HUMORAL IMMUNE CORRELATES OF PROTECTION FROM MOTHER-TO-FOETAL TRANSMISSION OF CYTOMEGALOVIRUS

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of

Master of Science in Medicine (By Research)

Johannesburg, July 2021

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Signature: S.Balla Date: 30 July 2021

For my parents, Ruben Balla and Susan Rall, and my husband, Azhar Abdul Habib

PUBLICATIONS

In utero human cytomegalovirus infection 1 is associated with increased levels of putatively protective maternal antibodies in nonprimary infection: evidence for boosting but not protection

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Published online 9 Feb 2021

Clinical Infectious Diseases

https://academic.oup.com/cid/advance-article-abstract/doi/10.1093/cid/ciab099/6131727

ABSTRACT

Human cytomegalovirus (HCMV) infects approximately 60% of the world's population. Although largely asymptomatic, it causes substantial morbidity and mortality in infants infected via congenital exposure and is the leading infectious cause of birth defects. Congenital infection occurs in the context of both primary and non-primary infection. The transmission risk is higher in primary infection, suggesting that underlying immunity may offer partial protection. The HCMV viral envelope is studded with numerous glycoproteins, including glycoprotein B and pentamer, both targets of neutralizing antibody responses. A previous study of primary maternal HCMV infection showed that antibodies specific for particular epitopes on pentamer were higher in non-transmitters, suggesting that such antibodies could be protective. However, maternal correlates of protection have not been clearly defined, especially in non-primary infection.

We compared key anti-HCMV antibody responses in mothers who transmitted HCMV to their children with those of non-transmitting controls in maternal non-primary infections. These responses included total antigen-specific antibody targeting pentamer and glycoprotein B, as well as antibodies targeting particular pre-defined epitopes of each of these antigens.

Unlike a previous study, we found that pentamer-directed antibody levels were significantly higher in transmitting mothers at birth compared to control non-transmitting mothers. The total epitope-specific antibodies targeting gB and pentamer were also higher in transmitters. There was no evidence that higher levels of any HCMV-specific antibodies correlated with reduced risk of congenital HCMV infection in non-primary maternal infection. Also, total gB antibody levels were higher in HIV-1 infected mothers however, this was only apparent in non-transmitters.

These data indicate boosting of antibody levels by maternal HCMV reactivation/re-infection, ultimately resulting in fetal infection, and not protection.

267 words

ACKNOWLEDGEMENTS

I would like to express my utmost gratitude to the following individuals for their contribution to my work:

Professor Lynn Morris for the opportunity to do my Masters Research Project in her prodigious laboratory.

Professor Penny Moore, my supervisor, for being the rock that I need. Your unwavering support and guidance has helped me in ways beyond the lab. I am grateful for everything that I have learned from you and I look forward to working with you in the future.

Associate Professor Jeffrey Dorfman, for your role as my co-supervisor. Thank you for sharing your plethora of knowledge with me, and for the careful experimental planning, I truly have learnt a lot from you.

The HIV Virology Section (Sharon, Rebecca, Charissa) for the abundant advice and tremendous contribution that every one of you had towards the progress of my Masters Research Project. For being available to help and guide me around the lab, the pieces of advice and for the day-to-day joyous ambience.

My parents for the faith and support they have given me throughout my years of studying. For trusting in my scientific knowledge and showing an interest in my Research.

My husband and pillar of strength for sharing my interest in science, for being the shoulder when it was too tough to handle, for pushing me beyond my comfort zone and for reassuring me that it will all be alright.

My siblings for the untiring love and surplus of questions about my research despite not quite understanding what I was doing.

Funding for this project was provided by the NRF through Penny Moore's SARChI Chair in Virus-Host Dynamics.

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ABBREVIATIONS

APS	Ammonium Persulphate
cCMV	Congenital Cytomegalovirus
dH_2O	Deionized Water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
FBS	Fetal Bovine Serum
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HIV	Human Immunodeficiency Virus
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
SDS	Sodium Dodecyl Sulphate
SNHL	Sensorineural Hearing Loss
TEMED	Tetramethylethylene- Diamine

SYMBOLS AND UNITS

°C	Degrees Celsius
g	Unit of Relative Centrifugal Force, 1g = Standard Acceleration Due To Gravity At The Earth's Surface.
hr	Hour
kDa	Kilo Daltons
min	Minute
mL	Millilitre
r.p.m	Rotations Per Minute
sec	Second
µL	Microlitre

CHAPTER 1: INTRODUCTION

1.1 EPIDEMIOLOGY OF CYTOMEGALOVIRUS INFECTION

1.1.1 Prevalence of Cytomegalovirus and Vulnerable Populations

For thousands of years Herpesviruses have coexisted with their hosts (Davison, 2011). The human betaherpesvirus 5 (HHV-5), more commonly referred to as human cytomegalovirus (HCMV), is one of the most genetically complex of all pathogenic human viruses, and the largest genome in the family Herpesviridae. HCMV is almost universally distributed and is estimated to infect more than 60% of the world's population by the age of 50 years (**Figure 1.1**) (Cannon, Schmid and Hyde, 2010).

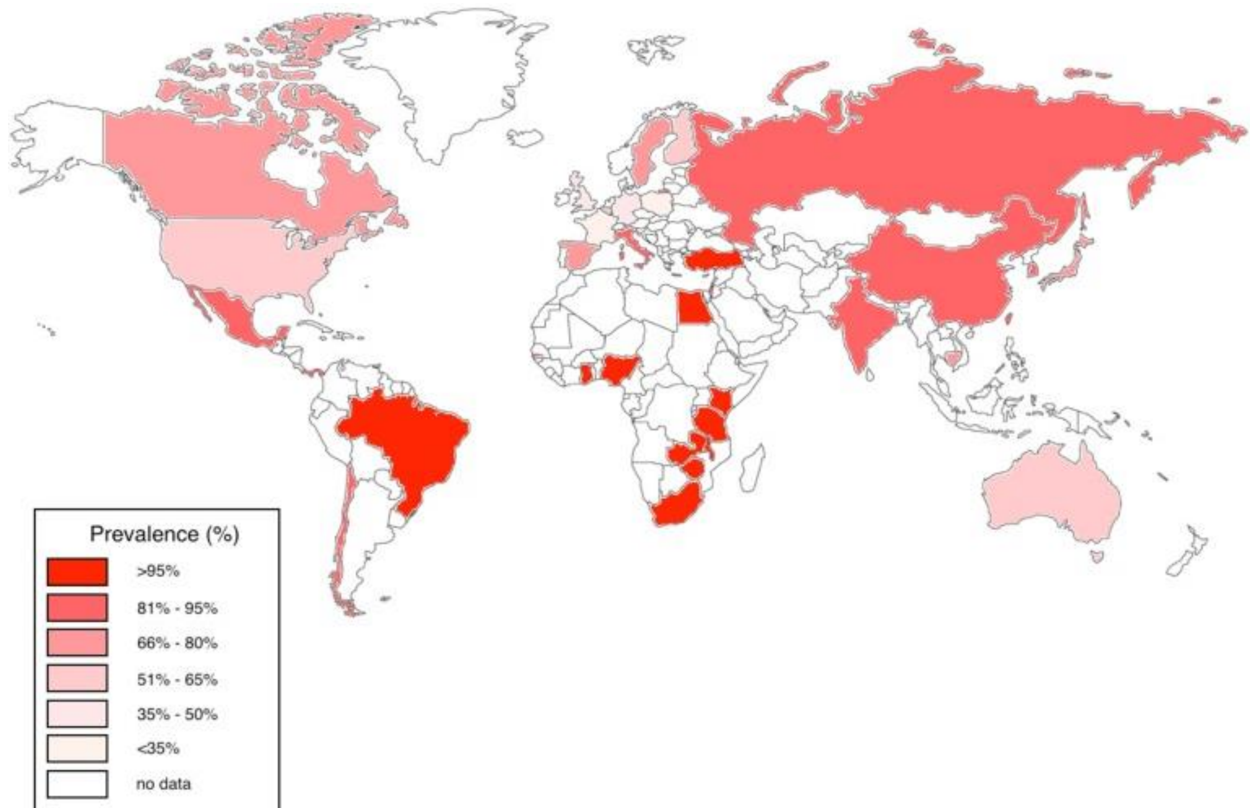


Figure 1.1: Global HCMV seroprevalence rates in adults. Schematic summary of studies of HCMV prevalence among adults (16–50 years old) published between the years 2005 and 2015 from the following countries: Australia, Belgium, Brazil, Canada, Cambodia, Chile, China, Finland, France, Gambia, Germany, Ghana, India, Israel, Italy, Japan, Kenya, Mexico, Nigeria, Panama, South Africa, Spain, Sweden, Taiwan, Tanzania, Turkey, UK, USA, Zambia, and Zimbabwe (Adland *et al.*, 2015).

HCMV can be transmitted via direct contact with infected bodily fluids such as saliva, urine, breast milk, blood transfusion, organ transplants, or through the placenta to a foetus (Fields, Knipe and Howley, 2013). It has been consistently reported that prevalence rates in women are slightly higher than in men (Hecker *et al.*, 2004; Staras *et al.*, 2006; Bate, Dollard and Cannon, 2010). While the prevalence of infection increases with age in all groups, the overall occurrence and median age that HCMV is initially acquired differs based on demographic factors (Fields, Knipe and Howley, 2013). Generally, in developing countries the prevalence of infection is higher, and HCMV is generally acquired earlier compared to more developed countries such as the United States and those of Europe (Fields, Knipe and Howley, 2013). The prevalence of HCMV is higher particularly in the lower socioeconomic strata, among non-white individuals, as well as in immigrant populations from developing countries (Griffiths, Baboonian and Ashby, 1985; Gratacap-Cavallier *et al.*, 1998; Staras *et al.*, 2006; Kenneson and Cannon, 2007; Cannon, Schmid and Hyde, 2010; Manicklal *et al.*, 2013). Although HCMV prevalence has been reported to be higher in non-white individuals, this is likely to be a reflection of discordant living circumstances, rather than racial factors. As a consequence of these conditions, maternal seroprevalence may be over 80% in low to middle income countries whereas as many as 50% of women are seronegative in economically developed countries (Ross *et al.*, 2010).

A hallmark of the members of *Herpesviridae,* including HCMV, is that the immune system never completely clears infection (Fields, Knipe and Howley, 2013). Thus, the virus becomes latent and usually remains in the body for the lifetime of the host (Schleiss and Heineman, 2005). HCMV establishes this latency in epithelial/endothelial and myeloid cells. Reactivation of HCMV can occur, once again resulting in active infection, characterising HCMV as an opportunistic disease (Noriega *et al.*, 2012). Active infection can result in severe consequences including, but not limited to, retinitis, organ failure and lung tissue inflammation (Kinzler and Compton, 2005). Healthy infected adults often are not aware of their infection since they are largely asymptomatic. However, primary HCMV infection (first exposure and infection of the pathogen), reinfection or reactivation are all cause for concern in individuals with a compromised ability to mount an immune response (Fields, Knipe and Howley, 2013)..

Key populations vulnerable to clinical disease caused by HCMV include HIV-1 infected people who are progressing towards disease (both with and without treatment), as well as organ and stem cell transplant recipients (Dasari, Smith and Khanna, 2013). In fact, 75% of patients are diagnosed with clinical HCMV disease in the first year after transplantation (Streblow, Orloff and Nelson, 2007). Another vulnerable HCMV target is the developing foetus. Congenital HCMV infection (cCMV) of the foetus is the most common clinically significant congenital infection across the globe (Boppana and Britt, 1995; Schleiss and Heineman, 2005; Lilleri *et al.*, 2007; Boppana, Ross and Fowler, 2013; Manicklal *et al.*, 2013; Britt, 2017). cCMV infection impacts approximately 1 in every 150 (0.7%) live born infants with higher rates reported in low and middle income countries (Kenneson and Cannon, 2007; Manicklal *et al.*, 2013; Permar, Schleiss and Plotkin, 2018). Various factors influence the clinical consequences of cCMV, including gestational age at time of infection, whether the maternal infection is primary or non-primary, as well as the viral load present within the newborn following transmission (Fields, Knipe and Howley, 2013). This is the focus of our study.

1.1.2 Transmission Patterns in Adults and Children

Patterns of transmission from child-to-child and from adult-to-child are the result of shedding HCMV in saliva, urine, and breast milk as well as lack of general hygiene practices (such as coughing or sneezing without covering the mouth). Shedding in urine and saliva, which is persistent and recurrent, leads to transmission predominantly among children compared to transmission from children to adults (Cannon, Schmid and Hyde, 2010).

Transmission from mother to foetus or infant plays a significant role in maintaining HCMV prevalence in humans. There are three main transmission pathways - transplacental, intrapartum and postnatal transmission/routes (Pass and Anderson, 2014). While this study focuses of congenital CMV infection, via transplacental and intrapartum routes, all three transmission pathways are discussed. Intrapartum transmission occurs during the birthing process since viral particles are often present in the cervix or vagina (Stagno *et*

al., 1982). Both intrapartum and postnatal transmission pathways are associated with maternal shedding cycles. Vaginal or cervical virus shedding occurs in about 10% of women, and between 2-28% of women shed HCMV close to delivery time, with higher rates in younger women (Stagno *et al.*, 1982). A study of HCMV transmission in pregnant women estimated that 50% of babies who were born to mothers with positive vaginal viral cultures, but were not breastfed, acquired the virus (Reynolds *et al.*, 1973; Fields, Knipe and Howley, 2013).

While intrapartum contact contributes to a portion of infections in babies, the most common pathway of mother-to-child transmission is through breast-feeding. Many infants that acquire HCMV in the first year of life are born to mothers that are HCMV antibody positive and breastfeed their infants (Stagno *et al.*, 1980). These infected infants shed the virus through saliva and urine for years after breastfeeding. This offers ample opportunity for infection of caregivers as well as other babies, thus, raising the significance of viral delivery through breast milk (Pass and Anderson, 2014). This results in a very high force of infection in most low- and middle- income settings. For example, in one Malawi study, 85% of children were CMV infected by 18 months of age (Miles *et al.*, 2008). HCMV infection that occurs through these two transmission routes (intrapartum and breast milk) correlates with little to no clinical illness, except in the case of very low birth weight premature newborns. Despite this, these modes of transmission remain key to the epidemiology of infection (Fields, Knipe and Howley, 2013).

Although breastfeeding is a very common route of maternal transmission, transplacental (intrauterine) transmission is the most medically significant mode of transmission (Stagno *et al.*, 1980). The transmission rate through the placenta during pregnancy, during primary infection, is roughly 20% during the first trimester and increases with gestational age to approximately 75%. Although third trimester transmission occurs more frequently, first or second trimester transmission is linked with higher risk of clinical disease (Pass *et al.*, 2006; Fields, Knipe and Howley, 2013). Intrauterine HCMV transmission does not only occur in mothers with a primary infection. Transplacental transmission to the infant also occurs during the course of non-primary

infections in seropositive (previously exposed) mothers. The relative contribution of reinfection, persistent infection, and reactivation of latent HCMV infection is not well studied (see section 1.2 below). In addition to infection, the comparatively high presence of HCMV observed in the placenta has also been correlated with stillbirth (Iwasenko *et al.*, 2011) and HCMV is likely a major cause of stillbirths. Neutralizing antibodies (nAbs), discussed further below, may prevent transplacental transmission and resulting infection of the foetus (Maidji *et al.*, 2010; Nigro and Adler, 2011).

1.2 PRIMARY AND NON-PRIMARY INFECTION

Primary infection typically begins with viral replication in the mucosal epithelium at a site of entry following direct contact with infectious secretions from another individual with either a primary infection or non-primary (recurrent/reinfection) infection (Fields, Knipe and Howley, 2013). Primary HCMV infection in high resource settings often occurs in adulthood and pregnant women of reproductive age, while in low resource settings primary infection usually occurs at a young age (Kim, 2010; Manicklal *et al.*, 2013). This is a consequence of generally high HCMV prevalence in low resource settings (Griffiths and Baboonian, 1984; Stagno *et al.*, 1986). Experimentally, non-primary HCMV infection is defined as detecting active HCMV infection in a previously infected person after a minimum of four weeks without detectable HCMV during active surveillance (Ljungman, Griffiths and Paya, 2002). This results from either reactivation of latent virus or reinfection by exogenous (horizontally transmitted) virus (Boppana *et al.*, 2001; Ross *et al.*, 2010; Yamamoto *et al.*, 2010). Reinfection can only be experimentally established with the detection of an HCMV strain that is different from the original strain that caused the patient's first infection, while reactivation is presumed to occur when the two strains of HCMV are the same (Ljungman, Griffiths and Paya, 2002). In practice, this is usually not investigated, and re-infections are rarely properly distinguished from reactivations.

Recent studies have compared the risk of maternal-to-foetal transmission in primary versus non-primary maternal infection, and suggest that the transmission risk is 30-40% in primary infection but only 3-4% in non-primary infection (Leruez-Ville *et al.*, 2017; Simonazzi *et al.*, 2017; Permar, Schleiss and Plotkin, 2018). This partial protection from

in utero transmission from previously exposed women suggests the presence of underlying immunity that often, but not always, curtails active HCMV replication in the placenta (Permar, Schleiss and Plotkin, 2018). This underlying immunity is discussed further below. Despite the reduced risk of transmission in secondary infection, the number of infants that are congenitally infected in pregnancies after primary infection is significantly less than the number of congenitally infected infants born as a result of non-primary infection (Britt, 2017). This is a consequence of the much higher numbers, globally, of secondary infection (Britt, 2017). Indeed, 70–80% of all infants with cCMV infection are born to mothers with non-primary infection during pregnancy (Wang *et al.*, 2011),. A study suggested correlates of protection from foetal transmission in maternal primary infection (section 1.6.3 below), and I used their measures in this thesis. However, correlates of protection in the setting of non-primary infection have not yet been clearly defined, and require further investigation (Permar, Schleiss and Plotkin, 2018).

1.3 CLINICAL CONSEQUENCES OF CONGENITAL HCMV INFECTION

While cCMV infection may not be as well-known as the other common causes of paediatric disabilities, it is the leading infectious cause of birth defects and permanent sequelae (Permar, Schleiss and Plotkin, 2018). Sequelae include neurodevelopmental delay, brain disease, growth restriction, non-genetic sensorineural hearing loss (SNHL), and other intellectual disabilities, with SNHL being the most frequent (Kenneson and Cannon, 2007). Most children that exhibit symptoms of cCMV will have a combination of deficits, most commonly, mental retardation, cerebral palsy, SNHL, and impaired vision (Fields, Knipe and Howley, 2013). Up to 50% of symptomatic children and 10% of otherwise asymptomatic children at birth with cCMV infection have SNHL (Yamamoto *et al.*, 2011). A significant number of these children experience a delay in the onset of hearing loss as well as continual hearing deterioration during childhood (Ross *et al.*, 2006). The frequency of SNHL in children born to mothers with primary CMV infection versus non-primary infection has been reported to be 11–15% and 7–10%, respectively (Ross *et al.*, 2006; Foulon *et al.*, 2008). In addition to SNHL, cCMV infection is likely a significant but often under-appreciated cause of stillbirths and neonatal deaths (Iwasenko *et al.*, 2011; Chawana *et al.*, 2019). For these reasons, developing an effective human

CMV vaccine that is able to prevent congenital infection through elicitation of protective immune responses is a high public health priority (Schleiss, Permar and Plotkin, 2017).

1.4 MATERNAL HIV-1 INFECTION IS ASSOCIATED WITH AN INCREASED RISK OF CONGENITAL HCMV INFECTION

Pregnant women with human immunodeficiency virus (HIV-1) infection have an increased risk of HCMV transmission *in utero*, thus, enhancing the risk of cCMV infection (Manicklal *et al.*, 2014; Pathirana *et al.*, 2019). It seems plausible that HIV-1 infected women are less likely to have an immune response that prevents or retards foetal transmission of HCMV. Studies conducted before antiretroviral therapy (ART) was readily available reported the HCMV transmission rates as high as 11% (Doyle, Atkins and Rivera-Matos, 1996; Kovacs *et al.*, 1999; Mwaanza *et al.*, 2014). About 90% of HIV-infected women of childbearing age are co-infected with HCMV. Manicklal *et al* (2013) and Duryea *et al* (2010) both reported an estimated 3% of HIV-1 exposed infants acquiring cCMV infection compared to only 1% of control HIV-1 unexposed infants (Duryea *et al.*, 2010; Manicklal *et al.*, 2013). Additionally, large cohort studies conducted in the United States, France, and Spain described cCMV infections in 2.3-4.6% of infants born to HIV-infected mothers. These reported values are all considerably higher than the expected rate of about 0.7% for infants born to HIV-uninfected mothers. In contrast, a Brazilian study did not identify a difference in cCMV infection rates in infants born to HIV-infected versus HIV-negative women (Mussi-Pinhata *et al.*, 1998).

cCMV infection rates have been reported to be higher in women with low $CD4^+$ T cell counts (Pass and Anderson, 2014). Gantt *et al* (2016) reported that the $CD4^+$ T cell count is inversely correlated with cCMV transmission risk (Gantt *et al.*, 2016). Additionally, Bialas *et al* (2015) used their recently developed rhesus monkey cCMV transmission model to show that pregnant monkeys with low peripheral $CD4^+$ T cell counts had increased rates of placental rhesus CMV transmission. They hypothesized that this observation was because of a delay in the maternal CMV-specific nAb response (Bialas *et al.*, 2015), thus, concluding that the effect HIV-1 has on $CD4^+$ T cells may also impair

the functional CMV-specific Ab responses which results in enhanced cCMV transmission (Bialas *et al.*, 2016). Pathirana *et al* (2019) did not observe an association between maternal $CD4^+$ T cell counts and risk of congenital transmission (Pathirana *et al.*, 2019).

It is important to note that maternal ART has been exceedingly effective in reducing intrauterine HIV-1 transmission. However, the burden of cCMV infection in HIV-exposed infants is still high in maternal populations receiving ART with an HCMV transmission rate of 1.5%–3.5% (Guibert *et al.*, 2009; Duryea *et al.*, 2010; Frederick *et al.*, 2012; Manicklal *et al.*, 2014; Gantt *et al.*, 2016; Pirillo *et al.*, 2017; Pathirana *et al.*, 2019). Therefore, it is vital to better understand the effect of maternal HIV-1 infection on cCMV infection, as the number of HIV-1 exposed, CMV-infected infants with lasting neurodevelopmental effects increases in the ART era.

1.5 STRUCTURE AND MORPHOLOGY OF THE HUMAN CYTOMEGALOVIRUS

1.5.1 Genome and Structure

HCMV has one of the largest viral genomes, consisting of ~236 kb double-stranded linear DNA, containing over 100 open reading frames (**Figure 1.2**) (Britt and Mach, 1996; Murphy *et al.*, 2003; Dolan *et al.*, 2004; Stern-Ginossar *et al.*, 2012).

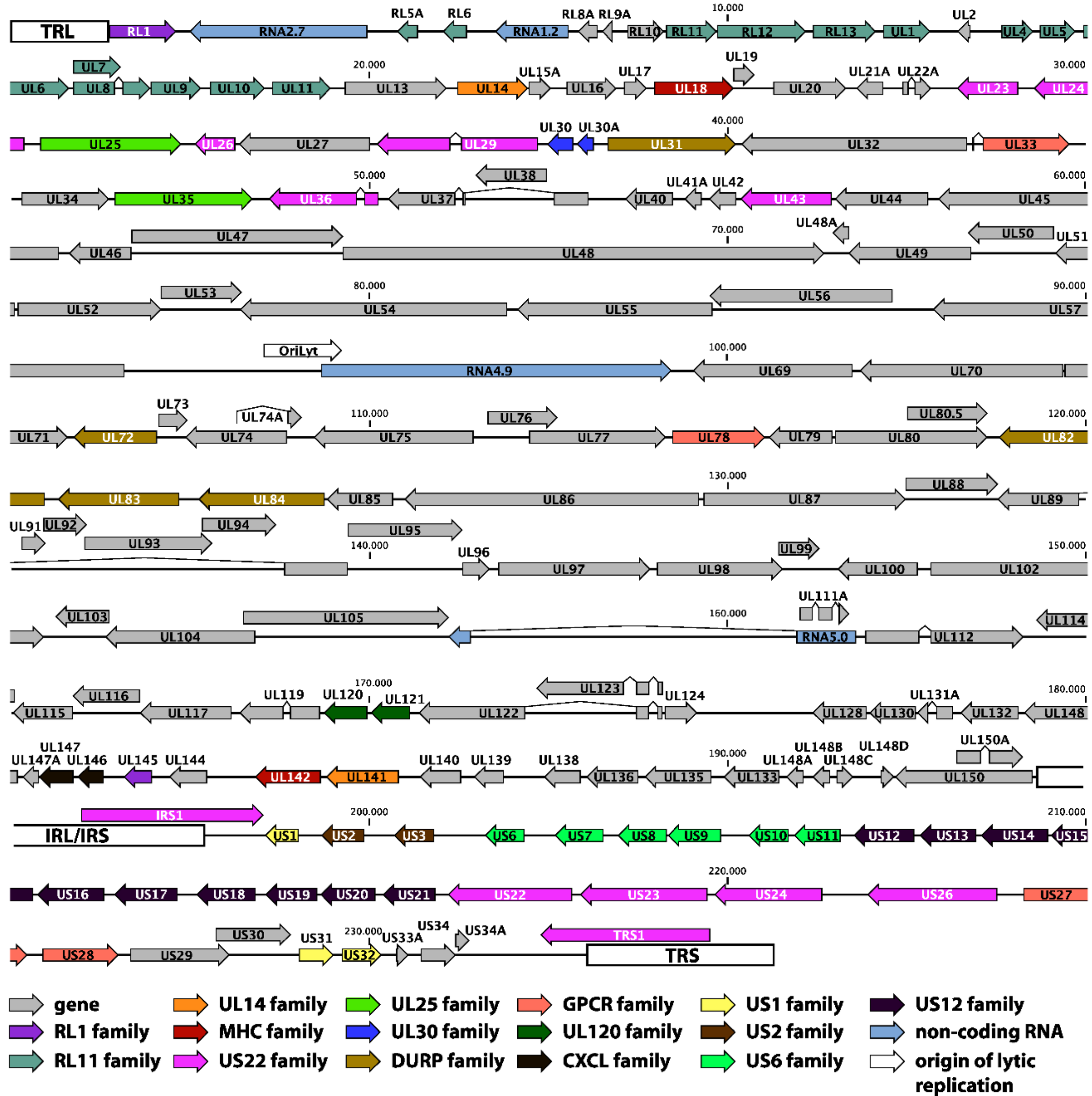


Figure 1.2: Annotated genome of HCMV strain Merlin (GenBank accession NC_006273). The double-stranded DNA genome is shown as a single line. The position of the nucleotides are given in base pairs. The terminal internal repeat regions (TRL, IRL/IRS and TRS) are represented in white boxes while the arrows indicate genes. The different gene families are shown in different colours, with a key below the genome (Sijmons, Van Ranst and Maes, 2014)

HCMV viral particles are ~200 nm in size and consist of an extremely stable proteinaceous icosahedral capsid comprised of 162 capsomeres, containing the genome (Fields, Knipe and Howley, 2013). Surrounding the capsid is a tegument layer containing more than 14 viral tegument proteins, followed by the outer envelope, which contains several glycoprotein complexes, described in more detail below (**Figure 1.3**) (Hahn *et al.*, 2004; Varnum *et al.*, 2004; Adler *et al.*, 2006; Ryckman *et al.*, 2006; Ryckman, Chase and Johnson, 2008; Straschewski *et al.*, 2011).

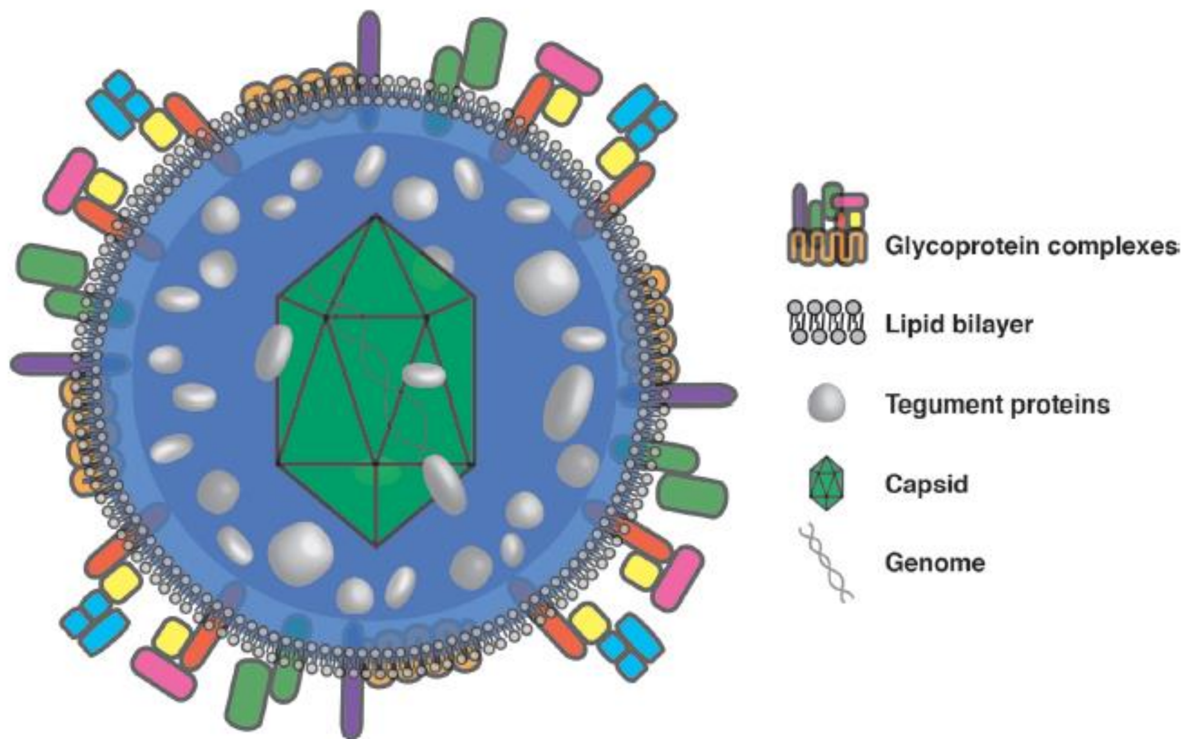


Figure 1.3: Schematic of the structure of Cytomegalovirus. The HCMV virion has a double-stranded DNA genome of over 200 kb contained within an icosahedral proteinaceous capsid (green). The tegument layer (blue) surrounding the capsid contains numerous tegument phosphoproteins (grey). The lipid bilayer surrounds the virion and is studded with several glycoprotein complexes (multi-coloured). A key of each structural component in on the right of the schematic (Gardner and Tortorella, 2016).

HCMV encodes over 100 protein products of which 66 are involved in known functions ranging from mediating viral entry and release, to influencing cell tropism and host

responses to infection products (Cunningham *et al.*, 2010; Fields, Knipe and Howley, 2013). Additionally, the great coding capacity and extensive mRNA splicing results in other minor viral matrix and envelope components involved in viral infection (Fields, Knipe and Howley, 2013).

1.5.2 Envelope Glycoproteins

HCMV harbours at least 20 viral proteins which are embedded in the viral envelope. These include glycoprotein B (gB; produced by the gene *ul55)*, glycoprotein H (gH; produced by the gene *ul75*), glycoprotein L (gL; produced by the gene *ul115*), glycoprotein O (gO; produced by the gene *ul74*), glycoprotein M (gM; produced by the gene *ul100*) and glycoprotein N (gN; produced by the gene *ul73*) (Gardner and Tortorella, 2016). The virion surface is studded with these glycoproteins that assemble to form various complexes such as the gM/gN dimer, the gH/gL/gO trimer, the gH/gL/UL128/UL130/UL131a pentameric complex (simply referred to as the "pentamer"), as well as the gB oligomer that later forms a homotrimer, discussed below (**Figure 1.4** and **Table 1.1**).

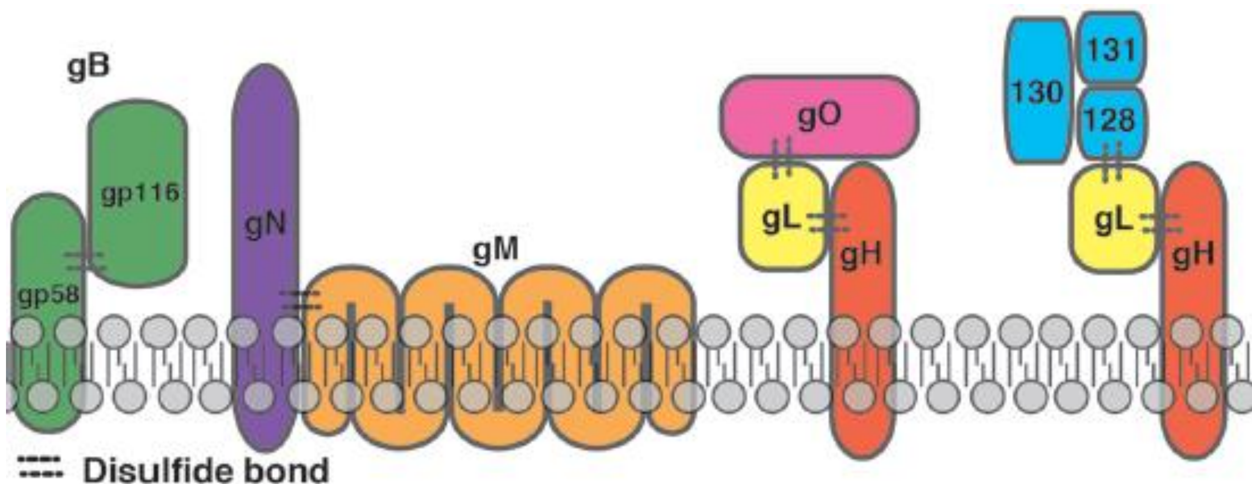


Figure 1.4: HCMV glycoprotein complexes embedded in the viral envelope. Each glycoprotein found in the viral envelope is designated a colour. The glycoprotein B oligomer (green) consists of two polypeptides gp116 and gp58. Glycoprotein N (purple) and glycoprotein M (orange) form the gN/gM dimer. Glycoprotein O (pink), glycoprotein L (yellow) and glycoprotein H (red) form the gH/gL/gO trimer. The UL128-131a/gL/gH pentameric complex (pentamer) is formed through the assembly of proteins UL128, UL130, and UL131a (blue), glycoprotein L

(yellow) and glycoprotein H (red). The parallel dashed lines represent disulfide bonds between the proteins (Gardner and Tortorella, 2016).

