Towards a screening programme for congenital cytomegalovirus

A thesis submitted to University College London for the degree of

Doctor of Philosophy (PhD)

By

Claire Elizabeth Atkinson

2015

Declaration of Authorship

I declare that the work in this thesis was carried out in accordance with the regulations of the University College London. The work is original, except where indicated by special references in the text and where I have consulted the work of others, this is always clearly stated. I confirm that no part of this thesis has been submitted for any other academic award.

Signed: Claire Elizabeth Atkinson

Abstract

The work in this thesis investigated the detection of cytomegalovirus (CMV) DNA from dried blood spots (DBS) by polymerase chain reaction. The hypothesis was that testing DBS could be formed into a suitable strategy for CMV detection with applications in newborn screening for congenital CMV (CCMV) and in resource limited settings. An iterative approach was taken to assay development in collaboration with clinicians with proven cases and controls. Specificity was greater than 99%, but initial sensitivity for CCMV diagnosis was only 74%. Serial testing of DBS created in the laboratory showed that CMV DNA remained detectable on DBS for 24 months; a clinically relevant timescale for retrospective diagnosis.

A significant association between DBS CMV viral load and the degree of sensorineural hearing loss (SNHL) was found. This relationship was non-linear suggesting an explanation for the clinical benefit of short term therapy in neonates with this chronic infection.

Sensitivity was increased to 90% for CCMV diagnosis by the development of a one tube nested PCR and this method detected all cases who developed SNHL on follow up. This suggests that DBS testing could be suitable for newborn screening.

The role of DBS for studies in resource limited settings was investigated. DBS were compared to plasma for CMV DNA detection. CMV acquisition in infants born to HIV-1 positive mothers was investigated with breast milk CMV viral load and maternal CD4 counts shown to be major determinants of infant CMV acquisition suggesting the possibility that restoring maternal immunity or reducing breast milk CMV levels may reduce transmission.

Overall these results support the working hypothesis and provide valuable guidance on the use of DBS in clinical cohorts. Large scale studies of selected and universal screening to allow intervention against CCMV are now warranted.

Word count 293

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Table of Contents

List of Figures

List of Tables

Abbreviations

ABR	Auditory Brainstem Response
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
ART	Antiretroviral Therapy
AUC	Area under Curve
BEST	Benefits of Extended Screening Testing Study
bp	base pair
BPSU	British Paediatric Surveillance Unit
CASG	Collaborative Antiviral Study Group
CCMV	Congenital Cytomegalovirus
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CDC	Centers for Disease Control and Prevention
CDV	Cidofovir
CHIC	CMV in Hearing Impaired Children
CHIMES	CMV & Hearing Mulitcenter Screening Study
CI	Confidence Interval
CMV	Cytomegalovirus
CNS	Central Nervous System
CRP	C reactive protein
Ct	Cycle Threshold
dBHL	Decibel Hearing Level
DBS	Dried Blood Spot
DNA	Deoxyribose nucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DP	Diphosphate

dsDNA	Double stranded DNA
E	Early
EBV	Epstein Barr virus
EGFR	Epidermal Growth Factor Receptor
FAM	6-carboxyfluorescein
FOS	Foscarnet
FRET	Fluorescence Resonance Energy Transfer
gB	Glycoprotein B
GCV	Ganciclovir
ge/ml	genomes/millilitre
GEE	General Estimating Equation
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
gO	Glycoprotein O
HAART	Highly Active Antiretroviral Therapy
HEU	HIV Exposed Uninfected
HHV6	Human Herpes Virus 6
HI	HIV Infected
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HR	Hazard Ratio
HSV	Herpes simplex virus
ICH	Institute of Child Health
IE	Immediate Early
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IRL	Internal Repeat Long
IRS	Internal Repeat Short
ITPG	Isopropyl β-D-1-thiogalactopyranoside
IU	International Unit
L	Late
LB	Lysogeny Broth
MHC	Major Histocompatibility Complex
MIEP	Major Immediate Early Promoter
mM	Millimolar
µM	Micromolar
MP	Monophosphate
MTCT	Mother to Child Transmission
NHS	National Health Service
NHSP	Newborn Hearing Screening Programme
NK	Natural Killer Cells
nM	Nanomolar
NPV	Negative Predictive Value
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PMTCT	Prevention of Mother to Child Transmission
PPV	Positive Predictive Value
RNA	Ribonucleic Acid
ROC	Receiver operating characteristic
SCID	Severe Combined Immunodeficiency
TBE	Tris/Borate/EDTA
TCID50	Tissue Culture Infective Dose
TLR	Toll Like Receptor
Tm	Melting Temperature

TNF	Tumour Necrosis Factor
TP	Triphosphate
TREC	T Cell Receptor Excision Circles
TRL	Terminal Repeat Long
TRS	Terminal Repeat Short
UK	United Kingdom
UL	Unique Long
ul	Microlitre
US	Unique short
USA	United States of America
UV	Ultraviolet
VGCV	Valganciclovir
VZV	Varicella Zoster Virus
WHO	World Health Organisation

List of publications

The following articles have been published from work in this thesis:

Peer reviewed papers

Walter, S., Atkinson, C., Sharland, M., Rice, P., Raglan, E., Emery, V. C., Griffiths, P. D., 2008. Congenital cytomegalovirus: Association between dried blood spot viral load and hearing loss. Arch Dis.Child Fetal Neonatal Ed 93, 280-285

Atkinson, C., Walter, S., Sharland, M., Tookey, P., Luck, S., Peckham, C., Griffiths, P., 2009. Use of stored dried blood spots for retrospective diagnosis of congenital CMV. J.Med.Virol. 81, 1394-1398.

Atkinson, C., Emery, V. C., Griffiths, P. D., 2014. Development of a novel single tube nested PCR for enhanced detection of cytomegalovirus DNA from dried blood spots. J.Virol.Methods 196, 40-44

Roxby, A. C., Atkinson, C., Asbjornsdottir, K., Farquhar, C., Kiarie, J. N., Drake, A. L., Wald, A., Boeckh, M., Richardson, B., Emery, V., John-Stewart, G., Slyker, J. A., 2014. Maternal valacyclovir and infant cytomegalovirus acquisition: a randomized controlled trial among HIV-infected women. PLoS.One. 9, e87855.

Slyker, J., Farquhar, C., Atkinson, C., Asbjornsdottir, K., Roxby, A., Drake, A., Kiarie, J., Wald, A., Boeckh, M., Richardson, B., Odem-Davis, K., John-Stewart, G., Emery, V., 2014. Compartmentalized cytomegalovirus replication and transmission in the setting of maternal HIV-1 infection. Clin.Infect.Dis. 58, 564-572.

Letters

Atkinson, C., Luck, S., Kadambari, S., Griffiths, P., 2013. Difficulties in diagnosing congenital CMV: how accurate is detection using dried blood spot? J.Perinatol. 33, 495.

Chapter 1

1 Introduction

1.1 History of Cytomegalovirus

Cytomegalovirus (CMV) has been identified as an important viral pathogen in humans for more than a century. In 1904 Ribbert *et al* first described large "protozoan like" inclusion bearing cells in sections of kidney and parotid glands of children that he first observed in 1881 (Ribbert H, 1904). During this period histopathologists frequently found large cells containing a central nuclear body surrounded by two well defined zones and named them "owl's eye inclusion bodies". Later in 1925, Von Glahn and Pappenheimer noted that Lipschuetz has seen similar inclusions in a man following varicella infection. They believed these unusual cells may not be due to an unknown protozoan infection and might be related to a group of viruses. By 1950, Wyatt el at described 25 cases of a rare lethal congenial infection and suggested the name, "generalised cytomegalic inclusion disease", although its viral aetiology was not known (WYATT, SAXTON et al., 1950).

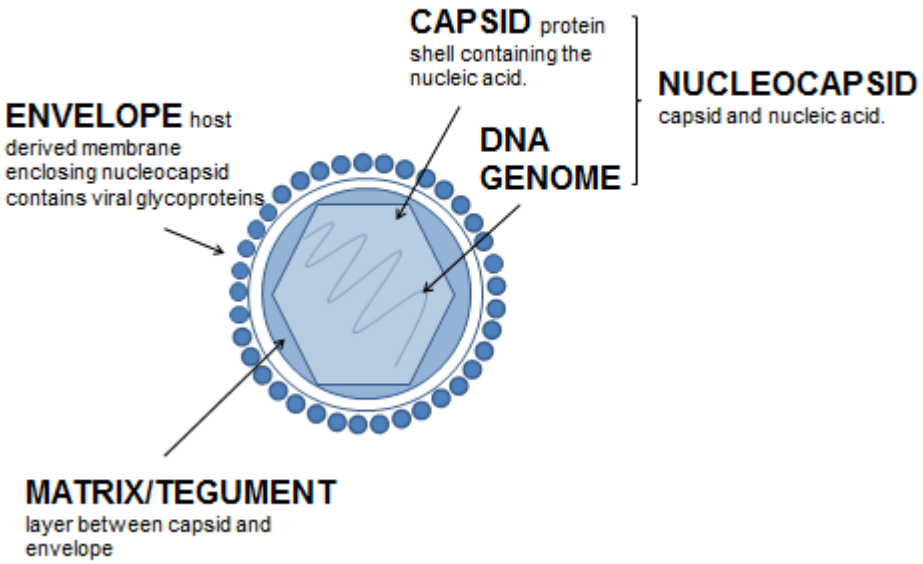
The development of human embryonic cell culture provided the breakthrough with three investigators independently isolating human cytomegalovirus (Smith, 1956;Weller, Macauley et al., 1957;Rowe, Hartley et al., 1956). Weller and co-workers proposed the term "cytomegalovirus" and subsequently isolated CMV from urine samples of infants with generalised disease (Weller, 1970).

Human cytomegalovirus or human herpes virus 5 is classified as a member of the *Betaherpesvirinae* subfamily*,* within the *Herpesviridae* family*.* This classification was originally based on the slow growth in cell culture and species specificity and this classification was later confirmed by genetic sequence homologies to other herpesvirus genomes (Roizman B & Pellett PE, 2001;Griffiths, Emery VC et al., 2009).

1.2 Virus Structure

Under the electron microscope CMV shows the appearance of a typical herpesvirus virion (figure 1.1). The virion is approximately 200-300nm in diameter which contains three identifiable regions, the capsid 100nm in diameter embedded in a proteinaceous matrix (the tegument), which is surrounded by a lipid envelope. The capsid exhibits icosahedral symmetry with 162 capsomers enclosing the large double stranded DNA genome (Chen, Jiang et al., 1999). The tegument contains a number of virus encoded key regulatory proteins which are mostly phosphorylated. The tegument is surrounded by a host cell derived lipid envelope containing more than 20 virus encoded surface glycoproteins (Mocarski, Shenk et al., 2007). These include glycoprotein B (gB), gH, gL, gM, gN and gO (Varnum, Streblow et al., 2004).

A)



B)

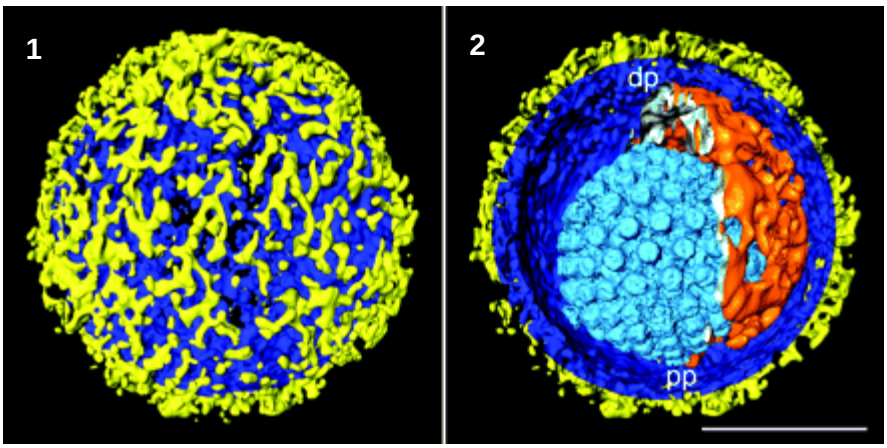


Figure 1-1 Representation of the structural features of Herpesviruses

A) schematic picture of a herpes virion (not to scale); courtesy of Dr R Milne)

B) Segmented surface rendering of a single herpes virion tomogram after denoising. ***(1)*** *Outer surface showing the distribution of glycoprotein spikes (yellow) protruding from the membrane (blue).* ***(2)*** *Cutaway view of the virion interior, showing the capsid (light blue) and the tegument (orange) inside the envelope (blue and yellow). pp, proximal pole; dp, distal pole. Scale bar, 100 nm (adapted from (Grunewald, Desai et al., 2003).*

1.3 Structure of the genome

The genetic content of CMV (strain Ad169) was first determined by Chee *et al;* in 1989 (Chee, Bankier et al., 1990). However, it is now known that *in vitro* cell cultured derived laboratory strains do not accurately represent the genetic content of wild type virus and clinical strains have been shown to include extra open reading frames (ORFs) and genetic content (Cha, Tom et al., 1996;Dolan, Cunningham et al., 2004).

CMV has a linear double stranded DNA (dsDNA) genome that is the largest of the human herpesviruses with approximately 230 kilobases (kb). The nucleotide sequence of several clinical isolates has been determined and it is estimated that CMV encodes for 165 ORFs (Dolan, Cunningham et al., 2004). An alternative proteomic analysis suggests that over 700 peptides or proteins may be encoded (Stern-Ginossar, Weisburd et al., 2012). The genome consists of unique long (UL) and unique short segments (US), each of which is flanked on one end by terminal repeated sequence (TRL and TRS) and internal repeats (IRL and IRS) on the opposing end which allow the virus to exist in four isomeric forms (figure 1.2) (Davison, Dolan et al., 2003;Dolan, Cunningham et al., 2004;Mocarski, Shenk et al., 2007) Individual genes are designated by location within the genome for example UL55 is the 55th gene in the UL region, according to the original report of the CMV strain Ad169 sequence (Chee, Bankier et al., 1990).

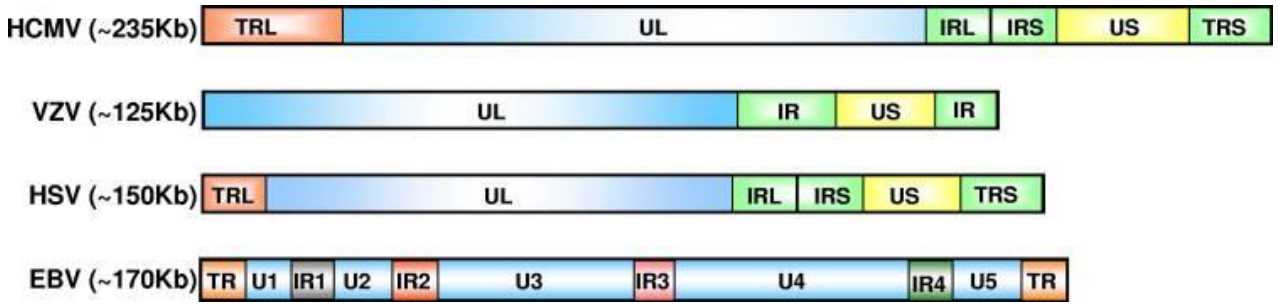


Figure 1-2: Comparative schematic genome organisations of human herpesviruses.

(HCMV) human cytomegalovirus; Varicella zoster virus (VZV); human simplex virus (HSV); Epstein-Barr virus (EBV). The lettering within the individual regions of the genome depicts the following features: terminal repeat long (TRL), unique long (UL), unique short (US), internal repeat long (IRL), internal repeat short (IRS), terminal repeat short (TRS), and internal repeat (IR).This figure is taken from (Crough & Khanna, 2009) with permission & is not to scale.

1.4 Cellular tropism

CMV has a strict host specificity; within the human host there seems to be broad cell tropism, rendering CMV capable of infecting most cell types, with CMV detected in endothelial cells, connective tissue cells, smooth muscle cells, neuronal cells, mucosal epithelial cells and cells of the monocyte/macrophage lineage (Sinzger, Digel et al., 2008).

In vitro primary cell cultures of human foreskin or embryonic lung fibroblasts are the most commonly used cell type for propagation of CMV as they yield relatively high titre viral stocks in cell culture supernantant. However primary cell lines have a finite life in cell culture limiting their use. The entry of CMV into certain cell types such as endothelial and epithelial cells is dependent on the unique long b' (ULb') region of the genome, encoding three genes (UL128-131) whose products interact to form a complex with gH/gL to promote fusion at the host cell plasma membrane (Vanarsdall & Johnson, 2012). It is now know that proteins encoded for in the UL128-131 locus of the CMV genome play an essential role in CMV entry into certain cell types with epithelial and endothelial cells requiring both gB and a pentameric form of gH/gL denoted gH/gL/UL128-131 for entry via a mechanism which involves macropinocytosis or endocytosis and low-pH-dependent fusion with endosomes (Ryckman, Jarvis et al., 2006;Wang & Shenk, 2005) but is redundant for entry into fibroblasts (Hahn, Revello et al., 2004;Wang & Shenk, 2005) and has resulted in the inability of isolates of CMV to replicate efficiently in endothelial cells (Sinzger, Schmidt et al., 1999) or to transfer virus from virus-infected cells to leukocytes (Hahn, Revello et al., 2004). All serially passaged non-endotheliotropic laboratory strains of CMV show mutations or deletions in the UL128–131A locus affecting at least one of the genes (Hahn, Revello et al., 2004;Akter, Cunningham et al., 2003)

Thus, an intact/wild-type UL128–131A locus is essential for infection of most of the host cells of CMV, with the exception of fibroblasts.

1.5 CMV replication

The lytic replication of CMV is very similar to other members of the herpesvirus family. The role of some gene products can be understood by analogy to other herpesviruses. The lytic cycle of CMV can be sub divided into a series of distinct steps; attachment, cell entry, gene transcription and DNA replication, followed by virion assembly and egress (Mocarski, Shenk et al., 2007) figure1.3.

1.5.1 Attachment and cell entry

CMV infects a wide spectrum of cell types; the main pathway of entry into host cells is mediated through a membrane fusion event involving multiple receptor-ligand interactions on the cell surface. The second pathway involves endocytosis of the enveloped capsid within the membrane of the endocytic vesicle (Ryckman, Jarvis et al., 2006). Many different cell surface molecules can serve as receptors for virus entry but only two viral complexes have been shown to be essential for entry glyocprotein B (gB) and the glycoprotein gH/gL dimer. Analogous studies of herpesviruses have shown that gB is important for viral fusion (Vanarsdall & Johnson, 2012) and interaction of gB with heparan sulphate glycoprotein is believed to initiate a signalling cascade which allows other interactions between cell surface molecules and viral glycoproteins to occur, such as the heterodimeric complexes of glycoproteins H, L, M, which ultimately leads to fusion and the delivery of the capsid

into the infected cell (Compton & Feire, 2007). gB has also been shown to interact with a number of cellular components including epidermal growth factor receptor (EGFR), intergrins. (Wang, Huong et al., 2003;Compton & Feire, 2007;Feire, Koss et al., 2004) and tetherin (Viswanathan, Smith et al., 2011) and that gB can bind and trigger Toll like receptor 2 (TLR-2) which facilitates subsequent replication (Boehme, Guerrero et al., 2006).

1.5.2 Viral Gene transcription and DNA replication

Once the virus nucelocapsid containing the viral genome and tegument is deposited into the cytoplasm it appears that the capsid is transported along cytoplasmic microtubules and is translocated to the nucleus where viral DNA is released to enter through nuclear pores as a linear molecule (Mocarski, Shenk et al., 2007). The double stranded DNA undergoes circularisation and the gene expression pathway of CMV then follows a standard template for herpesviruses (Fortunato & Spector, 1999). Immediate early (IE) gene expression, which occurs during the first 4 hours post infection, followed by early (E) gene expression and the synthesis of proteins key for viral replication. Finally late (L) gene synthesis occurs, producing structural proteins that are used to construct new virions (Mocarski, Shenk et al., 2007)

The major immediate early promoter (MIEP) drives the synthesis of two key proteins IE72 (UL123) and IE86 (UL122) which are two major immediate early transactivators which play an essential role in activation of E and L gene expression and DNA replication (Yee, Lin et al., 2007). Included in these early responses are activation of the gene products which are also known to influence replication via anti-apoptotic function, activation of phosphoinositide-3 kinase and induction of host cell antiviral

responses by blocking interferon (IFN)-inducible protein kinase R (Yu & Alwine, 2002;Sambucetti, Cherrington et al., 1989;Child, Hakki et al., 2004).

About 6 hours post infection, the E stage of CMV replication begins following expression of the major immediate early genes which primarily encode for viral proteins that are required for viral DNA replication or control a range of cellular responses that aid viral replication, facilitate cellular survival and escape from host immune surveillance. This results in a large proportion of the viral genome becoming transcriptionally active including expression of UL112/UL113 and UL54 (viral DNA polymerase) lasting around 18-24 hours post infection when the synthesis of viral DNA begins (Mocarski, Shenk et al., 2007).

The final set of genes to be expressed are the L genes, These encode virion structural proteins and are required for the assembly of an infectious particle. Little information is available regarding late gene expression in infected cells (Mocarski, Shenk et al., 2007). The entire replicative cycle *in vitro* is slow and estimated to take 48-72 hours in permissive human fibroblasts.

