Betaherpesvirus genetic variation and infection in HIV-1 infected and 'HIV-1 exposed' Zambian children

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Abstract

The betaherpesviruses human cytomegalovirus (HCMV) and human herpesvirus 6 (HHV-6) are investigated here as pathogens in Zambia, an HIV-1 endemic region where there was little previous data. In particular we assess the effects of these viruses on 'maternally HIV-1 exposed' infants: HIV-1 negative infants of HIV-1 positive mothers. HCMV and HHV-6 are serious causes of morbidity and mortality in HIV/AIDS and are linked with AIDS progression, so in this thesis they are investigated in both HIV-1 infected and uninfected Zambian children, along with maternally HIV exposed infants, an under studied and growing group in Southern Africa. The aim of this thesis is to employ qualitative and quantitative PCR assays to determine betaherpesvirus prevalence, loads and genotypes in three independent Zambian paediatric cohorts: Two retrospective cohorts (141 infants hospitalized with fever and 36 childhood HIV-1 positive respiratory mortalities), and also one prospective cohort (812 infants taking part in a population-based study designed to test the efficacy of a micronutrient fortified feed supplement to improve growth and health) in which relationships between betaherpesvirus infection, duration of breast feeding, infant growth and morbidity were investigated. Prevalence and loads were highest within the symptomatic cohorts, although lower levels of both viruses were also detected in sera from healthy infants taking part in the prospective study. High load HCMV infections were shown here to be significantly more prevalent in maternally HIV-1 exposed infants. Genotyping analysis focused of two hypervariable glycoproteins (gO and gN), which in HCMV have been shown to form seven linked genotypes. Here we identified a new genotype (gN4d) and demonstrated linkage with gO5, demonstrating now eight gO/gN linkages. Analyses of this data and that generated in other countries show these linkages are globally maintained. Conversely for HHV-6, whilst HHV-6B is the predominant strain for childhood infections in the U.S, Europe and Japan, in Zambia HHV-6A was identified in 84% of infant infections, suggesting emergence elsewhere. The prevalence of active betaherpesvirus infections through detection of viral sera-DNA was 34-40% for HCMV and 8-13% for HHV-6, showing that primary infection with HCMV occurs much earlier in this region than in European and North American countries. Active HCMV infections were associated with inhibitory effects on growth and a trend for increased morbidity in HIV-1 exposed infants as measured by an increased rate of hospital referrals. HCMV seroprevalence was associated with anaemia and stunting, and breast feeding increased HCMV transmission, particularly in HIV-1 exposed infants. A micronutrient supplement with iron reduced anaemia. In summary, genotypes of HCMV and HHV-6 were identified and characterised in infant infections in this region, and analyses shows association with morbidity and growth delays for HCMV infected children, particularly with maternal HIV-1, a newly identified potential hazard for this population.

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List of Abbreviations

AIDS	Acquired immune deficiency syndrome
BMI	Body mass index
CI HHV-6	Chromasomally integrated human herpesvirus-6
CIGNIS	Chilenje Infant Growth Nutrition and Infection Study
DNA	Deoxyribonucelic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCMV/HHV-5	Human cytomegalovirus/human herpesivurs-5
HHV-6	Human herpesvirus-6
HHV-6A	Human herpesvirus-6 variant A
HHV-6B	Human herpesvirus-6 variant B
HIV-1	Human immunodeficiency virus-1
KSHV/HHV-8	Kaposi's sarcoma associated herpesvirus/human herpesvirus-8
LSHTM	London School of Hygiene and Tropical Medicine
PBMCs	Perepheral blood mononuclear cells
PCR	Polymerase chain reaction
PMTCT	Prevention of mother to child transmission of HIV
UTH	University Teaching Hospital

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Dedication

To the memory of Jonathan Chikwanda

1.0 Introduction

1.1 Herpesvirus classification

Over 200 herpesviruses have been identified, mostly from mammals, birds and reptiles, which together form the family *Herpesviridae*. More recently herpesviruses have been found in both fish and amphibians, and also oysters, which through sequence analysis have been assigned to separate families: The *Alloherpesviridae* and *Malacoherpesviridae* respectively. Together these three families form the order *Herpesvirales* which likely diverged from a pre-historic common ancestor, with some structural and sequence similarities to large tailed DNA bacteriophages (*Caudovirales*)(McGeoch, Rixon, and Davison, 2006). Sequence analysis of the family *Herpesviridae*, has identified three sub-families: The *alpha-*, *beta-* and *gammaherpesvirinae*, derived from a common ancestor over 400 million years ago (McGeoch and Gatherer, 2005). The *alphaherpesvirinae* were originally defined due to their broad host range, rapid growth in culture and ability to establish latency in neuronal cells. The *betaherpesvirinae* have a more restricted host range but a broad cell-tropism. They replicate more slowly *in vitro*, spreading in tissue culture through cell-cell fusion forming syncytia and latency is established in haematopoietic progenitors and monocytes/macrophages. *Gammaherpesvirinae* establish latency in lymphoid tissue and the human gammaherpesviruses HHV-4 (EBV) and HHV-8 (KSHV)(Table 1) are both tumorigenic, a unique feature among the human herpesviruses. Together eight herpesviruses are known to infect humans as shown (Table 1). Data from Europe and North America has shown that two betaherpesviruses, HCMV and HHV-6, are pathogens in infants and are the main viral opportunistic infections in HIV/AIDS causing both morbidity and mortality (Clark et al., 1996; Knox and Carrigan, 1994; Kovacs et al., 1999). In this thesis these viruses are examined in Zambia, a developing country in Southern Africa, an HIV-1 endemic region. Interactions are examined in HIV-1 co-infected and maternally 'HIV-1 exposed' but uninfected children, an increasing and under-recognized, poorly characterized group.

Virus name	Vernacular name (Abbreviation)	Sub-family	Genome size (kbp)	Genome Accession No.
HHV-1	Herpes simplex virus 1 (HSV-1)	α	152	NC_001806
HHV-2	Herpes simplex virus 2 (HSV-2)	α	155	NC_001798
HHV-3	Varicella zoster virus (VZV)	α	125	NC_001348
HHV-4	Epstein Barr virus (EBV)	γ	172	NC_007605
HHV-5	Human cytomegalovirus (HCMV)	β	236	NC_006273
HHV-6	Human herpesvirus 6 variant A (HHV-6A)	β	159	NC_001664
	Human herpesvirus 6 variant B (HHV-6B)	β	162	NC_000898
HHV-7	Human herpesvirus 7 (HHV-7)	β	153	NC_001716
HHV-8	Kaposi's sarcoma associated herpesvirus (KSHV)	γ	138	NC_009333

Table 1: The human herpesviruses

1.2 Betaherpesviruses

Betaherpesviruses are species specific with three members of this subfamily infecting humans: Human herpesvirus 5 (HHV-5) (common name: human cytomegalovirus, HCMV), human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7) (Table 1). Along with similar growth kinetics and sites of latency, sequence comparisons show similar gene organization, betaherpesvirus specific genes and closer similarity between conserved genes (Davison et al., 2003; Gompels et al., 1995). HCMV and HHV-6 are widespread, causing life-long latent infections which are usually immunologically controlled in healthy individuals. In the immunosuppressed setting these infections can reactivate causing serious and life-threatening illness, affecting a wide range of tissues and organ systems.

Betaherpesvirus virions have a typical herpesvirus morphology and are composed of a double stranded DNA genome, encased in a 100-300 nm diameter icosahedral nucleocapsid (McGeoch, Rixon, and Davison, 2006; Roizman B, 2004). This comprises 162 capsomers: 150 hexamers and 11 pentamers with the final pentameric position being occupied by the portal complex (McGeoch, Rixon, and Davison, 2006). Betaherpesvirus nucleocapsids are surrounded by a large globular tegument which is subtly pleomorphic. This in turn is surrounded by a lipid envelope, which is derived from host cell membrane, and is studded with evenly distributed spike-like glycoproteins (Mocarski, 2007; Yamanishi, 2007).

HCMV has been widely studied initially due to its severe pathology in children who are congenitally infected, but later it emerged as one of the most important viral causes of morbidity and mortality in transplant recipients as reviewed (Britt, 2008). HCMV is also the main viral opportunistic infection in HIV/AIDS patients where it is a powerful marker of disease progression (Darwich et al., 2008; Kovacs et al., 1999; Nigro et al., 1996; Williams et al., 2001). Where there is a high prevalence of primary HCMV infections or high load reactivations during pregnancy there is a greater risk of transmitting HCMV from mother to child, transplacentally (Gouarin et al., 2002; Gouarin et al., 2001) or through breast milk (Murata et al., 2009). Factors which may affect transmission include immunity which is affected by nutrition, and viral strain variation which may determine virulence. Hypervariable loci in what is generally a highly conserved genome may encode virulence factors that drive tropism and can be used to investigate strain differences.

HHV-6 was discovered later than HCMV, and primary infection is common in infancy, where it causes fever (Hall et al., 1994; Portolani et al., 1993). Febrile episodes are occasionally accompanied by a rash termed *exanthem subitum* (*roseola infantum*) but fever is the primary symptom of infection. The virus exists as two strain variants, HHV-6A and HHV-6B, which are genomically distinct (Dominguez et al., 1999; Gompels, 2006). Primary infections causing fever in North America and Europe are predominantly found to be HHV-6B, although HHV-6A is detectable in a around 3% of cases (Dewhurst et al., 1993; Hall et al., 1994; Zerr et al., 2005). Both variants are linked with severe pathology in immunocompromised groups and several serious diseases in children and adults as reviewed (Gompels, 2004). HHV-6 productively infects the same cells as HIV (CD4 T-cells) (Lusso et al., 1991b) and can cause disseminated infections in HIV/AIDS (Clark et al., 1996).

This thesis investigates the prevalence, viral loads and genotypes of these two betaherpesviruses in three paediatric cohorts from Zambia in Southern African, and assesses the effects of infant and maternal HIV-1 infection on childhood HCMV and HHV-6 infections, and their effects on growth and morbidity. Betaherpesvirus prevalence, loads, genotypes and morbidity associations have not previously been defined for this population.

1.3 Human Herpesvirus 6

1.3.1 Introduction

HHV-6 was first isolated in 1986 and was named 'B-lymphotropic virus' as it was found in patients with lymphoproliferative disorders (Salahuddin et al., 1986), although it was later found to replicate mainly in T-lymphocytes. The virus exists as two distinct strain variants characterised by the reference strains U1102 (HHV-6A) and Z29 (HHV-6B) both isolated in AIDS patients: U1102 from Uganda (Downing et al., 1987) and Z29 from the Democratic Republic of Congo (then Zaire)(Lopez et al., 1988). These two African strains, along with HST from Japan (Isegawa et al., 1999), are the only fully annotated HHV-6 genomes to have been published: (U1102 (NC_001664)(Gompels et al., 1995), Z29 (NC_000898)(Dominguez et al., 1999) and HST (AB021506)(Isegawa et al., 1999)). Additional strains have been typed by restriction analysis (Ablashi, 2006b) and HHV-6 does not appear to display the general recombination-driven mosaic character that has been described for HCMV, both within (Haberland, Meyer-Konig, and Hufert, 1999) and between genes (Rasmussen, Geissler, and Winters, 2003; Yan et al., 2008), although very few clinical studies have rigorously genotyped HHV-6 at multiple loci. Studies from Europe and North America have identified HHV-6B primary infection as a major cause of high fever (Dewhurst et al., 1993; Hall et al., 1994) which has been shown to be responsible for up to 20-40% of infant hospitalizations from febrile illness (Hall et al., 1994; Portolani et al., 1993) with a small minority (6%) of these children being diagnosed as having *exanthem subitum* (rash)(Hall et al., 1994). A small proportion will have more serious complications, the most common being febrile seizures, but others such as respiratory, gastrointestinal and lymphatic complications have been documented (Asano et al., 1994; Hall et al., 1994; Okada et al., 1993). HHV-6A has also been identified in a minority of febrile paediatric primary infections, but is rare in Europe and North America, whereas in Zambia, in a cohort of hospitalized febrile infants, previous studies showed it may have a more equal prevalence to that of HHV-6B (Gompels, 2004; Kasolo, Mpabalwani, and Gompels, 1997). However, the prevalence of HHV-6 variants in the general population was not defined, and is an objective of this thesis. Although both variants are linked with neurological disease, HHV-6A appears in some cases to be more pathogenic, linked with myocarditis (Bigalke et al., 2007), and there is evidence for increased neurovirulence (De Bolle et al., 2005; Hall et al., 1998), with a linkage to encephalitis (Yao et al., 2009), syncytial giant cell hepatitis (Potenza et al., 2008), Multiple Sclerosis (Alvarez-Lafuente et al., 2004; Alvarez-Lafuente et al., 2006) and many case reports showing severe

disease, including fatalities, in adult immunocompromised patients as reviewed (Gompels, 2004).

1.3.2 HHV-6 Genome structure

HHV-6 has a double stranded DNA genome up to 162 Kbp in length (Gompels et al., 1995; Isegawa et al., 1999), composed of one unique region flanked by direct repeats. The U1102 HHV-6A reference genome contains 110 ORFs compared to 119 ORFs in Z29 HHV-6B. The two genomes display 90% identity with most variation being located in the direct repeats at the right-hand end of the unique region. This region, where nucleotide identity can drop to 72% (Dominguez et al., 1999), encodes the immediate early (IE) genes, envelope glycoproteins and the viral chemokine U83 (French et al., 1999). Isolated hypervariable loci are also found in more central positions such as U47 which shows 69.8% amino acid identity between reference strains U1102 and HST (Isegawa et al., 1999).

1.3.3 HHV-6 cellular tropism and receptors

Lytic infection *in vivo* occurs in CD4 positive T-lymphocytes, but HHV-6 can infect a range of different cell types *in vitro* such as haematopoietic cells (Kondo et al., 1991), macrophages (Kempf et al., 1997), dendritic cells (Asada et al., 1999; Hirata, Kondo, and Yamanishi, 2001; Kakimoto et al., 2002; Niiya et al., 2004), NK cells (Lusso et al., 1993) and γδ T-cells (Lusso et al., 1995), along with other structural cell types such as primary foetal astrocytes (He et al., 1996). Latency is established in monocytes and bone marrow progenitor cells (Luppi et al., 1999). The two laboratory reference strain variants replicate in different immortalized T-cell lines: HHV-6A grows in HSB-2 and J Jhan cells whereas HHV-6B grows in Molt-3 and MT-4 cells (Yamanishi, 2007). Also some *ex vivo* data suggest HHV-6A grows in both CD4+ve and CD8+ T-cells whereas HHV-6B is inefficient at infecting the latter (Grivel et al., 2003). In oligodendrocytes HHV-6A caused lytic infection whereas HHV-6B was nonproductive (Ahlqvist et al., 2005). In other neuronal cells HHV-6A has been shown to be more neurovirulent (De Bolle et al., 2005).

One cell receptor for HHV-6 entry is CD46 (Santoro et al., 1999), a complement regulator that is present in the membrane of all nucleated cells and interestingly, is also a receptor for the measles virus (Dorig et al., 1993; Naniche et al., 1993). HHV-6A interacts with CD46 through the viral gH/gL/gQ1/gQ2 glycoprotein complex (section 1.3.12)(Akkapaiboon et al.,

2004; Mori et al., 2003), although a 'fusion from without' assay using CD46-transfected CHO cells showed that HHV-6B strain HST does not use CD46, and that glycoprotein gB is also essential for syncytium formation in this model system (Mori et al., 2002). Interactions between other glycoproteins and other receptors have been demonstrated, such as between gB (U39) or gM (U100) and heparin sulphate (Pfeiffer et al., 1993), and there are likely further specific glycoprotein-receptor interactions.

1.3.4 HHV-6 transmission and epidemiology

HHV-6 is shed in saliva (Collot et al., 2002; Di Luca et al., 1995; Suga et al., 1998; Zhao et al., 1997) but it is rarely detected in urine (Ashshi, Klapper, and Cooper, 2003; Li et al., 2009) and there is no evidence of secretion in breast milk (Dunne and Jevon, 1993), which is a major route of transmission for HCMV(Murata et al., 2009). In a large study in North America, cumulative detection of HHV-6 in saliva also rose to 77% by 2 years of age (Zerr et al., 2005) showing that horizontal transmission by the oral route is likely, either from mothers or carers and also between children (Tanaka-Taya et al., 1996; van Loon et al., 1995). A different North American study found that after maternal antibody declined by around 6 months the seroprevalence then increased with a comparable 80% of children being infected by 2 years of age (Hall et al., 2006). In adults seroprevalence is between 70-100% (Bhattarakosol et al., 2001; Chua, Khairullah, and Hooi, 1996; Linhares et al., 1991; Tolfvenstam et al., 2000; Ward et al., 1993) but differences could be attributed to the different serological tests used, which may vary by up to 30% as described in one Danish study (Nielsen and Vestergaard, 1996). The prevalence of HHV-6 infections in Southern Africa is currently unknown, and this thesis aims to address this, assessing the prevalence of active infections in infant sera.

HHV-6 was thought to be transmitted congenitally in roughly 1% of neonates (Adams et al., 1998; Dahl et al., 1999; Hall et al., 2004; Hall et al., 1994) although more recent data suggests this is primarily due to vertical germ-line transmission of chromosomally integrated HHV-6 (Ward et al., 2006). The pathogenicity of these germ-line infections is unclear and so it is largely postnatal primary infections that are associated with disease, typically acute febrile illness, with occasional cases of fatal complications (Hall et al., 1994). Data suggests congenital infections may be influenced by 'germ-line' integration: In 1993 a group of Italian researchers used restriction fragment analysis and Southern blotting on PBMCs from one multiple sclerosis patient and two with lymphoproliferative disorders, and identified

fragments containing viral sequences that were larger than the 170Kb viral DNA (strain GS) from infected HSB-2 cell lines (Luppi et al., 1993). This was the first suggestion that the viral episome might in some cases integrate into the host genome. They then supported this with FISH (fluorescent *in situ* hybridization) analysis of the PBMCs (Peripheral Blood Mononuclear Cells), showing association of the HHV-6B genome with the telomeric repeats in the short arm of chromosome 17 (Torelli et al., 1995). Other FISH studies from Japan (Daibata et al., 1998; Daibata et al., 1999; Tanaka-Taya et al., 2004) and the U.K (Clark et al., 2006; Ward et al., 2006) also showed strong linkage between HHV-6 and the genome, resulting in 'chromosomal transmission'. The prevalence of chromosomally inherited HHV-6 has been shown to be 0.8% in the U.K (Leong et al., 2007). In North America it accounts for 86% of congenital transmission (Hall et al., 2008).

1.3.5 HHV-6 infection in immunocompetent children

As stated previously, maternal immunity to HHV-6 drops off in infants at about 6 months of age, from which point there is a steady accumulation of primary infections with up to 80% of infants being HHV-6 positive by two years of age (Hall et al., 2006; Zerr et al., 2005). Primary infection in North America (Dewhurst et al., 1993; Hall et al., 2006), Europe (Ward, Thiruchelvam, and Couto-Parada, 2005) and Japan (Tanaka-Taya et al., 1996; Yamamoto et al., 1994), is primarily with the HHV-6B variant although some primary infections with HHV-6A have been detected (van Loon et al., 1995). Primary infection always results in fever (Zerr et al., 2005), and whilst in many cases this will be self-resolving, HHV-6B has been shown to be responsible for between 20% (Italy (Portolani et al., 1993)) to 40% (US (Hall et al., 1994)) of infant hospitalizations. Episodes of high fever are sometimes accompanied by a rash (*exanthem subitum*) but primary HHV-6 infection in infants can give rise to more severe complications including pneumonia, convulsions, lymphadenopathy, hepatitis, bone marrow suppression, recurrent encephalitis and gastrointestinal problems (Gompels, 2004; Hall et al., 1994). Studies of primary infection with HHV-6 have been in children from North American, European and Japanese populations, where HHV-6B has been shown to be responsible for the vast majority of infections (irrespective of severity), with HHV-6A being identified in no more than 3% of cases (Dewhurst et al., 1993; Hall et al., 2006). Conversely, a study from Zambia (Southern Africa) found a considerably higher prevalence of HHV-6A (40%) in a small cohort of hospitalized febrile infants aged 6-34 months (Kasolo, Mpabalwani, and Gompels, 1997). The comparative prevalence of HHV-6A and B in the population as a whole was not addressed however, and so will be analysed in this thesis.

1.3.6 HHV-6 and neurological disease

HHV-6 has been associated with a wide range of diseases but due to its widespread presence within the population and a lack of studies measuring viral load, gene expression or using genotyping to distinguish between latent and lytic infection, causal disease correlations have been difficult to establish. HHV-6 encephalitis, indicated by detection of active infection in CSF, is an observed complication during primary infection (Suga et al., 1993; Ward et al., 2005) which is also seen in immunocompromised patients (Ogata, 2009; Ogata et al., 2010; Provenzale, van Landingham, and White, 2010), with extrahippocampal involvement in patients CSF PCR positive for HHV-6. Lobectomy studies detected replicating HHV-6B in hippocampal astrocytes of 60% of patients with mesial temporal lobe epilepsy (Theodore et al., 2008). Recent data also supports a link between HHV-6 and a subset of patients with MS (Multiple Sclerosis). Active HHV-6 infections (as shown by detection of IE viral RNA in peripheral blood) were demonstrated in 16% (all HHV-6A) of RRMS (relapse remitting Multiple Sclerosis) patients compared to 0% of controls (Alvarez-Lafuente et al., 2004; Alvarez-Lafuente et al., 2006). but the same group showed that HHV-6A does not play an active role in SPMS (secondary progressive MS)(Alvarez-Lafuente et al., 2007). Multiple Sclerosis is rare in black African populations, and the presentation is different to that seen in populations of European decent (Modi et al., 2001). Variation within HHV-6 isolates found in different populations may partly explain differences in MS frequency and presentation.

1.3.7 HHV-6 therapy

In vivo studies provide some evidence for GCV (Ganciclovir) as an inhibitor of HHV-6 disease after transplantation decreasing the rate of reactivations (Tokimasa et al., 2002) and it has also been shown to reduce HHV-6 viral loads in both CSF and blood, and also in transplant recipients (Zerr et al., 2002). The action of GCV is dependent on a viral kinase and that of HHV-6 (U69) is not as efficient at phosphorylating the prodrug as that of HCMV and mutations in this loci cause resistance reducing the efficacy further (Manichanh et al., 2001; Nakano et al., 2009). Foscarnet and Cidofovir have also been said to be potent inhibitors of HHV-6 replication *in vitro* (Reymen et al., 1995; Williams-Aziz et al., 2005) and *in vivo* (Pohlmann et al., 2007) where they are often reserved as second line drugs to GCV. Mutations have also been found in the HHV-6 DNA polymerase gene (U38) which cause resistance to Foscarnet (Bonnafous et al., 2007). Acyclovir and other thymidine-kinase dependent drugs

are only marginally effective against HHV-6 (Mel'nichenko, L'Vov N, and Galegov, 2009). There are currently no acceptable licensed drugs that have been developed specifically for HHV-6 through prospective studies.

1.3.8 HHV-6 latency

After primary infection (usually during infancy) HHV-6 establishes a latent infection that, as with all herpesvirus infections, remains for the lifetime of the host. During latency, the virus can be detected in certain cell types, such as monocytes/macrophages, salivary glands, lymph nodes, neuronal and kidney cells (Yoshikawa et al., 1999). PCR analysis shows that after *in vitro* infection of macrophages with HHV-6, viral DNA can be detected long after antigen and cytopathic effect assays revert to being negative (Kondo et al., 1991). The IE1 and IE2 loci in HHV-6, as well as being lytic genes, have been shown *in vitro* to encode transcripts through splicing, that are specific to latently infected but not re-activated macrophages (Kondo et al., 2002). Similar transcripts have been detected, suggesting latent infection in an astroglioma cell line (Yoshikawa et al., 2002). Latent infections will periodically reactivate in response to a range of stimuli such as stress or infection, especially where infections directly weaken immunity such as in HIV/AIDS, resulting in shedding of virus in blood and saliva (Di Luca et al., 1995). Establishment of a latent infection with one strain during childhood is not protective against super-infection with different strains (or variants) as shown in an infant study where infections with multiple variants were observed (van Loon et al., 1995).

1.3.9 HHV-6 lytic replication cycle

After entry to the cell, virions undergo de-envelopment and capsids are transported to the nucleus. Like all herpesviruses, HHV-6 expresses three temporal classes of lytic genes. Some differences were seen in splice patterns between HHV-6A and HHV-6B for IE loci such as U91 and U15-U20 (Mirandola et al., 1998). Splice variants have also been observed for U83, the only chemokine to be encoded by HHV-6 (Dewin, Catusse, and Gompels, 2006). Viral DNA is released into the nucleus where Early genes are expressed and DNA replication creates concatameric repeats of the circular episome, generated by rolling circle replication. Subsequent cleavage and packaging results in progeny capsids that are exported into the cytoplasm. Immature capsids are first enveloped at the inner nuclear membrane, are then de-enveloped, acquire tegument and are then re-enveloped probably in a membrane that is intermediate between the trans-golgi network and endosomes (Mori et al., 2008). This same

study showed release of virons in multi-vesicular bodies which fuse with the plasma membrane.

1.3.10 HHV-6 immunomodulation

Like all viruses that target and replicate in T-cells, HHV-6 is inherently immunomodulatory through the physical mechanism of lytic replication but HHV-6 also has a variety of signalling mechanisms through which it modulates immune cell function (Table 3, Page 37). Infection down-regulates the CD46 receptor and up-regulates CD4, both of which affect immunity (Grivel et al., 2003; Santoro et al., 1999). The virus can impair antigen presenting cells through suppression of IL-12 secretion, which is a T-cell stimulating factor (Smith et al., 2003; Smith et al., 2005) and can induce T-cell adhesion markers (CD2, CD4 and others)(Furukawa et al., 1994). HHV-6 infection induced HLA class I and MHC class II expression on DCs (Dendritic cells)(Kakimoto et al., 2002). Both HHV-6A and HHV-6B destroy thymic grafts in SCID mice (Gobbi et al., 1999). HHV-6 expresses one chemokine (U83)(French et al., 1999; Zou et al., 1999) and two chemokine receptors (U51 and U12)(Isegawa et al., 1998; Milne et al., 2000). The U83A chemokine is a broad receptor agonist binding CCR1, CCR4, CCR5, CCR6 and CCR8 (Dewin, Catusse, and Gompels, 2006). It has a very high affinity for CCR5 and can inhibit HIV infection by CCR5-tropic strains (Catusse et al., 2007)(for interactions with HIV see section 1.3.13). The U51A chemokine receptor from HHV-6A binds specific inflammatory modulators including CCL5, affecting the spread of virus infected cells by chemotaxis and also evading immune regulation by chemokine diversion and down-regulation of CCL5 (Catusse et al., 2008; Milne et al., 2000).

1.3.11 HHV-6 detection and diagnosis

HHV-6 infections can be detected in clinical specimens by a range of techniques such as serology, antigenaemia, whole virus isolation, or PCR (Ward, 2005). The major limitation of antibody and antigen detection assays for the detection of HHV-6 is that the immunodominant epitopes of HHV-6A and B cross react, and so these classical techniques cannot differentiate between the two strain variants (Chandran et al., 1992). Molecular techniques like PCR can be used to differentiate HHV-6A and HHV-6B, but as their genomes show considerable conservation, careful selection of targets within and around variable loci is essential. Strategies include: selecting primers to give PCR products with different sizes for the two

strain variants, or selecting primers with a 3' mismatch that exclusively amplify just one of the variants, or designing Real Time PCR assays with probes specific for just one strain variant. This thesis employs a PCR strategy which uses conserved primers flanking a hypervariable locus and then sequencing PCR products to identify the strain variant. This method is more powerful than those outlined above in that it does not rely on known fragment sizes and probe sequences that may vary, and the sequence data generated can be used for more detailed phylogenetic analysis incorporating data from other studies. When detecting HHV-6 DNA by PCR one must consider chromosomally integrated HHV-6 which is prevalent in up to 1% of cases (Ward et al., 2006).

1.3.12 HHV-6 glycoprotein complexes and variation

The identification of the first cellular receptor for HHV-6 was consistent with the broad cellular tropism discussed in section 1.3.3 (Santoro et al., 1999): CD46 is a type 1 glycoprotein and complement regulator that is found on all nucleated cells. For some strain variants this receptor is bound by the HHV-6 glycoprotein complex gH/gL/gQ1/gQ2: Interestingly, whilst the gH/gL/gQ1/gQ2 complexes of HHV-6A strains U1102 and GS (Akkapaiboon et al., 2004; Mori et al., 2003) and of HHV-6B strains Z29 and PL-1 (Pedersen et al., 2006; Santoro et al., 1999) bind CD46, the corresponding complex in HHV-6B strain HST does not (Mori et al., 2002), suggesting components of this complex play a role in the differential effects of infections both between HHV-6A and B, but also between individual strains within each variant group. A further level of control may lie in the fact that CD46 displays different glycosylation patterns on different cell types/tissues/locations (Johnstone et al., 1993). All herpesviruses contain certain conserved glycoproteins known to be involved in cell recognition, binding, entry and subsequent nuclear egress and cell-cell fusion processes. Glycoproteins gH and gL fall into this category but gQ1 and gQ2 are unique to the roseolaviruses (HHV-6 and HHV-7, but not HCMV). They are encoded by the spliced products of ORFs U97-U100, which show variation between HHV-6A and B of up to 72% (Isegawa et al., 1999). As stated in section 1.3.2, most variation is seen in the terminal regions of the genome, but some more centrally located loci are also highly variable. The U47 gene encodes a hypervariable glycoprotein, gO, which has homologues in all betaherpesviruses (Sadaoka, Yamanishi, and Mori, 2006) including HCMV (Huber and Compton, 1998). Previous data has suggested possible recombination between the two strain variants at this loci, with some U47 sequences displaying characteristics of both HHV-6A and HHV-6B (Gompels, 2006; Kasolo, Mpabalwani, and Gompels, 1997). HHV-6 glycoportein gO forms a

tripartite complex with gH/gL (Akkapaiboon et al., 2004; Mori et al., 2004) which is related to gH/gL/gQ1/gQ2 and may have a pivotal role in the determination of cell tropism differences between HHV-6A and HHV-6B. Neighbouring U47 is U46, which encodes another hypervariable glycoprotein (gN). This gene also has a homologue in HCMV (UL73)(Mach et al., 2000). It has been previously shown in our lab that linkage disequilibrium exists between glycoproteins gO and gN in HCMV (Mattick et al., 2004). The hypervariable U47 (gO) and U46 (gN) loci will be used to investigate HHV-6 strain variation in this thesis.

1.3.13 HHV-6 infection and HIV/AIDS

HHV-6 was first discovered in an AIDS patient with B-cell lymphoma and was initially thought to be a possible causative agent of AIDS-associated lymphoma (Ablashi, 2006a). Therefore, from its very discovery in 1986 (Salahuddin et al., 1986), HHV-6 has been closely linked with HIV and much work has since been undertaken investigating possible clinical and molecular links between the two viruses.

HHV-6 has been commonly detected by PCR at multiple tissue sites with increased HHV-6 viral load in HIV/AIDS patients in the absence of HAART (Clark et al., 1996). These HIV associated HHV-6 reactivations can result in disseminated infections (Knox and Carrigan, 1994) and have been responsible for fatalities due to pneumonitis (Knox et al., 1995) and encephalitis (Knox and Carrigan, 1995; Knox, Harrington, and Carrigan, 1995). Despite being first associated with B-lymphocytes, it was shown that HHV-6 can only infect B-cells when they are first infected with EBV (Flamand et al., 1993), whilst the native tropism of HHV-6 is primarily for T-cells (Lusso et al., 1988). HHV-6 and HIV are both T-cell tropic and can infect the same T-cell simultaneously (Lusso et al., 1989), and so how these two viruses interact both clinically and on a molecular level could be highly significant with respect to AIDS progression. HHV-6 gene products can activate HIV and also up-regulate the CD4 HIV receptor (Lusso et al., 1991a). In AIDS progression with depleted CD4 counts there is less HHV-6 in the blood, likely because there are fewer cells for replication, but this leads to an increase in disseminated organ infections (Clark et al., 1996; Emery et al., 1999; Fairfax et al., 1994). There may be variant specific effects as only the HHV-6A chemokine U83A has been shown to block infection by an R5-tropic HIV strain through the CCR5 co-receptor (Catusse et al., 2007; Dewin, Catusse, and Gompels, 2006). In addition, viral chemokine receptor

U51A expression, down regulates the human chemokine ligand CCL5 (RANTES) which can reduce CCR5 activity (Catusse et al., 2008).

Several lines of tissue culture and clinical evidence suggest that HHV-6 might accelerate AIDS progression: A comparison of HHV-6 IgM prevalence rates (indicative of HHV-6 reactivation) showed significantly higher prevalence in HIV positive symptomatic patients (60%), compared to HIV asymptomatic (40%) and HIV negative (19%) controls (Ablashi et al., 1998). This is supported by molecular studies where high load HHV-6B has been found in tissue containing high load HIV at autopsy (Emery et al., 1999). This correlation between viral loads may in part be due to the fact that both HHV-6A and HHV-6B can transactivate the HIV promoter, which may increase retroviral replication (Horvat, Wood, and Balachandran, 1989; Horvat et al., 1991). Such a mechanism might also re-activate HIV from its proviral state: Infection of the ACH-2 line of human leukemic T cells carrying latent HIV with HHV-6 resulted in reactivation of HIV as measured by Reverse Transcriptase activity (Isegawa 2007). HHV-6 may also facilitate HIV to infect cell-types for which it usually shows negligible tropism: In tissue culture, HHV-6 (either variant) facilitated infection of two haematopoietic progenitor cell lines (HIV does not otherwise infect stem cells)(Furlini et al., 1996), likely through the up-regulation of CD4 (Flamand et al., 1998). HHV-6A infection of syncytiotrophoblasts induces HIV replication, suggesting co-infection *in utero* may increase the likelihood of mother to child transmission of HIV (Csome 2002). In a recent animal study, HHV-6A was shown to accelerate AIDS in Macaques (Lusso 2007).

Conversely, there are also several studies suggesting a competitive and inhibitory relationship between HHV-6 and HIV: A study from Thailand following HHV-6 uptake in infants showed clearly delayed HHV-6 seroconversion in HIV positive, compared with HIV negative infants (Kositanont et al., 1999). There are also several *in vitro* studies showing inhibition of HIV by HHV-6 (Carrigan, Knox, and Tapper, 1990; Levy, Landay, and Lennette, 1990; Pietroboni et al., 1988) or HHV-6 gene products (Catusse et al., 2007).

As up to 100% of adult populations are HHV-6 positive (Bhattarakosol et al., 2001; Chua, Khairullah, and Hooi, 1996; Linhares et al., 1991; Tolfvenstam et al., 2000; Ward et al., 1993) and 80% of these primary infections occur before 2 years of age (Hall et al., 1994; Zerr et al., 2005), for countries in the above studies co-infections with HHV-6 are the norm in HIV/AIDS, with the two viruses displaying complex interactions that may be variant specific, and also differentially affected by the stage of disease progression (Dewin, Catusse, and

Gompels, 2006; Lusso et al., 1989; Milne et al., 2000). The prevalence of HHV-6 infections in Southern Africa, an HIV-1 endemic region, is not known. Determining HHV-6 prevalence and which strain variants are predominant in Zambia is an aim of this thesis.

1.4 Human Cytomegalovirus

1.4.1 Introduction

Human Cytomegalovirus (HCMV) is a member of the *Betaherpesvirinae* subfamily of the *Herpesviridae*. Its name (from the Greek *cyto,* "cell" and *megalo,* "large") is derived from the fact that in tissue culture, infection gives rise to large syncytia, the result of fusion of the initially infected cell with its neighbours. The virus establishes latency in myeloid and bone marrow progenitors (Khaiboullina et al., 2004; Sindre et al., 1996) from which it may reactivate repeatedly during the lifetime of the host. These reactivations are often clinically silent but virus is shed in very high titres in saliva and urine, facilitating transmission. Primary HCMV infection can occur in children or adults and is generally self-limiting in immunocompetent hosts, with acute symptomatic primary infections generally limited to the immunocompromised host (Jarvis and Nelson, 2007). Congenital HCMV infections are the leading infectious cause of birth defects (Cannon and Davis, 2005), such as sensorineural hearing loss (Verbeeck et al., 2008; Walter et al., 2008) and mental retardation (Cheeran, Lokensgard, and Schleiss, 2009). In immunocompromised individuals, such as transplant recipients and those with HIV/AIDS, HCMV infects a broad range of organ systems, and is a serious cause of morbidity and mortality, particularly in HIV co-infected children where it is a major pathogen (Chintu et al., 2002; Kovacs et al., 1999).

1.4.2 HCMV genome structure

The HCMV genome is the largest of all herpesviruses (~230-240 Kbp – GC 54-59%) and is the largest genome of any virus that infects humans (Davison et al., 2003; Dolan et al., 2004). Unlike HHV-6 which has just one unique region, HCMV has a class E isomerizing genome, composed of two unique regions (Unique long - U_L and Unique short - U_S) flanked by short inverted repeats, which facilitates four possible isoforms, present in the general population of viruses in equimolar amounts (Mocarski, 2007). The genome encodes between 166-252 open reading frames (Davison et al., 2003; Murphy et al., 2003b), although only approximately 45 are essential for replication in fibroblasts (Dunn et al., 2003). As with HHV-6 and other herpesviruses, HCMV genes are divided into three sequentially expressed kinetic classes, which are expressed (*in vivo* or *in vitro*) in a temporal cascade. These three classes of genes are interspersed throughout the genome (Landolfo et al., 2003). The U_L region of HCMV is essentially collinear with the unique region of HHV-6 and there are up to 70 ORFs with

sequence similarity to HHV-6 (Gompels et al., 1995). The function of many genes has not been determined directly, but has been proposed based on their homology to genes in other herpesviruses. Experimental verification remains the only sure way of confirming gene function, which might vary between different herpesviruses with some genes even being redundant. Clinical strains quickly acquire deletions and rearrangements when grown in tissue culture (Dolan et al., 2004). Deleted regions may encode virulence determinants not required for growth *in vitro* (Wang and Shenk, 2005b). The HCMV genome carries 30-50 ORFs with sequence characteristics indicative of possible glycoprotein genes. Whilst many of these genes are highly conserved between clinical strains, some of them display hypervariation and can be used for genotyping and for studying recombination (Bradley et al., 2008; Stanton et al., 2005; Yan et al., 2008).

1.4.3 HCMV cellular tropism and receptors

HCMV cellular tropism may be determined by viral membrane proteins and also host cell transcription factors (Sinzger, Digel, and Jahn, 2008). HCMV can and initiate gene expression in a variety of vertebrate cell types, including epithelial and endothelial cells, fibroblasts, smooth muscle cells, neurons, monocytes and macrophages (Isaacson, Feire, and Compton, 2007; Plachter, Sinzger, and Jahn, 1996; Sinzger et al., 1995), resulting in virus replication in a wide range of organ systems. HCMV infection in glandular and ductal epithelial cells in the salivary glands, kidneys, breast milk ducts, and genital organs allows infectious virus to be shed into a range of bodily fluids such as saliva, urine, cervical secretions and semen as reviewed (Mocarski, 2007). This broad host range may be supported by a single conserved interaction between the virus and a universal receptor, but the search for a definitive receptor for HCMV has proved problematic. A more likely explanation, is that over several hundred million years of co-evolution, the continuous jousting between virus and host has given rise to several viable virus-receptor interactions, as have been found with the *alpha-* and *gamma-*herpesviruses (Spear and Longnecker, 2003). Furthermore, the function of some of these interactions may be limited to 'tethering' of the virus, and distinct interactions may be necessary for entry (Isaacson, Feire, and Compton, 2007).

Serial passage of wild type HCMV strains in tissue culture quickly leads to loss of genetic material and highly passaged tissue culture strains often show marked differences. Sequence and infectivity analysis have identified a region comprising 19 ORFS (UL133-UL150) which is lost in the AD169 and Towne vaccine strains due to serial passage in fibroblasts (Cha et al.,

1996; Prichard et al., 2001). For AD169, subsequent culture in endothelial cells can rescue endothelial tropism however, suggesting loss of tropism was due to a much smaller, reversible mutation (Gerna et al., 2003). In the case of AD169 this was due to a frame shift mutation in UL131A (Davison et al., 2003) and the whole UL128-131 locus appears to be essential for endothelial cell tropism (Hahn et al., 2004; Wang and Shenk, 2005a), and is also implicated in dendritic, epithelial and leukocyte cell tropism (Gerna et al., 2005). There is some evidence to suggest that clinical isolates might often contain mixed populations of different strains, and that culturing conditions selects for strains with an appropriate tropism (Grazia Revello et al., 2001), although it seems clear that culture can also drive highly significant mutations, some of which are reversible in plaque-purified viruses (Gerna et al., 2003). All three UL128-UL131 gene products form a complex with gH-gL and evidence shows this complex drives endothelial and dendritic cell tropism (Gerna et al., 2005; Wang and Shenk, 2005b).

Studies in fibroblasts show that cellular platelet-derived growth factor-α receptor (PDGFR-α)(Soroceanu, Akhavan, and Cobbs, 2008) is a critical receptor for HCMV and is blocked by neutralizing antibodies to glycoprotein gB. Glycoprotein gO (UL74) has been shown to have a role in cell fusion (Paterson et al., 2002) and two recent studies suggest it has a chaperone function in transporting other glycoproteins to the cell surface (Jiang et al., 2008; Ryckman, Chase, and Johnson, 2010). The UL74 gene is hypervariable but there is no evidence for recent immune selection (Mattick et al., 2004) and so variation may have evolved as an adaption to replication in different cell types and may therefore play a role in tropism. Furthermore, the UL74 gene has been shown to be linked to another hypervariable glycoprotein gene, UL73 (gN) (Mattick et al., 2004), and in this thesis these two loci are used to investigate the genotypes present in Southern Africa, a region from which very little HCMV genotype data has been collected previously.

1.4.4 HCMV transmission and epidemiology

HCMV has no natural reservoir other than in humans, where transmission occurs by direct or in-direct person-to-person contact. High loads have been demonstrated in saliva and urine (>10^7copies/ml and >10^5copies/ml respectively)(Kearns et al., 2002; Yoshikawa et al., 2005) but the main route of transmission in infants is through breast milk (Jim et al., 2009; Kerrey et al., 2006; Schleiss, 2006a; Schleiss, 2006b). The virus can also be isolated from a broad range of other bodily fluids/excretions including blood, tears, oropharyngeal secretions, faeces, semen, cervical and vaginal secretions (Mocarski, 2007). Primary HCMV infections mainly occur during childhood or adolescence (later than HHV-6) and are usually asymptomatic (occasionally they can cause fever or mononucleosis), after which latency is established for the lifetime of the host. Even in healthy children, sub-clinical viral shedding is common, with rates (determined by viral culture) in North American day care centres, ranging from 15-30% in children under two years of age (Bale et al., 1999; Noyola et al., 2005). HCMV seronegative child care providers have a 20% risk of seroconverting over their first 12 months of work, which rises to 40% after their second year (Murph et al., 1991). In Africa, a study from the Gambia established that children shed more virus than their mothers and that loads in urine were the highest. They suggest that transmission through bed wetting between siblings, and to or from mothers, is the most common route of transmission (Bello, 1992). It has been suggested that another major route of HCMV infections is through sexual transmission, and studies have shown condom use is protective, suggesting semen is a major route of HCMV transmission (Carre et al., 1997; Robain et al., 1998). Condom use may well be associated with other more hygienic practices however, such as hand washing or fewer sexual partners. As well as being transmitted horizontally, HCMV can also be transmitted vertically either *in utero* or during birth. These congenital HCMV infections (see section 1.4.3) are the major cause birth defects in immunocompetent children where they can cause a range of physical and mental disabilities.

HCMV is endemic and within any given population seroprevalence rates increase steadily with age (Mocarski, 2007; Staras et al., 2006). There are differences in seroprevalence rates between different groups and the reasons for these observations are not clear. One study found that the average seroprevalence in the US was 54% for men and 64% for women. They also grouped by race and found higher seroprevalence among some ethnic minority groups (Staras et al., 2006). Another American study, looking at young women, found that those from ethnic minority American groups contract HCMV earlier (Colugnati et al., 2007). Previous studies

have found higher seroprevalance to be linked with lower socioeconomic status (Stagno et al., 1982). At the start of this thesis there was evidence for different seroprevalence rates in different countries, including three from West Africa (Table 2). A current hindrance is that different studies use different diagnostic kits for measuring HCMV IgG antibody which makes comparisons between different studies difficult. There is currently very little data on the prevalence of HCMV infections in Southern Africa which will be addressed in this thesis.

Country (City)	HCMV seroprevalence	Study population	Reference
Mali (Bamako)	58%	Adult healthy HIV-ve blood donors	(Maiga et al., 2003)
Tunisia (Sfax)	82%	Adult healthy HIV-ve blood donors	(Gargouri et al., 2000)
Burkina Faso	82%	Adult healthy HIV-ve blood donors	(Ledru et al., 1995)
Nigeria (Ibadan)	55%	Adult healthy blood donors	(Olaleye, Omilabu, and Baba, 1990)
India (Delhi)	95%	Adult healthy HIV-ve blood donors	(Kothari et al., 2002)
Turkey	94%	Adult healthy HIV-ve blood donors	(Ataman et al., 2007)

Table 2: Comparison of HCMV seroprevalence in different countries

1.4.5 HCMV congenital infection and maternal seroprevalence

Congenital HCMV is generally defined by the detection of viral DNA in blood spots on Guthrie cards (Mosca and Pugni, 2007). It is an extremely damaging infection initiated by either primary or reactivated infection in the mother during pregnancy, and is the major viral cause of paediatric mental and physical disability affecting 0.2-2.2% of newborns (Barbi et al., 2006; Stagno et al., 1986). Around 7-11% of infected foetuses are then born with symptoms (Griffiths and Walter, 2005; Kenneson and Cannon, 2007), with a neonatal mortality rate of 20-30% (Gaytant et al., 2002; Ross et al., 2006). Of those congenitally infected (both symptomatic and asymptomatic), up to 28% will develop late sequelea (Nigro et al., 2005). Symptoms may include growth retardation, hepatosplenomegaly, jaundice, pneumonia, gastrointestinal, retinal, and neurological disease such as sensorineural hearing loss (Adler, Nigro, and Pereira, 2007; Griffiths, 2004; Ogawa et al., 2007; Stagno et al., 1986; Vancikova and Dvorak, 2001). In the United States these infections have been estimated to cause around 400 infant deaths and a further 8000 permanent disabilities per year, affecting more children than several better-known childhood conditions such as Down Syndrome, Fetal Alcohol Syndrome and Spina Bifida (Cannon and Davis, 2005). The impact of HCMV is likely to be underestimated as children who develop late sequelae are not always followed up.

The seroprevalence of HCMV among women of child bearing age in North America has been shown to be higher in some ethnic minority groups (Colugnati et al., 2007). Evidence for

higher HCMV seroprevalence in ethnic minority groups was also found in the U.K, also independently linked with lower socioeconomic status and parity (Tookey, Ades, and Peckham, 1992). The main cause of congenital HCMV infection in populations of low seroprevalence is maternal primary infection during pregnancy and increased viral load (Gouarin et al., 2001; Lazzarotto et al., 2007; Satilmis et al., 2007; Stagno et al., 1982) suggesting that populations with high seroprevalence will have fewer primary infections during pregnancy and hence lower rates of congenital HCMV. However, data from high seroprevalence populations in Africa shows that the rate of congenital HCMV is actually higher probably due to prevalent high load maternal reactivations or secondary infections during pregnancy. In a study from the Gambia HCMV seroprevalence in pregnant mothers was 87% and they found that 14% of infants contracted congenital HCMV (Bello and Whittle, 1991). More recently rates of 3.9% (Kaye et al., 2008) and 5.4% (van der Sande et al., 2007) have been published but these are still significantly higher than the 1% of live births seen in Europe and North America (Alford et al., 1990; Kenneson and Cannon, 2007; Stagno and Whitley, 1985), where congenital infection was initially thought to be more common. In populations with reduced immunity due to HIV or malnutrition, early primary or congenital HCMV infections with high loads could be a significant cause of morbidity and mortality, which is investigated here.

1.4.6 HCMV treatment and prevention

HCMV drugs have been most extensively studied in immunocompromised patients as it is in this patient group that the virus causes the most severe disease. Ganciclovir, valganciclovir (oral ganciclovir), cidofovir, foscarnet and fomivirsen, have been licensed for the treatment of HCMV diseases in the U.K and U.S (Schreiber et al., 2009). Intravenous ganciclovir was shown to reduce developmental delays in congenitally infected infants with CNS disease (Oliver et al., 2009) and there is also evidence for prevention of sensorineural hearing loss (Lackner et al., 2009). Ganciclovir is administered intravenously, through a sustained release surgical implant, or as the pro-drug valganciclovir which is administered orally and has increased gastrointestinal absorption. The National Institute of Allergy and Infectious Diseases (NIAID) in the U.S has completed phase II trials on the bioavailability and dosing of valganciclovir vs intravenous ganciclovir in congenitally infected infants (www.clinicaltrials.gov - NCT00031434) and they are now recruiting for a phase III trial to assess the efficacy of valganciclovir to reduce the prevalence of mental retardation and hearing loss in congenitally infected infants (www.clinicaltrials.gov - NCT00466817).

Foscarnet and cidofovir are only given intravenously and fomivirsen as an intravitreous injection for the treatment of HCMV retinitis (Jabs and Griffiths, 2002). As HCMV infection is normally mild and self-resolving in the immunocompetent setting, the use of these drugs is reserved for only the most severe cases of HCMV infection in otherwise healthy patients. In the HIV/AIDS setting Ganciclovir prophylaxis is recommended for patients of all ages, with CD4 counts of less than 50 cells/μl (Masur, Kaplan, and Holmes, 2002), although in lower income countries availability of anti-HCMV drugs is highly restricted. In this setting, curative treatment with valganciclovir may be an economically more feasible intervention in the treatment of disseminated HCMV disease, especially pneumonia, associated with HIV/AIDS. In this setting, when pneumonia patients do not respond to antibacterial treatment, HCMV is strongly implicated (Chintu et al., 2002; Mocarski, 2007).

Artesunate has been shown to inhibit HCMV in vitro and in vivo (Kaptein et al., 2006). As an anti-malarial drug this is widely available in tropical low income countries where it could be an affordable alternative to treatment with the HCMV-specific drugs discussed above. HCMV synthesis is also sensitive to compounds that inhibit polyamine synthesis such as desferrioxamine (Cinatl et al., 1995). Then more recently 4-benzyloxy-gamma-sultone derivatives have been investigated as non-nucleoside inhibitors of HCMV (De Castro et al., 2009), and HCMV IE expression has been inhibited by dihydro-beta-agarofuran sesquiterpenes isolated from the plant genus Euonymus (Pusztai et al., 2008). Another plant with extracts showing anti HCMV activity is Sasa albo-marginata (Sakai et al., 2008). MicroRNAs are integral to CMV pathology, affecting both host and viral gene expression and so they may present a new class of antiviral target (Dolken, Pfeffer, and Koszinowski, 2009). Interestingly linked with HIV, HAART therapy has recently been suggested to have an inhibitory effect on HCMV viral load (Mihailescu et al., 2008), likely through restoring the host immune response to HCMV. This has positive implications for the treatment of HCMV disease in HIV/AIDS patients, in regions where HIV is endemic and anti-retrovirals are becoming more widely available. Another novel strategy is the linking of known drugs together such has been attempted for zidovudine and foscarnet via an actadecylglycerol residue (Schott et al., 2009). As well as treatment with antiviral drugs, other more specific therapies are also being investigated such as adoptive T-cell therapy (Brestrich et al., 2009).

HCMV has been identified as one of the most pressing viral targets for vaccine development (Arvin et al., 2004), largely due to the impact of congenital HCMV disease in North America. There is also data to suggest that HCMV seropositive individuals have a significantly lowered

life expectancy due to immune senescence (Hadrup et al., 2006; Khan et al., 2002). The mechanism may include the virus gradually swamping immune capacity, depleting the pool of naive T-cells available to fight other infections. The role of HCMV in immune senescence is now under further investigation and has implications for HIV positive populations where HCMV is more widespread (Kaye et al., 2008). An effective vaccine could hence have broad reaching benefits. In the 1970s the Towne strain of HCMV was generated through 125 serial passages *in vitro*. The resulting virus was highly attenuated and did not cause disease, but failed to illicit neutralizing antibody responses and could not protect seronegative parents from being infected by their seropositive children (Adler et al., 1995). Since then, many different vaccine strategies have been developed but at the time of writing this thesis none have yet been licensed.

1.4.7 HCMV latency

As with other herpesviruses, an understanding of latency is integral to elucidating the molecular mechanisms that determine pathology. HCMV has evolved latency switches that allow it to persist at very low levels in certain cell types, a characteristic that likely evolved as a means of avoiding the host immune response (Sinclair and Sissons, 2006). The main difference between lytic and latent HCMV infection is that the former is marked by high levels of IE gene expression whereas IE transcripts are not seen in undifferentiated monocytes in which latent HCMV DNA has been detected (Mendelson et al., 1996; Taylor-Wiedeman, Sissons, and Sinclair, 1994). Highly sensitive PCR strategies have demonstrated the presence of HCMV DNA in healthy HCMV-seropositive individuals (Larsson et al., 1998; Taylor-Wiedeman et al., 1991), suggesting that as few as 1 in 10,000 PBMCs may carry the latent HCMV episome (Sinclair and Sissons, 2006). Cell sorting experiments have then been used to identify peripheral blood monocytes as the major site in healthy carriers for the latent HCMV episome (Larsson et al., 1998; Taylor-Wiedeman et al., 1991). HCMV DNA is found in CD34 positive myeloid progenitors but only in monocytes and not PBNLs (Polymorphonuclear Leukocytes) (Taylor-Wiedeman et al., 1993; Taylor-Wiedeman et al., 1991) which are also derived from myeloid progenitors, also the site of latency for HHV-6 (Yoshikawa et al., 1999).

There has been no latent origin of replication identified for HCMV as there has been for EBV (Sinclair and Sissons, 2006). It has been difficult to identify transcripts that are associated with maintenance of this latent state, as naturally infected DNA positive myeloid cells are

present in such small numbers and it is not possible to enrich for them. Studies have therefore largely relied on infecting differentiating myeloid cultures (Kondo, Kaneshima, and Mocarski, 1994; Reeves et al., 2005a; Reeves et al., 2005b). Cultures will carry the genome for several weeks without producing infectious virions but these same cultures can also be re-activated to lytic infection. Microarray studies also failed to identify transcripts that were specifically associated with latency (Challacombe et al., 2004; Goodrum et al., 2002; Zhu et al., 1998). Southern blotting has identified an HCMV transcript (UL81-82/LUNA) that may affect inhibition of lytic gene expression through inhibiting the MIEP (Major Immediate Early Promoter)(Bego et al., 2005) and has been shown to be associated with acetylated histones in naturally latently infected cells (Reeves and Sinclair, 2009). Infection studies in human primary CD34 positive cells have compared wild-type and recombinant knockouts, and shown that UL138 is necessary for latency (Goodrum et al., 2007; Petrucelli et al., 2009). Cellular transcription factors have also been identified that both promote (NF-κB, CREB and Sp1) and repress (YY1 and ERF) MIEP (Sinclair and Sissons, 2006), possibly mediating HCMV transcription through the recruitment of enzymes that alter chromatin structures, a mechanism which has been suggested for other herpesviruses such as EBV (Amon and Farrell, 2005). It is the host cells balance of these transcription factors that either inhibit or promote lytic gene expression. Rather than the traditional view of latent and lytic infections as two clearly defined states of infection in phenotypically distinct cell types it may actually be more of a gradient. Both LUNA (Bego et al., 2005) and UL138 (Petrucelli et al., 2009) can be detected in small amounts during lytic infection and so the transition between latent and lytic states may be a more continuous and dynamic process than once thought (Reeves and Sinclair, 2009).

1.4.8 HCMV lytic replication cycle

After receptor attachment (see section 2.3) entry to the cell is facilitated by multiple interactions between virion surface glycoproteins and cell surface receptors. The virus plasma membrane fuses with that of the cell, releasing both tegument proteins and the capsid into the cytoplasm. Studies using microtubule-de-polymerizing drugs (nocodazole and colchicine) have suggested HCMV capsids are transported to nuclear pores via the microtubule network (Ogawa-Goto et al., 2003) from where viral DNA enters the nucleus. After entry into the nucleus the HCMV genome circularizes to form an episome. Viral DNA replication occurs within three days post infection through the 'rolling circle' mechanism, with the generation of long concatamers which are then cleaved for packaging in to progeny capsids (Mocarski,

2007). Nucleocapsids are assembled within the nucleus and egress probably occurs via the envelopment de-envelopement pathway through the nuclear membranes (Gibson, 1996; Mocarski, 2007), possibly initiating at invaginations of just the inner nuclear membrane (Buser et al., 2007; Dal Monte et al., 2002) although there remains some debate over the exact mechanism (Pignatelli et al., 2007). The capsids are then located to late endosomes where they are coated in tegument and undergo secondary envelopement in a membrane containing virion glycoproteins, before being released from the cell (Fraile-Ramos et al., 2007). As with transport of mature capsids to the nucleus, there is evidence to suggest that immature particles are transported along microtubules, away from the nucleus towards the cell membrane (Sampaio et al., 2005). Despite being non-essential in other herpesviruses, in betaherpesviruses such as HCMV, glycoprotein gN (UL73)(studied here) has been shown to be required for virion morphogenesis (Mach et al., 2007). Viral glycoproteins are present in large quantities on the cell membrane and this facilitates fusion with neighbouring cells. Thus cell free and cell-to-cell spread are distinct mechanisms with the latter important for intra-host spread and as a mechanism of immune evasion.

1.4.9 HCMV immunomodulation

There are over thirty HCMV genes with evidence for immunomodulatory functions either by similarity to other host or viral proteins or defined experimentally. These genes may affect classical and non-classical major histocompatibility complex protein function, leukocyte migration and activation, cytokine responses and host cell susceptibility to apoptosis. Interestingly, of all betaherpesvirus immunomodulatory genes only three have homologues in both betaherpesviruses (Table 3). The expression of MHC Class I homologues on the surface of MCMV infected cells may contribute to evasion of NK cell recognition (Farrell, Degli-Esposti, and Davis-Poynter, 1999). UL16 binds MICB, ULBP1 and ULBP2 and inhibits their interaction with NKG2D, suppressing NK clearance (Sutherland, Chalupny, and Cosman, 2001). Human NK cell receptors for MHC class I belong to the NKG2 and killer cell Ig-like receptor (KIR) families. Certain NKG2 and KIR genotypes in HIV positive patients may be more strongly associated with HCMV disease than others (Price et al., 2007). UL18 encodes an MHC Class I homologue but unlike endogenous MHC class I it binds leukocyte immunoglobulin-like receptor (LIR-1), which is expressed prominently on monocytes and B-cells, but also on subsets of NK and T-cells (Cosman, Fanger, and Borges, 1999). This may result in down regulation of DC, NK and T-cells, but there is also evidence for the activation of these same cells (Wagner, Ljunggren, and Achour, 2008; Wagner et al., 2007). HCMV can

down regulate MHC class II in cell culture, but other than US2 (Tomazin et al., 1999), other genes responsible for this are yet to be identified (Miller et al., 2001).

HCMV	HHV-6/ HHV-7	HCMV Gene Family; Gene Name	Function
RL11		RL11 family, IgG Fc-binding glycoprotein	Modulates antibody activity
UL16		Membrane glycoprotein	Inhibits natural killer (NK) cell cytotoxicity by downmodulating MICA-ULBP, NKG2D ligand
UL18		UL18 family, putative membrane glycoprotein; MHC class I homologue	LIR-1 ligand
UL20		T-cell receptor γ chain homologue	
UL21A			CC chemokine binding protein
UL33	U12	GPCR family, virion envelope protein	Constitutive signalling [HHV-6A, HHV-6B and HHV-7 are chemokine receptors]
UL40		Membrane glycoprotein	Signal peptide binds HLA-E and inhibits NK cell cytotoxicity via CD94:NKG2A
	U21	Putative membrane glycoprotein	Directs MHC class I to lysosomes
UL78	U51	GPCR family; chemokine receptor, envelope glycoprotein	
UL111		IL10 homologue	Latency associated
	U83	Secreted glycoprotein	CC chemokine, binds CCR5 and can block HIV entry
UL119	U85	IgG Fc-binding glycoprotein, virion envelope glycoprotein	Membrane protein related to OX-2; modulation of antibody activity
UL128		Putative secreted protein	Putative CC chemokine, endothelial and epithelial cell tropism, complexes with gH:gL
UL147		UL146 family; putative secreted glycoprotein	Putative CXC chemokine
UL146		UL146 family; secreted glycoprotein	hCXCR2-specific CXC chemokine
UL144		Membrane glycoprotein; TNF receptor homologue	Regulates lymphocyte activation via Band T-lymphocyte attenuator (BTLA)
UL142		UL18 family; putative membrane glycoprotein; MHC class I homologue	
UL141		UL14 family; membrane glycoprotein	Inhibits NK cytotoxicity by downregulating CD155
UL140		Putative membrane protein	Inhibits NK cytotoxicity
US2		US2 family; membrane glycoprotein	Degradation of MHC class I and, possibly, MHC class II
US3		US2 family; IE gene and membrane glycoprotein	Inhibits processing an dtransport of MHC class I and, possibly MHC class II
US6		US6 family; putative membrane glycoprotein	Inhibits transported of antigen processing (TAP)-mediated ER peptide transport
US8		US6 family; membrane glycoprotein	Binds to MHC class I
US10		US6 family; membrane glycoprotein	Delays trafficking of MHC class I
US11		US6 family; membrane glycoprotein	Selective degradation of MHC class I
US12-US21		US12 family; putative multiple transmembrane protein, GPCR?	
US27		GPCR family; virion envelope glycoprotein	
US28		GPCR family; membrane protein	Broad spectrum CC and CX3C chemokine receptor, mediates cellular activation and migration

Table 3: Betaherpesvirus immunomodulatory genes

Adapted from Mocarski (Mocarski, 2007)

As well as encoding proteins that mimic or interact with MHCs, HCMV also encodes several proteins that affect the immune inflammatory system which directs leukocytes to the site of infection. Some of these resemble chemokine receptors (US27, US28, UL33 and UL78) but only one (US28) has been shown to bind chemokines (Gao and Murphy, 1994), and for this reason is the best characterized, and has no homologues in the other betaherpesviruses (Mocarski, 2002). US28 binds RANTES, MCP-1, MIP-1α, MIP-1β but binds most strongly with Fractalkine (Casarosa et al., 2005; Kledal, Rosenkilde, and Schwartz, 1998) and can also signal constitutively (Casarosa et al., 2001). US28 can also act as a co-receptor for HIV into

otherwise unsusceptible cells (Ohagen et al., 2000) and also binds the KSHV chemokine, vMIP-2 (Kledal et al., 1997; Kuhn, Beall, and Kolattukudy, 1995). US28 might have a role in inflammatory response and leukocyte migration and is pro-inflammatory (Mocarski, 2002). US28 confers chemotactic mobility in smooth muscle cells (Streblow et al., 1999; Streblow et al., 2003), which is inhibited by US28 binding to Fractalkine. Fractalkine binding to US28 expressed on macrophages however produces strong migration suggesting that US28 signalling is both cell- and ligand- specific (Vomaske et al., 2009). HCMV also encodes chemokine-like genes: UL146 encodes vCXCL-1 which is another secreted homologue of human IL-8. This hypervariable chemokine (Bradley et al., 2008) binds CXCR2 but not CXCR1 (the human form binds both), but like IL-8 it can chemotax and degranluate neutrophils (Penfold et al., 1999). HCMV encodes two IgG Fc receptors, the first has two copies per genome, located within the repeat regions (TRL11 and IRL11), and the second is the spliced product of UL118/119 (Atalay et al., 2002). The HCMV ORF UL111A encodes a homologue of human IL-10 which is spliced and only has 25% amino acid sequence identity with the human form (Kotenko et al., 2000). Like human IL-10, it can inhibit the proliferation of mitogen-stimulated PBLs and reduced cell surface expression of MHC Class I and II on PBMCs (Spencer et al., 2002), although more recent work has shown is may have an immune stimulatory role with respect to B-cells, possibly showing differential effects of lytic and latent viral transcripts (Spencer et al., 2008). A different study has recently identified several isoforms, possibly with variable glycosylation patterns, but all are able to form a heterodimer with human IL-10 (Lin et al., 2008). The studies summarized here demonstrate a complex and convoluted interaction between virus and host, with a myriad of selective pressures being exerted in both directions. Viral proteins that subvert the host immune system can also promote virus dissemination and are potential virulence determinants.

1.4.10 HCMV detection and diagnosis

HCMV was first discovered in the 1930s in biopsy/autopsy studies, through histological identification of 'owls eye inclusions' in kidney, liver or lung tissue. This is a highly specific way of detecting HCMV organ involvement but it is not very sensitive as it only accounts for 20% of HCMV PCR-positive tissue (Mattes et al., 2000). The virus has since been detected by other classical techniques, such as virus isolation and serology-based techniques to detect either HCMV-specific antibody or antigens, in human sera. Such tools are useful in establishing the prevalence of HCMV within a given population, but are not suited to following the clinical course of infection, such as assessing reactivation frequency of this

chronic infection in a specific bodily fluid or tissue. Molecular techniques such as PCR (Polymerase Chain Reaction) and qPCR (quantitative PCR) are much better suited to this kind of investigation. They are highly sensitive and highly specific methods of detecting the presence and amount of HCMV within a range of different specimen types such as whole blood, sera, urine, saliva, breast milk and tissue. RT-PCR (Reverse Transcriptase PCR) can also be used to assess the transcription levels of specific genes. In this thesis, qualitative and quantitative PCR are used to detect HCMV DNA in DNA-extracted whole blood, lung tissue and sera. Detection in sera correlates with active infection, reduces interference from latent virus and also eliminates sensitivity problems associated with a high cellular background. There are commercial kits available for qPCR (eg: *COBAS AMPLICOR CMV Monitor Test* (Roche)) and also services (eg: *5500 Cytomegalovirus (CMV) Real-time qPCR* (ViraCor)) but commercial primer sequences are often undisclosed or contained within a mastermix and cannot be used for genotyping. Whilst commercial assays are highly specific, other in-house protocols are more sensitive and cost-effective (Boivin et al., 1998; Herrmann et al., 2004), and have the advantage, through careful primer design, of being able to genotype different HCMV strains (Beyari et al., 2005; Mattick et al., 2004; Paterson et al., 2002; Pignatelli, Dal Monte, and Landini, 2001).

1.4.11 HCMV glycoprotein complexes

Four glycoprotein complexes have been defined for HCMV: gCI (gB alone), gCII (gM/gN) (Gretch 1988), gCIII (gH/gL/gO) (Paterson 2004) and gH/gL/UL128-131 (Wang Shenk 2005), which are known to function in the direct mechanics of cell tethering (Isaacson, Feire, and Compton, 2007; Kari and Gehrz, 1992) entry (Feire, Koss, and Compton, 2004) and egress (Jiang et al., 2008) of progeny virions and tropism (Wang and Shenk, 2005a). However, genome analysis has suggested there may be as many as 50 HCMV glycoprotein genes, and so the real number and variety of these complexes may be considerably greater. There is evidence for cell binding and entry through glycoprotein gB (Soroceanu, Akhavan, and Cobbs, 2008) although previously the gH-gL complex has been implicated (Milne, Paterson, and Booth, 1998), with peptides from coiled-coil domains on both gB and gH being shown to block fusion (Lopper and Compton, 2004), suggesting HCMV has multiple entry mechanisms, possibly determining tissue/cell tropism or to circumvent entry inhibition due to host immunity. Hypervariable glycoproteins gO (UL74) and gN (UL73) are investigated in this thesis as potential determinants of virulence and/or tropism. Interestingly they both from complexes with more conserved proteins.

The gH-gL-gO complex was identified by immunoprecipitation and radiolabelling techniques (Huber and Compton, 1997; Li, Nelson, and Britt, 1997; Paterson et al., 2002) and shown to form in the ER maturing in the post Golgi (Huber and Compton, 1999; Theiler and Compton, 2002). Initially a gO mutant (generated through creation of random transposon libraries of bacmids containing mutant herpesvirus genomes) was capable of only minimal replication in fibroblast cell culture, suggesting a role in fibroblast tropism (Hobom et al., 2000). More recently studies using a different ΔgO mutant showed similar low titres in fibroblast cell culture, but normal quantities of extracellular virus (Wille et al., 2009). These results are in keeping with several studies showing a role for gO in cell-cell fusion and spread in both fibroblasts (Jiang et al., 2008) and astrocytes (Paterson et al., 2002), although retroviral vectors expressing AD169 gO in CHO epithelial cells showed no increase in the observed syncytia after co-transfection with gH/gL and gO (Kinzler and Compton, 2005), supporting the idea that HCMV gO has a function that is species or cell-type specific. Recent data suggests that the gH-gL-gO complex is primarily a cellular egress complex in which gO acts as a chaperone (Ryckman, Chase, and Johnson, 2009). Both gH-gL and gH-gL-UL128-UL130 are required for endothelial/epoithelial cell tropism, and gH-gL alone for fibroblast cell tropism with gO being required for correct transport of gH-gL to the cell surface and incorporation into progeny virions (Wille et al., 2009). There are other virion associated gO-containing complexes (such as gO-gL) and these appear to arise in the post-Golgi compartment and are also likely involved in egress (Theiler and Compton, 2002). Glycoprotein gO contains a cleavable signal sequence, with the gH/gL/gO complex shown to anchor by the trans-membrane domain of gH (Theiler and Compton, 2001).

The gM/gN complex (Mach et al., 2000) binds heparin sulphate (Kari and Gehrz, 1992) but has also been shown to function intracellularly in nuclear assembly and egress (Mach et al., 2005; Mach et al., 2007). This complex is conserved throughout the human herpesviruses where deletion of either or both components often has little effect on replication efficiency (Baines and Roizman, 1991; Masse et al., 1999), whereas in HCMV, deletion of gM results in a replication-incompetent virus (Hobom et al., 2000). Deletion of large parts of gM however, including C44 which forms a disulphide bridge with C90 on gN, has shown that large parts of gM are despensible (Mach et al., 2005), but cytoplasmic tails express trafficking signals and are essential for HCMV assembly and replication (Krzyzaniak, Mach, and Britt, 2007). Likewise, the C-terminal domain of gN is required for virion morphogenesis (Mach et al., 2007). The gM/gN complex is also a target for neutralizing antibodies (Shen et al., 2007;

Shimamura, Mach, and Britt, 2006). Certain gN genotypes may also have a latency associated functions inferred by them being more commonly detected in HCMV genomes extracted from monocytes taken from healthy blood donors (Pignatelli et al., 2006)

1.4.12 HCMV hypervariable glycoproteins

Compared to rampantly emergent RNA viruses, variation in the *herpesviridae* during a single replication cycle is restricted due to lower error rates in DNA-dependant DNA polymerases. The rapid drift associated with viruses such as influenza or HIV, is not seen in herpesviruses, but over millennia, high levels of variation in select loci has been observed at levels similar to flu or HIV. Evolutionary modelling and genomic analysis suggest recombination is a common process by which novel HCMV strains may diverge (Gompels et al., 1995; Mattick et al., 2004; McGeoch, Rixon, and Davison, 2006). The 14 known full length sequences of HCMV and studies identifying multiple loci in clinical isolates (Bale et al., 2001; Cunningham et al., 2009; He et al., 2006; Rasmussen et al., 2002; Rasmussen, Geissler, and Winters, 2003) support the model of mosaics of different strains in the environment. Studies looking at conserved loci, such as UL55 (gB), have hence failed to identify genotype specific clinical outcomes (Coaquette et al., 2004; Humar et al., 2003; Sarcinella et al., 2002). Whilst gB is a major virion glycoprotein responsible for cell recognition and binding, clinical differences between strains, such as virulence, tropism or disease outcome, are more likely to be determined by more variable loci. Such loci have been located towards the long unique and repeat boundaries (Cha et al., 1996; Murphy et al., 2003a), although interestingly there are two hypervariable genes (with variation mainly towards 5' termini), that are unique in that they are more centrally located (Gompels, 2006; Paterson et al., 2002). These genes are UL73 (gN) and UL74 (gO), discussed above with respect to their complex formation with gM and gH-gL respectively. These virion surface glycoproteins are encoded by neighbouring genes that overlap slightly (by 24 base pairs in AD169) at their respective 3' ends, being encoded by opposite strands of the double stranded HCMV genome (Figure 2, Page 61).

Further sequence analysis of the UL73 gene initially identified four genotypes (gN1, gN2, gN4 and gN4)(Pignatelli, Dal Monte, and Landini, 2001), but then phylogenetic analysis showed that gN4 can be divided into three tightly clustered genotypes (gN4a, gN4b and gN4c)(Pignatelli et al., 2003a), making seven genotypes in total (Mattick et al., 2004). Analysis of UL74 has identified eight genotypes (gO1a, gO1b, gO1c, gO2a, gO2b, gO3, gO4 and gO5)(Mattick et al., 2004). Furthermore sequence analysis of strains from Europe, North

America and China showed these overlapping hypervariable loci form seven linked groups (Chen et al., 2008; Mattick et al., 2004; Pignatelli et al., 2003a)(Table 4). This is in contrast to other herpesviruses, such as HHV-6 and HHV-8, where there is evidence for differences in African countries (Kasolo et al., 1998; Kasolo, Mpabalwani, and Gompels, 1997; Kasolo et al., 2007). Linkage between HCMV genes is rare (Rasmussen et al., 2002; Rasmussen, Geissler, and Winters, 2003) and so both HCMV gO and gN are good targets for analysis as regions which are likely to affect the virulence and tropism of different strains. At the start of this thesis nothing was known about HCMV genotypes in Africa and HCMV had not even been identified in Zambia – the study site for this thesis. In this thesis we use the gO/gN locus to investigate the strains present and genotype variation in three Zambian paediatric cohorts and compare these findings with those from other regions globally.

Glycoprotein gO/UL74	Glycoprotein gN/UL73	Reference strain	Reference strain accession number
gO1a	gN1	AD169	NC_006273
gO1b	gN3a	TR	AC146906.1
gO1c	gN4c	Toledo	AC146905.1
gO2a	gN3b	PH	AC146904.1
gO2b	gN2	HAN 36	GQ227771.1
gO3	gN4a	HAN 39	GQ227773.1
gO4	gN4b	Towne	AC146851.1
gO5	gN4c	Merlin*	GU179001.1

Table 4: Reference strains for the eight gO/gN linked genotypes

*Single example of gO5 at the start of this thesis, initially linked with gN4c, the same as gO1c, suggesting some redundancy in linkage. In this thesis this is investigated further.

1.4.13 HCMV infection and HIV/AIDS

HCMV primary infections and reactivations are a major cause of morbidity and mortality in those suffering from HIV/AIDS. A North American study has shown significant increases in HCMV seroprevalence associated with HIV (60% in HIV negatives vs 95% in HIV positives) (Stover et al., 2003). In populations where HCMV seroprevalence in the general population is very high, the effect of HIV infection on HCMV seroprevalence is not measurable, but there are correlations with HCMV viral loads, which are significantly reduced after HAART (Mihailescu et al., 2008). In Cambodia HCMV sera viral loads were linked with death in HIV/AIDS patients, independently of other opportunistic infections, CD4 count and HAART (Micol et al., 2009).

Studies have shown in children, that HCMV infections occur earlier among those who are HIV positive (Holland et al., 2000; Likitnukul, Bhattarakosol, and Poovorawan, 2003). In HIV positive children, HCMV is linked with disease progression along with impaired brain growth and progressive motor defects (Kovacs et al., 1999), pneumonitis (Williams et al., 2001) and infantile hepatitis (Shibata et al., 2005). Studies in the US found higher rates of HCMV disease in children who were HIV positive. A significant association was observed between HCMV shedding, lower CD4 counts and higher HIV p24 concentrations (Chandwani et al., 1996) along with a decrease in survival (Kitchen et al., 1997). Also within HIV positive children, a U.S study found that the prevalence of active HCMV infection was significantly higher in symptomatic than in asymptomatic HIV positive children, with active infection detected in 70% of AIDS mortalities (Frenkel et al., 1990). Active HCMV (and HHV-6) infections were also shown to be more common among children with HIV infection or those suffering from cancer (Leach et al., 2002). There are also cases of sudden infant deaths among HIV positive babies due to generalized and disseminated HCMV infection (Brady et al., 1988).

In the U.S, where co-infections of HCMV and HIV in children have mainly been studied, whilst extremely damaging, this is a relatively small population. In Africa studies are beginning to address the prevalence of HCMV and its potential impact on morbidity and mortality within the world's largest HIV positive paediatric populations. A study from South Africa found HCMV to be a major cause of pneumonia in HIV-infected infants (Jeena, Coovadia, and Chrystal, 1996) but at the start of this thesis there was little known about HCMV in Zambia. One autopsy study of HIV positive Zambian childhood respiratory mortalities found HCMV in lung tissue (by histopathology) in 42% for 0-5 month old infants (Chintu et al., 2002).

CD4 lymphopenia is likely a major cause of increased HCMV viral loads in HIV infected patients, with fewer circulating CD4 cells available to control HCMV viraemia. HIV and HCMV interactions can also occur within the same cell, where HCMV can transactivate the HIV LTR in cell lines carrying HIV proviral DNA (Davis et al., 1987; Yurochko, Huong, and Huang, 1999) or form a pseudotype altering the HIV tropism (Margalith et al., 1995). HCMV may also affect HIV when infecting a different/neighbouring cell, with cytokine release or antigen presentation possibly transactivating HIV proviral DNA (Clouse et al., 1989; Peterson et al., 1992), and with co-receptor (alternative receptor) up-regulation increasing HIV infection (Pleskoff et al., 1997). Antibody dependent enhancement could also facilitate HIV

entry into cells which are usually non-permissive, but that are expressing an HCMV-encoded Fc receptor such as RL11 (McKeating, Griffiths, and Weiss, 1990). Conversely, HIV has been shown to elicit effects on HCMV, with replication being shown to be up-regulated 2-3 fold in explanted lymphoid tissue co-infected with either CXCR4 or CCR5 tropic HIV (Biancotto et al., 2008). The two viruses have also been shown to co-infect explanted human cervical tissue (Fox-Canale et al., 2007). The molecular interactions between HCMV and HIV are bidirectional, and monitoring of viral co-infections in HIV positive patients, is probably equally as important as the monitoring of HIV.

Many HIV positive patients will be co-infected with both HCMV and HHV-6 although there is little data on this from Southern Africa. Elsewhere, in severely immunocompromised patients, co-infections with both betaherpesviruses are common, resulting in severe pathology (Caselli et al., 2006; DesJardin et al., 1998; Dockrell et al., 1997; Humar et al., 2002). The various and sometimes contradictory *in vitro* interactions described above could be due to differential effects of different betaherpesvirus strain variants interacting with HIV in different cell types or patient groups. Interactions between HCMV and HIV may be further complicated by the presence of other chronic infections such as those caused by the gammaherpesviruses (EBV and KSHV), known to be causal in HIV associated malignancies and pathology (Birx, Redfield, and Tosato, 1986; Chang et al., 1994).

1.5 HIV-1 exposed uninfected infants

1.5.1 Introduction

Southern Africa is the centre of the global HIV pandemic, with rates of infection with HIV being as high as 33% among pregnant women in Botswana (Creek et al., 2009). In this region HIV-1 predominates (Chilongozi et al., 2008) with seroprevalence among women of child bearing age being over 20% in neighbouring Zambia (CSO_Zambia, 2007). Maternal immunity is very important for controlling HCMV infections and so infants who's mothers are immune suppressed are likely to receive less protection through maternal antibody and possibly increased risk of HCMV transmission through breast milk. In recent years the roll out of ART (antiretroviral therapy) to HIV-1 positive African mothers has dramatically reduced the rate of MTCT (mother-to-child-transmission) of HIV-1 so that now less than 10% of infants born to HIV-1 positive mothers actually contract the virus (Coetzee et al., 2005; Guay et al., 1999; Jackson et al., 2003). In Zambia this means the remaining 90% are HIV-1 exposed but uninfected and this accounts for 18% of the infant population as a whole. As the HIV-1 pandemic expands, and as ART becomes more widely available, the number of infants who are HIV-1 exposed but uninfected is also set to expand, yet causes of morbidity and mortality in this group have been less well studied than in HIV-1 infected infants. At the start of this thesis there was data showing that compared to HIV-1 unexposed infants, HIV-1 exposed infants suffer poorer health and development (Makasa et al., 2007; Marinda et al., 2007; Otieno et al., 2006; Thea et al., 1993) but the reasons for this were not well defined. Some of these studies suggested poorer nutrition and increased exposure to infections as possible causes.

1.5.2 Infection and morbidity in HIV-1 exposed infants

Infants in tropical or sub-tropical regions of the globe are naturally exposed to a great number of pathogens – viruses, bacteria, fungi and parasites – than those in temperate countries. Opportunistic infections have been well studied in HIV-1 infected infants but much less so in those who are HIV-1 exposed, in whom they may also be more prevalent and/or more persistent. Increased morbidity from infectious diseases such as malaria (Otieno et al., 2006) and protozoal and bacterial diarrhoea (Thea et al., 1993) is common in low income settings, along with viral diarrhoea caused by a wide range of species: Rota-, Noro-, Adeno-, Entero- and Astro-viruses (Abba et al., 2009; Silva et al., 2008). HCMV also infects the gut, and can

cause lethal enteritis, often in immune compromised patients but also in those who are otherwise healthy (Middleton, 1996; Page et al., 1998). HCMV infections may affect nutrient uptake, either directly through a localized gut infection, or indirectly by diverting energy resources away from the digestive tract to the immune system. Data on morbidity associated with common childhood viral infections in HIV-1 exposed infants is scarce, but the betaherpesviruses are known to be associated with increased morbidity and mortality in HIV-1 infected infants (Kositanont et al., 1999; Kovacs et al., 1999) and hence the rationale for part of this thesis, to look at their effects on HIV-1 exposed infants and interactions with other markers of morbidity including anaemia, reported fever, rate of hospital referrals and survival. Anaemia is common in normal birth weight Zambian infants and increases with age, so that by 6 months of age up to 50% of infants are anaemic (van Rheenen et al., 2008). In this region plasmodium falciparum malaria is endemic, and severe anaemia is a common complication linked with infant mortality (Biemba et al., 2000). HCMV is also known to cause anaemia in congenitally and perinatally infected infants (Distefano et al., 2004) possibly through disruption of the differentiation of haematopoetic stem cells (He et al., 2003). Primary infection with HHV-6 can sometimes cause anaemia (Kagialis-Girard et al., 2006) but more significantly, it is a major cause of fever in infants and has been reported as responsible for up to 40% of infant hospitalizations in North America and Europe (Hall et al., 1994; Portolani et al., 1993). The possible effects of HCMV and HHV-6 infections on HIV-1 exposed infants are not known and will be investigated in this thesis.

