nitrophenol phosphate disodium (Sigma). Absorbance was measured at 405_{nm} using MultiSkan II plus plate reader (LabSystems).

Sip and gbs1356 specific IgG antibodies had the highest signal when using 1:200 dilutions of post-immunisation sera bound to 5 µg/mL of antigen, Figure 10.8. Similarly, for gbs2106 and gbs0393, 1:200 dilutions of sera showed saturation of IgG specific antibody level at 1.0 µg/mL of protein antigen, Figure 10.9.

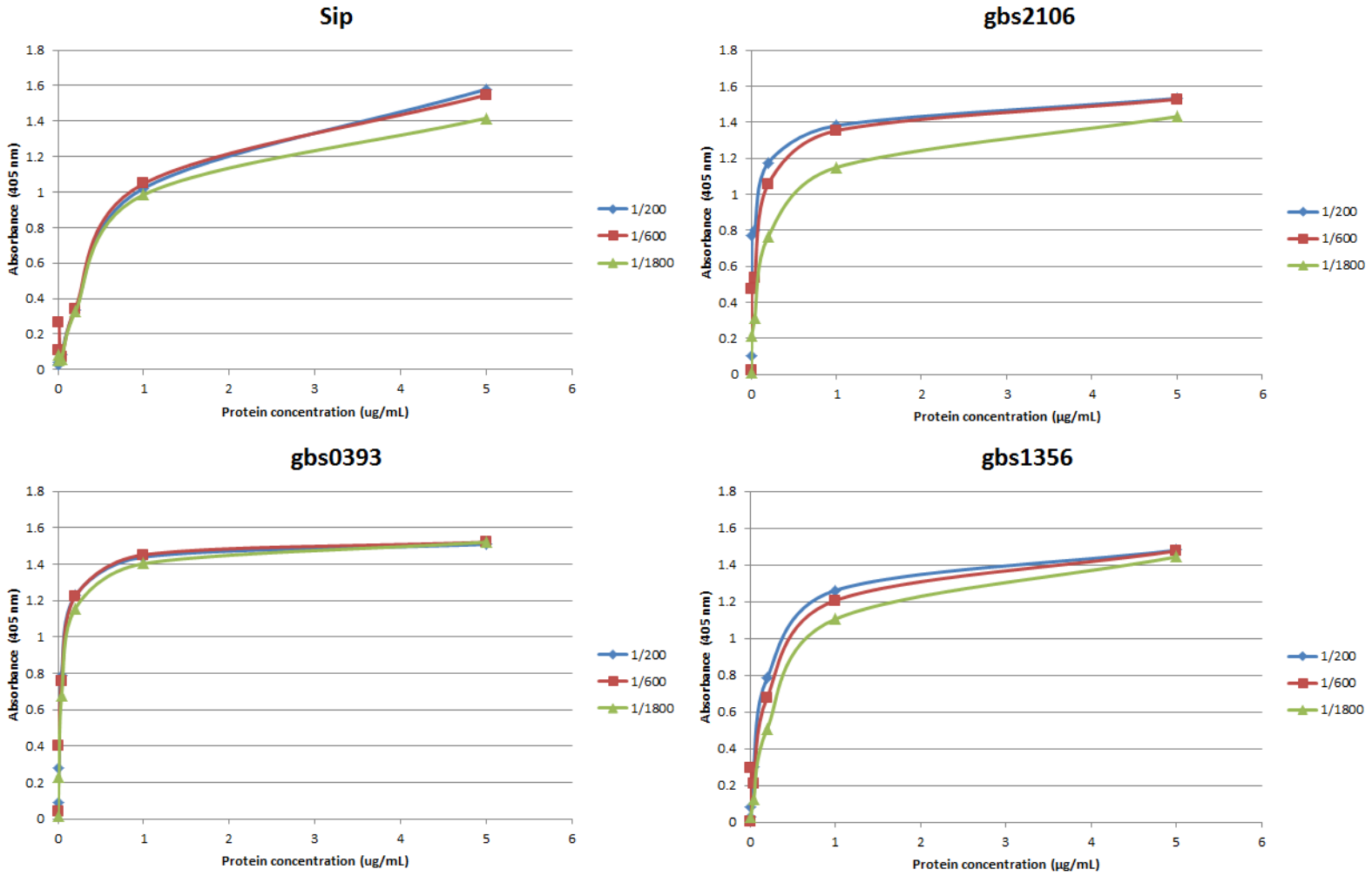

Figure 10.9 Sensitivity assay of post-immunisation sera following immunisation with surface immunogenic protein (Sip), gbs2106, gbs0393 and gbs1356.

The antibody kinetics for each antigen was measured to determine seroconversion in the respective immunised rabbits. Protein concentration that yielded the highest signal, Sip (5 µg/mL), gbs2106 (1.25 µg/mL), gbs0393 (1.25 µg/mL) and gbs1356 (5 µg/mL) were absorbed onto plate overnight at 4^{o}C. The assay was performed similar to the sensitivity assay using 1:200 and 1:800 dilutions of the pre- and post-immunisation sera.

Figure 10.10 displaying the antibody kinetics for Sip and gbs1356 whereby the highest response was achieved after the primary injection. The subsequent booster doses marginally increased IgG antibodies level. A similar immune response was observed for gbs2106 and gbs0393, Figure 10.11. A seroconversion >30-fold was achieved for each respective antigen post-immunisation.

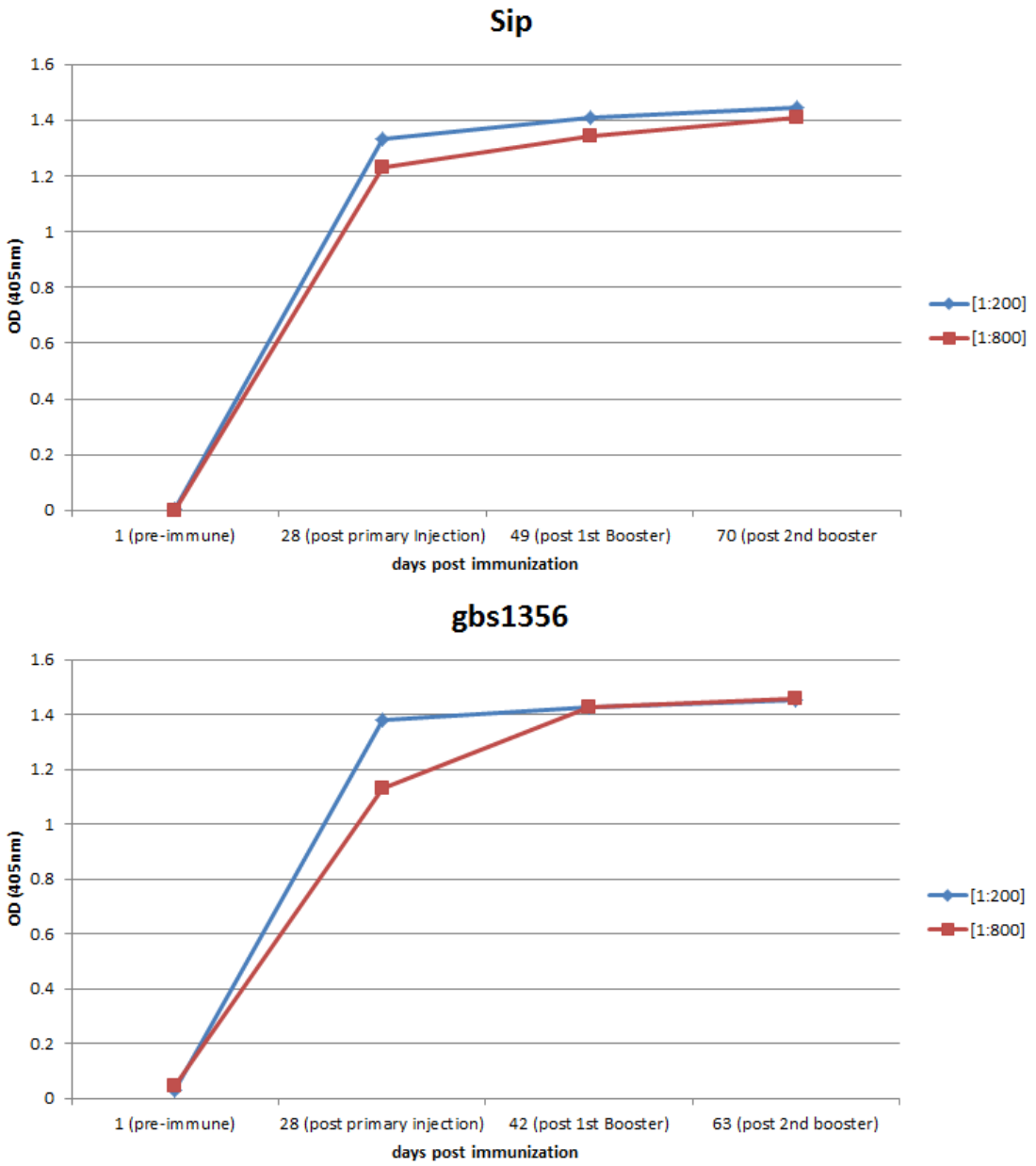

Figure 10.10 IgG response against group B Streptococcus recombinant proteins surface immunogenic protein (Sip) and gbs1356.