The CMV genome contains one origin of lytic replication (*ori*Lyt) which is structurally complex and DNA synthesis is dependent on its transcriptional activation (Pari & Anders, 1993). Viral DNA synthesis is dependent on a large multiprotein complex including UL54-UL44 (DNA polymerase catalytic subunit; polymerase accessory protein), UL57 (single stranded DNA binding protein), and the heterotrimeric complex of helicase-primase (HP) which consists of UL105 (HP1), UL70 (HP2), UL102 (HP3) (Anders DG, Kerry JA et al., 2007). The CMV genome replicates using a rolling circular mechanism producing a concatameric structure, which needs to be cleaved into unit genomes lengths prior to encapsidation (Mocarski, Shenk et al., 2007).

1.5.3 **Virion Assembly and Egress**

Studies of alphaherpesviruses have provided much greater understanding of the mechanisms of viral capsid assembly and DNA packaging during herpesvirus replication (Newcomb, Homa et al., 2001;Heymann, Cheng et al., 2003;Brown & Newcomb, 2011). Capsid maturation, encapsidation and release from cells are crucial functions which are carried out by L and E genes. Viral DNA is encapsidated in the nucleus and matures by moving to the cytoplasm (Mocarski, Shenk et al., 2007). Studies suggest herpesvirus nucleocapsids are transported out of the nucleus through a process of envelopment and de-envelopment across the nuclear membranes. This process is called nuclear egress (Mettenleiter, Klupp et al., 2009;Mettenleiter, Muller et al., 2013). This process occurs in different subcellular compartments; in the first step (envelopment) the viral capsid starts at the inner nuclear membrane where transfection studies have shown that a complex of two proteins, whose homologs in CMV are encoded by the genes UL50 and UL53, facilitate disruption of the nuclear lamina by recruiting cellular protein kinase C (PKC), and/or the viral protein kinase UL97 to phosphorylate lamins (Mettenleiter, Klupp et al., 2009). This process delivers viral particles to the perinuclear space where they undergo a de-envelopment event. A secondary process (final envelopment) occurs in the cytoplasm in close proximity to the Golgi apparatus where the bulk of the tegument proteins are added to the nucelocapsid as it travels through the cytoplasm (Mocarski, Shenk et al., 2007). Once the virions are packaged, they are shed from the host cell through an exocytosis mechanism, the vacuoles containing the enveloped infectious virions are transported to the plasma membrane, where they fuse, resulting in the release of mature virions from the infected cell into the extracellular space (Mettenleiter, Muller et al., 2013).

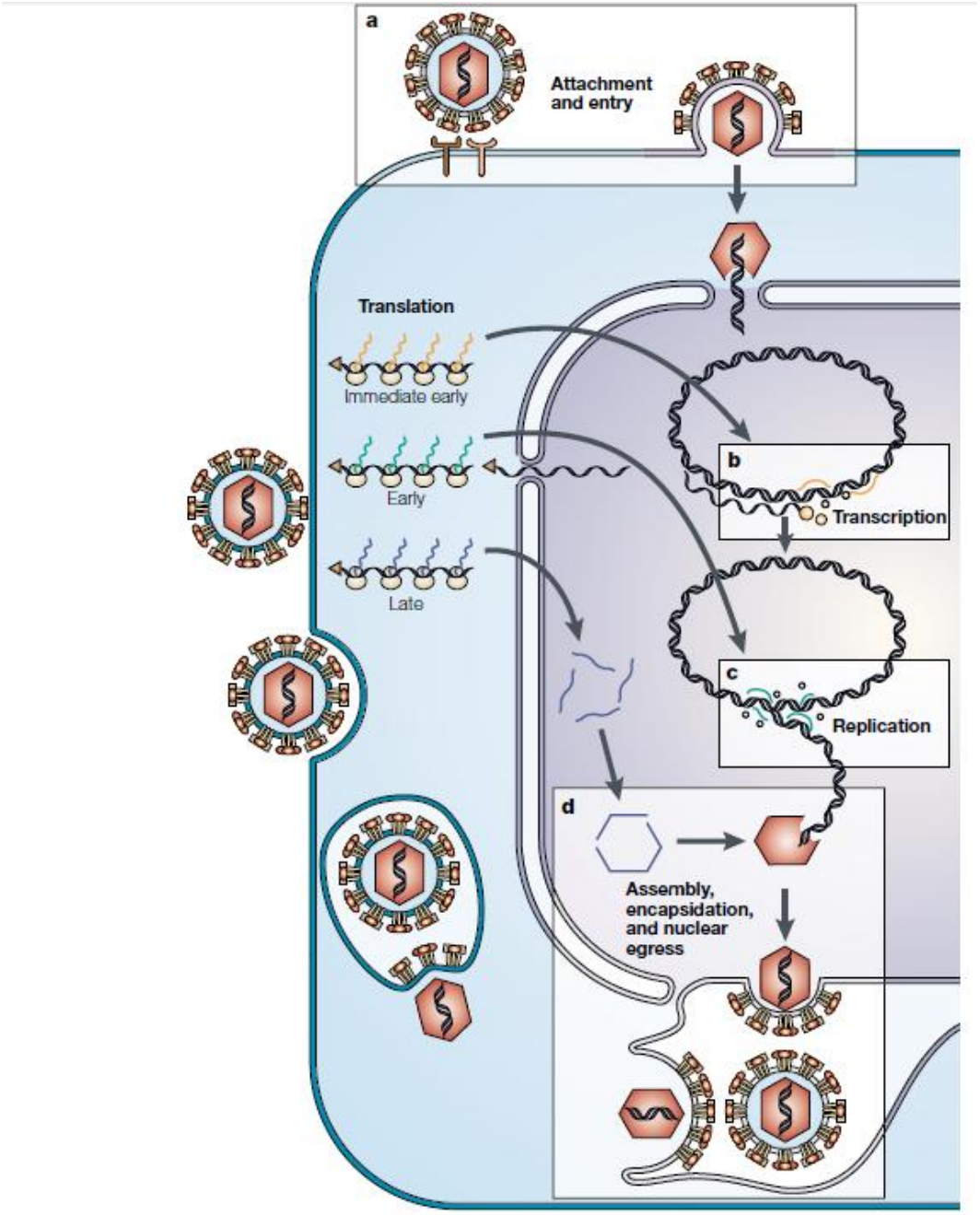


Figure 1-3 Herpesvirus replication cycle:

a) Attachment and entry b) Gene expression, three classes of viral genes are transcribed and translated. Immediate-early proteins (IE) (yellow) participate in further transcription. C) DNA replication, early proteins (E) (green) synthesise new viral DNA molecules using circularised DNA as a template d) Capsid assembly and nuclear egress. Late proteins (L) (blue) assemble into capsids, which incorporate newly replicated viral DNA. Nucleocapsids leave the nucleus by budding through the inner nuclear membrane into the perinuclear space. Through a complex process of de- and re-envelopment, mature virions reach exocytic vesicles, which fuse with the plasma membrane and release new virions into the extracellular space (Coen & Schaffer, 2003), reproduced with permission).

1.6 Latency and virus reactivation

Latency, or the ability of virus DNA to persist for the lifetime of the host after primary infection without extensive gene transcription and is an important biological property of all herpesviruses. The concept of CMV latency, *in vivo* is not at a single cell level and is more likely to include both sites of low level productive infection and true latent infection. Viral latency can be operationally defined as the maintenance of the viral genome in the absence of production of infectious virions but with the ability of the viral genome to reactivate under certain conditions (Sinclair, 2008). The ability to reactivate from latency is a common feature of CMV and is likely to occur in healthy individuals but is normally controlled by the host immune response. However, in the immunocompromised individual this can lead to disease and is a well-established cause of mobidity (Rubin, 2001;Griffiths, Emery VC et al., 2009) Genomic viral DNA has been detected in various cell types including monocytes and macrophages (Taylor-Wiedeman, Sissons et al., 1991;Soderberg, Larsson et al., 1993), lymphocytes (Schrier, Nelson et al., 1985), endothelial cells (Sinzger, Grefte et al., 1995) and CD34+ bone marrow progenitor cells (Mendelson, Monard et al., 1996). Although the exact list of sites of latency are yet to be determined, cells of myeloid lineage appear to be a major candidate (Sinclair, 2008) with active viral replication related to the state of cell differentiation (Soderberg-Naucler, Fish et al., 1997b). The viral genome is maintained as closed circular DNA that persists as an episome in latently infected cells and is not integrated into the host DNA (Bolovan-Fritts, Mocarski et al., 1999).

The mechanisms that favour the establishment of latency are unknown, but at least three possible pathways have been proposed. The first is that the virus enters the

cell in a latent state with no de novo gene expression. The second is that following entry the virus enters the lytic stage but is interrupted driving the virus into latency. The third is the expression of a subset of 'latent' genes unrelated to lytic infection but necessary for the establishment of latency (Kondo, Kaneshima et al., 1994;Goodrum, Jordan et al., 2002;Reeves, Lehner et al., 2005;Crough & Khanna, 2009). Repression of the viral MIEP is a key event in latency (Sinclair, 2010;Reeves, 2011) In addition signalling events can induce reactivation from latency, these stimuli include pro-inflammatory cytokines such as tumour necrosis factor TNF-α and interferon-ɣ (Soderberg-Naucler, Fish et al., 1997a). Reactivation of CMV has also been shown in response to inflammation or stress but the precise underlying mechanisms are unknown (Prosch, Wendt et al., 2000).

1.7 Immune responses to CMV

Following initial infection the human immune system devotes a large and continuous portion of its response to limit CMV replication (Elkington, Walker et al., 2003). The underlying reasons why the host devotes a large portion of its response to CMV is not known, but is likely to reflect the persistent nature and sporadic low-level reactivations of the virus and the resulting immune control. It may have a detrimental effect on health contributing to immune senescence in the elderly (Pawelec, Derhovanessian et al., 2009).

Due to the strict host specificity of CMV, the investigation of functional immune responses in human disease is restricted. Animal models are frequently used with murine cytomegalovirus the most commonly used model. However this is not without limitations.

The human immune response to CMV infection is generated by cells of both the innate and adaptive immune systems; with the innate immune system being the first line of defence against viral infection. This response is essential for the early detection of CMV as it enters the host cell to establish infection. The host recognises the virus as foreign and activates several mechanisms and pathways of innate immune response. These include inflammatory cytokines and interferon which help establish an antiviral state (Marshall & Geballe, 2009) and leads to up regulation of co-stimulatory molecules that are crucial for priming the adaptive immune response and also includes recruitment of professional antigen presenting cells (APCs), phagocytes and NK cells (Isaacson, Juckem et al., 2008) Innate immunity in the perinatal period is an important host defence against CMV infection, due to the immaturity of the adaptive immune response (Gibson, Piccinini et al., 2004). Studies have shown that CMV recognition by the innate response is mediated by TLR-2 which recognises gB and gH, leading to activation of the NF-κB-dependent signal transduction pathway (Boehme, Guerrero et al., 2006).

Following primary infection CMV is processed by APCs which stimulate the antigen specific host immune response, this adaptive response uses both humoral and cellular immunity to control CMV infection (Jackson, Mason et al., 2011) and to ultimately drive the virus into latency. Suppression of this immune control can lead to CMV reactivation or symptomatic primary infection, resulting in CMV disease (Steininger, 2007;Limaye, Kirby et al., 2008). Likewise, in the immature host CMV can have a devastating effect when acquired in utero.

A whole range of proteins within CMV elicit a specific antibody response. These include the structural tegument proteins pp65, pp150 and pp28, capsid proteins

UL86 and UL48.5, the envelope glycoproteins gB, gH and gM and non-structural proteins such as the IE1 protein (Britt & Boppana, 2004;Gandhi & Khanna, 2004). Neutralising antibodies predominantly target the glycoproteins gB, gH/gL and gM/gN with the gB epitope antigen domain1 being the dominant target (Britt, Jarvis et al., 2005). gH/gL can also associate with UL128, UL130, and UL131A to form a pentameric complex (Ryckman, Rainish et al., 2008;Wang & Shenk, 2005;Adler, Scrivano et al., 2006) with antibodies targeting this complex contributing to a large fraction of the neutralising activity in CMV hyperimmune globulin (Fouts, Chan et al., 2012) Overall antibodies against CMV may be crucial in restricting viral dissemination and restricting the severity of clinical disease (Gerna, Sarasini et al., 2008).

In the context of T-cell responses; broadly targeted CMV specific T cells dominate the memory compartments of seropositive healthy adults, comprising on average 10% of the overall cluster of differentiation (CD) 4+ and CD8+ memory compartments in peripheral blood. It is now known that the CD4 and CD8 arms of the adaptive immune response target virtually the whole CMV proteome (Sylwester, Mitchell et al., 2005) with certain proteins more frequently targeted including pp65, IE1, IE2, UL48 and pp150.

CMV specific CD8 T cells in healthy individuals are characterised by a late stage phenotype which shows high expression of CD57 and low levels of CD27 and CD28 with high levels of perforin and granzyme A (Appay, Dunbar et al., 2002).

The CMV-specific adaptive immune responses are difficult to monitor as primary infection in healthy adults is usually asymptomatic. However Wills *et al,* have shown that healthy CMV seropositive donors have high frequencies of CMV specific memory cytotoxic T lymphocyte precursors in their peripheral blood that strongly

recognise tegument protein pp65 (Wills, Carmichael et al., 1996). Thus, the majority of studies have been carried out in CMV-naive kidney transplant recipients who received a kidney from a CMV positive donor. In general following peak CMV replication CD4+ CMV specific T cells circulate and secrete Th1 cytokines including IFN-γ and TNF-α (van Leeuwen, Remmerswaal et al., 2004). This is subsequently followed by the appearance of CMV-specific CD8+T cells in the peripheral blood co-expressing CD45RA and CD45R0 surface cell phenotype, and the cell cycle associated nuclear marker Ki67. CD28 expression is lost with variable expression of CD27 (van de Berg, van et al., 2008) and cells can express both perforin and granzyme B, making them capable of lysing CMV infected cells (Hertoghs, Moerland et al., 2010). Following CMV infection, virus-specific CD8+ T cells express CD45RA− CD45R0+ characteristic of memory T cells and gradually lose CD27 and re-acquire CD45RA expression, but still retain their human leucocyte antigen (HLA) class I-restricted cytolytic potential (La & Diamond, 2012)

1.8 Immune evasion

CMV has evolved a multitude of immune evasion strategies. Multiple genes have been identified which subvert host immune surveillance and interfere with both the innate and adaptive host immune responses preventing viral clearance (see table 1.1 (Miller-Kittrell & Sparer, 2009). The host immune response is dependent on MHC class I presentation of antigenic peptides on the host cell's surface to cytotoxic T cells (Cresswell, Ackerman et al., 2005). CMV down regulates this MHC class I response from the IE to L stages of infection.

During immediate early expression pp65 phosphorylates the IE-1 protein, selectively blocking the processing and presentation of IE derived peptides (Gilbert, Riddell et al., 1996). In addition CMV expresses US2, US3, US6, US10 and US11 that down regulate MHC class 1 expression by either interference with antigen processing, export or egress to the Golgi apparatus (Jones & Sun, 1997;Jones, Wiertz et al., 1996;Ahn, Gruhler et al., 1997;Furman, Dey et al., 2002;Wiertz, Jones et al., 1996). US2 and US3 also target the MHC class II pathway resulting in down regulation of class II cell surface display.(Miller, Cebulla et al., 2001).

Natural killer cells of the innate immune response will recognise and kill cells that fail to display class I MHC. The significance of this NK response is reflected in the evasion strategies CMV has evolved to impede NK cell activity (Wilkinson, Tomasec et al., 2008). These include a range of MHC class I homologues and other gene products that are capable of modulating both inhibitory and stimulatory NK receptors. These include UL40 which increases cell surface expression of HLA E protecting infected cells by providing an inhibitory signal to NK cells (Tomasec, Braud et al., 2000). Other CMV genes which have been shown to manipulate the NK cell response are UL16, UL83, UL141 and UL142 (Dunn, Chalupny et al., 2003;Chapman, Heikeman et al., 1999;Browne & Shenk, 2003;Tomasec, Wang et al., 2005;Wills, Ashiru et al., 2005) and a micro RNA miR-UL112 (Stern-Ginossar, Elefant et al., 2007) which block the effect of stimulatory NK signals.

Table 1-1 *CMV genes involved in immune evasion; mechanism of evasion and effect on host immune response.*

Mechanism of evasion	CMV Gene product	Effect on immune system
MHC Class I down-regulation	US2, US3, US6, US11	Decreased presentation of CMV antigens to CD8+ T cells Decreased presentation of CMV antigens to CD4+ T cells
CMV-IE-1 phosphorylation	UL83 (pp65)	T cells cannot target first genes expressed upon reactivation
MHC Class I homolog	UL18	Inhibition of macrophage cell lysis
Inhibitory receptors, down regulation of ligands	UL16, UL142	Evasion of NK cells
Up regulation of HLA-E and gpUL18	UL40	Evasion of NK cell mediated lysis
Chemokine receptor	US28	Immune homing interference
IL-10 homolog	UL111a	Immune suppression
Inhibitors of apoptosis	UL36, UL37	Decrease in phagocytosis of infected cells by APCs
Down regulation of MHC class I-related chain B expression	MicroRNA (miR-UL112)	Decreased recognition by NK cells and T cells via NKG2D

1.9 Transmission of CMV

Transmission of CMV occurs at mucosal sites by direct or indirect person to person contact. Sources of CMV include urine, saliva, breast milk, cervical and vaginal secretions and semen, blood, and tears. CMV transmission can also occur via blood products and organ allografts (Stagno, 2001). In most individuals CMV infections are sub-clinical, however virus secretion can persist for years after congenital, perinatal, post natal and primary infections in adults. In particular urine and saliva of young children may contain high viral loads and are a major source of CMV infection (Murph & Bale, Jr., 1988;Staras, Flanders et al., 2008). Perinatal transmission of CMV is common with virus acquired from the birth canal or breast milk (Stagno, 2001;Schleiss, 2006), with 96% of seropositive mothers having CMV DNA detectable in mature breast milk during lactation (Hamprecht, Maschmann et al., 2001). It is also suggested that CMV can remain viable on environmental surfaces, therefore giving another possible mode of transmission (Stowell, Forlin-Passoni et al., 2012).

In solid organ transplant recipients, CMV can be transmitted via the donor organ. CMV seronegative patients (R-) receiving an organ from a seropositive donor (D+)are at the highest risk of CMV infection and resulting CMV disease (Pereyra & Rubin, 2004). Typing of CMV has shown that even when the recipient is seropositive to CMV they are at risk of becoming infected with a different strain from the donor organ (Grundy, Lui et al., 1988) or can reactivate latent CMV. However, pre exsiting immunity is associated with lower risk of disease but is modulated by type of allograft, level of immunosurpression, HLA matching and the level of latent CMV in the organ (Stratta, Pietrangeli et al., 2010).

In contrast seropositive allogeneic bone marrow transplant patients receiving bone marrow from a seronegative donor are at highest risk of CMV infection (Rubie, Attal

et al., 1993). Typing of CMV has shown that virus is reactivated in the host and that transfer of cellular immunity from a seropositive donor may also offer some protection against CMV replication (Wimperis, Brenner et al., 1986;Riddell, Watanabe et al., 1992).

1.10 Epidemiology of CMV infection

Infection with CMV is widespread across all global regions with an overall CMV IgG (against CMV antigens) seroprevalence rate of approximately 60% in adult populations. Seroprevalence generally increases with age and can vary widely depending on ethnicity and socioeconomic status, with highest rates (up to 100%) seen in developing countries throughout Africa and Asia (Ho, 1990;Cannon, Schmid et al., 2010). In women of childbearing age, CMV seroprevalence in the UK has been found to be 54% and independently associated with increasing parity, older age, lower social class, and being single at antenatal booking (Tookey, Ades et al., 1992) with similar rates observed in the USA (Staras, Dollard et al., 2006). In contrast seroprevalence in women of childbearing age in developing countries is >90% (Kenneson & Cannon, 2007) and has been linked to household size, hygiene standards and child care practices (Stagno, 2001).

1.11 Clinical manifestations

CMV disease can follow primary infection, re-infection (with another strain) or reactivation. For the majority of patients primary (and recurrent) CMV infection has been described as ‘asymptomatic’ (Griffiths, Emery VC et al., 2009), however data is limited on the proportion of primary infections that are symptomatic.

In renal and liver transplant patients analysis in the context of donor and recipient serostatus showed that, the majority (78%) of patients that were CMV seronegative at time of transplant developed CMV viraeima (primary infection), with the CMV seropositive recipients developing CMV viraeima in 54% of cases (reactivation/re-infection) (Atabani, Smith et al., 2012).

In immunocompetent patients an infectious mononucleosis, similar to the syndrome associated with Epstein Barr Virus (EBV) may occur and has been attributed to up to 20% of mononucleosis like cases (Klemola, Von Essen et al., 1970). Abnormal liver function, splenomegaly and reduced white cell counts have also been observed (Griffiths, Emery VC et al., 2009) However when the immune system is compromised for example with HIV infection, with acquired immunodeficiency syndrome (AIDS), following transplantation and in the neonate CMV can become pathogenic causing a range of clinical manifestations (Emery, 2001) see figure 1.4).

Viral replication and load have been shown to be important factors in pathogenesis, with a direct association between CMV viral load and disease (Cope, Sweny et al., 1997;Hassan-Walker, Kidd et al., 1999;Emery, Sabin et al., 2000;Regoes, Bowen et al., 2006). A threshold relationship between CMV viruria and disease has been described in renal transplant patients where the quantity of CMV in urine was directly linked to the risk of CMV disease in a non-linear fashion (Cope, Sweny et al., 1997).