1.5.3 Development and breast feeding in HIV-1 exposed infants

HIV-1 infected infants are known to suffer impaired development but maternally HIV-1 exposed infants have been less well studied. At the start of this thesis there were some preliminary reports which suggested that maternally HIV-1 exposed but uninfected infants also had poor development as recently reviewed (Filteau, 2009a). The factors contributing to this are not well defined although some hypotheses have been put forward. For example, during the thesis data was published from Zambia showing that HIV-1 exposed infants suffer from growth faltering (Makasa et al., 2007). One contributing factor proposed was that HIV-1 positive mothers are generally much less likely to breast feed or stop breast feeding early compared with HIV-1 negative mothers (Arpadi et al., 2009). As well as improving growth, breast feeding has other developmental and immunological benefits that are less well understood. For example breast feeding is strongly associated with reductions in gastrointestinal and respiratory infections (Cesar et al., 1999; Howie et al., 1990), and

improved neurological development (Anderson, Johnstone, and Remley, 1999). The benefits of breast feeding are intuitive to many mothers but for those who are HIV-1 positive, and being faced with a 5-10% chance (and some may think it is greater) of transmitting HIV-1 to their child, it is not surprising that they either do not breast feed at all, or terminate breast feeding much earlier than they would do otherwise (Fadnes et al., 2009). It is now an undisputed fact that for HIV-1 positive mothers, the longer they breast feed the more likely they are to transmit HIV-1 to their child (Coovadia and Bland, 2007). The position of the World Health Organization is that exclusive breast feeding is recommended in the 'first months of life' (they do not specify to an exact age), unless available complementary feeding to breast milk is at AFASS standards: 'acceptable, feasible, affordable, sustainable and safe'(Filteau, 2009a). What constitutes AFASS complementary nutrition for HIV-1 exposed infants is not clear and how this affects childhood infections, particularly common virus diseases and breast milk transmitted agents or their strain variants is not known and this is investigated in here in comparison to other cohorts which can be affected by HCMV and HIV. HCMV is known to be widely transmitted through breast milk and so is investigated in this thesis for effects on a range of outcomes with respect to HIV-1 exposure, duration of breast feeding and micronutrient fortification.

1.6 Aims and objectives

This thesis aims to investigate genotype variation of betaherpesvirus infections in Zambian children. Assays will be designed and set up to screen two retrospective cohorts for both HCMV and HHV-6. These assays will then be optimized to screen a large population based cohort taking part in a complementary feeding micronutrient intervention. Prevalence of active infections and also seroprevalence will be analysed for effects on other markers of development and health, with a focus on maternally HIV-1 exposed infants. These aims will be met by the following objectives:

a) Set up qualitative and quantitative PCR assays for measuring loads and genotyping of betaherpesviruses using the hypervariable loci which encode glycoproteins gO and gN
b) Use of the above assays to screen three cohorts of Zambian children:
   - HIV-1 infected respiratory mortalities
   - Hospitalized fevers
   - CIGNIS – Chilenje Infant Growth Nutrition and Infection Study
c) Analyse betaherpesvirus genotypes present in this southern African region and comparing with other regions globally
d) Analyse the effects of betaherpesvirus infection (both active infection and seroprevalence) on markers of development and health, including measures of virus infection and vaccine immunity, with respect to micronutrient interventions and maternal HIV-1 exposure

2.0 Materials and Methods

2.1 Study Sites

The three cohorts studied in this thesis were recruited at either UTH (University Teaching Hospital) or the Chilenje Clinic, both of which are in Lusaka, Zambia. Zambia has a population of roughly 11.7 million (World Health Statistics 2007)(Shibuya, 2007) and UTH is a 2000-bed referral hospital, serving approximately 60% of this population (Kasolo, 1999). Most of the patients attending UTH however come from within Lusaka, a capital city with a population of just over 1.3 million (Central Statistical Office Zambia – 2000 census)(CSO_Zambia, 2000). Initial screening for betaherpesviruses was conducted at the UTH Virology Laboratory, with representative aliquots being taken back to LSHTM (London School of Hygiene and Tropical Medicine) for Real Time PCR and genotyping analysis.

2.2 Patients and Samples

This thesis presents analysis of three independent paediatric cohorts from Lusaka:

a) 141 whole blood samples taken from children hospitalized with fever (excluding malaria and bacterial pneumonia). These were collected or submitted for routine clinical screening to UTH. All children in this cohort were resident in Lusaka (Kasolo, 1999) and were aged between 6 and 34 months. DNA extraction was performed roughly 10 years prior to the work presented in this thesis.
b) 36 lung tissue samples taken from HIV-1 positive paediatric respiratory mortalities (UTH, Zambia)(Chintu et al., 2002). No other clinical or demographic data are available for this cohort.
c) Paired sera samples (6 and 18 months) from a cohort of 812 Zambian infants taking part in CIGNIS (Chilenje Infant Growth, Nutrition and Infection Study: UTH and Chilenje clinic, Lusaka, Zambia). This cohort also included a sub-set of infants who presented with fever herein termed the CIGNIS 'morbidity' samples. All CIGNIS infants were defined as HIV-1 infected, HIV-1 exposed (infants with negative or unknown HIV-1 status, who's mother's are HIV-1 positive), or HIV-1 unexposed (mother HIV-1 negative and infant HIV-1 negative or unknown).

2.3 Ethics

Ethical approval was granted for work on all three cohorts. The hospitalized fever cohort originated from a separate study of spent bloods for which ethical clearance was awarded by the ethical review board of The London School of Hygiene and Tropical Medicine (LSHTM), along with the joint University Teaching Hospital(UTH)/ University of Zambia(UNZA) ethics committee. The respiratory mortalities cohort originated from a Zambian study into the causes of paediatric respiratory failure for which ethical clearance was awarded by the joint UTH/UNZA ethics committee. The original study allowed for detection of HCMV by histopathology in lung tissue samples taken at autopsy. Permission was granted by the principal investigators of this study (Prof C Chintu and Dr F Kasolo) to follow up on this analysis for both HCMV and HHV-6 using molecular diagnostics. The CIGNIS study was awarded ethical clearance by both the LSHTM and UTH/UNZA ethics committees.

2.4 DNA extraction

DNA extractions were performed with a QIAamp DNA blood extraction kit (QIAGEN, Crawley, U.K) according to the manufacturers protocol. For the hospitalized fever cohort 200µl of whole blood were extracted and eluted in 200µl dH_2O. For the respiratory mortalities cohort, a small piece of tissue about $2mm^2$ was homogenized with a surgical blade and then DNA-extracted, eluted in 100µl of dH_2O. For the CIGNIS cohort 200µl of serum was DNA-extracted and eluted in 50µl dH_2O. DNA extractions from clinical material were monitored using a qualitative assay for the human house keeping gene GAPDH, adapted from a previously published quantitative assay (Asahi-Ozaki et al., 2006).

2.5 DNA storage and transport

Prior to DNA extraction all clinical material was stored at -80°C at the virology unit laboratory, UTH. Power-cuts are rare at this facility but freezer temperatures are monitored daily. Freezer break downs were dealt with swiftly by shifting samples to where there is spare capacity. To our knowledge the samples in this study were at no point thawed for an extended period allowing sample degradation. Extracted DNA was stored at the same facility at -20°C. For transportation of DNA from Zambia to the UK, samples were precipitated in 1/10 volume of 3M sodium acetate, and 2 volumes of 100% ethanol, then transported at room temperature.

Upon arrival they were chilled to -20°C, centrifuged at 13,000rpm for 10 minutes after which the ethanol was removed and they were allowed to air-dry for 10 minutes. Samples were then re-suspended in the appropriate volume of nuclease free water.

2.6 Qualitative PCR

Qualitative PCR reactions were made up in 25µl: 12µl GoTaq® Green Master Mix (Promega, Southampton, U.K), 2.5µl of each primer (10µM), 1-5µl of template DNA made up to 7ul with dH_2O. Thermal cycling parameters were 95°C for 2 min, then 40 cycles of 95°C for 30 sec, annealing at 59 °C for 30 sec and extension at 72 °C for 2 min, followed by a final extension step of 72°C for 5 min. Generally 20µls of product was run on an Ethidium Bromide-stained 1-2% agarose gel where positive bands were extracted using a MinElute Gel Extraction Kit (QIAGEN, Crawley, U.K) (Manufacturer's_Protocol, 2008) and eluted in 15µl of nuclease-free water ready for sequencing. All PCR assays included both positive and negative controls. Extracted DNA from virus-infected tissue culture supernatants of the AD169 laboratory adapted strain of HCMV, or the U1102 strain of HHV-6, were used as positive controls. Two negative controls were included with every assay, one reagent control, and one control containing water kept in the sample storage room.

2.7 PCR Primers and assay sensitivity

Primers were designed with the assistance of Primer3 software (Rozen and Skaletsky, 2000) and then adjusted manually after cross-checking across multiple genotypes using ClustalW (Chenna et al., 2003) where necessary (to minimise miss-matches between known genotypes). All primers and probes were synthesized by and purchased from Sigma-Aldrich (Dorset, UK), with sequences detailed in Appendix A. Genomic locations of primers and targets were as shown (Figure 2). The sensitivity of all primer pairs was determined through assaying serial ten-fold dilutions of cloned PCR products representing each target. The sensitivity of individual primer pairs was as shown for both HCMV and HHV-6 (Figure 1)

2.8 PCR contamination prevention

Facilities at the UTH and LSHTM laboratories each comprise three separate rooms for PCR. At the UTH samples are DNA-extracted in a category 2 hood in room one. PCR reagents are stored and prepared in room two. DNA is then added to samples back in room 2 with thermocyclers and gel running equipment all being located in room three. At the LSHTM the set up is similar, with three separate rooms for reagent storage and set-up, DNA storage and addition, and thermocycling and electrophoresis. All reagents were aliquoted to prevent repeat

freeze thaws and reduce risk of contamination. The protocol in place should any contamination be detected was the disposal of all reagents, thorough cleaning of all surfaces and pipettes in all 3 labs with vircon followed by 70% ethanol, and re-stocking with new reagents. Two incidents of contamination were noted, likely arising from carriage of PCR product clones into the reagent set-up room. On both occasions the above protocol was implemented successfully and at no point was there any evidence to suggest that clinical samples had become contaminated.

2.9 Cloning

PCR products were cloned into the pCR®4-TOPO® vector using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Paisley, U.K) (Manufacturer's_Protocol, 2006). Briefly, cloning reactions (PCR product, vector, salt and water) were set up in 6µl and incubated at room temperature for 5 minutes. 2µl of this reaction were then transfected in to 25µl of TOP10 Chemically Competent Cells (Invitrogen, Paisley, U.K). The transfection reaction was incubated on ice for 30 mins, followed by a 30 second heat shock at 42°C, after which 250µl of SOC medium were added prior to incubation for 1 hour at 37°C in a shaking incubator (200rpm). 5-50µl aliquots were then plated on selective plates (Ampicillin 5µg/µl) and incubated over night at 37°C. Selected colonies were then re-spread and after 24 hours subsequent double-colony purified clones were selected and dropped in to 3ml cultures (Ampicillin 5µg/µl). After a further 16 hour incubation, plasmid DNA was extracted from 1.5mls of a 3ml culture using a Miniprep (Invitrogen, Paisley, U.K) (Manufacturer's_Protocol, 2005). The remainder was used to make a glycerol stock (500ul sterile glycerol and 500ul culture) and stored at -80°C. From the Miniprep DNA was eluted in 50µl of nuclease free sterile water and DNA concentrations were measured using a Nanodrop spectrophotometer (NanoDrop Products, Wilmington, DE, USA, supplied in U.K by Thermo Fisher Scientific, Loughborough).

2.10 Preparation of plasmid standard dilution curves

Plasmid copy numbers were calculated (Appendix B) and aliquots prepared containing $2x10^6$ copies/µl. 10 fold serial dilutions (10µl in 90µl) were then prepared down to $2x10^{-1}$ copies/µl. Reactions were then set up as described previously but with 5µl of template (so highest dilution of $2x10^6$ copies/µl is equivalent to 10^7 absolute copies) and 3µl dH_2O (instead of 1µl

template and 7µl dH_2O, as previously). Using 5µl template improved reproducibility assaying serial dilutions. Sensitivities of assays for HCMV gB, gO and gN along with HHV-6 gO and gN are shown (Figure 2).

2.11 Sequencing

Sequencing reactions for both forward and reverse strands of PCR products were set up in 10µl using 1µl of sequencing mix, 3µl of sequencing buffer (ABI v3.1), 1µl of primer (1µM), 4µl of nuclease free water and 1µl of template DNA (For faint bands this was sometimes varied up to 3µl, reducing the amount of water accordingly). Thermocycling parameters were 94°C for 4 min, then 25 cycles of 96°C for 20 sec, 50°C for 10 sec and 72°C for 4 min. Sequencing reactions were done in 96-well plate format and products were then ethanol precipitated as follows: 62.5µl of molecular grade ethanol, 24.5µl of dH2O and 3µl of 3M sodium acetate were added to reach well (for multiple samples a mastermix of these three components made first and then 90µl were put in each well). The plate was then covered in an adhesive film and vortexed for 10 seconds and then left at -20°C for 20 minutes. This was followed by centrifugation at 3000g for 30 minutes after which plates were carefully inverted and the ethanol allowed to drain by gravity. The inverted plate was then spun for 15 seconds at 50g to remove residual ethanol. 150µl of ice-cold 70% ethanol were then added to each well, the plate was covered with adhesive film and vortexed, and then a second centrifugation step at 3000g for 15 minutes. Ethanol removal was repeated in an identical manner and then 10µl of HiDye Formamide (Applied Biosystems, Warrington, U.K) were added to each well. Plates were read on a 3730 DNA Analyser (Applied Biosystems, Warrington, U.K).

2.12 Bioinformatics

Forward and reverse sequences were edited and compiled using *ChromasPro* software and grouped with established genotypes using *BLAST* (NCBI). Nucleotide polymorphisms were only accepted if present on both strands. Translations and alignments were constructed and analysed using the European Molecular Biology Open Software Suite (*EMBOSS*) and other online applications (eg. *Transeq, ClustalW, Boxshade etc...*). Phylogenetic analysis was undertaken using the *Phylip3.67* suite of applications. Primarily alignments had to be generated in the phylip format (.ph) and then saved as plain text files (.txt). From these files 100 bootstraps were generated (seqboot.exe) which were then fed into phylogeny programmes to generate maximum likelihood (*proml.exe*) and parsimony (*protpars.exe*) trees. *Consense.exe* was then used to generate the consensus tree among 100 bootstraps. Distance-based phylogenies of larger alignments were also generated (*filtch.exe*). Resulting phylogenies were then viewed in *treeview* and edited in *microsoft paint*.

2.13 Statistical analyses

Nominal data were compared using Pearson's Chi-Squared unless there was a predicted value of <5 in which case Fishers exact test was used. Continuous variables were compared using a t-test for the equality of the means assuming equal variance. Analysis was undertaken in either Graphpad or SPSS 16. Additional statistical analysis was performed using STATA by the CIGNIS study statistician, Kathy Baisley (KB)

2.14 Real Time PCR

HCMV DNA copy numbers were measured using a Real Time Taqman assay for HCMV gB using the previously described FAM-TAMRA labelled probe and primers (gB1 and gB2)(adapted from (Mattes et al., 2005)). A FAM-TAMRA labelled probe was also designed to bind the HHV-6 U47 PCR product (derived from primers U47F and U47R) to be used in a Real Time Taqman assay for HHV-6. The U47 primers were chosen so as to provide a control for PCR product contamination in the primary screen with U46 primers. To facilitate comparison of HCMV and HHV-6 copy numbers between blood, serum and tissue cohorts a Taqman assay for quantification of the house keeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used (adapted from(Asahi-Ozaki et al., 2006)). PCR product

clones (TOPO-TA Cloning Kit, Invitrogen) were used to make 10-fold dilution series from 10^6 down to 10^{-1} copies/5µl (template volume) for HCMV gB, HHV-6 U47 and GAPDH. These dilution series were assayed in duplicate with average Ct values for dilutions 10^6-10^2 being used to construct standard curves. HCMV gB, U47 and GAPDH assays had sensitivity cut offs of 10^1, 10^2 and 10^2 copies/5µl respectively, determined by the lowest dilution for which two positive results were obtained. Reactions were made up in 25µl in 96-well plate format: 12µl of TaqMan® Universal PCR Master Mix (ABI), 1µl of each primer (10µM) and 1µl of FAM-TAMRA labelled probe, 5µl of nuclease free water and 5µl of template DNA. All Real Time Taqman assays were run on a PRISM® 7000 (ABI) using the following cycling conditions: 50°C for 2 min, 95°C for 10 min and 60 cycles of 95°C for 15 sec and 60°C for 30 sec. Where possible, samples were run in duplicate with the average HCMV or HHV-6 viral load being calculated, and where necessary, combined with GAPDH results to give a final value of copes/10^6 cells. For sequences of all primers and probes used in Real Time PCR see Appendix A.

2.15 HCMV serology

A serological screen was undertaken using an anti-cytomegalovirus IgG ELISA kit (DiaSorin S.p.A, Saluggia, Italy) as per the manufacturer's instructions. In brief, samples were diluted 1:101 and 100µl was added to each well (which are coated with HCMV strain AD169) along with controls. The plate is then incubated for 1 hour at 37°C, followed by 5 washes with buffer. The secondary antibody (mouse anti-human IgG conjugated to horseradish preoxidase) is then added and the plate is incubated again for 1 hour at 37°C. After a further 5 washes with buffer 100µl of substrate (chromogen) is added followed by a 30 minute incubation at room temperature away from direct light. After addition of 200 µl of strop reagent absorbance is read within 30 minutes using a photometer at 450/630 and 405/630 nm. This assay was set up and conducted by an MSc project student, Kunda Musonda (KM).

3.0 HCMV DNA detection, loads & genotypes in three Zambian paediatric cohorts

3.1 Introduction

The aim of this chapter was to develop and implement PCR based assays for detecting active HCMV infections in Zambian children. This virus has not been well studied in Africa, particularly in southern regions at the centre of the HIV-1 pandemic where co-infections may cause serious morbidity and mortality as shown elsewhere (Kovacs et al., 1999). HCMV has not been studied before in Zambia and the prevalence of active infections during childhood, and the genotypes present, are not known. PCR is a highly sensitive and specific technique for detecting viral infections and through the design of conserved primers flanking hypervariable loci, sequencing of PCR products will be used to genotype HCMV at the hypervariable UL73 (glycoprotein gN) and UL74 (glycoprotein gO) genes. PCR assays were employed initially to screen the two retrospective cohorts: DNA-extracted blood from 141 infants hospitalized with fever (6-34 months of age), and DNA-extracted lung tissue samples from 36 HIV-1 positive children (0-16 yrs of age) who died from respiratory disease. After these analyses, a more sensitive assay (gB) was then used to screen DNA-extracted sera samples from a large prospective population-based cohort of healthy Zambian infants taking part in CIGNIS (Chilenje Infant Growth Nutrition and Infection Study), at both 6 and 18 months of age. PCR detection of HCMV in DNA-extracted sera is indicative of active infection as cell associated virus is excluded. After primary detection, all positive samples were subject to genotyping analysis using assays that target the two linked hypervariable loci, UL74 and UL73. HCMV viral loads will be determined using a Taqman based Real Time PCR assay (Mattes et al., 2005). The prevalence of active HCMV infections and loads will be analysed with respect to HIV-1 infection and exposure and genotypes present in this region will be compared with data from other regions globally.

3.2 HCMV PCR assay design and strategy

Assays were required for a high sensitivity primary qualitative screen for HCMV along with assays targeted for the sequencing of two hypervariable loci (UL73 and UL74). With the aim of obtaining good sequence data from samples with minimal levels of viral DNA, multiple sets of nested primer pairs were designed for both hypervariable targets, to increase sensitivity

and broaden sequence coverage (Figure 1). Alignments of the specific loci of eight published reference strains (AD169 NC_001347, Merlin NC_006273, Towne AC146851, Toledo AC146905, FIX AC146907, TR AC146906, PH AC146904 and TB40/E EF999921) were used in the design all HCMV assays, but primer sequences were largely based on that of the reference strain AD169, with exceptions and mismatches noted below.

As very little HCMV genotyping had been done previously in Southern Africa, a conserved target, UL55 (gB), was chosen to allow detection of all known genotypes and any novel HCMV strains that might be unique to this region. For this qualitative assay, primer sequences were taken from a previously published Real Time PCR assay (Mattes et al., 2005) and checked against an alignment of the eight published HCMV reference strains along with selected other human herpesviruses (Appendix C). These primers should broadly detect all HCMV strains but should not cross-react with other human herpesviruses which may be present in the clinical samples. The assay was highly sensitive with a cut off of 1 absolute copy (Figure 1) and was used as the primary qualitative screen for HCMV. Sensitivity analysis was undertaken against the reference strain AD169 only, as the primer sequences relatively conserved across all reference strains: Reference strain Towne contained one mismatch against both the forward and reference primers. Reference strain FIX contained 3 mismatches against the reverse primer (gB2) but a highly related strain, Toledo, contained just one mismatch against the gB2 primer (Appendix C).

The first of the two hypervariable targets was UL73, which encodes the hypervariable glycoprotein gN. An alignment was constructed of the same eight reference strains detailed above, plus two additional strains (Can4 and Can10) so that all seven UL73 genotypes were represented (Mattick et al., 2004). Primers were then designed for broad detection across this hypervariable locus, with mismatches kept to a minimum (Appendix D). External forward primer U73OF was identical across all published reference strains, and the reverse primer U73OR had just one mismatch against reference strain Towne. The internal primer pair (U73F and L73R) has been previously used in our lab (Mattick et al., 2004). U73F contains up to four mismatches, with the corresponding reverse primer L73R having up to eight mismatches, with these clustered towards the 5' terminus of the primer. A second internal primer pair (gN-Up/gN-lw)(Pignatelli et al., 2003a) contained two mismatches in the forward primer, one of which was in the 3' terminal position and may exclude detection of strains like PH (gN3b) and TR (gN3a). The reverse primer gNlw contained 1 mismatch against Toledo and Merlin reference strains but not on the 3' terminal nucleotide. Three nested assays were

most sensitive using U73F/L73F or gNUp/gNlw internal to U73OF/U73OR, or gNUp/gNlw internal to U73F/L73R, all with the same cut off of 10 absolute copies (Figure 1).

The second hypervariable HCMV target was UL74 which encodes the hypervariable glycoprotein gO. An alignment was constructed of the eight reference strains as previously, plus three additional reference strains (DM7, SW475 and SW1715) so that all eight UL74 genotypes were represented (Appendix E). External primers (GO1O/GO2O) contained one mismatch against strain PH in the forward primer (GO1O), but none in the reverse primer (GO2O). The first internal primer pair (gOUp/gOlw) contained no mismatches in the forward primer (gOUp), but 4 in the reverse (gOlw). A further internal primer pair (GO1/GO2) was available which has been previously used in our lab (Mattick et al., 2004; Paterson et al., 2002). The GO1 primer contained up to 4 mismatches and the GO2 primer up to 6 mismatches. None of the mismatches were at the 3' terminal nucleotide. The most sensitive assays using these gO primers were two nested assays using gO-Up/gO-lw internal to GO1O/GO2O, or GO1/GO2 internal to gOUp/gOlw, both with a cut off of 1 absolute copy (Figure 1).

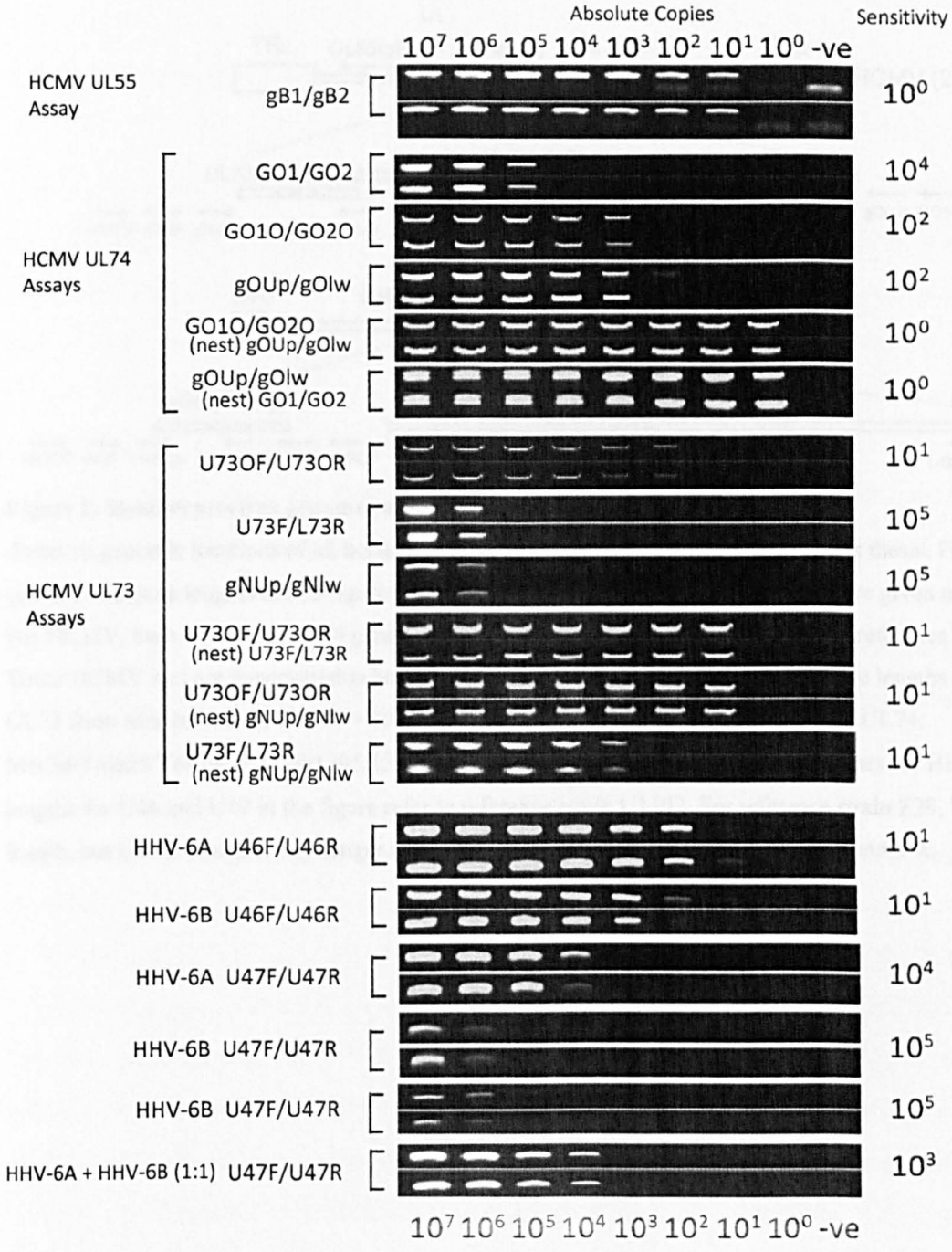


Figure 1: PCR assay sensitivity

10-fold serial dilutions of plasmid controls containing PCR products for the respective primer pairs including nested reactions. It is noted that sensitivity in clinical samples containing excess background genomic DNA may differ. The U47F/U47R primers were up to two logs less sensitive against HHV-6B, than HHV-6A, and so this dilution series was repeated twice to confirm. The rationale for the use of different PCR assays is explained in chapters 3 and 4 for HCMV and HHV-6 respectively.

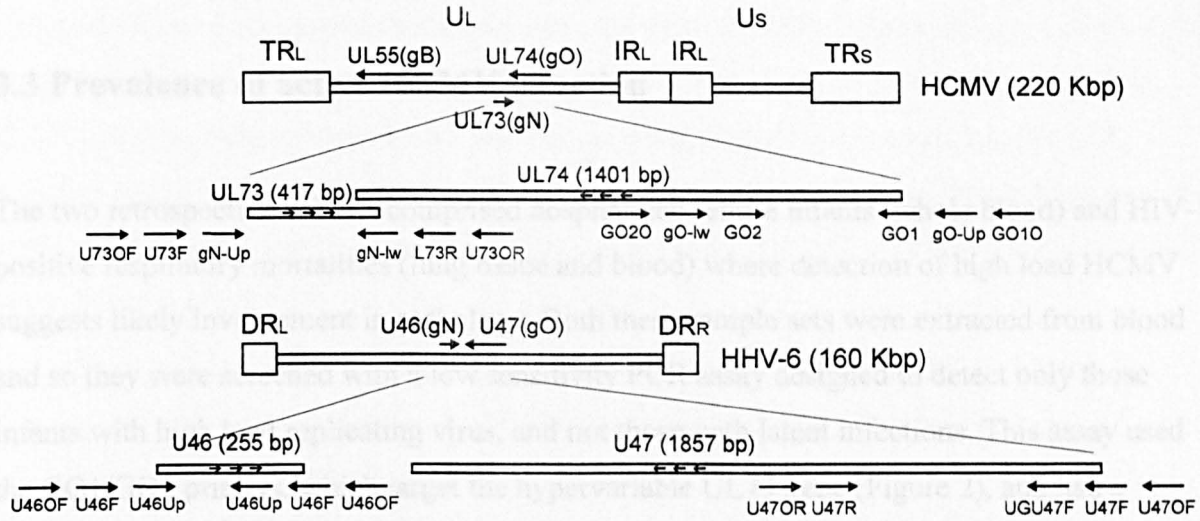


Figure 2: Betaherpesvirus genomes and primer locations

Relative genomic locations of all betaherpesvirus PCR targets and primers used in this thesis. For legibility the genome and gene lengths in this figure are not to scale but gene lengths in base pairs are given in parentheses. For HCMV, both UL73 and UL74 gene lengths are taken from the laboratory adapted reference strain AD169. These HCMV loci are hypervariable however and other available reference strains have lengths as follows: UL73 from Merlin/Toledo/Towne = 408/408/411bp respectively. Correspondingly for UL74: Merlin/Toledo/Towne = 1419/1395/1374bp. There are two published reference genomes for HHV-6: The gene lengths for U46 and U47 in the figure refer to reference strain U1102. For reference strain Z29, U46 is equal in length, but U47 is considerably longer at 2217bp. PCR product sizes are given in Appendix A.

3.3 Prevalence of active HCMV infection

The two retrospective cohorts comprised hospitalized febrile infants (whole blood) and HIV-1 positive respiratory mortalities (lung tissue and blood) where detection of high load HCMV suggests likely involvement in pathology. Both these sample sets were extracted from blood and so they were screened with a low sensitivity PCR assay designed to detect only those infants with high load replicating virus, and not those with latent infections. This assay used the GO1/GO2 primers, which target the hypervariable UL74 gene (Figure 2), and had a sensitivity of 10^4 absolute copies (Figure 1). We employed this assay with a view to obtaining direct genotype data from the sequencing of PCR products. Using this assay, the prevalence of active HCMV infections was 10% in the hospitalized fever cohort but 94% within the respiratory mortalities cohort (10% (14/141) vs 94% (34/36), $P < 0.001$)(Table 6). Using this assay, sequencing of the resulting PCR products enabled detection of strain variation within the N-terminal hypervariable region of UL74 (data presented in section 3.8).

For screening the CIGNIS cohort comprising DNA-extracted sera taken from healthy Zambian infants, a more sensitive assay was required, as preliminary data from use of the GO1/GO2 assay did not yield any positives. Furthermore, we considered the possibility that primers designed over such a variable region may not detect novel variants. Searching the literature identified an HCMV Real Time PCR assay that targets the highly conserved UL55 (glycoprotein gB) gene (Mattes et al., 2005). We used their gB1/gB2 primers (Figure 2) for qualitative PCR and in our hands this assay was highly sensitive, detecting 1 absolute copy (Figure 1). This level of sensitivity was more appropriate for detecting active but likely much lower load infections in the sera of otherwise healthy infants. Of 812 infants successfully enrolled onto CIGNIS, 518 were screened with the HCMV gB assay at month 6. Of the remaining 294 infants: 99 did not provide enough blood for a virology aliquot to be taken, 112 are not yet screened and 83 were screened but negative control indicated contamination. A total of 396 infants were screened at month 18. Of the remaining 416 infants, 161 had withdrawn, 12 died, 54 did not provide enough sera, 61 are yet to be screened, 42 were contaminated and 86 await updated records from Zambia. Work is ongoing to complete this dataset. The prevalence of active HCMV infections as determined by detection of viral DNA in sera was 40% at month 6 and 34% at month 18. All of these samples were taken only from healthy infants. Mothers presenting at the clinic with sick children were asked to return when

the child was well. On occasion samples were taken from febrile infants and so in addition to the 6 and 18 month 'healthy' samples, there was also a small set of 23 samples taken from CIGNIS infants who presented with fever on an unscheduled visit to the clinic (6> <18 months). HCMV sera-DNA prevalence was significantly higher in these 'morbidity samples' (65% (15/23)), compared with either month 6 ((200/518): P = 0.011) or month 18 ((133/396): P = 0.002)(Figure 3a).

3.4 Prevalence of active HCMV infection by infant HIV-1 status

Where HIV-1 data was available, prevalence of active HCMV infections was compared between HIV-1 negative and positive infants (Table 6). In CIGNIS all infants completing the study are screened for HIV-1 by the standard WHO three-test protocol at 18 months of age. Of 178 infants with unknown HIV-1 status, 157 withdrew, 11 died and 10 HIV-1 serology records are missing or were not done. In the hospitalized fever and respiratory mortalities cohorts, HIV-1 diagnosis was by PCR to detect proviral DNA. Among the hospitalized fevers 33% (47/141) of infants were found to be HIV-1 positive but HCMV DNA prevalence did not differ significantly by infant HIV-1 status (Table 6). All 36 infants in the respiratory mortalities cohort were selected as HIV-1 positive and this was confirmed using the proviral DNA assay. For the CIGNIS infants 18 months HIV-1 serology was available for 411 infants screened for HCMV at 6 months of age, and all 396 infants screened for HCMV at 18 months of age (Table 5). Prevalence of active HCMV infection did not differ significantly by infant HIV-1 status in either the healthy infants or among the 23 infants with morbidity (Figure 3b). On the CIGNIS study the number of HIV-1 infected infants is understated as HIV-1 status was not known for any infants who withdrew from the study.

Cohort	Age	HIV-1 uninfected	HIV-1 infected	P	Totals*
CIGNIS (healthy infants)	6m	40% (156/392)	53% (10/19)	.265	40% (200/518)†
	18m	33% (128/383)	39% (5/13)	.705	34% (133/396)
CIGNIS (morbidity)	6-18m	62% (8/13)	100% (2/2)	.524^	65% (15/23)†

Table 5: HCMV sera-DNA prevalence using gB assay in the CIGNIS cohort stratified by infant HIV-1 status

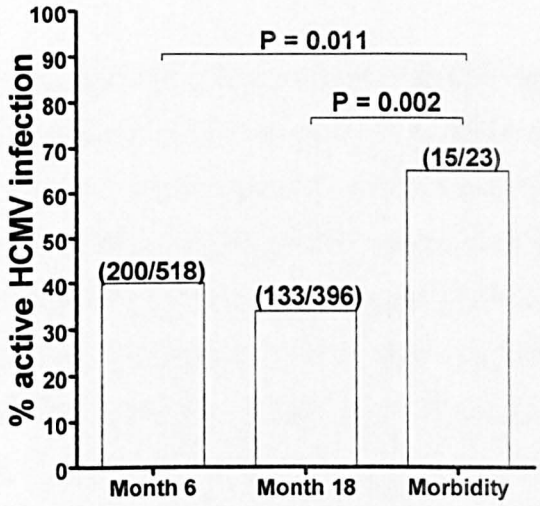
Significance was by Pearson Chi Squared or Fishers exact test (^) if there was an expected value < 5. *The totals column includes infants of unknown HIV-1 status (*). ND = Not done, NA = Not applicable. Significance between totals was as follows: †P = 0.011

Cohort	Age	HIV-1 uninfected	HIV-1 infected	P	Totals*
Fever (blood, n=141)	6-34m	7% (7/94)	15% (7/47)	.163	10% (14/141)‡
Respiratory (lung, n=36)	0-16yr	ND	94% (34/36)	NA	94% (34/36)‡

Table 6: HCMV sera-DNA prevalence using GO1/GO2 primers in the Fever and Autopsy cohorts stratified by infant HIV-1

Significance was by Pearson Chi Squared or Fishers exact test (^) if there was an expected value < 5. *The totals column includes infants of unknown HIV-1 status (*). ND = Not done, NA = Not applicable. Significance between totals was as follows: †P = 0.011 ‡P <0.001

A)



B)

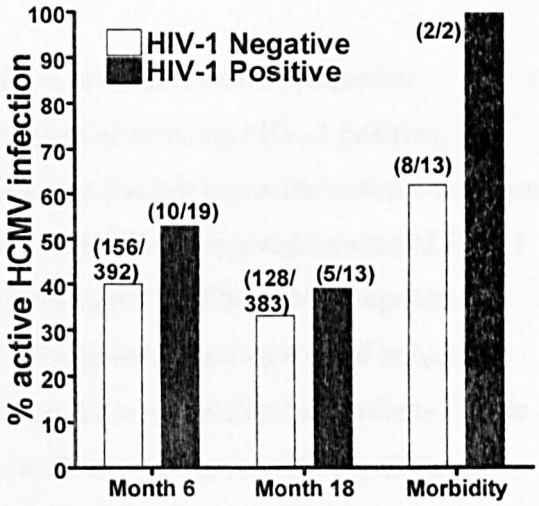


Figure 3: Prevalence of active HCMV infections was higher in samples from febrile infants

HCMV sera DNA prevalence at a) month 6, month 18, and in the small subset of samples from infants who presented with fever and donated an extra sera sample and b) sub-stratified by infant 18 month HIV-1 status. Significance was by Pearson Chi Squared.

3.5 Prevalence of active HCMV infection by maternal HIV-1 status

In the population based study, CIGNIS, maternal HIV-1 serostatus was requested at recruitment. Of 812 mothers, 177 (22%) reported themselves as being HIV-1 positive, 564 (70%) as HIV-1 negative and the remaining 71 (9%) either did not know their status or chose not to disclose it (Figure 4a). Infants were defined as either HIV-1 exposed (maternal HIV-1 seropositive) or HIV-1 unexposed (maternal HIV-1 seronegative). The HIV-1 exposed grouping includes those with unknown HIV-1 status. We assessed prevalence of detectable HCMV sera-DNA in both HIV-1 exposed, and unexposed groups of Zambian infants (Table 7). At 6 and 18 months, the prevalence of HCMV sera-DNA was not significant different between HIV-1 exposed and HIV-1 unexposed infants (Table 7)(Figure 4b). These results show no link between the prevalence of detectable HCMV sera-DNA and HIV-1 exposure.

Cohort	Age	HIV-1 unexposed	HIV-1 exposed	P	Totals*
CIGNIS (population study)	6	37% (132/359)	43% (41/96)	.287	40% (200/518)
	18	35% (95/274)	28% (22/78)	.285	34% (133/396)
CIGNIS (Morbidity)	6-18m	69% (9/13)	63% (5/8)	1.0^	65% (15/23)

Table 7: Prevalence of active HCMV infections is unaffected by HIV-1 exposure

Significance was by Pearson Chi Squared or Fishers exact test (^) if there was an expected value < 5. *The totals column includes subjects with mothers of unknown HIV-1 status.

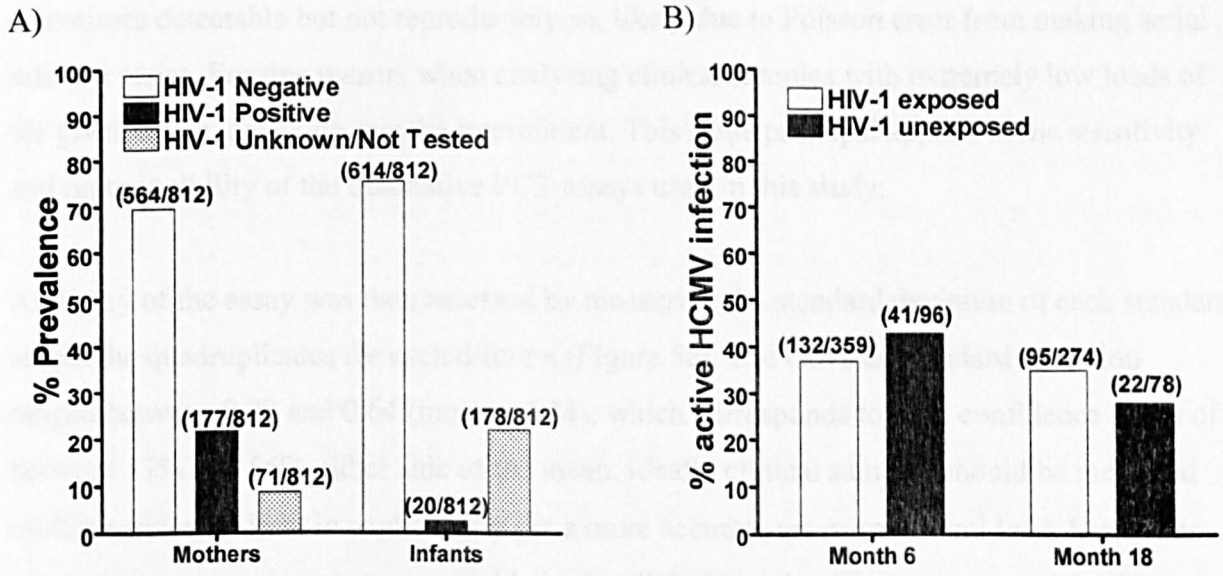


Figure 4: Distribution of HIV-1 status in mothers and infants plus prevalence of active HCMV was unaffected by HIV-1 exposure

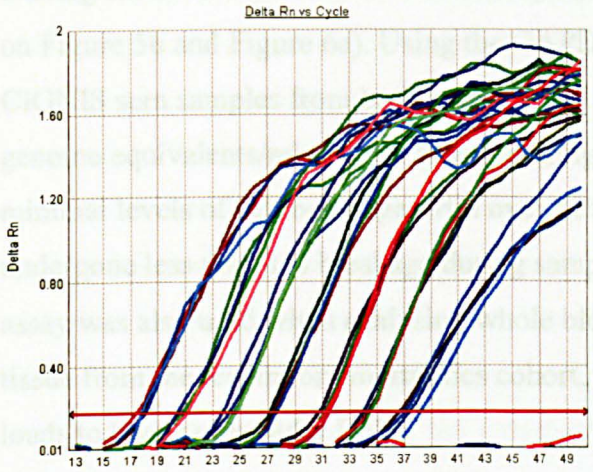
a) Distribution of infants and their mothers on the CIGNIS study by HIV-1 status as determined by sera-antibody and b) prevalence of active HCMV infection determined by detection of HCMV sera-DNA, between HIV-1 unexposed and HIV-1 exposed infants at 6 and 18 months.

3.6 HCMV and GAPDH Real Time Taqman PCR set up and qualitation

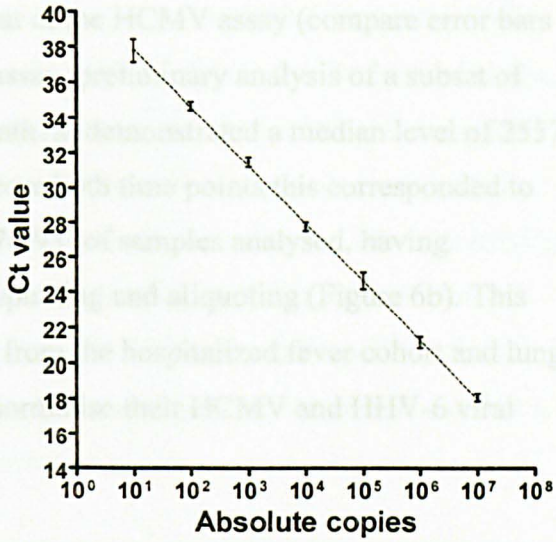
Since detection by qualitative PCR of HCMV sera-DNA does not distinguish between normal primary infections that are immune controlled and severe infections with higher viral loads, a quantitative PCR assay was employed to investigate viral loads. The hypothesis was that infants with impaired immunity (either through HIV-1 infection or exposure) may be less able to control HCMV infections resulting in higher sera-DNA loads. We employed the Real Time Taqman PCR assay referred to in section 3.2 (Mattes et al., 2005), using gB1/gB2 primers which target the highly conserved UL55 gene. A conserved target was essential for this assay as a Taqman probe might not bind consistently over variable sequence. As shown in appendix C, the gB3 probe has just one mismatch with HCMV reference strains FIX and Toledo, but is otherwise identical in all HCMV strains known at the start of this thesis. Sensitivity of this assay was assessed using a plasmid standard of cloned gB1/gB2 PCR product form reference strain AD169. Plasmid copy number was measured and dilution series were prepared ranging from $2x10^6$ through to $2x10^{-3}$ absolute copies/µl. 5µl of template from each dilution was then used giving a series from 10^7 through to 10^{-2} absolute copies. It was determined that standards with ≥10 copies in 5µl of template were detectable in all 4 replicates (Figure 5a). Standards containing 1, 0.1 and 0.01 absolute copies were not detectable and so the cut off for the assay was defined as 10 absolute copies. In subsequent standard series, 1 absolute copy was sometimes detectable but not reproducibly so, likely due to Poisson error from making serial dilution series. For this reason, when analysing clinical samples with extremely low loads of the given target, detection may be intermittent. This same principal applies to the sensitivity and reproducibility of the qualitative PCR assays used in this study.

Accuracy of the assay was then assessed by measuring the standard deviation of each standard across the quadruplicates for each dilution (Figure 5c). The Ct value standard deviation ranged between 0.22 and 0.64 (mean = 0.34), which corresponds to 95% confidence limits of between 17% and 56% either side of the mean. Ideally clinical samples should be measured multiple times (at least in triplicate) to get a more accurate measure of viral load. Due to the constraints on sample volumes available in the CIGNIS study, this was not possible. The cut off of 10 absolute copies corresponded to a sera-DNA load of 500 copies/ml sera. With many CIGNIS samples being only borderline detectable we grouped samples either side of a 1000 copies/ml, a level which has been previously associated with pathology (Li et al., 2003; Mattes et al., 2005).

A)



B)



C)

A	B	C	D	E	F
Copies	Mean Ct	Standard Deviation	Copies*	95% confidence limits†	
10000000	18.065	0.22	9368308	8015167	10949889
1000000	21.158	0.32	1094820	874574	1370532
100000	24.615	0.48	99308	71393	138139
10000	27.755	0.24	11229	9538	13220
1000	31.460	0.28	858	707	1040
100	34.643	0.23	94	80	111
10	37.750	0.64	11	7	17

Figure 5: HCMV Real Time Taqman PCR assay sensitivity and accuracy

Qualitation of HCMV Real Time Taqman assay showing a) amplification plots of replicate standards from 10^7 copies down to 10^1 copies (left to right) and b) the resulting standard curve generated from mean Ct values for each dilution with bars indicating one standard deviation. The best-fit linear regression line ($r^2 = 0.999$; slope = -3.317) is shown as a dashed line. Standards were constructed from a plasmid containing cloned PCR product using primers gB1 and gB2 (Appendix A). *Linear regression of column A vs column B gives a Y intercept of 41.19 and a slope of -3.317 so the calculated absolute copies from the mean Ct values is given by $10^{((\text{column B}-41.19)/-3.317)}$. †calculated by adding or subtracting 1.96 standard deviations to the mean Ct value and then re-calculating copy number using same equation.

A consideration when measuring HCMV sera-DNA with quantitative PCR is the possibility of significant levels of cell breakage occurring during sample handling leading to leakage of intracellular/latent viral genomes that might artificially inflate the detectable sera-DNA viral load. To control against this we used Real Time Taqman assay to quantify human genomic DNA, targeting the GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) housekeeping gene (Asahi-Ozaki et al., 2006), to measure how many host cellular genome equivalents were present in the sera and from this calculate breakage. Compared with the HCMV Real Time Taqman assay, sensitivity was one log lower, with no detection observed in replicate dilutions

containing 10 absolute copies and a mean standard deviation across all dilutions of 0.79, making the accuracy of the GAPDH assay half that of the HCMV assay (compare error bars on Figure 5b and Figure 6a). Using the GAPDH assay, preliminary analysis of a subset of CIGNIS sera samples from both month 6 and month18, demonstrated a median level of 25570 genome equivalents/ml of sera. When looking across both time points this corresponded to minimal levels of cell breakage, with over 82% (76/93) of samples analysed, having undergone less than 1% breakage during sample spinning and aliquoting (Figure 6b). This assay was also used when analysing whole blood from the hospitalized fever cohort and lung tissue from the respiratory mortalities cohort, to normalise their HCMV and HHV-6 viral loads to levels of cellular DNA.

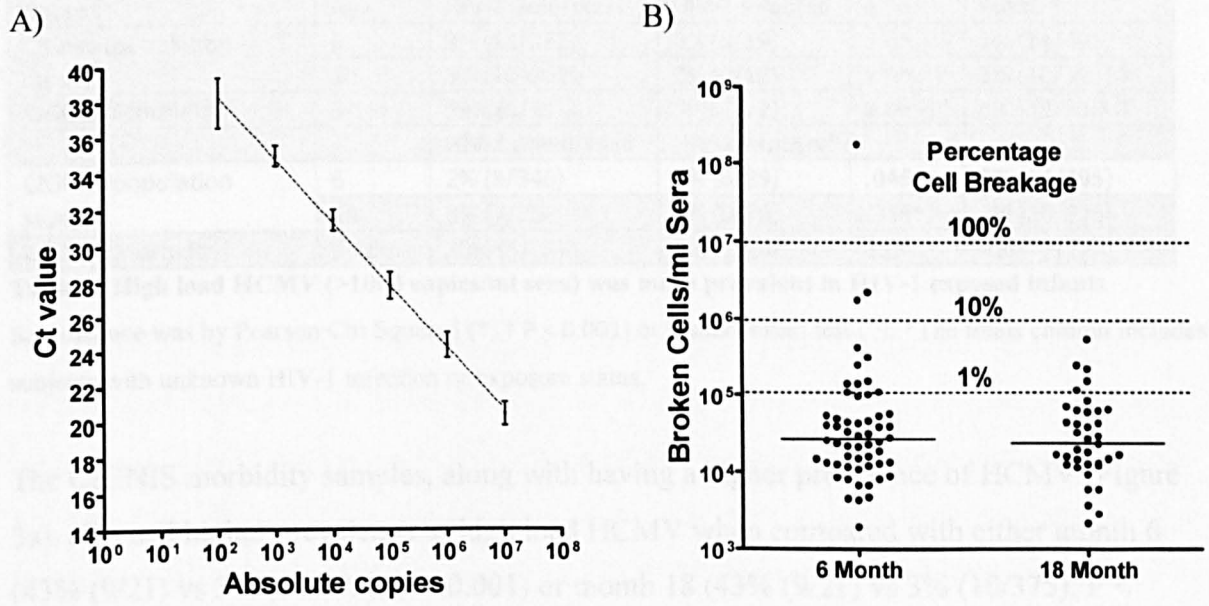


Figure 6: GAPDH Real Time Taqman PCR assay sensitivity and accuracy

Qualitation of GAPDH Real Time Taqman assay showing a) a standard curve of mean Ct values from replicate 10-fold serial dilutions with error bars indicating one standard deviation. The best-fit linear regression line (r^2 = 0.999; slope = -3.515) is shown as a dashed line. Standards were constructed from a plasmid containing cloned PCR product using primers GAPup and GAPdown primers (Appendix A), and b) levels of human cellular DNA detected in CIGNIS sera. Solid lines indicate median loads whilst the dotted lines indicate calculated levels for 1, 10 and 100% cell breakage based on 1ml of sera being derived from roughly 2mls of blood, which would contain approximately 10^7 cells.

3.7 CIGNIS HCMV DNA loads by HIV-1 status

In the CIGNIS study, samples found to be positive by the first round qualitative screen for HCMV, were then analysed further using a Taqman Real Time PCR assay. HCMV sera-DNA was detected in 200 month 6 and 133 month 18 samples (section 3.2), of which 178 and 133

respectively were screened by the HCMV Real Time PCR assay to determine viral load. In the CIGNIS study, 92% of both month 6 and month 18 samples had HCMV viral loads of <1000 copies/ml sera. Here, detection of high load HCMV sera-DNA (>1000 copies/ml sera – associated with pathology in other studies (Li et al., 2003; Mattes et al., 2005)) is compared with both HIV-1 infection and exposure. As previously, the HIV-1 exposed group includes both HIV-1 negative and unknown infants, born to HIV-1 positive mothers. When stratifying by HIV-1 exposure, detection of high load HCMV sera-DNA was significantly associated with HIV-1 exposed infants at baseline (2% (8/346) vs 7% (6/89), P = 0.046)(Table 8 and Figure 7). By month 18 this was no longer significant but analyses may be complicated as it only included those infants who had completed the study.

Cohort	Age	HIV-1 uninfected	HIV-1 infected	P	Totals*
CIGNIS (population study)	6	3% (11/371)	0% (0/19)	1.0^	3% (14/495) †
	18	3% (10/362)	0% (0/12)	1.0^	3% (10/375) ‡
CIGNIS (Morbidity)	6-18m	39% (5/13)	50% (1/2)	1.0^	43% (9/21)† ‡
		HIV-1 unexposed	**HIV-1 exposed**		
CIGNIS (population study)	6	2% (8/346)	7% (6/89)	**.046^**	3% (14/495)
	18	3% (7/257)	0% (0/75)	.356^	3% (10/375)
CIGNIS (Morbidity)	6-18m	39% (5/13)	38% (3/8)	1.0^	43% (9/21)

Table 8: High load HCMV (>1000 copies/ml sera) was more prevalent in HIV-1 exposed infants

Significance was by Pearson Chi Squared (†, ‡ P < 0.001) or Fishers exact test (^). *The totals column includes subjects with unknown HIV-1 infection or exposure status.

The CIGNIS morbidity samples, along with having a higher prevalence of HCMV (Figure 3a), also had higher prevalence of high load HCMV when compared with either month 6 (43% (9/21) vs 3% (14/495); P < 0.001) or month 18 (43% (9/21) vs 3% (10/375); P < 0.001)(Table 8) although median viral loads did not differ significantly (Figure 8). Only healthy infants were sampled at months 6 and 18, and so the minority of infants who had high load HCMV likely had residual viraemia post active infection and were not perceived by the mother or study clinician to be unwell. In the morbidity samples, the prevalence of high load infections between HIV-1 exposed and un-exposed infants did not differ significantly (Table 8 and Figure 7).

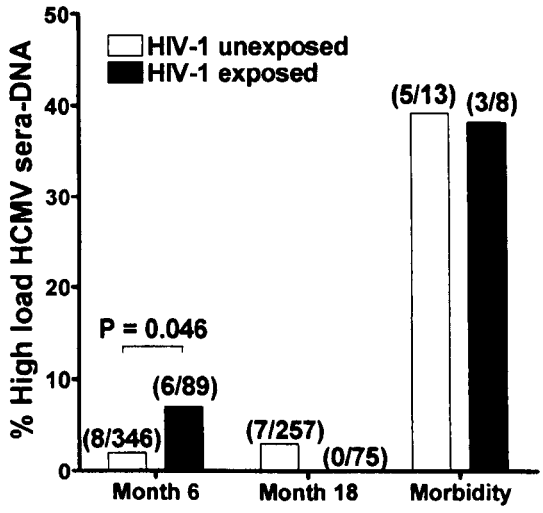


Figure 7: High load HCMV at month 6 is 3 times as prevalent among HIV-1 exposed infants
Prevalence of high load (>1000 copies/ml sera) HCMV infections by maternal HIV-1 status. No month 18 high load HCMV infections were detected in HIV-1 exposed infants. Significance is by Fishers exact test.

3.8 HCMV DNA loads in CIGNIS morbidity, hospitalized fever and respiratory mortalities cohorts

Direct comparisons between the population-based study (CIGNIS) and two retrospective cohorts were not always possible, as whilst all CIGNIS samples were DNA-extracted sera, the hospitalized fever samples were DNA-extracted whole blood and the respiratory mortalities samples were DNA-extracted lung tissue (mixed with blood). Using some estimates to control for volume differences some initial comparisons were made. As one volume of sera is generally spun from two volumes of blood, CIGNIS and hospitalized fever cohorts were compared using copies/ml sera or 0.5 times copies/ml blood respectively (Figure 8). The median HCMV load in the morbidity samples (non-hospitalized febrile infants) was significantly lower (2 logs) than in the hospitalized fever cohort (1283 copies/ml sera vs 154253 copies/ml sera ((½ the load measured in one ml of blood), which were collected from hospitalized febrile infants (Figure 8). The detection of higher loads in blood than in sera could be due to the detection of latent HCMV genomes as well as cell-free virus, but could also represent higher levels of replicating HCMV in these infants with hospitalized fever compared with febrile children who were not hospitalized.

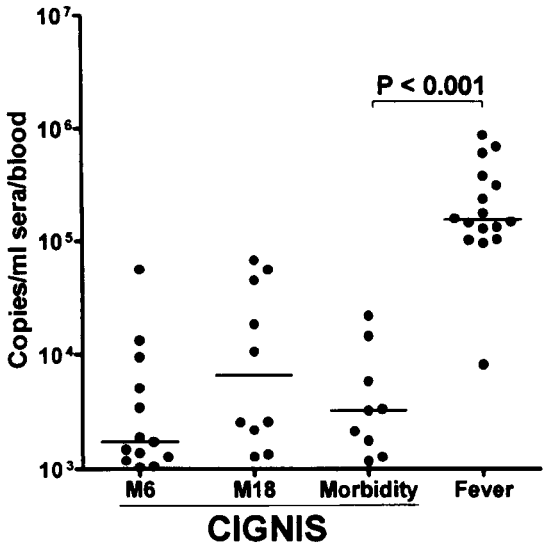


Figure 8: High load HCMV prevalence between cohorts

Distribution of HCMV high loads (>1000 copies/ml) within CIGNIS month 6, month 18 and morbidity samples, as well as those from the hospitalized febrile infant cohort. As these hospitalized fever samples were DNA-extracted whole blood (and CIGNIS samples were sera), hospitalized fever loads were halved as one volume of sera is derived from roughly two volumes of blood. Significance is by Fishers exact test.

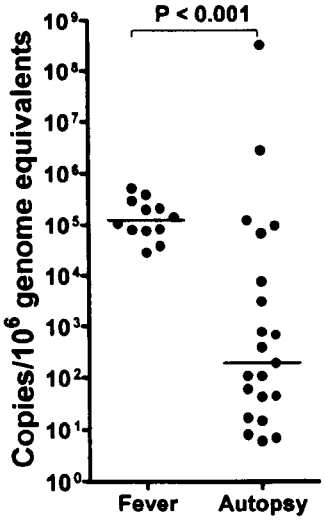


Figure 9: HCMV loads in hospitalized fever and respiratory mortalities cohorts

HCMV loads/10^6 genome equivalents. 5µl of sample was assayed with a Real Time Taqman PCR assay targeting the HCMV UL55 gene and then also assayed with a second Real Time Taqman assay for the endogenous GAPDH gene. Lines represent median values with the significance between them measured by Fishers exact test.

The respiratory mortality samples comprised DNA-extracted lung tissue mixed with blood, and were all from HIV-1 positive children (aged 0-16yrs) who died of respiratory disease. HCMV loads in this cohort were compared to those from the hospitalized fever cohort through normalization to cell number. The Real Time Taqman PCR assay for the endogenous GAPDH gene was used to measure the number of genome equivalents in each sample. HCMV load results are hence given as a ratio of HCMV copies/10^6 genome equivalents (Figure 9) (Griscelli et al., 2001). Overall the hospitalized fever samples clustered more tightly, and had a significantly higher median load of $8.06x10^5$ copies/10^6 genome equivalents ($P < 0.001$). Levels of HCMV and GAPDH gave a much broader range of values up to 10^8 copies/10^6 genome equivalents reflecting differences of viral loads within lung tissue as well as blood inflitration. HCMV loads were detected in both cohorts at levels consistent with active infection (Li et al., 2003; Mattes et al., 2005).

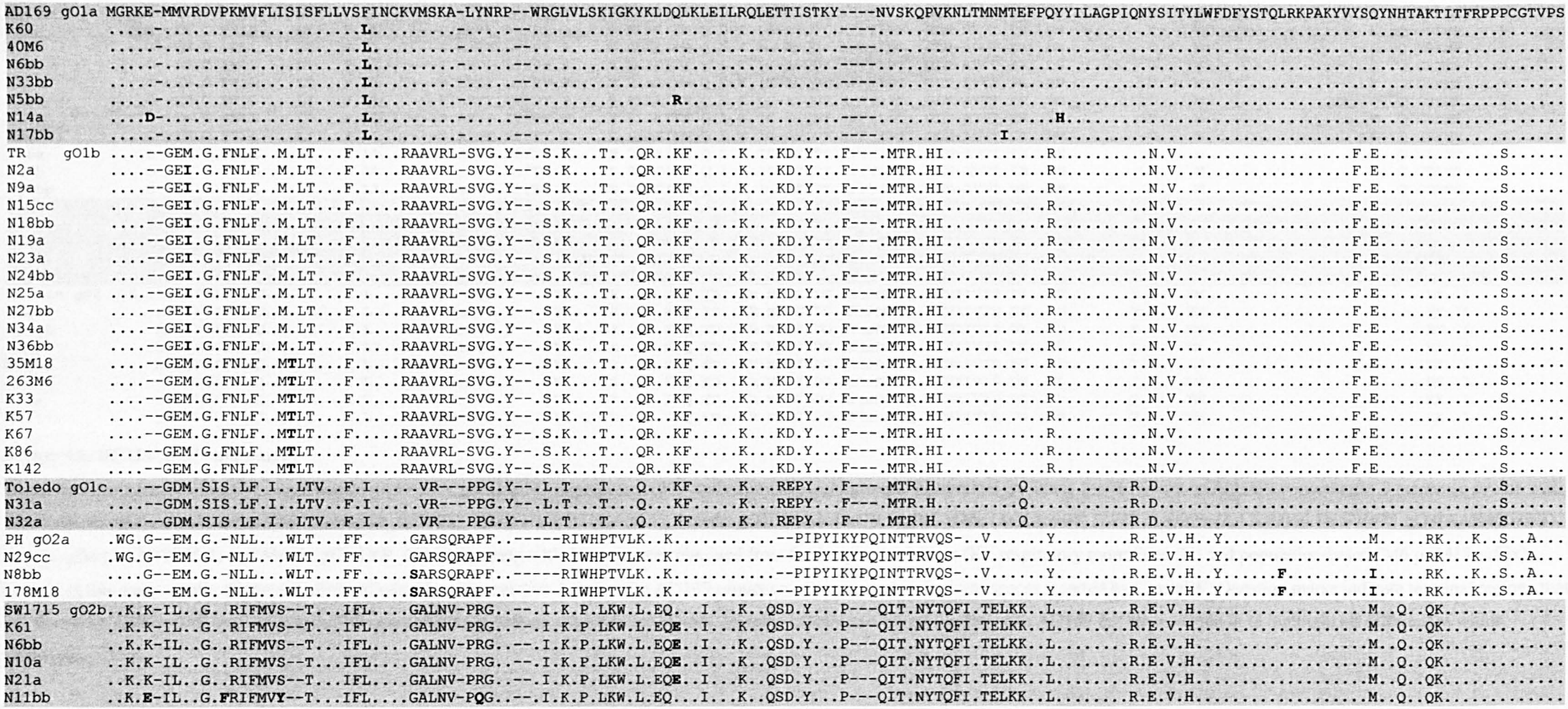
3.9 HCMV genotype analysis

Having identified HCMV in this paediatric population in an HIV-1 endemic area, the next question was whether the same or different strains exist in Zambia compared with elsewhere. To answer this, the hypervariable linked genes UL73 (glycoprotein gN) and UL74 (glycoprotein gO) were investigated. Samples from all three cohorts, found to be positive for HCMV through the primary screen, were subsequently analysed by PCR assays targeting the ~0.45 Kb hypervariable N-terminal region of the 1.4 Kb UL74 gene, along with the entire ~0.42 Kb of the smaller neighbouring UL73 gene. Sequence data was most readily obtained from the two symptomatic cohorts due to higher viral DNA loads. All sequences were confirmed in both forward and reverse directions with selected novel sequences confirmed using a high fidelity thermal polymerase.

3.9.1 HCMV UL74 sequence alignment

A nested PCR assay (GO1O-gOUp-gOlw-GO2O)(Figure 2) was used to amplify and sequence the N-terminal hypervariable region of the UL74 gene in all samples HCMV DNA positive from initial screens. Although the first round reaction was less sensitive, re-constructions suggested the nested assays should be as sensitive as the primary screen (Figure 1). In practice however, this was not the case, with genotypes being primarily detected in the respiratory mortalities and hospitalized fever cohorts where loads were higher. Of 396 HCMV DNA positive samples, a total of 56 UL74 sequences were determined: 44 from the

respiratory mortality samples, 8 from the hospitalized fever samples and 4 from the CIGNIS samples. Alignments of the UL74 sequences were constructed with 8 reference strains representing the eight established HCMV gO genotypes, with data from this study being grouped accordingly (Figure 10). Results show that the majority of sequences belonged to the gO1b (32% - 18/56) and gO3 (18% - 10/57) groups, but with representatives of all eight genotypes being detected. Interestingly, all gO5 sequences identified from Zambia, contained a 2 base pair N-terminal deletion, taking the accepted start methionine out of frame, suggesting leaky transcription and internal initiation on the first downstream methionine, which may result in a lower level of expression of HCMV gO in these strains (Figure 11).


AD169 gO1a MGRKE-MMVRDVPKMVFLISISFLLVSFINCKVMSKA-LYNRP--WRGLVLSKIGKYKLDQLKLEILRQLETTISTKY----NVSKQPVKNLTMNMTEFPQYYILAGPIQNYSITYLWFDFYSTQLRKPAKYVYSQYNHTAKTITFRPPPCGTVPS
K60
40M6
N6bb
N33bb
N5bb
N14a
N17bb
TR gO1b
N2a
N9a
N15cc
N18bb
N19a
N23a
N24bb
N25a
N27bb
N34a
N36bb
35M18
263M6
K33
K57
K67
K86
K142
Toledo gO1c
N31a
N32a
PH gO2a
N29cc
N8bb
178M18
SW1715 gO2b
K61
N6bb
N10a
N21a
N11bb

Continued…

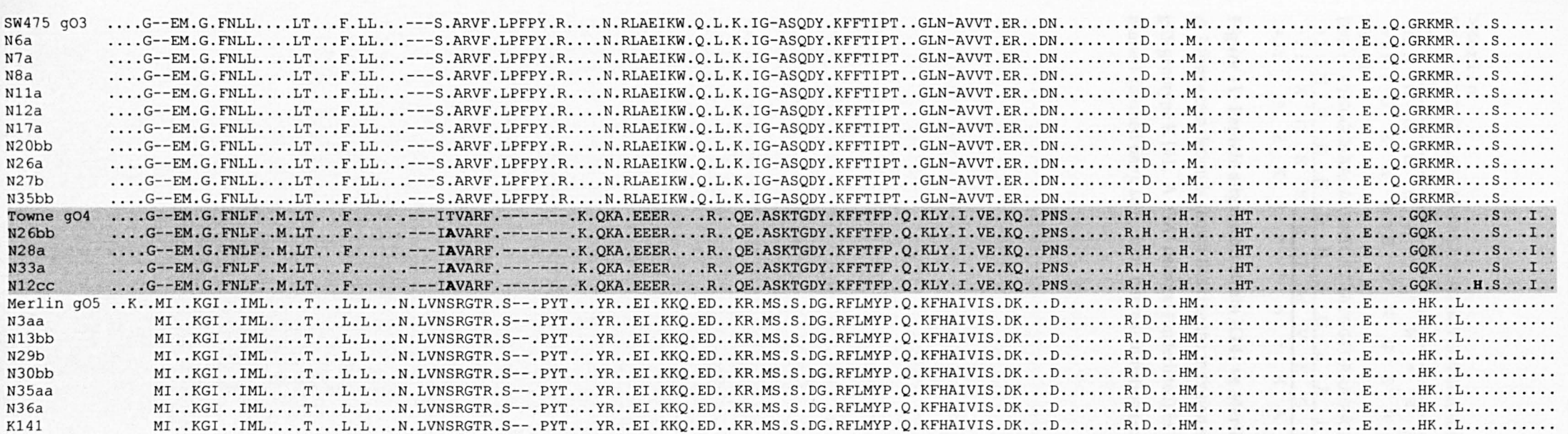


Figure 10: HCMV UL74 alignment

N-terminal amino acid ClustalW alignment of all HCMV gO (UL74) sequences in this study showing eight groups: N-terminal (residues 1-148 relative to AD169) sequence for 8 reference strains with GenBank accession numbers (AD169-gO1a (NP_040008), TR-gO1b (AC146906.1), Toledo-gO1c (AAN40079.1), PH-gO2a (AC146904) SW1715-gO2b (AAN40066.1), SW475-gO3(AAN40072.1), Towne-gO4 (AAN40080.1) and Merlin-gO5 (YP_081522)) along with 56 sequences obtained from the hospitalized fever (K), respiratory mortalities (N) and population-based (M6 or M18 suffix) cohorts in this study. In the alignment, dots indicate identities relative to reference AD169 sequence, whereas dashes indicate spaces inserted by ClustalW. Question marks indicate terminal regions that are unknown. Differences from each group's representative genotype sequence are in bold. In some cases these are an identity with AD169 but have written in to highlight all differences within genotypes.

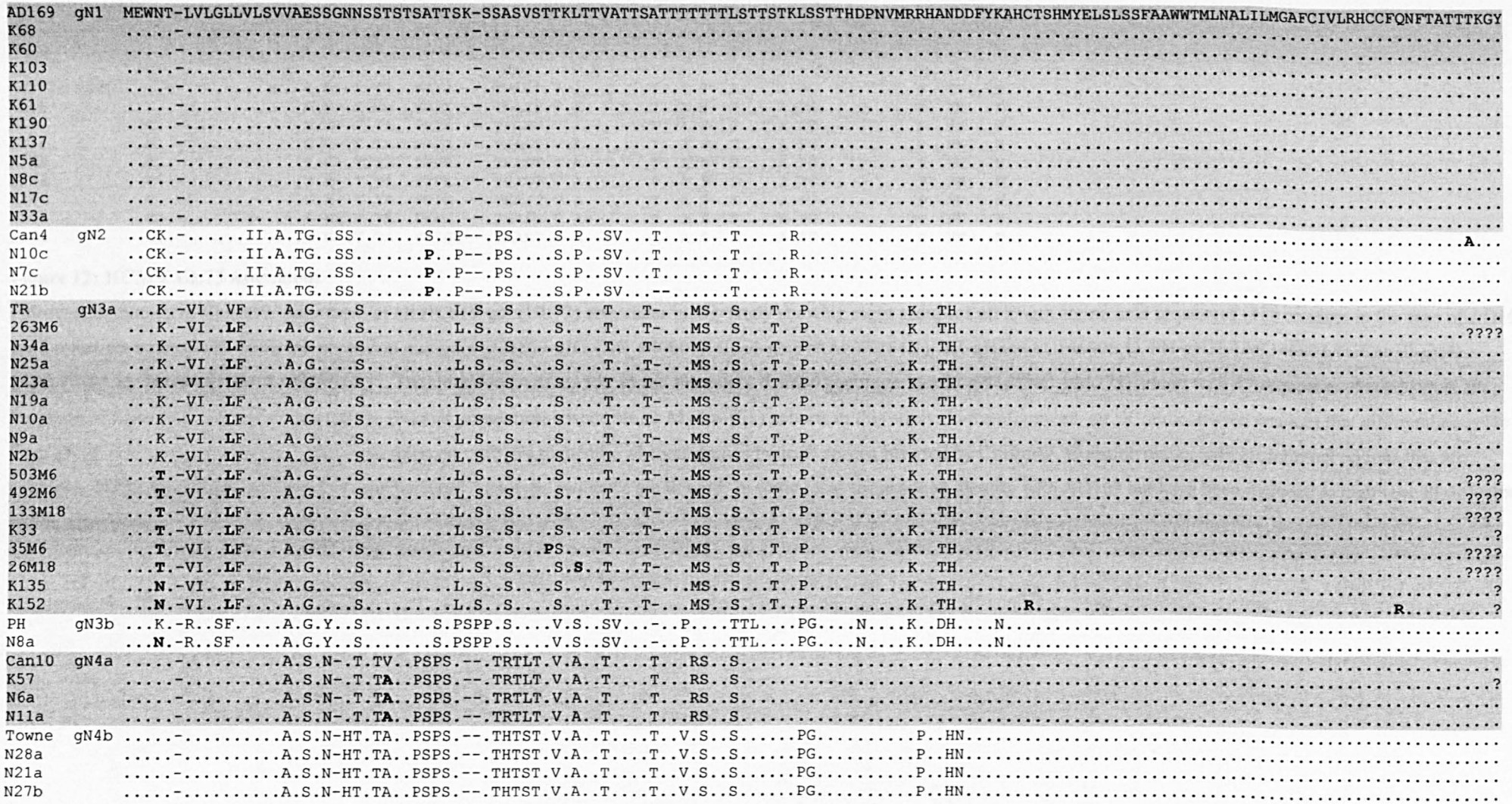
```
>Merlin
DNA: ATGGGGAAAAAAGAGATGATAATGGTGAAAGGCATTCCTAAAATTATGCTC
 +1: M  G  K  K  E  M  I  M  V  K  G  I  P  K  I  M  L
 +2:  W  G  K  K  R  *  *  W  *  K  A  F  L  K  L  C  S
 +3:   G  E  K  R  D  D  N  G  E  R  H  S  *  N  Y  A  P
>K141
DNA: ATGGGGAAAAAAGATGATAATGGTGAAAGGCATTCCTAAAATTATGCTCCT
 +1: M  G  K  K  D  D  N  G  E  R  H  S  *  N  Y  A  P
 +2:  W  G  K  K  M  I  M  V  K  G  I  P  K  I  M  L  L
 +3:   G  E  K  R  *  *  W  *  K  A  F  L  K  L  C  S  *
```

Figure 11: Peptide alignment: strain K141 vs Merlin

A 3 frame translation is shown flanking the initiation site in both Merlin reference strain and Zambian reference g05 genotype K141. A 'GA' deletion (underlined) in K141-like sequences introduces a stop codon (*) into frame 1 (dashed line), suggesting internal initiation (solid line, K141).

3.9.2 HCMV UL73 sequence alignment

A nested PCR assay (U73OF-U73F-L73R-U73OR)(Figure 2) was used to amplify and sequence entire hypervariable UL73 gene in all samples HCMV DNA positive from initial screens. Of 396 HCMV DNA positive samples, a total of 47 UL73 sequences were determined: 28 from the respiratory mortality samples, 12 from the hospitalized fever samples and 6 from the CIGNIS samples. Alignments of the UL73 sequences were constructed with 7 reference strains representing the seven established HCMV gN genotypes, with data from this study being grouped accordingly (Figure 12). The most prevalent genotype was gN3a (34% - 16/47) which is linked to gO1b, the most prevalent UL74 genotype. The second most prevalent UL73 genotype was gN1 (23% - 11/47), although four of these sequences were from multiply infected samples and two from putative novel linkages (Table 10). The alignment of all available UL73 sequences identified representative members of all established genotypes (Figure 12). Novel sequences were identified that group with the reference strain Merlin, which has previously been grouped with gN4c (Dolan et al., 2004; Stanton et al., 2005). Here analysis of multiple sequences similar to the Merlin gN identifies seven conserved amino acid substitutions (see Xs in Figure 14) compared with the gN4c reference Toledo, defining a new gN genotype termed gN4d. These Merlin-like gN4d sequences are as divergent from gN4c as the other neighbouring genotype, gN4b, which is also defined by seven amino acid substitutions. This grouping was further confirmed by phylogenetic analysis (section 3.7). These new Merlin-like gN4d genotypes from Zambia were mainly from the respiratory mortalities cohort (N1c, N12c, N18a, N24a, N29a, N30a and N36a) but there was also one from the hospitalized fever cohort (K141). A second notable change was a double threonine deletion in sequence N21b, reducing an 8T to a 6T polythreonine motif.


AD169 gN1 MEWNT-LVLGLLVLSVVAESSGNNSSTSTSATTSK-SSASVSTTKLTTVATTSATTTTTTTLSTTSTKLSSTTHDPNVMRRHANDDFYKAHCTSHMYELSLSSFAAWWTMLNALILMGAFCIVLRHCCFQNFTATTTKGY
K68
K60
K103
K110
K61
K190
K137
N5a
N8c
N17c
N33a
Can4 gN2
N10c
N7c
N21b
TR gN3a
263M6
N34a
N25a
N23a
N19a
N10a
N9a
N2b
503M6
492M6
133M18
K33
35M6
26M18
K135
K152
PH gN3b
N8a
Can10 gN4a
K57
N6a
N11a
Towne gN4b
N28a
N21a
N27b

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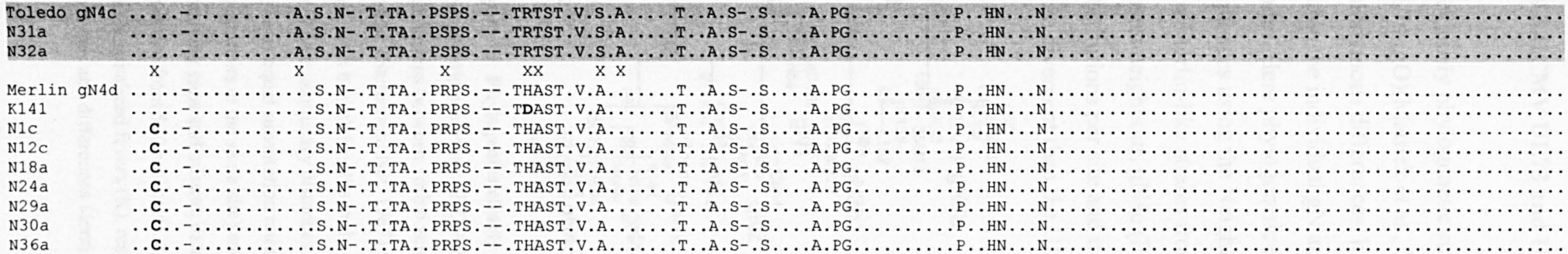


Figure 12: HCMV UL73 Alignment

Full-length amino acid ClustalW alignment of all HCMV gN (UL73) sequences in this study showing eight groups: Full length amino acid sequences (138 residues in the case of AD169) for 8 reference strains with GenBank accession numbers (AD169-gN1 (NP_040007), Can4-gN2 (AAL77764.1), TR-gN3a (AC146906.1), PH-gN3b (AC146904.1), Can10-gN4a (AAL77775.1), Towne-gN4b (AAG23510.1), Toledo-gN4c (AAG23509.1) and the newly defined genotype Merlin-gN4d (NC_006273), along with 47 sequences obtained from the hospitalized fever (K), respiratory mortalities (N) and population-based (M6 or M18 suffix) cohorts in this study. In the alignment, an 'X' indicates the residues that differentiate gN4c from gN4d, dots indicate identities relative to reference AD169 sequence, whereas dashes indicate spaces inserted by ClustalW. Question marks indicate terminal regions that are unknown. Differences from each group's representative genotype sequence are in bold. In some cases these are an identity with AD169 but have been included to highlight all differences within genotypes.

3.9.3 HCMV UL73 and UL74 phylogenetic analyses

Previously phylogenetic studies of variation in both UL73 (gN) (Pignatelli et al., 2003a) and UL74 (gO)(Mattick et al., 2004) through analyses of an excess of nonsynonymous to synonymous differences, have demonstrated evidence for positive selection. Interestingly, within the individual gN and gO genotypes they are all conserved forms in tightly defined clades. Here phylogenetic evolutionary distances have been investigated between amino acid sequences using the Neighbor-Joining method (Saitou and Nei, 1987), confirming that the new Merlin-like strains from Zambia, form a new genotype (gN4d), clearly distinct from the closest neighbour, gN4c (Figure 13). Branch lengths represent number of amino acid substitutions per site and it is evident that the N-terminal of UL74 (gO) is up to three times more divergent than the UL73 (gN) gene.

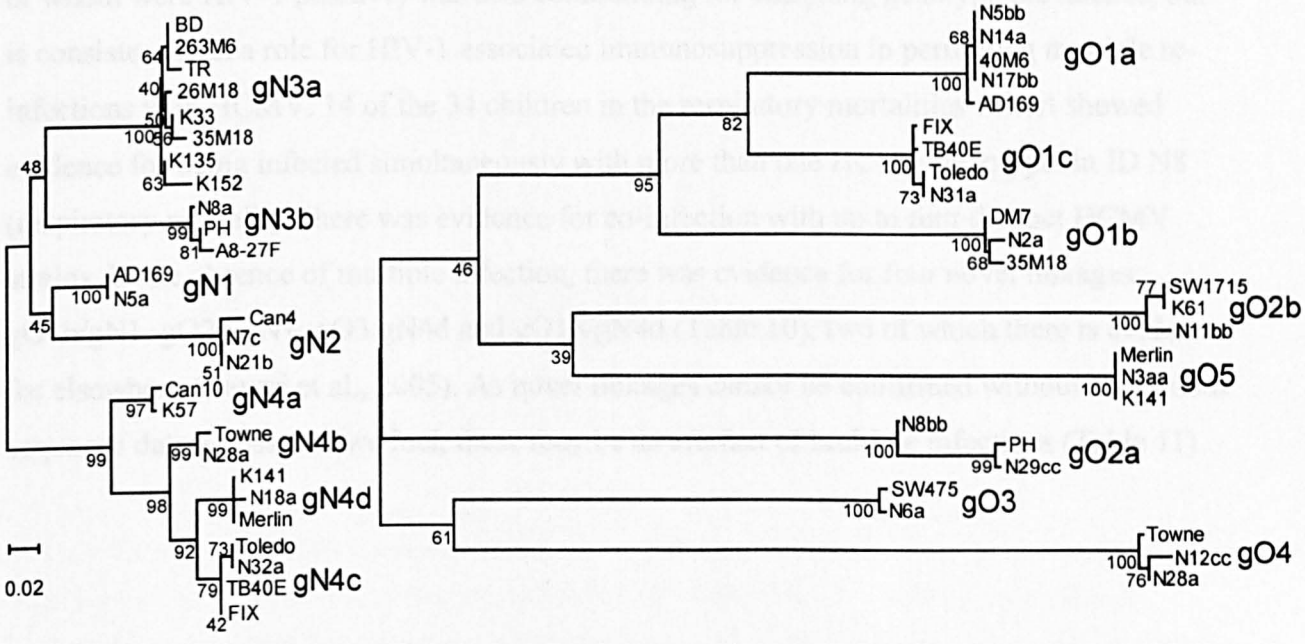


Figure 13: Phylogenies clearly demonstrate eight distinct HCMV gN and gO genotypes

Phylogenetic analyses of HCMV N-terminal gO and full-length gN defines the gO5/gN4d linkage. Evolutionary relationships between amino acid sequences are shown using distance based trees using the Neighbor-Joining method (Saitou and Nei, 1987) generated using *MEGA4.1* software (Tamura et al., 2007). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl, 1965) (assumes equal substitution rates between sites, and amino acid frequencies but corrects for multiple substitutions at the same site) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option) and bootstrap values are from 500 replicates. The reference sequences are as in Figure 10 and Figure 12 along with sequences from hospitalized fevers (K), respiratory mortalities (N) and CIGNIS (M6 or M18 suffix), representing the genotypes and differences identified.

3.9.4 HCMV gO/gN linkages and multiple infections in Zambian children

Previously seven HCMV gO/gN linkages had been defined (Mattick et al., 2004) but the new gN4d genotype detected in Zambia linked with gO5 in five samples (N13, N29, N30, N36 and K141) and also the reference strain Merlin, forming a new eighth linked genotype, gO5/gN4d, that has not been defined previously (Table 9). The two most prevalent linkages were gO1b/gN3a and gO1a/gN1 but analysis was confounded by the difficulties of genotyping low load samples. Analysis of genotypes in samples where only single infections were detected showed evidence for seven out of the eight genotype linkages in Zambia (Table 9), but evidence for gO2a/gN3b, gO2b/gN2 and gO4/gN4b were only seen in multiply infected samples. The high prevalence of multiple infections in the respiratory mortalities cohort (all of whom were HIV-1 positive) was also confounding for analysing genotype prevalence, but is consistent with a role for HIV-1 associated immunosuppression in permitting multiple re-infections with HCMV. 14 of the 34 children in the respiratory mortalities cohort showed evidence for being infected simultaneously with more than one HCMV genotype. In ID N8 (respiratory mortality) there was evidence for co-infection with up to four distinct HCMV strains. In the absence of multiple infection, there was evidence for four novel linkages: gO1b/gN1, gO2b/gN1, gO3/gN4d and gO1b/gN4d (Table 10), two of which there is evidence for elsewhere (Beyari et al., 2005). As novel linkages cannot be confirmed without continuous sequence data across the two loci, these may be an artefact of multiple infections (Table 11).

