Group-B Streptococci: developing a correlate of protection for future vaccine trials with the help of pregnant Gambian women and their infants

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PhD Thesis 2016

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Declaration

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February 2016

Abstract

Background: Vertical GBS transmission is the prerequisite for early-onset disease. Disease protection is associated with maternally-derived anti-GBS antibody. This study investigates the correlation between serotype-specific functional antibody in cord sera and maternal/infant GBS colonisation, as a surrogate marker of GBS colonisation and thus disease protection.

Methods: 750 Gambian mother/infant pairs were followed to day 60-89 postpartum. Maternal/infant GBS colonisation was determined by culture and polymerase chain reaction. Cord and infant serum were collected for functional antibody concentrations, analysed by flow cytometry.

Results: Maternal colonisation was 33.7%; serotype (ST) V was most prevalent. Functional antibody concentration was lower in colonised- than in non-colonised mother/infant pairs at birth (STIa ($p<0.001$), STII ($p<0.001$), STIII ($p<0.001$) and STV ($p<0.001$)). Mother-colonised/infant-non-colonised pairs had significantly lower functional antibody concentration than non-colonised mother/infant pairs against STIa ($p=0.001$), STII ($p=0.001$), STIII ($p<0.001$) but not STV ($p=1.0$). Persistently colonised infants had lower functional antibody concentration than non-colonised infants against STII ($p=0.04$) and STV ($p=0.01$). Reduced infant colonisation risk was associated with functional antibody concentration above the 75th centile against STIa ($p<0.001$), STII ($p<0.001$), STIII ($p=0.01$) and STV ($p<0.001$). A ST-dependent threshold was observed above which infants were non-colonised at birth.

Conclusions: Higher concentrations of functional maternally-derived antibodies are associated with a decreased risk of GBS colonisation in infants up to day 60-89 of life. Enhancing maternally-derived antibody concentrations through vaccination may reduce infant colonisation and so reduce the risk of GBS disease.

Acknowledgements

I acknowledge the following people for their support, encouragement and collaboration on this project:

Imperial College London

Prof. Beate Kampmann (Principal Supervisor)

Public Health England, Porton Down

Prof. Andrew Gorringe (Co-supervisor)

Mrs. Fiona Warburton (Statistician)

Dr. Stephen Taylor

Mrs. Lauren Allen

Miss. Jessica Birt

Mrs. Holly Humphries

Mr. Stephen Thomas

St. George's, University of London

Prof. Paul Heath (Co-supervisor)

Medical Research Council (MRC) Unit, The Gambia

Dr. Martin Antonio

Dr. Ed Clarke

Mrs. Saffiatou Darboe

Mr. Sheikh Jarju

Mr. Amadou Faal

Mr. Mustapha Jaiteh

Miss. Francess Safo

Mr. Ebenezer Foster-Nyarko

Thanks

I would like to express my special appreciation and thanks to my supervisor Professor Beate Kampmann for your encouragement and supporting me personally through thick and thin. I would also like to thank my co-supervisors: Professor Paul T. Heath and Professor Andrew Gorringe for your constant support, encouragement and advice. Without the support of these three leaders in science there would not be a PhD candidate here today. Thanks also to Dr. Stephen Taylor, Mr. Stephen Thomas and Mrs. Lauren Allen for all your help in setting me up to run the assays at Porton Down and for your unfailing humour as I discovered yet another flow cytometer problem and Mrs. Saffiatou Darboe and Mr. Sheikh Jarju for your expertise in teaching me the laboratory techniques at MRC. Thanks are also due to Dr. Anna Battersby and Dr. Robin Basu Roy for being my source of support in the Gambia and Dr. Gabriella Watson for covering the fieldwork whilst I was travelling. Thanks also to my field and laboratory staff who have worked extremely hard to make the project such a success; to Professor Carol Baker for allowing me access to her standards and original pathogen collection used in the development of the immunological assays and Dr. Monica Fabrini and Dr. Immaculata Margit y Ros from Novartis Vaccine Laboratories for allowing access to their OPkA methods. A special thanks goes to my family. Words cannot express how grateful I am to my husband for all of the sacrifices that you've made on my behalf as I left you with the children for 18 months to conduct the fieldwork. Your long stints in proofreading this thesis and provision of food have not gone unnoticed. To my daughters Megan and Krystal, thank you for not creating havoc in the house whilst I was away and letting Papa think he was looking after you.

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Abbreviations

ABC - ATP binding cassette
ACP – alpha C protein
AMP – anti-microbial peptide
ANC - antenatal clinic
AOR - adjusted odds ratio
APC – antigen presenting cell
APH - antepartum haemorrhage
BB – blocking buffer
BCG – Bacille Calmette Guerin
BibA – GBS immunogenic bacterial adhesion
BSA – Bovine Serum Albumin
C3c – complement fragment 3c
CAMP – Christie, Atkinson, Munch, Peterson
CCNA – Colombia Colistin Naladixic acid Agar
CDA – complement deposition assay
cfb – CAMP factor B
CFU – colony-forming units
CI - confidence interval
CIP – complement inhibitor protein
CO_2 – carbon dioxide
CoV - coefficient of variance
CPS – capsular polysaccharide
CSF - cerebrospinal fluid
CspA – cell surface protease A
Ct – cycle threshold
DMF - N,N-Dimethylformamide
DNA – deoxyribonucleic acid
DTP – Diptheria, Tetanus, Pertussis
ECM – extra cellular matrix
ELISA - enzyme-linked immunoabsorbent assay
EO – early onset
FbsA/B – fibrinogen-binding proteins A and B
Fc – fragment crystalline
FCI – Fetal Clone I
FcRn – fragment crystalline receptor
FI – Fluorescence Intensity
FI-C – Fluorescence Intensity – conjugate
FI-C' – Fluorescence intensity – complement
FITC - fluorescein isothiocyanate
FL 1 – fluorescence
fOPA - fluorescence-based opsonophagocytosis assay
FS – forward scatter
GBS – Group B Streptococcus
GM - geometric mean

GMC – geometric mean concentration
H_2O_2 – hydrogen peroxide
Hb – haemoglobin
HBSS – Hanks buffered salt solution
HBV – Hepatitis B Vaccine
Hib – Haemophilus influenza B
HIV – human immunodeficiency virus
HL-60 – human promyelocytic leukaemia cells
HvgA – hypervirulent GBS adhesion
IAP – intrapartum antibiotics
IBA – IgG surface-binding assay
IgA/G/M – immunoglobulin A/G/M
IL – interleukin
ING – Infant Nutrition Group
IQR - interquartile range
JFPH – Jammeh Foundation for Peace Hospital
LBW - low birth weight
Lmb – laminin-binding protein
LMIC - Low and middle income countries
LO – late onset
LPS – lipopolysaccharide
LTA – lipoteichoic acid
MBL – mannose-binding lectin
MHC – major histocompatibility complex
MRC – Medical Research Council
O_2 – oxygen
OPkA – Opsonophagocytosis killing assay
OPV – oral polio vaccine
OR - odds ratio
PavA - pneumococcal adhesion and virulence protein A
PbP – penicillin-binding protein
PBS - phosphate buffered saline
PCR – polymerase chain reaction
PCV13 – Pneumococcus Conjugate Vaccine 13 valent
PHE – Public Health England
PilB - pilus protein island B
PMN – polymorphoneuclear leukocytes
PPH - postpartum haemorrhage
RABA - radioantigen binding assays
RCD – reverse cumulative distribution curve
RPMI – Rosewell Park Memorial Institute medium
Rt-PCR – Real-time Polymerase Chain Reaction
SD - standard deviation
SHRS – standard human reference serum
SHT – streptococcal histadine triad
Sod – superoxide dismutase

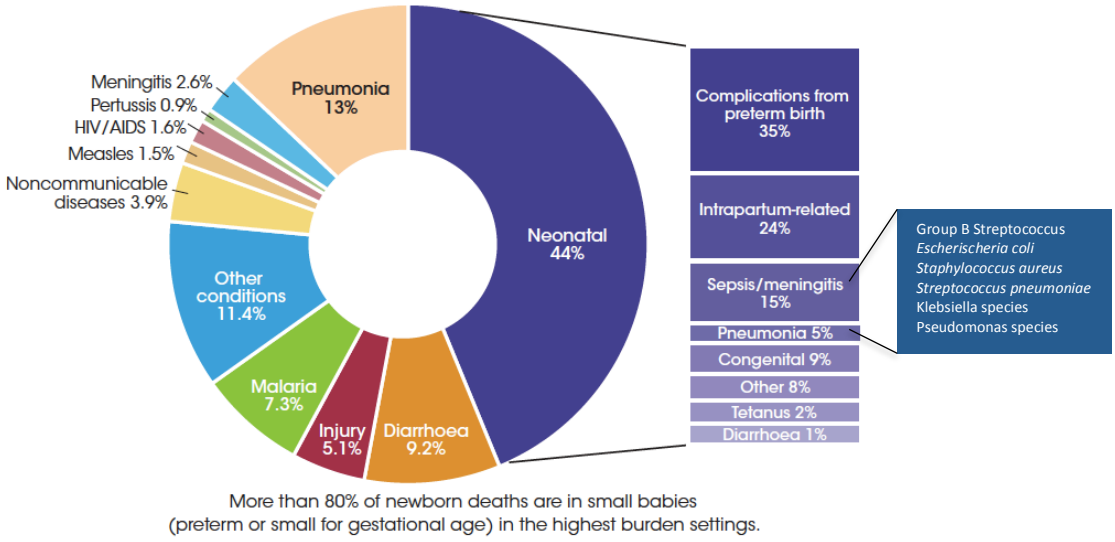
Srr1 – serine-rich repeat domain protein 1
SS – side scatter
ST – serotype
st-sequence type
STGG – Skim milk-tryptone-glucose-glycerol
TBA - traditional birth attendant
TD – T-cell dependent
THB – Todd Hewitt Broth
TI – T-cell independent
TNFα – tumour necrosis factor-alpha
UK – United Kingdom
USA - United States of America
UV – ultraviolet
WHO – World Health Organisation

Chapter 1 - Background

1.1. General Introduction

Globally, neonatal mortality remains unacceptably high, with little change in the death rate in the first 28 days of life, despite reductions in under-5 mortality of up to 50% since 1990.(1) In 2014, neonatal deaths accounted for 44% of all deaths in children under 5 with neonatal infection accounting for over a third of all deaths.(1) (Figure 1).

Figure 1 - Causes of neonatal mortality as a proportion of all under-5 mortality



Adapted from World Health Organisation, Every Newborn 2015(1) and Waters et al. 2011(2) pie chart demonstrates % of neonatal deaths, first pull out indicates causes of neonatal deaths, second pull out indicates main causes of neonatal sepsis/meningitis and pneumonia.

Group B Streptococcus (GBS) remains the leading cause of neonatal mortality in the United Kingdom (UK) (0.5/1000 live births)(3) and the United States of America (USA) (0.34-0.37/1000 live births)(4), with long-term adverse neurodevelopmental outcomes in up to 50% of survivors.(5) In low- and middle-income countries, little is known about the role of GBS in these deaths.(6)

GBS acquisition occurs through vertical transmission in 15%-50% of infants born to a vaginally/rectally colonised mother. Maternal colonisation is a prerequisite

for early onset (EO) and a risk factor for late onset (LO) disease.(4, 7) EO-GBS disease can be prevented by intrapartum antibiotics (IAP) and, in a number of resource-rich countries EO-GBS disease has become significantly less common following the introduction of IAP strategies.(4, 8) These strategies include both universal and risk-based screening of pregnant women. In the United States of America (USA) women over 35 weeks of pregnancy, or those in labour prior to 35 weeks are screened for GBS and if GBS is identified they are offered high dose intravenous benzylpenicillin in labour.(7) This strategy is thought to be the reason for the rapid decline in EO-GBS disease and GBS-associated mortality since the first introduction of guidelines in 1996. However, there is much debate about the efficacy of IAP protection and a recent Cochrane review found high levels of potential bias in studies reporting efficacy of IAP as an intervention to reduce GBS disease.(9) Whilst the report found evidence for a reduction in EO-GBS disease, there was no evidence of a reduction in all cause or GBS-associated mortality or LO-GBS disease.(9) The United Kingdom (UK) National Screening Committee examined the evidence for the prevention of GBS disease and, in contrast to the USA, recommended that routine screening of all pregnant women should not be the part of UK antenatal care.(3) In resource-poor countries IAP use poses additional logistical and financial problems and is poorly implemented.(10, 11)

A maternal vaccine would be effective in preventing EO- and LO-GBS disease via placental anti-GBS antibody transfer, as well as protecting the mother against puerperal GBS sepsis. There are several capsular polysaccharide (CPS) conjugate vaccine candidates for maternal vaccination in development to prevent both EO- and LO-GBS disease. These CPS-conjugate vaccines include serotypes (STs) Ia, Ib and III,

based on epidemiological data from the UK, USA and South Africa.(12, 13) However, several obstacles exist in moving the most advanced vaccine into phase III clinical trials. The first is that, given the relative rarity of GBS disease in Europe and the USA, large numbers of infants would need to be recruited to determine vaccine efficacy (at an incidence rate of 1/1000 live births it is estimated that 60,000 infants would be required to show an effective vaccine). Secondly, there is practically no evidence outside of the USA, Europe and South Africa regarding disease-causing and colonising STs. This poses additional dilemmas in establishing vaccine efficacy in those areas of the world where the burden of neonatal infection is highest. Finally, obstacles exist in determining what concentration of antibody is required to protect the infant for the duration of the at-risk period (first three months of life), as there are currently no internationally recognised standards from which to interpret individual study results.

As colonisation is the prerequisite for EO-GBS disease, this thesis seeks to introduce studies that attempt to address the efficacy of maternal antibody in reducing and eliminating colonisation in the infant by determining the functional antibody concentration that prevents infant colonisation.

1.2. Group B Streptococcus

GBS is a Gram-positive bacterium that causes invasive infection and can be fatal to both mothers and their infants.(4, 11) GBS are oxidase-negative, catalase-negative cocci occurring in chains and are facultative anaerobes that are serologically classified on the basis of cell wall polysaccharide antigens.(14) GBS is an encapsulated bacterium and to date 10 STs have been identified.(15) GBS can

colonise the vagina, rectum and the intestines of pregnant women and can be vertically transmitted to their neonates during delivery. Up to 30% of women carry GBS in the vagina or rectum without it causing problems or symptoms.(8, 16, 17) However, GBS may cause fatal infections including meningitis, sepsis and pneumonia throughout the neonatal period.(18, 19) Vaginal colonisation in labour is the major risk factor for neonatal GBS disease.

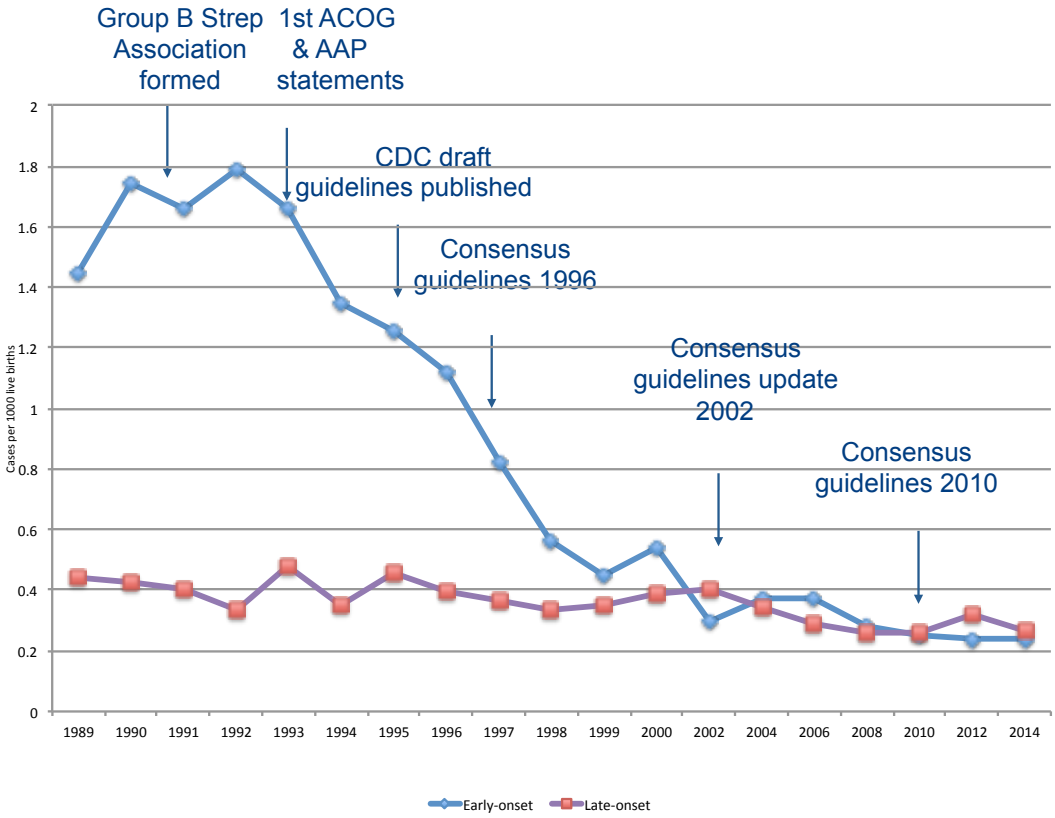
1.2.1. Global epidemiology of invasive GBS disease

GBS invasive disease has two forms, EO, within six days of life, which commonly presents as pneumonia or sepsis and LO, which presents after day six, often with meningitis and focal infections (4).

1.2.1.1. GBS in high-income countries

In the USA and Europe, GBS-STs causing invasive disease are predominantly ST Ia, Ib, II, III and V.(8, 20) EO-GBS disease is due to several STs (Ia, Ib, II, III and V in 70% of cases) while LO-GBS disease is predominantly due to STIII.(20) Since the introduction of IAP in the USA rates of EO-GBS disease have declined from 1.7 per 1000 live births in the 1990s to 0.34-0.37 per 1000 live births in 2014 (Figure 2).(4) However, over the same time period IAP has had little effect on LO-GBS disease (Figure 2).

Figure 2 - Incidence of early- and late-onset invasive GBS disease in the USA



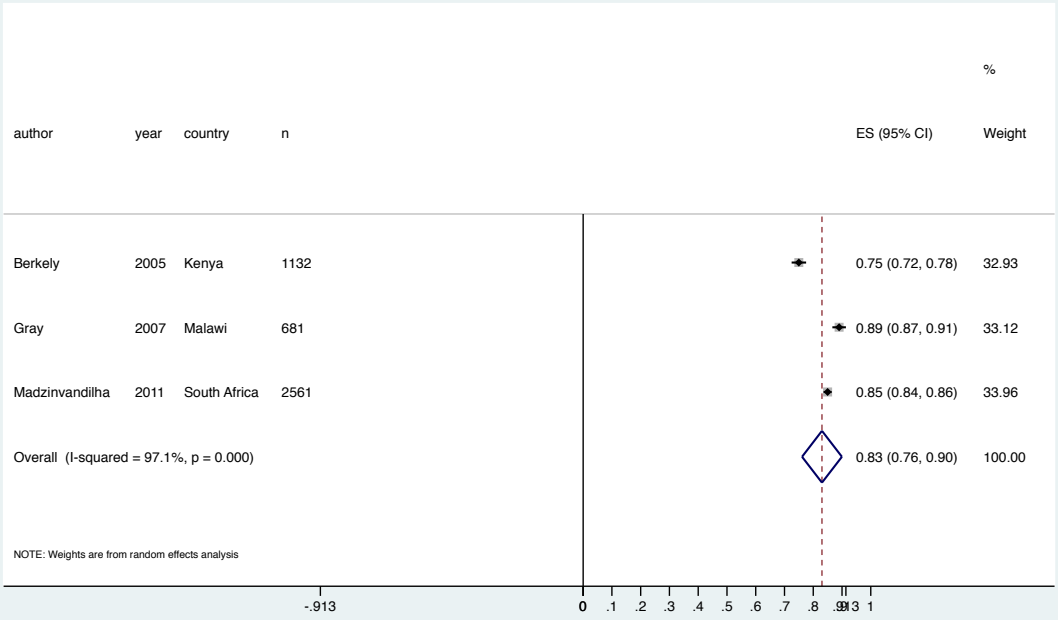
Active Bacterial Core surveillance areas 1990-2014 and activities for prevention of GBS disease (adapted from Active Bacterial Core Surveillance 2015)(21). Blue line= early onset disease, purple line=late onset disease

1.2.1.2. GBS in low and middle-income countries (LMIC)

Although the epidemiology of GBS in high-income countries is well documented, its contribution to the burden of neonatal infection in LMIC has proven more difficult to assess.(10) Six studies from five countries (Malawi, South Africa, Kenya, Nigeria, Zimbabwe)(22-27) report estimates of invasive neonatal disease with an overall pooled disease rate of 4% of neonatal infection (95% CI 1-6). Estimates of LO-GBS disease in LMIC range from 1-4 per 1000 live births.(28) Three studies report STs of disease-causing isolates (22, 24, 26) and a random effects meta analysis demonstrated GBS ST III, Ia and V accounted for 84% of EO- and 96% of LO-GBS disease in African countries, although high heterogeneity (I^2 of 99.7%) prevents any

extrapolation of the data to other populations (Figure 3). Conversely, Asian studies have reported a much lower incidence.(29-31)

Figure 3 - Random effects meta-analysis of percentage of GBS disease caused by GBS ST Ia, III and V in African countries 2000-2015



Berkely et al. included all children admitted with fever between August 1998 and Juiy 2002; Gray et al. included all children with a positive blood culture for GBS between May 2004 and June 2005; Madzinvandiha et al. included all positive GBS cultures identified in the laboratory between January 2004 and December 2008 as part of a prospective cohort trial of chlorhexidine wash in labour to prevent GBS disease. All studies used BACTEC automated blood culture systems to identify positive cultures followed by latex agglutination to identify GBS.

Under-identification and under-reporting of cases and deaths appears very likely in some study settings, especially where it is difficult to access healthcare. A substantial proportion of births in sub–Saharan Africa (42-65%), India (53%) and Bangladesh (85%) for example, occur outside health institutions. In contrast, South Africa, which reports one of the highest incidences, also has a high proportion of hospital-based births (89%).(28) Despite GBS colonisation rates in the Gambia in the

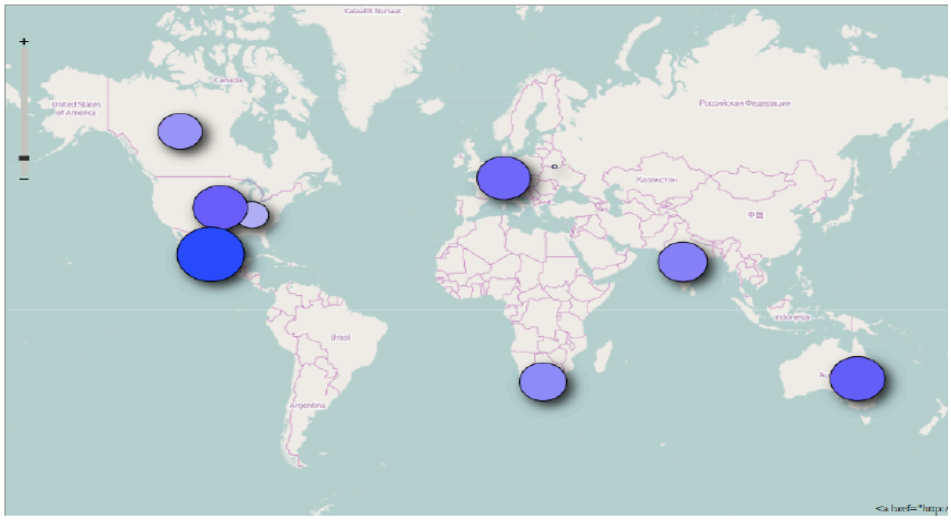
late 1990's of 22%(17), in a separate study, Mulholland et al. (1999) reported no neonatal GBS disease.(32) However, this study excluded neonates dying before day two of life - a number of whom may have succumbed to GBS disease. These fatalities would result in inaccurate measures of EO-GBS disease incidence.

1.2.2. Global epidemiology of colonising GBS

To estimate the true burden of GBS disease, it may be useful to determine the maternal colonisation rate by country, as maternal colonisation is the main risk factor for EO-GBS disease. In contrast to GBS invasive disease incidence studies, maternal GBS colonisation studies reported similar colonisation rates to those of high-income countries (Middle East/North Africa, 22%; Asia/Pacific, 19%; Sub-Saharan Africa, 19%; India/Pakistan, 12%; and Americas, 14%) (33) and European pooled incidence of 21.3%.(16) It is important to note that there is some variation in colonising GBS STs by geographical region with GBS ST Ia, III and V most prevalent in the USA and Europe, GBS STIII and V in African countries with GBS STIII, V and VIII in Asia.(34) (Figure 4)

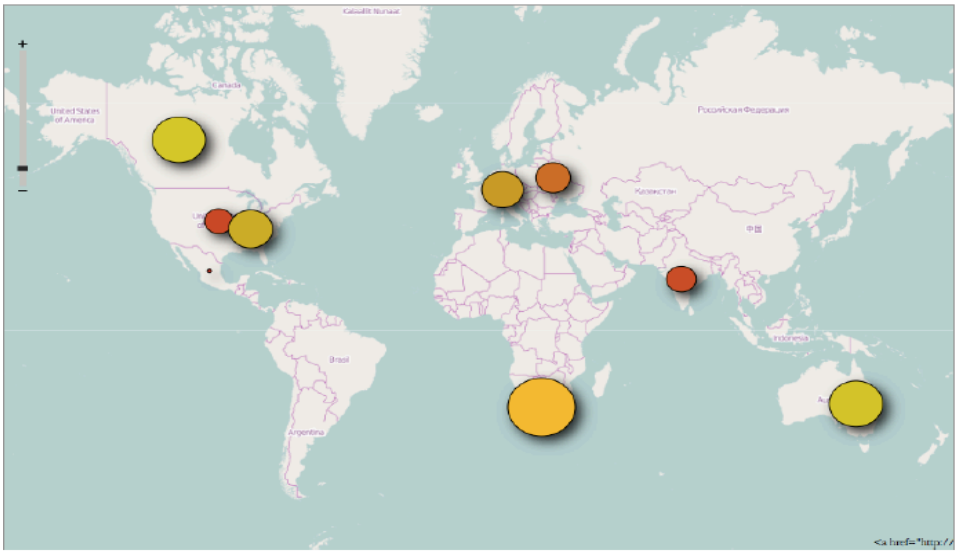
Figure 4 - Differences in global colonising serotypes

A. Percentage of pregnant women by country colonised with STs Ia, Ib, III



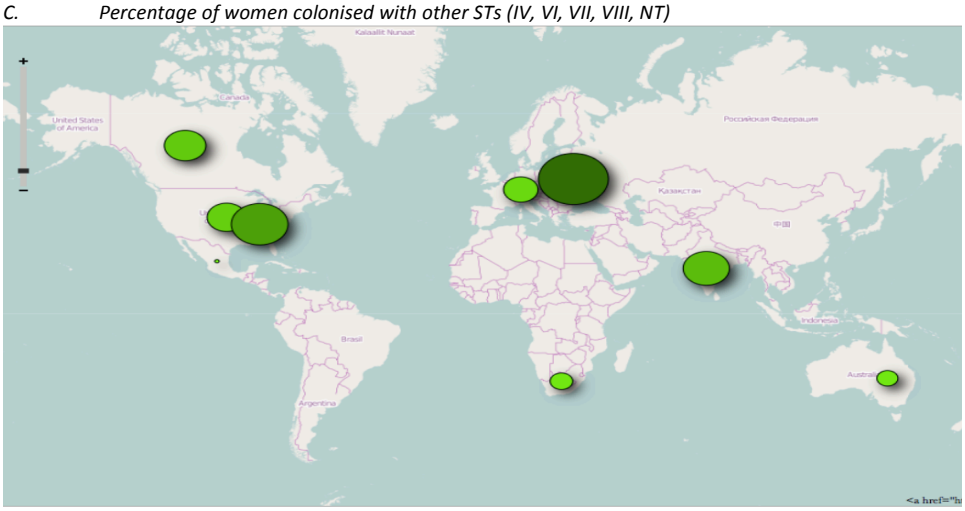
STIa, STIb and STIII coverage 32.5 53.3 74.2

B. Percentage of pregnant women by country colonised with STII & V



STII and STV coverage 22.8 31.4 40.0

C. Percentage of women colonised with other STs (IV, VI, VII, VIII, NT)



Other serotypes 3.05 21.6 40.1

Adapted from Ippolito et al (2010)(34). Size of circles represent increasing percentage of associated colonising STs.

1.2.2.1. Epidemiology of colonising GBS-STs in Africa

A literature search of all African studies that reported colonising GBS STs, used carefully defined microbiology methods and included selective culture media, identified four colonisation studies, two from South Africa(12, 26), one from Gambia(17) and one from Zimbabwe.(25) All studies identified reported colonisation rates of GBS in pregnant women on at least one occasion. Colonisation rates in pregnant women varied between African countries (22% in Gambia to 30.9% in South Africa).(12, 17) In a random effects meta-analysis overall colonisation rate was 26% (CI 21-31%), although data were quite heterogeneous (I^2=85%). Colonising GBS ST prevalence also varied between countries with GBS STs Ia, III and V most common in South Africa and Zimbabwe (South Africa: Ia (39%), III (33%) and V (12%); Zimbabwe: III (42%), V (37%) and Ia (11%).(12, 25)%)).(12, 25) The only West African Study (from the Gambia) indicated STV predominance (38%) followed by II and I with low GBS STIII (6%).(17) Overall, random effects meta analysis of colonising GBS STs in African studies demonstrated predominance of GBS STIa (30% CI 15-44), followed by III (26% CI 2-50), V (22%, CI 1-43) II (16% CI 1-31) with low prevalence of STIb (5% CI 3-8).

1.3. Disease pathogenesis in GBS infection

EO-GBS infection may occur intrapartum by inhalation of the GBS bacteria as the infant passes through the birth canal or ascending transmission following premature rupture of the mother's membranes prior to labour.(35) The GBS is then able to multiply in the amniotic fluid and colonise the foetal skin and mucous membranes.(35, 36) It is also thought that GBS at high enough inoculum may cross

the placenta, possibly explaining why some infants born by caesarian section may also develop EO-GBS disease.(37) EO-GBS disease presents within 24 hours in 90% of cases(38), primarily due to aspiration into the infant lungs causing pneumonia and subsequent septicaemia in 27-87% of cases.(39) LO-GBS disease may occur from postnatal exposure to GBS or via breast milk and presents up to three months of age, most commonly as meningitis in 40-60%.(4, 10, 40)

1.3.1. GBS colonisation of maternal rectum and vagina and cellular invasion

The first step in the pathogenesis of GBS infection is the ability of the bacteria to adhere to mucosal surfaces and compete with normal flora. Various studies have demonstrated that GBS can efficiently adhere to the vaginal and rectal mucosal surfaces (41), placenta (42), respiratory epithelium (43, 44) and blood-brain-barrier.(41, 45) Following adherence to epithelial cells, three mechanisms have been determined for GBS that facilitate host cell invasion, subsequent systemic dissemination and the ability to cross the blood-brain-barrier: intracellular invasion, cytolytic injury and induction of host inflammatory responses (Figure 5).(46) The process involves the ability of GBS to mediate host-cell reactions and to interfere with innate immune clearance mechanisms via a combination of surface and secreted virulence factors that are described in more detail below.

1.3.1.1. Surface Proteins that aid GBS adherence and cellular invasion

In order to maintain colonisation and cell invasion in the presence of the host immune response, GBS appears to have developed a series of adaptations to its surface proteins that aid epithelial adherence, promote cellular invasion and prevent complement deposition. Alpha C protein (ACP), GBS immunogenic bacterial adhesion

(BibA), pili, Fibrinogen-binding proteins A and B (FbsA/B), serine-rich domain repeat protein 1 (Srr1), laminin-binding protein (Lmb), pneumococcal adhesion and virulence protein A (PavA) and lipoteichoic acid (LTA) mediate GBS binding to the cell surface and fibrinogen and fibronectin in the extracellular matrix (ECM) and enhance cellular invasion (Figure 5).(47)

GBS secretes hyaluronate lysase, which causes damage to placental tissue that may promote EO-GBS disease.(48) Lung epithelial cell damage is thought to be caused by β-haemolysin/cytolysin, which results in interleukin 8 (IL8) secretion causing further cellular damage and invasion of lung cells.(49) This mechanism is inhibited by human surfactant and therefore may be why there is increased risk of GBS disease observed in premature infants who are known to be surfactant-deficient.(50) It is also possible that GBS is able to enter epithelial cells by endocytosis and transcytosis, which can enable GBS to cross the blood-brain barrier causing subsequent meningitis.(51, 52)

1.3.1.2. Hypervirulence

A substantial proportion of LO-GBS meningitis is thought to be associated with GBS STIII.(53) There are a limited number of GBS STIII clonal complexes. The main sequence type (st) associated with neonatal meningitis is st-17 and it is therefore designated as "the hypervirulent clone". A novel surface protein found on st-17 (hypervirulent GBS adhesion, HvgA) has been implicated in more efficient adherence to epithelial cells and microvascular endothelial cells such as those of the blood-brain-barrier. This protein may contribute to the evolution of GBS meningitis in neonates.(54)

1.3.2. Interference with opsonophagocytosis

Polymorphonuclear lymphocyte (PMN)-mediated killing is critical in the host defense against GBS.(55) For effective killing, the organism needs to be opsonised with complement and specific antibody. Complement deposition is critical for opsonophagocytosis of GBS.(55) In the case of GBS, several secreted and surface factors interact to impair the classical and alternative complement pathway, thus impairing uptake by PMN and intracellular killing.

1.3.2.1. The GBS capsular polysaccharide (CPS)

Human isolates of GBS express a CPS, a major virulence factor that helps the microorganism evade opsonophagocytosis.(56) Isolates of GBS can be divided into ten CPS STs (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX), each antigenically and structurally unique. In 1974, Baker et al. demonstrated that while all GBS STs were capable of causing neonatal infection, GBS STIII isolates were significantly increased amongst infants with GBS meningitis.(57)

The CPS of GBS is poorly immunogenic, especially GBS STIII, and aids bacterial evasion by masking surface proteins. All GBS STs contain a terminal sialic acid molecule, similar to mammalian host cell sialic acid that impairs surface deposition of opsonically active complement C3 on the bacterial surface blocking the alternative complement pathway.(58)

1.3.2.2. Surface proteins that interfere with complement deposition

In addition to aiding cellular adhesion and invasion, surface proteins have been implicated in the ability of GBS to evade opsonisation with complement. Three routes can activate the complement pathway: the classical pathway, the lectin binding pathway and the alternative pathway that differ in their effector

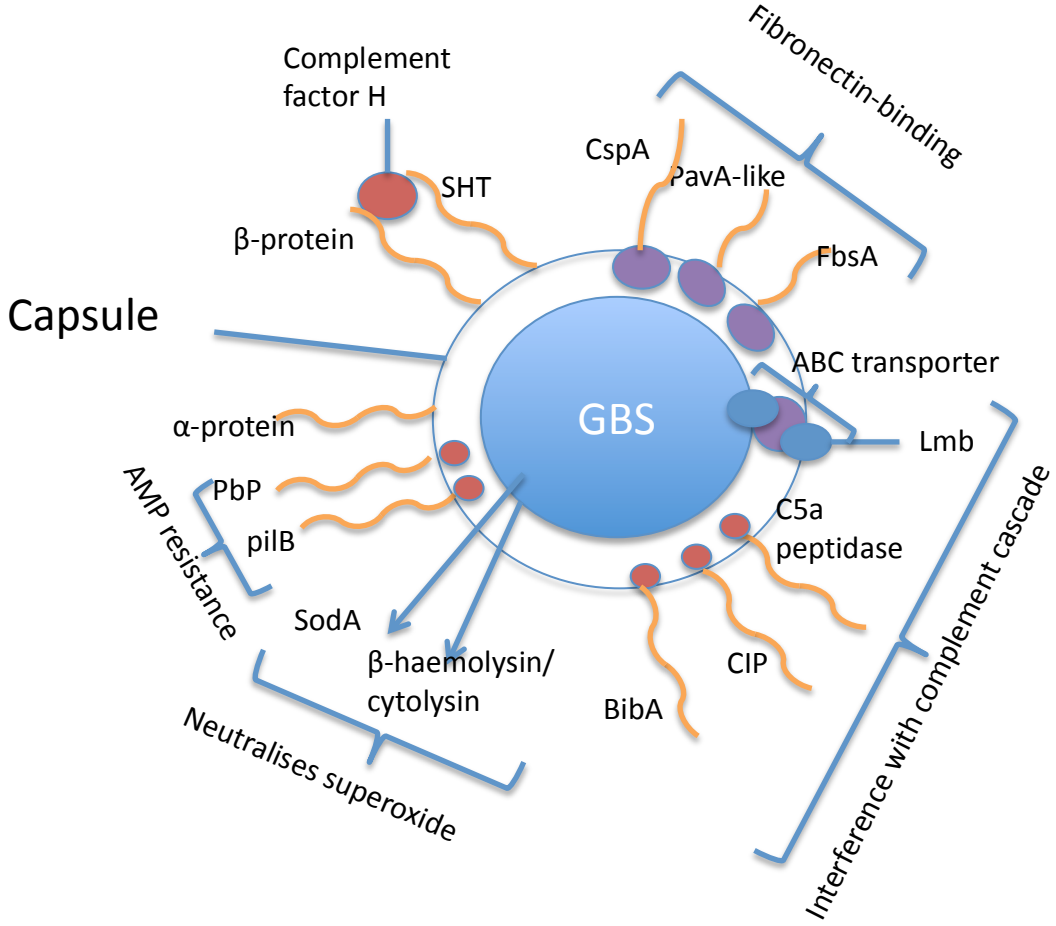
mechanisms and pathways of activation. The final common pathway is the cleavage of C3 to form C3b that attaches to the bacteria surface. Regulation of complement is tightly controlled by factor I, factor H and C4b binding protein. Several proteins on the surface of GBS have been implicated in inactivation or evasion of the components of these complement pathways. For example, in the absence of C5a-peptidase, which cleaves C5a (a chemoattractant of neutrophils,) lower levels of neutrophils were found both in *in vitro* models (59) and in neonatal disease.(35) C5a peptidase is produced by most GBS STs and is therefore a potential target against which a vaccine could be directed. When conjugated with GBS STIII in a CPS protein-conjugate vaccine it induced antibody responses that are cross protective in mice for other GBS STs.(60) However, C5a peptidase is not expressed by GBS STV (61), limiting its use where GBS STV is most prevalent. Additionally, a recently discovered complement inhibitory protein (CIP) was found to inhibit both the classical and lectin binding pathway but not the alternative pathway.(62)

Surface proteins, such as α- and β-proteins (present on GBS ST Ia, Ib, II and V) are also thought to mediate complement deposition. Although a definitive role for these proteins in the promotion of virulence is as yet unclear, β-protein may bind to the crystallised portion of IgA at mucosal surfaces and to protein factor H, required for complement C3b deposition.(58) Maruvada et al describe a streptococcal histidine triad (SHT) surface protein present in GBS STIII that also binds factor H.(63) This may account for the ability of GBS STIII to bind to factor H in the absence of the β-protein.

Other surface proteins such as BibA (which binds to C3b and inhibits the classical complement pathway), Lmb and cell surface protease ((CspA), which binds

to ECM fibrinogen) reduce complement deposition onto the bacterial surface.(47) Figure 5 outlines the GBS capsule and surface proteins involved in evasion of innate immunity.

Figure 5 - The GBS capsule, surface proteins and secreted toxins



ABC – ATP-binding cassette; AMP – antimicrobial peptide; BibA – Group B Streptococcus immunogenic bacterial adhesion A; CIP – complement inhibitory protein, CspA – cell surface protease A; FbsA – fibrinogen-binding protein; Lmb – laminin-binding protein; PavA- pneumococcal adhesion and virulence protein A; PbP – penicillin-binding protein; pilB – pilus protein island B; SHT – streptococcal histidine triad; SodA – superoxide dismutase.

1.3.2.3. Secreted toxins that cause local inflammation and interfere with phagocytic killing

GBS secretes a variety of toxins and virulence factors (invasins), promoting an inflammatory response that may account for the rapid progression of GBS disease in neonates.

Once phagocytosis has occurred, GBS is further able to prevent killing by neutralising the result of the phagocyte oxidative burst. GBS has both an endogenous supply of glutathione(64) and secretes the enzyme superoxide dismutase A (SodA) (65) to neutralise superoxide ions. GBS also secretes a carotinoid pigment that is genetically similar to β-haemolysin/cytolysin that is able to neutralise the effective of superoxides.(66)

The activity of antimicrobial peptides (AMP) may be suppressed by GBS due to D-alanine residue in the GBS cell wall that reduces the surface's negative charge and thus affinity to cationic peptides such as AMPs. Penicillin binding protein (PbP) in the GBS cell surface additionally enhances resistance to AMP secreted by phagocytes. Pilus protein island B (pilB) is thought to prevent AMP-mediated killing and increase resistance to macrophages and neutrophils.(47)

1.4. The role of breast milk in late-onset GBS infection

It has been suggested that GBS typically colonises the infant's oropharyngeal mucosa through contact with maternal vaginal/rectal secretions at the time of birth.(67) However, Butter and De Moor also demonstrated infant nasopharyngeal colonisation at the same time as GBS was cultured from the mother's breast milk, suggesting this as another mechanism of acquisition.(68) Despite this finding, the majority of breastfed infants of mothers colonised with GBS do not develop LO-GBS disease (69), even after proven GBS mastitis and several months of breastfeeding. Breast milk is known to have many protective properties, including antibody that should protect infants from both EO- and LO-GBS disease.(40)

Therefore, there appears to be a contradiction between the possibility that

breast milk might cause LO-GBS disease in some infants yet protects the majority from acquiring GBS disease. The mechanisms by which GBS is transmitted through breast milk, or how breast milk may protect from infection, are not fully understood. This knowledge gap may partly result from the fact that breast milk of GBS-colonised mothers is not routinely screened, even in donor breast milk in neonatal breast milk banks.(70) It is however important to understand what factors in breast milk may be involved in either protection from disease or transmission of GBS infection. This is especially true for premature infants who are a high-risk group and in LMICs where breastfeeding is the only sustainable infant feeding option.

1.5. GBS vaccine development

Various monovalent and trivalent vaccines have undergone clinical trials since the early 1970's. Here I describe the developments leading up to the proposed phase III trial of the current pentavalent CPS-protein-conjugate vaccine.

1.5.1. Animal Studies

Three decades of research have been spent assessing the immunogenicity, safety and effectiveness of GBS vaccine formulations in animal and healthy human models. Studies of CPS protein-conjugate vaccines against GBS ST Ia and Ib (71) identified protection from invasive disease in mouse pups. Similar findings were present for monovalent GBS ST II (72), III (73-77) and V (78) vaccines and a tetravalent vaccine (79) with good *in vitro* opsonophagocytosis mediated by neonatal mouse serum following maternal vaccination. These mouse-neonatal challenge models have also been useful to observe the concentration and function of transplacentally acquired antibody.(76, 77) Additionally, animal models have served

to derive protective concentrations of antibody that would possibly confer protection from invasive disease.(73-77)

1.5.2. Human Studies

Originally, GBS vaccine development was based on a CPS vaccine. However, the CPS vaccine was poorly immunogenic with GBS ST Ia and III, especially if women had low pre-immunisation antibody concentrations and less than 60% of pregnant women developed an antibody response post-vaccination.(80) A protein-conjugate vaccine against GBS overcomes the poor immunogenicity of the GBS-CPS by inducing a T-cell dependent immune response.(81)

Monovalent tetanus-toxoid conjugate vaccines to GBS ST Ia, Ib (82), II, III (83) and V have been tested in healthy, non-pregnant women. Maximal antibody responses to all GBS STs peaked at 4-8 weeks post-vaccination with antibody persistence between 26-52 weeks post-vaccination.(84) The tetanus-toxoid conjugate GBS vaccine against STIII has also been tested in a small sample of pregnant women between 30-32 weeks gestation. In this study, infants were found to have lower geometric mean concentration (GMC) of antibody at one month and two months than at birth (50% and 30% respectively), although opsonophagocytosis was observed up to two months of age.(85)

1.5.3 Surface protein vaccines

CPS- conjugate vaccines are not without their drawbacks and some have argued that there is increased risk of interaction with other vaccines and possible impairment of vaccine responses when conjugate vaccines are used. There is also the possibility of the emergence of non-vaccine GBS STs following vaccination containing limited GBS STs, as seen with *Streptococcus pneumoniae* (86) and a loss of

natural boosting due to decreased exposure to GBS in vaccinated women if the vaccine has an impact on maternal colonisation. A further limitation to the development of a CPS-protein conjugate vaccine is the lack of global GBS ST data. It is likely that a multivalent CPS-protein conjugate vaccine will be needed. However, without epidemiological data about colonising and disease-causing GBS STs, the applicability of a single global vaccine to different epidemiological settings remains unclear.

One way of overcoming these limitations would be to develop a surface protein vaccine. The ideal surface protein candidate would be one present on the surface of all GBS STs such as the pilus proteins or ACP and resistance to proteases, immunity, group B (Rib) proteins. A phase I trial of an ACP-Rib protein-only vaccine, which could protect against up to 95% of GBS isolates is currently underway in Northern Ireland (Neostrep 2015).(87)

The use of genomics to identify candidate proteins has revolutionised vaccine development in the past ten years. Work such as that at the GlaxoSmithKline (GSK, formerly Novartis) laboratories in Siena has identified four proteins that are common to GBS disease causing STs: the pilus proteins (88, 89) and the Sip protein.(90) It has been suggested that, by extrapolating the data from mouse models, pilus proteins could protect against 94% of GBS strains.(89)

1.6. Neonatal Immunity and GBS

1.6.1. Neonatal immune response

The physiological demands placed on the neonatal immune system include: to protect against infection; avoid harmful Th1 responses that would induce an

alloimmune reaction between mother and infant and mediate the transition to extra-uterine life and colonisation with microorganisms on the skin and gastrointestinal tract.(91, 92) The bias towards Th2 responses (81, 91) means that the neonatal B cells do not respond optimally to the GBS CPS and therefore little antibody is produced to neutralise, opsonise, induce phagocytosis or activate complement, all vital steps in preventing GBS disease.

1.6.2. Neonatal T- and B-Cell responses

Although infant immune responses are present at birth, early life responses are thought to be weaker and of shorter duration than mature immune responses, leaving infants vulnerable to infections in the absence of stimulation through vaccination(93). Infant vulnerability to encapsulated bacteria such as GBS is mainly due to a failure to generate T-cell independent B cell responses to bacterial polysaccharides.(94) These responses mature over time, but remain limited in children under 18-24 months of age. (95-98) Neonates have reduced marginal zone macrophages that are required for antigen recognition and subsequent antibody production, which reduces B cell activation resulting in fewer plasma cells(99). The reduction in plasma cell numbers is also associated with decreased plasma cell survival, which may also be why there is limited antibody persistence following antigen challenge and limited immune memory(93). Additionally, neonatal B cells express lower levels of CD40 and other co-stimulatory molecules, which limits the response of the neonatal immune system to CD40L and IL10 expressed by T cells(100). (Table 1) In addition to T-cell functional and structural immaturity, infants are deficient in IL-1 and tumour necrosis factor α (TNFα) that are needed to optimise responses to CPS.(101, 102)

Table 1 - Characteristics of neonatal B cell responses compared to healthy adults *(adapted from Siegrist and Aspinall)(93)*

Cell type	Characteristics
Naive B cells	Decreased expression of cell-surface receptors (CD21, CD40, CD80, CD85)
Plasma cells	Limited IgG responses to protein antigens below 12 months of age Limited IgG responses to most CPS below 18-24 months of age Limited persistence of IgG antibodies below 12 months of age
Memory B cells	Effective memory B-cell priming at and before birth Progressive diversified IgG repertoire Limited affinity maturation below 4-6 months of age

The neonatal adaptive immune response is impaired during early life, with lower peak antibody levels, affinity and concentration of IgG2 (the predominant subclass associated with opsonisation of the bacterial CPS compared to healthy adults.(92, 103, 104)

1.6.3. The role of complement in innate immunity to GBS

The complement cascade can be activated by the classical, alternative or mannose binding lectin (MBL) pathways as outlined above. Binding of the fragment crystalline (Fc) portion of IgM and IgG antibody to the C1q component of C1 activates the classical pathway whilst MBL binds directly to the bacterial surface in the absence of antibody forming a C1-like complex that initiates the MBL pathway. The alternative pathway is activated when Factor B binds to circulating C3b directly to the bacterial surface, resulting in C3 convertase. Common to all three pathways is the cleavage of C3 to C3a and C3b. C3a is a potent chemoattractant. C3b binds to cell surface complement receptors or via C3 convertase to form C5 convertase to enhance opsonisation of GBS. GBS opsonisation with complement results in

phagocytosis without antibody. However, once antibody binds to Fc receptors signal activity between complement receptors increases and phagocytosis increases exponentially.(105) Thus, both antibody and complement play a role in immunity to GBS infection, posing unique challenges for the neonate.

1.6.3.1. Neonatal complement defects

Several factors impair the neonatal complement-mediated immune response to GBS, including suboptimal concentration of neonatal complement.(106, 107) As complement is vital for the opsonisation of GBS STIII(108), this lack of complement during the neonatal period contributes to increased infant susceptibility to disease. Infant B cells express less CD21 (complement receptor 2) compared to adults, which limits the neonatal response to the GBS CPS-complement complex.(81, 106, 109)

The antibody-complement interaction is also impaired, with neonates being unable to fully activate the alternative complement pathway in the absence of antibody against STIII.(55, 108) However, there may be differences between GBS STs and the ability of the CPS to activate the alternative complement pathway, as it has been found that GBS STIa does not need antibody to activate complement.(110, 111) Taken together the relative lack of complement and functional deficits exacerbate the inadequate neonatal immune response to CPS bacteria such as GBS.

1.7. The importance of maternal antibody in GBS disease protection

To consider maternal antibody as a means of protection from neonatal disease is not a new concept. Mothers in the Faroe Islands who survived measles virus in 1846 were found to have infants who did not contract the disease (112) and mothers who had received the vaccinia vaccine in 1879 had infants who did not

contract smallpox (113), demonstrating the benefit of passive protection in the form of maternally-derived antibody. The World Health Organisation (WHO) now recommends maternal vaccines in pregnancy for tetanus, pertussis and inactivated influenza virus. Additional vaccines such as pneumococcal and meningococcal polysaccharide vaccines, inactivated polio, hepatitis A and hepatitis B are also considered safe during pregnancy.(114)

The importance of maternally-derived antibody in preventing GBS disease was first demonstrated over 30 years ago by the increased risk of infant disease in infants born to women with negligible anti-GBS antibody concentrations.(115) Studies have shown that human sera containing a high concentration of CPS-specific antibody promoted efficient opsonisation and phagocytosis *in-vitro* and affirmed the importance of CPS GBS ST-specific antibodies in disease protection.(116)

1.7.1. Placental antibody transfer

Studies comparing maternal and foetal antibody concentrations demonstrate that anti-GBS CPS antibody is transferred with a high degree of efficiency after 30 weeks gestation.(117)

Neonatal IgG antibody is found to be higher at birth than in the mother indicating active transfer across the placenta.(118-120) IgG binds to the placenta via receptors to the Fc portion of the antibody and these receptors can be saturated if maternal concentrations are very high.(121) Several studies have demonstrated significant negative correlations between maternal concentrations of IgG and placental transfer ratios for both total IgG and specific IgG against viruses, LPS and other antigens.(122-124) Problems with placental transfer of antibody may also occur with high maternal antibody concentrations such as the

hypergammaglobulinaemic state found in human immunodeficiency virus (HIV) infected women.(125, 126) African studies have reported lower cord/maternal placental transfer ratios of total IgG compared to the UK which may reflect the comparatively high concentrations of maternal antibody in African populations (113, 122, 127), possibly due to increased pathogen exposure in African women.(113) Maternal age, weight, parity, and type of delivery do not influence placental antibody transfer.(128)

Therefore, the difference in invasive disease prevalence between the Gambia and resource rich settings could also be due to higher concentrations of transplacentally acquired protective antibody. Suara et al. (1998) reported high mean maternal GBS STIII antibody concentrations in women that could account for the relative rarity of GBS STIII GBS-disease in The Gambia (GMC) 1.1 ± 0.5µg/mL).(129)

1.7.1.1. Subclasses of IgG and placental transfer

There are several reports of reduced placental transfer of antibody to encapsulated bacteria, for example IgG1 antibodies to *H. influenzae* type b are transferred in greater amounts than IgG2.(130) This is concerning as IgG2 is the predominant IgG subclass required for protection against encapsulated bacteria. Compared to IgG1 and IgG3, IgG2 is often detected in lower amounts in cord blood than in maternal blood and IgG2 shows less affinity for the FcRn receptor.(131, 132) It is therefore vital that not only is total antibody concentration high but also that sufficient amounts of IgG2 cross the placenta to provide effective antibody-mediated killing to prevent GBS disease.

Lower total antibody concentrations for GBS ST Ia and III have been

demonstrated in GBS-infected infants, including lower IgG subclasses, especially IgG2.(125, 133-135) As the functional IgG response against GBS STIII is mainly due to opsonisation with the IgG2 subclass, it is possible that the deficient transfer of this antibody to the foetus is a reason for enhanced susceptibility.(136)

1.8. Maternal antibody and maternal vaccination

Boosting naturally-acquired antibody through maternal vaccination is a useful strategy in neonatal disease prevention, as neonatal vaccination would not protect infants from EO-GBS disease. The past 30 years have been spent in developing a vaccine to be given in pregnancy to elicit high levels of functional, GBS ST-specific IgG that would cross the placenta and protect neonates from disease.(137, 138) It is estimated that a pentavalent vaccine containing GBS STs Ia, Ib, II, III and V would cover 94-100% of invasive isolates globally and a trivalent vaccine that would cover 87% of invasive disease (ST Ia, Ib and III)(13, 116). The development of other candidates continues.

1.8.1. Conundrums in measuring vaccine efficacy

Whilst the vaccine study data highlighted in section 1.4. are encouraging, in order to assess vaccine efficacy in preventing neonatal disease, antibody concentrations must be determined that confer protection from disease. However, determining a protective antibody concentration is not easily achieved, as protective antibody concentrations vary by organism (116), by GBS ST (139, 140) and the assessment of immunogenicity varies by research methods.(116, 139)

The association between GBS ST-specific capsular antibody concentrations and invasive GBS disease in newborns was initially characterised in 1976 by Baker

and colleagues.(115) In the majority of subsequent studies lower GBS ST-specific capsular antibodies were found in infants with EO- and LO-GBS disease compared with controls.(141, 142) Different specific antibody concentrations that could be associated with protection from disease have been defined in different studies as well as for different GBS STs (Table 2). In a meta-analysis undertaken to compare the proportions of cases and controls with antibody levels ≥2 μg/mL, the odds of invasive GBS disease was 6.56 (95% CI: 2.10–20.55) and 2.38 (95% CI: 1.20–4.70) times greater in infants whose mothers had antibody levels <2 μg/mL for GBS STs III and Ia, respectively.(143) A threshold of 1 μg/mL has also recently been proposed as a correlate of protection against disease caused by GBS STs Ia and III.(116) Thresholds are much higher in other large studies including the DEVANI project, based on European sera (144), but there has been significant controversy regarding appropriate methods to be used to measure the antibody in order to derive such thresholds reliably.(145) A recent study from South Africa suggested that maternal concentrations of >3μg/mL for GBS STIa and >1μg/mL for GBS STV were associated with significantly reduced maternal colonisation with these GBS STs (Table 2). Further, the authors suggested that the opsonophagocytosis killing (OPkA) titre correlated more strongly with decreased rectovaginal acquisition of GBS, indicating the need for both a quantitative and a functional assessment of antibody when estimating protection.(146) Interpretation of all of these results is difficult due to differences in methods and the lack of available standardised reference sera. A standardised approach to establish antibody concentration and function and its role in reducing maternal colonisation and neonatal disease is urgently required in order to achieve licensure of any future vaccines based on immunogenicity criteria.(147)

Table 2 - Proposed protective antibody concentrations for GBS ST Ia, III and V

Author	Country	Year of Study	Method of antibody assessment	Proposed protective concentration	OR/RR (95% CI) of disease protection
Baker et al (116)	USA	1998-1999	ELISA	STIa, STIII, and STV ≥1µg/mL	OR: if Antibody ≥0.5µg/mL: STIa: 0.11 (0.01-0.74) III: 0.09 (0.00-0.72) V: 0.29 (0.01-3.10)
Edwards et al(148)	USA	1979	OPkA	STIII ≥10µg/mL	2.0 $\log_{10}$ reduction in CFU/mL compared to antibody deficient sera
Baker et al(83)	USA	2003	OPkA	STIII ≥1µg/mL	>1.0 $\log_{10}$ reduction in CFU/mL
Baltimore (149)	USA	1981	RABA	STIII ≥2µg/mL	
Feldman (141)	UK	1990	ELISA	STIII ≥2µg/mL	GMC of STIII lower in GBS disease cases
Klegerman(142)	USA	1983	ELISA	STIa ≥1µg/mL	GMC of STIII lower in GBS disease cases
Lin (140)	USA	2001	ELISA	STIII ≥10 µg/mL	OR: 0.09 (0.01-0.78)
Lin (139)	USA	2004	ELISA	STIa ≥5 µg/mL	OR: 0.12 (0.02–0.93)
DEVANI study(144)	Europe	2014	ELISA	STIa ≥5 µg/mL STIII ≥10 µg/mL No correlate for STV	
Kwatra(150)*	South Africa	2015	Fluorescence based micro-bead immunosorbent assay	≥3 µg/mL STIa ≥3 µg/mL STIII ≥1 µg/mL STV	OR: STIa : 0.91 (0.68-1.21) STIII: 0.47 (0.26-0.84) STV: 0.39 (0.15-1.01)
Dangor(151)	South Africa	2015	Fluorescence based micro-bead immunosorbent assay	STIa ≥6 µg/mL STIII ≥3 µg/mL	Used Bayesian modelling to impute the degree of protection based on specific threshold

GBS=Group B Streptococcus; ELISA=enzyme-linked immunoabsorbent assay; OPkA=opsonophagocytosis killing assay; RABA=radioantigen binding assay; DEVANI=design of a vaccine to immunize neonates against GBS through a durable maternal immune response; ST=serotype; OR=odds ratio; RR=risk ratio; CFU/mL=colony-forming units per millilitre; GMC=geometric mean concentration.

1.9. Assays used to predict immunity to GBS disease

A major barrier to the conduct of efficacy trials - which would have to measure prevention of invasive neonatal GBS disease as the primary endpoint- is the large sample size required, as described above. As an alternative, a vaccine could be licenced based on immunogenicity data, if a threshold of protective antibody was defined.

An enzyme-linked immunoabsorbent assay (ELISA) measures antibody concentrations and has been used in the majority of vaccine studies to date in order to estimate the antibody concentration required to reduce the risk of GBS disease. However, an antibody ELISA does not provide information on the functional ability of the antibody to neutralise GBS. Therefore, an ELISA alone may not be sufficient in predicting functional immunity from GBS infection.(143) A possible solution to this may be the development of an effective functional antibody assay that could be used as an *in vitro* correlate of protection. An example of this is the opsonophagocytosis killing assay (OPkA) (148, 152), which mimics the *in vivo* process of the killing of the bacterium by host effector cells following opsonisation by specific antibodies. Passive protection of mice by sera from adults immunised with GBS CPS-based vaccines has been shown to correlate with high functional antibody titres measured by OPkA.(149, 153) However, OPkA assays are laborious to perform and require large volumes of test sera. Reliance on human donor phagocytes introduces an extra variable, though this can be addressed with the use of the human phagocytic cell line human promyelocytic leukaemia cells (HL-60).(154) An OPkA that measures the uptake of fluorescently-labelled bacteria by flow cytometry

could potentially substitute for a conventional OPkA requiring cumbersome colony counts.(155) A fluorescence opsonophagocytosis assay (fOPA) has also been developed that measures both the uptake of bacteria and the oxidative burst activity of the HL-60 cells in a 96-well format.(156) For large seroepidemiological studies however, both the OPkA and fOPA are still too laborious and require too much serum volume from each vaccinee. This is a critical issue in studies where sample volume is at a premium, such as in neonatal studies. Other assays, including an antibody-mediated complement C3b/iC3b deposition assay (125) have been developed that are less labour intensive and less variable as they do not rely on human phagocytes and require small serum volumes.

1.10. Conclusion

Since there are geographical differences in GBS ST distribution, the potential global effectiveness of GBS vaccines currently in development is unknown. In particular, there are no recent GBS ST-specific data from West Africa in order to predict the potential local impact of a maternal vaccine. The effect of GBS ST-specific antibody transfer to the infant and subsequent antibody function on infant colonisation, and the natural history of antibody in infant blood have not been evaluated but are important for future vaccination strategies. The work presented in this thesis aims to address both of these gaps in knowledge.

Chapter 2 – Hypotheses, aims and objectives

2.1. Hypotheses

1. GBS colonisation in The Gambia is predominantly due to ST Ia, Ib, III and V (addressed in specific aim 1);

2. GBS ST-specific maternally-derived antibody in cord blood is inversely associated with GBS-colonisation in infants (addressed in specific aim 2);

3. Higher levels of GBS ST-specific antibody in infants are associated with better ability to kill GBS *in vitro,* representing a laboratory surrogate for protection from disease (addressed in specific aim 3).

2.2. Aims

Specific Aim 1: *To describe the ST-specific prevalence of GBS colonisation in women and their infants in a field setting in the Gambia;*

We postulate that ST prevalence of GBS colonisation in Gambian women and their infants is similar to other African countries and thus that a pentavalent vaccine candidate would provide more than 80% coverage of circulating GBS isolates.

Specific Aim 2: *To examine the relationship between maternal GBS ST-specific antibody and GBS colonisation in neonates*;

We postulate that increased concentration of anti-GBS ST-specific antibody will reduce infant colonisation at birth and that infants with higher concentration of anti-GBS antibody at birth will have reduced risk of GBS colonisation at six days and up to day 60-89 of life.

Specific Aim 3: *To develop an opsonophagocytosis assay and correlate the results to the complement deposition assay in order to determine if a concentration of antibody exists above which an infant is no longer colonised with GBS.*

We postulate that higher concentrations of antibody bound to complement determine infant colonisation by promoting increased opsonophagocytic killing. It is anticipated that there will be an antibody threshold above which an infant is no longer GBS colonised and that this threshold can be used as a marker of protection from colonisation and thus disease.

Chapter 3 - Materials and Methods

In this Chapter, I describe how I addressed the specific aims of the project, including study setting, design, participant sensitisation and recruitment and the laboratory assays and data analysis techniques used.

3.1. Field study set up, sensitisation and recruitment

3.1.1. Study setting

The Gambia is a low-income country with an annual birth rate of 43.1/1000 population, neonatal mortality rate of 28/1000 live births (157) and neonatal sepsis rate of 4.42/1000 live births.(32) The study occurred at two sites in urban costal Gambia: the antenatal clinic (ANC) at Faji Kunda Hospital, and, in order to increase recruitment following successful Wellcome Trust Clinical Research Training Fellowship funding from 1st September 2014, at the ANC at Jammeh Foundation for Peace Hospital (JFPH). Together these two hospitals have approximately 12,500 births annually and were selected as they reflect the antenatal care usually available to Gambian women. All women were provided with serological testing for HIV and malaria antigen during pregnancy prior to recruitment. If participants were HIV positive at screening, they were referred for specialist ongoing care.

3.1.2. Ethical considerations

The Medical Research Council (MRC) Scientific Coordinating Committee and the Gambian Government/MRC Joint Ethics Committee gave ethical approval for the study on 1st September 2013 (SCC 1350). An amendment was added on 1st August 2014 prior to recruitment roll out to JFPH (Appendix 1).

3.1.3. Consent

Informed consent to enter the study was sought from mothers on behalf of their infants and from the mothers themselves. A field worker fluent in both English and the mother's local language (e.g. Mandinka, Wolof, Fula, Jolla) undertook informed consent. Pregnant women were encouraged to discuss with their partners for their agreement. If there was disagreement between the partners, the mother was deemed ineligible for study entry. All consent forms were designed according to standards set by MRC and were approved by the Gambian Government/MRC Joint Research Ethics Committee (Appendix 2).