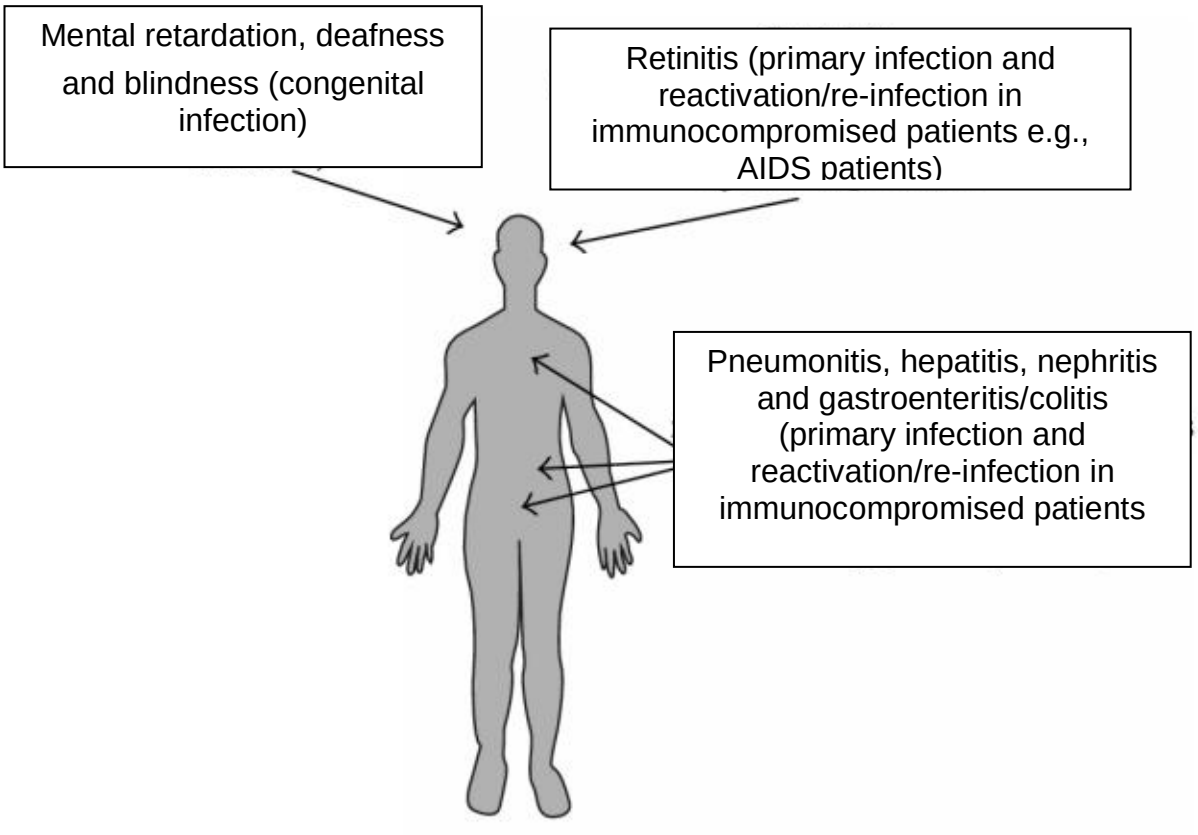


Figure 1-4: CMV-induced disease pathology.

CMV causes disease in individuals where the immune system is compromised (adapted from Poole et al; 2014)

1.11.1 **CMV in transplantation**

In transplant recipients CMV infection can have both direct and indirect effects (figure 1.5) with direct effects being associated with high CMV viral loads and the detection of the virus in the affected organ. CMV has also been associated with other indirect clinical conditions which can occur without CMV but whose incidence is increased when CMV is present (figure1.5).

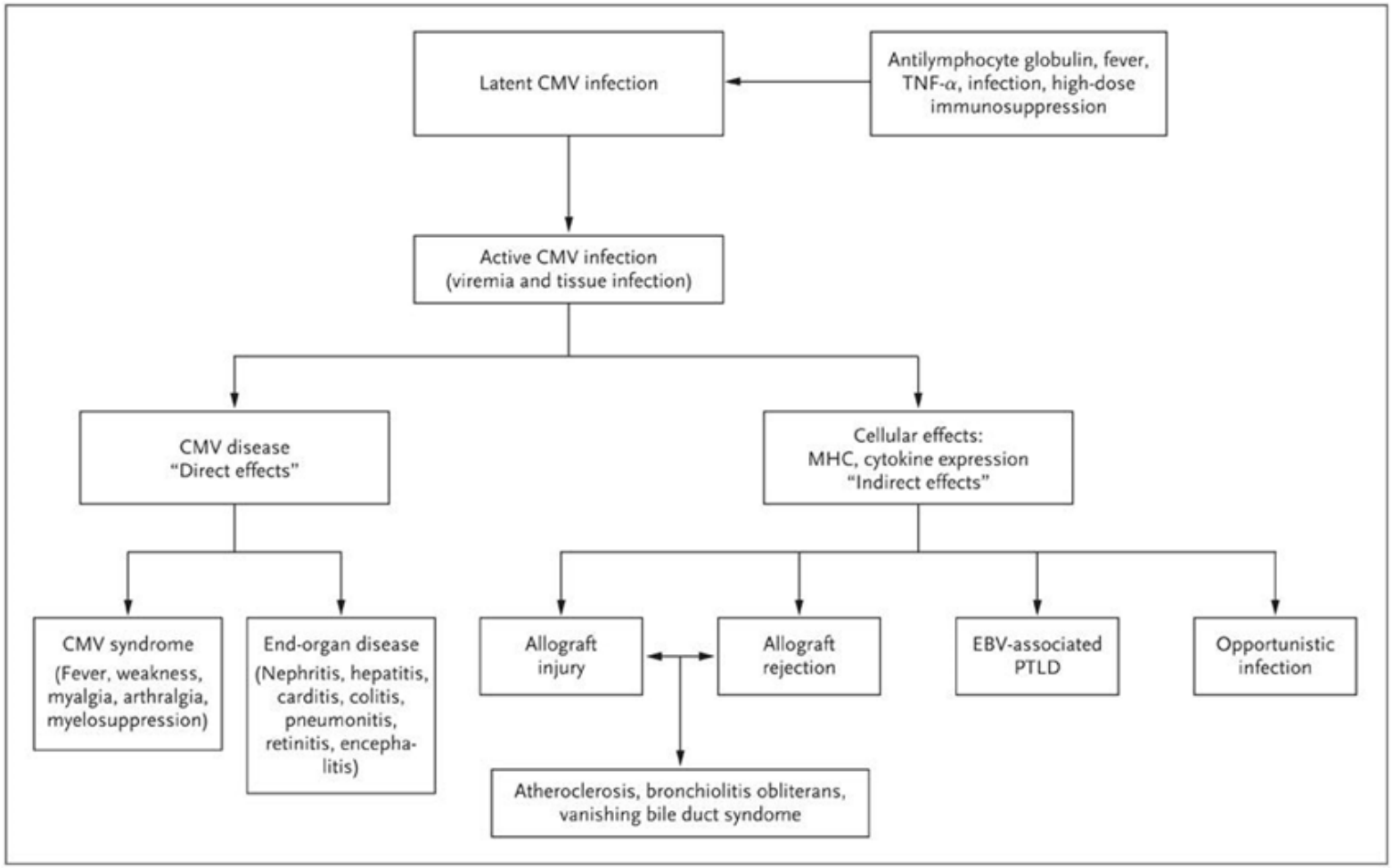


Figure 1-5: Overview of the direct and indirect effects of CMV in transplantation.

MHC: major histocompatibility complex, EBV: Epstein–Barr virus, and PTLD: post-transplantation lymphoproliferative disorder. (Reproduced with permission from(Fishman, 2007).

1.11.2 **CMV and HIV**

Prior to the introduction of highly active antitretroviral therapy (HAART), CMV was one of the most frequent opportunistic infections in HIV-positive patients in developed countries and a major cause of mortality and morbidity (Bowen, Griffiths et al., 1996;Gallant, Moore et al., 1992) with up to 9% of AIDS patients developing CMV disease (Gallant, Moore et al., 1992) and up to 44% of patients developing a CMV-associated disease during the course of their HIV infection predominately CMV retinitis (Hoover, Saah et al., 1993) Other manifestations include gastrointestinal disease (colitis, esophagitis and gastritis), neurological disease (meningoencephalitis, neuritis or polyradiculopathy), and pulmonary disease (pneumonitis). The frequency of clinical manifestations of CMV infection was shown to be related to the $CD4^+$ T-cell count with CMV disease rarely observed until the $CD4^+$ T-cell count dropped below 100 cells/µl or when other signs of severe immunodeficiency, such as other opportunistic infections, were present (Crowe, Carlin et al., 1991).

With the introduction of HAART in the mid-1990s, there was a sharp decline in CMV disease (Ledergerber, Egger et al., 1999); (Brodt, Kamps et al., 1997). A natural history study showed that asymptomatic CMV viraemia disappeared in patients given HAART, presumably due to the re-appearance of CMV specific immune responses (Deayton, Sabin et al., 2002)

1.11.3 **HIV and CMV infant acquisition**

An increased prevalence of CCMV in children infected with HIV compared with those who are uninfected has been reported (Doyle, Atkins et al., 1996) with the French perinatal cohort reporting a threefold higher risk of developing symptomatic CCMV in HIV infected newborns compared to HIV uninfected newborns with CCMV (Guibert, Warszawski et al., 2009). However data on the incidence of CCMV in HIV-positive mothers in resource-limited settings is lacking.

Vertical transmission of CMV in HIV infected mothers to their infants has been shown to be more frequent, with one African study in Kenyan infants reporting that 17% of HIV infected mothers had detectable viraemia (Slyker, Lohman-Payne et al., 2009b) and a cumulative incidence of CMV DNA detected in the plasma of 90% of HIV-exposed but uninfected infants and 93% of infants who had acquired HIV-1 in utero by 3 months of age (Slyker, Lohman-Payne et al., 2009). In the setting of maternal HIV-1 infection maternal CD4 measurements, HIV-1 RNA viral load, CMV viral load and death have been reported to correlate with subsequent infant disease progression and mortality, though the precise mechanisms are unknown (Obimbo, Mbori-Ngacha et al., 2004;Ioannidis, Tatsioni et al., 2004;Newell, Coovadia et al., 2004;Abrams, Wiener et al., 2003;Slyker, Lohman-Payne et al., 2009b). In Vitro, HIV-1 replication has been shown to be up-regulated in syncytiotrophoblast cells of the placenta when co-infected with CMV suggesting that interactions betweenHIV-1 and CMV may contribute to virus transplancental transmission (Toth, Mosborg-Petersen et al., 1995).

1.12 Treatment of CMV infection

1.12.1 Antiviral therapy

There are currently four antiviral drugs available for the treatment of CMV infections: ganciclovir (GCV), valganciclovir (VGCV), foscarnet (FOS), and cidofovir (CDV). Their mechanisms of action are summarised in figure 1.6

Ganciclovir

GCV is a deoxyguanosine analogue and in 1988 was the first drug to be approved for the treatment of CMV. Since then, it has remained the first-line treatment for CMV infections. VGCV is a prodrug (L valyl-ester formulation) of GCV that can be given orally. After oral administration VGCV is rapidly converted to GCV by intestinal and hepatic esterases.

In a CMV infected cell, GCV is selectively phosphorylated by the viral protein kinase homologue pUL97 (the product of the UL97 gene). Following this initial phosphorylation cellular kinases convert GCV monophosphate (GCV MP) into an active GCV triphosphate (GCV TP), which potently inhibits the CMV DNA polymerase by direct competition with deoxyguanosine triphosphate on the enzyme binding site. GCV TP is also actively incorporated into the viral DNA, where it acts as a chain terminator (Biron, Stanat et al., 1985;Balfour, Jr., 1999;Sullivan, Talarico et al., 1992)

Foscarnet and Cidofovir

FOS and CDV are also potent inhibitors of the viral DNA *pol*. However due to their toxicity profiles, they are usually reserved for treatment of patients who have failed or who do not tolerate GCV therapy. CDV is a nucleotide analogue of cytidine that is phosphorylated into its active form by cellular enzymes (Cihlar & Chen, 1996). Once it is in its active diphosphate form, CDV inhibits CMV DNA *pol* by a mechanism similar to that of GCV.

FOS is a pyrophosphate analogue and differs from CDV and GCV by the fact that it does not require any step to convert it into an active form. FOS mode of action is that it binds to and blocks the pyrophosphate binding site on the viral polymerase, preventing incorporation of incoming deoxynucleoside triphosphates (dNTPs) into viral DNA (Chrisp & Clissold, 1991).

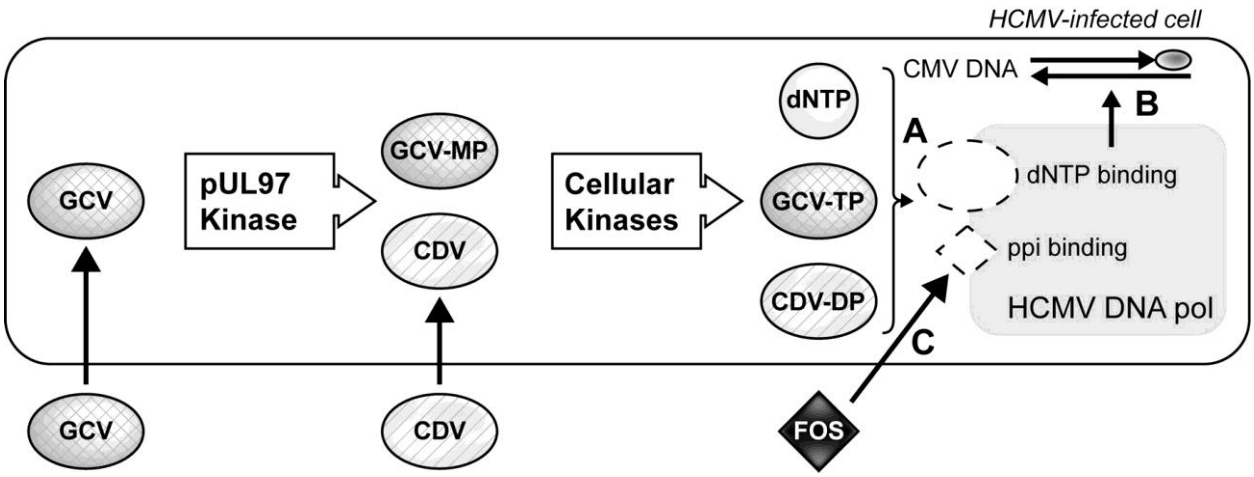


Figure 1-6: ***Mechanisms of action of systemic antivirals ganciclovir, foscarnet and cidofovir against CMV infection.***

GCV and CDV, once phosphorylated, compete with dNTPs for the binding site on the DNA pol ***(A)*** *and are incorporated into CMV DNA* ***(B)****, thus inhibiting viral DNA replication. FOS directly inhibits viral DNA replication by blocking the pyrophosphate (ppi) binding site****(C)****, thus preventing ppi cleavage from incoming dNTPs and subsequent incorporation of the nucleotide into viral DNA.*
MP: monophosphate, DP: diphosphate, and TP: triphosphate.
(Reproduced with permission from(Gilbert & Boivin, 2005).

1.12.2 Other treatments under development with activity against CMV infection

Several experimental agents with much lower toxicity compared to GCV, FOS and CDV have recently been developed and evaluated in clinical trials
These drugs include maribavir, brincidofovir and letermovir and CMV immunoglobulin.

Maribavir

Maribavir (originally named 1263W94) is an inhibitor of the UL97 protein kinase of CMV which actively inhibits inhibits viral DNA assembly and egress of viral capsids from the nucleus of infected cells (Biron, Harvey et al., 2002). Maribavir originally showed promise in a phase 2 clinical trial in allogeneic stem cell transplant patients (Winston, Young et al., 2008) but failed to meet study goals in two phase 3 trials. One in allogeneic HSCT recipients and the other in liver transplant recipients. Both studies showed a good safety profile for maribavir but its efficacy for CMV prevention was sub-optimal (Marty, Ljungman et al., 2011;Marty & Boeckh, 2011). The failure of the trial has been suggested to be related to study design and the dose selected for the study (Marty & Boeckh, 2011). Resistance to maribavir has already been seen (Avery, Marty et al., 2010)

Brincidofovir

Recently brincidofovir a lipid prodrug of CDV that produces high intracellular levels of CDV diphosphate has been shown to prevent CMV disease in stem cell transplantation (Marty, Winston et al., 2013). The addition of a lipid side chain has improved the absorbency of the prodrug across plasma membranes limiting the amount of drug in the general circulation and its associated renal toxicity.

Letermovir

Letermovir inhibits the viral terminase enzyme complex UL56 (Goldner, Hewlett et al., 2011). This inhibition allows the CMV DNA to be produced but stops it from being packaged into infectious particles. The drug has proven to be well tolerated and demonstrated clinical efficacy when used prophylactically in a phase 2 trial in stem cell transplantation, meeting all primary endpoints (Chemaly, Ullmann et al., 2014).

Cytomegalovirus immune globulin

CMV immune globulin is used in combination with an antiviral agent for prophylaxis of high-risk transplant recipients and has been approved by the United Stated Food and Drug Administration when given in conjunction with GCV. Recent data have evaluated the efficacy of CMV immunoglobulin in pregnant women with primary CMV infection to reduce the rate of vertical transmission and improve neonatal outcome with no significant difference reported compared to recipients of placebo (Revello, Lazzarotto et al., 2014)

1.13 Prevention

1.13.1 Vaccine

The development of a safe and effective vaccine against CMV infection is one of the highest health priorities as the Institute of Medicine has reported that a vaccine able to protect against cytomegalovirus infection would be highly cost effective(Stratton, Durch et al., 2001) The estimated costs of medical and educational care for children affected by CCMV in the USA were $1.9 billion per year, whereas the investment needed to develop a CMV vaccine would be approximately $360 million.

However CMV is a highly diverse virus with immune evasion strategies that allow it re-infect and reactivate from latency, so it represents a more complex target than other viruses for which vaccines have been developed.

Several CMV candidate vaccines have been evaluated (Cheeran, Lokensgard et al., 2009). In a clinical trial a live attenuated vaccine produced from the Towne strain of CMV induced protection from severe cytomegalovirus disease but not CMV re-infection following renal transplantation; vaccine development was subsequently discontinued (Plotkin, Higgins et al., 1994).

Pass *et al*. published the first results of a phase 2, randomised, double-blind, placebo-controlled clinical trial of a recombinant CMV gB vaccine with MF59 adjuvant. The vaccine protected 50% of seronegative women against primary CMV infection and the authors concluded that the vaccine has potential to decrease cases of maternal and congenital CMV infection (Pass, Zhang et al., 2009). The same vaccine or placebo was given to both seronegative and seropositive candidates awaiting solid organ transplantation. The vaccine reduced both the level of viraemia and the need for pre-emptive treatment post transplant and the correlate of

protective immunity was found to be the antibody titre against gB (Griffiths, Stanton et al., 2011).

Another phase 2, randomised, placebo-controlled clinical trial used two DNA plasmids (gB and pp65) in seropositive patients undergoing stem cell transplants; the need for preventative treatment was reduced and the correlate of protection was the number of ELISPOT forming cells (Kharfan-Dabaja, Boeckh et al., 2012). These results suggest that a CMV vaccine can both protect against primary CMV infection and boost natural immunity in the seropositive host. However, future studies, such as a phase 3 clinical study, are needed to confirm the efficacy of these vaccines.

1.13.2 **Preventative Programmes**

Preventative programs for congenital CMV infection have been developed by the Centers for Disease Control and Prevention (CDC) and the American College of Obstetricians and Gynaecologists in the United States to educate pregnant women to reduce their exposure to CMV. Saliva and urine from young children are significant sources of CMV infection for pregnant women (Adler, 1991;Staras, Flanders et al., 2008) and educating pregnant women in good personal hygiene practices (i.e. hand washing, not sharing food utensils and not kissing young children on the mouth) has reduced the risk of CMV infection when compared to non-pregnant mothers attempting conception (Adler, Finney et al., 2004). A recent randomised controlled trial showed that hygiene information given to CMV seronegative pregnant women significantly prevents maternal infection, with the cmv conversion rate of 1.2% in the intervention group vs 7.6% in the comparison group (Revello, Tibaldi et al., 2015).

Sexual transmission from a seropositive male partner should also be considered as a potential route by which women may be infected with CMV (Fowler & Pass, 2006) and condoms can be used to prevent infection.