Table 1.1: Glycoprotein complexes of Cytomegalovirus

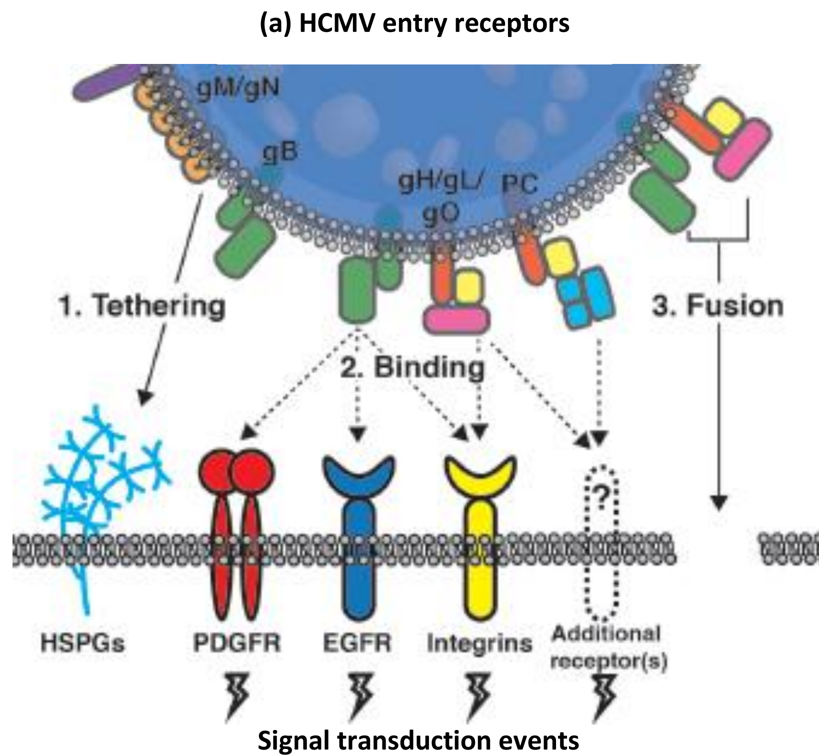
Glycoprotein	ORF	type of protein	Potential N-linked glycosylation sites	Complex(es)
gB (gp58/116)	UL55	Transmembrane type I	18	Oligomer, gB:gH/gL*
gH	UL75	Transmembrane type I	6	gH/gL, gH/gL/gO, pentamer, gB:gH/gL
gL	UL115	Soluble	1	gH/gL, gH/gL/gO, pentamer, gB:gH/gL*
gO	UL74	Soluble	18	gH/gL/gO
gM	UL100	Transmembrane type III	1	gM/gN
gN	UL73	Transmembrane type I	2	gM/gN
UL128	UL128	Soluble	0	Pentamer
UL130	UL130	Soluble	3	Pentamer
UL131a	UL131a	Soluble	1	Pentamer

*the "**:**" in gB:gH/gL refers to a noncovalent interaction while the "**/**" refers to a disulfide linkage (Gardner and Tortorella, 2016).

Although the exact functions of each glycoprotein complex are not completely understood, these complexes have crucial roles in the life cycle of HCMV and allow for attachment and entry into the numerous cell types (Hobom *et al.*, 2000; Dunn *et al.*, 2003; Gardner and Tortorella, 2016). In particular, the glycoproteins gB, gH, and gL, conserved across all herpesviruses, have crucial functions in attachment and entry, and are known targets of nAbs (Connolly *et al.*, 2011; Eisenberg *et al.*, 2012; Gardner and Tortorella,

2016). While other glycoprotein complexes are involved in the entry process, such as the gM/gN complex that tethers HCMV to the host surface, we focused on gB and pentamer because of the previous study suggesting their important in the context of primary infection (Lilleri *et al.*, 2013),

HCMV has a broad cellular tropism, a characteristic that is determined by the multiple glycoproteins/complexes able to mediate entry. The virus uses multiple receptors (**Figure 1.5 A**) and specific pathways (**Figure 1.5 B**) to gain entry into a wide range of cell targets including epithelial/endothelial cells, fibroblasts, monocytes/macrophages, smooth muscle cells, neurons, stromal cells, and liver hepatocytes (Vanarsdall and Johnson, 2012). More specifically, fibroblast entry occurs by pH-independent fusion at the cell surface using gB and gH/gL/gO (Compton, Nepomuceno and Nowlin, 1992) while entry into epithelial/endothelial, dendritic and monocytic cells occurs by endocytosis or macropinocytosis and then pH-dependent fusion, using gB, gH/gL/gO, and pentamer (Hahn *et al.*, 2004; Gerna *et al.*, 2005; Patrone *et al.*, 2005; D Wang and Shenk, 2005; Adler *et al.*, 2006; Ryckman *et al.*, 2006; Wille *et al.*, 2010; Straschewski *et al.*, 2011; Nogalski *et al.*, 2013; Zhou, Lanchy and Ryckman, 2015). These varied entry pathways highlight the involvement of very distinct glycoprotein complexes which shows the complexity of HCMV (**Figure 1.5**). A more detailed summary of these glycoprotein complexes follows below.



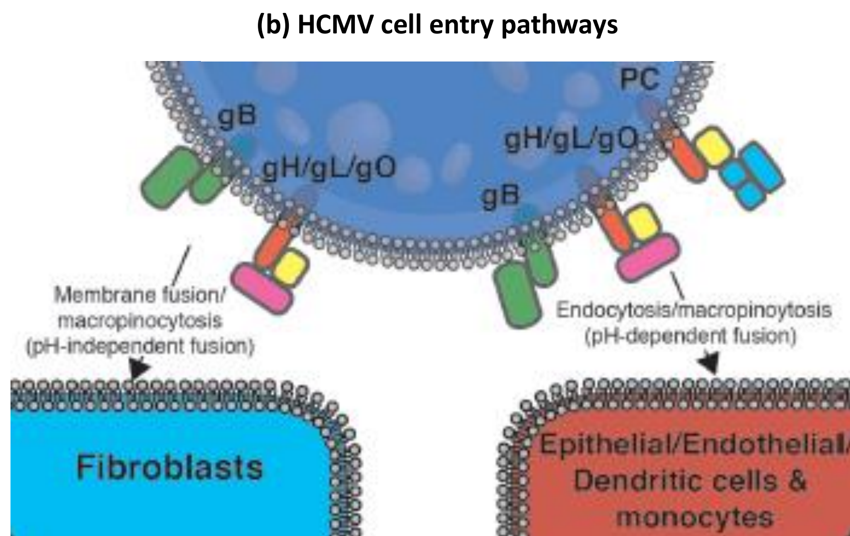


Figure 1.5: Schematic showing cytomegalovirus attachment and entry schematic. (a) HCMV uses several receptors and complexes for cell attachment and entry. (1) Initially HCMV gB and gM/gN complexes interact with heparan sulphate proteoglycans (HSPGs), tethering to the target host cell of all cell types. (2) Binding occurs with several cell surface receptors including integrins, epidermal growth factor receptors (EGFR) and platelet-derived growth factor receptors

α (PDGFRα). (3) Membrane fusion is initiated by gB and gH/gL complexes (gH/gL/gO and/or pentameric complex -PC). **(b)** HCMV enters fibroblast cells through pH-independent membrane fusion at the cell surface or macropinocytosis using gB and/or gH/gL/gO. HCMV enters epithelial/endothelial, dendritic cells and monocytes through pH-dependent fusion following endocytosis/macropinocytosis (Gardner and Tortorella, 2016) using gB and/or gH/gL/gO and/or pentamer. Although gB is used to enter multiple cells, it is predominantly used to enter fibroblast cells while pentamer (PC) is used to enter predominately endothelial cells.

1.5.3 Glycoprotein B

Initially the nascent gB is synthesized as a 160 kDa monomer, which is 906 amino acids long, that is then cleaved by the cellular furin endoprotease within the *trans*-Golgi network into the two polypeptides, gp58 and gp116 (**Figure 1.6**) (Spaete *et al.*, 1988; Britt and Vugler, 1989; Burke and Heldwein, 2015). These two polypeptides are then linked together by a covalent disulfide bond, forming a heterodimer (sometimes referred to as monomer) (**Figure 1.4**). Thus, each monomer consists of a large ectodomain surface subunit (gp116), a hydrophobic membrane-proximal region, and a small type 1 transmembrane domain (gp58) (**Figure 1.6**). The ectodomain of each monomer contains five antigenic domains (AD1-5) each eliciting varying immune responses of both neutralizing and non-neutralizing antibody responses (**Figure 1.6**) (Burke and Heldwein, 2015).

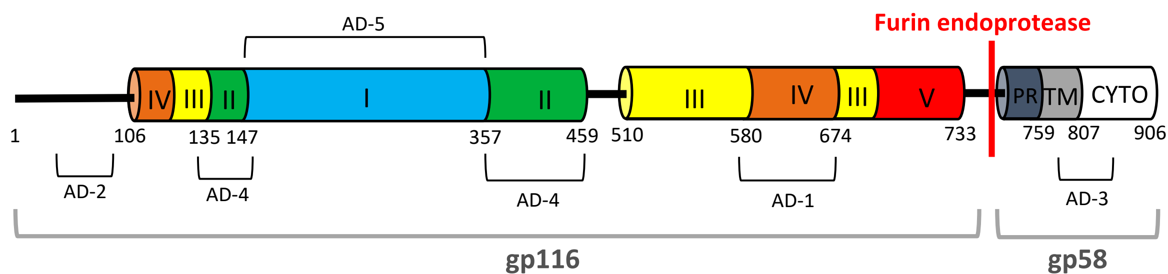


Figure 1.6: Linear schematic of HCMV glycoprotein B. The colours and roman numerals indicate structural domains. The numbers indicate the first and last amino acid corresponding to each colour-coded structural domain. The furin endoprotease cleavage site is shown in red. The gp116 and gp58 regions are shown in grey. AD: Antigenic domains (1-5). PR: Membrane proximal region. TM: transmembrane domain. Cyto: Cytoplasmic domain. Adapted from (Heldwein *et al.*, 2006).

Although studies continue to explore the multifunctional nature of gB, it is clear that gB is important for cell-to-cell spread as well as cell–cell fusion, both involving membrane fusion (Fields, Knipe and Howley, 2013). gB binds to cell surface heparan sulfate proteoglycans which tethers HCMV to the host cell surface, a feature that is ubiquitous to several herpesviruses. gB then interacts with multiple cellular receptors (**Figure 1.5**), such as platelet-derived growth factor receptor-α (PDGFRα) and integrins, for viral attachment (Feire, Koss and Compton, 2004; Wang *et al.*, 2005; Soroceanu, Akhavan and Cobbs, 2008; Chan, Nogalski and Yurochko, 2009; Feire *et al.*, 2010; Cobbs *et al.*, 2014).

Structural comparisons of the closely related sequence homologs of gB from Herpes Simplex Virus-1 or Epstein Barr Virus (other members of *Herpesviridae*), have not only indicated that HCMV gB monomers form a homotrimer on the envelope (**Figure 1.7**) (Connolly *et al.*, 2011) but also that HCMV gB is a class III fusion protein (Harrison, 2008; Backovic and Jardetzky, 2009; Sharma *et al.*, 2013; Burke and Heldwein, 2015; Chandramouli *et al.*, 2015). This means that HCMV gB has structural features akin to Herpes Simplex Virus-1 gB, thus, it falls into the Class III category (Backovic and Jardetzky, 2009). HCMV gB is referred to as a "fusion protein" because this protein effects fusion of the viral membrane with the cell membrane, resulting in fusing the two membranes and entry of the virus into the host cell.

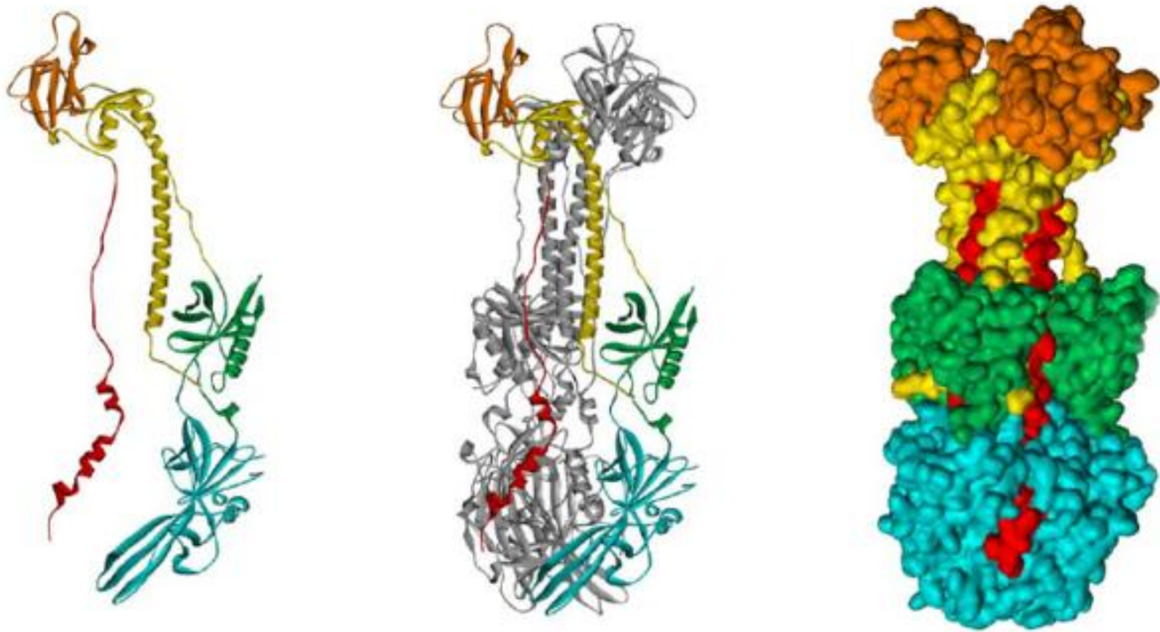


Figure 1.7: Architecture of HCMV glycoprotein B ectodomain. A ribbon diagram of the ectodomain of the gB monomer (left), the gB homotrimer with two of the three monomers shown in grey (middle) and accessible surface representation of the gB trimer (right). All three representations are domain colour-coded according to Figure 1.6 (Heldwein *et al.*, 2006).

This means that gB undergoes a conformational change from monomer to homotrimer. The re-ordering of the domains within these gB subunits and the energy released from this refolding results in the fusion of the viral envelope and host cell membranes during entry, either directly at the plasma membrane of fibroblasts, or through endocytosis/macropinocytosis in endothelial/epithelial cells (Isaacson, Juckem and Compton, 2008; Burke and Heldwein, 2015). This ultimately results in release of the nucleocapsid and matrix proteins into the cytoplasm of the host cell.

1.5.4 gH/gL Complexes

Apart from gB, other glycoproteins assemble to form complexes that mediate entry into any cell type (Heldwein *et al.*, 2006; Backovic *et al.*, 2009). gH is a transmembrane protein

(**Table 1.1**) that is important for attachment and is part of the core fusion machinery (Gardner and Tortorella, 2016). gH requires gL as a chaperone for correct and complete maturation (Britt, 2007), meaning gH/gL dimerization in the endoplasmic reticulum increases export of these glycoproteins and ultimately allows HCMV to target a full range of host cells (Ryckman *et al.*, 2008; Ryckman, Chase and Johnson, 2010; Vanarsdall, Chase and Johnson, 2011; Gardner *et al.*, 2016). Similar to gB, the gH/gL complex influences cell attachment and entry by mediating fusion along with gB. Cell fusion of HCMV to the host cell occurs more efficiently when both gH/gL and gB are involved as opposed to gB alone (Isaacson, Juckem and Compton, 2008), which is seen in other herpesviruses (Connolly *et al.*, 2011).

The majority of gH/gL exists as the unmodified heterodimer glycoprotein complex (“unmodified”, i.e. does not contain gO and is not part of a pentamer). However, when associated with the highly glycosylated UL74-encoded gO (Ryckman, Chase and Johnson, 2010; Wille *et al.*, 2010), via a disulfide bond to gL, it forms a trimeric complex that binds to various cellular receptors (**Figure 1.4** and **1.5**) (Kabanova *et al.*, 2016). This allows for entry into both fibroblasts and endothelial/epithelial cells. The function of gH on its own, associated with gL through a disulfide bond to form the gH/gL dimeric complex, or in other multiple-protein entry complexes suggests that it is key in infection of various cell types (Ryckman *et al.*, 2008; Macagno *et al.*, 2010; Kabanova *et al.*, 2016).

1.5.5 The gH/gL/UL128-131A Pentameric Complex (pentamer) - UL128 (to make pentamer) and gO (to make trimer) compete for binding to gL

In addition to the gH/gL/gO trimer, gH/gL assembles with the UL128, UL130, and UL131A gene products to form the gH/gL/UL128-131A pentameric complex (**Figure 1.4**), more simply referred to as pentamer (Ryckman *et al.*, 2008). This assembly occurs through a disulfide bond between the soluble protein UL128 and gL (Gardner and Tortorella, 2016). A recent study showed that both gO (from the trimeric complex) and UL128 compete for the same binding site on gL to generate a disulfide bond (**Figure 1.8**) (Ciferri *et al.*, 2015). The protein UL148 may regulate the pentamer to gH/gL/gO ratio which subsequently regulates the cell tropism of emerging virions (Li *et al.*, 2015).

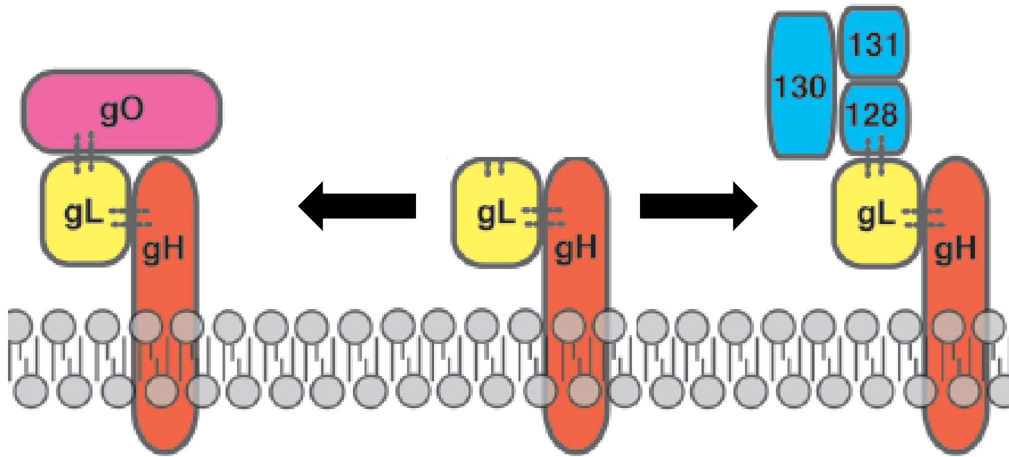


Figure 1.8: Formation of gH/gL/gO trimer and pentamer from gH/gL. The gH/gL complex interacts with either gO or with the UL proteins (UL128-131A) to form the mutually exclusive complexes gH/gL/gO and pentamer, respectively. adapted from (Gardner and Tortorella, 2016).

The gH/gL/gO to pentamer ratio varies considerably between the different strains of HCMV (Zhou *et al.*, 2013). HCMV laboratory strain passaging of AD169 and Towne, resulted in selection of viral particles that did not express pentamer (Cha *et al.*, 1996; Hahn *et al.*, 2004; Ryckman *et al.*, 2006). Mutants without pentamer can replicate in fibroblasts better than the wild-type virus strains (Wang and Shenk, 2005; Ryckman *et al.*, 2006; Straschewski *et al.*, 2011) but cannot infect epithelial/endothelial cells, monocytes, or macrophages (Hahn *et al.*, 2004; Wang and Shenk, 2005; Straschewski *et al.*, 2011). Mutants without the gH/gL/gO complex have stark defects in replication even though these mutants contain high levels of pentamer (Hobom *et al.*, 2000; Dunn *et al.*, 2003; Wille *et al.*, 2010). Thus, the levels of infection of fibroblasts and epithelial/endothelial cells are associated with gH/gL/gO abundance on the virus surface.

Pentamer not only enhances infection in epithelial/endothelial cells (D Wang and Shenk, 2005; Ryckman, Chase and Johnson, 2008) and monocytes/macrophages (Ryckman *et al.*, 2006; Nogalski *et al.*, 2013) but also enhances viral interactions with neutrophils, dendritic cells, as well as several other cell types (Revello and Gerna, 2010; Martinez-

Martin *et al.*, 2018). This suggests that pentamer may be essential in the pathogenesis of HCMV. Along with gB, pentamer is the focus of this study.

1.6 ANTIBODY RESPONSES TO HCMV INFECTION

Antibodies (Abs), otherwise known as immunoglobulins, are protective proteins that are produced by the immune system as part of the humoral immune response to the presence of an antigen. When a host becomes infected with a pathogen, such as HCMV, the body produces Abs that target various epitopes on multiple viral antigens (Rhorer *et al.*, 2009). While some Abs are only able to bind to the virus (non-neutralizing Abs), a subset of Abs are able to both bind and *neutralize* the virus, or inactivate it by blocking viral infectivity. These Abs are referred to as neutralizing Abs (nAbs) (Rhorer *et al.*, 2009).

1.6.1 Neutralizing Antibody Responses Specifically Towards Glycoprotein B and Pentamer

What is crucial in developing an HCMV vaccine is to define which glycoprotein targets are essential to this protection, since various glycoproteins are involved in host cell entry and have neutralizing epitopes (Compton and Feire, 2007). It may be crucial to elicit Ab responses against multiple epitopes for protection against cCMV infection mediated by vaccines, with the best-established targets being the neutralizing epitopes within gB and pentamer (Permar, Schleiss and Plotkin, 2018). Thus, we have focused on gB and pentamer based on the data discussed below.

Several studies suggest that gB-directed Abs are important to prevent viral spread from mother-to-child (Permar, Schleiss and Plotkin, 2018). gB has long been recognized as a target of neutralizing antibodies (Cranage *et al.*, 1986). Anti-gB IgG mAbs have been shown to protect human trophoblast progenitor cells, the precursors to placental cells, by blocking placental trophoblast infection while anti-pentamer IgG Abs could not protect trophoblast progenitor cells (Schleiss, 2017). In fact, an HCMV mutant strain without pentamer was still able to infect human trophoblast progenitor cells. This suggested that pentamer is not important for viral entry into the placenta (Zydek *et al.*, 2014). However, this is likely because pentamer is required only for endothelial/epithelial cell entry while

gB aids entry into all cell types. In addition to this, a weak correlation was observed between protection against transmission and binding of maternal IgG to gB, in the setting of non-primary HCMV infection in women co-infected with HIV-1. However, no association was observed with pentamer, the gH/gL dimer, or the gH/gL/gO trimer (Bialas *et al.*, 2016).

Despite the data above, pentamer is also recognized as a target with the most potent neutralizing Abs (Lilleri *et al.*, 2013). Lilleri *et al* (2013) characterized maternal sera into transmitters and non-transmitters, with primary HCMV infection occurring just before or during pregnancy. It was shown that adsorption with pentamer led to a drastic decrease in neutralizing activity within epithelial cells at all the studied time points after infection. Lilleri *et al* (2013) also showed that the IgG levels for Abs specific to pentamer, but not gB, were higher in maternal non-transmitters to the transmitters within 30 days after HCMV infection (Lilleri *et al.*, 2013).

Similarly, Fouts *et al* (2012) showed a large number of nAb responses in hyper-immunoglobulin of humans was pentamer-specific and unable to recognize the other major entry glycoproteins including gB (Fouts *et al.*, 2012). Several other studies have shown that nAbs predominantly target pentamer during HCMV infection of epithelial/endothelial cells (Genini *et al.*, 2011; Fouts *et al.*, 2012; Freed *et al.*, 2013). Consistent with this, immunizing with pentamer elicited a strong nAb response in mouse, rabbit, as well as rhesus macaque models (Fu *et al.*, 2012; Wussow *et al.*, 2013; Wen *et al.*, 2014). Together these data suggest the significance of pentamer-specific Abs.

A study conducted by Macagno *et al* (2010) isolated a panel of human HCMV-nAbs by means of memory B-cell immortalization as well as neutralization screening of different target cells infected with HCMV. This panel of mAbs, representing those mAbs elicited during a natural infection, were then categorized into two groups. Group 1 consisted of 10 mAbs with the capacity to bind a broad range of targets, including gH and gB. These antibodies were also capable of neutralizing HCMV infection in the nanomolar concentrations of all the cell types tested, including epithelial/endothelial and fibroblast

cells. Group 2 consisted of 17 highly potent mAbs binding more specifically to novel epitopes of pentamer and were capable of neutralizing HCMV infection at picomolar concentrations of all cell types except fibroblasts (Macagno *et al.*, 2010). Thus, this data supports the notion that eliciting nAbs that target both pentamer and gB of HCMV are effective for neutralizing HCMV infection, which could ultimately result in vaccine design and development based on these key antigenic targets.

In our study we synthesized and conducted experiments using five mAbs from the panel of 27 mAbs isolated by Macagno *et al* (2010). These antibodies were used to define epitopes on pentamer and gB for which we measured epitope-specific antibodies. The three selected pentamer epitopes were chosen because they are targets of potently neutralizing antibodies since these regions consist of the fibroblast and endothelial/epithelial cell receptors binding sites for viral entry into these cell types (Ciferri *et al.*, 2015). Likewise, the two gB epitopes we chose were chosen because they are two distinct neutralizing antigenic sites.

1.6.2 Current State of Vaccine Development

Although vaccine development for HCMV has been slow, there is an optimism based on evidence indicative of immune correlates of protection reducing transmission of HCMV to the foetus in seronegative women. If this foetal disease were to be even modestly reduced, it would have a significantly positive impact as a public health goal (Permar, Schleiss and Plotkin, 2018). Advances in the field of HCMV vaccines have been made possible by: (i) describing maternal immune correlates of protection against congenital transmission, subsequent to primary and non-primary infection in humans as well as in animal models; and (ii) by describing the epitopes found on viral proteins that are targets of nAbs (Permar, Schleiss and Plotkin, 2018).

Hence, research thus far suggests that one of the avenues for protection against cCMV is via vaccines eliciting antibody responses against the best-established targets, gB and pentamer. The three primacies for HCMV vaccine development remain: 1) testing new vaccine candidates in the early phases by comparing natural immunity responses and

partially efficacious vaccine responses; 2) describing immune correlates of cCMV transmission in seropositive women and recipients of vaccines; and 3) using animal models to show certain immune responses are able to block HCMV infection of the placenta (Permar, Schleiss and Plotkin, 2018). Our study fits into the second category, exploring immune correlates of protection against mother-to-child transmission of HCMV in seropositive women.

HCMV vaccine development first started in the 1970s (Plotkin, Farquhar and Horberger, 1976), but have only now progressed in earnest. The target populations are women of childbearing age, with immunization before or during early pregnancy to prevent transmission, and solid organ or hematopoietic cell transplant patients that risk either primary infection from the transplanted organ or reactivation from latent infection because of immune suppression (Permar, Schleiss and Plotkin, 2018).

There is more advanced research studies available on gB compared to pentamer, with a gB-based vaccines already tested in a randomized, placebo-controlled, phase 2 clinical trial (Baraniak *et al.*, 2018; Nelson *et al.*, 2018; Schleiss, 2018). In the clinical trial, a subunit vaccine consisting of gB in an adjuvant known as MF59 showed protection from HCMV infection in young mothers, even though the overall efficacy was only ~50% and the elicited mAbs were poorly neutralizing (Pass, 2009; Pass and Anderson, 2014). A few hypotheses were proposed for these observations: using an undesired antigenic domain, injecting the post-fusion conformation of gB, or possibly the sequence diversity among gB proteins that occurs in natural circulating HCMV strains (Renzette *et al.*, 2011, 2017). The results of this trial suggested that preventing primary maternal infection through vaccination may be an achievable goal. Although a few pharmaceutical companies presently have HCMV vaccine development programs that are active, no licensed HCMV vaccine that prevents maternal or cCMV infection is available (Pass and Anderson, 2014; Gerna and Lilleri, 2020).

1.7 GAPS IN OUR KNOWLEDGE

Most studies have investigated maternal immune correlates solely in primary infection (Lilleri *et al.*, 2013). However, since most cases of congenital CMV worldwide are from cases in which the maternal infection was non-primary, further research in the context of non-primary congenital infection is necessary. Improved understanding of what is needed for foetal protection against CMV infection, including in HIV infected mothers, is key for developing an effective preconception vaccine.

With all of the evidence indicating that natural immunity is at least partly protective against congenital CMV infection, what remains to be investigated is how can that immunity be reproduced or improved on through the use of vaccination. In order to do this well, it would be best to define the targets of protective immunity.

1.8 RESEARCH QUESTION

1.8.1 Hypothesis

Antibodies that target proteins gB and pentamer and/or epitopes on pentamer are associated with protection from *in utero* transmission of non-primary maternal HCMV infection.

1.8.2 Aim

This study aimed to evaluate the levels of antibody against gB and pentamer in non-primary infected maternal sera, analyse the levels of these antibodies, and identify any differences in these levels between samples from transmitting and non-transmitting mothers as well as between HIV-infected and uninfected mothers. This could allow us to test for antibody correlates of protection from mother-to-child transmission in non-primary CMV infection, as potential vaccine targets for future study.

1.8.3 Objectives

1. Measuring levels of maternal antibody targeting HCMV glycoprotein B and the HCMV pentamer by ELISA to assess whether the level of antibody are different between HCMV transmitting and non-transmitting mothers, and whether maternal HIV-1 infection impacts these levels.

2. Measuring levels of maternal antibodies targeting specific epitopes within HCMV glycoprotein B and the HCMV pentamer assess whether the level of antibody distinguishes HCMV transmitting and non-transmitting mothers, and whether maternal HIV-1 infection impacts these levels.

CHAPTER 2: MATERIALS AND METHODS

2.1 STUDY PARTICIPANTS

2.1.1 Maternal Samples

In this research project we used serum samples from mothers who did, and who did not transmit HCMV to their foetus. Blood samples from pregnant women enrolled at Baragwanath Hospital maternity department, or at several obstetric clinics linked to Baragwanath Hospital maternity department at time of labor were collected between July 2014 and December 2016, as part of a birth cohort study (Madhi *et al.*, 2020). This was under Protocol M140203, "Establishing a sero-correlate of protection from invasive group B streptococcus disease in newborns and infants aged ≤90 days." The pregnant women had to be ≥18 years of age and deliver a live birth at Chris Hani Baragwanath Academic Hospital, Soweto, to be eligible for the study under Protocol M140203 (Madhi *et al.*, 2020). Explicit permission for study of antibodies to CMV was obtained during the consent process for M140203. Mothers enrolled in M140203 who either did or who did not transmit CMV to their children *in utero* were identified in a sub-study of M140203 entitled "Epidemiology of congenital cytomegalovirus (CMV) in a high HIV prevalent setting in Soweto, South Africa" study M151161 (Principal Investigators: Dr. Jayani Pathirana and Prof Shabir Madhi). Children with congenital CMV were matched to well children with no history of hospitalization in the first three weeks of life who had a negative CMV PCR test in saliva within 3 days of birth and a confirmatory negative PCR test in saliva or urine at three weeks of age. The control infants were matched to congenital CMV cases by gestational age at birth, birth weight, gender and maternal HIV-1 infection status. We compared the mothers of cases (transmitters) to mothers of controls (non-transmitters).

To ascertain whether mothers transmitted CMV to their infants, a total of 3,274 children were enrolled, of which 2,908 mothers consented that their infants be screened for congenital CMV infection by PCR of saliva samples collected within the first 48 hours of birth (**Figure 2.1**). Results were obtained for 2,685 newborn infants and of these, 92 were positive i.e. 92 mothers transmitted CMV *in utero* as mentioned above. These CMV positive infants were enrolled in a secondary cohort set up to identify and study CMV transmission (M151161). Of the 92 infected children, consent for the secondary study was obtained for 62 infants, of whom 46 were confirmed as CMV infected cases (Pathirana and Madhi, unpublished data) (**Orange box, Figure 2.1**). Each of the 46 cases

were matched to 2 non-transmitting maternal controls for gestational age at birth, birth weight, gender and maternal HIV-1 infection status. (**Pink box, Figure 2.1**). Infants were not matched on date of birth, with enrolment of some controls born several months after the birth of the case.