3.1.4. Funding

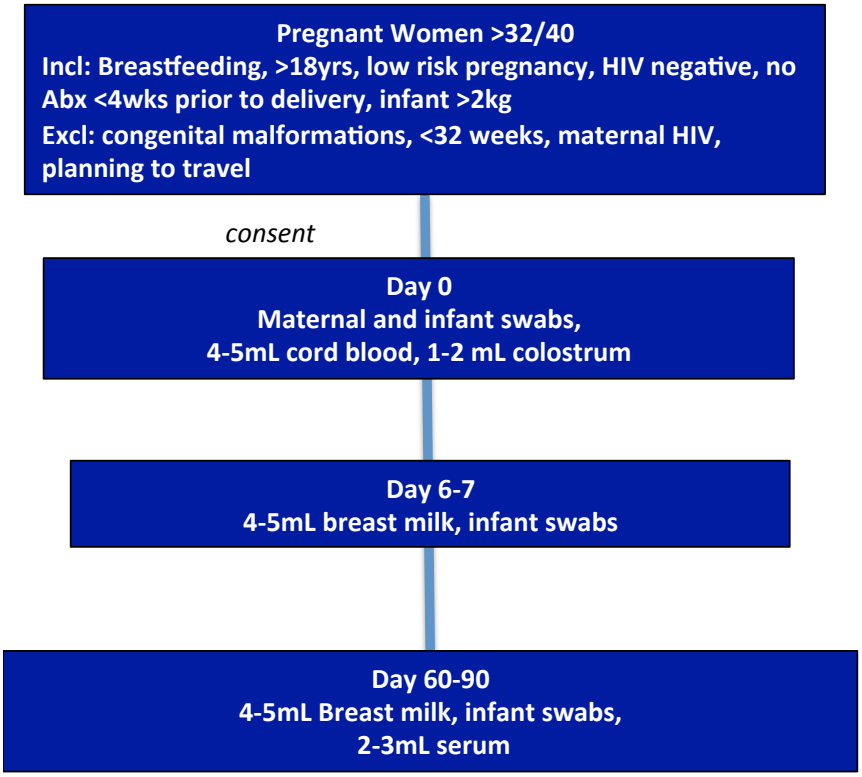
This research was funded by a Wellcome Trust Institutional Strategic Support Fund grant (year 1) and a Wellcome Trust Clinical Research Training Fellowship and an Al Thrasher Foundation Award (Years 2&3). The positive control sera and the bacteria used for the opsonophagocytosis assays were kindly donated by Prof. Carol Baker, Professor of Pediatric Infectious Diseases, Baylor College of Medicine, Houston, Texas, USA . The bacteria used for the flow cytometric assays were kindly donated by Prof. Androulla Efstratiou, Professor of Microbiology, Public Health England, Colindale, UK.

3.1.5. Study Design

This work represents a prospective cohort study of pregnant women attending ANC in the Banjul area of The Gambia and their infants (0-89 days) for assessment of GBS colonisation and GBS ST-specific antibody in cord and infant serum at two time points: birth and day 60-89 (See Figure 6 for study design). Mothers were sensitised at 32 weeks of pregnancy and screened for eligibility.

All eligible mothers knew their HIV status either by early antenatal blood test result or by rapid test at 32 weeks. HIV-positive mothers were excluded from enrolment because of the effect of HIV on the maternal immune system. Mothers were required to give birth at one of the health facilities. Rectovaginal swabs were taken in labour for maternal GBS colonisation and cord blood sample at birth for functional antibody testing. Infant nasopharyngeal and rectal swabs and maternal breast milk for culture were taken on day one (birth), day six and day 60-89. A venous blood sample for antibody concentration was taken from infants at day 60-89.

Figure 6 - Study design



3.1.6. Recruitment

If these women fulfilled the eligibility criteria they were encouraged to discuss with their partners. Follow up occurred within six days of initial contact for written consent. Screening and recruitment took place between 1st January 2014 and 31st December 2014. Mothers were approached to complete a screening questionnaire and the infant checked for any abnormalities requiring medical intervention. Women and infants were recruited until the sample size of 750 women was reached. Infants were followed up at home until day six and then asked to return to clinic when the infant was 60-89 days old for final follow up visit and vaccinations. If an infant died during the study a verbal autopsy was carried out to assess the potential cause of death.

3.1.7. Inclusion/Exclusion criteria

Inclusion criteria:

- Breastfeeding women
- age 18-45 years
- pregnancy over 32 weeks gestation
- deemed low risk singleton pregnancy (no evidence of pre-eclampsia, cardiomyopathy, maternal gestational diabetes, placenta praevia, twin pregnancy).
- giving birth in the Banjul area
- infants birthweight ≥2kg
- remaining in the Banjul area for first 90 days postpartum
- Healthy infants over 32 weeks of gestation assessed by Ballard score.

Exclusion criteria:

- Women planning to move from the area within 3 months postpartum
- congenital abnormalities
- infants requiring neonatal resuscitation or transfer to a neonatal unit
- extremely preterm infants (under 32 weeks gestation)
- known maternal HIV infection

3.1.8. Demographic and clinical information

Background demographic and maternal clinical information was obtained at screening including: HIV status, haemoglobin concentration, use of medication and vaccination in pregnancy, ethnic origin, gravida, number of previous still births/spontaneous abortions, number of living children, maternal weight, blood pressure, illnesses during pregnancy, number of antenatal attendances, maternal

education level, diet, compound location, type of cattle at the compound, midwife or traditional birth attendant delivery, clinical attendances for other reasons.(158) Infant clinical information was obtained at birth and at each follow up visit (See Appendix 3 for example case report form). Each child was issued with a Gambian Maternal and Child Health Record including details of illnesses and anthropometry, which were reviewed at each visit. The Gambia follows the WHO vaccination schedule and children receive Bacille Calmette Guerin (BCG), Hepatitis B vaccine (HBV) and oral polio virus (OPV) at birth and Pentavalent Diptheria, Tetanus, Pertussis, HBV, *H. influenzae* (DTP-HBV-Hib), OPV, Pneumococcal conjugate vaccine 13 valent (PCV13) and rotavirus vaccines at 2, 3 and 4 months. As the vaccine supply chain was sometimes uncertain in West Africa at the time of the study, all study participants received their vaccinations in the study clinic at follow up. All study subjects receiving vaccines were provided with contact information and asked to contact the study team if there were any adverse reactions following vaccination.

3.1.9. Sample collection

The participant's perineum was wiped with surgical spirit and then rectovaginal swabs (Copan, UK) were collected in skim-milk tryptone, glucose glycerol (STGG) medium on presentation to labour ward by the midwifery and field nursing team. Cord blood (4-5mL) was collected using sterile technique before separation of the placenta using sterile needle and syringe in serum separator tubes (BD, UK). Colostrum and breast milk were collected by hand expression with the help of the nursing staff. Both breasts were wiped with an alcohol wipe prior to expression. Infant nasopharyngeal (calcium alginate swabs, Sterilin, UK) and rectal swabs (Copan, UK) in STGG were collected by the field nursing team. Detailed

standard operating procedures were developed for this purpose (See Appendix 4). Infant venous blood (2-3mL) was collected using sterile technique into serum separator tubes (BD, UK) using sterile needle and syringe at clinic at day 60-89. Swabs and cord blood were kept at 4°C and transported to the clinical microbiology laboratories within six hours. The swab samples were vortexed and separated into two aliquots (one containing the original swab) and stored at -70 °C. The serum samples were centrifuged for ten minutes at 2200 rpm to separate the serum and then separated into 1mL aliquots prior to freezing at -70°C. Breast milk samples were centrifuged at 3200 rpm for 30 minutes to separate whey and lipid layer. The lipid layer was removed using a sterile scalpel and stored separately in 1mL aliquots of whey at -70°C.

3.2. Microbiological and molecular quantification of GBS colonisation

3.2.1. Media preparation

3.2.1.1. Skim-milk tryptone glucose glycerol (STGG) – transport and storage medium

To make the STGG, 3g of Oxoid (Oxoid UK) tryptone soya broth, 0.5g Glucose and 2g skim milk powder was added to 10mL glycerol and 100mL double distilled water and vortexed briefly. The resulting suspension was then dispensed in 1 mL aliquots into 1.5mL SARSTED® (Sarstedt, Germany) cryotubes and autoclaved for 10 minutes at 121°C. The suspension was cooled and refrigerated (4-8°C) until use. Each new batch was tested for sterility prior to use.

3.2.1.2. Preparation of Todd–Hewitt Broth (THB)

To make the THB, 36.4g of Todd-Hewitt broth (Oxoid, UK) was dissolved in

200mL of distilled water and then made up the volume to 1L. The suspension was sterilised at 115°C for 10 minutes and stored at 4°C until required. Sterility and performance were checked weekly using standard culture.

3.2.1.3. Preparation of Colombia-Colistin-Nalidixic Acid enrichment media (CCNA)

Ellner et al. (159) developed a selective Staphylococcal/Streptococcal medium, made by adding colistin and nalidixic acid antibiotic supplements to Columbia Agar Blood Base (CCNA). The CCNA inhibits *Streptococcus albus*, Micrococcus species, Proteus, Klebsiella and Pseudomonas species allowing unrestricted growth of *Staphylococcus aureus*, haemolytic streptococci and enterococci.

For each 1L of medium, 39g of dehydrated Colombia agar base (Oxoid, UK) was added to 1L of distilled water (as per manufacturer's instructions) and mixed until dissolved. The solution was then autoclaved at 121°C for 15 minutes and cooled to 45-50°C. Once the solution had cooled sufficiently 15mg nalidixic acid and 10mg colistin were reconstituted in 10mL 95% ethanol and added together with 50mL of defibrinated sheep blood (5%) with mixing. The resulting agar was then poured into 90mm diameter petri plates to a depth of 4mm and allowed to set. The CCNA plates were refrigerated at 4-8°C until use. Prior to use the plates were dried in the oven at 50°C for 10 minutes.

NOTE: Blood agar plates were made in the same manner but without the addition of nalidixic acid and colistin. Quality control and performance were undertaken weekly.

3.2.2. Isolation of GBS from swabs and breast milk from culture

3.2.2.1. Pre-culture

The protocol was adapted from the Public Health England (PHE) protocol for the pre-incubation of swabs in enrichment broth prior to plating on solid media to enhance the yield of GBS.(160) Swab and breast milk samples were processed within seven days of collection in batches of 100. For swabs, the aliquots that did not contain the original swab tip were thawed on wet ice and transported to the microbiology laboratory. Samples were vortexed for 10-20s and 100µL of vortexed specimen was transferred to 2.5mL THB for 24 hours pre-culture at 37°C in 5% CO_2 incubator. Breast milk was thawed, vortexed and 100µL added to THB as for swabs. After pre-incubation, the THB was aliquoted into 1mL vials and stored at -70°C for further processing.

3.2.2.2. Serial dilutions

After pre-incubation, the THB-sample suspension was briefly vortexed, then 100µL of THB-sample suspension was added to 900µL of THB to create a 1:10 dilution in a cryovial. Two further dilutions of 1:100 and 1:1000 suspensions were made for each sample.

3.2.2.3. Plating samples

A CCNA plate was divided into four segments and 10µL of neat suspension and each of the three dilutions were dispensed into each of the four quadrants and incubated overnight at 37°C in 5% CO_2. From the primary plate, presumptive beta haemolytic streptococcus colonies were streaked onto a blood agar plate and reincubated at 37°C in 5% CO_2 for 24 hours. Beta haemolytic streptococci appeared

colourless or grey, about 2mm in diameter with or without a zone of beta-haemolysis surrounding.

3.2.2.4 Catalase test

As Staphylococcal species can reduce diatomic oxygen to hydrogen peroxide (H_2O_2) or superoxide before converting H_2O_2 back to oxygen and water using catalase, the catalase test enables differentiation between Streptococcus and Staphylococcal species.

A drop of H_2O_2 was pipetted to a glass slide and a colony of the test organism was added. The droplet was observed for the evolution of O_2 gas bubbles. If O_2 gas bubbles were seen, the catalase test was considered to be positive. All specimens that were catalase test negative were identified by Lancefield grouping using Streptex® (Oxoid, UK). Growth was semi-quantified from the 1:1000 dilution as: sparse, $\leq 10^3$ colony-forming units (CFU)/mL; average, $>10^3$ & $< 10^5$ CFU/mL; or abundant, $\geq 10^5$ CFU/mL.

3.2.2.5. Lancefield grouping

Streptex® (Oxoid, UK), a commercially available rapid latex agglutination test system, was used in the qualitative detection and identification of the Lancefield groups of Streptococci.

Following the second 24-hour incubation, one colony of presumed Streptococci was vortexed in 70µL phosphate buffered saline (PBS) (pH 7.4) and incubated for 10 minutes as per manufacturer's instructions. One drop (10uL) of a solution containing polystyrene latex particles to each of the Lancefield Groups (A, B, C, D, E, F, G) was added with 10µL of presumptive positive solution to a reaction card

and incubated for 2 minutes. A clear area of latex agglutination within two minutes indicated a positive test.

3.2.2.6. Storage of positive GBS isolates

Once the GBS was identified by latex agglutination, one or more colonies from the confirmed CCNA plate were streaked as a lawn onto a blood agar plate. The growth was examined for purity after overnight incubation. Ten colonies of GBS were harvested using a sterile loop into STGG broth for storage at -70°C for future whole genome sequencing and ten colonies were harvested into PBS for real-time (Rt) (quantification) and gel-based (serotyping) polymerase chain reaction (PCR) and stored at -70°C.

3.2.3. GBS Quantification by real-time polymerase chain reaction (Rt-PCR)

3.2.3.1. PCR optimisation

As sample collection involved the use of plain swabs and calcium alginate swabs the first ten samples collected of each swab type that tested positive on culture for GBS were tested using Rt-PCR to determine whether the calcium alginate in the nasopharyngeal swabs might inhibit the magnesium in the PCR-mastermix solution. As the PCR results were concordant with the culture results I continued to use calcium alginate swabs for nasopharyngeal swab collection as these swabs were readily available in the Gambia.

3.2.3.2. Nucleic acid extraction

A 1mL suspension of GBS in PBS from the positive isolates was thawed on wet ice, then boiled in a water bath for 20 minutes and cooled to room temperature to release the deoxyribonucleic acid (DNA) for extraction. Following boiling, the samples were vortexed briefly and 300µl of the positive suspension was added to an

equal volume of lysis solution [300µl AL buffer (Qiagen, Germany), 30µl proteinase K (Sigma-Aldrich, USA)], and incubated for 10 min at 56°C. Following incubation, 300µl of pure ethanol was added and the samples vortexed before DNA was purified using DNeasy Tissue kit (Qiagen, Germany) as per manufacturers' instructions and eluted in a volume of 100µl. Two 200µl aliquots of the purified DNA solution were stored at -70°C for PCR.

3.2.3.3. Rt-PCR

The Christie, Atkinson, Munch, Peterson factor B (*CAMP factor B; cfb)*-gene was used as the target with primers as described in Diaz et al (161) and a TaqMan® (Thermoscientific, UK) probe. Primers and probes are outlined in Table 3. Rt-PCR was performed using a LightCycler (Roche, Switzerland). The following PCR conditions were applied: 0.5 mM primer, 0.2 mM probe, 2.0 mM $MgCl_2$, 0.05 mM dH_2O (2µl template in a final volume of 50µl), 22.5µL Mastermix (Roche), 2.5mL sample DNA. A cycle threshold (Ct) value of 40 was used as the lower limit of quantification for positive fluorescence detection signal of target amplification. Two positive controls and six negative controls were included in all runs. Purified DNA from a known GBS strain was used as positive control in a dilution corresponding to $1x10^7$ bacterial genomes. We used sterile water as a negative control. Each isolate was tested in duplicate.

Table 3 - Sequences of primers and probes and cycling conditions for Rt- and gel-based PCR

PCR Target	GenBank accession number	Sequence of primers and probes	Cycling Conditions	Reference
Cfb	X72754	Primer 1: ATC CTG AGA CAA CAC TGA CA (position 263–282) Primer 2: TTG CTG GTG TTT CTA TTT TCA (position 340–320) TaqMan probe: 6-FAM–ATC AGA AGA GTC ATA CTG CCA CTT C–TAMRA (position 293–317)	50 °C (2 min); 95°C (10 mins); 45 cycles of 95°C (15 s), 60 °C (60 s)	Diaz (161)
CpsI-Ia-6-7-F	AB028896	GAATTGATAACTTTTGTGGATTGCGATGA	95 °C (15 min); 35 cycles of 95°C (60 s), 54 °C (60 s), 72°C (2 min); 72°C (10 min).	Imperi (162)
CpsI-6-R	AF337958	CAATTCTGTCGGACTATCCTGATG		
CpsI-7-R	AB028896	TGTCGCTTCCACACTGAGTGTTGA		
CpsI-F	AF163833	CAATCCTAAGTATTTTCGGTTCATT		
cpsL-R	AB028896	TAGGAACATGTTCATTAACATAGC		
cpsG-F	AF163833	ACATGAACAGCAGTTCAACCGT		
cpsG-R	AB028896	ATGCTCTCCAAACTGTTCTTGT		
cpsG-2-3-6-R	AF163833	TCCATCTACATCTTCAATCCAAGC		
cpsN-5-F	AB028896	ATGCAACCAAGTGATTATCATGTA		
cpsN-5-R	AF337958	CTCTTCACTCTTTAGTGTAGGTAT		
cpsJ-8-F	AB050723	TATTTGGGAGGTAATCAAGAGACA		
cpsJ-8-R	AB050723	GTTTGGAGCATTCAAGATAACTCT		
cpsJ-2-4-F	AF163833	CATTTATTGATTCAGACGATTACATTGA		
cpsJ-2-R	AF349539	CCTCTTTCTCTAAAATATTCCAACC		
cpsJ-4-R	AB050723	CCTCAGGATATTTACGAATTCTGTA		
cpsI-7-9-F	AY375363	CTGTAATTGGAGGAATGTGGATCG		
cpsI-9-R	AF355776	AATCATCTTCATAATTTATCTCCCATT		
cpsJ-Ib-F	AB050723	GCAATTCTTAACAGAATATTCAGTTG		
cpsJ-Ib-R	AY376403	GCGTTTCTTTATCACATACTCTTG		

3.2.3.4. Standard Curve

In order to determine the quantity of GBS DNA corresponding to the recorded Ct value from the Rt-PCR, a standard curve was developed using a GBS-positive sample of known bacterial genome concentration of $1x10^7$. The positive isolate was diluted to 1:10, 1:100, 1:1,000 and 1:10,000 concentrations corresponding to bacterial genome concentrations of 10^6, 10^5,10^4, 10^3 to give a five point standard curve of known concentrations. The results were then input into a four-parameter logistical regression curve using the following formula:

$$F(x) = ((A-D)/(1+((x/C)^B))) + D$$

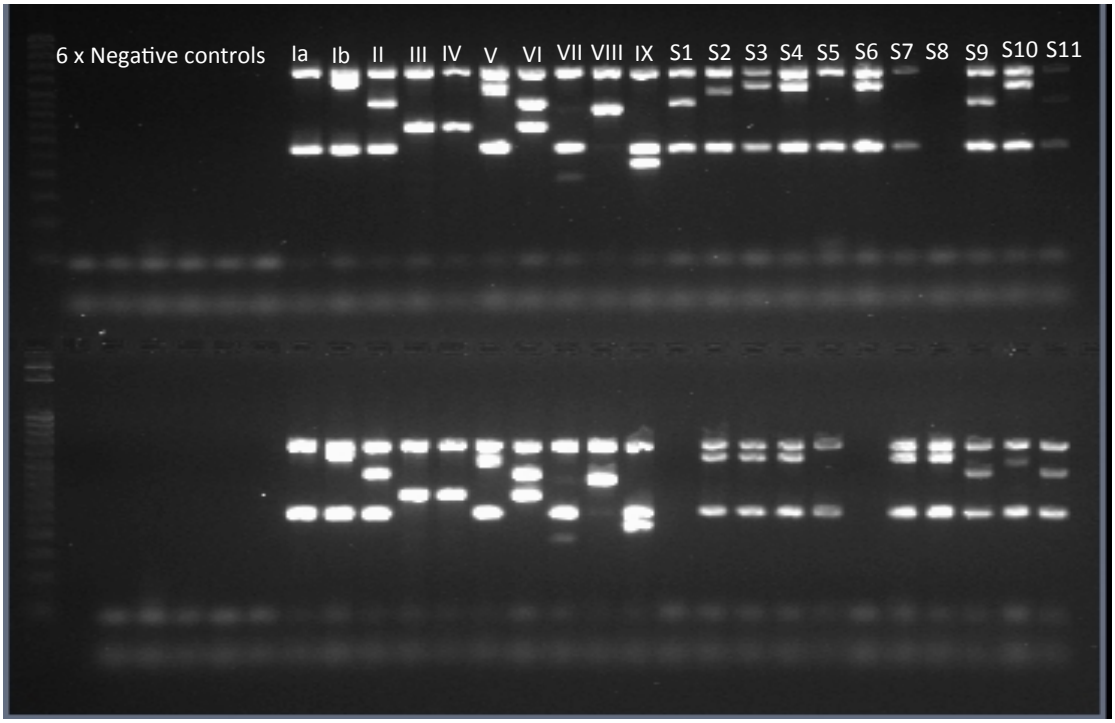
Where F(x) is the unknown concentration, A=minimum, B=slope, C=inflection, D=maximum.

3.2.3.5. Multiplex PCR and post-reaction electrophoresis for GBS serotyping.

The cps genes for each of the GBS STs were used as the target with primers and probes described by Imperi et al.(162) Primers are outlined in Table 3. PCR was performed using a Thermocycler (Roche). The following PCR conditions were applied: 0.5 mM primers cps 1-6-7-F, cps-G-R,cps-G-2-3-6-R, cpsJ-8-F, cpsJ-8-R, cpsI-7-9-F; 0.3 mM primers cpsI-6-R, cpsI-7-R, cpsL-F, cpsL-R, cpsG-F, cpsN-5-F, cpsN-5-R, cpsJ-2-4-F, cpsJ-2-R, cpsJ-4-R, cpsI-9-R, cpsJ-Ib-F, cpsJ-Ib-R, 0.05 mM dH_2O (3.1µl template in a final volume of 50µl), 12.5µL Mastermix (Roche), 2.5mL sample DNA.

A 10µL sample of each PCR product was mixed with 2µL loading buffer and 10µL of the resulting sample with 15µL of 50-bp ladder (cat# 70538-3) were then pipetted in a 1.5% (wt./vol.) high resolution agarose-1000 gel (Life Technologies, UK) and analysed by horizontal electrophoresis at 100V for 90 minutes. By using GBS reference strains representing all recognized GBS STs, ultraviolet (UV) transillumination of the amplified products on the agarose gel showed a two or three band pattern, specific and characteristic of each GBS ST (Figure 7). As an internal positive control, a 688 base pairs band corresponding to the amplification of a conserved fragment of the cpsL gene was used. As a negative control, sterile water was added instead of DNA to the template. Each isolate was tested in duplicate.

Figure 7 - Gel electrophoresis of the multiplex PCR amplification products



Direct analysis of amplicon size and band pattern allowed the determination of the molecular STs of the GBS reference strains used: lane 1: NCTC 11078 (A909,Ia); lane 2: NCTC 8187 (H36B, Ib); lane 3: NCTC 11079 (18RS21, II); lane 4: ATCC BAA-22 (M781, III); lane 5: CNCTC 1/82 (IV); lane 6: ATCC BAA-611 (2603, V); lane 7: strain Prague 118754 (VI); lane 8: Prague 4/89 (7271, VII); lane 9: NCTC 9828 (M9, VIII); lane 10: strain ISS5611 (IX). Lanes 11-21 samples. M1: molecular marker 50 bp Step Ladder (Promega); M2: molecular marker 1 kb plus DNA Ladder (Life Technologies).

3.3. Functional immunoassay methods

3.3.1. Bacteria

GBS strains H092040676 (Ia), H090820125 (Ib), H092120162 (III) H090320548 (II) and H091780506 (V) were used in the immunological assays. Starting from the GBS strain master seed, 1mL of frozen culture was removed from the -70°C freezer to a class II microbiological safety cabinet and allowed to thaw at room temperature. 10µL of stock suspension was then streaked onto 5% horse blood Colombia agar plates using a sterile inoculating loop and incubated at 37°C in 5% CO_2 overnight. The growth from the whole plate was then transferred using a sterile inoculating loop to 10mL of THB and the optical density at 600nm (OD_{600nm}) was determined. The

$OD_{600nm}$was used to calculate the volume of inoculum to be added to 50mL of THB in an Erlenmeyer non-baffled shake-flask to obtain a final pre-incubation OD_{600nm} of 0.1. The resulting inoculum was incubated at 37°C with shaking at 180 rpm until a final OD_{600nm} of 1.0 was reached. The aim was to achieve mid-log phase culture growth (1.5-2 hours). At this point 100µL of culture was serially diluted 1:10 to 10^{-6}. 100µL from each dilution was spread onto the horse blood Colombia agar plates and incubated overnight at 37°C in 5% CO_2. Colonies were counted for each plate and averaged. The total viable count for each GBS ST was then calculated using the dilution factor and expressed as CFU/mL. Following growth the bacteria were split into two 25mL flasks and centrifuged at 3060g for five minutes. The supernatant was discarded and the remaining pellets were each suspended in 10mL of PBS. These suspensions were centrifuged at 3060g for five minutes and the supernatant discarded subsequently. The pellets were re-suspended in 9.5mL of PBS and 500µL of 40% formaldehyde, giving a final formaldehyde concentration of 2%. These suspensions were incubated at room temperature for one hour. Following incubation the killed cultures were vortexed to break up any remaining cocci in chains. To test for viability 10µL of each suspension was streaked onto a horse blood Colombia agar plate and incubated overnight at 37°C in 5% CO_2. Once total killing was confirmed the suspensions were aliquoted into 1mL volumes and stored at -20°C until use. An OD_{600nm} was taken for each suspension and used to calculate the bacteria required for each assay.

3.3.2. IgG surface-binding assay (IBA)

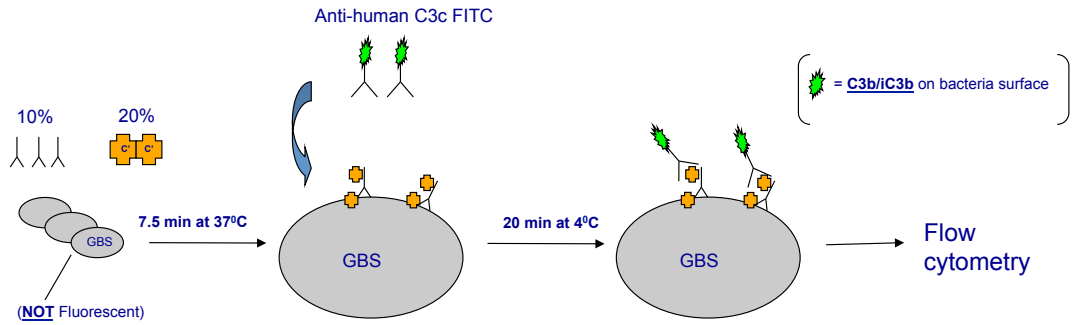
Surface deposition of ST-specific antibody was measured on the surface of formaldehyde-fixed GBS using a flow cytometric assay performed in 96-well

microtitre plates. This assay is the first step developed to assess functional antibody activity as it measures antibody binding to the surface of whole GBS but not antibody-mediated complement deposition (described below). Briefly, 2µl of each test serum was added to 198µl of ST Ia, Ib, II, III or V GBS bacteria at 5.14 x 10^7 CFU/mL in blocking buffer (BB) (1% bovine serum albumin (BSA) in PBS). This was incubated at 25°C for 30 minutes with shaking (900 rpm), then centrifuged. Supernatant was removed and the pellet washed twice with 200µl of BB. Alexa Fluor® 488 Goat Anti-Human IgG (heavy and light) (Life Technologies) (1:500) in BB was added and samples incubated for 20 minutes at 4°C, before being washed twice more with BB and analysed by flow cytometry.

3.3.3. Complement deposition assay (CDA)

The initial CDA methods have been described previously. (125, 163, 164) 5µl heat-inactivated test serum was added to the relevant wells of a standard U-bottom 96-well microtitre plate, followed by 35µl fixed bacteria at a concentration of 5.14x10^6 in PBS 1% BSA and 10µl IgG-depleted human plasma.(165) Samples were incubated at 37°C for seven minutes with shaking at 900rpm, centrifuged at 3060g for five minutes and each well washed with 200µl PBS 1% BSA, centrifuged again and the pellets re-suspended in 200µl rabbit anti-C3c- fluorescein isothiocyanate (FITC) [Abcam, UK (1:500 dilution in PBS 1% BSA)] and incubation continued at 4°C for 20 minutes. The plate was centrifuged at 3060g for five minutes and each well washed twice with 200µl PBS 1% BSA, and finally centrifuged again and the pellets re-suspended in 200µl PBS 1% BSA. The plates were stored in the dark at 4°C until analysed by flow cytometry. (Figure 8)

Figure 8 - Summary of complement deposition assay



3.3.4. Flow cytometry

Assays were analysed using a Beckman Coulter Cyan flow cytometer equipped with a Cytek 96-well microtitre plate reader. Protocols were initially set-up to analyse profiles of bacterial events using bacteria only control samples and the bacteria identified on the cytometer by the forward scatter (FS), measuring the size of the cell, and side scatter (SS), measuring the granularity and internal structural complexity. An analysis 'gate' was drawn around the population of interest (single and diplococci) and a relevant histogram plot created to analyse the fluorescence given off by the bacterial population. For each sample, 10,000 individual events were analysed for fluorescence and a horizontal gate was drawn to include 10% of the bacteria plus conjugate control sample population for IBA or 10% of the bacteria plus conjugate plus complement control sample population for the CDA. A fluorescence intensity (FI) was calculated for each sample, which involved the multiplication of the percentage of bacteria moving into the horizontal gate (%-gated), by the mean fluorescence of that population (X-mean). The result was expressed as the average FI (average FI taken for duplicate test samples) of the test serum sample minus the average FI of the bacteria plus conjugate only control for IBA, expressed as FI-C or

bacteria plus conjugate plus complement control for the CDA, expressed as FI-C' (Figure 9).

3.3.5. Opsonophagocytic killing assay (OPkA)

The following methods are adapted from the GSK vaccine research laboratories standard operating practice for opsonophagocytosis killing assay.(156)

3.3.5.1. Bacteria

For the OPkA, GBS strains 515 02/2012(Ia), H36Ib 08/2013 (Ib), COH1 11/2013 (III) 5401 10/2013 (II) and CJBIII 03/2009 (V) were grown up to an OD of 1.0 dispersed in 10% glycerol in 1mL aliquots into cryo-vials for flash freezing in a 95% ethanol dry ice bath and stored at -70°C until use. Bacteria were enumerated by viable count on CCNA and incubated at 37°C in 5% CO_2 overnight before counting.

Figure 9 – Flow diagram of calculation for fluorescence intensities

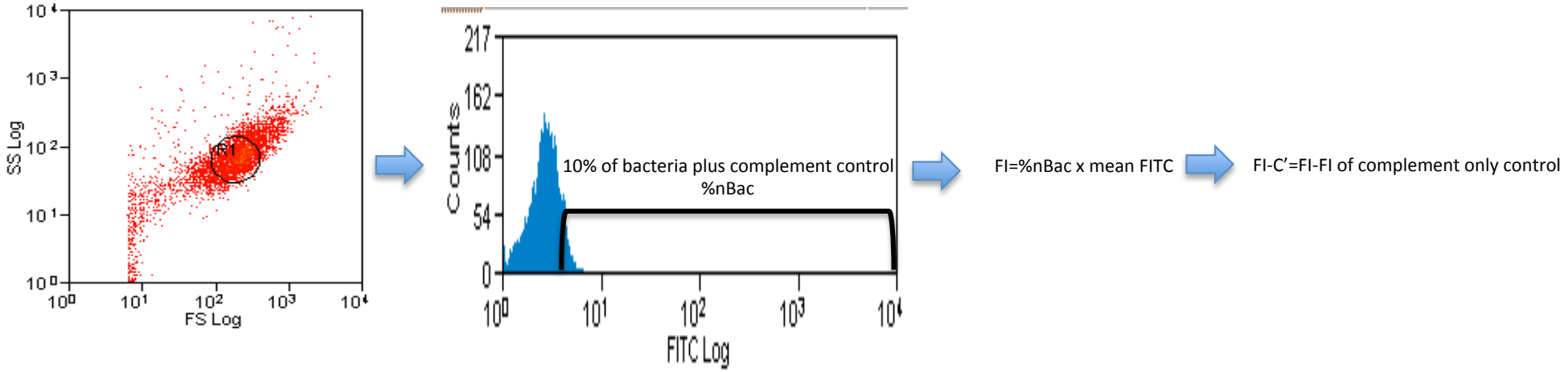


Diagram outlines gating of bacterial population, followed by gating to include 10% of the bacteria plus complement control followed by calculation of the fluorescence index and then subtracting the complement only control to give a final value FI-C' for the test sample. SS=side scatter; FS=forward scatter; FITC=fluorescein isothiocyanate; FI=fluorescence intensity; Bac=bacteria; FI-C'=FI-complement control.

3.3.5.2. Phagocytes

HL-60 cells (CCL240; American Type Culture Collection, Manassas, VA, USA) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (without phenol red; Gibco) supplemented with 20% fetal bovine serum (LabTech) and 1% L-glutamine (Gibco). The cells were subcultured daily to maintain the cell density between 1×10^5 and 1×10^6 cells mL^{-1}. The cells were differentiated into granulocytes in the same medium containing 0.8% (vol/vol) N,N-dimethylformamide (DMF; Sigma) for 5 days.

3.3.5.3. OPkA protocol

Prior to use, a 1mL vial of bacteria was thawed at room temperature, diluted in 9mL of PBS and centrifuged at 1800g for 10 minutes. The pellet was resuspended in 1mL of PBS, diluted 1:10, 1:20, 1:30 and 1:40 in Hanks Buffered Salt Solution (HBSS) containing phenol red (HBSS-red) and then diluted 1:2 in HBSS-red plus 10% normal rabbit serum (Sigma-Aldrich USA).

100µl heat-inactivated test sera diluted 1:3 with HBSS-red were added to the relevant wells of a standard U-bottom 96-well microtitre plate and a five step serodilution preformed by transferring 50µL serum into 50µL HBSS-red. 12.5µL of each dilution was then dispensed into a second 96-well microtitre plate, followed by 25µl fixed bacteria at a concentration of $5.x10^5$ CFU/mL in HBSS-red, 75µL HL60 cells at a concentration of 2.6×10^7 cells/mL and 12.5µl human complement. Samples were incubated at 37°C for 1 hour with shaking at 600rpm in a Thermomixer (Eppendorf, Germany). Two positive controls and six negative controls were added to each plate. The following negative controls were used: two wells with bacteria, complement and phagocytes but without human serum; two wells with bacteria,

positive control serum and complement but without phagocytes and two wells with bacteria, serum, phagocytes and heat inactivated complement. Before and after the one hour incubation, (T_0 and T_{60}), each reaction was diluted in sterile water (1:100 at T_0 by adding 25µL of reaction to 225µL sterile water , 1:20 and 1:200 at T_{60} by adding 25µL to 25µL of water and then 25µL of the resultant solution to 225µL water (1:20) and repeating this (1:200)). 10µL of each dilution was subsequently plated by tilt method onto CCNA plates and incubated overnight at 37°C in 5% CO_2. The following day colonies on the T_0 and T_{60} plates were counted using an automated colony counter. The opsonophagocytic activity was determined as the mean log_{10} reduction in GBS CFU/mL after 60 minutes of incubation at 37°C compared to T_0 ($LogT_0$-$LogT_{60}$). The OPkA titre was expressed as the reciprocal serum dilution leading to 50% killing of bacteria:

$$killing(\%)=[(mean\ CFU/mL\ T_0\text{-}mean\ CFU\ T_{60})/mean\ CFU/mL\ T_0]100$$

3.4. Sample size calculation

Hypothesis 1: GBS ST-specific maternal-derived antibody in serum determines GBS-colonisation in infants;

Based on estimates from the pilot study of 100 women in The Gambia which established a colonisation rate of approximately 24%, 50% transfer of colonisation from mother to infant (12% infants colonised) and 6% prevalence of GBS STIII (lowest GBS ST found in original study),(17) a sample size of 750 women would be required to give 180 colonised women/90 colonised infants and at least 10 women/5 infants colonised with GBS STIII and 570 non-colonised women. 10 mothers for each GBS ST

would give 80% power to detect a correlation of 70% or greater between GBS ST-specific antibody and colonisation.

Hypothesis 2: *Higher levels of GBS ST-specific antibody in infants are associated with better ability to kill GBS in-vitro and this represents a laboratory surrogate for protection from disease*;

Based on the pilot study, all infants were expected to be seropositive. Thus 150 samples would give over 90% statistical power to compare antibody concentration to IBA/CDA/OPkA assays.

Hypothesis 3: *GBS colonisation in The Gambia is predominantly due to ST Ia, III and V*

Using the sample size of 150 colonised mothers calculated for hypothesis 1, the standard error and 95% confidence intervals (CI) were calculated for GBS ST prevalence between 6-40%. For a GBS ST prevalence predicted for GBS STIII, a sample size of 150 colonised mothers would allow us to detect 6% prevalence of this GBS ST with a standard error of 1.8%. Table 4 outlines the standard error and 95% CIs calculated.

Table 4 - Standard error and 95% CIs for different GBS ST prevalence

Prevalence	Standard Error	95% CI
6%	0.018	2.4-9.6%
10%	0.023	5.4-14.6%
20%	0.030	14-26%
30%	0.035	23-37%
40%	0.038	32.6-47.4%

3.5. Clinical data management and statistical analysis

A study-specific, password protected relational Filemaker Pro database (Filemaker Inc. version 10.0v3) was developed to collect clinical and demographic data. All personal identifiers were excluded from the database and all study participants were

assigned a unique patient identification number. STATA version 12 (StataCorp 2013) and GraphPad Prism version 6.0 (GraphPad Software Inc, La Jolla, California) were used for statistical analysis. Additional statistical support was sought from Fiona Warburton at Public Health England who verified all my statistical calculations once I had completed the analysis.

3.5.1. Epidemiological analyses

Descriptive statistics were calculated which included the prevalence of colonisation at individual time points expressed as a proportion of the total number of participants. To determine the individual risk factors for maternal and infant colonisation logistic regression was performed; analysis of variance (ANOVA) was used for the single variable analyses of the transmission dynamics at birth. Adjusted odds ratios (AOR) were then calculated using variables from the single variable analysis with a p-value <0.2 using a backwards-stepwise regression procedure. The log-rank test was used to examine differences in duration of colonisation between GBS STs. Assuming that the vertical transmission rate is 50% (4) I calculated observed vs. expected statistics for risk of infant colonisation by GBS ST. For comparison between our study and the previous study conducted in the Gambia in 1994 (17), I calculated 95% CIs of the prevalence of GBS colonisation in both studies. If the CIs did not overlap then the prevalence of colonisation was interpreted as statistically different.

3.5.2. Antibody analyses

Details of the flow cytometric analyses are outlined in 3.3.4.

Due to the skewed distribution of the GBS ST-specific FI-C' values, log transformations were required to conform to regression assumptions. Potential

differences in antibody concentrations between colonised and non-colonised mothers and infants were evaluated by Student's t test after log transformation of data. I compared four groups (mother colonised/infant non-colonised; mother colonised/infant colonised; mother non-colonised/infant colonised and neither mother nor infant colonised) using ANOVA following log-transformation of data. Comparison of log-transformed serum FI-C' values at birth (cord blood) and at 60-89 days was performed using a paired t test. To determine which variables were independently associated with the acquisition of GBS, logistical regression was used. Deming regression (166) was used to determine the correlation between bacterial concentration and cord and infant antibody concentration. Deming regression is an errors-in-variable model that is an extension of linear regression. It takes into account possible errors in both the x and y-axis. To ascertain the relationship between cord/infant GBS ST-specific functional antibody response and the covariates maternal age and infant sex, I used multiple linear regression. Missing data were excluded from analysis. For all comparisons, $p<0.05$ was considered to be significant.

Chapter 4 – Epidemiological study

4.1. Introduction

In the African setting, information on GBS colonisation and disease prevalence remains sparse.(6) Maternal colonisation is the leading risk factor for both EO- and LO-GBS disease(4), yet in LMIC little is known about other risk factors that may affect not only maternal colonisation but also transmission from mother to child. Limited knowledge about maternal or infant colonising GBS STs in African countries indicate that GBS STV may be important as both a colonising and invasive disease ST (12, 26), similar to the USA in the 1990s.(167)

An example of this is the study by Suara et al (1994) undertaken in the Gambia, which examined GBS colonisation in pregnant women and their infants and demonstrated prevalence of maternal colonisation of 22% (30/136 mothers) and 13% in their infants with GBS STV the predominating ST.(17) However, interpreting this data is difficult, as selective media was not used to isolate GBS. Hence, there was the potential for overgrowth of other gut microflora in this study that may have led to an underestimation of GBS colonisation in this population. Additionally, sampling was limited to the vagina. We now know that rectovaginal sampling gives the highest yield for GBS.(168)

Whilst the main purpose of the study was to determine the role of antibody in prevention of colonisation with GBS, the description of the study cohort and comparison with other African studies is valuable. Additionally, detailed characterisation of the cohort is essential in order to understand the dynamics of transfer from mother to child and factors that may affect antibody concentration in this population and is indeed a strength of this study.

This Chapter describes the study cohort including demographics and risk factors for GBS colonisation and transfer from mother to child. Further, in this Chapter I describe the prevalence of colonising GBS STs in Gambian women and their infants from delivery to day 60-89 and the effect of different risk factors on colonisation over the entire study period.

4.2. Study design and participants

The study took place between 1st January 2014 and 31st December 2014 in two government health centres as described in Chapter 3. The results of this study have been published and all figures and tables are reproduced from Le Doare et al (2016) unless otherwise indicated.(158)

Field workers explained the purpose of the study to eligible participants in their local language (Mandinka, Wolof, Fula, Jola, Mangago) and each participant received an information sheet and was asked to sign an informed consent form, or in case of illiteracy, the information sheet and consent form were read to the participant in local language and the participant asked to provide a thumb-print to confirm their consent and the consent form was then countersigned by an impartial witness (Appendix 2). The study was approved by the Joint Gambian Government/Medical Research Council Research Ethics Committee, SCC 1350 prior to field activities (Appendix 1).

Following recruitment, demographic and clinical data were collected from each participating mother (Appendix 3). Since HIV is a potential confounding factor for immune response, each mother was offered a confirmatory HIV test prior to enrolment. Women found to be HIV positive were referred for specialist ongoing

care. All sick infants were also offered an HIV test and had blood cultures and malaria films taken as part of routine care. Further information about recruitment is outlined in 3.1.6. If an infant died during the study a verbal autopsy was carried out to assess the potential cause of death(169).

4.3. Methods

The microbiological methods used for this part of the study are outlined in Chapter 3. The primary outcome was prevalence of GBS ST-specific colonisation in mothers and in infants at birth, six days and days 60-89 using microbiological culture and molecular techniques. Secondary outcomes were detection of GBS in breast milk; infant acquisition and loss of GBS colonisation during the study follow up period and infant GBS disease (sample obtained from sterile site), as ascertained by positive microbiological culture and confirmatory PCR. Infant GBS disease was defined as infant presenting with features of systemic sepsis or meningitis (tachycardia, tachypnoea, irritability, poor feeding with or without fever) and a positive blood culture (eligible infants had 1-2mL of blood taken using aseptic technique and cultured in a paediatric blood bottle in a BACTEC machine). Swabs were considered negative if no GBS was evident by culture and PCR and positive if GBS was found on culture and PCR. If swabs were negative on culture but positive on PCR, conventional PCR was performed to determine GBS ST. If the second PCR resulted in the identification of a GBS ST the samples were deemed positive. If no GBS ST was identified or the DNA did not amplify, samples were deemed negative.

4.3.1. Data analysis approach

Descriptive statistics were calculated, which included the prevalence of colonisation at individual time points expressed as a proportion of the total number of participants. Logistical regression was also performed for univariate analyses such as individual risk factor and maternal colonisation status; ANOVA was used for the univariate analyses of the transmission dynamics at birth. AORs were then calculated using variables from the univariate analysis with a p-value <0.2 using backwards-stepwise logistic regression. The log-rank test was used to examine differences in duration of colonisation between GBS STs. Further details can be found in section 3.6.1. Assuming an expected breast milk colonisation rate similar to the observed maternal rectovaginal colonisation rates of 8.4% (STIa), 6.8% (STIb), 16.5% (STII), 10.1% (STIII) and 54.9% (STV) observed vs. expected statistics for breast milk colonisation were calculated by GBS ST. For comparison between our study and the study conducted in 1994, 95% CIs for both studies were calculated.

4.4. Demographics

A total of 750 mothers and their infants were recruited to the study. All mothers met the inclusion criteria except one, who was admitted to hospital on day three postpartum severely unwell and subsequently tested positive for HIV-1, despite a negative test at 20 weeks gestation. She and her infant were referred to routine clinical services for further management and samples excluded from further analysis. Of the remaining eligible women and infants, 723 completed the day six visit (median age at follow up 8 days, interquartile range (IQR) 7-9) and 684 completed 60-89 day follow up (median age at follow up 62 days, IQR 60-63). The

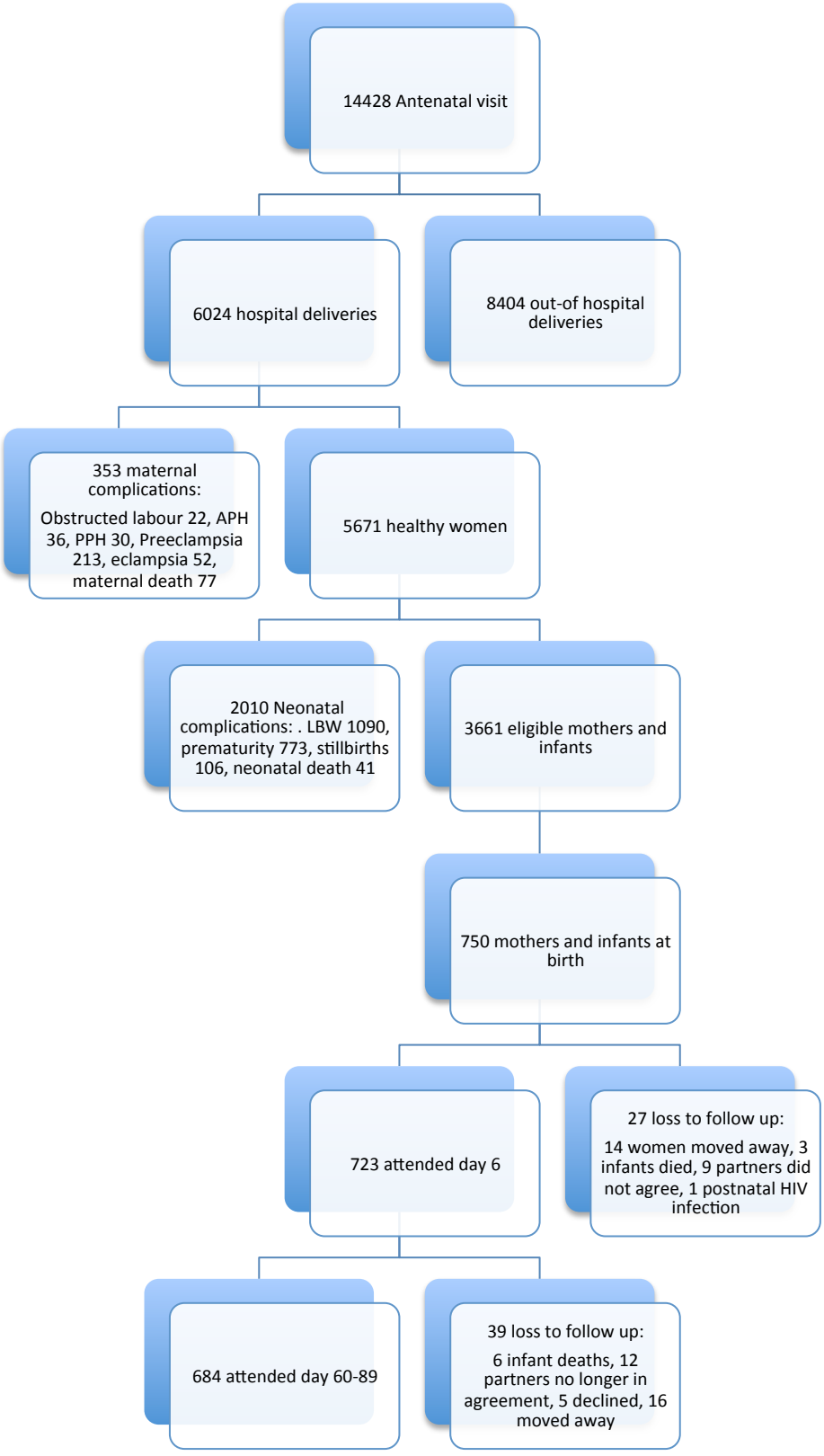
main reasons for not completing follow up were: 30 women moved out of area, 21 women whose partners were not in agreement with continued study participation left the study, one postnatal HIV infection, five declined for other reasons and nine infants died during the follow up period. All infants were exclusively breastfed for the duration of the study. Participant flow diagram and demographic characteristics of mother-infant pairs are outlined in 10 and Table 5.

Table 5 - Mother and infant demographics and odds ratios of maternal GBS colonisation at delivery

		Total	Colonised	Non-colonised	p-value	OR (95% CI)
Maternal Age yrs.	<20	110	40	70	0.78	1
	20-24	262	81	181		0.78 (0.5-1.3)
Mean: 25 (IQR 18-41)	25-29	203	71	132		0.94 (0.6-1.5)
	30+	175	61	114		0.99 (0.6-1.7)
Ethnicity	Mandinka	315	115	200	0.38	1
	Wolof	108	36	72		0.9 (0.5-1.4)
	Fula	126	45	81		1 (0.6-1.5)
	Jola	118	33	85		0.7 (0.4-1.0)
	Other	81	23	58		0.7 (0.4-1.2)
	Missing	2	1	1		
Maternal Weight (kg)	<50	27	7	20	**0.03**	1
	50-70	424	128	296		1.2 (0.5-3.0)
	70-90	148	61	87		2.0 (0.8-5.0)
	>90	145	56	91		1.8 (0.7-4.4)
	Missing	6	3	3		
Maternal illness in pregnancy	Yes	128	45	83	0.32	1
	No	504	165	339		1.1 (0.7-1.7)
	Missing	118	43	75		
Maternal Hb below 10g/dL	No	99	42	57	**0.04**	1
	Yes	651	211	440		0.5 (0.3-0.9)
Maternal antibiotics in early	No	505	166	339	0.74	1
pregnancy	Yes	128	44	84		1.1 (0.7-1.6)
	Missing	117	74	43		
Gravida	1	208	67	141	0.44	1
Median: 2 (IQR 1-4)	2-4	370	121	249		1 (0.7-1.5)
	5+	169	63	116		1.3 (0.8-2.0)
	Missing	3	1	2		
Parity	0-1	212	68	144	0.59	1
Median: 1 (IQR 0-4)	2-4	468	164	304		1.1 (0.8-1.6)
	5+	67	20	47		1.1 (0.7-1.7)
	Missing	3	1	2		
Living children	0	230	80	150	0.17	1
Median: 1 (IQR 0-3)	1-4	465	155	311		0.9 (0.7-1.3)
	5+	53	17	36		0.9 (0.5-1.7)
	Missing	2	2	0		
Spontaneous abortion	0	649	213	436	0.09	1
Median 1(IQR 0-2)	1	53	20	33		1.2 (0.7-2.2)
	2+	14	7	7		1.5 (0.8-2.7)
	Missing	34	16	18		
Stillbirths	0	641	207	434	0.05	1
	1	75	29	46		0.9 (0.4-2.1)
	2+	28	15	13		3.6 (1.2-10.5)
	Missing	6	2	4		
Number of ANC visits	1	303	85	218	**0.01**	1
	2+	447	168	279		1.5 (1.1-2.1)
Attendance by midwife or TBA	Midwife	436	168	268	**0.001**	1
	TBA	254	67	187		0.5 (0.4-0.8)
	Missing	60	37	23		
Birth Season	Dry Cool	280	55	225	**0.001**	1
	Dry Hot	175	85	90		3.9 (2.5-5.9)
	Wet	295	113	18		2.5 (1.7-3.7)
Infant Sex	Male	369	122	247	0.59	1.1 (0.8-1.5)
	Female	381	131	250		
Birth Weight (kg)	2.0-3	260	73	187	**0.04**	1
	3.1-4	464	169	295		1.5(1.1-2.0)
	>4.0	26	11	15		1.9 (0.8-4.3)
Gest. Age at birth (wks.)	<34	56	18	38	**0.03**	1
	34-37	231	83	148		0.7 (0.4-1.1)
	38+	463	152	311		1.1 (0.7-1.7)

IQR = inter-quartile range; ANC=antenatal clinic; TBA=traditional birth attendant

Figure 10 - Study flow diagram



The recruitment process at each stage of the field study with reasons for exclusion or loss to follow up. Loss to follow-up was 3.6% at end of day 6 and 8.8% by the end of 3 months of life. APH = antepartum haemorrhage, PPH =postpartum haemorrhage, LBW=low birth weight (<2kg).

4.5. Antibiotic treatment in pregnancy

Over the course of the study, 77 women received antibiotics during pregnancy: 34 for urinary tract infection; 10 for pneumonia; 14 for vaginal discharge; nine for malaria and 10 for non-malarial fever. Of these, 73 women received amoxicillin (median time prior to delivery 42 days [IQR 18-63 days]); 16 received antimalarial therapy (median 38 [IQR 31-41]) and three received erythromycin. In the three months of follow up, 51 infants required antibiotic therapy: 34 received ampicillin and gentamicin for presumed neonatal sepsis (median 18 days old [IQR 4-34]); ten received amoxicillin or cloxacillin for skin infections (median 20 days [IQR 10-58]); seven received other antibiotics (median 18 days [IQR 11-23].

4.6. Prevalence and risk factors for maternal colonisation

The overall prevalence of rectovaginal GBS colonisation at delivery was 33.7% (253 women). In univariate analysis maternal weight ≥50kg, maternal haemoglobin <10g/dL, more than one previous stillbirth, more than one ANC visit, midwife delivery, delivery during the dry hot or wet season and gestation under 34 weeks were associated with increased risk of GBS colonisation (Table 5). In adjusted analysis, maternal haemoglobin <10g/dL, history of more than one previous stillbirth, attendance at delivery by a midwife rather than a traditional birth attendant (TBA) and giving birth during the dry hot or wet season were associated with increased odds of maternal GBS colonisation at delivery (Table 6).

Table 6 - Multivariable analysis: risk factors for maternal GBS colonisation at time of delivery

		AOR (95% CI)	p-value
Maternal Weight (kg)	<50	1	
	50-70	1.0 (0.4-1.6)	0.96
	71-90	1.7 (0.6-4.5)	0.30
	>90	1.4 (0.3-4.7)	0.68
Number of ANC visits	1	1	
	2+	1.3 (0.9-1.8)	0.16
Maternal Hb above 10g/dL	No	1	
	Yes	0.7 (0.4-0.9)	**0.04**
Attendance by midwife or TBA	Midwife	1	
	TBA	0.6 (0.4-0.9)	**0.03**
Previous Stillbirths	0	1	
	1	0.9 (0.4-2.0)	0.86
	2+	**5.4 (1.6-18.0)**	**0.009**
Birth Season	Dry Cool	1	
	Dry Hot	**2.0 (1.3-3.1)**	**0.004**
	Wet	**1.8 (1.2-2.7)**	**0.008**

ANC =antenatal clinic, Hb=haemoglobin; TBA = traditional birth attendant; AOR=adjusted odds ratio

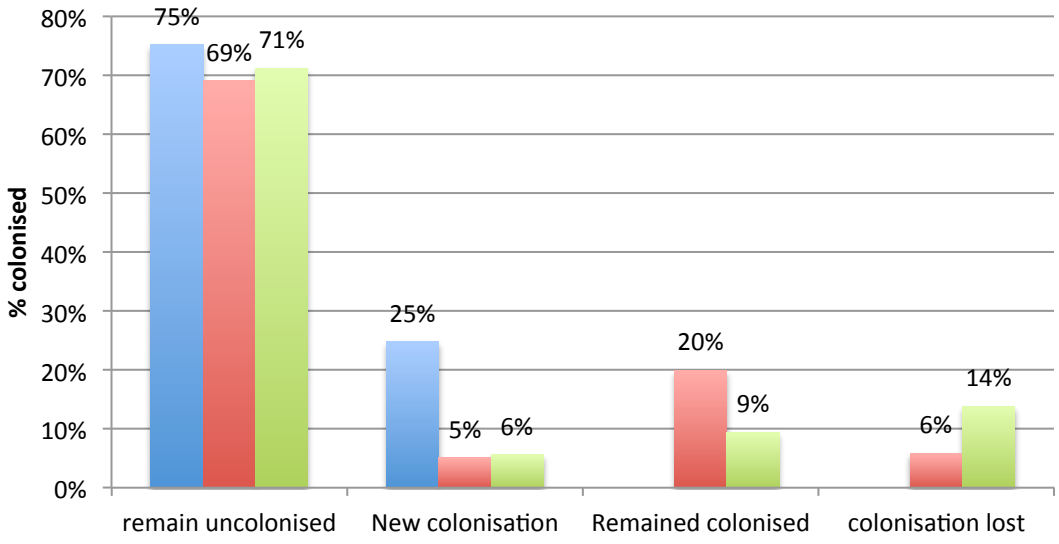
4.7. GBS colonisation dynamics at birth

There were 253 colonised mothers and 186 colonised infants at birth, of whom 146 were born to colonised women (57.0%). Table 7 describes the number of colonised infants at birth, day six and at day 60-89 according to colonisation site. Figures 11 and 12 demonstrate colonisation dynamics at each time point.

Table 7 - Dynamics of colonisation by site and time point (%total)

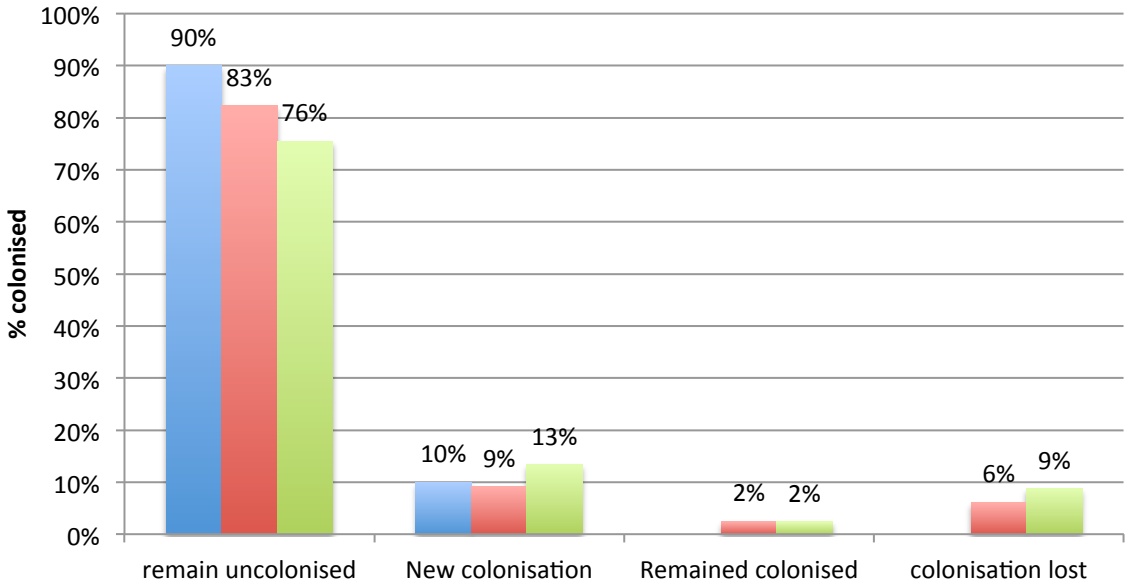
Birth n=750; 253 colonised mothers (%total)	
Mother and infant colonised	146 (19.5% total; 57.7% born to colonised mothers)
Infants colonised, mothers non-colonised	40 (5.3)
Total colonised infants	186 (24.8)
Mothers colonised, infants non-colonised	107 (14.3)
Neither mother nor infant colonised	457 (60.9)
Site of colonisation at birth n=186 (% colonised infants)	
Nasopharyngeal and rectal	96 (51.6)
Rectal only	68. (36.6)
Nasopharyngeal only	22 (11.8)
Site of colonisation day 6-9; n=722 (% of the 181 colonised infants)	
Nasopharyngeal and rectal	57 (31.5)
Rectal only	95 (52.5)
Nasopharyngeal only	29 (16.0)
Site of colonisation day 60-89; n=684 (% the 94 colonised infants)	
Nasopharyngeal and rectal	11 (11.7)
Rectal only	57 (60.6)
Nasopharyngeal only	26 (27.7)

Figure 11 - Infant GBS colonisation status over time



Bars represent percentage of infants colonised/uncolonised at each of the time points; blue bar=birth, red = day 6 compared to birth and green = day 60-89 compared to day 6. N=750 at birth; n=723 at day 6 and n=684 at day 60-89.

Figure 12 - Breast milk colonisation over time



Bars represent percentage of breast milk colonised/uncolonised at each of the time points; blue bar=colostrum on day 1, red = day 6 compared to colostrum and green = day 60-89 compared to day 6. N=750 at birth; n=723 at day 6 and n=684 at day 60-89.

Compared to mother/infant pairs who were both non-colonised at birth, mother/infant pairs were more likely to both be colonised if mother's weight was

above 70kg, infants were born at less than 37 weeks, born during the dry hot or wet seasons, mothers with more than one previous stillbirth, mothers attending ANC more than once, infants delivered by midwives, infants born with a birth weight below 3.1kg or receiving breast milk colonised with GBS. (Table 8)

Table 8 - Characteristics of mother/infant pairs according to colonisation status at birth

	Total	M+I+	M+I-	M-I+	M-I-	p-value
Maternal weight (kg)						
<50	27	2	5	4	16	**0.04**
50-70	424	74	54	21	275	
70-90	148	36	25	3	84	
90+	145	32	23	9	81	
Missing	6	3	0	3	0	
Stillbirths						
0	641	119	87	32	403	
1	75	20	9	6	40	**0.03**
2+	28	5	10	2	11	
Missing	6	2	1	0	3	
Number of ANC visits						
1	303	58	27	15	203	
2+	447	88	80	25	254	**0.002**
Midwife delivery	436	108	60	20	248	**<0.001**
TBA	254	29	38	12	175	
Missing	60	9	9	8	34	
Season						
Dry Cool	280	22	33	17	208	
Dry Hot	175	66	19	3	87	**<0.001**
Wet	295	58	55	20	162	
Birth weight (kg)						
2.0-3	260	44	29	20	167	**0.02**
3.1-4	464	95	74	18	277	
>4.0	26	7	4	2	13	
Gestation (weeks)						
<34	56	13	5	4	34	**0.04**
34-37	231	57	26	12	136	
38+	463	76	76	24	287	
Breast milk colonised						
Yes	64	31	18	7	8	**<0.001**
No	600	101	77	31	391	
Missing	86	14	12	2	58	
Spontaneous abortion						0.09
0	649	121	92	31	405	
1	53	14	6	4	29	
2+	14	7	1	0	6	
Missing	34	11	0	5	18	
Ethnicity						0.10
Mandinka	315	63	52	23	177	
Wolof	108	23	13	8	64	
Fula	126	28	17	2	79	
Jola	118	21	12	3	82	
Other	81	11	12	4	54	
Missing	2	0	1	0	1	
Maternal Hb <10g/dL						0.11
No	99	24	18	2	55	
Yes	651	122	89	33	403	
Maternal Age						0.96
<20	110	23	17	2	68	
20-24	262	43	38	19	162	
25-29	203	46	25	11	121	
30+	175	34	27	8	106	
Mother ill						0.48
Yes	128	24	21	3	80	
No	504	97	68	25	314	
Missing	118	25	18	12	63	
Maternal antibiotics in pregnancy						
No	505	99	92	0	314	0.91
Yes	128	23	24	0	81	
Gravida						0.44
1	208	39	28	13	128	
2-4	370	67	54	17	232	
5+	169	39	25	10	95	
Missing	3	1	0	0	2	
Parity						0.94
0-1	212	39	29	13	131	
2-4	468	96	68	24	280	
5+	67	10	10	3	44	
Missing	3	1	0	0	2	
Living children						0.66
0	230	43	37	14	136	
1-4	465	94	61	25	285	
5+	53	7	9	1	36	
Missing	2	2	0	0	0	
Infant Sex						0.71
Male	369	72	50	23	224	
Female	381	74	57	17	233	

M+I+=mother and infant colonised; M+I-=mother colonised, infant non-colonised; M-I+=mother non-colonised, infant colonised, M-I-=neither mother nor infant colonised; TBA = traditional birth attendant; ANC=antenatal clinic; Hb=haemoglobin.