Overall, it is likely that both virus acquired from young children and sexually are responsible for reinfection of seropositive mothers with new or different strains of CMV and promoting awareness of congenital CMV (CCMV) and preventative measures to all prospective mothers remains a key health target (Nyholm & Schleiss, 2010)

1.14 Congenital CMV

1.14.1 Epidemiology of Congenital CMV

CMV is the most common congenital virus infection, affecting about 2 to 22 newborns per 1000 every year depending on the population (Kenneson & Cannon, 2007). The epidemiology has a complex relationship with maternal infection; CCMV can result from primary infection, reinfection (by another strain), or by reactivation of latent virus. Significant differences in seroprevalance exist between populations, which correlate closely to socioeconomic status and race (Cannon, Schmid et al., 2010;Kenneson & Cannon, 2007;Stagno, Dworsky et al., 1982) and as a result up to 50% of women of child-bearing age may be susceptible to primary CMV infection in developed countries (Cannon, 2009). Primary infection has been reported to occur in 1 to 8% of initially seronegative pregnant women (Rahav, 2007) however, primary CMV infection in the mother is rarely diagnosed during pregnancy as the infection is mild or asymptomatic (Stagno, Reynolds et al., 1977). Viral transmission from mother to fetus occurs at the uterine-placental interface (Maidji, McDonagh et al.,

2006). The risk of fetal infection is highest in maternal primary infection with an overall transmission rate of rate of 32% (Kenneson & Cannon, 2007). This risk in primary infection has been reported to increase with gestational age in some studies, up to 77% in the third trimester (Bodeus, Hubinont et al., 1999), however severe sequelae are considered to be more common if the fetus becomes infected early in gestation. The risk of CCMV is significantly lower in infection resulting from reactivation or re-infection in the mother with a reported transmission rate of 1.4% (Kenneson & Cannon, 2007) as preconceptional immunity against CMV provides partial but incomplete protection against intrauterine transmission (Boppana, Rivera et al., 2001). There is a 69% reduction in risk of having a baby with CCMV in seropositive women compared with seronegative women from the same community (Fowler, Stagno et al., 2003).

Therefore the prevalence of CCMV varies widely around the world. Two recent systematic reviews reported an overall prevalence of 0.64% and 0.7% (Kenneson & Cannon, 2007;Dollard, Grosse et al., 2007). In general the incidence of CCMV infection increases with maternal seroprevalence, with regions where maternal seroprevalance is low reporting rates of CCMV to be 0.6-0.7%, whilst in highly seropositive populations rates of CCMV infection have been reported to be 1-5%, the difference reflecting the increased chance of reactivation or reinfection within the seropositive mother (Kenneson & Cannon, 2007;Dollard, Grosse et al., 2007). However the full clinical burden of CCMV in a high seoprevalence setting is yet to be defined. Estimates of the prevalence of CCMV infection and sequelae in infected children in high (90%)- and low (50%)-seroprevalence settings are summarised in figure 1.7 with estimated numbers taken from systemic reviews of the literature.

1.14.2 **Clinical Manifestations of Congenital CMV & Burden of Disease**

No recent data exist for the burden of CCMV in the UK. Systematic reviews of published work indicate that, in the USA approximately 13% of infected infants show severe clinical sequelae at birth with neurological damages, sensorineural hearing loss (SNHL), visual impairments or mental retardation (Dollard, Grosse et al., 2007). Organ damage in the fetus is thought to be caused by virus replication and placental dysfunction (Adler & Marshall, 2007). The effects of CMV can include growth retardation, prematurity, splenomegaly, hepatomegaly, thrombocytopenia, microcephaly, pneumonitis, jaundice and cerebral calcifications.

Within this symptomatic group there is a perinatal mortality rate of around 0.5%, with 70-80% of the surviving babies having major neurological sequelae with poor prognosis (Dollard, Grosse et al., 2007)

Children who are born without symptoms at birth (asymptomatic) are at risk of developing late onset disease. The majority (90%) of CCMV infected infants appear asymptomatic at birth but sequelae can develop months or years later affecting 14% overall (Dollard, Grosse et al., 2007). Although the risk for an infected fetus is higher if the mother has primary infection, the seroprevelance of CMV is so high worldwide that many cases of CCMV are born to ‘immune’ mothers (Cannon, Schmid et al., 2010). This complex epidemiology has implications for the deployment of vaccines to prevent disease caused by CCMV.

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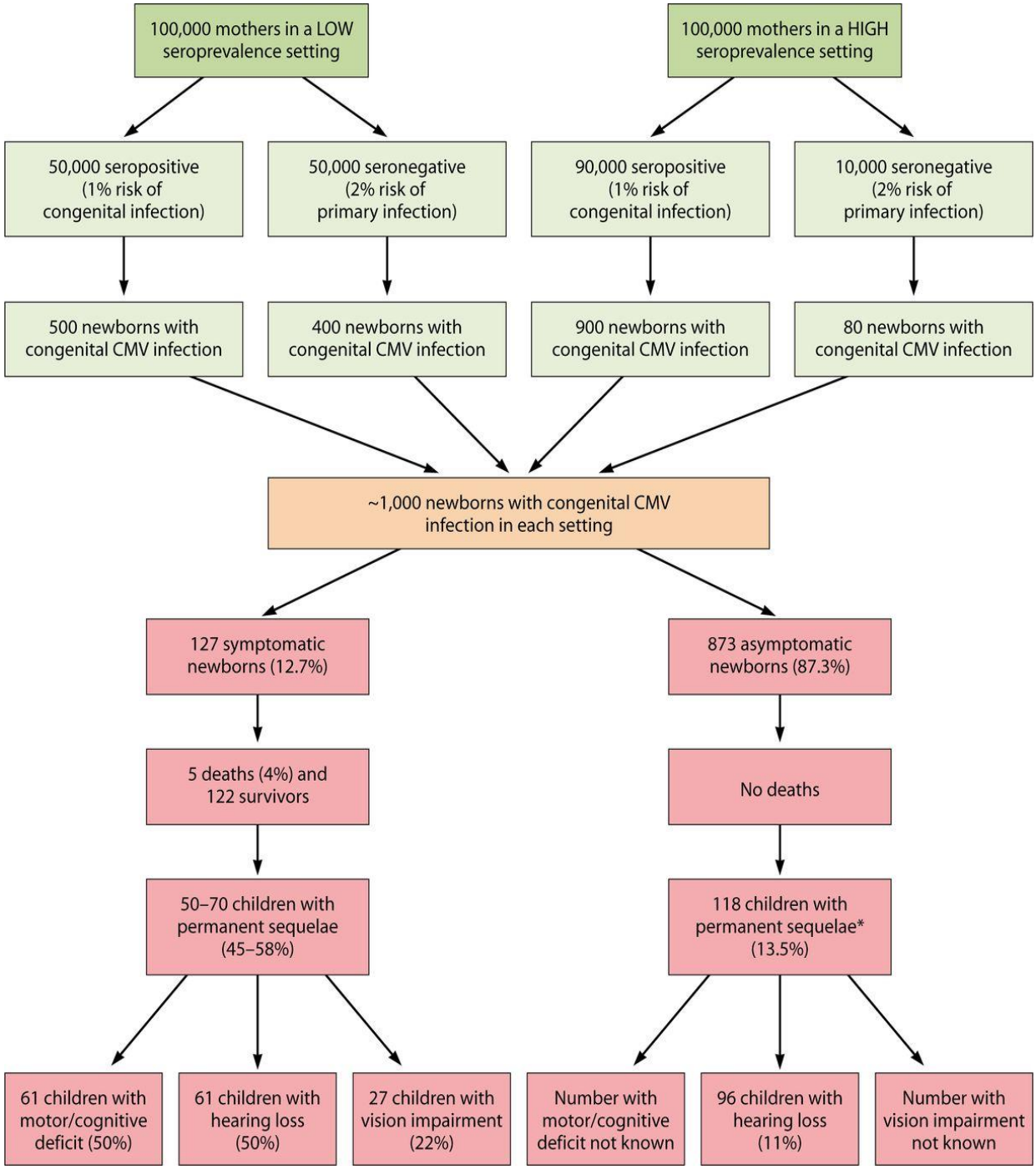


Figure 1-7*: Estimates of the prevalence of CCMV infection and sequelae in infected children in high (90%) and low (50%) seroprevalence settings*.

The following assumptions are made: risk of primary infection 2%, risk of intrauterine transmission in primary infection 40% and in CMV positive mothers 1%. Sequelae rates are based on estimates from (Dollard, Grosse et al., 2007). Proportions within each category do not correspond to 100% because a child may have >1 complication. The figure does not take into account the effect of maternal human immunodeficiency virus infection, racial or ethnic backgrounds which may increase the risk of CMV vertical transmission and sequelae in infected infants. (Reproduced with permission from (Manicklal, Emery et al., 2013).

* most of the children in the asymptomatic group will have hearing loss. There are insufficient data to accurately estimate the number of children with cognitive/motor deficits and vision impairment

1.14.3 Hearing loss

Hearing loss (HL) is an heterogeneous trait with many known genetic and environmental causes including infection (Nance, 2003). Establishing the incidence of congenital deafness in a population is complex and can vary widely, for example HL loss linked to congenital rubella infection, which can be epidemic in nature. Other environmental causes of hearing loss include prematurity, head trauma, subarachnoid haemorrhage, and drug ototoxicity.

Genetic causes are classified according to the pattern of inheritance, clinical features, or the identification of the genetic mutation.

Newborn hearing screening

Two screening techniques are commonly used:

- Automated auditory brain-stem response which measures average neural response to a large number of repeated sound signals of the same pitch and intensity
- spontaneous or sound-induced otoacoustic emissions which detects sound produced by movements of outer hair cells of the cochlea.

1.14.4 Sensorineural hearing loss

Sensorineural hearing loss is the result of damage to the hair cells within the cochlea or the hearing nerve (or both). SNHL hearing loss changes both the ability to hear quiet sounds and the quality of the sound that is heard. SNHL is permanent as once the cochlea hair cells become damaged, the damage is irreversible.

In the UK, childhood HL is defined as a bilateral SNHL of 40 dB or more, whilst in the USA a SNHL of 35 dB or more in either ear is typically the threshold used to identify patients who are referred for confirmatory testing. A combined reported incidence of HL for the UK and USA is 1.86 per 1000 newborns, increasing to 2.7 per 1000 children at 4 years of age (Nance, 2003) see figure 1.8.

SNHL is the most common long term complication of CCMV, with an overall prevalence of 10–15% (Dahle, Fowler et al., 2000;Dollard, Grosse et al., 2007;Ross, Fowler et al., 2006). In children who are symptomatic at birth with cytomegalic inclusion disease the prevalence is higher at 30-40%. SNHL from CCMV can be either unilateral or bilateral and can vary greatly from mild to profound deafness.

Approximately half of SNHL attributable to CCMV infection are late-onset or progressive but the pathological mechanisms involved are unknown. CMV DNA has been detected in the perilymph of children undergoing cochlear implantation up to the age of 7 years (Bauer, Parizi-Robinson et al., 2005;Sugiura, Yoshikawa et al., 2004;Di, Cattani et al., 2009) supporting the hypothesis of ongoing CMV replication in the inner ear (Di, Cattani et al., 2009). It is estimated that up to half of the children with CCMV related SNHL would not be detected by the newborn hearing screening (Fowler, Dahle et al., 1999). An alternative approach to preventing SHNL would be to identify neonates at birth by screening them for CCMV. The Collaborative Antiviral Study (CASG) showed in a randomised controlled trial that intravenous delivery of the anti-viral drug GCV reduces CMV replication and hearing deterioration in infants with symptomatic CCMV and central nervous system involvement (Kimberlin, Lin et al., 2003). A trial of orally administered VGCV in symptomatic infants comparing 6 weeks treatment with 6 months did not improve hearing outcome in the short term

but reported modestly improved hearing and developmental outcomes in the long term (Kimberlin, Jester et al., 2015). Thus early diagnosis of CCMV infection could potentially allow for anti-viral treatment of CCMV to control hearing loss. However the side effects of GCV such as neutropenia can be severe, although reversible.

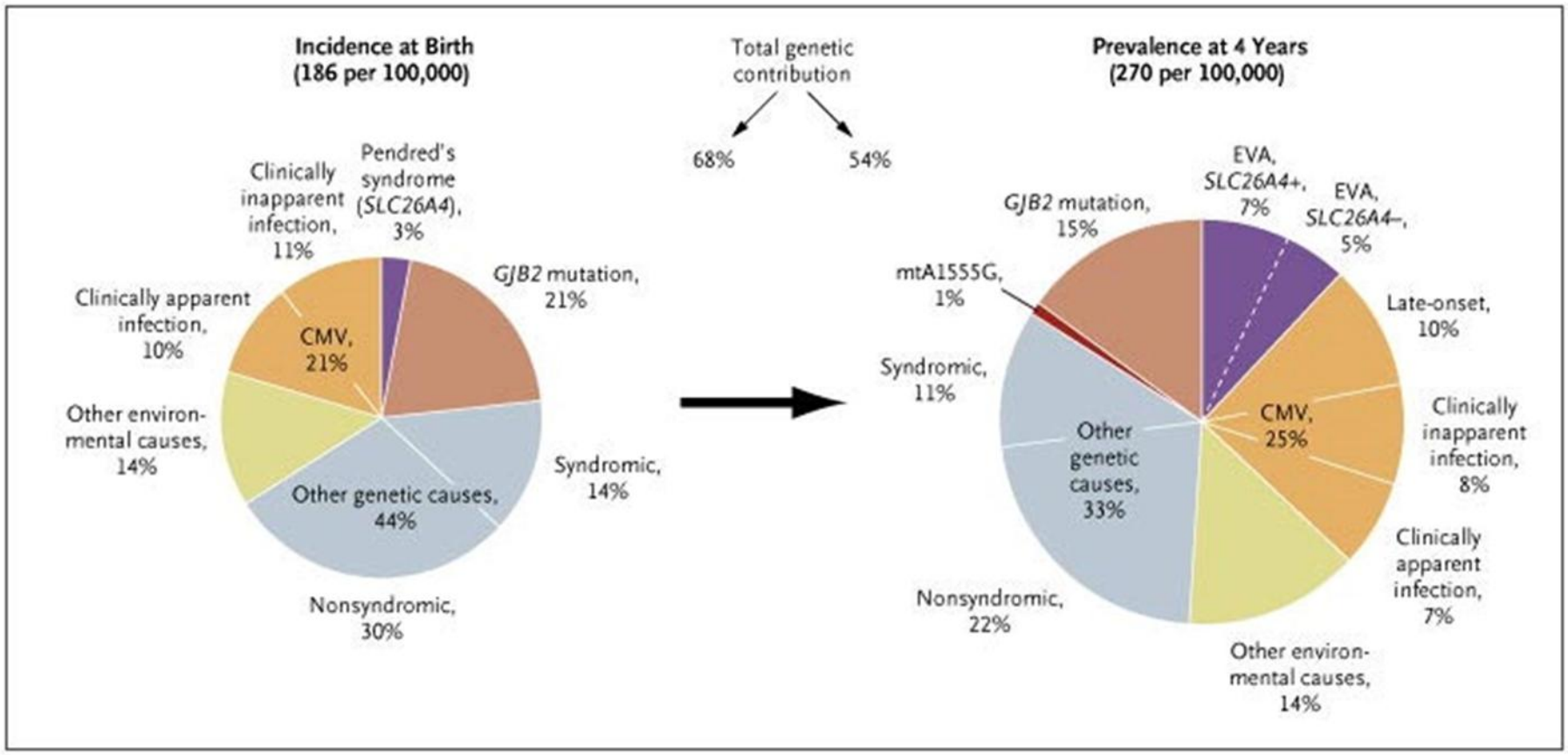


Figure 1-8: **The incidence of deafness at birth in the UK and United States, and its prevalence at four years of age** (reproduced with permission from (Nance, 2003).

Abbreviations: CMV: cytomegalovirus, mtA1555G: the mitochondrial A1555G mutation, and EVA enlargement of the vestibular aqueduct.

1.14.5 **Diagnosis of CCMV**

The asymptomatic nature of CCMV infection and progressive SNHL presents a diagnostic conundrum. Fowler et al reported in 1992 that after a mean follow up of 4.7 years 25% of children born to mothers with primary infection developed one or more sequelae. In children born to mothers who experienced non-primary infection (reinfection or reactivation) 8% of children developed one or more sequelae attributable to CCMV. SNHL is observed in approximately 10-15% of children with CCMV (Fowler, Stagno et al., 1992). The majority of children with hearing loss show delayed onset or progressive hearing loss during childhood. (Dahle, Fowler et al., 2000). Thus the presence of symptoms at birth cannot be used as a guide to choose who to test for CCMV; instead, a routine screening programme would be necessary.

Classically; the diagnosis of CCMV infection was by the isolation of virus from urine or saliva within the first 3 weeks of life. This timeline was established as the earliest age that perinatal CMV infection could be detected in urine by cell culture was 3 weeks.

CMV DNA detection by polymerase chain reaction (PCR) in urine, saliva and blood has recently been described as an alternative to cell culture for the diagnosis of CCMV (Coll, Benoist et al., 2009;Dollard, Schleiss et al., 2010;Gandhi, Fernandez-Alvarez et al., 2010)

However, early postnatal acquisition of infection is common, with CMV from perinatal infection being detected in blood as early as 12 days of life (Hamprecht, Maschmann et al., 2001) , so the timing of the sample is pivotal to the diagnosis (Peckham, Johnson et al., 1987).

Retrospective diagnosis

Diagnosis is not possible from urine or saliva samples taken after 14 days of age because perinatal infection cannot be excluded in these later samples. It is not possible, therefore, to determine whether late onset sequelae such SNHL and developmental delay identified in late infancy are due to CCMV or other factors such as an underlying genetic predisposition in a child with perinatal infection.

Dried blood spots (DBS) are taken routinely 5–8 days after birth from every baby born in the United Kingdom. Small amounts of blood are collected on Guthrie cards and used in newborn screening assays to identify biochemical and genetic disorders including phenyleketonuria, congenital hypothyrodism and cystic fibrosis. In 1994, Shibata et al. first reported the successful detection of CMV DNA from DBS using PCR (Shibata, Takano et al., 1994). In 2000 Barbi et al compared detection of CMV DNA from DBS using PCR with virus isolation for the diagnosis of CCMV and found 100% concordance between the two methods. These early results and the timing of the DBS showed DBS could potentially be used to identify congenitally infected newborns and secondly used to retrospectively confirm congenital CMV in children who present with unexplained SNHL and or neurological damage (Barbi, Binda et al., 2000).

Diagnosis of CMV infection in the mother and fetus

The majority of maternal primary infections are asymptomatic, those that do report symptoms give a generalised illness with glandular fever or flu-like illness (Stagno, 2001). Maternal primary infection is proven by CMV IgG seroconversion (CMV IgG negative to CMV IgG positive). CMV IgM may also be present but interpretation can be difficult as IgM can persist for months after primary infection and can be present after reactivation and reinfection (Lazzarotto, Varani et al., 1999;Lazzarotto, Gabrielli et al., 2004) Therefore, a positive CMV IgM and or IgG should be further investigated by using an avidity assay to determine the binding maturity of the CMV IgG antibodies. CMV IgG antibodies that exhibit low-affinity (those that bind less tightly with their target protein) are an indication that infection occurred <20 weeks prior to testing(Grangeot-Keros, Mayaux et al., 1997). As antibodies mature their binding affinity increases (affinity maturation), a high CMV IgG avidity index can exclude a recent primary infection and when detected before 12 to 16 weeks of gestation indicates a significantly lower risk of CCMV (Lazzarotto, Spezzacatena et al., 1999) The combination of low-avidity CMV IgG antibodies together with a positive CMV IgM strongly indicates a maternal primary CMV infection in the preceding 3 or 4 months (Lazzarotto, Varani et al., 2000).

Once CMV infection is established in the mother, fetal infection and prognosis can be closely monitored by 2 to 4 weekly fetal ultrasound (US) examinations and amniotic fluid viral load testing (Yinon, Farine et al., 2010) as cerebral ultrasound abnormalities are strongly associated with a poor prognosis

(Benoist, Salomon et al., 2008). Amniotic fluid CMV viral load confirms fetal infection but timing of amniotic fluid sampling is critical to its diagnostic sensitivity, with amniocentesis carried out after 20 weeks gestation and at least 7 weeks post maternal infection (Liesnard, Donner et al., 2000;Donner, Liesnard et al., 1994;Revello & Gerna, 2002). The first time requirement is to allow for fetal kidney function to be well established so that CMV appears in the amniotic fluid; the second is to allow for a presumed delay while CMV passes from the maternal to the fetal compartments.

1.14.6 **Treatment of congenital CMV infection**

Prenatal treatment

GCV cannot be used in pregnancy due to its mutagenic potential in animals. Valaciclovir has been given to pregnant women with primary CMV infection and proven fetal infection which appears safe and decreases the circulating viral load (Jacquemard, Yamamoto et al., 2007).

CMV hyper immune globulin has also being used in a non-randomised trial to prevent CMV transmission to the fetus and improve neonatal outcome with some benefit reported (Nigro, Adler et al., 2005). However a recent randomised controlled trial showed no significant reduction of intrauterine transmission of CMV (Revello, Lazzarotto et al., 2014).

Treatment of the Neonate

Currently GCV and VGCV are the preferred antivirals for the treatment of CCMV (Faulds & Heel, 1990) with a 6-week course of intravenous GCV or oral VGCV considered for neonates born with central nervous system involvement (CNS) (Gandhi, Fernandez-Alvarez et al., 2010).

The efficacy of GCV for the prevention of progressive hearing loss in infants with proven congenital CMV CNS disease (microcephaly, other neurological findings, neuroimaging abnormalities, or hearing loss) was evaluated in a randomized trial by Kimberlin *et al* in 2003. Results showed that after 12 months of follow-up, a higher rate of normal hearing, improved hearing or prevention of worsening of hearing in those with a baseline hearing deficit, was seen in children who had a 6-week course of intravenous GCV, compared to children with no therapy (Kimberlin, Lin et al., 2003). A secondary analysis on the same study population also showed neurological benefits following GCV treatment with children who received GCV having fewer developmental delays at 6 and 12 months than untreated infants (Oliver, Cloud et al., 2009). However, side effects of GCV include bone marrow toxicity, particularly neutropenia, thrombocytopenia and anaemia.