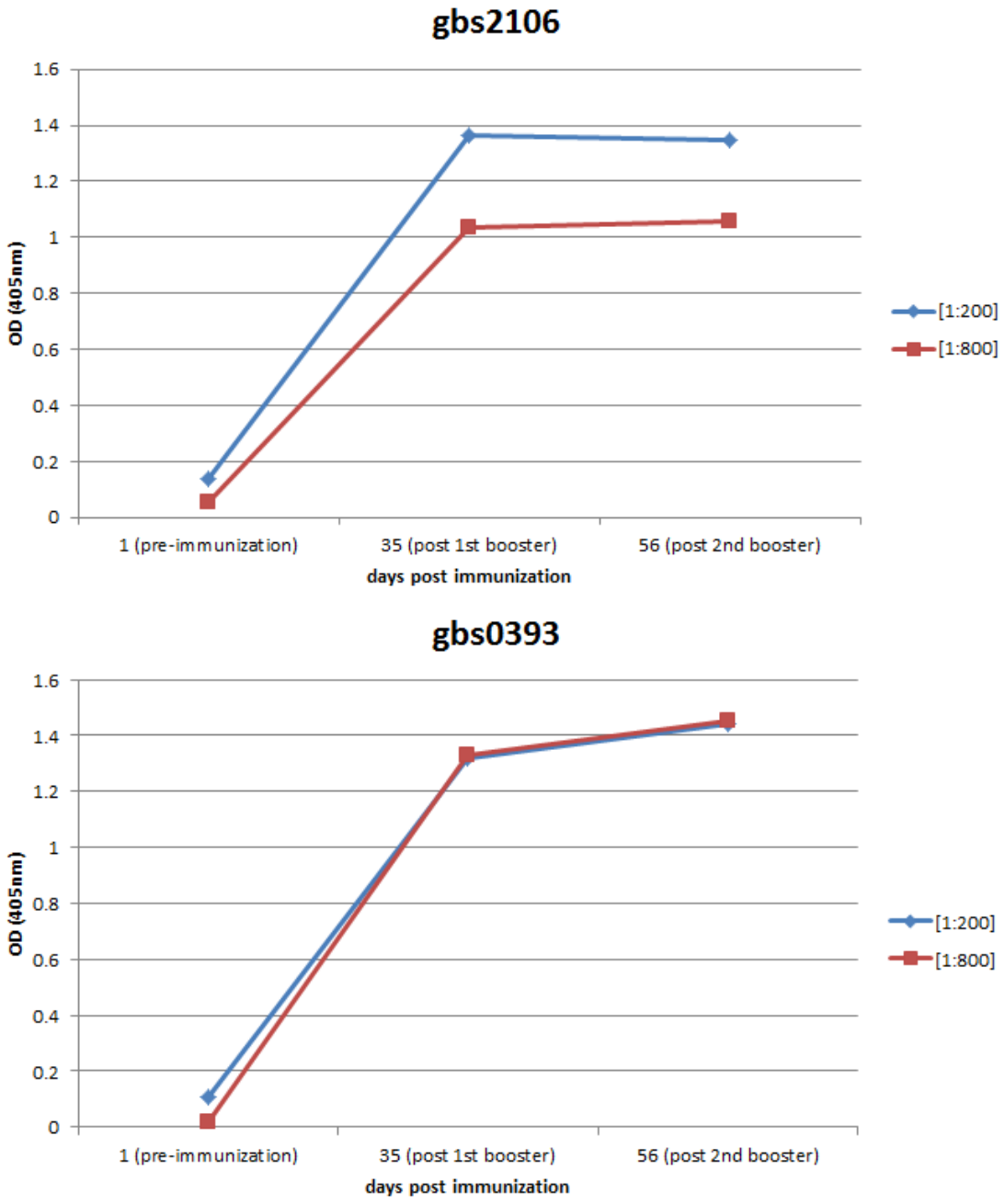

Figure 10.11 IgG response against group B Streptococcus recombinant proteins gbs2106 and gbs0393.

Cross-reactivity of the post-immunisation sera was evaluated among the immunising protein antigens. Highest cross-reactivity of 19% was observed between Sip and gbs1356, Figure 10.12.

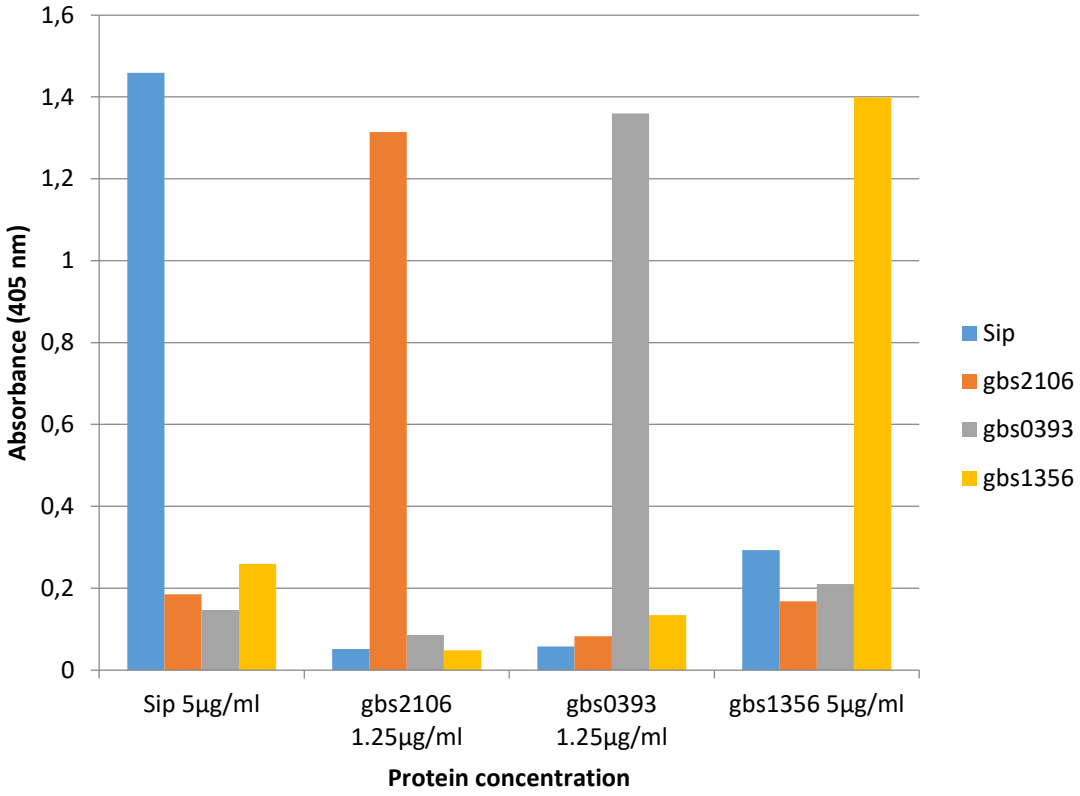

Figure 10.12 Cross-reactivity IgG antibodies from rabbits immunised with either surface immunogenic protein (Sip), gbs2106, gbs0393 or gbs1356.

In vitro expression on of proteins on the surface of GBS

Anti-GBS surface protein sera generated in rabbits were used to assess *in vitro* expression of Sip, gbs0393, gbs1356 and gbs2106 on the surface of GBS as described in the methods section. GBS NEM316 of serotype III from which the recombinant peptides were cloned was used a control. The target protein was defined as expressed on the surface of GBS when antibody binding from post-immunisation serum were 2-fold

higher compared to pre-immunisation serum as measured by their median fluorescence intensity (FI). Amongst the evaluated proteins, post-immunisation with >2-fold IgG binding to the surface of GBS compared to that of control sera was detected for Sip, gbs2106 and gbs0393 proteins. The fluorescence intensity (FI) signal detected on GBS when probing with pre-immunisation sera from rabbits immunised gbs0393 and gbs2106 were similar to that detected for unstained bacteria, Figure 10.13. Whilst, for Sip and gbs1356 proteins, pre-immunisation FI values were higher compared to unstained bacterial cells, which inevitably reduced the resolution power for inferring surface localisation of proteins. Antibody binding on the surface of GBS was 6-fold higher when using Sip specific post-immunisation sera despite the high binding signal of the relative pre-immunisation sera, and therefore Sip was characterised as exposed on the surface of GBS, Figure 10.13.