In adjusted analysis, compared to mother/infant pairs who were both non-colonised at birth, mother/infant pairs were more likely both to be colonised if birth occurred in the dry hot or wet seasons, infants were born to mothers with one or more previous stillbirths or abortions, or infants born before 34 weeks gestation. Compared to mother/infant pairs where neither was colonised, mother/infant pairs where the mother was colonised but not the infant were more likely if the mother had had more than one previous stillbirth, or if the mother gave birth during the wet season. Infant colonisation in the absence of maternal colonisation (n=40) was associated with gestation before 34 weeks. (Table 9)

Most notably, compared to mother/infant pairs at birth where both remained non-colonised, infants born to mothers who had GBS cultured in their breast milk were significantly more likely to be colonised with GBS. (Table 9)

Table 9 - Multivariable analysis of maternal/infant pairs by GBS colonisation status at time of delivery

	M+I+* OR (95% CI)	M+I-* OR (95% CI)	M-I+* OR (95% CI)
Season			
Dry Cool	1	1	1
Dry Hot	8.3 (3.9-17.6)	1.8 (0.8-3.9)	0.4 (0.1-1.4)
Wet	3.5 (1.8-6.8)	2.3 (1.3-4.3)	1.1 (0.5-2.5)
Breast milk colonised			
Yes	21.0 (8.6-51.3)	11.5 (4.5-	7.5 (2.0-27.5)
No	1	29.4)	1
		1	
Gestation (weeks)			
<34	1	1	1
34-37	0.9 (0.9-1.0)	1.0 (0.9-1.1)	0.9 (0.8-1.0)
38+	0.4 (0.2-1.0)	0.5 (0.3-1.6)	0.9 (0.8-1.0)
Midwife delivery	1	1	1
TBA	0.9 (0.5-1.6)	1.0 (0.6-1.8)	0.6 (0.2-1.3)
Number of antenatal clinic visits			
1	1	1	1
2+	1.1 (0.7-1.9)	1.2 (0.8-2.1)	1.3 (0.6-2.8)
Stillbirths			
0	1	1	1
1	2.5 (1.0-8.7)	2.0 (1.0-4.7)	1.4 (0.3-5.9)
2+	2.4 (0.9-4.1)	2.2 (0.9- 2.1)	ND
Spontaneous abortion			
0	1	1	1
1	0.9 (0.5-1.4)	0.8 (0.3-2.1)	1.0 (0.2-5.5)
2+	1.2 (1.1-2.3)	0.3 (0.0-2.2)	2.0 ND
Gravida	1	1	1
1	1.0 (0.9-1.0)	1.1 (0.6-2.0)	1.0 (0.8-1.2)
2-4	1.0 (0.5-2.0)	0.8 (0.4-1.8)	0.8 (0.3-2.3)
5+			

*M+I+=mother and infant colonised; M+I-=mother colonised, infant non-colonised; M-I+=mother non-colonised, infant colonised, M-I-=neither mother nor infant colonised; TBA=traditional birth attendant.*M-I- base group, adjusted odds ratios are compared to this group.*

4.8. GBS colonisation dynamics at day six and day 60-89

Infants born to colonised mothers remained at increased risk of colonisation up to day six if born during the dry hot season, but not at day 60-89. These infants also had increased risk of colonisation if born before 34 weeks gestation at both six days and day 60-89. A birth weight over 3.1kg was associated with a decreased risk of colonisation at both time points after birth. (Table 10).

The association between breast milk colonisation and infant colonisation remained significant up to 89 days regardless if GBS was isolated in the maternal rectovaginal tract at delivery or not. (Table 10)

Infants born to non-colonised mothers had an increased risk of being GBS colonised on day six if born during the wet season and up to 89 days if born during the dry hot season. These data suggest that infant colonisation is most prevalent during the wet season in this setting as infants born during the dry hot season would be 60-89 days of age during the wet season (Table 10).

Table 10 - Univariate analysis of infant GBS colonisation dynamics over time compared to birth

Mother GBS colonised at delivery		Birth OR (95% CI)	Day 6 OR (95% CI)	day 60-89 OR (95% CI)
Birth Season	Dry Cool	1	0.2 (0.1-0.6)	0.1 (0.0-0.4)
	Dry Hot	4.6 (2.2-9.7)	1.9 (1.0-4.1)	0.6 (0.2-1.3)
	Wet	1.4 (0.7-2.8)	0.7 (0.3-1.5)	0.3 (0.1-0.7)
Living Children	0	1	0.5 (0.3-0.9)	0.2 (0.1-0.4)
	1-4	0.9 (0.5-1.6)	0.4 (0.2-0.7)	0.1 (0.0-0.3)
	5+	0.9 (0.3-2.6)	0.6 (0.2-1.8)	0.2 (0.0-0.9)
Gestation at birth (wks.)	<34	1	0.4 (0.1-1.4)	ND
	34-37	0.9 (0.3-2.4)	0.3 (0.1-0.9)	0.2 (0.1-0.7)
	38+	0.4 (0.1-1.0)	0.2 (0.1-0.5)	0.0 (0.0-0.1)
Infant Weight (kg)	2.0-3	1	0.5 (0.2-1.0)	0.1 (0.0-0.3)
	3.1-4.0	1.2 (0.4-3.8)	0.7 (0.3-0.9)	0.1 (0.1-0.3)
	>4.0	0.6 (0.1-2.3)	0.3 (0.1-0.7)	0.1 (0.0-0.6)
BM colonised	Yes	0.9 (0.5-1.7)	0.4 (0.2-0.8)	0.3 (0.1-0.7)
	No	1	0.4 (0.3-0.6)	0.1 (0.1-0.2)
Infant Sex	Male	1	0.4 (0.3-0.7)	0.1(0.1-0.3)
	Female	0.7 (0.4-1.2)	0.4 (0.2-0.6)	0.1(0.1-0.3)
Mother not GBS colonised at delivery				
Birth Season	Dry Cool	1	1.4 (0.5-4.3)	0.8 (0.2-2.8)
	Dry Hot	1	1.3 (0.3-5.3)	4.7 (1.7-13.5)
	Wet	2.1 (0.8-6.0)	4.1 (1.6-10.5)	0.2 (0.0-1.9)
Living Children	0	1	1.8 (0.5-5.7)	1.7 (0.5-5.6)
	1-4	1.0 (0.3-2.9)	2.2 (0.8-5.9)	0.7 (0.2-2.3)
	5+	2.0 0.8 (0.1-7.1)	1	0.8 (0.1-7.3)
Gestation at birth (wks.)	<34	1	3.3 (0.3-34.4)	1
	34-37	1.6 (0.2-13.4)	2.5 (0.3-20.6)	1.4 (0.2-13.0)
	38+	1.1 (0.1-9.0)	2.2 (0.3-17.3)	1.3 (0.2-10.8)
Infant Weight (kg)	2.0-3.0	1	1.2 (0.4-3.8)	0.6 (0.1-2.3)
	3.1-4.0	0.5 (0.2-1.4)	1.2 (0.5-2.9)	0.6 (0.2-1.7)
	>4.0	0.9 (0.2-2.3)	1.7 (0.9-4.9)	0.4 (0.3-2.2)
BM colonised	Yes	14.2 (4.0-50.8)	6.9 (2.5-19.1)	3.3 (1.2-11.0)
	No	1	2.0 (0.9-4.2)	1.0 (0.4-2.5)
Infant Sex	Male	1	1.4 (0.6-3.1)	0.8 (0.3-2.0)
	Female	0.3 (0.1-1.0)	1.1 (0.5-2.5)	0.5 (0.2-1.5)

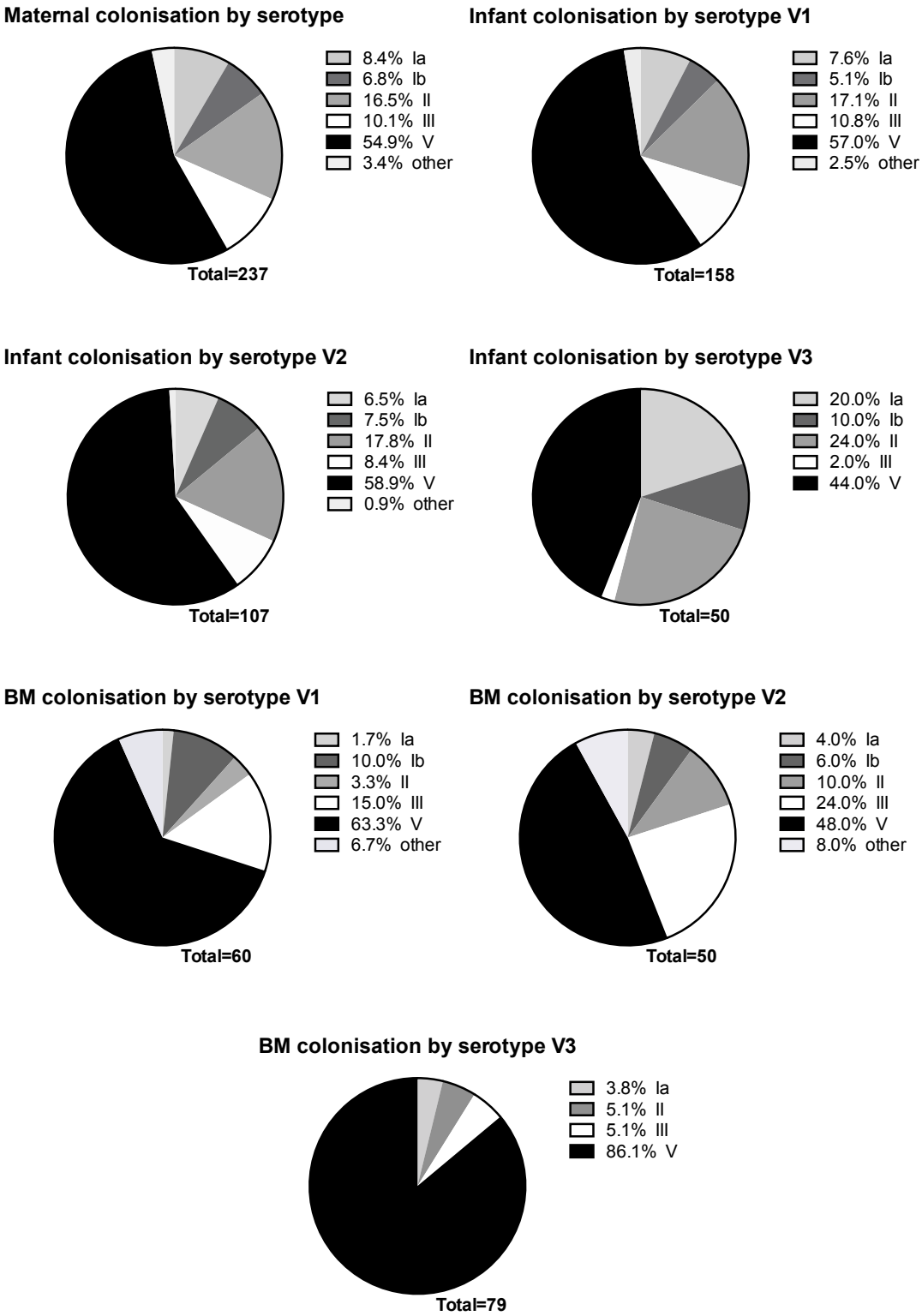
GBS= Group B Streptococcus; CI=95% confidence interval; OR=odds ratio; wk=weeks; kg=kilograms; BM=breast milk

4.9. Serotype distribution

The predominant maternal rectovaginal colonising GBS ST was GBS STV in 54.9% of colonised mothers. Infants were more likely to be colonised with GBS STV at birth (p=0.004), day six (p=0.004) and day 60-89 (p=0.009) than any other GBS ST. At birth, infants were more likely to be colonised with GBS STs III and V if their mothers were colonised with GBS STs III and V (x^2=24.4, p<0.001) compared to mothers colonised with other GBS STs. (Figure 13).

Mothers' breast milk was more likely to be colonised with GBS STV (p<0.001) at birth and day 60-89 than any other GBS ST. At birth colostrum was more likely colonised with GBS ST III (p<0.001) and V (p=0.02) than Ia, or II (Figure 13).

Figure 13 - Colonising serotype distribution by isolation site and time point



Pie charts demonstrating percentage by GBS serotype of maternal rectovaginal colonisation at delivery; breast milk and infant colonisation by time point; BM= breast milk; Ia=serotype Ia, Ib=serotype Ib, II=serotype II, III=serotype III, V=serotype V. N=750 at birth; n=723 at day 6 and n=684 at day 60-89.

4.10. Neonatal sepsis

During the study period, 51 infants (6.8%) presented with signs of infection (68 per 1000 live births): 18 with signs of pneumonia, 22 with signs of other febrile illness, ten with skin infections and one with signs of meningitis. One child presenting on day six of life with irritability and poor feeding, had a positive cerebrospinal fluid (CSF) culture for GBS and was treated for 21 days with ampicillin and gentamicin (1.3 per 1000 live births). This infant was born to a mother who had GBS STV cultured from both rectovaginal swab and breast milk at birth and day six of life and the infant swab was positive at birth and day six for GBS STV.

Nine infants died during the study period. Based on verbal autopsy, six deaths were due to pneumonia (median age at death 7 days [IQR 2-35]); three were due to sepsis (median age 6 days [IQR 1-48]). Blood cultures were GBS negative in all cases. One infant was born to a colonised mother and was also colonised with GBS at the time of birth but was non-colonised on day 6. This infant died of presumed sepsis on day 48 of life. No other infants who died or their mothers were colonised with GBS at time of birth or day 6 of life. All infants died prior to the final follow-up appointment. There was no bacteriological confirmation of GBS disease as a cause of death in any case.

4.11. Discussion

In this longitudinal study undertaken in Gambian mothers and their infants, the first colonisation study in the Gambia for 25 years and the first longitudinal study undertaken in this population, colonisation of Gambian mothers and their infants was assessed and demonstrated high prevalence of GBS colonisation in mothers at

birth and in their infants up to 89 days of life. In addition, infants were more likely to be colonised if GBS was isolated from their mother's breast milk. The overall prevalence of maternal GBS in this population was higher than in the original study by Suara et al (1992) at 33% vs. 22%.(17)22%.(17) However, the previous study likely under-identified GBS colonisation, as selective medium was not used in the microbiology laboratory at the time the study was undertaken by Suara et al. The prevalence of GBS colonisation in this study is similar to the recently reported pooled prevalence in South African women (30.4%).(12)

Our finding that mothers with more than one stillbirth demonstrated an increased odds of having a colonised infant are in concordance with Monari et al, who identified stillbirth as a risk factor for invasive EO-GBS disease.(170) This phenomenon has also been noted in non-human primates (171), but there are currently little human data on the association between stillbirth, GBS colonisation and invasive disease. A recent meta-analysis of GBS and stillbirths was unable to identify a global link between the two due to the scarcity of data and differences in reporting methods and definitions.(172)

The prevalence of infant colonisation in this study is similar to previous studies in Europe (173-176) but higher than those from other African countries.(177) Selective media, known to increase the yield of GBS, was used and this could account for the differences between this study and those using less sensitive culture techniques. To our knowledge this is the first longitudinal study of infant colonisation and demonstrates that GBS colonisation rates remain high up to 89 days of life. The overwhelming risk factor for infant colonisation at all time points was GBS colonisation of their mother at the time of birth. Delivery before 34 weeks also

increased the odds of GBS colonisation at birth. Similar studies have indicated that risk factors for infant colonisation include maternal rectovaginal colonisation, preterm delivery, low birth weight and maternal urinary tract infection (4, 137, 173, 176).(4, 137, 173, 176). However, unlike these studies low birth weight was not identified as a risk factor, probably because only infants weighing more than 2 kg were recruited.

Several case reports suggest that isolation of GBS in breast milk is implicated in late onset GBS disease.(178, 179) Our data support this suggestion, since GBS colonisation in breast milk was also identified as a risk factor for neonatal colonisation at delivery and for persistence of infant colonisation throughout the first three months of life. This is an important finding in a population such as this with high rates of breastfeeding. It may be that GBS enters the mammary glands from a colonised infant following infant suckling. Conversely, it is also possible that gut microbiota may pass into the breast via the entero-mammary circulation.(180) Whether it is also a risk factor for LO-GBS disease in this population is not clear, since cases of LO-GBS disease were not observed and the study was not powered to measure clinical disease as an endpoint. Conversely, it might be a protective factor through induction of immunity.(40) The relevance of this finding requires further study.

It is interesting to note that risk factors for maternal colonisation include midwife delivery and increasing number of antenatal visits. This phenomenon has not been previously noted in LMIC where hygienic birth practices may be compromised. GBS has been implicated in nosocomial outbreaks globally (181, 182), although the number of reported outbreaks in resource-rich countries has greatly

reduced since the implementation of GBS screening programmes. It is conceivable that unclean birth practices, especially in times of clean water shortage, such as the hot, dry and wet seasons, may facilitate the fecal-oral spread of GBS between colonised and non-colonised mothers and their infants. It is also possible that increasing hospital exposure by midwives may increase the risk of nosocomial transmission.

A large proportion of women were colonised with GBS STV, similar to the previous smaller study by Suara et al, which identified 32 colonised pregnant women of whom 12 (30%) were colonised with GBS STV.(17) STV has been identified as an important neonatal and adult colonising and disease-causing GBS ST globally.(12, 28, 183) Additionally, in our cohort, STV was the predominant GBS ST cultured in breast milk and GBS STs Ib and V were associated with longer median colonisation duration compared to other GBS STs. A study of pregnant women from South Africa found high prevalence of GBS colonisation throughout pregnancy, with GBS STV and III colonisation lasting longer than other GBS STs (STV mean duration 8.6 weeks; 95%CI 6.8-10.4 vs. STIII 9.2 weeks; 95% CI 8.1–10.2).(12) The difference in GBS ST distribution and duration of colonisation between these studies may be due to population differences between South Africa and the Gambia. There are no other longitudinal infant colonisation studies to compare with the results of this study. Our results may also indicate differences in maternally-derived antibody that might prevent colonisation of certain GBS STs in these diverse populations. In the most recent study from South Africa, the authors indicate that it is the acquisition of a new GBS ST that is associated with EO-GBS disease rather than the persistent GBS ST, possibly due to absence (or low levels) of maternal antibody to this new GBS

ST.(146)

In summary, this study has identified a number of factors that may affect maternal colonisation and transmission from mother to infant, such as previous stillbirths and spontaneous abortions, increased hospital contact and season of birth. It will be important to bear these factors in mind when analysing antibody data. These data may also have implications for our understanding of the relationship between GBS and stillbirths and for prevention of LO-GBS disease.

Chapter 5 – Optimisation of opsonophagocytosis killing assay and antibody-mediated complement deposition assay

As outlined in Chapter 1, higher GBS ST-specific antibody concentration has been associated with decreased risk of neonatal GBS infection(116). However, there is evidence from pneumococcal and meningococcal studies that functional antibody activity may correlate more closely with protection from disease,(184, 185) as ELISAs give information on antibody concentration without information on functional activity. As antibody function is required for bacterial killing,(148) it was deemed important to investigate functional activity of anti-GBS antibody. As outlined in Chapter 1, the current assessment of functional antibody activity against GBS is undertaken using opsonophagocytic killing assays (OPkA).

However, there are many issues surrounding this assay in large-scale neonatal studies due to its reliance on human cells, the labour intensity of the assay and the volume of serum required (in excess of 100uL in some cases). One of the hypotheses of this study is that higher concentrations of functionally active anti-GBS antibody at birth and at day 60-89 are inversely correlated with infant GBS colonisation.

Measurement of antibody-mediated deposition of C3b/iC3b onto GBS bacteria may be a more suitable field assay compared to the current "Gold Standard", the OPkA. Thus, correlation with the OPkA against each GBS ST should first be established. In this Chapter, the optimisation of the OPkA and CDA and their correlation is described.

5.1. Optimisation of functional assays

As part of the training and preparation for this project, I spent two months in the GSK vaccine research laboratories (at the time of the study, Novartis Vaccine Laboratories, Siena, Italy), learning their OPkA methods. This was followed by a further six months in the immunoassay research laboratories at Public Health England, Porton Down where I refined both the OPkA and the CDA used to assess functional antibody concentrations.

5.2. Optimisation of the opsonophagocytosis assay (OPkA)

Prior to undertaking the OPkA a number of optimisation steps were required. These included: establishing a representative panel of sera to test the assays; defining the appropriate concentration of baby rabbit complement for use with each GBS ST; determining the concentration of human IgG-depleted complement required in each assay and finally correlating the killing titres obtained with IgG-depleted human and rabbit complement. The OPkA as described in Chapter 3 was used to determine a killing titre in experiments using either baby rabbit or human complement.

5.2.1. Panel of sera

For both optimisation experiments a panel of 30 sera was selected with: high (defined as 75-99 centile for geometric mean (GM) FI-C' values, n=10); medium (defined as 25-75th centile for GM FI-C' values, n=10) and low (10-25th centile for GM FI-C' values n=10) values for each of the GBS STs Ia, III and V. (Table 11)

Table 11 – GM FI-C' values used in panel sera for OPkA optimisation experiments

GBS ST	High (GM FI-C'; 95% CI) n=10	Medium (GM FI-C'; 95% CI) n=10	Low (GM FI-C'; 95% CI) n=10
Ia	7956 (4218-8607)	4445 (5324-7622)	2191(1000-3090)
III	9348 (7083-9976)	3784 (2741-4561)	2301 (1000-3189)
V	9067 (8219-15391)	4841 (3869-6466)	2520 (1000-3145)

5.2.2. Assay parameters

Each OPkA contained a standard human reference serum (SHRS) positive control and three negative controls (without serum, without cells and without complement). As antibody-independent killing was observed in the negative control with serum and complement but without cells, an antibody titre of double the upper limit of the titre observed in this control well was considered positive (>1:30). Titres below the limit of detection were allocated an arbitrary value of 15.

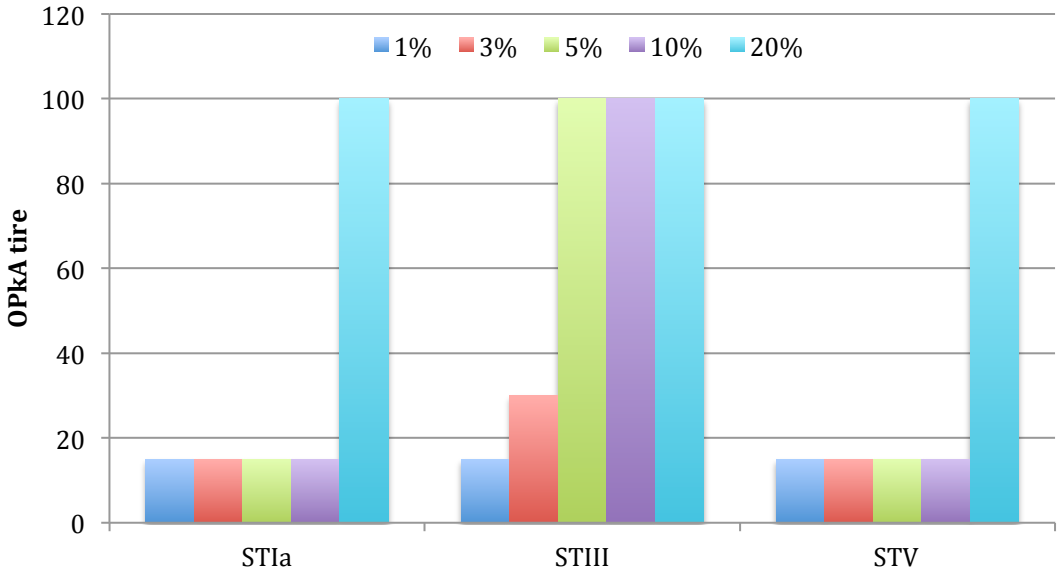
5.2.3. Rabbit complement concentration

The assay was performed as described in the methods section but with concentrations of rabbit complement at 1%, 3%, 5%, 10% and 20%. The assay was performed for GBS STs Ia, III and V using SHRS sera raised against each of the GBS STs used. Optimal killing of GBS STs Ia and V in the negative controls was observed with 10% complement concentration, whilst optimal killing in the GBS STIII negative controls was observed at 3%. (Figure 14a)

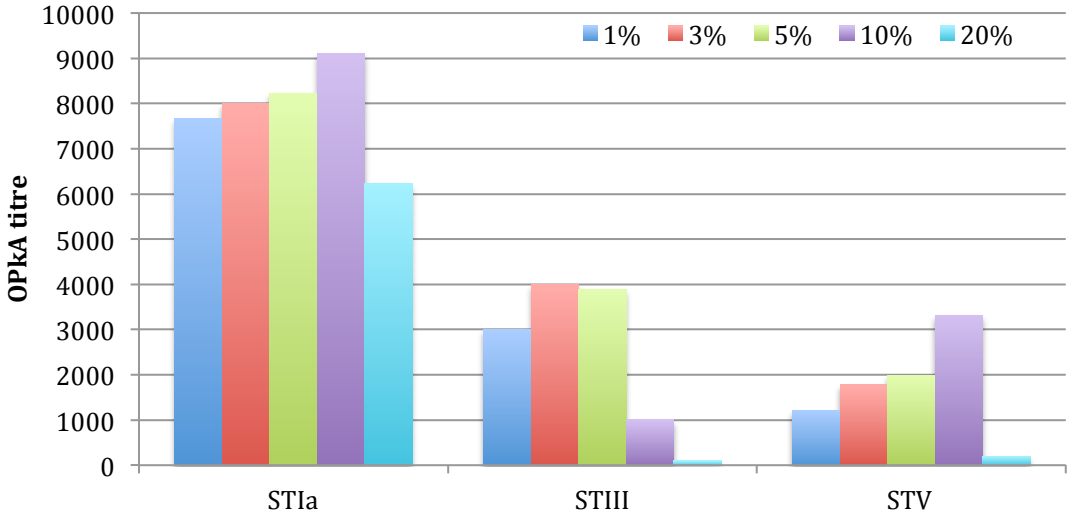
GBS STIa and III had higher killing titres than V at all concentrations of complement. There was also an increased OPkA titre with increasing complement concentration until a point was reached when killing appeared to drop off dramatically in the GBS STIII OPkA. (Figure 14b)

Figure 14 - OPkA titres in positive sera and negative controls

A. OPkA titres in the complement-only control wells (no serum) with each concentration of rabbit complement for GBS STs Ia, III and V.



B. OPkA titre comparing rabbit complement concentrations with SHRS sera for GBS STs Ia, III and V



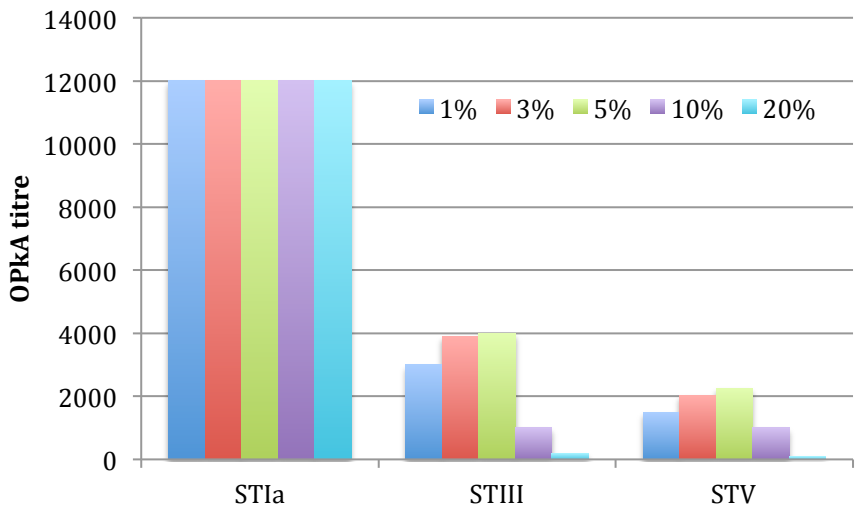
Bar graph shows effect of different complement concentrations on OPkA antibody titres for (A) Negative control of serum and complement but no cells and (B) SHRS serum against the homologous serotype. STIa=serotype Ia, STIII=serotype III and STV = serotype V. N=5 replicate wells for each SHRS serotype.

5.2.4. Human complement concentration

The assay was repeated using the same approach as described in the methods section but with IgG-depleted human plasma as the complement source instead of rabbit complement and concentrations of at 1%, 3%, 5%, 10% and 20%.

The assay was performed for GBS STs Ia, III and V using SHRS sera raised against each of the GBS STs used. Figure 15 demonstrates an increased OPkA titre with increasing complement concentration. With GBS STIII the killing titre dropped dramatically between 10% and 20% of complement. When determining the different concentrations of complement within the assay, GBS STs III and V optimal killing was observed with 5% complement concentration. Titres above the upper limit of the assay were observed for GBS STIa at all concentrations of human complement, suggesting an enhanced antibody/complement independent killing mechanism may be responsible for killing of this GBS ST.

Figure 15 - OPkA comparing human complement concentrations with SHRS for GBS ST Ia, III and V



Bar graph shows effect of different complement concentrations on OPkA antibody titres for SHRS sera against the homologous serotype. STIa=serotype Ia, STIII=serotype III and STV = serotype V. The upper limit for the killing titre against STIa was 1:12,000 and greater than 50% killing was observed at all complement concentrations. N=5 replicates for each of the three SHRS serotypes at each complement concentration.

5.2.5. Correlation between rabbit and human complement OPkA

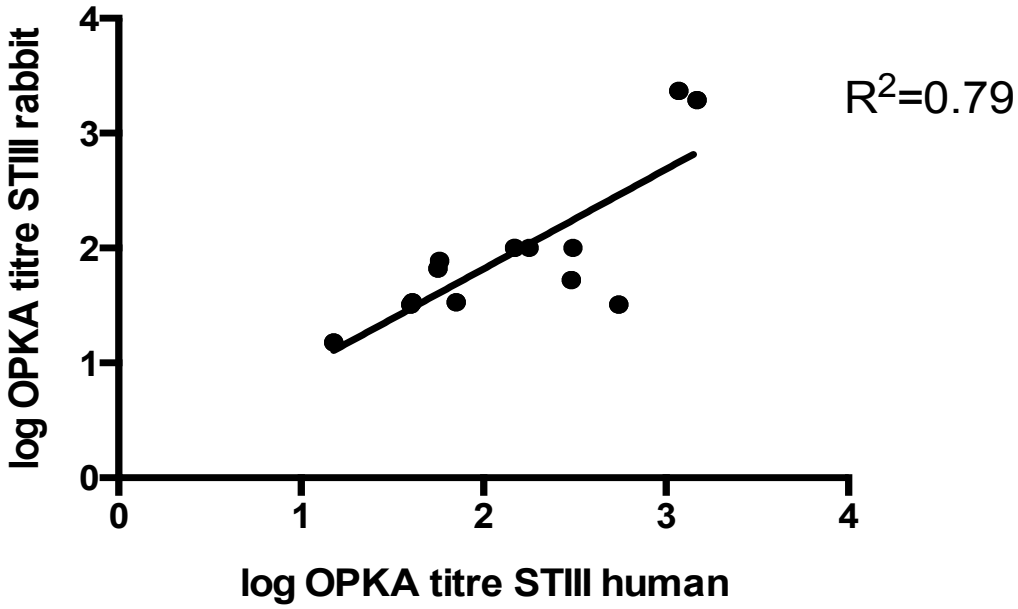
As the OPkA methods developed by GSK used baby rabbit complement and the CDA methods used human complement it was necessary to investigate whether

killing as measured by a human complement OPkA correlated with the standard rabbit complement OPkA.

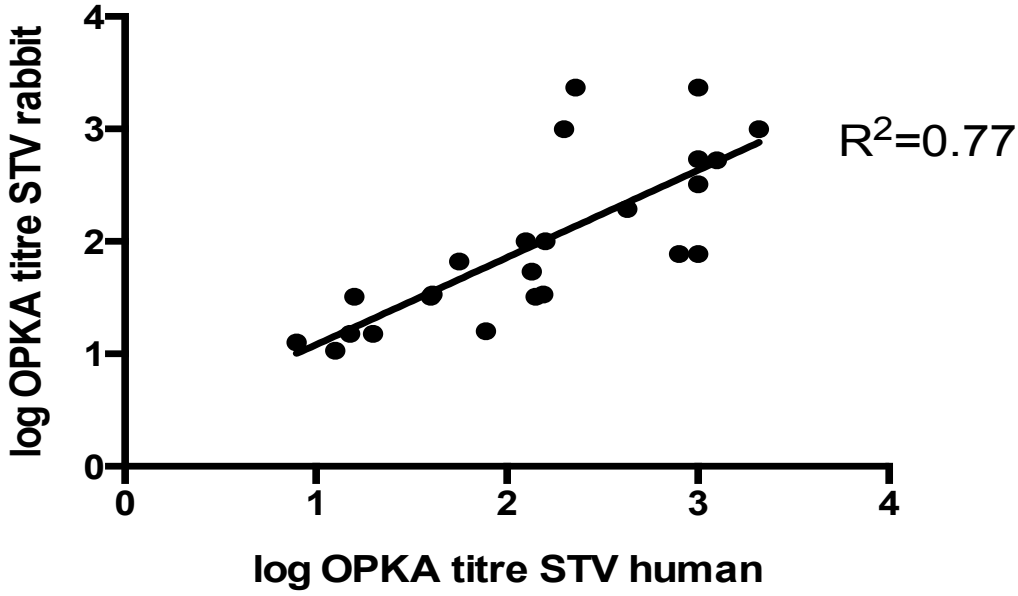
Overall, there was good correlation between baby rabbit and human complement for GBS STIII (R^2=0.79) and GBS STV (R^2=0.77). However, there was only a weak correlation between the rabbit and human complement OPkA for GBS STIa (R^2=0.02). Figure 16 illustrates the correlations observed.

Figure 16 - Example of the correlation between OPkA using human and rabbit complement with A. GBS STIII and B. GBS STV

A. Rabbit complement OPkA vs. human complement OPkA against GBS STIII (n=20)



B. Rabbit complement OPkA vs. human complement OPkA against GBS STV (n=20)



Scatter plots with regression line using log transformed OPkA antibody titres against STIII and STV GBS of human vs. baby rabbit complement, OPkA – opsonophagocytosis killing assay, STIII – serotype III; STV- serotype V. rabbit = baby rabbit complement; human = human exogenous complement. N=20 for each serotype.

5.3.Antibody-mediated complement deposition assay (CDA) optimisation

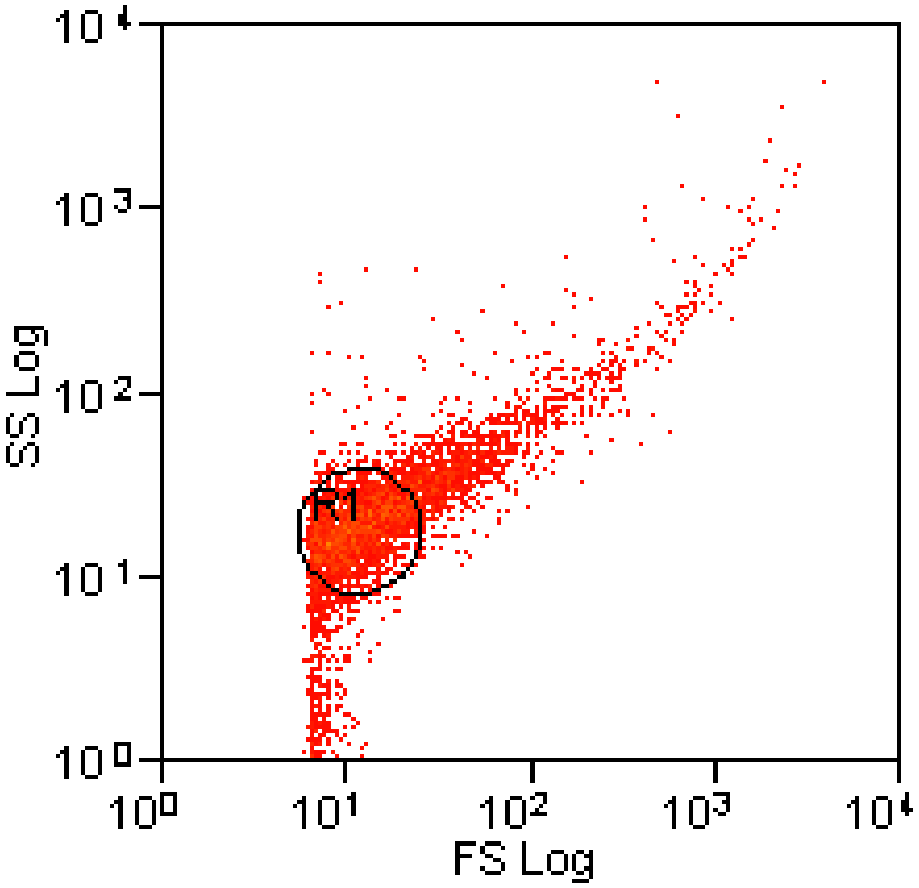
Having optimised the OPkA the next step was to optimise the CDA to measure antibody-mediated C3b/iC3b complement deposition onto the surface of whole bacteria (Chapter 2). The same panel of sera outlined in 5.2.1. above was used to optimise the CDA. There were several steps in the optimisation process: agreeing the gating strategy, assessment of each of the assay component effects on FI, assessment of representativeness of bacterial strains used and assay reproducibility (linearity, interassay variability, positive and negative predictive values, sensitivity and specificity).

5.3.1. Gating strategy for antibody-mediated complement deposition

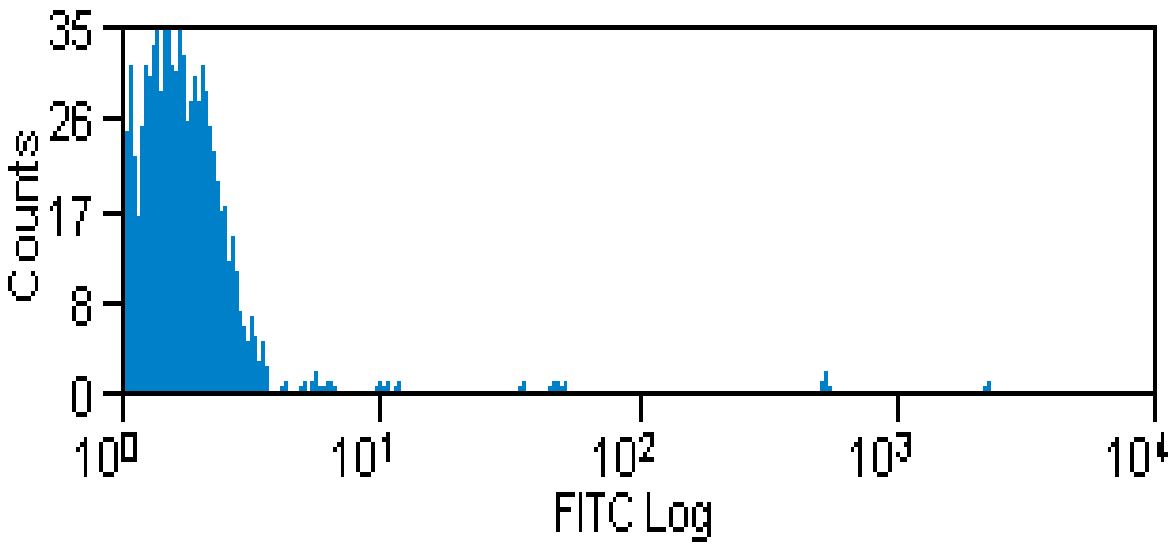
In order to correctly position the gate to determine antibody-mediated C3b/iC3b deposition it was necessary to identify the level of background florescence. Figure 17 demonstrates how this was performed using bacteria, conjugate and complement. A gate was set around the bacterial population following optimisation of the voltage in the bacteria-only control (600 Hz).

Figure 17 - Example of gating strategy for bacteria, conjugate and complement control

A.



B.

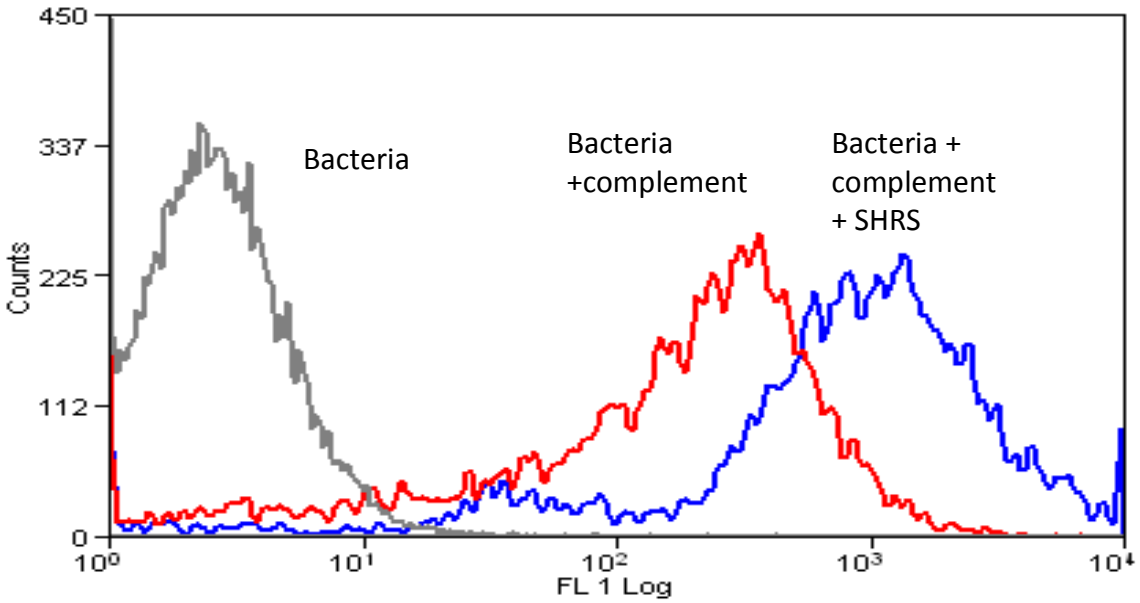


Representative dot plot (A) and histogram (B) from a bacteria, conjugate and complement only control are shown. (A) gating (R1) used to identify bacteria based on size (FS) and complexity (SS); R1 is the gate through which the opsonized bacteria are counted; (B) histogram of bacteria, conjugate and complement. FITC=Fluorescein isothiocyanate. FITC indicates the florescence of the anti-rabbit C3c FITC conjugate bound to the surface of GBS bacteria.

5.3.2. Gating strategy for controls

As mentioned in the methods section, a number of controls were incorporated into the assay in order to ensure accurate gating. Figure 18 demonstrates examples of the fluorescence of each of these controls. Gates are initially set on the bacteria-only population as an internal negative control to identify fluorescence due to conjugate binding. These gates were then applied to the serum samples from the Gambian cohort.

Figure 18 - Overlay plot demonstrating positive and negative controls using CDA



Representative histogram from a bacteria, conjugate and complement only control and standard human reference sera (SHRS) control are shown. Grey = bacteria only, red = bacteria+complement+ conjugate control, dark blue = SHRS vaccinated pool positive control. FL 1 Log = log_{10} fluorescence of each sample.

5.3.3. The fluorescence Intensity

Following gating (as outlined above) a fluorescence intensity value (FI) was assigned to each sample, which was calculated by multiplying the number of events

in the gate (R1) by the mean fluorescence.

For the measurement of antibody-mediated C3b/iC3b deposition an arbitrary lower limit of quantification of 2000 FI-C' was used as at this level error bars were above 5% variance. Any FI-C' below 2000 was given an arbitrary measurement of 1000 FI-C' for the analysis. Where no antibody-mediated C3b/iC3b deposition was detected, these data were given a value of ND and excluded from analysis. The GM FI-C' and 95% CI onto each GBS ST were calculated and the percentage of cord blood and infant sera with FI-C' values above 2000 was recorded.

5.3.4. Gating strategy for interassay control.

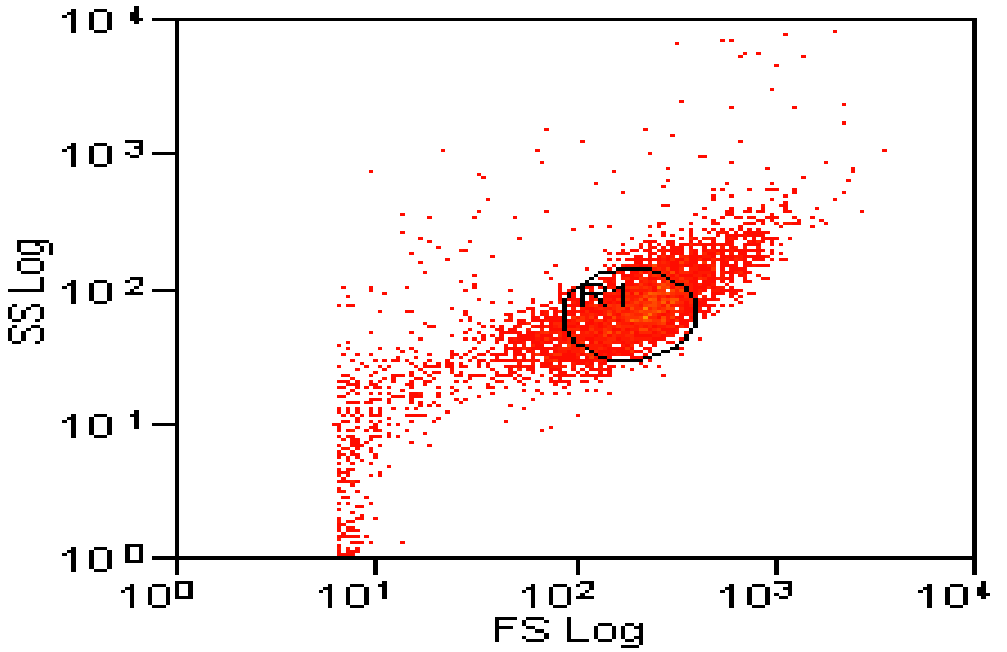
In addition to the positive and negative internal controls used in each assay, each assay contained four wells of pre-activated zymosan (1.8×10^6 particles/well; CompTech, USA): two wells with zymosan and conjugate and two wells with zymosan, conjugate and complement only. Zymosan is used to induce antibody-independent complement deposition in the absence of sera. In this assay zymosan is used to assess inter-assay variability over the nine months duration of the study. Data from each day were normalised to the C3b/iC3b bound to the preactivated zymosan control to account for day-to-day variation within the assay. Figure 19 demonstrates a representative plot for a zymosan and complement control. Over the course of the study, the mean standard deviation of FI for zymozan controls was 8,831 (coefficient of variance (CoV), 31.6%).

Table 12 – Mean and standard deviation FI and coefficient of variance of zymosan by month of study and overall

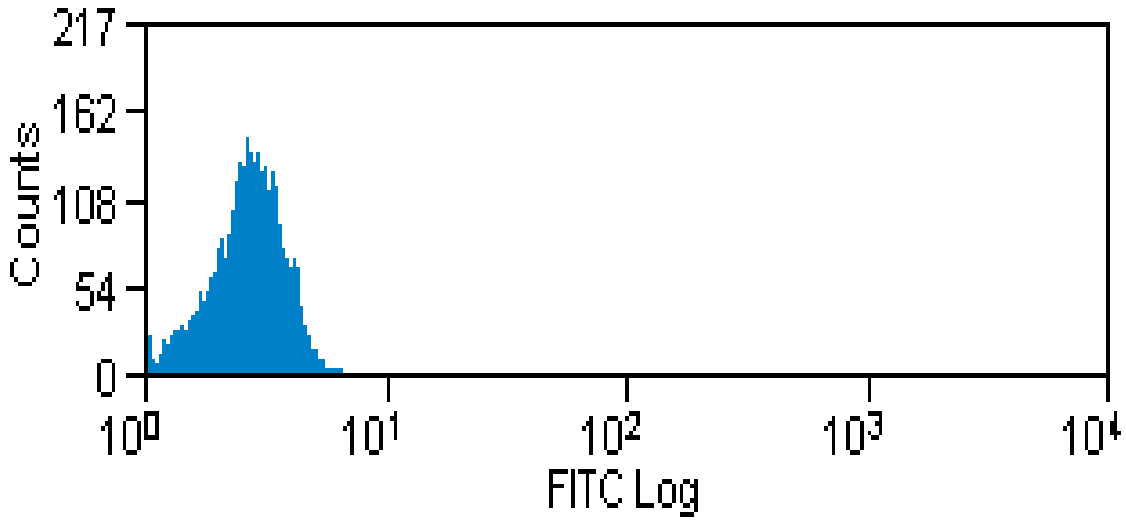
Zymosan	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Overall
SD	8934	8410	8482	8980	9948	8890	8991	8549	8293	8831
Mean	28999	27778	29776	28888	24447	27602	29993	28872	25559	27990
Cov	30.8%	30.3%	28.5%	31.1%	40.7%	32.2%	30.0%	29.6%	32.4%	31.6%

Figure 19 - Scatter plot and histogram demonstrating zymosan gating based on size and complexity

A.



B.



Representative dot plot and histogram from a zymosan, conjugate and complement only control are shown. (A) gating strategy used to identify zymosan based on size (FS) and complexity (SS). R1 is the gate through which the opsonized zymosan is counted; (B) histogram of zymosan, conjugate and complement only control. FITC=Fluorescein isothiocyanate. FITC indicates the florescence of the anti-rabbit C3c FITC conjugate bound to the surface of zymosan.

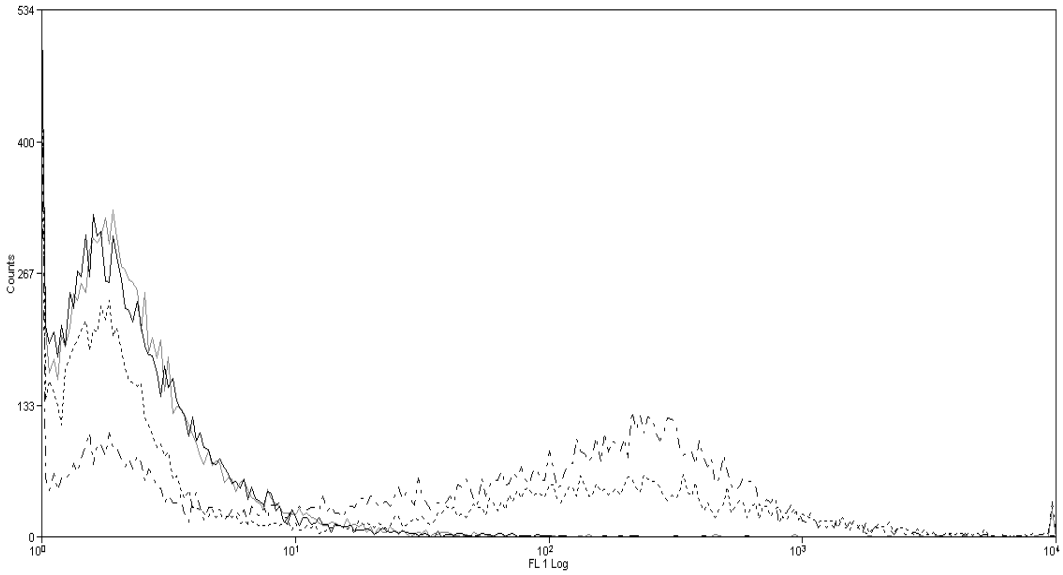
5.3.5. Effect of different assay components on fluorescence background

It was important to assess the variability of the different components of the assay during the course of the CDA analysis. These components included different

human complement and conjugate batches and different batches of 1% BSA in PBS buffer. Figure 20 demonstrates the differences observed over the nine months of analysis.

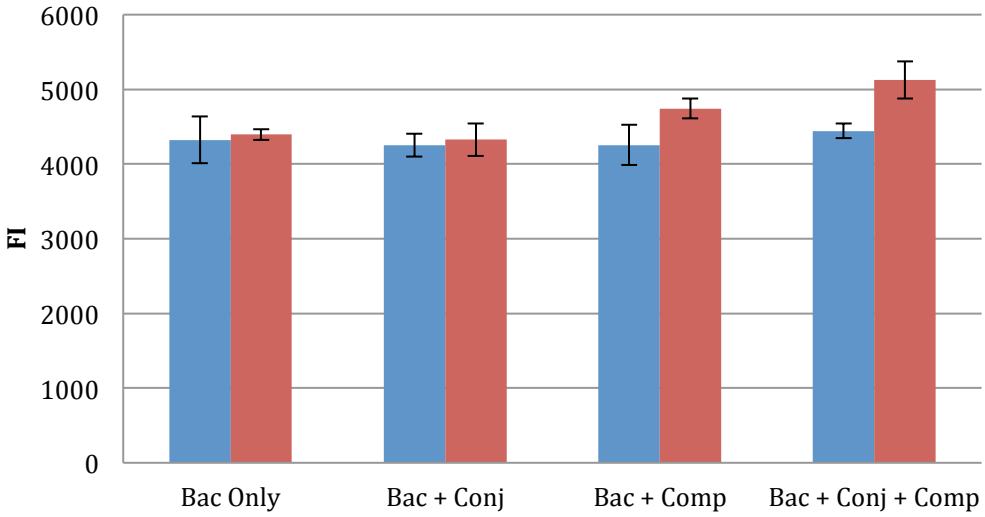
Figure 20 - Differences in assay components during the study

A. Bacteria with 1% BSA in PBS



The overlay plot shows the fluorescence log (FL 1 log) of the different bacteria controls in 1% BSA in PBS used during the experiments. Solid black line = March, solid grey line = May; dotted line = July; dashed line = September.

B. Conjugate



Bar chart of different conjugate batches used during the experiments. Blue bar = conjugate batch 4, used until May 2015; Red bar = conjugate batch 5, used from May to October 2015. Black lines represent error bar from duplicate samples. Bac Only = bacteria only; Bac + Conj = bacteria plus conjugate; Bac + Comp = bacteria plus complement; Bac + Conj + Comp = bacteria plus conjugate plus complement. FI – Fluorescence intensity.

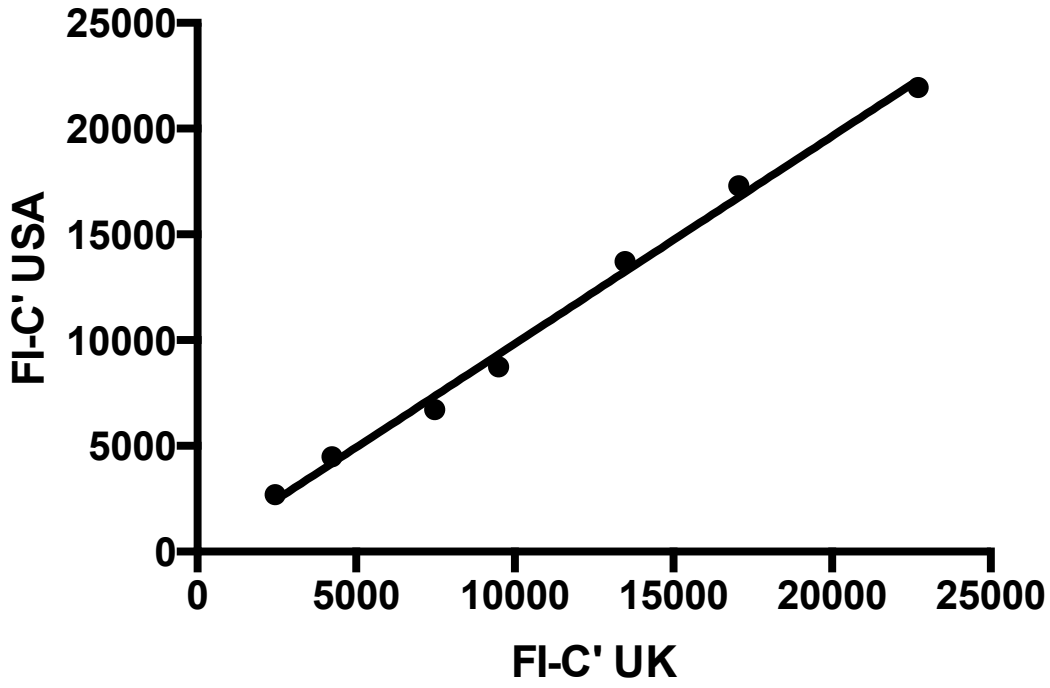
The variability between buffer batches, complement batches and conjugate batches was normalised to the C3b/iC3b bound to the pre-activated zymosan control to account for batch-to-batch variation within the assay, as outlined in 5.3.4. During the course of the nine months of the study the mean standard deviation (SD) of the FI was 5509, CoV (CoV=SD/mean) 34.3% for the conjugate control and 4434 (39%) for the bacteria control.

5.3.6. Comparison of two strains of GBS STIII in positive and negative controls

The GBS isolates used in this study were geographically diverse United Kingdom clinical isolates from neonatal blood cultures. GBS strains H092040676 (STIa), H090820125 (STIb), H090320548 (STII), H092120162 (STIII), and H091780506 (STV) were kindly provided by Prof .Androulla Efstratiou, Public Health England, Colindale, UK.

In order to determine if these bacteria were representative of bacteria used in other assays the CDA experiment was repeated using SHRS and GBS STIII strain COH-1 kindly provided by Prof. Carol Baker, Baylor College of Medicine, Texas, USA. There was little difference in the FI for any of the negative controls or in the FI-C' with the SHRS between H092120162 and COH-1 STIII. (Figure 21)

Figure 21 - Comparison of FI-C' between two strains of GBS STIII



Scatter plot with regression line comparing FI-C' of USA GBS invasive strain with UK GBS invasive strain. N= 10 samples. FI-C' fluorescence intensity minus complement control. USA= GBS STIII strain COH-1; UK= GBS STIII strain H092120162.

5.3.7. Assay reproducibility

In order to determine reproducibility of the CDA, intra- and inter-assay precision calculations were performed using a panel of one rabbit and 13 human sera against a GBS STII strain. The CoV for the intra-assay precision (three assays performed on one day) was 22% and CoV was 26% for the inter-assay precision (one assay performed on three separate days). Linearity of the assay was assessed using SHRS. The assay was highly linear, with a mean R^2 of 0.96 for the five GBS STs tested.

5.4. Assay optimisation with pilot sera

During a pilot phase to test the experimental set up, 100µL test serum was provided from two difference sources:

1. A cohort of 100 mother-infant pairs from the Gambia – these sera were from the initial pilot study and were used to optimise the CDA by comparing it to the IBA.

2. A cohort of 60 mother-infant pairs with samples taken from pregnant woman at 30 weeks gestation, cord blood and from infants at 12, 24 and 52 weeks of age from upper River Region, The Gambia kindly made available from the Infant Nutrition Group (ING) at MRC – used to determine the concentration of antibody in mothers and their infants in the Gambia by CDA and to assess changes in GBS ST-specific antibody distribution since the previous study undertaken in 1996 using CDA.

5.4.1. Comparison of IBA and CDA in the pilot Gambian study cohort

Before I could measure antibody-mediated C3b/iC3b deposition onto the surface of whole bacteria it was necessary first to measure antibody binding to the surface of whole bacteria without the addition of complement. This is an important step in assay development as antibody bound to the surface of the bacteria may be bound directly to surface proteins and not mediate C3b/iC3b deposition. In order to determine the relationship between antibody bound to the surface of whole bacteria and antibody-mediated C3b/iC3b deposition the FI-C of the IBA to the FI-C' of the CDA was compared in the first 100 mother-infant pairs. With the exception of GBS STIb, the GM FI-C of IBA was lower than GM FI-C' of CDA, but this was not significant. This is likely due to the different conjugates used between the CDA and IBA. However, the correlation between IBA and CDA was $>R^2=0.7$ ($p<0.001$) for all GBS STs. The results of the GM FI-C' (95% CI) of the IBA compared to the CDA are shown in Table 13.

Table 13 - GM (95% CI) of IBA FI-C vs. CDA FI-C' against GBS ST Ia, Ib, II, III and V

GBS ST	GM FI-C IBA n=100	GM FI-C' CDA n=100	R^2	P
Ia	1851 (1538-2227)	2437 (2181-2723)	0.70	0.01
Ib	6197 (4111-9343)	3732 (2839-4904)	0.94	<0.001
II	4363 (2852-6676)	8133 (5923-11168)	0.90	<0.001
III	4698 (2213-7183)	5261 (4140-6685)	0.88	<0.001
V	5165 (3519-7580)	5799 (4544-7402)	0.78	0.02

Table demonstrates FI-C and FI-C' values from the first 100 cord samples as outlined in 5.4. above. GM=geometric mean, FI-C=fluorescence intensity minus conjugate; FI-C'=fluorescence intensity minus complement; IBA=IgG surface-binding assay; CDA=antibody-mediated complement deposition assay.

Based on assay optimisation described in 5.3. the CDA was used to assess functionally active antibody against all GBS STs using human complement at a concentration of 20%.

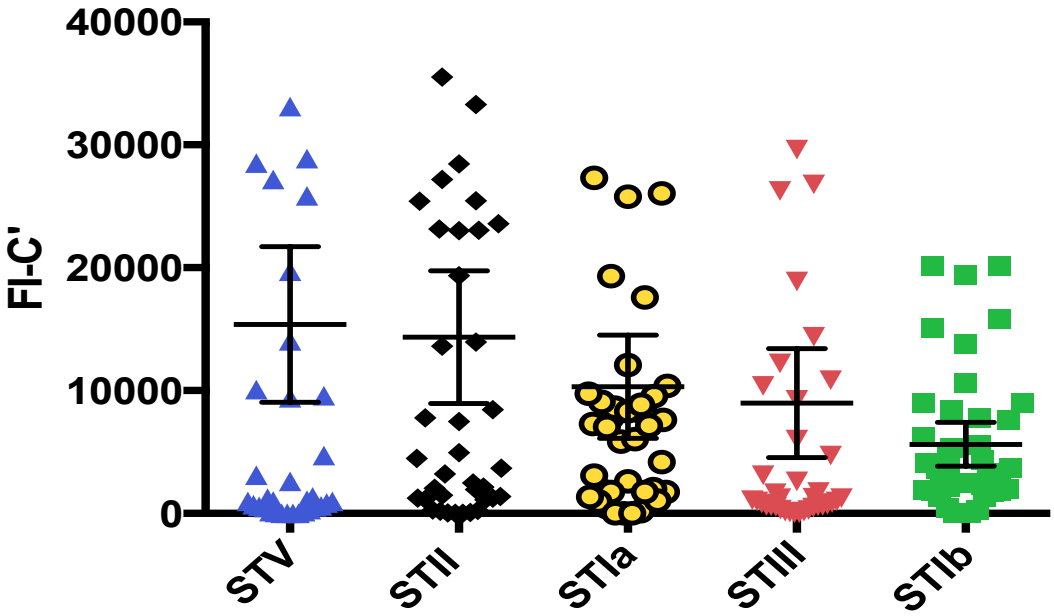
5.4.2. Changes in ST-specific antibody distribution in the Gambia

Longitudinal data from the mother infant cohort described in 5.4. (n=60) above demonstrated a high concentration of antibody-mediated complement deposition against GBS STV, the predominant colonising and invasive disease causing GBS ST in the Gambia. Further, the analysis demonstrated measurable GM FI-C' against at least one of the GBS STs tested. The analysis of infant sera also demonstrated a decline in functional antibody activity against GBS STV until 12 weeks, followed by a transcient increase in antibody-mediated C3b/iC3b deposition at 24 weeks and subsequent decline. One possibility is that this indicates waning maternal antibody concentrations followed by increasing infant antibody

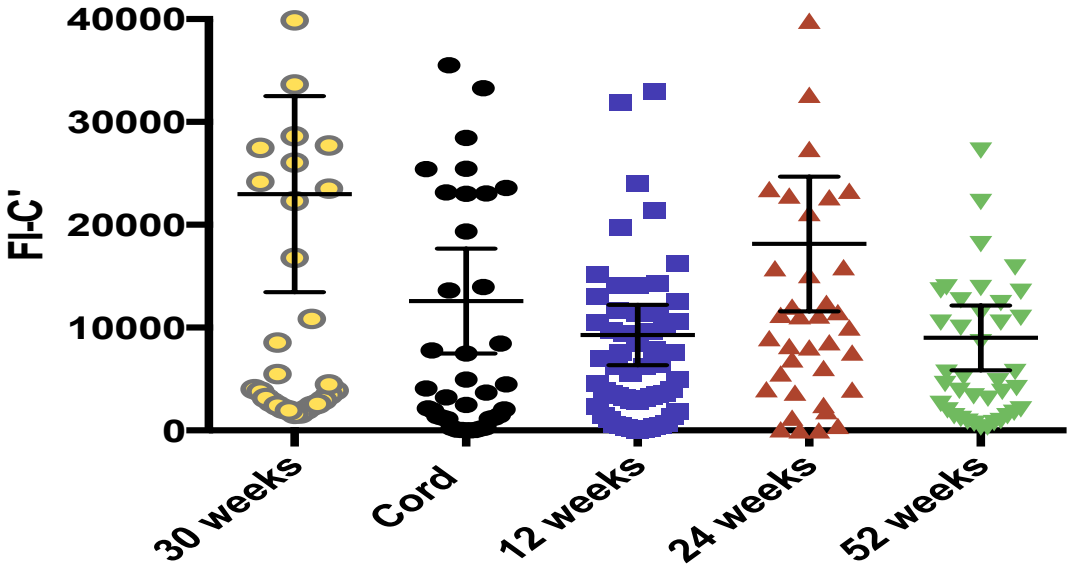
concentrations in response to infant GBS colonisation and subsequent early challenge. (Figure 22)

Figure 22 - Antibody-mediated C3b/iC3b deposition onto GBS (95% CI) in Gambian cord and infant serum

A – Antibody-mediated C3b/iC3b deposition onto GBS STs Ia, Ib, II, III and V in cord blood



B. Antibody-mediated C3b/iC3b deposition onto GBS STV in maternal and infant serum from 30 weeks gestation to 1 year of life.