A randomized multicentre placebo-controlled trial (CASG112; NCT00466817) commenced in 2008 to compare the clinical benefit of 6 weeks versus 6 months of VGCV in symptomatic infants to define the role of prolonged antiviral therapy and address if a reduction in ongoing viral replication in end organs may contribute to improved long-term outcome. The results reported did not improve hearing outcome in the short term but reported modestly

improved hearing and developmental outcomes in the long term (Kimberlin, Jester et al., 2015).

1.14.7 **Screening for congenital CMV**

Screening is defined as, ‘a systematic application of a test to asymptomatic individuals at risk of a specific disorder to trigger further investigation or preventative action’ (Wald, 2008). CCMV may be appropriate to include in national newborn screening programs because of its incidence (it is more common than other disorders tested for by programs) and the fact that it is a major cause of disability. To date the most significant obstacles to the implementation of screening for CCMV include the lack of a standardised high-throughput screening test and a protocol for follow-up of CMV-infected children. However the possibility of prompt diagnosis of CCMV should not be underestimated as it is crucial in minimising the impact of CCMV disease. The CCMV disease burden in the USA in 1990’s was estimated to be 1.86 billion dollars annually (>300,000 US dollars per child)(Arvin, Fast et al., 2004). Universal newborn CMV screening would identify infants at risk of CMV disease including hearing loss, and could lead to early intervention such as cochlear implants which may reduce the impact of hearing loss on speech, language and social development. Thus, early diagnosis could allow interventions to compensate for hearing loss. This is very important as it could prevent further deterioration in hearing and language skills which in turn could mean the difference between the child being moderately deaf and profoundly deaf with implications for schooling and social development. A major barrier to a screening programme for CCMV is that to date no approved

and validated intervention for CCMV exists. Although GCV therapy has been shown to prevent progression of hearing loss in symptomatic babies the side effects can be severe and there is no evidence base to support its use as a treatment in asymptomatic children. However screening would provide early detection of CCMV infected infants and therefore provide the opportunity for early intervention and enhanced monitoring of affected infants for late onset disease. The development of a suitable screening test could help recruit children into a randomised controlled trial to determine if GCV is effective at reducing SNHL.

The most obvious platform for congenital CMV screening would be the neonatal DBS, which are routinely used to screen for other inherited conditions. Detection of CMV DNA from DBS has been suggested for screening but this approach has not yet been demonstrated to be sensitive enough for use in a large-scale newborn screening programme. However, methods for the extraction and detection of CMV DNA are dynamic and rapidly improving as DNA extraction methods and PCR assays are refined. Therefore it is envisaged that if a sufficiently sensitive and specific test did become available, the costs and logistics of adding a PCR-based test to the newborn screening programme would require careful consideration.

1.15 Aims of this thesis

1.15.1 Setting the scene

The work in this part time PhD thesis began in 2005. Therefore it is important to 'set the scene' of diagnostic testing for CCMV as the field of viral molecular diagnostic testing has dramatically changed during the course of this thesis with PCR now the method of choice for much viral diagnostics. However this was not the case in 2005, where the method of choice for the diagnosis of congenital CMV was restricted to cell culture with the isolation of CMV from urine samples taken within the first 21 days of life being the 'gold standard' diagnostic test. This method was used for both diagnostic testing and large epidemiological studies (Adler, 2007). However this method does not lend itself to high throughput and relies on virus remaining viable prior to inoculation in cell culture. With the development of PCR, Shibata *et al* were the first to report that CMV DNA could be detected from DBS using PCR in 1994 (Shibata, Takano et al., 1994) followed by Barbi et al in 1996 and 2000 when their study compared detection of CMV DNA from DBS using PCR with virus isolation for the diagnosis of CCMV and found 100% concordance between the two methods. (Barbi, Binda et al., 2000). These early results and the timing of the newborn DBS suggested that DBS could be used to retrospectively confirm congenital CMV in children with compatible symptoms, but still had many unanswered questions regarding the DBS as a sample matrix and its applicability for screening.

Therefore the aim of this thesis is to study several aspects of detection of CMV from DBS. I will systematically address the concerns surrounding DBS as a sample type and their potential to be used in general for both retrospective diagnosis of CCMV and to facilitate modern diagnostic testing in resource limited settings. Finally I will apply this knowledge to assess the feasibility of using DBS for screening newborns for CCMV. I will use an iterative approach of devising an assay, using it to test defined cohorts of patients, redefining the assay and testing it on additional cohorts. Overall, I hope to facilitate development of a testing programme for CCMV in the UK by devising a highly sensitive high throughput assay that is suitable for both detection of CMV in clinical cohorts and in newborn screening.

Evaluation of CMV DNA detection from DBS

Detection of CMV DNA can vary depending on the method of DNA extraction from the cards (Soetens, Vauloup-Fellous et al., 2008), the amplification method used and the region of the CMV genome being detected (Barbi, Binda et al., 2006). I have addressed these issues by evaluating and optimising DNA extraction and PCR methods to develop a DBS assay for the detection of CMV DNA (Chapter 3) Secondly, the assay developed has been used to address concerns raised in the literature over the stability of CMV DNA in DBS over time and the risk of cross contamination under standard UK storage conditions (Chapter 3). Finally, the DBS sample was systematically evaluated against 'gold standard' reference plasma samples for the diagnosis of CMV infection in a clinical cohort with a high prevalence of CMV (Chapter 5).

The introduction of a national diagnostic service for the retrospective diagnosis of CCMV from DBS

This topic is addressed in several ways. Firstly, the sensitivity and specificity of the assay was optimised in a clinical cohort of children with laboratory confirmed CCMV (Chapter 3). The optimised DBS assay developed in chapter 3 and 4 was then used to investigate the role of CCMV in SNHL and a diagnostic algorithm for the retrospective diagnosis of CCMV from children with SNHL was developed and adopted for use in the UK (Chapter 4). Finally the DBS assay was introduced into the routine diagnostic service of the laboratory for the retrospective diagnosis of CCMV in children with compatible symptoms (Chapter 6).

The use of DBS in clinical cohorts for the detection of CMV infection

The applicability of the DBS sample type, to facilitate modern diagnostic testing methods in resource limited settings was addressed, DBS samples were analysed for the detection of CMV infection in two cohorts of infants born to HIV positive mothers in resource limited settings. The results were used to investigate the role of maternal CMV levels in multiple compartments during the antenatal/postpartum period and their association with infant CMV acquisition (chapter 7).

Potential for the use of DBS in newborn screening for CCMV

The results of the previous studies were used to address the feasibility of screening newborns for CCMV by the laboratory analysis of the newborn DBS. Firstly the developed methods were adapted to be suitable for larger

volume testing and the resulting methods tested in two large clinical cohorts (Chapter 6 and 7). Secondly the sensitivity of the DBS assay was addressed by the development of a one-step nested PCR with high analytical performance that is suitable for high throughput (Chapter 6). Finally the potential of the high throughput nucleic acid extraction and one step nested protocol for newborn screening was determined (Chapter 6).

Chapter 2

2 General Materials and Methods

2.1 Study Timeline

The work in this part time thesis was undertaken over a period of 8 years (including a year of maternity leave). It is therefore important to understand the study timeline in relation to the results and development work. The work started in 2005 and developed from an interest in diagnostic testing whilst working full time as a diagnostic scientist in the Department of Virology at the Royal Free Hospital, London and has been performed alongside this full time diagnostic role . As part of this job I had undertaken testing for CCMV and developed an interest in this area. 'Proof of concept' papers had just been published for the detection of CMV DNA from dried blood spots in the retrospective diagnosis of CCMV, but there was no offer of this test routinely in the UK. Alongside this interventions; including the use of antivirals in the treatment of CCMV had recently been described and the awareness of CCMV was increasing therefore there was a need for an accurate and reliable diagnostic test for CCMV in the UK. This test was offered routinely from 2005 onwards with the aim of setting up an assay suitable for screening for CCMV.

This study has taken an iterative approach and as new methods have been developed they have been assessed and validated with the outcome of assay improvement.

2.2 Clinical Collaborations

Clinical collaborations were set up with the following groups.

British Paediatric Surveillance Unit: Congenital CMV Study

The Unit was set up in 1986. It is a joint initiative of the Royal College of Paediatrics and Child Health, Public Health England (PHE) and the Institute of Child Health (ICH) to support research into rare childhood disorders. Children born in the UK with suspected or laboratory diagnosed CCMV were reported between 2001-2002 by paediatricians via the British Paediatric surveillance unit (BPSU) notification system (BPSU 17th Annual report, 2002-2003). CCMV cases were confirmed on the basis of PCR or virus isolation from urine, blood, saliva or tissue taken at biopsy within 3 weeks of birth. 'Possible' CCMV cases were infants with positive samples only after 3 weeks of age and symptoms compatible with CCMV which were not accounted for by any other diagnosis. The DBS of children reported via the BPSU notification system were retrieved from storage after obtaining the parents' consent. All residual DBS material was tested under code with local ethical approval. At time of analysis the clinical presentation (CMV status) and outcome of the children was not known.

CMV in hearing impaired children (CHIC study)

An independent ethically approved study 'CMV in Hearing Impaired Children' (CHIC) was established to look at the relationship between CCMV and SNHL. The study was conducted over 14 months and recruited patients from 5 national health service (NHS) sites (Royal Ear Nose and Throat Hospital, London, Bradford Teaching Hospitals NHS Trust, Queen's Medical Centre Nottingham, Milton Keynes General NHS Trust and Northampton General

NHS Trust) From April 2005 to June 2006 potentially eligible participants were identified by informing Consultant audiological physicians (community and hospital-based), Consultant paediatricians, neonatologists, virologists, and fetal medicine Consultants of the study and asking them to report any children with CCMV or unexplained SNHL. Signed informed consent was obtained from the parents/guardians to allow retrieval and testing of the child's residual DBS for CMV DNA and data collection from the child's notes. All testing of residual material was done under code. At time of analysis the clinical presentation (CMV status, hearing loss) and outcome of the children was not known

Collaborative studies with the University of Washington, USA

A collaborative study with the University of Washington, Seattle USA was undertaken on samples from Kenya. All work performed by them is acknowledged in each chapter, all other work is my own.

Participants

All studies were approved by the University of Washington Institutional Review Board and the Ethics and Research Committee of Kenyatta National Hospital, and written informed consent was obtained from all mothers on behalf of themselves and their infants. A cohort of infants born to HIV infected women were used to study acute CMV acquisition and DBS detection methods. Specimens were selected from a larger perinatal HIV transmission cohort, details of which have been presented elsewhere (Lohman-Payne, Slyker et al., 2009;Lohman, Slyker et al., 2005;Obimbo, Mbori-Ngacha et al., 2004;John-Stewart, Mbori-Ngacha et al., 2009;Gichuhi, Obimbo et al., 2005;Lohman, Slyker et al., 2003).

2.3 Dried Blood Spots

Clinical diagnostic samples

Based on my preliminary results routine diagnostic testing of DBS samples for retrospective diagnosis of CCMV was introduced in 2005. DBS samples were received into the laboratory for the retrospective diagnosis of congenital CMV from throughout the UK.

Laboratory prepared test DBS

Newborn screening standard Whatman 903 cards (Whatman, GE Healthcare Maidstone, England) were obtained from NHS supplies within the Royal Free Hospital, London, UK.

Test DBS were prepared from either CMV positive or negative anticoagulated (sodium citrate) whole blood samples from solid organ transplant recipients whose viral loads were previously determined for diagnostic purposes by quantitative real time PCR in our laboratory. DBS were prepared by applying 50µl of whole blood with a wide range of CMV viral loads onto the Whatman 903 card. The prepared cards were left to air dry overnight and then stored at room temperature, touching each other to mimic UK newborn screening laboratory storage conditions.

Quality Control Molecular Diagnostic DBS proficiency panels

Quality Control for Molecular Diagnostics (QCMD) is an independent international external quality assessment proficiency testing organisation. QCMD provides a CMV DBS panel as part of its congenital infection external

quality assurance programme. Blood spots are manufactured and represent a wide range of CMV viral loads. In this study the 2007 and 2011 CMV DBA panels were analysed.

2.4 Extraction of viral DNA from DBS samples

Unless stated, total nucleic acid was extracted from a semicircle of DBS (10mm diameter). On occasions where a semicircle was not available the total sample was used.

Control measures

Strict control measures to avoid false positive results were applied to all DBS processing (Kwok & Higuchi, 1989) of 3 separate rooms for PCR with strict protocol. To prevent contamination by carryover of CMV DNA the scissors or punch were washed thoroughly with 0.1M hydrochloric acid and 70% ethanol prior to and after cutting each DBS. Negative (blank) DBS were included as an extraction control and subjected to every step of the process to ensure no contamination occurred.

QiAmp DNA blood Mini kit

Total nucleic acid was extracted from the DBS using the commercial extraction system Qiagen mini blood kit (Qiagen, Hilden, Germany) A semicircle of DBS was cut into 3 pieces and added to 180µ ATL buffer. The tube was vortexed for 10 seconds and then incubated at 85ºC for 10 minutes. The tube was centrifuged briefly (6,000xg for 1 minute) to remove any droplets from the lid and allowed to cool. Once cool, 20µl of proteinase K

(>600 mAU/ml) were added and the tube incubated at 56 ºC for 1 hour. 200µl of lysis buffer AL were added, mixed by vortexing and incubated at room temperature for 10 minutes, 200µl of absolute alcohol was added and the tube vortexed thoroughly. The lysate was then transferred to a QIAamp spin column and placed over a 2ml collection tube, after centrifugation at 6,000xg for 1 minute. The column was placed onto a new 2ml collection tube and washed by the addition of 500µl of Buffer AW1. The spin column was then centrifuged at 6,000xg, 1 minute, after which the collection tube was changed and 500µl Buffer AW2 were added, the column centrifuged at 14,000xg, 3 minutes. An extra spin was performed (6,000xg for 1 minute) to ensure complete removal of buffer. The spin column was then placed on a 1.5ml microcentrifuge tube and the resulting nucleic acid was eluted into 60ul of AE buffer by centrifuging at 6,000xg for 1 minute. The eluted nucleic acid was stored at -20ºC.

EasyMAG extractions

Semi-automated nucleic acid extractions were performed using the magnetic particle based extraction system the NucliSENS EasyMAG (Biomerieux, Marcy l'Etoile, France) with manual pre-treatment. Briefly half a DBS was added to 2mls of EasyMAG lysis buffer in a 5ml lidded tube and 20µl of proteinase K solution >600mAU/ml (Qiagen, Hilden, Germany) added. The mixture was rocked on a plate shaker at room temperature in a horizontal position for a minimum of 30 minutes. The tube was then spun in a centrifuge at 1500 x g for 30 seconds to remove any paper fibres. The resulting supernatant was then added to the EasyMAG and processed as per

manufacturer's instructions running the "off board lysis protocol" (generic protocol, version 2.0.1) with an elution volume of 60µl.

QIAsymphony Extractions

Automated nucleic acid extractions were performed on the QIAsymphony SP workstation (Qiagen, Hilden, Germany) using the QIAsymphony DNA mini kit (magnetic particle based automated extraction) with manual pre-treatment. Briefly, half a DBS was added to 400ul of ATL lysis buffer and 20µl of proteinase K solution >600mAU/ml (Qiagen, Hilden, Germany) in a 5ml lidded tube. The tube was then incubated in a waterbath for 1 hour at 56ºC. The tube was then spun in a centrifuge at 1500 x g for 30 seconds to remove any paper fibres. The resulting supernatant was then transferred to a 2ml tube and loaded onto the QIAsymphony SP workstation. Extraction was performed as per manufacturer's instructions running the VirusBlood-V5_DSP protocol with an elution volume of 60µl

2.5 Quantitative TaqMan Real time PCR

Principle of the TaqMan assay

Traditionally a PCR is run to an end point, analysis is then performed to detect the amplified product for example by running DNA on an agarose gel after the reaction has finished and visualising the product via intercalating dye. In contrast, real-time PCR allows the accumulation of amplified product to be detected and measured as the PCR progresses, that is, in “real time”. The TaqMan assay (Heid, Stevens et al., 1996) is based on two principles; *Taq* polymerase exhibits a 5’ to 3’ exonuclease activity (Holland, Abramson et al., 1991) and the fluorescence resonance energy transfer (FRET) (Cardullo, Agrawal et al., 1988). In the TaqMan assay the *Taq* polymerase cleaves a target specific internal probe. This oligonucleotide probe is labelled at the 5’ end with a reporter fluorophore and a quencher dye at the 3’ end. When the probe is intact fluorescence of the reporter is quenched due to its proximity to the quencher. During the combined annealing/extension step of the amplification reaction, the probe hybridises to the target and the specific 5'—>3' exonuclease activity of *Taq* cleaves off the reporter. The reporter dye energy is no longer transferred to the quencher (no FRET) resulting in a measurable fluorescent signal. This fluorescent signal is proportional to the amount of amplified product in the sample and increases with every PCR cycle. The TaqMan instrument reads the increase of fluorescence over PCR cycle (Rn) and constructs an amplification plot (figure 2.1) An arbitrary threshold is set for the PCR and the cycle where Rn exceeds the threshold is determined. The cycle number at which this occurs is called the threshold

cycle, or Ct. The Ct of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescent signal above background. Due to this relationship Ct can be used to quantify the genome copy numbers of target in a sample

Quantitation using a standard curve

Absolute quantitation was performed by generating a standard curve.
A serial dilution of known copy number template (plasmid that contained the cloned region of interest) was used.

Linear regression of the Log_{10} starting quantity of template versus Ct were then plotted to give the standard curve. The plot was then used for extrapolating the quantity of the target in the sample (figure 2.2)

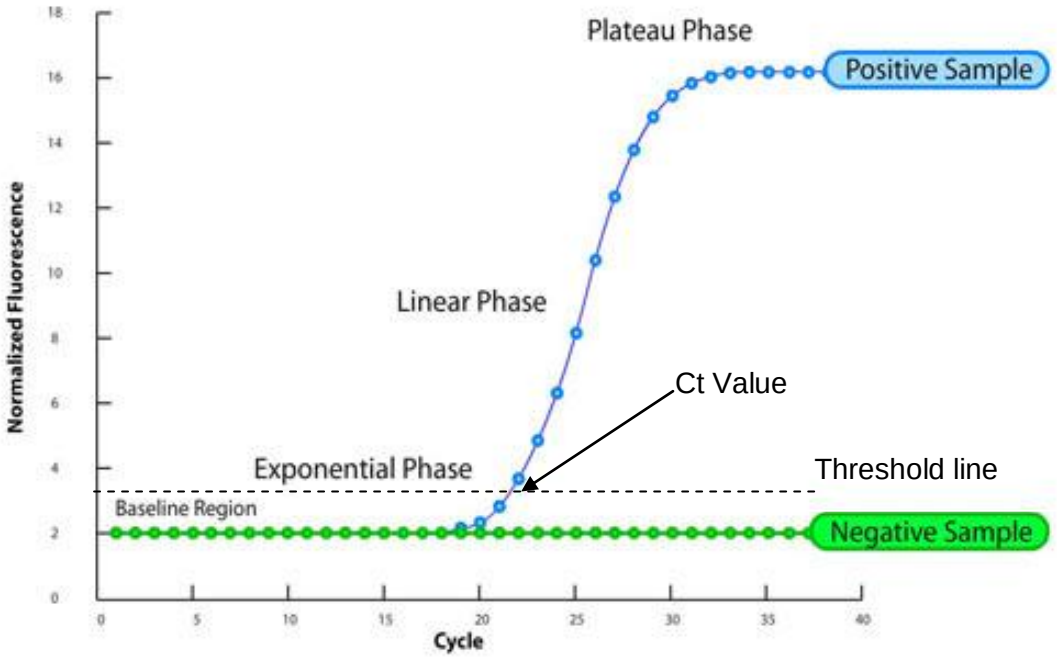


Figure 2-1*: TaqMan Amplification plot.*

The threshold line and crossing point for Ct value are shown. Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable. The increase in fluorescence (signal >background) is then plotted to construct an amplification curve. With increasing cycling a non-exponential 'plateau' phase is reached until completion of cycling.