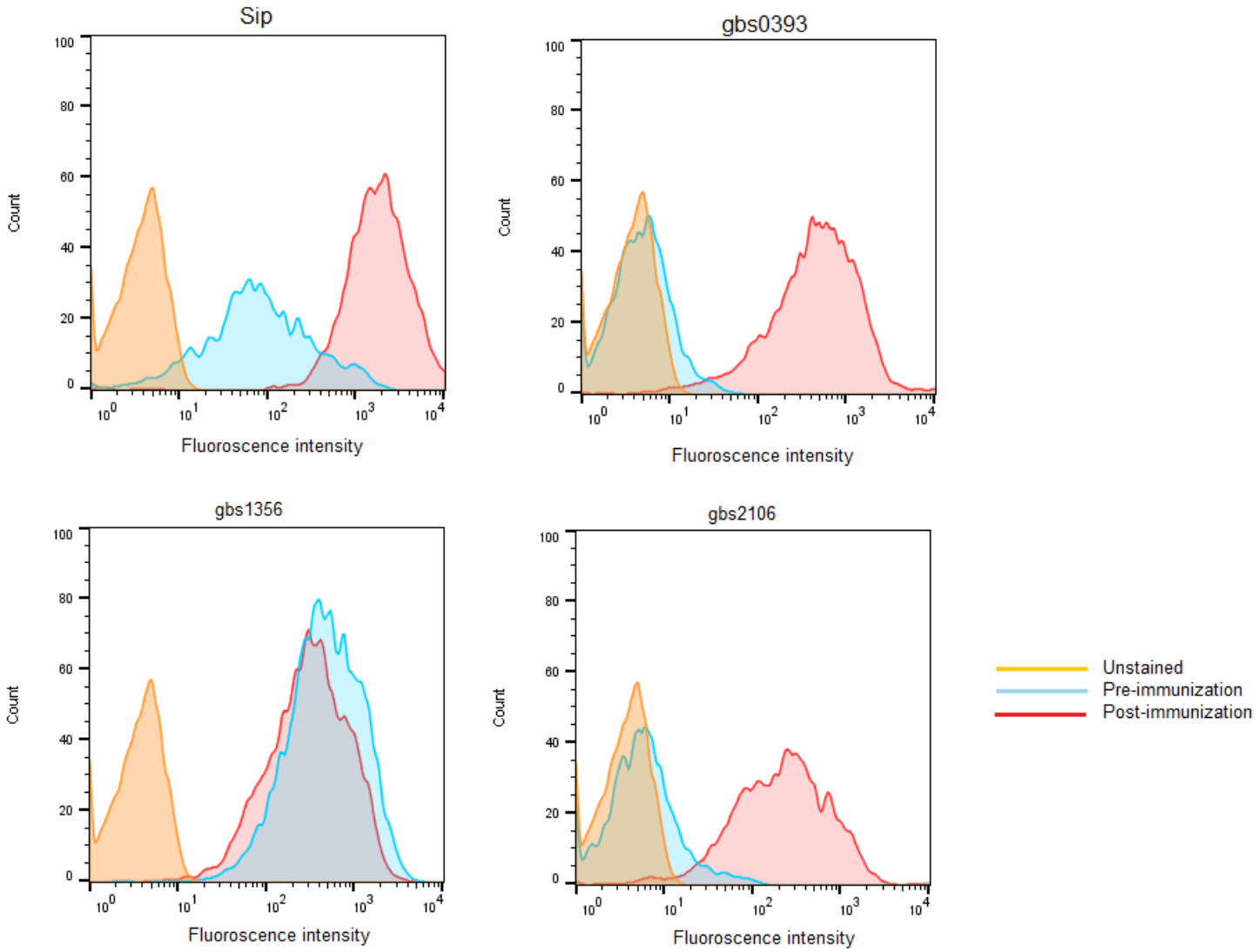

Figure 10.13 Evaluation of in vitro expression of proteins on the surface of group B Streptococcus

10.7 Manuscripts in press

This work entails serological analysis of GBS surface proteins in four different study cohorts, separated into chapters 5-8. The manuscript entitled "**Natural acquired group B *Streptococcus* capsular polysaccharides and surface protein antibodies in HIV-infected and HIV-uninfected children**" comprises of the work included in chapters 4 and 5. The second manuscript which is entitled "**Reduced trans-placental transfer of antibodies against group B *Streptococcus* surface proteins in HIV-infected mother-newborn dyads**" comprises of work described in chapter 7. Reprints of both manuscripts are included below.

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Natural acquired group B *Streptococcus* capsular polysaccharide and surface protein antibodies in HIV-infected and HIV-uninfected children

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ABSTRACT

Group B *Streptococcus* (GBS) is a major cause of invasive disease in young infants and also in older immunocompromised individuals, including HIV-infected persons. We compared naturally acquired antibody titres to GBS polysaccharide and surface protein antigens in HIV-uninfected and HIV-infected children aged 4–7 years.

A multiplex Luminex immunoassay was used to measure IgG concentrations against GBS capsular polysaccharides (CPS) for serotypes Ia, Ib, III and V; and also extracellular localizing proteins which included cell-wall anchored proteins: Fibrinogen binding surface Antigen (FbsA), GBS Immunogenic Bacterial Adhesin (BibA), Surface immunogenic protein (Sip), gbs0393, gbs1356, gbs1539, gbs0392; and lipoproteins gbs0233, gbs2106 and Foldase PsrA.

HIV-infected children (n = 68) had significantly lower IgG GMT compared to HIV-uninfected (n = 77) children against CPS of serotype Ib ($p = 0.012$) and V ($p = 0.0045$), and surface proteins Sip ($p < 0.001$) and gbs2106 ($p = 0.0014$). IgG GMT against GBS surface proteins: FbsA, gbs1539, gbs1356, gbs0392, gbs0393 and Foldase PsrA were significantly higher in HIV-infected children ($p < 0.004$). Moreover, amongst HIV infected children, IgG GMT to GBS surface proteins were higher in those with $CD4^+$ lymphocyte counts <500 cell/μL compared to those who had $CD4^+$ lymphocyte count ⩾500 cell/μL with the exception of Sip.

The increased susceptibility to invasive GBS disease in HIV-infected individuals could be due to the lower serotype specific capsular antibody and possibly due to lower antibody to some of the GBS proteins such as Sip and gbs2106.

1. Introduction

Group B *Streptococcus* (GBS) is an important cause of invasive disease in neonates, but also causes disease in pregnant women, the elderly and immunocompromised individuals such as those with HIV-infection [1]. Prior to widespread use of intrapartum antibiotic prophylaxis in the USA, the age-group distribution of invasive GBS disease was 46% in neonates (⩽1 month age), 2% in children aged 1–17 years and 33% in those ⩾18 years [2]. Notably, the majority of invasive GBS disease in adults was associated with underlying conditions such as diabetes mellitus, cancer and HIV-infection [2]. HIV infection increases susceptibility to invasive bacterial infection in children, particularly encapsulated bacteria such as *Haemophilus influenzae* type b and *Streptococcus pneumoniae* [3]. There is, however, limited data on invasive GBS infection in HIV-infected children, with only few reports among severely immunocompromised children [3–7].

An inverse association has been observed for the risk of developing invasive GBS disease during early-infancy and the level of serotype-specific capsular polysaccharide antibodies derived from trans-placental transmission [8]. In healthy infants, serotype-specific capsular IgG concentration to the dominant invasive serotypes (Ia, II and III) gradually decline post-partum, reaching approximately 10% of cord-blood IgG concentrations by 6 months of age [8]. Nevertheless, invasive GBS disease is uncommon beyond three months of age in non-immunocompromised children. This could be due to immunological responses to other GBS virulence factors including surface proteins, which might protect against disease, as well as underlying immunological responses elicited by colonization during early life.

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0264-410X/

GBS surface proteins bacterial adhesion protein A (BibA), fibrinogen binding protein (FbsA) and surface immunogenic protein (Sip) have been identified as protective immunogens in murine models [9], including influencing the risk of GBS acquisition and clearance of colonization. BibA, an anti-phagocytosis protein that inhibits a complement regulator (C4 bp) was shown to confer 69% protection against a lethal challenge dose of GBS serotype-III in mice models [10,11]. FbsA induces fibrinogen aggregation to mask GBS from opsonophagocytosis [12] and Sip, a surface exposed protein occurring in almost all GBS strains was reported to confer serotype-independent protection in 89% of mice challenged with a lethal dose of either GBS serotype Ia/c, Ib, II-II and V-VI [13].

The aim of this study was to determine whether differences in antibody titres to capsular polysaccharide (CPS) and surface protein epitopes may play a role in susceptibility to invasive GBS disease in HIV-infected children. To explore this, we compared natural acquired serum IgG antibody to serotype-specific CPS and selected GBS protein antigens between HIV-infected and HIV-uninfected children. The surface proteins evaluated, included known virulence proteins together with other novel proteins which were identified using a bioinformatics approach [14].

2. Materials and methods

2.1. Study population

We retrospectively tested available serum samples from a group of 77 HIV-uninfected and 68 HIV-infected children who had previously participated in a study on the durability of antibody responses to an investigational 9-valent pneumococcal conjugate vaccine (PCV) as detailed [15]. These children were enrolled between December 2004 to April 2005 and serum samples were collected and stored at −70 °C, before processing for this study. The sera collected were analysed for the prevalence of IgG antibody concentration for select GBS surface proteins and serotype specific CPS.

2.2. In silico selection of GBS immunogenic surface proteins

Using GBS NEM316 as the reference strain for serotype III, surface proteins were predicted using an adaptation of the Giombini et al. [16] protocol that performs automated identification of bacterial surface proteins from inserted genome and proteome sequences. A systemic process was adopted rather than a filtration method to analyse all proteins for potential cell surface features using available bioinformatics tools. SingalP was used to screen for proteins that contain a signal peptide at their N-terminal sequence required for protein export [17]. Proteins with signal peptides were cross-validated using TargetP to assess their final subcellular localization [18]. Transmembrane spanning proteins were identified using TMHMM and prodiv-HMM [19]. A cut-off $\leqslant 3$ membrane helices per protein was permitted since proteins with more than 3 membrane spanning regions tend to be insoluble [20]. Extracellular localization of the proteins was further predicted using pSORTb and LipoP that screens for cell-wall anchor motifs (LPxTG or LxxC) [21,22]. Proteins that were identified as extracellular and/or cell-wall surface anchored proteins were screened for the presence of B-cell epitope regions using BepiPred and an average threshold score of 0.4 was considered significant for immunogenic proteins [23]. The selected proteins were varied according to subcellular localization and degree of heterogeneity to evaluate the association of these features to antibody response and the influence of HIV infection thereupon. GBS proteins with surface localization motifs; Sip, gbs0393, gbs0392, gbs1356, gbs2106, gbs1539 were selected to be cloned, expressed and for immunogenicity testing and Foldase PsrA was included as a representative lipoprotein.