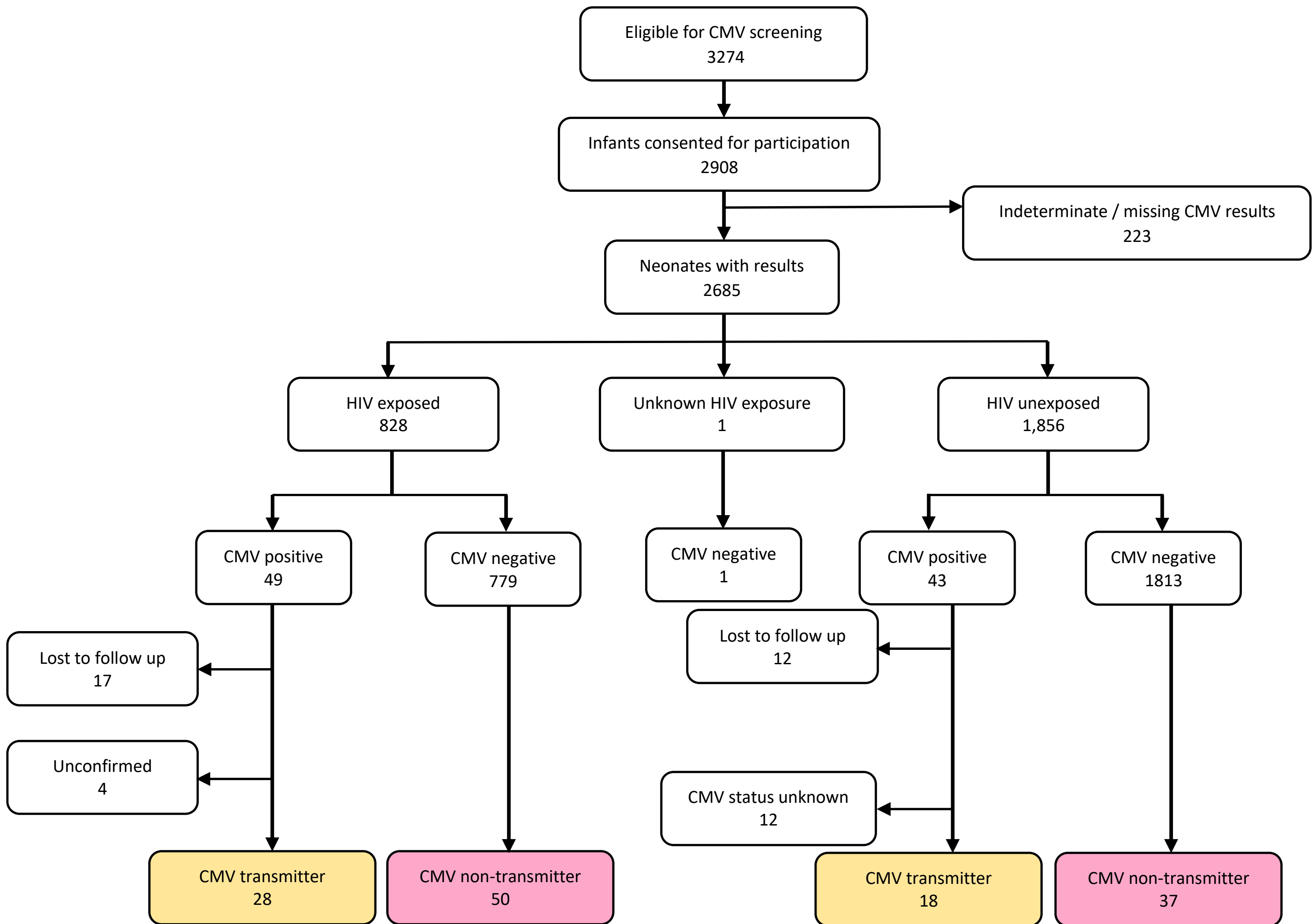


Figure 2.1: Schematic of participant screening and enrolment. Of the maternal sera, 46 mothers transmitted HCMV to their infants while 87 mothers did not transmit HCMV to their infants. (Schematic adapted from Jayani Pathirana, study period 6 May- 8 December 2016) (Pathirana *et al.*, 2019)

Of the 46 cases, 41 were tested for CMV-specific IgG and CMV-specific IgM antibody levels in the laboratory by Dr. Jeffrey Dorfman. All of the cases had high levels of CMV IgG and 38 out of 41 cases were CMV IgM negative, indicating that these 38 cases were non-primary infections while the other three cases were primary infections or recent re-infections with a new strain (data not shown). The remaining 5 cases were not tested and may thus be primary or non-primary infections. All samples, including IgM positive samples, were included in this study. Ethical approval for this study was obtained from the University of the Witwatersrand (M180617).

2.1.2 Control Samples

CMV negative and positive control samples for ELISAs were obtained from adult donors. A volume of 10 mL blood was collected from 25 individuals who are staff performing research at NICD or RMPRU, under Protocol M180617. These samples were tested for sero-positivity to CMV using the clinical ELISA assay described in section 2.6 below. CMV seronegative samples were used in our assays as negative controls, while CMV seropositive samples as positive controls in selected assays.

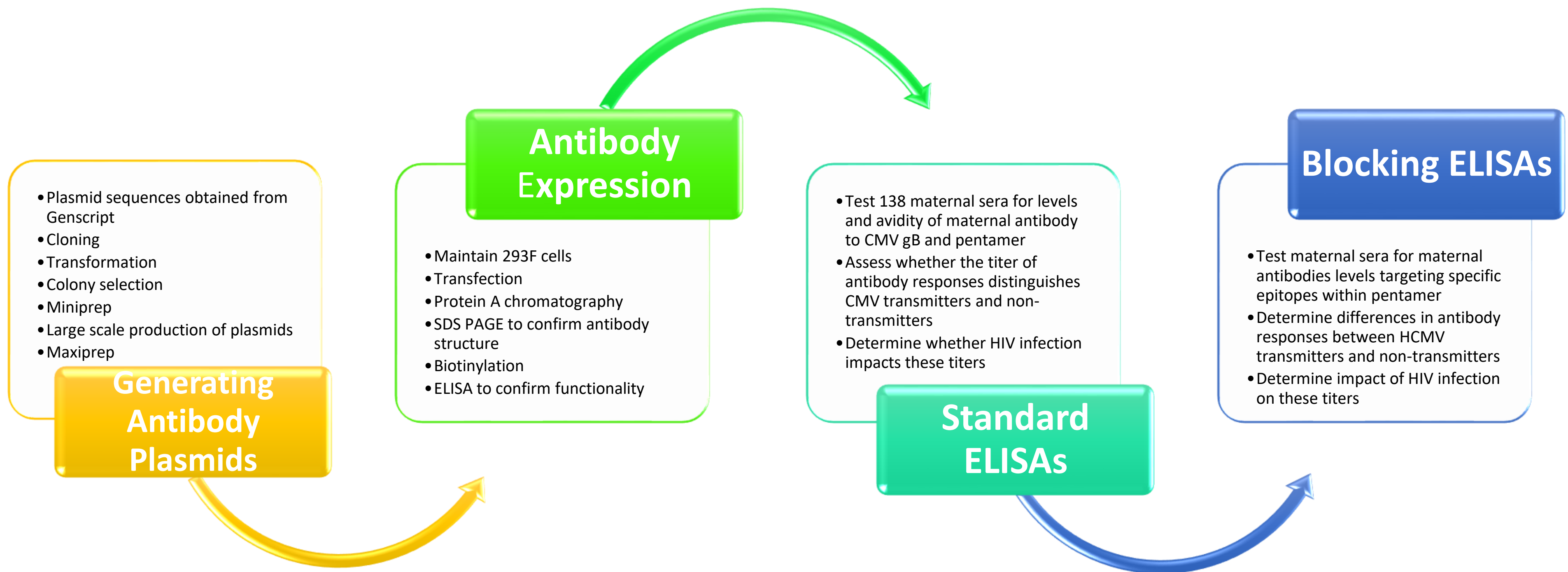


Figure 2.2: Flow diagram illustrating the approach taken in this study to synthesize monoclonal antibodies for standard and blocking ELISA experiments. Plasmid sequences were used to transform bacterial cells, which were subsequently miniprepped and sequenced. After sequence selection, large-scale production of the bacterial colonies was performed followed by maxiprep. The samples were then transfected in 293F cells to produce the desired antibodies, which were purified. A portion of the produced antibodies was biotinylated. Both biotinylated and unbiotinylated antibodies were used in downstream ELISA experiments.

2.2 SYNTHESIS AND SUB-CLONING OF MONOCLONAL ANTIBODY GENES

2.2.1 Cloning Monoclonal Antibody Plasmids

For this study, five monoclonal antibodies (mAbs), namely 11B12, 8I21, 10P3 (targeting pentamer), 10C6 and 6B4 (targeting gB), were expressed (**Figure 2.2**). The antibody heavy and light chain sequences for the five mAbs, previously isolated and described by Macagno *et al* (2010), were synthesized and cloned into a pUC57 shuttle vector by Genscript (USA).

Upon receipt, these sequences underwent a restriction endonuclease digestion (Thermo Scientific, USA) for one and half hours at 37 °C as per the manufacturer's instructions (**Table 2.1**), to excise the desired insert (300-400 bp) from the shuttle vector (2.7 kb).

Note: This procedure was performed by another scientist (Bronwen Lambson) in the laboratory for the pentamer mAbs, while I conducted this procedure for the gB mAbs.

Table 2.1: Restriction endonuclease digestion instructions

Reagent	Volume (µL)	Heavy chain	Light chain
Plasmid (1 µg)	8		
10x green buffer*	5		
Enzyme 1	1	AgeI (BshTI)	AgeI (BshTI)
Enzyme 2	1	Sal I	BsiWI (pfl23II) (Kappa) or Xho I (Lambda)
Water	35		
Total volume	**50**		

*10x restriction endonuclease digestion green buffer lot# 00650355.

The CMVR VRC03 Heavy Chain Expression Vectors (NIH AIDS Reagent Program, USA lot# 110161), containing the Kanamycin antibiotic gene, also underwent restriction endonuclease digestion (Thermo Scientific, USA) following the same protocol as above

(**Table 2.1**). Since nucleic acids have a uniform charge distribution, DNA is able to be separated and analysed using electrophoretic techniques. The digested heavy and light chain plasmids as well as the vectors were electrophoresed on a 1% agarose gel (Appendix 1). A volume of 4 µL Marker VIII ladder was used and 50 µL of the plasmids and vector were added to the wells. To isolate the desired intact DNA from the agarose gel after electrophoresis, a gel extraction technique was performed. The bands corresponding to the plasmids and vector were excised and purified using the QIAquick® Gel Extraction Kit protocol (Qiagen, Hilden, Germany) as per the manufacturer's instructions (Appendix 1).

2.2.2 DNA Ligation

The inserts were (ligated) sub-cloned into the backbone vector of CMVR VRC03 Heavy Chain Expression Vectors (NIH AIDS Reagent Program, USA cat# ARP-12037, lot# 110161) and CMVR VRC03 Light Chain Expression Vector (NIH AIDS Reagent Program, USA cat# ARP-12038) each containing the Kanamycin antibiotic gene. To achieve this, we followed a standard ligation reaction using the Rapid DNA Ligation Kit (Roche Applied Science, cat# 11635379001) as per the manufacturer's instructions (Appendix 1). In a sterile reaction vial, the vector and insert DNA were dissolved in a 1 x concentration of DNA Dilution Buffer (mixed thoroughly beforehand) (**Table 2.2**). The ligation reaction occurs at a ratio of 1 vector to 3 insert.

Table 2.2: Reaction vial mixture

Reagent	**Volume (µL)**
Insert	1
Vector	3
DNA Dilution Buffer	2
dH_2O	4
	10

The T4 DNA Ligation Buffer (labeled vial 1) was thoroughly mixed. Note that the contents of vial 1 should be mixed immediately before use. To the reaction vial, 10 µL T4 DNA Ligation Buffer was added and mixed thoroughly, after which 1 µL T4 DNA Ligase (Vial 3) was added and mixed. The reaction tube of each antibody chain (heavy and light) was incubated in a PCR machine for 30 minutes at 25 °C.

2.2.3 Transformation of Antibody Plasmid Constructs into JM109 Cells

The plasmids of the heavy and light chains of each antibody were transformed into *E. coli* JM109 cell suspensions. For each antibody construct, a volume of 2 µL of plasmid was added to 50 µL of JM109 Competent cells (Zymo Research, USA) that had been thawed on ice for 10 minutes. A volume of 500 µL of room temperature Super Optimal broth with Catabolite repression (S.O.C) outgrowth medium (Invitrogen, USA) was added to the cells to allow for bacterial growth in an Infors HT minitron incubation shaker (United Scientific, South Africa) at 37 °C, 150 r.p.m. for one hour. From each tube, 200 µL of the bacterial growth culture was plated onto pre-warmed Luria-Bertani (LB) agar plates (10 g/L casein enzymic hydrolysate, 5 g/L yeast extract, 10 g/L sodium chloride, pH 7.5±0.2 at 25 °C) containing the antibiotic Kanamycin (KAN). These plates were incubated overnight at 37 °C to allow for colony formation.

2.2.4 Transformation of XL10 Gold Ultracompetent Cells

When transformation of JM109 cells were not successful, XL10 gold ultracompetent cells (Agilent Technologies, USA) were used as they have a higher transformation efficiency. Transformation using these cells is as described in above in section 2.2.1, with 2 µL of previously thawed β-mercaptoethanol added to the cells, and followed by incubation on ice for 30 minutes, with frequent gentle mixing. After the addition of the plasmids, the cells were heat shocked at 42°C for 30 seconds and 450 µL room temperature S.O.C outgrowth media (Invitrogen, USA) was added. To allow for bacterial growth, the tubes were placed in an Infors HT minitron incubation shaker (United Scientific, South Africa)

at 37 °C, 150 r.p.m. for one hour. From the tubes, 50 µL of the bacterial outgrowth was plated onto pre-warmed LB/KAN agar plates and incubated overnight at 37°C.

2.2.5 Colony Selection

A total of four bacterial colonies for each plasmid were arbitrarily selected and picked using a pipette tip. Each of the colonies were grown overnight in 10 mL of LB containing the Kanamycin antibiotic (50 µg/mL) at 37 °C. The cells were then pelleted for 20 minutes at 6500 *g*. For rapid, small-scale isolation of plasmid DNA from the bacteria, the supernatant was removed and the plasmid DNA was miniprepped using the QIAprep® Spin Miniprep Kit protocol (Qiagen, Hilden, Germany) as per the manufacturer's instructions (Appendix 1). This minipreparation of the plasmid DNA is based on the alkaline lysis procedure. The DNA concentrations were determined using the Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific, USA) by measuring the absorbance at 260 nm.

2.2.6 Sequencing of plasmid inserts

Sequences of the plasmids were confirmed to assure that the correct antibody with the correct sequence is being produced. After determining the concentrations, 300 ng of DNA from each miniprep was used in a Sanger sequencing reaction, using 2 pmol/µL 1012 Forward primer (**Table 2.3**), to ensure that the plasmids contained correct antibody heavy and light chain sequences.

Table 2.3: Sanger sequencing reagents and cycle conditions

Reagents	×1 (µL)	Cycling: each cycle generates new sequencing product	
*BigDye™ Terminator v3.1 Ready Reaction Mix	1	1 cycle	
ABI buffer	1.5	96 °C	5 min
1012 Forward primer 2 pmol/µL	3	25 cycles	
DNA 300 ng	**X	96 °C	10 sec
Distilled water	Up to 4.5	50 °C	5 sec
Total Volume	10	60 °C	4 min
		4 °C	hold

* BigDye™ Terminator v3.1 Ready Reaction Mix (Pink mix) and ABI buffer supplied by BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Scientific, USA cat# 4337458)

** X used to denote variable volume

The sequencing products were cleaned up using the sodium acetate method. A fresh mix of 1 mL of 100% anhydrous ethanol (EtOH) and 40 µL sodium acetate (3 M NaAc) pH 4.6 was prepared. Into each of the sequencing reaction tubes, 50 µL of the EtOH/NaAc mix was resuspended by pipetting up and down. The tubes were then centrifuged for 30 minutes at 2000 *g*. Thereafter, tubes were inverted in a paper towel, centrifuged for one minute at 150 *g*, and then rinsed but not resuspended with 100 µL of ice-cold 70% ethanol. The tubes were further centrifuged for five minutes at 2000 *g* followed by inversion in a paper towel and centrifuged for 1 minute at 150 *g*. The PCR products were then sequenced using the 3500 Genetic Analyzer (Applied Biosystems Hitachi, Japan) and chromatograms analysed using the Sequencher 5.4.1 programme.

2.3 LARGE-SCALE PRODUCTION OF ANTIBODY PLASMIDS

After sequence verification, a single clone for each antibody was re-transformed on agar plates (50 µg / mL) made up of Luria-Bertani medium and kanamycin antibiotic (LB/KAN)

and grown overnight at 37 °C. A single colony from each plate was used to inoculate 3 mL of KAN broth starter culture and after 7 hours, 125 µL of cells were transferred to 120 mL KAN broth. These cultures were grown overnight at 37°C and the cultures were harvested by centrifugation for 15 minutes at 6500 *g*. This was followed by removal of the supernatant from each culture and maxiprep of the DNA using the QIAprep® Spin Maxiprep Kit protocol (Qiagen, Hilden, Germany) as per the manufacturer's instructions (Appendix 1).

To verify the sequence each of the heavy and light chain plasmids, 300 ng of DNA from each maxiprep was Sanger sequenced as described above.

2.4 EXPRESSION OF ANTI-CMV MONOCLONAL ANTIBODIES

2.4.1 Transfection of 293-F Cells

293-F mammalian suspension cells were cultured in FreeStyle™ 293 Expression Medium (Gibco, USA) without serum or antibiotics. Cells were passaged once a week. Since these cells double overnight, they were manually counted and passaged the day before transfection, by adding 20 µL of cells to 80 µL Trypan Blue dye and applying to the disposable hemocytometer. After counting, an appropriate volume of FreeStyle™ 293 Expression Medium (Gibco, USA) was added to the flask to achieve an overall of two million cells, ready for transfection.

Each cell culture had a total volume of 100 mL at a cell count of $2x10^6$ cells/mL (two million cells). The cell culture flasks were incubated at 37 °C.

Table 2.4: Template used for 100 mL transfection

mAb name	Plasmid	ng/µL	µg/µL	Flask number	Volume to add 50 µg of each
10P3	Heavy chain	4947.18	4.947	1	10.1
	Light chain	1738.02	1.738		28.8
					Total 100 µg DNA

3:1 ratio of PEI MAX to DNA. The mAb in grey is used as an example.

2.4.2 Purification of Monoclonal Antibodies

Seven days post-transfection, the antibody-rich supernatant was harvested, and cellular material and debris were largely removed by spinning down the cells at 2000 *g*, 4 °C for 20 minutes. The antibody-rich supernatant was then filtered using a steritop and stericup filter (Millipore Express, USA) and purified using Protein A affinity column chromatography (Thermo Scientific, USA; Appendix 1). This purification method relies on trapping the target onto the agarose-linked stationary phase, in this case by exploiting the binding of the Fc region of the mAbs to the protein A of the stationary phase. The mobile phase carrying all the remaining molecules that did not become trapped was discarded as the flow-through using PBS.

To each of the antibodies, in the stericup, 1 mL of Protein A bead solution was added. This was left to shake in the cold room overnight. The antibodies with the Protein A beads were then put through a column. At this stage the antibodies are captured onto the resin beads and the remaining liquid of the supernatant was allowed to pass through the column. The column, containing the antibody and resin, was washed twice with 1x PBS. The column was then plugged to prevent the elution buffer from running through into the waste collecting bucket. The column environment was made acidic by adding 7.2 mL of a 0.1 M glycine buffer pH, adjusted to 2.5 with 1 M hydrochloric acid, to elute the bound antibodies. A 50 mL falcon tube containing 800 µL of an alkaline 1M Trizma neutralization buffer, (pH adjusted to 8 with 1M hydrochloric acid) was placed under the plugged column. After removal of the plug, the antibodies were eluted into the falcon tube. The eluted antibodies were then transferred to a Vivaspin® protein concentrator (Sartorius stedim,

France), with a molecular weight cut off of 30 000. The eluted antibody was concentrated by centrifuging the protein concentrator tube at 2000 *g*, until approximately 1 mL was left in the concentrator. The concentrated antibodies were further centrifuged with 1x PBS twice for buffer exchange. The protein concentrations were determined using the Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific, USA) measuring at 280 nm. The concentration of each antibody was adjusted to approximately 1 mg/mL using 1x PBS.

2.5 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS ANALYSIS OF PURIFIED CMV-SPECIFIC MONOCLONAL ANTIBODIES

To confirm expression of the antibody heavy and light chains, and to assess the purity, each expressed mAb was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This technique makes use of the negative charge of the detergent SDS, which denatures the secondary, tertiary and quaternary structures of proteins. This enables separation of the negatively charged molecules through an acrylamide matrix based on their molecular weight, as they migrate towards a positively charged electrode.

2.5.1 Gel Preparation

This technique requires the formation of two gels, a “resolving gel”, which separates proteins according to their size, topped by a “stacking gel”, which stacks proteins at the same level before they enter the resolving gel. Before preparing the resolving gel, the glass casting apparatus was cleaned and assembled, ensuring that the bottom of the glass cast was completely covered to exclude air. A 12% acrylamide resolving gel was prepared by combining the reagents as per the manufacturer’s instructions (Invitrogen NuPAGEstruc Sigma Aldrich, St Louis, USA) (**Table 2.5**). The 10% Ammonium persulphate (APS; Sigma, SA lot# MKBK1247V) solution was prepared fresh before being added and the Tetramethylethylene-diamine (TEMED; Sigma, SA lot# 112K0586) was added last as it catalyzes the polymerization of the gel.

Table 2.5: Reagents used to prepare 15 mL of the 12% resolving gel

Reagent	**Volume (mL) of a 12% gel**
Deionized H_2O	6.5
40 % Acrylamide (Sigma, SA lot# 037K6053)	4.5
1.5 M Tris HCl pH 8.8	3.8
10% SDS	0.15
10% APS	0.15
TEMED	0.015

The resolving gel solution was carefully poured into the glass cast (leaving approximately 1.5 cm at the top for the stacking gel) using a 5 mL serological pipette, followed by absolute isopropanol to exclude air, which would prevent the resolving gel from setting was excluded. Additionally, the isopropanol ensured that the gel was level and uniform. The resolving gel was allowed to set for 30 minutes before the stacking gel was prepared.

The stacking gel contains less acrylamide, and is used to allow proteins to compact together into one band prior to entering the resolving gel for separation. The stacking gel was prepared by combining reagents as per the manufacturer's instructions using the recipe below (**Table 2.6**).

Table 2.6: Reagents used to prepare the stacking gel

Reagent	**Volume (mL) of a 12% gel**
Deionized H_2O	6.2
40 % Acrylamide	1.2
0.5 M Tris HCl pH 6.8	2.4
10% SDS	0.10
10% APS	0.10
TEMED	0.010

After the setting of the resolving gel, the isopropanol was removed carefully and the top of the cast was dabbed with a paper towel to ensure no residual isopropanol was left. Any isopropanol that did not evaporate would interrupt the continuous layer of the two gels. The stacking gel was then poured over the resolving gel, and a 1 mm × 10 comb inserted into the stacking gel for the formation of wells, and the gel allowed to set for 30 minutes.

2.5.2 Antibody Sample Preparation

Approximately 10 to 15 µg of protein is required for a distinct band, thus, 15 to 23 µL of each antibody, depending on the concentration, was aliquoted into a 2 mL micro tube (Sarstedt, Germany lot# 5082711). The antibodies were exposed to reducing conditions to break the disulphide bonds holding the heavy and light chains together, allowing the chains to run separately through the resolving gel. This was done by adding 1 µL of reducing agent (Invitrogen 10×) and 1 µL of 1 M Dithiothreitol to each sample. Furthermore, 4 µL of NuPAGE® LDS Sample Buffer (4×) loading dye (Novex, USA Lot# 1621180) was added to each sample to allow for easier identification of the samples when loaded onto the gel. The glycerol present in the loading dye allowed the samples to settle into the well. Each sample was incubated at 95°C for approximately five minutes to denature proteins, after which samples were spun down at 1500 r.p.m. for a few seconds to collect any evaporated droplets in the tube lids. In addition to each monoclonal antibody sample, a Novex® Sharp Pre-stained Protein Standard ladder (Invitrogen, USA lot# 1767882) was added into the first well to size the mAb chain bands, with sizes ranging from 260 kDa 3.5 kDa.

2.5.3 Assembly of Gel Apparatus and Protein Sample Loading

While the gel was still in its casting glass, the buffer chamber (reservoir) was filled with 1 L of 1× SDS Running Buffer, diluted 10-fold using distilled water from a 10× SDS Running Buffer stock prepared as per the recipe below (**table 2.7**).

Table 2.7: Reagents used to prepare a 10× protein running buffer

Reagent	Mass/Volume
SDS	10 g
Tris	30.3 g
Glycerine	144.1 g
dH_20	800 mL

After, loading of samples, the gel was electrophoresed for 15 minutes at 100 V constant voltage to allow the mAbs to stack before entering the resolving gel. Thereafter, the gel was run at 200 V constant voltage for 45 minutes.

2.5.4 Staining and De-Staining the Gel

Gels were removed from the casting apparatus and washed several times in distilled water to remove any salts present from the running buffer. Thereafter gels were stained by incubation with coomassie brilliant blue protein safe stain (TaKaRa, USA lot# AH70830A) for approximately 1 hour. The gel was gently agitated in the stain during incubation for constant mixing of the stain. Distilled water was then used to wash off unbound residual stain. The gel was further incubated in water and gently agitated overnight. Lastly, the gel was photographed and the band sizes were determined and recorded.

2.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

2.6.1 Preparation of Wash and Blocking Buffers

Monoclonal antibodies were assayed against commercially acquired CMV proteins to confirm their functionality. The ELISA wash buffer (PBS/Tween20) was prepared by adding 0.05% Tween20 and 10× PBS (lab stocks) to deionized H_20 as per the recipe below (**Table 2.8**).

Table 2.8: Wash buffer recipe used for ELISA

Reagents	Wash buffer
Deionized H_2O	900 mL
Tween 20 (0.05%)	0.5 mL
10× PBS	100 mL

The blocking (dilution) buffer, used for diluting both primary and secondary antibodies as well as blocking the ELISA plate after coating, was prepared by adding 5% of low fat liquid skim milk to PBS/Tween20 as per the recipe below (**Table 2.9**).

Table 2.9: Blocking buffer recipe used for ELISA

Reagents	Blocking buffer
PBS/Tween20 (wash buffer)	100 mL
Low fat powdered skim milk (5%)	5 g

2.6.2 ELISA Antigens

Two commercially acquired CMV antigens, gB (Sinobiological, China lot# AA45920.1), and pentamer (Native Antigen Company, UK lot# 17082309) were used to coat the Costar® 3590 96-well EIA/RIA (ELISA) plates (Corning Incorporated, USA) at 1.25ug/ml (pentamer) or at 0.625ug/mL (gB). Plates were incubated overnight at 4°C.

2.6.3 ELISA Assay

Following coating, the ELISA plate was washed 4 times with wash buffer, using the automated ELx405 plate washer, to remove any unbound antigen (gB or pentamer). Subsequently 200 µL of blocking buffer (containing 5% low fat skim milk) was added for 1 hr at 37°C to prevent any non-specific binding to the ELISA micro-titer well, improving assay sensitivity by reducing background interference, and improving the signal-to-noise

ratio. The ELISA plate was then washed 4 times to remove the blocking buffer. Each mAb was diluted to 10 µg/mL using blocking buffer, added to the relevant wells and incubated for a further 1 hr at 37°C. The ELISA plate was then washed 4 times to remove any unbound primary antibody. The secondary antibody, anti-human IgG (Fc specific) Peroxidase Ab produced in goat (Sigma Aldrich, USA lot# 116M4809V), was diluted to 1 in 3,000 in blocking buffer, added to each well at 100 µL and incubated for 1 hr at 37°C. Subsequently, the ELISA plate was washed four times and the 1-step™ Ultra TMB-ELISA substrate (Thermo Scientific, USA lot# RF22249510) was added at 100 µL for 5 minutes, allowing any positive reactions to turn blue. As the substrate solution is light sensitive, extra care was taken at this step to sure that light was blocked using aluminium foil. This was followed by the addition of 25 µL of 1M H_2SO_4 stop solution, which converted all the positive (blue) wells to yellow. The ELISA plate was analysed using the spectrophotometer at 450 nm. The labeled-mAbs were used in the downstream blocking ELISAs at 0.3 µg/mL and samples were repeated if the duplicate extinction values were greater than 15%.

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2.6.2 Statistical Analysis

The Mann Whitney test was used to compare the antibody levels to glycoprotein B and pentameric complex present in maternal serum samples of transmitting and non-transmitting mothers. GraphPad® Prism (GraphPad Software, La Jolla, USA) software was used for the analysis.

2.7 BIOTINYLATION OF MONOCLONAL ANTIBODIES

2.7.1 Labeling the Monoclonal Antibodies

Each mAb was biotinylated for the blocking ELISAs (described in section 2.8 below). This was achieved by dissolving 2.2 mg of 10 mM Sulfo-NHS-LC-Biotin in 400 µL ultrapure water. Of the freshly prepared biotin solution, 27 µL was added to 1 mg of each mAb (1.3-1.7 mL) in and incubated in the fridge overnight. Subsequently, buffer exchange and removal of unreacted biotin reagent was conducted using desalting columns. The desalting columns were each placed into 15 mL collection tubes which were centrifuged

at 1000 g for 2 minutes. Columns were equilibrated with 2.5 mL of PBS added to the top of the resin bed followed by centrifugation at 1000 g for 2 minutes. The flow-through was discarded and this step was repeated 3 times. The columns were then placed in new 15 mL collection tubes and the newly biotinylated mAbs in the original reaction mix were applied directly onto the center of the resin bed. The mAbs were allowed to absorb into the resin for 5 minutes followed by centrifugation at 1000 g for 2 minutes. The collected flow-through solution contained purified biotinylated mAbs. These biotinylated mAbs are henceforward referred to as labeled-mAbs.

2.7.2 Confirming Biotin Incorporation by ELISA

Following a similar procedure as described in section 2.6, gB (Sinobiological, China), or pentamer (Native Antigen Company, UK) were used to coat the high binding Costar® 3590 96-well EIA/RIA (ELISA) plates (Corning Incorporated, USA) at a starting concentration of 2.5 µg/mL or 1.25 µg/mL, respectively. This was incubated overnight at 4°C, then the ELISA plate was washed 4 times with wash buffer. A volume of 200 µL of blocking buffer (containing 5% low fat skim milk) was added to the ELISA micro-titer wells and incubated for 1 hr at 37°C. The ELISA plate was then washed 4 times to remove the dilution (blocking) buffer. Each labeled-mAb was diluted to 10 µg/mL in dilution buffer, added to the relevant wells and incubated for 1 hr at 37°C. The ELISA plate was then washed 4 times to remove any unbound labeled-mAb. The anti-biotin secondary antibody (Pierce™ Biotin (HRP) Goat Polyclonal Antibody, Thermo Scientific lot# OB16682412) was diluted to 1 in 1000 in dilution buffer, added to each well at 100 µL and incubated for 1 hr at 37°C. Subsequently, the ELISA plate was washed 4 times and the 1-step™ Ultra TMB-ELISA substrate (Thermo Scientific, USA lot# RF22249510) was added at 100 µL for 5 minutes, allowing any positive reactions to turn blue. This was followed by the addition of 25 µL of 1M H_2SO_4 stop solution. The ELISA plate was analysed using the spectrophotometer at 450 nm.

2.8 BLOCKING ELISA EXPERIMENTS

To measure antibody level to epitopes on pentamer and gB, blocking ELISA experiments were conducted. These ELISA experiments were conducted similarly to that of the ELISAs described in section 2.6 above, with one additional step. The Costar® 3590 96-well EIA/RIA (ELISA) plates (Corning Incorporated, USA) were coated overnight with either pentamer (Native Antigen Company, UK) at 1.25µg/ml or gB (Sinobiological, China) at 0.625 µg/mL. As in section 2.6, the ELISA plates were washed with washing buffer and blocking buffer was added for an hour to block any spaces not coated by the antigens. Following another washing step, the maternal sera were added as 4-fold dilutions in blocking buffer starting at 1 in 10. This was incubated for an hour after which the plates were washed and 0.3 µg/mL of labeled-mAb in blocking buffer was added for an hour (the additional step). After another washing step, a 1 in 5000 dilution of Pierce™ anti-biotin HRP (Thermo Scientific, USA, lot# OB16682412) was added to detect any labeled-mAbs attached to the coated plate. After the final washing step, the substrate and stop solutions were added (as in section 2.6) and the ELISA plates were analysed using the spectrophotometer at 450 nm.

If there are large amounts of antibodies which bind to the same site as the mAb, or very close by in the maternal sera, then these serum antibodies will block most of the antigenic sites. The labeled-mAbs with have little to no sites to bind, which in turn means little to no binding of the Pierce™ anti-biotin HRP to the labeled-mAbs. This results in a low intensity colour being produced since there are fewer Pierce™ anti-biotin HRP molecules to detect. The converse is also true. Small amounts of serum antibodies specific for the site of interest (defined by the biotinylated mAb employed) in the maternal sera translates to most antigenic sites available to the biotin labeled-mAbs, which ultimately results in a high intensity colour being produced (**Figure 2.3**). Maternal samples were repeated if the difference in extinction values between duplicate samples were greater than 15%.