Dot and whisker plot of antibody-mediated C3b/iC3b deposition against: (A) STIa, STIb, STII, STIII and STV in cord blood and (B) STV from 30 weeks gestation to 1 year of life. Horizontal lines represent mean and 95% confidence intervals. N=100 samples. Yellow circles = maternal serum at 30 weeks gestation; black circles = cord blood; blue squares = infant serum at 12 weeks; red triangles = infant serum at 24 weeks; green triangles = infant serum at 52 weeks. FI-C' = fluorescence intensity minus the bacteria+conjugate+complement control. STIa= serotype Ia, STIb = serotype Ib, STII = serotype II, STIII = serotype III and STV=serotype V, cord=cord blood.

5.5. Correlation between CDA and OPkA and assay robustness

In order to determine the correlation between the CDA and OPkA the Pearson correlation coefficient was calculated for each pairwise comparison of results from matched panel samples as outlined above (5.2.1. (Table 11)). In addition the operating characteristics of the CDA were assessed as a 'test' for correctly classifying the paired OPkA titre as <30 (<50% killing) or ≥30 (50% or greater killing observed). For this analysis, OPkA was treated as the 'gold standard', and CDA assessed as the predictor variable at threshold titers of ≥30. For each pairwise set of comparisons, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and positive and negative likelihood ratios (LR+ and LR−) were calculated. Likelihood ratios provide an indexed measure of the accuracy of a positive or negative test result by combining the sensitivity and specificity of the test into a single statistic. The higher the LR+ (or lower the LR-), the more likely it is that the test result correctly agrees with the gold standard.

The sensitivity, specificity and predictive values were calculated using the following calculations:

	OPKA +	OPKA -	
CDA +	True positive (A)	False positive (B)	**(A+B)**
CDA -	False negative (C)	True negative (D)	**(C+D)**
	(A+C)	**(B+D)**	

Sensitivity = A/(A+C)
Specificity = B/(B+D)
PPV = A/(A+B)
NPV = C/(C+D)
LR+ = Sensitivity/(1-specificity)
LR- = (1-sensitivity)/Specificity

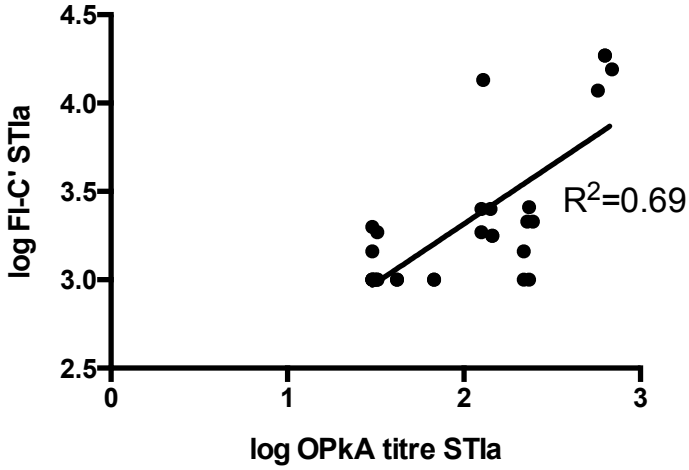
Because high titres of antibody-independent killing were observed against GBS STIa in the OPkA using human complement and yet results from the IBA and CDA correlated well with rabbit complement OPkA, the results from the optimised OPkA using baby rabbit complement were used in the correlation analysis with the CDA.

5.5.1. Results of correlation between CDA and OPkA

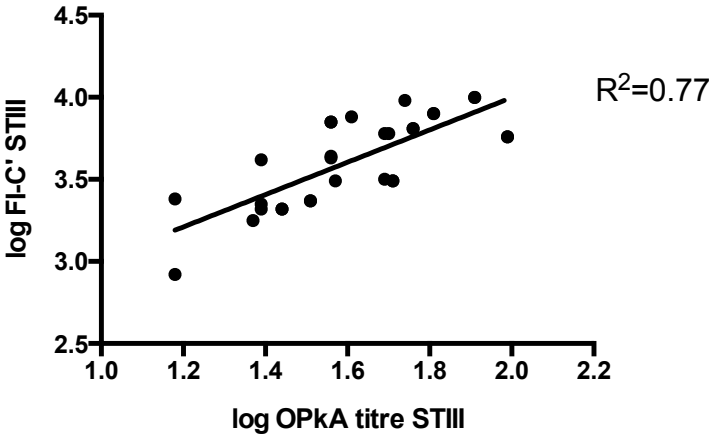
A total of 90 individual sera were included in this analysis (30 for each of the three GBS STs Ia, III and V as described in section 5.2.1). The correlation of the CDA compared to the OPkA significant for GBS STs Ia, III and V (Figure 23).

Figure 23 - Correlation between CDA and OPkA

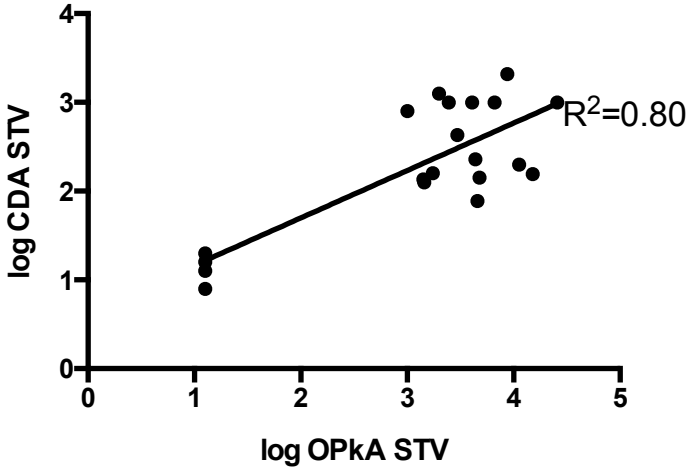
A. CDA vs. rabbit OPkA GBS STIa (n=30)



B. CDA vs. rabbit OPkA GBS STIII (n=30)



C. CDA vs. rabbit OPkA GBS STV (n=30)



Scatter plots with regression line of log transformed CDA and OPkA values obtained with a panel of n=30 sera for (A) STIa, (B) STIII, (C) STV. FI-C=fluorescence intensity minus complement only control. R^2 = Pearson correlation coefficient.

5.5.2. CDA sensitivity, specificity, positive and negative predictive values

When sensitivity, specificity, PPV and NPV of the CDA was calculated (*n* = 90), the sensitivity of the CDA was 80.7% and specificity was 90.0% with a weighted positive likelihood ratio of 8.07 (2.7-23.8) indicating that overall the CDA is a useful test for predicting opsonophagocytic killing. Table 14 indicates the positive and negative predictive values, likelihood ratios and sensitivity and specificity overall for the CDA.

Table 14 - Positive and negative predictive values, sensitivity and specificity of the CDA overall and for GBS STs Ia, III and V

	Sensitivity % 95% CI	Specificity 95% CI	PPV 95% CI	NPV 95% CI	LR+	LR-
Overall	80.7 (67.7-89.5)	90.0 (72.3-97.4)	93.9 (82.1-98.4)	71.1 (53.9-84.0)	8.07 (2.7-23.8)	0.2 (0.1-0.4)
STIa	60.0 (36.0-80.0)	89.0 (50.7-99.0)	92.3 (62.1-99.6)	50.0 (25.5-74.5)	5.4 (0.8-35.5)	0.5 (0.3-0.8)
STIII	95.2 (74.1-99.8)	87.5 (46.7-99.3)	95.2 (74.1-99.8)	87.5 (46.7-99.3)	7.6 (1.2-47.8)	0.1 (0.0-0.4)
STV	87.5 (60.4-97.8)	92.3 (62.1-99.6)	99.3 (66.0-99.7)	85.7 (56.2-97.4)	11.4 (1.7-75.5)	0.1 (0.0-0.5)

The sensitivity, specificity, positive and negative predictive values remained high for GBS STs V and III. For GBS STIa the sensitivity was reduced compared to other STs although specificity remained high. This would indicate that for STIa, the assay would be able to correctly identify participants without antibody-mediated complement deposition (specificity 89.0%) but the test would have a high false negative rate. This could mean an underestimate of protection by categorising infants who have antibody as being antibody-negative. This is also demonstrated by

the lowest positive likelihood ratio and wide CI spanning 1 for STIa (LR+ 5.4 (0.8-35.5)).

5.6. Discussion

This optimisation data demonstrate a good correlation between the OPkA using baby rabbit complement and the CDA for GBS STs Ia, III and V. Although I was only able to establish a correlation between human and rabbit complement for GBS STs III and V, the use of human over rabbit complement is still preferred when assessing functional antibody concentrations as species-specific interactions can affect the results obtained. Baker et al have used endogenous complement in their OPkA assays.(83, 84, 186) However, adult sera may contain naturally acquired antibody against GBS that would activate complement and interfere with the assay results. The use of IgG-depleted human complement in the CDA reduces this risk and is a strength of the assay design.(165) Other investigators have used baby rabbit sera as a complement source(146, 154, 156) to overcome some of these difficulties as baby rabbit sera is both readily available and easily pooled which can aid standardisation of assays. In addition, baby rabbit serum lacks the intrinsic bactericidal activity seen in human serum. Rabbit serum is recommended by both the WHO and the CDC as a complement source when assessing human bactericidal responses to serogroup A and C meningococcus (187) and pneumococcus(184, 188). However, baby rabbit complement has been reported to induce higher bactericidal titres against serogroup B (189) and C (190)meningococcal strains compared to human complement. In our study, there was a good correlation between the IBA and CDA suggesting that the responses with human complement could be used in

functional assays.

Following optimisation, the CDA demonstrated good inter-and intra-assay variability and the use of zymosan to control for variations in the assay components showed an acceptable CoV, which suggests that the assay is robust in determining antibody-mediated C3b/iC3b deposition onto the surface of GBS. The CDA has many advantages over the OPkA, including avoiding the need for human cells and using fixed whole bacteria. These elements have the advantage of making the assay more robust over time and reducing the interassay variability often seen in OPkA. Additionally, the CDA would have advantages over an assay measuring IgG bound to the CPS as the use of whole fixed bacteria would also allow measurement of functional activity of IgG in a protein-based vaccine. The CDA uses smaller quantities of serum (5µL vs. 30µL in the OPkA), which would be useful when serum is at a premium such as in neonatal studies.

The pilot study demonstrated the ability of the CDA to identify functional antibody in sera up to 52 weeks of age. Infant antibody declined up to six months of age but thereafter increased. This corresponds to the time of increase in the neonatal adaptive immune response and may suggest early colonisation with GBS inducing antigen-specific antibody, as is also seen in pneumococcal colonisation in Gambian infants.(191) This work was performed using sera from women with natural immunity to GBS rather than vaccine-induced immunity. Therefore, the Gambian sera will have antibody to both the CPS and to proteins on the bacterial surface and different results may be obtained with sera obtained from indivuduals who are vaccinated with a CPS-protein conjugate vaccine.

The correlation between the CDA and the OPkA results from matched samples both overall and by GBS ST suggests that the CDA is robust in predicting OPkA and could be a useful marker of bacterial killing *in vitro*. Testing the result by GBS ST is important, as results from other pathogens, such as meningococcus, have demonstrated stronger correlations between functional assays with different sources of complement and some serogroups are correlate better than others.(192) The CDA was able to predict opsonophagocytic killing for the three GBS STs tested. The sensitivity (80.7%) and specificity (90%) of the CDA overall were good. However, some sensitivity was lost for GBS STIa compared to the other two GBS STs due to antibody-independent killing, which could lead to an under-estimation of killing in sera where functionally active antibody is present.

The PPV of the CDA was high overall and for all of the GBS STs, demonstrating that the test is robust in predicting opsonophagocytic killing. In terms of evaluating killing ability, the risk of underestimating protection from killing with a less sensitive assay is preferable to overestimating protective rates. These findings suggest that CDA could be used as an alternative to OPkA to predict antibody-mediated killing of GBS in a Gambian population. Taken together with the results of the pilot study, these results indicate that the CDA may be a useful assay to identify functionally active anti-GBS antibody against ST Ia, Ib, II, III and V.

Chapter 6 – Serotype-specific antibody-mediated complement deposition in cord blood and infant sera.

6.1. Introduction

As outlined in Chapter 5, it has been established that the CDA can be used to predict opsonophagocytic killing for GBS STs Ia, III and V. The next step is to establish the correlation between functional antibody concentration and infant GBS colonisation. In order to establish this, it was first necessary to characterise the antibody-mediated C3b/iC3b deposition in the cohort, their decline over time and any cross-reactivity between GBS STs.

As there are no antibody reference standards available for assays investigating GBS, it was necessary to establish cross-reactive and functional activity of each of the GBS ST-specific antibodies in order to establish differences in the functional behaviour of each GBS ST-specific antibody against GBS *in vitro*.

In this Chapter, antibody-mediated C3b/iC3b deposition onto the surface of whole GBS bacteria is quantified using cord blood and infant sera obtained at 60-89 days of age. Cross-reactivity between GBS ST-specific antibodies is also assessed to determine the dominant GBS ST-specific response in cord blood and serum. Additionally, longevity of GBS ST-specific antibodies up to 89 days of age in infants is determined.

6.2. Methods

The CDA was used to measure antibody-mediated C3b/iC3b deposition onto the surface of whole bacteria. This method is described in Chapter 3. For the analysis

I calculated reverse cumulative distribution curves according to Reed et al 1995.(193)

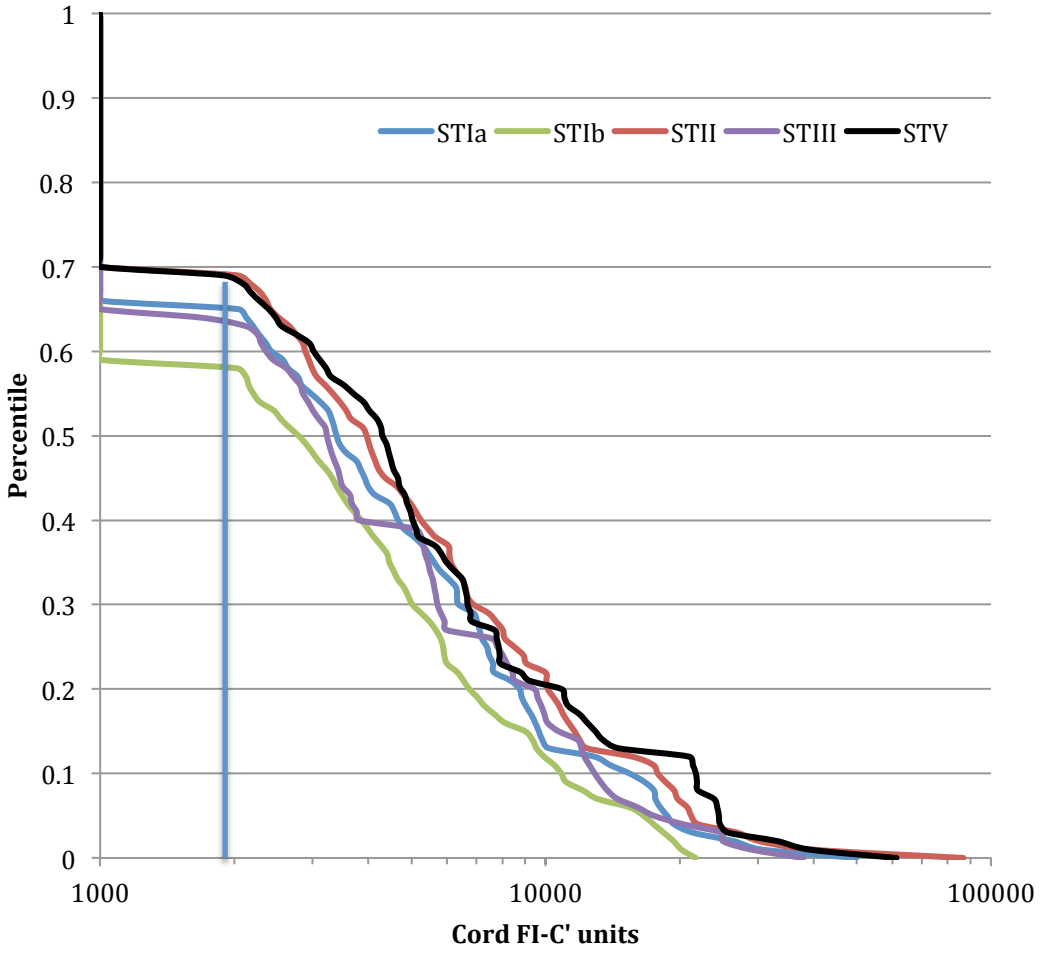
To assess cross-reactivity between antibodies and dominant GBS ST-specific response, the percentage of subjects with GM FI-C' values greater than or equal to the following lower limit of quantification points was assessed: 2000 FI-C', 5,000 FI-C', 10,000 FI-C' and 20,000 FI-C'. All results were adjusted for maternal and infant factors that might be associated with decreased cord antibody concentrations such as maternal malaria, infant birth weight, sex and season of birth (194, 195).

6.3. Distribution of GBS ST-specific antibody-mediated C3b/iC3b deposition in cord blood.

Using 2000 FI-C' as a lower limit of quantification, 405 cord sera (54.0%) had C3b/iC3b deposition against at least one GBS ST. Figure 24 demonstrates the reverse cumulative distribution of C3b/iC3b deposition onto GBS ST Ia, Ib, II, III and V in cord sera.

The predominant antibody-mediated C3b/iC3b deposition in cord sera was against the GBS STs with the highest prevalence of colonisation, outlined in Chapter 4; namely GBS STs V and II, followed by Ia and III with relatively low levels of antibody-mediated C3b/iC3b deposition onto Ib. This is in accordance with our finding that STIb is less prevalent in colonisation samples than the other four GBS STs.

Figure 24 - Reverse cumulative distribution curve (RCD) of antibody-mediated C3b/iC3b deposition against GBS ST Ia, Ib, II, III and V GBS in cord sera.



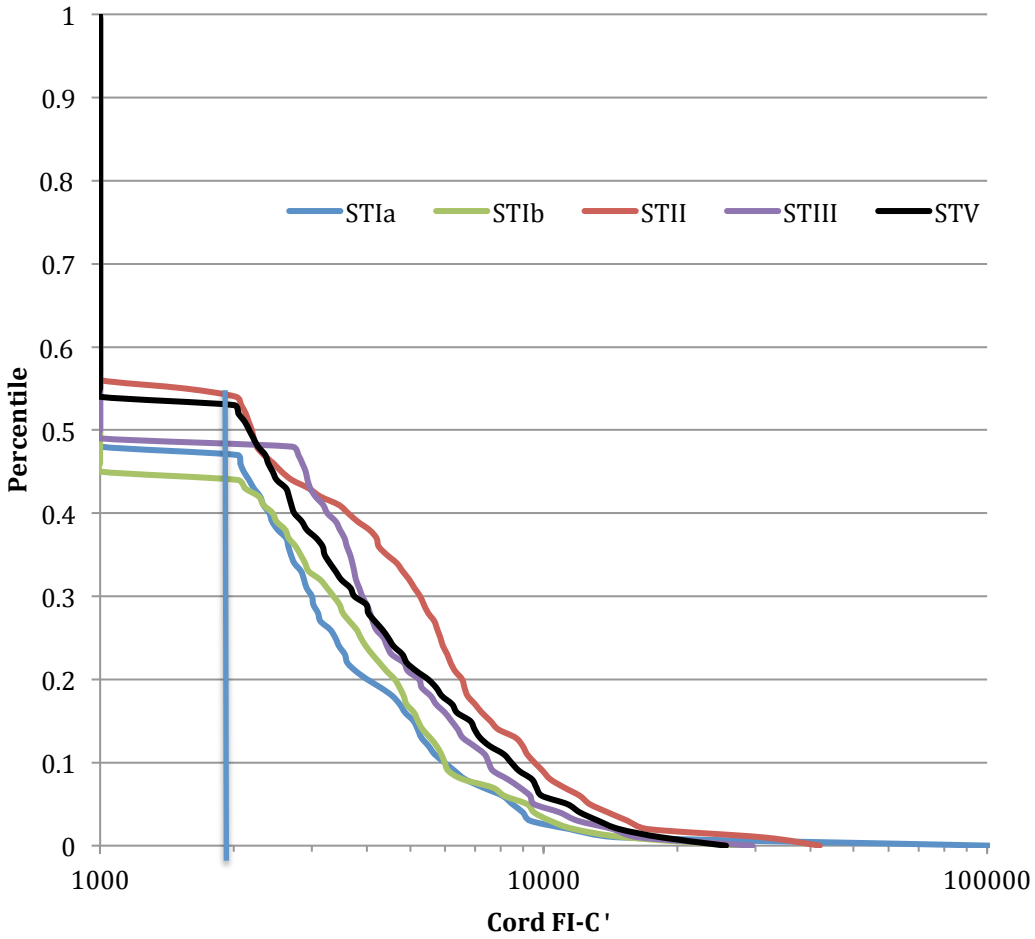
Reverse cumulative distribution curve of FI-C' detected in 750 cord sera. Y-axis represents percentile of cord sera with FI-C' concentration above the lower limit of quantification for each of the serotypes: STIa=serotype Ia (n=487), STIb=serotype Ib (n=442), STII = serotype II (n=525), STIII=serotype III (n=480), STV=serotype V (n=525). X-axis is the associated FI-C' value for that percentile of the population. FI-C' is the florescence of the sample minus the complement-only control. Vertical line represents 2000 FI-C' unit lower limit of quantification point.

6.4. Distribution of ST-specific antibody-mediated C3b/iC3b deposition in infant sera at day 60-89.

At day 60-89, 273 infants (43.0% of infant sera) had concentrations above 2000 FI-C' against at least one GBS ST. Figure 25 shows the reverse cumulative distribution of antibody-mediated C3b/iC3b deposition obtained with infant sera.

The predominant GBS STs with highest antibody-mediated C3b/iC3b deposition in infant sera were similar but not identical to that of cord sera. More infants had antibody-mediated C3b/iC3b deposition against GBS STII, followed by V and III with low antibody-mediated C3b/iC3b deposition onto GBS STs Ia and Ib. This corresponds to the predominant colonising GBS STs: V (57.0%), II (17.1%) and III (10.8%) (Chapter 4).

Figure 25 - Percentage of infant sera at day 60-89 with C3b/iC3b-mediated antibody deposition against GBS ST Ia, Ib, II, II and V



Reverse cumulative distribution curve of FI-C' detected in 684 infant sera. Y-axis represents percentile of cord sera with FI-C' concentration above the lower limit of quantification for each of the serotypes: STIa=serotype Ia (n=301), STIb=serotype Ib (n=282), STII = serotype II (n=351), STIII=serotype III (n=308), STV=serotype V (n=333). X-axis is the associated FI-C' value for that percentile of the population. FI-C' is the florescence of the sample minus the complement-only control. Vertical line represents 2000 FI-C' unit lower limit of quantification point.

6.5. Differences in concentration of antibody-mediated C3b/iC3b deposition onto GBS by ST in cord and infant sera

The percentage of cord and infant sera with antibody-mediated C3b/iC3b deposition above and below the 2000 FI-C' lower limit of quantification varied by GBS ST. A larger proportion of cord sera had antibody-mediated C3b/iC3b deposition against GBS ST V (77%), II (79%), III (75%) and Ia (75%) than Ib (63%) ($p<0.001$).

By day 60-89, the number of infant sera with FI-C' above 2000 was reduced compared to cord sera for all GBS STs: Ia (-39.6%), Ib (-33.0%), II (-34.2%), III (-39.1%) and V (-34.6%). Table 15 summarises those sera above 2000 FI-C', those where fluorescence was detected but below 2000 FI-C' and those where no fluorescence was detected.

Table 15 - Number (%) of subjects with detectable antibody-mediated C3b/iC3b deposition in cord and infant sera.

	Cord N with FI-C'=525/750			**Infants at three months N with FI-C'=412/628**		
	FI-C'			FI-C'		
	≥2000	<2000	ND	≥2000	<2000	ND
Ia	394 (53)	93 (12)	263 (35)	238 (38)	63 (10)	327 (52)
Ib	330 (44)	112 (15)	308 (41)	220 (35)	62 (10)	346 (55)
II	415 (55)	110 (15)	225 (30)	273 (43)	78 (12)	277 (44)
III	396 (53)	84 (11)	270 (36)	241 (38)	67 (11)	320 (51)
V	405 (54)	120 (16)	225 (30)	265 (42)	68 (11)	295 (47)

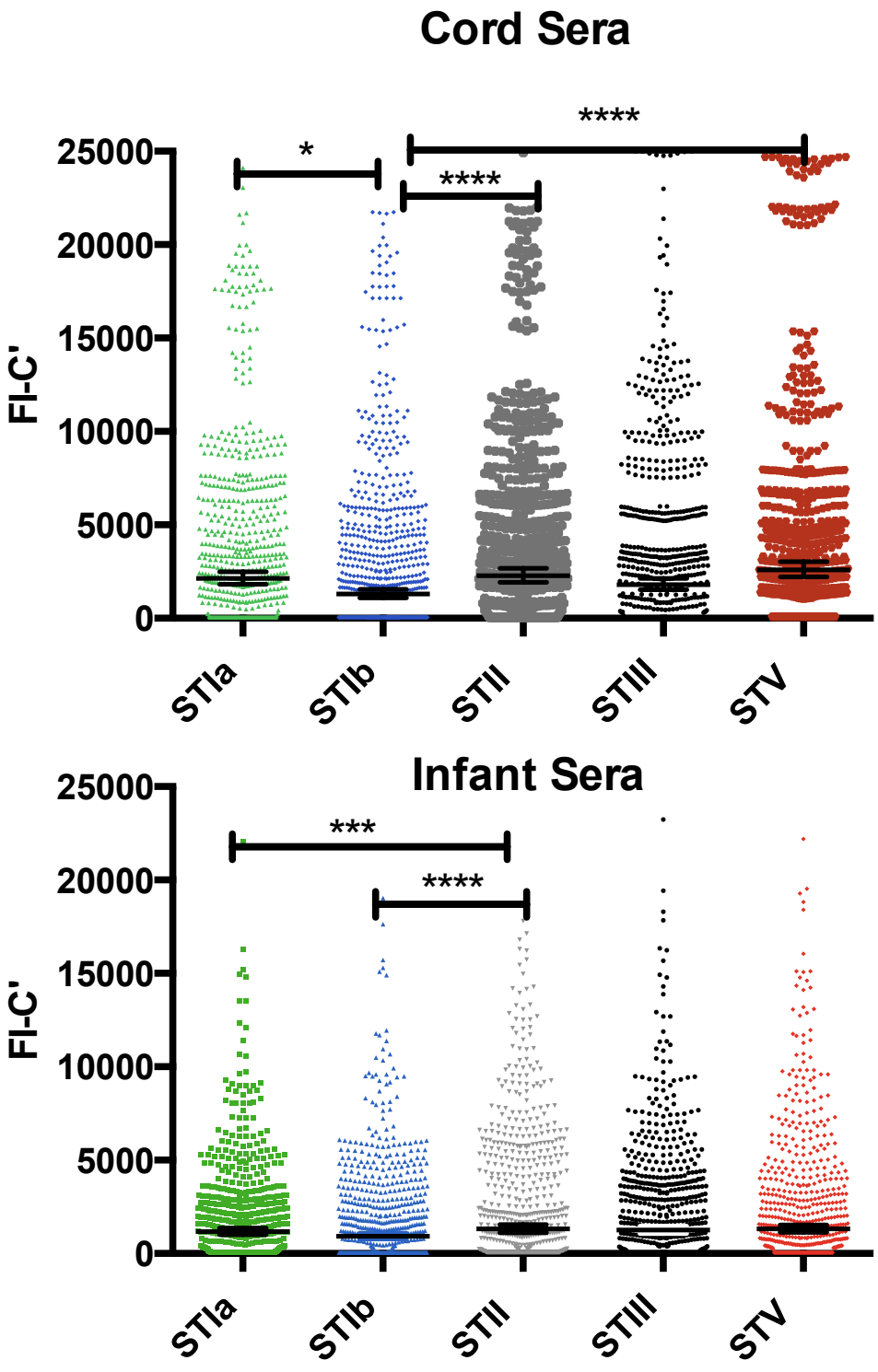
N=number; Ia = serotype Ia; Ib = serotype Ib; II=serotype II; III=serotype III; V=serotype V; FI-C' florescence minus complement only control. ND= not detected

The GM FI-C' (95% CI) antibody-mediated C3b/iC3b deposition also varied between GBS STs with highest GM FI-C' against STV [FI-C' 3835 (3494-4208)],

followed by STII [FI-C’ 3754 (3428-4111], STIII [FI-C’ 3197 (2930-3488)] then STIa [FI-C’ 3318 (3040-3621)] and STIb [FI-C’2736 (2521-2971)].

Compared to other GBS STs, the GM FI-C’ for STIb was lower than for STIa ($p=0.04$), STII ($p<0.001$) and STV ($p<0.001$) but not than STIII ($p=0.12$). There were no significant differences between the GM FI-C’ C3b/iC3b concentrations between any of the other GBS STs. Figure 26 summarises the GM FI-C’ and 95% CI for C3b/iC3b deposition in cord and infant sera.

Figure 26 - Antibody-mediated C3b/iC3b deposition in cord and infant sera



*Line and error bars represent the GM FI-C' and 95% confidence interval for antibody-mediated C3b/iC3b deposition onto the surface of whole GBS bacteria against serotype Ia (STIa), serotype Ib (STIb), serotype II (STII), serotype III (STIII) and serotype V (STV). Student t-tests were used to determine differences in GMC FI-C' between serotypes. *p=0.04, ***p=0.01, ****p<0.001 in cord sera n=525 (top figure) and infant sera n=412 (bottom figure).*

A multiple linear regression model was calculated to predict antibody-mediated C3b/iC3b deposition based on maternal age, weight, parity, malaria during pregnancy and infant birth weight, season of birth and infant sex. A significant regression equation was found ($F_{8,435}=4.99$, $p<0.001$, $R^2=0.58$. Increasing antibody-mediated C3b/iC3b deposition was associated with increasing birth weight (as a surrogate for gestational age) or birth during the wet season and decreasing antibody was associated with maternal malaria during pregnancy and maternal tetanus vaccination in pregnancy after taking account of maternal age, maternal weight, parity and sex of baby. (Table 16)

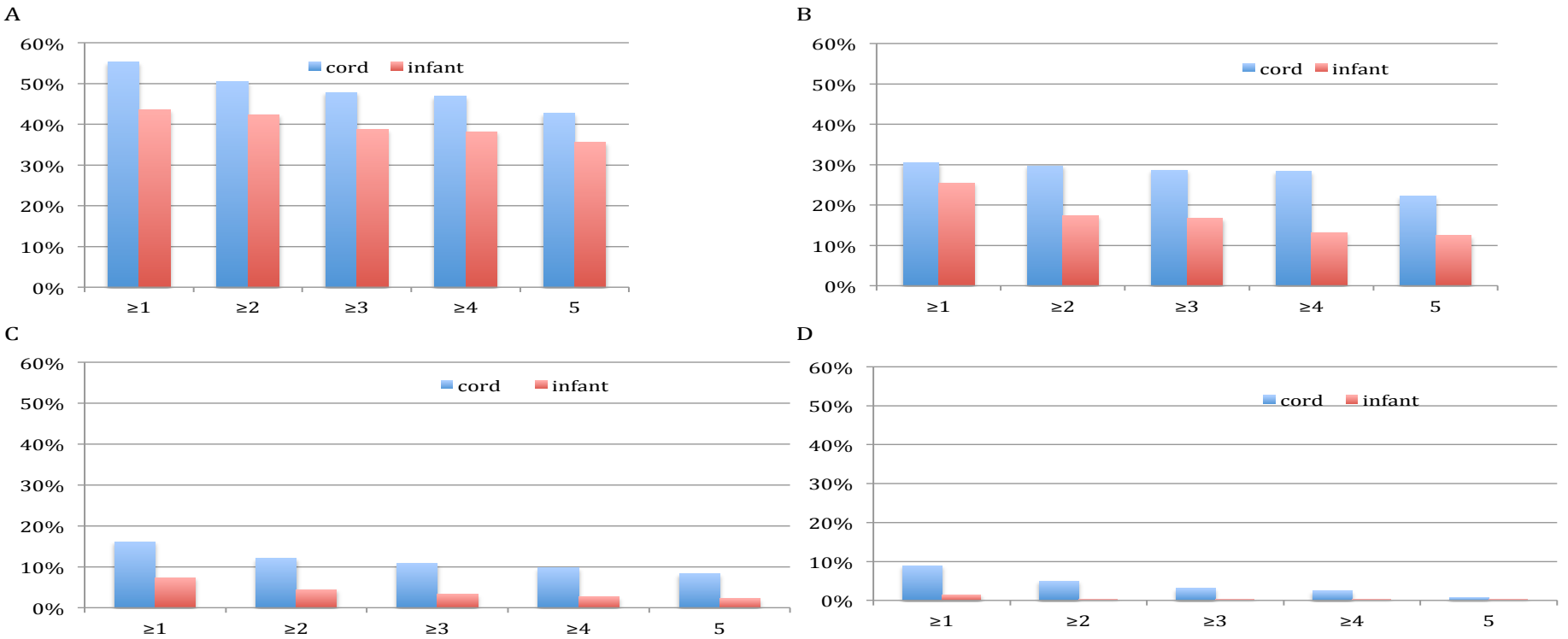
Table 16 – Multivariable linear regression model of factors associated with antibody-mediated C3b/iC3b deposition in cord blood

Variable		Coefficient	95% CI for coefficient	p-value
Maternal factors				
Age		0.007	-0.004 to 0.019	0.223
Weight (kg)		-0.000	-0.005 to 0.004	0.83
Parity		-0.021	-0.056 to 0.015	0.25
Malaria in pregnancy		-0.338	-0.576 to -0.099	**0.006**
Two tetanus vaccinations in pregnancy		-0.197	-0.330 to -0.064	**0.004**
Infant factors				
Birth weight		.1290985	.0130307 .2451663	**0.029**
Season of birth	Dry hot	.1860007	.0577072 .3142943	**0.005**
	Wet	.2085752	.0939361 .3232143	**<0.001**
Sex	Female	-.0194428	-.1114994 .0726138	0.678

6.6. Cross-reactivity between GBS STs

Although 34% of mothers were found to be colonised with GBS, antibody-mediated C3b/iC3b deposition was detected in 70% of all cord samples and 56% of infant samples to at least one GBS ST as shown in Figure 27. Additionally, 57% of cord samples and 44% of infant samples demonstrated antibody-mediated C3b/iC3b deposition against all five GBS STs tested. Cross-reactivity between GBS STs was examined using different lower limit of quantifications as described above. As expected, at low lower limit of quantifications, a high amount of cross-reactivity was observed. However, at a lower limit of quantification above 10,000 FI-C' functional antibody against a predominant GBS ST was identified . There was also greater divergence between the cord and infant sera values at these higher FI-C' values.

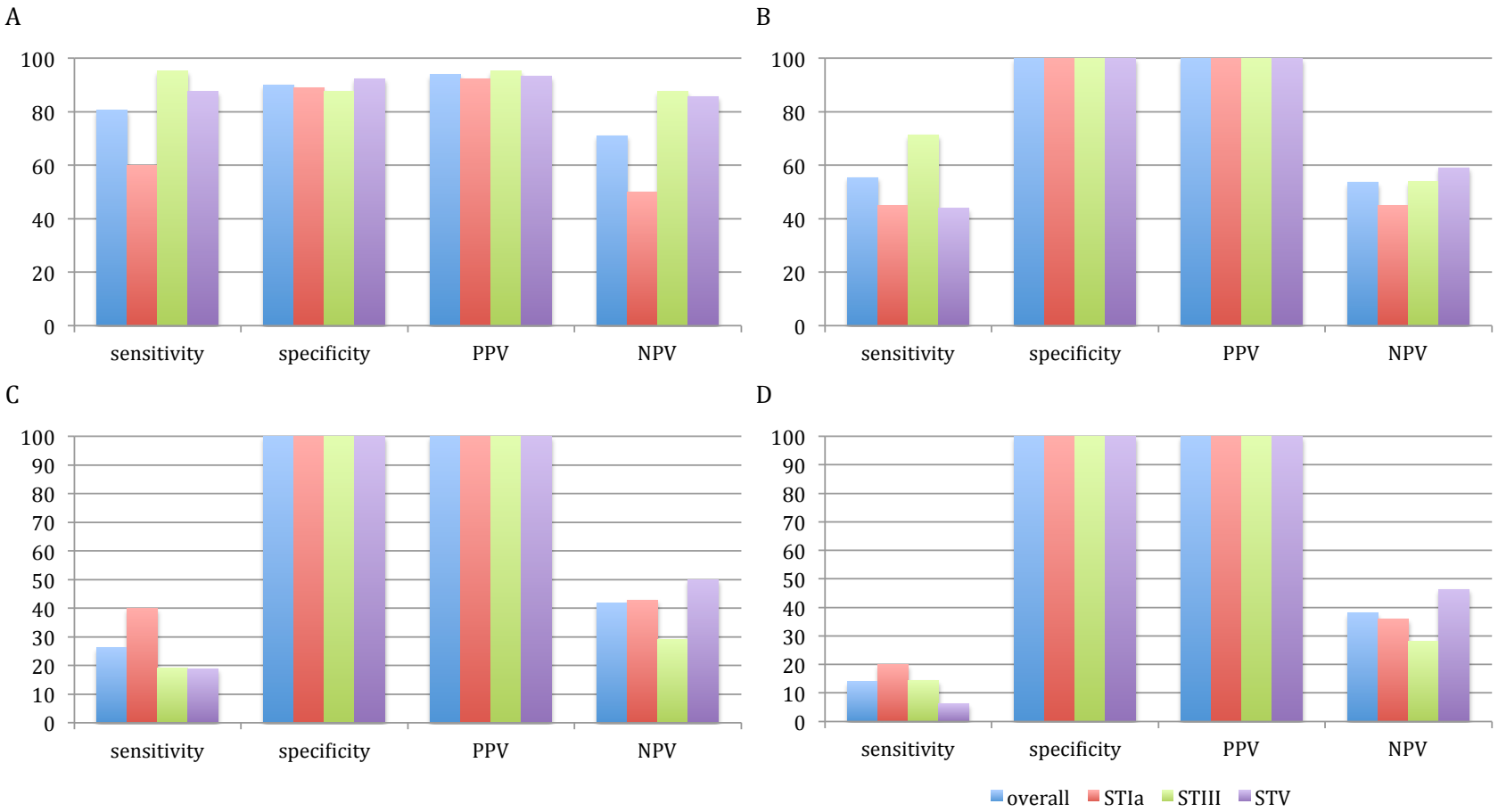
Figure 27 - Percentage of sera with antibody-mediated C3b/iC3b deposition detected to at least one GBS ST using different lower limits of quantification



Bar graph demonstrating percentages of cord and infant sera that show antibody-mediated C3b/iC3b deposition onto ≥1 GBS strain tested using a lower limit of quantification FI_C= value of ≥2,000 (A), ≥5,000 (B), ≥10,000 (C), and ≥20,000 (D). Samples that were assigned a not-determined value were classified as not having measurable functional antibody levels. Cord, n =525; infant, n=412.

To determine whether these different lower limit of quantifications affected the ability of the CDA to predict opsonophagocytic killing, the sensitivity, specificity, PPV and NPV of the CDA were calculated using the lower limit of quantifications described above. With increasing lower limit of quantification the sensitivity of the CDA declined. With lower limit of quantification above FI-C' 10,000 the sensitivity of the test dropped to below 50%. Figure 28 illustrates these parameters at each of the different lower limit of quantification. Based on these results an FI-C'=2000 was selected as the lower limit of quantification point for the CDA.

Figure 28 - Sensitivity, specificity, PPV and NPV (%) of CDA in prediction of OPkA titre using different lower limits of quantification

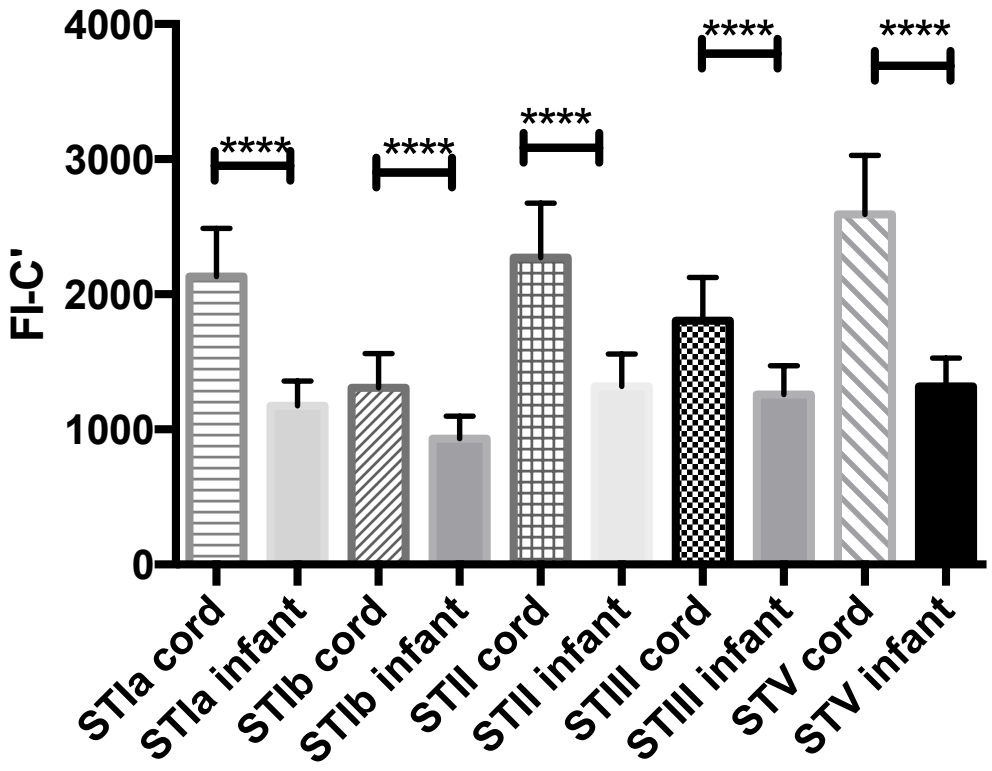


Bar chart presenting percentage sensitivity, specificity, positive and negative predictive values overall, and for STIa, STIII and STV with lower limit of quantification: (A) FI-C' 2000, (B) FI-C" 5000, (C) FI-C' 10,000, (D) FI-C' 20,000. N=30 samples for each serotype

6.7. Antibody-mediated C3b/iC3b deposition between birth and day 60-89

Antibody-mediated C3b/iC3b deposition declined rapidly between birth and day 60-89. By day 89 antibody-mediated C3b/iC3b deposition was reduced by 50-71% dependent on GBS ST. In a chi-squared analysis to determine if rate of antibody decline was the same for all GBS STs, the percentage decline in GM FI-C' forGBS ST Ia (-45%), II (-42%) and V (-49%) antibody was markedly greater than the GM FI-C' for Ib (-29%) and III (-30%) antibody (X^2 (4, N=525) =18.6; p=0.001). The results are summarised in Figure 29.

Figure 29 - GM FI-C' antibody mediated C3b/iC3b deposition at birth and day 60-89



*Bars represent GM FI-C' concentration with 95% confidence interval; patterned bars are cord sera, solid bars are infant sera; FI-C' fluorescence minus complement control; Student t-tests were calculated using GMC FI-C' in cord vs. infant serum in n=525 serum samples; **** p=<0.001*

6.8. Discussion

A high proportion of cord (70%) and infant sera (55%) demonstrated antibody-mediated C3b/iC3b deposition against at least one GBS ST. The predominant antibody–mediated C3b/iC3b deposition were onto GBS ST V and II, which correspond to the predominant colonising GBS STs. The low concentration of antibody-mediated C3b/iC3b onto GBS STIb in cord and infant sera also corresponded to the low prevalence of this GBS ST in colonised women and infants. The finding of higher antibody-mediated C3b/iC3b deposition compared to colonisation is similar to a Thai study by Herbert et al. (2015) which demonstrated antibody-mediated C3b/iC3b deposition in 91% of cord serum compared to a GBS colonisation rate of 12% in Thailand.(164) Similar to the present Gambian study, antibody-mediated C3b/iC3b deposition corresponded to known carriage rates in Thailand.

The number of cord sera with antibody-mediated C3b/iC3b deposition above 2000 FI-C' varied by GBS ST but antibody-mediated C3b/iC3b deposition onto the surface of GBS STV was greater than for other GBS STs. Concordantly, the GM FI-C' concentration was also highest for GBS STs V and II and lowest for Ib in cord sera, in line with the higher proportion of GBS STs V and II colonised women. In adjusted analysis, increasing birth weight and birth during the dry hot or wet season but not tetanus vaccination were associated with increased cord GM FI-C' for all GBS STs in cord blood. Maternal malaria in pregnancy was associated with decreased GM FI-C'.

The results suggest high cross-reactivity between the different GBS STs, possibly as a result of repeated episodes of colonisation by different GBS STs. In contrast, the study by Herbert et al. demonstrated similar numbers of sera with

antibody-mediated C3b/iC3b deposition against at least three GBS STs but a much lower percentage with antibody-mediated C3b/iC3b deposition against all five GBS STs (7% vs. 40% in our study). This may be due to differences in circulating GBS STs or due to assay sensitivity issues. Our finding of a predominant GBS ST at higher GM FI-C' values is in keeping with studies from South Africa demonstrating that new, rather than persistent colonisation with GBS in pregnant women is associated with higher antibody concentrations(146). It is important to note that our study used naturally immune sera and so the cross-reactivity seen reflects natural antibody and results may be different in sera from vaccinated women.

Antibody-mediated C3b/iC3b deposition decline between birth and day 89 appears to vary between GBS STs with the quickest declines shown for GBS STIa, STII and STV. Our results could reflect higher initial antibody-mediated C3b/iC3b deposition for GBS STs II and V compared to other GBS STs and so a more rapid slope of decline. The finding that antibody-mediated C3b/iC3b deposition to different GBS STs declines at different rates was also reported in a population of non-pregnant vaccinated adults in the USA. In this study, the rate of antibody decline between 4 weeks and 52 weeks post-vaccination was most marked in GBS STIII (-59%), followed by Ia (-42%) and V (-38%). However, encouragingly, despite different rates of antibody decline, this USA study indicated that functional activity persisted for up to two years post-vaccination with at least a $\log_{10}$ reduction in bacteria CFU/mL. In contrast, in our study a decline in functional activity was observed that varied between GBS STs between birth and day 60-89. This could imply that different rates of antibody decline would lead to colonisation with subsequent diverse GBS STs at different rates.

Chapter 7 - Association between functional antibody and maternal and infant colonisation in a Gambian cohort

7.1. Introduction

Anti-capsular antibody against GBS is thought to be the most important protective mechanism against invasive disease.(115) Antibody to GBS CPS passively transferred from mother to infant via the placenta has been shown to reduce the risk of neonatal EO-GBS disease by 70-90%, dependent on GBS ST.(116)

It is less clear, however, whether presence of high levels of functional antibody can reduce the likelihood of colonisation from mother to infant at birth and in the subsequent first months of life. Kwatra et al (2015) demonstrated that higher OPkA titres against GBS STs Ia and III in early pregnancy were associated with absence of maternal colonisation with these two GBS STs during later pregnancy.(146) If naturally-acquired maternal anti-CPS antibody passed to the infant could be shown to reduce infant colonisation from both the colonising and cross-reactive GBS STs, this would significantly reduce the risk of infants developing invasive EO- or LO-GBS disease. In turn, this proof of principle would have important implications for the likelihood of success of GBS vaccines given to pregnant women in order to prevent both infant colonisation and ultimately invasive disease.

Data in Chapter 5 and 6 shows that the CDA is capable of predicting opsonophagocytic killing in vitro and that Gambian mothers have relatively high concentrations of anti-GBS functional antibody in cord blood. High levels of cross-reactivity between GBS STs have also been demonstrated that may be an advantage in protection from invasive disease following challenge from different GBS STs.

In this Chapter the relationship between functional antibody concentration against GBS STs Ia, II, III and V and the infant risk of GBS-acquisition with the homologous GBS ST up to the age of 89 days is examined as is the association between functional antibody and postnatal clearance of GBS.

7.2. Methods

The patient population, microbiological and molecular methods for collecting samples are described in Chapters 3 and 4. I used the CDA as described in Chapters 3 and 5 to measure antibody-mediated C3b/iC3b deposition onto the surface of GBS bacteria STIa, II, III and V by flow cytometry. Results are reported as GM FI-C', using a lower limit of quantification of 2000 FI-C' units (Chapter 5).

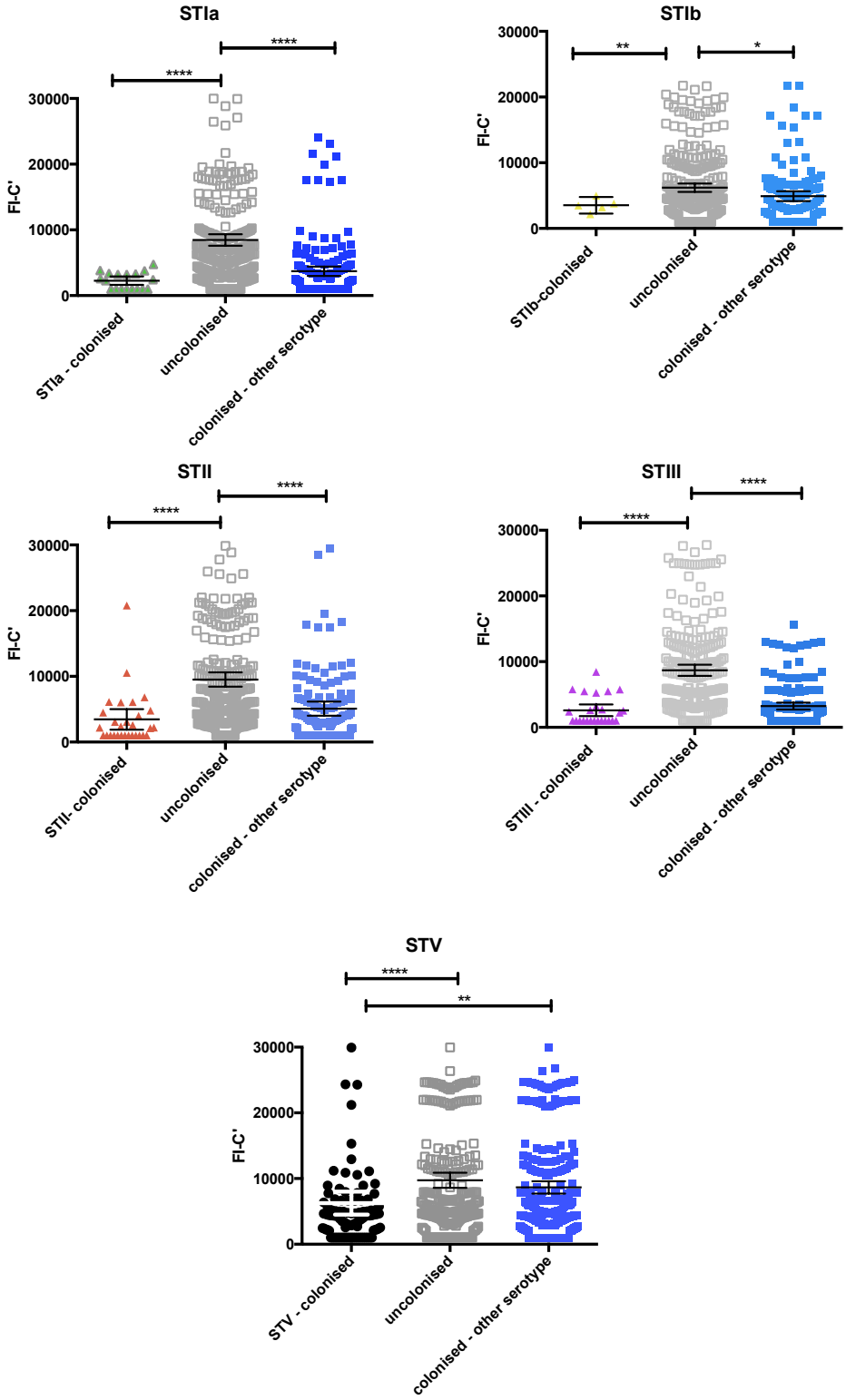
Potential differences in antibody-mediated C3b/iC3b deposition between sera from colonised and non-colonised mothers and infants were evaluated by student's t test after log transformation of data. Four groups were compared (mother colonised/infant non-colonised; mother colonised/infant colonised; mother non-colonised/infant colonised and neither mother nor infant colonised) using ANOVA following log-transformation of data. Comparison of log-transformed antibody-mediated C3b/iC3b deposition in serum at birth (cord blood) and at 60-89 days was performed using a paired t test. Logistic regression analysis was performed to determine which variables were independently and significantly associated with the acquisition of GBS. The correlation between bacterial concentration and functional GBS ST-specific antibody was evaluated using Deming linear regression and 95% CIs (PRISM, La Jolla). For all comparisons, $p < 0.05$ was considered to be significant.

7.3. Antibody-mediated C3b/iC3b deposition in cord blood and maternal colonisation at delivery

Overall, 525 serum samples from cord blood had detectable antibody-mediated C3b/iC3b deposition against any GBS ST. Colonised mothers were more likely to present during the wet season, be over 70kg in weight, non-anaemic, with more than one previous stillbirth, and more than one ANC attendance. These characteristics are described in Chapter 4.

Compared to mothers who were non-colonised with any GBS ST at delivery, mothers colonised with GBS STs Ia, Ib, II, III or V had significantly lower GM FI-C' antibody-mediated C3b/iC3b deposition against the homologous colonising GBS ST. (Figure 30)

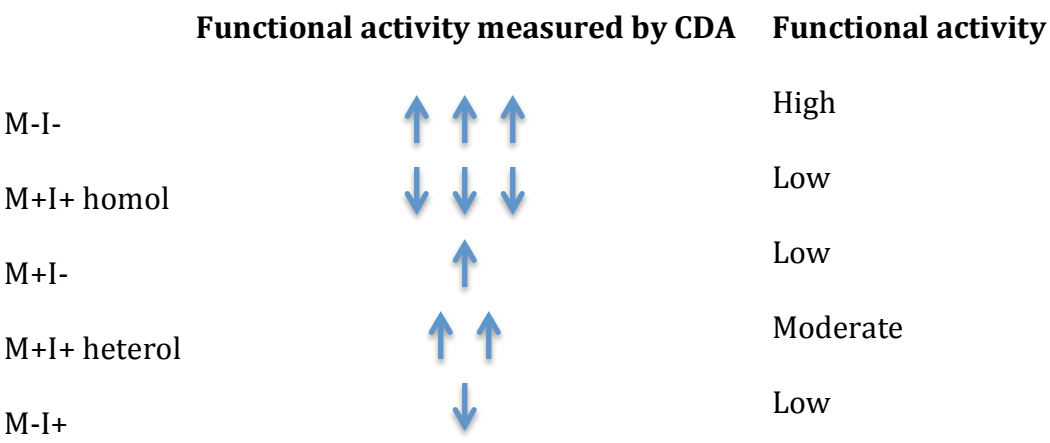
Figure 30 – GM-FI-C' antibody-mediated C3b/iC3b deposition (95% CI) in cord blood onto GBS ST Ia, Ib, II, III and V



*Dot and whisker plot demonstrating GMC antibody-mediated C3b/iC3b deposition against GBS STIa, Ib, II, III and V in cord blood (n=525). GM FI-C' [95% CI] compared between mothers colonised with homologous serotype (coloured shapes), non-colonised mothers (gray shapes) and mothers colonised with other serotypes (blue squares). ANOVA was calculated to compare groups *p<0.05, **p<0.01,****P < .001.*

A multivariable regression analysis, summarised in Table 17 showed that mothers who were colonised with the homologous GBS ST at delivery had significantly lower antibody-mediated C3b/iC3b deposition against GBS STs Ia, II, III and V when adjusted for maternal age, previous abortions, number of stillbirths, maternal anaemia, maternal weight, number of ANC visits, gestation at birth and season of delivery compared to mothers who remained non-colonised. This was true whether the mother was colonised with the homologous GBS ST or if they were colonised with another GBS ST. For GBS STIb, there was no association between mothers colonised with the homologous GBS ST or another GBS ST, probably due to the low numbers of mothers colonised with GBS STIb. An example of the potential model of functional antibody activity in relationship to the colonisation status of both mothers and infants is described in Figure 31.

Figure 31 -Potential model of functional antibody activity against homologous GBS ST



M-I-=mother/infant non-colonised; M+I+ homol = mother/infant pair both colonised with homologous serotype; M+I-=mother colonised/infant non-colonised; M+I+ heterol = mother/infant colonised with heterologous serotype; M-I+=mother non-colonised/infant colonised heterologous serotype.

Table 17- Multiple regression model demonstrating the reduction in antibody-mediated C3b/iC3b deposition associated with maternal colonisation with homologous GBS ST compared to non-colonised mothers

	STIa FI-C', (95% CI)	STIb FI-C', (95% CI)	STII FI-C', (95% CI)	STIII FI-C', (95% CI)	STV FI-C', (95% CI)
Mother colonised with homologous GBS ST	-6174 (-9273 to -3074)****	-1924 (-6287 to 2438)	-5866 (-9106 to -2626)****	-6731 (-9067 to -4394)****	-3982 (-6004 to -1959)****
Mother colonised with another GBS ST	-5166 (-6466 to -3866)****	-885 (-1931 to 160)	-4871 (-6582 to -3160)****	-5476 (-6626 to -4326)****	-4435 (-6574 to -2290)****

*All GM FI-C' figures demonstrate the reduction in GM FI-C' compared to mother/infant group where neither was colonised. Model adjusted for maternal age, weight, anaemia, gestation at delivery, previous stillbirths/spontaneous abortions, number of antenatal clinic visits and season at delivery.****=p<0.0001*

7.4. Antibody-mediated C3b/iC3b deposition in cord blood and maternal and infant colonisation dynamics at delivery

There were 186 colonised infants at birth and antibody-mediated C3b/iC3b deposition was detected in 525/750 cord samples. Infants were more likely to be colonised if born to a colonised mother, where maternal weight was below 70kg, mother had more than one previous stillbirth or attended ANC more than once. Additionally, infants were more likely to be colonised if born at less than 37 weeks, during the dry hot or wet seasons, with a birth weight below 3.1kg or delivered by midwives. These characteristics are described in Chapter 4. Due to the low number of mother/infant pairs both colonised with STIb (n=7) or mothers colonised with STIb when infants remained non-colonised (n=7) It was not possible to draw conclusions about the relationship between antibody-mediated C3b/iC3b deposition and colonisation with STIb.

There was a significant difference between mother/infant both colonised, mother/infant both non-colonised, mother colonised/infant non-colonised, mother/infant colonised with a different GBS ST, mother non-colonised/infant colonised for GBS STs Ia ($F=17.92$, $p<0.001$), II ($F=12.09$, $p<0.001$), III ($F=23.65$, $p<0.001$) and V ($F=7.93$, $p<0.001$) in cord blood.

7.4.1. Antibody-mediated C3b/iC3b deposition if both mothers and infants were colonised

Antibody-mediated C3b/iC3b deposition was lower if mother/infant were both colonised than if mother/infant were both non-colonised with the homologous GBS ST for Ia, II, III and V. Table 18 outlines the GM FI-C' values for each of the groups. If both mother and infant were colonised with the homologous GBS ST, GM

FI-C' antibody-mediated C3b/iC3b deposition was significantly higher than if mother/infant were colonised with different GBS STs for II (p=0.002) and III (p=0.04) but not for Ia (p=0.26) or V (p=1). There was no difference between GM FI-C' antibody-mediated C3b/iC3b deposition when comparing mother and infant colonised pairs and those pairs where mother was non-colonised and infant was colonised with the homologous GBS ST as the antibody measured (p=1.0 for all GBS STs)(Figure 32).

Table 18 - GM FI-C' and 95% CI by GBS ST and mother/infant colonisation group

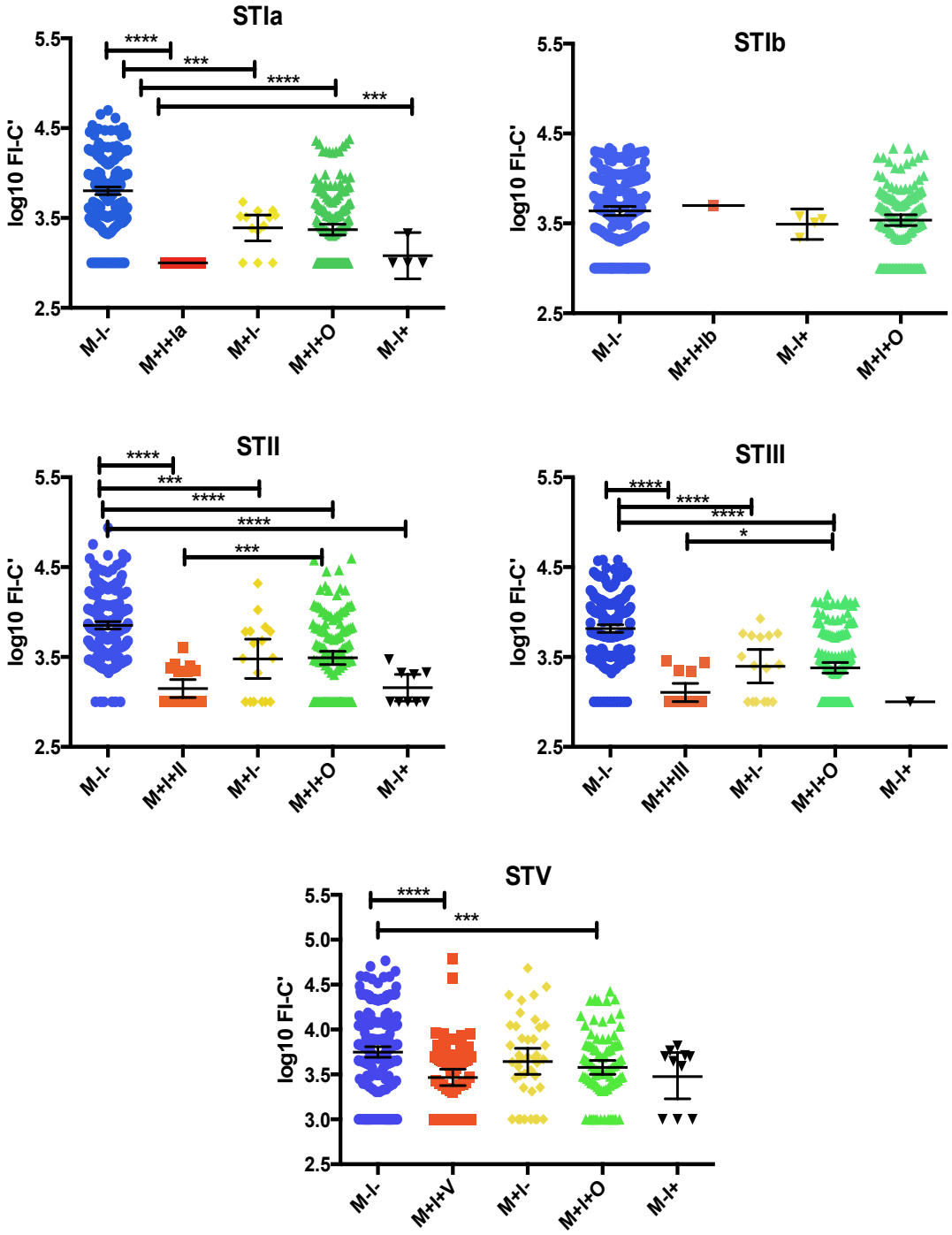
	STIa	STII	STIII	STV
M-I-	6309 (5754-7079)	7079 (6457-7762)	6607 (5888-7244)	6026 (5248-6918)
M+I+ homologous GBS ST	1000 (1000-1000)	1413 (1122-1778)	1288 (1000-1585)	3090 (2455-3802)
M+I-	2455 (1778-3388)	3020 (1820-5012)	2512 (1585-3802)	3677 (3388-6457)
M-I+	1202 (661-1995)	1445 (1023-2042)	1000 (1000-1000)	3090 (1778-5495)
M+I+ other GBS ST	2344 (1995-2512)	3090 (2630-3631)	2399 (2089-2754)	3981 (3162-4786)

M-I-=neither mother nor infant colonised; M+I+ mother and infant colonised; M+I-=mother only colonised; M-I+ infant only colonised; ST=serotype

7.4.2. Antibody-mediated C3b/iC3b deposition if neither mother nor infant was colonised.

Compared to all other groups, GM FI-C' antibody-mediated C3b/iC3b deposition was significantly higher for all GBS STs if the mother/infant pair were non-colonised (Figure 32). In a multivariable regression analysis, mother/infant pairs who remained non-colonised had significantly higher GM FI-C' antibody-mediated C3b/iC3b deposition than when mother/infant pairs were both colonised, when adjusted for maternal age, maternal weight, previous abortions/stillbirths, maternal anaemia, number of ANC visits, birth season and gestation ($P<0.0001$)(Table 16).