2.3. Recombinant GBS surface proteins

GBS NEM316 serogroup III (CDC) was grown overnight on 5% sheep blood agar and subsequently cultured overnight in Todd Hewitt broth (Merck, South Africa) at 37 °C with shaking. Genomic DNA was extracted and purified with the QIAgenDNAamp kit (QIAgen, Netherlands). Genes encoding immunogenic proteins were amplified by PCR with manually-designed primers that truncated the signal peptides and cell-wall anchor motifs, Table 1. PCR mixture was inclusive of 1.0 μM of each primer (Inqaba Biotec, South Africa), 25–50 ng gDNA and Phusion Flash Enzyme mix (2.5 U DNA polymerase, DMSO, 50 μM MgCl, 400 μμM per dNTPs) (Thermo Scientific) and the reaction cycles were: an initial denaturation cycle at 98 °C for 2 min, 30 cycles of 98 °C for 5 s, 50–55 °C for 30 s, 72 °C for 15 s/kb and a final extension cycle at 72oC for 5 min. The gene amplicons were then cloned into pETite C-His vector (Lucigen, USA) and propagated in HI *E. Coli* competent 10G cells (Lucigen, USA) as per the manufacturer's recommendations. Gene sequencing of the cloned insert was performed to confirm the integrity and orientation of the cloned fragment, prior to protein expression and purification. His tagged proteins were purified with Ni^{2+} affinity chromatography, and visualized with SDS PAGE to confirm their purity and polypeptide molecular weight and were subsequently stored in PBS with 50% glycerol at −70 °C. FbsA-6313, gbs0233-NEM316, BibA-COH1 and BibA-NEM316 proteins of serotype II were obtained from Valneva Austria GmbH (Vienna, Austria). Sequence comparisons of the BibA protein of NEM316 and COH1 strains have highly identical flanking sequences and a variable internal region [24]. To represent both BibA variants in a multiplex assay with minimum cross reactivity the complete protein from GBS-COH1 strain was used and only the variable fragment of the polypeptide from the NEM316 strain was included.

2.4. Luminex assay for the quantification of antibodies to GBS antigens

We developed a multiplex Luminex assay to quantify the IgG titres against GBS CPS (Norvatis Vaccines, now part of GSK) and 11 surface proteins. CPS were coupled to carboxylated microbeads with a cross-linking reagent 4-(4,6-dimethoxy-[1,3,5]-triazi-2-yl)-4-methyl-morpholinium (DMTMM) as described previously [25]. Purified proteins were desalted by gel filtration with PD-10 desalting columns (GE healthcare, Germany). Desalted proteins were immobilized onto carboxylated beads using the 2-step carbodiimide reaction [26], at a protein concentration of 50 μg/ml per unique bead region. Conjugated beads were blocked with PBSFN buffer (PBS, 10% fetal bovine serum, 0.05% NaN_3) and stored at 4 °C.

Pooled purified IgG from adult humans (Polygam; National Bioproducts, South Africa) was used as a reference serum to measure IgG antibodies. Polygam calibrated with a standard homotypic GBS was used as reference serum to extrapolate IgG geometric mean concentration (GMC) to CPS reported as micrograms per ml (μg/ml). IgG geometric mean titres (GMT) for the protein antigens were reported as arbitrary units (U/ml) as extrapolated from Polygam sera which was assigned a value of 100 U/ml. In house high and low control sera were prepared from pooling sera from HIV-uninfected pregnant women that were either colonized or uncolonized with GBS respectively. These controls were included in the multiplex assay to assess inter-assay variation. All serum samples, controls and the reference serum were diluted 1:100 with PBSFN for immunological analysis. Samples with titres above the detection limits were further diluted. Competitive inhibition assays were conducted for all proteins in a multiplex assay with 5 μg of

Table 1
Primer sequences of target peptides flanked by att-L/R homologous recombination sites (indicated as bold letters).

	Forward primer	Reverse primer
Foldase protein	**GAAGGAGATATACATATG**GTTGTTACAATGAAGGGCGACAC	**GTGATGGTGGTGATGATG**TTGTGATAAGATGCCTGCAAATGC
Sip	**GAAGGAGATATACATATG**GCACAAGAAACAGATACGACGTGG	**GTGATGGTGGTGATGATG**TGATACGTGAACGTGGTCATAGTG
gbs0392	**GAAGGAGATATACATATG**GTCATCCCAGTAGACCCAGCAGTACC	**GTGATGGTGGTGATGATG**TGGGAGCGTCTTTTCAGCACCTG
gbs0393	**GAAGGAGATATACATATG**GCTGTTACAACCACACCTGCAGTTAATC	**GTGATGGTGGTGATGATG**TGGCAGTCCTTTAGCTGGAGC
gbs1356	**GAAGGAGATATACATATG**GTAGGGAGAACTGTTGCGACAAGTG	**GTGATGGTGGTGATGATG**ACCGTCCGCATTTTCATTGTCCTT
gbs1539	**GAAGGAGATATACATATG**GCTGATACAAGTGATAAGAATACTGACACG	**GTGATGGTGGTGATGATG**TCCCGTAGATGCTATTACAGTATCTG
gbs2106	**GAAGGAGATATACATATG**GCAGATAAAGTTCGCGTAGCC	**GTGATGGTGGTGATGATG**CCAAGCTGATAAACCTTGAGCACG

Sip, Surface immunogenic protein.

the homologous antigen incubated with 1:100 Polygam solution at room temperature for 1 h. Sample analysis was performed with the Bioplex 200 (Bio-Rad) instrument as previously described [27].

2.5. Data analysis

Stata software (version 13.0 Stata-Corp, Tx USA) was used for statistical analysis. For statistical comparisons antibody titres were $\log_{10}$ transformed and the difference between the groups were tested by two tailed *Student's t*-test. A more conservative p value $\leqslant 0.01$ was considered significant to allow for multiple comparisons in lieu of undertaking any correction for multiple comparisons. The IgG geometric mean concentrations (GMC) for CPS or titres (GMT) for surface proteins among the HIV infected children with $CD4^+$ count < 500 cell/μl and $CD4^+$ count $\geqslant$ 500 cell/μl were compared using Mann-Whitney *U* test.

2.6. Ethics

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Approval number: M121019), who granted a waiver of further parental consenting for the additional testing of the archived samples.

3. Results

Of the 2094 predicted proteins of the GBS NEM316 genome, 32 proteins were identified as cell anchored proteins (possessing LysM or LPxTG domains) and 25 as lipoproteins (possessing LxxC domains). The BepiPred screening for linear immunogenic epitope regions revealed 17 proteins that had an average score greater than the cut-off value of 0.4. These included known virulence proteins such as streptococcal C5a peptidase and surface immunogenic protein (Sip). Table 2 describes the surface protein features as determined using bioinformatics software.

The quantitative multiplex assay compared well with singleplex assays for all antigens measured in triplicate using the reference sera, with all correlations $R^2 > 0.9$ and coefficient of variation $\leqslant$10%. The specificity of the assay was high for FbsA, gbs0393, gbs2106, BibA-COH1, BibA-NEM316, and gbs1356 antigens with >95% of the signal being absorbed in the presence of the homologous antigens, Table 3. The specificity of the assay was slightly lower for Foldase, gbs0392, gbs1539 and Sip with 76.3–86% of antibody binding inhibited by the respective antigen, and with only 32% inhibited by recombinant Gbs0233. Cross reactivity >20% was reported for some antigens, Table 3.

3.1. Demographics

The demographic profiles of the HIV-infected and HIV-uninfected children were similar for gender and age (3.9–7.1 years). This comprised of 55.9% males of which 41 were HIV-uninfected (median age = 5.1 years) and 40 were HIV-infected (median age = 5.2 years), and 44.1% females; 36 HIV-uninfected (median age = 5 years) and 28 HIV-infected (median age 5.9 years). $CD4^+$ lymphocyte count data was available for 57 of the HIV-infected children with a median of 566 cells/μl (range 0–1897 cells/μl), including 30 (52%) with $CD4^+$ count $\geqslant$ 500 cells/μl and 27 (47%) < 500 cells/μl. Of the HIV infected children 12 (21%, 6 male and 6 female) were on antiretroviral (ARV) treatment, that comprised of two nucleoside reverse transcriptase inhibitors and one non-nucleoside reverse transcriptase inhibitor. The median $CD4^+$ count of the HIV-infected children receiving ARV treatment was 442 cells/μL (range 255–1094 cells/μL).

3.2. IgG antibodies against GBS antigens

The differences in antibody concentrations/titres between HIV-infected and HIV-uninfected children were epitope dependent. IgG GMC (μg/ml) against CPS were lower in HIV-infected compared to HIV- uninfected children particularly for serotype Ia (0.078 vs 0.061, p = 0.01) and V (2.971 vs 1.952, p = 0.005), Table 4. In contrast, GMTs were higher against the majority of the protein epitopes in HIV-infected compared to HIV-uninfected children, including the lipoprotein Foldase PsrA, and the cell wall anchored proteins FbsA, Gbs1356, Gbs0393, Gbs1539 and Gbs0392 (p < 0.005), Table 4. Titres against the lipoprotein Gbs2106, and the lysM domain cell-wall anchored protein Sip were, however, significantly higher in HIV-uninfected compared to HIV-infected children (p < 0.001; Table 4). None of the evaluated GBS antigen specific IgG levels were associated with colonization with *Streptococcus pneumonia* or PCV vaccine status (data not shown).