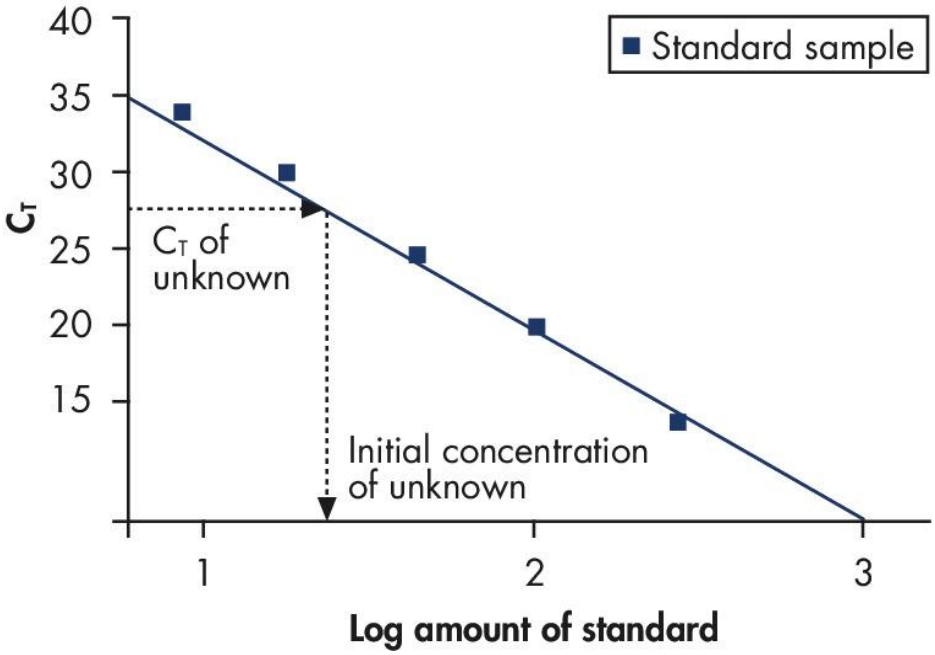


Figure 2-2: ***Standard curve generated by plotting Ct vs concentration (log_{10})***

The concentration of the unknown can be extrapolated against the known Ct data for each standard. The dotted arrow corresponds to the sample concentration which reaches the threshold at Ct 27

2.5.1 **Quantitative CMV gB PCR**

A singleplex real-time amplification was performed on the TaqMan 7000/7500 (Applied Biosystems, Warrington ,UK) with primers and probes specific for a highly conserved region of CMV glycoprotein B (gB) UL55 as previously described (Mattes, Hainsworth et al., 2005).

gB1: *5' GAGGACAACGAAATCCTGTTGGGGA 3'*
gB2: *5' TCGACGGTGGAGATACTGCTGAGG 3'*
Probe: *5' FAM-CAATCATGCGTTTGAAGAGGTAGTCCA-TAMRA*

Forward primer (gB1) and the reverse primer (gB2) corresponded to nucleotides 1942 to 1964 and 2066 to 2031 of the CMV gB ORF. The 150bp product was detected using a TaqMan probe labelled at the 5' end with 6-FAM and the 3' end with TAMRA

DNA amplification was performed in 30ul total reaction volumes. Each reaction contained 10µl of DNA extract 15ul of "Universal Mastermix" (Applied Biosystems, Warrington,UK) 0.25µM of gB1 and gB2 and 0.1µM of probe. PCR cycling conditions were 10 minutess at 95ºC followed by 50 cycles of 15s at 95ºC and 1 minute at 60ºC with data acquisition during the 60ºC extension.

Quantitation was achieved using serial dilutions of cloned target to produce a standard curve to determine the CMV viral load. CMV viral loads were calculated using the Sequence Detection System software version 2.1.1.

The theoretical limit of detection of the assay was 100 genomes/ml (1 genome/reaction).

A minimum of 3 no template controls and extraction water controls were run on each individual run to control for contamination. A known concentration of CMV strain Ad169 was run in triplicate on each run to control for inter-assay variation of viral load. All DBS were analysed for CMV DNA in triplicate.

2.5.2 **β globin PCR**

A singleplex real-time amplification was performed on the TaqMan 7000/7500 (Applied Biosystems, Warrington ,UK) with primers and probes specific for a highly conserved 101bp region of the human β globin gene (GenBank Sequence Database accession numbers U01317 β-globin gene). as previously described (Lo, Tein et al., 1998).

β globin 1:*354- 5' GTGCACCTGACTCCTGAGGAGA 3'*
β globin 2: 455- *5' CCTTGATACCAACCTGCCCAG 3'*
β globin Probe: 402- *5' VIC-AAGGTGAACGTGGATGAAGTTGGTGG-TAMRA 3'*

The PCR cycling conditions were 95 °C for 10 minutes (Taq Activation), followed by 50 cycles at 95 °C for 15 seconds, and at 60°C for 1 minute. The total PCR volume was 25 µl, which contained 300nM of each primer, 100 nM probe, 12.5µl TaqMan Universal Master Mix (Applied Biosystems, Warrington UK) and 5ul sample input.

Data was analysed using Sequence Detection System software Version 1.3 (Applied Biosystems, Warrington, UK).

Generation of a β globin quantitation plasmid standard

To accurately quantify the amount of cellular DNA, a plasmid containing the 101bp β globin PCR target region was cloned and quantified.

Amplification of the β globin target region

Amplification of the target region was performed by PCR. Reactions were set up in a reaction volume of 50µl, which contained 10X buffer containing 1.5mM magnesium chloride ($MgCl_{2)}$, 375µM of each dNTP, 300nM of primers and 2.5 units of *pfuUltra* high fidelity DNA polymerase (Stratagene, Cheshire, UK). PCR cycling conditions were 95ºC for 3 minutes followed by 40 cycles of 90ºC for 1 minute, 55ºC for 30 seconds and 72ºC for 3 minutes, followed by a final extension at 72ºC for 10 minutes.

Gel electrophoresis

Agarose gel electrophoresis was used to confirm the presence and correct size of 101bp PCR product. A 1.5% agarose gel was made by dissolving 1.5g molecular grade agarose (Sigma Aldrich, Dorest, UK) in 100ml Tris Borate EDTA (TBE) buffer (Sigma Aldrich, Dorest, UK) by heating in a microwave. Once cooled, 10mg/ml ethidium bromide (Sigma Aldrich, Dorest, UK) was added. PCR products were mixed with 5X loading dye (Invitrogen, Paisley, UK) and loaded onto the gel with the DNA mass hyperladder 1 (Bioline, London, UK). The gel was run for approximately 45 minutes at 100 volts. All bands on gels were visualised using a UV transilluminator (Biorad, Hertfordshire, UK).

Purification of PCR product

Purification of the PCR product was performed with the QIAQuick Gel extraction kit (Qiagen, Hilden, Germany). The resulting DNA was eluted into nuclease free water.

A-tailing

To enable efficient TA cloning, purified products were added to a mix of 250µM dATP, 10X buffer containing 2.5mM $MgCl_2$ and 2.5 units of Amplitaq gold polymerase (Qiagen, Hilden, Germany). The mix was heated to 95ºC for 10 minutes followed by 72ºC for 20 minutes.

PCR cloning

The TOPO TA cloning kit (Invitrogen, Paisley, UK) was used to clone the amplified PCR product. TOPO TA cloning uses the *Topoisomerase I* to ligate the PCR product with the A nucleotide overhang into the vector which contains a T nucleotide overhang. The ligation reaction consisted of 4µl PCR product, 1µl salt solution and 1µl pCR-2.1 TOPO vector. The ligation reaction was incubated at room temperature for 5 minutes and then stored on ice ready for transformation. 2µl of the ligation was transferred into a vial of 50µl TOP10F' *E. coli* cells (Applied Biosystems, Warrington, UK) and incubated on ice for 15 minutes. Cells were then heat shocked (42ºC for 45 seconds) and cooled on ice. 250µl SOC medium (Invitrogen, Paisley, UK) was added to the cells and the resulting mix incubated at 37ºC for 1hour on an orbital shaker. Cells were plated onto lysogeny broth (LB) agar plates containing 50µg/ml ampicillin (Invitrogen, Paisley, UK) and 100mM Isopropyl β-D-1-

thiogalactopyranoside (ITPG) (Invitrogen, Paisley, UK) and incubated overnight at 37ºC.

LB Agar plates

500ml of LB agar was made from LB powder (Invitrogen, Paisley, UK) in deionised water according to the manufacturer's instructions with the addition of 7.5g agar (Sigma Aldrich, Dorest, UK). The resulting mixture was autoclaved and allowed to cool prior to the addition of ampicillin (Invitrogen, Paisley,UK) and ITPG (Invitrogen, Paisley,UK), poured into petri dishes, allowed to solidify and stored at +4°C in the dark for the maximum of 1 week.

Miniprep

A single transformed white colony was selected from the LB plates and added to 2mls LB broth containing 50µg/ml ampicillin and incubated overnight at 37ºC on an orbital shaker. The resulting plasmid DNA was extracted from the bacterial cells using the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

LB Broth

500ml of broth was made from LB powder(Invitrogen, Paisley, UK) in deionised water according to the manufactures instructions. The resulting broth was autoclaved and allowed to cool and stored at room temperature.

Determination of plasmid concentration

To determine the plasmid DNA yield the DNA concentration was determined by measuring the plasmid sample absorbance at 260nm on the Nanodrop

2000c (Thermo Scientific, Willington, USA) and using the relationship that an A_{260} of 1.0 = 50µg/ml pure dsDNA.

Concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50µg/ml

The plasmid concentration was calculated from the DNA concentration using the following equation

$$n = \frac{cDNA\ x\ N_A}{l_{DNA}\ x\ M_{bp}}$$

c_{DNA} [ng µl^{-1}] = DNA Concentration

l_{DNA} = length of DNA fragment in base pairs

N_A = Avogadro constant (6.022 × 10^{23})

M_{bp} = average weight of a double-stranded base pair (660 g mol^{-1} = 6.6 × 10^{11} ng mol^{-1})

2.5.3 Real Time PCR data analysis

Qualitative Data analysis

Due to limited sample being available a single extraction was carried out on each DBS. The resulting extract was run in triplicate, DBS were counted positive if a positive sigmoid curve signal was seen in the real time PCR assay. Qualitative data from triplicate testing of DBS were analysed as follows; DBS were counted positive if ≥2 of the triplicates gave a positive

signal in the PCR assay. For samples that were confirmed with the UL69 PCR assay the sample was counted positive if ≥2 wells were positive in both the initial screening gB assay and UL69 assay. For any discrepant results the sample was repeated (if sufficient DBS was available) if the same results were found then the UL69 result was taken as the final result (figure 2.3)

Quantitative Data Analysis

In the quantitative data analysis, detected CMV viral load and mean loads were calculated from the amount of blood contained in the area of card tested. CMV viral loads were converted into CMV genomes per ml of original blood sample. Negative (undetected) samples were assigned the minimum detected load (100 copies).

CMV loads were also reported in genomes/10^6 cells. CMV loads were normalised against a β globin standard. The total number of cells detected was determined and a ratio calculated for normalisation. The resulting ratio was then used to convert the number of CMV genomes to the number of CMV genomes/10^6 cells using the following equation.

$$n = \frac{Number\ of\ cells}{10^6\ cells}\ x\ CMV\ genome\ detected$$

n = CMV genomes/10^6 cells

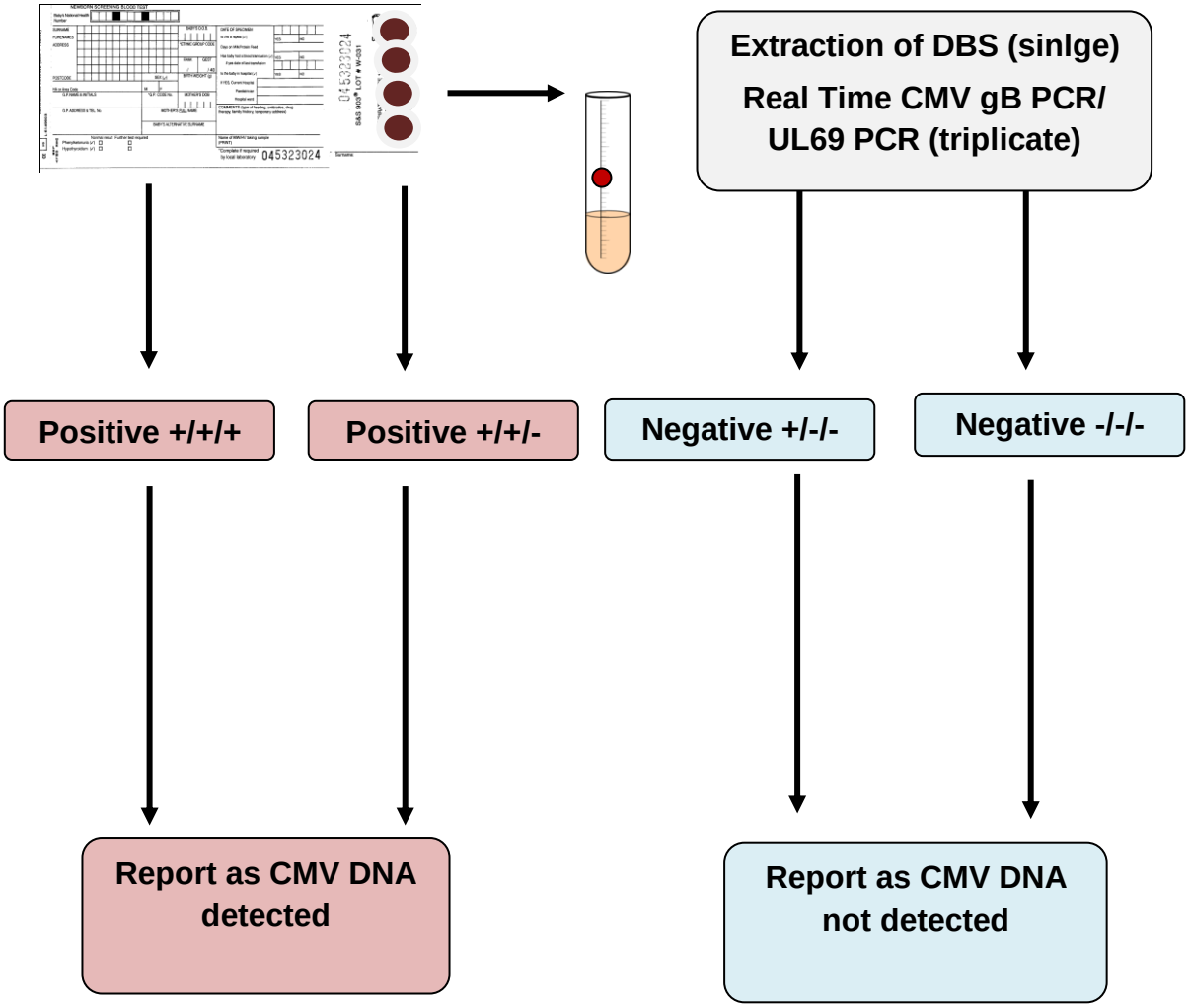


Figure 2-3: ***Flow diagram for interpretation of triplicate testing of DBS results***

DBS were counted positive if ≥2 of the triplicates gave a positive signal in the PCR assay. For any discrepant results the sample was repeated (if sufficient DBS was available) if the same results were found then the UL69 result was taken as the final result.

2.6 Statistical analysis

Each chapter contains details of the statistical analysis performed.

STATA SE version 13.0 and GraphPad Prism version 5 was used for statistical analysis. These included Wilcoxon signed rank test to compare two related values, Student's T test to compare independent values, one way ANOVA (Kruskal-Wallis) to determine differences between groups. Linear regression and Spearman's rank correlation was used to analyse relationship between two variables.

Generalised estimating equations were used to examine associations

And Cox proportional hazard regression was used to identify correlates. All generalised estimating equations models used robust standard errors and exchangeable correlation matrices.

Receiver operator characteristic were used to examine sensitivity/ specificity and predict cut off thresholds.

Logistic regression was used to model relationships between DBS CMV viral load and SNHL and to predict the probability of CMV transmission by 1 year at different maternal baseline CD4 counts and 2-week breast milk CMV DNA levels.

Unless stated CMV and HIV viral loads were $\log_{10}$-transformed to normalise distribution GraphPad Prism version 5 was used for plotting graphs

Chapter 3

3 Evaluation of dried blood spots for the retrospective diagnosis of congenital CMV

3.1 Introduction

The gold standard for diagnosis of congenital cytomegalovirus infection is virus isolation from urine or saliva before the first 3 weeks of life (Lazzarotto, Varani et al., 1999;Stagno, 2001). Recently, CMV DNA detection by polymerase chain reaction (PCR) has become the method of choice for many diagnostic laboratories. This newer molecular approach has many benefits over virus isolation including high sensitivity and specificity, no requirement for cell culture, no need for viable virus to be present in the specimen and reduced labour and turnaround times.

Early postpartum and perinatal acquisition of CMV is common and infection arises via contact with maternal secretions or ingestion of breast milk. Although mainly asymptomatic, postpartum and perinatal infection with CMV can produce a variety of clinical symptoms. These symptoms appear to be transient and to have no effect on neonatal outcome (Vollmer, Seibold-Weiger et al., 2004). Therefore it is essential to distinguish between CCMV and perinatal infection to ensure long term follow-up of CCMV infected infants.

Only 10% of babies congenitally infected with CMV will be symptomatic at birth, therefore the majority of infected babies will be asymptomatic, have no diagnostic investigations performed and no samples stored for future analysis. Due to the possible early acquisition of virus, samples taken after 21 days of life will not be able to differentiate between perinatal infection and congenitally acquired CMV. Therefore, to determine if late onset sequelae such as SNHL and developmental delay identified in late infancy are due to CCMV or other

factors such as an underlying genetic predisposition is a major problem, unless early samples are available.

Guthrie cards or DBS are taken routinely as part of the newborn screening programme 5–8 days after birth from every baby born in the United Kingdom. The concept that capillary blood, obtained from pricking the heel or finger and blotted onto filter paper, could be used for early identification of disease to facilitate the pre-symptomatic treatment of congenital diseases in newborns led to mass neonatal screening for metabolic diseases being introduced in Scotland by Robert Guthrie in 1963, with national screening for phenylketonuria beginning in 1969-70. All babies in England are screened for phenylketonuria, congenital hypothyroidism, sickle cell disease, cystic fibrosis and medium-chain acyl-CoA dehydrogenase deficiency (MCADD). In the USA, 28 diseases are tested for using DBS depending on the State (MMWR Weekly, 2012)

In 1994, Shibata *et al* were the first to report that CMV DNA could be detected from DBS using PCR (Shibata, Takano et al., 1994). In 2000 Barbi et al compared detection of CMV DNA from DBS using PCR with virus isolation for the diagnosis of CCMV and found 100% concordance between the two methods. (Barbi, Binda et al., 2000) These early results and the timing of the newborn DBS suggested that DBS could be used to retrospectively confirm congenital CMV in children who present with unexplained SNHL and/or neurological damage consistent with CCMV.

DBS samples are, by definition, small-volume collections with each spot holding a maximum of 80ul of whole blood on a standard Whatman 903

screening card (Whatman, GE Healthcare) The sensitivity of CMV DNA detection from DBS compared to detection of CMV by virus isolation in urine or saliva reported in the literature ranges from 28-100% depending on the methods used and the population tested (reviewed by Snijdewind *et al* (Snijdewind, van Kampen et al., 2012). The studies which showed the highest sensitivities for detection of CMV DNA from DBS were performed on children who were symptomatic at birth. (Barbi, Binda et al., 2000;Scanga, Chaing et al., 2006;Vauloup-Fellous, Ducroux et al., 2007) Lower sensitivities were reported in cohorts of children with asymptomatic as well as symptomatic cases. (Soetens, Vauloup-Fellous et al., 2008); (Yamamoto, Mussi-Pinhata et al., 2001;Boppana, Ross et al., 2010).

However, the use of DBS for retrospective diagnosis of CCMV has raised some critical questions. It has also been shown that sensitivity of DNA detection from DBS depends on methodology with DNA extraction, the amplification method used and the region of the CMV genome being detected (Soetens, Vauloup-Fellous et al., 2008);(Scanga, Chaing et al., 2006;Barbi, Binda et al., 2006); (de Vries, Claas et al., 2009) with the different methodologies and area of DBS tested making direct comparison between studies impossible. Also the stability of viral DNA within the DBS matrix is not known. Johansson *et al*; selected 32 infants for retrospective analysis of CMV DNA in DBS samples stored since birth. The PCR was performed 12-18 years after DBS collection and showed that CMV DNA can be detected in DBS up to 18 years old (Johansson, Jonsson et al., 1997),but these cards could be from children with the highest viral load at time of sampling and CMV DNA could still be detectable despite significant degradation.

The detection of CMV DNA may also be influenced by the DBS storage conditions, for example, storage for prolonged periods at room temperature in non-sterile conditions with potential cross contamination of cards stored adjacent to those from babies with congenital CMV been reported (Johansson, Jonsson et al., 1997)

To date, no studies have addressed the stability of CMV DNA in DBS or the potential for cross contamination between cards stored in the UK. The work in this chapter sought to address these important issues systematically.

3.2 Aims

Overall the work in this chapter uses an iterative approach to optimise CMV DNA detection from the DBS sample matrix. To achieve this I had the following aims

- Optimise the existing methodology for nucleic acid extraction and CMV detection from the DBS
- Investigate the stability of CMV DNA on DBS in long term storage and the possibility of cross contamination between stored DBS.

A major obstacle to developing and moving forward CCMV diagnosis is the lack of available samples from proven cases. At present DBS are only taken as part of routine neonatal care. This leaves a ‘catch 22’ situation, as a variety of neonatal samples including urine and saliva is required for full validation but no samples are available. Therefore my final aim was to

- Establish and maintain clinical collaborations to access DBS samples from children with confirmed CCMV or suspected CCMV to provide a basis for testing the methodology of DNA extraction, and real time PCR detection in a clinical cohort

3.3 Materials and methods

3.3.1 Dried Blood Spot analysis

Preparation of study DBS

Newborn screening standard Whatman 903 cards (Whatman, GE Healthcare Maidstone, England) were obtained from NHS supplies within the Royal Free Hospital, London, UK.

Test DBS were made from either CMV positive or negative anti-coagulated (sodium citrate) whole blood samples from solid organ transplant recipients whose viral loads were previously determined for diagnostic purposes by quantitative real time PCR in our laboratory (viral loads 15,259 and 10,265 genomes/ml respectively). These loads were chosen to represent viral loads obtained from neonates with CCMV in our laboratory prior to this study.