Figure 32 – Antibody-mediated C3b/iC3b deposition comparing mother and infant colonisation groups



Dot and whisker plots representing the mean and 95% confidence intervals of log_{10} FI-C' values of antibody-mediated C3b/iC3b deposition onto the surface of whole GBS STs Ia, Ib, II, III and V bacteria (n=525). FI-C' – fluorescence intensity minus complement control; M-I- - neither mother nor infant colonised; M+I+(Ia/Ib/II/III/V) - both mother and infant colonised ***with the homologous GBS ST;*** *M+I- - mother colonised with homologous ST, infant non-colonised with any ST; M+I+O – mother and infant colonised* ***with different GBS ST****; M-I+ - infant colonised with homologous ST, mother non-colonised with any ST. M-I-=neither mother nor infant colonised; M+I+ mother and infant colonised; M+I-=mother only colonised; M-I+ infant only colonised; ST=serotype. ANOVA was used to compare groups; *p<0.05; ***P<0.01; ****p<0.001.*

7.4.3. Antibody-mediated C3b/iC3b deposition if mother colonised but infant non-colonised at birth

Compared to mother/infant pairs who both remained non-colonised at delivery, mothers who were colonised but had infants who were non-colonised had significantly lower GM FI-C' antibody-mediated antibody deposition to GBS ST Ia ($p=0.001$), II ($p=0.001$), III ($p<0.001$) but not than V ($p=1.0$). When comparing mothers and infants who were both colonised with mothers who were colonised but whose infants were non-colonised, there was a trend towards higher GM FI-C' antibody-mediated C3b/iC3b deposition for all GBS STs but this was not significant (GBS ST Ia $p=0.44$; II $p=0.09$; III $p=0.23$; V $p=0.32$). There was no difference between mothers colonised and infants who were non-colonised compared to mothers/infant pairs where both were colonised with other GBS STs or where infant was colonised but mother was non-colonised.

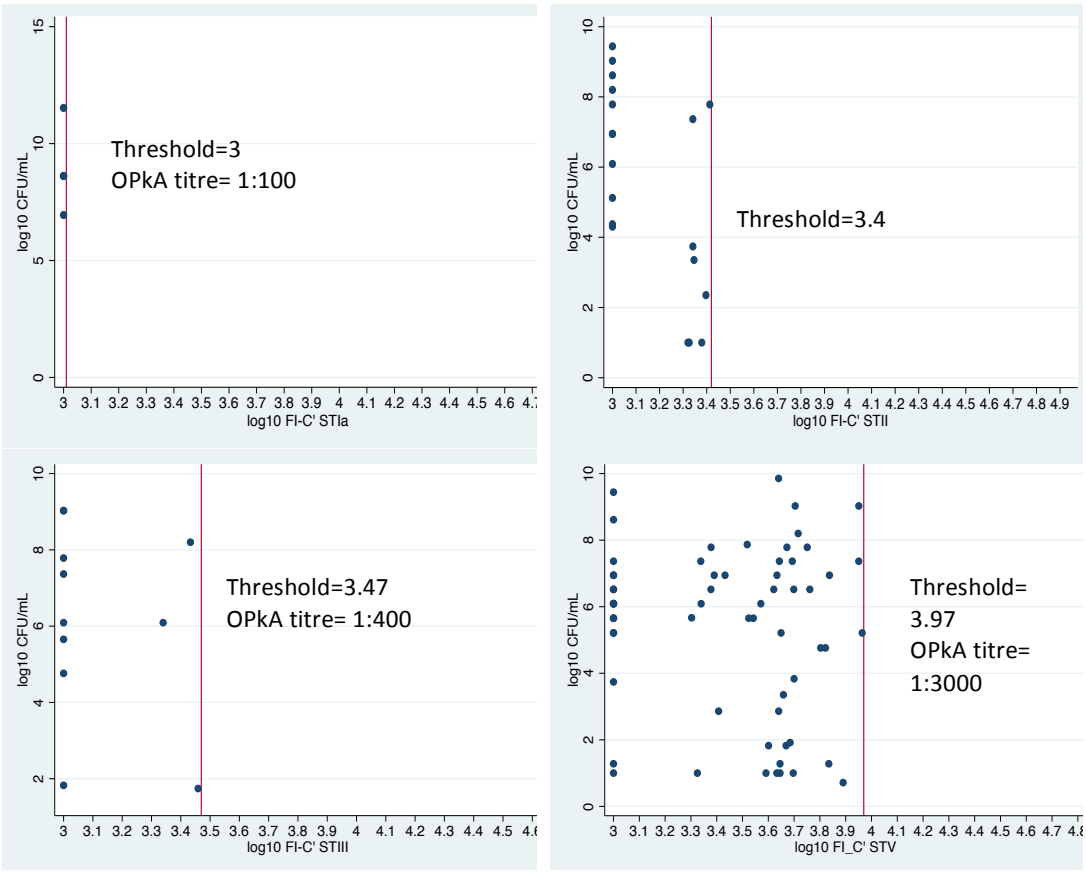
7.4.4. Antibody-mediated C3b/iC3b deposition if infant was colonised but mother was non-colonised

Compared to mother/infant pairs who were non-colonised at birth, GM FI-C' antibody-mediated C3b/iC3b concentration was lower in infants who were colonised when mothers were non-colonised for GBS STs Ia ($p=0.001$) and II ($p<0.001$) but not for III ($p=0.22$) or V ($p=0.42$). There was no difference between any of the other groups and GM FI-C' antibody-mediated C3b/iC3b deposition compared to infants colonised in the absence of maternal colonisation.

7.5. Using CDA and OPkA to estimate correlates of protection against colonisation

There was no correlation between $\log_{10}$ GBS CFU/mL from rectovaginal or infant swabs and GM FI-C' antibody-mediated C3b/iC3b deposition in cord blood for GBS STs III or V. There was insufficient data to analyse the correlation between GBS STs Ia or Ib bacterial concentration and antibody-mediated C3b/iC3b deposition. There was a moderate correlation between GBS CFU/mL from infant swabs at birth and antibody-mediated C3b/iC3b deposition in cord blood for GBS STII (R^2=0.36, F=9.67, P=0.006). In addition, plotting the bacterial concentration in infant swabs with the antibody-mediated C3b/iC3b deposition in cord blood indicated that there was a threshold above which no bacteria were found by culture or PCR. These thresholds were observed at $\log_{10}$ FI-C'=3 (3000 FI-C' units) for GBS STIa, $\log_{10}$ FI-C'=3.4 (2951 FI-C' units) for GBS STIII and $\log_{10}$ FI-C'=3.97 (9332 FI-C' units) for GBS STV (Figure 33).

Figure 33 - Infant bacterial colonisation and functional antibody threshold



Scatter plots of log_{10} GBS CFU/mL from infant swabs and log_{10} FI-C' for GBS STs Ia, Ib, II, III and V. Vertical red line represents threshold above which there is no bacterial colonisation is seen. STIa n=3; STII n=20; STIII=10; STV n=62.CFU/mL – colony-forming units per mililitre; FI-C' fluorescence intensity minus C3b/iC3b only control; ST – serotype.

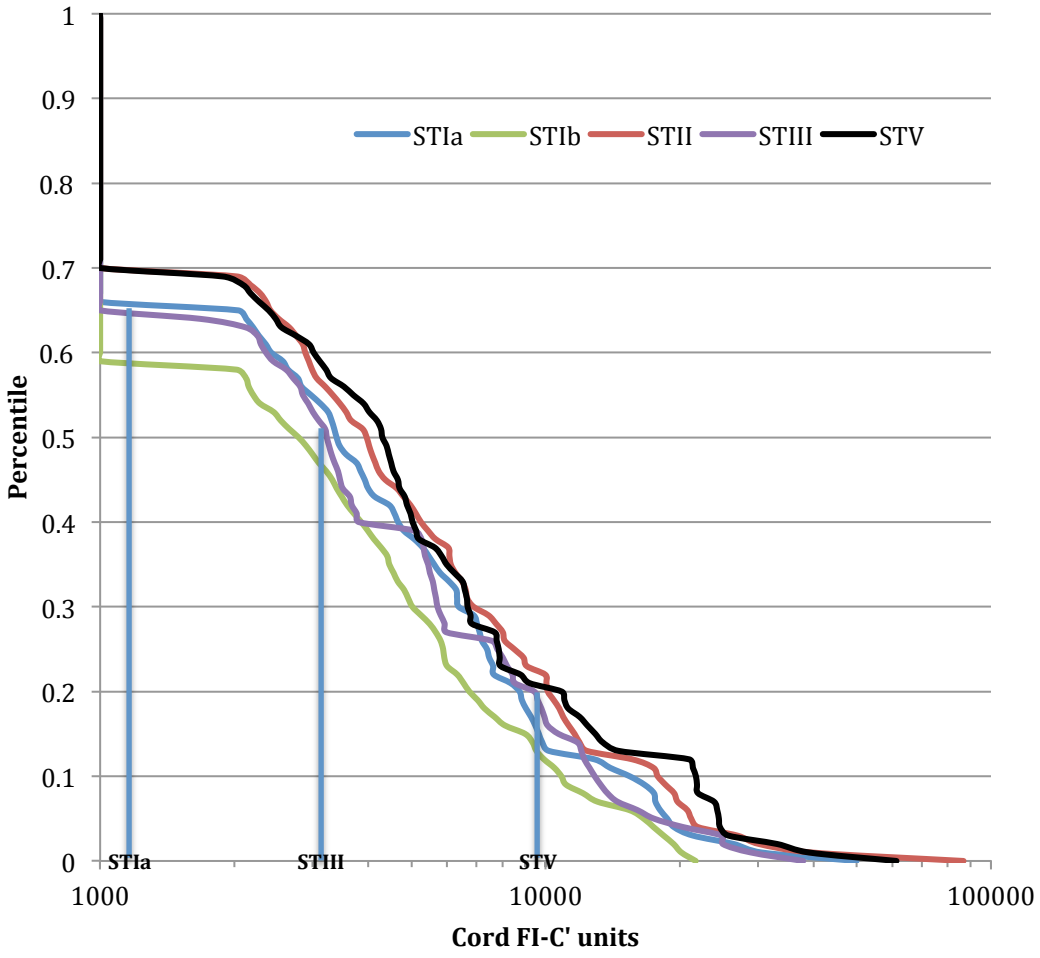
Using the correlation coefficients calculated in Chapter 5, it was possible to estimate the potential OPkA titre that corresponded to the observed threshold for GBS STs Ia, III and V. Table 19 demonstrates the log_{10} and antilogged FI-C' values for each GBS ST and the associated OPkA titre.

Table 19- Log_{10} and antilog of FI-C' and corresponding OPkA titre.

GBS ST	Log_{10} FI-C'	FI-C'	OPkA titre
STIa	3.01	1000	1:100
STIII	3.47	2951	1:400
STV	3.97	9332	1:3000

Using the RCD curve calculated in Chapter 6, 65% of cord blood would have OPkA titres above this level for GBS STIa, 52% for GBS STIII and 20% for GBS STV. Figure 34 demonstrates the RCD curve for cord blood with the associated FI-C' values corresponding to absence of infant bacterial colonisation.

Figure 34 – Reverse cumulative distribution curve of cord sera with associated FI-C' values corresponding to absence of infant colonisation at birth with that GBS ST.

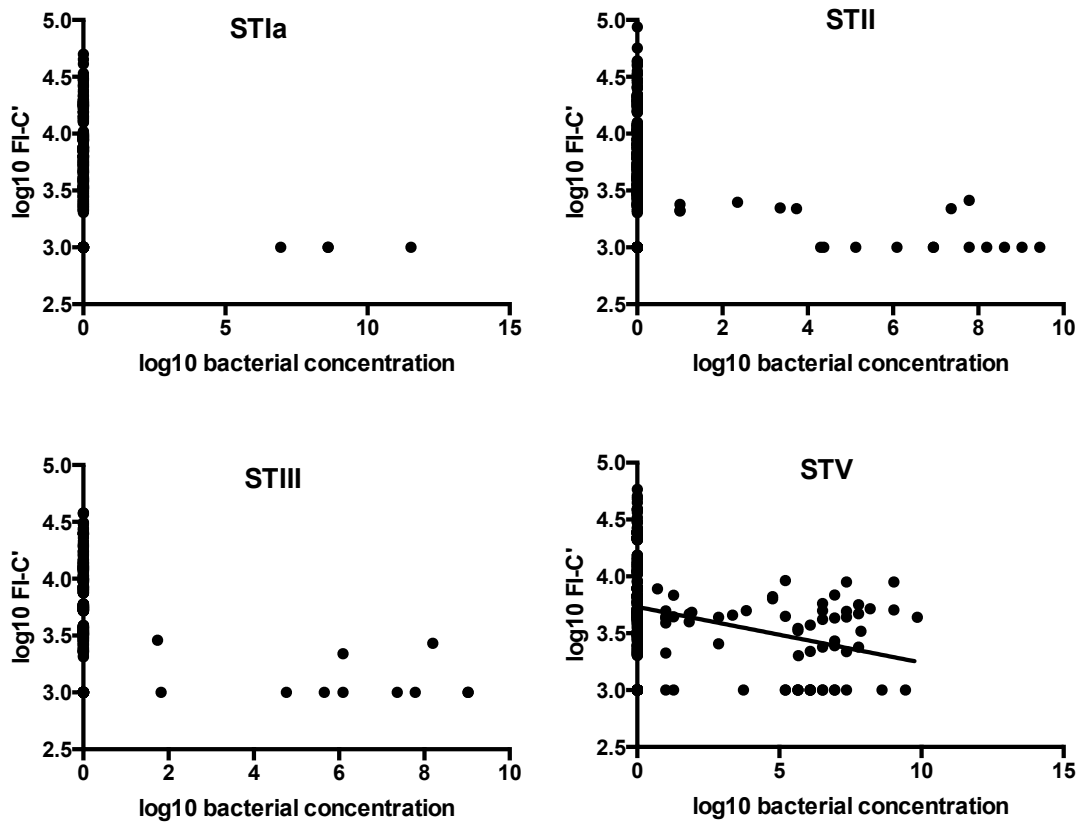


Reverse cumulative distribution curve of 750 cord serum samples. Y axis represents percentile of cord sera with FI-C' concentration over the lower limit of detection for each of the STs. X-axis represents FI-C' for that population percentile. Vertical line at FI-C'=1000 represents FI-C' value associated with absence of infant colonisation with GBS STIa; Vertical line at FI-C'=2951 represents FI-C' value associated with absence of colonisation with GBS STIII; Vertical line at FI-C'=9332 represents FI-C' value associate with absence of colonisation with GBS STIII. STIa=serotype Ia (n=487), STIb=serotype Ib (n=442), STII = serotype II (n=525), STIII=serotype III (n=480), STV=serotype V GBS (n=525). FI-C' is the florescence of the sample minus the complement-only control.

In order to investigate further the relationship observed between bacterial concentration and functional cord antibody in the scatter diagrams the differences were examined between cord sera containing very low (below 25th centile); low (25th to 50th centile); median (50th to 75th centile) and high (above 75th centile) FI-C' values for each of the four GBS STs and this was compared to infant colonisation at birth with the homologous GBS ST. Compared to those infants who had very low FI-C' values, infants with high homologous GBS ST antibody-mediated C3b/iC3b deposition were less likely to be colonised with GBS STs Ia ($F=10.3$, $p<0.001$), II ($F=38.15$, $p<0.001$), III ($F=9.14$, $p=0.01$) and V ($F=20.18$, $p<0.001$). Additionally, compared to those infants with very low FI-C' in cord blood, infants with low or median FI-C' were less likely to be colonised with GBS STV ($P<0.001$).

Deming linear regression was then performed to investigate the relationship between bacterial concentration and antibody-mediated C3b/iC3b deposition against the homologous colonising GBS ST. For each cord sample the paired bacterial concentration for the homologous GBS ST was plotted. The scatter plots in Figure 35 demonstrate a significant negative correlation between functional antibody concentration and bacterial concentration demonstrated by negative slope and 95% CI that do not span zero. This indicates that as functional antibody decreases, bacterial concentration increases. Table 20 outlines the slopes, 95% CIs and statistical significance value for each of the GBS STs.

Figure 35 – Deming regression plots for GBS ST Ia, II, III and V comparing cord serum with CFU/mL from infant swabs at birth



Deming regression of log10 FI-C' against log10 bacterial concentration for STIa, STII, STIII and STV. Log_{10} bacterial concentration on the x axis (CFU/mL), log_{10} FI-C' on the y axis; STIa=serotype Ia, STII=serotype II, STIII= serotype III, STV=serotype V.

Table 20 – Relationship between antibody-mediated C3b/iC3b deposition and bacterial concentration (CFU/mL) for GBS ST Ia, II, III and V

Serotype	Intercept (95% CI)	Slope (95% CI)	P
Ia	3.70 (3.64-3.75)	-0.09 (-0.15 to -0.03)	0.0009
II	3.75 (3.7-3.79)	-0.1 (-0.14 to -0.07)	<0.0001
III	3.69 (3.65-3.74)	-0.1 (-0.14 to -0.05)	<0.0001
V	3.73 (3.68-3.78)	-0.05 (-0.07 to -0.03)	<0.0001

7.6. Clearance of colonisation and antibody-mediated C3b/iC3b deposition

To better understand the role of antibody kinetics on bacterial colonisation, four groups of infants were compared: those who were never colonised, those who were intermittently colonised (either at birth or day 6-9 or day 60-89), those

colonised at two time points (either birth and day 6-9, or birth and day 60-89, or day 6-9 and day 60-89) and those who remained colonised throughout the study using ANOVA. There was insufficient longitudinal data to assess the impact of antibody-mediated C3b/iC3b deposition on colonisation with GBS STIb.

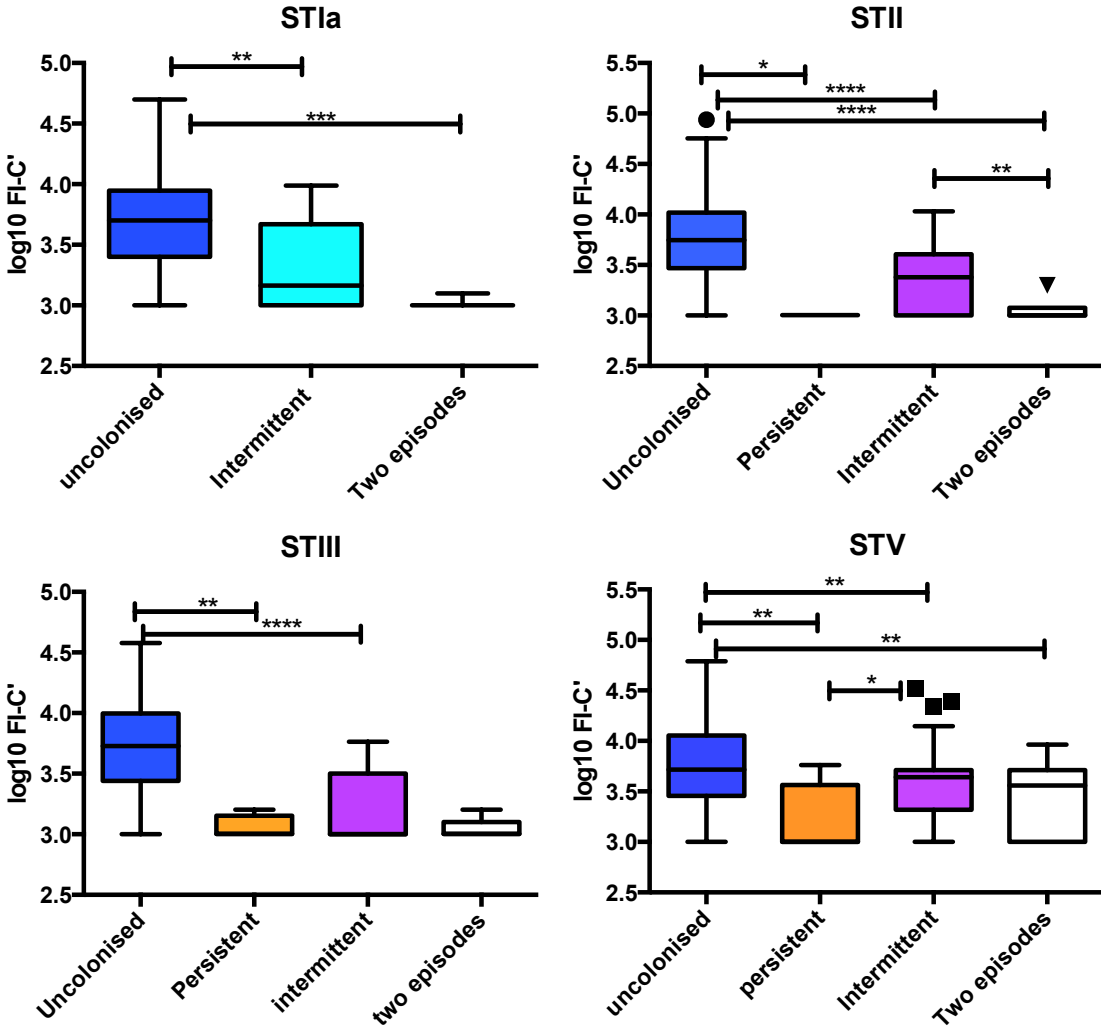
7.6.1. Persistent colonisation from birth to day 60-89

Infants who were persistently colonised with the same GBS ST from birth to day 60-89 had significantly lower FI-C' antibody-mediated cC3b/iC3b deposition values than those infants who remained non-colonised for GBS ST II ($p=0.04$) and V ($p=0.01$). No infants were persistently colonised with GBS STIII and only one infant was persistently colonised with GBS STIa. There was no statistical difference in antibody-mediated C3b/iC3b deposition between persistently and intermittently colonised infants (GBS STII $p=0.23$; GBS STV $p=0.34$)(Figure 36).

7.6.2. Intermittent colonisation between birth and day 60-89.

Infants who were intermittently colonised had significantly lower antibody-mediated C3b/iC3b deposition for GBS STs Ia ($p=0.005$), II ($p<0.001$), III ($p<0.001$) and V ($p=0.02$) compared to infants who remained non-colonised from birth to day 60-89. Infants colonised on one occasion had significantly higher antibody concentrations for GBS STs II ($p=0.002$) and V ($P=0.05$) but not for the other GBS STs (STIa $p=0.38$, STIII $p=0.31$) compared to infants colonised on two occasions. There was no significant difference between infants colonised on two occasions and infants who were persistently colonised (GBS ST V $p=0.49$, Ia $p=0.34$, II $p=0.49$, III $p=0.36$) (Figure 36).

Figure 36 -Median and standard deviation of antibody-mediated C3b/iC3b deposition in cord blood comparing non-colonised, intermittently and persistently colonised infants



*Box and whisker plot with Tukey correction demonstrating median antibody-mediated C3b/iC3b deposition in non-colonised infants, intermittently colonised infants (one occasion), infants colonised on two occasions and persistently colonised infants with GBS STIa, II, III and V. FI-C'= fluorescence intensity minus complement control, n=684. ANOVA was calculated to determine differences between the four groups, *p<0.05, **p<0.03, ***p<0.01, ****p<0.001.*

7.7. Discussion

This study has demonstrated an inverse correlation between maternal and infant colonisation and functional antibody in cord blood. The data support the notion that the risk of a colonised mother giving birth to a colonised infant diminishes as naturally-acquired antibody increases. This proof of concept supports

the idea that vaccinating mothers in pregnancy against GBS could have the same effect, provided the vaccine-induced antibody levels are similar and the antibody similarly functional in comparison with naturally acquired antibody.

This study has the advantage of measuring functional antibody against whole GBS bacteria rather than total anti-CPS antibody concentration and so enables a more detailed analysis of the physiological effect of antibody on bacterial killing that may be important to reduce sustained bacterial colonisation in the infant.

Our data show that mothers with higher GM FI-C' antibody-mediated complement deposition are less likely to be colonised with GBS themselves than mothers with lower GM FI-C' antibody-mediated complement deposition in cord blood. In turn, infants born to mothers with higher GM FI-C' antibody-mediated complement deposition are themselves less likely to be colonised at birth or to acquire GBS colonisation during the first weeks of life. This is an important finding as reducing maternal colonisation has the effect of reducing infant colonisation and therefore should impact on EO- and LO-GBS disease.

Apart from the study presented in this thesis, there are currently no data available that investigate the role of cord antibody in protecting infants longitudinally from colonisation at birth and up to 89 days of age.

Interestingly, our results differ from previous studies that have demonstrated higher anti-CPS IgG concentrations in pregnant women who were colonised with GBS than in non-colonised women. With the exception of the study by Baker et al. (1980)(117), these studies measured antibody by ELISA or radioactive binding assay (RABA). Neither of these assays tell us anything about antibody function, nor were the studies designed to assess whether antibody reduces the risk of colonisation in

the mother and transmission to the infant at birth, as shown in this study. Two studies have used OPkA to measure antibody in longitudinal cohort studies of pregnant women. The Baker study (111) which measured antibody via RABA and OPkA in cord and maternal sera to GBS STIII in 10 colonised women, found that although antibody measured by RABA was low in non-colonised women, irrespective of colonisation status most mothers had low levels of functional antibody concentration with little variation in antibody activity between 28 weeks gestation and delivery. The Baker study also found that mothers colonised with GBS STIII had higher OPkA titres against GBS STIII than non-colonised mothers.(117) This apparent increase in functional antibody associated with colonisation could represent different timing of acquisition and therefore different antibody kinetics that would not be detected when sampling occurred on only one occasion using maternal/cord serum and vaginal colonisation at birth, as was the case with our study design. A much larger longitudinal study of 507 pregnant women by Kwatra et al(146) attempted to describe such kinetics. Their study found that women who were never colonised during pregnancy had higher functional antibody measured by OPkA than those women who had new acquisition of colonisation during the study. Further, they found that although women who were newly acquired GBS during pregnancy had higher antibody measured by ELISA at that timepoint than non-colonised women there was no an increase in OPkA titre. Further, the study identified that women who became colonised during pregnancy had an increase in both ELISA and OPkA titre between 28 weeks of pregnancy and delivery. It is likely that this rise in antibody concentration and function prevents further maternal colonisation with this GBS ST. This study was not designed to follow up mothers longitudinally, and

therefore cannot predict which mothers had been persistently colonised and those that were newly colonised by the time they gave birth. However, the fact that increased functional activity was seen in non-colonised compared to colonised mothers and an inverse relationship between cord functional antibody and transfer of colonisation between mother and child also demonstrates that timing of acquisition is likely to be important in transfer of functional antibody from mother to child and thus protection from infant colonisation. One could argue though that ultimately, what matters for protection is the amount of functional antibody reaching the infant at the time of birth and hence the exact kinetics of colonisation during pregnancy might be less relevant. The kinetics of vaccine-induced versus naturally-acquired antibody might be very different and this requires further exploration in future studies.

This study also demonstrated that compared to mother/infants pairs who are non-colonised at birth, mother/infant pairs where both are colonised had significantly lower antibody-mediated C3b/iC3b deposition. The same was true if the infant was colonised but not the mother. Interestingly, if the mother/infant pair had discordant GBS STs then the FI-C' was higher than if both mother/infant were colonised with the same GBS ST for II and III but not for Ia or V. This is an important finding as it may suggest that waning maternal immunity with subsequent colonisation of a different GBS ST has occurred. Further, this data suggests that the immune response to the different GBS STs may vary and it will therefore be highly important to analyse vaccine responses to multiple GBS STs prior to Phase III vaccine trials if we are to ensure protective titres are achieved for each GBS ST.

Where mothers are colonised but infants are non-colonised the GM FI-C' was lower than if both mother and infant remain non-colonised. However, there was no difference between mother/infant colonised and mother colonised/infant non-colonised for any GBS ST. This could again be due to the challenge with a new GBS ST and a developing maternal immune response or due to persistent colonisation and therefore waning immunity.

This study has demonstrated that functional antibody above the 75th centile against GBS STs Ia, II, III and V correlates with absence of bacteria identified by culture or PCR in infants. This data suggest that there is a potential threshold of functional antibody above which the infant is no longer colonised. This finding could be very useful in developing an assay that can ultimately be correlated with vaccine efficacy in prevention of neonatal disease.

Our data demonstrates that infants who remain colonised from birth up to day 60-89 have significantly lower FI-C' values in cord blood and rapid decline in activity up to day 89 of life. Infants who cleared colonisation by the end of the first week of life had higher FI-C' values in cord blood than infants who remained colonised for two visits but cleared by the final visit. This would suggest that higher antibody-mediated C3b/iC3b deposition contributes to on-going killing in the infant up to day 89 of life.

To date, nearly all published work has focused on the role of antibody in protecting infants from invasive disease.(115, 116, 139, 140, 142, 196-203). These studies are mostly cross-sectional in nature and therefore cannot demonstrate the effect of maternal antibody during the entire period of risk for EO- and LO-GBS disease. There are currently no published studies of antibody persistence in infants

and our data add a new dimension. Edwards et al. (2012) demonstrated OPkA activity in healthy adult sera up to 18 months post-vaccination with GBS STs Ia and III CPS-conjugated vaccines, although the antibody concentration declined rapidly by four-weeks post- vaccination. Baker et al. 2002 also demonstrated good functional antibody activity two months after vaccination of pregnant women.(85) The GBS ST-specific kinetics of antibody decline in both infants and mothers are important to clarify further, as this will have implications for the vaccine strategy, for example the timing of vaccination in pregnancy and the need for further doses in subsequent pregnancies.

Our data suggests that interventions such as maternal vaccination should have an impact on reduction of maternal and infant colonisation at birth and up to day 89 of life in infants. Estimating a threshold of protection from colonisation is problematic, as this is only a surrogate for protection from invasive disease, but our results demonstrate that it might be possible to develop an immune correlate of protection from colonisation, if well standardised assays were to become available. Of course, such potential correlates require verification in the context of protection from disease.

Chapter 8. General Discussion

Based on the available published literature at the time of initiating this project, the hypothesis was formulated that maternal antibody could directly influence maternal and infant colonisation with GBS at birth and up to 89 days of life, in line with the estimated half life of maternal antibody in general. If this hypothesis could be proven, it would support the use of colonisation as a surrogate marker of protection from infant disease. If maternal vaccination against GBS and the resulting antibody is transferred across the placenta, this could prevent colonisation during the first three months of life, after which the risk of an infant developing invasive disease reduces.

As a first step, the prevalence and risk factors for maternal and infant colonisation with GBS at birth and the factors associated with postnatal acquisition in a Gambian population were investigated. This step was important in order to establish the current epidemiological situation of GBS in the Gambia, given that a range of GBS STs needs to be covered by a vaccine. If the GBS STs causing colonisation and disease in the Gambia were different to those found in other countries then the vaccine candidates currently in development might not be effective in a West African population. A longitudinal prospective cohort study of Gambian mother/infant pairs was thus performed to measure the prevalence of maternal/infant colonisation, the predominant GBS STs and the colonisation dynamics from delivery and up to day 89 of life. These findings were subsequently used to determine the possible predisposing factors to be included in the model to explain the correlation between maternal antibody and infant colonisation in line with our original hypothesis.

After successful completion of the field study, where microbiological and serological samples from 750 mother/infant pairs were obtained, the antibody-mediated C3b/iC3b deposition onto the surface of whole GBS bacteria was determined in order to measure the functional activity of this maternally-derived antibody in opsonising GBS. As opsonisation and subsequent phagocytosis and killing are key steps in eradicating GBS from the circulation, it is vital that the functional activity of the antibody is determined if an effective correlate of protection is to be developed.

Whilst relatively well standardised OPkA are routinely used to measure killing of pneumococci, no universally accepted, standardised OPkA currently exists for GBS. The CDA used in this work has many advantages over an OPkA, including avoiding the need for human cells and the viable counts of bacteria. These elements have been found to make the assay more robust and reduce the interassay variability often seen in OPkA between different laboratories. Additionally, the CDA could have advantages over an assay measuring IgG bound to the CPS, as is the case in a CPS ELISA, since the use of whole fixed bacteria would also allow measurement of functional activity of IgG in the context of a protein-based vaccine. Given that a good correlation between OPkA used in industry and the CDA was demonstrated for GBS STs Ia, III and V, the CDA was used for further analysis of functional antibody activity.

In this final Chapter I will reflect on the sentinel findings of the analysis of antibody-mediated C3b/iC3b deposition and correlation to maternal and infant colonisation and implications for the development of future vaccines in West Africa.

8.1. Epidemiology of GBS colonisation in the Gambia in 2014

One of the unique strengths of our cohort was the longitudinal follow up of mother/infant pairs from birth and up to 89 days. This showed that GBS STV remained the predominant GBS ST in the Gambia followed by GBS STs II and III with an overall colonisation rate of 33.7%. This is significantly higher than reported in the original Suara et al study from 1994 (present study, 33% colonised (29.63-36.37) vs. Suara, 22% colonised (95% CI 15.04-28.96); p=0.007).(17) Several possible explanations for this difference, include:

a) Advances in our knowledge of the relatively fastidious nature of the GBS bacteria have allowed the development of special growth conditions that may increase the recovery of GBS from swabs. Protocols used by the CDC and PHE recommend the use of selective media to reduce overgrowth from the faster growing enterobacteriaceae that inhabit the maternal rectum and vagina.(4, 160). These were not used in the earlier study.

b) the original Suara study sampled only from the vagina. The maternal anatomy during labour is such that bacteria can easily cross from the rectum to the vagina and it is now recommended that rectovaginal swabs rather than only vaginal swabs be taken to increase the likelihood of detecting GBS colonisation.(4, 168, 204)

c) This study used more rigorous culture and molecular techniques to identify GBS in line with recommendations by the CDC and PHE. (4, 160)

Thus, as a consequence, our results are a more robust estimate of the colonisation prevalence in Gambian women in 2014 compared to previous estimated and are in line with estimates from South Africa and Zimbabwe where selective media was also used.(12, 25)

The large number of infants recruited and the thorough clinical characterisation meant that a number of risk factors for colonisation could be assessed. In line with other studies, premature infants (gestation below 34 weeks) were at increased risk of GBS colonisation.(205, 206) This could be because of the limited time available for maternal antibody to cross the placenta in those born before 34 weeks. The reduced skin barrier, permeable infant gut and immature immune system also provide a more favourable environment for bacterial colonisation (and subsequent invasion). Alternatively, the fact that the mother is colonised with GBS could contribute to premature labour resulting in a GBS-colonised premature infant.(207, 208)

Interestingly, it was found that mothers and their infants were more likely to be colonised with GBS if birth occurred during the dry hot or wet seasons. Climate has been shown to affect human disease, both by influencing bacterial virulence factors and host immune response.(209, 210). One study demonstrated an increased incidence of streptococcal pyoderma in humid climates(211) and a recent Spanish study found an increased risk of GBS colonisation with temperatures above 23.5°C and humidity above 77.3% (OR 1.38; 95% CI 1.04–1.82).(212) Such a finding could be an important risk factor in equatorial countries such as the Gambia as increasing the core body temperature may increase the capacity of GBS to adhere to vaginal epithelial cells.(213) Additionally, in countries such as the Gambia, access to clean water is difficult, especially during the hot and wet seasons and hygienic practices may therefore be suboptimal. Our analysis could not fully investigate this, as information was not collected on maternal behavior or hygiene during pregnancy or

the study follow up period, but future research could address these questions more specifically than was possible during this project.

An association between GBS colonisation and previous stillbirth was also observed. It is known that mothers who are GBS colonised in pregnancy are at increased risk of having a GBS-infected infant, hence prophylactic antibiotics are offered to these women in resource rich settings, but the association between GBS and stillbirth is still unclear. To date, the associations are limited to case reports and case series.(214-217) Stillbirth is a significant public health concern with rates of up to 28.3 per 1000 total births in sub-Saharan Africa in 2009.(218) Infections are thought to account for approximately 10-25% of stillbirths in LMICs.(219) Despite this significant global burden, the issue of stillbirth has finally received some international focus within the development of the post-2015 Sustainable Development Goals.(1) A recent systematic review identified 17 studies describing stillbirth associated with GBS culture.(172) However, inconsistent and unstandardised reporting and diagnostic differences prevented assessment of the results to inform future maternal intervention strategies. Antenatal screening and treatment has substantially reduced the burden of EO- GBS disease in countries where it has been introduced but would be unlikely to reduce stillbirths occurring prior to screening at 35 weeks. However, if a maternal vaccine in pregnancy were introduced, then protection of the fetus and infant would occur that would prevent not only postpartum GBS disease but also GBS-related stillbirths. The findings of this current study strengthen the association between maternal GBS colonisation and stillbirth.

An increased risk of infant colonisation was also demonstrated if the infant

received breast milk from which GBS was cultured. Isolation of GBS in breast milk has been implicated in several case reports of LO-GBS disease (178, 179). Our data support this suggestion, since GBS colonisation in breast milk was also identified as a risk factor for neonatal colonisation, both at delivery and for persistence of infant colonisation throughout the first three months of life. This could be due to passage of GBS antenatally from mother to infant and continued passage via breast milk. In populations such as the Gambia, where breastfeeding rates are high, this is an important finding. It may be that GBS enters the mammary glands from a colonised infant following infant suckling, although there is also some suggestion that normal gut microbiota (of which GBS may be an example) may pass into the mother's breast via the entero-mammary circulation(180). Whether it is also a risk factor for LO-GBS disease in our population is not clear, since no cases of LO-GBS disease were observed and our study was not powered to measure clinical disease as an endpoint. Conversely, breastfeeding might be a protective factor through induction of immunity.(40) The relevance of this finding is currently being examined in a follow up study to better understand immune factors in breast milk that might be associated with infant colonisation.

Finally, delivery by a midwife was also identified as a risk factor for GBS colonisation, compared to those delivered by TBA, which was somewhat surprising. No other studies have highlighted this risk factor, but it is important as improving maternal and neonatal outcomes involves women giving birth in hospital facilities. There is no current satisfactory explanation for this finding in our population. However, the constraints placed on hospitals in LMICs could lead to reduced hygiene, especially when supply of gloves and clean water is scarce. Alternatively,

infants who are delivered by midwives may have had more complicated births, requiring more manual intervention than those delivered by TBAs. Nosocomial outbreaks of GBS have been reported in several hospitals globally (181, 182). Future studies should monitor GBS colonisation in mothers delivering with the help of midwives in hospitals to determine whether nosocomial spread might be a risk factor and therefore a create a need for hospital-based interventions.

Taken together, our data provide further evidence for the significant risk factors previously described(205) but also suggest some novel risk factors which have significance and require further investigation.

8.2. Maternally-derived functional antibody and colonisation

Most of the previous work examining maternally-derived antibody focused on reducing the risk of neonatal invasive disease by attempting to establish a concentration of antibody above which an infant does not acquire disease. Fewer studies have focused on the relationship between maternal or infant colonisation and antibody. This matters because maternal colonisation is the major risk factor for infant disease, thus investigating factors that can reduce maternal and/or subsequent infant colonisation should reduce the risk of invasive disease. Using prevention of colonisation as a potential correlate of protection has advantages in that colonisation is common but disease is rare. Additionally, studies of *H. influenzae*(220)*, S. pneumoniae*(221) and *N. meningitidis*(222) demonstrate that increasing antibody concentrations in serum are associated with reduced risk of colonisation with these bacteria. If the same is true of GBS colonisation, then increasing maternally-derived antibody through vaccination could interrupt GBS

colonisation and subsequent disease. However, the disadvantage of using colonisation as a surrogate is that no account is taken of the virulence and disease-causing potential between different GBS strains. Thus a correlate based on colonisation would need to be assessed using these strains in addition to the more frequent colonising strains.

This study uniquely describes maternally-derived antibody and infant colonisation from birth up to 89 days of life. In particular, it focused on the dynamics of transmission of colonisation and the effect of antibody on these dynamics over a 89-day period. The data demonstrate that compared to all other mother/infant groups, **the lowest functionally active antibody level is found in mother/infant pairs where both are colonised.** Infants receiving the lowest concentration of functionally active antibody via cord blood are also less likely to clear GBS colonisation between birth and day 60-89. Further, the results demonstrate that for GBS STs Ia, III and V, a threshold of antibody could be identified above which no infant was GBS-colonised at birth. Taken together this strengthens the argument that increasing maternal antibody by vaccination may reduce transmission of GBS from a colonised mother (and thus impact on EO-GBS disease) and, even if GBS is acquired at birth, improves the rate of clearance of colonisation in the baby (and thus impact on LO-GBS disease).

Increasing the concentrations of specific antibodies in infants through maternal vaccination has been shown to protect infants from diseases such as tetanus, pertussis and influenza.(223-225) This is therefore likely to be a useful strategy to prevent GBS disease. However, as outlined in the introduction to this thesis, the large number of pregnant women needed for a phase III vaccine trial to

establish formal efficacy is a barrier to progress in this area. Therefore, the ability to identify a reliable immune correlate of protection against GBS disease would facilitate vaccine licensure.(147)

Identifying biomarkers that correlate with protection against disease has hastened the development and introduction of new vaccines for pneumococcal and meningococcal diseases.(184, 226) The current paucity of data relating to correlates of protection against GBS disease has recently been described in an excellent review.(143) The major ongoing difficulty identified in this and other reviews is to establish a strain-specific threshold of protection from infant disease or colonisation of mother or infant, in part because there is a lack of standardisation between assays currently in use, which hampers interpretation across studies.(143)

The majority of GBS studies to date have focused on anti-CPS antibody concentration but have not discussed the functional component of the antibody, which may be important for clearance of the GBS. Binding of C3b/iC3b and opsonophagocytosis characterise the immune function that is responsible for protection and could therefore be used as a correlate of protection from GBS disease. ELISAs quantify the antibody response but do not provide information on antibody function. It is important to note that the ELISAs are specific for the CPS, whilst OPkA and CDA recognise both protein and polysaccharide antigens on the surface of the bacteria. There is a need to establish both concentration and function of antibody in order to determine a correlate of protection from GBS disease, which can then be correlated to the antibody concentration measured by ELISA as a useful surrogate of protection from GBS disease, which is easier to measure. Functional assays may also allow a better estimation of the duration of protection following

vaccination than ELISAs.(184, 185, 227) The need to assess functional antibody activity has already been shown for the assessment of vaccines against pneumococcus and has been used as a route to change future vaccine formulations(228): although initial licensure of the seven-valent pneumococcal vaccine was based on efficacy trials, it was accepted that efficacy trials would prove impractical for the licensure of higher valency formulations and so the assessment of opsonophagocytosis as a marker of antibody function proved essential for defining correlates of protection for subsequent vaccines.(229) Our findings add weight to the need to measure functional activity by demonstrating that antibody-mediated C3b/iC3b deposition reduces the risk of maternal and infant colonisation and enhances infant clearance of GBS colonisation.

However, the field remains confused with regards to the "right" assay to use: the review by Dangor et al (2015)(196) identified 47 studies describing correlates of protection using 34 different quantitative and 12 different functional assays. There are substantial differences in the methods of quantitative assays; some assays use microtitre plates coated with GBS CPS, some measure GBS ST-specific antibody in monoplex assays whilst others measure antibody in multiplex assays. Some measure IgG antibody via direct ELISA, some via indirect ELISA, some by measuring competitive binding. Equally, variations in the methods have been observed for functional antibody assays: some of the OPkA assays use multiple dilutions whilst others use a single point dilution and read the results from a standard curve; some measure killing whilst others measure internalisation of the bacteria into the phagocyte but not killing. Further, the assays use different technologies such as the Luminex platform or flow cytometry to determine antibody concentration. Finally,

the lack of standardised reference ranges for IgG despite the availability of reference sera (Prof. Baker, Baylor College of Medicine, Houston, Texas and Biota pharmaceuticals Inc., Australia) makes the selection of a threshold of protection difficult at present. Taken together, these methodological differences between assays and lack of reference sera make interpretation of results between groups problematic. However, irrespective of the assay used, protection from invasive disease is consistently associated with high maternal antibody concentration, and this study reports the same effect on infant colonisation in a West African setting for the first time. Standardised methodology and an internationally accepted reference serum are urgently required to define the threshold of protection from maternal and infant colonisation and infant disease further and is now a recognized research priority.

Although the initial analysis of antibody did not appear to demonstrate an absolute correlation between absence of colonisation and maternally-derived functional antibody, the examination of sera with high compared to low antibody-mediated C3b/iC3b deposition revealed that at concentrations above the 75th centile for GBS STs Ia, III and V, infants were no longer GBS colonised. Thresholds between 1μL/mL and 10μL/mL have been postulated for protection from invasive disease depending on GBS ST.(196) It is not possible to compare the results of our CDA with these ELISA results directly, although the two studies from South Africa and this study all demonstrate that increasing antibody is associated with risk reduction for colonisation. However, it will only be through standardisation of the current methods that a more robust estimate will be determined.

8.3. Limiting factors

This study has several limitations. The results are obviously limited by the sensitivity of detection of GBS on selective media, estimated at 85% (230) and by the fact that the GBS ST was identified by conventional PCR. Thus it may be that low levels of colonisation in infants are underestimated where DNA was below the limit of detection.

Only one culture-confirmed case of GBS invasive disease was identified in this cohort. This may represent an underestimation of the burden of GBS disease in this population. Several infants died within the first four hours of birth and so were not recruited or died in the first few days of life following EO-pneumonia without a culture-confirmed diagnosis. It is possible that these infants may have succumbed to EO-GBS disease. Whilst it is not possible to correlate these deaths with GBS, all three infants who died of early onset pneumonia had antibody-mediated C3b/iC3b deposition below the limit of detection. There are no other data from the Gambia regarding the burden of GBS disease. Although more information may have been gathered from a larger cohort, this study used a sample size that could be feasibly attained during a single-handed PhD project and with an endpoint of colonisation. There is little other evidence from the Gambia for GBS disease. However, a retrospective review of positive blood and CSF cultures from the MRC hospital over eight years from infants under 89 days of age identified 17 positive cultures. This is consistent with our disease estimate of 1.3/1000 live births.(231)

Sampling at four hours post-birth may indicate contamination with maternal secretions rather than established colonisation at birth. However, the fact that few infants had lost colonisation by day six in our cohort indicates that such colonisation

was persistent over the first week of life.

The low numbers of mother/infant pairs colonised with GBS STIb precludes detailed analysis of this GBS ST. This is disappointing as GBS STIb is an important cause of infant disease in the USA and is one of the GBS STs being targeted by the current vaccine candidates. However, the environment in which this work was conducted did not allow acquisition of a large number of women colonised with GBS ST1b, as it is not a dominant GBS ST in The Gambia.

The lack of standardised protocols available for functional assays was also a limitation of this study, as it is for others using functional assays against GBS. The absence of a standardised assay to assess functional antibody activity means that the results of our study should be interpreted with caution, as it is not possible to compare some aspects with other studies at present.(147) There were a number of reagents used, which differ from other studies that may have affected our results. IgG-depleted human plasma was used as a complement source for our assays (165) because of the possibility of increased immunogenicity when using baby rabbit serum. There have been different approaches to the use of complement sources in functional assays, with some groups preferring endogenous complement that has not been IgG depleted (83, 84, 186), whilst others use baby rabbit serum (146, 154, 156). The use of a pooled IgG-depleted human plasma may have affected our results compared to baby rabbit serum, which may give higher activity due to the lack of species-specific complement regulatory protein activity. This may have been why our CoV was 34%, which was marginally higher than the 30% CoV used in industrial assays to assess OPkA in pneumococcus (184) or 22% CoV in complement control in standardised bactericidal assays for *H. influenza.*(228) It will be vital going forward

that assay standardisation is undertaken in order to demonstrate reliable predictions of protection from colonisation and disease.

Interestingly, a large amount of cross-reactivity between serotypes with functionally active antibodies was observed. Cross-reactive antibody has been shown to provide additional protection against invasive pneumococcal disease (232) in vaccinated populations and closely related GBS STs may possibly behave in a similar way. Our work was performed using sera from women with natural immunity to GBS rather than vaccine-induced immunity. Therefore, the sera from the Gambian women will have antibody to both the CPS and to proteins on the bacterial surface. Different results may be obtained with sera obtained from CPS-vaccinated individuals. It may also be possible that the assay is not sufficiently sensitive at FI-C' lower limit of quantification points to detect GBS ST-specific functional activity at these concentrations. I found that the assay sensitivity dropped with higher FI-C' limits of quantification and hence used this lower limit of quantification for our assay. In future, it may be necessary to revise this lower limit of quantification, especially once we are able to standardise the assay in direct comparison with other functional assays such as the OPkA.

Although the overall sensitivity and specificity of the CDA were good, it is important to note that some sensitivity was lost for GBS STIa when comparing CDA to OPkA and compared to the other two GBS STs, which could lead to an under-estimation of killing in sera where functionally active antibody is present.(233) As antibody-independent complement-mediated killing varies by GBS ST, it will be important to undertake GBS ST-specific functional antibody studies when assessing

protection from disease and colonisation in the future, as the protective thresholds are likely to be different.

Finally, the assumption has been made that the reduction in colonisation is due to functional serum antibody concentration. It may be that protection from colonisation at the mucosal surface is more complicated and includes innate and adaptive factors associated with breast milk or the infants' developing microbiome (40). However, in other vaccine studies, increased serum antibody concentration has been associated with decrease in infant colonisation for pneumococcus, meningococcus (A, C, W, Y) and *H. influenzae* as outlined above and the same may be true of GBS. To measure mucosal immunity is extremely challenging, as there are few methods available and to assess this aspect was beyond the scope of this study. However, samples have been stored to assess the microbiome in future work.

8.4. Future work

Work carried out in this thesis has led to a detailed characterisation of the seroepidemiology of maternal and infant colonisation with GBS in the Gambia. The results have gone some way to establish a putative threshold of protection from maternal and therefore infant colonisation throughout the at- risk period for EO- and LO-GBS disease by demonstrating the inverse relationship between functional antibody concentration and maternal and infant colonisation. The next step would include the development of a widely available panel of international standard sera and the standardisation of assay protocols between laboratories and between studies. The value of standardising protocols and reference sera has already been demonstrated in the evaluation of several vaccines.(188, 234-236)

It may be possible to achieve licensure of GBS vaccines in the next few years if standardised protocols for an anti-GBS CPS IgG ELISA quantification assay and functional assays (OPkA/CDA) and freely available standardised reference sera are developed between all participating academic and commercial organisations. This will enable seroepidemiological studies to reliably describe thresholds of protection against invasive GBS disease.(147) I have recently initiated a large multinational consortium of academia, industry, WHO and regulatory authorities to develop standardized protocols for quantitative and functional GBS assays which is currently under review with the Bill and Melinda Gates Foundation and for which I am the principle investigator. Using colonisation as a surrogate marker for protection from invasive disease may provide a useful adjunct to these studies and might replace the need for large efficacy trials in the future, once validated in the field.

In addition the use of whole genome sequencing of maternal/infant strains will allow us to better understand the host-pathogen interaction and will provide more information on difference virulence between colonising and disease-causing strains. I have recently begun a collaboration with the Wellcome Trust Sanger Institute to undertake whole genome sequencing of the colonising isolates isolated in this study.

8.5. Concluding remarks

In summary, I have shown that antibody-mediated C3b/iC3b deposition is inversely related to infant colonisation at birth and to clearance of colonisation up to 89 days of life. I have identified that mothers are more likely to transfer GBS to their infants if functional cord antibody is low and that infants are then more likely to

become colonised postnatally themselves.

I have identified differences in functional antibody concentration against the different GBS STs associated with disease and colonisation. This warrants further research and may hold the key to the rapid development and implementation of effective vaccines for the prevention of neonatal GBS disease, which remain a major goal to decrease morbidity and mortality during the most vulnerable period of life.

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Appendix 1 – Ethical approvals

Scientific Coordinating Committee
MRC Unit: The Gambia, Fajara
PO Box 273 Banjul, The Gambia
West Africa
Switchboard (+220) 4495442/6 Ext 2308
Fax (+220) 4495919/4496513
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Intranet: http://mrcportal/Committees/SCC/SitePages/Home.aspx
Webpage:https://mrcportal.mrc.gm/Committees/SCC/SitePages/Home.aspx

7 February 2014

Professor Beate Kampmann
Vaccinology Theme Leader
MRC Unit, The Gambia
Fajara

Dear Professor Kampmann

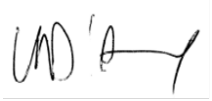
L2014.08v2, Re SCC 1350v3: Group-B streptococci in pregnant women and their infants in the Gambia- A fresh look at prevalence, serotypes and antibody is required

Thank you for submitting your response letter dated 6 February 2014 addressing the issues raised by the SCC at its meeting held on 3 February 2014.

I am pleased to approve your request which will be forwarded to the Ethics Committee for consideration at its meeting on 28 February 2014.

With best wishes

Yours sincerely



Professor Umberto D'Alessandro
Chairman, Scientific Coordinating Committee

Additional documents submitted for review:-

- Consent Form (adult), version 2.0 – 27 January 2014
- Informed Consent Document (child), version 3.0 – 8 January 2014
- SCC Application Form: SCC 1350

The Gambia Government/MRC Joint
ETHICS COMMITTEE

C/o MRC Unit: The Gambia, Fajara
P.O. Box 273, Banjul
The Gambia, West Africa
Fax: +220 – 4495919 or 4496513
Tel: +220 – 4495442-6 Ext. 2308
Email: ethics@mrc.gm

11 March 2014

Professor Beate Kampmann
Vaccinology Theme Leader
MRC Unit, The Gambia
Fajara

Dear Professor Kampmann

L2014.08, Re SCC 1350v3: Group-B streptococci in pregnant women and their infants in the Gambia- A fresh look at prevalence, serotypes and antibody is required

Thank you for submitting your letters dated 21 January and 6 February 2014 for consideration by the Gambia Government/MRC Joint Ethics Committee at its meeting held on 28 February 2014.

We are pleased to approve your request.

With best wishes

Yours sincerely

Mr Malamin Sonko
Chairman, Gambia Government/MRC Joint Ethics Committee

Documents submitted for review:-

- Consent Form (adult), version 2.0 – 27 January 2014
- Informed Consent Document (child), version 3.0 – 8 January 2014
- SCC Application Form: SCC 1350

The Gambia Government/MRC Joint Ethics Committee:

Mr Malamin Sonko, Chairman
Professor Ousman Nyan, Scientific Advisor
Ms Naffie Jobe, Secretary
Mrs Tulai Jawara-Ceesay
Dr Ahmadou Lamin Samateh
Dr Roddie Cole
Prof. Umberto D'Alessandro
Dr Stephen Howie
Dr Kalifa Bojang
Dr Ramatoulie Njie
Dr Momodou L. Waggeh
Dr Siga Fatima Jagne

Appendix 2 – Patient information sheet and consent forms

Identification code: SOP-CTS-015, version 3.0
Attachment 2c (Child CT)

PARTICIPANT INFORMATION SHEET

Version 3 Date 08/01/14

Study Title: Group-B streptococci in pregnant women and their infants in the Gambia- A fresh look at prevalence, serotypes and antibody is required

SCC:	1350v3	Protocol:	

Sponsor:

What is informed consent?

You and your child are invited to take part in a research study. Before you decide, you need to understand why the research study is being done and what it will involve. Please take time to read the following information or get the information explained to you in your language. Listen carefully and feel free to ask if there is anything that is not clear or you do not understand. You may also wish to consult your spouse, family members, friends or others before deciding to take part in the study.

If you decide that you and your child join the study, you will need to sign or put a thumbprint on a consent form saying you agree for both of you to be in the study. You will receive a copy of the signed consent form.

Why is this study being done?

Group-B Streptococcal (GBS) is a bacterium which is often carried by healthy women and in a very small percentage can cause death and sickness in their babies. The illness could be prevented by giving a vaccine to pregnant women. The idea of vaccinating the mother is so that she can pass on the protection (antibody) during the pregnancy to the baby, just like we already do with tetanus vaccination. A vaccine is currently being developed but we do not yet know if this vaccine will be useful in The Gambia because we do not know which serotype of GBS are most common in The Gambia.

This project aims to understand what type of GBS is most commonly carried by pregnant women in The Gambia and what antibody Gambian mothers already make and transmit to their infants in their blood and breastmilk, even without the vaccine being available.

The results of the study will be made available to your community.

What does this study involve?

If you decide to take part, we will ask you to deliver your baby at Faji Kunda Health Centre. At birth we will ask you to take swabs from your vagina and rectum when you are in labour and to take swabs from your baby's mouth and rectum when they have been born. We will also ask for a

SCC:		

sample of cord blood to be taken by our team at the time of birth and a small sample of your breastmilk, which you can express to look for antibodies. If you are not aware of your HIV status, we need you to take a blood test to check this. Before we do the test, it will be fully explained to you. If you do not want to do an HIV test, we will not force you to take a test. If you do take the test, you will be told the result and given advice about the meaning of it. If you are found to be HIV infected, your baby will not be able to continue in the study. You will be referred to a government approved clinic for treatment, and your baby will also be tested for the infection. The study also involves you agreeing to attend the Faji Kunda Health Centre for an appointment at around 1 week and 2-3 months afterwards.

We will ask you to attend the Faji Kunda MRC Clinic at around 1 week to check you and your baby's health and to take swabs from your baby's mouth and rectum and some breastmilk from you.

We will then ask you to attend the Faji Kunda MRC Clinic again when your baby is 2-3 months old and at the time of the first normal vaccinations. At that time we would take another set of swabs from your baby's mouth and rectum and a small blood sample from your baby and another sample of your breastmilk.

In case the investigator discovers that you or your child is sick and decides that either of you cannot participate in the study because of that, you (or your child) will receive immediate care at the study site and then be referred to the appropriate health facility.

If the research study needs to be stopped, you will be informed and your child will have the normal medical care.

What will happen to the samples taken in this study?

We will test the swabs we take from you and your baby to test for GBS and other bacteria.

We will use the blood and breastmilk samples to test for antibodies to GBS that might protect your baby from infection. We might also wish to save some blood for tests of other antibodies against bacteria or viruses against which vaccines are currently being developed and some might be useful also for pregnant women in the future. The samples may also be used in laboratories of other organisation that might include vaccine companies in order to check our results.

What harm or discomfort can you expect in the study?

We aim to minimise any discomfort you or your baby may experience during the study. Taking a rectovaginal swab is not painful and should take only a few seconds. We will ask you to express a small amount of breastmilk (5ml) at each visit.

We will take a blood test from your baby at 2-3 months (less than a teaspoon full).

What benefits can you expect in the study?

At the study visits you will have the opportunity to discuss any concerns about your baby's health. We hope that what we find out in this study will help to stop babies in The Gambia and other parts of the world from becoming sick in the future.

Version		Date	

SCC:		

Will you be compensated for your child's/ward's participation in the study?

You will not get paid for the participation of yourself or your child in the study, but you will get either transport by MRC or get your transport costs to the clinic for each study visit reimbursed.

Are there other products or treatment?

We are not offering any treatment.

What happens if you refuse to participate in the study or change your mind later?

You are free to let your child and yourself participate or not in the study and you have the right to stop participating at anytime without giving a reason. This will not affect the medical care that you or your child would normally receive.

In case you decide to withdraw yourself or your child's participation during the study we will not work on any samples without your permission, but any information already generated from the samples will be kept.

Should any new information become available during the study that may affect your own or your child's participation, you will be informed as soon as possible.

What compensation will be available if you or your child are injured during the study?

We will be responsible to provide for treatment caused by the research study. If medical treatment is required as an emergency, please refer to your health centre or clinic and contact the field worker who gave his/her telephone number to you or contact [Name] on [Phone number].

How will you/your baby's information be kept and who will be allowed to see it?

All information that is collected about you and your baby in the course of the study will be kept strictly confidential. You and your baby's personal information will only be available to the study team members and might be seen by some rightful persons from the Ethics Committee, Government authorities and sponsor.

Who should you contact if you have questions?

Please feel free to ask any question you might have about the research study. If you have any queries or concerns you can contact Dr Kirsty Le Doare on [Phone number], and you can always call the study staff. We will give you their telephone numbers on a card for you to keep. If you are unable to attend the clinic on the designated times then please call the Sukuta Health on [Phone number], and inform the person on duty. The field staff at the MRC Sukuta clinic will then try and arrange for blood to be collected at an alternative time.

Who has reviewed this study?

This study has been reviewed and approved by a panel of scientists at the Medical Research Council and the Gambia Government/MRC Joint Ethics Committee, which consists of scientists and lay persons to protect the rights and wellbeing of you and your baby.

Version		Date	

SCC:	

CONSENT / ASSENT FORM

Participant's Name _____

Participant's Identification Number: |__|__|__|__|__|__|__|__|__|__|

I, _____ **OR** _____
(Printed name of parent) (Printed name of guardian)

☐ have read the written information **OR**

☐ have had the information explained to me by study personnel in a language that I understand and

- confirm that my choice to let my baby participate is entirely voluntarily
- confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided,
- understand that I grant access to data about my baby to authorised persons described in the information sheet,
- have had time to consider to let my baby take part in this study.
- confirm that I agree to have stored samples of blood from my baby used for future antibody research
- confirm that I agree that samples taken from my baby may be shipped to countries outside the Gambia for future study
-

Tick as appropriate

I agree to allow my child take part in this study Yes ☐ No ☐

I agree to further research on my child's samples as described in the information sheet Yes ☐ No ☐

Participant/parent/guardian signature/thumbprint* _____

Date (dd/mmm/yyyy) Time (24hr)

Printed name of impartial witness* _____

Signature of impartial witness* _____ _____

Version		Date	

SCC:		

Date (dd/mmm/yyyy) Time (24hr)

Printed Name of Person obtaining consent

I attest that I have explained the study information accurately in _____ to, and was understood to the best of my knowledge by, the participant/parent/guardian and that he/she has freely given consent to participate *in the presence of the above named impartial witness (where applicable).

Signature of Person obtaining consent

Date (dd/mmm/yyyy) Time (24hr)

**Only required if the participant is unable to read or write.*
A copy of this informed consent document has been provided for the participant.

CONSENT FORM

Participant Identification Number: |__|__|__|__|__|__|__|__|__|__|__|__|__|__|

I, _____
(Printed name of participant)

☐ have read the written information **OR**

☐ have had the information explained to me by study personnel in a language that I understand and

- confirm that my choice to participate is entirely voluntarily,
- confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided,
- understand that I grant access to data about me to authorised persons described in the information sheet,
- confirm that I agree to samples taken during this study being taken outside of the Gambia for further study
- have received time to consider to take part in this study.

Tick as appropriate

I agree to take part in this study	Yes ☐	No ☐
I agree to have an HIV test	Yes ☐	No ☐
I agree to have a rectovaginal swab taken when I am in labour	Yes ☐	No ☐
I agree to further research on my samples as described in the information sheet	Yes ☐	No ☐

Participant's signature/ thumbprint* _____

_____
Date (dd/mmm/yyyy) Time (24hr)

Printed name of witness* _____

Printed name of person obtaining consent _____

I attest that I have explained the study information accurately in _____ to, and was understood to the best of my knowledge by, the participant. He/she has freely given consent to participate *in the presence of the above named witness (where applicable).