Amongst HIV-infected children, CPS IgG GMCs were positively correlated with the surface proteins IgG GMTs, with the exception of FbsA, Sip and gbs1356, Table 5. Conversely, HIV-infected children with CD4 counts < 500 cells/μl had higher IgG GMC/GMT compared to children with $CD4^+$ counts $\geqslant$ 500 cells/μl for proteins gbs1356, gbs1539, gbs0233 and Foldase PsrA, and a similar trend for gbs0393, FbsA and BibA proteins, Fig. 1. Similarly for CPS antigens, IgG titres were higher in HIV-infected children with CD4 counts < 500 cells/μ compared to those who had with $CD4^+$ counts $\geqslant$ 500 cells/μl, however the differences were not statistically significant (data not shown).

4. Discussion

Low circulating antibodies to GBS polysaccharides has been associated with increased risk of developing invasive GBS disease in infants [28–30]. Increased bacterial infection, inclusive of GBS, occurs in HIV-infected individuals due to immunopathologies associated with HIV infection [3]. The increased susceptibility to invasive GBS disease in HIV-infected children is suggested to be a consequence of inadequate humoral immune responses to capsular polysaccharide [31,32]. This was particularly evident for serotypes Ia and V, in which their respective IgG concentrations were shown to be lower among HIV-infected children. Also, we identified lower

Table 2
Subcellular localization characteristics and putative functions of group B *Streptococcus* surface proteins.

Protein name	Recombinant peptide Length (aa)	Protein surface features						Putative function/pathogenicity
		SignalP	TargetP	TMHMM	pSORT	LipoP	BepiPred score	
FbsA (gbs1087)	340	Yes	Secreted	0	LPXTG	–	0.55	Induces fibrinogen aggregation, Adhesion
BibA-COH1 (SAN2207)	460	Yes	Cytoplasmic membrane	1	LPXTG	–	0.45	Complement inhibitor, Adhesion
Gbs0233	308	No	Secreted	0	–	LXXC	−0.03	Lipoprotein, ABC transporter
BibA-1(gbs2018)	300	Yes	Cytoplasmic membrane	1	LPXTG	–	0.45	Complement inhibitor, Adhesion
Gbs0392	119	Yes	Secreted	1	LPXTG	–	0.89	Unknown function
Gbs0393	834	Yes	Secreted	2	LPXTG	–	0.44	Adhesin, surface antigen protein
Gbs1356	846	Yes	Cytoplasmic membrane	2	LPXTG	–	0.40	Adhesin, Agglutinin receptor
Gbs2106	189	Yes	Secreted	1	–	LXXC	0.57	Unknown function, Hypothetical lipoprotein
Gbs1539	156	Yes	Secreted	2	LPXTG	–	0.97	Unknown function
Foldase PsrA protein (gbs0827)	272	Yes	Secreted	0	–	LXXC	0.33	Peptidyl-prolyl cis-trans isomerase activity
Sip (gbs0031)	414	Yes	Secreted	0	LysM	–	0.57	Immunogenic protein

aa, amino acid.

Table 3
Specificity absorption assay for group B *Streptococcus* surface proteins, homologous inhibition is indicated in italic, and heterologous cross-reactivity ⩾ 20% is marked as bold and italic.

	Foldase	**Gbs0392**	**FbsA**	**Gbs1539**	**Gbs0393**	**Gbs2106**	**BibA-NEM316**	**BibA-COH1**	**Gbs1356**	**sip**	**Gbs0233**
Foldase	*76.3*	5.8	11.6	−60.5	13.7	2.9	−8.3	−2.4	16.9	3.4	−29.1
Gbs0392	5.1	*80.7*	−2.9	−21.3	20.3	−10.8	−7.3	−7.1	0.3	−0.7	−2.9
FbsA	−19	−20.2	*99.9*	−10.9	−14	−5.1	−6.9	1.9	−31.4	−6.6	9.6
Gbs1539	−20.4	9.7	−10.4	*80.3*	−36	−18.1	−10.2	16.7	3.2	−18.4	−8.4
Gbs0393	18.3	***24.1***	13.5	−20.1	*99.9*	11.3	1.5	9.5	***54***	11.4	18.2
Gbs2106	17.1	1.5	2.4	9.	6.7	*97.8*	−9.6	−5.6	2	−9	16.4
BibA-NEM316	19.1	−13.9	4.3	28.6	18.9	9.1	*93.2*	***28.2***	19.9	11	−19.8
BibA-COH1	12	−23.8	−1.6	8.9	12.1	0.7	0	*95.2*	3.5	−1.2	−39.7
Gbs1356	***39.4***	−12.4	0.3	***53.7***	***33.5***	9.7	6.6	6.8	*95*	10.5	−24
sip	***30.9***	−12.1	−8.1	8.3	18.6	−4.9	−1.9	−3.4	***39***	*86.8*	−40.3
Gbs0233	0.1	−30.3	−6.4	16.1	−.2.3	−5.4	13.4	−5.5	−18.4	−13.2	*31.9*

FbsA, Fibronogen binding surface antigen.
BibA, gbs2018 (NEM316 strain) or SAN2207 (COHI strain).
Sip, Surface immunogenic protein.

IgG titres to Sip and gbs2106 proteins, which have also been identified as virulence factors in animal model studies [13], but for which an association to invasive disease in humans has not been established. Nonetheless, IgG titres against GBS surface antigens in both HIV-infected and HIV-uninfected children shows that humoral immune response occurred to these epitopes, likely due to GBS colonization during childhood [33].

HIV infection causes immuno-pathogenesis associated with dysregulated and dysfunctional $CD4^+$ lymphocytes, in particular, the hyper-activation of B cells and hypergammaglobulinaemia [34]. The proper immunological response to individual antigens may be affected by HIV infection depending on the mechanism and pathway of antigen recognition by the immune system. Since protein antigens require activating signals from T lymphocytes to activate B cells [35], the higher protein antibody titres in HIV-infected children compared to HIV-uninfected children to most surface proteins may be a result of an aberrant response, due to hypergammaglobulinaemia that commonly occurs with HIV infection and frequently results in high antibody titres of lower functionality [36,37]. This is further corroborated by the inverse relationship between antibody titres to protein antigens and $CD4^+$ lymphocyte counts in HIV-infected children. Similar to these findings, serum IgG against pneumococcal surface proteins in HIV-infected adults were also found to be elevated compared to HIV-uninfected adults [38].

The frequency of GBS colonization remains high beyond infancy, although often occurs asymptomatically, however similar serotypes are isolated in both invasive cases and in healthy colonized individuals [5,33,39]. The frequent colonizing serotypes in this population has been reported to be Ia, Ib, II, III and V [5,31]. Factors influencing GBS acquisition and/or transition to invasive disease are poorly understood. GBS colonization information among the study participants was not available, which presented a limitation in our analysis, albeit increased invasive disease was reported amongst HIV-infected and HIV-exposed uninfected infants [40,41] and also in immunocompromised children [3–6].

The evaluated GBS proteins were differed based on the localization of the protein on the surface of the bacteria (lipoprotein or cellwall anchored), and also according to their function as indicated by shared homology of proteins with known function. There was no clear relationship to HIV-status between the presumed function of the protein or in relation to whether it was mapped to an extra cellular location. This was in contrast to that observed for pneumococcal protein antigens in the same study population, in which a selection of membrane and cell-wall associated pneumococcal antigens, had a distinct dichotomy with respect to HIV status, where the HIV-infected group had high titres associated with cell membrane anchored proteins and low titres to cell-wall anchored proteins and vice versa [27]. The author's hypothesised that these differences could have been due to some cross reactive

Table 4
Comparison of the geometric mean concentration (GMC, μg/ml) of group B *Streptococcus* capsular polysaccharides and geometric mean titres (GMT, U/ml) of select surface protein antigens between HIV-uninfected and HIV-infected children between the age of 4–7 years.

	Function	Antigen	HIV-uninfected GMT (95% CI)	HIV-infected GMT (95% CI)	p value
Capsular polysaccharides		Ia	0.078 (0.066–0.093)	0.061 (0.047–0.079)	0.1048
		Ib	0.755 (0.62–0.92)	0.472 (0.34–0.65)	0.0119
		III	0.439 (0.38–0.51)	0.375 (0.32–0.44)	0.1541
		V	2.971 (2.49–3.55)	1.952 (1.54–2.47)	0.0045
Lipoproteins		Gbs0233	180.04 (141.39–229.26)	255.43 (194.80–334.92)	0.0560
	–	Gbs2106	139.11 (94.84–204.05)	53.10 (33.74–83.58)	0.0014
		Foldase PsrA	162.24 (127.04–207.19)	304.56 (212.89–435.70)	0.0037
Cell wall anchored proteins	Adhesin	FbsA	51.70 (38.70–68.96)	150.84 (103.44–219.94)	<0.001
		BibA-COH1	113.83 (93.29–138.91)	142.84 (113.61–179.58)	0.1361
		BibA-NEM316	34.57 (25.71–46.49)	30.71 (23.17–40.69)	0.5662
	Putative Adhesin	Gbs1356	288.46 240.75–345.62)	738.38 (567.43–960.84)	<0.001
		Gbs0393	214.27 (180.93–253.77)	360.64 (275.06–472.86)	0.0011
		Sip	196.41 (160.98–239.65)	77.03 (56.87–104.32)	<0.001
	–	Gbs1539	133.60 (97.61–182.85)	429.85 (294.93–626.49)	<0.001
		Gbs0392	190.61 (103.44–219.94)	345.62 (257.62–462.11)	0.0023

FbsA, Fibronogen binding surface antigen.
BibA, gbs2018 (NEM316 strain) or SAN2207 (COHI strain).
Sip, Surface immunogenic protein.