DBS were prepared by applying 50µl of whole blood onto the Whatman 903 card. The prepared cards were left to dry overnight and then stored at room temperature, touching each other to mimic normal UK storage conditions. DBS were subjected to DNA extraction and amplification by real time PCR every month for up to 24 months. A total of 9 sample sets were prepared: two positive DBS sets and seven negative DBS sets.

Initially, DBS were prepared from the positive blood (15,259 ge/ml) and three negative samples for a period of 16 months. These DBS were tested in triplicate for 16 consecutive months. After reviewing the results for 16 months of storage a second sample set was prepared to extend the study to 24 months. The second sample set was prepared from a CMV positive blood

with a lower viral load (10,265 ge/ml) and four CMV negative bloods. These DBS were tested in triplicate for 24 consecutive months.

Preparation of study DBS for determination of the limit of detection

CMV PCR positive whole blood samples (as described previously) with known viral loads were used to generate DBS of known CMV viral load. 12 DBS cards were produced in total with viral loads that ranged from 214 – 129,071 ge/ml. The resulting DBS were left to dry overnight before being stored under conditions to mimic UK Newborn Screening standard storage. After 1 month of storage DBS were subjected to DNA extraction and amplification by real time PCR.

Determining the number of CMV genomes per DBS

For analysis it is assumed that each DBS contains 0.38µl of whole blood per mm² (absorption of whole blood into Whatman 903® card) (QCMD, CMV DBS09 Instruction manual, QCMD, Glasgow, UK).

Therefore for the following studies the following equation is used to incorporate the area of DBS and therefore blood volume in the DBS analysis.

Ir[1000/Ar0.38]=SC

Where: *Ir* = Initial results (genomes/ml)

Ar = area of DBS used for extraction in mm²

SC = sample concentration in genomes/ml

The CMV input per semicircle of DBS was determined using the equation:

CMV load = ×/1000(Ar (0.38)

Where × = CMV viral load in genomes/ml, *Ar* = area of DBS in mm²

Whatman 903® cards are standardised to meet United States Federal Drug Agency and medical device regulations. The range of diameters quoted for each DBS is 15-17mm (Whatman Neonatal brochure 042009). For all experimental calculations a DBS diameter size of 16mm was assumed. This

corresponds to a total DBS area of 201.96 mm^2 or an area 100mm^2 per semicircle DBS.

Quality Control Molecular Diagnostic DBS proficiency panel

An external quality assurance panel of 9 DBS samples was distributed in 2007 by the QCMD (QCMD, Glasgow. UK) Seven samples were derived from whole blood, negative for CMV DNA and IgG antibody, and spiked with cultured *in vitro* CMV strain Towne in various concentrations or clinical material (7.3 x 10^2 – 9.6 ×10^5 genomes/ml). Two additional samples were CMV-negative whole blood. The panel was distributed to 33 European and South African laboratories.

The DBS samples were extracted and tested in triplicate, the DBS was counted positive if ≥2 of the triplicates gave a positive signal in the real time PCR assay.

British Paediatric Surveillance Unit: Congenital CMV study

The DBS of children reported via the British Paediatric Surveillance Unit (BPSU) notification system were retrieved from storage after obtaining the parents' consent. Children with diagnosed or suspected congenital CMV born in the UK between 2001 - 2002 were reported by paediatricians via the BPSU notification system (BPSU 17th Annual report, 2002 - 2003). CCMV cases were confirmed on the basis of PCR or virus isolation from urine, blood, saliva or tissue taken at biopsy within 3 weeks of birth. 'Possible' CCMV cases were infants with positive samples only after 3 weeks of age and symptoms compatible with CCMV which were not accounted for by any other diagnosis. Consent for DBS retrieval was obtained from the parents of 63 children, and 55 DBS were retrieved and analysed under code for the presence of CMV DNA. The cards had been stored in standard UK storage conditions and all were collected within the first 21 days of life.

DBS sample preparation

A semicircle of DBS was used for analysis. The area was measured and then cut with scissors to ensure consistency in sample input across all extraction methods. All DBS samples were then cut into 3 pieces for extraction.

Strict control measures to avoid cross contamination were applied.

Scissors were cleaned with 0.1M HCl prior to and after cutting each DBS to remove any possible DNA cross contamination.

Negative (blank) DBS were cut with the same scissors and processed alongside the DBS samples in every step of the process.

Disposable gloves were worn and changed after each DBS had been handled

DNA Extraction: Heat Shock Protocol

DNA was extracted from DBS using a protocol described by Barbi *et al; (Barbi, Binda et al., 2000)*. Blood was eluted from the DBS sample by incubating the semicircle of DBS at 4°C overnight in 25µl of minimum essential medium without supplement (Gibco, UK) and then by heating in a heating block the resulting supernatant and DBS mixture at 55°C for 60 min followed by 100°C for 7 min. The samples were then cooled on the bench to room temperature and centrifuged at 6708xg (10,000rpm) for 3 min; the resulting supernatant was removed from the DBS pieces and frozen at −80°C overnight. After thawing, 10µl of supernatant was used as input into the PCR.

DNA Extraction: Biomerieux Nuclisens EasyMag DBS protocol

As part of this study a protocol was developed for the extraction of total nucleic acid using the semi-automated Nuclisens EasyMag system (Biomerieux, Basingstoke. UK). A DBS pre-treatment lysis step was developed and added to the automated methodology. Briefly, blood was eluted from the DBS in 2ml EasyMag lysis buffer with the addition of 20µl proteinase K (>600 units/ml) (Qiagen, Hilden, Germany). The mixture was

incubated at room temperature for 30 minutes on a plate rocker. The total resulting supernatant was removed from the DBS pieces using a pastette and used as sample input into the EasyMag system.

The Nuclisens EasyMag generic 1.0 protocol was used for extraction with a final elution that generated 60µl of total nucleic acid extract.

PCR for C Reactive Protein Gene

To confirm the CMV negative results were not due to inhibition of the PCR (a false negative result) a real time PCR was performed for a human gene. A positive signal in the PCR would confirm both the presence of amplifiable human DNA and the absence of inhibition. The PCR was modified from Wardinger et al 2000. (Wandinger, Jabs et al., 2000) A 101bp region was amplified that spanned the first exon and part of the intron within the C reactive protein (CRP) gene.

Amplification was carried out using the following primers and probe
CRP forward primer: 5'CTTGACCAGCCTCTCTCATGC 3'
reverse primer 5'TGCAGTCTTAGACCCCACCC3'.
Probe 5' FAM-TTTGGCCAGACAGGTAAGGGCCACC-TAMRA

Primers were purchased from Invitrogen UK and the probe was purchased from Applied Biosystems UK. The PCR was carried out in a volume of 50ul; which contained 25µl TaqMan universal mastemix, 20nM of each primer and 100nM of probe. Cycling conditions were 10 minutes at 95°C followed by 45

cycles of 95°C for 15 seconds and 60°C for 1 minute. The resulting fluorescence was measured in real time using the ABI TaqMan 7000 sequence detection system software version 1.3 (Applied Biosystems, UK).

3.3.2 Statistical analysis

GraphPad prism 5 was used for statistical analysis.

To compare the genome number of CMV DNA detected from a DBS with the equivalent volume of whole blood a Wilcoxon signed rank test was used.

Linear regression analysis was performed to investigate the relationship between the amount of CMV DNA detected from a DBS and the CMV viral load in the whole blood sample.

For the stability study DBS samples were split into groups according to the age of DBS at the time of analysis, to provide similar and adequate numbers for reasonable comparison. A one-way ANOVA (Kruskal–Wallis) was used to analyse differences between groups. Linear regression was used to analyse trends in viral load with age of DBS.

The mean cycle threshold values for the CMV positive DBS samples were compared using a Wilcoxon signed rank test.

3.4 Results

To validate the use of DBS for the retrospective diagnosis of CCMV, it was necessary to evaluate and improve the assay by testing known positive and negative controls repeatedly. This established the stability of CMV DNA in DBS over time and the risk of cross-contamination under standard UK storage

conditions. Furthermore it established the sensitivity and specificity of CMV PCR of DBS for retrospective diagnosis of CCMV.

3.4.1 Initial assay validation

DBS were obtained through the BPSU: congenital CMV study. A total of 55 DBS were retrieved as part of this study, 31 from children with confirmed CCMV infection and 24 from children with possible CCMV infection.

Nucleic acid from all 55 DBS was extracted using the QiaAmp DNA mini kit DBS protocol and tested in triplicate using the gB real time PCR assay.

A sample was considered positive if a signal in ≥2 of the triplicates gave a positive signal above a fixed fluorescent threshold (set above baseline) in the DBS assay.

Initial results with a 5µl nucleic acid extract input into the PCR gave a positive result in 18/55 giving an overall detection of 32.7%. When this was analysed into possible and confirmed CCMV infection, 17 samples from the confirmed CCMV group (55%) and 1 possible CCMV infection (4%) gave a positive result.

Increasing the input to 10µl, 27/55 (49%) samples tested positive for CMV DNA, corresponding to an increase in overall detection of 16.3%. When this was analysed into possible and confirmed CCMV infection, 23 samples from confirmed CCMV (74%) group and 4 possible CCMV infections (17%) gave a positive result. This corresponded to an overall increase in detection of 19% in the confirmed CCMV group and 13% in the possible CCMV group.

The sensitivity of the test for the detection of CMV DNA from a DBS was found to be 74.2% (95% CI: 55.38% - 88.11%) with a 10ul input.

When the viral loads were analysed the additional CCMV cases identified corresponded to the DBS with the lowest CMV viral load (CMV viral load range 124 – 241 genomes per DBS semicircle).

When the input was increased to 15µl no extra positive signals were observed suggesting that the limit of detection for the assay had been achieved.

A 10µl sample input was chosen for future work.

CRP assay

All DBS samples tested had a detectable signal for the CRP gene, thereby validating both the DBS sample and extraction protocol for removing inhibitors.

3.4.2 Sensitivity of CMV DNA detection from DBS

The CMV viral load (genomes/ml) was calculated for both the DBS sample and the equivalent volume of whole blood. Samples which showed a CMV viral load >100 genomes/ml were classed as positive (theoretical limit of detection of the PCR assay) as this equates to 1 genome per reaction in the PCR. The lowest CMV viral load tested which gave a positive signal in the DBS assay was 1,702 ge/ml. The relationship between whole blood viral load and the detection of CMV DNA from the DBS sample matrix was further investigated. Assuming that each DBS contains 0.38µl of whole blood per mm² (absorption of whole blood into Whatman 903® card) (QCMD, CMV

DBS09 Instruction manual, QCMD, Glasgow, UK). When the results were compared a significant difference was seen between the whole blood and DBS viral load (p= 0.001) (See table 3-1) with the mean viral load recovered in the DBS sample lower than the whole blood viral load. On further investigation a linear relationship was found between the CMV viral load recovered from a DBS and that found in whole blood (R^2 =0.904) (See figure 3-2) and all DBS samples with a CMV viral load < 3 $\log_{10}$ were negative in the gB PCR assay.

From this data set a DBS with a viral load of 1,702 ge/ml of whole blood was required to give a positive result from a semicircle of DBS in the CMV DBS assay.

Table 3-1: Comparison of CMV viral loads in paired whole blood and 'test' DBS samples.

The table shows the CMV viral load in the whole blood sample used to make the 'test' dried blood spots (DBS). The resulting mean CMV viral load obtained from triplicate testing of the test DBS, log_{10} genome/ml difference between the two results obtained and standard deviation.

Whole blood CMV viral load (genomes/ml)	Mean CMV viral load obtained in DBS assay (genomes/ml)	Log_{10} difference between mean viral load results (genomes/ml) ±SD
214	<100*	-
398	<100*	-
863	<100*	-
893	<100*	-
1702	874	0.40 ±0.21
2026	957.6	0.72±0.36
2554	877.8	0.73±0.24
3601	1900.6	0.80±0.18
15904	5498.6	0.34±0.15
31921	14826	0.61±0.07
72619	15215.2	0.84±0.14
129071	38965.2	0.62±0.09

*Samples which showed no amplifaction signal were assigned the theoretical detection limit of the PCR

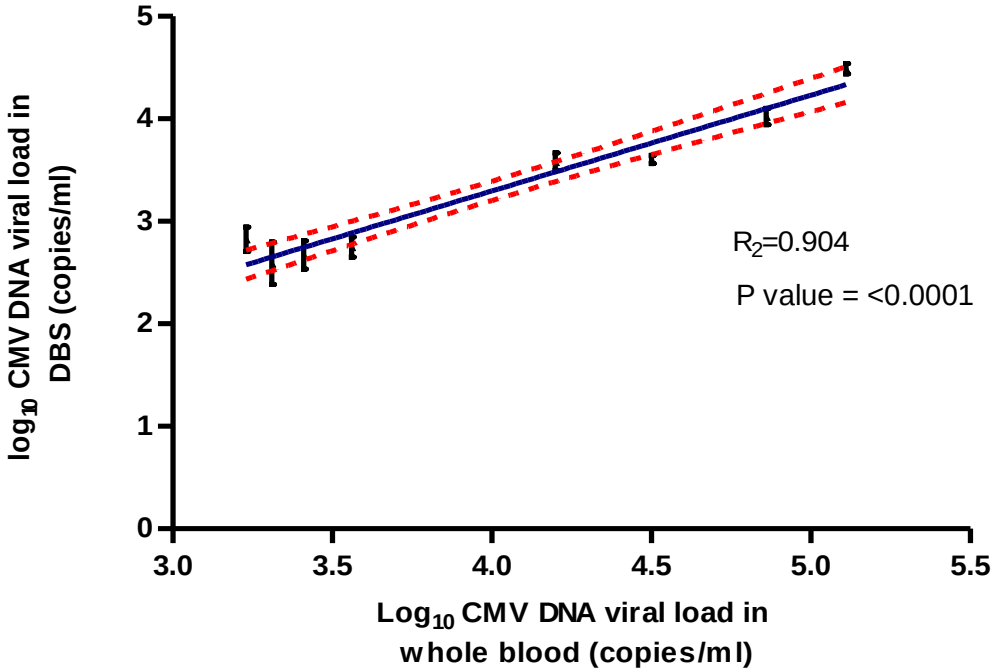


Figure 3-1: Correlation between CMV viral loads obtained from whole blood and paired DBS samples

Mean CMV viral load results were obtained from paired whole blood and dried blood spot (DBS) samples (triplicate testing). A linear relationship was seen with the amount of CMV recovered from a DBS increasing with the whole blood CMV viral load (R^2 = 0.904). Data were analysed using linear regression. The line of best fit (blue) and 95% confidence band (red) are shown. R^2 and P values were determined in Graphpad Prism.

3.4.3 Stability of CMV DNA in DBS over time

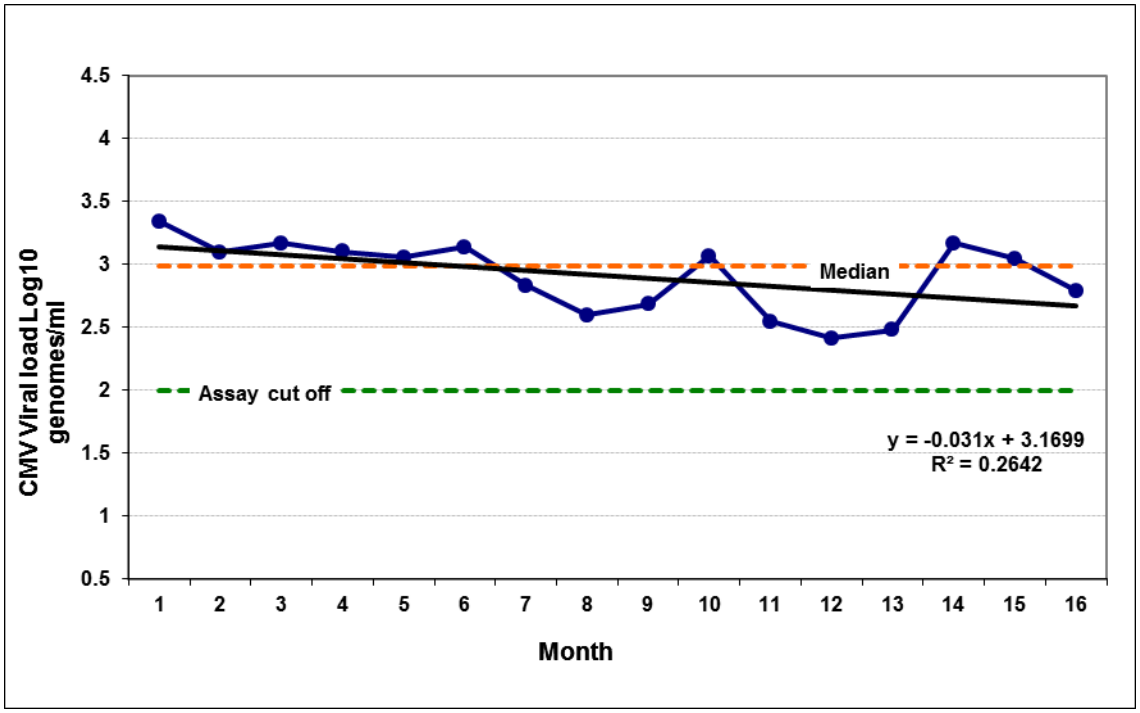
To investigate the stability of CMV DNA on DBS, CMV DNA positive and negative study cards were produced from whole blood samples from adult solid organ transplant patients with or without CMV viraemia. Two DBS sample sets from whole blood samples with CMV viral loads 15,259 ge/ml and 10,265 ge/ml were used. A total of seven negative DBS sample sets were tested in parallel over the 24 month period.

Initially, 24 DBS were prepared from the 15,259 genomes/ml positive blood and three negative samples. These were tested in triplicate for 16 consecutive months. Initial results showed that CMV DNA was still detectable after 16 months of storage. (see figure 3-2a). Therefore, the study period was extended to 24 months. A second sample set was prepared from a CMV positive blood with a lower viral load (10,265 ge/ml) and four negative bloods.

CMV DNA was still detectable on the second positive DBS sample set after 24 months of storage despite a moderately low CMV viral load in the original whole blood sample used for initial card preparation (390/DBS input). On further analysis of viral load both positive cards showed a decline in viral load over the 24 month period. There was a significant difference between the median log viral load detected after 18-24 months of storage compared to 0-5 months (median log 2.3 vs 3.0 respectively; p=0.0007) (see figure 3-2b).

Linear regression was used to plot the slope of decline. This was used to predict the time point the DBS would become undetectable in the assay. It is predicted that the DBS CMV viral loads 15,259 ge/ml and 10,265 ge/ml would become undetectable after 35 months and 32 months of storage.

Analysis of the seven CMV negative blood test cards showed consistently negative results over the 24 month study period. No cross contamination between cards was seen despite being stored in close contact with CMV positive DBS giving 100% specificity for the study cards.



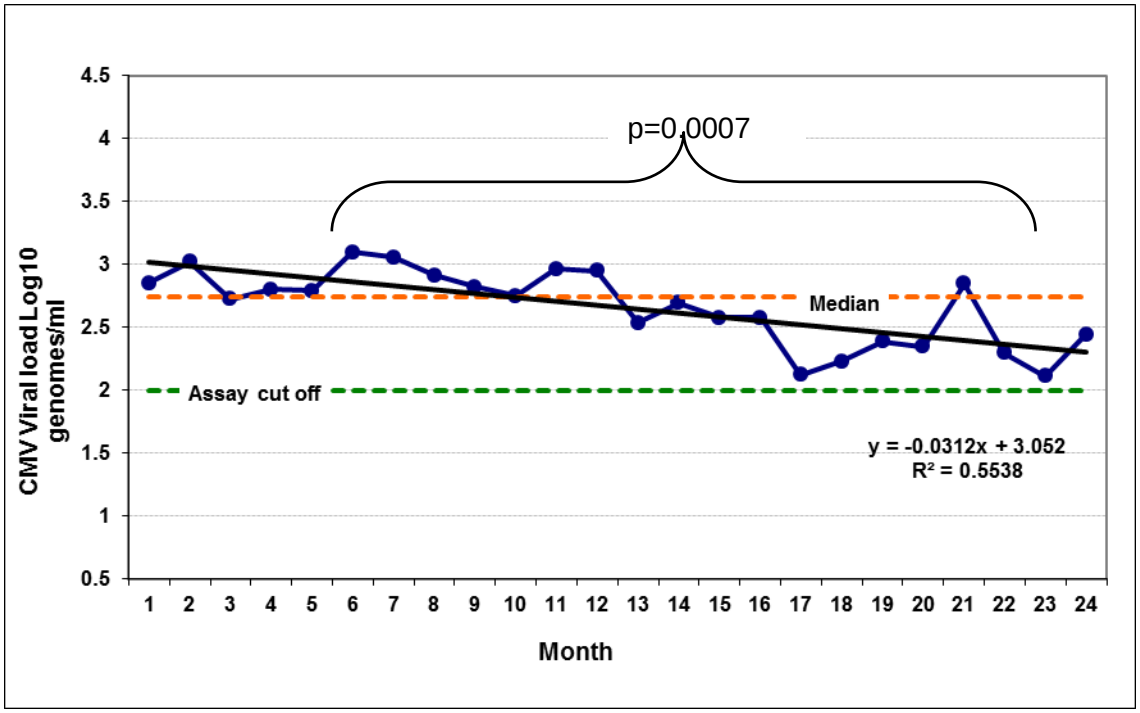


Figure 3-2: Log_{10} CMV viral load (genome/ml) in two simulated DBS over time in storage.