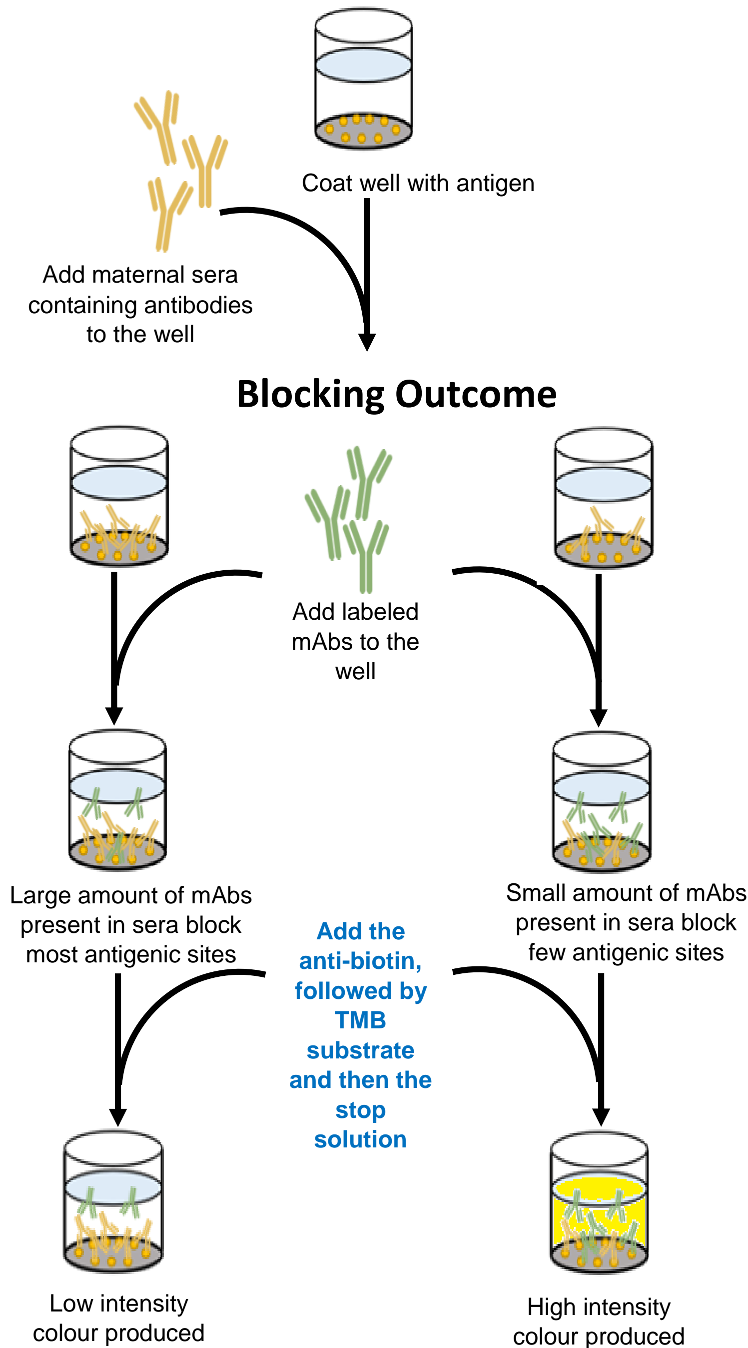


Figure 2.3: Schematic of a blocking ELISA. The orange circles represent the antigen (gB or pentamer), the yellow antibody structures represents the maternal antibodies present in maternal sera and the green antibody structures represent biotinylated (labeled) antibodies. Anti-biotin is added and bound to the biotin molecules attached to the labeled antibodies. The addition of TMB substrate allows for a colour change from a clear solution to blue. The degree of colour intensity is depended on the amount of bound labeled antibody is bound to the antigen. The addition of an acid (stop solution) results in a colour change from blue to yellow, and the intensity of the yellow colour is measured by spectrophotometry.

CHAPTER 3: RESULTS

The main objective of this study was to explore humoral immune correlates of protection from mother-to-child HCMV transmission. To achieve this, we set out to fulfill two main aims. The first aim sought to assess a difference in key anti-CMV immune responses, using maternal sera, between mothers who transmit HCMV to the foetus (transmitters) versus mother who do not transmit HCMV to the foetus (non-transmitters). Additionally, we tried to determine whether or not a difference exists between HIV-positive and negative mothers because previous data (summarized in the introduction) has shown that maternal HIV-1 infection is associated with an increased risk of HCMV transmission, which enhancing the risk of cCMV infection (Ellington, Clarke and Kourtis, 2016; Reitter *et al.*, 2016).

3.1 OPTIMIZATION FOR ALL ELISA TYPES: COATING CONCENTRATION OF PENTAMER AND GLYCOPROTEIN B ANTIGENS

We sought to test levels of antibodies recognizing HCMV glycoprotein B (containing a polyhistidine tag; gB-His) and the pentameric UL128-UL131A/gH/gL complex (pentamer) (Ryckman *et al.*, 2008; Fouts *et al.*, 2012). We tested levels of antibodies targeting these antigens, and separately measured levels of antibodies targeting specific epitopes within these two antigens- gB-His and pentamer. Both gB and pentamer antigens are involved in HCMV entry into cells and are the targets of neutralizing antibodies (Ryckman *et al.*, 2008; Macagno *et al.*, 2010).

To measure both total and epitope-specific maternal antibody levels to HCMV gB-His and pentamer by means of ELISA assays, the ELISA conditions required optimization. We first optimized the coating conditions for these two target antigens. More specifically, the concentration of the antigen coated on the wells of the 96-well ELISA plates as well as the buffer used to produce the optimal coating concentration were investigated. The coating concentration was determined by titrating the antigens from 2.5 down to 0.078 µg/mL in phosphate buffered saline (PBS) (**Figure 3.1**). Two HCMV immune sera (positive control samples) and one HCMV non-immune serum (negative control), provided as laboratory stocks, were added at dilutions 1 in 900 for pentamer and 1 in 2000 for gB-His. These HCMV immune and non-immune sera are consistently used as

controls in downstream experiments and are simply referred to as positive and negative controls, respectively.

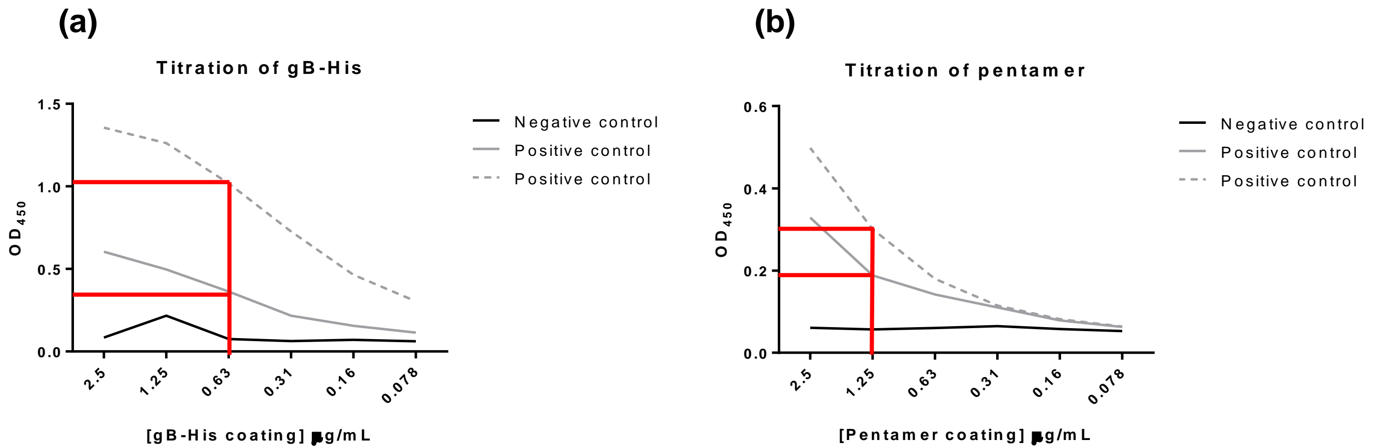


Figure 3.1: Determining coating concentration of antigens for ELISA experiments. The antigens glycoprotein B (containing a polyhistidine-tag) **(a)** and pentamer **(b)** were titrated down 2-fold from 2.5ug/mL. The positive (grey solid and dashed) and negative (black) sera were added at dilutions 1 in 900 for pentamer and 1 in 2000 for glycoprotein B. The optimal coating concentration, indicated by the red lines, were 0.625 µg/mL for glycoprotein B coating **(a)** and 1.25 µg/mL for pentamer coating **(b)**, based on the OD_{450} readings. Note that there are no patient ID numbers for the control sera, thus are referred to simply as “positive control” or “negative control”.

We looked for the window between the negative and positive sample to find the optimal coating concentration for pentamer and gB-His. This data shows that, based on the OD_{450} readings, the optimal coating concentration for pentamer and gB-His are 1.25 µg/mL and 0.625 µg/mL, respectively, in PBS. These selected coating concentrations allowed us to effectively coat each well of the 96-well ELISA plates while using the lowest amount of antigen that would permit the ELISAs to function optimally.

To further optimize the coating concentration, we compared PBS coating to carbonate buffer pH 9.6 coating. Since adsorption occurs through passive hydrophobic interactions

between amino acid side chains and the plastic of the wells, which are influenced by pH, we selected the carbonate buffer pH 9.6 to see if we could achieve better coating and stabilization of the antigens. One positive and one negative control (sera) were tested against pentamer coated at 1.25 µg/mL in PBS as well as in carbonate buffer pH 9.6. Additionally, four randomly selected maternal serum samples as 5-fold dilutions were also tested (**Figure A1 in Appendix 2**). The OD_{450} values of all four maternal sera as well as the positive control were higher when pentamer was coated using carbonate buffer. There was a slight increase in the background with the negative control serum, but, the window between the positive and negative control sera appeared larger. Based on this data, 1.25 µg/mL of pentamer and 0.625 µg/mL of gB-His prepared with carbonate buffer pH 9.6 were used in all subsequent ELISA experiments.

3.2 MEASURING TOTAL ANTIGEN-SPECIFIC ANTIBODY LEVELS TO GLYCOPROTEIN B AND PENTAMER

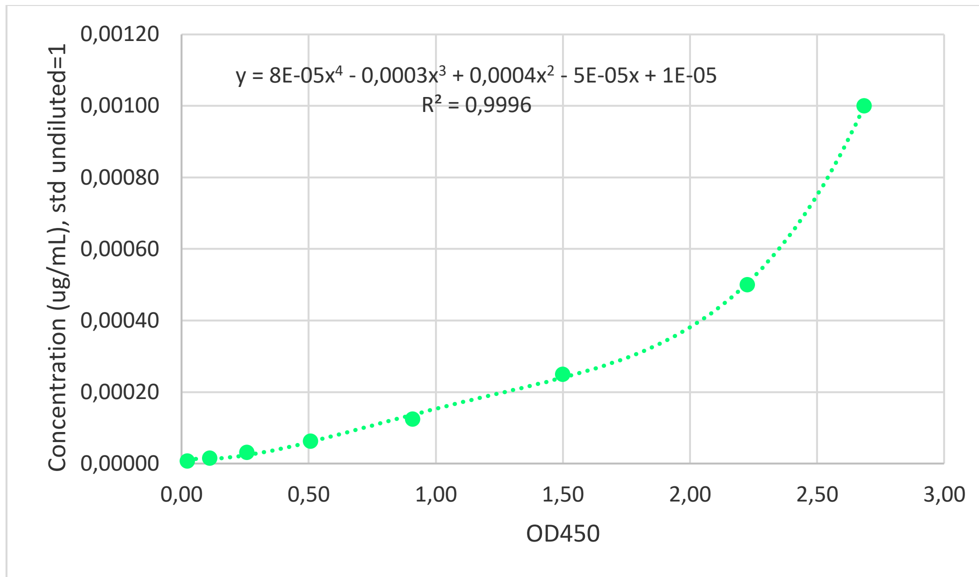
3.2.1 Determination of Antibody Levels for Total Antigen-Specific Antibody (gB-His and Pentamer)

A standard sample 45660, arbitrary chosen to have 1 unit per mL of mAb, was diluted 2-fold from 1 in 250 for pentamer and 1 in 1000 for gB-His and included in all the experiments. This standard sample allowed us to plot a graph that generated an equation (polynomial equation order 4). The definition of 1 unit of antibody was such that sample 45660 had 1 unit/ml of antibody specific for gB and 1 unit of antibody/ml of antibody specific for pentamer. Note that the arbitrary units for antibodies to gB and to pentamer are thus unrelated. Thus, the only relationship between the unit measures is that 1 unit/ml corresponds to the level found in sample 45660 for each antigen. The OD_{450} readings of each sample (described below) was inputted as x values into the polynomial equation. Next, these values (the y values) were multiplied by the dilution factor, allowing us to determine a level for each sample.

3.2.2 Establishing a Negative Threshold Value for Total Antigen-Specific Antibody (gB and Pentamer)

After determining both the coating concentrations for gB-His and pentamer as well as the standard sample 45660, we sought to establish a negative threshold value below which samples would be presumed to be negative i.e. indicating whether or not a maternal sample has detectable anti-HCMV mAbs to pentamer and gB-His. We defined positive as significantly different from a set of sera from individuals who were unexposed to HCMV. To obtain CMV unexposed samples, we recruited 25 participants of varying age and gender, and screened them for prior CMV exposure using ta commercial CMV IgG kit (Euroimmun catalogue # EI 2570-9601 G). This test kit provides a semi-quantitative *in vitro* assay for human IgG Abs in serum (or plasma) for diagnosing prior HCMV infection.

We determined six of the participant samples out of the 25 study participants we recruited were negative for HCMV antibodies using this assay kit and are presumed to have been unexposed. We also had laboratory stocks of two previously identified HCMV negative samples. We thus had this set of eight known negative samples. These were used in an ELISA for pentamer and gB-His to determine the negative threshold value. Included in the experiment were two maternal sera used as positive controls, 48046 and 49495, with high and low OD_{450} responses, respectively. The standard sample 45660 was diluted 2-fold from 1 in 250 for pentamer and 1 in 1000 for gB-His, as mentioned above, and included in all the experiments (**Figure A2 in Appendix 2**). The OD_{450} readings of each sample (x values) was inputted into the polynomial equation to generate the corresponding y values. These were multiplied by the dilution factor, allowing us to determine the concentration value for each of the eight samples (**Figure 3.2** and **Table 3.1** shows relevant calculation for anti-gB as an example; calculation of anti-pentamer not shown). The threshold values were determined by taking an average of the calculated antibody level plus two standard deviations. We expected just over 95% of the real negatives to be within 2 standard deviations of the mean if they are distributed normally. Thus, any sample that falls out of that range is unlikely to be a true negative sample.



OD_{450} value from a known negative sample:

Sample code	OD_{450} value
04140219	0.0207

Substitute the OD_{450} values as the x values:

$y = 8E\text{-}05x^4 - 0.0003x^3 + 0.0004x^2 - 5E\text{-}05x + 1E\text{-}05$

$y = 8E\text{-}05(0.1174)^4 - 0.0003(0.1174)^3 + 0.0004(0.1174)^2 - 5E\text{-}05(0.1174) + 1E\text{-}05$

y = 0.000013

Multiply by the dilution factor (x 1500) to get the antibody level

y = 0.000014 x 1500

y = **0.0207**

(These are all the values seen in column 4 of Table 3.1)

(Note: Antibody levels for samples 10140219 and J were calculated using a slightly

Figure 3.2: Sample 45660 used to generate a standard graph. Maternal serum sample 45660 was used as the standard in when testing maternal sera against pentamer and gB. Sample 45660 was used to generate a standard curve by plotting the OD_{450} values as the x-values and the dilution factor as the y-values. A Polynomial equation (order 4) was generated (top). The OD_{450} values of each maternal sample was inputted into the equation (bottom green square) to determine an antibody level (arbitrary units).

Table 3.1: Determination of the concentration values of each known negative sample tested against gB-His

Sample code	OD_{450} value	Antibody level (arbitrary units)*	Antibody level multiplied by dilution factor of 1500	Average of the eight values in column 4	The value of two standard deviations	Threshold value (average plus two standard deviations)
04140219	0.1174	0.000013	0.0207	0.019	0.0166	0.0353
06140219	0.0489	0.000016	0.0177			
10140219	0.02615	0.000018	0.0268			
14140219	0.11205	0.000009	0.0135			
19140219	0.02945	0.000009	0.0133			
23140219	0.000650	0.000010	0.0150			
S	0.0566	0.000016	0.0152			
J	0.02705	0.000018	0.0267			

* OD_{450} value inputted as x values in Polynomial equation (as shown in figure 3.2)

The negative threshold values derived from this method (in arbitrary units) were 0.0054 and 0.035 against pentamer and gB-His, respectively. Any maternal samples that produced a reading at or below 0.0054 for pentamer and 0.035 for gB-His were regarded as a negative sample.

3.2.3 Determining Total Antigen-Specific Antibody Levels in Maternal Sera

We next sought to determine the concentration of Abs in the maternal sera against both pentamer and gB-His. Each of the 137 maternal serum samples received were tested in two 5-fold dilutions against pentamer (1:500 and 1:2500) as well as against gB-His (1:1500 and 1:7500). Included in each ELISA experiment was a negative control (HCMV non-immune serum) and a standard sample 45660 diluted 2-fold sample 45660, as above from 1 in 1000 to 1 in 128000. We determined the Ab level similarly to how we determined the negative threshold values above: We plotted a standard graph to get the equation. The OD_{450} readings were inputted as x values into the equation and by multiplying by the

dilution factor, we determined the antibody level for each of the maternal samples, using the sample 45660 from the standard curve, setting its antibody level undiluted to be 1 arbitrary unit for each antigen (see above for fuller explanation) (**Table A1 in Appendix 2**).

3.2.4 Relationship between Total Antibody Levels and Transmitter/Non-Transmitter Status

From the determined concentration values, we compared the values from each case (transmitting mothers) to the values from the controls [non-transmitting mothers of their cCMV-negative controls matched for maternal HIV-1 status, child gender and gestational age at birth (±2wks)] (Pathirana *et al.*, 2019). We utilized a mixed effects linear regression model for statistical analysis. This statistical modal comprises both fixed effects and random effects. Essentially the model looks for one fixed effect, in our case the difference between cases (transmitters) and controls (non-transmitters) and a random effect, which allows each set of one transmitter with its grouped non-transmitters to be different from other sets. The model effectively calculates how different each case is from its own controls for all samples and then uses a random effects component as a way to systematically adjust for the differences between groups – where a group is one case ant its controls. This will tell us if cases are consistently different from controls and if so, by how much.

This model fitted our data best since we are comparing mothers of congenital CMV cases to the mothers of their cCMV-negative controls. Additionally, we did not have the same number of controls for each case so using another statistical test such as a paired t test, which only matches 1 to 1, would mean that for some of our other statistical options we would have to randomly dump one of the controls and unnecessarily lose statistical power.

Using the mixed effects model allowed us to compare the concentration values and determine if there is a significant difference in the levels of maternal Abs between transmitting mothers and non-transmitting mothers (**Figure 3.3**).

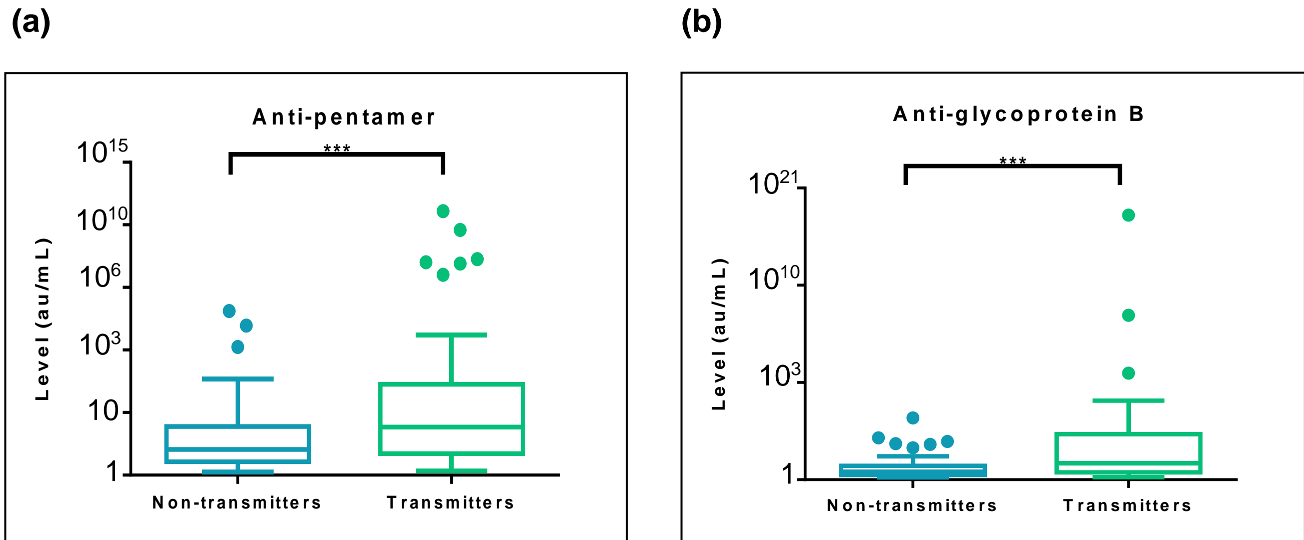


Figure 3.3: Transmitters versus non-transmitters against pentamer and glycoprotein B. The 45 mothers that transmitted HCMV to their infants are referred to as transmitters (turquoise) while 78 mothers that did not transmit HCMV to their infants are non-transmitters (blue). A significant difference in the level of maternal antibodies was seen when the samples were tested against **(a)** pentamer (P value summary: ***; P ≤ 0.001) and against **(b)** glycoprotein B-His (P value summary: ***; P ≤ 0.001). Note that the data points are within the boxes and the “outliers” are shown as dots

From this data we observed a significant difference in maternal Ab levels between cases (transmitters) and controls (non-transmitters). More specifically, on average there was 1.75 times the levels of anti-pentamer Ab in transmitters than non-transmitters (95% CI 1.28-2.40; p=0.0005). Likewise, on average there was 1.71 times the levels of anti-gB Ab in transmitters than non-transmitters (95% CI 1.34-2.36; p=0.0010), as seen in **Figure 3.3**.

Table 3.2: Fold difference in antibody levels, transmitters/non-transmitters

Monoclonal antibodies	P value	Fold
Anti-pentamer mAbs	0.0005	1.75 (95% CI 1.28-2.40)
Anti-gB mAbs	0.001	1.71 (95% CI 1.24-2.36)

3.3 EPITOPE-SPECIFIC ELISA SETUP I: PRODUCTION AND TESTING OF ANTI-PENTAMER MONOCLONAL ANTIBODIES

3.3.1 Anti-Pentamer Antibody Selection and Transfection

Our second aim was to explore possible differences in antibody levels targeting epitopes defined by mAbs such as that done by Lilleri *et al* (2013) and Macagno *et al* (2010). We chose three epitopes from the HCMV pentamer on the basis that these three seemed most strongly associated with transmitter status in a study of mothers with primary HCMV infection. We also chose two epitopes on glycoprotein B for comparison.

We selected a total of five mAbs (10P3, 11B12, 8I21, 10C6, 6B4) based on information provided by Macagno *et al* (2010), with each mAb targeting a different epitope of interest. (**Table 3.3**). For ease of understanding, I show the analysis of the anti-pentamer (section 3.3.2) and anti-gB mAbs (section 3.3.6) separately.

Table 3.3: Characterization of antibodies for epitope-specific ELISAs

mAb name	Ig Isotype	Target
10P3	IgG3, λ	UL130, UL131A (Part of pentamer)
11B12	IgG1, κ	gH (Part of pentamer)
8I21	IgG1, κ	gH, gL, UL128, UL130 (Part of pentamer)
10C6	IgG3, λ	gB
6B4	IgG1, κ	gB

Antibodies are colour-coded throughout the study for ease of identification (10P3 in green, 11B12 in blue, 8I21 in orange, 10C6 in red and 6B4 in purple).

Plasmids encoding the antibody heavy and light chain sequences for the anti-pentamer mAbs were synthesized by Genscript (USA). The sequences were then sub-cloned into CMVR heavy chain expression vectors ((Sacks *et al.*, 2019) by first digesting the CMVR vector and pUC57 plasmids (provided by Genscript) containing the mAb sequences, separately. Next the digested plasmids and vector were run on a 1% agarose gel and cut out (data not shown). The insert and vector were then ligated together. Sequencing was

conducted to confirm correct production of the mAb sequences, which in turn translates to the correct amino acid sequences (**Figure 3.4**). The amino acid sequences indicate the three complementarity-determining regions (CDRs) as well as the four framework regions. These framework regions provide the scaffold holding the CDRs in place to come into contact with the antigen (pentamer or gB-His in our case). The CDRs consist of a high ratio of different amino acid residues in a given position (Janeway *et al.*, 2001).

The sequences below allow us to determine the variability between the published sequences of our mAbs in the CDRs of the heavy and light chains. While there is some variability in the CDRH1 between all the mAbs, more variability exists in CDRH2 and CDRH3. Most of the variability in the light chain between mAbs is in CDRL1 and CDRL3.

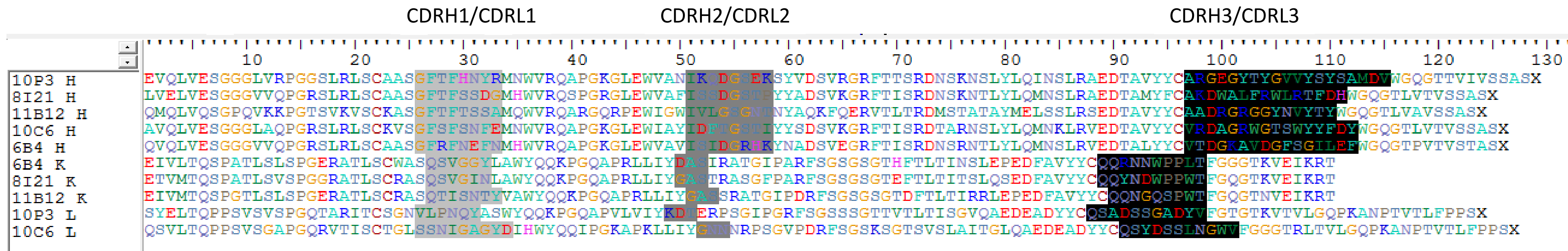


Figure 3.4: Amino acid sequence of the variable regions of HCMV monoclonal antibodies. The mAb heavy chain sequences listed first are designated H while the light chain sequences listed second are designated L (lambda chain) or K (kappa chain). The complementarity-determining regions 1 on the heavy and light chains (CDRH1/CDRL1) are shown in light grey, CDRH2/CDRL2 are shown in dark grey and CDRH3/CDRL3 are shown in black. The four framework regions are located between the three CDRs. The constant regions are not shown.

3.3.2 Purification of Anti-Pentamer Antibodies

We transfected of 293-F mammalian suspension cells with the heavy and light chains of each antibody and purified antibody from the cell supernatant with Protein A chromatography. The concentration of each antibody was determined by absorbance at 260 nm and adjusted using PBS (**Table 3.4**). This allowed for the determination of protein yield, which is indicative of successful protein expression. Additionally, a rough approximation of the purity of the expressed antibody was indicated by the A260/A280 absorbance ratio. A ratio value of 0.57 is indicative of a high purity antibody sample (Glasel, 1995, Sambrook and Russell, 2001) and in this study, the ratio values of the mAbs were between 0.52 and 0.56. We obtained approximately 15 mg of 11B12, 2 mg of 8I21 and 1 mg of 10P3.

Table 3.4: Yield of anti-pentamer monoclonal antibodies

Antibody	**Antibody target**	**Final concentration (mg/mL)**	**A260/A280**	**Volume (mL)**	**Yield (mg)**
10P3	UL130, UL131A	1.10	0.52	1.29	1.42
11B12	gH	0.67	0.56	23.0	15.4
8I21	gH, gL, UL128, UL130	0.66	0.54	2.40	1.58

The values in this table are calculated as the average of three readings taken.

3.3.3 SDS-PAGE Characterization of Anti-Pentamer Antibodies

The mAbs were electrophoresed on a reducing SDS-PAGE, along with an anti-HIV mAb (**Figure 3.5**). The DH511 anti-HIV mAb, previously expressed in the laboratory, was used as a comparison between all the mAbs as well as to ensure that the SDS PAGE was made correctly by comparing this gel to another gel that DH511was run on. This was to confirm the expression and purity of both heavy and light chains, which can be identified by the size of their protein bands. The reducing agent enabled size estimation of the polypeptides of expressed proteins. It is expected that the heavy chains for antibodies appear as bands at a size of approximately 55 kDa while the light chains appear as bands at a size of approximately 27 kDa.

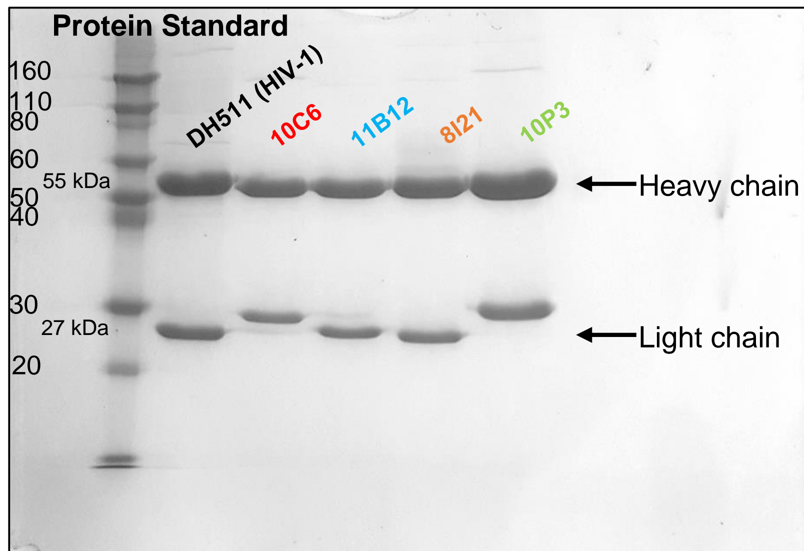


Figure 3.5: Heavy and light chains of antibodies directed against pentamer and glycoprotein B. The mAbs 10P3, 11B12, 8I21 (anti-pentamer) and 10C6 (anti-gB) were denatured into their heavy and light chains, which separated at bands corresponding to the sizes 55kDa and ~30 kDa, respectively. An HIV mAb DH511 was used as a positive control (at 1 mg/mL). The molecular weight marker used was supplied by Invitrogen consisting of 12 pre-stained protein bands that range in molecular weight from 3.5-260 kDa.

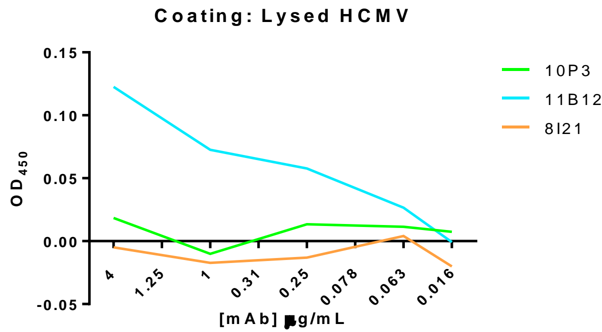
The heavy and light chains for all expressed antibodies appeared as bands at the corresponding size. Very little material of other molecular weights was detected, confirming expression of the chains with minimal impurities. The slight increase in size to 30 kDa seen for the light chains of 10P3 and 10C6 are likely due to the fact that these mAbs have longer amino acid chains, as seen by the amino acid sequence of 10P3 and 10C6 in **Figure 3.5**, and/or perhaps heavier glycosylation than the other antibodies we analysed.

3.3.4 Confirming Functionality of Anti-Pentamer Antibodies– Binding to Lysed HCMV

The expressed mAbs (11B12, 8I21, 10P3) were then tested to determine their reactivity for lysed HCMV. This was done using the same commercial kit as that used in section 3.2.1 i.e. the CMV commercial IgG kit (Euroimmun lot# EI 2570-9601 G) (**Figure 3.6**). As expected, the positive control serum at a dilution of 1:250 and 1:1000 bound to the lysed virus-coated wells at an OD_{450} value of almost 2.5 and 1.0, respectively. The negative control serum was indeed negative at both dilutions (OD_{450} = 0.0015).

The concentration of the anti-pentamer mAbs 11B12, 8I21 and 10P3 were titrated down from 4 µg/mL to 0.016 µg/mL. The controls showed that the CMV commercial IgG kit worked as intended. Only 11B12 of the 3 pentamer mAbs recognized the lysed virus-coated wells, although very poorly at an OD_{450} value of <0.20 at 4 µg/mL. The fact that anti-sera recognize the ELISA wells of the kit, but not the anti-pentamer mAbs may suggest that the epitopes on pentamer for these mAbs could have been altered/destroyed during the lysing process of the lysed whole virus utilized by this commercial IgG kit. Other explanations are possible, including that the antibodies did not function. We tested this below with the purified pentamer preparation that we purchased (section 3.3.5 below).

(a) **(b)**



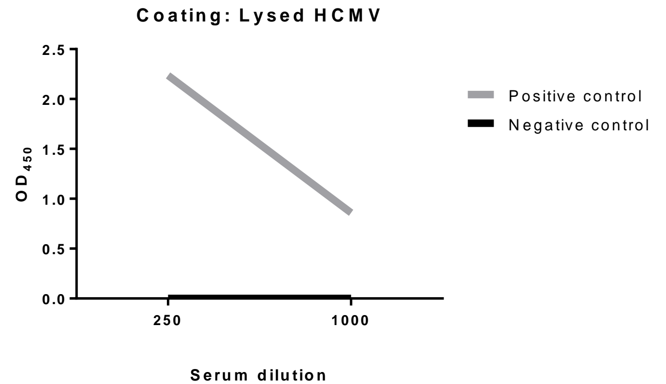


Figure 3.6: Anti-pentamer antibodies tested against whole lysed CMV commercial ELISA kit. (a) The anti-pentamer 10P3 in green, 11B12 in blue and 8I21 in orange were titrated 4-fold from 4 µg/mL to 0.016 µg/mL **(b)** Immune serum (grey) was used as the positive control and non-immune serum (black) was used as the negative control.

The 11B12 mAb was the only anti-pentamer mAb to show any binding.