Sample	Cohort	UL74	UL73	HIV Status	Putative Linkage
N5	Autopsy	1a	1	+	gO1a/gN1
K60	Fever	1a	1	-	gO1a/gN1
N14	Autopsy	1a	ND	+	gO1a/?
N22	Autopsy	1a	ND	+	gO1a/?
40M6	CIGNIS	1a	ND	-	gO1a/?
156M18	CIGNIS	1a	ND	-	gO1a/?
321M18	CIGNIS	1a	ND	-	gO1a/?
K68	Fever	ND	1	+	?/gN1
K166	Fever	ND	1	-	?/gN1
K180	Fever	ND	1	-	?/gN1
N2	Autopsy	1b	3a	+	gO1b/gN3a
N9	Autopsy	1b	3a	+	gO1b/gN3a
N15	Autopsy	1b	3a	+	gO1b/gN3a
N19	Autopsy	1b	3a	+	gO1b/gN3a
N23	Autopsy	1b	3a	+	gO1b/gN3a
N25	Autopsy	1b	3a	+	gO1b/gN3a
N34	Autopsy	1b	3a	+	gO1b/gN3a
263M6	CIGNIS	1b	3a	-	gO1b/gN3a
35M6	CIGNIS	1b	3a	-	gO1b/gN3a
503M6	CIGNIS	ND	3a	-	?/gN3a
492M6	CIGNIS	ND	3a	+	?/gN3a
133M18	CIGNIS	ND	3a	-	?/gN3a
26M18	CIGNIS	ND	3a	+	?/gN3a
N31	Autopsy	1c	4c	+	gO1c/gN4c
N32	Autopsy	1c	4c	+	gO1c/gN4c
178M18	CIGNIS	2a	ND	-	gO2a/?
K3	Fever	ND	3b	+	?/gN3b
K146	Fever	ND	3b	+	?/gN3b
K152	Fever	ND	3b	+	?/gN3b
776M6	CIGNIS	2b	ND	-	gO2b/?
329M18	CIGNIS	2b	ND	-	gO2b/?
K57	Fever	3	4a	+	gO3/gN4a
K54	Fever	ND	4a	+	?/gN4a
K141	Fever	5	4d	-	gO5/gN4d
N13	Autopsy	5	4d	+	gO5/gN4d
N29	Autopsy	5	4d	+	gO5/gN4d
N30	Autopsy	5	4d	+	gO5/gN4d
N3	Autopsy	5	ND	+	gO5/?
N1	Autopsy	ND	4d	+	?/gN4d

Table 9: HCMV UL73/UL74 linkages in Zambia

HCMV UL73 and UL74 genotypes detected in all three cohorts, showing HIV-1 status of the infant and evidence for seven gO/gN linked genotypes, as differentiated by white and grey shading. ND = Not Determined. Samples in which only one genotype was determined are included but with a question mark against the corresponding genotype in the linkage column.

Sample	Cohort	UL74	UL73	HIV Status	Putative Linkage
K190	Fever	1b	1	-	gO1b/gN1 (novel)
K61	Fever	2b	1	+	gO2b/gN1 (novel)
N20	Autopsy	3	4d	+	gO3/gN4d (novel)
N18	Autopsy	1b	4d	+	gO1b/gN4d (novel)
N24	Autopsy	1b	4d	+	gO1b/gN4d (novel)
K33	Fever	1b	1/3a	-	multiple gN
N10	Autopsy	2b	2/3a	+	multiple gN
N21	Autopsy	2b	2/4b	+	multiple gN
N17	Autopsy	1a/3	1	+	multiple gO
N33	Autopsy	1a/4	1	+	multiple gO
N11	Autopsy	2b/3	4a	+	multiple gO
N27	Autopsy	1b/3	4b	+	multiple gO
N28	Autopsy	1b/4	4b	+	multiple gO
N26	Autopsy	3/4	4b	+	multiple gO
N36	Autopsy	1b/5	4d	+	multiple gO
N12	Autopsy	3/4	4d	+	multiple gO
N35	Autopsy	3/5	ND	+	multiple gO
K142	Fever	1b/3	4c	-	multiple gO
N6	Autopsy	1a/3	1/4a	+	multiple gO and gN
N7	Autopsy	2b/3	2/4a	+	multiple gO and gN
N8	Autopsy	1b/2a/3	1/3b	+	multiple gO and gN

Table 10: HCMV UL74/UL73 novel and multiple linkages

HCMV novel gO/gN linkages and multiply infected samples from the hospitalized fever (Fever) and respiratory mortalities (Autopsy) cohorts, showing HIV-1 status of the infant and putative linkage. White and grey shading groups samples as novel, multiple gN, multiple gO or multiple gO and gN. ND = Not Determined.

Sample ID	AD169		TR-BAC		Toledo		PH-BAC		Can4		ZVS		Towne		Merlin	
	gO1a	gN1	gO1b	gN3a	gO1c	gN4c	gO2a	gN3b	gO2b	gN2	gO3	gN4a	gO4	gN4b	gO5	gN4d
K33		x	x	X												
N10				X					x	x						
N21									x	x				x		
N17	x	x									x					
N33	x	x											x			
N11									x		x	x				
N27			x								x			x		
N28			x										x	x		
N26											x		x	x		
N36			x												x	x
N12											x		x			x
N35											x				x	
K142			x			x					x					
N6	x	x									x	x				
N7									x	x	x	x				
N8		x	x				x	x			x					

Table 11: HCMV UL73/UL74 multiple linkages

The 14 respiratory mortality and 2 hospitalized fever samples for which definitive evidence of multiple infections was detected. The eight reference strains are given horizontally showing established gO/gN linkages. Crosses indicate detection of that genotype by sequence analysis. Grey boxes indicate the detection of gO/gN genotypes that correspond to one the eight established genotypes. At the time of writing, the Can4 and ZVS reference linkages (gO2b/gN2 and gO3/gN4a) had not been confirmed by publication of a whole genome.

3.10 Discussion

Detection of HCMV sera-DNA indicates active infection. Among healthy Zambian infants, the prevalence of these active infections was 40% at month 6 and 34% at month 18, suggesting many primary HCMV infections in Zambia occur in early childhood. Prevalence was higher (65%) in the 23 morbidity sera samples that were submitted by CIGNIS participants reporting to the clinic with fever (9 of these infants were later admitted to hospital). These findings from Zambia are similar to another recent study of African infants in the Gambia, in which 90% of infants were shown to have undergone their primary HCMV infection by 12 months of age (Kaye et al., 2008). In this study over 75% of infants were infected before they were 6 months of age. Exposure to HCMV was similar in both HIV-1 positive and negative Zambian infants. With respect to maternal HIV-1 status however (where numbers afforded power for analysis) whilst overall detection of sera-DNA was not significantly different, crucially, the prevalence of high load HCMV at month 6 was over three times greater among maternally HIV-1 exposed infants, and this may affect morbidity and development as assessed further in chapter 5.

In this study, two symptomatic Zambian paediatric cohorts were also screened for HCMV DNA. These were both retrospective studies of symptomatic children, one detecting HCMV in DNA-extracted whole blood from Zambian infants hospitalized with fever, and the other from a set of DNA-extracted lung tissue samples taken from HIV-1 positive children who died of respiratory disease. HCMV DNA prevalence in the retrospective hospitalized fever cohort was 10%, considerably lower than in the population-based study (CIGNIS), but screening of both retrospective cohorts was undertaken before that of the prospective cohort, using less sensitive primers with the goal of identifying infections in which HCMV was contributing to pathology. Indeed whilst prevalence was lower, viral loads were much higher, with all 16 infections detected having loads of over 10,000 copies/ml sera. The highest HCMV DNA prevalence was seen within the respiratory mortalities, where 94% of children were found to be positive. It was also in this cohort that the highest viral load was identified, although due to variable proportions of tissue and blood, the range of normalised viral loads (copies/10^6 cell genome equivalents) within this cohort was much broader than for the hospitalized fever cohort. These respiratory mortality samples were previously screened by histopathology, which identified HCMV as the 3rd most common diagnosis (Chintu et al., 2002). The PCR results presented here suggest that HCMV may in fact be ubiquitous in HIV-1 associated paediatric respiratory disease in Zambia, underlying more common diagnoses

such as tuberculosis and other bacterial pneumonias, and possibly undermining treatment for these diseases.

Previous analyses of relatively conserved loci (such as UL55, UL75 or UL115) (Rasmussen and Cowan, 2003; Rasmussen et al., 2002), along with certain more variable loci (such as UL139, UL144 and UL146) (Bradley et al., 2008; He et al., 2006; Stanton et al., 2005) have failed to demonstrate clear linkage disequilibrium between different genes, suggesting a mosaic morphology for the HCMV genome. However, we have genotyped two hypervariable loci (the UL73 and UL74 genes; glycoproteins gN and gO respectively) which have been shown to be linked in isolates from the U.S, Europe and Japan (Cunningham et al., 2009; Mattick et al., 2004; Yan et al., 2008). A likely explanation for this linkage is that, as they are neighbouring genes and overlap, they are less likely to be separated by recombination. Due to their hypervariability however, there is also the possibility that they interact, either directly or indirectly, and may in some way affect the biology of different strains, possibly as virulence determinants, or with respect to host cell tropism as has been suggested for UL74 (Ryckman, Chase, and Johnson, 2010). Here we have identified all seven UL73 and eight UL74 established genotypes in Zambia, and through phylogenetic analysis have identified a new UL73 genotype, gN4d, which links with the gO5 group, as exemplified by the Merlin reference strain (Dolan et al., 2004).

The data in this thesis is the most comprehensive for HCMV genotypes in Africa. The results are further compared to all sequences published and also on the NCBI (National Centre for Biotechnology Information) database (Table 12). The prevalence of these UL74/UL73 linked genotypes appears to differ by geographical region, although different studies have focussed on different HCMV disease patient groups (Figure 14). The gO4/gN4b linkage is significantly more prevalent in Japan (14/55 - 25%) than elsewhere (vs Europe and North America: 9/79 P = 0.034, vs Southern Africa: 2/51 P = 0.002), but this study cohort was comprised exclusively of symptomatic post-natal and congenital infections (Yan et al., 2008), and so the link may be with the patient group or the region. In Southern Africa it is gO1a/gN1 and gO1b/gN3a that predominate, but they are no more prevalent here than elsewhere. A previous study from Malawi investigated both UL73 and UL74 in a study looking at transmission of HCMV within family groups, identified three novel UL73/UL74 combinations (Beyari et al., 2005). We have also shown some evidence of novel linkages, including two of those suggested by the Malawian study (gO1b/gN1 and gO2b/gN1)(Table 12). The prevalence of these 'novel' gO/gN linked genotypes (12/51 – 24%) is significantly higher in this HIV-1 endemic region

than in Japan (5/55 P = 0.043) or Europe and North America (1/79 Chi squared is not calculable). For Southern Africa, these possibly represent artefacts of multiple infections in cohorts with prevalent HIV/AIDS, but these same multiple infections may also be facilitating recombination and the emergence of novel strains.

In Europe and North America, a more balanced distribution of all genotypes is observed. Analysing the geographical distribution of different genotypes is hampered by the fact that these sequences were obtained from a considerable range of different clinical specimens from patients with different disease presentations. To completely confirm geographic differences in the distribution of HCMV UL73/UL74 genotypes, population-based studies of which genotypes cause primary infection in healthy children are required. We have done this here for Zambia on the CIGNIS study, and whilst genotyping has proved difficult due to very limited samples volumes, we have found that 6/12 samples from healthy Zambian infants were likely from the gO1b/gN3a genotype (both loci were not typed), which is supported by a high prevalence of this same genotype in the two symptomatic cohorts. Whether this trend would continue with higher numbers, or whether asymptomatic primary infections in other regions would be predominantly gO1b/gN3a is unknown. Broadly, all eight genotypes are found globally, which is distinct from what was been shown for the related betaherpesvirus HHV-6 (Kasolo, Mpabalwani, and Gompels, 1997)(Investigated here in chapter 4), and also for the gammaherpesvirus KSHV (Kasolo et al., 1998; Kasolo et al., 2007). The variation exhibited by HCMV UL73 and UL74 has been shown to have arisen in our distant past and is not the result of current immune selection as displayed by the more rapidly mutating RNA viruses (Mattick et al., 2004). Possible forces of selection include geography, innate immunity or cell tropism factors.

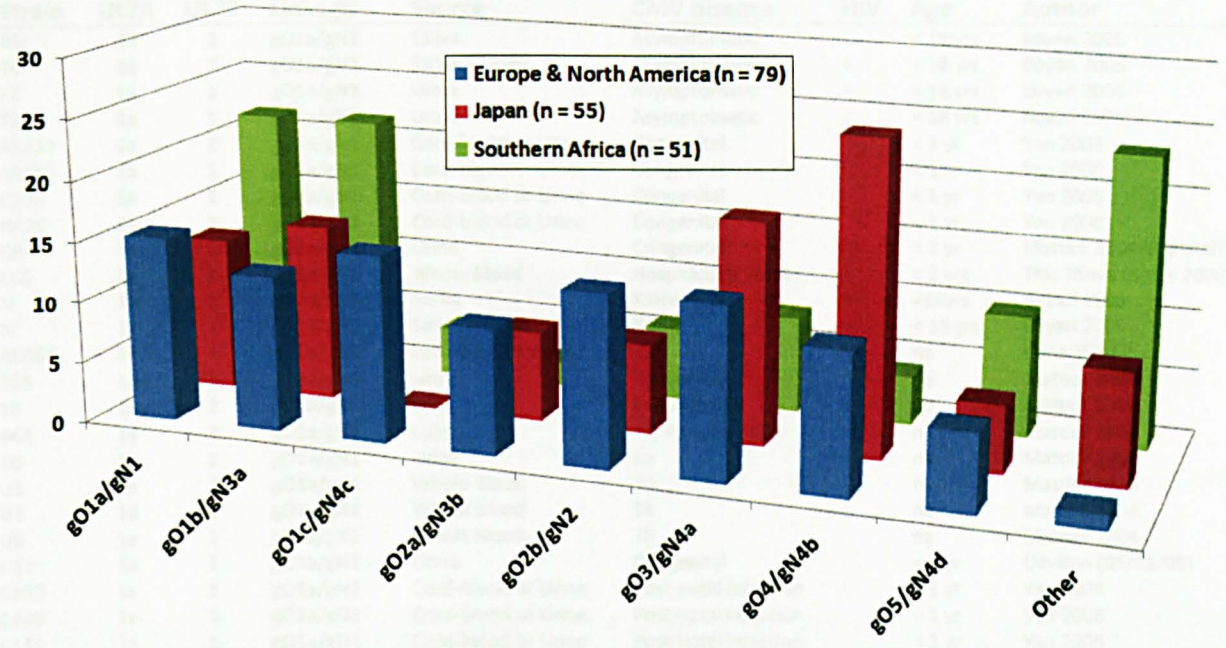


Figure 14: Global distribution of UL74/UL73 linkages

Histogram showing prevalence of HCMV gO/gN linkages in three genetically distinct regions. 'Other' refers to novel linkages (generally not confirmed by read-through sequence but suggested by independent detection) outside of the 8 established linked groups.

Strain	UL74	UL73	Linkage	Source	CMV disease	HIV	Age	Author
B5r	1a	1	gO1a/gN1	Saliva	Asymptomatic	-	< 18 yrs	Beyari 2005
T4	1a	1	gO1a/gN1	Saliva + Urine	Asymptomatic	+	< 18 yrs	Beyari 2005
F2	1a	1	gO1a/gN1	Urine	Asymptomatic	+	< 18 yrs	Beyari 2005
T3	1a	1	gO1a/gN1	Urine	Asymptomatic	-	< 18 yrs	Beyari 2005
ASA12	1a	1	gO1a/gN1	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
ASA59	1a	1	gO1a/gN1	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
C106	1a	1	gO1a/gN1	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
N42C	1a	1	gO1a/gN1	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
GR	1a	1	gO1a/gN1	Urine	Congenital		< 1 yr	Mattick 2004/Pignatelli 2001
K60	1a	1	gO1a/gN1	Whole Blood	Hospitalized Fever	-	< 2 yrs	This Thesis (Bates 2008)
Xi	1a	1	gO1a/gN1	Saliva	KSHV	+	>18yrs	Beyari 2005
Bi	1a	1	gO1a/gN1	Saliva + Urine	KSHV	+	< 18 yrs	Beyari 2005
AD169	1a	1	gO1a/gN1	Lab-adapted strain	na		na	Chee 1990
33B	1a	1	gO1a/gN1	Urine	Congenital		na	Mattick 2004
3E	1a	1	gO1a/gN1	Lung	Pneumonitis	+	na	Mattick 2004
64A	1a	1	gO1a/gN1	Lung	TR, Pneumonitis		na	Mattick 2004
TD	1a	1	gO1a/gN1	Urine	na		na	Mattick 2004
U1	1a	1	gO1a/gN1	Whole Blood	TR		na	Mattick 2004
U3	1a	1	gO1a/gN1	Whole Blood	TR		na	Mattick 2004
U6	1a	1	gO1a/gN1	Whole Blood	TR		na	Mattick 2004
U11	1a	1	gO1a/gN1	Urine	Congenital		< 1 yr	Davison (05/11/09)
C102	1a	1	gO1a/gN1	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
C140	1a	1	gO1a/gN1	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
C141	1a	1	gO1a/gN1	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
W1	1a	1	gO1a/gN1	Lung biopsy	na	+	na	Davison (01/06/09)
HAN40	1a	1	gO1a/gN1	Bronch. lavage	na		na	Davison (01/06/09)
N5	1a	1	gO1a/gN1	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N6	1a	1	gO1a/gN1*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N17	1a	1	gO1a/gN1*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N33	1a	1	gO1a/gN1*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
C154	1a	3a	gO1a/gN3a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK16	1a	3a	gO1a/gN3a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
S01	1a	3a	gO1a/gN3a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
HAN11	1a	3a	gO1a/gN3a	Bronch. lavage	na		na	Davison (01/06/09)
J60298	1a	4c	gO1a/gN4c	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
263M6	1b	3a	gO1b/gN3a	Sera	Asymptomatic	-	< 1 yr	This Thesis (Bates 2008)
35M6	1b	3a	gO1b/gN3a	Sera	Asymptomatic	-	< 1 yr	This Thesis (Bates 2008)
TR	1b	3a	gO1b/gN3a	na	AIDS retinitis	+	na	Murphy 2003
C177	1b	3a	gO1b/gN3a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK32	1b	3a	gO1b/gN3a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK72	1b	3a	gO1b/gN3a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK82	1b	3a	gO1b/gN3a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
Y01	1b	3a	gO1b/gN3a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FL	1b	3a	gO1b/gN3a	na	Congenital		< 1 yr	Mattick 2004/Pignatelli 2001
TS	1b	3a	gO1b/gN3a	Urine	Growth delay		na	Mattick 2004/Pignatelli 2001
Ci	1b	3a	gO1b/gN3a	Saliva	KSHV	+	< 18 yrs	Beyari 2005
15A	1b	3a	gO1b/gN3a	Urine	na	+	na	Mattick 2004
C83	1b	3a	gO1b/gN3a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
J60250	1b	3a	gO1b/gN3a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
U02	1b	3a	gO1b/gN3a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
HAN32	1b	3a	gO1b/gN3a	Bronch. lavage	na		na	Davison (01/06/09)
HAN28	1b	3a	gO1b/gN3a	Bronch. lavage	na		na	Davison (01/06/09)
CINCY	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	na	Davison (01/06/09)
W2	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	na	Davison (01/06/09)
N2	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N9	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N15	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N19	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N23	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N25	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N34	1b	3a	gO1b/gN3a	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
ML	1b	3a	gO1b/gN3a	Urine	Urinary infection		na	Mattick 2004/Pignatelli 2001
PS	1b	3a	gO1b/gN3a	Urine	Urinary infection		na	Mattick 2004/Pignatelli 2001
K33	1b	3a	gO1b/gN3a*	Whole Blood	Hospitalized Fever	-	< 2 yrs	This Thesis (Bates 2008)
B1	1b	1	gO1b/gN1	Saliva + Urine	Asymptomatic	-	>18yrs	Beyari 2005
K190	1b	1	gO1b/gN1	Whole Blood	Hospitalized Fever	-	< 2 yrs	This Thesis (Bates 2008)
Ri	1b	1	gO1b/gN1	Saliva + Urine	KSHV	+	>18yrs	Beyari 2005
N18	1b	4d	gO1b/gN4d	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N24	1b	4d	gO1b/gN4d	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
Toledo	1c	4c	gO1c/gN4c	Lab-adapted strain	Congenital		< 1 yr	Murphy 2003
HAN20	1c	4c	gO1c/gN4c	Bronch. lavage	na		na	Cunningham 2009
HAN33	1c	4c	gO1c/gN4c	Bronch. lavage	na		na	Davison (01/06/09)

HAN25	1c	4c	gO1c/gN4c	Bronch. lavage	na		na	Davison (01/06/09)
HAN3	1c	4c	gO1c/gN4c	Bronch. lavage	na		na	Davison (01/06/09)
VR1814	1c	4c	gO1c/gN4c	amniotic fluid	na		na	Davison (05/11/09)
FIX	1c	4c	gO1c/gN4c	na	Pregnancy primary		na	Murphy 2003
A12	1c	4c	gO1c/gN4c	Urine	na	+	na	Mattick 2004
DM	1c	4c	gO1c/gN4c	Urine	Liver Disease		< 1 yr	Mattick 2004
TB40E	1c	4c	gO1c/gN4c	na	na		na	Slnzger 2008
U2	1c	4c	gO1c/gN4c	Whole Blood	TR		na	Mattick 2004
U5	1c	4c	gO1c/gN4c	Whole Blood	TR		na	Mattick 2004
N31	1c	4c	gO1c/gN4c	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N32	1c	4c	gO1c/gN4c	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
ASA16	2a	3b	gO2a/gN3b	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK19U	2a	3b	gO2a/gN3b	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
1152B	2a	3b	gO2a/gN3b	na	na		na	Mattick 2004
A8	2a	3b	gO2a/gN3b	Urine	Congenital (symp)		< 1 yr	Mattick 2004
GB	2a	3b	gO2a/gN3b	na	na		na	Mattick 2004
U01	2a	3b	gO2a/gN3b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
U06	2a	3b	gO2a/gN3b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
N8	2a	3b	gO2a/gN3b*	Lung biopsy	Respiratory Disease	+	< 18 yrs	This Thesis (Bates 2008)
PH	2a	3b	gO2a/gN3b	Whole Blood	TR		na	Murphy 2003
HAN38	2a	3b	gO2a/gN3b	Bronch. lavage	na		na	Cunningham 2009
HAN13	2a	3b	gO2a/gN3b	Bronch. lavage	na		na	Cunningham 2009
HAN8	2a	3b	gO2a/gN3b	Bronch. lavage	na		na	Davison (01/06/09)
HAN1	2a	3b	gO2a/gN3b	Bronch. lavage	na		na	Davison (01/06/09)
R2	2a	1	gO2a/gN1	Urine	Asymptomatic	-	< 18 yrs	Beyari 2005
ASA68	2b	2	gO2b/gN2	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
C164	2b	2	gO2b/gN2	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
N66	2b	2	gO2b/gN2	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
Can4	2b	2	gO2b/gN2	Urine	TR		na	Mattick 2004/Pignatelli 2001
Can7	2b	2	gO2b/gN2	Urine	TR		na	Mattick 2004/Pignatelli 2001
C134	2b	2	gO2b/gN2	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
AF1	2b	2	gO2b/gN2	amniotic fluid	na		na	Davison (05/11/09)
U8	2b	2	gO2b/gN2	Urine	Congenital		< 1 yr	Davison (05/11/09)
HAN36	2b	2	gO2b/gN2	Bronch. lavage	na		na	Davison (01/06/09)
HAN31	2b	2	gO2b/gN2	Bronch. lavage	na		na	Davison (01/06/09)
HAN24	2b	2	gO2b/gN2	Bronch. lavage	na		na	Davison (01/06/09)
HAN21	2b	2	gO2b/gN2	Bronch. lavage	na		na	Davison (01/06/09)
HAN15	2b	2	gO2b/gN2	Bronch. lavage	na		na	Davison (01/06/09)
HAN12	2b	2	gO2b/gN2	Bronch. lavage	na		na	Davison (01/06/09)
HAN2	2b	2	gO2b/gN2	Bronch. lavage	na		na	Davison (01/06/09)
N7	2b	2	gO2b/gN2*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N10	2b	2	gO2b/gN2*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N21	2b	2	gO2b/gN2*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
K61	2b	1	gO2b/gN1	Whole Blood	Hospitalized Fever	+	< 2 yrs	This Thesis (Bates 2008)
F1	2b	1	gO2b/gN1	Urine	Asymptomatic	+	>18yrs	Beyari 2005
J60284	2b	3a	gO2b/gN3a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
ASA15	3	4a	gO3/gN4a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK03	3	4a	gO3/gN4a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK20	3	4a	gO3/gN4a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK31	3	4a	gO3/gN4a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
C49	3	4a	gO3/gN4a	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
ZVs	3	4a	gO3/gN4a	Saliva	Congenital		< 1 yr	Mattick 2004/Pignatelli 2001
K57	3	4a	gO3/gN4a	Whole Blood	Hospitalized Fever	+	< 2 yrs	This Thesis (Bates 2008)
ME	3	4a	gO3/gN4a	na	na		na	Mattick 2004
TM	3	4a	gO3/gN4a	Urine	Congenital		< 1 yr	Mattick 2004
U4	3	4a	gO3/gN4a	Whole Blood	TR		na	Mattick 2004
BO	3	4a	gO3/gN4a	Saliva	na		< 18 yrs	Mattick 2004/Pignatelli 2001
C145	3	4a	gO3/gN4a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
J60236	3	4a	gO3/gN4a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
U03	3	4a	gO3/gN4a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
J60249	3	4a	gO3/gN4a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
J60248	3	4a	gO3/gN4a	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
6397	3	4a	gO3/gN4a	Urine	Congenital		< 1 yr	Davison (01/06/09)
HAN39	3	4a	gO3/gN4a	Bronch. lavage	na		na	Davison (01/06/09)
HAN34	3	4a	gO3/gN4a	Bronch. lavage	na		na	Davison (01/06/09)
HAN29	3	4a	gO3/gN4a	Bronch. lavage	na		na	Davison (01/06/09)
HAN27	3	4a	gO3/gN4a	Bronch. lavage	na		na	Davison (01/06/09)
HAN16	3	4a	gO3/gN4a	Urine	Congenital		< 1 yr	Davison (01/06/09)
N6	3	4a	gO3/gN4a*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N7	3	4a	gO3/gN4a*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N11	3	4a	gO3/gN4a*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N20	3	4d	gO3/gN4d	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
ASA19	4	4b	gO4/gN4b	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008

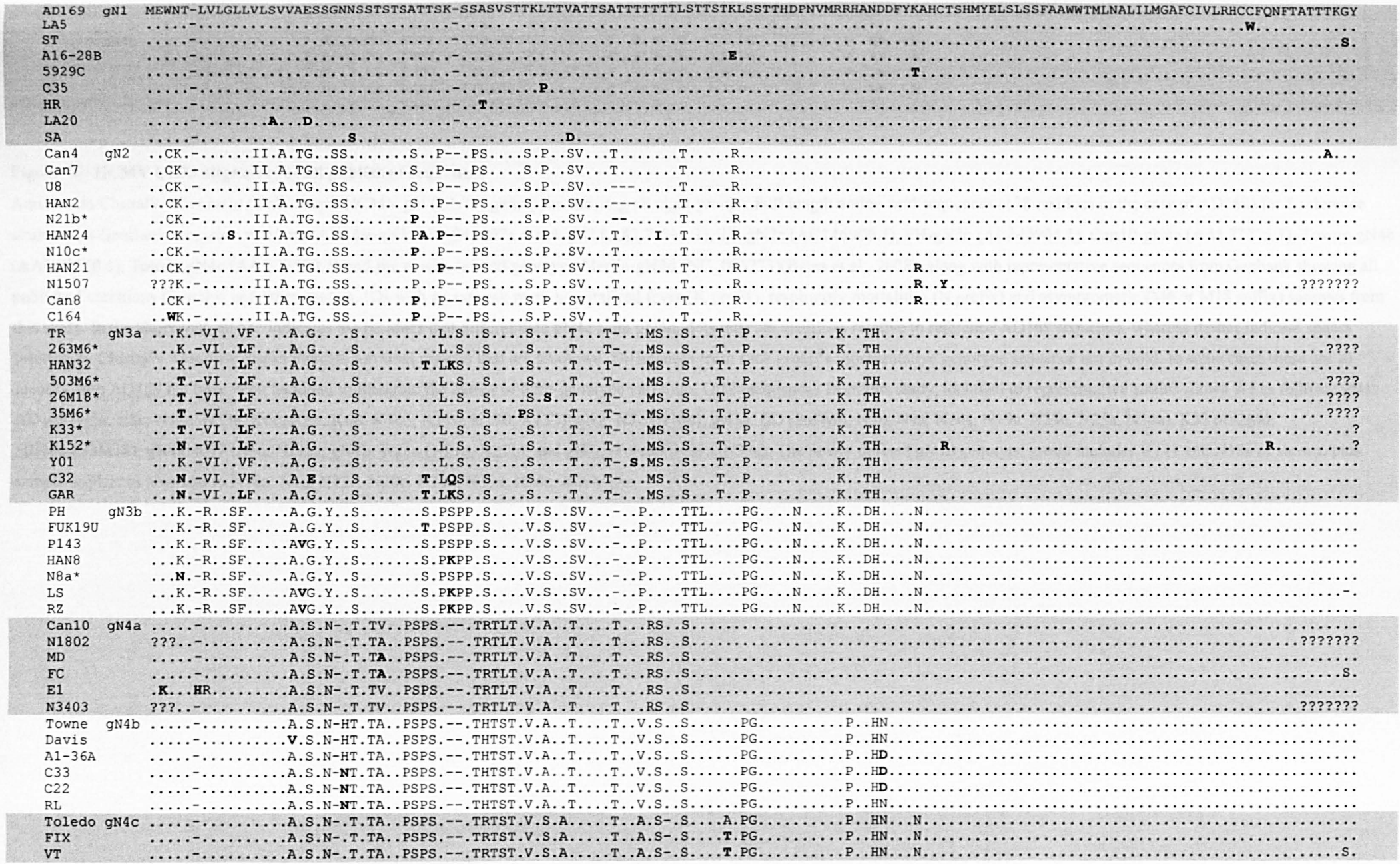
FUK74	4	4b	gO4/gN4b	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
X01	4	4b	gO4/gN4b	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
LV	4	4b	gO4/gN4b	Urine	Congenital		< 1 yr	Mattick 2004/Pignatelli 2001
Davis	4	4b	gO4/gN4b	Lab-adapted strain	na		na	Mattick 2004/Pignatelli 2001
Towne	4	4b	gO4/gN4b	Lab-adapted strain	Congenital		na	Rasmussen 2003
3301	4	4b	gO4/gN4b	Urine	Congenital		< 1 yr	Cunningham 2009
HAN23	4	4b	gO4/gN4b	Bronch. lavage	na		na	Davison (01/06/09)
HAN22	4	4b	gO4/gN4b	Bronch. lavage	na		na	Davison (01/06/09)
HAN17	4	4b	gO4/gN4b	Bronch. lavage	na		na	Davison (01/06/09)
1B	4	4b	gO4/gN4b	Urine	TR		na	Mattick 2004
70A	4	4b	gO4/gN4b	Whole Blood	na		> 18 yrs	Mattick 2004
C110	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
C122	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
C14	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
C170	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
C185	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
C196	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
J60223	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
J60299	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
N22	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
C135	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
U07	4	4b	gO4/gN4b	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
N26	4	4b	gO4/gN4b*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N28	4	4b	gO4/gN4b*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
B5u	4	1	gO4/gN1	Urine	Asymptomatic	-	< 18 yrs	Beyari 2005
E4	4	3a	gO4/gN3a	Urine	Asymptomatic	-	< 18 yrs	Beyari 2005
Ei	4	3a	gO4/gN3a	Saliva	KSHV	+	> 18yrs	Beyari 2005
ASA01	5	4d	gO5/gN4d	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
FUK28	5	4d	gO5/gN4d	Cord-blood or Urine	Congenital		< 1 yr	Yan 2008
Merlin	5	4d	gO5/gN4d	Urine	Congenital		< 1 yr	Dolan 2004
3157	5	4d	gO5/gN4d	Urine	Congenital		< 1 yr	Cunningham 2009
JP	5	4d	gO5/gN4d	Prostate tissue	AIDS mortality	+	na	Cunningham 2009
HAN30	5	4d	gO5/gN4d	Bronch. lavage	na		na	Davison (01/06/09)
HAN19	5	4d	gO5/gN4d	Bronch. lavage	na		na	Davison (01/06/09)
C149	5	4d	gO5/gN4d	Cord-blood or Urine	Post-natal infection		< 1 yr	Yan 2008
K141	5	4d	gO5/gN4d	Whole Blood	Hospitalized Fever	-	< 2 yrs	This Thesis (Bates 2008)
N13	5	4d	gO5/gN4d	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N29	5	4d	gO5/gN4d	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N30	5	4d	gO5/gN4d	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)
N36	5	4d	gO5/gN4d*	Lung biopsy	AIDS mortality	+	< 18 yrs	This Thesis (Bates 2008)

Table 12: Summary of all published UL73/UL74 genotypes

HCMV UL73 and UL74 genotypes detected in this study combined with all available data globally. Where available, sample source and disease presentation are indicated along with HIV-1 status and age. Grey boxes highlight samples displaying evidence for novel linkages. Asterisks mark samples in which additional genotypes were identified. Samples ASA01, FUK28 and C149 were published as gN4c (Yan et al., 2008) but re-analysis here has shown they are in fact members of the new gN4d clade. TR = transplant recipient. All HCMV UL73 and UL74 sequences from this study were published in 2008 as referenced (Bates et al., 2008). For sequences that are not as yet published, the date of submission to Genbank is given in brackets.

Analysing all available sequence data for HCMV UL73, 59 different variants have been identified, divided between the 8 genotype groups (Figure 15). Of these, 11 have been detected solely in this study, with particular attention being drawn towards the genotype gN3a, which accounts for over half of these novel strains. This analysis also determined a new gN genotype gN4d which includes the Merlin reference strain, and revealed a new gO/gN linkage. An alignment was also constructed combining unique UL74 sequences from this study with all those available (Figure 16), identifying a total of 38 unique UL74 variants globally, and of these, 7 have been found exclusively in this study. We have also identified a unique gO N-terminal truncation from a 2bp deletion resulting in internal initiation required to express the gO glycoprotein. Analysis here of recent sequence depositions in Genbank show that at least another strain shows a similar deletion: HAN19 (Genbank submission, Davison). All these sequences could be investigated using biological assays as described above.

In this chapter evidence for HCMV multiple infections and possible recombinations is presented for this paediatric population. We have also observed that active HCMV infections, as detected by sera-DNA, appear more frequent and at an earlier age than elsewhere. However, analysis suggests this is unlikely to be due to different strain variants in this region as all genotypes detected elsewhere were detected in this population. This is distinct from that observed for related betaherpesvirus HHV-6, as described in the next chapter. At the start of this study it was not known whether HCMV, or what strain genotypes of HCMV, were present in Zambia. In this study we have identified HCMV as common infection in Zambian infants, with high loads being associated with pathology. The high prevalence of HCMV in end-stage AIDS in Zambian children suggests possible application of anti-CMV drugs in the future, with a special focus on AIDS-associated pneumonia for which other bacterial diagnoses and related treatments are not successful.


AD169 gN1 MEWNT-LVLGLLVLSVVAESSGNNSSTSTSATTSK-SSASVSTTKLTTVATTSATTTTTTTLSTTSTKLSSTTHDPNVMRRHANDDFYKAHCTSHMYELSLSSFAAWWTMLNALILMGAFCIVLRHCCFQNFTATTTKGY
LA5
ST
A16-28B
5929C
C35
HR
LA20
SA
Can4 gN2
Can7
U8
HAN2
N21b*
HAN24
N10c*
HAN21
N1507
Can8
C164
TR gN3a
263M6*
HAN32
503M6*
26M18*
35M6*
K33*
K152*
Y01
C32
GAR
PH gN3b
FUK19U
P143
HAN8
N8a*
LS
RZ
Can10 gN4a
N1802
MD
FC
E1
N3403
Towne gN4b
Davis
A1-36A
C33
C22
RL
Toledo gN4c
FIX
VT

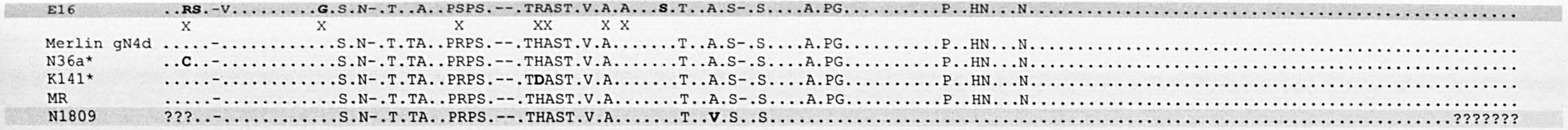


Figure 15: HCMV UL73 alignment of all published sequences

Amino acid ClustalW alignment of all unique HCMV gN (UL73) genotypes showing all eight groups: Full length amino acid sequences (138 residues in the case of AD169) for 8 reference strains with GenBank accession numbers (AD169-gN1 (NP_040007), Can4-gN2 (AAL77764.1), TR-gN3a (AC146906.1), PH-gN3b (AC146904.1), Can10-gN4a (AAL77775.1), Towne-gN4b (AAG23510.1), Toledo-gN4c (AAG23509.1) and the newly defined genotype Merlin-gN4d (NC_006273)(Bates et al., 2008), along with representative sequences from Genbank showing all published variations from this and other studies. IDs with an asterisk mark hospitalized fever (K prefix), respiratory mortalities (N prefix) and asymptomatic (M6 or M18 suffix) samples from this study. In the alignment, an 'X' indicates the residues that differentiate gN4c from gN4d, dots indicate identities relative to reference AD169 sequence, whereas dashes indicate spaces inserted by ClustalW. Question marks indicate terminal regions that are unknown. Differences from each group's representative genotype sequence are in bold. In some cases these are an identity with AD169 but have been included to illustrate the extent of intra-genotype variation. Other sequences from this study, identical to representative strains shown are as follows: gN1: AD169 (N5a, N8c, N17a, N33a, K60, K61, K68, K103, K110, K190, K137); gN2: N7c (N10c); gN3a: BD (263M6, N2b, N9a, N10a, N19a, N23a, N25a, N34a), K33 (492M6, 503M6,133M18); gN4a: K57 (N6a, N11a); gN4b: N28a (N21a, N27b), and gN4c: Toledo (N31a, N32a). The newly defined gN4d genotype group includes K141 and N18a as shown, plus sample sequences identical to N18a: N1c, N12c, N20c, N24a, N30a, N36a, and N29a.

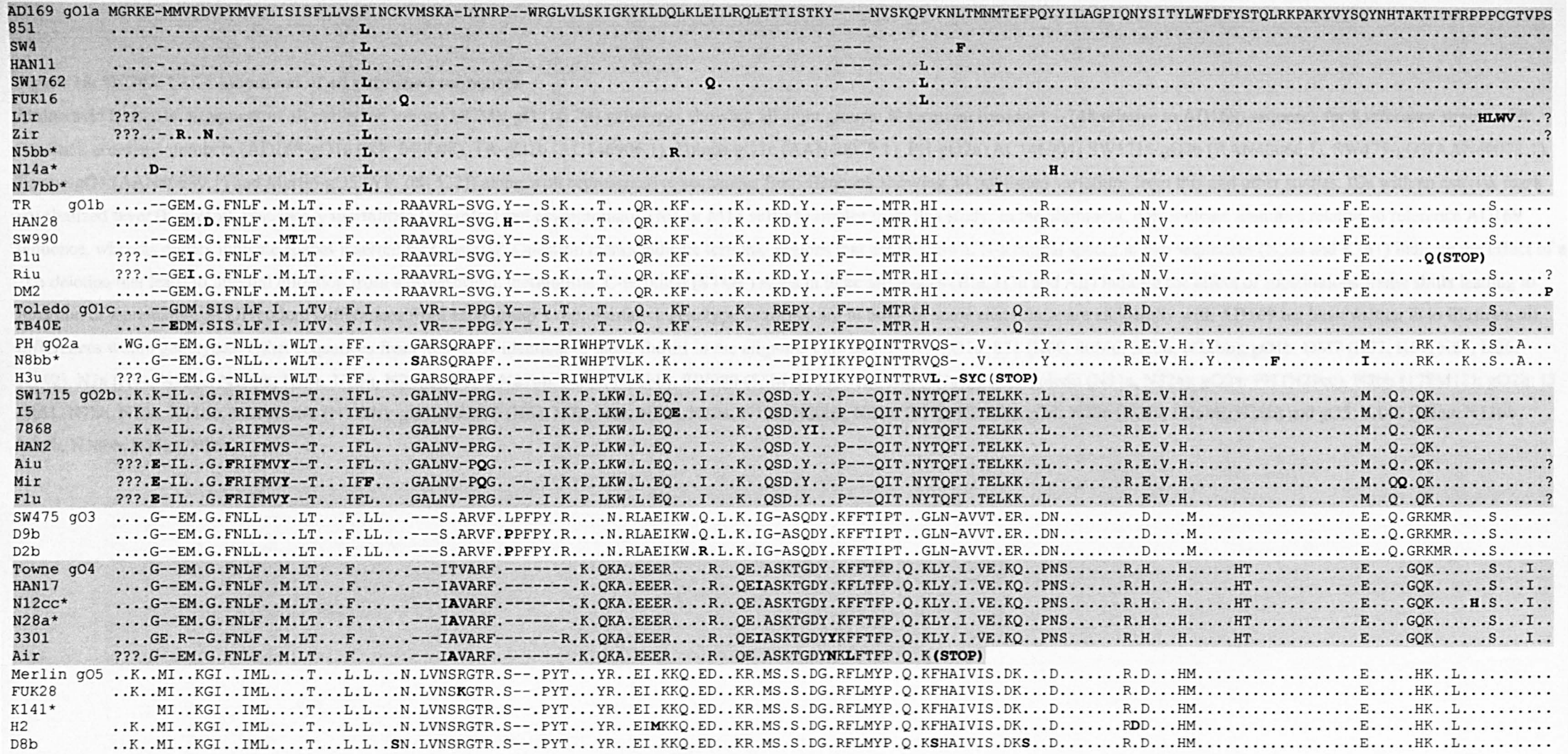

AD169 gO1a MGRKE-MMVRDVPKMVFLISISFLLVSFINCKVMSKA-LYNRP--WRGLVLSKIGKYKLDQLKLEILRQLETTISTKY----NVSKQPVKNLTMNMTEFPQYYILAGPIQNYSITYLWFDFYSTQLRKPAKYVYSQYNHTAKTITFRPPPCGTVPS
851
SW4
HAN11
SW1762
FUK16
L1r
Zir
N5bb*
N14a*
N17bb*
TR gO1b
HAN28
SW990
B1u
Riu
DM2
Toledo gO1c
TB40E
PH gO2a
N8bb*
H3u
SW1715 gO2b
I5
7868
HAN2
Aiu
Mir
F1u
SW475 gO3
D9b
D2b
Towne gO4
HAN17
N12cc*
N28a*
3301
Air
Merlin gO5
FUK28
K141*
H2
D8b

Figure 16: HCMV UL74 alignment of all published sequences

Amino acid ClustalW alignment of all published unique HCMV gO (UL74) genotypes showing all eight groups: N-terminal (residues 1-148 relative to AD169) sequence for 8 reference strains with GenBank accession numbers (AD169-gO1a (NP_040008), TR-gO1b (AC146906.1), Toledo-gO1c (AAN40079.1), PH-gO2a (AC146904) SW1715-gO2b (AAN40066.1), SW475-gO3(AAN40072.1), Towne-gO4 (AAN40080.1) and Merlin-gO5 (YP_081522)) along with representative sequences from Genbank showing all published variations from this and other studies. IDs with an asterisk mark hospitalized fever (K prefix), respiratory mortalities (N prefix) and asymptomatic (M6 or M18 suffix) samples from this study. In the alignment, dots indicate identities relative to reference AD169 sequence, whereas dashes indicate spaces inserted by ClustalW. Question marks indicate terminal regions that are unknown. N-terminal spaces in two sequences (N3aa and K141) indicate the effect of a 2bp deletion that leads to internal initiation from a downstream methionine. C-terminal **(STOP)** signs in three sequences (Blu, H3u and Air) indicate the effect of substitutions/frame shifts leading to insertion of a premature STOP codon. Differences from each group's representative genotype sequence are in bold. In some cases these are an identity with AD169 but have written in to illustrate all differences within genotypes. Other sequences from this study identical to those shown in the alignment are as follows: gO1a: 851 (K60, 40M6, N6bb, N22bb); gO1b: DM7 (K33, K57, K67, K86, K142), N2a (N9a, N15cc, N18bb, N19a, N23a, N24bb, N25a, N27bb, N34a, N36bb), SW990 (35M18, 263M6), Riu (N2a); gO1c: Toledo (N31a, N32a); gO2a: PH (N29cc), N8bb (178M18); gO2b: I5 (K61, N7bb, N10a, N11bb, N21a), Aiu (N11bb); gO3: SW475 (N6a, N7a, N8a, N11a, N12a, N17a, N20bb, N26a, N27b, N35bb); gO4: N28a (N33a, N26bb, N28b) and gO5: K141 (N3aa, N13bb, N29b, N30bb, N35aa, N36a).

4.0 HHV-6 DNA detection, loads & genotypes in three Zambian paediatric cohorts

4.1 Introduction

The previous chapter presented the design and implementation of PCR assays for measuring the prevalence of HCMV active infections, loads and genotypes in three Zambian paediatric cohorts. In this chapter we describe assays for the detection of another betaherpesvirus, HHV-6, and present a similar analysis of the prevalence of active infections, loads and genotypes present in the same three Zambian paediatric cohorts. Like for HCMV, the prevalence of active HHV-6 infections during infancy has not been well studied in Africa. We employed a PCR strategy to investigate HHV-6 prevalence and loads in the three cohorts. Assays were also designed to investigate strain variation through sequencing the U47 (glycoprotein gO) and U46 (glycoprotein gN) genes, homologues of HCMV UL74 and UL73 studied in the chapter 3. In HHV-6 these loci are less variable than in HCMV, showing divergence of up to 7%. U46 is only published for three reference strains and no other sequence data is known. The design of primers in the conserved flanks of these variable loci allows the genotyping of strains with U46 or U47 genes similar to known sequences, as well as detecting potential novel strain variants or recombinants. In this chapter we also investigate relationships between the prevalence and loads of active HHV-6 infections, with HIV-1 infection and exposure.

4.2 HHV-6 PCR assay design and strategy

PCR assays were required for the detection and genotyping of HHV-6 in whole blood, lung tissue and sera from paediatric clinical specimens. Primers were designed using alignments of the U46 and U47 loci from the two HHV-6 reference strains U1102 (NC_001664) and Z29 (NC_000898), representing HHV-6A and HHV-6B respectively. All primers (Appendix A) were designed to detect both strain variants although at some sites mismatches were unavoidable: Primers U47OF, U46Up and U46R each contained one mismatched base pair (Appendix F), with primer U47F containing two mismatches (Appendix G). Clones for testing the sensitivity of internal primers were constructed using PCR products generated using the external U47OR/R and U46OF/R primers so that the effects of the mismatches in the U47F and U46R primers could be properly assessed. The U47F/U47R primers had a sensitivity of

10^3 absolute copies when measured using serial dilutions of clones of PCR products amplified from HHV-6A (strain U1102), but a lower sensitivity of 10^5 absolute copies when using clones containing PCR products derived from HHV-6B (strain N11)(Figure 1). This particularly low sensitivity was tested twice in duplicate using different clones and was consistent. A possible explanation may be the double mismatch in HHV-6B within the forward primer. We used this assay to screen the two retrospective cohorts (whole blood from141 hospitalized febrile infants and lung tissue from 36 HIV-1 positive respiratory mortalities) as this low sensitivity assay would only detect active infections likely involved in pathology. Primers targeting U46 (U46F/U46R) were considerably more sensitive, with an absolute cut off of 10 copies, which did not vary between the two strain variants. This assay was employed to measure the prevalence of sera-DNA in the CIGNIS cohort, in which all routine samples were taken from healthy children. Detection of HHV-6 DNA in sera is indicative of active infection although loads are likely to be lower when compared with the two symptomatic cohorts. Strain variation was investigated in all three cohorts using U47 and U46 primers.

4.3 Prevalence of active HHV-6 infection

The prevalence of HHV-6 infections was first assessed in the two retrospective cohorts: DNA-extracted blood from 141 infants hospitalized with fever (6-34 months of age), and DNA-extracted lung tissue samples from 36 HIV-1 positive children (0-16 yrs of age) who died from respiratory disease. These two cohorts were screened with a low sensitivity PCR assay (U47F/U47R primers). Using this assay HHV-6 had a blood-DNA prevalence of 5% in the fever cohort and a lung tissue-DNA prevalence of 72% in the respiratory mortalities cohort (Table 13). We then compared these prevalence rates with Zambian infants taking part in the CIGNIS study, who were screened for HHV6 by PCR in DNA-extracted sera at both 6 and 18 months of age. Of the 812 infants enrolled in the study, 610 month 6 and 375 month 18 samples were screened for HHV-6 using the more sensitive U46F/R PCR assay. The overall HHV-6 prevalence in the CIGNIS study was 8% at month 6, increasing significantly to 13% at month 18 (46/611 vs 52/399 $P < 0.001$)(Table 13). In addition to the 6 and 18 month samples, there was also a small set of 23 samples taken from CIGNIS infants who presented with fever at a non-scheduled time during the study. Prevalence of HHV-6 detection in sera-DNA in this set was 57%, significantly higher than in either the month 6 or month 18 sets (57% (13/23) vs 8% (46/611) $P < 0.001$ or 13% (52/399) $P < 0.001$). Overall,

the prevalence of HHV-6 active infections was highest in the respiratory mortalities and febrile samples from CIGNIS participants.

4.4 Prevalence of active HHV-6 infection by infant HIV-1 status

In the hospitalized fever and respiratory mortalities cohorts, HIV-1 diagnosis was by detection of proviral DNA using PCR on DNA-extracted blood and lung tissue respectively. HIV-1 was detected in 33% (47/141) of the hospitalized febrile infants and was confirmed in all 36 respiratory mortalities (they were selected as HIV-1 positive). The prevalence of HHV-6 DNA did not differ significantly by infant HIV-1 status in the hospitalized febrile infants. The prevalence of HHV-6 infection in the respiratory mortalities cohort was extremely high (72% (26/36)) and was significantly greater than the prevalence seen in HIV-1 positive children within the hospitalized fever or CIGNIS cohorts ($P < 0.001$)(Table 13), although did not differ significantly from the HHV-6 prevalence in HIV-1 positive infants in the CIGNIS morbidity samples (Table 13). In the main CIGNIS cohort HIV-1 diagnosis was by serology at 18 months. Among the 480 infants screened for HHV-6 at 6 months of age the HIV-1 prevalence was 4% (19/480). Infants with unknown HIV-1 status were largely those who had been withdrawn from the study before they could undergo their 18 month screen for HIV-1. Of 327 infants who were screened for HHV-6 at both time points, just 4 (1.2%) were HHV-6 sera-DNA positive at both month 6 and month 18. One of these infants was HIV-1 positive (the other three were negative and born to HIV-1 negative mothers) and was admitted to hospital with protein energy malnutrition, tuberculosis and diarrhoea. This child died 11 days after submitting a morbidity sample which was also positive for HHV-6. Another child who submitted a morbidity sample died of pneumonia 7 months after being tested positive for HHV-6.

Cohort	Age	HIV-1 uninfected	HIV-1 infected	P	Totals*
CIGNIS	6m	8% (38/461)	16% (3/19)[3]	.215^	8% (46/611)[1]
	18m	13% (49/384)	14% (2/14)	.697^	13% (52/399)[1,2]
CIGNIS (Morbidity)	6-18m	46% (6/13)	66% (2/3)	1.0^	57% (13/23)[2]
Fever (blood, n=141)	6-34m	3% (3/94)	9% (4/47)[4]	.222^	5% (7/141)
Autopsy (lung, n=36)	0-16yr	ND	72% (26/36)[3,4]	ND	72% (26/36)

Table 13: HHV-6 sera-DNA prevalence vs infant HIV-1

Significance was by Pearson Chi Squared or Fishers exact test (^) if there was an expected value < 5. *The totals column includes infants of unknown HIV-1 status . Significance between totals was as follows: [1]P = 0.005 [2,3,4] P < 0.001

4.5 Prevalence of active HHV-6 infection by HIV-1 exposure

Maternal HIV-1 status was requested at recruitment on the CIGNIS study, which was primarily powered to look at HIV-1 exposed but uninfected infants. At both 6 and 18 months, the prevalence of HHV-6 sera-DNA was not significantly affected by HIV-1 exposure (Table 14). As stated in the previous section, there was a significant increase in HHV-6 sera-DNA prevalence at month 18 (13%) from month 6 (8%) (P = 0.005). This was only significant among HIV-1 unexposed infants (9% (36/275) vs 13% (36/426); P = 0.048) with the number of HIV-1 exposed infants being lower. This result differed to the HCMV results, where there were no significant differences between the prevalence of active HCMV infections between the two time points, possibly reflecting differences in the rate of uptake and also the frequency of detectable reactivations between the two betaherpesviruses.

Cohort	Age (m)	HIV-1 unexposed	HIV-1 exposed	P	Totals*
CIGNIS	6	9% (36/426)[3]	6% (7/117)	.381	8% (46/611)[1]
	18	13% (36/275)[3]	13% (10/78)	.950	13% (52/399)[1,2]
CIGNIS (Morbidity)	6-18m	42% (5/12)	63% (5/8)	.410^	57% (13/23)[2]

Table 14: HHV-6 sera-DNA prevalence vs maternal HIV-1

Significance was by Pearson Chi Squared or Fishers exact test (^) if there was an expected value < 5. *The totals column includes infants and mothers of unknown HIV-1 status. Significance between totals was as follows: [1] P = 0.005, [2] P < 0.001, [3] P = 0.048

4.5 HHV-6 Real Time Taqman PCR set up and qualitation

In chapter 3 we describe the use of two Taqman-based Real Time PCR assays for quantifying both HCMV and also the human house-keeping gene GAPDH. For HCMV we selected a previously published assay that targeted the highly conserved UL55 gene (glycoprotein gB), to facilitate the detection of possible novel strain variants that might be missed using primers designed to detect the highly variable gN and gO genes. For HHV-6 the gO and gN genes (U47 and U46) are also variable but less so than in HCMV, and preliminary sequence analysis was showing lower levels of variation (section 4.8). For these reason we chose to design a Taqman probe for use with the U47F/U47R primers, which had the smallest product size (208bp), most appropriate for use in a Real Time assay.

Sensitivity of the HHV-6 U47 Real Time assay was assessed by measuring the probability of detection using dilution series of plasmid standards of cloned HHV-6A (U1102) PCR product. It was determined that standards with ≥100 copies in 5µl of template were detectable

in 100% of 10 dilution series. Standards containing 10 absolute copies were detectable in 70% (7/10) of cases, whilst 5µl of template containing just one copy was detectable in only 10% (1/10). Accuracy of the assay was then assessed by measuring the standard deviation of each standard across the ten repeats (Figure 17). Values had a standard deviation of up to 45% (at 10^1 copies) to 38% (at 10^7 copies), and so the viral loads measured using this assay were only accurate to roughly half a log. For the CIGNIS cohort, detection of 10 absolute copies corresponded to a load of 500 copies/ml sera with our extraction and assay protocol, but with many samples having undetectable or very low loads we have grouped loads as for HCMV, > or < than 1000 copies. For hospitalized fever and respiratory mortalities cohorts levels of HHV-6 DNA were much higher and are expressed as copies/ml whole blood or copies/10^6 cell genome equivalents as determined by the GAPDH Real Time assays described in chapter 3. Interestingly the U47F/U47R primers were several logs more sensitive when used in the Real Time assay (with different cycling conditions and the addition of a specific probe) than in the qualitative PCR assay, suggesting further optimization of the qualitative assay may improve sensitivity.

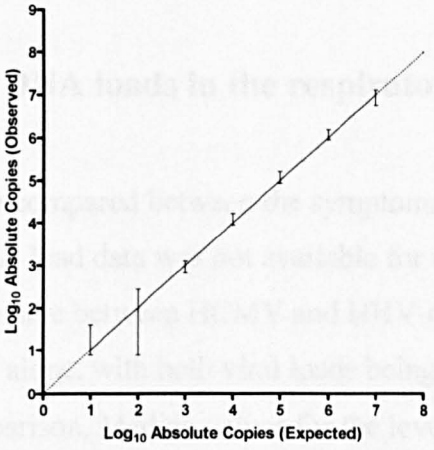


Figure 17: HHV-6 Real Time Taqman Standards

Error bars indicate standard deviation. Measurements were taken using the formula y = (x-40.4)/-3.315 which was derived from the averages of the five highest standards (10^3 - 10^7 absolute copies).

4.6 CIGNIS HHV-6 DNA loads by HIV-1 status

HHV-6 viral loads were measured and compared across the CIGNIS cohort and correlated with HIV-1 status in infants and their mothers. All samples found to be positive by the first round qualitative screen for HHV-6, were then analysed further using a Taqman Real Time PCR assay. A set of 91 HHV-6 positive samples (46 month 6 and 45 month 18) were assayed

along with 13 HHV-6 positive CIGNIS morbidity samples. In the CIGNIS study, many samples had low HHV-6 levels of <1000 copies/ml sera. Overall higher load infections were more commonly detected at month 6 (13% (6/46)) than at month 18 (2% (1/45))(P value incalculable)(Table 15). Due to low numbers sub-stratifying by infant HIV-1 status or maternal exposure was inconclusive, although there is the suggestion that HIV-1 exposed infants may be more likely to present with high load HHV-6 infections at 18 months. This is contrary to the effect seen for HCMV which showed high load infections were more common in exposed infants but only at the earlier time point.

Age	HIV-1 uninfected	HIV-1 infected	P	Total*
Month 6	13% (5/38)	0% (0/3)	1.0^	13% (6/46)
Month 18	2% (1/44)	ND	ND	2% (1/45)
Morbidity	0% (0/6)	0% (0/2)	ND	0% (0/13)
Age	**HIV-1 unexposed**	**HIV-1 exposed**	**P**	**Total***
Month 6	14% (5/35)	14% (1/7)	1.0^	13% (6/46)
Month 18	0% (0/33)	13% (1/8)	.195^	2% (1/45)
Morbidity	0% (0/5)	0% (0/5)	ND	0% (0/13)

Table 15: High HHV-6 load stratified by both maternal and infant HIV-1 status

Significance was by Pearson Chi Squared or Fishers exact test (^). *The totals columns include subjects with unknown HIV-1 infection or exposure status.

4.7 HHV-6 and HCMV DNA loads in the respiratory mortalities cohort

For HCMV, DNA loads were compared between the symptomatic cohorts. Due to limited sample volumes, HHV-6 DNA load data was not available for the hospitalized fever cohort and so comparisons are made here between HCMV and HHV-6 loads detected in the respiratory mortalities cohort alone, with both viral loads being normalized relative to cellular genome equivalents for comparison. Median values for the level of both viruses were similar although some HCMV infections had considerably higher loads, possibly representing active pulmonary HCMV infections at time of death in these children.

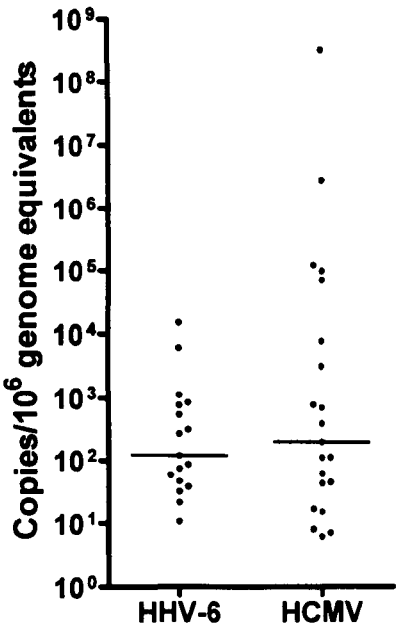


Figure 18: HHV-6 vs HCMV loads in respiratory mortalities cohort

Comparison of HHV-6 and HCMV viral loads in respiratory mortalities cohort. As DNA was extracted from variable quantities of lung tissue (interspersed with blood) viral loads were normalized to cell number using the GAPDH Real Time Taqman assay as described previously (Section 3.5). Bars represent median values.

4.8 HHV-6 genotype analysis

Genotypic analysis of HCMV focussed on the two linked hypervariable genes, UL73 and UL74, which encode for the glycoproteins gN and gO respectively. The HHV-6 genome encodes homologues of these two glycoproteins (U46 and U47 respectively) and so these were the focus of genotyping analysis presented here for HHV-6. Although less variation is shown in these genes than for their homologues in HCMV, they differentiate between HHV-6A and B, as well as showing intra-strain variation as previously for U47 (Gompels, 2006). When comparing amino acid sequences for HCMV gO (UL74), the N-terminal region analysed here varies by up to 34% and in the full length HCMV gN (UL73), variation was up to 22%. The N-terminal region of HHV-6 gO (U47) varies by just 14% and then in gN (U46), variation was 6% at the amino acid level. The CIGNIS cohort was screened using primers that target the U46 gene (U46F/U46R). PCR products were hence sequenced directly from this primary screen. Further primer pairs were designed to detect the U47 gene, including the U47F/U47R primers that were used in the primary screen of the two retrospective cohorts (section 4.2). Confirmation for loci was shown using the hypervariable HHV-6 specific gene U83 (David Clark, personal communication). Within all three cohorts, samples were not always detected by both U46 and U47 primers (Table 17) with analysis sometimes inhibited

by limited sample volumes and/or low HHV-6 viral loads. Amongst all samples positively genotyped as HHV-6A or HHV-6B, the relative proportions of the two variants were then analysed. Samples in which both variants were detected at different loci were considered dual infections, although with complete genomic sequence only known for four reference strains globally (U1102, Z29, AJ and HST) the possibility of the existence of recombinants (as seen for HCMV) cannot be ruled out. In the CIGNIS cohort, the predominant variant detected was HHV-6A (85%), which was significantly more prevalent than either HHV-6B (3%, $P < 0.001$) or co-infections with both variants (12%, $P < 0.001$)(Table 14). HHV-6B was significantly more likely to be detected as a co-infection than HHV-6A (80% (8/10) vs 12.3% (8/65), $P = < 0.001$). In contrast, the respiratory mortalities cohort showed 74% of infections as HHV-6B alone. This was significantly greater than the prevalence of HHV-6A infections (15%, $P < 0.001$) or co-infections (11%, $P < 0.001$).

Cohort	Age (m)	HHV-6A	HHV-6B	HHV-6A+B
CIGNIS	6	88% (30/34)	6% (2/34)	6% (2/34)
	18	82% (27/33)	0% (0/33)	18% (6/33)
CIGNIS (m6 + m18)	6 +18	85% (57/67)[a,b]	3% (2/67)[a]	12% (8/67)[b]
CIGNIS (morbidity)	6-18	100% (6/6)	ND	ND
Fever	6-34	14% (1/7)	28% (2/7)	43% (3/7)
Autopsy	0-18yrs	15% (4/27)[c]	74% (20/27)[c,d]	11% (3/27)[d]

Table 16: Prevalence of HHV-6 strain variants A and B in all three cohorts

The total of 67 infants in the CIGNIS cohort from 6 and 18 months includes just one infant (ID37) who was genotyped at both time points (variant A at month 6, but co-infected by month 18). ND = Not Detected. [a,b,c,d] $P < 0.001$.

Sample ID	Cohort	U46 (gN)	U47 (gO)	Overall Genotype	HIV status
1M6	CIGNIS	A	ND	A	-
2M6	CIGNIS	A	A	A	-
3M6	CIGNIS	ND	A	A	-
5M6	CIGNIS	ND	A	A	-
21M6	CIGNIS	A	ND	A	-
37M6†	CIGNIS	ND	A	A	-
47M6	CIGNIS	ND	A	A	-
53M18	CIGNIS	A	ND	A	-
76M18	CIGNIS	ND	A	A	-
77M18	CIGNIS	A	A	A	-
78M18	CIGNIS	A	A	A	-
91M18	CIGNIS	A	ND	A	-
99M18	CIGNIS	ND	A	A	-
109M18*	CIGNIS	ND	A	A	-
118M6*	CIGNIS	A	ND	A	-
129M18	CIGNIS	A	A	A	-
138M18	CIGNIS	A	A	A	-
143M18	CIGNIS	ND	A	A	-
158M18	CIGNIS	A	ND	A	-
163M18	CIGNIS	A	A	A	-
168M18	CIGNIS	A	A	A	-
169M6	CIGNIS	ND	A	A	-
170M18	CIGNIS	ND	ND	A	-
175M18	CIGNIS	A	ND	A	-
179M18	CIGNIS	A	ND	A	-
184M18	CIGNIS	ND	ND	A	-
187M18	CIGNIS	ND	ND	A	-
206M18	CIGNIS	A	A	A	-
225M18	CIGNIS	ND	A	A	-
227M18	CIGNIS	A	ND	A	-
250M18	CIGNIS	ND	A	A	-
278M18	CIGNIS	ND	A	A	-
340M6	CIGNIS	A	ND	A	-
346M18	CIGNIS	A	ND	A	-
397M6	CIGNIS	A	ND	A	-
444M6	CIGNIS	ND	A	A	-
449M6	CIGNIS	A	A	A	-
455M6	CIGNIS	ND	A	A	-
458M6	CIGNIS	A	A	A	+
462M6	CIGNIS	ND	A	A	-
470M6	CIGNIS	ND	A	A	-
491M6	CIGNIS	A	ND	A	-
493M6	CIGNIS	A	A	A	-
494M6	CIGNIS	A	A	A	-
495M18	CIGNIS	A	ND	A	-
500M18	CIGNIS	A	ND	A	-
501M6	CIGNIS	A	A	A	-
503M6	CIGNIS	A	A	A	-
504M6	CIGNIS	ND	A	A	-
507M18	CIGNIS	A	ND	A	-
507M6	CIGNIS	A	A	A	-
514M6	CIGNIS	ND	A	A	-
538M6	CIGNIS	ND	A	A	-
556M6	CIGNIS	ND	A	A	-
659M6	CIGNIS	A	ND	A	-
694M6	CIGNIS	A	ND	A	-
756M6	CIGNIS	A	ND	A	-
K67	Fever	ND	A	A	-
N1	Autopsy	A	ND	A	+
N10	Autopsy	A	A	A	+
N29	Autopsy	A	A	A	+
N30	Autopsy	A	A	A	+
151M18	CIGNIS	A	A,B	A,B	-
174M18	CIGNIS	ND	A,B	A,B	-
219M18	CIGNIS	ND	A,B	A,B	-
337M6	CIGNIS	A	A/B	A/B	+
37M18†	CIGNIS	A	A,B	A,B	-
39M18	CIGNIS	A	A	A,B	-
467M6	CIGNIS	ND	A,B	A,B	-
82M18	CIGNIS	A	A/B	A,B	-
K35	Fever	ND	A	A,B	+
K63	Fever	ND	A	A,B	+
K66	Fever	B	A	A,B	+
N2	Autopsy	A,B	B	A,B	+
N25	Autopsy	B	A,B	A,B	+
N32	Autopsy	ND	A,B	A,B	+
321M6	CIGNIS	B	B	B	-
540M6	CIGNIS	B	B	B	-
N3	Autopsy	B	B	B	+
N5	Autopsy	B	B	B	+
N8	Autopsy	B	B	B	+
N9	Autopsy	B	B	B	+
N11	Autopsy	B	B	B	+
N12	Autopsy	B	B	B	+
N13	Autopsy	B	B	B	+
N14	Autopsy	ND	B	B	+
N15	Autopsy	B	B	B	+
N16	Autopsy	ND	B	B	+
N17	Autopsy	B	B	B	+
N18	Autopsy	B	B	B	+
N19	Autopsy	B	B	B	+
N20	Autopsy	ND	B	B	+
N22	Autopsy	B	B	B	+
N23	Autopsy	B	B	B	+
N26	Autopsy	B	B	B	+
N28	Autopsy	B	B	B	+
N33	Autopsy	B	B	B	+
N35	Autopsy	B	B	B	+

Table 17: HHV-6 genotypes

HHV-6 genotypes detected at two different loci (U46, U47). Overall genotypes were denoted as 'A' (only A detected at one or multiple loci) 'B' (only B detected at one or multiple loci), or 'A,B' (Different variants detected at different loci). ND indicates that the genotype of a given loci was 'Not Determined'. Of four samples HHV-6 positive at both time points, two were genotyped at just one time point (*), one was genotyped at both time points (†) and one was not genotyped at either time point. HHV-6 sequences from this study were published in 2009 (Bates et al., 2009).

4.8.1 HHV-6 U46 sequence alignment

The primary screen for HHV-6 targeted the U46 gene (glycoprotein gN) using primer pair U46F/U46R. U46 sequence data was obtained from the sequencing of this PCR product, although further primer pairs and nested sets were also used for some samples (Appendix F). HHV-6 U46 sequence was obtained from 23/27 respiratory mortality samples, 1/7 hospitalized fever samples, 39/98 CIGNIS samples (M6 and M18 combined) and 4/13 CIGNIS morbidity samples. Sequences were aligned using *ClustalW* software for both U46 (Figure 19) and U47 (Figure 20). With respect to U46, all but one CIGNIS sample (321M6) were found to be HHV-6A. Little variation was observed, seen in just four of these sequences. However, single nucleotide substitutions were identified in two samples that led to amino acid changes: 449M6 (K→R) and 226morb (F→S). In two other samples (324morb and 500M18) a thymidine deletion was observed that results in a frame shift resulting in premature termination suggesting a truncated glycoprotein gN with a novel 9 residue C-terminus. This deletion reduced a 7T to a 6T poly T motif, and was observed in two independent samples and in both directions. Such a truncation may result in a gN phenotype that is either knocked out or weakened. The U46 sequence obtained from respiratory mortalities sample N29 contained a two amino acid deletion, but all other U46 sequences from the respiratory mortalities cohort were identical to the reference strain Z29. The one hospitalized fever sample that was typed for U46 was also HHV-6B, but contained a single nucleotide substitution encoding a L→P change. Some sequences had double peaks at selected residues, indicative of possible dual infections as shown in reconstructions of HHV-6A + B PCR using cloned genes (as indicated in figure 19 and figure 20).

```
U1102     MSCKKSARQSLYVSLCLFYILVFAAATEVDFYSPECHSHTYEIVLNSFSSIWLLINLFLLLCSFAIFLKYWCYKTFASET
1M6       ................................................................................
2M6       ................................................................................
21M6      ................................................................................
77M18     ................................................................................
37M18     ................................................................................
39M18     ................................................................................
53M18     ................................................................................
82M18     ................................................................................
91M18     ................................................................................
118M6     ................................................................................
129M18    ................................................................................
138M18    ................................................................................
150morb   ................................................................................
151M18    ................................................................................
158M18    ................................................................................
163M18    ................................................................................
168M18    ................................................................................
175M18    ................................................................................
179M18    ................................................................................
227M18    ................................................................................
337M6     ................................................................................
340M6     ................................................................................
346M18    ................................................................................
458M6     ................................................................................
491M6     ................................................................................
493M6     ................................................................................
494M6     ................................................................................
495M18    ................................................................................
501M6     ................................................................................
503M6     ................................................................................
507M6     ................................................................................
507M18    ................................................................................
546morb   ................................................................................
694M6     ................................................................................
756M6     ................................................................................
N1        ................................................................................
N2(A)     ................................................................................
N10       ................................................................................
N24       ................................................................................
N30       ................................................................................
N29       ..........................................................--..........STOP
78M18     ...........................................X....................................
206M18    ............X...................................................................
659M6     .....................................X..........................................
226morb   ...............................................S................................
449M6     ....R...........................................................................
324morb   ..........................................................YCYVLSRFS
500M18    ..........................................................YCYVLSRFS
321M6     .....G...R.....W............M....S..............................................
540M6     .....G...R.....W............M....S..............................................
K66       .....G...R.....W....P.......M....S..............................................
N2(B)     .....G...R.....W............M....S..............................................
N3        .....G...R.....W............M....S..............................................
N5        .....G...R.....W............M....S..............................................
N8        .....G...R.....W............M....S..............................................
N11       .....G...R.....W............M....S..............................................
N12       .....G...R.....W............M....S..............................................
N13       .....G...R.....W............M....S..............................................
N14       .....G...R.....W............M....S..............................................
N15       .....G...R.....W............M....S..............................................
N18       .....G...R.....W............M....S..............................................
N19       .....G...R.....W............M....S..............................................
N20       .....G...R.....W............M....S..............................................
N21       .....G...R.....W............M....S..............................................
N22       .....G...R.....W............M....S..............................................
N23       .....G...R.....W............M....S..............................................
N25       .....G...R.....W............M....S..............................................
N26       .....G...R.....W............M....S..............................................
N27       .....G.X.R.X...W............M....S..............................................
N28       .....G...R.....W............M....S..............................................
N33       .....G...R.....W............M....S..............................................
N34       .....G...R.....W............M....S..............................................
N35       .....G...R.....W............M....S..............................................
Z29       .....G...R.....W............M....S..............................................
```

Figure 19: HHV-6 U46 alignment

HHV-6 U46 amino acid sequence alignment (excluding the four C-terminal residues VKGY) showing two reference strains (U1102 – HHV-6A, and Z29 – HHV-6B) along with those from the respiratory mortalities (N), hospitalized fever (K) and CIGNIS ('M6', 'M18' or 'morb' suffix) cohorts. In the alignment, dots indicate identities relative to reference U1102 sequence (HHV-6A). X's indicate double peaks that could not be resolved in either direction consistent with dual infection. Question marks indicate terminal regions that are unknown

4.8.2 HHV-6 U47 sequence alignment

The U47 gene was also amplified and sequenced using three sets of primers. U47 DNA sequence data was obtained for, 26/27 respiratory mortality samples, 4/7 hospitalized fever samples and 45/98 HHV-6 positive CIGNIS samples (M6 and M18 combined) and just 1/13 morbidity samples. DNA sequence was edited as described and an amino acid sequence alignment was constructed using *ClustalW* software (Figure 20). As with U46, many sequences were identical to either the HHV-6A or HHV-6B reference sequences (U1102 and Z29 respectively). A similar proportion of sequences showed novel amino acid substitutions within both U46 (16% (10/73)) and U47 ((15% (13/85)). However this rate of detection of novel sequences for both HHV-6 loci was significantly lower than for their respective HCMV homologues: HCMV UL73 ((46% (22/48)) and HCMV UL74 (52% (29/56)). Of 15 substituted amino acids with the U47 sequences, just two were previously shown in febrile Zambian infants (Kasolo et al., 2007), suggesting broader variation within the population at large. Sequence for ID 514M6 contained a C→A point mutation (on both forward and reverse strands) that introduces an in-frame stop codon, resulting in a HHV-6A protein of 173 residues, compared to 618 for the full length HHV-6 gO.

```
U1102       EESLRMAMSKFSNSNLTRSLTSFTSKNFFNYTSFVYFLLYNTTSCVPSNDQYFKQSPKPINVTTSFGRA
2M6         ...........................................................................
5M6         ...........................................................................
37M18       ...........................................................................
39M18       ...........................................................................
47M6        ...........................................................................
76M18       ...........................................................................
77M18       ...........................................................................
78M18       ...........................................................................
82M18Gi     ...........................................................................
99M18       ...........................................................................
109M18      ...........................................................................
129M18      ...........................................................................
138M18      ...........................................................................
143M18      ...........................................................................
163M18      ...........................................................................
169M6       ...........................................................................
225M18      ...........................................................................
250M18      ...........................................................................
278M18      ...........................................................................
444M6       ...........................................................................
449M6       ...........................................................................
455M6       ...........................................................................
459M6       ...........................................................................
462M6       ...........................................................................
467M6       ...........................................................................
470M6       ...........................................................................
493M6       ...........................................................................
494M6       ...........................................................................
501M6       ...........................................................................
503M6       ...........................................................................
504M6       ...........................................................................
507M6       ...........................................................................
538M6       ...........................................................................
546morb     ...........................................................................
K35         ...........................................................................
K63         ...........................................................................
K66         ...........................................................................
K67         ...........................................................................
N10         ???????....................................................................
N29         ??????????.................................................................
N30         ??????????.................................................................
N25(A)      ??????????.................................................................
37M6        ..........S.............S..................................................
39M18Gi     ....................A.......................................................
151M18      ...............P...........................................................
168M18      ..................................F........................................
174M18      ...................P.......................................................
206M18      ....................S......................................................
219M18      ....................P.............S........................................
458M6Gi     ................................G...........................................
556M6       ..............D........M.....................................................
3M6         ...........................................................................X......
514M6       ............*
321M6       .......I.............X.XXXX..................XX......EH.....
N32         ??????????.........P..PEI.............A...R......EH.....?????????
467M6Gi     D...Q..I............P..PEI.....C...........IR......EH.....
37M18Gi     D...Q..I............P..PEI.................IR......EH.....
151M18(B)   D...Q..I............P..PEI.................IR......EH.....
174M18(B)   D...Q..I............P..PEI.................IR......EH.....
219M18(B)   D...Q..I............P..PEI.................IR......EH.....
337M6       D...Q..I............P..PEI.................IR......EH.....
540M6       D...Q..I............P..PEI.................IR......EH.....
N2          D...Q..I............P..PEI.................IR......EH.....
N3          D...Q..I............P..PEI.................IR......EH.....
N5          D...Q..I............P..PEI.................IR......EH.....
N8          D...Q..I............P..PEI.................IR......EH.....
N9          ?????..I............P..PEI.................IR......EH.....
N11         D...Q..I............P..PEI.................IR......EH.....
N12         D...Q..I............P..PEI.................IR......EH.....
N13         D...Q..I............P..PEI.................IR......EH.....
N14         ??????????????????????????????????????????????S....EH.....
N15         D...Q..I............P..PEI.................IR......EH.....
N16         D...Q..I............P..PEI.................IR......EH.....
N17         D...Q..I............P..PEI.................IR......EH.....
N18         D...Q..I............P..PEI.................IR......EH.....
N19         D...Q..I............P..PEI.................IR......EH.....
N20         D...Q..I............P..PEI.................IR......EH.....
N22         ??????????.........P..PEI.................IR......EH.....
N23         D...Q..I............P..PEI.................IR......EH.....
```

```
N25(B)      D...Q..I.............P..PEI.................IR......EH.............
N26         ??????????..........P..PEI.................IR......EH.............
N28         D...Q..I.............P..PEI.................IR......EH.............
N33         D...Q..I.............P..PEI.................IR......EH.............
N35         D...Q..I.............P..PEI.................IR......EH.............
Z29         D...Q..I.............P..PEI.................IR......EH.............
```

Figure 20: HHV-6 U47 alignment

HHV-6 U47 amino acid sequence alignment of a 69 amino acid section (residues 161-229 of U47 from both references strain), showing two reference strains (U1102 – HHV-6A, and Z29 – HHV-6B) along with those from the respiratory mortalities (N), hospitalized fever (K) and CIGNIS ('M6', 'M18' or 'morb' suffix) cohorts. The suffix 'Gi' indicates that the detection was on extracted DNA that had been archived using *Genomiphi*. The Suffixes '(A)' and '(B)' indicate where HHV-6A and B were detected in different amplifications of the same sample. In the alignment, dots indicate identities relative to reference U1102 sequence (HHV-6A). Question marks indicate terminal regions that are unknown.

4.9 Discussion

In this chapter we have shown for the first time active HHV-6 infections in a population of infants in Zambia. HHV-6 sera-DNA prevalence in the CINGIS cohort was 8% at 6 months, rising to 13% at 18 months of age. This is broadly consistent with a US study on cumulative detection of HHV-6 secretion in saliva by PCR, in which infection occurred at a rate of 4.5%/month (Zerr et al., 2005). HHV-6 active infections were significantly more prevalent within CIGNIS morbidity samples from infants hospitalized during the study. The overall rate of detection of HHV-6 active infections was significantly lower than that of HCMV, reflecting possibly slower uptake and shorter periods of active infection, or this could in part be explained by differences in assay sensitivity. No significant differences were seen in the prevalence of HHV-6 active infections by either infant or maternal HIV-1 status, suggesting equal exposure but outcomes may be different. HHV-6 analysis of the two retrospective cohorts was undertaken with a PCR assay designed to detect only higher load infections that might be responsible for disease. In hospitalized febrile infants, there was no significant difference in the prevalence of HHV-6 (DNA extracted whole blood) between HIV-1 negative and positive infants. Conversely, prevalence of HHV-6 DNA detection was very high (72%) in lung tissue samples from HIV-1 positive children who died of respiratory disease. HHV-6 loads in the respiratory mortalities study were high with a median of just over 100 copies/10^6 genomes equivalents, although a previous study did not find any difference between HHV-6 loads in lung tissue from patients with interstitial pneumonia and controls (Yamamoto et al., 2005). The respiratory mortalities studied here were all from children with end-stage AIDS however and, like HCMV, HHV-6 may be an important underlying infection which may respond to treatment. Chapter 5 contains further analysis on the clinical outcomes of this population-based study.

The predominant strain variant detected in healthy Zambian infants is HHV-6A. This finding is the exact opposite of that seen in studies in Europe or North America, where 97% of infant primary infections are caused by HHV-6B (Dewhurst et al., 1993; Hall et al., 2006; Zerr et al., 2005), and where HHV-6A is considered more prevalent in older individuals, especially those on immunosuppressive therapy or with HIV/AIDS (Ablashi et al., 1998; Iuliano et al., 1997; Secchiero et al., 1995). In contrast to the population-based CIGNIS study, symptomatic HHV-6 infections in Zambia, notably those associated with end-stage AIDS, were predominantly HHV-6B. It has been shown previously that HHV-6A products can infer an inhibitory effect on HIV-1 CCR5-tropic strains, through the blocking of this co-receptor with the viral chemokine U83A and also stimulating the immune response which may protect against HIV-1 (Catusse et al., 2007). Interestingly, the same chemokine in HHV-6B (U83B) does not bind CCR5 and chemattracts a different cellular subset (Luttichau et al., 2003). These differences in the two HHV-6 strain variants, and the exclusive interaction of the A variant with the HIV-1 co-receptor, suggest that the findings here that HHV-6A predominates in and HIV-1 endemic region could have significant effects on the course of disease progression.