Table 5
Multiple linear regression of the geometric mean concentration (GMC, μg/ml) of group B *Streptococcus* capsular polysaccharides and the geometric mean titres (GMT, U/ml) of surface proteins among HIV-uninfected and HIV-infected children between the age of 4–7 years.

	Ia		Ib		III		V	
	HIV-Uninfected Coeff (p value)	HIV-Infected	HIV-Uninfected Coeff (p value)	HIV-Infected	HIV-Uninfected Coeff (p value)	HIV-Infected	HIV-Uninfected Coeff (p value)	HIV-Infected
FbsA	0.16 (0.423)	0.10 (0.604)	0.08 (0.644)	0.12 (0.410)	0.23 (0.307)	0.50 (0.076)	0.20 (0.307)	0.14 (0.465)
gbs0233	0.14 (0.399)	0.35 (0.006)	0.22 (0.120)	0.26 (0.011)	0.05 (0.796)	0.67 (0.001)	0.23 (0.151)	0.41 (0.003)
BibACOH1	0.13 (0.329)	0.30 (0.005)	0.09 (0.431)	0.18 (0.043)	0.02 (0.878)	0.46 (0.006)	0.04 (0.784)	0.36 (0.002)
BibANEM	0.51 (0.009)	0.44 (0.001)	0.11 (0.542)	0.30 (0.004)	-0.03 (0.891)	0.79 (<0.001)	0.19 (0.340)	0.55 (<0.001)
gbs2106	0.44 (0.082)	0.64 (0.003)	0.34 (0.133)	0.48 (0.004)	0.27 (0.351)	1.21 (<0.001)	0.13 (0.607)	0.81 (<0.001)
gbs0393	0.09 (0.443)	0.21 (0.101)	0.17 (0.083)	0.19 (0.066)	0.17 (0.180)	0.49 (0.014)	0.19 (0.093)	0.34 (0.016)
Sip	0.10 (0.475)	0.17 (0.262)	0.20 (0.094)	0.11 (0.344)	0.05 (0.758)	0.33 (0.144)	0.13 (0.338)	0.13 (0.423)
Foldase	0.25 (0.132)	0.29 (0.090)	0.17 (0.246)	0.32 (0.018)	0.18 (0.334)	0.72 (0.006)	0.31 (0.057)	0.49 (0.007)
Gbs1539	0.18 (0.390)	0.24 (0.183)	0.14 (0.441)	0.19 (0.193)	0.10 (0.692)	0.61 (0.032)	0.18 (0.389)	0.35 (0.073)
gbs1356	0.29 (0.015)	0.21 (0.104)	0.28 (0.007)	0.15 (0.142)	0.27 (0.054)	0.32 (0.103)	0.29 (0.014)	0.23 (0.096)
gbs0392	0.36 (0.026)	0.44 (0.001)	0.24 (0.091)	0.33 (0.002)	0.43 (0.021)	0.78 (<0.001)	0.30 (0.066)	0.54 (<0.001)

FbsA, Fibronogen binding surface antigen.
BibA, gbs2018 (NEM316 strain) or SAN2207 (COHI strain).
Sip, Surface immunogenic protein.

epitopes from other bacteria in HIV infected individuals due to increased gut microbial translocation in this population. Similarly, increased cross-reactive epitopes in HIV-infected children may also be responsible for the higher GMTs observed in this study despite the lack of an apparent dichotomy.

The study limitations included the use of Polygam as a reference serum, which is pooled IgG from human adults and thus may result in an overestimation of IgG titres with cross-reactive antibodies induced by other species, especially for antigens that had low specificity. However we conceived that since the assay was performed consistently throughout, any cross-reactive antibodies detected would result in a proportional increase and therefore would not greatly affect the difference between the study groups compared. Furthermore, some of the study participants received PCV vaccine and/or were colonized with *S. pneumonia*, albeit no cross-reactivity was evaluated between GBS surface antigens and pneumococcal specific antibodies. However it is noteworthy that GBS specific IgG levels were not associated with PCV vaccination status or colonization with *S. pneumonia* in the respective children.

The process of reverse vaccinology in vaccine design and development has been used successfully in identifying essential virulence factors of *N. meningitidis* and *S. agalactiae* which are

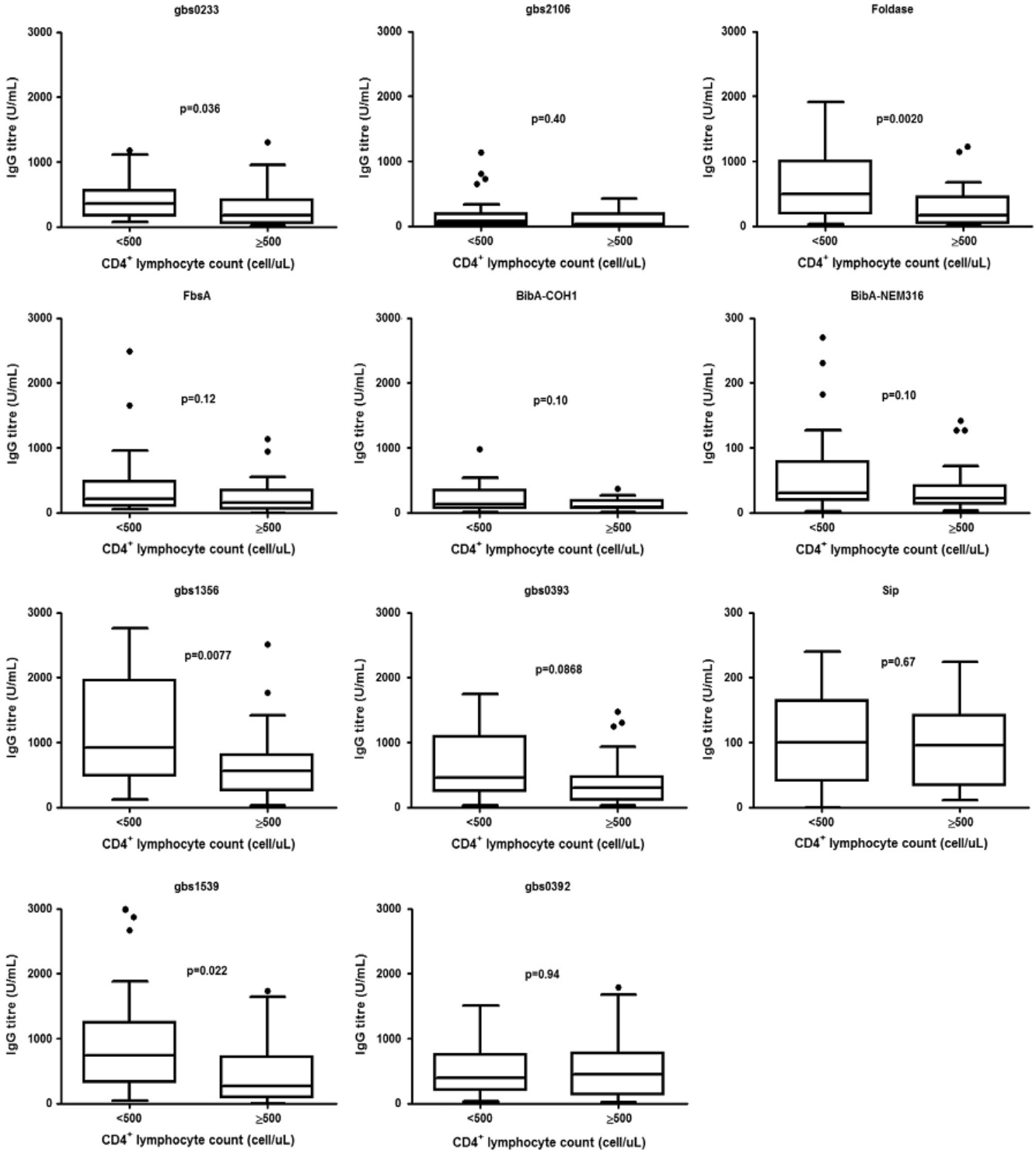

Fig. 1. IgG titres (U/mL) against group B *Streptococcus* surface proteins among HIV infected children comparison between those with $CD4^+$ count < 500 cells/ml and those with $CD4^+$ count ⩾ 500 cell/μl.

conserved amongst several serotypes thus making them possible universal vaccines [20,42]. Using reverse vaccinology we were able to identify novel immunogenic proteins localized on the surface of GBS. This study revealed that natural antibody responses to GBS surface antigens are produced post-infancy, irrespective of HIV status. However differences in the antigen specific titres should be investigated as a possible cause of a dysfunctional immune response or increased GBS colonization in HIV infected children. Furthermore characterization of antibody avidity and functionality should be evaluated to determine if the risk of invasive GBS diseases beyond infancy is a factor of immunosuppression resulting from a dysfunctional and dysregulated immune system. Should the attenuated response to these epitopes, particularly serotype-specific CPS and select GBS surface proteins, be responsible for the increased risk in susceptibility to GBS in severely immunocompromised HIV infected individuals, these epitopes may have potential as future vaccine candidates [43–46].