Signature of person obtaining consent _____

_____
Date (dd/mmm/yyyy) Time (24hr)

** Only required if the participant is unable to read or write.*

Appendix 3 – Case report forms

SCC:	1350

Imperial College London

MRC The Gambia Unit

GAMI Study Visit 1 (Birth) – Case report form (CRF)

NB/ COMPLETE THIS FORM BEFORE TAKING SWABS AND CORD BLOOD

Study Information

Subject ID: **T I___I___I___I**

Today's Date: I___I ___I / I___I___I/ I___I___I

Time of visit (24h): I___I ___I : I___I___I

Mother's name:

Last *First*

Study mother medical information

	(Tick yes or no)	If no give details:
Was the HIV rapid test done after delivery? *(NB. There is no need to document HIV test results from during the pregnancy or earlier)*	Yes I___I No I___I	

Please document HIV rapid test result	Yes I___I No I___	

	SO (negative):	I___I
	S1 (HIV1+ve):	I___I
	S2 (HIV2 +ve):	I___I

If HIV rapid test indicates a possible +ve status, if not already done so, take mother's blood for serology testing. Send sample to MRC lab as URGENT. Continue to proceed to complete the study visit activities, and review serology result at next visit.

	(Tick yes or no)	
Was HIV serology testing done after delivery?	Yes I___I No I___I	
HIV serology result	SO (negative):	I___I
	S1 (HIV1+ve):	I___I
	S2 (HIV2 +ve):	I___I

SCC:	1350

Imperial College London

MRC The Gambia Unit

Study infant medical information

Feeding history	(Tick yes or no)	If no give details:
Is the infant feeding well?	Yes I___I No I___I	
Please indicate the feeding type:	Exclusive breastfeeds I___I Formula milk I___I Please specify which formula if known…… Mixed breast and bottle feeds I___I Expressed breast milk (EBM) by bottle I___I Supplemental water I___I Other supplemental feed I___I Please specify……	

History of infectious episodes	(Tick yes or no)	If yes give details:
Is the umbilical stump red inflamed discharging or offensive smelling?	Yes I___I No I___I	
Is the infant jaundiced?	Yes I___I No I___I	
Symptoms or signs of significant illness or infection (Fever, hypothermia, poor feeding, floppiness, hypoglycaemia)?	Yes I___I No I___I	
Any admission to hospital in the past 1 –week?	Yes I___I No I___I	
Any episodes of the folllowing?:		
Malaria	Yes I___I No I___I	
Septicaemia	Yes I___I No I___I	
Pneumonia	Yes I___I No I___I	
Meningitis	Yes I___I No I___I	
Worms	Yes I___I No I___I	
Diarrhoea	Yes I___I No I___I	
Otitis media	Yes I___I No I___I	
Tonsilitis	Yes I___I No I___I	
Upper respiratory tract infection	Yes I___I No I___I	
Any intravenous or oral antibiotics therapy?	Yes I___I No I___I	

SCC: 1350

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MRC The Gambia Unit

Study infant anthropometry

Weight (gms): |__|__|__|__|__|

Length (cm): |__|__|__|.|__|

Head circumference (cm): |__|__|__|.|__|

Study infant vital signs

Respiratory rate (breaths per minute) |__|__|__|__|

Heart rate (beats per minute) |__|__|__|__|

Oxygen saturations (%): |__|__|__|__|

Temperature (^{o}C) |__|__|__|.|__|

If the vital signs are abnormal the infant should be reviewed by the study clinician

Eligibility assessment for continued enrolment

In order to continue to be eligible for inclusion in the study, the mother and infant must meet the inclusion criteria and none of the exclusion criteria.

	Inclusion criteria	**Inclusion criteria met? (Tick yes or no)**
1.	Ability to comply with the study procedures as judged by a member of the research team?	**Yes** |__|__| **No** |__|__|

	Exclusion criteria	**Exclusion criteria met? (Tick yes or no)**
1.	Significant congenital abnormalities?	**Yes** |__|__| **No** |__|__|
2.	Mother HIV positive on rapid test AND confirmed on serology? *(Study clinician will inform you of a positive test result)*	**Yes** |__|__| **No** |__|__|
3.	Symptoms or signs of significant illness or infection (Fever, hypothermia, poor feeding, floppy, hypoglycaemia)?	**Yes** |__|__| **No** |__|__|
4.	Planning to move from the area within the next 2 months that would mean she cannot attend clinic	**Yes** |__|__| **No** |__|__|
5.	Enrollment in other studies requiring blood or breast milk sampling?	**Yes** |__|__| **No** |__|__|
6.	Any other condition or situation meaning that participation would not be in the best interests of the infants?	**Yes** |__|__| **No** |__|__|
7	Any reason that would prevent the study endpoints being assessed in the infant effectively as judged by the investigator?	**Yes** |__|__| **No** |__|__|

Do any of the vital signs or examination findings exclude the infant from the study according to the criteria above?	**Yes I___I No I___I** ***If yes, do not proceed to taking nasopharyngeal and rectal swabs. If in doubt discuss the findings with study clinician***

Is the subject eligible for continued enrolment?	**Yes I___I No I___I** ***If yes, proceed to rectal and nasopharyngeal swab taking and obtain a breast milk sample from the mother*** ***If no, do not proceed to swab the baby & discuss with study clinician*** ***Give an appointment for visit 2***
Is the child aged 1-2days of age?	**Yes I___I No I___I** **If no, do NOT proceed to swab the baby.**

Individual completing form

Name: _____
Last *First*

Signature: _____

Appendix 4 – Standard operating procedures for field samples

1. Standard operating procedure 3 – breast milk collection and processing
2. Standard operating procedure 4 – collection of cord blood
3. Standard operating procedure 5 – nasopharyngeal and rectal swab collection
4. Standard operating procedure 6 – rectovaginal swab collection

SCC: 1350

Imperial College London

MRC The Gambia Unit

GAMI Study

Standard Operating Procedure 3.

SOP Title:	Breast Milk Collection and processing
SOP Version:	02
Category:	Clinical
Prepared by:	Dr Kirsty Le Doare, WT Clinical Research Fellow
Signature and date:	
Reviewed by:	Dr Ed Clarke, Head of Infant Immunology
Signature and date:	
Amended from:	
Date effective:	14 November 2013

1. BACKGROUND

According to GAMI study protocol (dated 14 Jan 2014), 2-5 mls of breast milk will be collected from mothers who have given verbal consent to participate in the study.

2. PURPOSE AND SCOPE

This standard operating procedure (SOP) describes the process of collection of breast milk from mothers who have given consent to participate in this longitudinal study.

The aim of breast milk collection is to explore the role of antibody in protecting newborn infants from colonisation with Group B streptococcus.

This SOP applies to the study nurses, government midwives, and study ciinicians involved in this procedure.

3. ABBREVIATIONS

MRC	Medical Research Council; representing MRC Unit The Gambia
SOP	Standard Operating Procedure

4. RESPONSIBLE PERSONS

The study team, including the government midwives identify pregnant women at the labour ward of Faji Kunda health centre who meet the study inclusion criteria. A study information sheet is provided and explained. The woman is advised that full written consent will be sought by the study team after she has recovered suitably from the delivery should she wish to take part.

5. PROCEDURE

5.1. Confirm that written consent is given for breast milk sample to be taken and stored.

5.2. Explain the procedure to the mother.

5.3. Make available properly labeled breast milk specimen containers as follows: 2 x clear universal containers. (These containers are available on the labour ward in pre-prepared packs. All containers are marked with a black line to indicated the fill level)

5.4. As the mother to wash her hands and provide sterile cotton wool and 0.02% chlorhexidine to the mother.

5.5. Show the mother how to clean the nipple area and areola of the breast from which milk will be taken.

5.6. Ask the mother to express milk from each breast into separate containers.

5.7. Ask her to manually express her milk. Remove the first 0.5ml that will be introduced on a tissue and discarded appropriately. Ask her to directly express into the sterile plastic tube (universal container) to collect approximately 5mls and place in a cold box.

5.8. Put a sticker on the laboratory form, the corresponding CRF section and the tube.

5.9. Complete the *field sample log* sheet kept at the clinic.

5.10. Ensure that the specimen remains on wet ice in the cold box and is transported to the lab within six hours of collection to the designated laboratory staff. Complete the appropriate *sample log book* kept at the laboratory.

5.11. Thank the mother and the attending midwife/midwives.

6. LABORATORY HANDLING

6.1. Store breast milk in the fridge and process at time of delivery.

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6.2 Following receipt of the completed Breast milk sample from the Faji Kunda Health Centre team, take the right breast samples and centrifuge for 30 mins at 3200 rpm. Remove the fat layer with a scalpel and store separately.
6.3. Aliqout Whey into 1mL aliquots and label tubes.
6.4. Freeze at -70 degrees.
6.5. Record the freezer location in the Sample log book, sign and date the record.
6.6. Take the left breast and dilute 5mL breastmilk in 5mL sterile phosphate buffered saline (PBS)
6.7. Centrifuge at 805g for 20 minutes at 20°C.
6.8. Remove the fat layer with a scalpel.
6.9. Wash the cell pellet in 5mL PBS and recentrifuge. Then repeat this wash step.
6.10. Resuspend in 7% fetal bovine serum in PBS.
6.11. Count cells using a hemocytometer.
6.12. Freeze immediately at -70°C.

7. REFERENCES

SCC 1350 – Feb 2014

- Study Protocol - Group-B Streptococcal Antibody in Mothers and Infants (GAMI) January 2014

8. ATTACHMENTS

- Participant Information Sheet and consent form (English), version 1.0
- Breast Milk collection form

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SCC:	1350

Imperial College London

MRC The Gambia Unit

GAMI Study

Standard Operating Procedure 4.

SOP Title:	Cord blood Collection
SOP Version:	01
Category:	Clinical
Prepared by:	Dr Kirsty Le Doare, Clinical Research Fellow
Signature and date:	
Reviewed by:	Dr Ed Clarke, Head of Infant Immunology
Signature and date:	
Amended from:	SOP by Aderonke Odutola for study protocol SCC 1173 (Dr Martin Ota, Principal Investigator)
Date effective:	14 November 2013

1. BACKGROUND

According to GAMI study protocol (dated 14 Jan 2014), 5 mls of cord blood will be collected during delivery from the newborn of mothers who have given verbal consent to participate in the study at the Faji Kunda Health centre.

2. PURPOSE AND SCOPE

This standard operating procedure (SOP) describes the process of collection of cord blood from newborns of mothers who have given consent to participate in this longitudinal study.

The aim of cord blood collection is to explore the role of antibody in protecting newborn infants from colonisation with Group B streptococcus.

This SOP applies to the study nurses, government midwives, and study ciinicians involved in this procedure.

3. ABBREVIATIONS

MRC	Medical Research Council; representing MRC Unit The Gambia
SOP	Standard Operating Procedure

4. RESPONSIBLE PERSONS

The study team, including the government midwives identify pregnant women at the labour ward of Faji Kunda health centre who meet the study inclusion criteria. A study information sheet is provided and explained. The woman is advised that full written consent will be sought by the study team after she has recovered suitably from the delivery. The cord blood sample will be stored safely, but discarded should she decide not to partake in the study.

5. PROCEDURE

5.1 Confirm that verbal consent is given for cord blood sample to be taken and stored.

5.2 Confirm the delivery is a potentially uncomplicated (not high-risk) case.

5.3 Explain to the pregnant woman in labour what you are about to do and remind her the reason for collecting the cord blood.

5.4 Make available properly labeled blood sample tubes as follows: 2 x yellow top. (These tubes are available on the labour ward in pre-prepared packs. All tubes are marked with a black line to indicated the fill level)

5.5 Make available sterile alcohol swabs and a 10ml syringe with a green needle.

5.6 Put on a pair of sterile gloves as soon as delivery of the baby is imminent.

5.7 Immediately following the delivery of the baby but before the placenta separates, identify the large thin-walled opening of umbilical vein lying superiorly.

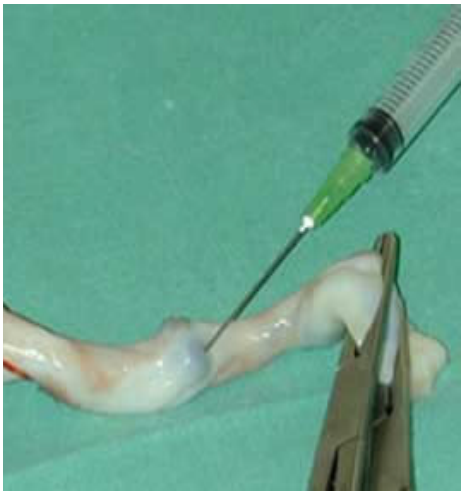
5.8 Remove any used gloves and put on a pair of new gloves.

5.9 Ensure that the cord is lying low between the mother's legs to avoid back flow of blood.

5.10 Clean cord properly with alcohol swab (or gauze soaked in spirit) and allow to dry. This is to avoid contamination e.g. with maternal blood.

5.11 Puncture the umbilical vein close to the positioned clamp (**this is to allow for repositioning of clamp in the event of significant bleeding after venepuncture**) on the portion of the cord attached to the placenta with a sterile/new needle and syringe and withdraw about 10mls of blood.

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5.12 Put blood into tubes up to the black *"fill line"* in the following order: yellow top.



5.13 If significant bleeding from venepuncture site reposition clamp in front of the site or asked attending midwife to if other than you.

5.14 Store blood in the provided cool box.

5.15 Transport the cord blood in a cold box (packed with ice-packs) within 6 hours of collection to the infant immunology laboratory at MRC, Fajara

5.16 Complete the study cord blood collection form completely leaving no blank space.

5.17 Thank the mother and the attending midwife/midwives.

6. LABORATORY PROCESSING

6.1. Centrifuge at 1000g for 10 minutes.
6.2. Aliquot the separated serum into 1mL aliquots.
6.2a Correctly label each tube and date.
6.3. Freeze at -70 degrees C.
6.4. Record the freezer location in the sample log book, sign and date the record.

7. REFERENCES

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- Study Protocol - Group-B Streptococcal Antibody in Mothers and Infants (GAMI) January 2014

8. ATTACHMENTS

- Participant Information Sheet and consent form (English), version 1.0
- Cord blood collection form

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GAMI Study

Standard Operating Procedure 5.

SOP Title:	Nasopharyngeal and rectal swab collection
SOP Version:	01
Category:	Clinical
Prepared by:	Dr Kirsty Le Doare, Clinical Research Fellow
Signature and date:	
Reviewed by:	Dr Ed Clarke, Head of Infant Immunology
Signature and date:	
Amended from:	SOP by Dr Brenda Kwambana and approved by Dr Martin Antonio
Date effective:	1 March 2014

1. BACKGROUND

According to GAMI study protocol (dated 14 Jan 2014), nasopharyngeal and rectal swabs will be taken from infants during the first 3 months of life. It has been suggested that GBS initially colonizes the infant's oropharyngeal mucosa when contact with maternal vaginal secretions occur at the time of birth (1). Group-B Streptococcus is an enteric pathogen and it is thought that gut translocation is the primary route for invasive disease in the newborn period. Although the specific mechanisms are poorly understood, attachment to the nasopharyngeal epithelial surface and gut mucosal surface is thought to be an essential step in the development of mucosal and invasive disease [2,3]. Hence, sampling of the nasopharynx and rectum can provide invaluable insights into the epidemiology and mechanisms of pathogenicity of GBS.

Purpose and Scope

This standard operating procedure (SOP) describes the process of collection of nasopharyngeal and rectal swabs from infants of mothers who have given consent to participate in this longitudinal study.

2. ABBREVIATIONS

NPS	Nasopharyngeal swab
RS	Rectal Swab
SOP	Standard Operating Procedure

STGG	Skim milk-tryptone-glucose-glycerol (STGG) transport medium

3. RESPONSIBLE PERSONS

The study team, including the government midwives identify pregnant women at the antenatal clinic or the labour ward of Faji Kunda health centre who meet the study inclusion criteria. A study information sheet is provided and explained. The study team seeks full written consent after the mother has recovered suitably from the delivery. A NPS and RS sample is collected within the first 48 hours of life. Two further NPS and RS samples are collected during the first 3 months of life. All operators must be qualified nurses or healthcare assistants.

4. FIELD PROCEDURE

4.1. Ensure that a clean environment is attained to avoid contaminating the sample.

4.2. Prepare sterile gloves, sterile calcium alginate fiber tip swabs and a 2 vials containing 1ml of STTG medium in each.

4.3. Check that the subject's ID is included in the list of NPS and RS swabs to be taken

4.4. Check that the ID number on the subjects identity card and subject study folder and the ID on the NPS and RS form all coincide. If there is any discrepancy between the ID on any of the records then the field worker should not proceed with the collection of the sample, but consult with the field supervisor/coordinator.

4.5. Explain the procedure to the mother

4.6. Use only calcium alginate fiber tip swab

4.7. Put on a pair of sterile gloves

NPS SWAB:

4.8. Position the infant in a supine position (on their back), with the infant's head furthest away from you, and the infant's legs pointing towards you. If a firm flat surface cannot be sought, this can be done on the knees of the nurse carrying out the procedure (as shown in the figure below). The infant should be briefly swaddled for the short period of the procedure. This will allow the infant to be kept still, and reduce any discomfort.

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Trained field nurse prepares baby B, removes any excess mucus from nose and calms baby.



Nurse collects baby B's NPS sample by carefully inserting the swab in the posterior nasopharynx, waiting 5 seconds and then rotating it 360 degrees before careful removal and immediate placement in STGG



Field worker collects meta data from baby B's mother (or guardian). The baby is also weighed and her height is measured.

Figure 1. Collection of NPS from an infant

4.9. Measure the distance which the swab should be passed, which is about half the distance from the nostril to the ear lobe.

4.10. Tip the infant's head slightly backward and pass the swab directly backwards, parallel to the floor of the nasopharynx. The swab should pass without resistance until it reaches the posterior pharynx.

4.11. If resistance is encountered, remove the swab and pass through the other nostril.

4.12. Once the swab is in place, leave in position for five seconds to saturate the tip before removing it slowly.

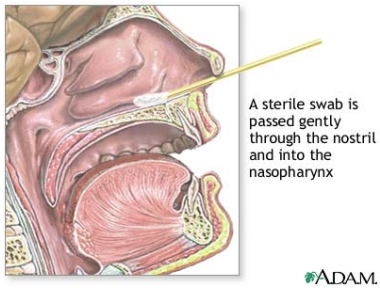


Figure 2. Passage of a sterile swab through the nasal cavity to the nasopharynx

4.13. Place the tip in a vial containing 1ml of STGG transport media. Using a pair of scissors wiped with alcohol, cut off the excess wire handle, leaving the tip in the transport media. Then tighten the cap and label the vial and place on wet ice.

4.14. Dispose what remains of the wire loop into "sharps" box

4.15. Remove any used gloves and dispose of into a yellow clinical waste bag.

4.16. Keep a record of the presence or absence of nasal mucous and whether or not the procedure was adequately carried out.

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4.17. Ensure that the specimen is placed in the cool box provided (containing 2 ice packs) and is transported to the lab within six hours of collection

RS SWAB:

4.18. Position the infant in a supine position (on their back), with the infant's head furthest away from you, and the infant's legs pointing towards you. If a firm flat surface cannot be sought, this can be done on the knees of the nurse carrying out the procedure. The infant should be briefly swaddled for the short period of the procedure. This will allow the infant to be kept still, and reduce any discomfort.

4.19. Hold the infant's legs up and pass the swab directly backward so that the tip is just inside the rectum (about 0.5cm). The swab should pass without resistance.

4.20. If resistance is encountered, remove the swab immediately.

4.21. Once the swab is in place, leave in position for five seconds to saturate the tip before removing it slowly.

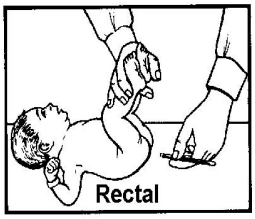


Figure 2. Passage of a sterile swab to the rectum

4.22. Place the tip in a vial containing 1ml of STGG transport media. Using a pair of scissors wiped with alcohol, cut off the excess wire handle, leaving the tip in the transport media. Then tighten the cap and label the vial and place on wet ice.

4.23. Dispose what remains of the wire loop into "sharps" box

4.24. Remove any used gloves and dispose of into a yellow clinical waste bag.

4.25. Keep a record of the presence or absence of faeces/meconium and whether or not the procedure was adequately carried out.

4.26. Ensure that the specimen is placed in the cool box provided (containing 2 ice packs) and is transported to the lab within 90 minutes of collection

4.27. Report any problems such as bleeding or excessive pain (crying in babies) arising during the procedure to the Research Clinician immediately.

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5. LABORATORY PROCEDURES (AT THE MRC FAJARA)

5.1. Prepare STGG transport medium as below:

Oxoid tryptone soya broth	3.0g
Glucose	0.5g
Oxoid skim milk powder	2.0g
Glycerol	10.0g(1.25g=1ml)
Double distilled water	100.0 ml

5.2 Dispense in 1.0 ml amount in Nunc or Nalgene cryotubes and autoclave for 10minutes at 121°C.

5.3 Tighten the lids before storage.

5.4 Store at 4-8°C or room temperature.

5.5 NB/ Prior to use vortex to re-suspend the pellet for a minimum of 10-15 seconds.

5.6 Transfer to Faji Kunda Health Centre team in cool box with 2 ice packs for NPS and RS to be taking according to field procedure.

5.7 Following receipt of the completed NPS and RS sample from the Faji Kunda Health Centre team, vortex the specimen for 10-20 seconds. Aliquot 0.5mL of STGG into a separate cryotube. Correctly label and date each samples and store in −70°C freezer.

5.8 Record the freezer location on the Sample Collection form and sign and date the record.

See appendix 1. for quality control of STGG medium.

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6. APPENDIX 1.

Quality control of STGG medium

Appearance:
- The solution should be tan in colour with a precipitate at the bottom of the tube.

Sterility:
- Select at random 1 vial from each batch. Vortex until the precipitate is suspended and plate 100 µL each on Sheep Blood agar. Incubate overnight at 37 °C in 5% CO2 and examine the plate for any growth. There should be no growth whatsoever. If there is any growth, discard the batch and prepare fresh medium.

Performance:
- Vortex a vial to suspend the precipitate and inoculate it with a representative vaccine strain of pneumococcus. Freeze at -70°Cfor 48 hours. Thaw out specimen at room temperature, vortex again, subculture 100 µL onto GBA plate and streak. Incubate overnight at 37°C in 5% CO2. The organism should show good growth.

7. ATTACHMENTS

- Participant information sheet and consent form (English)

8. REFERENCES

1. Kotiw M, Zhang GW, Daggard G, Reiss-Levy E, Tapsall JW, Numa A. Late-onset and recurrent neonatal Group B streptococcal disease associated with breast-milk transmission. Pediatr Dev Pathol. 2003 May-Jun;6(3):251-6.
2. Beisswenger C, Lysenko ES, Weiser JN. Early bacterial colonization induces toll-like receptor-dependent transforming growth factor beta signaling in the epithelium. Infection and immunity **2009** May;77(5):2212-20.
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GAMI Study

Standard Operating Procedure 6.

SOP Title:	rectovaginal swab collection
SOP Version:	01
Category:	Clinical
Prepared by:	Dr Kirsty Le Doare, Clinical Research Fellow
Signature and date:	
Reviewed by:	Dr Ed Clarke, Head of Infant Immunology
Signature and date:	
Amended from:	SOP by Dr Brenda Kwambana and approved by Dr Martin Antonio
Date effective:	1 March 2014

1. BACKGROUND

According to GAMI study protocol (dated 14 Jan 2014), rectovaginal swabs will be taken from women in labour. Group-B Streptococcus is an enteric pathogen and colonization in the mother is a prerequisite for transfer of colonization to the infant. Yield of bacterial growth is highest when combining rectal and vaginal swabs. Hence, sampling of the vagina and rectum can provide invaluable insights into the epidemiology of GBS.

Purpose and Scope

This standard operating procedure (SOP) describes the process of collection of rectovaginal swabs from mothers who have given consent to participate in this longitudinal study.

2. ABBREVIATIONS

RVS	Rectovaginal Swab
SOP	Standard Operating Procedure
STGG	Skim milk-tryptone-glucose-glycerol (STGG) transport medium

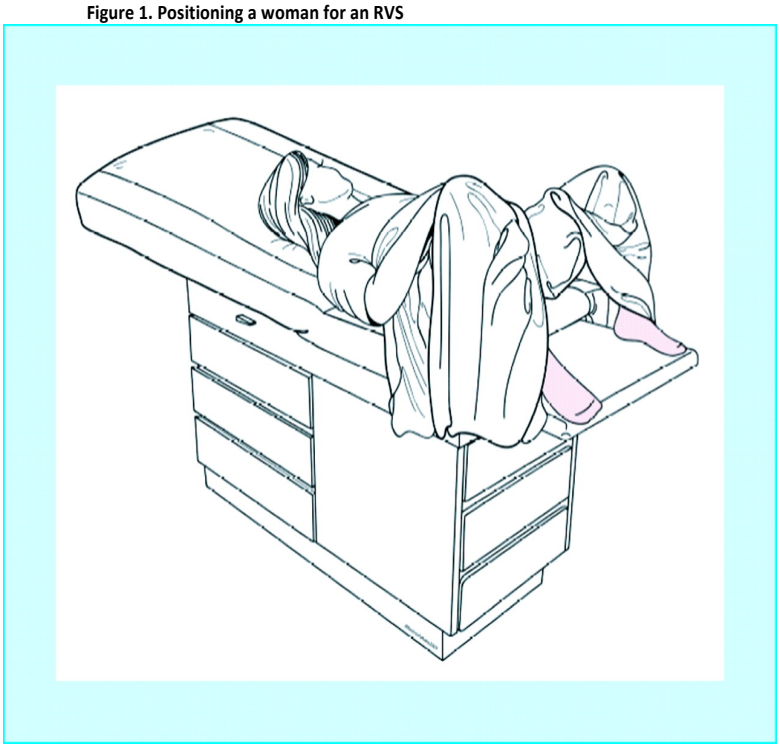
3. RESPONSIBLE PERSONS

The study team, including the government midwives identify pregnant women at the antenatal clinic or the labour ward of Faji Kunda health centre who meet the study inclusion criteria. A study information sheet is provided and explained. The study team seeks full written consent after the mother has recovered suitably from the delivery. An RVS sample is collected prior to delivery. All operators must be qualified nurses or healthcare assistants.

4. FIELD PROCEDURE

4.1. Explain the procedure to the mother.
4.2. Check that the subject's ID is included in the list of RVS swabs to be taken
4.3. Check that the ID number on the subjects identity card and subject study folder and the ID on the RVS form all coincide. If there is any discrepancy between the ID on any of the records then the field worker should not proceed with the collection of the sample, but consult with the field supervisor/coordinator.
4.4. Ensure that a clean environment is attained to avoid contaminating the sample.
4.5. Use only plain swab sticks for RVS.
4.6. Put on a pair of sterile gloves
4.7. Position the mother in a supine position (on her back), with her head furthest away from you with her knees bent and open (Figure 1).

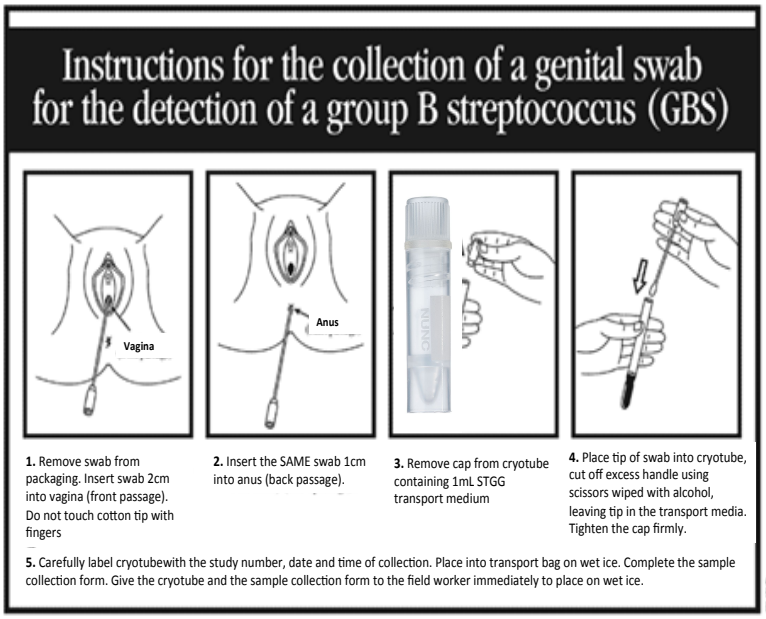
Figure 1. Positioning a woman for an RVS



4.8. Remove the protective covering over the vaginal swab by peeling off the protective wrapping around the swab stick. Do not let your fingers touch the swab.
4.9. Pass the swab into the lower vagina and leave for 5 seconds. Then remove slowly. See Figure 2. Do not let your gloved fingers touch the mother's skin.
4.10. Pass the tip of the same swab into the anus and leave for 5 seconds. Then remove slowly. There should be no resistance. If there is any resistance, remove the swab immediately.

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Figure 2 – taking a combined rectovaginal swab



4.11. Place the tip in a vial containing 1ml of STGG transport media. Using a pair of scissors wiped with alcohol, cut off the excess wire handle, leaving the tip in the transport media. Then tighten the cap and label the vial and place on wet ice.

4.12. Put the sticker on the laboratory form, the corresponding CRF section and the tube.

4.13. Complete the *sample log sheet* kept at the clinic.

4.14. Ensure that the specimen remains on wet ice and is transported to the lab within six hours of collection to the designated staff at the laboratory and complete the *sample log book* kept at the laboratory.

4.15. Dispose what remains of the wire loop into “sharps” box

4.16. Remove any used gloves and dispose of into a yellow clinical waste bag.

4.17. Thank the mother for her co-operation.

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5. LABORATORY PROCEDURES (AT THE MRC FAJARA)

5.1 Prepare STGG transport medium as below:

Oxoid tryptone soya broth		3.0g
Glucose		0.5g
Oxoid skim milk powder	2.0g	
Glycerol		10.0g(1.25g=1ml)
Double distilled water	100.0 ml	

5.2 Dispense in 1.0 ml amount in Nunc or Nalgene cryotubes and autoclave for 10minutes at 121°C.

5.3 Tighten the lids before storage.

5.4 Store at 4-8°C or room temperature.

5.5 NB/ Prior to use vortex to re-suspend the pellet for a minimum of 10-15 seconds.

5.6 Transfer to Faji Kunda Health Centre team in cool box with 2 ice packs for RVS to be taking according to field procedure.

5.7 Following receipt of the completed RVS sample from the Faji Kunda Health Centre team, vortex the specimen for 10-20 seconds, aliquot 0.5mL of STGG from sample into a separate cryovial, correctly label both tubes and store in –70°C freezer.

5.8. Record location of sample in freezer and sign and date the Sample Collection form.

See appendix 1. for quality control of STGG medium.

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6. APPENDIX 1.

Quality control of STGG medium

Appearance:

- The solution should be tan in colour with a precipitate at the bottom of the tube.

Sterility:

- Select at random 1 vial from each batch. Vortex until the precipitate is suspended and plate 100 µL each on Sheep Blood agar. Incubate overnight at 37 °C in 5% CO2 and examine the plate for any growth. There should be no growth whatsoever. If there is any growth, discard the batch and prepare fresh medium.

Performance:

- Vortex a vial to suspend the precipitate and inoculate it with a representative vaccine strain of pneumococcus. Freeze at -70°Cfor 48 hours. Thaw out specimen at room temperature, vortex again, subculture 100 µL onto GBA plate and streak. Incubate overnight at 37°C in 5% CO2. The organism should show good growth.

7. APPENDIX 2.

Sample processing

To prepare 100ml of nutrient broth (Glycerol broth 16%):

- Nutrient broth 84ml
- Glycerol 16ml

Mix well and distribute in 5mls amounts in screw-cap tubes or bottles. Sterilise by autoclave at 115°C for 20 minutes. This medium should be within the range Ph 7.2-7.6 at room temperature. Put in the fridge for longer storage

To perform PCR

- Aliquot 7.5µl to each labelled PCR plate well.
- Add 2.5µl of sample or control to the appropriate tubes/wells (use 2.5µl UV treated water to negative control and do not add anything to the blank).
- Place PCR tubes into the real-time instrument (ABI).

8. ATTACHMENTS

- Participant information sheet and consent form (English)

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9. REFERENCES

1. Kotiw M, Zhang GW, Daggard G, Reiss-Levy E, Tapsall JW, Numa A. Late-onset and recurrent neonatal Group B streptococcal disease associated with breast-milk transmission. Pediatr Dev Pathol. 2003 May-Jun;6(3):251-6.
2. Beisswenger C, Lysenko ES, Weiser JN. Early bacterial colonization induces toll-like receptor-dependent transforming growth factor beta signaling in the epithelium. Infection and immunity **2009** May;77(5):2212-20.
3. Reddy MS, Bernstein JM, Murphy TF, Faden HS. Binding between outer membrane proteins of nontypeable Haemophilus influenzae and human nasopharyngeal mucin. Infection and immunity **1996** Apr;64(4):1477-9.
4. International Conference on Harmonisation of Technical Requirements for the Registratio of Pharmaceutical fo Human Use (ICH). Harmonised Trapartite Guideline for Good Clinical Practice E6 (R1). 1996
5. United Kingdom. British Association of Research Quality Assurance (BARQA). Good Clinical laboratory Practice: GCLP. T Stiles, V Grant and N Mawbey. Third Impression, 2007. BARQA March 2003.

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Appendix 5 – Published papers to date

1. Risk factors for Group B Streptococcus colonization and disease in Gambian women and their infants, Journal of Infection, (2016), Jan 4
2. Placental transfer of anti-group B Streptococcus immunoglobulin G antibody subclasses from HIV-infected and uninfected women to their uninfected infants. AIDS, 2016 Jan 28;30(3):471-5.
3. Anti-Group B streptococcus antibody in infants born to mothers with human immunodeficiency virus (HIV) infection, Vaccine, 2015 29;33 (5) 621-7
4. Antibody-mediated complement C3b/iC3b binding to group B Streptococcus in paired mother and baby serum samples in a refugee population on the Thailand-Myanmar border. Clin Vaccine Immunol. 2015 Mar;22(3):319-26
5. Breast milk and Group B streptococcal infection: vector of transmission or vehicle for protection? Vaccine. 2014 May 30;32(26):3128-32
6. An overview of global Group-B Streptococcal Epidemiology, (2013) Vaccine, 31 (4) D7-D12

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Journal of Infection (2016) xx, 1–12

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Risk factors for Group B Streptococcus colonisation and disease in Gambian women and their infants

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Accepted 24 December 2015
Available online ■ ■ ■

KEYWORDS
Neonatal infection;
Group B Streptococcus;
Vaccines;
Immunity

Summary *Objectives:* To determine risk factors for GBS colonisation in Gambian mothers and in their infants from birth to day 60–89 of age.
Methods: Swabs and breastmilk from mothers/infant pairs were collected and cultured on selective agar. Negative samples were analysed for GBS DNA via real-time PCR. Positive isolates were serotyped using multiplex PCR and gel-agarose electrophoresis.
Results: Seven hundred and fifty women/infant pairs were recruited. 253 women (33.7%) were GBS-colonised at delivery. The predominant serotypes were: V (55%), II (16%), III (10%), Ia (8%) and Ib (8%). 186 infants were colonised (24.8%) at birth, 181 (24.1%) at 6 days and 96 at day 60–89 (14%). Infants born before 34 weeks of gestation and to women with rectovaginal and breastmilk colonisation at delivery had increased odds of GBS colonisation at birth. Season of birth was associated with increased odds of persistent infant GBS colonisation (dry season vs. wet season AOR 2.9; 95% CI 1.6–5.2).
Conclusion: GBS colonisation is common in Gambian women at delivery and in their infants to day 60–89 and is dominated by serotype V. In addition to maternal

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http://dx.doi.org/10.1016/j.jinf.2015.12.014
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Please cite this article in press as: Le Doare K, et al., Risk factors for Group B Streptococcus colonisation and disease in Gambian women and their infants, J Infect (2016), http://dx.doi.org/10.1016/j.jinf.2015.12.014

colonisation, breastmilk and season of birth are important risk factors for infant GBS colonisation.

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Introduction

Group B Streptococcus (GBS) is a leading cause of neonatal infection worldwide and transmission occurs mainly from mother to child during the peripartum period.[1] In the African setting, information on GBS disease prevalence remains sparse.[2] Under-identification and under-reporting of GBS cases and deaths appear likely, especially where it is difficult to access healthcare. Maternal colonisation is the leading risk factor for both early and late onset GBS disease,[1] yet in resource-poor countries little is known about maternal colonisation rates. Little is also known about maternal or infant colonising serotypes in African countries but reports indicate that serotype (ST) V may be important as both a colonising and invasive disease serotype,[3,4] as it was in the USA in the 1990s.[5]

The current leading candidate for maternal vaccination is a trivalent capsular polysaccharide protein conjugate vaccine containing ST Ia, Ib and III which is based on data on invasive disease serotypes in the USA, UK[6] and South Africa.[3] Here, we report the results of a longitudinal prospective cohort study to investigate the prevalence of colonising and invasive disease serotypes of Gambian women and their infants from delivery to three months of age.

Methods

Study design and participants

We undertook a prospective longitudinal cohort study in two government health centres offering antenatal care to women in the Fajara area of costal Gambia, a low-income country with an annual birth rate of 43.1/1000 population, neonatal sepsis rate of 4.42/1000 live births[7] and neonatal mortality rate of 28/1000 live births.[8] The combined birth rate of these two health centres is approximately 12,500 births annually. The health centres were selected to be representative of the level of care usually available to Gambian women.

The eligibility criteria for maternal participation in the study included all pregnant women over the age of 18 years who had a negative HIV test and were deemed to be at low risk for pregnancy complications (no evidence of pre-eclampsia, cardiomyopathy, maternal gestational diabetes, placenta praevia, twin pregnancy). Women were invited to deliver at the health centre and offered a confirmatory HIV test prior to enrolment. Women found to be HIV positive were referred for specialist ongoing care. Mothers were excluded if they were not planning to breastfeed or were unable to remain in the Fajara area for the first three months postpartum. Healthy infants over 32 weeks of gestation assessed using the Ballard score and weighing over 2.5 kg were included. Infants were excluded if they had obvious congenital abnormalities or required resuscitation at the time of delivery requiring transfer to a neonatal unit. Mother and infant pairs were recruited to the study between 1st January 2014 and 31st December 2014 if both mother and infant met the inclusion criteria. All eligible women and infants were recruited until the predefined sample size was reached. Participants were followed up daily at home until day 6 and then asked to return to clinic when the infant 60–89 days old for final follow up visit and vaccinations. If an infant died during the study a verbal autopsy was carried out to assess the potential cause of death.

Ethics statement

Field workers explained the purpose of the study to eligible participants in their local language (Mandinka, Wolof, Fula, Jolla, Mangago) and each participant signed an informed consent form, or in case of illiteracy, thumb-printed and the consent form was signed by an impartial witness. The study was approved by the joint Gambian Government/Medical Research Council Research Ethics Committee, SCC 1350 V4.

Procedures

Sampling

Rectovaginal swabs were taken from enrolled women presenting in labour to one of the health centres and cord blood was taken after delivery but prior to separation of the placenta. A screening questionnaire was completed after four hours postpartum and the infant checked for any abnormalities requiring medical intervention. The questions included ethnic origin, gravida, parity, maternal weight, blood pressure, haemoglobin concentration, use of medication/traditional medicines/antibiotics and vaccination in pregnancy, any illnesses in pregnancy, number of antenatal attendances, HIV status, education, diet, compound location and presence of cattle at the compound. Nasopharyngeal and rectal swabs were taken from all eligible infants at four hours after birth. Mothers were provided with soap and asked to wash their hands and wipe their breasts with alcohol wipes before hand expressing colostrum/milk within the first 12 h after birth, at day 6 and between days 60 and 89. Nasopharyngeal and rectal swabs were also taken from infants at day 6 of life and again at 60–89 days of life. Infants who were unwell before day 6 were assessed at home and referred for treatment as necessary. All sick infants had a blood culture taken on admission to hospital. At each visit a standardized questionnaire was completed in the local language documenting infant anthropometry, feeding, vaccinations, signs and symptoms of infant illnesses, use of antibiotic/traditional medicine and vital signs.

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Laboratory procedures

Copan (for rectal and rectovaginal samples) and Dacron (for nasopharyngeal samples) swabs were collected in skim-milk tryptone glucose glycerol (STGG) transport medium, stored at 4 °C and transported to the Medical Research Council laboratories, The Gambia within 4 h of collection. On arrival the samples were vortexed briefly and immediately frozen at −70 °C until processing.

All swab specimens were inoculated into Todd−Hewitt broth supplemented with colistin and nalidixic acid and were processed for isolation of GBS using standard laboratory procedures.[1] Presumptive positive GBS samples were identified by latex agglutination (Oxoid). Five colonies from positive samples were harvested into phosphate buffered saline (PBS) and subjected to real-time polymerase chain reaction (PCR).[9] Negative samples were also subjected to confirmation by real-time PCR. All GBS positive isolates were then serotyped using conventional PCR and identified using gel PCR and agarose electrophoresis.[10]

Outcomes

The primary outcome was prevalence of ST-specific GBS colonisation in mothers and infants at birth, six days and between days 60−89 using microbiological culture and molecular techniques. Secondary outcomes were detection of GBS in breastmilk; infant acquisition and loss of GBS colonisation during the study follow up period and infant GBS disease (sample obtained from sterile site), as ascertained by positive microbiological culture and confirmatory PCR. Swabs were considered negative if no GBS was evident by culture and PCR and positive if GBS was found on culture and PCR. If swabs were negative on culture but positive on PCR, conventional PCR was performed to determine serotype. If the second PCR resulted in the identification of a GBS serotype the samples were deemed positive. If no serotype was identified or the DNA did not amplify, samples were deemed negative.

Statistical analysis

Calculated on the basis of the previously observed 24% colonisation rate,[11] the intended sample size was 750 mothers, to provide at least 180 colonised women for serotype analysis (95% confidence interval (CI) 150−202 women) and 90 colonised infants (95% CI 72−107 infants). The sample size of 180 colonised women was chosen to ensure at least 10 samples of the least prevalent ST based on historical data from The Gambia (ST III (6%)),[11] in order to allow longitudinal colonisation analysis.

Statistical analyses were completed using STATA version 12 (StataCorp 2013, Texas) and GraphPad Prism version 6.0 (GraphPad Software Inc, La Jolla, California). Descriptive statistics included the prevalence of colonisation at individual time points expressed as a proportion of the total number of participants. Odds ratios and 95% confidence intervals (CI) were calculated to determine risk of maternal and infant colonisation at birth in a single variable analysis. Adjusted odds ratios were then calculated using any variables from the single variable analysis with a p-value <0.2 using a backwards-stepwise procedure.

Analyses of the changes in infant colonisation over time were undertaken using longitudinal logistical regression. Adjusted odds ratios were then calculated using any variables from the single variable analysis with a p-value <0.2 using a backwards-stepwise procedure. New acquisition of GBS was defined as detection of a new serotype by culture or PCR that was not previously present. Clearance of colonisation was defined as a negative GBS culture or PCR for a specific serotype following a positive sample at the previous visit for the same ST. The log-rank test was used to examine differences in duration of colonisation between serotypes. Using an expected vertical transmission rate of 50%[1] we calculated observed vs. expected statistics for risk of infant colonisation by ST. For comparison between our study and the study conducted in 1994, we calculated 95% confidence intervals for both studies. P-values <0.05 were considered significant.

Role of the funding source

The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all the data and the corresponding author had final responsibility for the decision to submit for publication.

Results

Demographics

Between 15th January 2014 and 31st January 2015 we recruited a total of 750 mothers and their infants to the study. All mothers met the inclusion criteria except one, who was admitted to hospital on day three post-partum severely unwell and subsequently tested positive for HIV1, despite a negative test at 20 weeks of gestation. She and her infant were referred for further management and samples excluded from further analysis. Of the remaining eligible women and infants, 722 completed the day 6 visit (median age at follow up 8 days, IQR 7−9) and 684 completed 60−89 days follow up (median age at follow up 62 days, IQR 60−63). The main reasons for not completing follow up were: 31 women moved out of area, 20 women whose partners were not in agreement with continued study participation, one maternal HIV, nine infant deaths, five declined for other reasons. Participant flow diagram and demographic characteristics of mother−infant pairs are outlined in Supplementary Fig. 1.

Antibiotic treatment

Over the course of the study, 77 women received antimicrobials during pregnancy: 34 for urinary tract infection; 10 for pneumonia; 14 for vaginal discharge; 9 for malaria and 10 for non-malarial fever. Of these, 73 women received amoxicillin (median time prior to delivery 42 days [IQR 18−63 days]); 16 received antimalarial therapy (median 38 [31−41]) and three received erythromycin. In the three months of follow up, 51 infants required antibiotic therapy: 34 received ampicillin and gentamicin for presumed neonatal sepsis (median 18 days old [IQR 4−34]); 10 received amoxicillin or cloxacillin for skin infections (20 days [10−58]); 7 received other antibiotics (18 days [11−23]). Antibiotic therapy was included in adjusted analyses and had no impact on colonisation of mother or infant at any time point.

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Table 1 Mother and infant demographics and odds ratios for maternal GBS colonisation at delivery.

		Total	Colonised	Non-colonised	p-value	OR (95% CI)
Maternal	<20	110	40	70	0.78	1
age yrs	20–24	262	81	181		0.78 (0.5–1.3)
Mean: 25	25–29	203	71	132		0.94 (0.6–1.5)
(IQR 18–41)	30+	175	61	114		0.99 (0.6–1.7)
Ethnicity	Mandinka	315	115	200	0.38	1
	Wolof	108	36	72		0.9 (0.5–1.4)
	Fula	126	45	81		1 (0.6–1.5)
	Jola	118	33	85		0.7 (0.4–1.0)
	Other	81	23	58		0.7 (0.4–1.2)
	Missing	2	1	1		
Maternal	<50	27	7	20	0.03*	1
weight (kg)	50–70	424	128	296		1.2 (0.5–3.0)
	70–90	148	61	87		2.0 (0.8–5.0)
	>90	145	56	91		1.8 (0.7–4.4)
	Missing	6	3	3		
Maternal illness	Yes	128	45	83	0.32	1
in pregnancy	No	504	165	339		1.1 (0.7–1.7)
	Missing	118	43	75		
Maternal Hb below	No	99	42	57	0.04*	1
10 g/dL	Yes	651	211	440		0.5 (0.3–0.9)
Maternal antibiotics	No	505	166	339	0.74	1
in pregnancy	Yes	128	44	84		1.1 (0.7–1.6)
	Missing	117	74	43		
Gravida	1	208	67	141	0.44	1
Median: 2 (IQR 1–4)	2–4	370	121	249		1 (0.7–1.5)
	5+	169	63	116		1.3 (0.8–2.0)
	Missing	3	1	2		
Parity	0–1	212	68	144	0.59	1
Median: 1 (IQR 0–4)	2–4	468	164	304		1.1 (0.8–1.6)
	5+	67	20	47		1.1 (0.7–1.7)
	Missing	3	1	2		
Living children	0	230	80	150	0.17	1
Median: 1 (IQR 0–3)	1–4	465	155	311		0.9 (0.7–1.3)
	5+	53	17	36		0.9 (0.5–1.7)
	Missing	2	2	0		
Spontaneous abortion	0	649	213	436	0.09	1
Median 1(IQR 0–2)	1	53	20	33		1.2 (0.7–2.2)
	2+	14	7	7		1.5 (0.8–2.7)
	Missing	34	16	18		
Stillbirths	0	641	207	434	0.05**	1
	1	75	29	46		0.9 (0.4–2.1)
	2+	28	15	13		3.6 (1.2–10.5)
	Missing	6	2	4		
Number of ANC visits	1	303	85	218	0.01*	1
	2+	447	168	279		1.5 (1.1–2.1)
Attendance by midwife	Midwife	436	168	268	0.001*	1
or TBA	TBA	254	67	187		0.5 (0.4–0.8)
	Missing	60	37	23		
Birth season	Dry cool	280	55	225	0.000*	1
	Dry hot	175	85	90		3.9 (2.5–5.9)
	Wet	295	113	18		2.5 (1.7–3.7)
Infant sex	Male	369	122	247	0.59	1.1 (0.8–1.5)
	Female	381	131	250		
Birth	2.5–3	260	73	187	0.04*	1
weight (kg)	3.1–4	464	169	295		1.5 (1.1–2.0)
	>4.0	26	11	15		1.9 (0.8–4.3)

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Gest. age at birth (wks.)	<34	56	18	38	0.03*	1
	34–37	231	83	148		0.7 (0.4–1.1)
	38+	463	152	311		1.1 (0.7–1.7)

IQR = inter-quartile range; ANC = antenatal clinic; TBA = trained birth attendant. * p-values <0.05 were considered significant. ** p-value = 0.0475.

Prevalence and risk factors for maternal colonisation

The overall prevalence of recto-vaginal GBS colonisation at delivery was 33.7% (253 women). In single variable analysis maternal weight >50 kg, maternal haemoglobin <10 g/dL, more than one previous stillbirth, more than one antenatal clinic visit, midwife delivery, giving birth during the dry hot or wet seasons, birth weight over 3 kg and gestation under 34 weeks were associated with increased risk of GBS colonisation (Table 1). In adjusted analysis, maternal haemoglobin <10 g/dL, history of more than one previous stillbirth, attendance at delivery by a midwife rather than a trained birth attendant and giving birth during the wet or dry hot season were associated with increased odds of maternal GBS colonisation at delivery (Table 2).

GBS colonisation dynamics at birth

There were 253 colonised mothers and 186 colonised infants at birth, of whom 146 were born to colonised women (57.7%). Table 3 describes the number of colonised infants at birth, day 6 and at 60–89 days of age according to colonisation site. Fig. 1a and b demonstrate colonisation dynamics at each time point.

Compared to mother/infant pairs who were not colonised at birth, mother/infant pairs were more likely to both be colonised if mother's weight was below 50 kg, infant was born at less than 37 weeks of gestation, born during the dry hot or wet seasons, mothers with more than one previous stillbirth, mothers attending antenatal clinic more than once, infants delivered by midwives, infants born with a birth weight below 3.3 kg or receiving breastmilk colonised with GBS (Table 4).

In adjusted analysis, compared to mother/infant pairs who were not colonised at birth, mother/infant pairs were more likely to both be colonised if birth occurred in the dry hot or wet seasons, mothers with one or more previous stillbirths or spontaneous abortions, infants born before 34 weeks of gestation. Compared to mother/infant pairs where neither was colonised, mother/infant pairs where the mother was colonised but not the infant were more likely if the mother had more than one previous stillbirth, or if the mother gave birth during the wet season. Infant colonisation in the absence of maternal colonisation (n = 40) was associated with gestation before 34 weeks (Table 5).

Most notably, compared to mother/infant pairs at birth where neither were colonised, infants born to mothers who had GBS cultured in their breastmilk were more likely to be colonised with GBS (Table 5).

Table 2 Multivariable analysis: maternal GBS colonisation at time of delivery.

		AOR (95% CI)	p-value
Maternal weight (kg)	<50	1	
	50–70	1.0 (0.4–1.6)	0.96
	70–90	1.7 (0.6–4.5)	0.30
	>90	1.4 (0.3–4.7)	0.68
Number of ANC visits	1	1	
	2+	1.3 (0.9–1.8)	0.16
Maternal Hb below 10 g/dL	No	1	
	Yes	0.7 (0.4–0.9)	0.04*
Attendance by midwife or TBA	Midwife	1	
	TBA	0.6 (0.4–0.9)	0.03*
Previous stillbirths	0	1	
	1	0.9 (0.4–2.0)	0.86
	2+	5.4 (1.6–18.0)	0.009*
Birth season	Dry cool	1	
	Dry hot	2.0 (1.3–3.1)	0.004*
	Wet	1.8 (1.2–2.7)	0.008*

ANC = antenatal clinic, Hb = haemoglobin; TBA = trained birth attendant. * p-values <0.05 were considered significant.

GBS colonisation dynamics at birth, day 6–9 and day 60–89

Infants born to colonised mothers had an increased risk of colonisation at birth and day 6–9 if born during the dry hot season or if born before 34 weeks of gestation. For all variables the risk of colonisation decreased over time (Table 6).

Infants born to non-colonised mothers had an increased risk of being GBS colonised on day 6–9 and at day 60–89 if born during the wet season. At birth infants were more likely to be colonised if breastmilk was colonised and they remained more likely to be colonised at both 6–9 days and day 60–89.

Serotype distribution

The predominant maternal rectovaginal colonising serotype was ST V in 54.9% of colonised mothers. Mothers' breastmilk was more likely to be colonised with ST V ($p < 0.001$) at all time points than any other serotype. Infants were more likely to be colonised with ST V at birth ($p = 0.004$), day 6–9 ($p = 0.004$) and day 60–89 ($p = 0.009$) than any other serotypes. At birth, infants were more likely to be colonised with ST III and V if their mothers were colonised with ST III and V ($x^2 = 24.4$, $p < 0.001$) compared to mothers colonised with other serotypes. Fig. 2 outlines the percentage of different serotypes at different time points in infants and in breastmilk.

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Table 3 Dynamics of colonisation by site and time point.

Birth n = 750; 253 colonised mothers (% total)	
Mother and infant colonised	146 (19.5% total; 57.7% born to colonised mothers)
Infants colonised, mothers not colonised	40 (5.3)
Total colonised infants	186 (24.8)
Mothers colonised, infants not colonised	107 (14.3)
Neither mother nor infant colonised	457 (60.9)
Site of colonisation at birth (% colonised infants)	
Nasopharyngeal and rectal	96 (51.6)
Rectal only	68. (36.6)
Nasopharyngeal only	22 (11.8)
Site of colonisation day 6–9; n = 722 (% of the 181 colonised infants)	
Infant rectal colonisation only	95 (52.5)
Infant nasopharyngeal colonisation only	29 (16.0)
Infant nasopharyngeal and rectal	57 (31.5)
Site of colonisation day 60–89; n = 684 (% the 94 colonised infants)	
Infant rectal colonisation only	57 (60.6)
Infant nasopharyngeal colonisation only	26 (27.7)
Infant nasopharyngeal and rectal	11 (11.7)

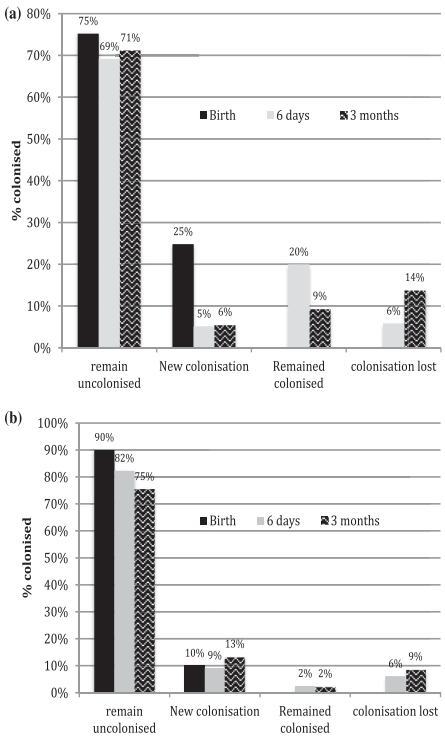


Figure 1 a – infant GBS colonisation status over time. Bars represent percentage of infants colonised at each of the time points; black bar = birth, grey = day 6 and patterned = three months. b – breastmilk colonisation over time. Bars represent percentage of infants colonised at each of the time points; black bar = birth, grey = day 6 and patterned = three months.

Neonatal sepsis

During the study period, 51 infants (6.8%) presented with signs of infection (68 per 1000 live births): 18 with signs of pneumonia, 22 with signs of other febrile illness, ten with skin infections and one with signs of meningitis. One child presenting on day 6 of life with irritability and poor feeding, had a positive CSF culture for GBS and was treated for 21 days with ampicillin and gentamicin (invasive GBS disease incidence: 1.3 per 1000 live births). This infant was born to a mother who had ST V cultured from both rectovaginal swab and breastmilk at birth and day 6 of life and the infant swab was also positive at birth and day 6 for ST V. The CSF sample was not typed due to laboratory constraints.

Nine infants died during the study period. Based on verbal autopsy, six deaths were due to pneumonia (median age at death 7 days [IQR 2–35]); three due to sepsis (median age 6 days [1–48]). Blood cultures were negative in all cases. One infant was born to a colonised mother and was also colonised with GBS at the time of birth but was not colonised on day 6. This infant died of presumed sepsis on day 48 of life. No other infants who died or their mothers were colonised with GBS at time of birth or day 6 of life. All infants died prior to the final follow-up appointment. It is therefore, not possible to determine whether any of these deaths were due to GBS disease.

Discussion

In this first colonisation study of Gambian mothers and their infants for 25 years and the first longitudinal study undertaken in this population, we demonstrate high prevalence of GBS colonisation in mothers at birth and in their infants up to 90 days of life. In addition, we demonstrate that infants were more likely to be colonised if GBS was isolated from their mother's breastmilk. The overall prevalence of maternal GBS in our population is higher than in the original

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Table 4 Characteristics of mother infant pairs according to colonisation status at birth.

	Total	M+I+	M+I−	M−I+	M−I−	p-value
Maternal age						0.96
<20	110	23	17	2	68	
20−24	262	43	38	19	162	
25−29	203	46	25	11	121	
30+	175	34	27	8	106	
Ethnicity						0.10
Mandinka	315	63	52	23	177	
Wolof	108	23	13	8	64	
Fula	126	28	17	2	79	
Jola	118	21	12	3	82	
Other	81	11	12	4	54	
Missing	2	0	1	0	1	
Maternal weight (kg)						0.04*
<50	27	2	5	4	16	
50−70	424	74	54	21	275	
70−90	148	36	25	3	84	
90+	145	32	23	9	81	
Missing	6	3	0	3	0	
Mother ill						0.48
Yes	128	24	21	3	80	
No	504	97	68	25	314	
Missing	118	25	18	12	63	
Maternal Hb < 10 g/dL						0.11
No	99	24	18	2	55	
Yes	651	122	89	33	403	
Maternal antibiotics in pregnancy						
No	505	99	92	0	314	0.91
Yes	128	23	24	0	81	
Gravida						0.44
1	208	39	28	13	128	
2−4	370	67	54	17	232	
5+	169	39	25	10	95	
Missing	3	1	0	0	2	
Parity						0.94
0−1	212	39	29	13	131	
2−4	468	96	68	24	280	
5+	67	10	10	3	44	
Missing	3	1	0	0	2	
Living children						0.66
0	230	43	37	14	136	
1−4	465	94	61	25	285	
5+	53	7	9	1	36	
Missing	2	2	0	0	0	
Spontaneous abortion						0.09
0	649	121	92	31	405	
1	53	14	6	4	29	
2+	14	7	1	0	6	
Missing	34	11	0	5	18	
Stillbirths						0.03*
0	641	119	87	32	403	
1	75	20	9	6	40	
2+	28	5	10	2	11	
Missing	6	2	1	0	3	
Number of ANC visits						0.002*
1	303	58	27	15	203	
2+	447	88	80	25	254	
Midwife delivery	436	108	60	20	248	<0.001*
TBA	254	29	38	12	175	
Missing	60	9	9	8	34	
Infant sex						0.71
Male	369	72	50	23	224	
Female	381	74	57	17	233	
Season						<0.001*
Dry cool	280	22	33	17	208	
Dry hot	175	66	19	3	87	
Wet	295	58	55	20	162	
Birth weight (kg)						0.02*
2.5−3	260	44	29	20	167	
3.1−4	464	95	74	18	277	
>4.0	26	7	4	2	13	
Gestation (weeks)						0.04*
<34	56	13	5	4	34	
34−37	231	57	26	12	136	
38+	463	76	76	24	287	
Breastmilk colonised						<0.001*
Yes	64	31	18	7	8	
No	600	101	77	31	391	
Missing	86	14	12	2	58	

M+I+ = mother and infant colonised; M+I− = mother colonised, infant not colonised; M−I+ = mother not colonised, infant colonised, M−I− = neither mother nor infant colonised; TBA = trained birth attendant; ANC = antenatal clinic.

study by Suara et al. (1992) (Suara 22% colonised (95% CI 15.04−28.96) vs. present study 33.7% colonised (29.63−36.37); p = 0.007).[11] However, the previous study likely under-identified GBS colonisation as selective medium was not used. An additional feature that may account for a lower colonisation rate was the collection of vaginal but not rectal swabs, which are known to have a higher yield for GBS.[12] The prevalence of GBS colonisation in our study is similar to the reported pooled prevalence in South African women (30.4%).[3]

Our finding that mothers with more than one stillbirth demonstrated an increased odds of having a colonised infant are in concordance with Monari et al., who identified stillbirth as a risk factor for invasive EOGBS disease.[13] This phenomenon has also been noted in non-human primates,[14] but there are currently little human data on the association between stillbirth, GBS colonisation and invasive disease.

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Table 5 Multivariable analysis of maternal/infant pairs by GBS colonisation status at time of delivery.[a]

	M+I+ OR (95% CI)	M+I− OR (95% CI)	M−I+ OR (95% CI)
Season			
Dry cool	1	1	1
Dry hot	8.3 (3.9−17.6)	1.8 (0.8−3.9)	0.4 (0.1−1.4)
Wet	3.5 (1.8−6.8)	2.3 (1.3−4.3)	1.1 (0.5−2.5)
Breastmilk colonised			
Yes	21.0 (8.6−51.3)	11.5 (4.5−29.4)	7.5 (2.0−27.5)
No	1	1	1
Gestation (weeks)			
<34	1	1	1
34−37	0.9 (0.9−1.0)	1.0 (0.9−1.1)	0.9 (0.8−1.0)
38+	0.4 (0.2−1.0)	0.5 (0.3−1.6)	0.9 (0.8−1.0)
Midwife delivery	1	1	1
TBA	0.9 (0.5−1.6)	1.0 (0.6−1.8)	0.6 (0.2−1.3)
Number of antenatal clinic visits			
1	1	1	1
2+	1.1 (0.7−1.9)	1.2 (0.8−2.1)	1.3 (0.6−2.8)
Stillbirths			
0	1	1	1
1	2.5 (1.0−8.7)	2.0 (1.0−4.7)	1.4 (0.3−5.9)
2+	2.4 (0.9−4.1)	2.2 (0.9−2.1)	ND
Spontaneous abortion			
0	1	1	1
1	0.9 (0.5−1.4)	0.8 (0.3−2.1)	1.0 (0.2−5.5)
2+	1.2 (1.1−2.3)	0.3 (0.0−2.2)	2.0 ND
Gravida			
1	1	1	1
2−4	1.0 (0.9−1.0)	1.1 (0.6−2.0)	1.0 (0.8−1.2)
5+	1.0 (0.5−2.0)	0.8 (0.4−1.8)	0.8 (0.3−2.3)

M+I+ = mother and infant colonised; M+I− = mother colonised, infant not colonised; M−I+ = mother not colonised, infant colonised, M−I− = neither mother nor infant colonised. 1 = adjusted odds ratios are compared to this group; TBA = trained birth attendant.
[a] M−I− = base group.

This finding could be important in reducing adverse pregnancy outcomes especially following introduction of any new vaccine.

The prevalence of infant colonisation in our study is similar to previous studies in Europe[15−18] but higher than those from other most African countries,[19] probably due to the differences in microbiological methods. To our knowledge this is the first longitudinal study of infant colonisation and demonstrates that GBS colonisation rates remain high up to three months of life. We demonstrate that the overwhelming risk factor for infant colonisation at all time points was having a mother who was GBS colonised at the time of birth. We also established that delivery before 34 weeks of gestation increased the odds of GBS colonisation at birth. Similar studies have indicated that risk factors for infant colonisation include maternal recto-vaginal colonisation, preterm delivery, low birth weight and maternal urinary tract infection.[1,15,18,20] However, unlike these studies we did not identify low birth weight as a risk factor, probably because we only recruited infants weighing more than 2.5 kg.

Several case reports suggest that isolation of GBS in breastmilk is implicated in late onset GBS disease.[21,22] Our data suggest that GBS colonisation in breastmilk is a risk factor for neonatal colonisation, both at delivery and for persistence of infant colonisation throughout the first three months of life. This is an important finding in a population such as this with high rates of breast-feeding. The mechanism for this is not clear; it is possible that GBS enters the mammary glands following suckling from a colonised infant, although there is also evidence that normal gut microbiota may pass into the breast via the entero-mammary circulation.[23] Whether it is also a risk factor for late onset disease in this population is not known. Conversely, it might conceivably be a protective factor through induction of immunity.[24] The relevance of this finding requires further study.

It is interesting to note that risk factors for maternal colonisation include midwife delivery and increasing number of antenatal visits. This phenomenon has not been previously noted in resource-poor settings where hygienic birth practices may be compromised. GBS has been implicated in nosocomial outbreaks in several hospitals globally,[25,26] although the number of reported outbreaks in resource-rich countries has greatly reduced since the implementation of GBS screening programmes. It is conceivable that unclean birth practices, especially in times of clean water shortage, such as the dry hot season or during the wet season may

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Table 6 Single variable analysis of infant GBS colonisation dynamics over time compared to birth.