Here we have genotyped HHV-6 primarily based on the analysis of two genes, U46 and U47, which encode the variable glycoproteins gN and gO respectively. Variation within the two glycoprotein genes is less than in the HCMV homologues and many strains show identical sequence to the two reference strains U1102 and Z29. The U47 gene has been analysed previously and so it was possible to combine sequences obtained here with those that have been previously published (Gompels, 2006)(Figure 21). This data shows mosaics of the U47 gene that resemble aspects of both strain variants, providing evidence for recombination in this population where both strain variants are prevalent. The biological role of glycoproteins gO and gN (in both HHV-6 and HCMV) are worthy of further investigation which may shed light on function of this variation.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	VARIANT
Z29	D	Q	I	S	N	L	S	P	F	T	P	E	I	S	L	L	I	R	E	H	T	F	B
467M6Gi	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	B
Zam3	E	R	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	B/A
Zam14	E	R	M	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	B/A
Zam15	E	R	M	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	B/A
KF	E	R	M	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	B/A
Zam50	E	R	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	B/A
Zam317	E	R	M	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	Q	.	.	B/A
Zam12	E	R	M	.	.	.	.	.	.	.	.	.	N	.	.	.	V	P	.	Q	.	.	A/B
Zam352	E	R	M	.	.	.	.	S	.	.	S	.	.	.	.	.	V	P	.	Q	.	.	A/B
Zam325	E	R	M	.	.	.	.	S	.	.	S	.	.	.	.	.	L	P	K	.	.	.	A/B
Zam5	E	R	M	.	.	.	.	S	.	.	S	.	.	.	.	.	V	P	K	Q	.	.	A/B
Zam7	E	R	M	.	.	.	.	S	.	.	S	.	N	.	.	.	V	P	K	.	.	.	A/B
Zam27	E	R	M	.	.	.	.	S	.	.	S	.	N	.	.	.	V	P	K	Q	.	L	A/B
GS	E	R	M	.	.	.	.	S	.	.	S	.	N	.	.	.	V	P	K	Q	I	.	A/B
Zam59	E	R	M	.	.	.	.	S	.	.	S	K	N	.	.	.	V	P	K	Q	.	L	A
Zam25Ly	E	R	M	.	.	.	.	S	.	.	S	K	N	.	.	.	V	P	K	Q	.	.	A
Zam23	E	R	M	.	.	.	.	S	.	.	S	K	.	.	.	.	V	P	K	Q	.	.	A
458M6Gi	E	R	M	.	.	.	.	S	.	.	S	K	N	G	.	.	V	P	K	Q	.	.	A
219M18	E	R	M	.	.	.	.	.	.	.	S	K	N	.	S	.	V	P	K	Q	.	.	A
168M18	E	R	M	.	.	.	.	S	.	.	S	K	N	.	.	F	V	P	K	Q	.	.	A
174M18	E	R	M	.	.	.	P	S	.	.	S	K	N	.	.	.	V	P	K	Q	.	.	A
151M18	E	R	M	.	.	P	.	S	.	.	S	K	N	.	.	.	V	P	K	Q	.	.	A
556M6	E	R	M	.	D	.	.	S	.	M	S	K	N	.	.	.	V	P	K	Q	.	.	A
39M18Gi	E	R	M	.	.	.	.	S	.	A	S	K	N	.	.	.	V	P	K	Q	.	.	A
206M18	E	R	M	.	.	.	.	S	S	.	S	K	N	.	.	.	V	P	K	Q	.	.	A
U1102	E	R	M	.	.	.	.	S	.	.	S	K	N	.	.	.	V	P	K	Q	.	.	A

Figure 21: HHV-6 U47 global alignment

Alignment (adapted and including data from (Gompels, 2006)) for an N-terminal portion of the HHV-6 U47 gene showing only variable residues. Column numbers refer to amino acids 161, 165, 168, 173, 174, 175, 178, 182, 183, 184, 185, 186, 187, 193, 198, 199, 206, 207, 214, 215, 223 and 226 relative to the Z29 HHV-6B reference strain. Representative examples of all available variants are shown and other sequences from this study, identical to representative strains shown are as follows: HHV-6B: Z29 (HST, 37M18Gi, 540M6), Zam3 (Zam13, Zam18, Zam25 Zam31, MBE, L9, MAR, BOU) , Zam5 (AJ) and HHV-6A: U1102 (ZamB35, ZamB63, ZamB66, ZamB67, 37M18, 39M18, 76M18, 77M18, 78M18, 82M18Gi, 99M18, 109M18, 129M18, 138M18, 143M18, 163M18, 169M6, 225M18, 278M18, 444M6, 449M6, 455M6, 459M6, 462M6, 467M6, 470M6, 493M6, 494M6, 501M6, 503M6, 504M6, 507M6, 514M6, 538M6

Prior to this study, there was no HHV-6 strain variant data from a population based cohort in Africa. This despite the fact that the two major reference strains used to type HHV-6 variants were isolated from AIDS patients in Uganda (HHV-6A strain U1102) and the Democratic Republic of Congo (HHV-6B strain Z29). Whilst in Europe and North America HHV-6B is responsible for over 97% of primary infections during infancy (Dewhurst et al., 1993; Hall et al., 1994), HHV-6A is thought to be a later infection and is associated more with neurological disorders (De Bolle et al., 2005; Hall et al., 1998) and there are many case reports of HHV-6A infections in immunosuppressed patient groups as reviewed (Gompels, 2004). In Zambia however, it was shown previously that HHV-6A and HHV-6B were of roughly equal prevalence in febrile infants (Kasolo, Mpabalwani, and Gompels, 1997). The most important finding here is that HHV-6A, not HHV-6B, is the predominant infection in healthy Zambian infants. This is juxtaposed against the predominance of HHV-6B in a group of HIV-1 positive children who died from respiratory disease, and raises questions as to whether our understanding of the epidemiology of HHV-6 infections from European, North American and Japanese studies, can be applied globally. Primary HHV-6 infections occur during infancy and are largely acquired from the mother or from other close family members (van Loon et al., 1995). Here we show that HHV-6A is endemic to Southern Africa, and may more recently have spread to other parts of the globe, where it may be responsible in part for some emerging diseases?

5.0 Betaherpesvirus infections in HIV-1 exposed infants and effects on micronutrient fortification

5.1 Introduction

CIGNIS (Chilenje Infant Growth Nutrition and Infection Study) is a placebo controlled trial with the objective of testing the efficacy of a micronutrient fortified feed supplement to improve immunity and reduce stunting and morbidity in HIV-1 negative Zambian infants born to HIV-1 positive mothers. These 'HIV-1 exposed' infants have been previously shown to suffer from impaired growth compared to 'HIV-1 unexposed' infants (HIV-1 negative infants of HIV-1 negative mothers)(Makasa et al., 2007). Due to the expanding HIV-1 pandemic, and with programmes to reduce mother-to-child transmission now in place in many countries, this HIV-1 exposed group is also expanding, but the reasons for impaired growth are not fully understood.

The previous two chapters have established baselines in this population for betaherpesvirus prevalence and strain variants present. We have shown, as elsewhere, that both HCMV and HHV-6 are pathogens associated with severe morbidity in HIV-1 infected infants (Kositanont et al., 1999; Kovacs et al., 1999). We have also shown higher prevalence of high load HCMV (but not HHV-6) sera-DNA in HIV-1 exposed infants, and in this chapter the effect of betaherpesvirus infections on these infants will be investigated further, as affected by a micronutrient fortification tested to improve development in HIV-1 exposed but uninfected infants. HCMV and HHV-6 sera-DNA detection (indicative of active infections) and also HCMV sera-antibody detection at 18 months (a measure of overall seroprevalence) will be analysed with respect to other markers of immunity, nutrition and growth. HCMV seroprevalence was assayed by KM, an MSc project student (methods), and his results are further analysed here. Other members of the CIGNIS team have been involved in collecting a variety of data that are analysed here: Level of maternal education, family's socioeconomic status (determined by Asset Index Score), duration of breast feeding, haemoglobin levels, reported fever (defined as fever reported in the 3 days prior to sera sampling), responses to polio vaccination, rate of referrals and finally anthropometric data. Betaherpesvirus sera-DNA, HCMV sera-antibody and HIV-1 infection and exposure were cross tabulated with all nominal variables and significance of associations was assessed using Pearson's Chi Squared.

The appendixes H-Q contain further statistical analyses performed by the study statistician, KB, which are related to some of the conclusions discussed.

5.2 Effects of HIV-1 infection and exposure on markers of morbidity and growth

All mothers were asked at recruitment for their antenatal HIV-1 status, which in Zambia is routinely tested in the third trimester of pregnancy. Responses were scored as 'positive', 'negative' or 'don't know/declined'. All infants who complete the study were tested for HIV-1 at 18 months of age. This is standard procedure in Zambia and complies with guidelines laid out by the Ministry of Health. With this data, infants were defined as either HIV-1 infected (HIV-1 positive infant, irrespective of maternal HIV-1 status), HIV-1 exposed (HIV-1 negative infants (including unknowns) of HIV-1 positive mothers) and HIV-1 unexposed (HIV-1 negative infants (including unknowns) of HIV-1 negative mothers. The CIGNIS study was population based and designed to study the effects of a micronutrient fortified feed supplement on morbidity and growth in HIV-1 exposed infants. Of a total of 812 mothers[1] taking part in the study, 564 (69.5%) were HIV-1 negative, 177 (21.8%) were HIV-1 positive and 71 (8.7%) either didn't know or chose not to disclose their status. With successful implementation of single dose Nevirapine therapy in Lusaka, rates of mother-to-child-transmission have been reduced significantly. We found 8% (12/152) of infants who were born to HIV-1 positive mothers, were HIV-1 positive when they completed the study at 18 months of age. There were 20 HIV-1 positive infants in total, 6 were born to HIV-1 negative mothers (likely due to postnatal infection) and 2 were born to mothers with unknown antenatal HIV-1 status. Correlations between infant HIV-1 status or HIV-1 exposure and other outcomes are summarized in table 18 and table 19 respectively (Pages 119 and 120).

HIV-1 is known to be transmitted vertically through breast milk (de Martino et al., 1992). Mothers were surveyed and asked if they were breast feeding at recruitment. Their answers were scored as either 'yes' (currently breast feeding), 'no' (stopped breast feeding before 6 months) or 'never' (study infant has never been breast fed). Low adherence to breast feeding among HIV-1 positive mothers is well documented (Fadnes et al., 2009) and this is likely a major cause of growth faltering among HIV-1 exposed infants (Arpadi et al., 2009). This is at the crux of the CIGNIS study which aims to improve growth and reduce morbidity in HIV-1

[1] There were actually only 799 mothers but the 13 pairs of twins enrolled onto the study were treated as 26 individual cases

exposed infants through an intervention with fortified feed supplement. In keeping with previous findings, HIV-1 exposed infants were significantly less likely to have been breast fed, with 27% (44/165) having never breast fed (compared to 0.5% (3/558) of HIV-1 unexposed) and only 43% (71/165) breast feeding for 6 months or longer (compared to 93% (538/558) of HIV-1 unexposed infants)(Table 19, Figure 22). Among HIV-1 infected infants, 75% (15/20) were still being breast fed at 6 months, demonstrating the known link between breast feeding and MTCT (Table 18, Figure 22).

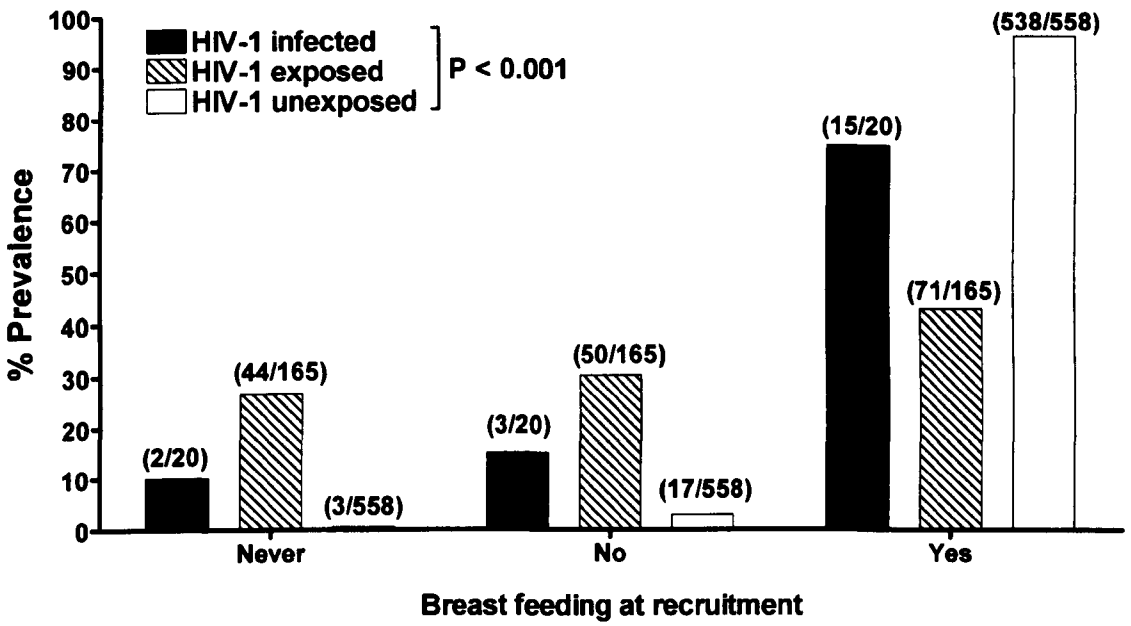


Figure 22: Maternal HIV-1 infection associated with earlier cessation of breast feeding
Prevalence of HIV-1 infected, exposed and unexposed infants by breast feeding duration. Significance is by Pearson Chi Squared.

In this cohort, maternal education level bore no correlation to HIV-1 infection (Table 18) or exposure (Table 19). Whilst HIV-1 exposed infants were significantly more likely to be from the low socioeconomic group (41% (67/165) vs 31% (175/558)(P = 0.027), the numbers of HIV-1 exposed infants did not differ significantly between middle and higher socioeconomic groups (Table 19).

No study to date has assessed the uptake of oral polio vaccine in HIV-1 exposed but negative infants. Here we have measured vaccine titres in 18 month old Zambian infants by neutralization assay, and analysed them with respect to HIV-1 infection or exposure. Polio neutralizing antibody titres were available for 11 HIV-1 positive infants on the CIGNIS study. 35% of these infants failed to mount a protective response, and those who did mounted lower responses than among HIV-1 uninfected infants (Figure 23a). In contrast, the effects of HIV-1

exposure were not so severe, but still HIV-1 exposed infants were less likely to mount high response and more likely to have low or un-protective responses (Figure 23b). This suggests that humoral immunity in HIV-1 exposed infants could be impaired compared to that of HIV-1 unexposed infants.

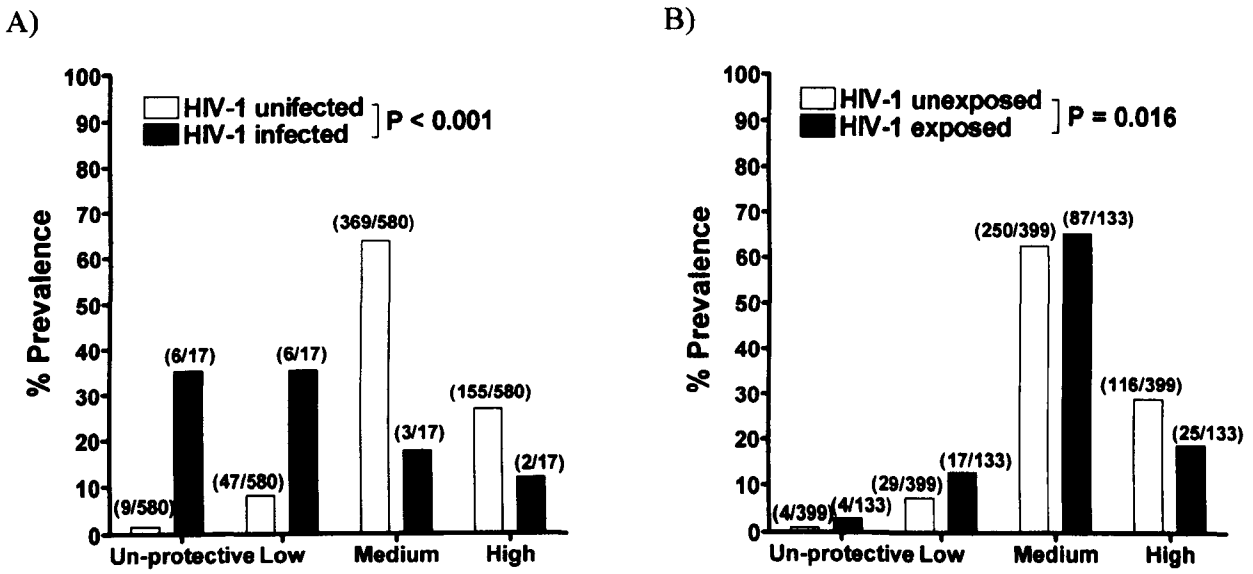


Figure 23: Polio vaccine failures and lower responses were associated with HIV-1 infection and exposure

Percentage prevalence of un-protective, low, medium and high, polio neutralizing antibody titres by a) infant HIV-1 status and b) HIV-1 exposure. Infant HIV-1 status was determined by detection of antibody at 18 months of age. HIV-1 exposure was defined by maternal antenatal HIV-1 status reported on recruitment as positive and infants 18 month antibody test being negative. Polio vaccine uptake was monitored by a neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) and high ($^1/_{1024}$-$^1/_{2048}$). Significance is by Pearson Chi Squared.

	Category/ month	Infant HIV-1 sera-Ab at 18 months				
		HIV-1 Negative		HIV-1 Positive		P
Demographics						
Gender	Male	47% (290/325)		50% (10/20)		.807
	Female	53% (324/614)		50% (10/20)		
Maternal Education	None	5% (28/614)		10% (2/20)		.427
	Primary	36% (162/614)		35% (7/20)		
	Secondary	40% (243/614)		35% (7/20)		
	Tertiary	28% (169/614)		15% (3/20)		
	University	2% (12/614)		5% (1/20)		
Socio-economic status	Low	32% (198/614)		35% (7/20)		.352
	Medium	38% (236/614)		50% (10/20)		
	High	29% (180/614)		15% (3/20)		
Breast feeding at baseline	Never	7% (42/614)		10% (2/20)		.584
	No	9% (58/614)		15% (3/20)		
	Yes	84% (514/614)		75% (15/20)		
Morbidity						
Polio Ab neutralization titre (12 months)	Un-protective	2% (9/580)		35% (6/17)		<.001***
	Low	8% (47/580)		35% (6/17)		
	Medium	64% (369/580)		18% (3/17)		
	High	27% (114/580)		12% (2/17)		
Fever	6	13% (81/614)		10% (2/20)		.677
	18	11% (53/467)		0% (0/17)		.141
Anaemic	6	37% (225/605)		63% (12/19)		.022**
	18	29% (176/607)		63% (10/16)		.004***
HCMV sera-DNA	6	40% (156/392)		53% (10/19)		.265
	18	33% (128/383)		39% (5/13)		.705
HHV-6 sera-DNA	6	8% (38/461)		16% (3/19)		.249
	18	13% (49/384)		14% (2/14)		.697
HCMV antibody	18	84% (376/450)		78% (7/9)		.644
Referral Rate		0.18		0.55		.002**
Died		0% (0/614)		5% (1/20)		<.001***
Growth						
Stunted	6	13% (71/557)		12% (2/17)		.905
	18	20% (105/524)		29% (4/14)		.433
		HIV-1 Negative		HIV-1 Positive		
		N =	Mean	N =	Mean	P
BMI-for-age	6	556	0.0186	17	0.0076	0.970
	18	524	0.1203	14	-0.1043	0.435
Weight-for-age	6	556	-0.4537	17	-1.0035	0.069*
	18	526	-0.5674	15	-0.7220	0.626
Length/height-for-age	6	557	-0.8131	17	-1.5888	0.007**
	18	524	-1.0817	14	-1.1579	0.809
Triceps skinfold-for-age	6	557	-0.1353	17	-0.7671	0.016**
	18	525	0.1739	15	-0.1440	0.194
Subscapular skinfold-for-age	6	557	0.4473	17	-0.0335	0.110
	18	525	0.5174	15	-0.2133	0.006**
Arm circumference-for-age	6	557	0.3215	17	-0.2341	0.048**
	18	524	0.0789	15	-0.5153	0.041**
Head circumference-for-age	6	557	0.6481	17	0.3782	0.288
	18	525	0.5454	15	0.5607	0.954

Table 18: HIV-1 infected infants have low polio vaccine response, increased anaemia, are stunted and have less fat

Distribution of infants by HIV-1 status (antibody test at 18 months) with respect to maternal education (as surveyed on recruitment), socioeconomic status (determined by Asset Index Score), breast feeding category (Never = not breast fed at baseline, No = breast feedings stopped before 6 months and Yes = breast feeding at and beyond 6 months), Polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

	Category/month	HIV-1 exposure (maternal reported status at recruitment)				
		HIV-1 unexposed		HIV-1 exposed		P
Demographics						
Gender	Male	49% (273/558)		45% (74/91)		.357
	Female	51% (285/558)		55% (91/165)		
Maternal Education	None	5% (25/558)		7% (11/165)		.106
	Primary	26% (145/558)		33% (55/165)		
	Secondary	38% (213/558)		36% (60/165)		
	Tertiary	30% (166/558)		21% (35/165)		
	University	2% (9/558)		2% (4/165)		
Socio-economic status	Low	31% (175/558)		41% (67/165)		.086*
	Medium	39% (219/558)		35% (57/165)		
	High	29% (164/558)		25% (41/165)		
Breast feeding at baseline	Never	0.5% (3/558)		27% (44/165)		<.001***
	No	3% (17/558)		30% (50/165)		
	Yes	93% (538/558)		43% (71/165)		
Morbidity						
Polio Ab neutralization titre (12 months)	Un-protective	1% (4/399)		3% (4/133)		.016**
	Low	7% (29/399)		13% (17/133)		
	Medium	63% (250/399)		65% (87/133)		
	High	29% (116/399)		19% (20/133)		
Fever	6	12% (65/558)		13% (22/165)		.559
	18	11% (40/359)		8% (8/104)		.310
Anaemic	6	38% (210/550)		37% (60/162)		.792
	18	29% (121/417)		31% (43/141)		.739
HCMV sera-DNA	6	37% (132/359)		43% (41/96)		.287
	18	35% (95/274)		28% (22/78)		.285
HHV-6 sera-DNA	6	9% (36/426)		6% (7/117)		.381
	18	13% (36/275)		13% (10/78)		.950
HCMV antibody	18	85% (263/311)		82% (81/99)		.517
Referral Rate		0.21		0.35		.007*
Died		0.5% (3/558)		5% (8/165)		<.001***
Stunted	6	11% (58/513)		18% (26/148)		.044**
	18	18% (65/369)		30% (34/112)		.003***
Growth						
		HIV-1 unexposed		HIV-1 exposed		
		N =	Mean	N =	Mean	P
BMI-for-age	6	512	0.0973	148	-0.1866	.010**
	18	369	0.1759	112	-0.0037	.117
Weight-for-age	6	512	-0.3752	148	-0.7413	.001***
	18	371	-0.4745	112	-0.8679	.003***
Length/height-for-age	6	513	-0.7799	148	-1.0424	.008**
	18	369	-1.0019	112	-1.4058	.001***
Triceps skinfold-for-age	6	513	-0.0841	148	-0.3022	.027**
	18	370	0.2551	112	0.0321	.029**
Subscapular skinfold-for-age	6	513	0.5287	148	0.1211	<.000***
	18	370	0.5934	112	0.3990	.077*
Arm circumference-for-age	6	513	0.4386	148	-0.0610	<.000***
	18	369	0.1934	112	-0.2656	<.000***
Head circumference-for-age	6	513	0.7254	148	0.3730	<.000***
	18	370	0.6519	112	0.2648	.001***

Table 19: HIV-1 exposed infants are stunted and have consistently lower anthropometric mean z-scores

Distribution of infants by HIV-1 exposure with respect to maternal education (as surveyed on recruitment), socioeconomic status (determined by Asset Index Score), breast feeding category (Never = not breast fed at baseline, No = breast feedings stopped before 6 months and Yes = breast feeding at and beyond 6 months), Polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

HIV-1 positive infants showed significantly higher levels of anaemia at both time points and did not show the decrease over the 12 month duration of the study seen with the HIV-1 negative children, with prevalence of anaemia at month 18 remaining over 60% among HIV-1 positives (Figure 24a), whereas for the population as a whole the prevalence of anaemia dropped significantly from 39% (310/798) to 30% (186/626) during the course of the study ($P < 0.001$). With respect to maternal HIV-1 status, prevalence of anaemia did not differ significantly at either time point (Figure 24b).

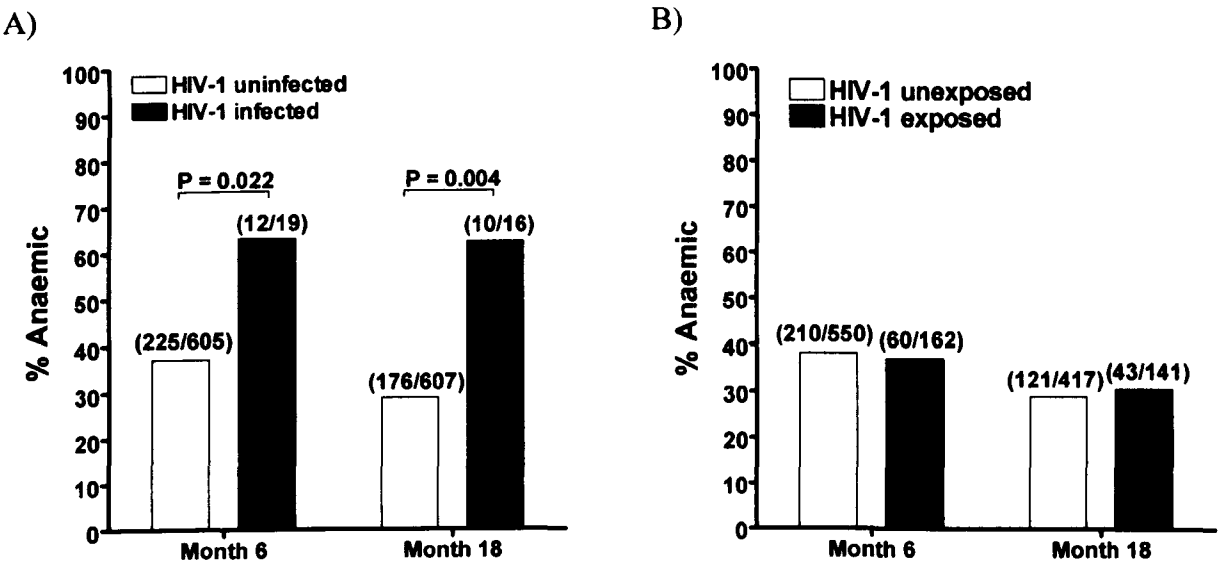


Figure 24: Prevalence of anaemia is higher in HIV-1 infected infants but does not differ by maternal HIV-1 exposure.

Anaemia was defined by a sera-haemoglobin concentration of less than 10.5 g/L. Significance is by Pearson Chi Squared.

The primary outcome of the CIGNIS intervention was growth, and so infants taking part in the study had anthropometric measurements taken at 6, 9, 12, 15 and 18 months of age. Over the 12 months of the study, BMI (Body Mass Index)-, weight-, length-, triceps skinfold-, arm circumference- and head circumference-for-age z-scores all changed significantly describing the infants growth relative to their age. Here baseline (month 6) and endpoint (month 18) measurements are analysed with respect to HIV-1 infection and exposure. At baseline, HIV-1 positive infants showed a trend for lower weight, but had significantly lower length, triceps skinfold and arm circumference mean z-scores. Their BMI and head circumference mean z-scores did not differ significantly at recruitment. By 18 months of age, many of these differences were resolved, the only significant differences being lower mean z-scores for arm circumference and sub-scapular skinfold (Table 18). Compared with those who were HIV-1 infected, HIV-1 exposed infants had poorer growth outcomes, having lower mean z-scores of

many anthropometric measures persisting to month 18. The exceptions just outside of significance were BMI and subscapular skinfold at month 18 (Table 19). The effects on length can also be expressed through prevalence of stunting (length-for-age z-score of less than -2), with HIV-1 exposed infants being almost twice as likely to be stunted by the time they are 18 months old (Table 19).

A total of 152 infants were referred at least once during the course of the study. The three most common primary diagnoses were acute diarrhoea (n = 40), malaria (n = 32) and pneumonia (n = 29). The remaining 51 primary diagnoses included 6 infants with protein energy malnutrition, 5 with skin infections and 4 with measles and a range of other infectious conditions. Referrals for treatment of physical injuries or deformities (Burns, Hernia, Phimosis and Talipes) were excluded from the analyses. Referral rates for sub-groups as the total number of referrals per 100 person years and were compared by the study statistician, KB, using the Cox regression methods and the Breslow method for ties. The referral rate was 300% higher among HIV-1 infected verses HIV-1 uninfected children (0.55 vs 0.18, P = 0.002)(Table 18), consistent with higher rates of morbidity due to HIV-1 infection (Appendix J, section 9.10.1). The rate of referrals was over 50% greater among HIV-1 exposed than among HIV-1 unexposed infants (0.35 vs 0.21, P = 0.007)(Table 19), also suggestive of higher rates of serious morbidity (Appendix J, section 9.10.2).

There were a total of twelve deaths on the study (Table 20) and initially it looks as if mortalities are significantly higher among HIV-1 exposed infants than unexposed (Table 19). This is misleading however, as the HIV-1 status was only known for one of these twelve children (The mother of this child was actually HIV-1 negative from her antenatal record card), and so it is not known how many of the eight HIV-1 exposed infants were actually HIV-1 infected. Therefore, with the current data set we cannot answer the question whether HIV-1 exposed but negative infants have a higher mortality rate than HIV-1 unexposed infants, but follow up studies may shed light on this as we track infants with HIV-1 positive mothers who we know were HIV-1 negative at 18 months and so were genuinely exposed to HIV-1 but uninfected in infancy.

Sample ID	Age at death (months)	Maternal HIV-1 status	Infant HIV-1 status	Final diagnosis
664	7	Positive	Unknown	Meningitis
743	16	Positive	Unknown	Acute diarrhoea
177	8	Positive	Unknown	Pneumonia
242	13	Positive	Unknown	Pneumonia
610	10	Positive	Unknown	Pneumonia
247	18	Positive	Unknown	Malaria
313	8	Positive	Unknown	Malaria
614	6	Positive	Unknown	Malaria
69	13	Negative	Unknown	Acute diarrhoea
672	8	Negative	Unknown	Pneumonia
746	6	Negative	Unknown	*Generalized con?*
70	9	Negative	HIV-1 positive	PEM (oedematous)

Table 20: CIGNIS infant mortalities

Age at death, maternal and infant HIV-1 status of the twelve infants who died during the course of the study showing final diagnosis. PEM = Protein Energy Malnutrition

We have established here that, compared with HIV-1 unexposed, HIV-1 exposed infants stop breast feeding earlier and are severely stunted with impaired growth. Whilst at 6 months, HIV-1 infected infants had significantly lower z-scores (compared with HIV-1 uninfected infants), by 18 months of age they had made up these differences and differed only in arm circumference and subscapular skinfold. For HIV-1 exposed infants however, all z-scores were significantly lower at 6 months, and by 18 months all but two were still significantly lower than for HIV-1 unexposed infants. Analysis of additive effects of HIV-1 exposure and breast feeding duration on developmental outcomes will be presented elsewhere by other members of the CIGNIS team.

5.3 Effects of betaherpesvirus active infection on markers of morbidity and growth

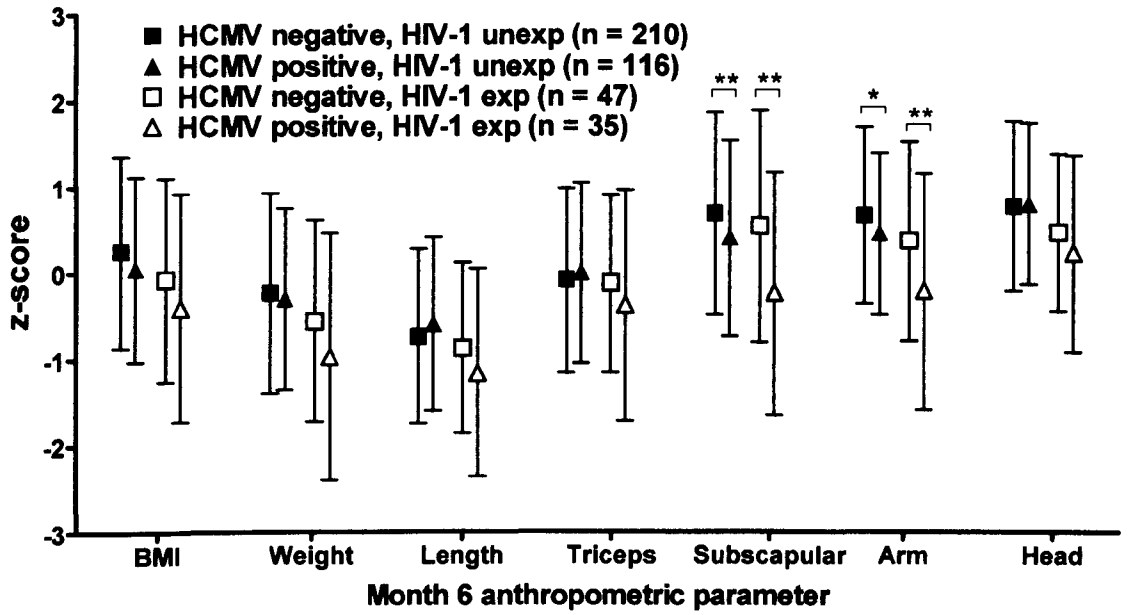
As shown in chapters 3 and 4, the epidemiology of both HCMV and HHV-6 appears to differ in Zambia as compared with Europe, North America and Japan. In these regions studies have shown that whilst congenital infection with HCMV is an important cause of morbidity (Alford et al., 1990; Ogawa et al., 2007), primary infection with HCMV during infancy is rare. Here we have found HCMV sera-DNA to be readily detectable in up to 40% of infants. For HHV-6, sera-DNA was less prevalent, possibly due to slower uptake or shorter periods of viraemia associated with primary infection or reactivation, but also partly due to the assay being less sensitive. Whilst HIV-1 exposure did no correlate with detection of betaherpesvirus sera-DNA, there was a trend for high load HCMV infections to be more commonly detected in HIV-1 exposed infants. We concluded in chapter 3 that whilst both HIV-1 exposed and unexposed infants are equally exposed to HCMV, those with HIV-1 positive mothers may be less capable of controlling either primary infection or reactivations, giving rise to transmission of high load viral infection to their children.

In this chapter HCMV and HHV-6 sera-DNA detection (indicative of active infection) are analysed for effects on markers of morbidity and growth in infants taking part in the CIGNIS study. No correlations were seen between active betaherpesvirus detection and either maternal education, socioeconomic status or duration of breast feeding (Table 22, Page 130). Anaemia was common in infants taking part on the CIGNIS study but did not differ with respect to detection of active betaherpesvirus infections (Table 22 and Table 26), suggesting active infection with these viruses does not affect haemoglobin levels. Furthermore no significant correlation was seen between prevalence of anaemia and betaherpesvirus loads (data not shown). Looking at possible interactions between the two betaherpesvirus, HCMV active infection at month 6 correlated with decreased prevalence of active HHV-6 infections at 18 months (Table 22). A possible interpretation of this is that early infection with HCMV may be indicative of earlier infection with HHV-6, and hence a decreased prevalence of active HHV-6 at the later time point, although HHV-6 detection at month 6 was unaffected. Broadly levels of detection of HHV-6 were lower, suggesting less persistent periods of viraemia during primary infection or less frequent reactivations. Conversely, active HHV-6 at month 6 correlated with high HCMV at month 18, suggesting those infants with early HHV-6 primary infections may have weakened immunity and hence more likely to shed HCMV at the later time point (Table 23, Page 131).

Active infections with HCMV or HHV-6 may affect anthropometric parameters, and so both month 6 and 18 month betaherpesvirus active infections were analysed for any effects on mean z-scores. At 6 months, BMI, subscapular skinfold and arm-circumference were significantly lower among infants with active HCMV infection than among those without (Table 22). These findings held when adjusted for maternal education, socioeconomic status and duration of breast feeding (Appendix H, section 9.8). The trend for lower weight in infants with an active HCMV infection did not hold when adjusted. Whilst the effect on arm circumference did persist to 18 months, detection at active infections at that time point did not correlate, suggesting that in older infants active HCMV infections cause less morbidity, possibly reflecting better developed immunity by 18 months (Table 23). These effects were not observed in older children, as HCMV active infection at 18 months did not correlate with any anthropometric outcomes (Table 23). The arm circumference of infants with active HCMV at 6 months was still significantly lower at 18 months but there was no other evidence to suggest that active HCMV infection at 6 months affected long term development, except a trend for increased referrals. Inversely, there was no evidence to suggest that impaired growth at 6 months was associated with active HCMV infection at 18 months.

Sub-stratifying by HIV-1 exposure showed greater decreases in 6 month subscapular skinfold and arm circumference among HIV-1 exposed than among HIV-1 unexposed infants, but the effect on BMI lost significance in these sub groups (Figure 25a)(Table 24, Page 132). Furthermore, active HCMV infection had a differential effect on length at 18 months: In HIV-1 unexposed infants, active HCMV infection was associated with increased length, but among HIV-1 exposed infants it was associated with impaired growth and >3-fold increase in the prevalence of stunting (Figure 25b)(Table 24). Among HIV-1 exposed infants detection of active HCMV infection at an early age was associated with some short and some long-term growth defects.

A)



B)

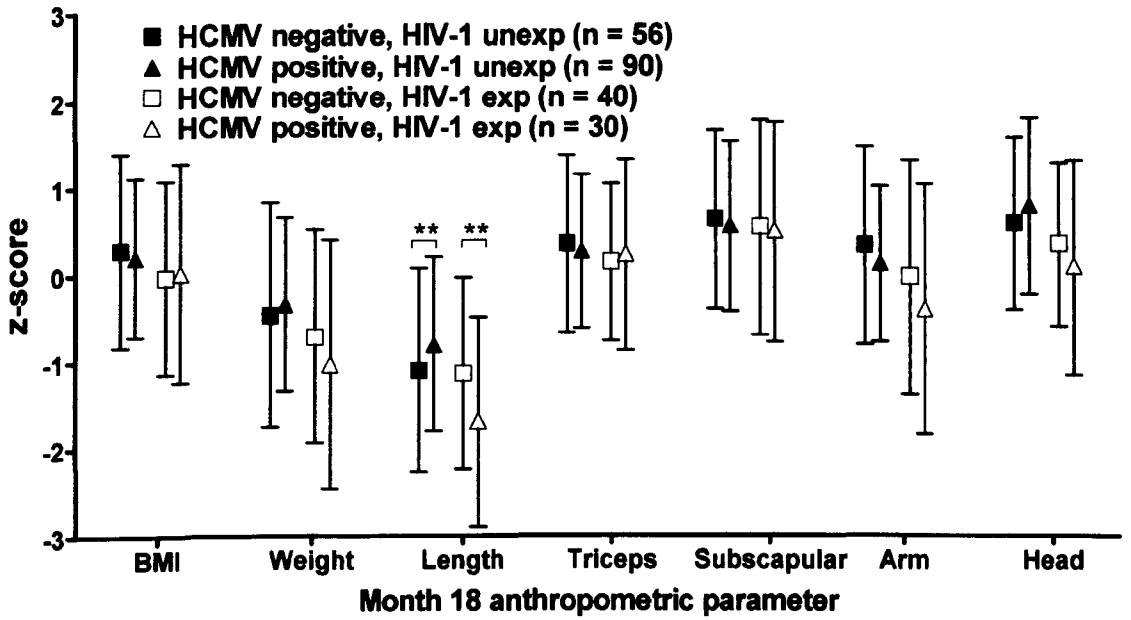
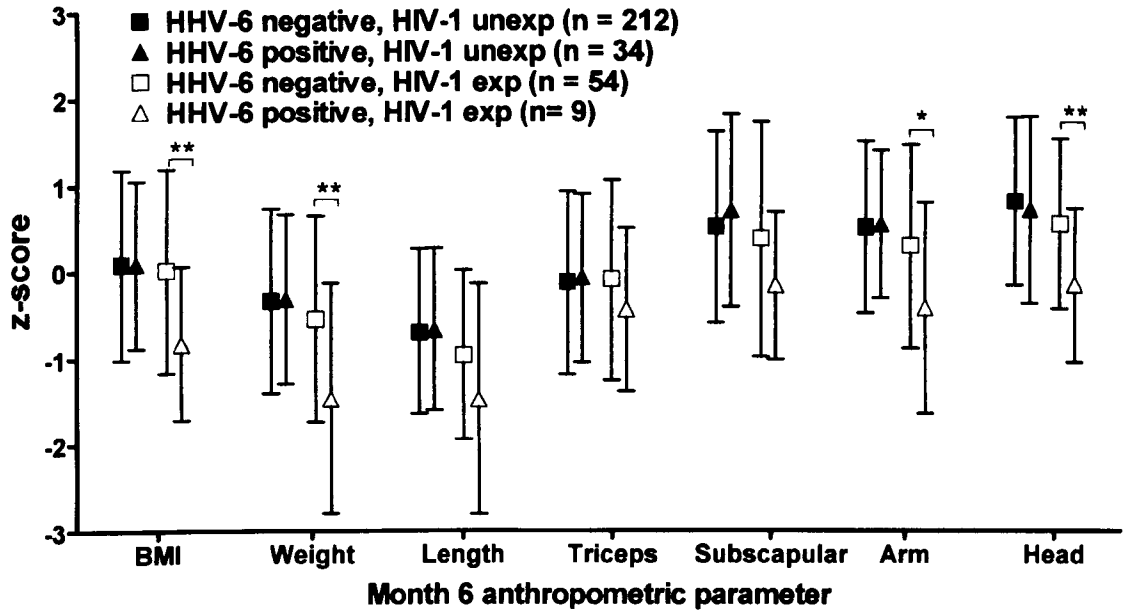


Figure 25: Effect of M6 HCMV active infection on anthropometry is greater in HIV-1 exposed infants

Anthropometric mean z-scores at a) 6 months and b) 18 months stratified by both HCMV active infection (at 6 months) and HIV-1 exposure. Bars represent 1 standard deviation from the mean. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005 by T-test. ND = not done, NA = not applicable

A)



B)

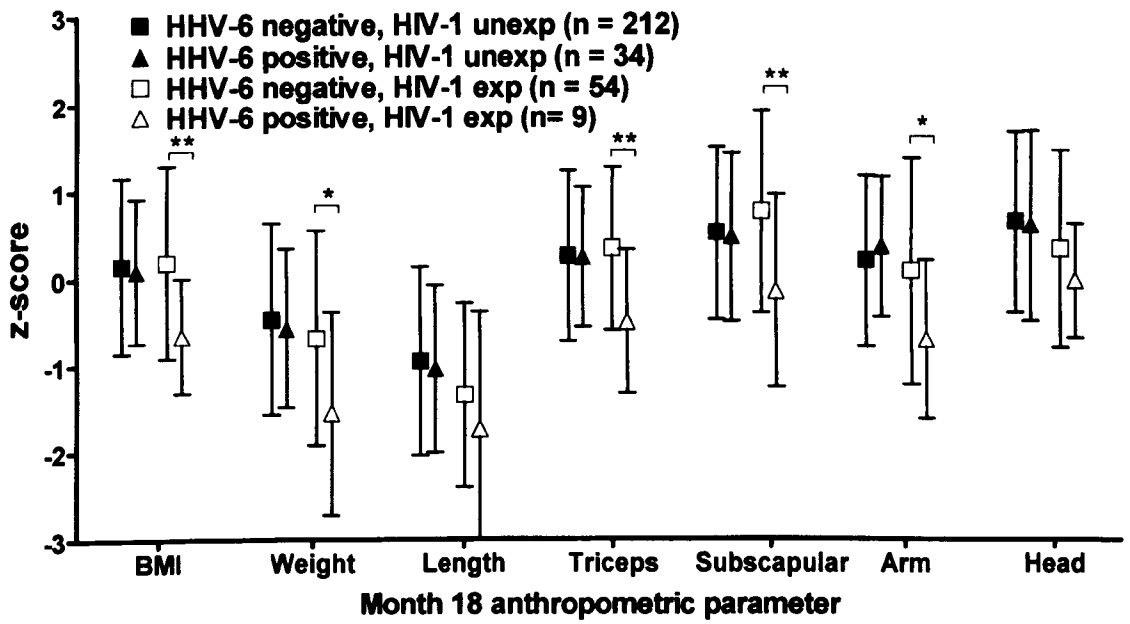


Figure 26: Effect of M18 HHV-6 active infection to impair growth among HIV-1 exposed infants only

Anthropometric mean z-scores at a) 6 months and b) 18 months stratified by both HHV-6 active infection (at 18 months) and HIV-1 exposure. Bars represent 1 standard deviation from the mean. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

Unlike for HCMV, active HHV-6 infections (at either time point) did not correlate with anthropometric baseline data or outcomes in the population as a whole (Table 25 and Table 26: Pages 133-134), but among HIV-1 exposed infants, active HHV-6 infection at month 18 was associated with wasting (Table 27, Page 135). This effect was significant for BMI, weight and head circumference at month 6 (Figure 26a) and BMI, triceps and subscapular skinfold at month 18 (Figure 26b), although at this latter time point there were only nine HIV-1 exposed infants who were HHV-6 positive and so power is low but the direction of the effect was the same for all parameters. Detection of HHV-6 at baseline did not show these effects on anthropometric parameters (at either time point) except triceps skinfold mean z-score was lower in HIV-1 exposed infants with an active HHV-6 infection at month 6. However there were only three HIV-1 exposed infants infected with HHV-6 at month 6 (Data not shown).

HCMV sera-DNA detection at 6 months was also analysed to see if these early active infections had an effect on hospital referral rates over the course of the study. Among the population as a whole, active HCMV at month 6 was linked with a trend for increased referrals (Appendix J, section 9.10.3) and adjusted analysis by the study statistician suggested this effect was primarily within HIV-1 exposed infants (Appendix J, section 9.10.4).

Of twelve children who died during the study, eight had HIV-1 positive mothers and HIV-1 infection was suspected (although not proven) by the medical officer in several of these cases. Four of these infants were screened for HCMV active infection at baseline, and three had active HCMV (Table 21). ID70 was HIV-1 positive (HIV-1 DNA PCR on admission – although mother was antenatally HIV-1 negative) and died of protein energy malnutrition 10 months after recruitment, and was also positive for HHV-6 active infection. ID313 was HIV-1 exposed and died from malaria 12 months after HCMV detection. The third child, ID743 was also HIV-1 exposed and died from acute diarrhoea, almost immediately after recruitment and detection of active HCMV. Histopathological data were not available but it is a possibility that this last child died with HCMV involvement in the gut as has been documented in HIV-1 positive infants elsewhere (Ukarapol et al., 2002; Zanolla et al., 2001).

Sample ID	Age at death (months)	Active HCMV month 6	Active HHV-6 month 6	Maternal HIV-1 status	Final diagnosis
664	13	NA	No	Positive	Meningitis
743	6	Yes	No	Positive	Acute diarrhoea
177	8	NA	NA	Positive	Pneumonia
242	13	No	No	Positive	Pneumonia
610	8	NA	No	Positive	Pneumonia
247	10	NA	NA	Positive	Malaria
313	18	Yes	No	Positive	Malaria
614	6	NA	No	Positive	Malaria
69	7	NA	NA	Negative	Acute diarrhoea
672	8	NA	No	Negative	Pneumonia
746	9	NA	NA	Negative	*Generalized con?*
70	16	Yes	Yes	Negative	PEM (oedematous)

Table 21: Active HCMV infection detected in 75% of infants who died

Age at death and maternal HIV-1 status along with HCMV and HHV-6 active infections in the twelve infants who died during the course of the study showing final diagnosis. PEM = Protein Energy Malnutrition, NA = sample was not available.

In summary, active HCMV infections at 6 months (but not at 18 months) were significantly associated with short term inhibitory effects on weight and peripheral fat. Among HIV-1 exposed infants early active infections and later active HHV-6 infections were associated with growth inhibition. Active HCMV (but not HHV-6) infection at month 6 showed a trend towards a higher rate of hospital referrals and was detected in 75% of infant mortalities.

	Category/ Month	HCMV sera-DNA detected at month 6				
		Negative		Positive		P
Demographics						
Gender	Male	48% (154/318)		45% (90/200)		.447
	Female	62% (164/318)		55% (110/200)		
Maternal Education	None	4% (13/318)		6% (12/200)		.518
	Primary	27% (87/318)		24% (48/200)		
	Secondary	37% (117/318)		42% (84/200)		
	Tertiary	29% (93/318)		27% (53/200)		
	University	3% (8/318)		2% (3/200)		
Socio-economic status	Low	33% (105/318)		32% (64/200)		.854
	Medium	38% (121/318)		41% (81/200)		
	High	29% (92/318)		28% (55/200)		
Breast feeding at baseline	Never	6% (19/318)		7% (14/200)		.124
	No	7% (21/318)		12% (23/200)		
	Yes	87% (278/318)		82% (163/200)		
Morbidity						
Polio Ab neutralization titre (12 months)	Un-protective	3% (7/229)		3% (4/160)		.581
	Low	9% (21/229)		12% (19/160)		
	Medium	62% (142/229)		65% (104/160)		
	High	16% (59/229)		21% (10/114)		
Fever	6	10% (33/318)		15% (29/200)		.159
	18	13% (30/235)		9% (13/149)		.221
Anaemic	6	42% (129/308)		40% (79/199)		.625
	18	29% (70/241)		28% (46/163)		.857
HHV-6 sera-DNA	6	7% (23/316)		11% (20/191)		.211
	18	17% (33/199)		8% (10/127)		.023**
HIV-1 infected	6	4% (9/245)		6% (10/166)		.265
HIV-1 exposed	18	20% (55/282)		24% (41/173)		.287
HCMV antibody	18	84% (158/189)		86% (100/116)		.540
Referral Rate		0.22		0.32		.080*
Died		0.3% (1/318)		1.5% (3/200)		.133
Growth						
Stunted	6	11% (32/293)		10% (17/172)		.725
	18	19% (43/224)		20% (28/138)		.799
		N =	Mean	N =	Mean	P
BMI-for-age	6	293	0.1533	171	-0.0973	0.023**
	18	224	0.1822	139	0.1081	0.516
Weight-for-age	6	293	-0.3179	172	-0.5103	0.091*
	18	225	-0.5196	139	-0.5807	0.640
Length/height-for-age	6	293	-0.7519	172	-0.7907	0.726
	18	224	-1.0745	139	-1.0778	0.978
Triceps skinfold-for-age	6	293	-0.1344	172	-0.1731	0.713
	18	225	0.2671	138	0.1632	0.316
Subscapular skinfold-for-age	6	293	0.6316	172	0.1956	0.000***
	18	225	0.5646	138	0.4325	0.251
Arm circumference-for-age	6	293	0.5724	172	0.2474	0.002***
	18	225	0.2355	138	-0.0397	0.024**
Head circumference-for-age	6	293	0.7010	172	0.6173	0.384
	18	225	0.5425	138	0.5554	0.906

Table 22: Active HCMV infection at month 6 correlates with lower BMI and lipodystrophy at month 6 and shows trends towards lower weight plus weakened humoral immunity

Table showing the distribution of infants by active HCMV infection (as determined by detection of HCMV DNA in sera by PCR) at month 6 with respect to maternal education (as surveyed on recruitment), socioeconomic status (determined by Asset Index Score), breast feeding category (Never = not breasf fed at baseline, No = breast feedings stopped before 6 months and Yes = breast feeding at and beyond 6 months), Polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and b) anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

	Category/ month	HCMV sera-DNA detected at month 18				
		Negative		Positive		P
Demographics						
Gender	Male	49% (128/264)		49% (65/133)		.942
	Female	52% (136/264)		51% (68/133)		
Maternal Education	None	4% (10/264)		3% (4/133)		.526
	Primary	27% (72/264)		22% (29/133)		
	Secondary	38% (99/264)		46% (61/133)		
	Tertiary	30% (79/264)		29% (38/133)		
	University	2% (4/264)		1% (1/133)		
Socio-economic status	Low	35% (91/264)		24% (32/133)		.096*
	Medium	37% (98/264)		45% (60/133)		
	High	28% (75/264)		31% (41/133)		
Breast feeding at baseline	Never	7% (18/264)		5% (6/133)		.658
	No	8% (22/264)		8% (11/133)		
	Yes	85% (224/264)		87% (116/133)		
Morbidity						
Polio Ab neutralization titre (12 months)	Un-protective	2% (5/254)		3% (4/131)		.346
	Low	10% (25/254)		10% (13/131)		
	Medium	59% (151/254)		66% (87/131)		
	High	29% (73/254)		21% 27/131)		
Fever	6	10% (27/264)		11% (14/133)		.926
	18	12% (30/255)		12% (14/118)		.978
Anaemic	6	38% (99/258)		38% (51/133)		.996
	18	30% (79/261)		33% (43/132)		.641
HHV-6 sera-DNA	6	7% (14/215)		15% (16/110)		.018**
	18	12% (31/264)		15% (20/132)		.340
HIV-1 infected	6	3% (8/263)		4% (5/133)		.705
HIV-1 exposed	18	24% (56/235)		19% (22/117)		.285
HCMV antibody	18	85% (161/190)		83% (90/109)		.623
Referral Rate		ND		ND		ND
Died		0% (0/264)		0% (0/133)		NA
Growth						
Stunted	6	11% (25/229)		10% (12/121)		.772
	18	17% (38/226)		24% (29/121)		.108
		N =	Mean	N =	Mean	P
BMI-for-age	6	228	0.0419	121	-0.0454	0.499
	18	226	0.1081	121	0.0599	0.665
Weight-for-age	6	228	-0.4431	121	-0.4994	0.654
	18	227	-0.5718	121	-0.6226	0.683
Length/height-for-age	6	229	-0.8141	121	-0.7902	0.853
	18	226	-1.0732	121	-1.0875	0.908
Triceps skinfold-for-age	6	229	-0.1810	121	-0.1235	0.632
	18	227	0.2093	121	0.1757	0.749
Subscapular skinfold-for-age	6	229	0.5374	121	0.3389	0.128
	18	227	0.4599	121	0.5201	0.602
Arm circumference-for-age	6	229	0.4428	121	0.3480	0.420
	18	227	0.1814	121	0.0660	0.318
Head circumference-for-age	6	229	0.6818	121	0.6624	0.861
	18	227	0.5395	121	0.5288	0.927

Table 23: Active HCMV infection at month 18 shows trends towards lower polio vaccine responses and higher prevalence of HHV-6 at month 6

Table showing the distribution of infants by active HCMV infection (as determined by detection of HCMV DNA in sera by PCR) at month 6 with respect to maternal education (as surveyed on recruitment), socioeconomic status (determined by Asset Index Score), breast feeding category (Never = not breasf fed at baseline, No = breast feedings stopped before 6 months and Yes = breast feeding at and beyond 6 months), Polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and b) anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

HIV-1 unexposed	Category/ month	HCMV sera-DNA detected at month 6					HIV-1 exposed	Category/ month	HCMV sera-DNA detected at month 6				
		Negative		Positive		P			Negative		Positive		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protective	0% (0/159)		2% (2/103)		.291	Polio Ab neutralization titre (12 months)	Un-protective	5% (2/43)		0% (0/34)		.558
	Low	9% (15/159)		9% (9/103)				Low	12% (5/43)		12% (4/34)		
	Medium	62% (98/159)		65% (67/103)				Medium	72% (31/43)		71% (24/34)		
	High	29% (46/159)		25% (25/103)				High	12% (5/43)		18% (6/34)		
Fever	6	10% (22/227)		11% (15/132)		.615	Fever	6	11% (6/55)		20% (8/41)		.237
	18	15% (24/162)		9% (9/100)		.168		18	5% (2/44)		11% (3/28)		.315
Anaemic	6	43% (95/221)		35% (46/132)		.131	Anaemic	6	42% (22/53)		40% (16/40)		.883
	18	29% (49/167)		27% (28/104)		.668		18	28% (13/46)		25% (9/36)		.741
HCMV sera-DNA	6	NA		NA		NA	HCMV sera-DNA	6	NA		NA		NA
	18	27% (37/137)		46% (39/84)		.003***		18	23% (9/39)		38% (9/24)		.218
HCMV antibody	18	83% (112/135)		88% (65/74)		.349	HCMV antibody	18	86% (30/35)		80% (20/50)		.558
HHV-6 sera-DNA	6	8% (17/227)		12% (15/126)		.166	HHV-6 sera-DNA	6	7% (4/55)		8% (3/39)		.939
	18	15% (20/137)		11% (9/85)		.389		18	21% (8/39)		0% (0/24)		.018**
Referral Rate		0.19		0.27		.184	Referral Rate		0.38		0.54		.332
Died		0% (0/227)		0% (0/132)		NA	Died		2% (1/55)		5% (2/41)		.394
Development													
Stunted	6	11% (22/210)		7% (8/116)		.284	Stunted	6	13% (6/47)		23% (8/35)		.230
	18	21% (33/156)		12% (11/90)		.078*		18	15% (6/40)		43% (13/30)		.008**
		Negative		Positive					Negative		Positive		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	210	0.2510	116	0.0484	0.112	BMI-for-age	6	47	-0.0787	35	-0.4040	.243
	18	156	0.2814	90	0.2012	0.561		18	40	-0.0338	30	0.0147	.865
Weight-for-age	6	210	-0.2380	116	-0.3060	0.599	Weight-for-age	6	47	-0.5621	35	-0.9751	.153
	18	156	-0.4687	90	-0.3443	0.432		18	40	-0.7168	30	-1.0357	.319
Length/height-for-age	6	210	-0.7402	116	-0.5966	0.217	Length/height-for-age	6	47	-0.8757	35	-1.1620	.240
	18	156	-1.1047	90	-0.8062	0.044**		18	40	-1.1403	30	-1.6953	.049**
Triceps skinfold-for-age	6	210	-0.0906	116	-0.0066	0.493	Triceps skinfold-for-age	6	47	-0.1304	35	-0.3809	.340
	18	156	0.3502	90	0.2629	0.498		18	40	0.1353	30	0.2243	.710
Subscapular skinfold-for-age	6	210	0.6883	116	0.4007	0.033**	Subscapular skinfold-for-age	6	47	0.5415	35	-0.2403	.013**
	18	156	0.6312	90	0.5543	0.567		18	40	0.5433	30	0.4943	.872
Arm circumference-for-age	6	210	0.6633	116	0.4534	0.071*	Arm circumference-for-age	6	47	0.3677	35	-0.2157	.040**
	18	156	0.3384	90	0.1257	0.129		18	40	-0.0247	30	-0.3950	.273
Head circumference-for-age	6	210	0.7714	116	0.7973	0.818	Head circumference-for-age	6	47	0.4689	35	0.2246	.288
	18	156	0.5962	90	0.7989	0.129		18	40	0.3533	30	0.0927	.319

Table 24: Active HCMV at month 6 effects length differentially by HIV-1 exposure

Distribution of active HCMV in HIV-1 unexposed and exposed infants with respect to polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of development. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

	Category/ month	HHV-6 sera-DNA detected at month 6				
		Negative		Positive		P
Demographics						
Gender	Male	49% (278/564)		40% (19/47)		.243
	Female	51% (286/564)		60% (28/47)		
Maternal Education	None	6% (31/564)		2% (1/47)		.343
	Primary	29% (164/564)		26% (12/47)		
	Secondary	38% (214/564)		32% (15/47)		
	Tertiary	26% (144/564)		36% (17/47)		
	University	2% (11/564)		4% (2/47)		
Socio-economic status	Low	33% (186/564)		42% (20/47)		.348
	Medium	38% (215/564)		36% (17/47)		
	High	29% (163/564)		21% (10/47)		
Breast feeding at baseline	Never	6% (31/564)		9% (4/47)		.547
	No	10% (55/564)		6% (3/47)		
	Yes	85% (478/518)		85% (40/47)		
Morbidity						
Polio Ab neutralization titre (12 months)	Un-protective	2% (10/417)		3% (1/36)		.282
	Low	9% (38/417)		17% (6/36)		
	Medium	65% (271/417)		50% (18/36)		
	High	24% (98/417)		11% (11/36)		
Fever	6	12% (70/564)		9% (4/47)		.431
	18	11% (41/370)		8% (3/40)		.487
Anaemic	6	41% (229/555)		42% (19/45)		.900
	18	31% (132/432)		33% (13/39)		.719
HCMV sera-DNA	6	37% (171/464)		47% (20/43)		.211
	18	32% (94/295)		53% (16/30)		**.018****
HIV-1 infected		4% (16/439)		7% (3/41)		.249
HIV-1 exposed		22% (110/500)		16.3% (7/43)		.381
HCMV antibody	18	87% (287/331)		75% (18/24)		.111
Referral Rate		ND		ND		ND
Died		1.2% (7/564)		2.1% (1/47)		.607
Growth						
Stunted	6	11% (58/527)		13% (4/31)		.744
	18	20% (80/393)		17% (4/24)		.662
		N =	Mean	N =	Mean	P
BMI-for-age	6	526	0.0771	31	-0.1542	.297
	18	393	0.1184	24	0.1413	.919
Weight-for-age	6	526	-0.4137	31	-0.4994	.703
	18	396	-0.5824	24	-0.4113	.503
Length/height-for-age	6	527	-0.8128	31	-0.6719	.514
	18	393	-1.1115	24	-0.8338	.261
Triceps skinfold-for-age	6	527	-0.1762	31	-0.0326	.474
	18	395	0.1934	24	0.3054	.569
Subscapular skinfold-for-age	6	527	0.4368	31	0.4023	.881
	18	395	0.4899	24	0.7346	.260
Arm circumference-for-age	6	527	0.3668	31	0.2213	.490
	18	395	0.0573	24	0.1596	.665
Head circumference-for-age	6	527	0.6552	31	0.6061	.796
	18	395	0.5286	24	0.5275	.996

Table 25: Active HHV-6 infection at 6 months correlates with higher HCMV sera-DNA detection at 18 months

Table showing the distribution of infants by HHV-6 sera-DNA detection at 6 months with respect to maternal education (as surveyed on recruitment), socioeconomic status (determined by Asset Index Score), breast feeding category (Never = not breasf fed at baseline, No = breast feedings stopped before 6 months and Yes = breast feeding at and beyond 6 months), Polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and b) anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

	Category/ month	HHV-6 sera-DNA detected at month 18				
		Negative		Positive		P
Demographics						
Gender	Male	48% (167/347)		52% (27/52)		.610
	Female	52% (180/347)		48% (25/52)		
Maternal Education	None	4% (12/347)		8% (4/52)		.293
	Primary	25% (87/347)		25% (13/52)		
	Secondary	39% (136/347)		46% (24/52)		
	Tertiary	31% (108/347)		19% (10/52)		
	University	1% (4/347)		2% (1/52)		
Socio-economic status	Low	31% (107/347)		37% (19/52)		.664
	Medium	40% (137/347)		39% (20/52)		
	High	30% (103/347)		25% (13/52)		
Breast feeding at baseline	Never	6% (20/347)		8% (4/52)		.692
	No	9% (30/347)		6% (3/45)		
	Yes	86% (297/347)		87% (45/52)		
Morbidity						
Polio Ab neutralization titre (12 months)	Un-protective	2% (8/336)		2% (1/50)		.219
	Low	11% (36/336)		21%)		
	Medium	63% (210/336)		71% (10/14)		
	High	24% (82/336)		7% (1/14)		
Fever	6	11% (37/347)		8% (4/52)		.511
	18	12% (39/325)		10% (5/50)		.682
Anaemic	6	38% (131/341)		40% (21/52)		.786
	18	31% (105/344)		36% (18/50)		.435
HCMV sera-DNA	6	41% (117/283)		23% (10/43)		**.023****
	18	33% (112/345)		39% (20/51)		.340
HIV-1 infected		4% (12/347)		4% (2/51)		.867
HIV-1 exposed		22% (68/307)		22% (10/46)		.950
HCMV antibody	18	83% (213/257)		91% (39/43)		.196
Referral Rate		ND		ND		ND
Died		0% (0/347)		1.9% (0/51)		NA
Growth						
Stunted	6	11% (32/303)		10% (5/49)		.940
	18	19% (57/301)		23% (11/47)		.473
		N =	Mean	N =	Mean	P
BMI-for-age	6	302	0.0275	49	-0.1214	.401
	18	301	0.1089	47	-0.0249	.387
Weight-for-age	6	302	-0.4532	49	-0.5578	.544
	18	302	-0.5636	47	-0.7574	.263
Length/height-for-age	6	303	-0.8083	49	-0.8110	.987
	18	301	-1.0573	47	-1.2113	.370
Triceps skinfold-for-age	6	303	-0.1606	49	-0.1608	.999
	18	302	0.2212	47	0.0760	.321
Subscapular skinfold-for-age	6	303	0.4502	49	0.5292	.660
	18	302	0.4969	47	0.3623	.405
Arm circumference-for-age	6	303	0.4099	49	0.3710	.810
	18	302	0.1390	47	0.1483	.954
Head circumference-for-age	6	303	0.7065	49	0.4865	.148
	18	302	0.5461	47	0.4917	.736

Table 26: Active HHV-6 infection at 18 months correlates with decreased prevalence of HCMV infection at 6 months

Table showing the distribution of infants by HHV-6 sera-DNA detection at 18 months with respect to maternal education (as surveyed on recruitment), socioeconomic status (determined by Asset Index Score), breast feeding category (Never = not breasf fed at baseline, No = breast feedings stopped before 6 months and Yes = breast feeding at and beyond 6 months), Polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and b) anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

HIV-1 unexposed	Category/ month	HHV-6 sera-DNA detected at month 18					HIV-1 exposed	Category/ month	HHV-6 sera-detection at month 18				
		Negative		Positive		P			Negative		Positive		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protective	1% (3/232)		0% (0/36)		.378	Polio Ab neutralization titre (12 months)	Un-protective	3% (2/65)		0% (0/10)		.808
	Low	10% (22/232)		3% (1/36)				Low	12% (8/65)		10% (1/10)		
	Medium	63% (145/232)		61% (22/36)				Medium	66% (43/65)		60% (6/10)		
	High	27% (62/232)		36% (13/36)				High	19% (12/65)		30% (3.10)		
Fever	6	10% (24/239)		11% (4/36)		.771^	Fever	6	12% (8/68)		0% (0/10)		.587^
	18	13% (29/226)		14% (5/36)		.793^		18	8% (5/63)		0% (0/9)		1.0^
Anaemic	6	37% (87/236)		39% (14/36)		.815	Anaemic	6	38% (25/66)		40% (4/10)		1.0^
	18	29% (69/237)		33% (12/36)		.606		18	31% (21/68)		50% (5/10)		.287
HCMV sera-DNA	6	39% (76/193)		31% (9/29)		.389	HCMV sera-DNA	6	44% (24/55)		0% (0/8)		**.020^****
	18	34% (80/237)		39% (14/36)		.546		18	25% (17/68)		50% (5/10)		.134
HCMV antibody	18	83% (145/174)		88% (29/33)		.513	HCMV antibody	18	83% (44/53)		100% (7/7)		.580
HHV-6 sera-DNA	6	NA		NA		NA	HHV-6 sera-DNA	6	11% (6/53)		0% (0/8)		1.0^
	18	10% (19/198)		10% (3/29)		1.0^		18	NA		NA		NA
Referral Rate		ND		ND		ND	Referral Rate		ND		ND		ND
Died		0% (0/239)		0% (0/36)		NA	Died		0% (0/68)		0% (0/10)		NA
Development													
Stunted	6	9% (18/213)		6% (2/34)		1.0^	Stunted	6	15% (8/54)		33% (3/9)		.184^
	18	16% (34/212)		21% (7/34)		.509		18	28% (15/54)		33% (3/9)		.707^
		Negative		Positive					Negative		Positive		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	212	0.0844	34	0.0906	0.975	BMI-for-age	6	54	0.0128	9	-0.8289	**0.045****
	18	212	0.1489	34	0.0853	0.727		18	54	0.1843	9	-0.6633	**0.030****
Weight-for-age	6	212	-0.3399	34	-0.3174	0.908	Weight-for-age	6	54	-0.5483	9	-1.4689	**0.038****
	18	212	-0.4741	34	-0.5741	0.617		18	54	-0.6922	9	-1.5567	**0.055***
Length/height-for-age	6	213	-0.6940	34	-0.6688	0.885	Length/height-for-age	6	54	-0.9643	9	-1.4756	0.174
	18	212	-0.9563	34	-1.0441	0.657		18	54	-1.3461	9	-1.7489	0.318
Triceps skinfold-for-age	6	213	-0.1253	34	-0.0718	0.782	Triceps skinfold-for-age	6	54	-0.0943	9	-0.4389	0.401
	18	212	0.2589	34	0.2429	0.928		18	54	0.3413	9	-0.5044	**0.013****
Subscapular skinfold-for-age	6	213	0.5193	34	0.7106	0.351	Subscapular skinfold-for-age	6	54	0.3815	9	-0.1578	0.254
	18	212	0.5208	34	0.4759	0.806		18	54	0.7648	9	-0.1433	**0.033****
Arm circumference-for-age	6	213	0.5203	34	0.5541	0.852	Arm circumference-for-age	6	54	0.3007	9	-0.4167	**0.097***
	18	212	0.2077	34	0.3700	0.363		18	54	0.0889	9	-0.6978	**0.087***
Head circumference-for-age	6	213	0.8186	34	0.7218	0.598	Head circumference-for-age	6	54	0.5580	9	-0.1556	**0.046****
	18	212	0.6561	34	0.6135	0.826		18	54	0.3424	9	-0.0222	0.354

Table 27: Active HHV-6 at month 18 correlates with wasting in HIV-1 exposed infants

Distribution of active HCMV in HIV-1 unexposed and exposed infants with respect to polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of development. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005

5.4 Effects of HCMV seroprevalence on markers of morbidity and growth

The previous section has investigated correlations between detection of active betaherpesvirus infections with markers of morbidity and growth. Detection of active infection is not representative of overall exposure however, as our analysis represents just two 'snap shots' in infants at 6 and 18 months of age. In this section, results of a screen for HCMV antibody in 18 month old infants on the CIGNIS study will be analysed with respect to morbidity and anthropometric data.

Detection of HCMV antibody at 18 months correlated significantly with both maternal education and socioeconomic status (Table 28, Page 142). HCMV seroprevalence among infants born to university educated mothers was as low as 55% (Figure 27a) and among infants from the high socioeconomic group seroprevalence was 15% lower than among infants from lower or medium socioeconomic groups (Figure 27b). Sub-stratification by maternal HIV-1 status showed no significant differences in HCMV seroprevalence between HIV-1 exposed and unexposed infants, maternal education category or socioeconomic grouping (data not shown). There was also a significant correlation between HCMV transmission as measured by seroprevalence, and duration of breast feeding (Table 28).

There was a trend for non-protective or low polio neutralizing antibody titres to be less prevalent among HCMV seronegative infants, but only among those who were HIV-1 unexposed, possibly due to the association of HIV-1 exposure and weakened vaccine responses being stronger. Interestingly, the two HCMV seronegative, HIV-1 unexposed infants who failed to mount a protective polio response, were both positive for HCMV sera-DNA at 6 months of age showing that they had been exposed to HCMV but had not seroconverted (Table 29, Page 143).

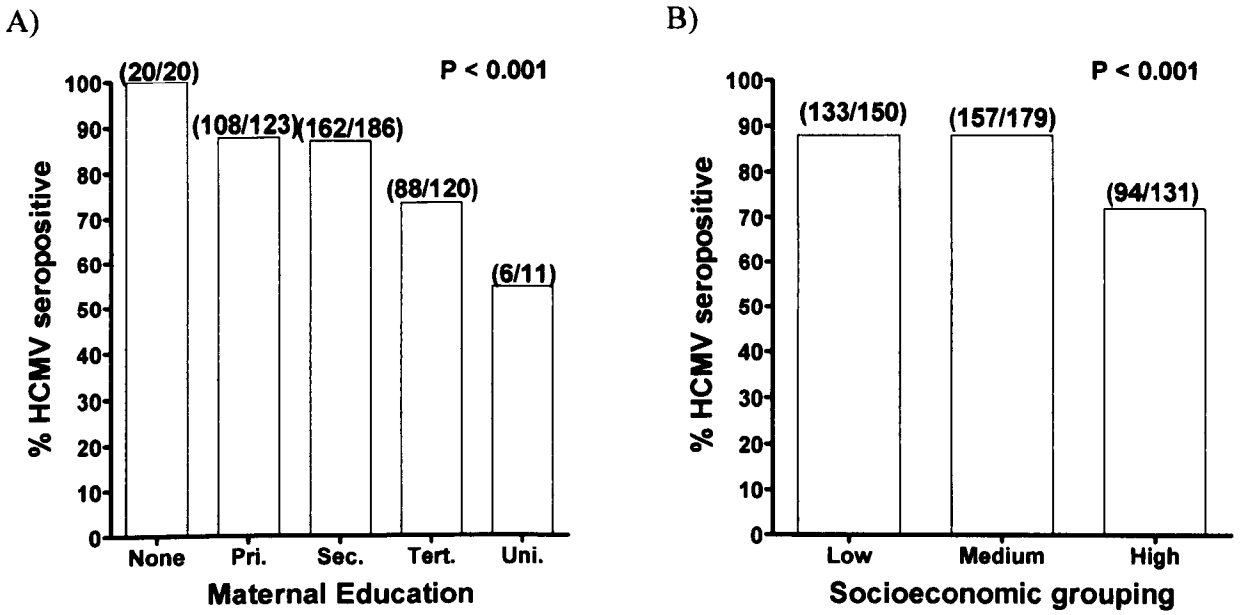


Figure 27: High maternal education and socioeconomic grouping are associated with lower HCMV seroprevalence

Prevalence of HCMV seropositive infants by a) level of maternal education and b) socioeconomic grouping. Significance is by Pearson Chi-Squared.

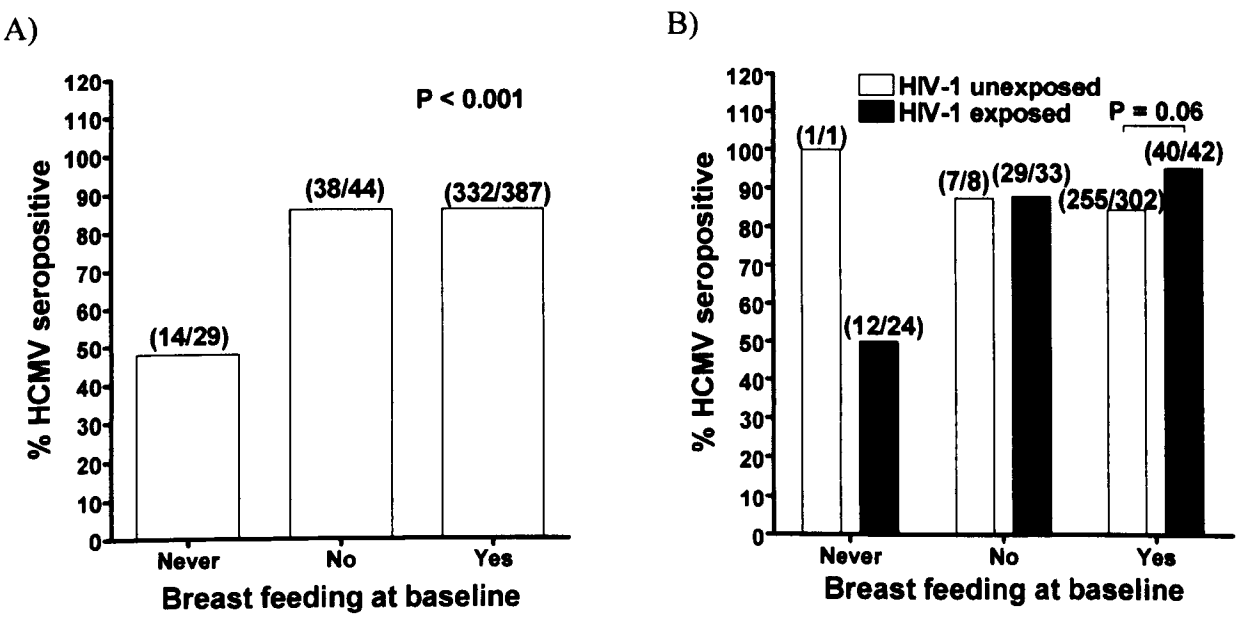


Figure 28: HCMV seroprevalence is higher in infants who have been breast fed

Histograms showing HCMV seroprevalence by a) breast feeding grouping alone and b) breast feeding grouping and HIV-1 exposure. Mother's were asked at recruitment (when infants were 6 months old) about their breast history: Never = not breast fed at all from birth, No = breast fed < 6 months but stopped before baseline, Yes = currently breast feeding at baseline. Significance is by Pearson Chi-Squared.

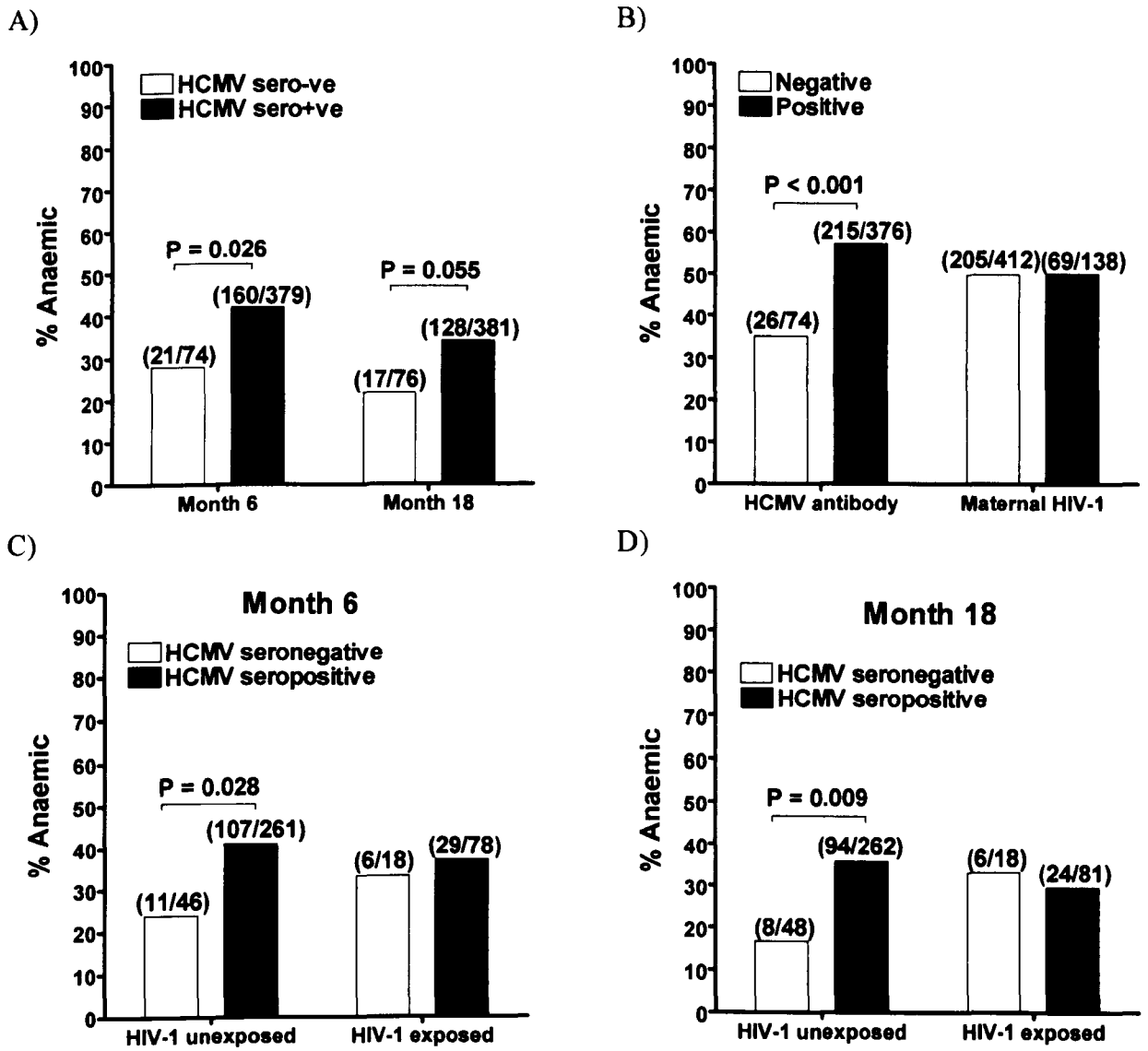


Figure 29: HCMV seroprevalence linked to increased anaemia but not among HIV-1 exposed infants

Prevalence of anaemia by a) HCMV seroprevalence and b) HCMV seroprevalence and HIV-1 exposure, combining both time points to define those anaemic at one or both. Additive effects of HCMV seroprevalence and maternal HIV-1 status on the prevalence of anaemia at both month 6 (c) and month 18 (d). Anaemia here is defined by a haemoglobin level of less than 10.5 g/L. Significance is by Pearson Chi-Squared.

HCMV seropositive infants were significantly more likely to be anaemic at 6 months of age, with this persisting as a strong trend at 18 months of age (Figure 29a)(Table 28). When grouping together infants who were anaemic at either or both time points, verses those who were not anaemic at either time point, HCMV seropositive infants were nearly twice as likely to be anaemic whereas the prevalence of anaemia across both time points was completely unaffected by maternal HIV-1 status (Figure 29b) as suggest previously (Figure 24b). Additive effects on the prevalence of anaemia were investigated by combining HCMV serology, with maternal HIV-1 data. Interestingly, significant increases in prevalence of anaemia (at both 6 and 18 months) due to infection with HCMV are seen only in HIV-1 unexposed infants (Figure 29c and d). The prevalence of anaemia in HIV-1 exposed infants was unaffected by HCMV seroprevalence suggesting that whilst overall HIV-1 exposure did not affect anaemia (Figure 24b), for the minority of infants who were HCMV seronegative, there was a trend for HIV-1 exposure to be linked with higher prevalence of anaemia, but within the low numbers of HCMV seronegative infants the difference was not significant.

We then looked at the effects of HCMV seroprevalence on anthropometric data (Table 28). Looking at infant growth measurements taken at 6 months of age, being HCMV seropositive at 18 months was associated with a trend towards lower triceps skinfold but this was not supported by trends towards decreases in any other parameter at baseline. By 18 months however, triceps skinfold did not differ by HCMV seroprevalence, but being HCMV seropositive was now significantly associated with lower length/height, lower weight, and a smaller head circumference. This effect on length held when adjusted for maternal education, socioeconomic status and duration of breast feeding (P = 0.015)(Appendix I, section 9.9.1) although the effect on weight and head circumference did not (Appendix I, sections 9.9.2 and 9.9.3). Concurrently, stunting was twice as prevalent in HCMV seropositive infants (22% (82/371) vs 11% (8/73) P = 0.03)(Table 28)(Figure 30a) and there was a trend for this effect to be greater within HIV-1 exposed infants (32% (25/77) vs 11% (2/18) P = 0.071) (Figure 30b). Whilst effects of HCMV seroprevalence on length at 18 months are significant in both HIV-1 exposed and unexposed infants, the correlation with reduced head circumference was observed exclusively among HIV-1 exposed infants (Figure 31)(Table 29).

Finally, HCMV seroprevalence did not have a significant effect of rate of hospital referrals (Table 28)(Appendix J, section 9.10.5). In summary, infants who were HCMV seropositive at 18 months of age were persistently anaemic and were significantly more likely to be stunted by 18 months and if HIV-1 exposed, have a lower head circumference.