3.3.5 Optimization of Monoclonal Antibodies: Binding To Purified Pentamer

It was hypothesized that the one of the possible reasons for the minimal or lack of binding to the whole-lysed virus was that the pentamer epitopes were hidden or damaged during the lysing process of the virus. The anti-pentamer antibodies are reported to bind to conformation-dependent epitopes. Thus, the anti-pentamer mAbs (11B12, 8I21, 10P3) were tested in an ELISA experiment to determine the ability of the mAbs to bind our commercially obtained purified pentamer antigen, which is presumably conformed properly, at least partially so.

Initially, to test binding of the mAbs to purified recombinant CMV pentamer, the three anti-pentamer mAbs were titrated down in 3-fold steps from 10 to 0.37 µg/mL for their ability to detectably bind to pentamer coated at 1.25 µg/mL (this concentration determined above in section 3.1) to determine optimal concentration of the mAbs i.e. the lowest detectable concentration for use in subsequent experiments. Anti-gB 10C6 mAb was

included and titrated, down in 3-fold steps, as a specificity control. Two positive and two negative controls (immune and non-immune sera, respectively) were also titrated 3-fold steps from 1 in 100 to 1 in 2700. Although the positive and negative controls behaved as expected, the anti-pentamer mAbs did not appear to titrate down (**Figure 3.7**).

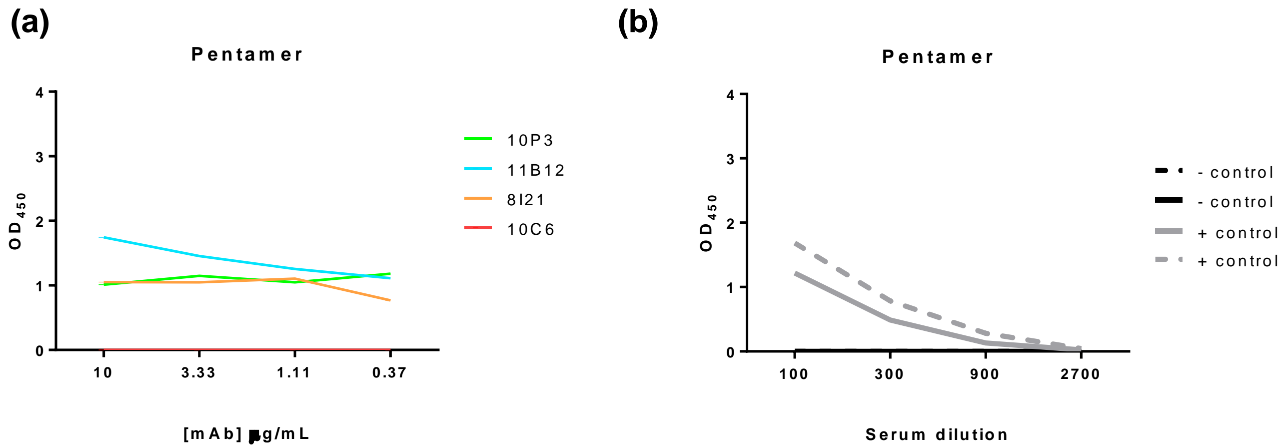


Figure 3.7: Titration of anti-pentamer monoclonal antibodies. (a) Anti-pentamer 10P3 (green), 11B12 (blue) and 8I21 (orange) were used to detect pentamer coated at 1.25 µg/mL. The mAbs were titrated down 3-fold from 10 µg/mL however no titration curve was detected. Anti-gB 10C6 (red) was included as a specificity control **(b)** Two immune sera (light and dark grey) used as positive controls and two non-immune sera (black and blue) used as negative controls were titrated 3-fold from a dilution of 1 in 100.

This result was possibly due the mAb concentrations being so high that we did not reach a concentration at which the binding was detectably reduced. Thus, we titrated the three anti-pentamer mAbs 3-fold starting from 1 µg/mL to 0.01 µg/mL against pentamer at 1.25 µg/mL. One positive and one negative control, at a fixed dilution of 1 in 900 (five repeats) was used (**Figure 3.8**).

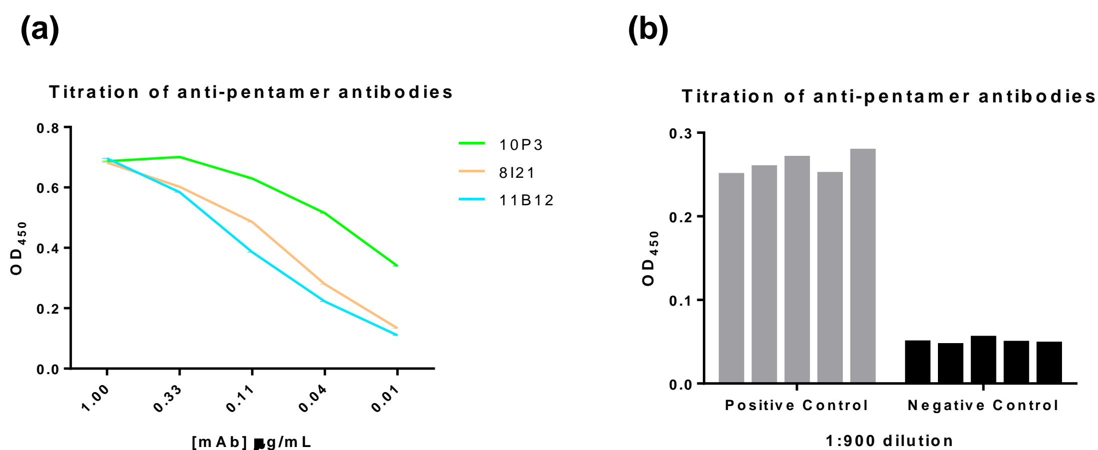


Figure 3.8: Titration of anti-pentamer monoclonal antibodies to lower concentrations than previous experiment. (a) Anti-pentamer 10P3 (green), 11B12 (blue) and 8I21 (orange) were used to detect pentamer coated at 1.25 µg/mL. The mAbs were titrated down 3-fold from 1 µg/mL. The lowest detectable concentration (optimal concentration) for all three anti-pentamer mAbs was 1 µg/mL. **(b)** One immune serum (grey) used as a positive control and one non-immune serum (black) used as a negative control were repeated five times at a fixed dilution of 1:900.

Both controls displayed profiles as expected. The lowest detectable concentration for all three mAbs were detected at OD_{450} ~0.70 at 1 µg/mL, thus this concentration was used in subsequent experiments. This confirmed that, in the prior experiment, we used saturating concentrations of mAb for all or almost all concentrations tested.

3.4 EPITOPE-SPECIFIC ELISA SETUP II: PRODUCTION AND TESTING OF ANTI- GB MONOCLONAL ANTIBODIES

3.4.1 Selection, Transfection and Purification of Monoclonal Antibody 10C6

As previously mentioned, the anti-gB mAb 10C6 was selected based on the mAbs identified by Macagno *et al* (2010). Anti-gB 10C6 was produced following the same procedure as the anti-pentamer mAbs. Sequencing was conducted to confirm correct production of the mAb sequence, which in turns translates to the correct amino acid sequences (**Figure 3.4**). Following transfection and purification, the concentration was determined and adjusted using PBS (**Table 3.5**). The high purity of 10C6 was indicated

by the A260/A280 absorbance ratio of 0.54 and we obtained approximately 17 mg of 10C6.

Table 3.5: **Yield of anti-gB 10C6**

Antibody	**Antibody target**	**Concentration after diluting with PBS (mg/mL)**	**A260/A280**	**Volume (mL)**	**Yield (mg)**
10C6	gB	0.64	0.54	26.20	16.77

The values in this table are calculated as the average of three readings taken.

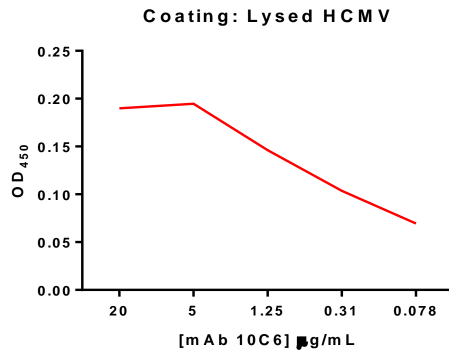
SDS PAGE was used to characterize and confirm the expression of both heavy and light chains of 10C6. The heavy and light chains appeared as bands at sizes corresponding that which was expected, 55 kDa and 30 kDa, respectively (**Figure 3.4**).

3.4.2 Confirming Functionality of Monoclonal Antibody 10C6– Binding to Lysed HCMV

As with the anti-pentamer mAbs, the anti-gB mAb 10C6 was tested for reactivity to lysed HCMV using the CMV commercial IgG kit in the same experiment as section 3.3.4, **Figure 3.6**. The positive and negative controls behaved as expected at a dilution of 1:250 and 1:1000. Both control sera were laboratory stocks (**Figure 3.9**).

The concentration of anti-gB 10C6 was titrated down from 20 µg/mL to 0.078 µg/mL. Anti-gB 10C6 very poorly recognized the lysed virus-coated wells, at an OD_{450} value of almost 0.20 at 20 µg/mL.

(a)



(b)

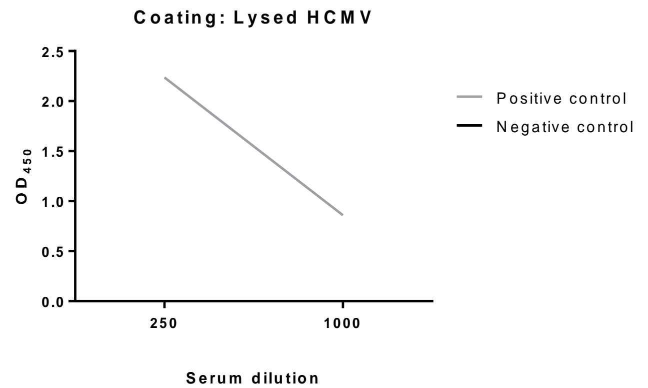


Figure 3.9: Monoclonal antibody 10C6 tested against whole lysed CMV commercial ELISA kit. (a) The anti-gB 10C6 in red was titrated 4-fold from 20 µg/mL to 0.078 µg/mL. **(b)** Immune serum (grey) was used as the positive control and non-immune serum (black) was used as the negative control.

This data shows that slight binding of anti-gB 10C6 occurs but only at high concentrations (20 µg/mL).

3.4.3 No Binding of Monoclonal Antibody 10C6 against Recombinant Glycoprotein B-His

Anti-gB 10C6 was also tested in an ELISA experiment to determine the ability of the mAb to bind gB-His coated at 2.5 µg/mL. This experiment followed the same format as the initial experiment described in section 3.3.4, in that two positive and two negative controls were titrated 3-fold from 1 in 100 to 1 in 2700. The three anti-pentamer mAbs were also include to ensure specificity of 10C6 to gB-His (**Figure 3.10**). All mAbs were titrated down in 3-fold steps from 10 to 0.37 µg/mL.

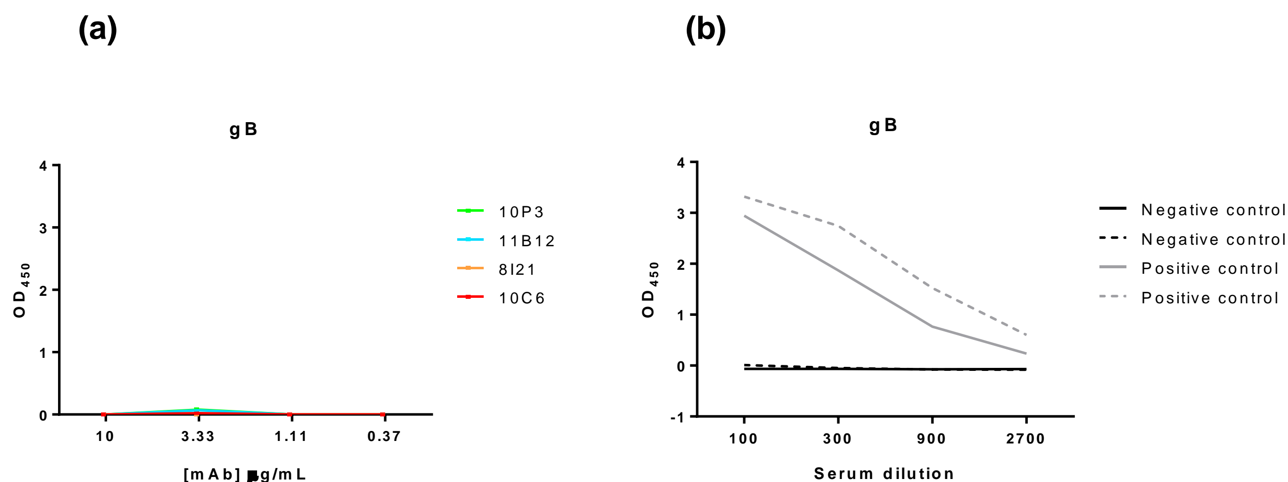


Figure 3.10: Monoclonal antibody 10C6 did not demonstrate binding to immobilized glycoprotein B measured at 450 nm. (a) Four mAbs were titrated against gB-His 3-fold from 10 µg/mL. No binding occurred for the anti-pentamer mAbs 10P3 (green), 11B12 (blue) and 8I21 (orange) to the gB-His as expected. The anti-gB mAb 10C6 (red) was expected to detect the coated gB-His but displayed the same profile as the anti-pentamer mAbs. **(b)** Two immune sera (grey solid and dashed) used as positive controls and two non-immune sera (black solid and dashed) used as negative controls were diluted 3-fold from 1:100.

The two positive controls displayed binding to recombinant gB-His at OD_{450} values ~3.0 when the sera were diluted 1 in 100. This showed success in both gB-His coating to the 96-well ELISA plates and binding of the secondary antibody (anti-human IgG, Fc specific, peroxidase Ab) to anti-gB present in the positive control serum. Both negative controls had OD_{450} values of ~0.01 at all four dilution points (100 to 2700). Anti-pentamer mAbs 10P3, 11B12 and 8I21 did not display any demonstrable binding to gB-His, as expected, with OD_{450} values of ~0.08 across all four dilution points. Surprisingly, the anti-gB 10C6 mAb also displayed a negative profile, not binding gB-His even at a concentration as high as 10 µg/mL.

It was hypothesized that the recombinant gB-His used could have possibly been folded in a specific orientation when coating the 96-well ELISA plates, thus blocking the specific binding site of 10C6. It is also possible, and probably most likely, that the epitope for 10C6

was never formed in the recombinant gB. Alternatively, the polyhistidine-tag could have been interfering with the binding site of 10C6. In order to test these possibilities, recombinant gB (gB-A) from a different supplier (Abcam, UK lot# ab43040) was tested. Positive and negative controls (sera), as well as two randomly selected maternal sera, one with high binding and one with low binding, and anti-pentamer 8I21 (**Figure A3 in Appendix 2**). The controls displayed binding profiles as expected, however, 10C6 did not bind to either gB-His supplied by Sinobiological or gB-A supplied by Abcam. The fact that gB-A has no polyhistidine tag eliminates the possibility that binding was hampered by the His tag. We concluded that, despite the heavy and light chains being the correct size, anti-gB mAb 10C6 was not suitable for our assay because this assay is based upon binding of a monoclonal antibody to the recombinant protein.

3.4.4 Production of Anti-gB 6B4 - Replacement for 10C6 in Our Experiments

Since the anti-gB 10C6 failed to bind gB-His (and gB-A) we decided on a different mAb that targets gB, based on the mAbs identified by Macagno *et al* (2010), referred to as 6B4 (Macagno *et al.*, 2010). Anti-gB 6B4 was produced following the same procedure as the other 4 mAbs and sequencing was done to confirm correct production of the mAb sequence (**Figure 3.3**). After transfection and purification, the concentration was determined and adjusted using PBS (**Table 3.6**). The purity of 6B4 was indicated by the A260/A280 absorbance ratio of 0.56 and we obtained 0.4 mg of 6B4.

Table 3.6: Yield of anti-gB 6B4 (Replacement for Non-functional 10C6 mAb)

Antibody	Antibody target	Concentration after diluting with PBS (mg/mL)	A260/A280	Volume (mL)	Yield after dilution (mg)
6B4	gB	0.67	0.56	0.59	0.396

The values in this table are calculated as the average of three readings taken.

SDS PAGE characterized and confirmed the expression of both heavy and light chains of 6B4. The heavy and light chains appeared as bands at the size corresponding that which was expected, 55 kDa and 27 kDa, respectively (Data not shown). Since the mAb 10C6 did not bind to any of the recombinant gB, we tested the newly synthesized anti-gB 6B4 against gB antigens from different suppliers at various coating concentrations starting at 2.5 µg/mL to 0.3 µg/mL (**Figure 3.11**). These gB antigens were gB-His (the same gB used in previous experiments) and gB-N (gB with no polyhistidine-tag) both supplied by Sinobiological as well as gB-A supplied by Abcam.

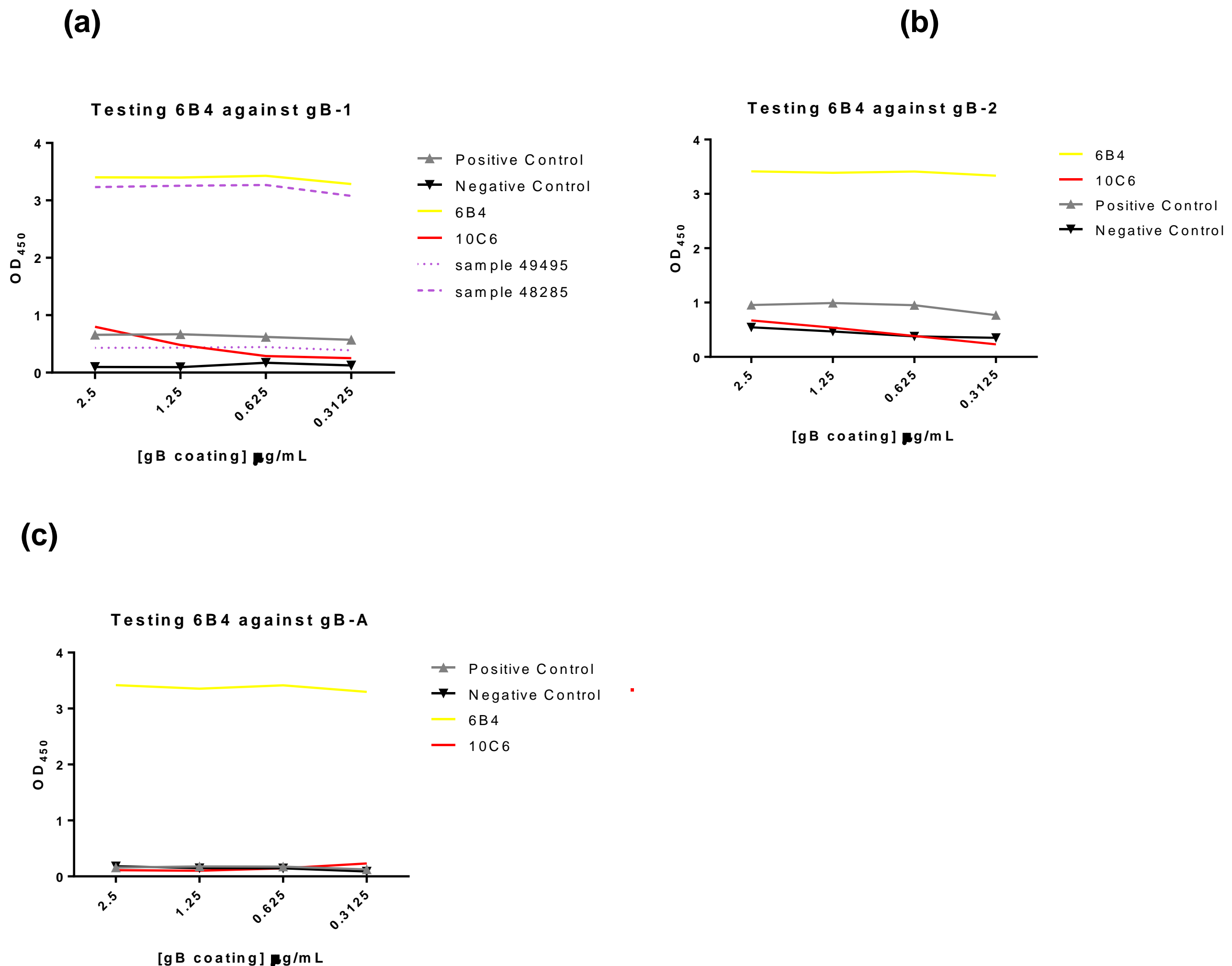


Figure 3.11: Testing monoclonal antibody 6B4 against glycoprotein B from different suppliers. All three gB antigens were titrated 2-fold from 2.5 µg/mL. Anti-gB 6B4 produced a reactivity response at OD_{450} values of greater than 3.0 against (a) gB-His (gB containing a polyhistidine-tag) supplied by Sinobiological, (b) gB-N (no polyhistidine-tag) supplied by Sinobiological and (c) gB-A supplied by Abcam. The positive control appeared to produce poor responses to gB-1 due to the fact that 6B4 produced very high OD_{450} values. Samples 11 and 23 were used as controls to ensure reproducible results. anti-gB 10C6 did not react to any of the recombinant gB antigens except for gB-2, where very slight binding was seen at a coating concentration of 2.5 µg/mL, which 10C6 was at a concentration of 20 µg/mL.

The positive and negative control sera produced the expected results, although the OD_{450} readings for the positive controls were much lower than observed in previous experiments. Anti-gB 10C6 was added to the experiment and showed only slight binding (OD_{450} < 1.0) against gB-His and gB-N at a coating concentration of 2.5 µg/mL, however, the concentration of 10C6 was 20 µg/mL. Anti-gB 6B4 showed binding to all three of the gB antigens (gB-His, gB-N and gB-A) at OD_{450} values greater than 3.0, thus, the polyhistidine-tag appeared not to interfere with binding. Additionally, samples 49495 and 48285 of were used as controls to ensure that gB-His produced similar results. This was important to make sure that values for antibody levels obtained in different experiments were as comparable as possible. gB-His did indeed produce similar results. Since 6B4 was able to bind all three gB antigens, the gB selected for downstream experiments was the same gB used in previous experiments (gB-His).

After confirming binding of anti-gB 6B4 to all three different gB (gB-His, gB-N and gB-A), 6B4 was titrated 3-fold starting at 1 µg/mL against gB-His coated at 0.625 µg/mL (**Figure 3.12**). We chose to continue using gB-His so that all the experiments were uniform in terms of gB coating. We were able to detect 6B4 mAb binding down to a concentration of 0.3 µg/mL.

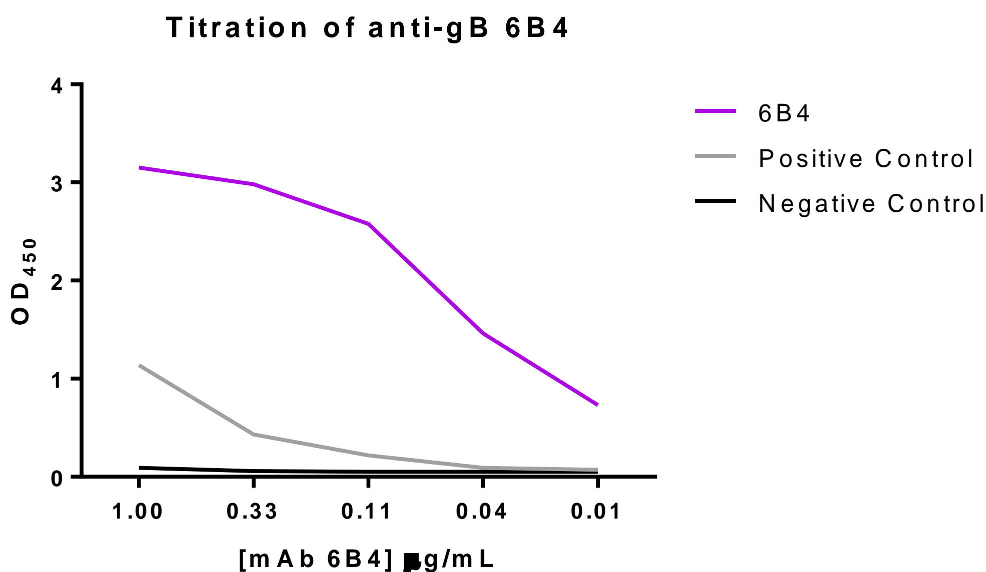


Figure 3.12: Titration of monoclonal antibody 6B4 against glycoprotein B. Anti-gB 6B4 was titrated 3-fold from 1 µg/mL to detect gB-His coated at 0.625 µg/mL (purple). The optimal concentration for 6B4 was determined as 0.33 µg/mL. This concentration was high enough for the window of detection to be high (OD(pos con)-OD(neg con)) – but not so high that it was in the saturating range (see text for fuller explanation).

Taken together, it was determined that the mAbs used in subsequent ELISA experiments as positive controls were anti-pentamer 11B12, 8I21, 10P3 at a concentration of 1 µg/mL and anti-gB 6B4 at a concentration of 0.3 µg/mL. These concentrations were chosen because they were the lowest concentrations that produced reasonably optimal OD_{450} reading, but not so high that the antibody levels were saturating and that saturation might interfere with our ability to detect blocking of antibody binding. The recombinant glycoprotein B used in subsequent experiments was gB-His (containing the polyhistidine-tag) because we were able to obtain enough of this protein to perform our blocking experiments.

3.5 EPITOPE-SPECIFIC ELISA SETUP III: BIOTINYLATION AND TESTING OF MONOCLONAL ANTIBODIES

After successfully creating the relevant mAbs, the second step in defining the specific epitopes was to conduct blocking ELISA experiments. The 137 maternal samples were tested in blocking ELISA experiments to determine the presence of Abs that bind to the same sites as the synthesized mAbs 11B12, 10P3, 8I21 and 6B4. This was conducted by first incubating the maternal sera with the coated antigen. Next the 96-well ELISA plates were washed and the biotinylated version of each mAb, described below, was incubated. Secondary anti-biotin HRP (anti-biotin) was used to detect any bound biotinylated mAbs. Detected biotinylated mAb, by means of detected a colour reaction, translates to a lack of Abs present in the maternal sera to block to the antigenic binding sites. The converse is true i.e. no colour reaction detected is due to Abs present in the maternal sera blocking the antigenic sites for the biotinylated mAbs.

The levels of the four mAbs (11B12, 8I21, 10P3 and 6B4) to epitopes within pentamer and gB-His were measured. This was achieved by adding biotin molecules to the mAbs by means of biotinylation experiments, allowing for detection by the anti-biotin. The biotinylated mAbs, referred to as labeled-mAbs, were then tested against pentamer and gB-His to ensure binding of the labeled-mAbs to the antigens. Additionally, the anti-biotin was tested at two different dilutions (1:5000 and 1:10000) against 11B12 to determine the lowest amount of anti-biotin that could be used for further experiments. Note that 1:5000 anti-biotin was used for the remaining labeled-mAbs (**Figure 3.13**).

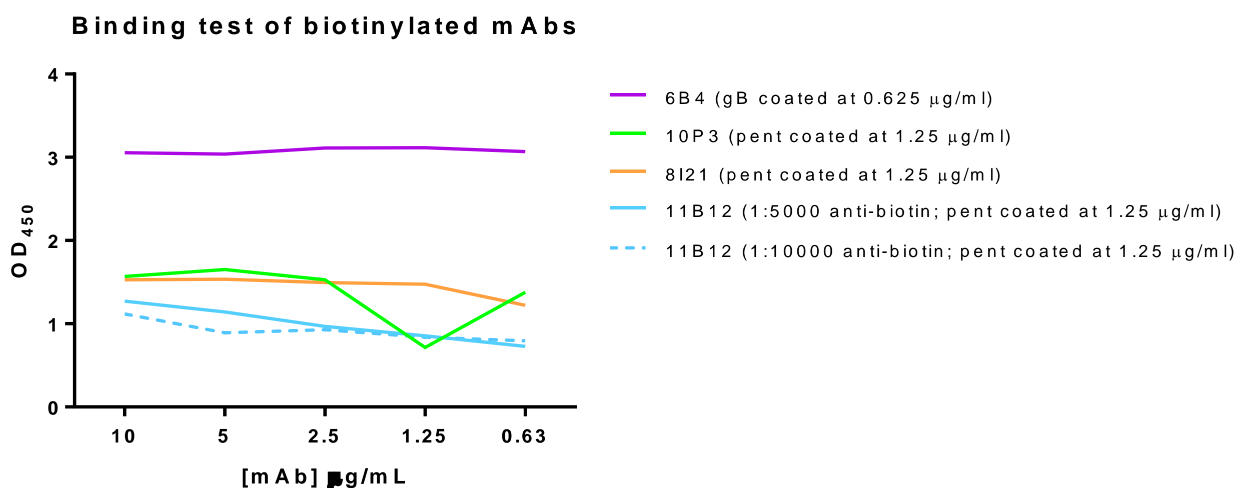


Figure 3.13: Determine binding of biotinylated monoclonal antibodies to pentamer and glycoprotein B to ensure no binding interference from the biotin molecules. All mAbs were titrated 2-fold from 10 µg/mL. Anti-gB 6B4 (purple) produced high reactivity responses to 0.625 µg/mL of gB-His. Reactivity responses were also seen for the anti-pentamer mAbs (10P3 in green, 8I21 in orange, 11B12 in solid and dashed blue) to 1.25 µg/mL of pentamer. Anti-biotin was tested at 1:5000 and 1:10000 against 11B12 and produced similar reactivity response profiles. Anti-biotin was used at 1:5000 against 10P3, 8I21 and 6B4.

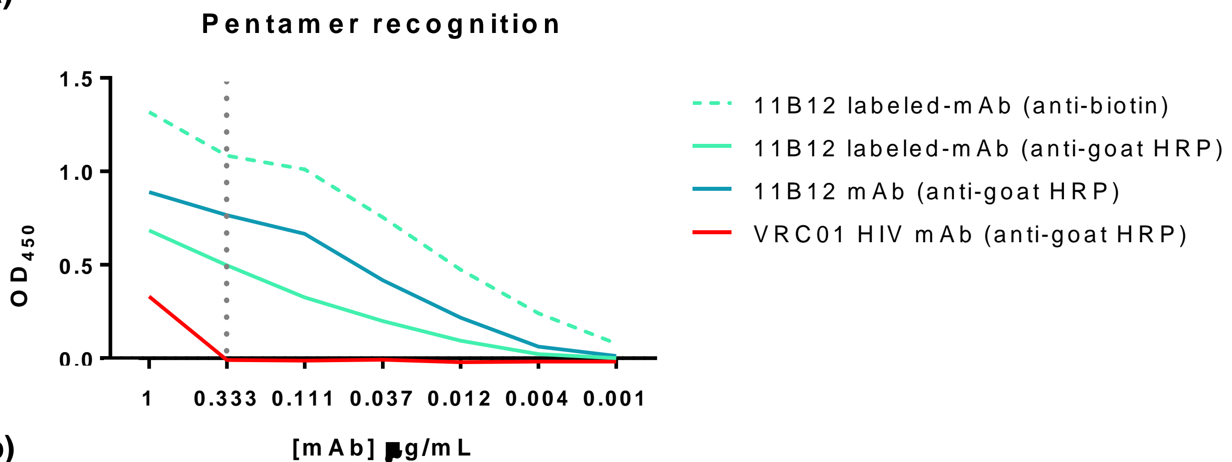
As expected, the labeled-mAbs 11B12, 8I21 and 10P3 were all able to bind pentamer but at lower OD_{450} values ($OD_{450} < 2$) than labeled-mAb 6B4 binding to gB-His ($OD_{450} \geq 3$). However, the labeled-mAbs did not titrate down until 1.25 µg/mL, where slight titration begins to occur for labeled-6B4, labeled-8I21 and the dip seen for labeled-10P3. This was likely due to the concentration of labeled-mAbs being too high to detect complete titration.

It is important to determine an ideal working concentration value for using the biotinylated antibodies in the epitope-specific ELISAs. This should be a concentration just below saturating concentrations in order to maximize the window between positive and negative If you get into the saturating range (too high concentration), then you lose sensitivity to see reduction in biotinylated mAb binding. In other words, too much antibody (high concentration) will be very difficult to block, and too little antibody (low concentration) will

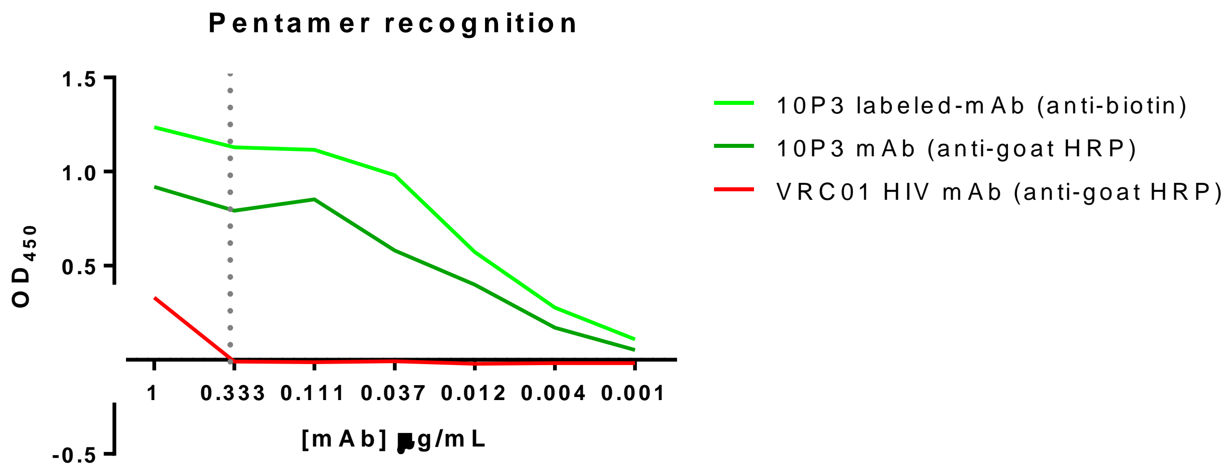
make it difficult to detect changes in binding. This ideal concentration was not achieved in this first experiment – all the concentrations tested were too high.