		Birth OR (95% CI)	Day 6 OR (95% CI)	Day 60–89 OR (95% CI)
Mother GBS colonised at delivery				
Birth season	Dry cool	1	0.2 (0.1–0.6)	0.1 (0.0–0.4)
	Dry hot	4.6 (2.2–9.7)	1.9 (1.0–4.1)	0.6 (0.2–1.3)
	Wet	1.4 (0.7–2.8)	0.7 (0.3–1.5)	0.3 (0.1–0.7)
Living children	0	1	0.5 (0.3–0.9)	0.2 (0.1–0.4)
	1–4	0.9 (0.5–1.6)	0.4 (0.2–0.7)	0.1 (0.0–0.3)
	5+	0.9 (0.3–2.6)	0.6 (0.2–1.8)	0.2 (0.0–0.9)
Gestation at birth (wks.)	<34	1	0.4 (0.1–1.4)	ND
	34–37	0.9 (0.3–2.4)	0.3 (0.1–0.9)	0.2 (0.1–0.7)
	38+	0.4 (0.1–1.0)	0.2 (0.1–0.5)	0.0 (0.0–0.1)
Infant weight (kg)	2.5–3	1	0.5 (0.2–1.0)	0.1 (0.0–0.3)
	3.1–4.0	1.2 (0.4–3.8)	0.7 (0.3–0.9)	0.1 (0.1–0.3)
	>4.0	0.6 (0.1–2.3)	0.3 (0.1–0.7)	0.1 (0.0–0.6)
BM colonised	Yes	0.9 (0.5–1.7)	0.4 (0.2–0.8)	0.3 (0.1–0.7)
	No	1	0.4 (0.3–0.6)	0.1 (0.1–0.2)
Infant sex	Male	1	0.4 (0.3–0.7)	0.1 (0.1–0.3)
	Female	0.7 (0.4–1.2)	0.4 (0.2–0.6)	0.1 (0.1–0.3)
Mother not GBS colonised at delivery				
Birth season	Dry cool	1	1.4 (0.5–4.3)	0.8 (0.2–2.8)
	Dry hot	1	1.3 (0.3–5.3)	4.7 (1.7–13.5)
	Wet	2.1 (0.8–6.0)	4.1 (1.6–10.5)	0.2 (0.0–1.9)
Living children	0	1	1.8 (0.5–5.7)	1.7 (0.5–5.6)
	1–4	1.0 (0.3–2.9)	2.2 (0.8–5.9)	0.7 (0.2–2.3)
	5+	2.0 (0.1–7.1)	1	0.8 (0.1–7.3)
Gestation at birth (wks.)	<34	1	3.3 (0.3–34.4)	1
	34–37	1.6 (0.2–13.4)	2.5 (0.3–20.6)	1.4 (0.2–13.0)
	38+	1.1 (0.1–9.0)	2.2 (0.3–17.3)	1.3 (0.2–10.8)
Infant weight (kg)	2.5–3.0	1	1.2 (0.4–3.8)	0.6 (0.1–2.3)
	3.1–4.0	0.5 (0.2–1.4)	1.2 (0.5–2.9)	0.6 (0.2–1.7)
	>4.0	0.9 (0.2–2.3)	1.7 (0.9–4.9)	0.4 (0.3–2.2)
BM colonised	Yes	14.2 (4.0–50.8)	6.9 (2.5–19.1)	3.3 (1.2–11.0)
	No	1	2.0 (0.9–4.2)	1.0 (0.4–2.5)
Infant sex	Male	1	1.4 (0.6–3.1)	0.8 (0.3–2.0)
	Female	0.3 (0.1–1.0)	1.1 (0.5–2.5)	0.5 (0.2–1.5)

facilitate the fecal-oral spread of GBS between colonised and non-colonised mothers and their infants.

A large proportion of women were colonised with ST V, similar to the previous smaller study by Suara et al., which identified 32 colonised pregnant women of whom 12 (30%) were colonised with ST V.[11] ST V has been identified as an important neonatal and adult colonising and disease causing serotype globally.[3,27,28] Additionally, in our cohort, ST V was the predominant serotype cultured in breastmilk and ST Ib and V were associated with longer median colonisation duration compared to other serotypes. A study of pregnant women from South Africa found a high prevalence of GBS colonisation throughout pregnancy, with ST V and ST III colonisation lasting longer than other serotypes (ST V mean duration 8.6 weeks; 95% CI 6.8–10.4 vs. ST III 9.2 weeks; 95% CI 8.1–10.2).[3] The difference in serotype distribution and duration of colonisation between these studies may be due to population differences between South Africa and the Gambia. There are no other longitudinal infant colonisation studies with which to compare our results. Our results may also indicate differences in maternally derived antibody that might prevent colonisation of certain serotypes in these diverse populations. In the most recent study from South Africa, the authors demonstrate that it is the acquisition of a new serotype that is associated with EOS rather than the persistent serotype, probably due to the absence of maternal antibody to this new serotype.[29]

Our study has several limitations. First, our results are limited by the sensitivity of detection of GBS on selective media, estimated at 85%[30] and by the fact that we identified the serotype by conventional PCR. This may imply that we have underestimated low levels of colonisation in infants where DNA was below the limit of detection. Second, we identified only one culture-confirmed case of GBS invasive disease. The low number of positive invasive isolates

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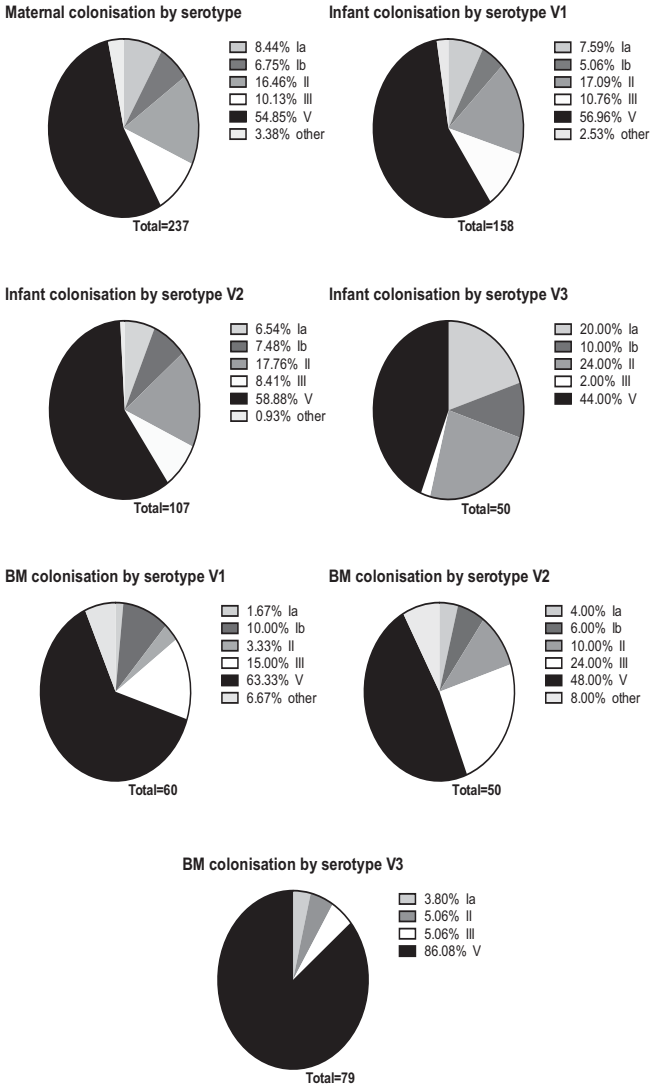


Figure 2 Colonising serotype distribution by isolation site and time point. Maternal rectovaginal colonisation at delivery; breastmilk and infant colonisation by time point; BM = breastmilk; Ia = serotype Ia, Ib = serotype Ib, II = serotype II, III = serotype III, V = serotype V.

may represent an underestimation of the burden of GBS disease in this population. Several infants died prior to recruitment or after recruitment but of early onset pneumonia without a culture-positive diagnosis and it is conceivable that they may have succumbed to early onset GBS disease. There are no other data from the Gambia regarding the burden of GBS disease. Although we recognize the limitations of its approach, the only other evidence for GBS disease in the Gambia is shown by a retrospective review of positive cultures from the MRC hospital over 8 years that identified 17 positive cultures from infants under 90 days of age which is consistent with our disease estimate of 1.3/1000 live births.[31] Finally, sampling at 4 h post-birth may indicate contamination with maternal secretions rather than established colonisation at birth. However, the fact that few infants lost colonisation at day 6 indicates that colonisation was persistent over the first week of life.

GBS capsular polysaccharide-protein conjugate vaccines are being developed, making the findings of our study important to ensure that appropriate serotypes are included in candidates that would be relevant to all African populations. Information about GBS colonisation and factors affecting acquisition and duration of colonisation will

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be helpful in considering strategies for reducing the burden of infant GBS disease worldwide.

Author contribution

KLD conceived the original idea, designed and performed the laboratory and data analysis and drafted the manuscript. SJ and SD performed and interpreted the laboratory analysis and contributed to the manuscript preparation. AG, PH and BK developed the original research idea and contributed to the manuscript preparation. FW advised on the statistical analysis and commented on drafts of the manuscript.

Funding

This work was supported by a Wellcome Trust Clinical Research Fellowship to KLD (WT104482MA) and the Thrasher Research Fund (BK: 12250). BK is also supported by grants from the UK MRC (MC_UP_A900/1122, MC_UP_A900/115) and the UK Medical Research Council (MRC) and the Department for International Development (DFID) under the MRC/DFID Concordat arrangement.

Conflict of interests

KLD, SJ, SD, AG declare no conflict of interests. PTH is an advisor to Novartis and Pfizer regarding GBS vaccines. BK is an advisor for Pfizer regarding GBS vaccines.

Acknowledgements

The authors would like to thank the study participants and field workers at Faji Kunda and Jammeh Foundation for Peace Hospitals and the lab staff Amadou Faal, Francess Sarfo and Mustapha Jaiteh at MRC Unit The Gambia. We would like to thank Martin Antonio, Ebenezer Foster-Nyarko and Edward Clarke for their support at the MRC Unit The Gambia.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jinf.2015.12.014.

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OPEN

CONCISE COMMUNICATION

Placental transfer of anti-group B *Streptococcus* immunoglobulin G antibody subclasses from HIV-infected and uninfected women to their uninfected infants

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Objectives: Placental antibody transfer is impaired in the context of HIV infection, which may render HIV-exposed, uninfected infants vulnerable to group B *Streptococcus* (GBS) disease. The GBS antibody response predominately consists of immunoglobulin G2 (IgG2) antibody. Thus we determined whether concentration and placental transfer of anti-GBS antibody subclasses was altered in HIV-infected compared with HIV-uninfected mothers.

Design: A retrospective analysis of anti-GBS antibody subclasses in 38 HIV-infected and 33 HIV-uninfected mothers and their uninfected infants.

Methods: Sera were analysed using a novel flow cytometric assay that quantified binding of IgG1, IgG2, IgG3 and IgG4 to serotype (ST)Ia, STIII and STV GBS bacteria.

Results: IgG2 binding to GBS STIa and V was lower in HIV-infected women compared with HIV-uninfected women. Moreover, IgG2 binding to GBS STIa was also lower in HIV-exposed, uninfected infants compared with unexposed infants. However, there were no statistically significant differences in the transplacental transfer ratio of IgG2 for any GBS serotype. The transplacental transfer of total IgG was reduced for GBS STIII and V and IgG1 subclass for STIII; placental transfer of all other subclasses was comparable in HIV-affected and HIV-unaffected pregnancies.

Conclusion: Anti-GBS IgG2 placental transfer is not affected by HIV infection. This is important for functional antibody against the capsular polysaccharide of GBS and provides confidence that maternal GBS vaccination may result in functional activity in HIV-infected and uninfected women.

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AIDS 2016, **30**:471–475

Keywords: antibody, group B Streptococcus, HIV

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Received: 21 June 2015; revised: 3 September 2015; accepted: 10 September 2015.

DOI:10.1097/QAD.0000000000000923

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Introduction

Opsonophagocytosis of group B *Streptococcus* (GBS) is mediated predominately by the immunoglobulin G2 (IgG2) subclass [1], which is poorly transported across the placenta compared with IgG1 [2,3]. This may reflect the lower affinity of IgG2 for the FcRn receptors on syncytiotrophoblasts of placental cells as compared with the other subclasses of IgG [4,5]. Studies comparing maternal and infant antibody concentrations demonstrate that anti-GBS capsular antibody is transferred across the placenta with a high degree of efficiency [6]. However, the proportionally higher concentration of antibody in infant compared with maternal serum at birth is thought to be mainly due to excess foetal IgG1 [2]. It has been demonstrated for GBS serotype (ST) Ia and STIII that not only is total anti-GBS antibody concentration lower in GBS-infected infants than in infants without infection born to colonized mothers [7,8], but that IgG2 is also lower in infants with GBS infection [9]. Recent studies have shown that maternal and placental transfer of total anti-GBS antibody is reduced in the context of maternal HIV-infection [10,11]. This might explain the greater reported incidence of early, and especially late onset, GBS disease observed amongst HIV-exposed, uninfected infants compared with unexposed infants [12]. A West African study identified hypergammaglobulinaemia as a risk factor for reduced placental transfer of IgG subclasses and this might be a further contributing factor to the observed excess of GBS morbidity amongst HIV-exposed infants [13]. Thus, we compared total and subclass anti-GBS antibody placental transfer in HIV-infected and HIV-uninfected South African women.

Methods

Samples were collected from mothers and infants enrolled in a cohort study investigating the influence of maternal HIV and mycobacterial sensitization on infant immune responses to Bacillus Calmette-Guérin (BCG) vaccination carried out between 2009 and 2011 [14]. The demographic details, $CD4^+$ cell count, viral load and antiretroviral medication have been previously described [14]. Informed consent was obtained from all mothers participating in the study. The study was approved by the Universities of Cape Town (382/2008) and Stellenbosch (N08/10/278), South Africa, and the National Health Service Research Ethics Committee, England (07/H0720/178).

Paired sera from 38 HIV-infected and 33 HIV-uninfected mothers and their uninfected infants were available to analyse immunoglobulin subclasses. Deposition of total IgG and IgG1, IgG2, IgG3 and IgG4 anti-GBS antibody subclasses onto the surface of formaldehyde-fixed GBS bacteria was measured using a flow cytometric assay performed in 96-well microtitre plates as previously described [10]. Mouse monoclonal antibodies: 4E3 antihuman IgG1 H&L, HP6014 antihuman IgG2 fd gamma, HP6050 antihuman IgG3 hinge heavy chain and mouse HP6025 antihuman IgG4 Fc (FITC) (Life Technologies, Carlsbad, California, USA) (1 : 500) in blocking buffer were added and samples incubated for 20 min at 4°C. Assays were analysed using a Beckman Coulter Cyan flow cytometer equipped with a Cytek 96-well microtitre plate loader. A fluorescence index (FI) was calculated for each sample, which involved the multiplication of the percentage of bacteria in the horizontal gate (%-gated), by the mean fluorescence of that population (X-mean). The final result for each test was expressed as the average FI of duplicate test samples minus the average FI of the bacteria and conjugate-only control. A standard unit (SU) measurement for each serum sample was then calculated by comparing to the serum FI response obtained with the positive control serum for each serotype which was given an arbitrary value of 1000 (kind gift from Professor Carol Baker, Baylor College of Medicine, Texas, USA) to give a result in SU/ml.

Statistical analyses were completed using STATA version 12 (StataCorp 2013, College Station, Texas, USA) and GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, California, USA). The sample size was calculated to demonstrate a 50% difference in IgG subclass concentrations between HIV-exposed and HIV-unexposed infants with the predefined assumption that antibody subclass concentrations would be lower in HIV-exposed infants. Using an α error of 5%, 80% test power and a 95% confidence interval, the sample size ranged from 10 to 30 in each group. Placental transfer was defined as the ratio of infant-to-mother GBS IgG concentration at birth. Missing data were excluded from analysis. We considered P values less than 0.05 as statistically significant.

Results

The paired sera from 71 mother–infant dyads (38 HIV-infected mothers and their uninfected infants and 33 HIV-uninfected mothers and their infants) at birth were available for analysis of total IgG, IgG1 and IgG2 for all serotypes. For serotypes ST1a and STV, sufficient sera was available from 60 mother–infant pairs to analyse subclasses IgG3 and IgG4. Sufficient sera were available from 40 mother–infant pairs for analysis of IgG3 and IgG4 for STIII.

Antibody subclass concentrations

Concentrations of anti-GBS IgG1 in mothers and infants were higher than concentrations of IgG2 for all GBS serotypes, independent of HIV status (Fig. 1).

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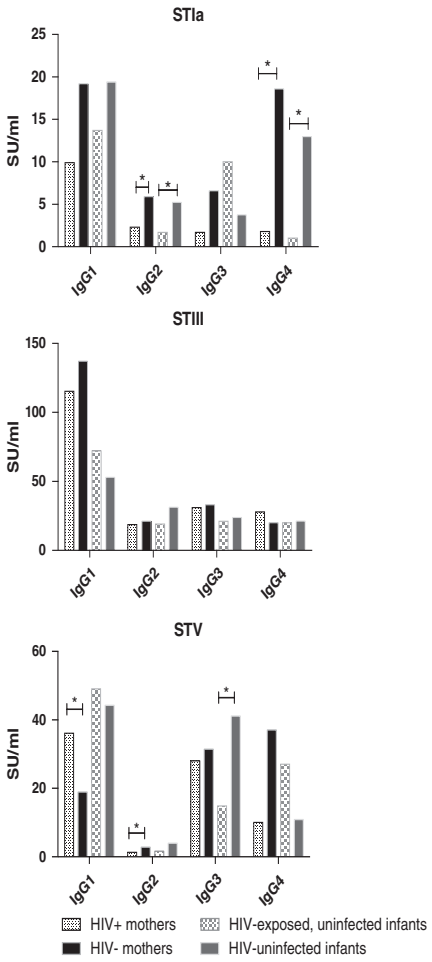


Fig. 1. Anti-group B *Streptococcus* subclass distribution in HIV-infected and HIV-uninfected mothers and their uninfected infants at birth. HIV−, HIV uninfected; HIV+, HIV infected; IgG, immunoglobulin G; ST, serotype; SU/ml, standard units per millilitre. Horizontal bars compare groups, $^{*}P < 0.05$.

For STIa, HIV-uninfected women had significantly higher geometric mean concentration (GMC) of subclass IgG2 and IgG4 than HIV-infected women (IgG2 2.3 [95% confidence interval (CI) 1.6–3.3] vs. 5.9 [4.0–8.9] SU/ml, $P = 0.02$; IgG4 1.8 [0.9–3.6] vs. 18.6 [6.0–57.8] SU/ml, $P = 0.03$) but comparable concentrations of IgG1 and IgG3 (Fig. 1). In HIV-unexposed compared with HIV-exposed, uninfected infants, there was a significantly higher concentration of IgG2 ($P = 0.03$) and IgG4 ($P = 0.03$). In addition, we noted a trend towards higher concentrations of anti-STIa IgG1 and lower concentration of IgG3 but this did not reach statistical significance (Fig. 1).

For STV, HIV-infected women had a trend towards higher concentrations of IgG1 than HIV-uninfected women ($P = 0.049$) and HIV-uninfected women had significantly higher concentrations of IgG2 than HIV-infected women ($P = 0.047$), although the concentration of IgG2 was low in both groups. HIV-unexposed infants had higher concentrations of anti-STV IgG3 antibody compared with HIV-exposed infants ($P = 0.039$). There was no statistical difference between HIV-exposed, uninfected infants and HIV-unexposed infants for any of the other IgG subclasses (Fig. 1).

There was no significant difference between anti-STIII IgG subclasses in HIV-infected and HIV-uninfected women and HIV-exposed, uninfected and HIV-unexposed infants.

Placental transport of total group B *Streptococcus* antibody

Total IgG transplacental antibody transfer ratio (TPR) was reduced in HIV-infected compared with HIV-uninfected women for GBS STIII [0.6 (0.3–1.1) vs. 1.3 (0.8–2.3) SU/ml, $P = 0.04$] and STV [1.2 (0.7–1.6) vs. 2.0 (1.1–3.0) SU/ml, $P = 0.04$] but not for STIa. (Table 1). There was no association between $CD4^{+}$ cell count or viral load and TPR.

Placental transport of subclasses group B *Streptococcus* antibody

TPR of IgG1 was decreased to HIV-exposed infants compared with HIV-unexposed infants [0.4 (0.1–1.4) vs.

Table 1. Geometric mean concentration (GMC) transplacental transfer ratios of total and subclass IgG1, IgG2, IgG3 and IgG4 against GBS STIa, III and V in HIV-infected and HIV-uninfected mothers to their infants [95% confidence interval (CI)].

	STIa			STIII			STV		
	HIV+	HIV−	*P*	HIV+	HIV−	*P*	HIV+	HIV−	*P*
Total	1.1 (0.8–1.6)	1.0 (0.6–1.6)	0.75	0.6 (0.3–1.1)	1.3 (0.8–2.3)	0.04	1.2 (0.7–1.6)	2.0 (1.1–3.0)	0.04
IgG1	1.0 (0.7–1.5)	1.4 (1.0–2.0)	0.44	0.4 (0.1–1.4)	1.3 (0.7–2.5)	0.04	1.0 (0.7–1.4)	1.4 (0.9–2.1)	0.14
IgG2	0.9 (0.5–1.5)	0.7 (0.5–1.2)	0.43	1.1 (0.4–3.2)	0.8 (0.4–1.4)	0.38	0.7 (0.5–1.1)	0.8 (0.6–1.1)	0.59
IgG3	0.3 (0–9)	1.0 (0.3–3.3)	0.5				0.9 (0.6–1.4)	1.3 (0.8–2.1)	0.23
IgG4	0.5 (0.2–1.1)	1.6 (0–4)	0.08				1.2 (0.5–2.9)	0.6 (0.3–1.4)	0.31

GBS, group B *Streptococcus*; HIV−, HIV-uninfected; HIV+, HIV infected; IgG, immunoglobulin G; ST, serotype.

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1.3 (0.7–2.5) SU/ml; $P = 0.04$] for STIII but not for STIa or STV (Table 1). The TPR for IgG2 was unaffected by maternal HIV infection status for any serotype. There were no statistical differences between antibody TPR for any other subclass or any other serotype of GBS between groups.

Discussion

Our findings demonstrate that whilst total transplacental transfer ratios are reduced in HIV-infected mothers for GBS STIII and V, the proportion of individual subclasses transferred from mother to infant is unaffected by maternal HIV infection, with the exception of IgG1 to GBS STIII. Importantly, there was no difference in the placental transfer of IgG2 that bounds to any of the GBS serotypes.

HIV-exposed, uninfected infants may be at increased risk of GBS infection, in particular late onset disease [10,12] The observation that there is no difference in the proportion of IgG subclass placental transfer (except for IgG1 for STIII) may mean that HIV-exposed, uninfected infants are at increased risk of disease as a result of their mother's reduced total antibodies that bind to GBS bacteria, rather than a selective deficit in IgG2 transfer. It may alternatively be due to impaired B cell function in the context of HIV infection that results from poor function of T helper cellular response and function of antibody in the context of HIV-infection [15].

The fact that IgG2 surface deposition was lower in HIV-exposed infants to STIa and in women to STV in HIV-infected women may indicate reduced antibody function due to HIV-infection. In this study we were unable to assess the opsonophagocytic ability of the antibody which would provide additional information on the functional ability of antibody to protect from GBS infection. In studies of pneumococcal vaccination, opsonophagocytosis was impaired in HIV-infected compared with HIV-uninfected individuals and the same may be true of GBS [16].

The serotypes we have selected in this study represent 77% of colonizing strains in South African pregnant women (STIII, 33%; STIa, 39%; STV, 12% [17]). They also represent 86.1% of early onset GBS disease and 100% of late onset disease (STIII 57.7% of early onset, 84.4% of late onset disease; STIa 22.6%/ 13.9%; STV 5.8%/1.9% [18]). Thus, the serotype-specific antibody observed corresponds to the predominant colonizing and disease-causing serotypes in South Africa.

Although patients with HIV disease develop high levels of total IgG in serum soon after infection [19], the IgG1 subclass is preferentially increased, whereas levels of total IgG2 may be normal or decreased [20]. We confirmed the relatively similar concentration of IgG2 antibody that binds to GBS STIII in the sera of HIV-infected and HIV-uninfected women. IgG2 responses to the bacterial capsular polysaccharide in humans may be driven in part by IFN-γ production [21], which may be impaired during HIV-infection [22]. However, HIV-infected patients in our cohort showed no selective deficit in IgG2 production. The differences in subclass distribution by GBS serotype may be as a result of different reactivity of the different serotypes in our assay. However, comparison with the larger study demonstrated good correlation between total antibody concentrations for all serotypes tested [10].

Maternal HIV infection has been associated with reduced placental transfer of antibodies against several common viral and bacterial antigens [23,24]. Additionally, the finding that HIV-exposed, uninfected infants are more likely to have sub-protective antibody levels to vaccine preventable diseases means that these infants are likely to be more prone to these diseases in early childhood. Our finding that the placental transfer of IgG2 subclass, important for the opsonization and killing of GBS, was not impaired indicates potential for a maternal vaccine to boost both maternal and infant immunity in areas of high HIV-prevalence.

Our sample size was limited and we are not able to draw conclusions on other possible effects of HIV-infection on placental transfer. Additionally, we cannot comment on the colonizing serotypes of the mothers. However, the fact that the antibody against the major GBS serotypes was detected in all women suggests prior colonization with GBS. Finally, we cannot comment on protective levels of antibody in this population, as there were no cases of GBS disease in our cohort.

In summary, the decreased concentration of serum IgG that bounds to the surface of GBS in HIV-infected women and their infants compared with non-HIV-infected dyads is not due to a selective defect in the generation of GBS-specific IgG2 antibody. Future efforts should address enhancing protective antibody concentrations through maternal vaccination against GBS during pregnancy to optimize IgG2 concentration in the neonate.

Acknowledgements

We thank the mothers and infants who participated in this study, Prof. Carol Baker for the donation of positive control sera and Prof. Androulla Efstraitou for supplying the GBS clinical isolates.

Contribution statement: K.L.D. developed the manuscript and original research idea. A.H., P.H., B.K., A.G., L.A., S.T. and C.J. developed the original idea and

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substantially contributed to the development of the manuscript.

Funding: K.L.D. is supported by a Wellcome Trust Clinical Research Training Fellowship (KLD2013) The Thomas Watt Eden Fellowship (Royal College of Physicians Grant Number 01012013); and the Gilead/BHIVA Registrar Award. B.K. is supported by the MRC (MR/K007602/1, MC_UP_A900/1122). C.J. was supported by the European Society for Pediatric Infectious Diseases and the Thrasher Research Fund.

Conflicts of interest

The authors do not have a commercial or other association that might pose a conflict of interest.

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Vaccine 33 (2015) 621–627

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Anti-Group B Streptococcus antibody in infants born to mothers with human immunodeficiency virus (HIV) infection☆

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ARTICLE INFO

Article history:
Received 26 August 2014
Received in revised form
10 December 2014
Accepted 12 December 2014
Available online 24 December 2014

Keywords:
Antibody
Group B Streptococcus
HIV-exposed-uninfected infants
HIV
Immunity

ABSTRACT

Background: HIV-exposed uninfected infants have increased infection risk and mortality compared to HIV-unexposed infants. HIV-exposed infants may be at increased risk of invasive GBS disease due to reduced maternal antibody against GBS.
Methods: We quantified antibodies that bind to the surface of whole Group B Streptococcus (GBS) of serotypes Ia, Ib, II, III and V using novel flow cytometry assays in South African HIV-infected and non-infected mothers and their uninfected infants. Antibody-mediated complement C3b/iC3b deposition onto GBS of these serotypes was also quantified by a novel flow cytometry assay.
Results: Geometric mean concentration (GMC) of both surface-binding anti-GBS antibody and antibody-mediated complement deposition onto GBS were reduced in HIV-infected women ($n=46$) compared to HIV-uninfected women ($n=58$) for ST1a (surface-binding: 19.3 vs 29.3; $p=0.003$; complement deposition: 2.9 vs 5.3 SU/mL; $p=0.003$), STIb (24.9 vs 47.6; $p=0.003$; 2.6 vs 4.9 SU/mL; $p=0.003$), STII (19.8 vs 50.0; $p=0.001$; 3.1 vs 6.2 SU/mL; $p=0.001$), STIII (27.8 vs 60.1; $p=0.001$; 2.8 vs 5.3 SU/mL; $p=0.001$) and STV (121.9 vs 185.6 SU/mL; $p<0.001$) and in their infants for STIa (complement deposition 9.4 vs 27.0 SU/mL; $p=0.02$), STIb (13.4 vs 24.5 SU/mL; $p=0.02$), STII (14.6 vs 42.7 SU/mL; $p=0.03$), STIII (26.6 vs 62.7 SU/mL; $p=0.03$) and STV (90.4 vs 165.8 SU/mL; $p=0.04$). Median transplacental transfer of antibody from HIV-infected women to their infants was reduced compared to HIV-uninfected women for GBS serotypes II (0.42 [IQR 0.22–0.59] vs 1.0 SU/mL [0.42–1.66]; $p<0.001$), III (0.54 [0.31–1.03] vs 0.95 SU/mL [0.42–3.05], $p=0.05$) and V (0.51 [0.28–0.79] vs 0.75 SU/mL [0.26–2.9], $p=0.04$). The differences between infants remained significant at 16 weeks of age.
Conclusions: Maternal HIV infection was associated with lower anti-GBS surface binding antibody concentration and antibody-mediated C3b/iC3b deposition onto GBS bacteria of serotypes Ia, Ib, II, III and V. This may render these infants more susceptible to early and late onset GBS disease.
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1. Introduction

The increasing numbers of HIV-exposed infants who remain uninfected is testament to the success of prevention of mother-to-child transmission programs in resource-poor settings in the face of a high disease burden [1]. Nevertheless, this group of infants appears to suffer from increased rates of lower respiratory tract infection and meningitis compared to HIV-unexposed infants and up to 4-fold higher mortality in the first year of life [2–4]. *Streptococcus agalactiae* (group B streptococcus, GBS) is a leading cause of neonatal pneumonia, sepsis and meningitis in many countries and five serotypes (Ia, Ib, II, III and V) account for the majority of disease [5]. Published data show that not only is GBS carriage increased in HIV-infected pregnant women compared with HIV-uninfected mothers [6], but that HIV-exposed, uninfected infants also appear to have an increased risk of late-onset GBS disease compared to HIV-unexposed infants [7].

☆ Preliminary data was presented at the European Society of Infectious Diseases meeting May 2014, Dublin, Ireland.

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http://dx.doi.org/10.1016/j.vaccine.2014.12.025
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Prevention of GBS infection in infants and adults through immunization is a theoretically attainable goal. Maternal transfer of antibodies is thought to prevent newborn GBS disease [8] and GBS vaccines offer the potential to prevent disease in low-income settings where prenatal screening and intrapartum antibiotics are generally not feasible. A number of studies have demonstrated an association between the risk of developing invasive GBS disease in the newborn and maternal anti-capsular antibody levels [9–12] with opsonophagocytosis the likely effector mechanism [13–15]. However, maternal HIV status can influence the efficiency of transplacental antibody transfer, resulting in reduced maternally-derived specific antibody concentration in the infant, even where the infant is subsequently HIV uninfected [16–19]. Data regarding the persistence of anti-GBS antibodies in populations with high HIV-prevalence and their function in vitro would provide insight into their protective potential in different populations. Thus our objective was to determine the association between maternal HIV infection status and total IgG binding to the surface of GBS bacteria of serotypes Ia, Ib, II, II and V and antibody-mediated deposition of C3b/iC3b onto the surface of these GBS strains as a potential surrogate for opsonophagocytosis in sera obtained from mothers and infants.

2. Methods

2.1. Study setting, eligibility and study measures

Samples were collected from mothers and infants enrolled in a cohort study investigating the influence of maternal HIV and mycobacterial sensitization on infant immune responses to BCG vaccination carried out between 2009 and 2011 [20]. In brief, 109 pregnant women presenting to a Community Health Center in Khayelitsha, Western Cape Province, South Africa who delivered a healthy infant over 37 weeks gestation, weighing over 2.5 kg, knew the result of their HIV test and were willing to provide written informed consent for themselves and their infants were recruited to the original study. The subset represented all mothers/infants with available samples. A venous blood sample was collected from the mother and infant within 24 h of delivery. All infants had a further venous blood sample collected at 16 weeks. HIV-exposed infants had an HIV polymerase chain reaction (Amplicor HIV-a DNA kit, version 1.5; Roche Molecular Systems Inc., Branchburg, NJ) performed at ages 4 and 16 weeks. The study was approved by the Universities of Cape Town (382/2008) and Stellenbosch (N08/10/278), South Africa, and the National Health Service Research Ethics Committee, England (07/H0720/178).

In 2009, the HIV prevalence among women attending antenatal clinics in the area was 31% [21]. During the study period, the Prevention of Mother to Child Transmission program consisted of dual therapy for mothers and infants, starting with the administration of zidovudine at 28 or more weeks' gestation, then zidovudine for 1 month to the infant and a single dose of nevirapine to both mother and infant. Mothers were eligible for highly active antiretroviral treatment if their CD4 count was less than 200 cells/μL. Exclusive infant feeding options were encouraged and mothers were provided with free formula for 6 months if they chose exclusive formula feeding.

2.2. Laboratory assays

The reproducibility of the assays was assessed between three different days and between three different operators with a panel of 18 sera. The assays had a coefficient of variation of less than 30%. Controls used were rabbit polyclonal sera (SSI) raised against serotypes Ia, Ib, II, III & V, and normal human donor sera.

2.2.1. GBS isolates and growth conditions

Group B Streptococcus isolates used in this study were H092040676 (Serotype Ia), H090820125 (Serotype Ib), H090320548 (Serotype II), H092120162 (Serotype III) and H091780506 (Serotype V), which were kindly provided by Professor Androulla Efstratiou, Public Health England, Colindale. Strains are subsequently referred to by their serotype.

GBS isolates used in the antibody surface binding and complement deposition assays were grown in Todd Hewitt broth at 37 °C with shaking (200 rpm). Once OD_{600nm} 1.0 was reached, cultures were centrifuged at 3060 × *g* for 5 min to pellet the bacteria. The pellet was re-suspended in the same volume of phosphate buffered saline (PBS, pH 7.4, Severn Biotech, UK) containing 2% formaldehyde and incubated at room temperature for 1 h. Bacteria were then washed in PBS by three rounds of centrifugation at 3060 × *g* for 5 min and resuspended. The final cell pellet was resuspended in 1 mL PBS, which was stored at 4 °C before use.

2.2.2. Sample preparation for antibody surface binding assay

Deposition of anti-GBS antibody onto the surface of whole GBS bacteria was measured on formaldehyde-fixed GBS using a flow cytometric assay performed in 96-well microtitration plates. Briefly, 2 μL of each test serum was added to 198 μL of serotype Ia, Ib, II, III or V GBS bacteria at 5.14×10^7 CFU/mL in blocking buffer (1% BSA in PBS). This was incubated at 25 °C for 30 min with shaking (900 rpm), then pelleted. Supernatant was removed and the pellet washed twice with 200 μL of blocking buffer. Alexa Fluor® 488 Goat Anti-Human IgG (H&L) (Life Technologies) (1:500) in blocking buffer was added and samples incubated for 20 min at 4 °C, before being washed twice more with blocking buffer.

2.2.3. Sample preparation for antibody-mediated complement C3b/iC3b deposition assay

Antibody-mediated C3b/iC3b deposition on the surface of whole GBS bacteria was measured on the surface of formaldehyde-fixed GBS using a flow cytometric assay performed in 96-well microtitration plates. Briefly, 35 μL serotype Ia, Ib, II, III and V GBS bacteria at 5.14×10^7 CFU/mL in blocking buffer (1% BSA in PBS) were added to 10 μL IgG-depleted human complement [22] and 5 μL of each test serum. Plates were incubated for 7.5 min at 37 °C with shaking (900 rpm) then pelleted. Supernatant was removed and the pellet washed once with 200 μL blocking buffer. Samples were resuspended in 200 μL blocking buffer containing 1:500 sheep anti-human C3c FITC (Abcam) and incubated as for the surface binding assay.

2.2.4. Sample acquisition

Assays were analyzed using a Beckman Coulter Cyan flow cytometer equipped with a Cytek 96-well microtitre plate reader. Protocols were initially set-up to analyze profiles of bacterial events using bacteria-only control samples and the bacteria identified on the cytometer by the forward scatter (FS), measuring the size of the cell, and side scatter (SS), the granularity and internal structural complexity. An analysis 'gate' was drawn around the population of interest (single and diplococci) and a relevant histogram plot created to analyze the fluorescence of the bacterial population. For each sample, 10,000 individual events were analyzed for fluorescence and a horizontal gate was drawn to include 10% of the 'no antibody' control population. A fluorescence index (FI) was calculated for each sample, which involved the multiplication of the percentage of bacteria in the horizontal gate (%-gated), by the mean fluorescence of that population (X-mean). The final result for each test was expressed as the average FI of duplicate test samples minus the average FI of the bacteria and conjugate-only control. A standard unit (SU) measurement for each serum sample was then calculated by comparing to the serum FI response

obtained with the positive control serum for each serotype which was given an arbitrary value of 1000 (kind gift from Professor Carol Baker, Baylor College of Medicine, Texas) to give a result in SU/mL.

2.3. Data management and statistical analysis

Sample size was determined for the cohort study; this sub-study had 80% power ($p = 0.05$) to investigate differences between antibody concentrations in HIV-exposed and HIV-unexposed infants of at least 30% with the pre-specified hypothesis that the concentrations would be lower in HIV-exposed infants. This assay is currently being formally compared with ELISA and opsonophagocytosis uptake assays in another study.

Statistical analyzes were completed using STATA version 12 (StataCorp 2013, La Jolla, CA) and GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA). Due to the skewed distribution of the anti-GBS antibody concentrations, log transformations were required to conform to regression assumptions. A paired *t*-test was used to determine any difference in anti-GBS-antibody concentrations to each GBS serotype between birth and at 16 weeks. Comparison of infant anti-GBS antibody concentrations by maternal HIV status was calculated using the Wilcoxon rank sum test. Correlation of infant anti-GBS antibody concentration to each GBS serotype at birth and at 16 weeks was assessed using Spearman correlation. Multiple linear regression was used to ascertain the relationship between infant anti-GBS antibody concentration to each GBS serotype and the covariates of maternal age, infant sex and maternal HIV. In addition, multiple linear regression was used to explore the relationship between maternal anti-GBS antibody concentration to each serotype and the covariates maternal age, maternal HIV status. Placental transfer was defined as the ratio of infant-to-mother anti-GBS IgG concentration at birth. Missing data were excluded from analysis. Decline in antibody concentration between birth and 16 weeks was calculated using ratio of means.

3. Results

3.1. Participant characteristics

Samples were analyzed from 104 women (46 (44%) HIV-infected and 58 (56%) HIV-uninfected women) and from 100 infants born to this group of women (46 (46%) HIV-exposed and 54 (54%) HIV-unexposed infants). One mother–infant pair was excluded from the final analysis because HIV-infection in the infant was detected at 4 weeks of age and the infant was referred for rapid initiation of antiretroviral treatment. All other infants born to HIV-infected mothers were HIV-uninfected. All infants were born after 37 weeks gestation. Follow-up samples at 16 weeks were available for 93 infants (93%; 38 HIV-exposed and 55 HIV-unexposed) at a mean postnatal age of 16.4 weeks (SD = 1.7).

All HIV-infected women chose exclusive formula replacement feeding. The median (IQR) CD4 count among the HIV-infected women was 411.5 (293.9–604.0) cells/μL and the median (IQR) viral load was 800 (357–6000) copies/mL Seven women had CD4 counts of less than 200 cells/μL; three of these were taking highly active antiretroviral treatment at enrollment and four were referred to commence highly active antiretroviral treatment following delivery. Further characteristics of the study cohort have been described previously [20]. At the time of the study, local GBS disease incidence in Cape Town was reported at 0.67/1000 live births in a population with 24% of these infants being HIV-uninfected, born to HIV-infected mothers [23].

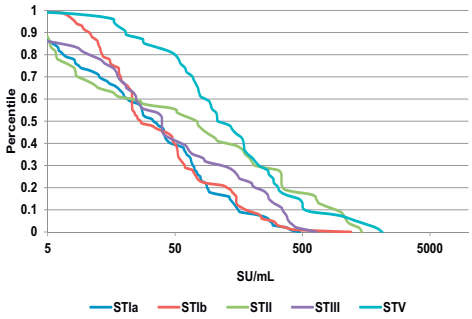


Fig. 1. Reverse cumulative distribution curve of maternal anti-GBS IgG surface binding by serotype concentrations (SU/mL). Curve demonstrates the percentage of the total population with each antibody concentration for each of the five GBS serotypes. ST, serotype; SU/mL, standard units/mL

3.2. Maternal anti-GBS-antibody to GBS serotypes Ia, Ib, II, III and V

All maternal sera had measurable geometric mean concentrations (GMC) of antibody to at least one serotype. The distribution of mothers' antibodies that bound to the surface of GBS serotypes Ia, Ib, II, III and V is shown in Fig. 1. The predominant surface binding anti-GBS-antibody was against serotype V, followed by II, III, Ib and Ia (Fig. 1).

Women with HIV infection had significantly lower GMC antibody concentrations to all five serotypes at delivery compared to HIV-uninfected women (Fig. 2 and Table 1). There were no associations between CD4 count and viral load on anti-GBS antibody concentration by serotype in HIV-infected women.

The concentrations of antibody-mediated complement C3b/iC3b deposition by GBS serotype are shown in Table 2. The GMC of antibody-mediated C3b/iC3b deposition was significantly greater in HIV-uninfected than HIV-infected women for all serotypes. The GMC of antibody-mediated C3b/iC3b deposition ranged from 4.9 SU/mL (serotype III) to 6.17 SU/mL (serotype V) in HIV-uninfected women and from 2.6 SU/mL (serotype Ib) to 3.4 SU/mL (serotype V) in HIV-infected women.

3.3. Association of maternal HIV infection with placental transfer of anti-GBS antibody

There was a positive correlation between paired maternal and infant surface binding antibody concentrations for all serotypes (Ia, $r^2 = 0.63$, $p < 0.001$; Ib, $r^2 = 0.71$, $p < 0.001$; II, $r^2 = 0.17$, $p < 0.001$; III, $r^2 = 0.35$, $p < 0.001$; V, $r^2 = 0.16$, $p < 0.001$). HIV-infected women had significant reductions in placental transfer of anti-GBS serotypes II and V compared with HIV-uninfected women (Table 3). There was no association between CD4 count or viral load on transplacental transfer ratios.

3.4. Infant anti-GBS-antibody by serotype at birth to GBS serotypes Ia, Ib, II, III and V

HIV-exposed uninfected infants had significantly lower GMCs of anti-GBS surface binding antibodies to all serotypes than unexposed infants (Fig. 3 and Table 1). In a multiple regression model maternal HIV-status remained significant ($p < 0.05$).

However, there were no significant associations found between infant anti-GBS-antibody concentrations to each serotype and maternal age or infant sex.

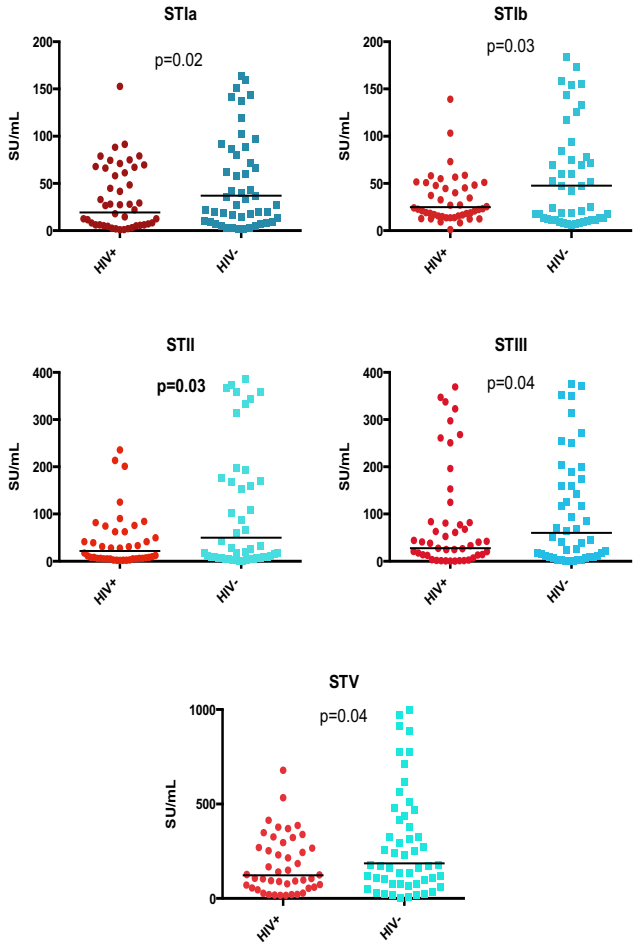


Fig. 2. Maternal anti-GBS surface binding IgG to GBS serotypes Ia, Ib, II, III and V concentrations at delivery (SU/mL). ST indicates GBS serotype. HIV indicates human immunodeficiency virus. Horizontal lines indicate GMC response.

Table 1
Geometric mean surface binding antibody concentrations (95% confidence intervals) in HIV-infected and HIV-uninfected mothers and their infants at birth and at 16 weeks of age.

	STIa	STIb	STII	STIII	STV
HIV+ mother	19.3 (12.7–29.3)	24.9 (19.6–31.6)	21.8 (13.4–35.7)	27.8 (15.9–48.6)	121.9 (88.0–169.0)
HIV− mother	37.0 (24.8–55.2)	47.6 (33.3–68.1)	50.0 (28.7–87.0)	60.1 (36.0–100.5)	185.6 (126.4–272.4)
HIV-exposed infants (birth)	9.4 (6.1–14.5)	13.4 (9.6–18.7)	14.6 (8.1–26.3)	26.6 (17.8–39.8)	90.4 (61.16–133.6)
HIV-unexposed infants (birth)	27.0 (16.5–44.3)	24.5 (16.4–36.5)	42.7 (23.4–77.8)	62.7 (38.1–103.1)	165.8 (112.0–245.4)
HIV-exposed infants (16 w)	7.0 (3.8–13.0)	8.7 (6.3–12.1)	17.6 (8.5–36.3)	48.0 (28.3–81.3)	68.54 (38.0–123.6)
HIV-unexposed infants (16 w)	21.6 (13.6–34.2)	14.9 (10.6–21.1)	68.3 (38.8–120.3)	97.4 (63.0–150.7)	180.2 (106.3–305.4)

STIa, GBS serotype Ia; STIb, GBS serotype Ib; STII, GBS serotype STII; STIII, GBS serotype III; STV, GBS serotype V; HIV+, HIV infected; HIV−, HIV uninfected; 16 w, 16 weeks of age.

Table 2
Geometric mean concentration of antibody-mediated C3b/iC3b deposition (95% confidence intervals) on GBS serotypes Ia, Ib, II, III and V in HIV-infected and HIV-uninfected mothers and their infants at birth.

	STIa	*p* value	STIb	*p* value	STII	*p* value	STIII	*p* value	STV	*p* value
HIV+ mother	2.9 (2.3–3.6)		2.6 (1.9–3.2)		3.1 (2.4–3.9)		2.8 (2.2–3.6)		3.4 (2.8–4.2)	
HIV− mother	5.3 (4.6–6.2)	0.003	4.9 (4.2–5.4)	0.003	6.2 (5.6–6.8)	0.001	5.3 (4.5–6.2)	0.001	6.2 (4.9–6.9)	<0.0001
HIV-unexposed infants	6.1 (5.9–6.3)		5.9 (4.6–6.2)		6.9 (5.6–7.2)		6.6 (6.4–6.7)		6.3 (5.8–6.9)	
HIV-exposed infants	3.8 (3.0–4.8)	0.02	3.1 (3.0–4.1)	0.02	4.2 (3.9–4.9)	0.02	3.9 (3.1–5.0)	0.03	4.2 (3.3–5.4)	0.04

STIa, GBS serotype Ia; STIb, GBS serotype Ib; STII, GBS serotype STII; STIII, GBS serotype III; STV, GBS serotype V; HIV+, HIV infected; HIV−, HIV uninfected.

Table 3
Influence of maternal HIV-infection on transplacental transfer of anti-GBS surface binding antibody to GBS serotypes Ia, Ib, II, III and V.

GBS serotype	HIV-infected mothers: infants (IQR)	HIV-uninfected mothers: infants (IQR)	% Reduction in HIV-infected	*p* value
STIa	0.66 (0.34–0.99)	0.6 (0.39–0.96)	0%	0.86
STIb	0.48 (0.17–0.88)	0.52 (0.32–0.78)	8%	0.48
STII	0.42 (0.22–0.59)	1.0 (0.42–1.66)	58%	<0.001
STIII	0.54 (0.31–1.03)	0.95 (0.4–3.05)	43%	0.05
STV	0.51 (0.28–0.79)	0.75 (0.26–2.9)	32%	0.04

STIa, GBS serotype Ia; STIb, GBS serotype Ib; STII, GBS serotype STII; STIII, GBS serotype III; STV, GBS serotype V; IQR, interquartile range; HIV+, HIV infected, HIV–, HIV uninfected; 16 w, 16 weeks of age.

Significantly lower GMC of antibody-mediated C3b/iC3b deposition was noted amongst HIV-exposed infants compared to HIV-unexposed infants for all serotypes (Table 2). Notably, the GMC of antibody-mediated C3b/iC3b deposition was higher in infants than in their mothers for all serotypes.

3.5. Infant anti-GBS antibody at 16 weeks of age

Anti-GBS surface binding antibody GMCs remained significantly lower at 16 weeks in HIV-exposed uninfected infants compared to unexposed infants for all serotypes (Table 1). HIV-exposed infants had significantly slower median rate of antibody decline compared to HIV-unexposed infants at 16 weeks compared to birth to STII (ratio of decline 1.03 [IQR −0.35 to 4.6] vs 0.21 SU/mL [−0.62 to 0.54], $p = 0.04$) and STIII (1.53 [−0.21 to 5.36] vs −0.09 SU/mL (−0.60 to 0.93), $p = 0.03$) but not to ST1a (−0.12 [IQR −0.64 to 0.13] vs −0.04 [−0.39 to 0.45]), ST1b (−0.52 [−0.65 to 0.46] vs −0.36 [−0.18 to 0.74]) or STV (−0.40 [−0.61 to 2.9] vs 0.14 [−0.52 to 2.7]).

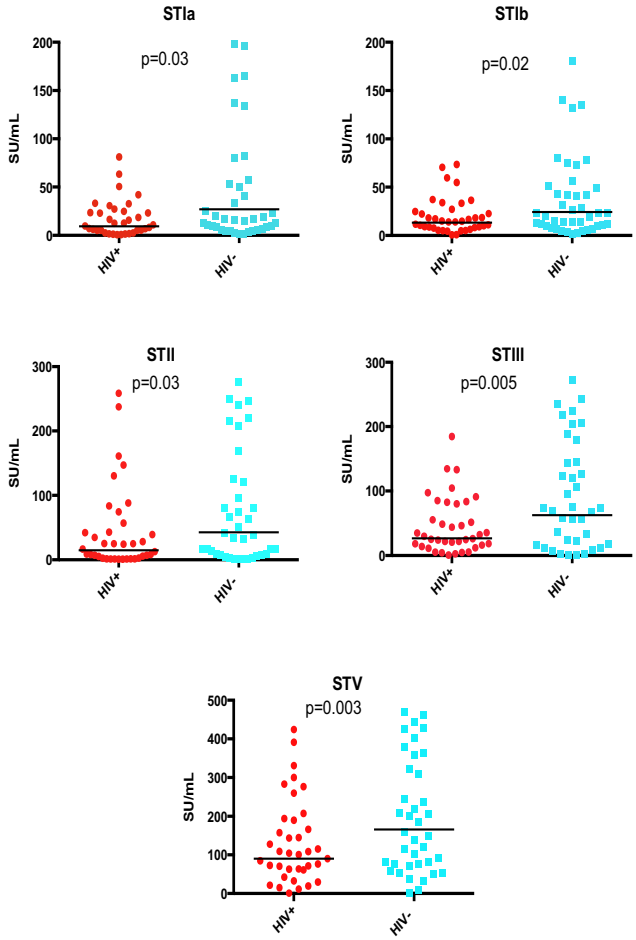


Fig. 3. Infant anti-GBS IgG serotype specific antibody concentrations (SU/mL). ST indicates GBS serotype. HIV indicates human immunodeficiency virus. Horizontal lines indicate GMC.

4. Discussion

Our findings demonstrate that total surface binding and functional maternal antibody concentration to each of the GBS serotypes (Ia, Ib, II, III and V) is lower at delivery in HIV-infected women compared to their HIV-uninfected peers. Further, for three of the five serotypes (II, III, V) the transplacental transfer ratio was significantly lower from HIV-infected women to their uninfected infants. It follows therefore that infants born to HIV-infected women had lower anti-GBS surface binding antibody concentrations at birth for all serotypes; this difference was still evident at 16 weeks of age. The implication of our findings is that HIV-exposed infants may be more vulnerable to early and late onset GBS disease than infants born to HIV-uninfected mothers.

Rectovaginal colonization with GBS is more frequent in HIV-infected women compared to HIV-uninfected women [6,24]. Maternal colonization is the major risk factor for infant acquisition at birth and for early onset GBS disease. Thus infants born to HIV infected mothers may have both increased susceptibility and increased exposure. It is generally understood that maternal colonization stimulates maternal immunity; thus the finding of lower maternal antibody concentrations despite higher colonization rates suggests impairment of anti-GBS-specific antibody production. This is also seen with polysaccharide-specific antibody production in HIV-infected adults, such as in pneumococcal disease [25]. The fact that HIV-infected women have higher rates of GBS colonization than HIV-uninfected women may mean that antibody production secondary to colonization with GBS is deficient in the context of maternal HIV-infection increasing the risk of invasive GBS disease in HIV-exposed infants.

It is difficult to correlate our anti-GBS antibody results with what is known regarding maternal colonization and infant GBS disease in this South African population. However, the highest concentrations of antibodies in women at delivery were to STV and III bacteria. The latter is consistent with the known predominance of STIII among carried strains in South African women (37%) but STV is carried by a minority of women (10%) [24,26]. In our cohort the lowest levels of antibody were to STIa bacteria, which is consistent with an increased incidence of disease due to this serotype [26]. Our findings may suggest differences in the immune response to different colonizing serotypes.

It is likely that lower maternal antibody concentrations and altered transplacental transfer of antibody in HIV infection play a role in the increased susceptibility to infectious diseases in infancy [19,27], but there is a lack of data on the impact of maternal HIV infection on neonatal infections in general, including GBS infections [28]. Infant anti-GBS surface binding antibody by serotype remains lower at 16 weeks in HIV-exposed, uninfected infants, who might therefore be more vulnerable to late onset GBS disease. This corresponds to recent data from Cape Town which suggest that 56% of GBS disease occurred after 7 days of life in a population where one in four infants were HIV-exposed [23]. Apart from the reduced passive immunity provided by maternal antibody in the context of maternal HIV infection it might be that there are differences in the immune response to infection of HIV-exposed, uninfected infants secondary to *in utero* HIV exposure, as some data suggest that these infants have altered lymphocyte differentiation and function despite remaining HIV-uninfected [29,30]. However, how this corresponds to clinical infection in the neonatal period is not known.

Our findings are consistent with studies that demonstrate the higher risk of infectious morbidity and mortality in HIV-exposed infants compared to HIV-unexposed infants [31–33]. Recent South African studies demonstrated a high prevalence of neonatal GBS disease in a population with a high HIV burden [23,24,34]. A Study from Malawi also demonstrated high rates of early and late onset GBS infection in a population with high HIV prevalence [35]. In contrast, one study from the USA did not find evidence of increased susceptibility to GBS infection in HIV-exposed, uninfected infants suggesting either regional screening and treatment differences for both HIV infection and GBS carriage or population-based differences in late onset GBS disease that might be unrelated to HIV-status [36].

Our study has some limitations. Firstly, we did not assess colonization in the mothers participating in the study and thus were unable to correlate antibody responses to maternal colonization. Whilst no infant developed early or late onset sepsis or meningitis during the study, our sample size was also too small to detect this as a valid endpoint and this was not the aim of the study.

The use of two novel flow cytometric assays that quantify antibody binding to the surface of GBS bacteria and antibody-mediated C3b/iC3b deposition on the bacterial surface may provide a more complete assessment of antibody function than the measurement of anti-polysaccharide IgG alone. However, the response measured is not wholly serotype-specific as part of the antibody binding is to cell wall proteins as well as the polysaccharide capsule. Despite this, we see a serotype-specific response with a clear differentiation between the distribution of antibody binding to the different serotypes.

The potential of a trivalent maternal GBS vaccine encompassing the major disease-causing serotypes (Ia, Ib, III) [8] to protect against neonatal disease remains promising. However, it is vital that ongoing evidence to support its use in vulnerable populations with high HIV-prevalence is collected in order to support immunization strategies in Sub-Saharan Africa.

Acknowledgements

We thank the mothers and infants who participated in this study. Professor Androulla Efstratiou, Public Health England, Colindale for access to GBS strains and Professor Carol Baker, Baylor College of Medicine, Texas for access to her Human positive serum samples. Mr. Mohammed Abdul-Khalid for his statistical advice and support.

Conflict of interest statement: PTH is an investigator for research studies done on behalf of St Georges, University of London (London, UK) and funded by Novartis vaccines (Siena, Italy) and serves as a consultant to Novartis vaccines on GBS vaccines. KLD is funded by a Wellcome Trust Global Health Fellowship (London, UK), Royal College of Physicians Thomas Watt Eden Fellowship (London, UK) and BHIVA/Gilead Registrar Award (London, UK). BK is funded by an MRC Program grant (London, UK) and an NIHR Senior Research fellowship (London, UK). CJ was funded by ESPID (Geneva, Switzerland) and The Thrasher Research Fund (Atlanta, USA). *Funding*: This work is supported by a Wellcome Trust Global Health Fellowship (Grant Number KLD2013); The Thomas Watt Eden Fellowship (Royal College of Physicians Grant Number 01012013); and the Gilead/BHIVA Registrar Award. *Contribution statement*: KLD developed the manuscript and original research idea. LA participated in the research. PTH, BK, ST, CJ developed the original idea and substantially contributed to the development of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.12.025.

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Antibody-Mediated Complement C3b/iC3b Binding to Group B Streptococcus in Paired Mother and Baby Serum Samples in a Refugee Population on the Thailand-Myanmar Border

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***Streptococcus agalactiae* (group B streptococcus [GBS]) is the leading cause of neonatal sepsis and meningitis. In this study, we determined antibody-mediated deposition of complement C3b/iC3b onto the bacterial cell surface of GBS serotypes Ia, Ib, II, III, and V. This was determined for 520 mother and umbilical cord serum sample pairs obtained at the time of birth from a population on the Thailand-Myanmar border. Antibody-mediated deposition of complement C3b/iC3b was detected to at least one serotype in 91% of mothers, despite a known carriage rate in this population of only 12%. Antibody-mediated C3b/iC3b deposition corresponded to known carriage rates, with the highest levels of complement deposition observed onto the most prevalent serotype (serotype II) followed by serotypes Ia, III, V, and Ib. Finally, neonates born to mothers carrying serotype II GBS at the time of birth showed higher antibody-mediated C3b/iC3b deposition against serotype II GBS than neonates born to mothers with no serotype II carriage. Assessment of antibody-mediated C3b/iC3b deposition against GBS may provide insights into the seroepidemiology of anti-GBS antibodies in mothers and infants in different populations.**

Group B streptococcus (GBS) (*Streptococcus agalactiae*) is a natural commensal of the vagina and rectum and is normally carried asymptomatically (1). However, it is also the leading cause of neonatal sepsis and meningitis in developed countries and is associated with a high mortality rate with early-onset disease (0 to 6 days postbirth) and high morbidity in survivors of late-onset GBS meningitis (2, 3). Prophylactic treatment with antibiotics has been introduced in the United States and Australia, greatly reducing the incidence of early-onset disease in those countries (4, 5). However, the incidence of late-onset disease (defined as occurring between 7 and 90 days after birth) has not decreased (5). In late-onset neonatal sepsis (LONS), GBS may be acquired horizontally from the community, making it harder to target the source of infection (6, 7).

Natural antibodies to GBS are transferred from mother to baby *in utero* beginning at 17 weeks (passive transport) and by active transport during the third trimester of pregnancy, and they are thought to protect the neonate from disease. Several clinical factors affect the likelihood of developing neonatal sepsis, including prematurity (8, 9), as premature babies have reduced specific antibodies due to the reduced time for transfer from mother to baby. Determination of the protective levels of antibody required to prevent GBS infection in neonates would greatly facilitate the evaluation and effective use of new vaccines. Effective vaccination would reduce the long-term costs of treatment of infected neonates; the health care costs of infants that acquire neonatal GBS infection are estimated to be roughly double those of control neonates matched for age and birth weight (10). Furthermore, effective vaccination will reduce the likelihood of emergence of antibiotic-resistant strains (11).

Functional immunity to GBS has been demonstrated in an opsonophagocytosis killing assay (OPKA) that measures the antibody and complement-dependent uptake and killing of bacteria by human phagocytes (12, 13). Human vaccine sera with positive OPKA activity have also been shown to passively protect mice in a maternal passive protection/infant challenge model (14). OPKA assays are generally laborious to perform and require large volumes of test sera. Reliance on human donor phagocytes introduces an extra variable, although this has been addressed with the use of the human phagocytic cell line HL60 (15). Additionally, this HL60-based OPKA has been adapted to measure the uptake of fluorescently labeled bacteria by flow cytometry, thus removing the extra plating and counting required for assay analysis (16). A fluorescence opsonophagocytosis assay (fOPA) was developed that measures both the uptake of bacteria and the functional respiratory activity of the HL60 cells in a 96-well format (17). However, OPKA and fOPA require considerable resources to perform

Received 5 January 2015 **Accepted** 8 January 2015

Accepted manuscript posted online 14 January 2015

Citation Herbert J, Thomas S, Brookes C, Turner C, Turner P, Nosten F, Le Doare K, Hudson M, Heath PT, Gorringe A, Taylor S. 2015. Antibody-mediated complement C3b/iC3b binding to group B streptococcus in paired mother and baby serum samples in a refugee population on the Thailand-Myanmar border. Clin Vaccine Immunol 22:319–326. doi:10.1128/CVI.00803-14.

Editor: D. L. Burns

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doi:10.1128/CVI.00803-14

large studies, and minimizing the sample volume required is important, particularly for infant studies.

We evaluated the antibody-mediated deposition of complement C3b/iC3b onto bacteria representing five GBS serotypes with 534 serum sample pairs from mother and cord blood samples from a population located on the Thailand-Myanmar border (18). This was evaluated using flow cytometry, and as opsonization of bacteria by antibody and complement is required for uptake and killing by phagocytic cells, this may be a surrogate for opsonophagocytosis.

MATERIALS AND METHODS

Sample collection. The study population was described by Turner et al. (18). In brief, mothers enrolled in the study were residents of the Maela refugee camp located on the Thailand-Myanmar border. Inhabitants were mostly Karen refugees, with a total population of approximately 43,000. Around 1,500 deliveries occur in the camp every year, and all antenatal care is provided by the Shoklo Malaria Research Unit. A total of 549 women were enrolled in the study at 28 to 30 weeks' gestation. Venous blood samples were collected from the mothers and umbilical cords at the time of birth. Of the sera collected, a total of 543 serum samples from mothers and 525 serum samples from umbilical cords were available for this study. Data were also collected on the GBS carriage status of the mother at the time of birth, which was assessed by culture of vaginal and rectal swabs (18).

Study ethics. All women gave informed consent to participate in the study. Ethical approval was granted by the ethics committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2009-011-03) and the Oxford Tropical Research Ethics Committee, Oxford University, United Kingdom (48 08).

GBS isolates and growth conditions. Group B streptococcus isolates used in this study were geographically diverse United Kingdom clinical isolates from neonatal blood cultures. H092040676 (serotype Ia), H090820125 (serotype Ib), H090320548 (serotype II), H092120162 (serotype III), and H091780506 (serotype V) were kindly provided by Androulla Efstratiou, Public Health England, Colindale. Throughout the article, strains are referred to by their serotype.

GBS isolates used in the complement deposition assay were grown in Todd-Hewitt broth at 37°C with shaking (200 rpm). Once an optical density at 600 nm (OD_{600}) of 1.0 was reached, cultures were centrifuged at 3,060 × *g* for 5 min to pellet the bacteria. The pellet was resuspended in the same volume of phosphate-buffered saline (PBS, pH 7.4; Severn Biotech, United Kingdom) containing 2% formaldehyde and incubated at room temperature for 1 h. Bacteria were then washed in PBS by 3 rounds of centrifugation at 3,060 × *g* for 5 min and resuspended. The final cell pellet was resuspended in 1 ml PBS, which was stored at 4°C before use.