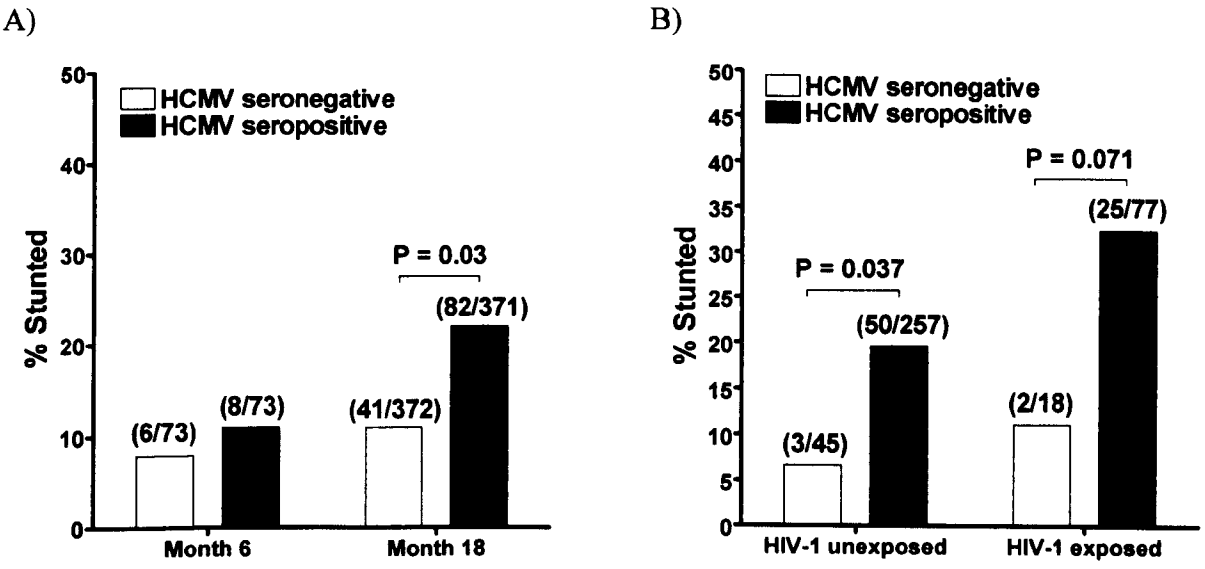
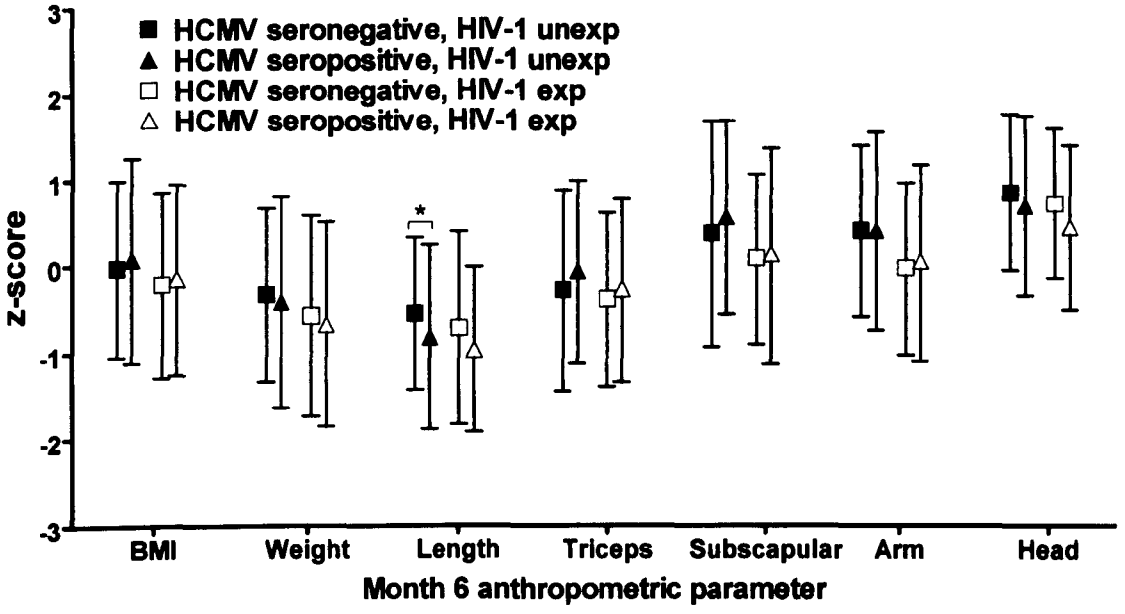


Figure 30: HCMV seroprevalence correlates with stunting with a trend for this to be exaggerated among HIV-1 exposed infants

Prevalence of stunting at a) both 6 and 18 months by HCMV seroprevalence and b) at 18 months alone sub-stratified by HIV-1 exposure. Significance is by Pearson Chi-Squared.

A



B

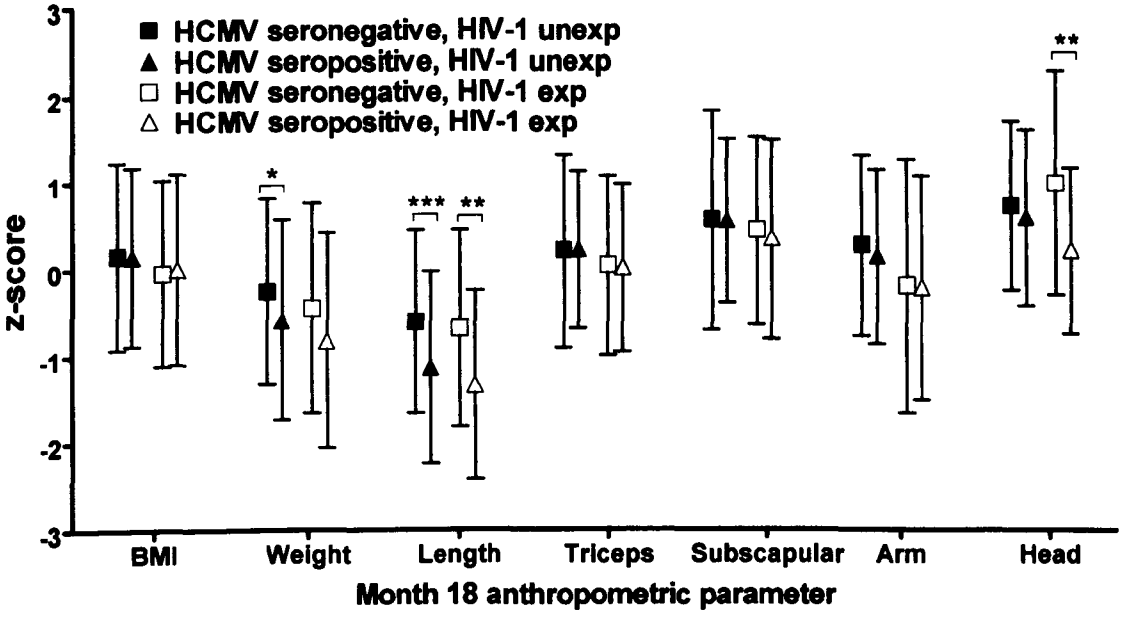


Figure 31: HCMV seropositive infants have a smaller head circumference only if HIV-1 exposed

Anthropometry mean z-scores at 18 months by HCMV antibody detection at 18 months sub-stratifying by HIV-1 exposure. Bars represent 1 standard deviation from the mean. Mean z-scores were compared by independent sample T test assuming equal variance. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

	Category/ month	HCMV sera-antibody detection at month 18				
		HCMV seronegative		HCMV seropositive		P
Demographics						
Gender	Male	45% (34/76)		47% (180/384)		.733
	Female	55% (42/76)		53% (204/384)		
Maternal Education	None	0% (0/76)		5% (20/384)		<.001***
	Primary	20% (15/76)		28% (108/384)		
	Secondary	32% (24/76)		42% (162/384)		
	Tertiary	42% (32/76)		23% (88/384)		
	University	7% (5/76)		2% (6/384)		
Socio-economic status	Low	22% (17/76)		35% (133/384)		<.001***
	Medium	29% (22/76)		41% (157/384)		
	High	49% (37/76)		25% (94/384)		
Breast feeding at baseline	Never	20% (15/76)		4% (14/384)		<.001***
	No	8% (6/76)		10% (38/384)		
	Yes	72% (55/76)		87% (332/384)		
Morbidity						
Polio Ab neutralization titre (12 months)	Un-protective	4% (3/75)		1% (5/369)		.444
	Low	9% (7/75)		8% (30/369)		
	Medium	63% (47/75)		64% (237/369)		
	High	24% (18/75)		26% (97/369)		
Fever	6	15% (11/76)		13% (49/384)		.685
	18	7% (4/59)		14% (42/294)		.118
Anaemic	6	28% (21/74)		42% (160/379)		.026**
	18	22% (17/76)		34% (128/381)		.055*
HCMV sera-DNA	6	34% (16/47)		39% (100/258)		.540
	18	40% (19/48)		36% (90/251)		.623
HHV-6 sera-DNA	6	12% (6/50)		6% (18/305)		.111
	18	8% (4/48)		16% (39/252)		.196
HIV-1 infected		3% (2/76)		2% (7/383)		.644
HIV-1 exposed		27% (18/66)		24% (81/344)		.517
Referral Rate		0.18		0.26		.334
Died		NA		NA		NA
Growth						
Stunted	6	8% (6/73)		11% (41/372)		.476
	18	11% (8/73)		22% (82/371)		.030**
		HCMV seronegative		HCMV seropositive		
		N =	Mean	N =	Mean	P
BMI-for-age	6	73	-0.0718	373	-0.0227	.741
	18	73	0.1229	371	0.0934	.823
Weight-for-age	6	73	-0.4304	373	-0.4989	.654
	18	73	-0.3392	372	-0.6482	.035**
Length/height-for-age	6	73	-0.6553	372	-0.8268	.188
	18	73	-0.7033	371	-1.1756	<.001***
Triceps skinfold-for-age	6	73	-0.3830	373	-0.1417	.079*
	18	70	0.1244	363	0.1325	.947
Subscapular skinfold-for-age	6	73	0.2474	373	0.4339	.222
	18	70	0.5209	363	0.4608	.653
Arm circumference-for-age	6	73	0.2579	373	0.3079	.730
	18	69	0.0828	363	0.0451	.790
Head circumference-for-age	6	73	0.8300	373	0.6193	.102
	18	70	0.8061	363	0.4882	.015**

Table 28: HCMV seroprevalence is associated with persistent anaemia and stunting and reduced head circumference at 18 months

Distribution of infants by HCMV seroprevalence (determined by ELISA at 18 months) with respect to maternal education (as surveyed on recruitment), socioeconomic status (determined by Asset Index Score), breast feeding category (Never = not breasf fed at baseline, No = breast feedings stopped before 6 months and Yes = breast feeding at and beyond 6 months), Polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

HIV-1 unexposed	Category/ month	HCMV sera-Ab detection at month 18					HIV-1 exposed	Category/ Month	HCMV sera-Ab detection at month 18				
		Negative		Positive		P			Negative		Positive		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protective	4% (2/47)		0.4% (1/255)		.073*	Polio Ab neutralization titre (12 months)	Un-protective	6% (1/18)		3% (2/76)		.880
	Low	11% (5/47)		8% (19/255)				Low	6% (1/18)		9% (7/76)		
	Medium	62% (29/47)		63% (160/255)				Medium	67% (12/18)		68% (52/76)		
	High	23% (11/47)		29% (75/255)				High	22% (4/18)		20% (15/76)		
Fever	6	15% (7/48)		23% (33/263)		.698	Fever	6	17% (3/18)		9% (7/81)		.307
	18	8% (3/37)		15% (30/205)		.287		18	7% (1/14)		10% (6/61)		.755
Anaemic	6	24% (11/46)		41% (107/261)		.028**	Anaemic	6	33% (6/18)		37% (29/78)		.760
	18	17% (8/48)		36% (94/262)		.009**		18	33% (6/18)		30% (24/81)		.757
HCMV sera-DNA	6	28% (9/32)		37% (65/177)		.349	HCMV sera-DNA	6	50% (5/10)		40% (20/50)		.558
	18	42% (14/33)		38% (65/173)		.599		18	33% (3/9)		26% (13/51)		.624
HCMV antibody	18	NA		NA		NA	HCMV antibody	18	NA		NA		NA
HHV-6 sera-DNA	6	9% (3/34)		7% (15/209)		.734	HHV-6 sera-DNA	6	20% (2/10)		3% (2/61)		.034**
	18	12% (4/33)		17% (29/174)		.513		18	0% (0/9)		14% (7/51)		.237
Referral Rate		0.15		0.20		.506	Referral Rate		0.32		0.52		.542
Died		NA		NA		NA	Died		NA		NA		NA
Development													
Stunted	6	4% (2/48)		10% (27/263)		.181	Stunted	6	11% (2/18)		14% (11/81)		.779
	18	7% (3/45)		20% (50/257)		.037**		18	11% (2/18)		33% (25/77)		.071*
		Negative		Positive					Negative		Positive		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	47	-0.0260	257	0.0725	0.594	BMI-for-age	6	16	-0.2150	77	-0.1506	.831
	18	44	0.1505	251	0.1413	0.957		18	16	-0.0488	73	-0.0023	.878
Weight-for-age	6	47	-0.3343	257	-0.4144	0.672	Weight-for-age	6	16	-0.5750	77	-0.6736	.761
	18	44	-0.2575	252	-0.5868	0.077*		18	16	-0.4506	73	-0.8214	.279
Length/height-for-age	6	47	-0.5509	257	-0.8233	0.098*	Length/height-for-age	6	16	-0.7175	77	-0.9713	.347
	18	44	-0.6055	251	-1.1342	0.003***		18	16	-0.6831	73	-1.3359	.034**
Triceps skinfold-for-age	6	47	-0.2828	257	-0.0667	0.206	Triceps skinfold-for-age	6	16	-0.3888	77	-0.2792	.706
	18	44	0.2093	252	0.2199	0.945		18	16	0.0369	73	0.0086	.917
Subscapular skinfold-for-age	6	47	0.3783	257	0.5682	0.302	Subscapular skinfold-for-age	6	16	0.0800	77	0.1257	.892
	18	44	0.5625	252	0.5563	0.970		18	16	0.4413	73	0.3445	.759
Arm circumference-for-age	6	47	0.4160	257	0.4132	0.988	Arm circumference-for-age	6	16	-0.0288	77	0.0436	.815
	18	43	0.2749	252	0.1444	0.433		18	16	-0.1938	73	-0.2144	.955
Head circumference-for-age	6	47	0.8591	257	0.7031	0.342	Head circumference-for-age	6	16	0.7306	77	0.4540	.293
	18	44	0.7332	252	0.5962	0.408		18	16	0.9950	73	0.2149	.007**

Table 29: HCMV seroprevalence is associated with increased anaemia only in the HIV-1 unexposed infants and decreases in head circumference are only in HIV-1 exposed

Distribution of HCMV seroprevalence in HIV-1 unexposed and exposed infants with respect to polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of development. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005

5.5 Effect modification of micronutrient fortified feed supplement by HIV-1 and HCMV infection and exposure

5.5.1 Effects of trial on morbidity and growth

Effects of HIV-1, HCMV and HHV-6 infection were assessed in infants who were being exposed to either a conventionally fortified or micronutrient fortified supplementary feed. Here initial results from the CIGNIS intervention are evaluated. The two feed supplements were primarily composed of maize meal (65%), beans (white and yellow)(15%), groundnuts (15%) and bambaranuts (5%). The micronutrient fortified feed supplement differed significantly in its micronutrient composition to the conventionally fortified feed supplement, with added vitamins, manganese, copper, selenium, calcium and phosphorus and significantly increased levels of iron, zinc and other core nutrients. The main analyses of these two fortified feed supplements are part of a separate study the CIGNIS team. In this thesis initial analysis examines the effects on betaherpesvirus infection.

In the following analyses of the effects of the intervention subject IDs 475-542 were excluded as they were not properly blinded. Therefore a total of 743 infants and their mothers were recruited on to the CIGNIS study and successfully randomized, with 373 on the conventionally fortified and 370 on the micronutrient fortified feed supplement. Demographics such as maternal education, socioeconomic grouping and duration of breast feeding were distributed evenly between the two arms of the study consistent with successful randomization and blinding (Appendix R, Page 239), although there was a trend for a slight gender imbalance between the two arms but no effects on gender were observed with respect to the intervention or to HIV-1 or betaherpesvirus infections. Further, there were a similar number of HIV-1 exposed infants recruited onto each arm of the study and differential effects on HIV-1 exposed and unexposed infants will be investigated.

At baseline the prevalence of anaemia was roughly 40% in both arms of the study (Appendix R, Page 239). The micronutrient fortified feed supplement was shown to significantly reduce the prevalence of anaemia to just 22% at 18 months compared with 40% in the conventionally fortified arm ($P < 0.001$). The intervention did not directly affect stunting or any other anthropometric outcomes and did not affect the prevalence of mortalities or the rate of hospital referrals (Appendix M Page 229 and Appendix R, Page 239, Table 1). Below the intervention is assessed for correlations with HIV-1 exposure and betaherpesvirus infections.

5.5.2 Effects between HIV-1 exposure and trial arms

Looking at HIV-1 unexposed and exposed infants as separate groups, the effect of the micronutrient fortified feed supplement to reduce anaemia was greater among HIV-1 exposed infants, who on the micronutrient arm had a slightly higher (but not significant) prevalence of anaemia at baseline (Appendix R, Table 2, Page 239). There was a trend among HIV-1 unexposed infants for head circumference to be not completely randomized at baseline but by the end of the study this difference had levelled out showing that the micronutrient fortification did not affect head circumference outcomes in either HIV-1 exposed or unexposed infants (Appendix R, Table 2, Page 239).

We also showed above that HIV-1 exposure is linked with stunting and decreases in all anthropometric outcomes (Table 19), but when analysing the two feed arms separately, we found significant reductions in BMI and weight only in the micronutrient fortified arm (Appendix R, Table 3, Page 239). Consistent with trends towards lower anthropometric outcomes in HIV-1 exposed infants on the micronutrient arm, there was also a trend for an increased rate of hospital referrals due to HIV-1 exposure but only in the micronutrient arm, together suggesting there could be some detrimental effect of the micronutrient feed supplement among HIV-1 exposed infants. The rate of hospital referrals was 2-fold higher among HIV-1 exposed infants on the micronutrient arm (0.21 vs 0.45, P = 0.004)(Appendix R, Table 3, Page 239 and Appendix N, Page 230) but HIV-1 exposed infants on the conventional arm were no more likely to be referred. Further sub-stratifications by HIV-1 exposure, breast feeding duration and socioeconomic status have been investigated by other members of the CIGNIS team who found that the micronutrient fortified feed supplement was actually beneficial for linear growth among HIV-1 exposed infants who were breast fed for less than 6 months. These results will be discussed further in section 5.6.

5.5.3 Effects between active HCMV infection and trial arms

Having seen in the previous analysis that the effects of HIV-1 exposure on growth were increased in the micronutrient fortified arm, we then looked to see if infants who had an active HCMV infection at baseline were affected differentially by the intervention. The previously documented beneficial effect of the intervention on anaemia was observed in both HCMV sera-DNA positive and negative infants (Table 30, Page 146). In infants who had an active HCMV infection at baseline, the micronutrient fortified feed supplement showed trends for increasing triceps skinfold and head circumference at 18 months, but these effects were also visible at baseline so could be due to skewed randomization. We then looked at the effects of active HCMV infection within each study arm (Table 31, Page 148) and this skewed randomization was also apparent, with negative effects on month 6 anthropometry linked with HCMV sera-DNA detection only on the conventionally fortified arm. This makes further analysis difficult without adjusted methods. Detection of active HCMV at baseline had no significant effects on length at 18 months, in either treatment arm (Table 31), also after sub-stratifying for HIV-1 exposure, adjusting for z-score at baseline, maternal education, socioeconomic status and duration of breast feeding (Appendix K, Page 220). In summary, the micronutrient fortified feed supplement reduced anaemia irrespective of active HCMV infection at baseline, but did not affect any other morbidity or growth parameters or the prevalence of mortalities or the rate of hospital referral (Table 30)(Appendix O, Page 231).

	Category/ month	HCMV sera-DNA month 6 negative						Category/ month	HCMV sera-DNA month 6 positive				
		Conventional		Micronutrient		P			Conventional		Micronutrient		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protect.	2% (2/110)		4% (4/102)		.821	Polio Ab neutralization titre (12 months)	Un-protect.	3% (2/69)		2% (1/67)		.732
	Low	8% (9/110)		9% (9/102)				Low	16% (11/69)		10% (7/67)		
	Medium	65% (71/110)		62% (63/102)				Medium	68% (47/69)		73% (49/67)		
	High	26% (28/110)		26% (26/102)				High	13% (9/69)		15% (10/67)		
Fever	6	10% (15/146)		11% (16/147)		.865	Fever	6	15% (13/87)		14% (12/85)		.878
	18	14% (14/103)		15% (16/108)		.799		18	7% (4/59)		11% (7/62)		.388
Anaemic	6	41% (58/142)		44% (65/141)		.595	Anaemic	6	38% (33/87)		44% (37/84)		.416
	18	42% (47/112)		19% (21/111)		<.000***		18	39% (27/70)		22% (15/68)		.035**
HCMV sera-DNA	18	28% (26/93)		29% (26/91)		.926	HCMV sera-DNA	18	47% (25/53)		45% (22/49)		.818
HCMV antibody	18	83% (80/96)		84% (75/89)		.863	HCMV antibody	18	89% (49/55)		82% (46/56)		.297
HHV-6 sera-DNA	6	7% (10/145)		4% (6/146)		.297	HHV-6 sera-DNA	6	5% (4/80)		8% (7/83)		.382
	18	20% (19/93)		14% (13/91)		.272		18	9% (5/55)		6% (3/49)		.571
HIV-1 infected		4% (4/115)		5% (5/111)		.693	HIV-1 infected		6% (4/72)		4% (3/68)		.756
HIV-1 exposed		19% (25/129)		17% (22/128)		.649	HIV-1 exposed		23% (17/75)		24% (18/76)		.882
Referral Rate		0.19		0.25		.396	Referral Rate		0.30		0.34		.641
Died		0.7% (1/146)		0% (0/147)		.315	Died		2.3% (2/87)		1.2% (1/85)		.574
Growth													
Stunted	6	11% (16/146)		11% (16/147)		.984	Stunted	6	13% (11/87)		7% (6/85)		.220
	18	17% (19/113)		22% (24/111)		.361		18	23% (16/71)		19% (13/68)		.620
		Conventional		Micronutrient					Conventional		Micronutrient		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	146	0.1949	147	0.1133	.536	BMI-for-age	6	87	-0.1625	85	0.0400	.287
	18	113	0.1971	111	0.1671	.839		18	71	0.0317	68	0.1878	.347
Weight-for-age	6	146	-0.2903	147	-0.3446	.690	Weight-for-age	6	87	-0.6695	85	-0.3473	.083*†
	18	113	-0.5138	112	-0.5254	.945		18	71	-0.7087	68	-0.4471	.172
Length/height-for-age	6	146	-0.7582	147	-0.7457	.916	Length/height-for-age	6	87	-0.9222	85	-0.6561	.200
	18	113	-1.0784	111	-1.0705	.960		18	71	-1.2034	68	-0.9468	.179
Triceps skinfold-for-age	6	146	-0.0950	147	-0.1736	.531	Triceps skinfold-for-age	6	87	-0.3613	85	0.0194	.027**†
	18	113	0.2039	112	0.3309	.332		18	71	0.0319	68	0.2984	.089*
Subscapular skinfold-for-age	6	146	0.7375	147	0.5264	.129	Subscapular skinfold-for-age	6	87	0.0523	85	0.3422	.127
	18	113	0.6014	112	0.5275	.602		18	71	0.2867	68	0.5825	.105
Arm circumference-for-age	6	146	0.5955	147	0.5493	.710	Arm circumference-for-age	6	87	0.1248	85	0.3728	.149
	18	113	0.2751	112	0.1954	.608		18	71	-0.1139	68	0.0366	.408
Head circumference-for-age	6	146	0.6702	147	0.7316	.590	Head circumference-for-age	6	87	0.4843	85	0.7534	.091*†
	18	113	0.5186	112	0.5666	.709		18	71	0.3686	68	0.7478	.040**

Table 30: Micronutrient fortification may increase head circumference in infants who had an HCMV active infection at 6 months

Effect of micronutrient fortified feed supplement sub-stratified by HCMV infection at 6 months, on prevalence of other betaherpesvirus active infections (detection of sera-DNA), HIV-1 infection (detection of antibody at 18 months), HIV-1 exposure (determined by maternal antenatal HIV-1 status), polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable † = groupings that were not randomized between treatment arms at baseline.

	Category/ month	Conventional fortification						Category/ month	Micronutrient fortification				
		HCMV sera-DNA month 6 negative		HCMV sera-DNA month 6 positive		P			HCMV sera-DNA month 6 negative		HCMV sera-DNA month 6 positive		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protective	2% (2/110)		3% (2/69)		.125	Polio Ab neutralization titre (12 months)	Un-protective	4% (4/102)		2% (1/67)		.281
	Low	8% (9/110)		16% (11/69)				Low	9% (9/102)		10% (7/67)		
	Medium	65% (71/110)		68% (47/69)				Medium	62% (63/102)		73% (49/67)		
	High	26% (28/110)		13% (9/69)				High	26% (26/102)		15% (10/67)		
Fever	6	10% (15/146)		15% (13/87)		.289	Fever	6	11% (16/147)		14% (12/85)		.466
	18	14% (14/103)		7% (4/59)		.184		18	15% (16/108)		11% (7/62)		.518
Anaemic	6	41% (58/142)		38% (33/87)		.662	Anaemic	6	44% (65/141)		44% (37/84)		.991
	18	42% (47/112)		39% (27/70)		.650		18	19% (21/111)		22% (15/68)		.611
HCMV sera-DNA	18	28% (26/93)		47% (25/53)		**.019****	HCMV sera-DNA	18	29% (26/91)		45% (22/49)		**.052***
HCMV antibody	18	83% (80/96)		89% (49/55)		.335	HCMV antibody	18	84% (75/89)		82% (46/56)		.737
HHV-6 sera-DNA	6	7% (10/145)		5% (4/80)		.573	HHV-6 sera-DNA	6	4% (6/146)		8% (7/83)		.174
	18	20% (19/93)		9% (5/55)		**.071***		18	14% (13/91)		6% (3/49)		.148
HIV-1 infected		4% (4/115)		6% (4/72)		.495	HIV-1 infected		5% (5/111)		4% (3/68)		.977
HIV-1 exposed		19% (25/129)		23% (17/75)		.576	HIV-1 exposed		17% (22/128)		24% (18/76)		.258
Referral Rate		0.19		0.30		.120	Referral Rate		0.25		0.34		.311
Died		0.7% (1/146)		2.3% (2/87)		NA	Died		0% (0/147)		1.2% (1/85)		NA
Growth													
Stunted	6	11% (16/146)		13% (11/87)		.698	Stunted	6	11% (16/147)		7% (6/85)		.338
	18	17% (19/113)		21% (15/70)		.435		18	22% (24/111)		19% (13/68)		.688
		HCMV sera-DNA month 6 negative		HCMV sera-DNA month 6 positive					HCMV sera-DNA month 6 negative		HCMV sera-DNA month 6 positive		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	146	0.1949	87	-0.1625	**.028****	BMI-for-age	6	147	0.1133	85	0.0400	.641
	18	113	0.1971	71	0.0257	.306		18	111	0.1669	68	0.1878	.894
Weight-for-age	6	146	-0.2903	87	-0.6695	**.020****	Weight-for-age	6	147	-0.3446	85	-0.3473	.987
	18	113	-0.5137	71	-0.6937	.334		18	112	-0.5274	68	-0.4471	.665
Length/height-for-age	6	146	-0.7582	87	-0.9222	.335	Length/height-for-age	6	147	-0.7457	85	-0.6561	.527
	18	113	-1.0781	71	-1.1733	.571		18	111	-1.0735	68	-0.9468	.495
Triceps skinfold-for-age	6	146	-0.0950	87	-0.3613	**.080***	Triceps skinfold-for-age	6	147	-0.1736	85	0.0194	.183
	18	113	0.2039	71	0.0319	.247		18	112	0.3309	68	0.2984	.822
Subscapular skinfold-for-age	6	146	0.7375	87	0.0523	**.000*****	Subscapular skinfold-for-age	6	147	0.5264	85	0.3422	.255
	18	113	0.6014	71	0.2867	.059*		18	112	0.5275	68	0.5825	.730
Arm circumference-for-age	6	146	0.5955	87	0.1248	**.002*****	Arm circumference-for-age	6	147	0.5493	85	0.3728	.229
	18	113	0.2751	71	-0.1139	**.024****		18	112	0.1954	68	0.0366	.360
Head circumference-for-age	6	146	0.6702	87	0.4843	.190	Head circumference-for-age	6	147	0.7316	85	0.7534	.867
	18	113	0.5186	71	0.3686	.326		18	112	0.5666	68	0.7478	.247

Table 31: HCMV active infections were linked with reduced arm circumference and subscapular skinfold at 6 months only within the conventionally fortified arm
Effect of micronutrient fortified feed supplement sub-stratified by HCMV seroprevalence, on prevalence of betaherpesvirus active infections (detection of sera-DNA), HIV-1 infection (detection of antibody at 18 months), HIV-1 exposure (determined by maternal antenatal HIV-1 status), polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

5.5.4 Effects between HCMV seroprevalence and trial arms

When looking at HCMV seronegative and seropositive infants, the effect of the micronutrient fortified feed supplement to reduce anaemia was similar in both arms, with greater significance among HCMV seropositive infants, the majority group (Figure 32 and Table 32, Page 151). Anthropometric outcomes were unaffected by the micronutrient fortified feed supplement irrespective of HCMV seroprevalence (Table 32). HCMV seroprevalence was shown previously to be associated with increases in anaemia and stunting, and decreases in weight, length and head circumference at 18 months (section 5.4). Interestingly the inhibitory effects of HCMV seroprevalence on head circumference were significant only in infants on the conventionally fortified arm but not observed for the micronutrient arm (Figure 33)(Table 33) suggesting a possible effect on head size of this intervention.

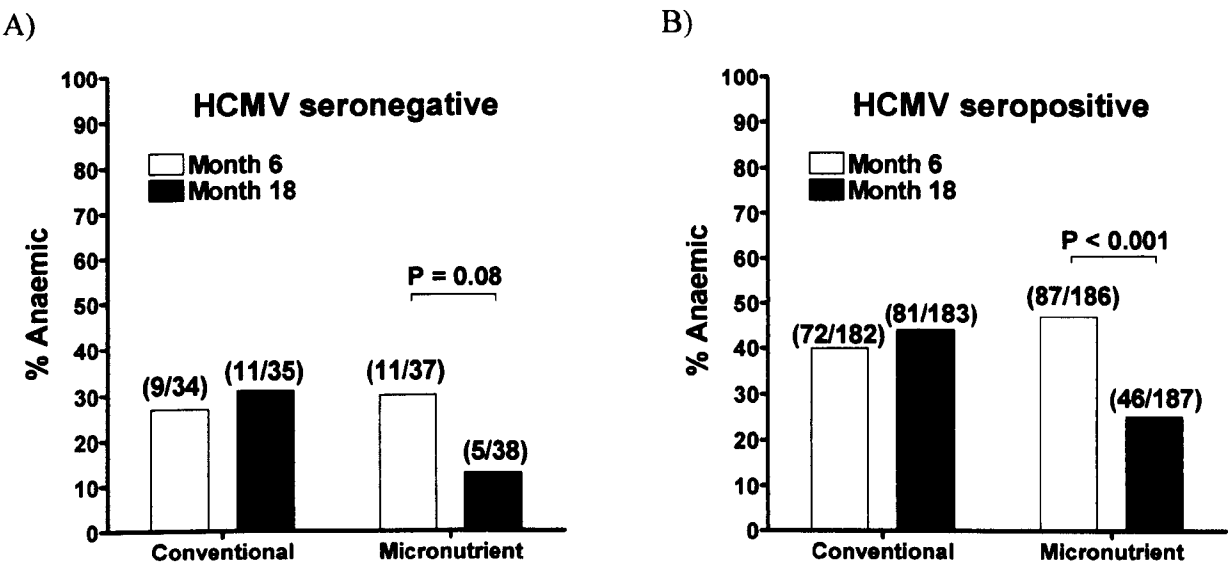


Figure 32: Micronutrient fortification reduces anaemia over the course of the study in both HCMV seronegative and HCMV seropositive infants

Prevalence of anaemia compared between time points sub-stratified by trial arm in a) HCMV seronegative and b) HCMV seropositive infants. Anaemia here is defined by a haemoglobin level of less than 10.5 g/L. Significance is by Pearson Chi-Squared.

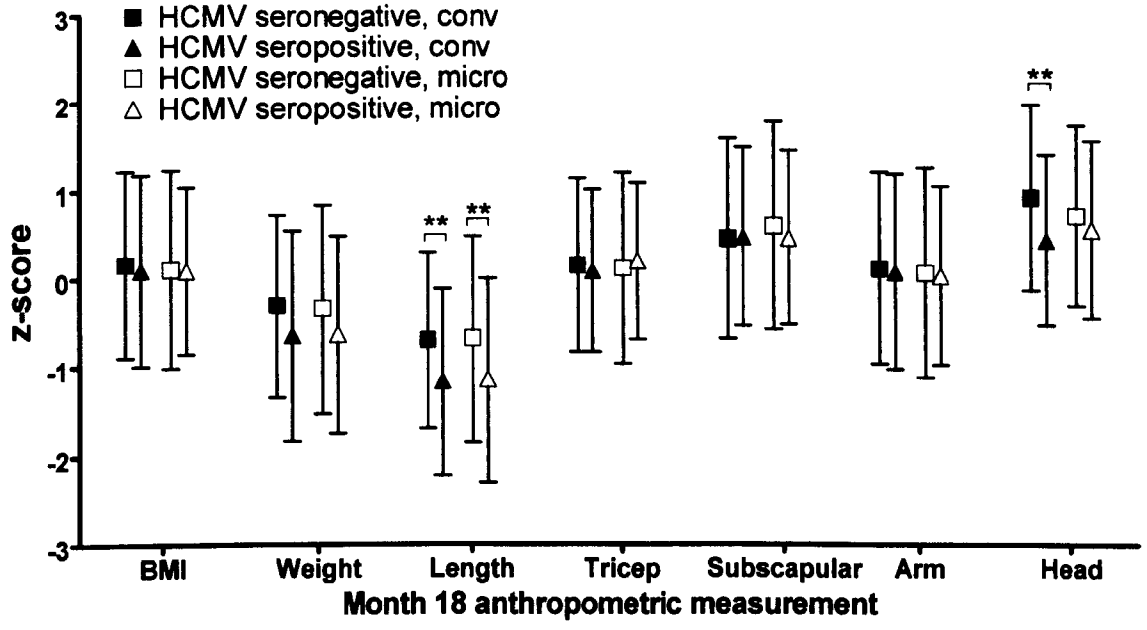


Figure 33: HCMV seropositive infants have smaller heads only within conventionally fortified arm

Anthropometric 18 month mean z-scores compared between HCMV seropositive and seronegative infants sub-stratified by the conventionally fortified and micronutrient fortified trial arms. Bars represent one standard deviation from the mean. Mean z-scores were compared by independent sample T test assuming equal variance Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

	Category/ month	HCMV seronegative						Category/ month	HCMV seropositive				
		Conventional		Micronutrient		P			Conventional		Micronutrient		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protective	3% (1/35)		5% (2/38)		.669	Polio Ab neutralization titre (12 months)	Un-protective	1% (2/181)		2% (3/181)		.181
	Low	6% (2/35)		13% (5/38)				Low	11% (20/181)		6% (10/181)		
	Medium	66% (23/35)		61% (23/38)				Medium	66% (119/181)		65% (117/181)		
	High	26% (9/35)		21% (8/38)				High	22% (40/181)		28% (51/181)		
Fever	6	14% (5/35)		13% (5/38)		.889	Fever	6	12% (22/184)		14% (26/189)		.604
	18	4% (1/26)		10% (3/30)		.373		18	14% (20/141)		15% (21/142)		.885
Anaemic	6	27% (9/34)		30% (11/37)		.760	Anaemic	6	40% (72/182)		47% (87/186)		.163
	18	31% (11/35)		13% (5/38)		**.059***		18	44% (81/183)		25% (46/187)		**<.001*****
HCMV sera-DNA	6	27% (6/22)		42% (10/24)		.306	HCMV sera-DNA	6	38% (49/129)		38% (46/121)		.996
	18	38% (8/21)		40% (10/25)		.895		18	35% (45/127)		36% (43/118)		.870
HHV-6 sera-DNA	6	9% (2/22)		11% (3/27)		.816	HHV-6 sera-DNA	6	6% (9/149)		4% (6/148)		.434
	18	10% (2/21)		8% (2/25)		.855		18	16% (20/128)		16% (19/118)		.919
HIV-1 infected		3% (1/35)		3% (1/38)		.953	HIV-1 infected		2% (4/184)		2% (3/188)		.682
HIV-1 exposed		30% (9/30)		21% (7/33)		.424	HIV-1 exposed		22% (37/166)		24% (40/168)		.742
Referral Rate		0.20		0.20		.948	Referral Rate		0.23		0.29		.405
Died		0% (0/35)		0% (0/38)		NA	Died		0% (0/184)		0% (0/189)		NA
Growth													
Stunted	6	9% (3/35)		8% (3/38)		.916	Stunted	6	10% (19/184)		12% (22/189)		.685
	18	12% (4/34)		11% (4/36)		.932		18	20% (37/182)		24% (42/179)		.471
		Conventional		Micronutrient					Conventional		Micronutrient		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	35	-0.0146	38	-0.1245	.663	BMI-for-age	6	184	0.0435	189	-0.0534	.441
	18	34	0.1600	36	0.1036	.830		18	182	0.0901	179	0.0852	.964
Weight-for-age	6	35	-0.4429	38	-0.4189	.924	Weight-for-age	6	184	-0.4710	189	-0.5213	.690
	18	34	-0.3129	36	-0.3475	.897		18	182	-0.6493	181	-0.6380	.926
Length/height-for-age	6	35	-0.7560	38	-0.5626	.385	Length/height-for-age	6	184	-0.8603	189	-0.8484	.922
	18	34	-0.7065	36	-0.6933	.960		18	182	-1.1745	179	-1.1566	.878
Triceps skinfold-for-age	6	35	-0.4474	38	-0.3237	.635	Triceps skinfold-for-age	6	184	-0.1648	189	-0.1193	.681
	18	34	0.1418	36	0.1081	.893		18	182	0.0755	181	0.1899	.232
Subscapular skinfold-for-age	6	35	0.2226	38	0.2703	.869	Subscapular skinfold-for-age	6	184	0.4693	189	0.3994	.570
	18	34	0.4447	36	0.5928	.595		18	182	0.4660	181	0.4555	.920
Arm circumference-for-age	6	35	0.2771	38	0.2403	.877	Arm circumference-for-age	6	184	0.3218	189	0.2943	.818
	18	33	0.1091	36	0.0586	.855		18	182	0.0681	181	0.0220	.681
Head circumference-for-age	6	35	0.8697	38	0.7934	.723	Head circumference-for-age	6	184	0.6277	189	0.6112	.877
	18	34	0.9144	36	0.7039	.400		18	182	0.4282	181	0.5485	.247

Table 32: Micronutrient fortification reduced anaemia at 18 months irrespective of HCMV seroprevalence

Effect of micronutrient fortified feed supplement sub-stratified by HCMV seroprevalence, on prevalence of betaherpesvirus active infections (detection of sera-DNA), HIV-1 infection (detection of antibody at 18 months), HIV-1 exposure (determined by maternal antenatal HIV-1 status), polio antibody titres (measured by neutralization assay with titres binned as un-protective (<$^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high (>$^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

	Category/ month	Conventional fortification						Category/ month	Micronutrient fortification				
		HCMV sero-ve		HCMV sero+ve		P			HCMV sero-ve		HCMV sero+ve		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protective	3% (1/35)		1% (2/181)		.651	Polio Ab neutralization titre (12 months)	Un-protective	5% (2/38)		2% (3/181)		.162
	Low	6% (2/35)		11% (20/181)				Low	13% (5/38)		6% (10/181)		
	Medium	66% (23/35)		66% (119/181)				Medium	61% (23/38)		65% (117/181)		
	High	26% (9/35)		22% (40/181)				High	21% (8/38)		28% (51/181)		
Fever	6	14% (5/35)		12% (22/184)		.701	Fever	6	13% (5/38)		14% (26/189)		.922
	18	4% (1/26)		14% (20/141)		.144		18	10% (3/30)		15% (21/142)		.492
Anaemic	6	27% (9/34)		40% (72/182)		.148	Anaemic	6	30% (11/37)		47% (87/186)		**.056***
	18	31% (11/35)		44% (81/183)		.159		18	13% (5/38)		25% (46/187)		.125
HCMV sera-DNA	6	27% (6/22)		38% (49/129)		.335	HCMV sera-DNA	6	42% (10/24)		38% (46/121)		.737
	18	38% (8/21)		35% (45/127)		.814		18	40% (10/25)		36% (43/118)		.738
HHV-6 sera-DNA	6	9% (2/22)		6% (9/149)		.586	HHV-6 sera-DNA	6	11% (3/27)		4% (6/148)		.127
	18	10% (2/21)		16% (20/128)		.465		18	8% (2/25)		16% (19/118)		.299
HIV-1 infected		3% (1/35)		2% (4/184)		.804	HIV-1 infected		3% (1/38)		2% (3/188)		.659
HIV-1 exposed		30% (9/30)		22% (37/166)		.359	HIV-1 exposed		21% (7/33)		24% (40/168)		.747
Referral Rate		0.17		0.24		.482	Referral Rate		0.19		0.28		.443
Died		0% (0/35)		0% (0/184)		NA	Died		0% (0/38)		0% (0/189)		NA
Growth													
Stunted	6	9% (3/35)		10% (19/184)		.752	Stunted	6	8% (3/38)		12% (22/189)		.501
	18	12% (4/34)		20% (37/182)		.242		18	11% (4/36)		24% (42/179)		**.099***
		HCMV sero-ve		HCMV sero+ve					HCMV sero-ve		HCMV sero+ve		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	35	-0.0146	184	0.0435	.794	BMI-for-age	6	38	-0.1245	189	-0.0534	.736
	18	34	0.1600	182	0.0901	.731		18	36	0.1036	179	0.0852	.918
Weight-for-age	6	35	-0.4429	184	-0.4710	.891	Weight-for-age	6	38	-0.4189	189	-0.5213	.650
	18	34	-0.3129	182	-0.6493	.124		18	36	-0.3475	181	-0.6380	.159
Length/height-for-age	6	35	-0.7560	184	-0.8603	.628	Length/height-for-age	6	38	-0.5626	189	-0.8484	.156
	18	34	-0.7065	182	-1.1745	**.017****		18	36	-0.6933	179	-1.1566	**.029****
Triceps skinfold-for-age	6	35	-0.4474	184	-0.1648	.151	Triceps skinfold-for-age	6	38	-0.3237	189	-0.1193	.290
	18	34	0.1418	182	0.0755	.705		18	36	0.1081	181	0.1899	.629
Subscapular skinfold-for-age	6	35	0.2226	184	0.4693	.260	Subscapular skinfold-for-age	6	38	0.2703	189	0.3994	.546
	18	34	0.4447	182	0.4660	.912		18	36	0.5928	181	0.4555	.461
Arm circumference-for-age	6	35	0.2771	184	0.3218	.817	Arm circumference-for-age	6	38	0.2403	189	0.2943	.802
	18	33	0.1091	182	0.0681	.845		18	36	0.0586	181	0.0220	.849
Head circumference-for-age	6	35	0.8697	184	0.6277	.182	Head circumference-for-age	6	38	0.7934	189	0.6112	.321
	18	34	0.9144	182	0.4282	**.009****		18	36	0.7039	181	0.5485	.400

Table 33: Micronutrient fortification may act to promote head growth in HIV-1 exposed infants

Effect of HCMV seroprevalence sub-stratified by micronutrient fortified feed supplement, on prevalence of betaherpesvirus active infections (detection of sera-DNA), HIV-1 infection (detection of antibody at 18 months), HIV-1 exposure (determined by maternal antenatal HIV-1 status), polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable

5.6 Discussion

In this chapter betaherpesvirus active infections (sera-DNA detection at 6 and/or 18 months of age) and HCMV seroprevalence (sera-Ab detection at 18 months of age) have been investigated for effects on markers of morbidity and growth in HIV-1 exposed or unexposed, as well as HIV-1 infected, Zambian infants. The study children were taking part in a nutritional intervention (CIGNIS) designed to test the efficacy of a micronutrient fortified feed supplement (over one that was conventionally fortified) to reduce morbidity and improve growth in HIV-1 exposed infants.

At recruitment, 22% (177/799) of mothers reported themselves has being HIV-1 positive. This rate is in keeping with national statistics which state that 23% of urban women of child-bearing age (15-49yrs) are reported to be HIV-1 positive (CSO_Zambia, 2007). All mothers in Lusaka are encouraged to have an HIV-1 screen during pregnancy and those who are positive are administered Nevirapine therapy for the prevention of MTCT (mother-to-child transmission)(Kuhn et al., 2009). Without intervention transmission rates vary between 21-43% but the administration of intrapartum Nevirapine has been shown to reduce rates by up to 50% (Guay et al., 1999; Jackson et al., 2003). Infant HIV-1 serostatus was determined for 152 infants born to HIV-1 positive mothers, and 8% (12/152) were HIV-1 positive at 18 months of age. There were eight other HIV-1 positive infants. Two of these infants had mothers of unknown status, and the remaining six had mothers who were HIV-1 negative according to the survey they responded to at recruitment. We do not know if these were pre- or post-natal maternal HIV-1 infections, but of the eight infants, one died quite early into the study due to protein energy malnutrition. Even if all eight infants were born to HIV-1 positive mothers, the resulting 'maximum' rate of MTCT on this study would be 13% (20/151) which remains consistent with reductions reported elsewhere as a result of Nevirapine prophylaxis (Guay et al., 1999; Jackson et al., 2003).

The study was not set up to focus on HIV-1 infected infants and so their numbers were low, but some significant correlations were observed. Anaemia was more common in HIV-1 infected infants throughout the study, and the micronutrient fortified feed supplement showed a trend to reduce anaemia among HIV-1 infected infants. Interestingly, whilst in HIV-1 infected infants some z-scores were significantly lower at baseline, by 18 months of age just two anthropometric parameters distinguished the HIV-1 infected from the uninfected infants: subscapular skinfold and arm circumference. This is consistent with previous studies showing

lipodystrophy in HIV-1 infected infants (Taylor et al., 2004). Compared to HIV-1 uninfected infants, the few who became HIV-1 infected (by 18 months of age) were significantly more likely to fail to mount a protective response to the polio vaccine. Protective responses were generally weaker as has been shown previously for both the polio and measles virus vaccines (Nair et al., 2009; Tejiokem et al., 2007). A possible mechanism for this is through HIV-1 infection and malfunction of Th2 T-cells which bind to and promote B-cell proliferation and antibody production (Becker, 2007; Parker, 1993).

In HIV-1 exposed infants, a significantly lower distribution of responses to the polio vaccine was also observed, clearly demonstrating that HIV-1 exposure is associated with weakened immunity. This combines with decreased adoptive immunity through breast milk, with HIV-1 positive mothers being significantly less likely to breast feed, or more likely to terminate breast feeding earlier than HIV-1 negative mothers. This is also a probable major cause of impaired growth and development in HIV-1 exposed infants and lies at the very centre of the rationale for the CIGNIS trial which seeks to compensate for reduced breast feeding and improve growth and development in HIV-1 exposed infants. The prevalence of stunting among HIV-1 exposed infants on this study was significantly greater, characterized not only by significantly lower mean z-scores for length, but also decreased weight and peripheral fat, with most differences persisting to 18 months. These results are consistent with what has been published with respect to infant growth and HIV-1 exposure (Makasa et al., 2007). Hospital referral rates were significantly higher among HIV-1 exposed infants suggesting a higher rate of serious morbidity. Mortalities were also more prevalent in HIV-1 exposed infants, but all five who died were of unknown HIV-1 status having not completed the study, and may well have been HIV-1 infected. More long term follow up after 18 month HIV-1 diagnosis will be required to establish whether mortality is greater in truly HIV-1 exposed uninfected infants as published previously (Marinda et al., 2007).

A serological screen for HCMV was undertaken on sera from 460 CIGNIS children at 18 months of age showing the overall prevalence of HCMV to be 83%. This rate is in much higher than that seen in European and North American seroprevalence studies (Emery, 2001; Staras et al., 2006) but is slightly lower than seen in studies from The Gambia (Kaye et al., 2008; Miles et al., 2007) and Kenya (Chakraborty et al., 2003). This relatively high 18 month HCMV seroprevalence is consistent with the prevalence of HCMV sera-DNA in this cohort. Interestingly HCMV sera-DNA prevalence did not correlate with HCMV seroprevalence, in that infants in whom HCMV sera-DNA was detected, were no more or less likely to be

HCMV seropositive than those who were negative for HCMV sera-DNA. One explanation for this is that exposure to HCMV by 18 months was almost universal in this population with some infants having antibody below the threshold of detection. This would mean that the serology data should be interpreted as 'detectable antibody at month 18' and it cannot be assumed that all infants without detectable antibody have not been exposed to the virus. Another explanation is the possible presence of false positives in the sera-DNA or seroprevalence analysis. All positives were scored from screens which contained the appropriate positive and negative controls although PCR is susceptible to transient contamination (especially so in tropical climates with limited air conditioning). Skewed randomization is also suggestive of contamination. Future studies should employ stricter controls and designed to allow enough sample for multiple screens. Due to limitations on sample volume on this large collaborative study a strategy of duplicate screens or extractions was not feasible. HHV-6 was less commonly detected than HCMV, although the assay used was less sensitive. A serological screen of 18 month sera is currently being undertaken. Preliminary results on a small subset of samples suggest 100% HHV-6 seroprevalence at 18 months (KM, personal communication). It is known from studies in the U.S that primary HHV-6 infection occurs in up to 80% of infants by two years of age (Zerr et al., 2005) and so if the earlier uptake in Zambia seen with HCMV is mirrored for HHV-6, we anticipate that all of the infants will be HHV-6 seropositive by 18 months of age. Our results suggest betaherpesvirus sera-DNAemia is common (significantly more so for HCMV than in HHV-6) and that these episodes of viral shedding in sera are normal in healthy infants but that they correlate with largely short term effects on morbidity and growth.

HCMV seroprevalence and active betaherpesvirus infections and were first analysed for possible correlations with demographic parameters that have been previously shown to be associated with HCMV such as maternal education, socioeconomic status and duration of breast feeding (de Martino et al., 1992; Dowd, Aiello, and Alley, 2009). HCMV seroprevalence was linked with lower socioeconomic status as previously, and also with increased duration of breast feeding which is a long established route of transmission for HCMV (Dworsky et al., 1983; Murata et al., 2009), but not HHV-6 (Kusuhara et al., 1997). These data suggest that breast feeding may be considered a risk factor for mother-to-child-transmission of both HIV-1 and HCMV, both of which contribute to impaired growth and increased morbidity and moralities.

Duration of breast feeding did not correlate with prevalence of active HCMV infection at 6 months, suggesting that the main window of transmission via breast milk is in early infancy. There are also other modes of transmission to consider with HCMV being shed in saliva and urine which are also known to contain high loads of HCMV and may influence transmission differentially in different populations (Mocarski, 2007). Furthermore, a study from The Gambia suggests that congenital infections are much more common in populations of high adult seroprevalence than thought previously (Kaye et al., 2008), possibly due to more frequent or higher load maternal reactivations (van der Sande et al., 2007), and this may contribute significantly as a mode of HCMV transmission in Zambia. Congenital or perinatal HCMV may be responsible for considerable levels of morbidity and impaired growth, with a back drop of high levels of maternal and infant immune suppression from either HIV-1 or other infections and also poor nutrition. In this study active HCMV infection at month 6 was a marker of morbidity as it was associated with a clear trend towards increased referrals.

HCMV seroprevalence was also associated with a trend towards decreased risk of polio vaccine failure but only amongst HIV-1 unexposed infants, likely due to the association of HIV-1 exposure and weakened polio vaccine responses being stronger. Anaemia is common in Zambian infants (van Rheenen et al., 2008), possibly due to a lack of dietary iron (Adetifa and Okomo, 2009) or common childhood infections such as malaria (Otieno et al., 2006) or viral infections. Here HCMV seropositive infants were more likely to be anaemic as has been shown previously (Distefano et al., 2004) possibly reflecting poorer dietary intake of iron linked with lower socioeconomic status or maybe through a direct effects of HCMV metabolising available iron (Crowe et al., 2004). By 18 months however this difference loses significance but this is not due to the intervention, in that HCMV seroprevalence is not associated with significant increases in anaemia at 18 months in either arm of the trial. Iron intake of mothers in the third trimester of pregnancy is linked to anaemia in infants, with low body iron at birth being associated with more long term iron deficiency (Iannotti et al., 2006). Anaemia may be a marker for MTCT of HCMV. It has already been shown to be an independent marker of MTCT of HIV-1 (Naniche et al., 2008) and is a marker of worse prognosis and disease progression in HIV-1 positive infants (Consolini et al., 2007).

It has been previously demonstrated that the prevalence of various infections can affect growth in infants, including changes in both BMI and head circumference (Prentice and Darboe, 2008). In the CIGNIS study active HCMV at month 6 was associated with lower BMI and lower peripheral fat, but these effects did not tend to persist throughout the study.

Conversely, HCMV seroprevalence is significantly associated with stunting and also lower weight at 18 months suggesting more long term inhibitory effects on growth. Interestingly, the detrimental correlation of HCMV seroprevalence and head size affected only HIV-1 exposed infants, suggesting these two infections may be combining to impair head growth. A plausible explanation may be that congenital HCMV is significantly more frequent among HIV-1 exposed infants and that the effects on head circumference are unique to congenital HCMV whereas those on length are also a result of postnatally acquired HCMV infection. Analysis of Bailey testing results is being undertaken by other members of the CIGNIS team and it will be interesting to see if the effect of HCMV and HIV-1 on head size also correlates with impaired mental development.

Finally we have assessed the impact of betaherpesvirus infections on the efficacy of a micronutrient fortified feed supplement to improve growth and reduce morbidity in Zambian infants. Overall the intervention significantly reduced the prevalence of anaemia likely due to the fact that the micronutrient fortified feed supplement contained nearly 40 times more iron than the conventionally fortified feed (250mg vs 6.5mg per Kg baked flour). These effects on anaemia were independent of HIV-1 exposure, HCMV seroprevalence or prevalence of active HCMV infections. The intervention did not have a significant overall effect on anthropometric outcomes, but sub-stratifying by HIV-1, HCMV and breast feeding groupings uncovered some interesting and significant effects: Analysis by the CIGNS team showed that the micronutrient fortified feed supplement was seen to benefit a sub-group: It improved linear growth among HIV-1 exposed infants who were not breast feeding at recruitment (Filteau, 2009b), and within this sub-group, HCMV seropositive infants were significantly longer if on the micronutrient enriched feed supplement than on the conventional supplement ($P = 0.022$)(Appendix L, analysis by KB, study statistician). This finding show's that the micronutrient fortified feed supplement had real benefits for a sub-group of children: HIV-1 exposed, HCMV seropositive and breast fed <6 months. But conversely, HIV-1 exposed, HCMV seropositive infants also showed a significantly greater rate of referrals ($P = 0.023$) (Appendix P, analysis by KB, study statistician), largely due to infectious causes. A total of 14 infants within this group were referred to hospital, 10 of whom were suffering with infections: five with malaria, two with skin infections, two with acute diarrhoea and one with pneumonia. The micronutrient fortified feed supplement may be promoting growth in this 'at risk' group but increased iron may be promoting infections resulting in increased hospitalizations. HIV-1 exposed, HCMV seropositive infants, account for around 15% of Zambian infant population. This is a sizeable group to be at risk of higher rates of referral in

response to micronutrient fortification of complementary feeds, especially if benefits to the majority of infants are marginal. These findings and the effects of HHV-6 in this respect are being analysed further by the CIGNIS team.

6.0 Discussion

6.1 Introduction

This thesis presents analysis of human betaherpesvirus infections, in one prospective and two retrospective, paediatric cohorts from Zambia, a country at the epicentre of the HIV-1 pandemic. Analysis of the two retrospective cohorts provided insight into the prevalence and loads of these two viruses in hospitalized fevers (n = 141) and HIV-1 positive respiratory mortalities (n = 36). The prospective cohort were taking part in a nutritional intervention study (Chilenje Infant Growth Nutrition and Infection Study - CIGNIS)(n = 812) which facilitated the first population based analysis of betaherpesvirus infections in this region, looking at viral sera-DNA prevalence and loads at both 6 and 18 months of age along with HCMV seroprevalence at 18 months. Data were collected from the CIGNIS cohort on infant and maternal HIV-1 status, demographics, prevalence of anaemia and reported fever, rates of hospital referrals and mortalities, and growth. This prospective cohort provided the largest data set and was used to assess the effects of betaherpesvirus infections on HIV-1 exposed but uninfected infants, a new and expanding group in this region known to suffer from severe growth retardation and increased morbidity (Makasa et al., 2007).

6.2 Betaherpesvirus prevalence in three Zambian paediatric cohorts

The prevalence and loads of HCMV and HHV-6 were first analysed in two retrospective Zambian paediatric cohorts: DNA-extracted lung tissue from HIV-1 positive paediatric respiratory mortalities, and DNA-extracted whole blood from infants hospitalized with fever. The respiratory mortalities displayed a significantly higher prevalence of HCMV than HHV-6 (94% vs 72%) although both were commonly detected, and loads normalized to cell number did not differ significantly between the two viruses. The higher prevalence of HCMV DNA correlates with the fact that it is known to infect the lungs and cause pneumonia and was previously commonly detected by histology in this cohort (Chintu et al., 2002). These two betaherpesviruses are not routinely monitored in Zambia, but are known to accelerate HIV-1 progression (Chakraborty et al., 2003; Kositanont et al., 1999; Kovacs et al., 1999; Nigro et al., 1996) and are important causes of morbidity and mortality in late stage AIDS which may respond to treatment.

This thesis has also established that active betaherpesvirus infections are common in healthy Zambian infants, and that the epidemiology of these infections in this region is quite distinct from that seen in other regions globally. CIGNIS was the first population based study of HHV-6 in this region but it was not designed to measure the cumulative incidence of primary HHV-6 infection as elsewhere. Prevalence of sera-DNA detection at two time points (8% at 6 months, 13% at 18 months) suggested possible earlier uptake of HHV-6 than in North America where 10% of infants were cumulatively infected by 6 months of age, rising to 80% by 18 months (Zerr et al., 2005). HHV-6 loads in the CIGNIS cohort were generally low and we did not identify any infants in whom we suspected chromosomally integrated HHV-6, which has been documented to have a prevalence of up to 1% in some European countries (Leong et al., 2007; Ward et al., 2006). Earlier uptake of HHV-6 would be consistent with our findings here from the first Zambian survey of HCMV seroprevalence which found 83% of infants to be seropositive by 18 months of age (Kunda Musonda, MSc Thesis), with active infections being detected in 40% of infants at 6 months of age. This is consistent with a much smaller study from Kenya which used exactly the same assay, but a more sensitive DNA extraction protocol (specific for viral DNA thus reducing background interference from human genomic DNA), to find 90% (n = 20) of HIV-1 exposed infants to be sera-DNA positive, although this study lacked an HIV-1 unexposed control group (Slyker et al., 2009). This early uptake has also been seen in other African countries (Bello, 1992; Kaye et al., 2008) but is in stark contrast to the epidemiology of HCMV infection elsewhere, where uptake is known to be much later with seroprevalence rates in North American children <2 yrs of age being reported between 15-30% (Bale et al., 1999; Noyola et al., 2005) or 30% in children from New Zealand aged 3-5 yrs old (O'Brien et al., 2009). The prevalence seen here in Zambia is similar to that recorded in elderly populations in the U.K (Vyse, Hesketh, and Pebody, 2009).

These early infections with HCMV in Africa are particularly interesting in light of a recent study from Malawi (Zambia's eastern neighbour), which found significantly lower (2-6 fold) median percentages of CCR7 positive naïve and central memory T-cells, but higher CCR7 negative effector and stable memory T-cells in Malawi. The study then assessed the effect of HCMV, with seropositive (36%) U.K adolescents having proportionately fewer truly naïve T-cells than those who were seronegative (64%). This comparison was not possible in the Malawian cohort as all 59 adolescents studied were HCMV seropositive (Ben-Smith et al., 2008). They suggest that in high disease burden countries, the greater natural exposure to many infections may be driving earlier ageing of the immune system, and that HCMV may

play a major role in this. Profound CD8 T-cell differentiation of CMV-specific T-cells was also observed in a study of 12 month old Gambian infants (Miles et al., 2007). Several studies have shown a link between HCMV seroprevalence and ageing as reviewed (Emery, 2001; Karrer et al., 2009) with HCMV seropositivity being linked with major differences in the magnitude of lymphoid subsets in healthy European adults (Chidrawar et al., 2009; Pita-Lopez et al., 2009). Data from the U.S shows that in seropositive adults, the percentage of HCMV-specific T-cells in the peripheral memory was around 10% for both CD4+ and CD8+ subsets (Sylwester et al., 2005). Furthermore many of these committed HCMV-specific memory cells are, to varying degrees, dysfunctional (Ouyang et al., 2004). Considering the number of pathogens most people have been exposed to by adulthood, this displays an extraordinary bias towards HCMV and is possibly the result of continual viral re-activation and immune stimulation maybe in concert with defects in apoptosis. The early uptake in infants and high seroprevalence of HCMV reported in this thesis may be significant factors contributing to ill health in Zambia, undermining efforts to combat a broad range of infectious diseases, due to an accelerated onset of immune senescence with age when compared with populations from temperate climates. Such geographical differences have also been observed in the prevalence of other herpesviruses. Interestingly VZV seroprevalence is generally lower in tropical compared to temperate countries (Kjersem and Jepsen, 1990; Saha et al., 2002), the complete opposite of that observed here for HCMV.

HIV-1 is endemic in Zambia and both HCMV (seroprevalence, sera-DNA loads and genotypes) and HHV-6 (sera-DNA loads and genotypes) have been analysed here on the prospective CIGNIS study within HIV-1 infected, HIV-1 exposed and HIV-1 unexposed healthy Zambian infants. This analysis showed trends, at both month 6 and month 18, for higher prevalence of HCMV and HHV-6 sera-DNA detection among HIV-1 positive infants but these differences were not significant with the low numbers of HIV-1 infected infants studied. Whilst prevalence of active HCMV or HHV-6 infections did not change significantly with respect to maternal HIV-1 status, high load HCMV infections were significantly (two-fold) more prevalent among HIV-1 exposed infants. The study was confounded however in that infants who were sick were less likely to give a blood sample and more likely to be withdrawn before completing the study. In a subgroup of 23 infants who were referred to hospital, the prevalence of both betaherpesviruses was significantly higher than in that of the general population, and in the case of HCMV higher loads were significantly more prevalent also.

6.3 Betaherpesvirus genotypes

6.3.1 HCMV genotypes

HCMV genes are mostly highly conserved between strains but a significant number of genes exhibit variation and have been extensively used for genotyping (Bradley et al., 2008; Pignatelli, Dal Monte, and Landini, 2001; Stanton et al., 2005). Linkage between these variable loci is rare due to the high frequency of recombination, a key characteristic of the HCMV genome (Haberland, Meyer-Konig, and Hufert, 1999; Rasmussen, Geissler, and Winters, 2003). Furthermore, efforts to link specific disease presentations or outcomes with specific genotypes have generally failed to draw firm conclusions (Arista et al., 2003; Dal Monte et al., 2004; Pignatelli et al., 2003b; Rosen et al., 1998; Rossini et al., 2005; Sarcinella et al., 2002; Trincado et al., 2000). Whilst linkage is rare, two neighbouring hypervariable glycoprotein genes, UL73 and UL74, have been shown to be linked forming eight linked genotypes (Mattick et al., 2004; Yan et al., 2008) and these were used to genotype HCMV in the three cohorts studied in this thesis. Here we have shown that all eight linked genotypes are present in Southern Africa as in other regions, which is distinct from similar studies on other herpesviruses (Kasolo et al., 2007). There may be some subtle trends for certain genotypes to be more prevalent in some regions but this analysis is confounded in that data comes from different clinical sources in different regions: The majority of Japanese sequence comes from congenital and infant primary infections whereas the majority of sequence data from Southern Africa comes from AIDS-related respiratory mortalities. The sequence data from North America and Europe is largely from congenital infections and transplant recipients. In Southern Africa, the epicentre of the AIDS pandemic, putative novel linkages (or mixed infections) were more commonly detected than elsewhere. Whilst this thesis does not present direct evidence of novel recombinants in this region, the combination of a high prevalence of multiple infections, lowered immunity due to HIV-1 or other factors, early HCMV primary infections, and the innate propensity for HCMV strains to recombine, make a strong argument for future studies isolate and sequence whole virus from this region. Whether the linkage between UL73 and UL74 is positional or functional (or both) will require further work using biological assays to exam interactions between these two proteins *in vitro* and *in vivo*. Sequence data from this thesis and elsewhere suggest the possible existence of HCMV strains with defective or truncated glycoproteins. Again, biological assays are required to determine the possible clinical relevance of such variants. I have constructed a C-terminally GFP-labelled gO1b clone as a potential starting point for such analysis. Both UL74 and UL73 are

glycoproteins thought to be involved in cell binding and/or virion egress. The use of labelled clones would allow transfection experiments mismatching gO and gN genotypes in assays looking at tropism, fusion, or intracellular localization. Such work may lead to the construction of recombinant viruses to examine the effects of gO/gN recombinants during natural infection *in vitro*.

6.3.2 HHV-6 genotypes

The molecular epidemiology of HCMV and HHV-6 are quite distinct. Whilst HCMV readily recombines with certain variable loci having up to 14 genotypes (Bradley et al., 2008), HHV-6 is generally known to exist as one of two distinct strain variants, with strong linkage across the entire length of the genome. These two strain variants are termed HHV-6A and HHV-6B. In Europe and North America infant primary infections are overwhelmingly with HHV-6B (Dewhurst et al., 1993; Hall et al., 1994; Zerr et al., 2005). In these regions HHV-6A is a later infection, is more neurotropic (Aberle et al., 1996; Hall et al., 1998) and more prevalent in immunocompromised patients (Ablashi et al., 1998; Iuliano et al., 1997; Nitsche et al., 2001; Secchiero et al., 1995). A previous study of febrile infants had suggested HHV-6A may be more common in Zambia (Kasolo, Mpabalwani, and Gompels, 1997) and in this thesis we confirm this to be true, with the first ever population based study of HHV-6 strain prevalence in this region, demonstrating that HHV-6A is actually the predominant HHV-6 primary infection in this region, being detected in 85% of active HHV-6 infections. This finding was supported by the fact that HHV-6A was detected at a similarly high prevalence and with sequence variation at two loci (U46 and U47). These data suggest that HHV-6A is the primary strain variant in Southern African maybe being better adapted to host genetic factors in these populations, and that elsewhere HHV-6A may be more emergent. Some leading researchers in the HHV-6 field have recently proposed, largely based on clinical data and growth in cell culture differences, the re-classification of HHV-6A and B to HHV-9 and HHV-10 respectively. The data presented in this thesis and previous data from our group (Gompels, 2006) provide some evidence for possible recombination between the two strain variants, and in light of the scarcity of complete genome sequences published on Genbank (there are only three), the proposed re-classification may be slightly premature and studies should be commenced, likely employing next generation sequencing technology, to fully sequence a diverse range of isolates from geographically distinct locations. Whilst this thesis does evidence a unique epidemiology for HHV-6A primary infections in infants from this Southern African region, geographical differences in seroprevalence (Sarmati et al., 2004) and

genotypes (Kasolo et al., 1998; Kasolo, Mpabalwani, and Gompels, 1997) are established for the gammaherpesvirus KSHV, and also for the alphaherpesvirus VZV (Quinlivan et al., 2002). In the case of KSHV, the presence of childhood endemic Kaposi's Sarcoma in Zambia demonstrates how infection with the same herpesvirus can have diverse clinical outcomes in different regions, possibly due to the interplay between host factors and key variable genes.

6.4 CIGNIS morbidity and mortality

CIGNIS is a placebo controlled trial designed to test the efficacy of a micronutrient fortified feed supplement to reduce morbidity and mortality and improve growth and development in HIV-1 exposed uninfected Zambian infants. With the roll out of PMTCT programmes this is an expanding group which have been previously shown to suffer higher morbidity and mortality (Marinda et al., 2007) and impaired growth (Makasa et al., 2007) compared to HIV-1 unexposed uninfected children, with premature cessation of breast feeding implicated as the key causal factor (Arpadi et al., 2009). This thesis assesses the effect of the micronutrient fortified feed supplement, HIV-1 infection and exposure and betaherpesvirus infections, on specific markers of morbidity such as prevalence of anaemia, reported fever and rate of hospital referrals, along with mortality.

HIV-1 infected infants showed significantly weaker neutralizing antibody responses to the polio vaccine and a higher prevalence of anaemia throughout the study, the latter of which was to some extent alleviated by the micronutrient fortified feed supplement. HIV-1 infected infants also suffered significantly higher rates of referral and associated mortalities although numbers were too low to assess the effect of the intervention on these outcomes. HIV-1 exposed infants (compared to HIV-1 unexposed infants) were significantly more likely to have never been breast fed or to terminate breast feeding early as shown previously (Arpadi et al., 2009). Associations with weakened polio vaccine responses were seen in both HIV-1 infected and exposed infants, although increased anaemia seen in HIV-1 infected infants was not observed in HIV-1 exposed infants, with the micronutrient fortified feed supplement reducing anaemia irrespective of HIV-1 exposure. HIV-1 exposed infants showed a general trend towards increased referrals but of great importance, this was significant only in the micronutrient arm of the study, suggesting that the micronutrient fortified feed supplement is combining with HIV-1 exposure to drive referrals. There may be positive effects among HIV-1 unexposed infants though, with a trend towards decreased mortalities in infants on the micronutrient fortified feed supplement.

Detection of active HCMV infections at baseline was associated with a trend towards increased referrals (irrespective of HIV-1 exposure). In this context, active HCMV infection at month 18 and active HHV-6 infection at either time point are scheduled to be analysed for associations with referral rates. HCMV seroprevalence at 18 months of age was associated with a trend towards stronger neutralizing antibody responses to the polio vaccine although any such benefit was negated among HIV-1 exposed infants. HCMV seroprevalence was also associated with a roughly 50% increase in the prevalence of anaemia throughout the study (at both time points). This could be an observation of a direct causal effect of HCMV metabolizing dietary iron (Crowe et al., 2004) but there could be additional indirect effects such as increased prevalence of other infections known to cause anaemia such as Malaria, among HCMV seropositive infants. The micronutrient fortified feed supplement reduced levels of anaemia in both HCMV seropositive and seronegative infants.

Broadly, the micronutrient fortified feed supplement did not significantly reduce the rate of referrals or the prevalence of reported fever, betaherpesvirus active infections or mortalities. The intervention was successful at reducing the prevalence of anaemia overall, but among HIV-1 exposed, HCMV seropositive infants the intervention appears to be driving hospitalization, with raised iron levels possibly causing oxidative stress and exacerbating infection.

6.5 CIGNIS growth

The CIGNIS study was not powered to examine HIV-1 infected children but even in this small group, there was some evidence of fat redistribution in the small group of HIV-1 infected infants on the study which has been previously documented (Taylor et al., 2004). Interestingly, persistent stunting or wasting were not associated with HIV-1 infection but it could be that significant numbers of this group were withdrawn from the study or died. Conversely, HIV-1 exposed infants showed significantly impaired growth by almost all anthropometric measures compared to HIV-1 unexposed infants as suggested in this region previously (Makasa et al., 2007).

The micronutrient fortified feed supplement did not appear to significantly affect growth parameters in either HIV-1 unexposed or exposed infants, the association of HIV-1 exposure with wasting was observed only on the micronutrient arm of the study. This effect is the result

of divergent trends, with the micronutrient reducing wasting in HIV-1 unexposed, but increasing it in HIV-1 exposed infants. This same divergent trend is seen for length. What component of the micronutrient fortified feed supplement could be responsible for this differential growth interaction with HIV-1 exposure? Of the markers assessed in this study the only significant effect of the micronutrient overall was its ability to reduce anaemia, likely through provision of a 40-fold increase in the concentration of dietary iron. An excess of iron can be detrimental and it is possible that HIV-1 exposed infants are less well equipped to maintain iron homeostasis, due to malfunctions in intestinal transport, extracellular and intracellular regulation (Collard, 2009), and that this affects growth as well as the prevalence of infections as discussed above. However, more detailed analyses by other members of the CIGNIS team found the micronutrient fortification was able to reduce stunting in a sub-group of HIV-1 exposed infants, those who stopped breast feeding before 6 months of age (Filteau, 2009b).

Active HCMV infections at baseline were associated with some short term decreases in length and peripheral fat and stunting at 18 months in HIV-1 exposed infants. HCMV seroprevalence was associated with long term stunting and wasting, and with breast milk shown here (as elsewhere) to be a common mode of transmission, extended breast feeding is a risk factor for MTCT of HCMV and associated growth inhibition. Interestingly, among HIV-1 exposed infants only, HCMV seroprevalence was also associated with a long term inhibition on head circumference, the developmental effects of which are under investigation through analysis of Bailey testing results, and initial findings show that decreased motor skills are associated with decreased head circumference and so it is possible that this will correlate with HCMV seroprevalence.

6.6 Future Work

There is still much work to be done on the CIGNIS project analysing data from Bailey testing, micronutrient and immunological data, and also long term follow up for possible effects of the intervention on mortalities. More broadly within the context of the betaherpesvirus infections presented in this thesis, there will be further multivariate adjusted analysis of HHV-6 and HCMV results with HIV-1 and the micronutrient intervention. More genotyping data will be collected for HCMV and particularly for HHV-6 which is a distinct strain variant causing infant primary infections in this region. Next generation sequencing technology could be used to complete several whole genome sequences for HHV-6 strains isolated in this

region and elsewhere, and could also be used to sequence the whole gO/gN locus of HCMV from archived strains and/or new cohorts. One could then assess whether increased prevalence of multiple infections, higher viral loads and reduced immune surveillance due to HIV, may be facilitating the emergence of novel recombinant HCMV or HHV-6 strains, which may be causing infant infections here.

Data from this thesis form the basis and rationale for possible functional assays to determine the role of hypervariable HCMV glycoproteins such as gO and gN. We have constructed a C-terminally labelled gO1b-GFP clone as an initial step in this direction, with a view to investigating the effects of gO/gN mismatches on cell-cell fusion or other functional assays. Results of such experiments may lead to the use of bacmids to construct recombinant viruses with 'mismatched' gO and gN genotypes for use in infection studies in different cell types or receptor blocking experiments to investigate virus-cell tropism. Immune-precipitation experiments could be used to look for interactions between gO or gN and other novel glycoproteins, many of which remain to be characterised.

Both HCMV and HHV-6 are significant pathogens in HIV/AIDS and use of real time PCR to monitor loads in different body fluids or compartments may be used to identify active infections which would respond to treatment should drugs such as oral gancyclovir become available in Zambia. With overwhelming betaherpesvirus lung involvement demonstrated here associated with late stage AIDS, treatment with anti-betaherpesvirus drugs may be appropriate for HIV/AIDS patients who are failing to respond to treatment for bacterial pneumonias. The rate of congenital HCMV infection in Zambia is not known and we hope that Guthrie cards may be available for a subset of the CIGNIS cohort to establish this and to investigate its effects on morbidity, growth and mental development.

Vaccine trials for HCMV have focussed entirely on populations with low HCMV seroprevalence, with adolescent females being the target group with a view to preventing primary infection during pregnancy and resulting congenital infection. These have been largely phase I and phase II trials, but if a vaccination strategy does eventually prove to be safe and effective, it may be of little relevance to populations of high HCMV seroprevalence. In countries like Zambia, vaccination would have to be administered in early infancy, and protection, likely with the need of periodical boosters, would have to be maintained throughout childhood and into the child bearing years. Another intervention may be the use of anti-betaherpesvirus drugs to treat HCMV viraemia in pregnant mothers to reduce the rate of

congenital infection, breast milk transmission and morbidity caused by infant primary infections, but an effect vaccine would be more economically viable.

7.0 Conclusions

This study of HCMV and HHV-6 infections in Zambian children has revealed some unique features of these infections. The distribution of HCMV genotypes differed little from that seen elsewhere but the predominance of HHV-6 variant A in this region, in healthy infants, is novel and suggests this is an emergent infection elsewhere. HCMV infection has been associated with increased morbidity and impaired growth in Zambian infants, and breast feeding is a risk factor for early transmission. Data suggest that these atypical early HCMV infections are factors in the poor development of HIV-1 exposed infants here. The micronutrient fortification did not affect these infections in HIV-1 exposed infants, although it did improve growth in those who were not breast fed.

8.0 References

Abba, K., Sinfield, R., Hart, C. A., and Garner, P. (2009). Pathogens associated with persistent diarrhoea in children in low and middle income countries: systematic review. *BMC Infect Dis* **9**, 88.

Aberle, S. W., Mandl, C. W., Kunz, C., and Popow-Kraupp, T. (1996). Presence of human herpesvirus 6 variants A and B in saliva and peripheral blood mononuclear cells of healthy adults. *J Clin Microbiol* **34**(12), 3223-5.

Ablashi, D. V. (2006a). Discovery and Classification of Human Herpesvirus-6 (HHV-6). 1 ed. *In* "Human Herpesvirus-6" (A. D. Krueger G, Ed.), Vol. 12, pp. 3-10. Elsevier, Oxford.

Ablashi, D. V. (2006b). Strain Variants of HHV-6. 1 ed. *In* "human Herpesvirus-6" (A. D. Krueger G, Ed.), Vol. 12, pp. 79-89. Elsevier, Oxford.

Ablashi, D. V., Marsh, S., Kaplan, M., Whitman, J. E., Jr., and Pearson, G. R. (1998). HHV-6 infection in HIV-infected asymptomatic and AIDS patients. *Intervirology* **41**(1), 1-9.

Adams, O., Krempe, C., Kogler, G., Wernet, P., and Scheid, A. (1998). Congenital infections with human herpesvirus 6. *J Infect Dis* **178**(2), 544-6.

Adetifa, I., and Okomo, U. (2009). Iron supplementation for reducing morbidity and mortality in children with HIV. *Cochrane Database Syst Rev*(1), CD006736.

Adler, S. P., Nigro, G., and Pereira, L. (2007). Recent advances in the prevention and treatment of congenital cytomegalovirus infections. *Semin Perinatol* **31**(1), 10-8.

Adler, S. P., Starr, S. E., Plotkin, S. A., Hempfling, S. H., Buis, J., Manning, M. L., and Best, A. M. (1995). Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age. *J Infect Dis* **171**(1), 26-32.

Ahlqvist, J., Fotheringham, J., Akhyani, N., Yao, K., Fogdell-Hahn, A., and Jacobson, S. (2005). Differential tropism of human herpesvirus 6 (HHV-6) variants and induction of latency by HHV-6A in oligodendrocytes. *J Neurovirol* **11**(4), 384-94.

Akkapaiboon, P., Mori, Y., Sadaoka, T., Yonemoto, S., and Yamanishi, K. (2004). Intracellular processing of human herpesvirus 6 glycoproteins Q1 and Q2 into tetrameric complexes expressed on the viral envelope. *J Virol* **78**(15), 7969-83.

Alford, C. A., Stagno, S., Pass, R. F., and Britt, W. J. (1990). Congenital and perinatal cytomegalovirus infections. *Rev Infect Dis* **12 Suppl 7**, S745-53.

Alvarez-Lafuente, R., De las Heras, V., Bartolome, M., Picazo, J. J., and Arroyo, R. (2004). Relapsing-remitting multiple sclerosis and human herpesvirus 6 active infection. *Arch Neurol* **61**(10), 1523-7.

Alvarez-Lafuente, R., de las Heras, V., Garcia-Montojo, M., Bartolome, M., and Arroyo, R. (2007). Human herpesvirus-6 and multiple sclerosis: relapsing-remitting versus secondary progressive. *Mult Scler* **13**(5), 578-83.

Alvarez-Lafuente, R., Garcia-Montojo, M., De las Heras, V., Bartolome, M., and Arroyo, R. (2006). Clinical parameters and HHV-6 active replication in relapsing-remitting multiple sclerosis patients. *J Clin Virol* **37 Suppl 1**, S24-6.

Amon, W., and Farrell, P. J. (2005). Reactivation of Epstein-Barr virus from latency. *Rev Med Virol* **15**(3), 149-56.

Anderson, J. W., Johnstone, B. M., and Remley, D. T. (1999). Breast-feeding and cognitive development: a meta-analysis. *Am J Clin Nutr* **70**(4), 525-35.

Applied_Biosystems (2003). Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR, pp. 8.

Arista, S., De Grazia, S., Giammanco, G. M., Di Carlo, P., and Iannitto, E. (2003). Human cytomegalovirus glycoprotein B genotypes in immunocompetent,

immunocompromised, and congenitally infected Italian populations. *Arch Virol* **148**(3), 547-54.

Arpadi, S., Fawzy, A., Aldrovandi, G. M., Kankasa, C., Sinkala, M., Mwiya, M., Thea, D. M., and Kuhn, L. (2009). Growth faltering due to breastfeeding cessation in uninfected children born to HIV-infected mothers in Zambia. *Am J Clin Nutr* **90**(2), 344-53.

Arvin, A. M., Fast, P., Myers, M., Plotkin, S., and Rabinovich, R. (2004). Vaccine development to prevent cytomegalovirus disease: report from the National Vaccine Advisory Committee. *Clin Infect Dis* **39**(2), 233-9.

Asada, H., Klaus-Kovtun, V., Golding, H., Katz, S. I., and Blauvelt, A. (1999). Human herpesvirus 6 infects dendritic cells and suppresses human immunodeficiency virus type 1 replication in coinfected cultures. *J Virol* **73**(5), 4019-28.

Asahi-Ozaki, Y., Sato, Y., Kanno, T., Sata, T., and Katano, H. (2006). Quantitative analysis of Kaposi sarcoma-associated herpesvirus (KSHV) in KSHV-associated diseases. *J Infect Dis* **193**(6), 773-82.

Asano, Y., Yoshikawa, T., Suga, S., Kobayashi, I., Nakashima, T., Yazaki, T., Kajita, Y., and Ozaki, T. (1994). Clinical features of infants with primary human herpesvirus 6 infection (exanthem subitum, roseola infantum). *Pediatrics* **93**(1), 104-8.

Ashshi, A. M., Klapper, P. E., and Cooper, R. J. (2003). Detection of human cytomegalovirus, human herpesvirus type 6 and human herpesvirus type 7 in urine specimens by multiplex PCR. *J Infect* **47**(1), 59-64.

Atalay, R., Zimmermann, A., Wagner, M., Borst, E., Benz, C., Messerle, M., and Hengel, H. (2002). Identification and expression of human cytomegalovirus transcription units coding for two distinct Fcgamma receptor homologs. *J Virol* **76**(17), 8596-608.

Ataman, S., Colak, D., Gunseren, F., Senol, Y., Colak, T., Aktekin, M. R., and Gultekin, M. (2007). [Investigation of cytomegalovirus seroepidemiology in Antalya with a population-based cross-sectional study and review of related data in Turkey]. *Mikrobiyol Bul* **41**(4), 545-55.

Baines, J. D., and Roizman, B. (1991). The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. *J Virol* **65**(2), 938-44.

Bale, J. F., Jr., Petheram, S. J., Robertson, M., Murph, J. R., and Demmler, G. (2001). Human cytomegalovirus a sequence and UL144 variability in strains from infected children. *J Med Virol* **65**(1), 90-6.

Bale, J. F., Jr., Zimmerman, B., Dawson, J. D., Souza, I. E., Petheram, S. J., and Murph, J. R. (1999). Cytomegalovirus transmission in child care homes. *Arch Pediatr Adolesc Med* **153**(1), 75-9.

Barbi, M., Binda, S., Caroppo, S., Calvario, A., Germinario, C., Bozzi, A., Tanzi, M. L., Veronesi, L., Mura, I., Piana, A., Solinas, G., Pugni, L., Bevilaqua, G., and Mosca, F. (2006). Multicity Italian study of congenital cytomegalovirus infection. *Pediatr Infect Dis J* **25**(2), 156-9.

Bates, M., Monze, M., Bima, H., Kapambwe, M., Clark, D., Kasolo, F. C., and Gompels, U. A. (2009). Predominant human herpesvirus 6 variant A infant infections in an HIV-1 endemic region of Sub-Saharan Africa. *J Med Virol* **81**(5), 779-89.