To address the fact that we were unable to titrate the labeled-mAbs, we conducted another titration experiment but this time we titrated the labeled-mAbs 3-fold from 1 µg/mL to determine the lowest detectable concentration for the downstream blocking ELISA experiments (**Figure 3.14**). Along with the labeled-mAbs, the unbiotinylated version of the mAb was added as positive controls and VRC01 HIV mAb as a negative control, both detected using 1:3000 anti-human IgG (Fc specific) peroxidase Ab produced in goat.

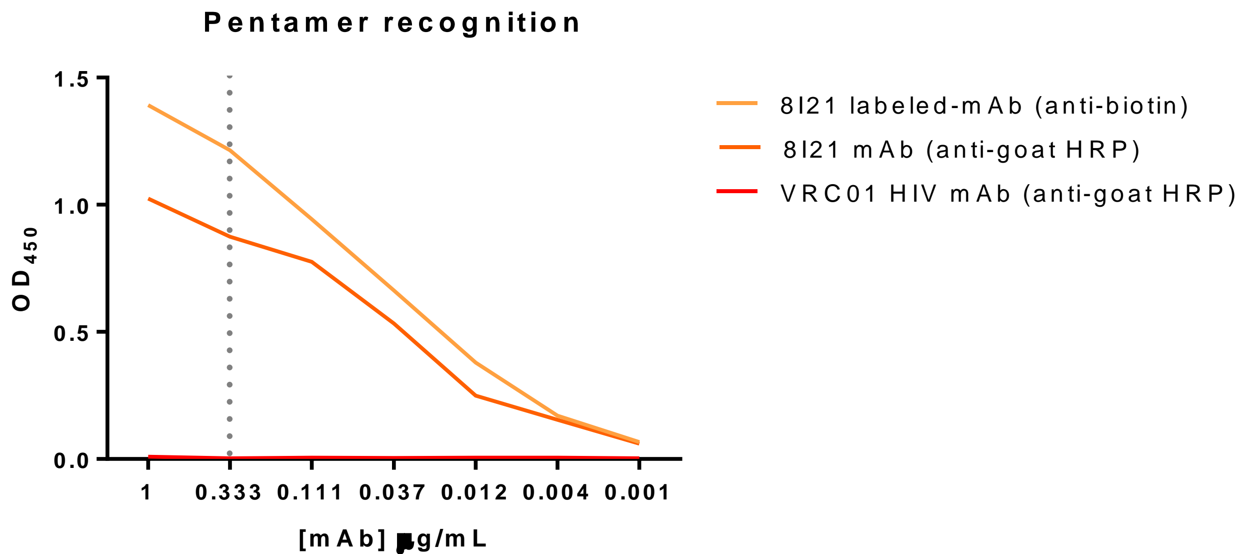
(a)



(b)



(c)



(d)

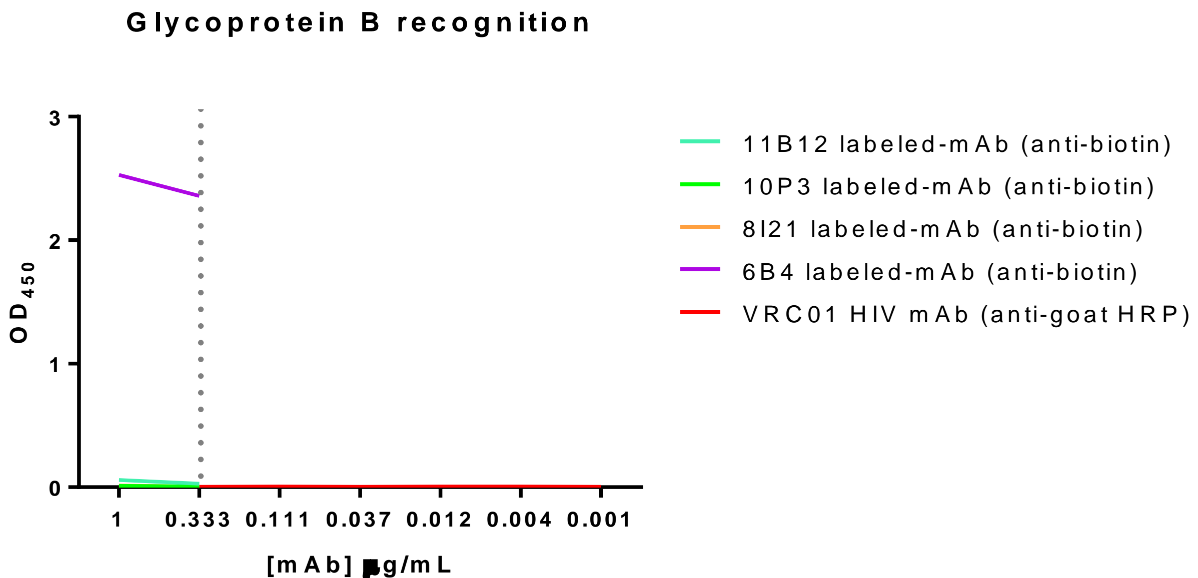


Figure 3.14: Titration of biotinylated monoclonal antibodies 11B12, 10P3, 8I21 and 6B4 against pentamer and gB-His to determine optimal concentration for blocking ELISA experiments. All the mAbs of **(a)**, **(b)** and **(c)** were titrated 3-fold from 1 µg/mL and tested against pentamer. The mAb VRC01 HIV (red) was used as a negative control in all four graphs. The optimal concentration is shown as a grey vertical dotted line. **(a)** Labeled-mAb 11B12 (light blue dashed) and unlabeled-mAb 11B12 (dark blue solid) were detected using the anti-biotin HRP while labeled-mAb 11B12 (light blue solid) was detected using the secondary anti-goat HRP. **(b)** Labeled-mAb 10P3 (light green) was detected using the anti-biotin HRP while unlabeled-mAb 10P3 (dark green) was detected using the secondary anti-goat HRP. Slight detection of VRC01

HIV was seen at 1 µg/mL of **(a)** and **(b)** likely due to experimental error. **(c)** Labeled-mAb 8I21 (light orange) was detected using the anti-biotin HRP while unlabeled-mAb 8I21 (dark orange) was detected using the secondary anti-goat HRP. **(d)** Labeled mAbs 11B12 (light blue), 10P3 (light green), 8I21 (light orange) and 6B4 (purple) were tested against glycoprotein B and detected using anti-biotin HRP. Only the anti-gB labeled-mAb 6B4 was detected. Note that this was the entirety of the experiment **(d)** as samples were limited at this point.

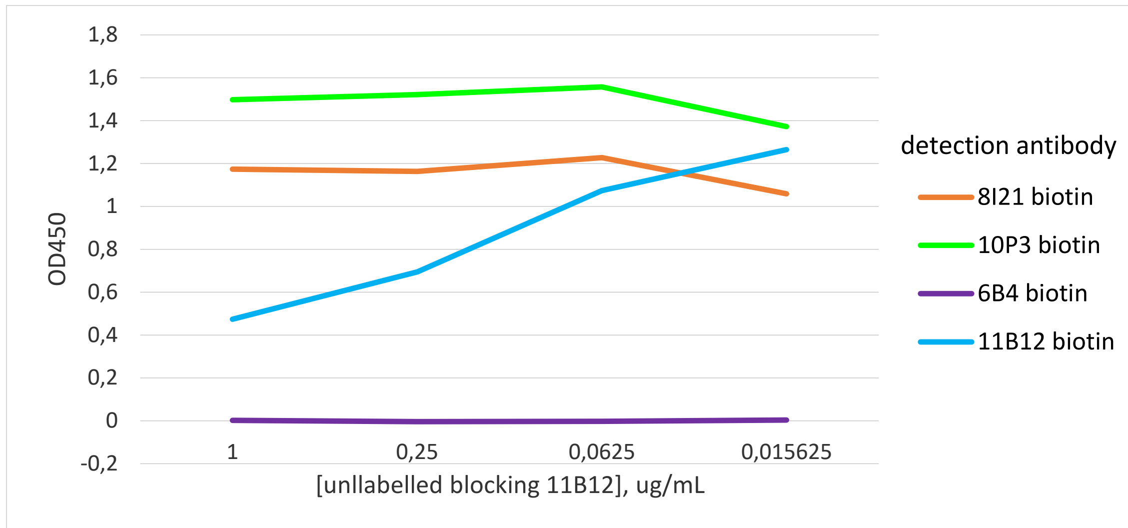
From **Figure 3.14 (a)** we observed a reduced binding signal, when the mAb 11B12 was biotinylated or the secondary anti-goat HRP just doesn't recognize it very well when it's biotinylated. When we utilized the anti-biotin we had a better signal of labeled-11B12. This was true for the rest of the labeled-mAbs (8I21, 10P3 and 6B4). We estimated that the ideal working concentration is 0.33 µg/mL for all four labeled-mAbs based upon the data in **Figure 3.14a** (for 8I21-biotin), **3.14b** (for 10P3-biotin), **3.14c** (for 11B12-biotin) and **3.14d** (for 6B4-biotin).

3.6 EPITOPE-SPECIFIC ELISA SETUP IV: ENSURING ELISA TEST SPECIFICITY AND DETERMINING OPTIMAL BIOTINYLATED ANTIBODY CONCENTRATIONS

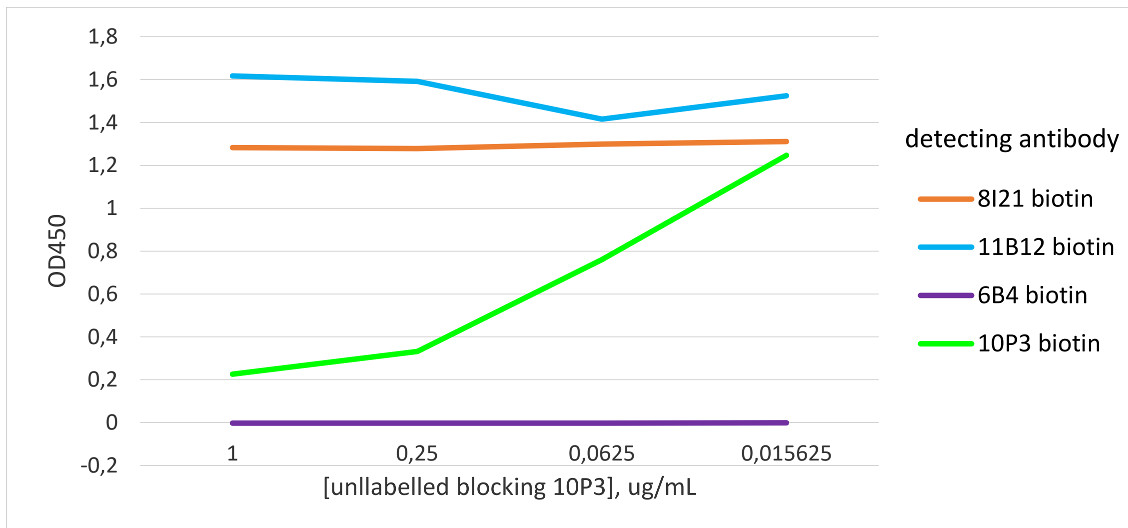
A set of control experiments measured the labeled-mAbs with their corresponding unlabeled-mAb. This was to ensure the specificity of the ELISA tests as well as ensure that changes in binding can be detected. The unlabeled version of mAbs 11B12, 10P3, 8I21 and 6B4 were titrated 4-fold from 1 µg/mL. The four labeled-mAbs were then added at a constant concentration of 0.3 µg/mL (**Figure 3.15**).

When blocking with unlabeled 11B12, the detected binding of labeled-11B12 increases in OD_{450} values as the concentration of unlabeled 11B12 decreases. Labeled-10P3 and labeled-8I21 had OD450 values > 1 at all concentrations of unlabeled 11B12, showing that the antigenic targets of labeled-10P3 and labeled-8I21 were not blocked by unlabeled-11B12. The labeled-mAb 6B4 did not show any blocking activity as expected since it is an anti-gB mAb and not anti-pentamer like 11B12, 10P3 and 8I21. These expected results were seen for all four blocking ELISA experiments.

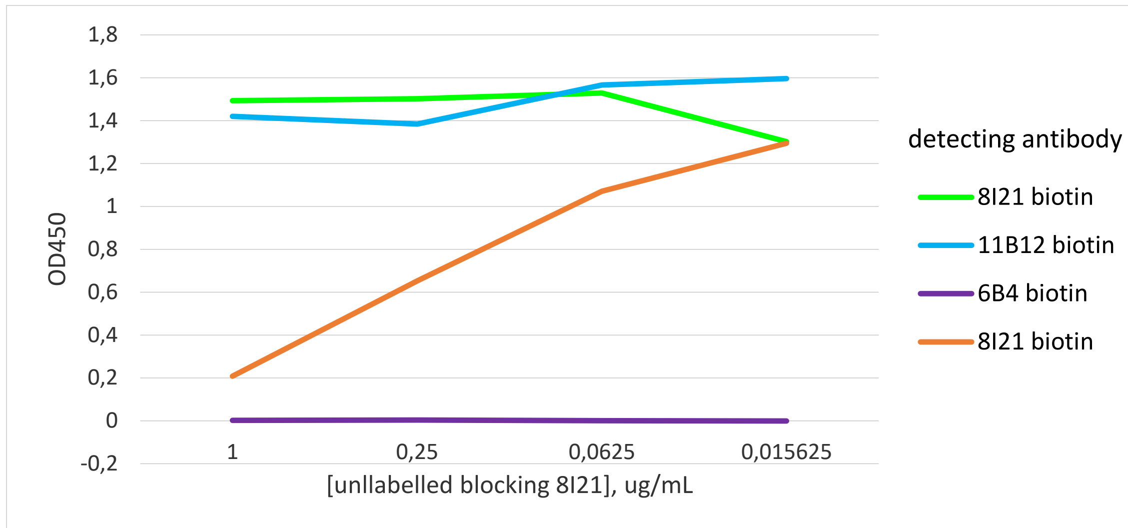
(a) Coating: Pentamer



(b) Coating: Pentamer



(c) Coating: Pentamer



(d) Coating: Glycoprotein B

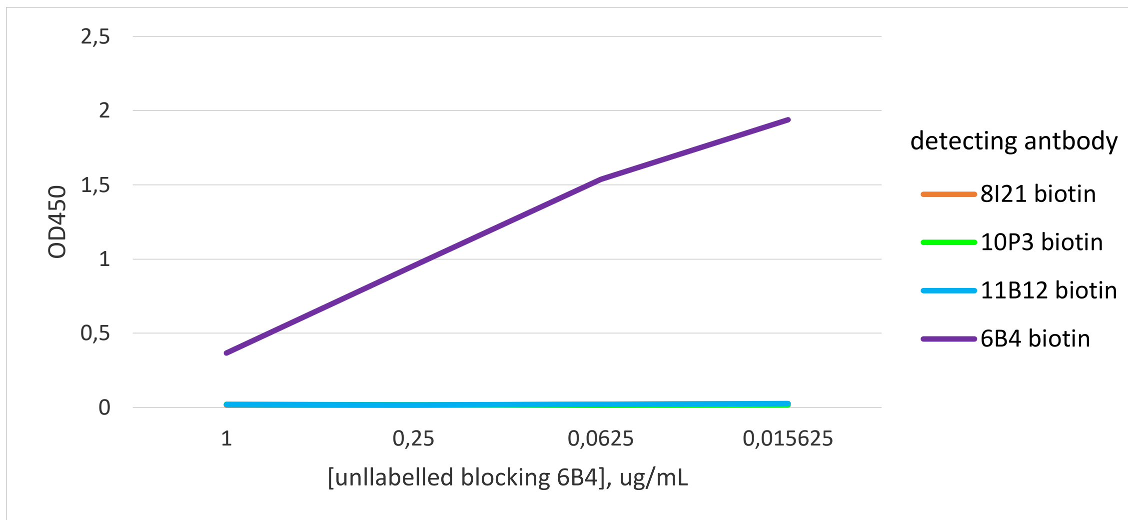


Figure 3.15: Assessing blocking by unlabeled monoclonal antibodies using the corresponding labeled (biotinylated) monoclonal antibodies. The unlabeled mAbs were titrated 4-fold from 1 µg/mL while the labeled-mAbs (biotin mAbs) were added at a constant concentration of 0.3 µg/mL. **(a)**, **(b)** and **(c)** were coated with pentamer at 1.25 µg/mL while **(d)** was coated with gB-His at 0.625 µg/mL. **(a)** Unlabeled-11B12 was used to block the labeled-mAbs. 8I21 biotin (orange) and 10P3 biotin (green) remained at fairly constant OD_{450} values of ~1.2 and ~1.5, respectively, thus were not blocked by the unlabeled-11B12. 11B12 biotin (blue) gradually increased in OD_{450} values as the concentration of unlabeled 11B12 decreased, allowing

labeled-11B12 to bind pentamer. No reactivity of 6B4 biotin (purple) since this mAb targets gB-His and not pentamer. **(b)** Unlabeled-10P3 was used to block the labeled-mAbs. 8I21 biotin and 11B12 biotin remained at fairly constant OD_{450} values of ~1.3 and ~1.6, respectively, thus were not blocked by the unlabeled-10P3. 10P3 biotin increased in OD_{450} values as the concentration of unlabeled 10P3 decreased, allowing 10P3 biotin to bind pentamer. No reactivity observed for 6B4 biotin as it targets gB-His. **(c)** Unlabeled-8I21 was used to block the labeled-mAbs. 10P3 biotin and 11B12 biotin remained at fairly constant OD_{450} values of around 1.5, thus were not blocked by the unlabeled-8I21. 8I21 biotin increased in OD_{450} values as the concentration of unlabeled 8I21 decreased. No reactivity observed for 6B4 biotin as it targets gB-His. **(d)** Unlabeled-6B4 was used to block the labeled-mAbs. 6B4 biotin increased in OD_{450} values as the concentration of unlabeled 6B4 decreased. No reactivity was observed for 10P3 biotin, 11B12 biotin and 8I21 biotin as they target pentamer and not gB-His.

3.7 EPITOPE-SPECIFIC ELISA SETUP V: ESTABLISHING A NEGATIVE THRESHOLD VALUE FOR THE MATERNAL SERA TESTED IN BLOCKING ELISA EXPERIMENTS

We tested samples from the (presumed) HCMV unexposed individuals, and found no detectable activity. Therefore, we could not calculate a threshold for positivity the same way as we did for the simple ELISA for total antigen-specific antibodies for gB and pentamer (**Figure A4 Appendix 2**). We therefore had to choose a different and more arbitrary method for determining a threshold. In fact, another published paper originated this procedure and used it for their blocking experiments so we did as well (Lilleri *et al.*, 2013).

We arbitrarily chose an ID_{50} of 1 as negative, i.e. if undiluted serum could have been used, it would block less than 50% of the binding of the biotinylated mAb. At this level, pure serum would be unable to substantially inhibit binding of the biotinylated antibody. All ID_{50} values calculated to be below 1 were thus assumed to be negative and all values below 1 were arbitrarily assigned a value of 0.5. This latter effect is to remove noise from the statistics.

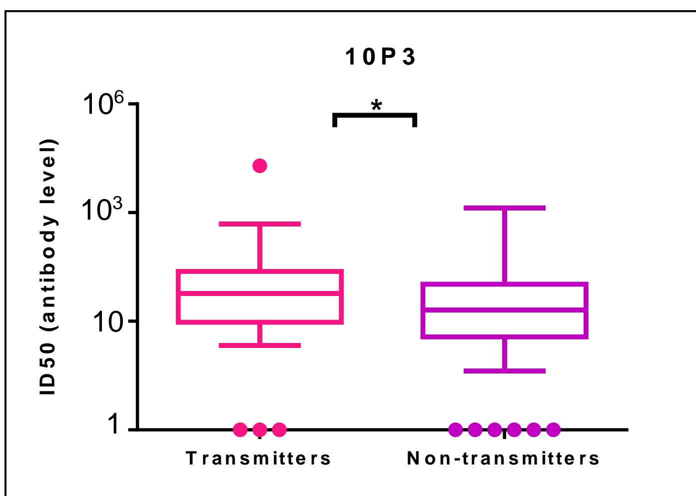
3.8 THE REAL EXPERIMENT: COMPARING EPITOPE-SPECIFIC ANTIBODY ACTIVITY WITH MATERNAL HCMV TRANSMITTER AND HIV STATUS

3.8.1 Transmitters Have Higher Levels of Antibodies Directed Against Three Epitopes in Pentamer

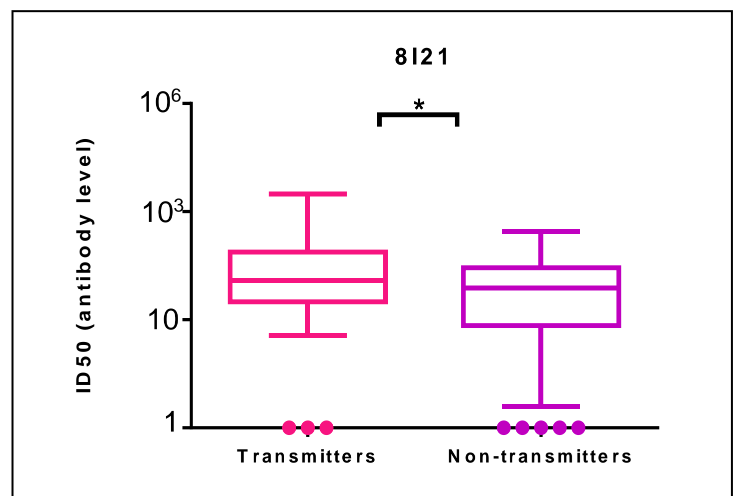
To answer the question of our second objective, that is, to define pentamer and gB epitopes by measuring the blocking activity of the mAbs (11B12, 10P3, 8I21 and 6B4), we compared maternal antibody levels targeting three epitopes on pentamer. These were defined by monoclonal antibodies 8I21 (Site found on gH,gL/UL128/UL130), 10P3 (site found on UL130/UL131) and 11B12, (site found on gH) (Macagno *et al.*, 2010; Lilleri *et al.*, 2013). We also compared antibody levels targeting one epitope on gB, defined by monoclonal antibody 6B4 (Macagno *et al.*, 2010). We tested the 137 maternal samples in blocking ELISA experiments (**Figure 3.16**).

The OD_{450} readings were then converted to percentage blocking values. We used GraphPad® Prism to generate individual ID_{50} values for each epitope/serum combination. An ID_{50} value is the dilution of serum at which 50% blocking is achieved of the biotinylated mAb. Then, we collect those ID_{50} values and compared them between paired mothers (usually 2 control non-transmitters per transmitter). We used a mixed effect model, as described in section 3.2.4, to directly compare each transmitter to the non-transmitter paired to it.

(a)



(b)



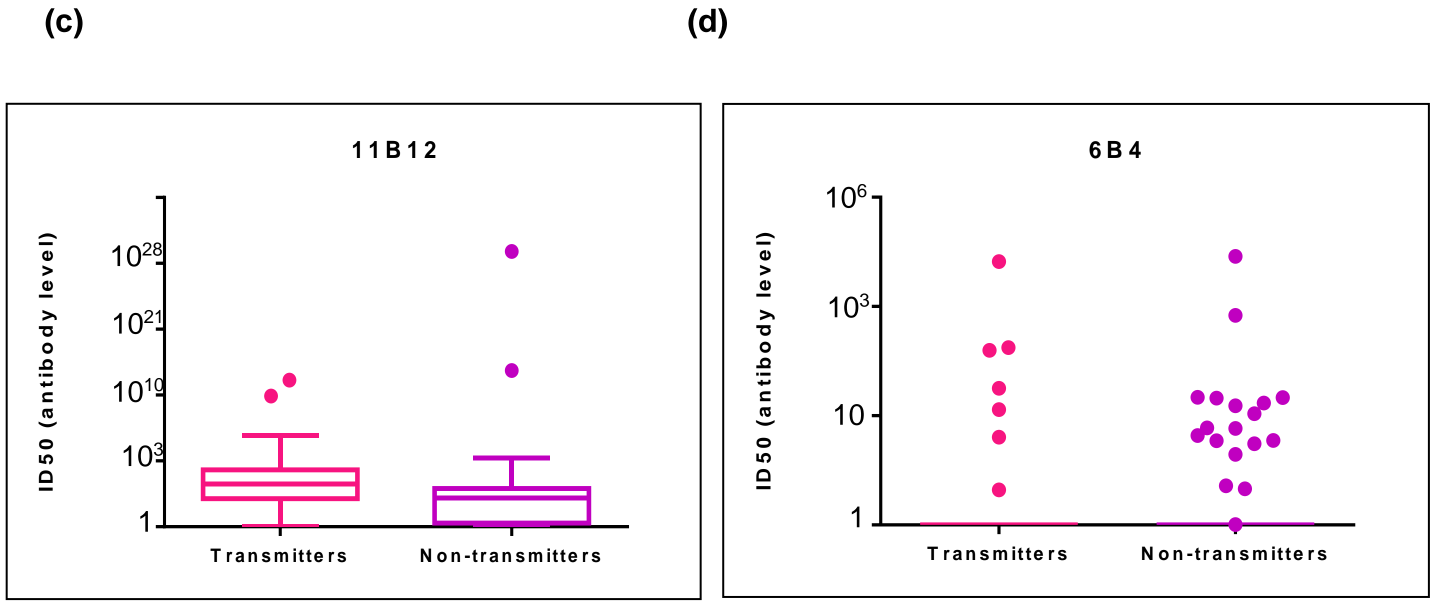


Figure 3.16: Levels of epitope-specific antibodies compared between transmitters versus non-transmitters using blocking ELISA experiments. The 45 mothers transmitting HCMV to their infants are referred to as transmitters (pink) while 78 mothers that are not transmitting HCMV to their infants are non-transmitters (purple). The maternal samples were tested to determine their ability to block the mAbs **(a)** 10P3, **(b)** 8I21, **(c)** 11B12 and **(d)** 6B4. A significant difference in the level of maternal antibodies was seen when the samples were tested for blocking against **(a)** 10P3 (P value summary: *; $P \leq 0.05$) and **(b)** 8I21 (P value summary: *; $P \leq 0.05$). **(d)** The median line falls on the x-axis therefore no box plot as such is shown. Note that the data points are within the boxes and the "outliers" are shown as dots

A significant difference towards the transmitters was seen regarding the anti-pentamer mAbs. More specifically, on average the transmitters had 1.71 fold (95% CI in **Table 3.7)** higher against the 8I21 epitope and 1.52 fold (95% CI in **Table 3.7)** higher against the 10P3 epitope compared to non-transmitters. No significant difference between transmitter and non-transmitters was seen for antibodies directed against the 11B12 epitope on pentamer (2.46 folder higher) or 6B4 epitope on gB (0.99 fold higher). These results suggest that the enhanced responses to epitopes on pentamer are epitope-specific and not just because of a generally stronger immune response in transmitters.

Table 3.7: Fold difference of transmitter/non-transmitter of monoclonal antibodies

Blocking activity	**P value**	**Fold difference in blocking activity transmitters/non-transmitters**
10P3	**0.0183**	*1.52 (95% CI 1.07-2.16)
8I21	**0.0374**	*1.71 (95% CI 1.03-2.28)
11B12	0.0552	2.46 (95% CI 0.98-6.19)
6B4	0.9723	0.99 (95% CI 0.64-1.53)

*More blocking activity of anti-pentamer mAbs seen in transmitters than non-transmitters.

3.8.2 Some of the Antibody Measures Were Higher in Mothers living with HIV-1

Mothers living with HIV-1 have a higher risk of transmitting HCMV (Manicklal *et al.*, 2013; Pathirana *et al.*, 2019), thus, we looked for an immune response that may be deficient that could explain this. From the data above, we analysed maternal HIV status by comparing the anti-CMV antibody levels between HIV-1 positive and negative mothers. Our data showed that two of the different antibody types measured were higher in those living with HIV-1 (**Table 3.8**). These measures were 11B12 blocking levels and total gB levels. We further stratified the data for these two parameters separately in transmitters and non-transmitters. When we looked at the non-transmitters, we found that the total gB antibody levels were higher in HIV-1 positive mothers/women living with HIV-1 (data not shown). However, there was no detectable difference based upon maternal HIV-1 status when analyzing the transmitting mothers. To note, for transmitters 17 samples are HIV-1 positive and 28 samples are HIV-1 negative, and for non-transmitters 44 samples are HIV-1 positive and 34 samples are HIV-1 negative.

Table 3.8: Ratio of antibody levels between mothers living with HIV-1 positive and negative mothers

Measure	Ratio PLWH*/HIV-1 neg (95% CI)	p value
Epitopes on pentamer		
8I21 blocking, ID_{50}	1.34 (0.91-1.99)	0.1388
10P3 blocking, ID_{50}	1.29 (0.89-1.88)	0.1770
11B12 blocking, ID_{50}	2.94 (1.18-7.35)	**0.0212**
Epitope on gB:		
6B4 blocking, ID_{50} (anti-gB)	1.02 (0.66-1.58)	0.9236
Total antigen-specific Ab levels:		
Total anti-pentamer	1.34 (0.95-1.90)	0.0946
Total anti-gB	1.50 (1.07-2.10)	**0.0176**

*people living with HIV-1

CHAPTER 4: DISCUSSION AND CONCLUSION

HCMV infection continues to be one of the most common congenital infections with severe effects worldwide and results in negative clinical symptoms of approximately one million newborns year after year (Permar, Schleiss and Plotkin, 2018). In particular, cCMV infection causes birth defects including long-term neurologic deficits, deafness, microcephaly, neurodevelopmental delay, and also fetal loss and infant mortality (Permar, Schleiss and Plotkin, 2018). The burden congenital CMV (cCMV) infection places on society is substantial, thus, HCMV vaccine development continues to be a major priority of public health research, with a variety of approaches for vaccine development (with gB and pentamer being vaccine candidates) being pursued to reduce this foetal disease (Permar, Schleiss and Plotkin, 2018; Gugliesi *et al.*, 2020).

The entry complex, gH/gL/pUL128-130-131 pentamer, and glycoprotein B are of particular interest because they both evoke strong neutralizing antibody responses (Permar, Schleiss and Plotkin, 2018) and are important players in viral entry into host cells (Fouts *et al.*, 2012; Lilleri *et al.*, 2013; Vanarsdall *et al.*, 2019). We sought to assess the level of mAb against pentamer and gB in both HCMV transmitting and non-transmitting mothers with mostly or all non-primary HCMV infection. We also aimed to assess the impact HIV-1 infection has on these mAb levels. We used maternal sera, derived from South African women from Soweto who were identified as HCMV transmitters to their children, comparing their levels to non-transmitting women. We tested the maternal sera for antibodies targeting HCMV pentamer and gB by standard ELISA. Separately, we tested for antibodies that target particular epitopes on pentamer and gB, using a blocking ELISA technique in which we measured the ability of sera to block binding of monoclonal antibodies to these key sites.

Since the majority of cCMV infections worldwide are from maternal non-primary infections (Manicklal *et al.*, 2013) and because this effect was previously unexplored in a population in which most maternal CMV infections are non-primary, we investigated a population of people exposed to HCMV before adulthood (Schoub *et al.*, 1993; Manicklal *et al.*, 2014). In our study, antibodies directed against key sites on the pentamer were considerably

higher in mothers who transmitted the virus (transmitters) than mothers who did not transmit HCMV (non-transmitters). This direction was also observed for total specific antibodies directed against pentamer and gB were also higher in transmitters. Antibodies blocking the 6B4 epitope on gB were not different between the groups, which suggests that this effect is specific to particular epitopes. The associations we observed are in the opposite direction as that reported in a key study of women with primary HCMV infection, described below (Lilleri *et al.*, 2013).

The study conducted by Lilleri *et al* (2013) has investigated antibody levels directed against key sites on these entry glycoproteins (pentamer and gB) in the context of primary maternal infection. Lilleri *et al* (2013) showed that non-transmitters had higher levels of antibody to the epitopes we studied on pentamer compared to the transmitters. Their studies also found that anti-gB Abs are produced by both non-transmitters and transmitters with the same kinetics and in similar amounts (Lilleri *et al.*, 2013). This previous work leads to the conclusion that antibodies to the pentamer epitopes may be protective i.e. help prevent mother to foetus transmission of HCMV. Our conclusions have led us to suggest otherwise – we were unable to find an association suggesting a maternal immune response that could protect from *in utero* transmission of HCMV.

We speculate that the dominant effect on antibody that we observed was due to boosting by the maternal infections (re-infections or reactivations). These reactivations and re-infections were possibly more common in transmitting mothers than non-transmitting mothers because, by choosing mothers who transmitted to their foetus, we selected for mothers who had reactivations or re-infections that resulted in foetal transmission.

We particularly considered that such a study might expose a critical immunity gap allowing transmission in the presence of an already existing immune response. If so, we possibly could have more clearly distinguished a directly protective antibody response from among the other responses found in previously exposed HCMV individuals. Critically, our study did not reveal such an effect.