Production of IgG-depleted human complement. Human complement used in the complement deposition assay was produced as described previously (19). Briefly, blood was collected from volunteers into containers with 50 μg/ml lepirudin (Movianto, United Kingdom). Plasma was then removed, pooled, and depleted of IgG by protein G Sepharose affinity chromatography. Aliquots were stored at −70°C until use.

Antibody-mediated complement C3b/iC3b deposition assay. Complement deposition assays were performed in 96-well plates, and each test serum sample was analyzed at a single dilution. To each well, 5 μl sera (10% final concentration) was added, followed by 10 μl IgG-depleted human complement (20% final concentration) and 35 μl fixed bacteria (1.8×10^6 CFU/well). The bacteria were resuspended in blocking buffer (BB) (PBS containing 1% bovine serum albumin [BSA]; Sigma, United Kingdom). Controls included samples of bacteria only and bacteria and complement only, which were made to the same volume using BB. Preactivated zymosan (1.8×10^6 particles/well; CompTech, USA) was also used as a control to normalize for day-to-day variation. The plate was incubated for 7.5 min at 37°C with shaking at 900 rpm. Plates were then centrifuged at 3,060 × *g* for 5 min, and the supernatant was removed, ensuring that the bacterial pellet was not disturbed. Pellets were washed with 200 μl BB and then centrifuged at 3,060 × *g* for 5 min, and the supernatant was removed, ensuring that the bacterial pellet was not disturbed. Each pellet was then resuspended in 200 μl BB containing rabbit anti-human C3c conjugated to fluorescein isothiocyanate (FITC) at 1/500 (Abcam, United Kingdom), which binds to both C3b and iC3b, and the plate was incubated at 4°C for 20 min. The plate was centrifuged at 3,060 × *g* for 5 min, and the bacterial pellet was washed twice in BB, as performed previously. Finally, the pellet was resuspended in 200 μl BB and kept at 4°C until analysis by flow cytometry.

Data collection and analysis. Samples were analyzed on a CyAn ADP flow cytometer (Beckman Coulter, Inc.) with an automated microsampler system (Cytek Development, Inc.). Data were acquired in a Summit 4.3 (Beckman Coulter, Inc.), and 5,000 events were obtained for each sample. This was performed by setting a gate that contained 10% of the events detected with the control containing bacteria and complement only. A fluorescence index (FI), which was calculated by multiplying the number of events in the gate noted above and the mean fluorescence intensity, was assigned to each sample. For each sample, the FI of the control containing bacteria and complement only was subtracted, giving a final fluorescence index minus complement-only control (FI−C′) value for each serum sample, which was used as a measure of antibody-dependent complement deposition on the bacterial surface. Antibody-independent C3b/iC3b deposition was similar between strains except for the serotype II isolate, which showed greater FI values. Data from each day were normalized to the C3b/iC3b bound to the preactivated zymosan control to account for day-to-day variation within the assay.

Each serum sample was analyzed in the antibody-mediated complement deposition assay (CDA) against each of the five GBS serotypes chosen. Each serum sample was analyzed in duplicate, and if the coefficient of variance (%CV) between the two replicates was >35%, then the serum was analyzed again. If the repeat FI−complement values were not within 50% of each other, then the serum was subjected to a further repeat analysis. If the repeats were within 50% of each other, the two C′ values were averaged; this was also the case if the analysis was repeated a third time. If all three repeats were not within 50% of each other, then the sample was given a not determined (ND) value for FI−C′. Graphs include all data except the samples that were assigned ND, unless otherwise stated.

Clinical data analysis and statistics. Clinical data were obtained from the mothers enrolled in the study and the neonates (18). These data included GBS carriage status of the mother, ethnic group, past obstetric history, recent antibiotic exposure, and any complications occurring during birth. Infants were also visited at 7 and 28 days postbirth and at unplanned sick visits, and the health of the neonate was checked specifically for signs of neonatal sepsis. All graphs were drawn in GraphPad Prism 6 (GraphPad Software, Inc.). Reverse cumulative distribution curves for CDA data were prepared as described by Reed et al. (20). The effects of clinical parameters on antibody-mediated C3b/iC3b deposition were evaluated using one-way analysis of variance (ANOVA).

RESULTS

Complement deposition assay. The CDA was optimized for incubation times, blocking buffer formulation, IgG-depleted human plasma concentration, shaking speed, bacterial concentration, and serum dilution. Reproducibility of the CDA was measured using a panel of 1 rabbit and 13 human sera against a serotype II strain. Intra-assay precision (three assays performed on 1 day) and interassay precision (one assay performed on 3 separate days) returned mean coefficients of variance of 22% and 26%, respectively. Interassay and complement variations over time were controlled by normalizing all data to the fluorescence index obtained with activated zymozan-complement controls, which were included in each assay. Over 67 assays, the mean standard deviation of FI for the zymozan controls was 8,830 (coeffi-

TABLE 1 Antibody-mediated C3b/iC3b deposition values above or below the lower limit of detection (FI−C′ < 2,000) or not determined due to a variable FI−complement value obtained in repeat analyses

Serotype	FI−C′ > 2,000 (% [*n*])[a]	FI−C′ < 2,000 (% [*n*])	FI−C′ not determined (% [*n*])
Ia	56.6 (605)	34.6 (370)	8.7 (93)
Ib	26.7 (285)	72.2 (771)	1.1 (12)
II	71.2 (760)	22.8 (244)	6.0 (64)
III	38.7 (413)	53.4 (570)	8.0 (85)
V	42.6 (455)	53.6 (572)	3.8 (41)

[a] *n* = number of samples analyzed. FI−C′ is the fluorescence index value obtained with the test serum minus the C3b/iC3b fluorescence obtained with the control containing bacteria and complement only.

cient of variance, 31.6%). Linearity of the assay was assessed using positive-control human sera (kind gift from Carol Baker), and it was shown to be highly linear, with a mean R^2 of 0.96 for the five serotypes tested.

Antibody-mediated C3b/iC3b deposition by paired mother and cord serum samples. Antibody-mediated C3b/iC3b deposition onto GBS isolates of serotypes Ia, Ib, II, III, and V for each serum sample was evaluated using high-throughput flow cytometry. A total of 1,045 serum samples were analyzed: 520 from mothers and 525 associated cord samples (5 sets of twins). Carriage and clinical data relating to the mothers and neonates are reported in Turner et al. (18). The GBS isolates were selected to represent the two most prevalent serotypes (II and Ia), two serotypes of medium prevalence (III and V), and one serotype of low prevalence (Ib) in this region. A value (fluorescence index obtained with test serum minus complement-only control value [FI−C′]) of C3b/iC3b deposition was assigned to each serum sample against each serotype tested. Sera with C3b/iC3b deposition values below the limit of detection of the assay (FI−C′, <2,000) were assigned a nominal FI−C′ value of 1,000 for data analysis. In addition, sera giving nonreproducible FI−C′ values in duplicate assays were recorded as not determined (ND) and excluded from the analysis. The number of sera assigned an FI−C′ value of 1,000 or ND varied among the GBS serotypes tested (Table 1).

Reverse cumulative distribution curves of antibody-mediated C3b/iC3b deposition observed against each serotype with mother and cord serum samples were compared (Fig. 1A). In the 549 mothers originally enrolled in the study, a carriage rate of 12% ($n = 66$) was determined using culture- and PCR-based methods (18). GBS serotype II was the most frequently carried, followed by serotypes Ia, III, V, and Ib. The level of antibody-mediated C3b/iC3b deposition observed against each serotype in the whole cohort corresponded with the carriage findings, with the highest number of sera showing measurable C3b/iC3b deposition to serotype II, followed by serotype Ia, serotype III, serotype V, and serotype Ib.

Reverse cumulative distribution curves were also used to compare the distribution of antibody-mediated C3b/iC3b deposition in mother and cord serum samples separately for each serotype (Fig. 1B). Antibody-mediated C3b/iC3b deposition levels for serotypes II and Ia were clearly higher in the cord samples than in samples from the mothers ($P = 0.009$ and $P = 0.037$, respectively). This trend was observed for serotypes III, V, and Ib, but these differences were not significant ($P > 0.05$). The same patterns were observed when geometric mean C3b/iC3b depositions were compared for the mother and cord data sets for each serotype (Fig. 2).

Although GBS carriage was only detected in 12% of mothers, antibody-mediated C3b/iC3b deposition was above the lower limit of detection in 97% of cord samples and 92% of mother samples to at least one serotype tested, as shown in Fig. 3. Moreover, 7% of cord samples and 4% of mother samples contained antibodies that mediated C3b/iC3b deposition to all five serotypes tested. The percentages of individuals with antibody-mediated C3b/iC3b deposition to ≥1 strain with FI−C′ values of >5,000, >10,000, and >20,000 are also presented, with fewer individuals possessing CDA activity at these higher levels. However, there is a greater divergence between values obtained for mother and cord samples as the cutoff value is increased.

Association of clinical data with antibody-mediated C3b/iC3b deposition. In this cohort, no neonates were found to have early-onset disease (EOD) due to group B streptococcal infection. Two neonates born to carriage-positive mothers showed signs of EOD, but GBS was not identified as the causative agent. Thus, it was not possible to evaluate the level of protective antibody-mediated complement deposition required to prevent disease.

The effects of a number of other clinical parameters on levels of antibody-mediated C3b/iC3b deposition were evaluated by one-way ANOVA. These included the mother's age, number of previous pregnancies, neonate's gestational age, and use of antibiotics. None of these parameters had a significant effect ($P > 0.05$) on the functional antibody levels in mother and cord samples in this study (data not shown). Sera from babies born at <37 weeks' gestation did not show lower functional antibody levels to any of the five GBS serotypes tested (data not shown).

GBS carriage was determined for each mother at the time of birth by culture of vaginal and rectal swabs (18). No carriage data were obtained from the neonate. For each serotype, antibody-mediated C3b/iC3b deposition in samples from mothers that carried GBS at the time of birth and their neonates was compared to that of mothers and neonates where GBS carriage was not detected. The antibody-mediated complement deposition in the cord samples from neonates with mothers carrying GBS serotype II was significantly higher ($P < 0.001$) than that determined in cord samples taken from neonates born to mothers that did not carry serotype II GBS (Fig. 4). A similar trend was observed when comparing sera from mothers who carried serotype Ia GBS and their neonates with samples from noncarriage mothers, shown in Fig. 5. This did not reach statistical significance due to the low number of mothers carrying this serotype. No significant differences were determined between mother and cord serum samples against serotypes III, V, and Ib, possibly due to the small number of mothers carrying these serotypes (18).

DISCUSSION

We developed a flow cytometry assay that measures antibody-mediated deposition of complement components (C3b/iC3b) that are required for opsonophagocytosis onto the surface of GBS serotypes Ia, Ib, II, III, and V. Maternal antibodies to GBS capsular polysaccharides have been shown to protect infants from early-onset GBS disease (21, 22), with opsonophagocytic killing the likely effector mechanism (15, 23). Opsonophagocytosis assays were developed for a range of bacteria (24–27) and have been widely used to assess immune responses to pneumococcal vac-

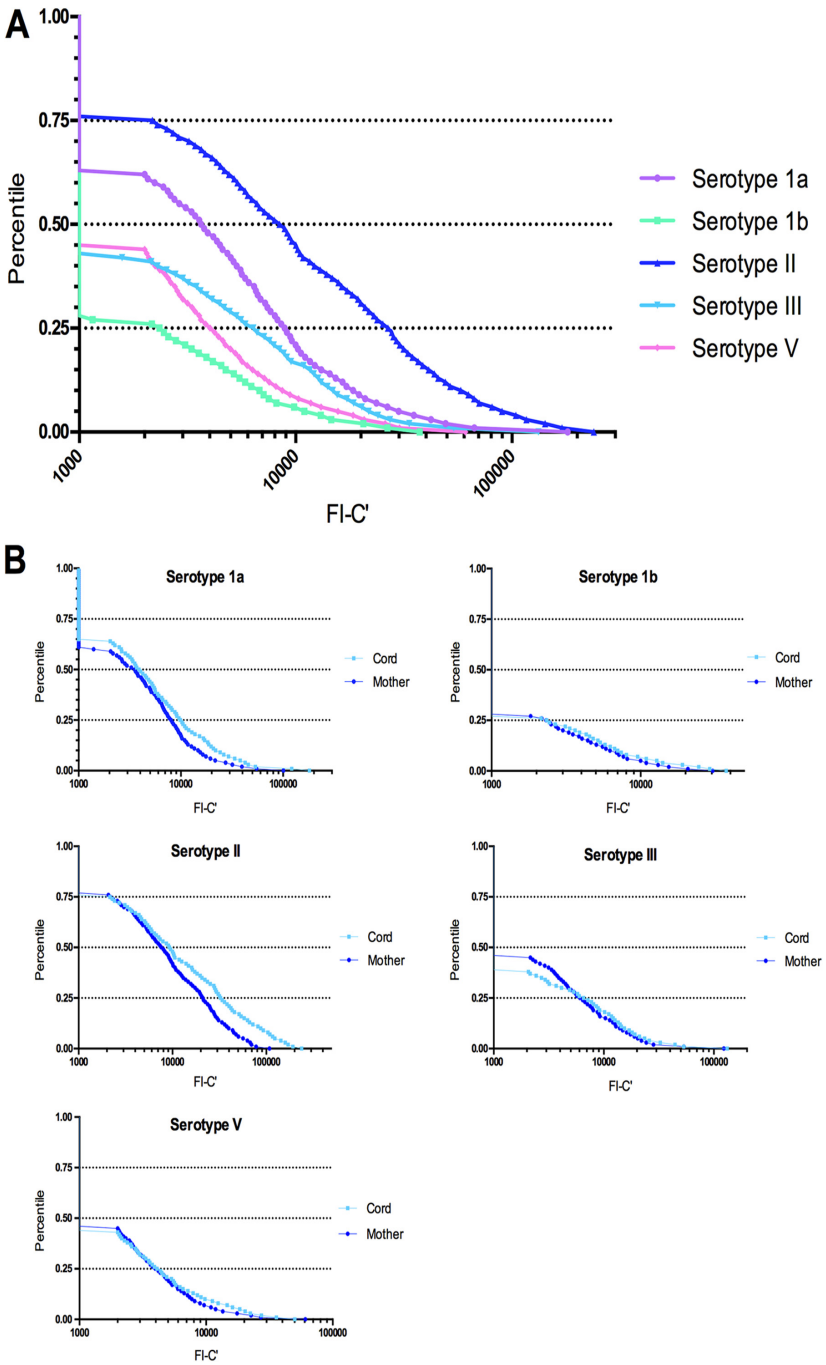


FIG 1 (A) Reverse cumulative distribution (RCD) curves show distribution of antibody-mediated C3b/iC3b deposition levels against GBS serotypes Ia (n = 975), Ib (n = 1,059), II (n = 1,007), III (n = 986), and V (n = 1,030) in serum samples taken from mothers and umbilical cords. (B) RCD curves show antibody-mediated C3b/iC3b deposition levels in mother and cord samples separately against GBS serotypes Ia (mother, n = 497; cord, n = 478), Ib (mother, n = 535; cord, n = 521), II (mother, n = 505; cord, n = 499), III (mother, n = 500; cord, n = 483), and V (mother, n = 518; cord, n = 509). FI-C′, fluorescence index minus complement only.

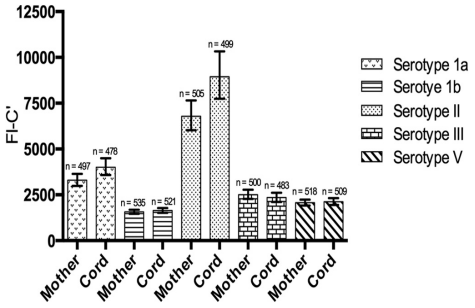


FIG 2 Geometric means of antibody-mediated C3b/iC3b deposition levels in mother and cord samples against serotypes Ia, Ib, II, III, and V GBS (n = the number of samples included in each group). Data do not include the values that were not assigned an FI−C′ value. Bars represent 95% confidence intervals.

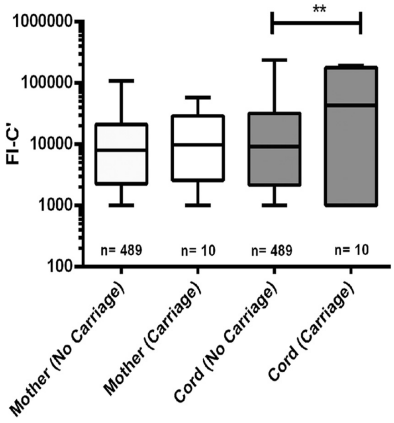


FIG 4 Box plot shows the effect of serotype II carriage on antibody-mediated C3b/iC3b binding to serotype II GBS in sera from mother and cord pairs taken from mothers who were carriage positive (serotype II only) versus those from mothers who were carriage negative. **, $P < 0.001$.

cines where a role in protection was demonstrated (28–30). However, these assays rely on a source of phagocytic cells, such as fresh human peripheral blood mononuclear cells (25, 27, 31) or differentiated HL60 cells (29, 30), and day-to-day variability in these cells can affect assay performance (32). The interaction of complement proteins with a number of pathogens, including *Neisseria meningitidis* (33) and *Haemophilus influenzae* (34), has been studied in detail and is key in protecting against disease. Complement interactions with *Streptococcus pneumoniae* are thought to be important, with individuals deficient in classic pathway components having increased susceptibility to disease (35) and serotypes with resistance to C3b/iC3b opsonization demonstrating greater invasive capacity (36). The potential role of opsonophagocytosis in protection from GBS disease was demonstrated using viable count assays that use log reduction in CFU determination as a primary readout (37–39).

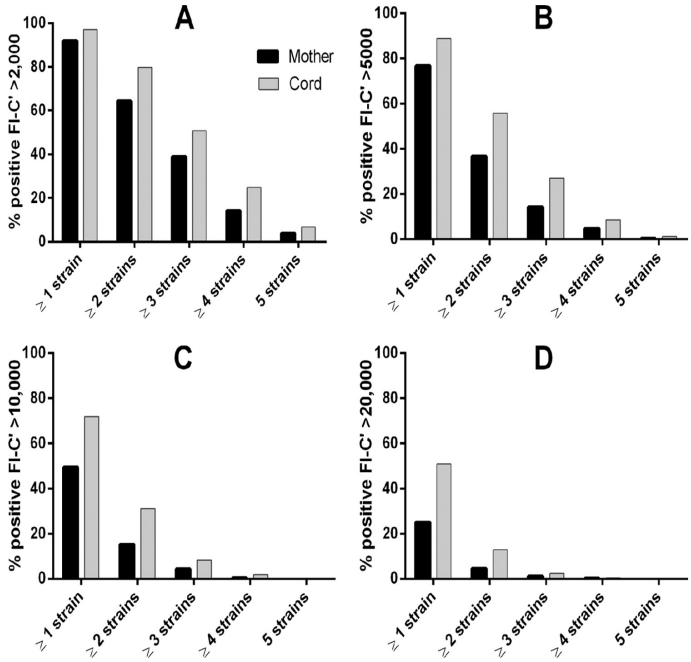


FIG 3 Percentages of mother and cord samples that show antibody-mediated C3b/iC3b binding against ≥1 GBS strain tested using a cutoff FI−C′ value of >2,000 (A), >5,000 (B), >10,000 (C), and >20,000 (D). Samples that were assigned a not-determined value were classified as not having measurable functional antibody levels along with the samples assigned an FI−C′ value of 1,000. Cord, n = 525; mother, n = 543.

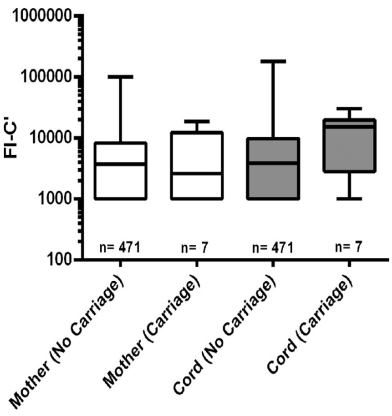


FIG 5 Box plot shows the effect of serotype Ia carriage on antibody-mediated C3b/iC3b binding to serotype Ia GBS. Comparison of functional antibody levels was done by comparing the mother and cord pairs taken from mothers who were carriage positive (serotype Ia only) versus those from mothers who were carriage negative.

In this study, a complement deposition assay was utilized as a possible surrogate for OPA. This flow cytometric assay measures the antibody-dependent deposition of the complement opsonins C3b/iC3b onto the surface of whole bacteria. This assay uses smaller volumes of test sera than OPKA and fOPA yet measures the total antibody-dependent complement deposition mediated by all anti-polysaccharide and anti-protein antibodies, including immunoglobulins other than IgG. Use of this single-dilution assay enables the screening of a large number of samples in a relatively short time. The assay uses fixed bacteria, which allowed a single batch of bacteria for each serotype to be used for the study, thus avoiding assay variability caused by growth-to-growth variation.

A total of 1,068 sera were analyzed against five GBS serotypes using the CDA, and antibody-mediated C3b/iC3b deposition was determined in mother and cord pairs from samples taken at the time of birth. Within this cohort of 549 mothers, none of the neonates developed GBS disease; therefore, it was not possible to determine the level of antibody-mediated C3b/iC3b deposition required to prevent disease. However, sera from neonates born to GBS serotype II carriage-positive mothers had significantly higher antibody-mediated complement deposition levels against serotype II GBS than sera from those born to mothers who were not carriers of serotype II. Coupled with the absence of invasive disease in this population, this may indicate that neonates at risk (born to carrier mothers) had adequate levels of antibodies to prevent disease. Greater antibody-mediated C3b/iC3b deposition in neonates born to carrier mothers was also observed for serotype Ia and was not observed for the other three serotypes tested likely due to the small numbers of carrier mothers. This phenomenon was observed previously, with infants born to GBS-colonized mothers having antibody concentrations to a GBS surface protein (Sip) that were higher than those found in infants born to mothers who had no colonization (40).

Antibody-mediated C3b/iC3b deposition above the lower limit of detection to at least one serotype was observed in 97% of cord samples and 92% of mother samples, with 7% of cord samples and 4% of mother samples containing antibodies that mediate C3b/iC3b deposition to all five serotypes tested. As only 12% of mothers were found to be carriers of GBS at the time of birth, this prevalence is greater than expected and may indicate that broad immunity is generated by repeated transient episodes of carriage of different GBS serotypes. No studies have evaluated the length of carriage of GBS, which may vary depending on the geographic region and ethnic population. The high proportion of individuals with functional anti-GBS antibodies observed in this population may explain the low incidence of GBS neonatal disease in this geographic region, with Southeast Asia showing some of the lowest rates of neonatal GBS disease (18, 41, 42). This requires further study of invasive disease epidemiology and antibody prevalence.

As only 32/525 infants in the study were <37 weeks' gestation, we expected consistently higher geometric mean antibody-mediated C3b/iC3b deposition levels in the cord samples than in samples from mothers due to the active transfer of IgG in the third trimester (43, 44). For the two most prevalent serotypes, the cord samples indeed showed greater complement deposition than the samples from mothers. However, in the serum samples from mothers, IgM may also contribute to the complement deposition observed and would not have been transferred to the neonates, which may explain why higher neonatal/maternal complement deposition ratios were not seen for the three least-carried serotypes.

Serum samples from premature and term neonates were compared in this study, but no difference was seen in complement deposition mediated by samples from the two groups. Prematurity was previously shown to affect antibody levels that are transferred from mother to baby (43). However, in this cohort, no neonates were born severely premature, since mothers were enrolled when already 28 to 30 weeks pregnant, and the low number of neonates born within this group ($n = 32$) may lack statistical power. In addition, antibody transfer occurs in the third trimester (≥27 weeks' gestation), which may be why we saw no difference between the neonates born prematurely (<37 weeks) and those born at term.

The antibody-mediated complement deposition assay is a very useful tool for assessing large numbers of sera against multiple serotypes/strains. It could potentially be used to assess why there are different incidences of disease in different ethnic groups and geographic regions. For instance, in the United States, a 3-fold-higher risk of GBS infection (late onset) was observed in the African American community, with an incidence of 0.42/1,000 live births versus an incidence of 0.11/1,000 live births in the nonblack community (6), despite presumably having the same circulating GBS strains. This has also been observed in southern Israel, where the Bedouin Arab population has a GBS EOD incidence of 0.07/1,000 live births, whereas the Jewish population has an incidence of 0.35/1,000 live births (45). It may be that in these higher-incidence populations, lower concentrations of functional antibodies are present in mothers, resulting in higher neonatal disease rates. Disease rates also vary in different geographic regions, with Africa thought to have the highest incidence of disease, followed by the Americas, Europe, and Southeast Asia (46). Geographic differences were also observed in England (0.72/1,000 live births), Scotland (0.42/1,000 live births), and Northern Ireland (0.9/1,000 live births) (9). Currently, little information is available to help understand these differences and why they occur to aid the rational introduction of a GBS vaccine or other therapy.

This study has opened up the potential to evaluate the levels of

antibody-mediated complement deposition against different GBS serotypes in large-scale seroepidemiological studies. Although the goal of evaluating the antibody-mediated complement deposition required to prevent disease was not attained in this study, this research has provided a baseline for future studies. This assay will enable some of the key unanswered questions, for instance, why different populations show different incidences of disease, to be studied. Use of this and other immunoassays in efficacy trials of new GBS vaccines may identify a correlate of protection that will facilitate vaccine development and evaluation.

ACKNOWLEDGMENTS

We are very grateful to Carol Baker for donating human positive-control sera for each serotype and to Androulla Efstratiou for kindly donating GBS strains.

We thank the mothers who participated in the study.

The Shoklo Malaria Research Unit is part of the Mahidol Oxford Research Unit, supported by the Wellcome Trust of Great Britain.

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Vaccine 32 (2014) 3128–3132

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Review

Breast milk and Group B streptococcal infection: Vector of transmission or vehicle for protection?

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ARTICLE INFO

Article history:
Received 7 February 2014
Received in revised form 30 March 2014
Accepted 2 April 2014
Available online 13 April 2014

Keywords:
Group-B streptococcus
Breast milk
Immunity
Antibody
Vaccination

ABSTRACT

Invasive Group-B streptococcal (GBS) disease is a leading cause of infant mortality and morbidity worldwide. GBS colonises the maternal rectum and vagina and transmission of bacteria from a colonized mother to her infant at birth is an important risk factor for GBS disease. GBS disease has also been associated with case reports of transmission via infected breast milk raising questions about mode of acquisition and transmission of this enteric pathogen and the development of neonatal disease. However, most breastfed infants remain unaffected by GBS in breast milk. Mechanisms associated with transmission of GBS in breast milk and potential factors that may protect the infant from transmission remain poorly understood. Understanding factors involved in protection or transmission of GBS infection via breast milk is important both for premature infants who are a high-risk group and for infants in the developing world where breastfeeding is the only sustainable infant feeding option. In this review we discuss the proposed mechanisms for GBS colonization in breast milk on one hand and its immune factors that may protect from transmission of GBS from mother to infant on the other. Innate and adaptive immune factors, including serotype-specific antibody and their significance in the prevention of infant disease are presented. We further report on the role of human oligosaccharides in protection from invasive GBS disease. Advances in our knowledge about breast milk and immunity in GBS disease are needed to fully appreciate what might mitigate transmission from mother to infant and protect neonates from this devastating disease and to contribute to the development of novel prevention strategies, including maternal immunization to prevent infant disease.

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1. Introduction

Streptococcus agalactiae (Lancefield Group B streptococcus; GBS) was first described as a cause of bovine mastitis by Nocard and Mollereau in 1887 [1]. Lancefield and Hare subsequently identified GBS in vaginal swabs in 1935 [2] and in 1938 Fry described three fatal cases in post-partum women [3]. Reports of neonatal disease from GBS were sporadic until the early 1960s when GBS became recognized as a leading cause of early neonatal sepsis in the USA [4]. By the 1970s it had become the dominant pathogen in the early neonatal period [5]. By the early 1980s GBS had become the most common cause of neonatal sepsis and meningitis in a number of developed countries [6–8]. In the past five years, late-onset (LO) GBS disease has been associated with case reports of transmission via infected breast milk [9] raising questions about mode of acquisition and transmission of this enteric pathogen and the development of neonatal disease.

Although GBS is not just a neonatal disease, the disease incidence and severity is highest during the first 90 days of life. Early onset (EO) GBS disease (disease presenting in the first six days of life) accounts for approximately 60–70% of all GBS disease. GBS serotypes Ia, Ib, II, III and V are responsible for most EO disease [10,11]. In contrast, serotype III predominates in LO disease, which may be acquired perinatally, nosocomially or from the community. [12]

In the USA EO disease rates have declined from 1.4 per 1000 live births in 1990 [13] to at 0.28 per 1000 live births in 2012 [14] mainly attributed to the implementation of universal screening for GBS rectovaginal colonization in pregnant women and intrapartum antibiotic prophylaxis. However, the incidence of LO disease has remained static at between 0.3 and 0.4 per 1000 births since 1990 [14]. This amounts to 28,100 cases and 1865 deaths annually in the USA [14]. Although the epidemiology of GBS in resource-rich

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http://dx.doi.org/10.1016/j.vaccine.2014.04.020
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countries is well documented, its contribution to the burden of neonatal infection in low/middle income countries has proved more difficult to assess. GBS has been reported as the predominant cause of neonatal sepsis in South Africa and Kenya [15–17] as well as an important cause of meningitis in Malawi and Kenya, but Asian studies have reported a much lower incidence [18–20]. A recent systematic review reported that the overall incidence of GBS in resource-poor settings ranged between 0 and 3.06 per 1000 live births [21].

GBS colonizes the rectum and vagina, and maternal colonization is a pre-requisite for EO disease and a risk factor for LO [22,23]. In resource-rich countries an estimated 20–30% of pregnant women are colonized with GBS [23,24], approximately 50% of their babies become colonized and 1% progress to develop invasive disease. EO disease may occur rapidly; signs of sepsis are evident at birth or within 12 h in over 90% of cases (98% within the first 12 h) [12]. Despite its rarity, LO disease, mostly presenting as meningitis, has devastating long term consequences in survivors with up to 50% suffering severe neurological sequelae [25].

It has been suggested that GBS initially colonizes the infant's oropharyngeal mucosa when contact with maternal vaginal secretions occur at the time of birth [26]. Butter and DeMoor demonstrated GBS in the nose and throat of infants at the same time as GBS was cultured from the mother's breast milk [27]. Fileron et al. reviewed cases of LOGBS disease associated with GBS in breast milk and found 48 LOGBS disease cases between January 1977 and March 2013 of which four had no other positive culture from mother or infant other than GBS-contaminated breast milk. [9].

Therefore, there appears to be a dichotomy between cases of LO disease through infected breast milk and the potential benefits of the components of breast milk which protect the majority of infants from invasive disease. The underlying mechanisms of GBS transmission or protection through breast milk, are not fully understood, but are important to elucidate, particularly in the context of premature infants who are a high risk group and for infants in the developing world where breastfeeding is the only sustainable infant feeding option. In this review we focus on the peculiarities of GBS that may aid transmission in breast milk and the role of immune parameters such as antibody in breast milk on the other hand that may help protect the breastfed infant from GBS disease.

2. Breast milk as vector of transmission of GBS

2.1. GBS in breast milk

Few studies have identified presence of GBS in breast milk, and methodological differences make comparisons difficult [28–32]. Low incidence is described in mothers of extremely preterm infants of 0.4% [31] and term infants of 0.82%. Higher incidence in raw milk ranged from 3.5% [30] to 10% [29] in donor breast milk. However, the concurrent incidence of GBS colonization in these mothers and the effect of intrapartum and postpartum antibiotic treatment were unknown.

The variety of delivery, treatment and storage methods of breast milk offers potential for GBS contamination. Human breast milk may contain 10^3 to 10^9 cfu/mL of GBS at any point, representing a reservoir of potential infection for the neonatal gut [33]. Breast milk directly from the mother (either through natural breast feeding or as expressed breast milk) is given raw and is rarely cultured in cases of neonatal infection. Expressed breast milk and bank milk may be frozen, which affects immune components and bank milk may also be pasteurized. Pasteurization is thought to eradicate important viral and bacterial infections [34] but also depletes milk of the majority of its cellular components and immunoglobulins [35] and may increase the bacterial growth rate [36].

Very recently, best practices on the use of breast milk in the context of prevention of GBS neonatal disease have been proposed, including the search for GBS in milk at the time of recurrent GBS neonatal disease and in cases of mastitis in mothers of high-risk preterm neonates admitted to neonatal intensive care units [37]. For these neonates, microbiological control of raw milk has also been proposed in the absence of mastitis [37]. However, the best strategy has yet to be developed as it does not appear that pasteurizing maternal milk changes the overall incidence of late onset GBS disease in preterm infants [38]. In a recent review article of cases of late onset GBS disease from breast milk, GBS was found in 0–2% of raw milk samples and 1.4% of pasteurized milk samples [9].

2.2. Proposed methods of acquisition

Two main mechanisms of acquisition have been proposed: following colonization of the neonatal oropharynx at the time of birth, mothers may develop colonization of the milk ducts through ascending infection from the neonate, due to the retrograde flow of milk associated with suckling. The infant is then reinfected as the concentration of bacteria increases in the breast milk [39]. This may occur with or without mastitis depending on additional factors such as milk stasis and bacterial load [40]. In most of the case reports of GBS disease associated with breast milk there is no sign of maternal mastitis, indicating silent maternal duct colonization [9]. However, recent studies in animal models and discovery of lactobacilli in breast milk after oral administration suggest that bacteria from the maternal digestive tract may also colonize the breast. [41] It has also been suggested that lactic acid bacteria may transfer from the mother's gut to breast milk and through the milk to the infant's digestive tract [42]. The epidemiological relationship between neonatal and maternal derived GBS isolates in breast milk has been confirmed by polymerase chain reaction (PCR) [43]. However, it is not clear whether the LO disease relates to infected breast milk or is a result of gut translocation from an already colonized infant.

GBS may infect the submucosa of the gastrointestinal tract either through a defect in the epithelial cell layer, or by concomitant infectious agents [33]. As neonatal gastric acid secretion is reduced, more bacteria may reach the intestinal mucosa. This is supported by findings that preterm infants fed with contaminated maternal milk via nasogastric tube have developed GBS disease [44].

3. Breast milk as vehicle of protection from GBS infection

3.1. Innate and adaptive immune properties of breast milk

Breast milk is the main source of non-pathogenic bacteria to the infant gastrointestinal tract. Intestinal bacteria are one of the most important stimuli for the development of mucosa-associated lymphoid tissue (MALT) in the neonatal small intestine [45] and produce organic acids that prevent growth of enteric pathogens. Additionally, breast milk and colostrum contain many components with antimicrobial and immunomodulatory properties that are believed to impair translocation of infectious pathogens [46]. Some of these substances compensate directly for deficiencies in the neonatal immune system and enhance survival of defense agents, including secretory IgA (SIgA), lactoferrin, lysozyme, IFN-γ; some adapt the gastrointestinal tract to extrauterine life, i.e. epidermal growth factor [47]; some prevent inflammation or enhance specific-antibody production, such as PAF-acetylhydrolase, antioxidants, interleukins 1, 6, 8, and 10, transforming growth factor (TGF), secretory leukocyte protease inhibitors (SLPI), and defensin 1 [46]. Breast milk also contains substantial amounts of intracellular adhesion molecule 1 and vascular adhesion molecule 1; low quantities of soluble

S-selectin, L-selectin and CD14, which may mediate differentiation and growth of B cells [46]. Natural autoantibodies, thought to be important in the selection of the pre-immune B cell repertoire and in the development of immune tolerance, are also detected in colostrum and in breast milk [48]. Recently, the beneficial effects of human oligosaccharides in prevention of neonatal diarrhoeal and respiratory tract infections have been highlighted [49,50].

Human breast milk is known to contain factors that can modulate toll-like receptor (TLR) signaling, including soluble TLR2, which can competitively inhibit signaling through membrane TLR2 [51], as well as a protein that inhibits TLR2-mediated and activates TLR4-mediated transcriptional responses in human intestinal epithelial and mononuclear cells by an as-yet-unknown mechanism [52]. It has been speculated that reduced TLR2 responsiveness at birth may facilitate the normal establishment of beneficial Gram-positive bifidobacteria intestinal flora. Lipids present in human milk have been shown to inactivate GBS in vitro, providing additional benefit to protect from invasive infection at the mucosal surfaces [53].

4. Antibody in breast milk

Neonates have low levels of SIgA and SIgM [54] thus protection from invasive pathogens at the mucosal surface relies on antibodies in breast milk. As antibody in breast milk is produced following antigenic stimulation of the maternal MALT and bronchial tree (bronchomammary pathway) [55], these antibodies are targeted to many infectious agents encountered by the mother both prior to birth and during the breastfeeding period.

It is currently hypothesized that SIgA represents the crucial primary protective component of breast milk [56,57]. SIgA protects against mucosal pathogens by immobilizing these, preventing their adherence to epithelial surfaces, or by neutralizing toxins or virulence factors. SIgA concentration is far higher in colostrum (12 mg/ml) than in that found in mature milk (1 mg/ml). A breastfed infant may ingest around 0.5–1.0 g of SIgA per day [40].

5. The role of SIgA in breast milk

SIgA production is enhanced by Interleukin-6 (IL-6) whilst the production of secretory components is enhanced by TNF-α and TGF-β causes class switching towards B cells producing IgA [47], all of which are present in breast milk.

SIgA antibodies present in breast milk are specific for numerous enteric and respiratory pathogens. In studies from resource-poor countries, breast milk-mediated protection against infections with *Vibrio cholerae*, *Campylobacter*, *Shigella*, *Giardia lamblia* and respiratory tract infections was significantly related to the content of SIgA antibody in breast milk against these pathogens [58–60]. This could support the hypothesis that similar protection could be obtained from SIgA antibody in breast milk to GBS in a highly breastfed population. However, maternal SIgA does not appear to enter the neonatal circulation, [61] except in preterm infants, where ingestion of milk rich in IgA to respiratory syncytial virus (RSV) resulted in increased serum IgA levels during the perinatal period [62], so its effectiveness is limited to the mucosal surface.

SIgA is more resistant to proteolysis than other immunoglobulins and is therefore able to function in the gastrointestinal tract [46]. This could account for the finding that the faeces of breast fed infants contains IgA by the second day of life, compared to 30% of formula-fed infants, where IgA is only found in faeces by one month of age [63].

Breast milk contains SIgA antibodies against bacterial-adhesion-site-like pili [46,64]. SIgA antibody in milk blocks adherence of *S. pneumoniae* and *Haemophilus influenza* to human retropharyngeal cells [64] and casein in vitro [65]. The neutralizing capacity of milk anti-poliovirus antibodies has also been reported [66,67].

The effect of third trimester maternal immunization with a single dose of licensed quadrivalent meningococcal vaccine on the potential protection of infants, including by breast milk demonstrated elevated *N. meningitidis*-specific IgA antibodies in breast milk up to six months post partum in vaccinated infants [68]. Similarly, in mothers who received pneumococcal polysaccharide vaccine (PSV) during the third trimester, the geometric mean concentration of IgA in breast milk was significantly higher two months postpartum than in women who received conjugate *H. influenzae* vaccine in the third trimester and remained higher at seven months post partum. [69]

6. Group B Streptococcal antibody in breast milk

As described above, high levels of breast milk SIgA could offer protection to neonates via interference of antibody with the carbohydrate-mediated attachment of GBS to nasopharyngeal epithelial cells. Through this mechanism, colonizing organism load may be reduced with a consequent reduction in morbidity and mortality caused by GBS in the neonatal period [70].

In transition milk, low or moderate IgA antibodies to CPS type III GBS, were detected in approximately 63% of a cohort of 70 Swedish women [71]. In a study of IgG antibody concentration in transition milk in 46 women from the USA, Weisman and Dobson [70] found concentrations of IgG to types Ia, II or III which were approximately 10% of those in maternal serum. Edwards et al. measured IgG and IgA in breast milk to type III GBS in 18 women with high and low antibody titers and found measurable levels of antibody in both groups up to 2 months post-delivery [72]. Detectable levels of CPS serotype III antibody in breast milk in women correlated with concurrently high levels in their serum. Whilst no studies demonstrate a correlation between GBS-antibody levels in breast milk and infant colonization, Berardi et al. report that GBS-positive breast milk is associated with heavy infant colonization [73].

To determine the effect of maternal immunization with GBS CPS-II and CPS-III antibody on postnatal protection from disease a rodent model has been used, where increased survival in pups exposed postnatally to breast milk with high titers of antibody compared to low titers was shown, supporting the beneficial added effect of breast milk antibody following vaccination [74,75].

7. Human oligosaccharides

Oligosaccharides prevent cell adherence for *S. pneumoniae* [76] and *Escherichia coli* (*E. coli*) [77]. Additionally, *E. coli* and *Campylobacter jejuni* toxin can be neutralized by oligosaccharides [49,78] and milk glycoconjugates prevent cell adherence of *Vibrio cholera* and *E. coli* [79,80]. Taken together, these studies suggest that the transfer of human milk oligosaccharides delivers real protection to infants against many bacterial and viral infections.

GBS type Ib and II polysaccharides are of interest as they are virtually identical to certain oligosaccharides present in human milk [75,81,82] which raises the possibility of cross-reactivity with other human glycoconjugates [83]. The results from murine models suggest that these oligosaccharides may act as receptor analogues that anchor the bacteria in the mucosal layer and prevent cell adhesion in the epithelial layer, thus preventing invasive disease.

8. Conclusion

Most neonatal infections occur via mucosal membranes in the respiratory, gastrointestinal, and urinary tracts, yet there is only limited protection at these vast mucosal surfaces during the

neonatal period. Breast milk provides considerable amounts of specific SIgA antibodies that are produced as a result of microbial and food antigens the mother has previously encountered. Such SIgA antibodies from breast milk provide protection to the neonate at the mucosal surface. Breast milk additionally contains high concentrations of non-specific protective molecules, such as lactoferrin that has bactericidal, viricidal, and fungicidal properties. Milk oligosaccharides might block adherence of microorganism at the mucosal surface by functioning as receptor analogues.

There is increasing data from recent publications that enhanced protection against diarrhea, respiratory tract infections, otitis media and *H. influenzae* infections, as well as wheezing illness may persist for years after breastfeeding. However, the role of breast milk antibody in protection from neonatal GBS disease remains poorly understood. Current research is evaluating transport, persistence and function of GBS antibodies and other immune-constituents in breast milk. These studies aim to identify protective factors involved in the passive transfer of immune components in breast milk and associated protection from colonization and infant disease. Additionally, research correlating neonatal colonization with antibody levels in breast milk would provide insight into possibly protective factors from disease.

Conflict of interest

None declared.

Contributors

KLD developed the research idea, undertook the literature review and prepared the first draft of the manuscript. BK developed the research idea and substantially contributed to the drafting and revision of the manuscript.

Funding

KLD is funded by a Wellcome Trust/Imperial Global Health Fellowship and the Royal College of Physicians Thomas Watt Eden Fellowship. BK is funded by the MRC and the NIHR.

Acknowledgements

We acknowledge the support of the Imperial College Biomedical Research Centre (BRC) for our work.

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Vaccine 31S (2013) D7–D12

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Review

An overview of global GBS epidemiology

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ARTICLE INFO

Keywords:
Group B streptococcus
Neonatal disease
GBS in pregnancy
GBS vaccine
Global epidemiology

ABSTRACT

Streptococcus agalactiae (group B streptococcus (GBS)), remains the leading cause of neonatal sepsis and meningitis in many countries and an important cause of disease in pregnant women, immunocompromised adults and the elderly. Intrapartum antibiotic strategies have reduced the incidence of early-onset neonatal GBS where applied, but have had no impact on late onset GBS infection and only a limited impact on disease in pregnant women. In low/middle income settings, the disease burden remains uncertain although in several countries of Southern Africa appears comparable to that of high-income countries. Disease may be rapidly fulminating and cases therefore missed before appropriate samples are obtained. This may lead to significant underestimation of the true burden and be a particular issue in many African and Asian countries; comprehensive epidemiological data from such countries are urgently required.

The available data suggest that the serotype distribution of GBS isolates is similar in Africa, Western Pacific, Europe, the Americas and the Eastern Mediterranean regions and has not changed over the past 30 years. Five serotypes (Ia, Ib, II, III, V) account for the majority of disease; conjugate vaccines including some or all of these serotypes therefore hold great promise for preventing this important disease.

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1. Historical trends

Streptococcus agalactiae (Lancefield group B streptococcus; GBS) was first described as a cause of bovine mastitis by Nocard and Mollereau in 1887 [1]. Lancefield and Hare subsequently identified GBS in vaginal swabs in 1935 [2] and in 1938 Fry described three fatal cases in post-partum women. This was notable because all previous severe streptococcal infections in that setting had been attributed to group A streptococcus [3]. Reports of neonatal disease from GBS were sporadic until the early 1960s when GBS became recognized as a leading cause of early neonatal sepsis in the USA [4,5]. By the 1970s it had become the dominant pathogen in the early neonatal period [6,7]. A similar (but slightly later) time trend was noted for GBS meningitis in the UK [8]. By the early 1980s GBS had become the most common cause of neonatal sepsis and meningitis in a number of developed countries [9–11].

Human isolates of GBS express a capsular polysaccharide (CPS), a major virulence factor that helps the microorganism evade host defense mechanisms [4]. Isolates of GBS can be divided into ten CPS serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX) each antigenically and

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0264-410X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.01.009

structurally unique. In 1974, Baker and Barrett demonstrated that whilst all GBS serotypes were capable of causing neonatal infection, type III isolates were significantly increased amongst infants with GBS meningitis [12].

Bisharat et al.'s work on GBS multilocus sequence typing (MLST) using a global collection of isolates demonstrated that capsular serotype does not strictly follow sequence type (ST) and that a single, bovine-derived ST (ST-17), appears to be overrepresented in neonatal disease [13]. Further work from the USA [14] and the UK [15] determined that ST-17 in types II and III GBS are closely related to ancestral bovine isolates. This sequence type appears to be associated with neonatal disease, irrespective of the capsular serotype, but not with adult disease [15].

2. Infants

2.1. Early and Late onset infections

GBS disease is not restricted to newborns, but its greatest impact, both in terms of severity and incidence, is in the neonatal period and up to the first 90 days of life. Early onset (EO) GBS disease is usually defined as infection presenting in the first six days of life and accounts for approximately 60–70% of all GBS disease. GBS serotypes Ia, II, III and V are responsible for most EO disease [16–18]. Maternal carriage of GBS in the gastrointestinal and/or genital tracts is a pre-requisite for EO disease, vertical transmission occurring during or just prior to birth. In developed countries an estimated 20–30% of pregnant women are colonized with GBS [19,20], approximately 50% of their babies become colonized and 1% progress to develop invasive disease. Disease may occur rapidly; signs are evident at birth or within 12 h in over 90% of cases (98% within the first 12 h) and presentation is typically with pneumonia or sepsis [21].

In contrast, late-onset (LO) disease is caused predominantly by serotype III, is acquired perinatally, nosocomially or from community sources, and in up to 50% of cases presents with meningitis [16,21–23]. Cases occurring later in infancy than 90 days are also described, but rare, and generally associated with extreme prematurity [24–28].

2.2. The burden of infant GBS disease – high income countries

The burden of GBS disease can be defined in terms of its incidence, morbidity and mortality. Multistate, population-based, active surveillance in the USA in 1990 identified an EO disease rate of 1.4 per 1000 live births and a LO rate of 0.4 per 1000 births [26]. This equated to 7600 cases and 310 deaths per year in the USA. Both EO and LOGBS disease occurred at significantly higher rates amongst African-American compared to Caucasian infants, as well as amongst low-birthweight or preterm infants [26,27].

In the UK, a multi-center study carried out between 1977 and 1978 recorded an incidence of invasive GBS disease in infants < 2 months of age of 0.3 cases per 1000 live births [29]. Through the late 1990s studies from different centers in England reported incidence figures ranging from 0.6 to 1.2 cases per 1000 live births [30–32]. In 2000–2001, national surveillance was enhanced; the overall incidence was 0.72 per 1000 live births (95% confidence interval 0.66–0.78), 0.47 per 1000 (0.42–0.52) for EO disease and 0.25 per 1000 (0.21–0.28) for LO disease [21]. Regional variation was marked; the incidence in Scotland was 0.42 per 1000 whilst in Northern Ireland it was 0.9 per 1000 live births. As with other studies, infants of low birthweight (<1500 g) were shown to have the highest rates of GBS disease [21].

National population-based surveillance studies have defined the incidence <3 months of age in a number of developed countries including Portugal (2001–2005, 0.54/1000) [11], Finland (1985–1994, 0.76/1000) [10] and Germany (2001–2003, 0.47/1000) [9]. Hospital based surveillance studies (which may be biased towards higher risk pregnancies) have generally yielded higher incidences such as in Australasia (EOGBS (<48 h of age): 2/1000 in 1991–1993, 1.3/1000 in 1993–1995, 0.5/1000 in 1995–1997) [33] and Spain (EOGBS (<3 days of age): 1.1/1000, 1996–1997; 0.7/1000, 2000–2001) [34]. Edmonds et al. recently conducted a systematic review to define the current global incidence of GBS disease in infants under 3 months [35]. The analysis was confined to data since the year 2000 and estimated an overall incidence of 0.53 (0.44–0.62) in the European region, 0.67 (range 0.54–0.80) in the Americas and 0.15 (range –0.03 to 0.07) in Australasia. IAP was frequent in developed countries and associated with lower incidence figures; those countries reporting no use of IAP had a 2.2-fold higher incidence of EOGBS compared with those reporting any use.

GBS disease is also associated with significant morbidity and mortality. GBS meningitis, in particular, results in long term neurodevelopmental impairment. Bedford et al. described 5-year neurodevelopmental outcomes following an acute episode of neonatal meningitis in 98 children, of whom 13% had severe, 17% moderate and 18% mild disability at 5 years. Overall 50% of those with GBS meningitis had neurodevelopmental impairment at 5 years of age [36]. A recent small series from the USA has shown similar rates of sequalae (up to 44% with moderate–severe impairment) [37].

Case fatality rates for GBS disease are estimated at between 9% in Oxford (1990–1996) [30] and 15% in London (1990–1999) [38], with a UK national figure of 10% in 2000–2001 [21]. In the USA between 1993 and 1998 the quoted case fatality rates for EO and LO disease were 4.7% and 2.8% respectively [39]. Case fatality rates were significantly higher in premature infants (<33 weeks gestation) 15.2% vs. 6.4% for term infants (>37 weeks gestation), relative risk of 6.7 in EOGBS (21, 39). Other national studies have revealed case fatality rates of 6.6% in Portugal [11], 4.3% in Germany [9] and 8% in Finland [10]. Overall current case fatality rates by region as estimated in the Edmond meta-analysis are 0.07 (range 0.04–0.1) in Europe and 0.11 (0.06–0.16) in the Americas [35].

2.3. Risk factors

Numerous maternal, obstetric and neonatal factors have been associated with or identified as risk factors for maternal GBS colonization, transmission of GBS from mother to baby, neonatal GBS colonization or neonatal disease with GBS (both EO and LO). Although some of these have provided useful insights into the pathophysiology of perinatal GBS disease, many of them are interrelated, studies have produced contradictory results and relatively few factors are sufficiently robust or clinically relevant enough to guide prevention strategies.

The gastrointestinal tract is the primary reservoir of GBS and the source of vaginal colonization in women. Local hygiene or sexual practices can increase the risk for vaginal colonization. Other factors associated with maternal colonization include ethnicity (women of Black race), use of tampons or intrauterine devices, obesity, absence of lactobacilli in the gastrointestinal flora and preterm delivery [40–42]. GBS bacteriuria during pregnancy is associated with heavy colonization, a further risk factor for perinatal transmission [43,44]. Mothers with GBS bacteriuria demonstrate a higher incidence of adverse obstetric outcomes: habitual abortion, intrauterine growth restriction, preterm labour, chorioamnionitis and premature rupture of membranes [43].

Factors associated with an increased risk of neonatal colonization include maternal colonization, male sex, Black race, prolonged rupture of membranes, prematurity, low levels of maternal anti-GBS antibodies and intrapartum fever. In one analysis, infants born

to GBS-colonized women had a 29-fold increased risk for EO disease compared to infants born to women who were not colonized [45]. In one case control study, prematurity (odds ratio 10.4, 95% confidence interval 3.9–27.6), rupture of the membranes more than 18 h before delivery (25.8, 10.2–64.8), rupture of the membranes before the onset of labour (11.1, 4.8–25.9), and intrapartum fever (10.0, 2.4–40.8) were all significant risk factors for EOGBS [46]. Infants born to mothers less than 20 years of age or of Black/Hispanic race [27], with heavy genital GBS colonization [47], low levels of anti-GBS capsular antibodies [48] or with a previous infant with EOGBS disease [49] are all associated with a higher risk of EO disease.

Risk factors for LO disease are less well documented. Male sex, Black race, maternal colonization and having a twin with LO disease are all associated with an increased risk for LOGBS disease [22,27,28,50,51]. LO disease has been noted in children born to HIV-infected mothers [52]. More recently it has become clear that the major risk factor for LO disease is extreme prematurity [25–28].

2.4. Intrapartum antibiotics (IAP)

National strategies for targeting pregnant women for IAP include either risk-based [53] or screening based strategies [54–56]. Following the publication of recommendations for both risk-based and screening-based approaches, the incidence of EOGBS disease in the USA decreased by almost 70% between 1996 and 1998 [39]. Death certificate analysis also documented a steeper decline in EO deaths temporally associated with prevention implementation, without similarly steep declines in other causes of deaths including LO deaths [57]. In an attempt to assess the relative effectiveness of the two strategies the CDC conducted a retrospective cohort study [58]. Adjusting for confounders, women who were screened for GBS prenatally had a 50% reduced risk of delivering a baby with GBS disease than did those exposed to the risk-based strategy. In one study from the USA between 2002 and 2010 the incidence of EO disease declined from 3.5 to 2.6 per 1000 admissions, whilst LO disease has increased from 0.8 to 1.1 per 1000 admissions [59].

However, even strict and universal implementation of guidelines does not eliminate EOGBS disease; it can occur despite administration of IAP, in infants of mothers who were negative on screening, and where no risk factors are evident in labour [60–62]. In 2005 it was estimated that 1425 cases of EOGBS occurred in the USA despite widespread use of IAP [63,64]. Most significantly, IAP has no impact on LOGBS infection where the burden of disease is substantial [64]. The majority of GBS meningitis occurs after the first week of life so this particular burden remains; in the USA GBS is now the most common cause of bacterial meningitis in children less than 5 years of age [63].

2.5. Low and middle income countries

Although the epidemiology of GBS in the developed world is well documented, its contribution to the burden of neonatal infection in low/middle income countries has proved more difficult to assess. GBS has been reported as the predominant cause of neonatal sepsis in southern Africa and Kenya [65–67] as well as an important cause of meningitis in Malawi and Kenya, but Asian studies have reported a much lower incidence [68–70]. In contrast, colonization studies from these countries have reported only limited variation in maternal GBS colonization rates, similar to those of high income countries (Middle East/North Africa, 22%; Asia/Pacific, 19%; Sub-Saharan Africa, 19%; India/Pakistan, 12%; and Americas, 14%) [71].

Two systematic reviews of GBS incidence have attempted to shed some light on this. Edmonds et al. showed that studies reporting use of any IAP had a lower incidence of EO disease, but found only one study from a middle-income country (South Africa) that reported IAP use and no low-income countries reported IAP use. Only 7 low-income countries reported data on GBS disease incidence, only 3 for EOGBS disease (Kenya, Bangladesh, Malawi). The mean incidence of EO disease was 0.43 (95% CI 0.37–0.49). Incidence was highest in studies from Africa (0.53, 95% CI 0.15–0.92]) whilst studies from Southeast Asia reported the lowest incidence (0.11, 95% CI 0.012–0.220). The incidence of LO disease was again highest in Africa (0.71, 95% CI 0.38–1.04). The case fatality ratio was three times higher in low-income countries (12.6%, 95% CI 10.8–14.9) than in high-income countries (4.6%, 2.1–9.1) [35].

Similar findings were reported by Dagnew et al., who conducted a systematic review and meta-analysis of GBS disease focusing on low and middle-income settings. They reported that the overall incidence of GBS in resource-poor settings ranged between 0 and 3.06 per 1000 live births [72]. Differences in incidence were observed both between and within geographic regions. Variation was greatest across sub-Saharan Africa, with studies from Nigeria and the Gambia reporting lower incidence rates (0–0.17 per 1000 live births [73,74]) than studies from southern Africa (1.80–3.06 per 1000 live births [75,76]). Variation was evident across studies from Latin America (0.39–1.15 per 1000 live births [77,78]), whereas studies from Asia and the Middle East reported a lower GBS incidence (0–0.26 per 1000 live births [68–70,79–83]). Reported case fatality rates (median, 20%; range, 10–60%) also varied within and between geographic regions [72].

Potential explanations for the variations highlighted in these reviews include fundamental differences in disease epidemiology due to differences in maternal GBS colonization, differences in strain virulence [84], maternal and infant anti-GBS antibody levels [74] or genetic susceptibilities. However, under-identification and under-reporting of cases and deaths appears very likely in some study settings, especially where it is difficult to access healthcare. A substantial proportion of births in sub-Saharan Africa (42–65%), India (53%) and Bangladesh (85%) for example, occur outside health institutions. In contrast, South Africa, which reports one of the highest incidences, also has a high proportion of hospital-based births (89%) [35]. Darmstadt et al. attempted to increase case ascertainment by capturing births in the community. Of 919 community assessments that resulted in a hospital referral only 54% complied within two days and compliance was lower in infants aged 0–6 days. Of 259 neonatal deaths, 62% were never assessed by a health worker and 59% occurred within the 1st two days of life, consistent with the time period of greatest risk for the development of GBS sepsis [68].

Differences in microbiological and sampling techniques may also contribute to differences in reported incidences [85]. Edmond et al. searched articles for relevant indicators of this, such as the proportion of specimens collected on the first day of illness or on the day of birth or care-seeking and referral on day one of life, but no data were available. Further, they sought to quantify laboratory techniques by searching for information on specimen volume, transport conditions and culture techniques, but data were disparate and could not be pooled [35]. Dagnew et al. also examined methods of GBS sample isolation and identification. Thirteen studies, including all studies from South and Southeast Asia and the Middle East used manual culture methods to isolate and identify GBS and seven used automated methods (BACTEC system). GBS incidence rates were shown to be higher amongst studies using automated culture methods: 0.80–3.06 vs. 0–0.39/1000, suggesting this as a further explanation [72].

2.6. GBS serotypes

In the USA and Europe, invasive GBS-serotypes are predominantly Ia, Ib, II, III and V [48,86–88] whilst a Gambian study reported

to GBS-colonized women had a 29-fold increased risk for EO disease compared to infants born to women who were not colonized [45]. In one case control study, prematurity (odds ratio 10.4, 95% confidence interval 3.9–27.6), rupture of the membranes more than 18 h before delivery (25.8, 10.2–64.8), rupture of the membranes before the onset of labour (11.1, 4.8–25.9), and intrapartum fever (10.0, 2.4–40.8) were all significant risk factors for EOGBS [46]. Infants born to mothers less than 20 years of age or of Black/Hispanic race [27], with heavy genital GBS colonization [47], low levels of anti-GBS capsular antibodies [48] or with a previous infant with EOGBS disease [49] are all associated with a higher risk of EO disease.

Risk factors for LO disease are less well documented. Male sex, Black race, maternal colonization and having a twin with LO disease are all associated with an increased risk for LOGBS disease [22,27,28,50,51]. LO disease has been noted in children born to HIV-infected mothers [52]. More recently it has become clear that the major risk factor for LO disease is extreme prematurity [25–28].

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serotype-V predominance [84]. The recent global review of invasive isolates showed that serotype III was the most frequently identified serotype in all regions with available data (48.9%), followed by serotypes Ia (22.9%), V (9.1%), Ib (7.0%) and II (6.2%). There was little change in ST distribution over the last 30 years [35]. There was however, little data from low/middle income countries.

3. Pregnancy-associated GBS disease

The incidence of pregnancy-associated GBS disease in the USA initially declined after the introduction of IAP strategies (0.29 per 1000 live births in 1993 vs. 0.11–0.14 per 1000 live births in 2005) [87]. In a recent study the incidence was shown to be 0.02/1000 in non-pregnant women vs. 0.49/1000 (range 0.36–0.64) in postpartum women [89]. This study highlighted that the majority of pregnancy-associated disease actually occurred in the postpartum period. The authors found that pregnant women had a relative risk for GBS disease of 5.0 (95% CI, 2.9–8.7) compared with non-pregnant women. The median age at onset was 28 years and half of the cases were associated with infection of the upper genital tract, placenta, or amniotic sac. Other manifestations included bacteremia without focus (31%), endometritis without foetal death (8%), chorioamnionitis without foetal death (4%), pneumonia (2%), and puerperal sepsis (2%). Amongst those for whom pregnancy outcome was known (368/409, 90%), 61% had a spontaneous abortion or stillborn infant, 30% had infants without apparent illness, 5% had live-born infants who developed clinical infections, and 4% had induced abortions. Most pregnancy-related cases (330/409, 81%) occurred in the absence of additional underlying conditions; 79 occurred with at least one additional underlying condition or risk factor including smoking, asthma, diabetes, obesity and alcohol or drug abuse [89]. The serotypes causing maternal GBS appear to be similar to those that cause EOGBS [90,91].

4. GBS in non-pregnant adults

Amongst adults, older age, black race, and underlying medical conditions are associated with higher rates of invasive GBS disease [92–101]. Common presentations of GBS disease in adults include skin and/or soft-tissue infection, bacteremia without focus, pneumonia, and osteomyelitis; serious clinical syndromes, such as meningitis and endocarditis are rare but are often associated with considerable morbidity and mortality [93–97,100,101]. The case fatality rate is markedly higher amongst adults than amongst neonates [94–98,100]. In contrast to neonates and pregnant women, disease incidence amongst non-pregnant adults increased significantly in the USA between 1990 and 2007 [102]. Amongst persons aged 15–64 years, incidence increased from 3.6 per 100 000 population in 1999 to 7.3 per 100 000 in 2007, a relative increase of 48% (95% CI, 32–65%); amongst those 65 years or older, incidence increased from 21.5 per 100 000 to 26.0 per 100 000, a relative increase of 20% (95% CI, 8–35%). These values translate to a 32% (95% CI, 22–43%) increase in the overall incidence of adult disease, which increased from 6.0 per 100 000 in 1999 to 7.9 per 100 000 in 2005. The case-fatality rate decreased from 23.7% in 1990 to 7.5% in 2007 [102]. The proportion with known outcome that died was highest in the oldest age groups. Seasonal variability was evident, with distinct peaks occurring each year during the later summer months. In contrast to pregnant women and neonates, in adult cases, serotype V predominated, accounting for 31% of cases, followed by serotypes Ia (24%), II (12%), and III (12%) [63]. Recently, however, a Portuguese study found that serotype Ia was responsible for the majority of invasive infections in all age groups [103].

The underlying medical conditions associated with invasive GBS disease in non-pregnant adults include diabetes mellitus (41%), heart disease (36%), and malignancy (17%). In one series, nearly 88% of adult cases had at least 1 underlying condition [100]. In the USA between 1998 and 2007, the proportion of patients with underlying diabetes increased significantly (from 36.5% to 44.4%), although the proportion of patients with cancer, heart failure, and atherosclerotic cardiovascular disease remained relatively stable [101].

5. Discussion

GBS is the leading cause of neonatal sepsis and meningitis in developed countries. It is also a recognized and increasing cause of disease in adults in such countries, especially pregnant women, the elderly and the immunocompromised. In resource-poor settings, however, the neonatal burden is less clear, although in some countries may be higher than in developed countries. The disease is often rapidly fulminating and many cases may be missed because of difficulties with obtaining samples before babies die. This issue is especially important in African and Asian countries. A failure to diagnose cases in the community, sick babies that then fail to reach healthcare facilities, inadequate culture methods, and widespread antibiotic usage may all lead to an underestimation of the GBS burden. More high-quality studies are urgently needed in these settings.

Comprehensive data on disease burden will enable appropriate preventative strategies to be devised and implemented. GBS vaccines in particular, hold great promise as they have the potential to prevent all neonatal GBS disease, early and late onset, to avoid the issues around screening and intrapartum antibiotic use and are likely to be the most cost effective approach of all. Available data suggest that a conjugate vaccine incorporating five serotypes may prevent over 85% of global GBS disease in infants under three months. Vaccine development is eagerly awaited.

Acknowledgements

Conflict of interest: PH serves as a consultant to Novartis vaccine re GBS vaccine development. He receives no personal funding for this. *Funding:* none.

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