Bates, M., Monze, M., Bima, H., Kapambwe, M., Kasolo, F. C., and Gompels, U. A. (2008). High human cytomegalovirus loads and diverse linked variable genotypes in both HIV-1 infected and exposed, but uninfected, children in Africa. *Virology* **382**(1), 28-36.

Becker, Y. (2007). The spreading of HIV-1 infection in the human organism is caused by fractalkine trafficking of the infected lymphocytes--a review, hypothesis and implications for treatment. *Virus Genes* **34**(2), 93-109.

Bego, M., Maciejewski, J., Khaiboullina, S., Pari, G., and St Jeor, S. (2005). Characterization of an antisense transcript spanning the UL81-82 locus of human cytomegalovirus. *J Virol* **79**(17), 11022-34.

Bello, C., and Whittle, H. (1991). Cytomegalovirus infection in Gambian mothers and their babies. *J Clin Pathol* **44**(5), 366-9.

Bello, C. S. (1992). Transmission of cytomegalovirus in the Gambia. *West Afr J Med* **11**(2), 140-5.

Ben-Smith, A., Gorak-Stolinska, P., Floyd, S., Weir, R. E., Lalor, M. K., Mvula, H., Crampin, A. C., Wallace, D., Beverley, P. C., Fine, P. E., and Dockrell, H. M. (2008). Differences between naive and memory T cell phenotype in Malawian and UK adolescents: a role for Cytomegalovirus? *BMC Infect Dis* **8**, 139.

Beyari, M. M., Hodgson, T. A., Kondowe, W., Molyneux, E. M., Scully, C., Porter, S. R., and Teo, C. G. (2005). Inter- and intra-person cytomegalovirus infection in Malawian families. *J Med Virol* **75**(4), 575-82.

Bhattarakosol, P., Pancharoen, C., Mekmullica, J., and Bhattarakosol, P. (2001). Seroprevalence of anti-human herpes virus-6 IgG antibody in children of Bangkok, Thailand. *Southeast Asian J Trop Med Public Health* **32**(1), 143-7.

Biancotto, A., Iglehart, S. J., Lisco, A., Vanpouille, C., Grivel, J. C., Lurain, N. S., Reichelderfer, P. S., and Margolis, L. B. (2008). Upregulation of human cytomegalovirus by HIV type 1 in human lymphoid tissue ex vivo. *AIDS Res Hum Retroviruses* **24**(3), 453-62.

Biemba, G., Dolmans, D., Thuma, P. E., Weiss, G., and Gordeuk, V. R. (2000). Severe anaemia in Zambian children with Plasmodium falciparum malaria. *Trop Med Int Health* **5**(1), 9-16.

Bigalke, B., Klingel, K., May, A. E., Kandolf, R., and Gawaz, M. G. (2007). Human herpesvirus 6 subtype A-associated myocarditis with 'apical ballooning'. *Can J Cardiol* **23**(5), 393-5.

Birx, D. L., Redfield, R. R., and Tosato, G. (1986). Defective regulation of Epstein-Barr virus infection in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related disorders. *N Engl J Med* **314**(14), 874-9.

Boivin, G., Handfield, J., Toma, E., Murray, G., Lalonde, R., Tevere, V. J., Sun, R., and Bergeron, M. G. (1998). Evaluation of the AMPLICOR cytomegalovirus test with specimens from human immunodeficiency virus-infected subjects. *J Clin Microbiol* **36**(9), 2509-13.

Bonnafous, P., Naesens, L., Petrella, S., Gautheret-Dejean, A., Boutolleau, D., Sougakoff, W., and Agut, H. (2007). Different mutations in the HHV-6 DNA polymerase gene accounting for resistance to foscarnet. *Antivir Ther* **12**(6), 877-88.

Bradley, A. J., Kovacs, I. J., Gatherer, D., Dargan, D. J., Alkharsah, K. R., Chan, P. K., Carman, W. F., Dedicoat, M., Emery, V. C., Geddes, C. C., Gerna, G., Ben-Ismaeil, B., Kaye, S., McGregor, A., Moss, P. A., Pusztai, R., Rawlinson, W. D., Scott, G. M., Wilkinson, G. W., Schulz, T. F., and Davison, A. J. (2008). Genotypic analysis of two hypervariable human cytomegalovirus genes. *J Med Virol* **80**(9), 1615-23.

Brady, M. T., Reiner, C. B., Singley, C., Roberts, W. H., 3rd, and Sneddon, J. M. (1988). Unexpected death in an infant with AIDS: disseminated cytomegalovirus infection with pancarditis. *Pediatr Pathol* **8**(2), 205-14.

Brestrich, G., Zwinger, S., Fischer, A., Schmuck, M., Rohmhild, A., Hammer, M. H., Kurtz, A., Uharek, L., Knosalla, C., Lehmkuhl, H., Volk, H. D., and Reinke, P. (2009). Adoptive T-cell therapy of a lung transplanted patient with severe CMV disease and resistance to antiviral therapy. *Am J Transplant* **9**(7), 1679-84.

Britt, W. (2008). Manifestations of human cytomegalovirus infection: proposed mechanisms of acute and chronic disease. *Curr Top Microbiol Immunol* **325**, 417-70.

Buser, C., Walther, P., Mertens, T., and Michel, D. (2007). Cytomegalovirus primary envelopment occurs at large infoldings of the inner nuclear membrane. *J Virol* **81**(6), 3042-8.

Cannon, M. J., and Davis, K. F. (2005). Washing our hands of the congenital cytomegalovirus disease epidemic. *BMC Public Health* **5**, 70.

Carre, N., Robain, M., Dussaix, E., Salmon-Ceron, D., and Meyer, L. (1997). Sexual factors associated with cytomegalovirus seropositivity in human immunodeficiency virus-infected men. The Seroco Study Group. *Sex Transm Dis* **24**(10), 582-6.

Carrigan, D. R., Knox, K. K., and Tapper, M. A. (1990). Suppression of human immunodeficiency virus type 1 replication by human herpesvirus-6. *J Infect Dis* **162**(4), 844-51.

Casarosa, P., Bakker, R. A., Verzijl, D., Navis, M., Timmerman, H., Leurs, R., and Smit, M. J. (2001). Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. *J Biol Chem* **276**(2), 1133-7.

Casarosa, P., Waldhoer, M., LiWang, P. J., Vischer, H. F., Kledal, T., Timmerman, H., Schwartz, T. W., Smit, M. J., and Leurs, R. (2005). CC and CX3C chemokines differentially interact with the N terminus of the human cytomegalovirus-encoded US28 receptor. *J Biol Chem* **280**(5), 3275-85.

Caselli, E., Bracci, A., Galvan, M., Boni, M., Rotola, A., Bergamini, C., Cermelli, C., Dal Monte, P., Gompels, U. A., Cassai, E., and Di Luca, D. (2006). Human herpesvirus 6 (HHV-6) U94/REP protein inhibits betaherpesvirus replication. *Virology* **346**(2), 402-14.

Catusse, J., Parry, C. M., Dewin, D. R., and Gompels, U. A. (2007). Inhibition of HIV-1 infection by viral chemokine U83A via high-affinity CCR5 interactions that block human chemokine-induced leukocyte chemotaxis and receptor internalization. *Blood* **109**(9), 3633-9.

Catusse, J., Spinks, J., Mattick, C., Dyer, A., Laing, K., Fitzsimons, C., Smit, M. J., and Gompels, U. A. (2008). Immunomodulation by herpesvirus U51A chemokine receptor via CCL5 and FOG-2 down-regulation plus XCR1 and CCR7 mimicry in human leukocytes. *Eur J Immunol* **38**(3), 763-77.

Cesar, J. A., Victora, C. G., Barros, F. C., Santos, I. S., and Flores, J. A. (1999). Impact of breast feeding on admission for pneumonia during postneonatal period in Brazil: nested case-control study. *Bmj* **318**(7194), 1316-20.

Cha, T. A., Tom, E., Kemble, G. W., Duke, G. M., Mocarski, E. S., and Spaete, R. R. (1996). Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J Virol* **70**(1), 78-83.

Chakraborty, R., Rees, G., Bourboulia, D., Cross, A. M., Dixon, J. R., D'Agostino, A., Musoke, R., Boshoff, C., Rowland-Jones, S. L., and Klenerman, P. (2003). Viral coinfections among African children infected with human immunodeficiency virus type 1. *Clin Infect Dis* **36**(7), 922-4.

Challacombe, J. F., Rechtsteiner, A., Gottardo, R., Rocha, L. M., Browne, E. P., Shenk, T., Altherr, M. R., and Brettin, T. S. (2004). Evaluation of the host transcriptional response to human cytomegalovirus infection. *Physiol Genomics* **18**(1), 51-62.

Chandran, B., Tirawatnapong, S., Pfeiffer, B., and Ablashi, D. V. (1992). Antigenic relationships among human herpesvirus-6 isolates. *J Med Virol* **37**(4), 247-54.

Chandwani, S., Kaul, A., Bebenroth, D., Kim, M., John, D. D., Fidelia, A., Hassel, A., Borkowsky, W., and Krasinski, K. (1996). Cytomegalovirus infection in human immunodeficiency virus type 1-infected children. *Pediatr Infect Dis J* **15**(4), 310-4.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**(5192), 1865-9.

Cheeran, M. C., Lokensgard, J. R., and Schleiss, M. R. (2009). Neuropathogenesis of congenital cytomegalovirus infection: disease mechanisms and prospects for intervention. *Clin Microbiol Rev* **22**(1), 99-126, Table of Contents.

Chen, H. P., Lin, J. C., Yang, S. P., Lan, Y. C., Weng, W. S., Tsai, C. H., Ho, D. M., Liu, C. Y., Cho, W. L., and Chan, Y. J. (2008). The type-2 variant of human cytomegalovirus glycoprotein N (gN-2) is not the rarest in the Chinese population of Taiwan: influence of primer design. *J Virol Methods* **151**(1), 161-4.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31**(13), 3497-500.

Chidrawar, S., Khan, N., Wei, W., McLarnon, A., Smith, N., Nayak, L., and Moss, P. (2009). Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. *Clin Exp Immunol* **155**(3), 423-32.

Chilongozi, D., Wang, L., Brown, L., Taha, T., Valentine, M., Emel, L., Sinkala, M., Kafulafula, G., Noor, R. A., Read, J. S., Brown, E. R., Goldenberg, R. L., and Hoffman, I. (2008). Morbidity and mortality among a cohort of human immunodeficiency virus type 1-infected and uninfected pregnant women and their infants from Malawi, Zambia, and Tanzania. *Pediatr Infect Dis J* **27**(9), 808-14.

Chintu, C., Mudenda, V., Lucas, S., Nunn, A., Lishimpi, K., Maswahu, D., Kasolo, F., Mwaba, P., Bhat, G., Terunuma, H., and Zumla, A. (2002). Lung diseases at necropsy in African children dying from respiratory illnesses: a descriptive necropsy study. *Lancet* **360**(9338), 985-90.

Chua, K. B., Khairullah, N. S., and Hooi, P. S. (1996). Seroepidemiology of human herpesvirus 6 in a population seen in the University Hospital, Kuala Lumpur, Malaysia. *Southeast Asian J Trop Med Public Health* **27**(1), 91-5.

Cinatl, J., Scholz, M., Weber, B., Cinatl, J., Rabenau, H., Markus, B. H., Encke, A., and Doerr, H. W. (1995). Effects of desferrioxamine on human cytomegalovirus replication and expression of HLA antigens and adhesion molecules in human vascular endothelial cells. *Transpl Immunol* **3**(4), 313-20.

Clark, D. A., Ait-Khaled, M., Wheeler, A. C., Kidd, I. M., McLaughlin, J. E., Johnson, M. A., Griffiths, P. D., and Emery, V. C. (1996). Quantification of human herpesvirus 6 in immunocompetent persons and post-mortem tissues from AIDS patients by PCR. *J Gen Virol* **77 (Pt 9)**, 2271-5.

Clark, D. A., Nacheva, E. P., Leong, H. N., Brazma, D., Li, Y. T., Tsao, E. H., Buyck, H. C., Atkinson, C. E., Lawson, H. M., Potter, M. N., and Griffiths, P. D. (2006). Transmission of integrated human herpesvirus 6 through stem cell transplantation: implications for laboratory diagnosis. *J Infect Dis* **193**(7), 912-6.

Clouse, K. A., Robbins, P. B., Fernie, B., Ostrove, J. M., and Fauci, A. S. (1989). Viral antigen stimulation of the production of human monokines capable of regulating HIV1 expression. *J Immunol* **143**(2), 470-5.

Coaquette, A., Bourgeois, A., Dirand, C., Varin, A., Chen, W., and Herbein, G. (2004). Mixed cytomegalovirus glycoprotein B genotypes in immunocompromised patients. *Clin Infect Dis* **39**(2), 155-61.

Coetzee, D., Hilderbrand, K., Boulle, A., Draper, B., Abdullah, F., and Goemaere, E. (2005). Effectiveness of the first district-wide programme for the prevention of mother-to-child transmission of HIV in South Africa. *Bull World Health Organ* **83**(7), 489-94.

Collard, K. J. (2009). Iron homeostasis in the neonate. *Pediatrics* **123**(4), 1208-16.

Collot, S., Petit, B., Bordessoule, D., Alain, S., Touati, M., Denis, F., and Ranger-Rogez, S. (2002). Real-time PCR for quantification of human herpesvirus 6 DNA from lymph nodes and saliva. *J Clin Microbiol* **40**(7), 2445-51.

Colugnati, F. A., Staras, S. A., Dollard, S. C., and Cannon, M. J. (2007). Incidence of cytomegalovirus infection among the general population and pregnant women in the United States. *BMC Infect Dis* **7**, 71.

Consolini, R., Bencivelli, W., Legitimo, A., Galli, L., Tovo, P., Gabiano, C., and De Martino, M. (2007). A pre-HAART follow-up study of the hematologic manifestations in children with perinatal HIV-1 infection: suggestions for reclassification of clinical staging. *J Pediatr Hematol Oncol* **29**(6), 376-83.

Coovadia, H. M., and Bland, R. M. (2007). Preserving breastfeeding practice through the HIV pandemic. *Trop Med Int Health* **12**(9), 1116-33.

Cosman, D., Fanger, N., and Borges, L. (1999). Human cytomegalovirus, MHC class I and inhibitory signalling receptors: more questions than answers. *Immunol Rev* **168**, 177-85.

Creek, T. L., Kim, A., Lu, L., Bowen, A., Masunge, J., Arvelo, W., Smit, M., Mach, O., Legwaila, K., Motswere, C., Zaks, L., Finkbeiner, T., Povinelli, L., Maruping, M., Ngwaru, G., Tebele, G., Bopp, C., Puhr, N., Johnston, S. P., Dasilva, A. J., Bern, C., Beard, R. S., and Davis, M. K. (2009). Hospitalization and Mortality Among Primarily Non-breastfed Children During a Large Outbreak of Diarrhea and Malnutrition in Botswana, 2006. *J Acquir Immune Defic Syndr*.

Crowe, W. E., Maglova, L. M., Ponka, P., and Russell, J. M. (2004). Human cytomegalovirus-induced host cell enlargement is iron dependent. *Am J Physiol Cell Physiol* **287**(4), C1023-30.

CSO_Zambia (2000). Projected Mid-year Population, pp. 6. Central Statistical Office Zambia.

CSO_Zambia (2007). HIV Prevalence Decline (C. S. O. Zambia, Ed.), pp. 1. Zambia, Central Statistical Office, Lusaka.

Cunningham, C., Gatherer, D., Hilfrich, B., Baluchova, K., Dargan, D. J., Thomson, M., Griffiths, P. D., Wilkinson, G. W., Schulz, T. F., and Davison, A. J. (2009). Sequences of complete human cytomegalovirus genomes from infected cell cultures and clinical specimens. *J Gen Virol*.

Dahl, H., Fjaertoft, G., Norsted, T., Wang, F. Z., Mousavi-Jazi, M., and Linde, A. (1999). Reactivation of human herpesvirus 6 during pregnancy. *J Infect Dis* **180**(6), 2035-8.

Daibata, M., Taguchi, T., Kubonishi, I., Taguchi, H., and Miyoshi, I. (1998). Lymphoblastoid cell lines with integrated human herpesvirus type 6. *J Hum Virol* **1**(7), 475-81.

Daibata, M., Taguchi, T., Nemoto, Y., Taguchi, H., and Miyoshi, I. (1999). Inheritance of chromosomally integrated human herpesvirus 6 DNA. *Blood* **94**(5), 1545-9.

Dal Monte, P., Pignatelli, S., Rossini, G., and Landini, M. P. (2004). Genomic variants among human cytomegalovirus (HCMV) clinical isolates: the glycoprotein n (gN) paradigm. *Hum Immunol* **65**(5), 387-94.

Dal Monte, P., Pignatelli, S., Zini, N., Maraldi, N. M., Perret, E., Prevost, M. C., and Landini, M. P. (2002). Analysis of intracellular and intraviral localization of the human cytomegalovirus UL53 protein. *J Gen Virol* **83**(Pt 5), 1005-12.

Darwich, L., Cabrera, C., Romeu, J., Martinez-Picado, J., Este, J. A., Tural, C., Bellido, R., Clotet, B., Angulo, A., Ruiz, L., and Bofill, M. (2008). The magnitude of interferon-gamma responses to human cytomegalovirus is predictive for HIV-1 disease progression. *J Acquir Immune Defic Syndr* **49**(5), 507-12.

Davis, M. G., Kenney, S. C., Kamine, J., Pagano, J. S., and Huang, E. S. (1987). Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. *Proc Natl Acad Sci U S A* **84**(23), 8642-6.

Davison, A. J., Dolan, A., Akter, P., Addison, C., Dargan, D. J., Alcendor, D. J., McGeoch, D. J., and Hayward, G. S. (2003). The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. *J Gen Virol* **84**(Pt 1), 17-28.

De Bolle, L., Van Loon, J., De Clercq, E., and Naesens, L. (2005). Quantitative analysis of human herpesvirus 6 cell tropism. *J Med Virol* **75**(1), 76-85.

De Castro, S., Garcia-Aparicio, C., Andrei, G., Snoeck, R., Balzarini, J., Camarasa, M. J., and Velazquez, S. (2009). 4-Benzyloxy-gamma-sultone derivatives: discovery of a novel family of non-nucleoside inhibitors of human cytomegalovirus and varicella zoster virus. *J Med Chem* **52**(6), 1582-91.

de Martino, M., Tovo, P. A., Tozzi, A. E., Pezzotti, P., Galli, L., Livadiotti, S., Caselli, D., Massironi, E., Ruga, E., Fioredda, F., and et al. (1992). HIV-1 transmission through breast-milk: appraisal of risk according to duration of feeding. *Aids* **6**(9), 991-7.

DesJardin, J. A., Gibbons, L., Cho, E., Supran, S. E., Falagas, M. E., Werner, B. G., and Snydman, D. R. (1998). Human herpesvirus 6 reactivation is associated with cytomegalovirus infection and syndromes in kidney transplant recipients at risk for primary cytomegalovirus infection. *J Infect Dis* **178**(6), 1783-6.

Dewhurst, S., McIntyre, K., Schnabel, K., and Hall, C. B. (1993). Human herpesvirus 6 (HHV-6) variant B accounts for the majority of symptomatic primary HHV-6 infections in a population of U.S. infants. *J Clin Microbiol* **31**(2), 416-8.

Dewin, D. R., Catusse, J., and Gompels, U. A. (2006). Identification and characterization of U83A viral chemokine, a broad and potent beta-chemokine agonist for human CCRs with unique selectivity and inhibition by spliced isoform. *J Immunol* **176**(1), 544-56.

Di Luca, D., Mirandola, P., Ravaioli, T., Dolcetti, R., Frigatti, A., Bovenzi, P., Sighinolfi, L., Monini, P., and Cassai, E. (1995). Human herpesviruses 6 and 7 in salivary glands and shedding in saliva of healthy and human immunodeficiency virus positive individuals. *J Med Virol* **45**(4), 462-8.

Distefano, A. L., Alonso, A., Martin, F., and Pardon, F. (2004). Human cytomegalovirus: detection of congenital and perinatal infection in Argentina. *BMC Pediatr* **4**, 11.

Dockrell, D. H., Prada, J., Jones, M. F., Patel, R., Badley, A. D., Harmsen, W. S., Ilstrup, D. M., Wiesner, R. H., Krom, R. A., Smith, T. F., and Paya, C. V. (1997). Seroconversion to human herpesvirus 6 following liver transplantation is a marker of cytomegalovirus disease. *J Infect Dis* **176**(5), 1135-40.

Dolan, A., Cunningham, C., Hector, R. D., Hassan-Walker, A. F., Lee, L., Addison, C., Dargan, D. J., McGeoch, D. J., Gatherer, D., Emery, V. C., Griffiths, P. D., Sinzger, C., McSharry, B. P., Wilkinson, G. W., and Davison, A. J. (2004). Genetic content of wild-type human cytomegalovirus. *J Gen Virol* **85**(Pt 5), 1301-12.

Dolken, L., Pfeffer, S., and Koszinowski, U. H. (2009). Cytomegalovirus microRNAs. *Virus Genes* **38**(3), 355-64.

Dominguez, G., Dambaugh, T. R., Stamey, F. R., Dewhurst, S., Inoue, N., and Pellett, P. E. (1999). Human herpesvirus 6B genome sequence: coding content and comparison with human herpesvirus 6A. *J Virol* **73**(10), 8040-52.

Dorig, R. E., Marcil, A., Chopra, A., and Richardson, C. D. (1993). The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell* **75**(2), 295-305.

Dowd, J. B., Aiello, A. E., and Alley, D. E. (2009). Socioeconomic disparities in the seroprevalence of cytomegalovirus infection in the US population: NHANES III. *Epidemiol Infect* **137**(1), 58-65.

Downing, R. G., Sewankambo, N., Serwadda, D., Honess, R., Crawford, D., Jarrett, R., and Griffin, B. E. (1987). Isolation of human lymphotropic herpesviruses from Uganda. *Lancet* **2**(8555), 390.

Dunn, W., Chou, C., Li, H., Hai, R., Patterson, D., Stolc, V., Zhu, H., and Liu, F. (2003). Functional profiling of a human cytomegalovirus genome. *Proc Natl Acad Sci U S A* **100**(24), 14223-8.

Dunne, W. M., Jr., and Jevon, M. (1993). Examination of human breast milk for evidence of human herpesvirus 6 by polymerase chain reaction. *J Infect Dis* **168**(1), 250.

Dworsky, M., Yow, M., Stagno, S., Pass, R. F., and Alford, C. (1983). Cytomegalovirus infection of breast milk and transmission in infancy. *Pediatrics* **72**(3), 295-9.

Emery, V. C. (2001). Cytomegalovirus and the aging population. *Drugs Aging* **18**(12), 927-33.

Emery, V. C., Atkins, M. C., Bowen, E. F., Clark, D. A., Johnson, M. A., Kidd, I. M., McLaughlin, J. E., Phillips, A. N., Strappe, P. M., and Griffiths, P. D. (1999). Interactions between beta-herpesviruses and human immunodeficiency virus in vivo: evidence for increased human immunodeficiency viral load in the presence of human herpesvirus 6. *J Med Virol* **57**(3), 278-82.

Fadnes, L. T., Engebretsen, I. M., Wamani, H., Semiyaga, N. B., Tylleskar, T., and Tumwine, J. K. (2009). Infant feeding among HIV-positive mothers and the general population mothers: comparison of two cross-sectional surveys in Eastern Uganda. *BMC Public Health* **9**, 124.

Fairfax, M. R., Schacker, T., Cone, R. W., Collier, A. C., and Corey, L. (1994). Human herpesvirus 6 DNA in blood cells of human immunodeficiency virus-infected men: correlation of high levels with high CD4 cell counts. *J Infect Dis* **169**(6), 1342-5.

Farrell, H. E., Degli-Esposti, M. A., and Davis-Poynter, N. J. (1999). Cytomegalovirus evasion of natural killer cell responses. *Immunol Rev* **168**, 187-97.

Feire, A. L., Koss, H., and Compton, T. (2004). Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. *Proc Natl Acad Sci U S A* **101**(43), 15470-5.

Filteau, S. (2009a). The HIV-exposed, uninfected African child. *Trop Med Int Health* **14**(3), 276-87.

Filteau, S. a. t. C. s. t. (2009b). Randomised trial of the efficacy of a micronutrient fortified locally produced infant food on growth and severe morbidity of Zambian Infants: the Chilenje Infant Growth, Nutrition and Infection Study (CIGNIS). London School of Hygiene and Tropical Medicine, London, U.K and Univeristy Teaching Hospital, Lusaka, Zambia.

Flamand, L., Romerio, F., Reitz, M. S., and Gallo, R. C. (1998). CD4 promoter transactivation by human herpesvirus 6. *J Virol* **72**(11), 8797-805.

Flamand, L., Stefanescu, I., Ablashi, D. V., and Menezes, J. (1993). Activation of the Epstein-Barr virus replicative cycle by human herpesvirus 6. *J Virol* **67**(11), 6768-77.

Fox-Canale, A. M., Hope, T. J., Martinson, J., Lurain, J. R., Rademaker, A. W., Bremer, J. W., Landay, A., Spear, G. T., and Lurain, N. S. (2007). Human cytomegalovirus and human immunodeficiency virus type-1 co-infection in human cervical tissue. *Virology* **369**(1), 55-68.

Fraile-Ramos, A., Pelchen-Matthews, A., Risco, C., Rejas, M. T., Emery, V. C., Hassan-Walker, A. F., Esteban, M., and Marsh, M. (2007). The ESCRT machinery is not required for human cytomegalovirus envelopment. *Cell Microbiol* **9**(12), 2955-67.

French, C., Menegazzi, P., Nicholson, L., Macaulay, H., DiLuca, D., and Gompels, U. A. (1999). Novel, nonconsensus cellular splicing regulates expression of a gene encoding a chemokine-like protein that shows high variation and is specific for human herpesvirus 6. *Virology* **262**(1), 139-51.

Frenkel, L. D., Gaur, S., Tsolia, M., Scudder, R., Howell, R., and Kesarwala, H. (1990). Cytomegalovirus infection in children with AIDS. *Rev Infect Dis* **12 Suppl 7**, S820-6.

Furlini, G., Vignoli, M., Ramazzotti, E., Re, M. C., Visani, G., and La, P. (1996). A concurrent human herpesvirus-6 infection renders two human hematopoietic progenitor (TF-1 and KG-1) cell lines susceptible to human immunodeficiency virus type-1. *Blood* **87**(11), 4737-45.

Furukawa, M., Yasukawa, M., Yakushijin, Y., and Fujita, S. (1994). Distinct effects of human herpesvirus 6 and human herpesvirus 7 on surface molecule expression and function of CD4+ T cells. *J Immunol* **152**(12), 5768-75.

Gao, J. L., and Murphy, P. M. (1994). Human cytomegalovirus open reading frame US28 encodes a functional beta chemokine receptor. *J Biol Chem* **269**(46), 28539-42.

Gargouri, J., Elleuch, H., Karray, H., Rekik, H., and Hammami, A. (2000). [Prevalence of anti-CMV antibodies in blood donors in the Sfax region (value in blood transfusion)]. *Tunis Med* **78**(8-9), 512-7.

Gaytant, M. A., Steegers, E. A., Semmekrot, B. A., Merkus, H. M., and Galama, J. M. (2002). Congenital cytomegalovirus infection: review of the epidemiology and outcome. *Obstet Gynecol Surv* **57**(4), 245-56.

Gerna, G., Percivalle, E., Lilleri, D., Lozza, L., Fornara, C., Hahn, G., Baldanti, F., and Revello, M. G. (2005). Dendritic-cell infection by human cytomegalovirus is restricted to strains carrying functional UL131-128 genes and mediates efficient viral antigen presentation to CD8+ T cells. *J Gen Virol* **86**(Pt 2), 275-84.

Gerna, G., Percivalle, E., Sarasini, A., Baldanti, F., Campanini, G., and Revello, M. G. (2003). Rescue of human cytomegalovirus strain AD169 tropism for both leukocytes and human endothelial cells. *J Gen Virol* **84**(Pt 6), 1431-6.

Gibson, W. (1996). Structure and assembly of the virion. *Intervirology* **39**(5-6), 389-400.

Gobbi, A., Stoddart, C. A., Malnati, M. S., Locatelli, G., Santoro, F., Abbey, N. W., Bare, C., Linquist-Stepps, V., Moreno, M. B., Herndier, B. G., Lusso, P., and McCune, J. M. (1999). Human herpesvirus 6 (HHV-6) causes severe thymocyte depletion in SCID-hu Thy/Liv mice. *J Exp Med* **189**(12), 1953-60.

Gompels, U. A. (2004). "Principles and practice of clinical virology - Chapter 2E Roseoloviruses: Human herpesvirus 6 and 7 p147-167

" 5th ed. (A. J. Zuckerman, Ed.) Wiley, Chichester.

Gompels, U. A., Nicholas, J., Lawrence, G., Jones, M., Thomson, B. J., Martin, M. E., Efstathiou, S., Craxton, M., and Macaulay, H. A. (1995). The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. *Virology* **209**(1), 29-51.

Gompels, U. K., FC (2006). HHV-6 Genome: Similar and different. 2nd ed. *In* "Human herpesvirus-6" (A. D. Krueger G, Ed.), Vol. 12, pp. 23–46. Elsevier Amsterdam.

Goodrum, F., Reeves, M., Sinclair, J., High, K., and Shenk, T. (2007). Human cytomegalovirus sequences expressed in latently infected individuals promote a latent infection in vitro. *Blood* **110**(3), 937-45.

Goodrum, F. D., Jordan, C. T., High, K., and Shenk, T. (2002). Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency. *Proc Natl Acad Sci U S A* **99**(25), 16255-60.

Gouarin, S., Gault, E., Vabret, A., Cointe, D., Rozenberg, F., Grangeot-Keros, L., Barjot, P., Garbarg-Chenon, A., Lebon, P., and Freymuth, F. (2002). Real-time PCR quantification of human cytomegalovirus DNA in amniotic fluid samples from mothers with primary infection. *J Clin Microbiol* **40**(5), 1767-72.

Gouarin, S., Palmer, P., Cointe, D., Rogez, S., Vabret, A., Rozenberg, F., Denis, F., Freymuth, F., Lebon, P., and Grangeot-Keros, L. (2001). Congenital HCMV infection: a collaborative and comparative study of virus detection in amniotic fluid by culture and by PCR. *J Clin Virol* **21**(1), 47-55.

Grazia Revello, M., Baldanti, F., Percivalle, E., Sarasini, A., De-Giuli, L., Genini, E., Lilleri, D., Labo, N., and Gerna, G. (2001). In vitro selection of human cytomegalovirus variants unable to transfer virus and virus products from infected cells to polymorphonuclear leukocytes and to grow in endothelial cells. *J Gen Virol* **82**(Pt 6), 1429-38.

Griffiths, P. D. (2004). "Principles and practice of clinical virology - Chapter 2C Cytomegalovirus p85-122

" 5th ed. (A. J. Zuckerman, Ed.) Wiley, Chichester.

Griffiths, P. D., and Walter, S. (2005). Cytomegalovirus. *Curr Opin Infect Dis* **18**(3), 241-5.

Griscelli, F., Barrois, M., Chauvin, S., Lastere, S., Bellet, D., and Bourhis, J. H. (2001). Quantification of human cytomegalovirus DNA in bone marrow transplant recipients by real-time PCR. *J Clin Microbiol* **39**(12), 4362-9.

Grivel, J. C., Santoro, F., Chen, S., Faga, G., Malnati, M. S., Ito, Y., Margolis, L., and Lusso, P. (2003). Pathogenic effects of human herpesvirus 6 in human lymphoid tissue ex vivo. *J Virol* **77**(15), 8280-9.

Guay, L. A., Musoke, P., Fleming, T., Bagenda, D., Allen, M., Nakabiito, C., Sherman, J., Bakaki, P., Ducar, C., Deseyve, M., Emel, L., Mirochnick, M., Fowler, M. G., Mofenson, L., Miotti, P., Dransfield, K., Bray, D., Mmiro, F., and Jackson, J. B. (1999). Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: HIVNET 012 randomised trial. *Lancet* **354**(9181), 795-802.

Haberland, M., Meyer-Konig, U., and Hufert, F. T. (1999). Variation within the glycoprotein B gene of human cytomegalovirus is due to homologous recombination. *J Gen Virol* **80 (Pt 6),** 1495-500.

Hadrup, S. R., Strindhall, J., Kollgaard, T., Seremet, T., Johansson, B., Pawelec, G., thor Straten, P., and Wikby, A. (2006). Longitudinal studies of clonally expanded CD8 T cells reveal a repertoire shrinkage predicting mortality and an increased number of dysfunctional cytomegalovirus-specific T cells in the very elderly. *J Immunol* **176**(4), 2645-53.

Hahn, G., Revello, M. G., Patrone, M., Percivalle, E., Campanini, G., Sarasini, A., Wagner, M., Gallina, A., Milanesi, G., Koszinowski, U., Baldanti, F., and Gerna, G. (2004). Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. *J Virol* **78**(18), 10023-33.

Hall, C. B., Caserta, M. T., Schnabel, K., Shelley, L. M., Marino, A. S., Carnahan, J. A., Yoo, C., Lofthus, G. K., and McDermott, M. P. (2008). Chromosomal integration of human herpesvirus 6 is the major mode of congenital human herpesvirus 6 infection. *Pediatrics* **122**(3), 513-20.

Hall, C. B., Caserta, M. T., Schnabel, K. C., Boettrich, C., McDermott, M. P., Lofthus, G. K., Carnahan, J. A., and Dewhurst, S. (2004). Congenital infections with human herpesvirus 6 (HHV6) and human herpesvirus 7 (HHV7). *J Pediatr* **145**(4), 472-7.

Hall, C. B., Caserta, M. T., Schnabel, K. C., Long, C., Epstein, L. G., Insel, R. A., and Dewhurst, S. (1998). Persistence of human herpesvirus 6 according to site and variant: possible greater neurotropism of variant A. *Clin Infect Dis* **26**(1), 132-7.

Hall, C. B., Caserta, M. T., Schnabel, K. C., McDermott, M. P., Lofthus, G. K., Carnahan, J. A., Gilbert, L. M., and Dewhurst, S. (2006). Characteristics and acquisition of human herpesvirus (HHV) 7 infections in relation to infection with HHV-6. *J Infect Dis* **193**(8), 1063-9.

Hall, C. B., Long, C. E., Schnabel, K. C., Caserta, M. T., McIntyre, K. M., Costanzo, M. A., Knott, A., Dewhurst, S., Insel, R. A., and Epstein, L. G. (1994). Human herpesvirus-6 infection in children. A prospective study of complications and reactivation. *N Engl J Med* **331**(7), 432-8.

He, J., McCarthy, M., Zhou, Y., Chandran, B., and Wood, C. (1996). Infection of primary human fetal astrocytes by human herpesvirus 6. *J Virol* **70**(2), 1296-300.

He, R., Ruan, Q., Qi, Y., Ma, Y. P., Huang, Y. J., Sun, Z. R., and Ji, Y. H. (2006). Sequence variability of human cytomegalovirus UL146 and UL147 genes in low-passage clinical isolates. *Intervirology* **49**(4), 215-23.

He, Z. X., Pan, S. N., Chen, J. L., Xiong, W., Li, K., Wang, Q. W., Zou, X. B., Huang, L. F., Chen, M. L., and Yang, M. (2003). [Effect of human cytomegalovirus on hematopoietic system]. *Zhonghua Er Ke Za Zhi* **41**(5), 321-4.

Herrmann, B., Larsson, V. C., Rubin, C. J., Sund, F., Eriksson, B. M., Arvidson, J., Yun, Z., Bondeson, K., and Blomberg, J. (2004). Comparison of a duplex quantitative real-time

PCR assay and the COBAS Amplicor CMV Monitor test for detection of cytomegalovirus. *J Clin Microbiol* **42**(5), 1909-14.

Hirata, Y., Kondo, K., and Yamanishi, K. (2001). Human herpesvirus 6 downregulates major histocompatibility complex class I in dendritic cells. *J Med Virol* **65**(3), 576-83.

Hobom, U., Brune, W., Messerle, M., Hahn, G., and Koszinowski, U. H. (2000). Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. *J Virol* **74**(17), 7720-9.

Holland, C. A., Ma, Y., Moscicki, B., Durako, S. J., Levin, L., and Wilson, C. M. (2000). Seroprevalence and risk factors of hepatitis B, hepatitis C, and human cytomegalovirus among HIV-infected and high-risk uninfected adolescents: findings of the REACH Study. Adolescent Medicine HIV/AIDS Research Network. *Sex Transm Dis* **27**(5), 296-303.

Horvat, R. T., Wood, C., and Balachandran, N. (1989). Transactivation of human immunodeficiency virus promoter by human herpesvirus 6. *J Virol* **63**(2), 970-3.

Horvat, R. T., Wood, C., Josephs, S. F., and Balachandran, N. (1991). Transactivation of the human immunodeficiency virus promoter by human herpesvirus 6 (HHV-6) strains GS and Z-29 in primary human T lymphocytes and identification of transactivating HHV-6(GS) gene fragments. *J Virol* **65**(6), 2895-902.

Howie, P. W., Forsyth, J. S., Ogston, S. A., Clark, A., and Florey, C. D. (1990). Protective effect of breast feeding against infection. *Bmj* **300**(6716), 11-6.

Huber, M. T., and Compton, T. (1997). Characterization of a novel third member of the human cytomegalovirus glycoprotein H-glycoprotein L complex. *J Virol* **71**(7), 5391-8.

Huber, M. T., and Compton, T. (1998). The human cytomegalovirus UL74 gene encodes the third component of the glycoprotein H-glycoprotein L-containing envelope complex. *J Virol* **72**(10), 8191-7.

Huber, M. T., and Compton, T. (1999). Intracellular formation and processing of the heterotrimeric gH-gL-gO (gCIII) glycoprotein envelope complex of human cytomegalovirus. *J Virol* **73**(5), 3886-92.

Humar, A., Kumar, D., Gilbert, C., and Boivin, G. (2003). Cytomegalovirus (CMV) glycoprotein B genotypes and response to antiviral therapy, in solid-organ-transplant recipients with CMV disease. *J Infect Dis* **188**(4), 581-4.

Humar, A., Kumar, D., Raboud, J., Caliendo, A. M., Moussa, G., Levy, G., and Mazzulli, T. (2002). Interactions between cytomegalovirus, human herpesvirus-6, and the recurrence of hepatitis C after liver transplantation. *Am J Transplant* **2**(5), 461-6.

Iannotti, L. L., Tielsch, J. M., Black, M. M., and Black, R. E. (2006). Iron supplementation in early childhood: health benefits and risks. *Am J Clin Nutr* **84**(6), 1261-76.

Isaacson, M. K., Feire, A. L., and Compton, T. (2007). Epidermal growth factor receptor is not required for human cytomegalovirus entry or signaling. *J Virol* **81**(12), 6241-7.

Isegawa, Y., Mukai, T., Nakano, K., Kagawa, M., Chen, J., Mori, Y., Sunagawa, T., Kawanishi, K., Sashihara, J., Hata, A., Zou, P., Kosuge, H., and Yamanishi, K. (1999). Comparison of the complete DNA sequences of human herpesvirus 6 variants A and B. *J Virol* **73**(10), 8053-63.

Isegawa, Y., Ping, Z., Nakano, K., Sugimoto, N., and Yamanishi, K. (1998). Human herpesvirus 6 open reading frame U12 encodes a functional beta-chemokine receptor. *J Virol* **72**(7), 6104-12.

Iuliano, R., Trovato, R., Lico, S., Luppi, M., Forastieri, G., Barsanti, L. A., Pizzigallo, A. M., Mecocci, L., Barozzi, P., Torelli, G., Mazzotta, F., and Ceccherini-Nelli, L. (1997). Human herpesvirus-6 reactivation in a longitudinal study of two HIV-1 infected patients. *J Med Virol* **51**(4), 259-64.

Jabs, D. A., and Griffiths, P. D. (2002). Fomivirsen for the treatment of cytomegalovirus retinitis. *Am J Ophthalmol* **133**(4), 552-6.

Jackson, J. B., Musoke, P., Fleming, T., Guay, L. A., Bagenda, D., Allen, M., Nakabiito, C., Sherman, J., Bakaki, P., Owor, M., Ducar, C., Deseyve, M., Mwatha, A., Emel, L., Duefield, C., Mirochnick, M., Fowler, M. G., Mofenson, L., Miotti, P., Gigliotti, M., Bray, D., and Mmiro, F. (2003). Intrapartum and neonatal single-dose nevirapine compared with zidovudine for prevention of mother-to-child transmission of HIV-1 in Kampala, Uganda: 18-month follow-up of the HIVNET 012 randomised trial. *Lancet* **362**(9387), 859-68.

Jarvis, M. A., and Nelson, J. A. (2007). Human cytomegalovirus tropism for endothelial cells: not all endothelial cells are created equal. *J Virol* **81**(5), 2095-101.

Jeena, P. M., Coovadia, H. M., and Chrystal, V. (1996). Pneumocystis carinii and cytomegalovirus infections in severely ill, HIV-infected African infants. *Ann Trop Paediatr* **16**(4), 361-8.

Jiang, X. J., Adler, B., Sampaio, K. L., Digel, M., Jahn, G., Ettischer, N., Stierhof, Y. D., Scrivano, L., Koszinowski, U., Mach, M., and Sinzger, C. (2008). UL74 of human cytomegalovirus contributes to virus release by promoting secondary envelopment of virions. *J Virol.*

Jim, W. T., Shu, C. H., Chiu, N. C., Chang, J. H., Hung, H. Y., Peng, C. C., Kao, H. A., Wei, T. Y., Chiang, C. L., and Huang, F. Y. (2009). High cytomegalovirus load and prolonged virus excretion in breast milk increase risk for viral acquisition by very low birth weight infants. *Pediatr Infect Dis J* **28**(10), 891-4.

Johnstone, R. W., Russell, S. M., Loveland, B. E., and McKenzie, I. F. (1993). Polymorphic expression of CD46 protein isoforms due to tissue-specific RNA splicing. *Mol Immunol* **30**(14), 1231-41.

Kagialis-Girard, S., Durand, B., Mialou, V., Pages, M. P., Galambrun, C., Bertrand, Y., and Negrier, C. (2006). Human herpesvirus 6 infection and transient acquired myelodysplasia in children. *Pediatr Blood Cancer* **47**(5), 543-8.

Kakimoto, M., Hasegawa, A., Fujita, S., and Yasukawa, M. (2002). Phenotypic and functional alterations of dendritic cells induced by human herpesvirus 6 infection. *J Virol* **76**(20), 10338-45.

Kaptein, S. J., Efferth, T., Leis, M., Rechter, S., Auerochs, S., Kalmer, M., Bruggeman, C. A., Vink, C., Stamminger, T., and Marschall, M. (2006). The anti-malaria drug artesunate inhibits replication of cytomegalovirus in vitro and in vivo. *Antiviral Res* **69**(2), 60-9.

Kari, B., and Gehrz, R. (1992). A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope. *J Virol* **66**(3), 1761-4.

Karrer, U., Mekker, A., Wanke, K., Tchang, V., and Haeberli, L. (2009). Cytomegalovirus and immune senescence: culprit or innocent bystander? *Exp Gerontol* **44**(11), 689-94.

Kasolo, F. C. (1999). London School of Hygiene and Tropical Medicine, London.

Kasolo, F. C., Monze, M., Obel, N., Anderson, R. A., French, C., and Gompels, U. A. (1998). Sequence analyses of human herpesvirus-8 strains from both African human immunodeficiency virus-negative and -positive childhood endemic Kaposi's sarcoma show a close relationship with strains identified in febrile children and high variation in the K1 glycoprotein. *J Gen Virol* **79 (Pt 12)**, 3055-65.

Kasolo, F. C., Mpabalwani, E., and Gompels, U. A. (1997). Infection with AIDS-related herpesviruses in human immunodeficiency virus-negative infants and endemic childhood Kaposi's sarcoma in Africa. *J Gen Virol* **78 (Pt 4)**, 847-55.

Kasolo, F. C., Spinks, J., Bima, H., Bates, M., and Gompels, U. A. (2007). Diverse genotypes of Kaposi's sarcoma associated herpesvirus (KSHV) identified in infant blood infections in African childhood-KS and HIV/AIDS endemic region. *J Med Virol* **79**(10), 1555-61.

Kaye, S., Miles, D., Antoine, P., Burny, W., Ojuola, B., Kaye, P., Rowland-Jones, S., Whittle, H., van der Sande, M., and Marchant, A. (2008). Virological and immunological correlates of mother-to-child transmission of cytomegalovirus in The Gambia. *J Infect Dis* **197**(9), 1307-14.

Kearns, A. M., Turner, A. J., Eltringham, G. J., and Freeman, R. (2002). Rapid detection and quantification of CMV DNA in urine using LightCycler-based real-time PCR. *J Clin Virol* **24**(1-2), 131-4.

Kempf, W., Adams, V., Wey, N., Moos, R., Schmid, M., Avitabile, E., and Campadelli-Fiume, G. (1997). CD68+ cells of monocyte/macrophage lineage in the environment of AIDS-associated and classic-sporadic Kaposi sarcoma are singly or doubly infected with human herpesviruses 7 and 6B. *Proc Natl Acad Sci U S A* **94**(14), 7600-5.

Kenneson, A., and Cannon, M. J. (2007). Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Rev Med Virol* **17**(4), 253-76.

Kerrey, B. T., Morrow, A., Geraghty, S., Huey, N., Sapsford, A., and Schleiss, M. R. (2006). Breast milk as a source for acquisition of cytomegalovirus (HCMV) in a premature infant with sepsis syndrome: detection by real-time PCR. *J Clin Virol* **35**(3), 313-6.

Khaiboullina, S. F., Maciejewski, J. P., Crapnell, K., Spallone, P. A., Dean Stock, A., Pari, G. S., Zanjani, E. D., and Jeor, S. S. (2004). Human cytomegalovirus persists in myeloid progenitors and is passed to the myeloid progeny in a latent form. *Br J Haematol* **126**(3), 410-7.

Khan, N., Shariff, N., Cobbold, M., Bruton, R., Ainsworth, J. A., Sinclair, A. J., Nayak, L., and Moss, P. A. (2002). Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *J Immunol* **169**(4), 1984-92.

Kinzler, E. R., and Compton, T. (2005). Characterization of human cytomegalovirus glycoprotein-induced cell-cell fusion. *J Virol* **79**(12), 7827-37.

Kitchen, B. J., Engler, H. D., Gill, V. J., Marshall, D., Steinberg, S. M., Pizzo, P. A., and Mueller, B. U. (1997). Cytomegalovirus infection in children with human immunodeficiency virus infection. *Pediatr Infect Dis J* **16**(4), 358-63.

Kjersem, H., and Jepsen, S. (1990). Varicella among immigrants from the tropics, a health problem. *Scand J Soc Med* **18**(3), 171-4.

Kledal, T. N., Rosenkilde, M. M., Coulin, F., Simmons, G., Johnsen, A. H., Alouani, S., Power, C. A., Luttichau, H. R., Gerstoft, J., Clapham, P. R., Clark-Lewis, I., Wells, T. N., and Schwartz, T. W. (1997). A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. *Science* **277**(5332), 1656-9.

Kledal, T. N., Rosenkilde, M. M., and Schwartz, T. W. (1998). Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. *FEBS Lett* **441**(2), 209-14.

Knox, K. K., and Carrigan, D. R. (1994). Disseminated active HHV-6 infections in patients with AIDS. *Lancet* **343**(8897), 577-8.

Knox, K. K., and Carrigan, D. R. (1995). Active human herpesvirus (HHV-6) infection of the central nervous system in patients with AIDS. *J Acquir Immune Defic Syndr Hum Retrovirol* **9**(1), 69-73.

Knox, K. K., Harrington, D. P., and Carrigan, D. R. (1995). Fulminant human herpesvirus six encephalitis in a human immunodeficiency virus-infected infant. *J Med Virol* **45**(3), 288-92.

Knox, K. K., Pietryga, D., Harrington, D. J., Franciosi, R., and Carrigan, D. R. (1995). Progressive immunodeficiency and fatal pneumonitis associated with human herpesvirus 6 infection in an infant. *Clin Infect Dis* **20**(2), 406-13.

Kondo, K., Kaneshima, H., and Mocarski, E. S. (1994). Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. *Proc Natl Acad Sci U S A* **91**(25), 11879-83.

Kondo, K., Kondo, T., Okuno, T., Takahashi, M., and Yamanishi, K. (1991). Latent human herpesvirus 6 infection of human monocytes/macrophages. *J Gen Virol* **72 (Pt 6),** 1401-8.

Kondo, K., Shimada, K., Sashihara, J., Tanaka-Taya, K., and Yamanishi, K. (2002). Identification of human herpesvirus 6 latency-associated transcripts. *J Virol* **76(8),** 4145-51.

Kositanont, U., Wasi, C., Wanprapar, N., Bowonkiratikachorn, P., Chokephaibulkit, K., Chearskul, S., Chimabutra, K., Sutthent, R., Foongladda, S., Inagi, R., Kurata, T., and Yamanishi, K. (1999). Primary infection of human herpesvirus 6 in children with vertical infection of human immunodeficiency virus type 1. *J Infect Dis* **180**(1), 50-5.

Kotenko, S. V., Saccani, S., Izotova, L. S., Mirochnitchenko, O. V., and Pestka, S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc Natl Acad Sci U S A* **97**(4), 1695-700.

Kothari, A., Ramachandran, V. G., Gupta, P., Singh, B., and Talwar, V. (2002). Seroprevalence of cytomegalovirus among voluntary blood donors in Delhi, India. *J Health Popul Nutr* **20**(4), 348-51.

Kovacs, A., Schluchter, M., Easley, K., Demmler, G., Shearer, W., La Russa, P., Pitt, J., Cooper, E., Goldfarb, J., Hodes, D., Kattan, M., and McIntosh, K. (1999). Cytomegalovirus infection and HIV-1 disease progression in infants born to HIV-1-infected women. Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted HIV Infection Study Group. *N Engl J Med* **341**(2), 77-84.

Krzyzaniak, M., Mach, M., and Britt, W. J. (2007). The cytoplasmic tail of glycoprotein M (gpUL100) expresses trafficking signals required for human cytomegalovirus assembly and replication. *J Virol* **81**(19), 10316-28.

Kuhn, D. E., Beall, C. J., and Kolattukudy, P. E. (1995). The cytomegalovirus US28 protein binds multiple CC chemokines with high affinity. *Biochem Biophys Res Commun* **211**(1), 325-30.

Kuhn, L., Semrau, K., Ramachandran, S., Sinkala, M., Scott, N., Kasonde, P., Mwiya, M., Kankasa, C., Decker, D., Thea, D. M., and Aldrovandi, G. M. (2009). Mortality and virologic outcomes after access to antiretroviral therapy among a cohort of HIV-infected women who received single-dose nevirapine in Lusaka, Zambia. *J Acquir Immune Defic Syndr* **52**(1), 132-6.

Kusuhara, K., Takabayashi, A., Ueda, K., Hidaka, Y., Minamishima, I., Take, H., Fujioka, K., Imai, S., and Osato, T. (1997). Breast milk is not a significant source for early Epstein-Barr virus or human herpesvirus 6 infection in infants: a seroepidemiologic study in 2 endemic areas of human T-cell lymphotropic virus type I in Japan. *Microbiol Immunol* **41**(4), 309-12.

Lackner, A., Acham, A., Alborno, T., Moser, M., Engele, H., Raggam, R. B., Halwachs-Baumann, G., Kapitan, M., and Walch, C. (2009). Effect on hearing of ganciclovir therapy for asymptomatic congenital cytomegalovirus infection: four to 10 year follow up. *J Laryngol Otol* **123**(4), 391-6.

Landolfo, S., Gariglio, M., Gribaudo, G., and Lembo, D. (2003). The human cytomegalovirus. *Pharmacol Ther* **98**(3), 269-97.

Larsson, S., Soderberg-Naucler, C., Wang, F. Z., and Moller, E. (1998). Cytomegalovirus DNA can be detected in peripheral blood mononuclear cells from all seropositive and most seronegative healthy blood donors over time. *Transfusion* **38**(3), 271-8.

Lazzarotto, T., Guerra, B., Lanari, M., Gabrielli, L., and Landini, M. P. (2007). New advances in the diagnosis of congenital cytomegalovirus infection. *J Clin Virol.*

Leach, C. T., Pollock, B. H., McClain, K. L., Parmley, R. T., Murphy, S. B., and Jenson, H. B. (2002). Human herpesvirus 6 and cytomegalovirus infections in children with human immunodeficiency virus infection and cancer. *Pediatr Infect Dis J* **21**(2), 125-32.

Ledru, E., Diagbouga, S., Ledru, S., Cauchoix, B., Yameogo, M., Chami, D., Sanou, O., and Chiron, J. P. (1995). A study of Toxoplasma and Cytomegalovirus serology in tuberculosis and in HIV-infected patients in Burkina Faso. *Acta Trop* **59**(2), 149-54.

Leong, H. N., Tuke, P. W., Tedder, R. S., Khanom, A. B., Eglin, R. P., Atkinson, C. E., Ward, K. N., Griffiths, P. D., and Clark, D. A. (2007). The prevalence of chromosomally integrated human herpesvirus 6 genomes in the blood of UK blood donors. *J Med Virol* **79**(1), 45-51.

Levy, J. A., Landay, A., and Lennette, E. T. (1990). Human herpesvirus 6 inhibits human immunodeficiency virus type 1 replication in cell culture. *J Clin Microbiol* **28**(10), 2362-4.

Li, H., Dummer, J. S., Estes, W. R., Meng, S., Wright, P. F., and Tang, Y. W. (2003). Measurement of human cytomegalovirus loads by quantitative real-time PCR for monitoring clinical intervention in transplant recipients. *J Clin Microbiol* **41**(1), 187-91.

Li, H., Meng, S., Levine, S. M., Stratton, C. W., and Tang, Y. W. (2009). Sensitive, qualitative detection of human herpesvirus-6 and simultaneous differentiation of variants A and B. *J Clin Virol* **46**(1), 20-3.

Li, L., Nelson, J. A., and Britt, W. J. (1997). Glycoprotein H-related complexes of human cytomegalovirus: identification of a third protein in the gCIII complex. *J Virol* **71**(4), 3090-7.

Likitnukul, S., Bhattarakosol, P., and Poovorawan, Y. (2003). Seroprevalence of cytomegalovirus infection in children born to HIV-1 infected women. *Asian Pac J Allergy Immunol* **21**(2), 127-30.

Lin, Y. L., Chang, P. C., Wang, Y., and Li, M. (2008). Identification of novel viral interleukin-10 isoforms of human cytomegalovirus AD169. *Virus Res* **131**(2), 213-23.

Linhares, M. I., Eizuru, Y., Tateno, S., and Minamishima, Y. (1991). Seroprevalence of human herpesvirus 6 infection in Brazilian and Japanese populations in the north-east of Brazil. *Microbiol Immunol* **35**(11), 1023-7.

Lopez, C., Pellett, P., Stewart, J., Goldsmith, C., Sanderlin, K., Black, J., Warfield, D., and Feorino, P. (1988). Characteristics of human herpesvirus-6. *J Infect Dis* **157**(6), 1271-3.

Lopper, M., and Compton, T. (2004). Coiled-coil domains in glycoproteins B and H are involved in human cytomegalovirus membrane fusion. *J Virol* **78**(15), 8333-41.

Luppi, M., Barozzi, P., Morris, C., Maiorana, A., Garber, R., Bonacorsi, G., Donelli, A., Marasca, R., Tabilio, A., and Torelli, G. (1999). Human herpesvirus 6 latently infects early bone marrow progenitors in vivo. *J Virol* **73**(1), 754-9.

Luppi, M., Marasca, R., Barozzi, P., Ferrari, S., Ceccherini-Nelli, L., Batoni, G., Merelli, E., and Torelli, G. (1993). Three cases of human herpesvirus-6 latent infection: integration of viral genome in peripheral blood mononuclear cell DNA. *J Med Virol* **40**(1), 44-52.

Lusso, P., De Maria, A., Malnati, M., Lori, F., DeRocco, S. E., Baseler, M., and Gallo, R. C. (1991a). Induction of CD4 and susceptibility to HIV-1 infection in human CD8+ T lymphocytes by human herpesvirus 6. *Nature* **349**(6309), 533-5.

Lusso, P., Ensoli, B., Markham, P. D., Ablashi, D. V., Salahuddin, S. Z., Tschachler, E., Wong-Staal, F., and Gallo, R. C. (1989). Productive dual infection of human CD4+ T lymphocytes by HIV-1 and HHV-6. *Nature* **337**(6205), 370-3.

Lusso, P., Garzino-Demo, A., Crowley, R. W., and Malnati, M. S. (1995). Infection of gamma/delta T lymphocytes by human herpesvirus 6: transcriptional induction of CD4 and susceptibility to HIV infection. *J Exp Med* **181**(4), 1303-10.

Lusso, P., Malnati, M., De Maria, A., Balotta, C., DeRocco, S. E., Markham, P. D., and Gallo, R. C. (1991b). Productive infection of CD4+ and CD8+ mature human T cell

populations and clones by human herpesvirus 6. Transcriptional down-regulation of CD3. *J Immunol* **147**(2), 685-91.

Lusso, P., Malnati, M. S., Garzino-Demo, A., Crowley, R. W., Long, E. O., and Gallo, R. C. (1993). Infection of natural killer cells by human herpesvirus 6. *Nature* **362**(6419), 458-62.

Lusso, P., Markham, P. D., Tschachler, E., di Marzo Veronese, F., Salahuddin, S. Z., Ablashi, D. V., Pahwa, S., Krohn, K., and Gallo, R. C. (1988). In vitro cellular tropism of human B-lymphotropic virus (human herpesvirus-6). *J Exp Med* **167**(5), 1659-70.

Luttichau, H. R., Clark-Lewis, I., Jensen, P. O., Moser, C., Gerstoft, J., and Schwartz, T. W. (2003). A highly selective CCR2 chemokine agonist encoded by human herpesvirus 6. *J Biol Chem* **278**(13), 10928-33.

Mach, M., Kropff, B., Dal Monte, P., and Britt, W. (2000). Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73). *J Virol* **74**(24), 11881-92.

Mach, M., Kropff, B., Kryzaniak, M., and Britt, W. (2005). Complex formation by glycoproteins M and N of human cytomegalovirus: structural and functional aspects. *J Virol* **79**(4), 2160-70.

Mach, M., Osinski, K., Kropff, B., Schloetzer-Schrehardt, U., Krzyzaniak, M., and Britt, W. (2007). The carboxy-terminal domain of glycoprotein N of human cytomegalovirus is required for virion morphogenesis. *J Virol* **81**(10), 5212-24.

Maiga, II, Tounkara, A., Coulibaly, G., and Kouriba, B. (2003). [Seroprevalence of the human cytomegalovirus among blood donors and AIDS patients in Bamako]. *Sante* **13**(2), 117-9.

Makasa, M., Kasonka, L., Chisenga, M., Sinkala, M., Chintu, C., Tomkins, A., and Filteau, S. (2007). Early growth of infants of HIV-infected and uninfected Zambian women. *Trop Med Int Health* **12**(5), 594-602.

Manichanh, C., Olivier-Aubron, C., Lagarde, J. P., Aubin, J. T., Bossi, P., Gautheret-Dejean, A., Huraux, J. M., and Agut, H. (2001). Selection of the same mutation in the U69 protein kinase gene of human herpesvirus-6 after prolonged exposure to ganciclovir in vitro and in vivo. *J Gen Virol* **82**(Pt 11), 2767-76.

Manufacturer's_Protocol (2005). PureLink™ Quick Plasmid Miniprep Kit. 250789 Version C ed. Invitrogen, Paisley, U.K.

Manufacturer's_Protocol (2006). TOPO TA Cloning® Kit for Sequencing. O ed, pp. Five-minute cloning of Taq polymerase-amplified PCR products for sequencing. Invitrogen, Paisley, U.K.

Manufacturer's_Protocol (2008). MinElute® Handbook. Qiagen, Crawley, U.K.

Margalith, M., D'Aquila, R. T., Manion, D. J., Basgoz, N., Bechtel, L. J., Smith, B. R., Kaplan, J. C., and Hirsch, M. S. (1995). HIV-1 DNA in fibroblast cultures infected with urine from HIV-seropositive cytomegalovirus (CMV) excretors. *Arch Virol* **140**(5), 927-35.

Marinda, E., Humphrey, J. H., Iliff, P. J., Mutasa, K., Nathoo, K. J., Piwoz, E. G., Moulton, L. H., Salama, P., and Ward, B. J. (2007). Child mortality according to maternal and infant HIV status in Zimbabwe. *Pediatr Infect Dis J* **26**(6), 519-26.

Masse, M. J., Jons, A., Dijkstra, J. M., Mettenleiter, T. C., and Flamand, A. (1999). Glycoproteins gM and gN of pseudorabies virus are dispensable for viral penetration and propagation in the nervous systems of adult mice. *J Virol* **73**(12), 10503-7.

Masur, H., Kaplan, J. E., and Holmes, K. K. (2002). Guidelines for preventing opportunistic infections among HIV-infected persons--2002. Recommendations of the U.S. Public Health Service and the Infectious Diseases Society of America. *Ann Intern Med* **137**(5 Pt 2), 435-78.

Mattes, F. M., Hainsworth, E. G., Hassan-Walker, A. F., Burroughs, A. K., Sweny, P., Griffiths, P. D., and Emery, V. C. (2005). Kinetics of cytomegalovirus load decrease

in solid-organ transplant recipients after preemptive therapy with valganciclovir. *J Infect Dis* **191**(1), 89-92.

Mattes, F. M., McLaughlin, J. E., Emery, V. C., Clark, D. A., and Griffiths, P. D. (2000). Histopathological detection of owl's eye inclusions is still specific for cytomegalovirus in the era of human herpesviruses 6 and 7. *J Clin Pathol* **53**(8), 612-4.

Mattick, C., Dewin, D., Polley, S., Sevilla-Reyes, E., Pignatelli, S., Rawlinson, W., Wilkinson, G., Dal Monte, P., and Gompels, U. A. (2004). Linkage of human cytomegalovirus glycoprotein gO variant groups identified from worldwide clinical isolates with gN genotypes, implications for disease associations and evidence for N-terminal sites of positive selection. *Virology* **318**(2), 582-97.

McGeoch, D. J., and Gatherer, D. (2005). Integrating reptilian herpesviruses into the family herpesviridae. *J Virol* **79**(2), 725-31.

McGeoch, D. J., Rixon, F. J., and Davison, A. J. (2006). Topics in herpesvirus genomics and evolution. *Virus Res* **117**(1), 90-104.

McKeating, J. A., Griffiths, P. D., and Weiss, R. A. (1990). HIV susceptibility conferred to human fibroblasts by cytomegalovirus-induced Fc receptor. *Nature* **343**(6259), 659-61.

Mel'nichenko, A. V., L'Vov N, D., and Galegov, G. A. (2009). [Inhibitory effect of foscarnet and acyclovir on reproduction of human herpesvirus 6 in cell culture]. *Antibiot Khimioter* **54**(1-2), 17-9.

Mendelson, M., Monard, S., Sissons, P., and Sinclair, J. (1996). Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors. *J Gen Virol* **77 (Pt 12)**, 3099-102.

Micol, R., Buchy, P., Guerrier, G., Duong, V., Ferradini, L., Dousset, J. P., Guerin, P. J., Balkan, S., Galimand, J., Chanroeun, H., Lortholary, O., Rouzioux, C., Fontanet, A., and Leruez-Ville, M. (2009). Prevalence, risk factors, and impact on outcome of cytomegalovirus replication in serum of Cambodian HIV-infected patients (2004-2007). *J Acquir Immune Defic Syndr* **51**(4), 486-91.

Middleton, P. J. (1996). Viruses that multiply in the gut and cause endemic and epidemic gastroenteritis. *Clin Diagn Virol* **6**(2-3), 93-101.

Mihailescu, R., Arama, V., Paraschiv, S., Streinu-Cercel, A., Otelea, D., Munteanu, D., Iosipenco, M., Chiotan, C., Benea, O. E., Mardarescu, M., Radulescu, M., Hristea, A., Ungurianu, R., Arama, S. S., Cercel, A. S., Calin, R., and Baicus, C. (2008). Impact of highly active antiretroviral therapy on cytomegalovirus viraemia in the absence of specific anti-cytomegalovirus therapy. *Rom J Intern Med* **46**(4), 305-11.

Miles, D. J., van der Sande, M., Jeffries, D., Kaye, S., Ismaili, J., Ojuola, O., Sanneh, M., Touray, E. S., Waight, P., Rowland-Jones, S., Whittle, H., and Marchant, A. (2007). Cytomegalovirus infection in Gambian infants leads to profound CD8 T-cell differentiation. *J Virol* **81**(11), 5766-76.

Miller, D. M., Cebulla, C. M., Rahill, B. M., and Sedmak, D. D. (2001). Cytomegalovirus and transcriptional down-regulation of major histocompatibility complex class II expression. *Semin Immunol* **13**(1), 11-8.

Milne, R. S., Mattick, C., Nicholson, L., Devaraj, P., Alcami, A., and Gompels, U. A. (2000). RANTES binding and down-regulation by a novel human herpesvirus-6 beta chemokine receptor. *J Immunol* **164**(5), 2396-404.

Milne, R. S., Paterson, D. A., and Booth, J. C. (1998). Human cytomegalovirus glycoprotein H/glycoprotein L complex modulates fusion-from-without. *J Gen Virol* **79 (Pt 4)**, 855-65.

Mirandola, P., Menegazzi, P., Merighi, S., Ravaioli, T., Cassai, E., and Di Luca, D. (1998). Temporal mapping of transcripts in herpesvirus 6 variants. *J Virol* **72**(5), 3837-44.

Mocarski, E. S., Jr. (2002). Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. *Trends Microbiol* **10**(7), 332-9.

Mocarski, E. S., Jr; Shenk, T; Pass, R.F (2007). Cytomegaloviruses. 5 ed. *In* "Fields Virology" (P. M. H. e. a. D.M Knipe, Ed.), Vol. 2, pp. 2701-2772. 2 vols. Lippincot, Williams and Wilkins, Philidelphia.

Modi, G., Mochan, A., Modi, M., and Saffer, D. (2001). Demyelinating disorder of the central nervous system occurring in black South Africans. *J Neurol Neurosurg Psychiatry* **70**(4), 500-5.

Mori, Y., Akkapaiboon, P., Yonemoto, S., Koike, M., Takemoto, M., Sadaoka, T., Sasamoto, Y., Konishi, S., Uchiyama, Y., and Yamanishi, K. (2004). Discovery of a second form of tripartite complex containing gH-gL of human herpesvirus 6 and observations on CD46. *J Virol* **78**(9), 4609-16.

Mori, Y., Koike, M., Moriishi, E., Kawabata, A., Tang, H., Oyaizu, H., Uchiyama, Y., and Yamanishi, K. (2008). Human herpesvirus-6 induces MVB formation, and virus egress occurs by an exosomal release pathway. *Traffic* **9**(10), 1728-42.

Mori, Y., Seya, T., Huang, H. L., Akkapaiboon, P., Dhepakson, P., and Yamanishi, K. (2002). Human herpesvirus 6 variant A but not variant B induces fusion from without in a variety of human cells through a human herpesvirus 6 entry receptor, CD46. *J Virol* **76**(13), 6750-61.

Mori, Y., Yang, X., Akkapaiboon, P., Okuno, T., and Yamanishi, K. (2003). Human herpesvirus 6 variant A glycoprotein H-glycoprotein L-glycoprotein Q complex associates with human CD46. *J Virol* **77**(8), 4992-9.

Mosca, F., and Pugni, L. (2007). Cytomegalovirus infection: the state of the art. *J Chemother* **19 Suppl 2**, 46-8.

Murata, H., Nii, R., Ito, M., Ihara, T., and Komada, Y. (2009). Quantitative detection of HCMV-DNA in saliva from infants and breast milk on real-time polymerase chain reaction. *Pediatr Int* **51**(4), 530-4.

Murph, J. R., Baron, J. C., Brown, C. K., Ebelhack, C. L., and Bale, J. F., Jr. (1991). The occupational risk of cytomegalovirus infection among day-care providers. *Jama* **265**(5), 603-8.

Murphy, E., Rigoutsos, I., Shibuya, T., and Shenk, T. E. (2003a). Reevaluation of human cytomegalovirus coding potential. *Proc Natl Acad Sci U S A* **100**(23), 13585-90.

Murphy, E., Yu, D., Grimwood, J., Schmutz, J., Dickson, M., Jarvis, M. A., Hahn, G., Nelson, J. A., Myers, R. M., and Shenk, T. E. (2003b). Coding potential of laboratory and clinical strains of human cytomegalovirus. *Proc Natl Acad Sci U S A* **100**(25), 14976-81.

Nair, N., Moss, W. J., Scott, S., Mugala, N., Ndhlovu, Z. M., Lilo, K., Ryon, J. J., Monze, M., Quinn, T. C., Cousens, S., Cutts, F., and Griffin, D. E. (2009). HIV-1 infection in Zambian children impairs the development and avidity maturation of measles virus-specific immunoglobulin G after vaccination and infection. *J Infect Dis* **200**(7), 1031-8.

Nakano, K., Nishinaka, K., Tanaka, T., Ohshima, A., Sugimoto, N., and Isegawa, Y. (2009). Detection and identification of U69 gene mutations encoded by ganciclovir-resistant human herpesvirus 6 using denaturing high-performance liquid chromatography. *J Virol Methods* **161**(2), 223-30.

Naniche, D., Lahuerta, M., Bardaji, A., Sigauque, B., Romagosa, C., Berenguera, A., Mandomando, I., David, C., Sanz, S., Aponte, J., Ordi, J., Alonso, P., and Menendez, C. (2008). Mother-to-child transmission of HIV-1: association with malaria prevention, anaemia and placental malaria. *HIV Med* **9**(9), 757-64.

Naniche, D., Varior-Krishnan, G., Cervoni, F., Wild, T. F., Rossi, B., Rabourdin-Combe, C., and Gerlier, D. (1993). Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus. *J Virol* **67**(10), 6025-32.

Nielsen, L., and Vestergaard, B. F. (1996). Competitive ELISA for detection of HHV-6 antibody: seroprevalence in a danish population. *J Virol Methods* **56**(2), 221-30.

Nigro, G., Adler, S. P., La Torre, R., and Best, A. M. (2005). Passive immunization during pregnancy for congenital cytomegalovirus infection. *N Engl J Med* **353**(13), 1350-62.

Nigro, G., Krzysztofiak, A., Gattinara, G. C., Mango, T., Mazzocco, M., Porcaro, M. A., Provvedi, S., and Booth, J. C. (1996). Rapid progression of HIV disease in children with cytomegalovirus DNAemia. *Aids* **10**(10), 1127-33.

Niiya, H., Azuma, T., Jin, L., Uchida, N., Inoue, A., Hasegawa, H., Fujita, S., Tohyama, M., Hashimoto, K., and Yasukawa, M. (2004). Transcriptional downregulation of DC-SIGN in human herpesvirus 6-infected dendritic cells. *J Gen Virol* **85**(Pt 9), 2639-42.

Nitsche, A., Muller, C. W., Radonic, A., Landt, O., Ellerbrok, H., Pauli, G., and Siegert, W. (2001). Human herpesvirus 6A DNA Is detected frequently in plasma but rarely in peripheral blood leukocytes of patients after bone marrow transplantation. *J Infect Dis* **183**(1), 130-3.

Noyola, D. E., Valdez-Lopez, B. H., Hernandez-Salinas, A. E., Santos-Diaz, M. A., Noyola-Frias, M. A., Reyes-Macias, J. F., and Martinez-Martinez, L. G. (2005). Cytomegalovirus excretion in children attending day-care centers. *Arch Med Res* **36**(5), 590-3.

O'Brien, T. P., Thompson, J. M., Black, P. N., Becroft, D. M., Clark, P. M., Robinson, E., Wild, C., and Mitchell, E. A. (2009). Prevalence and determinants of cytomegalovirus infection in pre-school children. *J Paediatr Child Health* **45**(5), 291-6.

Ogata, M. (2009). Human herpesvirus 6 in hematological malignancies. *J Clin Exp Hematop* **49**(2), 57-67.

Ogata, M., Satou, T., Kawano, R., Takakura, S., Goto, K., Ikewaki, J., Kohno, K., Ikebe, T., Ando, T., Miyazaki, Y., Ohtsuka, E., Saburi, Y., Saikawa, T., and Kadota, J. (2010). Correlations of HHV-6 viral load and plasma IL-6 concentration with HHV-6 encephalitis in allogeneic stem cell transplant recipients. *Bone Marrow Transplant* **45**(1), 129-36.

Ogawa-Goto, K., Tanaka, K., Gibson, W., Moriishi, E., Miura, Y., Kurata, T., Irie, S., and Sata, T. (2003). Microtubule network facilitates nuclear targeting of human cytomegalovirus capsid. *J Virol* **77**(15), 8541-7.

Ogawa, H., Suzutani, T., Baba, Y., Koyano, S., Nozawa, N., Ishibashi, K., Fujieda, K., Inoue, N., and Omori, K. (2007). Etiology of severe sensorineural hearing loss in children: independent impact of congenital cytomegalovirus infection and GJB2 mutations. *J Infect Dis* **195**(6), 782-8.

Ohagen, A., Li, L., Rosenzweig, A., and Gabuzda, D. (2000). Cell-dependent mechanisms restrict the HIV type 1 coreceptor activity of US28, a chemokine receptor homolog encoded by human cytomegalovirus. *AIDS Res Hum Retroviruses* **16**(1), 27-35.

Okada, K., Ueda, K., Kusuhara, K., Miyazaki, C., Tokugawa, K., Hirose, M., and Yamanishi, K. (1993). Exanthema subitum and human herpesvirus 6 infection: clinical observations in fifty-seven cases. *Pediatr Infect Dis J* **12**(3), 204-8.

Olaleye, O. D., Omilabu, S. A., and Baba, S. S. (1990). Cytomegalovirus infection among tuberculosis patients in a chest hospital in Nigeria. *Comp Immunol Microbiol Infect Dis* **13**(2), 101-6.

Oliver, S. E., Cloud, G. A., Sanchez, P. J., Demmler, G. J., Dankner, W., Shelton, M., Jacobs, R. F., Vaudry, W., Pass, R. F., Soong, S. J., Whitley, R. J., and Kimberlin, D. W. (2009). Neurodevelopmental outcomes following ganciclovir therapy in symptomatic congenital cytomegalovirus infections involving the central nervous system. *J Clin Virol* **46 Suppl 4**, S22-6.

Otieno, R. O., Ouma, C., Ong'echa, J. M., Keller, C. C., Were, T., Waindi, E. N., Michaels, M. G., Day, R. D., Vulule, J. M., and Perkins, D. J. (2006). Increased severe anemia in HIV-1-exposed and HIV-1-positive infants and children during acute malaria. *Aids* **20**(2), 275-80.

Ouyang, Q., Wagner, W. M., Zheng, W., Wikby, A., Remarque, E. J., and Pawelec, G. (2004). Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly. *Exp Gerontol* **39**(4), 607-13.

Page, M. J., Dreese, J. C., Poritz, L. S., and Koltun, W. A. (1998). Cytomegalovirus enteritis: a highly lethal condition requiring early detection and intervention. *Dis Colon Rectum* **41**(5), 619-23.

Parker, D. C. (1993). T cell-dependent B cell activation. *Annu Rev Immunol* **11**, 331-60.

Paterson, D. A., Dyer, A. P., Milne, R. S., Sevilla-Reyes, E., and Gompels, U. A. (2002). A role for human cytomegalovirus glycoprotein O (gO) in cell fusion and a new hypervariable locus. *Virology* **293**(2), 281-94.

Pedersen, S. M., Oster, B., Bundgaard, B., and Hollsberg, P. (2006). Induction of cell-cell fusion from without by human herpesvirus 6B. *J Virol* **80**(19), 9916-20.

Penfold, M. E., Dairaghi, D. J., Duke, G. M., Saederup, N., Mocarski, E. S., Kemble, G. W., and Schall, T. J. (1999). Cytomegalovirus encodes a potent alpha chemokine. *Proc Natl Acad Sci U S A* **96**(17), 9839-44.

Peterson, P. K., Gekker, G., Chao, C. C., Hu, S. X., Edelman, C., Balfour, H. H., Jr., and Verhoef, J. (1992). Human cytomegalovirus-stimulated peripheral blood mononuclear cells induce HIV-1 replication via a tumor necrosis factor-alpha-mediated mechanism. *J Clin Invest* **89**(2), 574-80.

Petrucelli, A., Rak, M., Grainger, L., and Goodrum, F. (2009). Characterization of a novel Golgi apparatus-localized latency determinant encoded by human cytomegalovirus. *J Virol* **83**(11), 5615-29.

Pfeiffer, B., Berneman, Z. N., Neipel, F., Chang, C. K., Tirwatnapong, S., and Chandran, B. (1993). Identification and mapping of the gene encoding the glycoprotein complex gp82-gp105 of human herpesvirus 6 and mapping of the neutralizing epitope recognized by monoclonal antibodies. *J Virol* **67**(8), 4611-20.

Pietroboni, G. R., Harnett, G. B., Farr, T. J., and Bucens, M. R. (1988). Human herpes virus type 6 (HHV-6) and its in vitro effect on human immunodeficiency virus (HIV). *J Clin Pathol* **41**(12), 1310-2.

Pignatelli, S., Dal Monte, P., and Landini, M. P. (2001). gpUL73 (gN) genomic variants of human cytomegalovirus isolates are clustered into four distinct genotypes. *J Gen Virol* **82**(Pt 11), 2777-84.

Pignatelli, S., Dal Monte, P., Landini, M. P., Severi, B., Nassiri, R., Gilloteaux, J., Papadimitriou, J. M., Shellam, G. R., Mertens, T., Buser, C., Michel, D., and Walther, P. (2007). Cytomegalovirus primary envelopment at large nuclear membrane infoldings: what's new? *J Virol* **81**(13), 7320-1; author reply 7321-2.

Pignatelli, S., Dal Monte, P., Rossini, G., Camozzi, D., Toscano, V., Conte, R., and Landini, M. P. (2006). Latency-associated human cytomegalovirus glycoprotein N genotypes in monocytes from healthy blood donors. *Transfusion* **46**(10), 1754-62.

Pignatelli, S., Dal Monte, P., Rossini, G., Chou, S., Gojobori, T., Hanada, K., Guo, J. J., Rawlinson, W., Britt, W., Mach, M., and Landini, M. P. (2003a). Human cytomegalovirus glycoprotein N (gpUL73-gN) genomic variants: identification of a novel subgroup, geographical distribution and evidence of positive selective pressure. *J Gen Virol* **84**(Pt 3), 647-55.

Pignatelli, S., Rossini, G., Dal Monte, P., Gatto, M. R., and Landini, M. P. (2003b). Human cytomegalovirus glycoprotein N genotypes in AIDS patients. *Aids* **17**(5), 761-3.

Pita-Lopez, M. L., Gayoso, I., Delarosa, O., Casado, J. G., Alonso, C., Munoz-Gomariz, E., Tarazona, R., and Solana, R. (2009). Effect of ageing on CMV-specific CD8 T cells from CMV seropositive healthy donors. *Immun Ageing* **6**, 11.

Plachter, B., Sinzger, C., and Jahn, G. (1996). Cell types involved in replication and distribution of human cytomegalovirus. *Adv Virus Res* **46**, 195-261.

Pleskoff, O., Treboute, C., Brelot, A., Heveker, N., Seman, M., and Alizon, M. (1997). Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. *Science* **276**(5320), 1874-8.

Pohlmann, C., Schetelig, J., Reuner, U., Bornhauser, M., Illmer, T., Kiani, A., Ehninger, G., Jacobs, E., and Rohayem, J. (2007). Cidofovir and foscarnet for treatment of human herpesvirus 6 encephalitis in a neutropenic stem cell transplant recipient. *Clin Infect Dis* **44**(12), e118-20.

Portolani, M., Cermelli, C., Moroni, A., Bertolani, M. F., Di Luca, D., Cassai, E., and Sabbatini, A. M. (1993). Human herpesvirus-6 infections in infants admitted to hospital. *J Med Virol* **39**(2), 146-51.

Potenza, L., Luppi, M., Barozzi, P., Rossi, G., Cocchi, S., Codeluppi, M., Pecorari, M., Masetti, M., Di Benedetto, F., Gennari, W., Portolani, M., Gerunda, G. E., Lazzarotto, T., Landini, M. P., Schulz, T. F., Torelli, G., and Guaraldi, G. (2008). HHV-6A in syncytial giant-cell hepatitis. *N Engl J Med* **359**(6), 593-602.

Prentice, A. M., and Darboe, M. K. (2008). Growth and host-pathogen interactions. *Nestle Nutr Workshop Ser Pediatr Program* **61**, 197-210.

Price, P., Witt, C., de Santis, D., and French, M. A. (2007). Killer immunoglobulin-like receptor genotype may distinguish immunodeficient HIV-infected patients resistant to immune restoration diseases associated with herpes virus infections. *J Acquir Immune Defic Syndr* **45**(3), 359-61.

Prichard, M. N., Penfold, M. E., Duke, G. M., Spaete, R. R., and Kemble, G. W. (2001). A review of genetic differences between limited and extensively passaged human cytomegalovirus strains. *Rev Med Virol* **11**(3), 191-200.

Provenzale, J. M., van Landingham, K., and White, L. E. (2010). Clinical and imaging findings suggesting human herpesvirus 6 encephalitis. *Pediatr Neurol* **42**(1), 32-9.

Pusztai, R., Hohmann, J., Redei, D., Engi, H., and Molnar, J. (2008). Inhibition of human cytomegalovirus IE gene expression by dihydro-beta-agarofuran sesquiterpenes isolated from Euonymus species. *In Vivo* **22**(6), 787-92.

Quinlivan, M., Hawrami, K., Barrett-Muir, W., Aaby, P., Arvin, A., Chow, V. T., John, T. J., Matondo, P., Peiris, M., Poulsen, A., Siqueira, M., Takahashi, M., Talukder, Y., Yamanishi, K., Leedham-Green, M., Scott, F. T., Thomas, S. L., and Breuer, J. (2002). The molecular epidemiology of varicella-zoster virus: evidence for geographic segregation. *J Infect Dis* **186**(7), 888-94.

Rasmussen, L., and Cowan, C. M. (2003). Neutralizing antibody to gB2 human cytomegalovirus does not prevent reactivation in patients with human immunodeficiency virus infection. *J Gen Virol* **84**(Pt 7), 1853-7.

Rasmussen, L., Geissler, A., Cowan, C., Chase, A., and Winters, M. (2002). The genes encoding the gCIII complex of human cytomegalovirus exist in highly diverse combinations in clinical isolates. *J Virol* **76**(21), 10841-8.

Rasmussen, L., Geissler, A., and Winters, M. (2003). Inter- and intragenic variations complicate the molecular epidemiology of human cytomegalovirus. *J Infect Dis* **187**(5), 809-19.

Reeves, M. B., Lehner, P. J., Sissons, J. G., and Sinclair, J. H. (2005a). An in vitro model for the regulation of human cytomegalovirus latency and reactivation in dendritic cells by chromatin remodelling. *J Gen Virol* **86**(Pt 11), 2949-54.

Reeves, M. B., MacAry, P. A., Lehner, P. J., Sissons, J. G., and Sinclair, J. H. (2005b). Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proc Natl Acad Sci U S A* **102**(11), 4140-5.

Reeves, M. B., and Sinclair, J. H. (2009). Analysis of latent viral gene expression in natural and experimental latency models of human cytomegalovirus and its correlation with histone modifications at a latent promoter. *J Gen Virol.*

Reymen, D., Naesens, L., Balzarini, J., Holy, A., Dvorakova, H., and De Clercq, E. (1995). Antiviral activity of selected acyclic nucleoside analogues against human herpesvirus 6. *Antiviral Res* **28**(4), 343-57.