Few studies have measured antibody levels comparing HCMV transmitting to HCMV non-transmitting mothers in the context of non-primary infection, and none of those have measured epitope-specific antibodies. A study conducted by Vanardall *et al* (2019) measured levels of antibody in maternal sera from a cohort of 14 pregnant Brazilian women. Previous studies conducted in this population of women indicated that majority of these women were infected by HCMV from childhood, indicating that most, if not all infections during pregnancy were non-primary infection (Yamamoto *et al.*, 2013), as is also found in South Africa, where we conducted our study (Pathirana *et al.*, 2019). The Vanarsdall *et al* (2019) study found no differences in pentamer-specific nAbs titers as well as no real differences in the total nAb titers in sera when they compared transmitters with non-transmitters (Vanarsdall *et al.*, 2019). They concluded that nAbs specific to pentamer do not correlate with mother-to-child transmission, a conclusion differing from that reported by Lilleri *et al* (2013). It is worth noting that this study investigated antibody levels early in pregnancy (first trimester). This is likely before any maternal HCMV reactivations/re-infections could lead to cCMV infection of the infant or a boost in maternal antibody levels. Importantly, this allowed Vanarsdall and co-workers to better explore protective effects because they measured antibody levels going into the period in which the foetus was susceptible to HCMV infection.

We measured the levels of maternal Abs targeting 3 epitopes on pentamer as well as gB, to further assess the impact of Abs levels in distinguishing HCMV transmitting and non-transmitting mothers. This allowed us to assess any of these responses as a target of protective immunity and therefore a good vaccine target. We observed that the transmitters had more blocking Abs for pentamer, particularly antibody responses targeting the 10P3 and 8I21 epitopes, than non-transmitters. However, the case is weaker for the antibody response targeting the 11B12 epitope with the p value being 0.055, slightly above what we consider to be a significant difference ($p \leq 0.05$). It is possible that there is an effect for the 11B12 epitope for which we lacked the statistical power to observe properly. No result was seen for the gB epitope defined by 6B4, which strengthens the argument that it's not just some sort of co-varying event that is happening to antibody levels to many epitopes, but rather that the effect is epitope-specific.

There are a few limitations of our study compared to the equivalent studies in primary infection. Other than the fact that our study was in the context of mostly or all non-primary infection while Lilleri *et al* (2013) was in the context of primary infection, we could not include the time intervals the way that the Lilleri *et al* (2013) study did (≤30 days, 30–60 days, >60 days after onset of infection) since we do not know when the CMV infection was that led to transmission. In other words, we were unable to pinpoint the timing of the maternal HCMV infection(s) that resulted in transmission to the foetus. However, we selected three epitopes (within pentamer) where the higher levels in non-transmitters with primary HCMV infection continued until 60 days after the onset of symptoms, and longer for one epitope (Lilleri *et al.*, 2013), to maximize our opportunities to detect such associations in this study. It is also conceivable that we got a different answer than Lilleri *et al* (2013) because we have so many HIV-1 positive mothers and the Lilleri *et al* (2013) study probably had very few (Lilleri *et al.*, 2013). However, that doesn't seem to be the case because when we analysed maternal HIV-1 negative pairs only we get the same answer (i.e. our conclusion didn't change, (Dorfman *et al.*, 2021). The way our study was designed (control infants matched by maternal HIV-1 status) could lead to somewhat obscured effects of HIV-1 status that are not independent of transmitter status. However, our observations are not consistent with the absence of a protective immune response in HIV-positive mothers that could explain their increased risk of *in utero* HCMV transmission. Last, this study utilized samples from a prior study of mothers of congenital cases and controls. In that study matching was done primarily on infant characteristics, not maternal characteristics. Thus, transmitting mothers were younger than non-transmitting mothers and had fewer previous pregnancies. We could not rule these variables out as confounders of our result.

Mothers living with HIV-1 are at higher risk (about 2.5-fold higher) of transmitting HCMV to their infant during pregnancy (Manicklal *et al.*, 2013; Pathirana *et al.*, 2019). Therefore, we considered that a gap in maternal HCMV control could result in this increased risk, and that such increased could be due to lower protective antibody levels. Only two of our measures were significantly different between the groups- total antigen-specific gB

antibodies and 11B12 epitope-specific antibodies. These differences were also in the direction that did not support the theory that the measured antibodies were protective.

In conclusion, the correlations we found in our study of mostly or all non-primary maternal infection were in the opposite direction compared to the observations made in the Lilleri *et al* (2013) study of primary maternal infection. Thus, an HCMV vaccine that elicits an antibody response which includes pentamer or gB could possibly reduce *in utero* HCMV transmission in settings with a high prevalence of HCMV exposure in women prior to pregnancy. However, if so, it is very unlikely that antibody would be the mechanism by which to achieve this reduction, or that antibody levels would be the direct correlate of protection. Further studies are needed to elucidate what maternal immune responses can mitigate the risk of congenital CMV infection. It is also worth noting that cell mediated immunity is thought to play a substantial role in preventing in utero transmission of HCMV, although there are no studies of which we are aware that address this point conclusively. In some ways, this is the major conclusion of this study's work – if not antibody responses, what else could be the key factor(s) in preventing in utero transmission of HCMV?

Overall, this study has aided in the understanding of HCMV transmission in the setting of non-primary maternal infection in South Africa. This data also serves as a valuable tool in future studies assessing HCMV and HIV-1 co-infection and has substantial implications regarding HCMV vaccine development.

CHAPTER 5: REFERENCES

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CHAPTER 6: APPENDICES

6.1 APPENDIX 1: GENERAL PROTOCOLS

6.1.1 Agarose gel electrophoresis

UltraPure^{TM} Agarose (Invitrogen, USA) was used in the preparation of 1% agarose gels for electrophoretic separation of DNA fragments based on molecular weight. The required mass of 1 g UltraPure^{TM} Agarose was dissolved in 1x Tris Acetate electrophoresis (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; Thermo Fisher Scientific, USA) using the microwave, supplemented with 0.5 µL/mL ethidium bromide (EtBr). After swirling the flask, the liquid was poured into the gel tray and a comb was inserted. After the gel had set in the tray, it was placed into the electrophoresis chamber and the comb was removed carefully. The chamber was filled with 1x TAE buffer covering the entire gel and wells. The 1% gels were resolved at 80 V. An image of all gels was taken using UV light in the ChemiDoc™ XRS+ imager and the exposure times were altered depending on EtBr-stained DNA band intensities.

6.1.2 Gel extraction

Gel extraction was carried out using the QIAquick® Gel Extraction Kit protocol (Qiagen, Hilden, Germany) and all centrifugation steps carried out at room temperature at 17900 *g* (Centrifuge 5424, Eppendorf, Germany). After excision of the product from the agarose gel using a clean sharp scalpel and an ultraviolet (UV) light box (UVP Inc., California, USA) for visualization, taking care to reduce exposure, the gel piece was placed into a previously weighed 1.5 mL microcentrifuge tube. The tube were then weighed again for determination of the gel mass and three times the equivalent volume of Buffer QG to gel mass was added to the tube. The gel was dissolved in solution at up to 50°C using a heating block for under ten minutes. If the solution did not turn yellow, 10 µL of 3 M sodium acetate (pH 7.0) was added and the tube was mixed by vortexing. One gel volume of isopropanol was added and the tube inverted to mix. The sample was then added to a QIAquick spin column in a 2 mL flow-through tube, centrifuged for one minute and the flow-through was discarded If multiple gel samples were to be run through one column, the steps were repeated from the addition of isopropanol up to this point, thereafter, 750 µL of Buffer PE (with ethanol) was added. The column was centrifuged for one minute.

The flow-through was discarded and to remove residual wash buffer, the column was further centrifuged for one minute. The column was then placed in a 1.5 mL microcentrifuge tube and 50 µL of EB elution buffer was applied to the filter. After letting this stand for a minute, the tube was centrifuged to elute the DNA and the concentration was determined using the Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

6.1.3 Minipreps

Minipreps of DNA plasmids were done according to the QIAprep® Spin Miniprep Kit protocol (Qiagen, Hilden, Germany). Bacterial colonies previously grown in 2 mL of LB/KAN at 37 °C were spun down for 20 minutes at 6500 *g* in the Centrifuge Universal 320R (Labotec, South Africa) and the supernatant removed. The bacterial pellet was fully resuspended in 250 µL resuspension Buffer P1 (containing RNase A to remove any bacterial RNA) and transferred to a 1.5 mL microcentrifuge tube. To the tube, 250 µL Lysis Buffer P2 containing LyseBlue reagent was added and the tube was inverted up to six times to mix for no longer than three minutes. Subsequently, 350 µL Neutralization Buffer N3 was added and the tubes were immediately inverted up to six times for mixing, seen by the colour change from blue to a milky white solution. After centrifugation (Centrifuge 5424, Eppendorf, Germany) for ten minutes at 17900 *g*, the supernatant was transferred to a 2 mL QIAprep ® Spin column and further centrifuged for a minute at 17900 *g*. The flow-through was discarded and the column was wash the using 750 µL Buffer PE. This was then centrifuged for a minute at 17900 *g* and the flow-through was again discarded. After a further one minute centrifugation to remove residual Buffer PE, since the ethanol could reduce the quality of the eluted DNA, the columns were placed into sterile 1.5 mL microcentrifuge tubes. The DNA was eluted using 50 µL EB Buffer by firstly letting it stand for 5 minutes and then centrifuging for another minute at 17900 *g*. The sample concentration was determined using the Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

6.1.4 Maxipreps

Maxipreps of DNA plasmids were done according to the QIAGEN® Plasmid Maxi Kit protocols (Qiagen, Hilden, Germany). Bacterial colonies previously grown in 120 mL of LB/KAN broth at 37°C were spun down for 20 minutes at 10000 *g* in the Centrifuge Universal 320R (Labotec, South Africa) and the supernatant removed. The pelleted bacterial cells were resuspended in 8 mL Resuspension Buffer P1 (containing RNase A). An equivalent volume of Lysis Buffer P2 was added and the tubes inverted thoroughly to mix for no longer than three minutes. Thereafter, 8 mL of Buffer S3 was added to the lysate and the tubes were vigorously mixed up to six times. The lysate was then transferred to the QIAfilter cartridge and incubated for ten minutes at room temperature. During the incubation time, the vacuum manifold and QIAGEN Plasmid *Plus* Maxi spin columns were prepared. The plunger was inserted into the QIAfilter cartridge and the lysate was filtered into a new tube. Subsequently, 5 mL BB Buffer was added to cleared lysate and this was transferred to the spin column using a tube extender. The DNA was washed using 0.7 mL Buffer ETR using a vacuum. A further washing step of the DNA was done with 0.7 mL PE Buffer using a vacuum. Residual was buffer was removed by centrifuging the sample for a minute at 10000 *g*. The column was placed into sterile 2 mL microcentrifuge tube and the DNA was eluted by centrifugation after adding 400 µL EB Buffer. The sample concentration was determined using the Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

6.1.5 Protein A chromatography

After the seven-day transfection, the transfected cells were transferred from the conical flask to a falcon tube (50 mL) that was then spun down at 4 °C for 20 minutes at 6500 *g*. The supernatant, containing the antibodies, was transferred to a stericup filter (Millipore Express). Separately, 1 mL of Protein A-Agarose was added to a falcon tube (15 mL) and made up to 15 mL using PBS. This was then centrifuged at 4 °C for 3 minutes at 6500 *g* the supernatant was discarded and PBS was added again making up 15 mL. After further centrifugation, the supernatant was discarded and 0.5 mL PBS was added to 1 mL Protein A-Agarose. To the falcon tube containing the filtered antibody, 1 mL of Protein A-Agarose was added and shaken in a cold room overnight. This was then filtered through a column

and washed twice with PBS, ensuring that the Protein A-Agarose beads were always immersed in liquid. The column was plugged at the bottom using a stopper. To a 50 mL falcon tube, 800 µL neutralization buffer was added and this tube was placed under the column. Subsequently, 7.2 mL of elution buffer was added to the column and the stopper was removed. This liquid was filtered into a filter falcon tube (50 mL) and centrifuged with PBS twice at 4 °C for 20 minutes at 6500 *g*, with the flow-through being discarded each time. The antibody was transferred to an Eppendorf tube and the concentration was determined using the Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

6.2 APPENDIX 2: FIGURES AND TABLES IN RESULTS SECTION

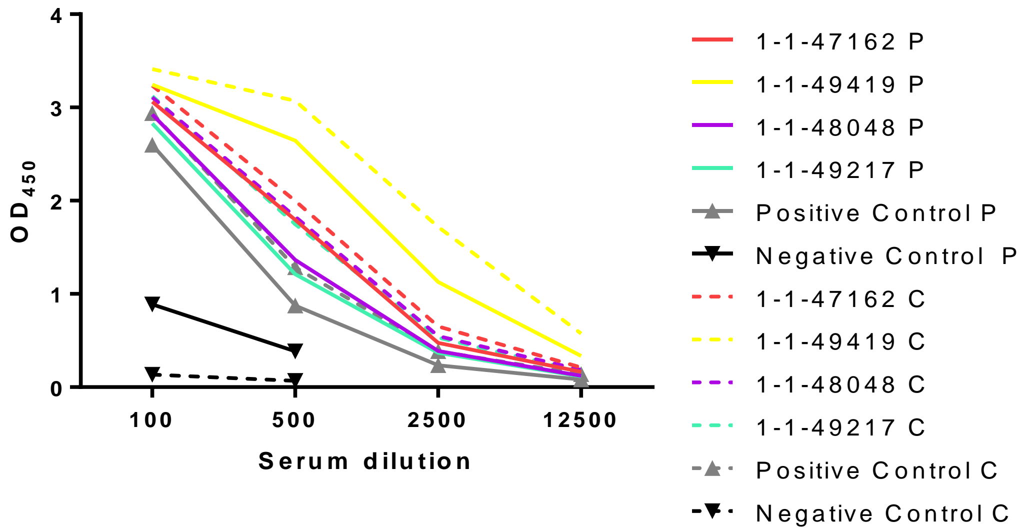
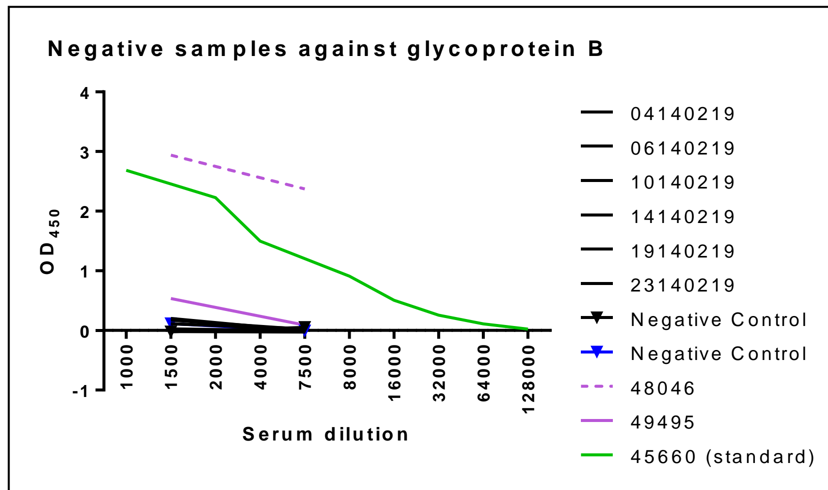


Figure A1: Phosphate buffer saline versus carbonate buffer pH 9.6. The positive (light upward triangles), negative (black downward triangle) and four maternal sera (red, yellow, purple, blue) were tested against pentamer prepared in PBS (solid lines, designated with P) and carbonate buffer pH 9.6 (dashed lines, designated with C). All the sera were diluted down 4-fold from 1 in 100.

(a)



(b)

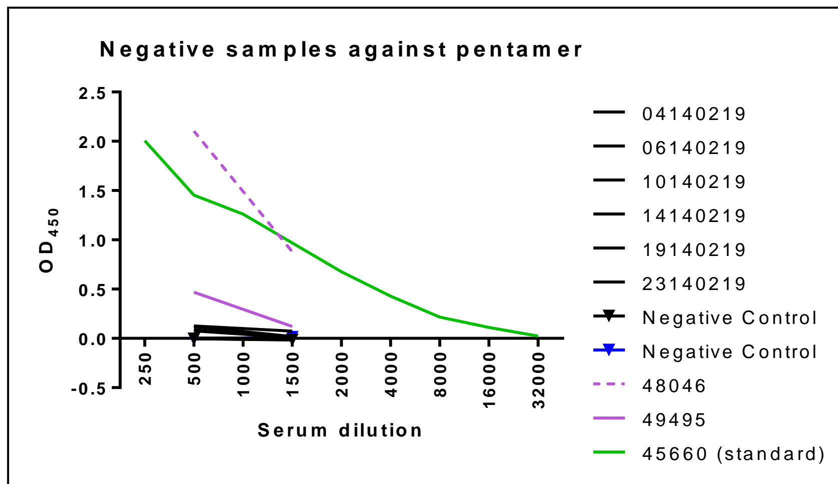
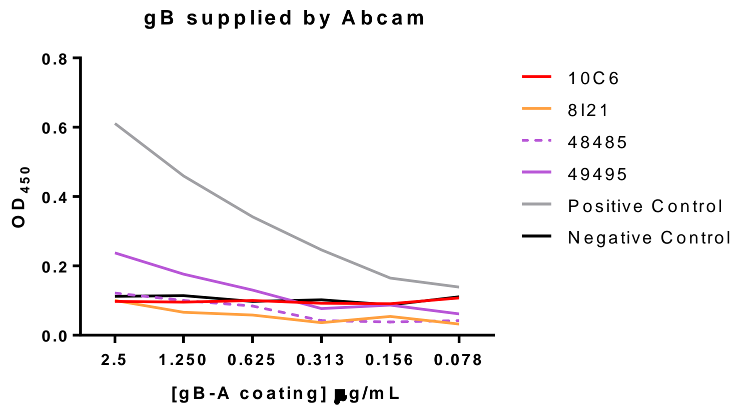


Figure A2: Standard ELISA experiments to determine a negative cut off value. (a) Negative samples (solid black, black and blue downward triangles) were added in two dilutions as 1 in 1500 and 1 in 7500 against gB-His. Two maternal samples (purple solid and dashed) were added as positive controls with varying reactivity responses. **(b)** Negative samples were added in two dilutions as 1 in 500 and 1 in 1500 against pentamer. Two maternal samples added as positive controls showed varying reactivity responses. Sample 45660 was added as a standard in 2-fold dilutions from **(a)** 1 in 1000 against gB-His and **(b)** 1 in 250 against pentamer.

(a)



(b)

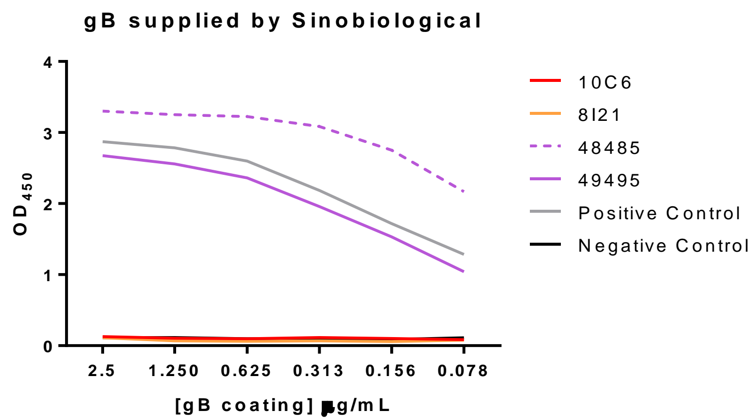
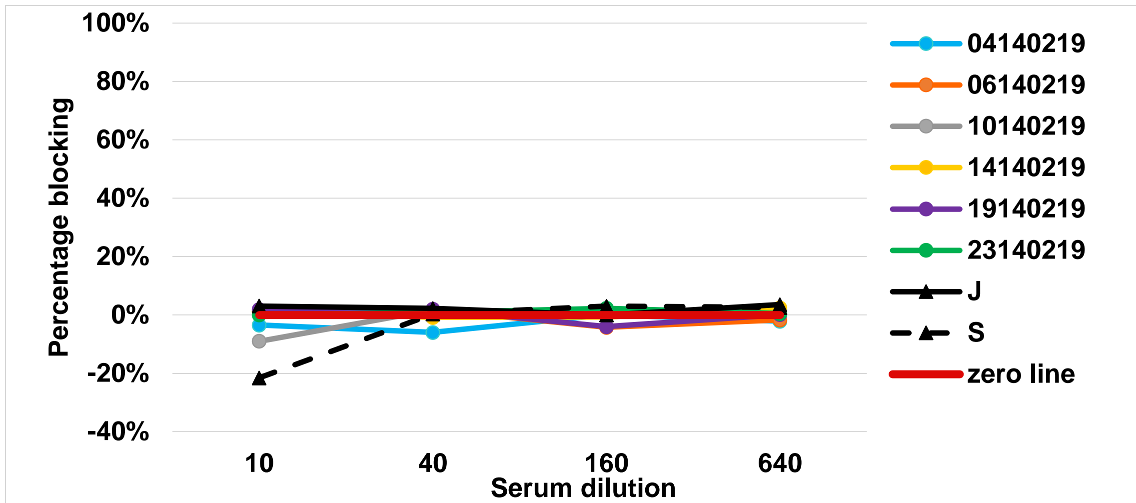
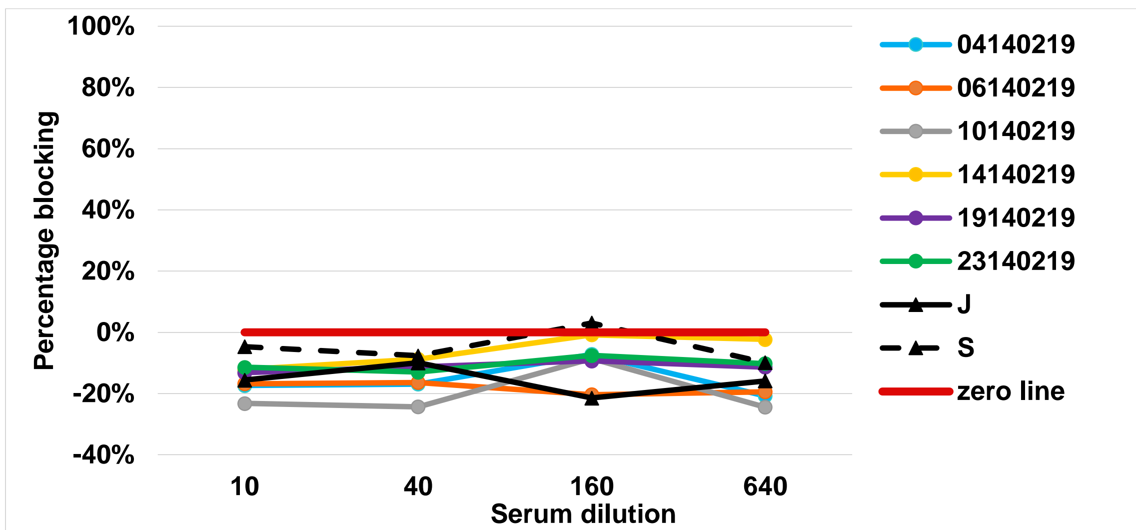


Figure A3: Testing monoclonal antibody 10C6 against glycoprotein B from different suppliers. Monoclonal antibodies 10C6 (red), 8I21 (orange), two maternal sera (purple sold and dashed), positive (grey) and negative (black) controls at 1 in 200 were tested against (a) gB-A supplied by Abcam and (b) gB-His supplied by Sinobiological, both titrated 2-fold from 2.5 µg/mL.

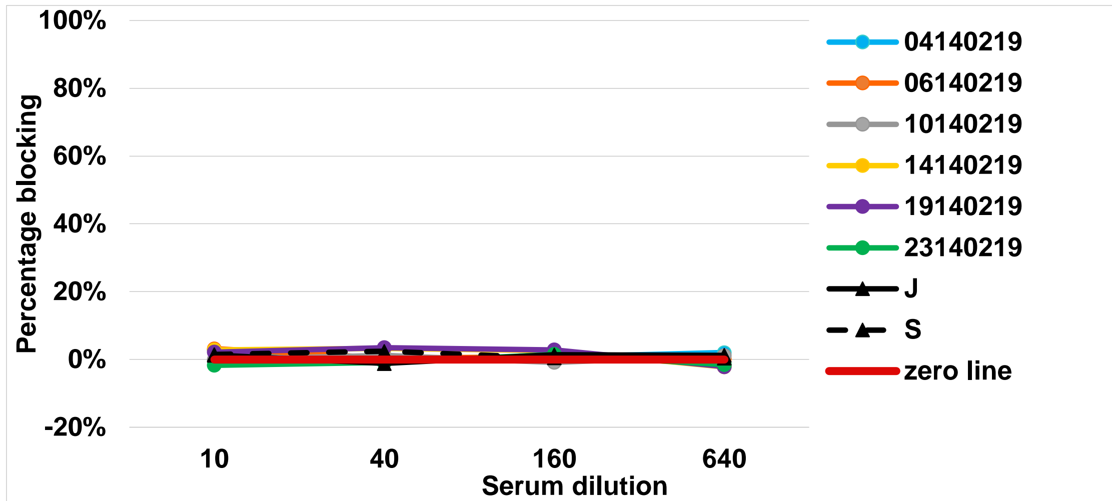
(a) 11B12



(b) 10P3



(c) 8I21



(d) 6B4

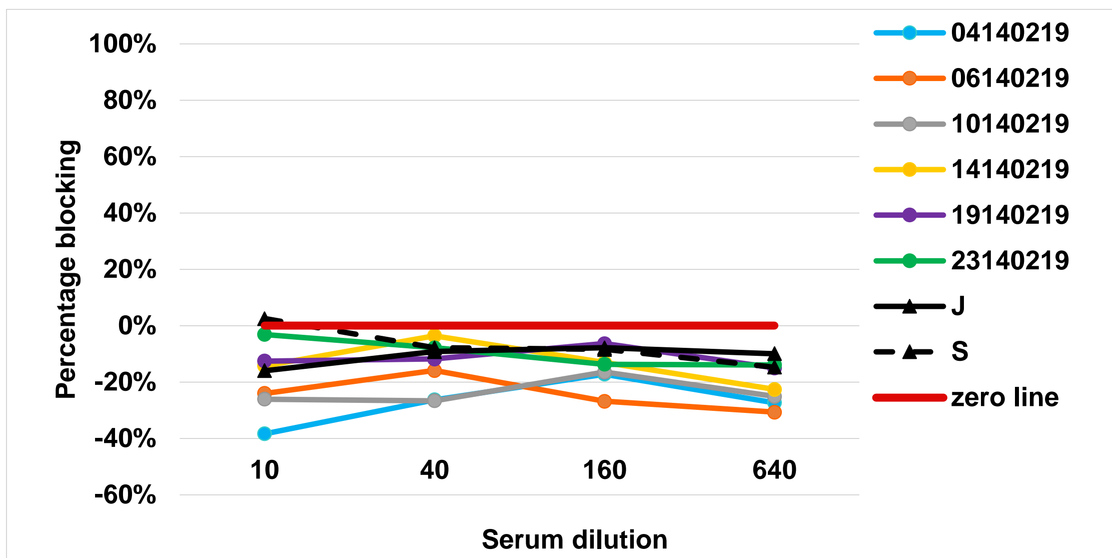


Figure A4: Testing samples from the (presumed) HCMV unexposed individuals in blocking ELISA experiments. All 8 HCMV negative samples (each with designated colour/pattern) were tested in blocking ELISAs against **(a)** 11B12, **(b)** 10P3, **(c)** 8I21 and **(d)** 6B4.No detectable activity was found. The zero line is shown in red.

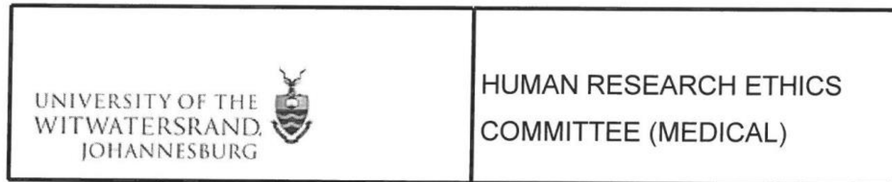
Table A1: Maternal antibody concentration against pentamer and glycoprotein B (His Tag)

Sample code	Concentration (au) against pentamer	Concentration (au) against gB	Sample code	Concentration (au) against pentamer	Concentration (au) against gB	Sample code	Concentration (au) against pentamer	Concentration (au) against gB
47162	0.52	0.11	**47378**	0.89	0.15	**47488**	0.05	0.22
45660	1.16	1.33	**47512**	0.38	0.26	**47395**	0.09	0.52
48048	0.48	0.21	**48067**	0.46	0.29	**46392**	0.15	1.24
47888	0.52	0.19	**48751**	2.05	0.12	**46219**	0.81	0.28
49661	0.21	0.10	**48789**	1.38	0.34	**47511**	1.05	1.27
48095	0.23	0.05	**48907**	0.29	0.33	**48857**	0.09	0.06
49217	0.44	0.13	**48913**	0.62	0.38	**46774**	0.42	0.27
49690	0.77	0.19	**49312**	0.20	0.14	**46811**	0.23	0.39
49643	1.02	0.10	**49347**	1.45	0.74	**46888**	1.22	0.06
49500	0.74	0.10	**49505**	0.36	0.15	**47061**	1.41	0.16
49495	0.07	0.06	**49750**	3.40	0.95	**47258**	0.77	0.14
49419	1.12	0.13	**49921**	0.49	0.29	**47268**	1.46	0.34
49145	0.53	0.15	**49946**	0.22	0.48	**50944**	0.33	0.11
49144	0.90	1.71	**50008**	0.22	0.28	**50954**	0.55	0.15
48859	0.52	0.33	**50260**	0.14	0.07	**50955**	0.18	0.06
47305	1.30	0.99	**50309**	0.36	0.26	**50985**	0.14	0.09
47743	0.84	0.73	**50439**	3.92	0.94	**53178**	0.77	0.12

46821	0.38	0.11	50455	2.24	0.84	53507	0.36	0.12
45154	0.32	0.14	50490	2.62	0.40	54098	0.52	0.23
48485	2.19	0.23	50500	4.22	1.02	50995	1.08	0.09
48528	0.29	0.12	50504	1.88	0.25	51028	0.13	0.16
48322	0.15	0.09	50506	0.18	0.07	47510	0.15	0.21
48285	0.82	0.86	50529	0.58	0.15	46694	0.93	0.43
48046	0.47	0.92	50564	3.45	0.60	50859	0.54	0.14
48043	0.82	0.36	50651	3.20	0.35	53152	0.39	0.12
47662	0.79	0.38	50652	0.75	0.34	44952	1.63	0.82
47931	0.74	0.79	50747	1.14	0.27	45015	1.02	0.28
47621	0.22	0.20	50770	1.49	0.12	45123	1.29	1.01
50178	0.13	0.08	51118	0.12	0.04	45422	0.96	0.24
50314	0.12	0.22	51248	0.16	0.07	45425	0.16	0.05
50236	0.79	0.01	51312	1.54	0.66	45606	0.38	0.10
50362	0.16	0.06	51360	1.18	0.94	45741	0.78	0.25
50436	0.44	0.29	51361	0.29	0.06	45825	0.38	0.15
49721	1.49	0.48	51433	0.79	0.29	45908	0.94	0.66
49155	0.55	0.18	51552	0.70	0.16	46002	0.27	0.08
48759	0.57	0.12	51626	0.71	0.29	46182	0.24	0.17
40561	0.15	0.05	51784	0.11	0.05	46480	0.39	0.09
42540	0.20	0.07	51873	0.13	0.03	46523	2.39	0.05
42924	0.35	0.08	51885	0.50	0.09	46556	0.37	0.20

43695	0.65	0.09	**51897**	0.17	0.07	**52319**	0.51	0.24
44404	3.38	5.44	**51931**	0.11	0.05	**52657**	0.40	0.25
44851	0.15	0.33	**51953**	0.68	0.37	**52679**	0.39	0.10
52716	0.68	0.24	**52025**	0.30	0.05	**52221**	0.18	0.16
52938	0.65	0.23	**52073**	0.95	0.28	**52245**	0.33	0.29
53126	0.25	0.11	**52076**	0.38	0.19	**52131**	0.58	0.20
52238	0.27	0.29	**52101**	0.53	0.14			

6.3 APPENDIX 3: ETHICS CLEARANCE CERTIFICATE



Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms S Balla et al
School of Pathology
Deparment of Virology
Centre for HIV and STI's
National Institute for Communicable Diseases

E-mail: sashkiab@nicd.ac.za

CC: Supervisor: Professors P Moore and J Dorfman <pennym@nicd.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 15/08/2018

REF: *R14/49*

PROTOCOL NO: **M180617** *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Humoral immune correlates of protection from mother-to-foetal transmission of cytomegalovirus*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

6.4 APPENDIX 4: PLAGIARISM DECLARATION

S. Balla 30 July 2021

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Sashkia Balla (Student number: 791249) am a student registered for the degree of Master of Science (MMed) in the academic year 2021.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: S. Balla Date: 5 March 2021

6.5 APPENDIX 5: EXAMINER 1 FEEDBACK

6.5.1 Examiner 1 Comments

SPECIFIC QUESTIONS TO BE ANSWERED BY THE EXAMINER OF A DISSERTATION SUBMITTED FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICINE

This questionnaire should be returned to the Postgraduate Office within 6 weeks of receipt of the thesis. PLEASE ANSWER [YES] OR [NO]:

1. Is the dissertation satisfactory as regards literary style and presentations? **[Yes]**

2. Does the dissertation reveal an adequate acquaintance with an understanding of the methods of research? **[Yes]**

3. Does the dissertation reveal originality of approach or involve original research? **[Yes]**

4. Is the dissertation adequate as the **sole ground** for the award of the degree? **[No]**
   - If **No** – kindly move to point numbered 5.
   - If **Yes** – kindly specify how you recommend award of the degree:

	Y/N
With distinction, and no corrections?	
With distinction, subject to minor corrections to the satisfaction of the Head of Department?	Y
Without distinction, with no corrections?	
Without distinction, subject to minor corrections to the satisfaction of Head of Department?	
Without distinction, subject to substantial amendments to the satisfaction of the Head of Department?	