Conflict of interest

All authors have reviewed the manuscript and approved submission for publication. The authors have declared no potential

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BRIEF REPORT

Reduced Transplacental Transfer of Group B *Streptococcus* Surface Protein Antibodies in HIV-infected Mother–Newborn Dyads

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We evaluated the effect of maternal HIV infection on transplacental antibody transfer specific to 8 group B *Streptococcus* (GBS) surface proteins among 81 HIV-uninfected and 83 HIV-infected mother–newborn pairs using a multiplex immunoassay. Significantly lower antibody titers were detected in HIV-infected mothers and HIV-exposed uninfected newborns compared to HIV-uninfected mother–newborn dyads. Maternal HIV infection was also associated with reduced transplacental transfer of antibodies for Sip (25.8%), Foldase (30.4%), gba0392 (36.5%), gbs0393 (32.9%), gbs1539 (39.2%), gbs2106 (35.7%), and BibA (19.4%); $P < .003$. This reduced transplacental antibody might contribute to increased susceptibility for invasive GBS disease in HIV-exposed uninfected infants.

Keywords. group B *Streptococcus*, surface proteins, antibodies, trans-placental transfer, HIV

Group B *Streptococcus* (GBS) is the most frequent cause of neonatal morbidity and mortality, with the highest incidence (1.21 cases per 1000 live births) observed in Africa [1]. Preterm delivery and low birth weight are risk factors associated with increased invasive GBS disease incidence in infants. In addition, maternal GBS disease and dissemination in utero may trigger premature birth, late miscarriages, and stillbirths [1]. Recently, maternal HIV infection has been shown to increase the risk of invasive GBS disease in infants with in-utero HIV exposure, even in the absence of them being HIV infected [2].

Increased susceptibility to bacterial and viral infections in HIV-exposed uninfected (HEU) infants is partly attributable to reduced maternally derived antibodies [2, 3]. Similarly, transplacental transfer of antibodies against GBS capsular polysaccharide is lower in HIV-infected women, [4], which might contribute to the heightened susceptibility to invasive GBS disease amongs HEU infants [2]. Hypergammaglobulinaemia, a common feature among HIV-infected individuals, has been proposed as a possible mechanism whereby Fc receptors (FcRn) on maternofetal syncytiotrophoblast that are essential in transporting IgG across the placenta are saturated by nonspecific antibodies, resulting in reduced transplacental transfer of antibodies [5].

Considering that protein antigens induce antibodies that are preferentially transported by FcRn [6], maternal HIV infection could affect the transplacental transfer of antibodies targeted at GBS surface proteins. The pangenome sequence of GBS consists of 396 conserved genes encoding surface-associated proteins, and a further 193 genes encoding variable-surface proteins[7], for which virulence characteristics and immunogenicity have only been described for a few. The aim of this study was to investigate the relationship between HIV infection and transplacental transfer of naturally occurring antibodies directed against 8 GBS surface proteins previously identified using computational algorithms [8].

METHODS

Study Population

Serum samples from mother–newborn pairs (who were previously recruited to participate in a study to evaluate the effects of maternal HIV infection on transplacental transfer of antibodies to GBS polysaccharide and pilus island epitopes) were analyzed [4]. Briefly, 164 women with confirmed HIV status during pregnancy, who delivered infants at ≥ 36 weeks' gestation age and birth weight of ≥ 2500 grams were enrolled. GBS colonization amongs the women was confirmed by a positive growth culture of lower vaginal and rectal swab using selective media as described [4]. Serum samples taken from maternal and cord blood was stored at –70°C until analyzed for this study.

Purification of Recombinant Proteins

Genes for GBS surface proteins gbs0392, gbs0393, gbs1356, gbs1539, gbs2106, Sip, and Foldase PsrA (gbs2107) were cloned from serotype III NEM316 strain into pETite vector (Lucigen, Middleton, WI). Cloned vectors were transformed into an *Escherichia coli* BL21 strain for protein production. Proteins were affinity purified in immobilized Ni^{2+} ion-affinity columns using 500 mM imidazole, 0.5 M NaCl, and 20 mM phosphate buffer solution, and stored in 50% glycerol at –70°C. Prior to performing immunological analysis, proteins were desalted

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using PD-10 desalting columns (GE Healthcare, Germany) as per manufacturer's instructions.

Quantification of Antibody Titers to GBS Surface Proteins

Unique microbead regions (Bio-Rad, Hercules, CA) were bound with 50 µg/ml of protein using a 2-step carbodiimide reaction [9]. The protein-coupled beads were blocked with phosphate-buffer saline, 10% fetal bovine serum, and 0.05% NaN_3, and stored in the dark at –4°C. Multiplex Luminex assay was performed as previously reported [8]. Immunoglobulin G (IgG) titers of paired maternal and cord serum samples were measured simultaneously. Serum IgG titers were measured in duplicates of 1:200 dilutions. Polygam (adult pooled IgG; National Bioproducts, South Africa) was used as a reference serum and assigned 100 arbitrary units per milliliter per antigen. IgG titers were extrapolated for each antigen and reported as units/mL (U/mL). Two controls were included in each immunoassay that consisted of pools of high- and low-titer serum samples from HIV-uninfected pregnant women. Participants with antibody levels below the detection limit were assigned a value one-quarter of the lower limit of detection.

Statistical Analysis

Data analysis was performed using STATA software (version 13.1, Stata Corp, College Station, TX) and GraphPad Prism (version 5.03, GraphPad Software, San Diego, CA). Maternal and cord antibody titers were $\log_{10}$-transformed for parametric statistical comparisons. Differences in geometric mean titer (GMT) between HIV-uninfected and HIV-infected mothers at delivery together with the respective GMT between HIV-unexposed and HEU newborns were tested with a 2-tailed Student *t* test. A *P* value < .05 was considered statistically significant after adjusting for GBS colonization, maternal age, gestational age and parity using multivariate regression.

Cord–maternal antibody ratios were calculated for each mother–newborn dyad to evaluate the effect of HIV infection on transplacental transfer of antibodies. Quantile regression was used to compare median ratios between HIV-uninfected and HIV-infected mother–newborn dyad, and adjusted for GBS colonization, colonizing serotype, maternal age, gestational age, and parity. Furthermore, a percentage difference in cord–maternal antibody ratios (CMRs) was calculated as $(1 - CMR_{HIV+}/CMR_{HIV-}) \times 100\%$ to measure the relative efficiency of transplacental antibody transfer between HIV-infected compared to HIV-uninfected women. Among HIV-infected mothers, the correlation between maternal antibody titers and maternal $CD4^+$ lymphocyte count and HIV-1 viral load was measured using univariate quantile regression.

All participants in the original study signed informed consent forms. This study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Approval number: M140902), who granted a waiver of further consent for the additional testing of the archived samples.

RESULTS

A total of 164 mother–newborn dyads were analyzed in this study. Detailed demographic information about the study participants is shown in Supplementary Table S1 [4]. Briefly, of the 164 mothers, 81 were HIV uninfected and 83 were HIV infected. $CD4^+$ lymphocyte counts were available for 71 (85.5%) of the 83 HIV-infected women, with a median of 423 cells/µL (range, 46–1268 cells/µL). HIV-1 RNA viral loads were detected in 79 HIV-infected women with a median of 96 copies/mL (range, 20–146 055 copies/mL). Using vaginal and rectal swabs, positive GBS growth at delivery were detected in 29.9% (49 of 164) women, including among 27.2% (22 of 81) HIV-uninfected and 32.5% (27 of 83; *P* = .45) HIV-infected women (Supplementary Table S1). Serotype distribution did not differ by HIV status, with the frequently colonizing serotypes being Ia (40.8%), III (28.6%), V (18.4%), II (8.2%), and Ib (4.1%) [4].

Maternal Antibodies to GBS Surface Proteins

All maternal serum samples had detectable antibody titers for the analyzed GBS surface proteins. IgG GMT against the GBS surface proteins analyzed did not differ by GBS colonization status among the women (Supplementary Table S2). HIV-infected women had lower antibody GMT (U/mL) compared to HIV-uninfected women, including after adjusting for cofactor variables, for proteins Sip (132.42 vs 186.72; *P* = .002), Foldase (162.06 vs 220.82; *P* = .035), and other putative proteins, including gbs2106 (153.62 vs 235.11; *P* = .002), gbs0393 (258.97 vs 381.35; *P* = .001), and gbs1356 (156.36 vs 215.49; *P* = .035) (Figure 1). Similar trends were observed for gbs0392 and gbs1539, with antibody GMT being higher among HIV-uninfected compared to HIV-infected women, albeit not statistically significant. No significant correlation was observed between $CD4^+$ lymphocyte count and HIV-1 RNA viral load with maternal antibody titers for all the GBS surface proteins (Supplementary Table S3 and S4).