Robain, M., Carre, N., Dussaix, E., Salmon-Ceron, D., and Meyer, L. (1998). Incidence and sexual risk factors of cytomegalovirus seroconversion in HIV-infected subjects. The SEROCO Study Group. *Sex Transm Dis* **25**(9), 476-80.

Roizman B, D. R., Fleckenstein B, Lopez C, Minson AC, Studdert MJ. (2004). International Committee on Taxonomy of Viruses (C. Büchen-Osmond, Ed.), Vol. 2008.

Rosen, H. R., Corless, C. L., Rabkin, J., and Chou, S. (1998). Association of cytomegalovirus genotype with graft rejection after liver transplantation. *Transplantation* **66**(12), 1627-31.

Ross, D. S., Dollard, S. C., Victor, M., Sumartojo, E., and Cannon, M. J. (2006). The epidemiology and prevention of congenital cytomegalovirus infection and disease: activities of the Centers for Disease Control and Prevention Workgroup. *J Womens Health (Larchmt)* **15**(3), 224-9.

Rossini, G., Pignatelli, S., Dal Monte, P., Camozzi, D., Lazzarotto, T., Gabrielli, L., Gatto, M. R., and Landini, M. P. (2005). Monitoring for human cytomegalovirus infection in solid organ transplant recipients through antigenemia and glycoprotein N (gN) variants: evidence of correlation and potential prognostic value of gN genotypes. *Microbes Infect* **7**(5-6), 890-6.

Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365-86.

Ryckman, B. J., Chase, M. C., and Johnson, D. C. (2009). HCMV TR strain glycoprotein O acts as a chaperone promoting gH/gL incorporation into virions, but is not present in virions. *J Virol*.

Ryckman, B. J., Chase, M. C., and Johnson, D. C. (2010). Human Cytomegalovirus TR Strain Glycoprotein O Acts as a Chaperone Promoting gH/gL Incorporation into Virions but Is Not Present in Virions. *J Virol* **84**(5), 2597-609.

Sadaoka, T., Yamanishi, K., and Mori, Y. (2006). Human herpesvirus 7 U47 gene products are glycoproteins expressed in virions and associate with glycoprotein H. *J Gen Virol* **87**(Pt 3), 501-8.

Saha, S. K., Darmstadt, G. L., Hanif, M., and Khan, R. (2002). Seroepidemiology of varicella-zoster virus in Bangladesh. *Ann Trop Paediatr* **22**(4), 341-5.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**(4), 406-25.

Sakai, A., Watanabe, K., Koketsu, M., Akuzawa, K., Yamada, R., Li, Z., Sadanari, H., Matsubara, K., and Muroyama, T. (2008). Anti-human cytomegalovirus activity of constituents from Sasa albo-marginata (Kumazasa in Japan). *Antivir Chem Chemother* **19**(3), 125-32.

Salahuddin, S. Z., Ablashi, D. V., Markham, P. D., Josephs, S. F., Sturzenegger, S., Kaplan, M., Halligan, G., Biberfeld, P., Wong-Staal, F., Kramarsky, B., and et al. (1986). Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* **234**(4776), 596-601.

Sampaio, K. L., Cavignac, Y., Stierhof, Y. D., and Sinzger, C. (2005). Human cytomegalovirus labeled with green fluorescent protein for live analysis of intracellular particle movements. *J Virol* **79**(5), 2754-67.

Santoro, F., Kennedy, P. E., Locatelli, G., Malnati, M. S., Berger, E. A., and Lusso, P. (1999). CD46 is a cellular receptor for human herpesvirus 6. *Cell* **99**(7), 817-27.

Sarcinella, L., Mazzulli, T., Willey, B., and Humar, A. (2002). Cytomegalovirus glycoprotein B genotype does not correlate with outcomes in liver transplant patients. *J Clin Virol* **24**(1-2), 99-105.

Sarmati, L., Carlo, T., Rossella, S., Montano, M., Adalgisa, P., Rezza, G., and Andreoni, M. (2004). Human herpesvirus-8 infection in pregnancy and labor: lack of evidence of vertical transmission. *J Med Virol* **72**(3), 462-6.

Satilmis, A., Gura, A., Ongun, H., Mendilcioglu, I., Colak, D., and Oygur, N. (2007). CMV seroconversion in pregnants and the incidence of congenital CMV infection. *Turk J Pediatr* **49**(1), 30-6.

Schleiss, M. R. (2006a). Acquisition of human cytomegalovirus infection in infants via breast milk: natural immunization or cause for concern? *Rev Med Virol* **16**(2), 73-82.

Schleiss, M. R. (2006b). Role of breast milk in acquisition of cytomegalovirus infection: recent advances. *Curr Opin Pediatr* **18**(1), 48-52.

Schott, H., Hamprecht, K., Schott, S., Schott, T. C., and Schwendener, R. A. (2009). Synthesis and in vitro activities of a new antiviral duplex drug linking Zidovudine (AZT) and Foscarnet (PFA) via an octadecylglycerol residue. *Bioorg Med Chem* **17**(1), 303-10.

Schreiber, A., Harter, G., Schubert, A., Bunjes, D., Mertens, T., and Michel, D. (2009). Antiviral treatment of cytomegalovirus infection and resistant strains. *Expert Opin Pharmacother* **10**(2), 191-209.

Secchiero, P., Carrigan, D. R., Asano, Y., Benedetti, L., Crowley, R. W., Komaroff, A. L., Gallo, R. C., and Lusso, P. (1995). Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. *J Infect Dis* **171**(2), 273-80.

Shen, S., Wang, S., Britt, W. J., and Lu, S. (2007). DNA vaccines expressing glycoprotein complex II antigens gM and gN elicited neutralizing antibodies against multiple human cytomegalovirus (HCMV) isolates. *Vaccine* **25**(17), 3319-27.

Shibata, Y., Kitajima, N., Kawada, J., Sugaya, N., Nishikawa, K., Morishima, T., and Kimura, H. (2005). Association of cytomegalovirus with infantile hepatitis. *Microbiol Immunol* **49**(8), 771-7.

Shibuya, T. B. K. (2007). World Health Statistics 2007. Electronic ed (M. Pinchuk, Ed.), Vol. 2009. WHO Press.

Shimamura, M., Mach, M., and Britt, W. J. (2006). Human cytomegalovirus infection elicits a glycoprotein M (gM)/gN-specific virus-neutralizing antibody response. *J Virol* **80**(9), 4591-600.

Silva, P. A., Stark, K., Mockenhaupt, F. P., Reither, K., Weitzel, T., Ignatius, R., Saad, E., Seidu-Korkor, A., Bienzle, U., and Schreier, E. (2008). Molecular characterization of enteric viral agents from children in northern region of Ghana. *J Med Virol* **80**(10), 1790-8.

Sinclair, J., and Sissons, P. (2006). Latency and reactivation of human cytomegalovirus. *J Gen Virol* **87**(Pt 7), 1763-79.

Sindre, H., Tjoonnfjord, G. E., Rollag, H., Ranneberg-Nilsen, T., Veiby, O. P., Beck, S., Degre, M., and Hestdal, K. (1996). Human cytomegalovirus suppression of and latency in early hematopoietic progenitor cells. *Blood* **88**(12), 4526-33.

Sinzger, C., Digel, M., and Jahn, G. (2008). Cytomegalovirus cell tropism. *Curr Top Microbiol Immunol* **325**, 63-83.

Sinzger, C., Grefte, A., Plachter, B., Gouw, A. S., The, T. H., and Jahn, G. (1995). Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. *J Gen Virol* **76 (Pt 4)**, 741-50.

Slyker, J. A., Lohman-Payne, B. L., John-Stewart, G. C., Maleche-Obimbo, E., Emery, S., Richardson, B., Dong, T., Iversen, A. K., Mbori-Ngacha, D., Overbaugh, J., Emery, V. C., and Rowland-Jones, S. L. (2009). Acute cytomegalovirus infection in Kenyan HIV-infected infants. *Aids* **23**(16), 2173-81.

Smith, A., Santoro, F., Di Lullo, G., Dagna, L., Verani, A., and Lusso, P. (2003). Selective suppression of IL-12 production by human herpesvirus 6. *Blood* **102**(8), 2877-84.

Smith, A. P., Paolucci, C., Di Lullo, G., Burastero, S. E., Santoro, F., and Lusso, P. (2005). Viral replication-independent blockade of dendritic cell maturation and interleukin-12 production by human herpesvirus 6. *J Virol* **79**(5), 2807-13.

Soroceanu, L., Akhavan, A., and Cobbs, C. S. (2008). Platelet-derived growth factor-alpha receptor activation is required for human cytomegalovirus infection. *Nature* **455**(7211), 391-5.

Spear, P. G., and Longnecker, R. (2003). Herpesvirus entry: an update. *J Virol* **77**(19), 10179-85.

Spencer, J. V., Cadaoas, J., Castillo, P. R., Saini, V., and Slobedman, B. (2008). Stimulation of B lymphocytes by cmvIL-10 but not LAcmvIL-10. *Virology* **374**(1), 164-9.

Spencer, J. V., Lockridge, K. M., Barry, P. A., Lin, G., Tsang, M., Penfold, M. E., and Schall, T. J. (2002). Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. *J Virol* **76**(3), 1285-92.

Stagno, S., Pass, R. F., Cloud, G., Britt, W. J., Henderson, R. E., Walton, P. D., Veren, D. A., Page, F., and Alford, C. A. (1986). Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. *Jama* **256**(14), 1904-8.

Stagno, S., Pass, R. F., Dworsky, M. E., Henderson, R. E., Moore, E. G., Walton, P. D., and Alford, C. A. (1982). Congenital cytomegalovirus infection: The relative importance of primary and recurrent maternal infection. *N Engl J Med* **306**(16), 945-9.

Stagno, S., and Whitley, R. J. (1985). Herpesvirus infections of pregnancy. Part I: Cytomegalovirus and Epstein-Barr virus infections. *N Engl J Med* **313**(20), 1270-4.

Stanton, R., Westmoreland, D., Fox, J. D., Davison, A. J., and Wilkinson, G. W. (2005). Stability of human cytomegalovirus genotypes in persistently infected renal transplant recipients. *J Med Virol* **75**(1), 42-6.

Staras, S. A., Dollard, S. C., Radford, K. W., Flanders, W. D., Pass, R. F., and Cannon, M. J. (2006). Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. *Clin Infect Dis* **43**(9), 1143-51.

Stover, C. T., Smith, D. K., Schmid, D. S., Pellett, P. E., Stewart, J. A., Klein, R. S., Mayer, K., Vlahov, D., Schuman, P., and Cannon, M. J. (2003). Prevalence of and risk factors for viral infections among human immunodeficiency virus (HIV)-infected and high-risk HIV-uninfected women. *J Infect Dis* **187**(9), 1388-96.

Streblow, D. N., Soderberg-Naucler, C., Vieira, J., Smith, P., Wakabayashi, E., Ruchti, F., Mattison, K., Altschuler, Y., and Nelson, J. A. (1999). The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell* **99**(5), 511-20.

Streblow, D. N., Vomaske, J., Smith, P., Melnychuk, R., Hall, L., Pancheva, D., Smit, M., Casarosa, P., Schlaepfer, D. D., and Nelson, J. A. (2003). Human cytomegalovirus chemokine receptor US28-induced smooth muscle cell migration is mediated by focal adhesion kinase and Src. *J Biol Chem* **278**(50), 50456-65.

Suga, S., Yoshikawa, T., Asano, Y., Kozawa, T., Nakashima, T., Kobayashi, I., Yazaki, T., Yamamoto, H., Kajita, Y., Ozaki, T., and et al. (1993). Clinical and virological analyses of 21 infants with exanthem subitum (roseola infantum) and central nervous system complications. *Ann Neurol* **33**(6), 597-603.

Suga, S., Yoshikawa, T., Kajita, Y., Ozaki, T., and Asano, Y. (1998). Prospective study of persistence and excretion of human herpesvirus-6 in patients with exanthem subitum and their parents. *Pediatrics* **102**(4 Pt 1), 900-4.

Sutherland, C. L., Chalupny, N. J., and Cosman, D. (2001). The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer cell functions. *Immunol Rev* **181**, 185-92.

Sylwester, A. W., Mitchell, B. L., Edgar, J. B., Taormina, C., Pelte, C., Ruchti, F., Sleath, P. R., Grabstein, K. H., Hosken, N. A., Kern, F., Nelson, J. A., and Picker, L. J. (2005). Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med* **202**(5), 673-85.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**(8), 1596-9.

Tanaka-Taya, K., Kondo, T., Mukai, T., Miyoshi, H., Yamamoto, Y., Okada, S., and Yamanishi, K. (1996). Seroepidemiological study of human herpesvirus-6 and -7 in children of different ages and detection of these two viruses in throat swabs by polymerase chain reaction. *J Med Virol* **48**(1), 88-94.

Tanaka-Taya, K., Sashihara, J., Kurahashi, H., Amo, K., Miyagawa, H., Kondo, K., Okada, S., and Yamanishi, K. (2004). Human herpesvirus 6 (HHV-6) is transmitted from parent to child in an integrated form and characterization of cases with chromosomally integrated HHV-6 DNA. *J Med Virol* **73**(3), 465-73.

Taylor-Wiedeman, J., Hayhurst, G. P., Sissons, J. G., and Sinclair, J. H. (1993). Polymorphonuclear cells are not sites of persistence of human cytomegalovirus in healthy individuals. *J Gen Virol* **74 (Pt 2)**, 265-8.

Taylor-Wiedeman, J., Sissons, J. G., Borysiewicz, L. K., and Sinclair, J. H. (1991). Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J Gen Virol* **72 (Pt 9)**, 2059-64.

Taylor-Wiedeman, J., Sissons, P., and Sinclair, J. (1994). Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J Virol* **68**(3), 1597-604.

Taylor, P., Worrell, C., Steinberg, S. M., Hazra, R., Jankelevich, S., Wood, L. V., Zwerski, S., Yarchoan, R., and Zeichner, S. (2004). Natural history of lipid abnormalities and fat redistribution among human immunodeficiency virus-infected children receiving long-term, protease inhibitor-containing, highly active antiretroviral therapy regimens. *Pediatrics* **114**(2), e235-42.

Tejiokem, M. C., Gouandjika, I., Beniguel, L., Zanga, M. C., Tene, G., Gody, J. C., Njamkepo, E., Kfutwah, A., Penda, I., Bilong, C., Rousset, D., Pouillot, R., Tangy, F., and Baril, L. (2007). HIV-infected children living in Central Africa have low persistence of antibodies to vaccines used in the Expanded Program on Immunization. *PLoS One* **2**(12), e1260.

Thea, D. M., St Louis, M. E., Atido, U., Kanjinga, K., Kembo, B., Matondo, M., Tshiamala, T., Kamenga, C., Davachi, F., Brown, C., and et al. (1993). A prospective study of diarrhea and HIV-1 infection among 429 Zairian infants. *N Engl J Med* **329**(23), 1696-702.

Theiler, R. N., and Compton, T. (2001). Characterization of the signal peptide processing and membrane association of human cytomegalovirus glycoprotein O. *J Biol Chem* **276**(42), 39226-31.

Theiler, R. N., and Compton, T. (2002). Distinct glycoprotein O complexes arise in a post-Golgi compartment of cytomegalovirus-infected cells. *J Virol* **76**(6), 2890-8.

Theodore, W. H., Epstein, L., Gaillard, W. D., Shinnar, S., Wainwright, M. S., and Jacobson, S. (2008). Human herpes virus 6B: a possible role in epilepsy? *Epilepsia* **49**(11), 1828-37.

Tokimasa, S., Hara, J., Osugi, Y., Ohta, H., Matsuda, Y., Fujisaki, H., Sawada, A., Kim, J. Y., Sashihara, J., Amou, K., Miyagawa, H., Tanaka-Taya, K., Yamanishi, K., and Okada, S. (2002). Ganciclovir is effective for prophylaxis and treatment of human herpesvirus-6 in allogeneic stem cell transplantation. *Bone Marrow Transplant* **29**(7), 595-8.

Tolfvenstam, T., Enbom, M., Ghebrekidan, H., Ruden, U., Linde, A., Grandien, M., and Wahren, B. (2000). Seroprevalence of viral childhood infections in Eritrea. *J Clin Virol* **16**(1), 49-54.

Tomazin, R., Boname, J., Hegde, N. R., Lewinsohn, D. M., Altschuler, Y., Jones, T. R., Cresswell, P., Nelson, J. A., Riddell, S. R., and Johnson, D. C. (1999). Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. *Nat Med* **5**(9), 1039-43.

Tookey, P. A., Ades, A. E., and Peckham, C. S. (1992). Cytomegalovirus prevalence in pregnant women: the influence of parity. *Arch Dis Child* **67**(7 Spec No), 779-83.

Torelli, G., Barozzi, P., Marasca, R., Cocconcelli, P., Merelli, E., Ceccherini-Nelli, L., Ferrari, S., and Luppi, M. (1995). Targeted integration of human herpesvirus 6 in the p arm of chromosome 17 of human peripheral blood mononuclear cells in vivo. *J Med Virol* **46**(3), 178-88.

Trincado, D. E., Scott, G. M., White, P. A., Hunt, C., Rasmussen, L., and Rawlinson, W. D. (2000). Human cytomegalovirus strains associated with congenital and perinatal infections. *J Med Virol* **61**(4), 481-7.

Ukarapol, N., Chartapisak, W., Lertprasertsuk, N., Wongsawasdi, L., Kattipattanapong, V., Singhavejsakul, J., and Sirisanthana, V. (2002). Cytomegalovirus-associated manifestations involving the digestive tract in children with human immunodeficiency virus infection. *J Pediatr Gastroenterol Nutr* **35**(5), 669-73.

van der Sande, M. A., Kaye, S., Miles, D. J., Waight, P., Jeffries, D. J., Ojuola, O. O., Palmero, M., Pinder, M., Ismaili, J., Flanagan, K. L., Aveika, A. A., Zaman, A., Rowland-Jones, S., McConkey, S. J., Whittle, H. C., and Marchant, A. (2007). Risk factors for and clinical outcome of congenital cytomegalovirus infection in a peri-urban West-African birth cohort. *PLoS One* **2**(6), e492.

van Loon, N. M., Gummuluru, S., Sherwood, D. J., Marentes, R., Hall, C. B., and Dewhurst, S. (1995). Direct sequence analysis of human herpesvirus 6 (HHV-6) sequences from infants and comparison of HHV-6 sequences from mother/infant pairs. *Clin Infect Dis* **21**(4), 1017-9.

van Rheenen, P. F., de Moor, L. T., Eschbach, S., and Brabin, B. J. (2008). A cohort study of haemoglobin and zinc protoporphyrin levels in term Zambian infants: effects of iron stores at birth, complementary food and placental malaria. *Eur J Clin Nutr* **62**(12), 1379-87.

Vancikova, Z., and Dvorak, P. (2001). Cytomegalovirus infection in immunocompetent and immunocompromised individuals--a review. *Curr Drug Targets Immune Endocr Metabol Disord* **1**(2), 179-87.

Verbeeck, J., Van Kerschaver, E., Wollants, E., Beuselinck, K., Stappaerts, L., and Van Ranst, M. (2008). Detection of perinatal cytomegalovirus infection and sensorineural hearing loss in belgian infants by measurement of automated auditory brainstem response. *J Clin Microbiol* **46**(11), 3564-8.

Vomaske, J., Melnychuk, R. M., Smith, P. P., Powell, J., Hall, L., DeFilippis, V., Fruh, K., Smit, M., Schlaepfer, D. D., Nelson, J. A., and Streblow, D. N. (2009). Differential ligand binding to a human cytomegalovirus chemokine receptor determines cell type-specific motility. *PLoS Pathog* **5**(2), e1000304.

Vyse, A. J., Hesketh, L. M., and Pebody, R. G. (2009). The burden of infection with cytomegalovirus in England and Wales: how many women are infected in pregnancy? *Epidemiol Infect* **137**(4), 526-33.

Wagner, C. S., Ljunggren, H. G., and Achour, A. (2008). Immune modulation by the human cytomegalovirus-encoded molecule UL18, a mystery yet to be solved. *J Immunol* **180**(1), 19-24.

Wagner, C. S., Riise, G. C., Bergstrom, T., Karre, K., Carbone, E., and Berg, L. (2007). Increased expression of leukocyte Ig-like receptor-1 and activating role of UL18 in the response to cytomegalovirus infection. *J Immunol* **178**(6), 3536-43.

Walter, S., Atkinson, C., Sharland, M., Rice, P., Raglan, E., Emery, V. C., and Griffiths, P. D. (2008). Congenital cytomegalovirus: association between dried blood spot viral load and hearing loss. *Arch Dis Child Fetal Neonatal Ed* **93**(4), F280-5.

Wang, D., and Shenk, T. (2005a). Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. *J Virol* **79**(16), 10330-8.

Wang, D., and Shenk, T. (2005b). Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. *Proc Natl Acad Sci U S A* **102**(50), 18153-8.

Ward, K. N. (2005). The natural history and laboratory diagnosis of human herpesviruses-6 and -7 infections in the immunocompetent. *J Clin Virol* **32**(3), 183-93.

Ward, K. N., Andrews, N. J., Verity, C. M., Miller, E., and Ross, E. M. (2005). Human herpesviruses-6 and -7 each cause significant neurological morbidity in Britain and Ireland. *Arch Dis Child* **90**(6), 619-23.

Ward, K. N., Gray, J. J., Fotheringham, M. W., and Sheldon, M. J. (1993). IgG antibodies to human herpesvirus-6 in young children: changes in avidity of antibody correlate with time after infection. *J Med Virol* **39**(2), 131-8.

Ward, K. N., Leong, H. N., Nacheva, E. P., Howard, J., Atkinson, C. E., Davies, N. W., Griffiths, P. D., and Clark, D. A. (2006). Human herpesvirus 6 chromosomal integration in immunocompetent patients results in high levels of viral DNA in blood, sera, and hair follicles. *J Clin Microbiol* **44**(4), 1571-4.

Ward, K. N., Thiruchelvam, A. D., and Couto-Parada, X. (2005). Unexpected occasional persistence of high levels of HHV-6 DNA in sera: detection of variants A and B. *J Med Virol* **76**(4), 563-70.

Wille, P. T., Knoche, A. J., Nelson, J. A., Jarvis, M. A., and Johnson, D. C. (2009). An HCMV gO-null mutant fails to incorporate gH/gL into the virion envelope and is unable to enter fibroblasts, epithelial, and endothelial cells. *J Virol.*

Williams-Aziz, S. L., Hartline, C. B., Harden, E. A., Daily, S. L., Prichard, M. N., Kushner, N. L., Beadle, J. R., Wan, W. B., Hostetler, K. Y., and Kern, E. R. (2005). Comparative activities of lipid esters of cidofovir and cyclic cidofovir against replication of herpesviruses in vitro. *Antimicrob Agents Chemother* **49**(9), 3724-33.

Williams, A. J., Duong, T., McNally, L. M., Tookey, P. A., Masters, J., Miller, R., Lyall, E. G., and Gibb, D. M. (2001). Pneumocystis carinii pneumonia and cytomegalovirus infection in children with vertically acquired HIV infection. *Aids* **15**(3), 335-9.

Yamamoto, K., Yoshikawa, T., Okamoto, S., Yamaki, K., Shimokata, K., and Nishiyama, Y. (2005). HHV-6 and 7 DNA loads in lung tissues collected from patients with interstitial pneumonia. *J Med Virol* **75**(1), 70-5.

Yamamoto, T., Mukai, T., Kondo, K., and Yamanishi, K. (1994). Variation of DNA sequence in immediate-early gene of human herpesvirus 6 and variant identification by PCR. *J Clin Microbiol* **32**(2), 473-6.

Yamanishi, K. M., Y; Pellet, P.E (2007). Human Herpesviruses 6 and 7. 5 ed. *In* "Fields Virology" (P. M. H. e. a. D.M Knipe, Ed.), Vol. 2, pp. 2819-2845. 2 vols. Lippincot, Williams and Wilkins, Philidelphia.

Yan, H., Koyano, S., Inami, Y., Yamamoto, Y., Suzutani, T., Mizuguchi, M., Ushijima, H., Kurane, I., and Inoue, N. (2008). Genetic linkage among human cytomegalovirus glycoprotein N (gN) and gO genes, with evidence for recombination from congenitally and post-natally infected Japanese infants. *J Gen Virol* **89**(Pt 9), 2275-9.

Yao, K., Honarmand, S., Espinosa, A., Akhyani, N., Glaser, C., and Jacobson, S. (2009). Detection of human herpesvirus-6 in cerebrospinal fluid of patients with encephalitis. *Ann Neurol* **65**(3), 257-67.

Yoshikawa, T., Asano, Y., Akimoto, S., Ozaki, T., Iwasaki, T., Kurata, T., Goshima, F., and Nishiyama, Y. (2002). Latent infection of human herpesvirus 6 in astrocytoma cell line and alteration of cytokine synthesis. *J Med Virol* **66**(4), 497-505.

Yoshikawa, T., Ihira, M., Taguchi, H., Yoshida, S., and Asano, Y. (2005). Analysis of shedding of 3 beta-herpesviruses in saliva from patients with connective tissue diseases. *J Infect Dis* **192**(9), 1530-6.

Yoshikawa, T., Suzuki, K., Ihira, M., Furukawa, H., Suga, S., Iwasaki, T., Kurata, T., Asonuma, K., Tanaka, K., and Asano, Y. (1999). Human herpesvirus 6 latently infects mononuclear cells but not liver tissue. *J Clin Pathol* **52**(1), 65-7.

Yurochko, A. D., Huong, S. M., and Huang, E. S. (1999). Identification of human cytomegalovirus target sequences in the human immunodeficiency virus long terminal repeat. Potential role of IE2-86 binding to sequences between -120 and -20 in promoter transactivation. *J Hum Virol* **2**(2), 81-90.

Zanolla, G., Resener, T., Knebel, R., and Verney, Y. (2001). Massive lower gastrointestinal hemorrhage caused by CMV disease as a presentation of HIV in an infant. *Pediatr Surg Int* **17**(1), 65-7.

Zerr, D. M., Gupta, D., Huang, M. L., Carter, R., and Corey, L. (2002). Effect of antivirals on human herpesvirus 6 replication in hematopoietic stem cell transplant recipients. *Clin Infect Dis* **34**(3), 309-17.

Zerr, D. M., Meier, A. S., Selke, S. S., Frenkel, L. M., Huang, M. L., Wald, A., Rhoads, M. P., Nguy, L., Bornemann, R., Morrow, R. A., and Corey, L. (2005). A population-based study of primary human herpesvirus 6 infection. *N Engl J Med* **352**(8), 768-76.

Zhao, J., Fan, H., Mu, G., Shen, X., and Cheng, X. (1997). Detection of human herpesvirus 6(HHV-6) DNA in salivary glands by the polymerase chain reaction. *Chin Med Sci J* **12**(2), 126-8.

Zhu, H., Cong, J. P., Mamtora, G., Gingeras, T., and Shenk, T. (1998). Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays. *Proc Natl Acad Sci U S A* **95**(24), 14470-5.

Zou, P., Isegawa, Y., Nakano, K., Haque, M., Horiguchi, Y., and Yamanishi, K. (1999). Human herpesvirus 6 open reading frame U83 encodes a functional chemokine. *J Virol* **73**(7), 5926-33.

Zuckerkandl, E. P., L. (1965). Evolutionary divergence and convergence in proteins. *In* "Evolving Genes and Proteins" (V. B. a. H. J. Vogel, Ed.), pp. 97-166. Academic Press, New York.

9.0 Appendices

9.1 Appendix A - Oligonucleotide primer sequence table

Primer name	Paired with	sequence 5'- 3'	Target		Reference	Product size (bp)
			Organism	Gene (Glycoprotein)		
GAPup	GAPdown	GCT CCC TCT TTC TTT GCA GCA AT	Human	GAPDH	Asahi-Ozaki et al. 2006	104
GAPdown	GAPup	TAC CAT GAG TCC TTC CAC GAT AC	Human	GAPDH	Asahi-Ozaki et al. 2006	104
GAPprobe	-	6-FAM-TCCTGCACCACCAACTGCTTAGCACC-TAMRA	Human	GAPDH	Asahi-Ozaki et al. 2006	-
UGU47F	U47R	TTTCCAACTCGAATCTGACAC	HHV-6A/B	U47 (gO)	Bates et al. 2009	177
U47F	U47R	CGAAGAATCCTTGCGGATG	HHV-6A/B	U47 (gO)	Bates et al. 2009	208
U47R	U47F	GGCTCGTCCAAAGGAAGTG	HHV-6A/B	U47 (gO)	Bates et al. 2009	208
U47probe	-	6-FAM-TTTCCAACTCGAATCTGACACG-TAMRA	HHV-6A/B	U47 (gO)	Bates et al. 2009	-
U47OF	U47OR	TGGGAGCACAGTTATTTGACA	HHV-6A/B	U47 (gO)	Bates Unpublished	322
U47OR	U47OF	TTGGTACTAGGGATATGTGGTGAT	HHV-6A/B	U47 (gO)	Bates Unpublished	322
U46F	U46R	TGTCTGCTGTTAATCACGTC	HHV-6A/B	U46 (gN)	Bates et al. 2009	312
U46R	U46F	GCGATCTAATAACCCTTCAC	HHV-6A/B	U46 (gN)	Bates et al. 2009	312
U46-Up	U46-lw	TAACGCTTAAGATGAGTTGTAAG	HHV-6A/B	U46 (gN)	Bates et al. 2009	251
U46-lw	U46-Up	CGTCTCTGAAGCAAAAGTC	HHV-6A/B	U46 (gN)	Bates et al. 2009	251
U46OF	U46OR	CGCTGTACATTTTTCCAGGTGAC	HHV-6A/B	U46 (gN)	Bates Unpublished	627/626
U46OR	U46OF	CACACAACCATCCAGATAACCTC	HHV-6A/B	U46 (gN)	Bates Unpublished	627/626
GO1	GO2	CTCCTCTGTCATGGGGAGAAA	HCMV	UL74 (gO)	Mattick et al. 2004	469
GO2	GO1	AGAAAGACAAGTCATGGAAGG	HCMV	UL74 (gO)	Mattick et al. 2004	469
GO1O	GO2O	CAACGGTAGATGAGCAGCAA	HCMV	UL74 (gO)	Bates et al. 2008	791
GO2O	GO1O	CATGGCGTTAACCAGGTAGAA	HCMV	UL74 (gO)	Bates et al. 2008	791
GO-Up	GO-lw	CGACCAGAATCAGCAGTGAG	HCMV	UL74 (gO)	Bates et al. 2008	742
GO-lw	GO-Up	TGTACAGTTGCGTTGTGCGTA	HCMV	UL74 (gO)	Bates et al. 2008	742
U73OF	L73OR	CGCGACAGTACCAGTTGAGA	HCMV	UL73 (gN)	Bates Unpublished	599
L73OR	U73OF	GGGACTATCTAGACTCGCTGCT	HCMV	UL73 (gN)	Bates Unpublished	599
U73F	L73R	TTCGGTCGGTCAACATCGTAA	HCMV	UL73 (gN)	Bates et al. 2008	394
L73R	U73F	CACCCACGTATGTAAACCTTAC	HCMV	UL73 (gN)	Bates et al. 2008	394
gN-Up	gN-lw	TGGTGTGATGGAGTGGAAC	HCMV	UL73 (gN)	Bates et al. 2008	420
gN-lw	gN-Up	TAGCCTTTGGTGGTGGTTGC	HCMV	UL73 (gN)	Bates et al. 2008	420
gB1	gB2	GAGGACAACGAAATCCTGTTGGGCA	HCMV	UL55 (gB)	Mattes 2004	149
gB2	gB1	TCGACGGTGGAGATACTGCTGAGG	HCMV	UL55 (gB)	Mattes 2004	149
gBP3	-	6-FAM-CAATCATGCGTTTGAAGAGGTAGTCCACG-TAMRA	HCMV	UL55 (gB)	Mattes 2004	
UP	BUP	GAAAAATTCGGTTAAGGCAG	HIV	GAG	Nanteza et al 1998	169
BUP	UP	TCTGATCCTGTCTGAAGAGCTG	HIV	GAG	Nanteza et al 1998	169
BU3	UP	TAGTATGGGCAAGCAGGGAGCT	HIV	GAG	Nanteza et al 1998	69
HPOL4235-2 OF	HPOL4538 OR	CCCTACAATCCCCAAAGTCA	HIV	POL	Bima Unpublished	324
HPOL4538 OR	HPOL4235-2 OF	TACTGCCCCTTCACCTTTCCA	HIV	POL	Bima Unpublished	324
HPOL4327 IF	HPOL4481 IR	TAAGACAGCAGTACAAATGGCAG	HIV	POL	Bima Unpublished	175
HPOL4481 IR	HPOL4327 IF	GCTGTCCCTGTAATAAACCCG	HIV	POL	Bima Unpublished	175

9.2 Appendix B – Equations and Calculations

i)* Mass of plasmid (μg) = Size of plasmid (bp) x Average mass of one base pair (μg)
(m) (n) (1.096×10^{-15} μg)

*Derivation of DNA mass formula

$$m = \left[n\right]\left(\frac{\cancel{1\ mole}}{6.023 \times 10^{23}\ molecules\ (bp)}\right)\left(\frac{660\ g}{\cancel{mole}}\right) = \left[n\right]\left(1.096 \times 10^{-21}\ g/bp\right)$$

n = DNA size (bp)
Avogadros number = 6.023e23 molecules/mole
Average MW of a double-stranded DNA molecule = 660 g/mole

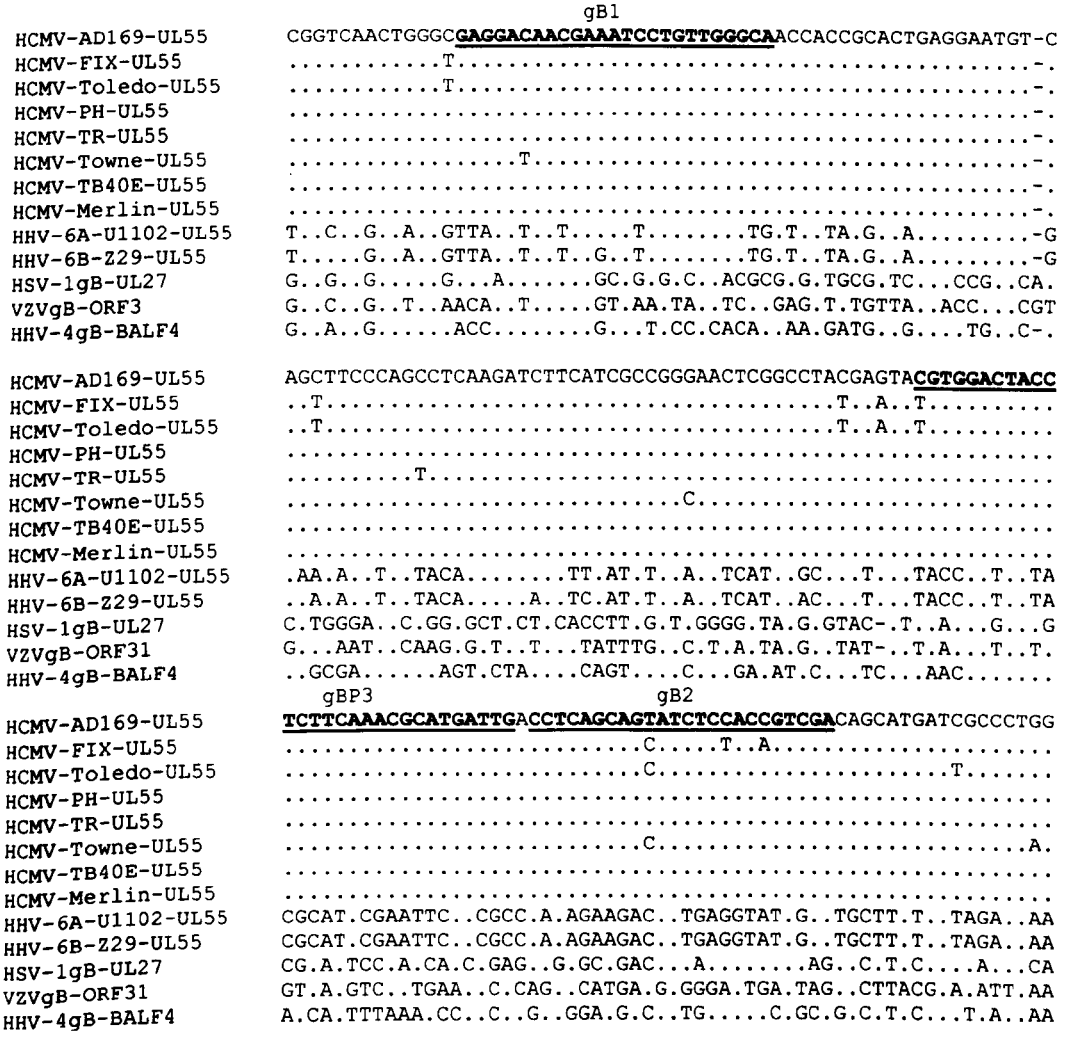
$$= \left[n\right]\left(1.096 \times 10^{-15}\ \mu g/bp\right)$$

ii) $\frac{\text{Concentration of Miniprep (μg/ μl)}}{\text{Mass of plasmid (μg)}}$ = Plasmid copies/μl

iii) $\frac{\text{Actual plasmid concentration (copies/ μl)}}{\text{Desired plasmid concentration (copies/ μl)}}$ = Dilution factor to achieve desired concentration

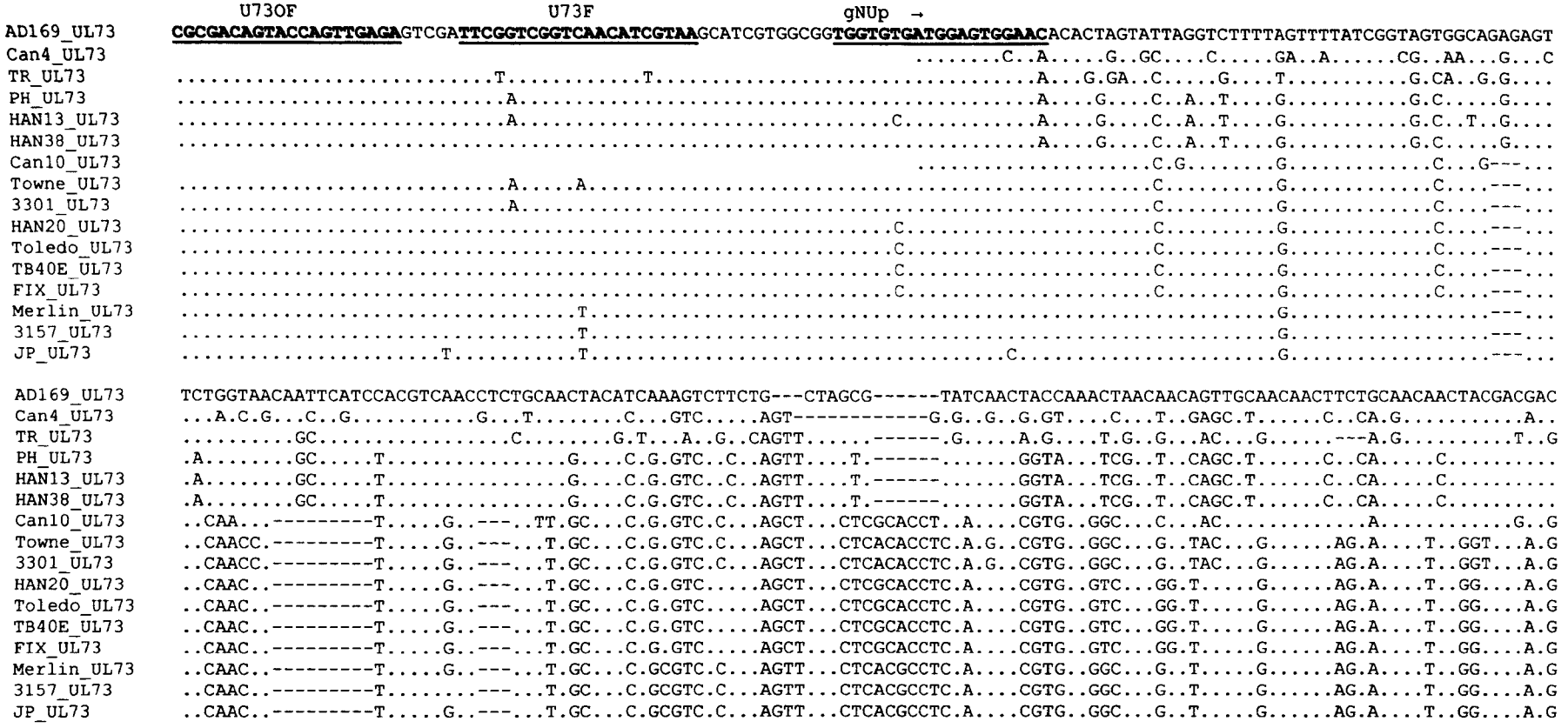
*Adapted from product support literature (Applied_Biosystems, 2003)

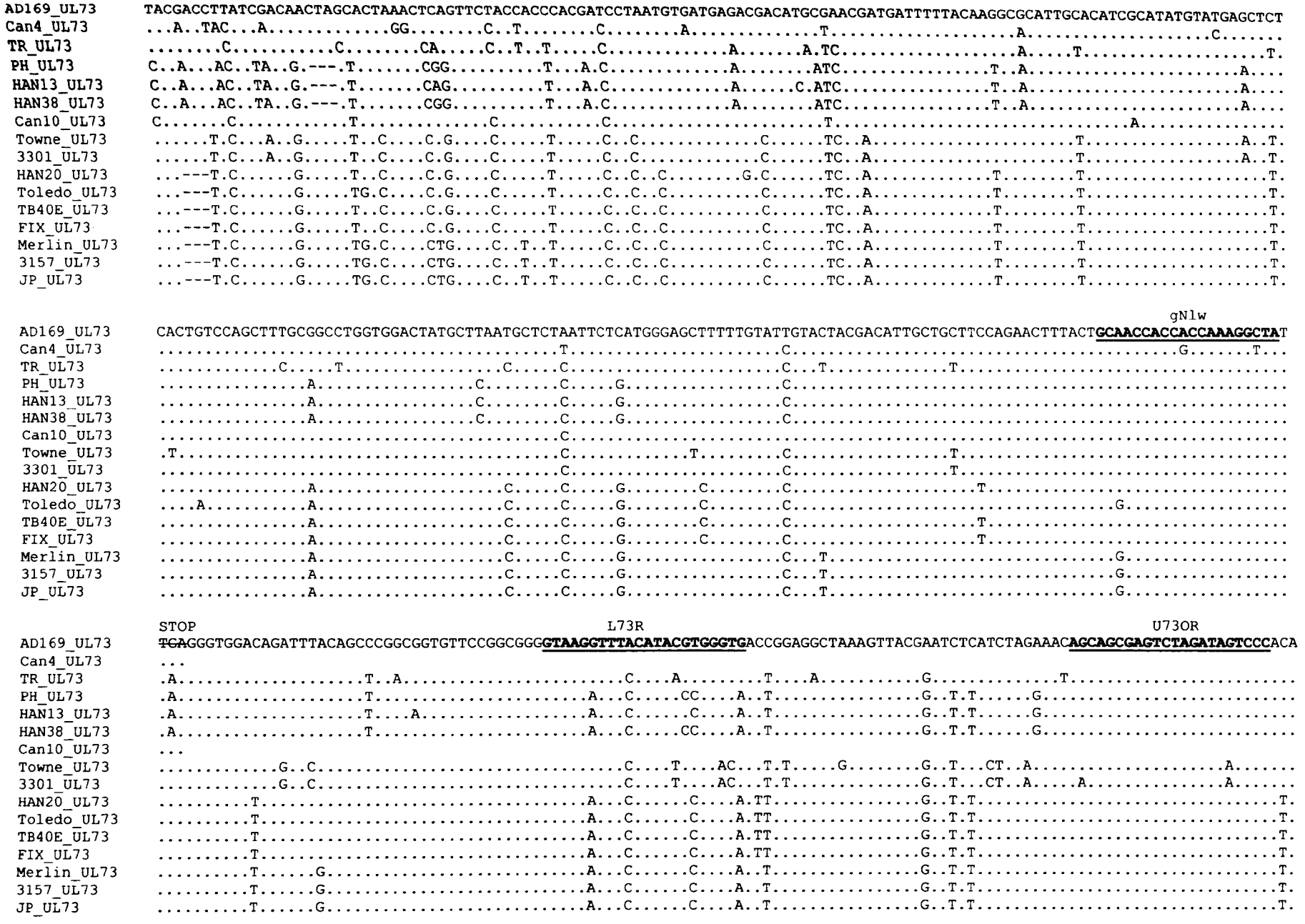
9.3 Appendix C – HCMV UL55 Oligonucleotide Primer Locations



The forward primer gB1 has one C-T mismatch against reference strain Towne. The reverse primer gB2 has a T-C mismatch against FIX, Toledo and Towne, with an additional C-T and C-A mismatch against FIX. Both gB2 and the probe (gPB3) were complementary to the coding strand shown. The pPB3 Taqman probe was labelled with the fluor 6-FAM (6-carboxyfluorescein) and quencher TAMRA (carboxy tetramethylrhodamine) at 5' and 3' ends respectively.

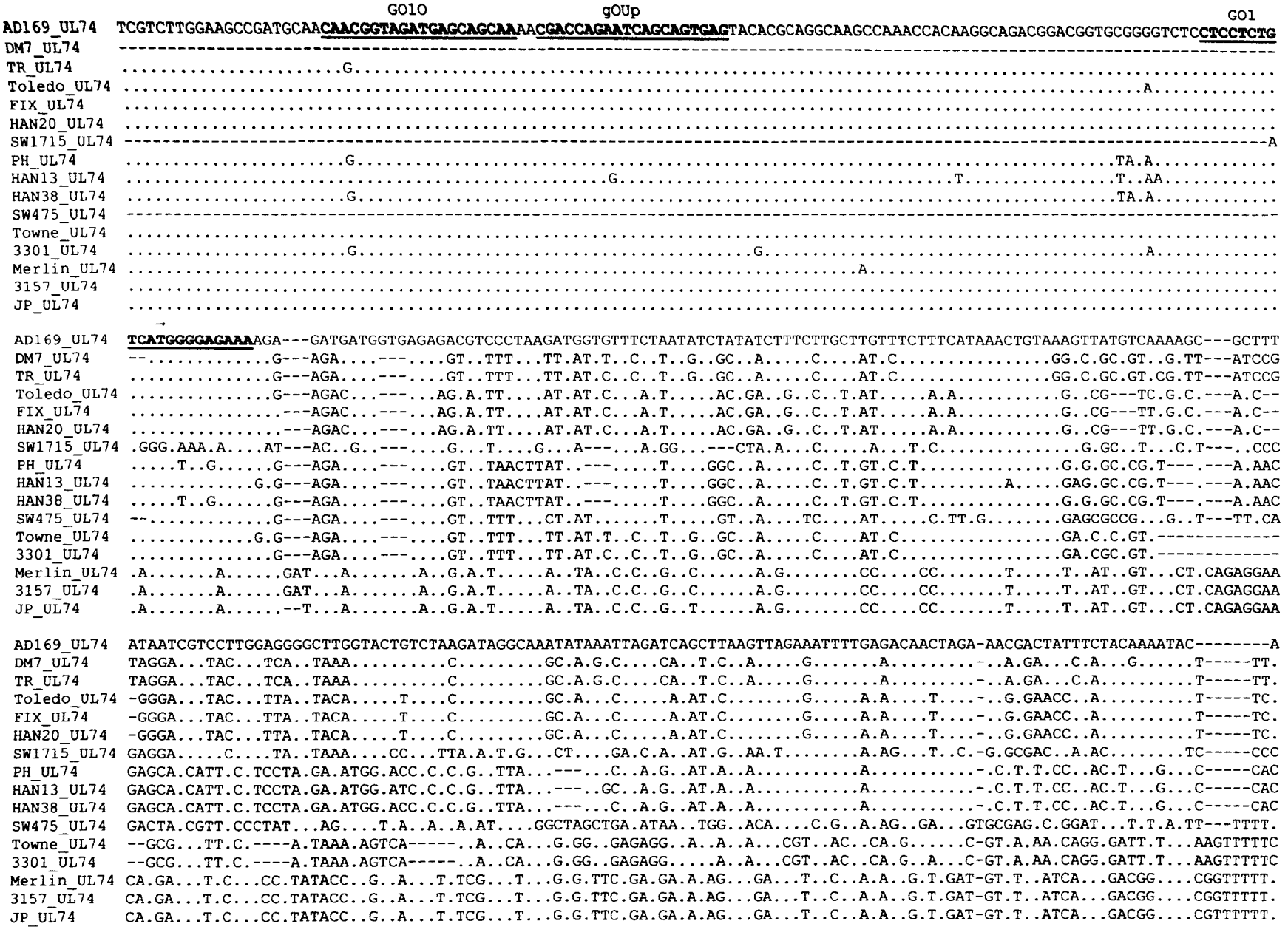
9.4 Appendix D – HCMV UL73 Oligonucleotide Primer Locations

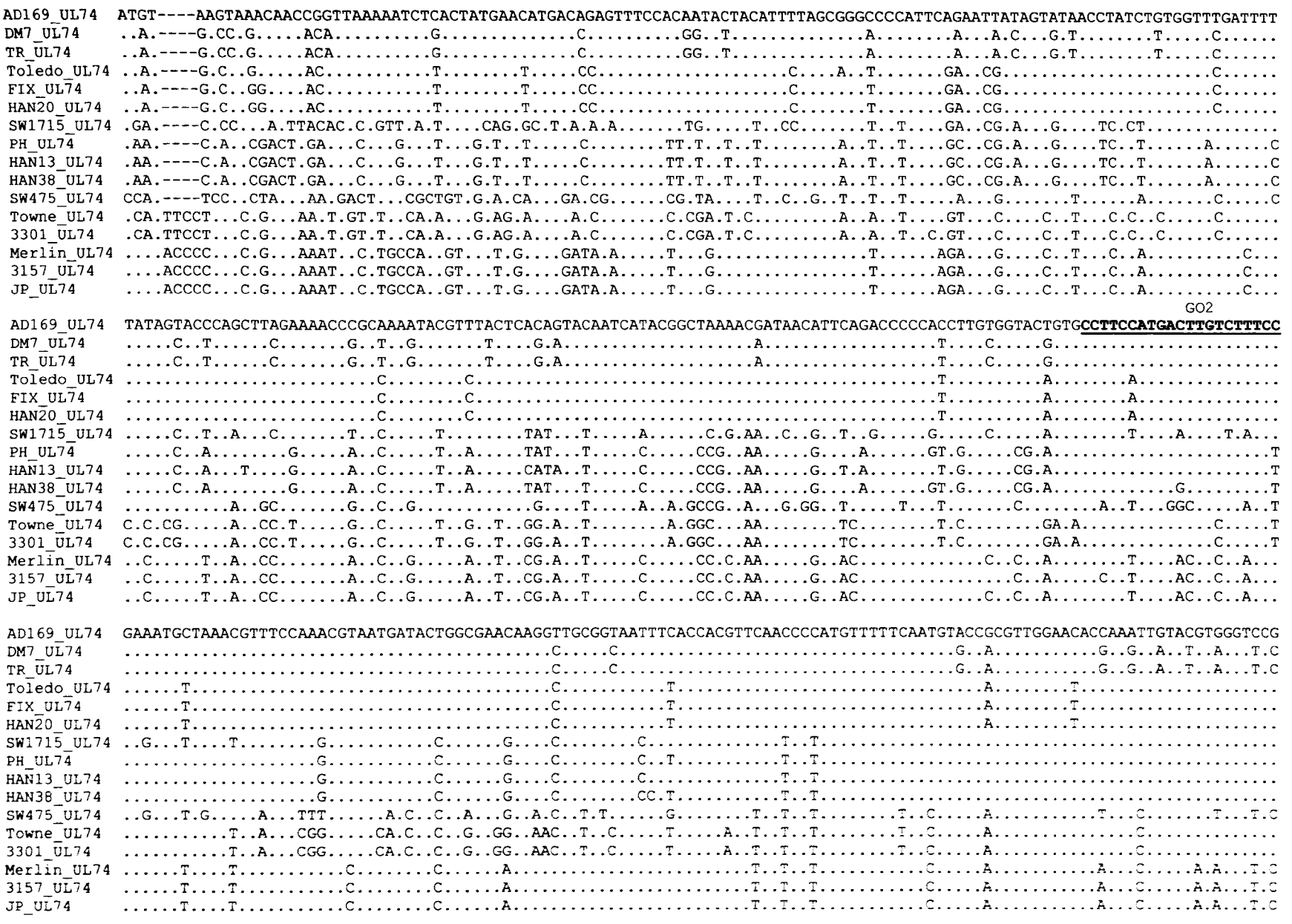



AD169_UL73 TACGACCTTATCGACAACTAGCACTAAACTCAGTTCTACCACCCACGATCCTAATGTGATGAGACGACATGCGAACGATGATTTTTACAAGGCGCATTGCACATCGCATATGTATGAGCTCT
Can4_UL73 ...A..TAC...A.....GG.....C..T.....C.....A.....T.....A.....C.....
TR_UL73 .....C.....C.....CA.....C..T..T.....C.....A.....A.TC.....A.....T.....T.
PH_UL73 C..A...AC..TA..G.---.T.....CGG.....T...A.C.....A.....ATC.....T..A.....A....
HAN13_UL73 C..A...AC..TA..G.---.T.....CAG.....T...A.C.....A.....C.ATC.....T..A.....A....
HAN38_UL73 C..A...AC..TA..G.---.T.....CGG.....T...A.C.....A.....ATC.....T..A.....A....
Can10_UL73 C.....C.....T.....C.....C.....T.....A.....
Towne_UL73 .....T.C...A..G.....T..C....C.G....C.....T.....C..C.....C.....TC..A.....T.....A..T.
3301_UL73 .....T.C...A..G.....T..C....C.G....C.....T.....C..C.....C.....TC..A.....T.....A..T.
HAN20_UL73 ...---T.C.....G.....T..C....C.G....C.....T.....C..C..C.....G.C.....TC..A.....T.....T.....T.
Toledo_UL73 ...---T.C.....G.....TG.C....C.G....C.....T.....C..C..C.....C.....TC..A.....T.....T.....T.
TB40E_UL73 ...---T.C.....G.....T..C....C.G....C.....T.....C..C..C.....C.....TC..A.....T.....T.....T.
FIX_UL73 ...---T.C.....G.....T..C....C.G....C.....T.....C..C..C.....C.....TC..A.....T.....T.....T.
Merlin_UL73 ...---T.C.....G.....TG.C....CTG....C..T..T.....C..C..C.....C.....TC..A.....T.....T.....T.
3157_UL73 ...---T.C.....G.....TG.C....CTG....C..T..T.....C..C..C.....C.....TC..A.....T.....T.....T.
JP_UL73 ...---T.C.....G.....TG.C....CTG....C..T..T.....C..C..C.....C.....TC..A.....T.....T.....T.
gN1w
AD169_UL73 CACTGTCCAGCTTTGCGGCCTGGTGGACTATGCTTAATGCTCTAATTCTCATGGGAGCTTTTTGTATTGTACTACGACATTGCTGCTTCCAGAACTTTACTGCAACCACCACCAAAGGCTATT
Can4_UL73 .....T.....C.....G.....T...
TR_UL73 .....C.....T.....C.....C.....C...T.....T.....
PH_UL73 .....A.....C.....C.....G.....C.....
HAN13_UL73 .....A.....C.....C.....G.....C.....
HAN38_UL73 .....A.....C.....C.....G.....C.....
Can10_UL73 .....C.....
Towne_UL73 .T.....C.....T.....C.....
3301_UL73 .....C.....C.....T.....
HAN20_UL73 .....A.....C.....C.....G.....C.....C.....T.....
Toledo_UL73 ....A.....A.....C.....C.....G.....C.....C.....G.....
TB40E_UL73 .....A.....C.....C.....G.....C.....C.....T.....
FIX_UL73 .....A.....C.....C.....G.....C.....C.....T.....
Merlin_UL73 .....A.....C.....C.....G.....C...T.....G.....
3157_UL73 .....A.....C.....C.....G.....C...T.....G.....
JP_UL73 .....A.....C.....C.....G.....C...T.....G.....
STOP
L73R
U73OR
AD169_UL73 TGAGGGTGGACAGATTTACAGCCCGGCGGTGTTCCGGCGGGGTAAGGTTTACATACGTGGGTGACCGGAGGCTAAAGTTACGAATCTCATCTAGAAACAGCAGCGAGTCTAGATAGTCCCACA
Can4_UL73 ...
TR_UL73 .A.....T..A.....C....A.....T....A.....G.....T.....
PH_UL73 .A.....T.....A...C.....CC....A..T.....G..T.T.....G.....
HAN13_UL73 .A.....T....A.....A...C.....C....A..T.....G..T.T.....G.....
HAN38_UL73 .A.....T.....A...C.....CC....A..T.....G..T.T.....G.....
Can10_UL73 ...
Towne_UL73 .....G..C.....C....T....AC...T.T.....G.....G..T...CT..A.....A.....
3301_UL73 .....G..C.....C....T....AC...T.T.....G..T...CT..A.....A.....A.....
HAN20_UL73 .....T.....A...C.....C....A.TT.....G..T.T.....T.
Toledo_UL73 .....T.....A...C.....C....A.TT.....G..T.T.....T.
TB40E_UL73 .....T.....A...C.....C....A.TT.....G..T.T.....T.
FIX_UL73 .....T.....A...C.....C....A.TT.....G..T.T.....T.
Merlin_UL73 .....T.....G.....A...C.....C....A..T.....G..T.T.....T.
3157_UL73 .....T.....G.....A...C.....C....A..T.....G..T.T.....T.
JP_UL73 .....T.....G.....A...C.....C....A..T.....G..T.T.....T.

Reference strains Can4 and Can10 are clinical isolates and no N- or C-terminal flanking sequence is available and so they have been left blank over these regions. The forward primer U73F contained two mismatches against strains TR and Towne. The gNUp forward primer has a detrimental 3' terminal C-A against Can4, TR and PH-like strains, with Can4 having an additional G-C mismatch just 4 nucleotides from the 3' terminus. The reverse primer gNlw has two mismatches against strain Can 4 and a A-G mismatch against reference strains Toledo and Merlin just 3 nucleotides from the 3' terminus. The primer, gNlw binds directly upstream from the gene's STOP codon. The second reverse primer, L73R, contains up to 5 mismatches against other reference strains but the 5 nucleotides at the 3' terminus are identical across all strains. Finally, the outer UL73 reverse primer (U73OR) has a 3' penultimate mismatch (G-A) against reference strain 3301. Reference strains HAN13, HAN38, 3301, HAN20, 3157 and JP were not available when primers were designed but are included here for reference.

9.5 Appendix E – HCMV UL74 Oligonucleotide Primer Locations

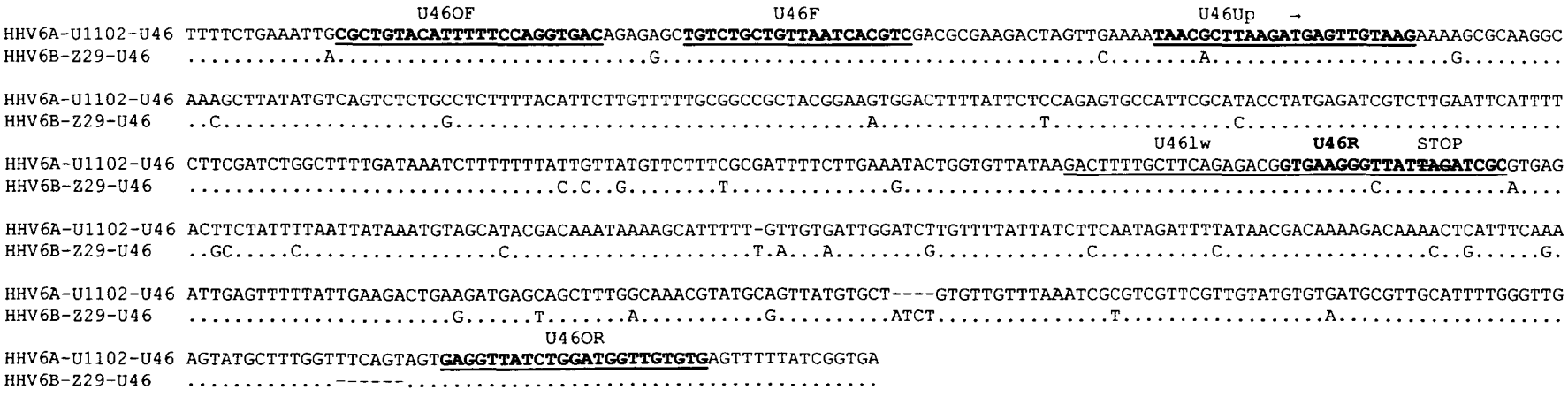



AD169_UL74 ATGT----AAGTAAACAACCGGTTAAAAATCTCACTATGAACATGACAGAGTTTCCACAATACTACATTTTAGCGGGCCCCATTCAGAATTATAGTATAACCTATCTGTGGTTTGATTTT
DM7_UL74 ..A.----G.CC.G.....ACA.....G.....C.....GG..T.....A.....A...A.C...G.T.....T.....C.....
TR_UL74 ..A.----G.CC.G.....ACA.....G.....C.....GG..T.....A.....A...A.C...G.T.....T.....C.....
Toledo_UL74 ..A.----G.C..G.....AC.....T.....T.....CC.....C....A..T.....GA..CG.....C.....
FIX_UL74 ..A.----G.C..GG....AC.....T.....T.....CC.....C.....T.....GA..CG.....C.....
HAN20_UL74 ..A.----G.C..GG....AC.....T.....T.....CC.....C.....T.....GA..CG.....C.....
SW1715_UL74 .GA.----C.CC...A.TTACAC.C.GTT.A.T....CAG.GC.T.A.A.A.....TG.....T..CC.....T..T....GA..CG.A...G....TC.CT.....
PH_UL74 .AA.----C.A..CGACT.GA...C...G...T...G.T..T.....C.....TT.T..T..T.....A..T..T....GC..CG.A...G....TC..T.....A.....C
HAN13_UL74 .AA.----C.A..CGACT.GA...C...G...T...G.T..T.....C.....TT.T..T..T.....A..T..T....GC..CG.A...G....TC..T.....A.....C
HAN38_UL74 .AA.----C.A..CGACT.GA...C...G...T...G.T..T.....C.....TT.T..T..T.....A..T..T....GC..CG.A...G....TC..T.....A.....C
SW475_UL74 CCA.----TCC..CTA...AA.GACT...CGCTGT.G.A.CA...GA.CG.....CG.TA....T..C..G..T..T..T.....A...G.....T.....A.....C.....C
Towne_UL74 .CA.TTCCT...C.G...AA.T.GT.T..CA.A...G.AG.A....A.C.....C.CGA.T.C.....A..A..T....GT...C....C..T...C.C..C.....C.....
3301_UL74 .CA.TTCCT...C.G...AA.T.GT.T..CA.A...G.AG.A....A.C.....C.CGA.T.C.....A..A..T..C.GT...C....C..T...C.C..C.....C.....
Merlin_UL74 ....ACCCC...C.G...AAAT..C.TGCCA..GT...T.G....GATA.A.....T...G.....T.....AGA...G....C..T...C..A.....C...
3157_UL74 ....ACCCC...C.G...AAAT..C.TGCCA..GT...T.G....GATA.A.....T...G.....T.....AGA...G....C..T...C..A.....C...
JP_UL74 ....ACCCC...C.G...AAAT..C.TGCCA..GT...T.G....GATA.A.....T...G.....T.....AGA...G....C..T...C..A.....C...
GO2
AD169_UL74 TATAGTACCCAGCTTAGAAAACCCGCAAAATACGTTTACTCACAGTACAATCATACGGCTAAAACGATAACATTCAGACCCCCACCTTGTGGTACTGTGCCTTCCATGACTTGTCTTTCC
DM7_UL74 .....C..T.....C.....G..T..G.....T....G.A.....A.....T....C.....G.....
TR_UL74 .....C..T.....C.....G..T..G.....T....G.A.....A.....T....C.....G.....
Toledo_UL74 .....C.....C.....T.....A.....A.....
FIX_UL74 .....C.....C.....T.....A.....A.....
HAN20_UL74 .....C.....C.....T.....A.....A.....
SW1715_UL74 .....C..T..A...C.....T..C.....T.....TAT...T.....A.....C.G.AA..C..G..T..G.....G.....C.....A.....T....A....T.A...
PH_UL74 .....C..A.....G.....A..C.....T..A.....TAT...T.....C.....CCG..AA.....G....A.....GT.G.....CG.A.....T
HAN13_UL74 .....C..A...T....G.....A..C.....T..A.....CATA..T.....C.....CCG..AA.....G..T.A.....T.G.....CG.A.....T
HAN38_UL74 .....C..A.....G.....A..C.....T..A.....TAT...T.....C.....CCG..AA.....G....A.....GT.G.....CG.A.....G.....T
SW475_UL74 .....A..GC.....G..C..G.....G....T.....A..A.GCCG..A...G.GG..T.....T...T.....C.....A..T...GGC.....A..T
Towne_UL74 C.C.CG.....A..CC.T.....G..C.....T..G..T..GG.A..T.....A.GGC...AA.....TC.....T.C.....GA.A.....C.....T
3301_UL74 C.C.CG.....A..CC.T.....G..C.....T..G..T..GG.A..T.....A.GGC...AA.....TC.....T.C.....GA.A.....C.....T
Merlin_UL74 ..C.....T..A..CC.....A..C..G.....A..T..CG.A..T.....C.....CC.C.AA.....G..AC.....C..C..A.....T....AC..C..A...
3157_UL74 ..C.....T..A..CC.....A..C..G.....A..T..CG.A..T.....C.....CC.C.AA.....G..AC.....C..A.....C..T....AC..C..A...
JP_UL74 ..C.....T..A..CC.....A..C..G.....A..T..CG.A..T.....C.....CC.C.AA.....G..AC.....C..C..A.....T....AC..C..A...
AD169_UL74 GAAATGCTAAACGTTTCCAAACGTAATGATACTGGCGAACAAGGTTGCGGTAATTTCACCACGTTCAACCCCATGTTTTTCAATGTACCGCGTTGGAACACCAAATTGTACGTGGGTCCG
DM7_UL74 .....C.....C.....G..A.....G..G..A..T..A...T.C
TR_UL74 .....C.....C.....G..A.....G..G..A..T..A...T.C
Toledo_UL74 .....C.....T.....A.....T.....
FIX_UL74 .....C.....T.....A.....T.....
HAN20_UL74 .....C.....T.....A.....T.....
SW1715_UL74 ..G...T....T.....G.....C.....G....C.....C.....T..T.....
PH_UL74 .....G.....C.....G....C.....C..T.....T..T.....
HAN13_UL74 .....G.....C.....G....C.....C.....T..T.....
HAN38_UL74 .....G.....C.....G....C.....CC.T.....T..T.....
SW475_UL74 ..G...T.G.....A...TTT.....A.C..C..A...G..A.C..T.T.....G.....T..T..T.....T..C.....A.....T...C.....T...T.C
Towne_UL74 .....T..A...CGG.....CA.C..C..G..GG..AAC..T..C.....T.....A..T..T..T.....T..C.....A.....C.....
3301_UL74 .....T..A...CGG.....CA.C..C..G..GG..AAC..T..C.....T.....A..T..T..T.....T..C.....A.....C.....
Merlin_UL74 .....T....T.....C.....C.....A.....T..T..T.....C.....A.....A...C.....A.A...T.C
3157_UL74 .....T....T.....C.....C.....A.....T..T..T.....C.....A.....A...C.....A.A...T.C
JP_UL74 .....T....T.....C.....C.....A.....T..T..T.....C.....A.....A...C.....A.A...T.C

```
                                                                                           gOlw                  GO2O
AD169_UL74   ACTAAGGTTAACGTAGATAGTCAAACGATTTATTTTCTAGGTTTAACCGCCCTGCTTTTACGTTACGCACAACGCAACTGTACACACAGTTTCTACCTGGTTAACGCCATGAGCCGGAAT
DM7_UL74     .AG..A..A.....G..............C..C...T....CC........T.....C..........G..............G................................A...
TR_UL74      .AG..A..A.....G..............C..C...T....CC........T.....C..........G..............G................................A...
Toledo_UL74  .....A..............................T.G..............A...C......................C.......................................
FIX_UL74     .....A..............................T.G..............A...C......................C.......................................
HAN20_UL74   .....A..............................T.G..............A...C......................C.......................................
SW1715_UL74  .....A.......................................G......................G...........C....G..................................
PH_UL74      .....A..............................T....................C..........G...........C.......................................
HAN13_UL74   .....A...................................................C..........G...........C.......................................
HAN38_UL74   .....A..............................T....................C..........G...........C.......................................
SW475_UL74   .....A..C..T..G........G.....C..C...T....CC..G...........C..........G.....T.....C....G..............................A...
Towne_UL74   .G...A..C..T..G.....C.....A.........T........G...........C..........G.....T..........G...C..............................
3301_UL74    .G...A..C..T..G.....C.....A.........T........G...........C..........G.....T..........G...C..............................
Merlin_UL74  .AC..A..C.....G........G..A..C..C...T.G..CC..........A........A.....G.....T.....C..T.G..............................A...
3157_UL74    .AC..A..C.....G........G..A..C..C...T.G..CC..........A........A.....G.....T.....C..T.G..............................A...
JP_UL74      .AC..A..C.....G........G..A..C..C...T.G..CC..........A........A.....G.....T.....C..T.G..............................A...
```

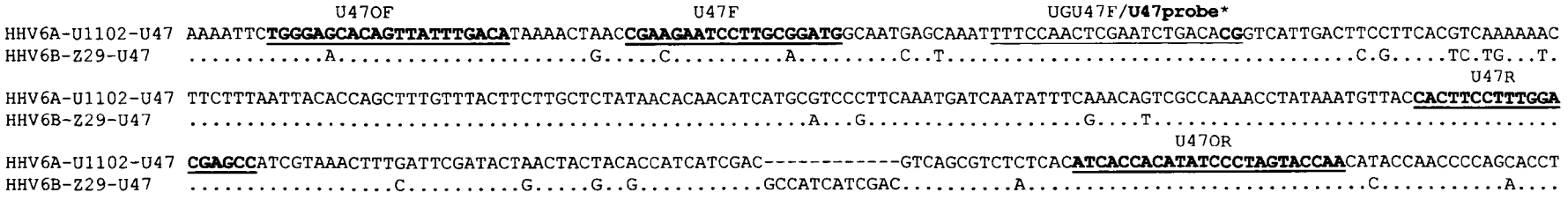
Reference strains DM7, SW1715 and SW475 are clinical isolates and only coding sequence has been published and is available. Where sequence is unavailable for these strains in the N-terminal flanking region the alignment has been left blank. The forward primer GO1O contains one A-G mismatch against reference strains TR and PH. The inner forward primer gOUp is identical across all reference strains. The GO1 primer bridges the UL74 start codon and contains a C-A mismatch against reference strain Merlin, a G-T and a A-G mismatch against PH and a G-A against SW1715 and Merlin. The first reverse primer GO2 has up to 7 mismatches (against reference strain SW475). The next reverse primer gOlw contains up to 4 mismatches (against reference strains Merlin, 3157 and JP). The outermost reverse primer GO2O has no mismatches. The arrow (→) indicates the start codon. Reference strains HAN13, HAN38, 3301, HAN20, 3157 and JP were not available when primers were designed but are included here for reference.

9.6 Appendix F – HHV-6 U46 Oligonucleotide Primer Locations



The forward primers U46OF and U46F contain no mismatches. The innermost forward primer, U46Up contains one G-A mismatch against reference strain Z29 and also bridges the gene's start codon. Inner reverse primers U46lw and U46R are adjacent with their being just one T-C mismatch in U46R. This primer also bridges the gene's STOP codon. The outermost reverse primer U46OR contains no mismatches.

9.7 Appendix G – HHV-6 U47 Oligonucleotide Primer Locations



The forward Primer U47OF has one G-A mismatch against reference strain Z29. U47F has two mismatches, one A-C near the 5' terminus and one G-A five nucleotides form the 3' terminus. The UGU47F primer and U47probe cover the same sequence, but differ in that the probe contains an additional G at the 5' terminus. The U47 Taqman probe was labelled with the fluor 6-FAM (6-carboxyfluorescein) and quencher TAMRA (carboxy tetramethylrhodamine) at 5' and 3' ends respectively. The reverse primers U47R and U47OR contained no mismatches.

9.8 Appendix H: HCMV sera-DNA at M6 – effects on growth

9.8.1 Association of HCMV DNA at M6 with BMI at M6:

```
. ttest _zbmi6,by(dcmv)
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     293    .1533106    .0659588    1.129033    .0234956    .2831256
       1 |     171   -.0973099    .0887689    1.160804   -.2725413    .0779214
---------+--------------------------------------------------------------------
combined |     464    .0609483    .0532019    1.146003   -.0435988    .1654953
---------+--------------------------------------------------------------------
    diff |            .2506205     .109786                .0348788    .4663623
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   2.2828
Ho: diff = 0                                     degrees of freedom =      462
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9886         Pr(|T| > |t|) = 0.0229          Pr(T > t) = 0.0114
```

Mean _zbmi at 6m is significantly lower among children with detectable DNA

```
. *adjusted for education etc

      Source |       SS       df       MS              Number of obs =     464
-------------+------------------------------           F(  6,   457) =    3.39
       Model |  25.9411231     6  4.32352052            Prob > F      =  0.0028
    Residual |   582.12766   457  1.27380232            R-squared     =  0.0427
-------------+------------------------------           Adj R-squared =  0.0301
       Total |  608.068783   463  1.3133235            Root MSE      =  1.1286
------------------------------------------------------------------------------
      _zbmi6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Idcmv_yn_1 |  -.2179099   .1095406    -1.99   0.047    -.4331756   -.0026443
   _Ieduc3_2 |   .1846498   .1289007     1.43   0.153    -.0686617    .4379614
   _Ieduc3_3 |    .393912    .149013     2.64   0.008     .1010763    .6867476
  _Isescat3_2 |   .0487253   .1285611     0.38   0.705    -.2039189    .3013696
  _Isescat3_3 |   .0734977   .1505554     0.49   0.626    -.2223689    .3693644
   _Ibfcat2_1 |   .3716934   .1524957     2.44   0.015     .0720137    .6713731
        _cons |  -.4059019   .1827524    -2.22   0.027    -.7650412   -.0467626
------------------------------------------------------------------------------
```

9.8.2 Association of HCMV DNA at M6 with weight at M6:

```
. ttest _zwei6,by(dcmv)
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     293   -.3179181    .0679918    1.163832   -.4517342    -.184102
       1 |     172   -.5102907    .0928319    1.217479   -.6935348   -.3270466
---------+--------------------------------------------------------------------
combined |     465   -.3890753    .0550134    1.186301   -.4971815    -.280969
---------+--------------------------------------------------------------------
    diff |            .1923726    .1137245               -.0311075    .4158527
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   1.6916
Ho: diff = 0                                     degrees of freedom =      463
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9543         Pr(|T| > |t|) = 0.0914          Pr(T > t) = 0.0457
Some evidence that _zwei at 6m is lower among children with detectable DNA (p=0.09)

. *adjusted for education etc

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  6,   458) =    5.70
       Model |  45.4004862     6  7.56674769            Prob > F      =  0.0000
    Residual |  607.591616   458  1.32661925            R-squared     =  0.0695
-------------+------------------------------           Adj R-squared =  0.0573
       Total |  652.992102   464  1.40731057            Root MSE      =  1.1518
------------------------------------------------------------------------------
      _zwei6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Idcmv_yn_1 |  -.1529556   .1116827    -1.37   0.171    -.3724297    .0665186
   _Ieduc3_2 |   .3333333   .1314261     2.54   0.012     .0750605    .5916062
```

```
  _Ieduc3_3 |   .4973645    .152068     3.27   0.001      .198527     .796202
 _Isescat3_2 |   .1852998   .1310877     1.41   0.158    -.0723081    .4429077
 _Isescat3_3 |   .2200178   .1536433     1.43   0.153    -.0819155    .5219511
  _Ibfcat2_1 |   .5272391   .1547035     3.41   0.001     .2232224    .8312557
       _cons |  -1.196161   .1861598    -6.43   0.000    -1.561994   -.8303279
------------------------------------------------------------------------------
```

9.8.3 Association of HCMV DNA at M6 with subscap. skinfold at M6:

***Unadjusted analysis - Association with raw subscapular skinfold measurement:**

```
. xi:regress subscap6 i.dcmv

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  1,   463) =   13.98
       Model |  43.1315277     1  43.1315277           Prob > F      =  0.0002
    Residual |  1428.45067   463  3.08520664           R-squared     =  0.0293
-------------+------------------------------           Adj R-squared =  0.0272
       Total |   1471.5822   464  3.17151337           Root MSE      =  1.7565
------------------------------------------------------------------------------
    subscap6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Idcmv_yn_1 |  -.6308496   .1687216    -3.74   0.000    -.9624045   -.2992948
       _cons |   8.282594   .1026144    80.72   0.000     8.080946    8.484241
------------------------------------------------------------------------------
.
```

***Unadjusted analysis - Association with subscapular skinfold Z score:**

```
. xi:regress _zss6 i.dcmv

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  1,   463) =   13.85
       Model |  20.4324219     1  20.4324219           Prob > F      =  0.0002
    Residual |  682.868536   463  1.47487805           R-squared     =  0.0291
-------------+------------------------------           Adj R-squared =  0.0270
       Total |  703.300958   464  1.51573482           Root MSE      =  1.2144
------------------------------------------------------------------------------
       _zss6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Idcmv_yn_1 |  -.4341985   .1166558    -3.72   0.000     -.663439    -.204958
       _cons |   .6289078   .0709487     8.86   0.000     .4894866    .7683291
------------------------------------------------------------------------------
```

***Adjusted analysis - association with raw subscapular skinfold measurement, adjusted for education, SES & breastfeeding (<6m, 6m+)**

```
. xi:regress subscap6 i.dcmv i.educ3 i.sescat3 i.bfcat2

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  6,   458) =    6.96
       Model |  122.968971     6  20.4948286           Prob > F      =  0.0000
    Residual |  1348.61323   458  2.94457037           R-squared     =  0.0836
-------------+------------------------------           Adj R-squared =  0.0716
       Total |   1471.5822   464  3.17151337           Root MSE      =   1.716
------------------------------------------------------------------------------
    subscap6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Idcmv_yn_1 |  -.5404824   .1663887    -3.25   0.001    -.8674624   -.2135024
  _Ieduc3_2 |   .2705099    .195803     1.38   0.168    -.1142738    .6552935
  _Ieduc3_3 |   .6243052   .226556     2.76   0.006      .179087    1.069523
 _Isescat3_2 |  -.0016917   .1952989    -0.01   0.993    -.3854846    .3821013
 _Isescat3_3 |   .0074496    .228903     0.03   0.974    -.4423808     .45728
  _Ibfcat2_1 |   1.01538   .2304825     4.41   0.000     .5624461    1.468315
       _cons |    7.0875   .2773471    25.55   0.000      6.54247    7.632531
------------------------------------------------------------------------------
```

***Adjusted analysis - association with subscapular skinfold Z score, adjusted for education, SES & breastfeeding (<6m, 6m+)**

```
. xi:regress _zss6 i.dcmv i.educ3 i.sescat3 i.bfcat2

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  6,   458) =    8.13
       Model |  67.6919282     6   11.281988           Prob > F      =  0.0000
    Residual |   635.60903   458  1.38779264           R-squared     =  0.0962
-------------+------------------------------           Adj R-squared =  0.0844
       Total |  703.300958   464  1.51573482           Root MSE      =   1.178
------------------------------------------------------------------------------
       _zss6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
```

```
 _Idcmv_yn_1 |  -.3651778   .1142287    -3.20   0.001    -.5896552   -.1407005
  _Ieduc3_2 |   .2055835   .1344221     1.53   0.127     -.058577    .4697441
  _Ieduc3_3 |   .4406464   .1555346     2.83   0.005     .1349965    .7462962
 _Isescat3_2 |   .0077643    .134076     0.06   0.954    -.2557161    .2712446
 _Isescat3_3 |   .0648401   .1571458     0.41   0.680    -.2439762    .3736563
  _Ibfcat2_1 |   .7920956   .1582301     5.01   0.000     .4811485    1.103043
       _cons |  -.3069072   .1904035    -1.61   0.108    -.6810801    .0672656
------------------------------------------------------------------------------
```

9.8.4 Association of HCMV DNA at M6 with arm circ. at M6:

***Unadjusted analysis - Association with raw arm circumference measurement:**

```
. xi:regress arm6 i.dcmv

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  1,   463) =    9.96
       Model |  16.0358364     1  16.0358364           Prob > F      =  0.0017
    Residual |  745.776714   463  1.61074884           R-squared     =  0.0210
-------------+------------------------------           Adj R-squared =  0.0189
       Total |  761.812551   464  1.64183739           Root MSE      =  1.2692
------------------------------------------------------------------------------
        arm6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Idcmv_yn_1 |  -.3846575   .1219108    -3.16   0.002    -.6242247   -.1450904
       _cons |   14.71024   .0741447   198.40   0.000     14.56454    14.85594
------------------------------------------------------------------------------
```

***Unadjusted analysis - Association with arm circumference Z score:**

```
. xi:regress _zac6 i.dcmv

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  1,   463) =    9.64
       Model |  11.3830307     1  11.3830307           Prob > F      =  0.0020
    Residual |  546.823792   463  1.18104491           R-squared     =  0.0204
-------------+------------------------------           Adj R-squared =  0.0183
       Total |  558.206822   464  1.20303194           Root MSE      =  1.0868
------------------------------------------------------------------------------
       _zac6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Idcmv_yn_1 |  -.3240839   .1043907    -3.10   0.002     -.529222   -.1189457
       _cons |   .5714676   .0634891     9.00   0.000      .446705    .6962301
------------------------------------------------------------------------------
```

***Adjusted analysis - association with raw arm circumference measurement, adjusted for education, SES & breastfeeding (<6m, 6m+)**

```
. xi:regress arm6 i.dcmv i.educ3 i.sescat3 i.bfcat2

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  6,   458) =    7.84
       Model |  70.9558215     6  11.8259703           Prob > F      =  0.0000
    Residual |  690.856729   458  1.50842081           R-squared     =  0.0931
-------------+------------------------------           Adj R-squared =  0.0813
       Total |  761.812551   464  1.64183739           Root MSE      =  1.2282
------------------------------------------------------------------------------
        arm6 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Idcmv_yn_1 |  -.3452011   .1190897    -2.90   0.004    -.5792311   -.1111712
  _Ieduc3_2 |   .4185092   .1401424     2.99   0.003     .1431073     .693911
  _Ieduc3_3 |   .6033277   .1621533     3.72   0.000     .2846709    .9219845
 _Isescat3_2 |   .1962686   .1397816     1.40   0.161    -.0784242    .4709614
 _Isescat3_3 |   .2657147   .1638332     1.62   0.106    -.0562432    .5876726
  _Ibfcat2_1 |   .5418345   .1649636     3.28   0.001     .2176551     .866014
       _cons |   13.73805   .1985061    69.21   0.000     13.34796    14.12815
------------------------------------------------------------------------------
```

***Adjusted analysis - association with arm circumference Z score, adjusted for education, SES & breastfeeding (<6m, 6m+)**

```
. xi:regress _zac6 i.dcmv i.educ3 i.sescat3 i.bfcat2

      Source |       SS       df       MS              Number of obs =     465
-------------+------------------------------           F(  6,   458) =    8.62
       Model |  56.6291373     6  9.43818955           Prob > F      =  0.0000
    Residual |  501.577685   458  1.09514778           R-squared     =  0.1014
-------------+------------------------------           Adj R-squared =  0.0897
       Total |  558.206822   464  1.20303194           Root MSE      =  1.0465
------------------------------------------------------------------------------
```

_zac6	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
_Idcmv_yn_1	-.2827993	.1014727	-2.79	0.006	-.4822091	-.0833894
_Ieduc3_2	.3728276	.1194111	3.12	0.002	.1381661	.6074892
_Ieduc3_3	.5474065	.138166	3.96	0.000	.2758887	.8189243
_Isescat3_2	.1514807	.1191037	1.27	0.204	-.0825767	.3855381
_Isescat3_3	.1881049	.1395973	1.35	0.178	-.0862256	.4624355
_Ibfcat2_1	.5545313	.1405605	3.95	0.000	.2783078	.8307548
_cons	-.3387473	.1691411	-2.00	0.046	-.671136	-.0063585

9.9 Appendix I: HCMV seroprevalence – effects on growth

9.9.1 Association of HCMV antibody at M18 with length at M18

***Unadjusted analysis - Association with raw length measurement:**

```
xi:regress length18 i.hcmv
```

Source	SS	df	MS		
Model	112.178035	1	112.178035	Number of obs =	444
Residual	4453.81355	442	10.0765012	F(1, 442) =	11.13
Total	4565.99158	443	10.3069787	Prob > F =	0.0009
				R-squared =	0.0246
				Adj R-squared =	0.0224
				Root MSE =	3.1744

length18	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
_Ihcmv_yn_1	-1.356117	.4064414	-3.34	0.001	-2.154914	-.5573186
_cons	79.56986	.3715296	214.17	0.000	78.83968	80.30005

***Unadjusted analysis - Association with length Z score:**

```
xi:regress _zlen18 i.hcmv
```

Source	SS	df	MS		
Model	13.6076854	1	13.6076854	Number of obs =	444
Residual	531.40255	442	1.20226821	F(1, 442) =	11.32
Total	545.010235	443	1.23027141	Prob > F =	0.0008
				R-squared =	0.0250
				Adj R-squared =	0.0228
				Root MSE =	1.0965

_zlen18	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
_Ihcmv_yn_1	-.4723188	.1403925	-3.36	0.001	-.7482385	-.1963991
_cons	-.7032877	.1283333	-5.48	0.000	-.9555069	-.4510684

***Adjusted analysis - association with length Z score, adjusted for education, SES & breastfeeding (<6m, 6m+)**

```
xi:regress _zlen18 i.hcmv i.educ3 i.sescat3 i.bfcat2
```

Source	SS	df	MS		
Model	82.0882412	6	13.6813735	Number of obs =	444
Residual	462.921994	437	1.05931806	F(6, 437) =	12.92
Total	545.010235	443	1.23027141	Prob > F =	0.0000
				R-squared =	0.1506
				Adj R-squared =	0.1390
				Root MSE =	1.0292

_zlen18	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
_Ihcmv_yn_1	-.3363102	.1373785	-2.45	0.015	-.606315	-.0663054
_Ieduc3_2	.4907333	.120562	4.07	0.000	.2537798	.7276867
_Ieduc3_3	.5476273	.1441021	3.80	0.000	.264408	.8308466
_Isescat3_2	.5328886	.1207856	4.41	0.000	.2954957	.7702816
_Isescat3_3	.478996	.1427444	3.36	0.001	.1984451	.759547
_Ibfcat2_1	.1157838	.1361658	0.85	0.396	-.1518375	.383405
_cons	-1.610427	.1890431	-8.52	0.000	-1.981974	-1.23888

***Adjusted analysis - association with length Z score, adjusted for education, SES & breastfeeding (<6m, 6m+) and length at M6**

```
. xi:regress _zlen18 i.hcmv _zlen6 i.educ3 i.sescat3 i.bfcat2
```

Source	SS	df	MS		
Model	346.5051	7	49.5007286	Number of obs =	443
Residual	189.046343	435	.434589293	F(7, 435) =	113.90
Total	535.551443	442	1.21165485	Prob > F =	0.0000
				R-squared =	0.6470
				Adj R-squared =	0.6413
				Root MSE =	.65923

_zlen18	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
_Ihcmv_yn_1	-.1888928	.0881969	-2.14	0.033	-.3622379	-.0155477

```
      _zlen6 |   .7915755   .0321712    24.61   0.000     .7283452    .8548059
   _Ieduc3_2 |    .186581   .0783885     2.38   0.018     .0325137    .3406483
   _Ieduc3_3 |   .3748958   .0925725     4.05   0.000     .1929507    .5568408
 _Isescat3_2 |   .2717904   .0782475     3.47   0.001     .1180003    .4255805
 _Isescat3_3 |   .2605946    .091856     2.84   0.005     .0800579    .4411314
  _Ibfcat2_1 |  -.1687652   .0882912    -1.91   0.057    -.3422957    .0047653
       _cons |  -.5137824   .1287736    -3.99   0.000    -.7668782   -.2606866
------------------------------------------------------------------------------
```

mean _zlen18 is significantly lower in children with detectable HCMV antibody, even when adjust for SES, education & breastfeeding

9.9.2 Association of HCMV antibody at M18 with weight at M18

***Unadjusted analysis - Association with raw weight measurement:**

```
. xi:regress weight18 i.hcmv

      Source |       SS       df       MS              Number of obs =     445
-------------+------------------------------           F(  1,   443) =    4.25
       Model |  8.14130103     1  8.14130103           Prob > F      =  0.0398
    Residual |  848.123241   443  1.91449942           R-squared     =  0.0095
-------------+------------------------------           Adj R-squared =  0.0073
       Total |  856.264542   444  1.92852374           Root MSE      =  1.3837
------------------------------------------------------------------------------
    weight18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Ihcmv_yn_1 |  -.3652532   .1771229    -2.06   0.040    -.7133587   -.0171476
       _cons |   10.27274   .1619445    63.43   0.000     9.954465    10.59101
------------------------------------------------------------------------------
```

***Unadjusted analysis - Association with weight Z score:**

```
. xi:regress _zwei18 i.hcmv

      Source |       SS       df       MS              Number of obs =     445
-------------+------------------------------           F(  1,   443) =    4.46
       Model |  5.82850118     1  5.82850118           Prob > F      =  0.0353
    Residual |   579.48578   443  1.30809431           R-squared     =  0.0100
-------------+------------------------------           Adj R-squared =  0.0077

       Total |  585.314281   444  1.31827541           Root MSE      =  1.1437
------------------------------------------------------------------------------
     _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Ihcmv_yn_1 |  -.3090477   .1464086    -2.11   0.035    -.5967895    -.021306
       _cons |  -.3391781   .1338622    -2.53   0.012     -.602262   -.0760941
------------------------------------------------------------------------------
```

***Adjusted analysis - association with weight Z score, adjusted for education, SES & breastfeeding (<6m, 6m+)**

```
. xi:regress _zwei18 i.hcmv i.educ3 i.sescat3 i.bfcat2

      Source |       SS       df       MS              Number of obs =     445
-------------+------------------------------           F(  6,   438) =    9.50
       Model |  67.4009186     6  11.2334864           Prob > F      =  0.0000
    Residual |  517.913362   438   1.1824506           R-squared     =  0.1152
-------------+------------------------------           Adj R-squared =  0.1030
       Total |  585.314281   444  1.31827541           Root MSE      =  1.0874
------------------------------------------------------------------------------
     _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Ihcmv_yn_1 |  -.1562734   .1451364    -1.08   0.282    -.4415238     .128977
   _Ieduc3_2 |   .4174637   .1272221     3.28   0.001     .1674221    .6675052
   _Ieduc3_3 |   .5967047    .152161     3.92   0.000     .2976482    .8957611
 _Isescat3_2 |   .4181968   .1274782     3.28   0.001     .1676518    .6687417
 _Isescat3_3 |   .4486008   .1507371     2.98   0.003     .1523429    .7448586
  _Ibfcat2_1 |   .1926846   .1438436     1.34   0.181     -.090025    .4753941
       _cons |  -1.254767   .1996085    -6.29   0.000    -1.647076    -.862457
------------------------------------------------------------------------------
```

***Adjusted analysis - association with weight Z score, adjusted for education, SES & breastfeeding (<6m, 6m+) and weight at M6**

```
. xi:regress _zwei18 i.hcmv _zwei6 i.educ3 i.sescat3 i.bfcat2

      Source |       SS       df       MS              Number of obs =     445
-------------+------------------------------           F(  7,   437) =  102.57
       Model |  363.858433     7  51.9797761           Prob > F      =  0.0000
```

```
    Residual |  221.455848    437  .506763954           R-squared     =  0.6216
-------------+------------------------------           Adj R-squared =  0.6156
       Total |  585.314281    444  1.31827541           Root MSE      =  .71187
------------------------------------------------------------------------------
      _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Ihcmv_yn_1 |  -.1138653   .0950302    -1.20   0.231    -.3006384    .0729079
       _zwei6 |   .7135963   .0295035    24.19   0.000     .6556098    .7715828
    _Ieduc3_2 |   .1190162   .0841954     1.41   0.158    -.0464621    .2844945
    _Ieduc3_3 |   .3366709   .1001912     3.36   0.001     .1397544    .5335874
  _Isescat3_2 |   .1992673   .0839434     2.37   0.018     .0342842    .3642504
  _Isescat3_3 |   .2526397   .0990126     2.55   0.011     .0580397    .4472397
   _Ibfcat2_1 |  -.2429364   .0958746    -2.53   0.012    -.4313691   -.0545037
        _cons |  -.2405023   .1372381    -1.75   0.080    -.5102311    .0292264
------------------------------------------------------------------------------
```

HCMV antibody significantly associated with lower mean weight at 18m (raw measurement & Z scores) in the univariate analysis, but not after adjusting for confounders.