5. If you have answered **"No"** to (4), do you recommend:

	Y/N
Substantial amendments, and re-examination?	
Major revision, extension or elaboration and re-examination?	
Do you wish to re-examine the revised dissertation?	
Reject the dissertation outright?	

6. The names of Examiners are confidential, but successful candidates may be told the names of their examiners with their consent. Would you agree to your name being divulged in this case? **[Yes]**

Kindly use the box below for your report. Please add additional pages should you require additional space for the report. **An examiner's report is mandatory.**

EXAMINER'S REPORT:

I hereby confirm the examination of the submitted MSc dissertation by Sashkia Balla (Student number 791249) entitled "Humoral immune correlates of protection from mother-to-foetal transmission of cytomegalovirus," and I provide the following report for review.

Summary:
I would like to take this opportunity to congratulate the student Sashkia Balla and the supervisors for a well-designed and executed study. In particular, I would like to credit the candidate with numerous iterations employed to optimize the ELISA and ensure the functionality of the assay, overall demonstrating the candidate's technical ability and expertise with the described methods. In view that the corrections are relatively minor in nature and that the data generated by the candidate is expertly presented and of high quality resulting in a publication. In addition, the candidate provided an excellent introduction to the topic and the results were more than adequately discussed, and contrasted against the current findings within the field, displaying the originality of the research. I therefore recommend the award of **Master in Medicine (by research) with distinction**, subject to the minor corrections to the satisfaction of Departmental Head. Congratulations!

Minor corrections and suggestions are provided below:

1. Pg 3, 2nd paragraph – pneumonitis – by definition refers to non-infectious cause of lung infections and thus in relation to active HCMV infection may not be correctly used.
2. Pg 4, 1st paragraph – Statement "HCMV include HIV-1 infected people who are progressing towards disease" suggest rephrasing this statement as "progressing towards disease" needs to be put into context (i.e. does this refer to advance disease HIV-1 patients (i.e. not on treatment, or with drug resistance causing immunological failure) or are HIV-1 patients infection and on treatment still viewed as vulnerable?
3. Statement – "Various factors influence the clinical consequences of cCMV, including gestational age at time of infection, whether the maternal infection is primary or non-primary, as well as the viral load present in bodily fluids of new-born from the mother." Suggest rephrasing of this statement "... viral load present within the new-born following transmission."
4. 2nd paragraph – "general hygiene practices" this is statement seems a bit contradictory in this context of this paragraph and perhaps clarification of "general" practices and the influence on transmission is required, as general contact is not considered to be a route of transmission.
5. 3rd paragraph – "There are three main transmission pathways - intrapartum, through breast milk (postnatal transmission), and transplacental transmission." Suggest rephrasing this sentence to "transplacetal, intrapartum and postnatal transmission/routes."
6. 3rd paragraph – "Both intrapartum and postnatal delivery (breast milk) pathways are associated with maternal shedding.." Suggest rephrasing "Both intrapartum and postnatal transmission pathways.."
7. Pg 5, 2nd paragraph – "cCMV infection that occurs through these two transmission routes (intrapartum and breast milk) correlates with little to no clinical illness, except in the case of very low birth weight premature newborns, but these modes of transmission remain key to the epidemiology of infection" Suggest revision and separation of this sentence in two.
8. Pg 6, 1st paragraph – "may prevent transplacental transmission and resulting infection of the fetus." Consistency in the use of spelling foetus vs fetus. (pg 89)
9. Pg 7, 1st paragraph – "A study suggested correlated of protection from foetal transmission" correlates
10. Pg 8, 1st paragraph – "These reported values are all considerably higher than the expected rate of about 0.7% for infants born to HIV-infected mothers." Revision HIV uninfected/non infected mothers?
11. 2nd paragpraph – Consistency in the referencing style used. "Gantt et al 2016 reported that..." and "Additionally, Bialas *et al* 2015 used their recently.." standardise the format as per the previous paragraph e.g. "Manicklal *et al* (2013) and Duryea *et al* (2010) both reported an..."

12. Pg 9, 1st paragraph – "Therefore, it is vital to better understand the effect of maternal HIV-1 infection on cCMV infection, as the number of HIV-1 exposed, CMV-infected infants with lasting neurodevelopmental effects increases in the ART era because of the successes of prevention of mother to child HIV- with HIV-1 (Bialas et al., 2016)." Long sentence, suggest revision.
13. Pg 12, 1st paragraph – "Additionally, the great coding capacity and extensive mRNA splicing results in other minor viral matrix and envelope components involved in viral infection." Suggest inclusion of literature references that support this statement or indicate if it just currently hypothesised to be the case.
14. Page 15, Figure 1.5 – "Although gB is used to enter multiple cell..." multiple cells
15. Pg 16, 1st paragraph – "Thus, each monomer consists of a large ectodomain surface subunit (gp116), a hydrophobic membrane-proximal region, a small type 1 transmembrane domain (gp58), and the cytoplasmic domain." Suggest moving gp58 to the end of the sentence as it includes PR, TM and cytoplasmic regions.
16. Pg 18, 1st paragraph – "This means that gB undergoes a conformational change from monomer to homotrimer and the energy released from this refolding results in the fusion of the viral envelope and host cell membranes during entry..." suggest clarification of this statement as the trimerization is not responsible for membrane fusion but rather the re-ordering of the domains within these gB subunits that result membrane fusion. Suggest rephrasing of this statement accordingly.
17. Pg 21, 3rd paragraph- "..mother to child.." (and pg 9, 49, 88 etc) check consistency in usage verses mother-to-child (pg 24, pg 26, pg 88). Correct throughout.
18. 3rd paragraph – "Anti-gB IgG mAbs have been shown to protect human trophoblast progenitor cells, the precursors to placental cells, by blocking placental trophoblast infection while anti-pentamer IgG Abs could not trophoblast progenitor cells (Schleiss, 2017)." Sentence requires correction.
19. Pg 22, 1st paragraph – "Lilleri also showed that the IgG levels for Abs specific to pentamer, but not gB, were higher in maternal non-transmitters to the transmitters within 30 days after HCMV infection (Lilleri *et al.*, 2013)" correct the literature format used (Lilleri verses Lilleri et al (2013) and the underlined section requires revision.
20. Pg 23, 2nd paragraph – "The three pentamer epitopes that we chose were chosen because they are targets.." suggest revision of this sentence.
21. Pg 26, 1st paragraph – "Antibodies to target proteins gB and pentamer..." that
22. Pg 28, 2nd paragraph – "a total of 3,274 children will enrolled, of which 2,908 mothers consented.." were.
23. Pg 31, Figure 2.2 – Reference made to Figure 2.2 under section 2.2.1 states "For this study, five monoclonal antibodies (mAbs), namely 11B12, 8I21, 10P3 (targeting pentamer), 10C6 and 6B4 (targeting gB), were synthesized (**Figure 2.2**)." Yet in Figure 2.2 there is no mention that the construct design was specific for production of antibodies. In addition "The samples were then transfected in to produce.." requires correction. Overall I felt that Figure 2.2 was unnecessary given the thoroughness of subsequent the methods section. Consider removal of Figure 2.2 or carry out corrections as suggested.
24. Pg 32, 1st paragraph – "For this study, five monoclonal antibodies (mAbs), namely 11B12, 8I21, 10P3 (targeting pentamer), 10C6 and 6B4 (targeting gB), were synthesized." Suggest rephrasing to "constructs were synthesised" or "were expressed/produced".
25. 2nd paragraph – would caution against the use of Fast Digest (as this refers to a commercial brand of restriction enzyme). Consider rephrasing to "restriction endonuclease digestion". (Change throughout)
26. Section 2.2.1 – suggested additions to this section - it is not explicitly stated that the variable fragments of the heavy and light encoding region were synthesised. In addition, only restriction of the heavy chain backbone plasmid is mentioned, the light chain vector should also be indicated.
27. Pg 34, 1st paragraph – The paragraph detailing the T4 ligation reaction should be revised into the 'past tense'.
28. Section 2.2.3 – heat shock transformation protocol has been omitted from this section.
29. Pg 38, section 2.5 – "and ensure not impurities or aggregations were.." suggest rephrasing this statement to "and to assess the purity (any aggregates are likely to be re-solubilised in the SDS-PAGE sample buffer thus information regarding aggregation is perhaps limited in this instance (further clarification of aggregate assessment maybe required if performed by the candidate).

30. Pg 40, 1st paragraph – "The stacking gel is much thinner and more fragile than the resolving gel. It contains less SDS..." Incorrect statement. The gels are the same thickness however, the stacker has a lower percentage of acrylamide and both gels contain a final concentration of 1% SDS.
31. Section 2.5.2 – DTT is not abbreviated nor is in the abbreviations list
32. Pg 50, Figure 3.1 and text below the figure – From the figure and the description provided it is unclear how the coating concentrations were selected based on the "window between negative and positive samples." Suggest inclusion of the description of the 'window' calculation and the minimal cut off values thereof. In addition, the positive samples used in the have no reference patient ID numbers as was used in the subsequent sections (e.g. section 3.2.2)
33. Pg 54, 1st paragraph – " Any maternal samples that produced a reading at or below 0.035 for pentamer and 0.0125 for gB-His were regarded as a negative sample." Below 0.035 for gB-His and 0.0125 for pentamer? In addition the cut-off for the negative control calculation for the pentamer is not shown. Consider inclusion in the appendix for instance.
34. Pg 55, section 3.2.4 – "The model tries to come up with a value for how different the cases are from the controls for everybody and then it.." suggest rephrasing to more scientific language.
35. Pg 56, Figure 3.3 – Limited data points are displayed on this figure. Suggest inclusion exclusively of the box and whisker plot as was done in the publication (Dorfman et al., Figure 1) or to overlay the box and whisker plot over the all the plotted data points if not too cumbersome.
36. Pg 57, section3.3 – "We also chose two epitope on glycoprotein B for comparison." Epitopes
37. Pg 58, 1st paragraph – "...which in turns translates to the correct amino acid sequences." In turn
38. Pg 58 2nd paragraph – "The sequences below allow us to determine the variability between the published sequences of our mAbs in the CDRs of the heavy and light chains." The amino acid alignments are not aligned to the to the published sequence in Figure 3.4. Therefore, this is an alignment of the selected antibody sequences and no comparison is made to previously published sequences. This confirmatory amino acid alignment could be moved to the appendix section. Suggest revision of this Figure accordingly.
39. Pg 62, section 3.3.4 – "...was nothing wrong with the CMV commercial IgG kit..."suggest rephrasing to more scientific language.
40. Pg 63, 1st paragraph – "This data shows that slight binding of anti-gB 10C6 occurs but only at high concentrations (20 μg/mL) while 11B12 was the only anti-pentamer mAb to show any binding." No data is shown for the 10c6 antibody binding to immobilized pentamer in Figure 3.6. Include data if available otherwise revise sentence accordingly.
41. Pg 64 1st paragraph & Figure 3.7 – "Two positive and two negative controls (immune and non-immune sera, respectively).." In this paragraph and in the figure legend (Fgiure 3.7) the candidate makes no mention of the incorporation of the 10c6 anti gB antibody specificity control within the assay. Suggest inclusion of a statement in this regard and correction of the Figure legend.
42. Pg 67, Figure 3.9a – The x-axis is incorrectly displayed and requires correction. In additional the Figure legend title states "Monoclonal antibodies tested..." where only the 10c6 single antibody was tested. Revise the figure legend accordingly.
43. Pg 67, Section 3.4.3 – **"Testing of Monoclonal Antibody 10C6 Against Recombinant Glycoprotein B-His– No Binding!"** Could be written in better scientific language, suggest revision.
44. Pg 68, Figure 3.10 – **"Monoclonal antibody 10C6 did not titrate down when tested against glycoprotein B measured at 450 nm"** Suggest rephrasing of this figure legend title, as there was no observable binding for titration to occur. Suggest rephrasing to "..not demonstrate binding to immobilised glycoprotein B..."
45. Pg 70 & 71, Figure 3.11 – Insufficient detail is provided concerning Samples 11 and 23 used. The candidate should be explicit in the description and the reason for their incorporation into the experiment and this should linked to the reference made in the text samples 49495 and 48285. The candidate also states "The positive and negative control sera produced the expected results." Which is perhaps not a true reflection of the data, considering such high OD readings where obtained for the positive control in the previous Figure 3.10 against the gB-His. At a minimum, acknowledgement of this discordance in the results should be stated, particularly if one assumes consistent usage of the positive control sera.
46. General comment regarding the sera samples used as positive and negative controls. It is unclear if the same samples were consistently used throughout the study as in certain ELISA experiments a

single positive and negative control was used (Fig 3.6, 3.9, 3.11, 3.12) whereas (3.10, 3.7) incorporated used two positive and two negative control and Figure 3.1 used two positive controls and a single negative. While the use of different positive and negatives controls would not affect the overall results, I would like bring this to the attention of the candidate particularly in instances where there has been a repeat of date (mentioned in comment 45 above) or for examples where perhaps the same control sera used for probing of the immobilized gB and pentamer (i.e. was this the same positive sera used). Suggest a clearer description of the positive and negative sera used throughout if it was consistently used, alternatively if there wasn't consistent usage of the control sera is this too should be stated.

47. Pg 76, Figure 3.14 – The full titration curve of antibody 6B4 against the immobilized gB is not shown in Figure 3.14d.
48. Pg 77, 1st paragraph – "From **Figure (a)** we observed a reduced.." Figure 3.14(a)
49. Pg 81, Section 3.8 – "To answer the question of our second aim.." second objective
50. Pg 81, Scetion 3.8.1 – "These were defined by monoclonal antibodies 8I21 (Site 7 gH,gL/UL128/UL130), 10P3 (site 4 on UL130/UL131) and 11B12, (site 9 on gH)..." It should be noted that this is the first time these antigenic sites have been described accordingly to 'numbered sites' Suggest inclusion of these numbered sites within the introduction, under description of the gB and pentamer antigens or the rephrasing of these targeted epitopes accordingly.
51. Similar to comment (35) the number of data points reflected on these box and whisker plot appear to be low, but this may formatting issue in the graphing software used. Suggest reformatting the figure where possible.
52. Pg 88, 2nd paragraph – "We observed that the transmitters had more blocking Abs for pentamer, particularly 10P3 and 8I21, than non-transmitters.." suggested revision "particularly antibody responses targeting the 10P3 and 8I21 epitopes." Also correct the 11B12 statement below this.
53. Section 6.1 appendix section – Suggest reporting the centrifugation speeds as x g,(as was done in the main methods section. In addition the numerical spacing using in the appendix sections differs from that use in the main methods section (13 000 vs 13000 etc).

Name of Candidate: Sashkia Balla..........

Name of Examiner: ....

Signature: Sashkia Balla..........

Date: 1 July 2021..........

Thank you for your help in the evaluation of our postgraduate students.

6.5.2 Examiner 1 List of Corrections

Introduction:

1. Pg. 3, 2nd paragraph, line 8 – pneumonitis – changed to "lung tissue inflammation"
2. Pg. 4, 1st paragraph, line 1 - sentence rephrased to: "Statement "HCMV include HIV-1 infected people who are progressing towards disease (both with and without treatment)"
3. Pg. 4, 1st paragraph, line 11 - rephrased as suggested.
4. Pg. 4, 2nd paragraph, line 2 – sentence rephrased to: "…the result of shedding HCMV in saliva, urine, and breast milk as well as lack of general hygiene practices (such as coughing or sneezing without covering the mouth)."
5. Pg. 4, 3rd paragraph, line 2 – rephrased as suggested.
6. Pg. 4, 3rd paragraph, line 5 – rephrased as suggested.
7. Pg. 5, 2nd paragraph, line 9 - sentence rephrased to: "cCMV infection that occurs through these two transmission routes (intrapartum and breast milk) correlates with little to no clinical illness, except in the case of very low birth weight premature newborns. Despite this, these modes of transmission remain key to the epidemiology of infection…"
8. Pg. 6, 1st paragraph (and pg. 89) – fetus – changed to "foetus"
9. Pg. 7, 1st paragraph, line 7 – rephrased as suggested.
10. Pg. 8, 1st paragraph, line 13 - sentence rephrased to: "These reported values are all considerably higher than the expected rate of about 0.7% for infants born to HIV-uninfected mothers."
11. Pg. 8, 2nd paragraph, lines 2 and 4 – Gantt et al 2016 and Bialas *et al* 2015 – changed to "Gantt *et al* (2016) and Bialas *et al* (2015)".
12. Pg. 9, 1st paragraph, line 5 - sentence rephrased/shortened to: "Therefore, it is vital to better understand the effect of maternal HIV-1 infection on cCMV infection, as the number of HIV-1 exposed, CMV-infected infants with lasting neurodevelopmental effects increases in the ART era."
13. Pg. 12, 1st paragraph, line 5 – literature reference "(Fields, Knipe and Howley, 2013)" included.
14. Pg. 15, Figure 1.5 – rephrased as suggested.

15. Pg. 16, 1st paragraph, line 6 - sentence rephrased to: "Thus, each monomer consists of a large ectodomain surface subunit (gp116), a hydrophobic membrane-proximal region, and a small type 1 transmembrane domain (gp58)"
16. Pg. 18, 1st paragraph, line 1 - sentence rephrased to: "This means that gB undergoes a conformational change from monomer to homotrimer. The re-ordering of the domains within these gB subunits and the energy released from this refolding results in the fusion of the viral envelope and host cell membranes during entry…"
17. Pg. 21, 3rd paragraph (and pg. 9, 49, 88 etc) – mother to child – changed to "mother-to-child"
18. Pg. 21, 3rd paragraph, line 5 – sentence rephrased to: "anti-pentamer IgG Abs could not protect trophoblast progenitor cells"
19. Pg. 22, 1st paragraph, line 6 – sentence rephrased to: "Lilleri *et al* (2013) also showed that the IgG levels for Abs specific to pentamer, but not gB, were higher in maternal non-transmitters compared to the transmitters, within 30 days after HCMV infection (Lilleri et al., 2013)" .
20. Pg. 23, 2nd paragraph, line 3 – sentence rephrased to: "The three selected pentamer epitopes were chosen because they are targets…"
21. Pg. 26, 1st paragraph, line 1 – rephrased as suggested.

Material and Methods:

22. Pg. 28, 2nd paragraph, line 2 – rephrased as suggested.
23. Pg. 31, Figure 2.2 – figure title and legend – rephrased to: "Figure 2.2: Flow diagram illustrating the approach taken in this study to synthesize monoclonal antibodies for standard and blocking ELISA experiments." and "…transfected in 293F cells…"
24. Pg. 32, 1st paragraph, line 2 – were synthesized - changed to "were expressed"
25. Pg. 32, 2nd paragraph (and throughout) – Fast Digest – "restriction endonuclease digestion".
26. Pg. 32, Section 2.2.1 – the methods contain the relevant details.
27. Pg. 34, 1st paragraph, line 1 - rephrased as suggested.

28. Section 2.2.3 – heat shock transformation protocol has been omitted from this section - there is no heat shock step in this protocol so no corrections have been made.
29. Pg. 38, section 2.5, line 1 – rephrased as suggested.
30. Pg. 40, 1st paragraph, line 6 – rephrased to: "The stacking gel contains less acrylamide, and is used to allow proteins to compact together into one band prior to entering the resolving gel for separation."
31. Section 2.5.2 – DTT – changed to "Dithiothreitol (DTT)" and added to abbreviations list.

Results:

32. Pg. 50, Figure 3.1 –figure legend – The coating concentration was based upon the OD_{450} readings. We looked at the readouts and decided to pick the lowest concentration at white the readout was near saturation (>approx. 90%). We did not use a calculation per se. In addition, there are no reference patient ID numbers for the samples, thus, these sample controls are simply referred to as "positive control" and "negative control"

    Note added to Figure 3.1 legend: "Note that there are no patient ID numbers for the control sera, thus are referred to simply as 'positive control' or 'negative control'."
33. Pg. 54, 1st paragraph, line 3 – sentence rephrased to: "Any maternal samples that produced a reading at or below 0.0054 for pentamer and 0.035 for gB-His were regarded as a negative sample.". The cut-off for the negative threshold calculation for the pentamer and gB are found in the appendix, Figure 2A.
34. Pg. 56, section 3.2.4, line 9 – sentence rephrased to: "The model effectively calculates how different each case is from its own controls for all samples and then uses a random effects component as a way to systematically adjust for the differences between groups – where a group is one case ant its controls. This will tell us if cases are consistently different from controls and if so, by how much.
35. Pg. 56, Figure 3.3 – Note added to figure legend: "This is the format of the figure, as the data points are within the boxes and the "outliers" are shown as dots." It is

worth mentioning that the Tukey method was used for the whiskers and dots in the plots (Krzywinski M, Altman N. Visualizing samples with box plots. Nat Methods 2014; 11:119–20). Additionally, the y-axes have been changed to logged values.

36. Pg. 57, section 3.3, line 5 - rephrased as suggested.
37. Pg. 58, 1st paragraph, line 7 - rephrased as suggested.
38. Pg. 58, 2nd paragraph – The sequences are published so this was simply included as conformation.
39. Pg. 62, section 3.3.4, line 2 - sentenced rephrased to: "The controls showed that the CMV commercial IgG kit worked as intended. "
40. Pg. 63, 1st paragraph, line 1 – sentence rephrased to: "The 11B12 mAb was the only anti-pentamer mAb to show any binding."
41. Pg. 64, 1st paragraph and Figure 3.7 – Figure legend rephrased to: "Anti-gB 10C6 (red) was included as a specificity control" and sentence rephrased to: "…lowest detectable concentration for use in subsequent experiments. Anti-gB 10C6 mAb was included and titrated, down in 3-fold steps, as a specificity control. Two positive and two negative controls (immune and non-immune sera, respectively)…"
42. Pg. 67, Figure 3.9a – The x-axis corrected and figure legend rephrased to: "Monoclonal antibody 10C6 tested…"
43. Pg. 67, Section 3.4.3 – heading - rephrased to: "No Binding of Monoclonal Antibody 10C6 Against Recombinant Glycoprotein B-His".
44. Pg. 68, Figure 3.10 – figure legend title - rephrased as suggested.
45. Pg. 70 and 71, Figure 3.11–Figure key changed to sample 49495 and sample 48285. Sentence rephrased to: "The positive and negative control sera produced the expected results, although the OD_{450} readings for the positive controls were much lower than observed in previous experiments."
46. There was consistent use of control sera, but as they did not have samples codes, they are referred throughout as positive or negative controls for simplicity. Sentence added to page 49, 3rd paragraph: "These HCMV immune and non-immune sera are consistently used as controls in downstream experiments and are simply referred to as positive and negative controls, respectively."

47. Pg. 76, Figure 3.14 – this was the entirety of the experiment. Note added to the figure legend: “Note that this was the entirety of the experiment as samples were limited at this point.”
48. Pg. 77, 1st paragraph, line 1 - rephrased as suggested.
49. Pg. 81, Section 3.8, line 1 - rephrased as suggested.
50. Pg. 81, Section 3.8.1, line 4 – sentence rephrased to: “These were defined by monoclonal antibodies 8I21 (Site found on gH,gL/UL128/UL130), 10P3 (site found on UL130/UL131) and 11B12, (site found on gH)…”
51. Pg. 81, Figure 3.16 - Note added to figure legend: “This is the format of the figure, as the data points are within the boxes and the “outliers” are shown as dots.”

Discussion:

52. Pg. 88, 2nd paragraph, line 4 - sentence rephrased to: “We observed that the transmitters had more blocking Abs for pentamer, particularly antibody responses targeting the 10P3 and 8I21 epitopes, than non-transmitters. However, the case is weaker for the antibody response targeting the 11B12 epitope…”
53. Pg. 108, Section 6.1 – rephrased as suggested.

6.6 APPENDIX 6: EXAMINER 2 FEEDBACK

6.6.1 Examiner 2 Comments

SPECIFIC QUESTIONS TO BE ANSWERED BY THE EXAMINER OF A DISSERTATION SUBMITTED FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICINE

This questionnaire should be returned to the Postgraduate Office within 6 weeks of receipt of the thesis. PLEASE ANSWER [YES] OR [NO]:

1. Is the dissertation satisfactory as regards literary style and presentations? **[Yes]**
2. Does the dissertation reveal an adequate acquaintance with an understanding of the methods of research? **[Yes]**
3. Does the dissertation reveal originality of approach or involve original research? **[Yes]**
4. Is the dissertation adequate as the **sole ground** for the award of the degree? **[Yes]**
   - If **No** – kindly move to point numbered 5.
   - If **Yes** – kindly specify how you recommend award of the degree:

	Y/N
With distinction, and no corrections?	
With distinction, subject to minor corrections to the satisfaction of the Head of Department?	Y
Without distinction, with no corrections?	
Without distinction, subject to minor corrections to the satisfaction of Head of Department?	
Without distinction, subject to substantial amendments to the satisfaction of the Head of Department?	

5. **If you have answered "No" to (4), do you recommend:**

	Y/N
Substantial amendments, and re-examination?	
Major revision, extension or elaboration and re-examination?	
Do you wish to re-examine the revised dissertation?	
Reject the dissertation outright?	

6. The names of Examiners are confidential, but successful candidates may be told the names of their examiners with their consent. Would you agree to your name being divulged in this case? **[Yes]**

Kindly use the box below for your report. Please add additional pages should you require additional space for the report. **An examiner's report is mandatory.**

EXAMINER'S REPORT:

Thank you for the opportunity to review and examine this excellent thesis. The study aimed to outline the significance of humeral immune response, specifically against pentamer and gB of human cytomegalovirus (hCMV), for the protection against in-utero mother-to-child transmission of hCMV among mothers with non-primary infection. I would award a distinction for Ms Balla's MSc based on the following:

- The thesis outlined a large amount of quality experimental work covering many techniques and is of high complexity for the level of MSc
- The candidate conducted a thorough literature review, logically contextualise the study and identified the relevant gap in current knowledge regarding congenital hCMV infection
- The research question was correctly framed given the knowledge gap, the objectives of the study were clearly defined and the experimental design was appropriate given the source study samples
- The study population and methodologies were clearly explained, with the aid of clearly labelled figures
- The data was suitably analysed, using the appropriate statistical tools and the results were clearly presented
- The discussion is excellent for the MSc level and the conclusion was supported by the data
- The majority of study findings were published in a high impact peer-reviewed publication during the course of MSc

I have only few minor corrects/comments which should be reviewed by head of department and does not require my further review:

Introduction:

- Page 5 refers to congenital CMV through breastfeeding. However, cCMV implies acquired in-utero (before birth) and therefore transmission cannot occur through breastfeeding. Overall, congenital CMV is central to the thesis and should be define clearly early on.
- Page 7 under 1.3 "Up to 50% of symptomatic children and 10% of otherwise asymptomatic children with cCMV infection have SNHL". Asymptomatic should not have hearing deficit, I think you mean asymptomatic at birth.
- Page 8 briefly described the association between CD4 T cell and cCMV infection (or the lack of inverse correlation in the local study). HCMV is a cell associated virus which universally establish persistence in its host. Thus, the role of cell mediated immunity should be highlighted in non-primary infection, as it would likely confound the study which looks purely as antibody response.
- Page 26 second objective only looking at pentamer epitopes, but the methods and results suggest gB epitopes were also assessed, just less successful than pentamer.

Material and Methods:

- Under maternal samples: Year and general source population demographics (only brief mention it is set in Soweto) should be described as this speaks to how study finding applies broadly.
- The timing (antenatal or post-natal) of maternal blood sample in relation to the infant diagnosis is unclear.
- CMV IgM can be positive in reactivation and re-infection and thus positive IgM does not confirm primary infection. In any case, it is unclear whether these IgM positive mothers were excluded from the study.
- Table 2.3 – under DNA "X" – is this standard way of expressing variable volume? Suggest an explanation in footnote or just put 300ng in that column.

Results:

- The numbers of transmitters and non-transmitters tested in each experiment should be stated in the results, as are the comparison between HIV-pos and HIV-neg women. See query under material and methods section.

Discussion and conclusion:

- Cell mediated immunity is understood to play a major role in CMV control. In the discussion, it should be highlighted at least as a plausible factor. Maternal CMV viral load were not measured, and this could also be a possible differentiator between transmitters and non-transmitters.
- Has the study considered using some of the antibody level as screening assay to gauge risk of in-utero transmission in high CMV prevalence settings where non-primary infections trasnmissions are common.

Name of Candidate: ...Sashkia Balla..

Name of Examiner: ......

Signature: Sashkia Balla ....

Date: ...... 1 July 2021

Thank you for your help in the evaluation of our postgraduate students.

6.6.2 Examiner 2 List of Corrections

Introduction:

- Page 5, 2nd paragraph, line 9 – sentence rephrased to: "HCMV infection that occurs through these two transmission routes (intrapartum and breast milk)…" Sentence added to page 4, 3rd paragraph: "While this study focuses of congenital CMV infection, via transplacental and intrapartum routes, all three transmission pathways are discussed."
- Page 7, 2nd paragraph, line 9 – rephrased as suggested.
- Page 8 briefly described the association between maternal $CD4^{+}$ T cell counts and cCMV infection that was observed in some but not all studies. This was to ensure that the reader is aware that antibody response is not the only aspect of immunity despite our study focusing purely on antibody response. It would be worth looking more in depth at cell mediated immunity as a separate study.
- Page 26 second objective – sentence rephrased to: "Measuring levels of maternal antibodies targeting specific epitopes within HCMV glycoprotein B…"

Material and Methods:

- Under maternal samples – 1st paragraph, line 2 and line 5 – changed to: "Blood samples from pregnant women enrolled at Baragwanath Hospital maternity department, or at several obstetric clinics linked to Baragwanath Hospital maternity department at time of labor were collected between July 2014 and December 2016, as part of a birth cohort study..." and "The pregnant women had to be ≥18 years of age and deliver a live birth at Chris Hani Baragwanath Academic Hospital, Soweto, to be eligible for the study under Protocol M140203..." To clarify, the samples we used were selected as a subset of a larger population study. More information on the maternal samples can be found in the paper by Madhi *et al* (2020).
- Samples were taken when the mother came in for delivery, and usually just after delivery. Below is an extract of Table 1 line 3 from the Clinical Infectious Diseases paper, published based on this dissertation, that describes the demographic information for mothers

Attribute	Transmitters [a], n=45	Non-transmitters [b], n=75	p value
Time from childbirth to sample collection, hours[d] Median (IQR) (range	2.9 (1.7-18.2) (-3.3 – 31.8)[e]	9.5 (1.4-20.0) (-9.2 – 54.6)[e]	0.4461

[a] mothers of congenital CMV cases
[b] mothers of matched CMV negative control infants
[d] negative value indicates sample collected before birth
[e] data missing for one study participant in each group (n=44 and n=74 shown)

- Page 29 – sentence included in paragraph: "All samples, including IgM positive samples, were included in this study."

  Page 86 and 89 – rephrased to: "mostly or all non-primary HCMV infection"

  We do not try to conclude that any material infection is primary, and we did not exclude the IgM positive samples (we are not making any strong conclusions about the IgM positive samples). Among the cases there were 4 IgM positives and among the controls there were 4 positives and 3 equivocal results. There are more controls than cases, however, it seems like the proportion is similar. Based upon this and based upon the fact that most studies (including our own – all IgG positive) see a very high seropositivity rate, it seems likely that many if not all the IgM positives are re-infections.
- Table 2.3 – "** X used to denote variable volume" added to footnote.

Results:

- Figure 3.3 legend and figure 3.16 legend have been edited to include the numbers of transmitters and non-transmitters tested in each experiment i.e. 45 transmitters and 78 non-transmitters. Page 83 – not added: "For transmitters, 17 samples are HIV-1 positive and 28 samples are HIV-1 negative, and for non-transmitters, 44 samples are HIV-1 positive and 34 samples are HIV-1 negative."

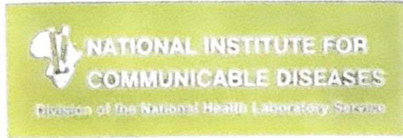
Discussion and Conclusion:

- Page 90, 1st paragraph – sentence added: "It is also worth noting that cell mediated immunity is thought to play a substantial role in preventing in utero transmission of HCMV, although there are no studies of which we are aware that address this point conclusively. In some ways, this is the major conclusion of this study's work – if not

antibody responses, what else could be the key factor(s) in preventing in utero transmission of HCMV? Regarding the comment on maternal HCMV viral load not being measured, thus, possibly being a differentiator between transmitters and non-transmitters, women with non-primary infection do not reliably she virus into any tissue, so it is not clear from what tissue one would measure such a viral load. Also, one would have to catch the virus during the infection that caused the transmission to the foetus.

- The study did look at the possibility of using some of the antibody level as screening assay to gauge risk of in-utero transmission in high CMV prevalence settings where non-primary infections transmissions are common. However, this would not be possible with this assay since there is no clear distinction between transmitters and non-transmitters. For example, looking at Figure 3.3, if a sample were to have an OD450 reading of 0.7, it would not be possible to determine if that sample was rom a transmitter or non-transmitter.

6.7 APPENDIX 7: HOD/HOS APPROVAL LETTER



Centre for HIV and Sexually Transmitted Infections
1 Modderfontein Road, Sandringham, 2031
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Tel: 011 386 6331

29 July 2021

The Postgraduate Office
Faculty of Health Sciences
University of the Witwatersrand

To whom it may concern,

Approval Letter: Corrections to MSc Dissertation – Sashkia Balla (Pathology/Virology)

This letter serves to confirm that all the required corrections have been made to the MSc Dissertation of Sashkia Balla (student number: 791249), to the satisfaction of his Supervisors and the Head of Department.

Yours Sincerely,

Prof. Penny Moore
Supervisor
NICD and University of the Witwatersrand

Prof Jeffrey Dorfman
Supervisor
RMPRU and University of the Witwatersrand

Dr. Florette Treurnicht
Head: Department of Virology
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