Infant Antibodies to GBS Surface Proteins

Of the 164 cord serum samples analyzed, 18 samples had antibody titers below the detection limit for gbs1539, 2 had no detectable titers to gbs1539 and gbs0392, and 1 had no detectable titres for gbs1539, gbs0393, Sip and gbs1356. Antibody GMT in HEU newborns (n = 83) were significantly lower compared to HIV-unexposed newborns (n = 81) for all proteins after multivariate regression controlling for cofactor variables, including for Sip (80.37 vs 153.70; *P* < .0001), Foldase (71.07 vs 142.27; *P* < .0001), BibA (127.46 vs180.42; *P* = .026), gbs2106 (82.91 vs 205.83; *P* < .0001), gbs0393 (113.50 vs 241.33; *P* < .0001), gbs1539

Table 1. Cord–Maternal Ratio of Antibody Titers Against Group B *Streptococcus* Surface Proteins in HIV-uninfected and HIV-infected Mother–Newborn Dyads

Surface Proteins	HIV-uninfected Mother–Newborn Dyad Median CMR (IQR) n = 81	HIV-infected Mother–Newborn Dyad Median CMR (IQR) n = 83	Reduction (%)[a]	*P* Value[b]
Sip	0.82 (0.70 – 1.03)	0.61 (0.46 – 0.79)	25.6	<.0001
BibA	0.88 (0.71 – 1.14)	0.71 (0.50 – 0.85)	19.3	.002
Foldase	0.65 (0.51 – 0.86)	0.45 (0.32 – 0.64)	30.8	.001
gbs2106	0.91 (0.73 – 1.11)	0.58 (0.42 – 081)	36.3	<.0001
gbs1356	0.51 (0.40 – 0.75)	0.32 (0.23 – 0.51)	37.2	.002
gbs0393	0.64 (0.52 – 0.85)	0.43 (0.27 – 0.63)	32.8	<.0001
gbs1539	0.39 (0.23 – 0.55)	0.24 (0.14 – 0.41)	38.5	<.0001
gbs0392	0.40 (0.27 – 0.63)	0.25 (0.16 – 0.42)	37.5	.027

Abbreviations: BibA, group B Streptococcus immunogenic bacterial adhesion; CMR, cord–maternal ratio; HIV, human immunodeficiency virus; IQR, interquartile range; Sip, surface immunogenic protein.

[a]CMR reduction calculated as $(1 - CMR_{HIV+}/CMR_{HIV-}) \times 100\%$.

[b]Quantile regression *P* value adjusted for overall colonization, colonizing serotype, maternal age, gestational age, and parity.

(73.12 vs 138.38; $P = .016$), gbs1356 (55.05 vs 104.06; $P = .001$), and gbs0392 (81.70 vs 130.2; $P < .0001$) (Figure 1).

Effects of HIV Infection on Transplacental Transfer of Antibodies

Maternal antibody titers correlated positively with cord antibody titers for all GBS surface proteins, $R^2 \geq 0.5$ and $P < .0001$. Cord–maternal antibody ratios were compared between HIV-infected and HIV-uninfected mother–newborn dyads using quantile regression and adjusted for cofactor variables. Maternal HIV infection was associated with lower ratios of transplacental transfer of antibodies for Sip (25.8%; $P < .001$), Foldase (30.4%; $P < .001$), gba0392 (36.5%; $P = .006$), gbs0393 (32.9%; $P < .001$), gbs1539 (39.2%; $P < .008$), gbs2106 (35.7%; $P < .001$), and BibA (19.4%; $P = .004$) (Table 1). Antibodies to proteins gbs1539 and gbs0392 had ≤0.40 median cord–maternal antibody ratios, irrespective of maternal HIV infection. No correlation was observed

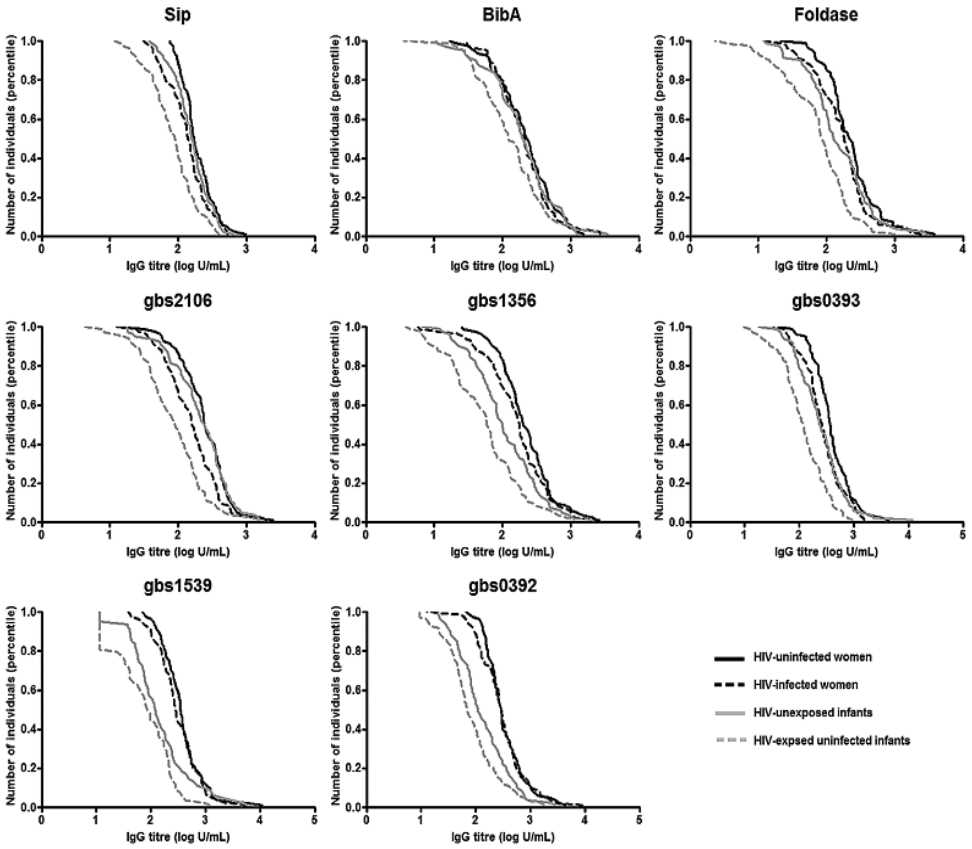

Figure 1. Reverse cumulative distribution of antibody titers against group B *Streptococcus* surface protein antigens in 81 HIV-uninfected (solid black line) and 83 HIV-infected (dotted black line) women at delivery together with their respective HIV-unexposed (solid gray line) and HIV-exposed uninfected (dotted gray line) newborn infants.

between cord–maternal antibody ratios with the maternal $CD4^+$ lymphocyte count and HIV-1 RNA viral loads (Supplementary Table S5 and S6).

DISCUSSION

In our study, we observed a 19%–39% reduction in the concentration of transplacental transfer GBS surface protein antibodies in HIV-infected compared to HIV-uninfected mother–newborn dyads. Low antibody GMT in HIV-infected mothers together with reduced efficiency of transplacental transfer of antibodies resulted in lower antibody GMT in HEU infants compared to HIV-unexposed infants. The results of this study are similar to that reported for antibody against *Haemophilus influenzae* type b, *Bordetella pertussis*, tetanus, and *Streptococcus pneumoniae* in HIV-infected mother–newborns [3].

A reduction in transplacental transfer of antibodies against GBS capsular polysaccharide for the predominantly invasive serotypes Ia, II, III, and V has been noted previously [4]. Maternally derived antibodies against these invasive serotypes play an important role in providing protection to infants [10]. It is therefore likely that the impediment of maternofetal antibody transfer against GBS surface proteins together with antibodies against GBS capsular polysaccharide (CPS) during intrauterine HIV exposure may, in part, contribute to the increased risk of developing invasive GBS disease in infants.

The GBS surface proteins Sip and BibA have been previously reported to confer protection in newborn pups challenged with a lethal dose of GBS, when born to mice dams immunized with the respective protein [11, 12]. The virulence and immunological role of the other evaluated GBS proteins are unknown, as these proteins were identified computationally; however, these proteins have emerged in other studies as potential GBS virulence factors. Using subtractive DNA hybridization, gbs1356 and gbs0393 were shown to be homologous to a family of adhesion proteins that play a role in the binding of GBS to host epithelial cells [13].

IgG_1, primarily induced by protein antigens, is efficiently transported across the placenta compared to IgG_2 induced by CPS. Therefore, maternal immunization using a protein-based vaccine might result in higher transplacental transfer of antibody concentrations to provide protection against invasive GBS disease in newborns [6]. Considerations, however, should be taken in selecting highly immunogenic protein antigens that are capable of crossing the placenta and provide protection to both the mother and the infant. Antibodies to the highly conserved proteins such as Sip (expressed by all GBS isolates), BibA (that exist in 3 different allelic forms [12]), and gbs2106 protein were efficiently transferred across the placenta in the absence of maternal HIV infection.

The prevalence of GBS colonization in HIV-infected women is similar to HIV-uninfected women, including for countries where there is a higher burden of HIV infection [14] and as observed in our study population. Antibody GMT did not differ by colonization status or colonizing serotype among HIV-uninfected and HIV-infected mothers for the investigated GBS surface protein antigens. Acquisition of GBS colonization occurs frequently during pregnancy [14]; therefore, the difference in the IgG level that may result from GBS colonization cannot be detected using a cross-sectional study [15]. Other limitations of the study include use of Polygam (National Bioproducts, South Africa) as reference serum, which does not allow quantification of absolute antibody concentrations comparable to other study populations.

Given that HEU infants are at high risk of invasive GBS due to reduced maternally derived antibodies, increasing maternal antibodies by vaccination has the potential to reduce the risk of invasive GBS disease. HIV-infected women, however, were reported to have lower antibody concentrations specific to CPS following immunization with a polysaccharide-protein glycoconjugate-based vaccine [10]. The poor immunogenic response in HIV-infected women may be circumvented by either adapting an alternate dosing schedule or dosing strength, or substituting for a GBS surface protein. Because it appears that the antibody response induced by proteins Sip, gbs2106, gbs1356, gbs0393, and Foldase are attenuated by HIV infection, this might contribute to the increased susceptibility to invasive GBS disease in HEU infants; therefore, further investigations are required to assess the potential inclusion of these proteins as vaccine candidates for protection against invasive GBS disease.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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