9.9.3 Association of HCMV antibody at M18 with head circ. at M18

***Unadjusted analysis - Association with raw head circumference measurement:**

```
.
. xi:regress head18 i.hcmv

      Source |       SS       df       MS              Number of obs =     445
-------------+------------------------------           F(  1,   443) =    5.53
       Model |  11.2406514     1  11.2406514           Prob > F      =  0.0191
    Residual |  899.729171   443  2.03099136           R-squared     =  0.0123
-------------+------------------------------           Adj R-squared =  0.0101
       Total |  910.969822   444  2.05173383           Root MSE      =  1.4251
------------------------------------------------------------------------------
      head18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Ihcmv_yn_1 |  -.4291833   .1824321    -2.35   0.019    -.7877231   -.0706435
        _cons |   47.87945   .1667987   287.05   0.000     47.55164    48.20727
------------------------------------------------------------------------------
```

***Unadjusted analysis - Association with head circumference Z score:**

```
. xi:regress _zhc18 i.hcmv

      Source |       SS       df       MS              Number of obs =     445
-------------+------------------------------           F(  1,   443) =    5.96
       Model |  5.88722156     1  5.88722156           Prob > F      =  0.0150
    Residual |  437.230986   443  .986977395           R-squared     =  0.0133
-------------+------------------------------           Adj R-squared =  0.0111
       Total |  443.118208   444  .998013981           Root MSE      =  .99347
------------------------------------------------------------------------------
      _zhc18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Ihcmv_yn_1 |  -.3106006   .1271747    -2.44   0.015    -.5605413   -.0606599
        _cons |   .8019178   .1162766     6.90   0.000     .5733956    1.03044
------------------------------------------------------------------------------
```

***Adjusted analysis - association with head circumference Z score, adjusted for education, SES & breastfeeding (<6m, 6m+)**

```
. xi:regress _zhc18 i.hcmv i.educ3 i.sescat3 i.bfcat2

      Source |       SS       df       MS              Number of obs =     445
-------------+------------------------------           F(  6,   438) =    6.59
       Model |  36.6654945     6  6.11091575            Prob > F      =  0.0000
    Residual |  406.452713   438  .927974231           R-squared     =  0.0827
-------------+------------------------------           Adj R-squared =  0.0702
       Total |  443.118208   444  .998013981           Root MSE      =  .96331
------------------------------------------------------------------------------
      _zhc18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Ihcmv_yn_1 |  -.2306002   .1285739    -1.79   0.074    -.4832988    .0220983
   _Ieduc3_2 |   .2442402   .1127039     2.17   0.031     .0227326    .4657478
   _Ieduc3_3 |   .5357743   .1347969     3.97   0.000     .2708453    .8007034
  _Isescat3_2 |   .2289476   .1129308     2.03   0.043      .006994    .4509011
  _Isescat3_3 |   .1097963   .1335354     0.82   0.411    -.1526536    .3722461
   _Ibfcat2_1 |   .2692612   .1274287     2.11   0.035     .0188136    .5197089
        _cons |   .1384244   .1768298     0.78   0.434     -.209116    .4859648
------------------------------------------------------------------------------
```

***Adjusted analysis - association with head circumference Z score, adjusted for education, SES & breastfeeding (<6m, 6m+) and head circumference at M6**

```
. xi:regress _zhc18 i.hcmv _zhc6 i.educ3 i.sescat3 i.bfcat2

      Source |       SS       df       MS              Number of obs =     445
-------------+------------------------------           F(  7,   437) =  108.69
       Model |  281.452357     7  40.2074796            Prob > F      =  0.0000
    Residual |  161.665851   437  .369944738           R-squared     =  0.6352
-------------+------------------------------           Adj R-squared =  0.6293
       Total |  443.118208   444  .998013981           Root MSE      =  .60823
------------------------------------------------------------------------------
      _zhc18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Ihcmv_yn_1 |  -.0787208   .0813952    -0.97   0.334    -.2386956     .081254
        _zhc6 |   .7624468   .0296404    25.72   0.000     .7041913    .8207022
   _Ieduc3_2 |   .0755927   .0714619     1.06   0.291    -.0648591    .2160445
   _Ieduc3_3 |   .2857622   .0856631     3.34   0.001     .1173993    .4541251
  _Isescat3_2 |   .0371113   .0716927     0.52   0.605    -.1037942    .1780167
  _Isescat3_3 |  -.0041856   .0844298    -0.05   0.960    -.1701247    .1617534
   _Ibfcat2_1 |   -.084367   .0816237    -1.03   0.302    -.2447909    .0760568
        _cons |   .0576347   .1116934     0.52   0.606    -.1618884    .2771578
------------------------------------------------------------------------------
```

HCMV antibody no longer significantly associated after adjusting for head circumference Z score at 6m

9.10 Appendix J: Effects of HIV-1 and HCMV infections on referral rate

9.10.1 Effect of HIV-1 infection on referral rate

```
*if exclude unknown - biased b/c had to survive to 18m to be known
  +-------------------------------------------------------+
  |   chiv_f    D          Y      Rate     Lower     Upper |
  |-------------------------------------------------------|
  | Negative   99   558.9432   0.17712   0.14545   0.21568 |
  | Positive    9    16.2519   0.55378   0.28814   1.06432 |
  +-------------------------------------------------------+

Cox regression -- Breslow method for ties
No. of subjects      =          574                 Number of obs    =       962
No. of failures      =          108
Time at risk         =  575.1950986
                                                    Wald chi2(1)     =      9.50
Log pseudolikelihood =   -680.14741                 Prob > chi2      =    0.0021
                          (Std. Err. adjusted for 743 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Ichiv_f_2 |   3.115142   1.148568     3.08   0.002     1.512292    6.416822
------------------------------------------------------------------------------

*if include unknown with positive
  +-------------------------------------------------------------+
  |            chiv2    D          Y      Rate     Lower     Upper |
  |-------------------------------------------------------------|
  |         Negative   99   558.9432   0.17712   0.14545   0.21568 |
  | Positive/unknown   51    81.2406   0.62776   0.47709   0.82602 |
  +-------------------------------------------------------------+

Cox regression -- Breslow method for ties
No. of subjects      =          743                 Number of obs    =      1188
No. of failures      =          150
Time at risk         =  640.1838638
                                                    Wald chi2(1)     =     35.08
Log pseudolikelihood =   -950.30622                 Prob > chi2      =    0.0000
                          (Std. Err. adjusted for 743 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Ichiv2_2 |   3.246976    .645646     5.92   0.000     2.198981    4.794425
------------------------------------------------------------------------------
```

9.10.2 Effect of HIV-1 exposure on referral rate

```
. *** (b) by maternal HIV overall
  +-------------------------------------------------------+
  |     mhiv_f    D          Y       Rate     Lower     Upper |
  |-------------------------------------------------------|
  |   Negative   91   435.7677   0.208827   0.170042   0.256458 |
  |   Positive   51   145.9877   0.349344   0.265498   0.459670 |
  | Don't know    8    58.4285   0.136919   0.068473   0.273785 |
  +-------------------------------------------------------+

Cox regression -- Breslow method for ties
No. of subjects      =          743                 Number of obs    =      1188
No. of failures      =          150
Time at risk         =  640.1838638
                                                    Wald chi2(2)     =     10.07
Log pseudolikelihood =   -963.73444                 Prob > chi2      =    0.0065
                          (Std. Err. adjusted for 743 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Imhiv_f_2 |   1.687752   .3303208     2.67   0.007     1.150044    2.476867
  _Imhiv_f_3 |   .6549621   .2273964    -1.22   0.223     .3316556    1.293436
------------------------------------------------------------------------------
```

9.10.3 Effect of HCMV sera-DNA at M6 on referral rate

.

```
. *** (a) all children

  +-------------------------------------------------------+
  | dcmvyn     D          Y      Rate     Lower     Upper |
  |-------------------------------------------------------|
  |      0    56   256.0768   0.21868   0.16829   0.28416 |
  |      1    49   154.7407   0.31666   0.23933   0.41898 |
  +-------------------------------------------------------+

Cox regression -- Breslow method for ties
No. of subjects      =          485                 Number of obs    =       780
No. of failures      =          105
Time at risk         =  410.8175426
                                                    Wald chi2(1)     =      3.07
Log pseudolikelihood =   -630.01148                 Prob > chi2      =    0.0799
------------------------------------------------------------------------------
             |                 Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Idcmvyn_1 |   1.456344   .3125847     1.75   0.080     .9562362    2.218008
------------------------------------------------------------------------------

Some evidence that children with detectable DNA more likely to be referred to hospital
```

9.10.4 Effect of HCMV sera-DNA at M6 on referral rate sub-stratified by HIV-1 exposure

	Referral rate (per 100 pers. Yrs.)		Unadjusted RR (95% CI)	p	Adjusted[1] RR (95% CI)	p
	HCMV negative	HCMV positive				
All children	53 / 251.0 (21.1)	35 / 154.1 (22.7)	1.07 (0.66–1.74)	0.77	1.12 (0.69–1.83)	0.64
Maternal HIV						
Negative	37 / 178.8 (20.7)	16 / 103.0 (15.6)	0.75 (0.41–1.36)	0.34	0.80 (0.43–1.47)	0.48
Positive	14 / 48.1 (29.1)	17 / 36.6 (46.4)	1.60 (0.72–3.58)	0.25	2.23 (0.92–5.42)	0.08

Active HCMV at 6 months shows a trend for risk of hospital referral in HIV-1 exposed infants

Referral rate is calculated for individual subjects as number of referrals to hospital per year. [1]adjusted for socioeconomic group and breastfeeding <6 months. By KB, CIGNIS statistician, LSHTM.

9.10.5 Effect of HCMV seroprevalence at M18 on referral rate

```
*rate overall.
  +-------------------------------------------------------+
  | scmv_~18     D          Y      Rate     Lower     Upper |
  |-------------------------------------------------------|
  |        0    11    60.7147   0.18118   0.10033   0.32715 |
  |        1    79   301.9577   0.26163   0.20985   0.32617 |
  +-------------------------------------------------------+

Cox regression -- Breslow method for ties
No. of subjects      =          421                 Number of obs    =       675
No. of failures      =           90
Time at risk         =  362.6723563
                                                    Wald chi2(1)     =      0.93
Log pseudolikelihood =   -529.23213                 Prob > chi2      =    0.3339
                       (Std. Err. adjusted for 421 clusters in subject_id)
------------------------------------------------------------------------------
             |                 Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iscmv_yn_~1 |   1.439886   .5431942     0.97   0.334     .6874071    3.016076
------------------------------------------------------------------------------

Referral rates is slightly higher in children with detectable HCMV antibody, but not
statistically significant

. *effect of HCMV antibody, within each maternal HIV group
  +-----------------------------------------------------------------------+
  |     mhiv_f   scmv_~18     D          Y       Rate      Lower      Upper |
  |-----------------------------------------------------------------------|
  |   Negative          0     7    46.1466   0.151691   0.072316   0.318187 |
  |   Negative          1    41   208.1178   0.197004   0.145057   0.267553 |
  |   Positive          0     4    12.3340   0.324306   0.121718   0.864084 |
  |   Positive          1    35    67.1239   0.521424   0.374379   0.726223 |
```

```
| Don't know          0     0     2.2341    0.000000          .          . |
|-------------------------------------------------------------------------|
| Don't know          1     3     26.7159   0.112292    0.036217   0.348171 |
+-------------------------------------------------------------------------+
```

Effect of HCMV antibody at 18m, in HIV negative mothers:

```
Cox regression -- Breslow method for ties
No. of subjects      =          300                 Number of obs    =       484
No. of failures      =           48
Time at risk         =  254.2643898
                                                    Wald chi2(1)     =      0.44
Log pseudolikelihood =   -264.82975                 Prob > chi2      =    0.5059
                          (Std. Err. adjusted for 300 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iscmv_yn_~1 |   1.293117   .4996476     0.67   0.506      .606376    2.757615
------------------------------------------------------------------------------
```

Effect of HCMV antibody at 18m, in HIV positive mothers:

```
Cox regression -- Breslow method for ties
No. of subjects      =           87                 Number of obs    =       139
No. of failures      =           39
Time at risk         =  79.45793228
                                                    Wald chi2(1)     =      0.37
Log pseudolikelihood =   -170.19167                 Prob > chi2      =    0.5424
                          (Std. Err. adjusted for 87 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iscmv_yn_~1 |   1.614098   1.268676     0.61   0.542      .3458501   7.533064
------------------------------------------------------------------------------
```

9.11 Appendix K: Effects of HCMV sera-DNA and intervention on growth

9.11.1 Length at M18

Stratified by HCMV DNA:

children with no detectable DNA:

```
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
Conventi |     113   -1.078407    .1067223    1.134474   -1.289864   -.8669504
Micronut |     111    -1.07045    .1153859    1.215665   -1.299118   -.8417828
---------+--------------------------------------------------------------------
combined |     224   -1.074464    .0783592    1.172774   -1.228884    -.920045
---------+--------------------------------------------------------------------
    diff |           -.0079566    .1570764               -.3175082     .301595
------------------------------------------------------------------------------
    diff = mean(Conventi) - mean(Micronut)                        t =  -0.0507
Ho: diff = 0                                     degrees of freedom =      222
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.4798         Pr(|T| > |t|) = 0.9596          Pr(T > t) = 0.5202
```

children with detectable DNA:

```
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
Conventi |      71    -1.20338     .127094    1.070913   -1.456861   -.9498994
Micronut |      68   -.9467647    .1415122    1.166939   -1.229224   -.6643052
---------+--------------------------------------------------------------------
combined |     139   -1.077842    .0951882    1.122252   -1.266058   -.8896258
---------+--------------------------------------------------------------------
    diff |           -.2566156    .1898528               -.6320363    .1188052
------------------------------------------------------------------------------
    diff = mean(Conventi) - mean(Micronut)                        t =  -1.3517
Ho: diff = 0                                     degrees of freedom =      137
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0894         Pr(|T| > |t|) = 0.1787          Pr(T > t) = 0.9106
```

Stratified by HCMV DNA, adjusted for Z-score at 6m, education, SES & BFing

children with no detectable DNA:

```
xi:regress _zlen18 i.treat _zlen6 i.educ3 i.sescat3 i.bfcat2 if dcmv==0

      Source |       SS       df       MS              Number of obs =     224
-------------+------------------------------           F(  7,   216) =   52.25
       Model |  192.836671     7  27.5480959            Prob > F      =  0.0000
    Residual |  113.877064   216  .527208631           R-squared     =  0.6287
-------------+------------------------------           Adj R-squared =  0.6167
       Total |  306.713736   223  1.37539792           Root MSE      =  .72609
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .0375975   .0975373     0.39   0.700    -.1546493    .2298443
      _zlen6 |   .8471233    .050404    16.81   0.000     .7477766     .94647
   _Ieduc3_2 |    .168706   .1246122     1.35   0.177    -.0769055    .4143175
   _Ieduc3_3 |   .3942089   .1392375     2.83   0.005     .1197707    .6686471
 _Isescat3_2 |   .3089169   .1254529     2.46   0.015     .0616483    .5561854
 _Isescat3_3 |   .4070025   .1451474     2.80   0.006     .1209158    .6930891
  _Ibfcat2_1 |  -.1893989   .1478743    -1.28   0.202    -.4808602    .1020624
       _cons |  -.7156839   .1910803    -3.75   0.000    -1.092305   -.3390632
------------------------------------------------------------------------------
```

children with detectable DNA:

```
xi:regress _zlen18 i.treat _zlen6 i.educ3 i.sescat3 i.bfcat2 if dcmv==1

      Source |       SS       df       MS              Number of obs =     138
-------------+------------------------------           F(  7,   130) =   43.40
       Model |  114.976746     7  16.4252494            Prob > F      =  0.0000
    Residual |  49.196678    130  .378435984           R-squared     =  0.7003
-------------+------------------------------           Adj R-squared =  0.6842
```

```
      Total |  164.173424   137  1.19834616           Root MSE      =  .61517
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |  -.0410019   .1062152    -0.39   0.700    -.251136    .1691322
      _zlen6 |    .739436    .053841    13.73   0.000    .6329179     .845954
   _Ieduc3_2 |   .3620201   .1280228     2.83   0.005    .1087422     .615298
   _Ieduc3_3 |   .4651104   .1507775     3.08   0.002    .1668151    .7634058
  _Isescat3_2 |   .3165401   .1245691     2.54   0.012    .0700951    .5629851
  _Isescat3_3 |   .3810984   .1426175     2.67   0.009    .0989468    .6632499
   _Ibfcat2_1 |    .207727   .1405344     1.48   0.142   -.0703036    .4857576
        _cons |  -1.171773   .1901899    -6.16   0.000   -1.548041   -.7955055
------------------------------------------------------------------------------
```

Stratified by maternal HIV & HCMV DNA:

HIV negative mother, no detectable DNA

```
      Source |       SS       df       MS              Number of obs =     159
-------------+------------------------------           F(  1,   157) =    1.33
       Model |  1.89625046     1  1.89625046           Prob > F      =  0.2514
    Residual |  224.687777   157  1.43113234           R-squared     =  0.0084
-------------+------------------------------           Adj R-squared =  0.0021
       Total |  226.584028   158  1.43407612           Root MSE      =  1.1963
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .2184177   .1897492     1.15   0.251    -.1563728    .5932083
        _cons |      -1.185   .1337503    -8.86   0.000    -1.449182   -.9208178
------------------------------------------------------------------------------
```

HIV negative mother, detectable DNA

```
      Source |       SS       df       MS              Number of obs =      92
-------------+------------------------------           F(  1,    90) =    0.57
       Model |  .608019743     1  .608019743           Prob > F      =  0.4504
    Residual |  95.2452237    90  1.05828026           R-squared     =  0.0063
-------------+------------------------------           Adj R-squared = -0.0047
       Total |  95.8532435    91  1.05333235           Root MSE      =  1.0287
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .1626288   .2145552     0.76   0.450    -.2636225    .5888802
        _cons |  -.9117778   .1533536    -5.95   0.000    -1.216442    -.607114
------------------------------------------------------------------------------
```

HIV positive mother, no detectable DNA

```
      Source |       SS       df       MS              Number of obs =      43
-------------+------------------------------           F(  1,    41) =    2.39
       Model |  2.6309828     1  2.6309828            Prob > F      =  0.1296
    Residual |  45.0777939    41  1.09945839           R-squared     =  0.0551
-------------+------------------------------           Adj R-squared =  0.0321
       Total |  47.7087767    42  1.13592326           Root MSE      =  1.0486
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |  -.4948485   .3198915    -1.55   0.130    -1.140882    .1511854
        _cons |  -.9018182   .2235517    -4.03   0.000     -1.35329   -.4503463
------------------------------------------------------------------------------
```

HIV positive mother, detectable DNA

```
      Source |       SS       df       MS              Number of obs =      34
-------------+------------------------------           F(  1,    32) =    1.75
       Model |  2.5821375     1  2.5821375            Prob > F      =  0.1957
    Residual |  47.3092154    32  1.47841298           R-squared     =  0.0518
-------------+------------------------------           Adj R-squared =  0.0221
       Total |  49.8913529    33  1.51185918           Root MSE      =  1.2159
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .5550175   .4199668     1.32   0.196    -.3004267    1.410462
        _cons |  -1.963684   .2789466    -7.04   0.000     -2.53188   -1.395489
------------------------------------------------------------------------------
```

Subgroup where we saw an effect on zlen at 18m: HIV positive mothers, breastfeeding less than 6m, stratified by HCMV DNA:

HIV positive mothers & not Bfing, with no detectable DNA:

```
      Source |       SS       df       MS              Number of obs =      24
-------------+------------------------------           F(  6,    17) =    9.67
       Model |  19.0156657     6  3.16927761            Prob > F      =  0.0001
    Residual |  5.57146768    17  .327733393           R-squared     =  0.7734
-------------+------------------------------           Adj R-squared =  0.6934
       Total |  24.5871333    23  1.0690058             Root MSE      =  .57248

------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .0563431   .2539298     0.22   0.827    -.4794019    .5920882
      _zlen6 |   .8389379   .1151548     7.29   0.000     .5959825    1.081893
   _Ieduc3_2 |    .130012   .3111235     0.42   0.681    -.5264012    .7864252
   _Ieduc3_3 |   .6930915   .3363875     2.06   0.055    -.0166241    1.402807
  _Isescat3_2 |   .0140946   .3476718     0.04   0.968    -.7194288    .7476179
  _Isescat3_3 |  -.2826425   .3982756    -0.71   0.488    -1.122931    .5576456
        _cons |   -.494002   .3939334    -1.25   0.227    -1.325129    .3371249
------------------------------------------------------------------------------
```

HIV positive mothers & not Bfing, with detectable DNA:

```
      Source |       SS       df       MS              Number of obs =      21
-------------+------------------------------           F(  6,    14) =   13.14
       Model |  28.8667247     6  4.81112078            Prob > F      =  0.0000
    Residual |  5.12558961    14  .366113544           R-squared     =  0.8492
-------------+------------------------------           Adj R-squared =  0.7846
       Total |  33.9923143    20  1.69961571            Root MSE      =  .60507

------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .5878893    .306295     1.92   0.076    -.0690483    1.244827
      _zlen6 |   .8201381   .1441625     5.69   0.000     .5109402    1.129336
   _Ieduc3_2 |   .9508052   .3938488     2.41   0.030     .1060835    1.795527
   _Ieduc3_3 |   1.074236   .3886774     2.76   0.015     .2406058    1.907866
  _Isescat3_2 |   .2947788   .3750167     0.79   0.445    -.5095519     1.09911
  _Isescat3_3 |  -.3130847   .4073449    -0.77   0.455    -1.186753    .5605832
        _cons |  -1.512388   .4326791    -3.50   0.004    -2.440393   -.5843837
------------------------------------------------------------------------------
```

There was some evidence that length is greater in children in MF arm, in subgroup of children with HIV pos mother, not Bfing, with detectable HCMV DNA

9.11.2 Weight at M18

Stratified by HCMV DNA:

children with no detectable DNA:

```
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
Conventi |     113   -.5138053     .118034    1.254718   -.7476745   -.2799361
Micronut |     112   -.5253571    .1197174     1.26697   -.7625852   -.2881291
---------+--------------------------------------------------------------------
combined |     225   -.5195556    .0838685    1.258027   -.6848277   -.3542834
---------+--------------------------------------------------------------------
    diff |            .0115518    .1681125               -.3197406    .3428443
------------------------------------------------------------------------------
    diff = mean(Conventi) - mean(Micronut)                        t =   0.0687
Ho: diff = 0                                     degrees of freedom =      223
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5274         Pr(|T| > |t|) = 0.9453          Pr(T > t) = 0.4726
```

children with detectable DNA:

```
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
Conventi |      71   -.7087324    .1380537    1.163261   -.9840719   -.4333929
Micronut |      68   -.4470588    .1305923    1.076892   -.7077221   -.1863955
---------+--------------------------------------------------------------------
combined |     139   -.5807194    .0954606    1.125464    -.769474   -.3919648
---------+--------------------------------------------------------------------
    diff |           -.2616736     .190353               -.6380836    .1147365
------------------------------------------------------------------------------
```

```
    diff = mean(Conventi) - mean(Micronut)                    t =  -1.3747
Ho: diff = 0                                     degrees of freedom =      137
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0857         Pr(|T| > |t|) = 0.1715          Pr(T > t) = 0.9143
```

Stratified by HCMV DNA, adjusted for Z score at 6m, education, SES & BFing

children with no detectable DNA:

```
      Source |       SS       df       MS              Number of obs =     225
-------------+------------------------------           F(  7,   217) =   64.03
       Model |   238.85891     7  34.1227015           Prob > F      =  0.0000
    Residual |  115.650845   217  .532953204           R-squared     =  0.6738
-------------+------------------------------           Adj R-squared =  0.6632
       Total |  354.509756   224  1.58263284           Root MSE      =  .73004

------------------------------------------------------------------------------
     _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .0900794   .0979741     0.92   0.359    -.1030233    .2831821
      _zwei6 |   .8350369   .0436669    19.12   0.000     .7489714    .9211024
   _Ieduc3_2 |   .1647528   .1253821     1.31   0.190    -.0823699    .4118755
   _Ieduc3_3 |   .3494835   .1403944     2.49   0.014     .0727722    .6261948
  _Isescat3_2 |   .2497903   .1253322     1.99   0.048     .0027661    .4968146
  _Isescat3_3 |   .3518137   .1453621     2.42   0.016     .0653113     .638316
   _Ibfcat2_1 |  -.3633285   .1497178    -2.43   0.016    -.6584158   -.0682412
        _cons |  -.3536646   .1873196    -1.89   0.060    -.7228633     .015534
------------------------------------------------------------------------------
```

children with detectable DNA:

```
      Source |       SS       df       MS              Number of obs =     139
-------------+------------------------------           F(  7,   131) =   43.87
       Model |  122.531543     7  17.5045062           Prob > F      =  0.0000
    Residual |  52.2687847   131  .398998357           R-squared     =  0.7010
-------------+------------------------------           Adj R-squared =  0.6850
       Total |  174.800328   138  1.26666904           Root MSE      =  .63166

------------------------------------------------------------------------------
     _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   -.119195   .1098638    -1.08   0.280    -.3365318    .0981417
      _zwei6 |   .7729896   .0495706    15.59   0.000     .6749272     .871052
   _Ieduc3_2 |    .048744    .129169     0.38   0.707    -.2067832    .3042712
   _Ieduc3_3 |   .3459919    .153613     2.25   0.026     .0421086    .6498751
  _Isescat3_2 |   .3470495   .1266923     2.74   0.007     .0964218    .5976771
  _Isescat3_3 |   .4403339   .1454914     3.03   0.003     .1525172    .7281506
   _Ibfcat2_1 |  -.0367305   .1438587    -0.26   0.799    -.3213173    .2478563
        _cons |  -.4515715   .1821957    -2.48   0.014    -.8119981   -.0911449
------------------------------------------------------------------------------
```

Stratified by maternal HIV & HCMV DNA:

HIV negative mother, no detectable DNA

```
      Source |       SS       df       MS              Number of obs =     159
-------------+------------------------------           F(  1,   157) =    1.43
       Model |  2.36321017     1  2.36321017           Prob > F      =  0.2339
    Residual |  259.791172   157  1.65472084           R-squared     =  0.0090
-------------+------------------------------           Adj R-squared =  0.0027
       Total |  262.154382   158  1.65920495           Root MSE      =  1.2864

------------------------------------------------------------------------------
     _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .2438323   .2040339     1.20   0.234    -.1591733    .6468379
        _cons |    -.58725   .1438194    -4.08   0.000    -.8713205   -.3031795
------------------------------------------------------------------------------
```

HIV negative mother, detectable DNA

```
      Source |       SS       df       MS              Number of obs =      92
-------------+------------------------------           F(  1,    90) =    0.62
       Model |  .629237373     1  .629237373           Prob > F      =  0.4329
    Residual |  91.2498181    90  1.01388687           R-squared     =  0.0068
-------------+------------------------------           Adj R-squared = -0.0042
       Total |  91.8790554    91  1.00965995           Root MSE      =  1.0069

------------------------------------------------------------------------------
     _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .1654421   .2100069     0.79   0.433    -.2517732    .5826574
```

```
       _cons |  -.4328889   .1501027    -2.88   0.005    -.7310941   -.1346837
------------------------------------------------------------------------------
```

HIV positive mother, no detectable DNA

```
      Source |       SS       df       MS              Number of obs =      44
-------------+------------------------------           F(  1,    42) =    3.37
       Model |  4.27814545     1  4.27814545           Prob > F      =  0.0735
    Residual |  53.3357727    42  1.26989935           R-squared     =  0.0743
-------------+------------------------------           Adj R-squared =  0.0522
       Total |  57.6139182    43  1.33985856           Root MSE      =  1.1269
------------------------------------------------------------------------------
      _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Itreat_1 |  -.6236364   .3397726    -1.84   0.074    -1.309325    .0620524
       _cons |  -.3768182   .2402555    -1.57   0.124    -.8616734     .108037
------------------------------------------------------------------------------
```

HIV positive mother, no detectable DNA

```
      Source |       SS       df       MS              Number of obs =      34
-------------+------------------------------           F(  1,    32) =    0.99
       Model |  1.9414722     1   1.9414722           Prob > F      =  0.3271
    Residual |  62.7392337    32  1.96060105           R-squared     =  0.0300
-------------+------------------------------           Adj R-squared = -0.0003
       Total |  64.6807059    33  1.96002139           Root MSE      =  1.4002
------------------------------------------------------------------------------
      _zwei18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Itreat_1 |   .4812632   .4836282     1.00   0.327    -.5038553    1.466382
       _cons |  -1.315263   .3212313    -4.09   0.000     -1.96959   -.6609365
------------------------------------------------------------------------------
```

9.11.3 BMI at M18

Stratified by HCMV DNA:

children with no detectable DNA:

```
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
Conventi |     113    .1970796    .1071444    1.138961   -.0152133    .4093726
Micronut |     111    .1671171    .1015494    1.069889   -.0341299    .3683641
---------+--------------------------------------------------------------------
combined |     224    .1822321    .0736904    1.102897    .0370135    .3274508
---------+--------------------------------------------------------------------
    diff |            .0299625    .1477046               -.2611199     .321045
------------------------------------------------------------------------------
    diff = mean(Conventi) - mean(Micronut)                        t =   0.2029
Ho: diff = 0                                     degrees of freedom =      222
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5803         Pr(|T| > |t|) = 0.8394          Pr(T > t) = 0.4197
```

children with detectable DNA:

```
Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
Conventi |      71    .0316901    .1211205     1.02058   -.2098771    .2732574
Micronut |      68    .1877941    .1121033    .9244278   -.0359651    .4115533
---------+--------------------------------------------------------------------
combined |     139    .1080576    .0826439    .9743566   -.0553544    .2714695
---------+--------------------------------------------------------------------
    diff |            -.156104    .1653916               -.4831546    .1709466
------------------------------------------------------------------------------
    diff = mean(Conventi) - mean(Micronut)                        t =  -0.9438
Ho: diff = 0                                     degrees of freedom =      137
    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1735         Pr(|T| > |t|) = 0.3469          Pr(T > t) = 0.8265
```

***Stratified by HCMV DNA, adjusted for Z score at 6m, education, SES & BFing**

children with no detectable DNA:

```
      Source |       SS       df       MS              Number of obs =     224
-------------+------------------------------           F(  7,   216) =   32.70
```

```
      Model |  139.554415     7   19.936345           Prob > F      =  0.0000
   Residual |  131.698669   216   .609716059          R-squared     =  0.5145
-------------+------------------------------          Adj R-squared =  0.4987
      Total |  271.253084   223  1.21638154           Root MSE      =  .78084
------------------------------------------------------------------------------
     _zbmi18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .0705824   .1050875     0.67   0.503    -.1365458    .2777106
      _zbmi6 |   .6918595   .0483801    14.30   0.000     .596502     .787217
   _Ieduc3_2 |   .1318378   .1341655     0.98   0.327    -.1326034    .396279
   _Ieduc3_3 |   .1898153   .1504219     1.26   0.208    -.1066674    .4862981
 _Isescat3_2 |   .1076496   .1338547     0.80   0.422     -.156179    .3714782
 _Isescat3_3 |   .1701265   .1552702     1.10   0.274    -.1359122    .4761652
  _Ibfcat2_1 |  -.2441667   .1605204    -1.52   0.130    -.5605535    .0722202
       _cons |   .0542771   .1955162     0.28   0.782    -.3310868    .4396411
------------------------------------------------------------------------------
```

children with detectable DNA:

```
      Source |       SS       df       MS              Number of obs =     138
-------------+------------------------------          F(  7,   130) =   21.50
       Model |  70.0530414     7  10.0075773           Prob > F      =  0.0000
    Residual |  60.5105992   130  .465466148          R-squared     =  0.5365
-------------+------------------------------          Adj R-squared =  0.5116
       Total |  130.563641   137  .953019274          Root MSE      =  .68225
------------------------------------------------------------------------------
     _zbmi18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |  -.0711162   .1183583    -0.60   0.549    -.3052739    .1630415
      _zbmi6 |   .6619408   .0558477    11.85   0.000     .5514528    .7724288
   _Ieduc3_2 |  -.2352765   .1391539    -1.69   0.093    -.5105759    .0400228
   _Ieduc3_3 |   .1146766   .1643002     0.70   0.486    -.2103717    .4397248
 _Isescat3_2 |   .1565689   .1381071     1.13   0.259    -.1166595    .4297973
 _Isescat3_3 |   .2208122   .1583628     1.39   0.166    -.0924897    .5341142
  _Ibfcat2_1 |  -.1604435   .1532274    -1.05   0.297    -.4635856    .1426985
       _cons |   .3035977   .1806845     1.68   0.095    -.0538649    .6610604
------------------------------------------------------------------------------
```

Stratified by maternal HIV & HCMV DNA:

HIV negative mother, no detectable DNA

```
      Source |       SS       df       MS              Number of obs =     159
-------------+------------------------------          F(  1,   157) =    0.84
       Model |  1.04521686     1  1.04521686           Prob > F      =  0.3613
    Residual |  195.730656   157  1.24669208          R-squared     =  0.0053
-------------+------------------------------          Adj R-squared = -0.0010
       Total |  196.775873   158  1.24541692          Root MSE      =  1.1166
------------------------------------------------------------------------------
     _zbmi18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .1621598   .1771004     0.92   0.361     -.187647    .5119667
       _cons |    .180625   .1248345     1.45   0.150    -.0659467    .4271967
------------------------------------------------------------------------------
```

HIV negative mother, detectable DNA

```
      Source |       SS       df       MS              Number of obs =      92
-------------+------------------------------          F(  1,    90) =    0.29
       Model |  .241111933     1  .241111933           Prob > F      =  0.5903
    Residual |  74.3305489    90  .825894988          R-squared     =  0.0032
-------------+------------------------------          Adj R-squared = -0.0078
       Total |  74.5716609    91  .819468801          Root MSE      =  .90879
------------------------------------------------------------------------------
     _zbmi18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .1024113   .1895401     0.54   0.590    -.2741432    .4789659
       _cons |   .1633333   .1354741     1.21   0.231    -.1058095    .4324762
------------------------------------------------------------------------------
```

HIV positive mother, no detectable DNA

```
      Source |       SS       df       MS              Number of obs =      43
-------------+------------------------------          F(  1,    41) =    2.84
       Model |  3.13124676     1  3.13124676           Prob > F      =  0.0995
    Residual |  45.1837439    41  1.10204254          R-squared     =  0.0648
-------------+------------------------------          Adj R-squared =  0.0420
       Total |  48.3149907    42  1.15035692          Root MSE      =  1.0498
------------------------------------------------------------------------------
```

_zbmi18	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
_Itreat_1	-.5398485	.3202672	-1.69	0.099	-1.186641	.1069442
_cons	.2431818	.2238143	1.09	0.284	-.2088203	.695184

HIV positive mother, detectable DNA

Source	SS	df	MS		Number of obs	= 34
Model	.335882611	1	.335882611		F(1, 32)	= 0.22
Residual	47.8027439	32	1.49383575		Prob > F	= 0.6386
					R-squared	= 0.0070
Total	48.1386265	33	1.45874626		Adj R-squared	= -0.0241
					Root MSE	= 1.2222

_zbmi18	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
_Itreat_1	.2001754	.4221516	0.47	0.639	-.6597193	1.06007
_cons	-.1468421	.2803978	-0.52	0.604	-.7179938	.4243096

9.11.4 Stunting at M18

Stratified by HCMV DNA:

children with no detectable DNA:

18 stunted	treat: Conventio	Micronutr	Total
No	94	87	181
	51.93	48.07	100.00
Yes	19	24	43
	44.19	55.81	100.00
Total	113	111	224
	50.45	49.55	100.00

Pearson chi2(1) = 0.8343 Pr = 0.361

children with detectable DNA:

18 stunted	treat: Conventio	Micronutr	Total
No	55	55	110
	50.00	50.00	100.00
Yes	16	13	29
	55.17	44.83	100.00
Total	71	68	139
	51.08	48.92	100.00

Pearson chi2(1) = 0.2457 Pr = 0.620

Stratified by HCMV DNA, adjusted for Z score at 6m, education, SES & BFing

children with no detectable DNA:

Logistic regression — Number of obs = 224; LR chi2(7) = 72.07; Prob > chi2 = 0.0000

Log likelihood = -73.513736 — Pseudo R2 = 0.3289

stunted18	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
_Itreat_1	1.543006	.6651188	1.01	0.314	.6629089	3.591546
_zlen6	.1528839	.0485164	-5.92	0.000	.0820805	.2847629
_Ieduc3_2	1.244853	.6117859	0.45	0.656	.4751087	3.261693
_Ieduc3_3	.3024915	.1947346	-1.86	0.063	.0856517	1.068293
_Isescat3_2	.9390758	.4875549	-0.12	0.904	.3394446	2.59796
_Isescat3_3	1.693998	1.021083	0.87	0.382	.5198022	5.520616
_Ibfcat2_1	2.829974	2.159243	1.36	0.173	.6343429	12.62528

children with detectable DNA:

```
Logistic regression                                     Number of obs   =        138
                                                        LR chi2(7)      =      86.80
                                                        Prob > chi2     =     0.0000
Log likelihood = -26.205735                             Pseudo R2       =     0.6235
------------------------------------------------------------------------------
   stunted18 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   2.549371   2.149987     1.11   0.267     .4881805     13.3133
      _zlen6 |   .0429277   .0295534    -4.57   0.000      .011136     .1654796
   _Ieduc3_2 |   .2588153   .2208699    -1.58   0.113     .0485942     1.378463
   _Ieduc3_3 |   .4352586    .418192    -0.87   0.387     .0662097     2.861364
 _Isescat3_2 |   .4356262   .3650352    -0.99   0.321     .0843035     2.251037
 _Isescat3_3 |   .0603376   .0702105    -2.41   0.016     .0061675     .5902945
  _Ibfcat2_1 |   .1629692   .1513232    -1.95   0.051     .0264078     1.005724
------------------------------------------------------------------------------
```

Stratified by maternal HIV & HCMV DNA:

HIV negative mother, no detectable DNA

```
Logistic regression                                     Number of obs   =        159
                                                        LR chi2(1)      =       0.39
                                                        Prob > chi2     =     0.5303
Log likelihood = -81.002588                             Pseudo R2       =     0.0024
------------------------------------------------------------------------------
   stunted18 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   1.278689   .5017932     0.63   0.531      .592556     2.759307
------------------------------------------------------------------------------
```

HIV negative mother, detectable DNA

```
Logistic regression                                     Number of obs   =         92
                                                        LR chi2(1)      =       0.49
                                                        Prob > chi2     =     0.4833
Log likelihood = -35.377791                             Pseudo R2       =     0.0069
------------------------------------------------------------------------------
   stunted18 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .6462585   .4051286    -0.70   0.486     .1891483     2.208056
------------------------------------------------------------------------------
```

HIV positive mother, no detectable DNA

```
Logistic regression                                     Number of obs   =         43
                                                        LR chi2(1)      =       0.23
                                                        Prob > chi2     =     0.6306
Log likelihood = -18.987923                             Pseudo R2       =     0.0061
------------------------------------------------------------------------------
   stunted18 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   1.490196   1.242111     0.48   0.632     .2909031     7.633759
------------------------------------------------------------------------------
```

HIV positive mother, detectable DNA

```
.
Logistic regression                                     Number of obs   =         34
                                                        LR chi2(1)      =       0.69
                                                        Prob > chi2     =     0.4071
Log likelihood = -22.691181                             Pseudo R2       =     0.0149
------------------------------------------------------------------------------
   stunted18 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .5555556   .3971769    -0.82   0.411      .136832     2.255627
------------------------------------------------------------------------------
```

9.12 Appendix L: Effects of intervention on growth among HCMV seropositive, HIV-1 exposed infants breast fed <6 months

9.12.1 length at M18

in all children with no detectable antibody

```
      Source |       SS       df       MS              Number of obs =      73
-------------+------------------------------           F(  1,    71) =    0.12
       Model |  .137877124     1  .137877124           Prob > F      =  0.7321
    Residual |  82.8593338    71  1.16703287           R-squared     =  0.0017
-------------+------------------------------           Adj R-squared = -0.0124
       Total |   82.997211    72  1.15273904           Root MSE      =  1.0803
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .0869925   .2530913     0.34   0.732    -.4176572    .5916421
       _cons |  -.7485714   .1826028    -4.10   0.000    -1.112671   -.3844717
------------------------------------------------------------------------------
```

in all children with detectable HCMV antibody

```
      Source |       SS       df       MS              Number of obs =     371
-------------+------------------------------           F(  1,   369) =    0.01
       Model |  .012308075     1  .012308075           Prob > F      =  0.9199
    Residual |   448.39303   369  1.21515726           R-squared     =  0.0000
-------------+------------------------------           Adj R-squared = -0.0027
       Total |  448.405339   370  1.21190632           Root MSE      =  1.1023
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |     .01152   .1144652     0.10   0.920    -.2135659    .2366059
       _cons |  -1.181413   .0812657   -14.54   0.000    -1.341215   -1.021611
------------------------------------------------------------------------------
```

in children of HIV positive mothers, breastfeeding <6m, no detectable HCMV

```
------------------------------------------------------------------------------
-> treat = Conventional porridge
    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+----------------------------------------------------------------
     _zlen18 |          8      -.4825    .4821631       -1.622635    .6576345
------------------------------------------------------------------------------

-> treat = Micronutrient-fortified
    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+----------------------------------------------------------------
     _zlen18 |          7   -.8085714    .3415889       -1.644409    .0272666
------------------------------------------------------------------------------
```

effect of diet group on _zlen at 18m, adjusted for baseline _zlen, education & SES, in children of HIV positive mothers, breastfeeding <6m,

with no detectable HCMV antibody:

```
. xi:regress _zlen18 i.treat _zlen6 i.educ3 i.sescat3 if mhiv_f==2 & bfcat2==0 & hcmv==0

      Source |       SS       df       MS              Number of obs =      15
-------------+------------------------------           F(  6,     8) =    8.62
       Model |  15.8628648     6   2.6438108           Prob > F      =  0.0039
    Residual |  2.45370852     8  .306713565           R-squared     =  0.8660
-------------+------------------------------           Adj R-squared =  0.7656
       Total |  18.3165733    14  1.30832667           Root MSE      =  .55382
------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |    .100187   .3224355     0.31   0.764    -.6433507    .8437246
      _zlen6 |   .7833414   .1869969     4.19   0.003     .3521257    1.214557
   _Ieduc3_2 |   .5609843   .6684728     0.84   0.426    -.9805168    2.102485
   _Ieduc3_3 |   .9993354   .9022472     1.11   0.300     -1.08125    3.079921
 _Isescat3_2 |   .0999833   .5708017     0.18   0.865    -1.216288    1.416254
 _Isescat3_3 |  -.6935022   .5991472    -1.16   0.280    -2.075138    .6881337
       _cons |  -.4716463   .8403178    -0.56   0.590    -2.409423     1.46613
```

```
-------------------------------------------------------------------------------
```

***children of HIV positive mothers, breastfeeding <6m, with detectable HCMV**

```
-------------------------------------------------------------------------------
-> treat = Conventional porridge

    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+---------------------------------------------------------------
     _zlen18 |         22        -1.6    .2710034       -2.163582   -1.036418
-------------------------------------------------------------------------------
-> treat = Micronutrient-fortified

    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+---------------------------------------------------------------
     _zlen18 |         20        -.954    .2339876       -1.443742   -.4642583
-------------------------------------------------------------------------------
```

***effect of diet group on _zlen at 18m, adjusted for baseline _zlen, education & SES, in children of HIV positive mothers, breastfeeding <6m,**

with detectable HCMV antibody:

```
. xi:regress _zlen18 i.treat _zlen6 i.educ3 i.sescat3 if mhiv_f==2 & bfcat2==0 & hcmv==1

      Source |       SS       df       MS              Number of obs =      41
-------------+------------------------------           F(  6,    34) =    8.77
       Model |  30.7486496     6  5.12477493            Prob > F      =  0.0000
    Residual |  19.8762529    34  .584595672           R-squared     =  0.6074
-------------+------------------------------           Adj R-squared =  0.5381
       Total |  50.6249024    40  1.26562256           Root MSE      =  .76459
-------------------------------------------------------------------------------
     _zlen18 |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+-----------------------------------------------------------------
   _Itreat_1 |   .6103258   .2550021     2.39   0.022     .0920991    1.128553
      _zlen6 |   .8530996    .135202     6.31   0.000      .578336    1.127863
   _Ieduc3_2 |   .3406602   .3159672     1.08   0.289    -.3014624    .9827828
   _Ieduc3_3 |   .6279317   .3305551     1.90   0.066    -.0438372    1.299701
 _Isescat3_2 |  -.0363417   .2943238    -0.12   0.902    -.6344796    .5617961
 _Isescat3_3 |  -.2812359   .3792457    -0.74   0.463    -1.051956    .4894842
       _cons |  -.7522426   .3109424    -2.42   0.021    -1.384154   -.1203316
-------------------------------------------------------------------------------
```

9.13 Appendix M: Effects of intervention on referral rate

***** (e) rate by treatment arm**

```
.
. *overall
  +-------------------------------------------------------------------------+
  |                    treat     D          Y      Rate     Lower     Upper |
  |-------------------------------------------------------------------------|
  |   Conventional porridge    69   322.2454   0.21412   0.16912   0.27110 |
  | Micronutrient-fortified    81   317.9385   0.25477   0.20491   0.31675 |
  +-------------------------------------------------------------------------+

Cox regression -- Breslow method for ties
No. of subjects      =          743                 Number of obs   =      1188
No. of failures      =          150
Time at risk         =  640.1838638
                                                    Wald chi2(1)    =      1.01
Log pseudolikelihood =   -968.90636                 Prob > chi2     =    0.3143

                          (Std. Err. adjusted for 743 clusters in subject_id)
-------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+-----------------------------------------------------------------
   _Itreat_1 |   1.192269    .208357     1.01   0.314     .8464885    1.679297
-------------------------------------------------------------------------------
```

9.14 Appendix N: Effects of HIV exposure and intervention on referral rate

9.14.1 Effects of treatment within HIV-1unexposed and exposed infants

```
. *effect of treatment arm, within each maternal HIV group
```

mhiv_f	treat	D	Y	Rate	Lower	Upper
Negative	Conventional porridge	46	219.1242	0.209927	0.157241	0.280266
Negative	Micronutrient-fortified	45	216.6435	0.207715	0.155088	0.278200
Positive	Conventional porridge	19	74.4915	0.255063	0.162693	0.399877
Positive	Micronutrient-fortified	32	71.4962	0.447576	0.316515	0.632906
Don't know	Conventional porridge	4	28.6297	0.139715	0.052437	0.372258
Don't know	Micronutrient-fortified	4	29.7988	0.134234	0.050380	0.357653

Effect of treatment arm, in HIV negative mothers:

```
Cox regression -- Breslow method for ties
No. of subjects      =          519                 Number of obs    =       850
No. of failures      =           91
Time at risk         =   435.767657
                                                    Wald chi2(1)     =      0.00
Log pseudolikelihood =   -553.09829                 Prob > chi2      =    0.9815
                        (Std. Err. adjusted for 519 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .9949989   .2156026    -0.02   0.982     .6506971     1.52148
------------------------------------------------------------------------------
```

Effect of treatment arm, in HIV positive mothers:

```
Cox regression -- Breslow method for ties
No. of subjects      =          157                 Number of obs    =       232
No. of failures      =           51
Time at risk         =  145.9877064
                                                    Wald chi2(1)     =      3.25
Log pseudolikelihood =    -251.9958                 Prob > chi2      =    0.0713
                        (Std. Err. adjusted for 157 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   1.761532   .5530078     1.80   0.071     .9520678    3.259215
------------------------------------------------------------------------------
```

Effect of treatment arm, in HIV unknown mothers:

```
Cox regression -- no ties
No. of subjects      =           67                 Number of obs    =       106
No. of failures      =            8
Time at risk         =  58.42850039
                                                    Wald chi2(1)     =      0.00
Log pseudolikelihood =   -32.752109                 Prob > chi2      =    0.9475
                        (Std. Err. adjusted for 67 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .9570646   .6377706    -0.07   0.947     .2592483    3.533187
------------------------------------------------------------------------------
```

9.14.2 Effects of HIV-1 exposure within treatment arms

```
*effect of maternal HIV within each trial arm
```

treat	mhiv_f	D	Y	Rate	Lower	Upper
Conventional porridge	Negative	46	219.1242	0.209927	0.157241	0.280266
Conventional porridge	Positive	19	74.4915	0.255063	0.162693	0.399877
Conventional porridge	Don't know	4	28.6297	0.139715	0.052437	0.372258

```
| Micronutrient-fortified     Negative   45   216.6435   0.207715   0.155088   0.278200 |
| Micronutrient-fortified     Positive   32    71.4962   0.447576   0.316515   0.632906 |
|-------------------------------------------------------------------------------------------|
| Micronutrient-fortified   Don't know    4    29.7988   0.134234   0.050380   0.357653 |
+-------------------------------------------------------------------------------------------+
```

Effect of maternal HIV, within the basal diet group:

```
Cox regression -- Breslow method for ties
No. of subjects      =          373                 Number of obs    =       582
No. of failures      =           69
Time at risk         =  322.2453585
                                                    Wald chi2(2)     =      1.55
Log pseudolikelihood =   -397.30745                 Prob > chi2      =    0.4618
                       (Std. Err. adjusted for 373 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Imhiv_f_2 |   1.232058    .327188     0.79   0.432     .7321229    2.073376
  _Imhiv_f_3 |   .6704823   .3276712    -0.82   0.413     .2572746    1.747341
------------------------------------------------------------------------------
```

Effect of maternal HIV, within the fortified diet group:

```
Cox regression -- Breslow method for ties
No. of subjects      =          370                 Number of obs    =       606
No. of failures      =           81
Time at risk         =  317.9385053
                                                    Wald chi2(2)     =     10.18
Log pseudolikelihood =    -460.9492                 Prob > chi2      =    0.0062
                       (Std. Err. adjusted for 370 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Imhiv_f_2 |   2.176068   .5950566     2.84   0.004      1.27323    3.719099
  _Imhiv_f_3 |   .6433506   .3179236    -0.89   0.372     .2442365    1.694668
------------------------------------------------------------------------------
```

9.15 Appendix O: Effects of HCMV sera-DNA and intervention on referral rate

```
. *effect of HCMV DNA at 6m within each treatment group
  +-------------------------------------------------------------------------------+
  |                   treat   dcmv_y~6    D          Y      Rate     Lower     Upper |
  |-------------------------------------------------------------------------------|
  |   Conventional porridge          0   25   131.3595   0.19032   0.12860   0.28166 |
  |   Conventional porridge          1   23    77.4155   0.29710   0.19743   0.44708 |
  | Micronutrient-fortified          0   31   124.7174   0.24856   0.17481   0.35344 |
  | Micronutrient-fortified          1   26    77.3251   0.33624   0.22894   0.49384 |
  +-------------------------------------------------------------------------------+
```

Effect of HCMV DNA at 6 m, in basal diet group:

```
Cox regression -- Breslow method for ties
No. of subjects      =          248                 Number of obs    =       392
No. of failures      =           48
Time at risk         =  208.7750257
                                                    Wald chi2(1)     =      2.42
Log pseudolikelihood =   -254.40394                 Prob > chi2      =    0.1199
                       (Std. Err. adjusted for 248 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Idcmv_yn_~1 |   1.555134   .4415014     1.56   0.120     .8914812    2.712834
------------------------------------------------------------------------------
```

Effect of HCMV DNA at 6 m, in fortified diet group:

```
Cox regression -- Breslow method for ties
No. of subjects      =          237                 Number of obs    =       388
No. of failures      =           57
Time at risk         =  202.0425169
                                                    Wald chi2(1)     =      1.03
Log pseudolikelihood =   -302.60063                 Prob > chi2      =    0.3107
                          (Std. Err. adjusted for 237 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Idcmv_yn_~1 |    1.37513   .4320589     1.01   0.311     .7428486    2.545581
------------------------------------------------------------------------------
```

9.16 Appendix P: Effects of HCMV seroprevalence and intervention on referral rate

9.16.1 Effect of HCMV seroprevalence within each trial arm

```
. *effect of HCMV antibody within each treatment group
  +-------------------------------------------------------------------------------+
  |                  treat   scmv_~18    D          Y       Rate      Lower      Upper |
  |-------------------------------------------------------------------------------|
  |   Conventional porridge          0    4    23.1102   0.173084   0.064961   0.461165 |
  |   Conventional porridge          1   38   156.5668   0.242708   0.176604   0.333555 |
  | Micronutrient-fortified          0    7    37.6044   0.186148   0.088743   0.390466 |
  | Micronutrient-fortified          1   41   145.3909   0.281998   0.207640   0.382985 |
  +-------------------------------------------------------------------------------+
```

Effect of HCMV antibody at 18m, in basal diet group:

```
Cox regression -- Breslow method for ties
No. of subjects      =          205                 Number of obs    =       322
No. of failures      =           42
Time at risk         =  179.6770673
                                                    Wald chi2(1)     =      0.49
Log pseudolikelihood =   -216.50684                 Prob > chi2      =    0.4825
                          (Std. Err. adjusted for 205 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iscmv_yn_~1 |   1.404665   .6795667     0.70   0.482     .5442117    3.625583
------------------------------------------------------------------------------
```

Effect of HCMV antibody at 18m, in fortified diet group:

```
Cox regression -- Breslow method for ties
No. of subjects      =          216                 Number of obs    =       353
No. of failures      =           48
Time at risk         =   182.995289
                                                    Wald chi2(1)     =      0.59
Log pseudolikelihood =   -250.26263                 Prob > chi2      =    0.4427
                          (Std. Err. adjusted for 216 clusters in subject_id)
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Iscmv_yn_~1 |   1.501174   .7943848     0.77   0.443     .5321034    4.235125
------------------------------------------------------------------------------
```

9.16.2 Effect of treatment within HCMV seroprevalence groups

```
*Effect of diet group on referrals, and association with HCMV
.
 *** (a) stratified by HCMV
```

hcmvy~18	treat	D	Y	Rate	Lower	Upper
0	Conventional porridge	6	29.3443	0.204469	0.091860	0.455123
0	Micronutrient-fortified	7	35.5893	0.196688	0.093768	0.412574
1	Conventional porridge	40	170.3601	0.234797	0.172229	0.320095
1	Micronutrient-fortified	46	160.7502	0.286158	0.214340	0.382040

```
*no detectable HCMV

Cox regression -- no ties

No. of subjects      =           76                 Number of obs    =       121
No. of failures      =           13
Time at risk         =  64.93366059
                                                    Wald chi2(1)     =      0.00
Log pseudolikelihood =     -54.3763                 Prob > chi2      =    0.9480
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   .9649478   .5284114    -0.07   0.948     .3298987    2.822455
------------------------------------------------------------------------------

*detectable HCMV

Cox regression -- Breslow method for ties

No. of subjects      =          384                 Number of obs    =       624
No. of failures      =           86
Time at risk         =  331.1102787
                                                    Wald chi2(1)     =      0.69
Log pseudolikelihood =   -498.40773                 Prob > chi2      =    0.4049
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |    1.22583   .2996976     0.83   0.405      .759143    1.979414
------------------------------------------------------------------------------
```

9.16.3 Effect of intervention stratified by HCMV seroprevalence and HIV exposure

```
*** (b) stratified by HCMV & maternal HIV
```

mhiv_f	hcmvy~18	treat	D	Y	Rate	Lower	Upper
Negative	0	Conventional porridge	2	15.6797	0.127553	0.031901	0.510015
Negative	0	Micronutrient-fortified	4	21.5168	0.185901	0.069772	0.495317
Negative	1	Conventional porridge	31	119.1540	0.260167	0.182967	0.369942
Negative	1	Micronutrient-fortified	24	117.2896	0.204622	0.137152	0.305283
Positive	0	Conventional porridge	2	7.7317	0.258676	0.064694	1.034299
Positive	0	Micronutrient-fortified	3	10.0342	0.298977	0.096426	0.926998
Positive	1	Conventional porridge	8	36.7310	0.217800	0.108921	0.435514
Positive	1	Micronutrient-fortified	20	30.5407	0.654863	0.422490	1.015045

***HIV positive, no detectable HCMV**

```
No. of subjects      =           20                 Number of obs    =        28
No. of failures      =            5
Time at risk         =  17.76591376
                                                    Wald chi2(1)     =      0.05
Log pseudolikelihood =   -14.300435                 Prob > chi2      =    0.8308
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   1.188938   .9629272     0.21   0.831     .2430897    5.815029
------------------------------------------------------------------------------
```

***HIV positive, detectable HCMV**

```
Cox regression -- Breslow method for ties

No. of subjects      =           72                 Number of obs    =       120
No. of failures      =           28
Time at risk         =  67.27173169
                                                    Wald chi2(1)     =      5.18
Log pseudolikelihood =   -114.18506                 Prob > chi2      =    0.0229
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   2.991643   1.440442     2.28   0.023     1.164303    7.68694
------------------------------------------------------------------------------
```

In children of HIV negative mothers, effect of treatment does not depend on HCMV status. In children of HIV positive mothers, risk of referral is higher in micronutrient arm if have detectable HCMV

9.16.4 Effect of intervention stratified by HCMV seroprevalence, HIV exposure and baseline anaemia

```
. *** (e) by maternal HIV, HCMV & hgb at baseline
```

mhiv_f	hcmvy~18	hgb_yn	treat	D	Y	Rate	Lower	Upper
Negative	0	0	Conventional porridge	1	8.4983	0.117671	0.016576	0.835353
Negative	0	0	Micronutrient-fortified	1	10.6639	0.093774	0.013209	0.665709
Negative	0	1	Conventional porridge	1	7.1814	0.139249	0.019615	0.988538
Negative	0	1	Micronutrient-fortified	3	9.8672	0.304037	0.098059	0.942688
Negative	1	0	Conventional porridge	16	73.7467	0.216959	0.132916	0.354142
Negative	1	0	Micronutrient-fortified	12	72.4079	0.165728	0.094118	0.291820
Negative	1	1	Conventional porridge	15	44.4244	0.337652	0.203559	0.560079
Negative	1	1	Micronutrient-fortified	11	42.9651	0.256022	0.141785	0.462300
Positive	0	0	Conventional porridge	1	5.3060	0.188467	0.026548	1.337944
Positive	0	0	Micronutrient-fortified	1	5.7084	0.175180	0.024676	1.243614
Positive	0	1	Conventional porridge	1	2.4257	0.412246	0.058070	2.926564
Positive	0	1	Micronutrient-fortified	2	4.3258	0.462342	0.115631	1.848645
Positive	1	0	Conventional porridge	7	22.3600	0.313059	0.149246	0.656674
Positive	1	0	Micronutrient-fortified	6	12.3313	0.486567	0.218596	1.083040
Positive	1	1	Conventional porridge	1	13.3771	0.074754	0.010530	0.530687
Positive	1	1	Micronutrient-fortified	14	16.1807	0.865228	0.512434	1.460912

```
*** (c) effect of diet in HIV positive mothers, by child hiv
.
```

chiv2	hcmvy~18	treat	D	Y	Rate	Lower	Upper
1	0	Conventional porridge	2	6.9651	0.287146	0.071815	1.148136
1	0	Micronutrient-fortified	2	9.1143	0.219435	0.054880	0.877398
1	1	Conventional porridge	5	30.1081	0.166068	0.069122	0.398983
1	1	Micronutrient-fortified	5	22.1081	0.226161	0.094134	0.543359
2	0	Conventional porridge	0	0.7666	0.000000	.	.
2	0	Micronutrient-fortified	1	0.9199	1.087054	0.153126	7.717071
2	1	Conventional porridge	3	6.6229	0.452976	0.146095	1.404485
2	1	Micronutrient-fortified	15	8.4326	1.778815	1.072387	2.950598

Rates of referrals is higher in micronutrient-fortified arm if child is HIV positive/unknown & has detectable HCMV

***HIV positive mother, HIV negative child, with detectable HCMV**

```
Cox regression -- no ties
No. of subjects      =           52                 Number of obs    =        80
No. of failures      =           10
Time at risk         =  52.21629021
                                                    Wald chi2(1)     =      0.24
Log pseudolikelihood =   -39.392671                 Prob > chi2      =    0.6277
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   1.363636   .8721721     0.48   0.628     .3892926    4.776623
------------------------------------------------------------------------------
```

***HIV positive mother, HIV positive or unknown child, with detectable HCMV**

```
Cox regression -- Breslow method for ties

No. of subjects      =           20                 Number of obs    =        40
No. of failures      =           18
Time at risk         =  15.05544148
                                                    Wald chi2(1)     =      5.07
Log pseudolikelihood =   -46.834164                 Prob > chi2      =    0.0244
------------------------------------------------------------------------------
             |               Robust
          _t | Haz. Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Itreat_1 |   3.805198   2.258861     2.25   0.024     1.188729    12.18069
------------------------------------------------------------------------------
```

9.17 Appendix Q: Breast feeding and risk of HCMV transmission

	% HCMV seropositive	Unadjusted OR (95% CI)	P
Duration of breast feeding			
Never	48% (14/29)	-	
< 6 months	86% (38/44)	6.79 (2.19-20.96)	< 0.001
> 6 months	86% (332/387)	6.48 (2.96-14.14)	< 0.001
Duration of breast feeding among HIV-1 exposed infants			
Never	50% (12/24)	-	
< 6 months	89% (29/33)	7.25 (1.94-27.04)	0.003
> 6 months	95% (40/42)	20 (3.91-102.1)	< 0.001

HCMV seroprevalence is linked to duration of breast feeding, especially among HIV-1 exposed infants.
Analysis by KB, study statistician

9.18 Appendix R: Conventional vs Micronutrient fortification

	Category/ month	Nutritional Intervention				
		Conventional fortification		Micronutrient fortification		P
Demographics						
Gender	Male	51% (191/373)		45% (166/370)		.084*
	Female	49% (182/373)		55% (204/370)		
Socioeconomic status	Low	34% (127/373)		33% (123/370)		
	Medium	41% (151/373)		37% (136/370)		.354
	High	26% (95/373)		30% (111/370)		
Breast feeding at baseline	Never	6% (22/373)		6% (21/370)		
	No	11% (39/373)		8% (29/370)		.453
	Yes	84% (312/373)		87% (320/370)		
Morbidity						
Polio Ab neutralization titre	Un-protective	2% (6/281)		3% (7/265)		
	Low	10% (28/281)		8% (21/265)		.790
	Medium	65% (182/281)		64% (170/265)		
	High	23% (65/281)		25% (67/265)		
Fever	6	11% (42/373)		14% (53/370)		.211
	18	10% (23/229)		12% (27/223)		.484
Anaemic	6	38% (140/368)		41% (149/362)		.389
	18	40% (114/286)		22% (63/282)		**<.001*** **
HCMV active infection	6	37% (87/233)		37% (85/232)		.876
	18	34% (63/183)		35% (58/167)		.952
HCMV seropositive		84% (184/219)		83% (189/227)		.829
HHV-6 active infection	6	6% (16/275)		5% (15/283)		.789
	18	15% (28/185)		13% (21/167)		.488
HIV-1 infected		3% (9/291)		3% (8/283)		.851
HIV-1 exposed		22% (74/332)		23% (74/329)		.950
Referral Rate		0.21		0.25		.314
Died		1.9% (7/373)		1.4% (5/370)		.570
Growth						
Stunted	6	14% (52/373)		11% (41/370)		.239
	18	19% (58/301)		20% (61/301)		.759
		Conventional fortification		Micronutrient fortification		
		N =	Mean	N =	Mean	P
BMI-for-age	6	372	0.0084	370	0.0118	0.970
	18	275	0.0907	268	0.1430	0.566
Weight-for-age	6	372	-0.5010	370	-0.4516	0.584
	18	275	-0.6149	271	-0.5222	0.372
Length/height-for-age	6	373	-0.8691	370	-0.8065	0.450
	18	275	-1.1176	268	-1.0446	0.464
Triceps skinfold-for-age	6	373	-0.1909	370	-0.1409	0.523
	18	275	0.1130	270	0.2183	0.188
Subscapular skinfold-for-age	6	373	0.4275	370	0.4099	0.845
	18	275	0.4823	270	0.5186	0.678
Arm circumference-for-age	6	373	0.2787	370	0.3354	0.500
	18	274	0.0478	270	0.0781	0.750
Head circumference-for-age	6	373	0.5646	370	0.7005	**0.072*†**
	18	275	0.4909	270	0.6088	0.178

Appendix R, Table 1: Micronutrient fortification reduced prevalence of anaemia but did not affect prevalence of betaherpesvirus active infections or HCMV seroprevalence and did not reduce stunting
Effect of micronutrient fortified feed supplement on prevalence of betaherpesvirus active infections (detection of sera-DNA), HCMV seroprevalence (detection of antibody at 18 months), HIV-1 infection (detection of antibody at 18 months), HIV-1 exposure (determined by maternal antenatal HIV-1 status), polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable † = groupings that were not randomized between treatment arms at baseline. From S.Filteau and CIGNIS study team.

	Category/ month	HIV-1 unexposed						Category/ month	HIV-1 exposed				
		Conventional fortification		Micronutrient fortification		P			Conventional fortification		Micronutrient fortification		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protective	2% (3/187)		0.6% (1/177)		.588	Polio Ab neutralization titre (12 months)	Un-protective	0% (0/64)		5% (3/56)		.308
	Low	9% (16/187)		7% (13/177)				Low	13% (8/64)		11% (6/56)		
	Medium	66% (123/187)		63% (112/177)				Medium	70% (45/64)		66% (37/56)		
	High	24% (45/187)		29% (51/177)				High	17% (11/64)		18% (10/56)		
Fever	6	11% (28/258)		13% (34/255)		.389	Fever	6	14% (10/74)		14% (10/74)		1.0
	18	10% (15/156)		13% (21/159)		.316		18	9% (4/46)		10% (4/41)		.864
Anaemic	6	39% (99/254)		38% (96/251)		.866	Anaemic	6	32% (23/73)		47% (34/72)		**.053*†**
	18	40% (75/189)		23% (43/190)		**<.001*** **		18	41% (27/66)		20% (12/60)		**.011****
HCMV sera-DNA	6	36% (58/162)		35% (58/164)		.934	HCMV sera-DNA	6	41% (17/42)		45% (18/40)		.679
	18	35% (44/127)		39% (46/119)		.514		18	26% (9/35)		25% (7/28)		.948
HCMV antibody	18	86% (129/150)		83% (128/154)		.487	HCMV antibody	18	80% (37/46)		85% (40/47)		.551
HHV-6 sera-DNA	6	6% (12/192)		6% (12/201)		.908	HHV-6 sera-DNA	6	4% (2/52)		2% (1/51)		.569
	18	15% (19/128)		13% (15/119)		.610		18	17% (6/35)		11% (3/28)		.469
Referral Rate		0.21		0.21		.982	Referral Rate		0.26		0.45		**.071***
Died		1.2% (3/258)		0% (0/255)		**.084***	Died		4.1% (3/74)		6.8% (5/74)		.467
Growth													
Stunted	6	14% (35/258)		9% (23/255)		.104	Stunted	6	16% (12/74)		19% (14/74)		.666
	18	17% (32/184)		18% (33/185)		.901		18	30% (18/60)		31% (16/52)		.930
		Conventional fortification		Micronutrient fortification					Conventional fortification		Micronutrient fortification		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	257	0.0219	255	0.1733	.140	BMI-for-age	6	74	-0.1231	74	-0.2500	.533
	18	184	0.0944	185	0.2570	.139		18	60	0.0140	52	-0.0242	.852
Weight-for-age	6	257	-0.4467	255	-0.3030	.176	Weight-for-age	6	74	-0.6604	74	-0.8222	.443
	18	184	-0.5653	187	-0.3851	.149		18	60	-0.8262	52	-0.9160	.709
Length/height-for-age	6	258	-0.7989	255	-0.7606	.682	Length/height-for-age	6	74	-0.9861	74	-1.0986	.511
	18	184	-1.0473	185	-0.9567	.440		18	60	-1.3598	52	-1.4588	.668
Triceps skinfold-for-age	6	258	-0.1208	255	-0.0471	.422	Triceps skinfold-for-age	6	74	-0.3627	74	-0.2418	.515
	18	184	0.1757	186	0.3335	.105		18	60	-0.0283	52	0.1017	.485
Subscapular skinfold-for-age	6	258	0.5250	255	0.5325	.942	Subscapular skinfold-for-age	6	74	0.1116	74	0.1305	.934
	18	184	0.5633	186	0.6232	.558		18	60	0.3747	52	0.4271	.808
Arm circumference-for-age	6	258	0.3672	255	0.5109	.137	Arm circumference-for-age	6	74	-0.0216	74	-0.1004	.702
	18	183	0.1257	186	0.2599	.218		18	60	-0.2380	52	-0.2975	.812
Head circumference-for-age	6	258	0.6509	255	0.8007	.095†	Head circumference-for-age	6	74	0.3350	74	0.4109	.654
	18	184	0.5657	186	0.7372	.101		18	60	0.2472	52	0.2852	.857

Appendix R, Table 2: Micronutrient fortification reduced anaemia at 18 months irrespective of HIV-1 exposure

Effect of micronutrient fortified feed supplement sub-stratified by HIV-1 exposure, on prevalence of betaherpesvirus active infections (detection of sera-DNA), HCMV seroprevalence (detection of antibody at 18 months), polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable † = groupings that were not randomized between treatment arms at baseline.

	Category/ month	Conventional fortification						Category/ month	Micronutrient fortification				
		HIV-1 unexposed		HIV-1 exposed		month			HIV-1 unexposed		HIV-1 exposed		P
Morbidity													
Polio Ab neutralization titre (12 months)	Un-protective	2% (3/187)		0% (0/64)		.398	Polio Ab neutralization titre (12 months)	Un-protective	0.6% (1/177)		5% (3/56)		.040**
	Low	9% (16/187)		13% (8/64)				Low	7% (13/177)		11% (6/56)		
	Medium	66% (123/187)		70% (45/64)				Medium	63% (112/177)		66% (37/56)		
	High	24% (45/187)		17% (11/64)				High	29% (51/177)		18% (10/56)		
Fever	6	11% (28/258)		14% (10/74)		.526	Fever	6	13% (34/255)		14% (10/74)		.968
	18	10% (15/156)		9% (4/46)		.851		18	13% (21/159)		10% (4/41)		.551
Anaemic	6	39% (99/254)		32% (23/73)		.245	Anaemic	6	38% (96/251)		47% (34/72)		.171
	18	40% (75/189)		41% (27/66)		.861		18	23% (43/190)		20% (12/60)		.668
HCMV sera-DNA	6	36% (58/162)		41% (17/42)		.576	HCMV sera-DNA	6	35% (58/164)		45% (18/40)		.258
	18	35% (44/127)		26% (9/35)		.319		18	39% (46/119)		25% (7/28)		.176
HCMV antibody	18	86% (129/150)		80% (37/46)		.359	HCMV antibody	18	83% (128/154)		85% (40/47)		.747
HHV-6 sera-DNA	6	6% (12/192)		4% (2/52)		.508	HHV-6 sera-DNA	6	6% (12/201)		2% (1/51)		.248
	18	15% (19/128)		17% (6/35)		.738		18	13% (15/119)		11% (3/28)		.784
Referral Rate		0.21		0.26		.432	Referral Rate		0.21		0.45		.004
Died		1.2% (3/258)		4.1% (3/74)		.100	Died		0% (0/255)		6.8% (5/74)		<.001***
Growth													
Stunted	6	14% (35/258)		16% (12/74)		.564	Stunted	6	9% (23/255)		19% (14/74)		.018**
	18	17% (32/184)		30% (18/60)		.036**		18	18% (33/185)		31% (16/52)		.042**
		HIV-1 unexposed		HIV-1 exposed					HIV-1 unexposed		HIV-1 exposed		
		N =	Mean	N =	Mean	P			N =	Mean	N =	Mean	P
BMI-for-age	6	257	0.0219	74	-0.1231	.338	BMI-for-age	6	255	0.1733	74	-0.2500	.008**
	18	184	0.0944	60	0.0140	.618		18	185	0.2570	52	-0.0242	.085*
Weight-for-age	6	257	-0.4467	74	-0.6604	.166	Weight-for-age	6	255	-0.3030	74	-0.8222	.002***
	18	184	-0.5653	60	-0.8262	.144		18	187	-0.3851	52	-0.9160	.006**
Length/height-for-age	6	258	-0.7989	74	-0.9861	.153	Length/height-for-age	6	255	-0.7606	74	-1.0986	.023**
	18	184	-1.0473	60	-1.3598	.053*		18	185	-0.9567	52	-1.4588	.009**
Triceps skinfold-for-age	6	258	-0.1208	74	-0.3627	.077*	Triceps skinfold-for-age	6	255	-0.0471	74	-0.2418	.173
	18	184	0.1757	60	-0.0283	.152		18	186	0.3335	52	0.1017	.114
Subscapular skinfold-for-age	6	258	0.5250	74	0.1116	.010**	Subscapular skinfold-for-age	6	255	0.5325	74	0.1305	.015**
	18	184	0.5633	60	0.3747	.213		18	186	0.6232	52	0.4271	.223
Arm circumference-for-age	6	258	0.3672	74	-0.0216	.007**	Arm circumference-for-age	6	255	0.5109	74	-0.1004	.000***
	18	183	0.1257	60	-0.2380	.031**		18	186	0.2599	52	-0.2975	.001***
Head circumference-for-age	6	258	0.6509	74	0.3350	.019**	Head circumference-for-age	6	255	0.8007	74	0.4109	.004***
	18	184	0.5657	60	0.2472	.037**		18	186	0.7372	52	0.2852	.006**

Appendix R, Table 3: Negative anthropometric outcomes due to HIV-1 exposure were more prevalent in infants on the micronutrient fortified feed supplement
Effect of HIV-1 exposure sub-stratified by treatment arm, on prevalence of betaherpesvirus active infections (detection of sera-DNA), HCMV seroprevalence (detection of antibody at 18 months), polio antibody titres (measured by neutralization assay with titres binned as un-protective ($<^1/_8$), low ($^1/_8$-$^1/_{32}$), medium ($^1/_{64}$-$^1/_{512}$) or high ($>^1/_{512}$)), prevalence of reported fever (in the 3 days prior to clinic visit) and prevalence of anaemia (as defined by Hb levels of < 10.5 g/L) at both the month 6 and month 18 visits and anthropometric markers of growth. Stunting was defined by a length-for-age z score of < -2.0. Mean z-scores were compared by independent sample T test assuming equal variance and P values for nominal variables were by Pearson Chi Squared. White boxes highlight significant findings, light grey indicates trends. Asterisks denote degree of significance * < 0.1, ** <0.05, *** <0.005. ND = not done, NA = not applicable