Depicted are the mean CMV DNA viral loads from triplicate testing over time. The median viral load detected (all samples) is shown as a dashed orange line and the assay cut off (100 genomes/ml) is shown as a dashed green line. There was a significant difference between the median log viral load detected after 18-24 months of storage compared to 0-5 months (median log 2.3 vs 3.0 respectively; p=0.0007).

3.4.4 Extraction optimisation and quality control

During the course of this study, a newer semi-automated extraction system (the EasyMag (Biomerieux, Basingstoke, UK) was validated for use in the diagnostic service of the laboratory. The system is based on a nucleic acid purification method developed by Boom *et al* 1990 (Boom, Sol et al., 1990) with enhanced magnetic silica technology. A full validation was performed for DBS extraction on the EasyMag system using the QCMD 2007 CMV DBS panel.

The DBS samples in the QCMD panel were used to evaluate three different extraction methods. The Qiagen mini blood DNA kit (original protocol), the EasyMag semi-automated extraction system and the heat shock protocol (method with reported 100% sensitivity).

The heat shock method was the most labour intensive protocol and gave the lowest recovery of CMV DNA (figure 3.3). On triplicate CMV testing it gave positive results in only 2 samples (29%) which corresponded to the highest viral loads ($9.6x10^5$ and $3.9x10^6$ genomes/ml).

Using the Qiagen Mini DNA extraction method three samples tested positive (43%). These corresponded to the three highest viral loads $8.8\ x10^4$, $9.6x10^5$ and $3.9x10^6$ ge/ml. When the DNA yields were quantified, the Qiagen kit recovered more CMV DNA than the heat shock method but less than the EasyMag protocol (see figure 3-4).

On triplicate testing the EasyMag extraction system showed the optimum extraction results with CMV DNA detected in 5 cards (71%). When the CMV yields were quantified, the Easymag recovered the highest quantity of CMV DNA among the 3 extraction methods tested (see figure 3-3).

All extracts when tested for CMV DNA by PCR failed to detect CMV DNA from the DBS with the lowest viral load (730 ge/ml). This corresponds to 27 genomes of CMV DNA per semicircle of DBS. The two negative DBS were reported negative in all three extraction protocols (see table 3-2). The EasyMag extraction protocol was adopted for use with DBS.

Table 3-2: Qualitative results of CMV DNA detection in the QCMD dried blood spot 2007 panel.

Dried blood spots (DBS) were tested using 3 different extraction methods, the expected results and the percentage of results reported back correctly to the QCMD from participating laboratories; Brackets () show the number of CMV positive wells per triplicate.

QCMD CMV DBS reported Viral load (c/ml)	Heat Shock Protocol	Qiagen Mini DNA Blood result	EasyMag result	Expected result (% correct qualitative results of all participants)
Negative	-	-	-	Negative (96)
Negative	-	-	-	Negative (96)
7.3×10^2	-	-	-	Positive (0)
7.3×10^2	-	-	-	Positive (7)
9.4×10^3	-	-	Positive (2/3)	Positive (48)
9.4×10^3	-	-	Positive (2/3)	Positive (52)
8.8×10^4	-	Positive (2/3)	Positive (3/3)	Positive (93)
9.6×10^5	Positive (3/3)	Positive (3/3)	Positive (3/3)	Positive (96)
3.9×10^6	Positive (3/3)	Positive (3/3)	Positive (3/3)	Positive (100)
Total number of positives correctly identified	2/7	3/7	5/7	

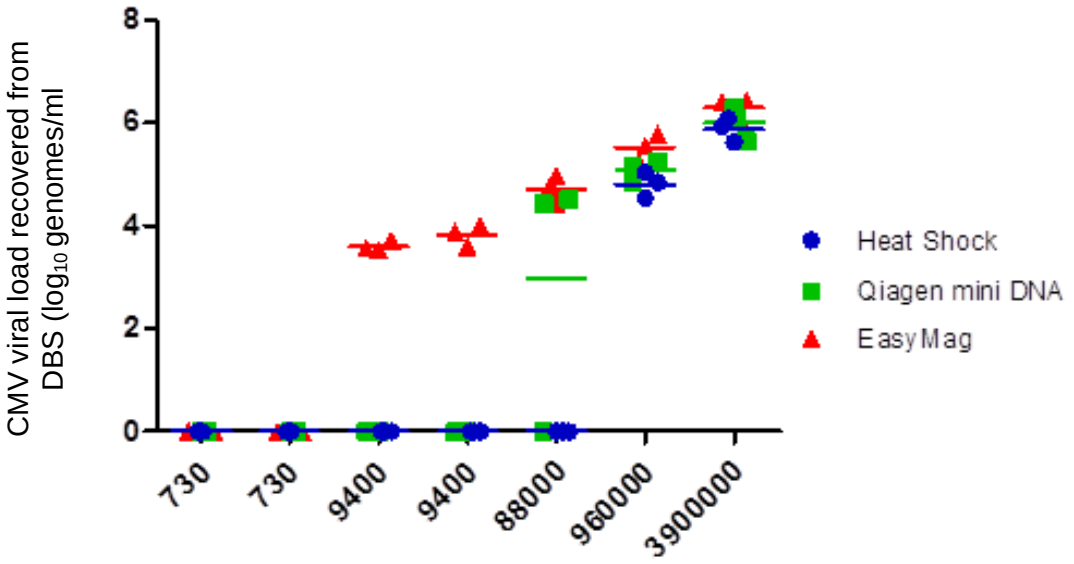


Table 3-3: The CMV viral loads detected from the 2007 QCMD CMV dried blood spot panel after extraction by either the heat shock protocol, Qiagen Mini DNA kit or the Biomerieux EasyMag semi-automated system.

DBS were tested in triplicate. Depicted are the CMV viral loads in genomes/ml obtained after adjusting for initial input (estimated to be 38µl of whole blood). Bars show the mean CMV DNA viral load of triplicate testing of each extraction protocol.

3.5 Discussion

The work in this chapter evaluated the use of DBS for retrospective diagnosis of CCMV. The existing methodology for nucleic acid extraction and detection of cytomegalovirus DNA from DBS was optimised to give the highest level of sensitivity.

To confirm the use of DBS for the retrospective diagnosis of CCMV, stability of CMV DNA on the DBS was investigated. In the standardised test, CMV DNA remained stable for 18 months and was still detectable for up to 2 years after preparation. This important observation provides the evidence for retrospective testing of DBS for CCMV inchildren up to 2 years of age. However a decline in DBS viral load was seen after 2 years of storage. These results show that CMV DNA is sufficiently stable to allow investigation of children who present with SNHL up to the age of 24 months. It is interesting to note that CMV DNA was still detectable in DBS samples received in the diagnostic virology laboratory in children up to 17 years of age (see 7.4.1). If the slope of decline in the linear regression analysis is forecast for the test DBS with the lowest viral load, the viral load would become undetectable after 32 months of storage.

Practically with CCMV DBS this cannot happen in all cases, because we would not be able to detect virus in children as old as 17 years without an extremely high viral load in the original sample. If a back calculation is performed using linear regression a CMV load of $>10^8$ ge/ml would be required. Reviewing blood (pre-treatment) CMV DNA viral load results received into the laboratory from congenitally infected neonates reveals that CMV loads in whole blood rarely are detected above 10^5ge/ml. It is therefore possible that CMV load in DBS declines initially and then remains stable at a lower viral load for a prolonged period of time. Long term studies could investigate this and the effect of different storage conditions on long term storage.

Cross contamination studies showed no contamination between positive and negative cards for the 24 month study period. Currently guidelines exist for the secure storage of newborn screening cards but not storage conditions. Informal enquiry has shown that most newborn screening laboratories store DBS in secure filing cabinets at room temperature. This important observation shows that a DBS testing positive for CMV is highly unlikely to be due to cross contamination during storage and validates the use of DBS for retrospective diagnosis of CCMV.

A Quality Control in Molecular Diagnostic DBS panel for CMV reported a false positivity of 9% across laboratories in 11% of datasets submitted (Barbi, MacKay et al., 2008). During my analyses no false positive results were seen and the three different extraction protocols reported no false positives with the QCMD 2007 DBS panel.

The results of this small study show that nucleic acid extraction methodology plays a critical role in the recovery of CMV DNA from the DBS. The three different extraction methods used in this study gave sensitivities of 29-71% in the QCMD samples. The most efficient was the EasyMag extraction method although the small size of this study did not have the power to confirm a statistical significant difference.

Optimal sensitivity was obtained when samples were tested in triplicate. Triplicate testing was shown in the initial validation on the BPSU samples to increase detection of CCMV by 9.7%. The sensitivity study showed a loss of CMV genomes recovered from the DBS when compared to the equivalent volume of whole blood and that DBS spotted with the lowest viral loads had the lowest detected loads reflecting the importance of CMV DNA concentration. Since this work was performed a study by De Vries *et al* in 2009 found significant differences between assay sensitivity dependent on extraction method with optimal sensitivity achieved when samples

were tested in triplicate supporting the critical role of the extraction methodology. (de Vries, Claas et al., 2009)

Sensitivity in the DBS was investigated by the use of DBS produced from positive whole blood with known CMV viral loads and cards received through the independent BPSU study. A viral load of 1,702 ge/ml of original whole blood was required to detect CMV DNA from a semicircle of DBS using the study protocol. This compares favourably with a recently published QCMD panel assessing DBS testing across numerous laboratories which found a 50% sensitivity threshold of 9.4×10^3 ge/ml (Barbi, MacKay et al., 2008). A study in 2007 reported a 95% sensitivity of 3.6 $\log_{10}$ genomes/ml (3,081 ge/ml) CMV DNA using the Qiagen Mini DNA kit and a whole DBS input. (Vauloup-Fellous, Ducroux et al., 2007) Potential variables between studies include the amount of DBS input, we have consistently used a semi-circle of DBS throughout these studies, as this amount is representative of the DBS that remains after newborn screening for metabolic diseases. This limited amount of sample and the CMV viral load of the neonate are likely to be a contributing factors in the cut off and sensitivity of the assay. However the sensitivity in the BPSU confirmed CCMV cohort was found to be 74%. The assay sensitivity will be addressed further by the development of a nested PCR in chapter 7.

Chapter 4

4 Retrospective diagnosis of congenital cytomegalovirus infection from dried blood spots in children with sensorineural hearing loss.

4.1 Introduction

Sensorineural hearing loss (SNHL) is permanent hearing loss as a result of damage to the hair cells within the cochlea or cranial nerve number VIII (or both). SNHL reduces the ability of the individual to hear sounds but also reduces the quality of the sound they hear. SNHL can be mild, moderate or severe and can result in total deafness.

CMV is the most common congenital virus infection, affecting about 0.2 to 2.2% of newborns every year depending on the population (Kenneson & Cannon, 2007;Kenneson & Cannon, 2007) with a worldwide average of 0.7% (Dollard, Grosse et al., 2007). Overall, approximately 12.7% of infected infants show severe clinical sequelae at birth including neurological damage with or without SNHL, visual impairment, mental retardation with or without SNHL (Dollard, Grosse et al., 2007). The majority (87.3%) of CCMV infected infants appear asymptomatic at birth but sequelae can develop months or years later affecting 13.5% overall (Dollard, Grosse et al., 2007). The most commonest of these is SNHL which affects 6-23% (Fowler & Boppana, 2006)

It is estimated that 5,000 babies born in the USA each year are destined to develop disease caused by CCMV. This makes CCMV the most common viral cause of SNHL and neurodevelopmental delay (Cannon & Davis, 2005;Cannon, 2009)

The prevalence of CCMV in the UK is thought to be comparable to that of the worldwide average of 0.7% with only one large study from the 1980s providing an estimate of 0.3%. (Peckham, Chin et al., 1983) Peckham *et al*, estimated that 12% of all cases of congenital bilateral SNHL were caused by CCMV in the UK (Peckham,

Stark et al., 1987). However these figures are likely to be an underestimate of the total burden of SNHL caused by CCMV as they report on bilateral severe hearing loss only.

Progressive SNHL with CCMV infection was first reported by Dahle *et al* in 1974 (Dahle, McCollister et al., 1974). A study by Fowler *et al* followed up a cohort of 307 children with asymptomatic congenital CMV and compared their hearing thresholds with 76 uninfected siblings and a cohort of children whose neonatal screen for CMV was negative. In the asymptomatic group 7.2% had SNHL and half of these infants had further deterioration of hearing at 18 months. Importantly 18% of these children had delayed onset SNHL detected at a median age of 27 months. Children with congenital CMV had no other risk factors for SNHL. Fluctuation of hearing thresholds was seen in 23%, and high frequency SNHL in 32% (Fowler, McCollister et al., 1997). In another study by Johansson *et al* SNHL was identified at birth in 5.2% of children identified with CCMV, but by 72 months the incidence of SNHL was 15.4% (Johansson, Jonsson et al., 1997). Overall in developed countries CCMV accounts for 21% of hearing loss at birth and 24% of hearing loss 4 years of age (Morton & Nance, 2006;Grosse, Ross et al., 2008).

The clinically silent asymptomatic infection and progressive nature of SNHL in CCMV infection has proven to be a major obstacle to CCMV diagnosis. Newborn hearing screening may miss or underestimate hearing loss in these asymptomatic children who may develop SNHL, so the presence of symptoms at birth cannot be used as a guide to choose who to test for CCMV; instead, a routine screening programme for CCMV infection would be necessary.

Detection of viruria and viraemia have been associated with the presence of SNHL in CCMV. In one study urine CMV titres during infancy were associated with the development of SNHL (Rivera, Boppana et al., 2002) and in a later study both urine and peripheral blood titres were associated with symptomatic CCMV infection (Boppana, Fowler et al., 2005). Viraemic infants were also found to be more likely to have SNHL by Bradford et al, on both study enrolment and six month follow up (Bradford, Cloud et al., 2005). Level of viraemia has also been associated with the presence of sequelae in CCMV, with different viral loads correlated to different risk of sequelae. In one study a low level DNAemia (<1000 genome copies/ 10^5 polymorphonuclear leukocyes) was highly predictive of absence of sequelae with a negative predictive value of 95% for sequelae at age 12 months with 19/20 infants with low level DNAemia having normal neurological outcomes(Lanari, Lazzarotto et al., 2006). However a later study by Ross et al could not confirm the association between systemic virus burden and SNHL in CCMV, but did suggest that asymptomatic children with a peripheral blood viral load of ≤3500 ge/mL appeared to be at lower risk for SNHL (Ross, Novak et al., 2009).

The majority of CCMV related SNHL occurs before the age of 3 years coinciding with speech and language development. Retrospective testing of DBS could provide a diagnosis for SNHL. This prompt recognition could lead to early intervention such as cochlear implants which would be expected to reduce the impact of hearing loss on speech, language and social development (Yoshida, Kanda et al., 2009). Thus, early diagnosis could allow interventions to compensate for hearing loss. This is very important as it could prevent further deterioration in hearing and language skills which in turn could mean the difference between the child being moderately deaf and profoundly deaf with implications for schooling and social development. It is also

possible that valganciclovir could be given at this age and a randomised placebo controlled trial to study this is in preparation (NCT01649869).

4.2 Aims

Overall, the goal of this chapter was to develop the laboratory methods to allow a testing programme to be established in the UK for the retrospective diagnosis of CCMV in older children presenting with SNHL. The following aims were addressed:

- To investigate the use of DBS for the retrospective diagnosis of CCMV in a clinical cohort of children with SNHL.

- To investigate the relationship between CMV viral load on DBS and severity of SNHL in CCMV.

- To determine whether the non-linear 'threshold' relationship demonstrated in transplant and AIDS patients. (Cope, Sweny et al., 1997;Cope, Sabin et al., 1997;Emery, Cope et al., 1999) is present in children with CCMV and SNHL.

- To establish and validate a new diagnostic approach for the use of DBS for retrospective diagnosis of CCMV by developing a diagnostic algorithm for investigating congenital CMV infection as a cause of sensorineural hearing impairment.

To address these aims a clinical collaboration was developed with an audiovestibular physician, Dr Simone Walter. An independent ethically approved study CHIC was

established to look at the relationship between CCMV and SNHL. The study was conducted over 14 months and recruited patients from 5 sites (Royal Ear Nose and Throat Hospital, London, Bradford Teaching Hospitals NHS Trust, Queen's Medical Centre Nottingham, Milton Keynes General NHS Trust and Northampton General NHS Trust) the results were published in 2008 (Walter, Atkinson et al., 2008). I developed the assays and performed all the virological testing while the clinical work and audiological assessments were performed by Dr S Walter. I was blind to the audiology results at time of testing the DBS and Dr Walter was blind to the DBS results until the clinical and virology results were completed.

4.3 Material and methods

4.3.1 CMV in hearing impaired children study Study Population

There were two groups of eligible children aged 0 -18years:

- Children known to have congenital CMV, with or without SNHL
- Children with unexplained SNHL

Identification of Eligible Participants

From April 2005 to June 2006 potentially eligible participants were identified by informing Consultant audiological physicians (community and hospital-based), Consultant paediatricians, neonatologists, virologists, and fetal medicine Consultants of the study and asking them to report any children with CCMV or unexplained SNHL. Signed informed consent was obtained from the parents/guardians to allow retriveal and testing of the child's DBS for CMV DNA and data collection from the child's notes. DBS were retrieved from 39 children with confirmed congenital CMV and 35 children with sensiorneural hearing loss of unknown cause. Exclusion criteria consisted of having a known cause of SNHL, being born in a country without newborn DBS screening and, for the unexplained SNHL group, having another cause of SNHL strongly suspected from their history or examination.

Case definitions

Cases were confirmed on the basis of PCR or virus isolation from urine, blood, saliva or tissue taken at biopsy within 3 weeks of birth. ‘Symptomatic congenital CMV’ was defined as CMV excretion within the first three weeks plus any of the following: petechiae, hepatosplenomegaly, jaundice with conjugated hyperbilirubinaemia,

microcephaly, seizures, chorioretinitis. Asymptomatic cases were infants with positive samples taken within 3 weeks of life, but with no clinical disease.

SNHL was defined as air conduction thresholds >20 decibel hearing loss (dBHL) for pure tones, or >30 dBnHL for clicks, tone pips or bursts on auditory brainstem response testing (ABR) when middle ear function was normal, and as bone conduction thresholds >20 dBHL for pure tones, or >30 dBnHL for clicks, tone pips or bursts on ABR when middle ear function was abnormal.

DBS analysis

A semiciricle of DBS was extracted using the QiaAmp DNA blood mini Kit (Qiagen, Hilden Germany). Standard control measures to avoid cross contamination were applied. Scissors were cleaned with 0.1M HCl prior to and after cutting each DBS. Negative (blank) DBS were included as an extraction control and subjected to every step of the process to ensure no contamination occurred.

The resulting nucleic acid extract was tested using an in-house real-time TaqMan PCR assay detecting a highly conserved region of glycoprotein B (as described in Chapter 2).

4.3.2 Development of CMV UL69 PCR

To confirm the detection of CMV DNA from the DBS. a real time PCR to amplify a second region of the CMV genome was developed; the UL69 region. The PCR serves a dual purpose; to confirm the presence of CMV DNA on the DBS and to validate the retrospective diagnosis of CCMV in a child with compatible symptoms.

The UL69 region was chosen as it was expected to be highly conserved and no amplification of this region had been performed previously; therefore limiting the possibility of contamination from amplified PCR product in the confirmatory assay.

Primer and probe design

The real time PCR was designed to amplify a conserved sequence in the CMV polymerase (UL69) gene. The primers and probe were designed with PRIMER EXPRESS software (version 1.5, Applied Biosystems, Foster City, CA). The UL69 sequence data was obtained from the GeneBank sequence database for the Merlin strain of CMV (Accession number AY446894). Primers were aligned to UL69 sequences (n=4) published in GeneBank to ensure that the selected primer and probe combinations were in conserved regions of the gene.

The primer and probe sequences were (5' TO 3')

UL69 Forward primer: CTGTCACACGACGAGCTCATG 3'

UL69 Reverse Primer: TTCCTCCAGCCAATCGAACT

TaqMan dual labelled UL69 Probe:

FAM-ACACCGACTACCTGTTGCACATCCGTCA- TAMRA

Primers were purchased from Invitrogen, Carlsbad, CA,USA and probe was purchased from Applied Biosystems.

Optimisation of assay condition

The real time PCR assay was developed and evaluated on ABI PRISM 7500 with Sequence Detection System software Version 1.3 (Applied Biosystems).

Primer and probe concentrations were optimised by a chessboard titration. Serial dilutions of each primer and probe (50nM – 900nM) were used to determine the combination of primer and probe concentrations that gave the minimum threshold cycle (Ct).

The PCR cycling conditions were optimised using a known copy number laboratory strain of CMV (Ad169). A serial dilution was run from $1x10^7$ to $1x10^2$ ge/ml. The PCR was performed at different extension temperatures between 55°C and 60°C to find the amplification conditions that gave the minimum reproducible Ct value with the PCR amplification efficiency closest to 100%. Amplification plots were visualised and calibration curves were constructed using ABI 7500 system SDS Software version 1.2

Amplification efficiency (*E*) was determined using the slope of the standard curve using the following formula:

E = 10^(-1/slope)

This was converted into a % amplification efficiency using the following formula

E %= (10^(-1/slope)-1)*100

Linear regression was used to determine the slope of best fit for the CMV Ad169 standard curve.

The PCR cycling conditions were 95 °C for 10 min (Taq Activation), followed by 50 cycles at 95°C for 15 s, and at 55°C for 1 min. The total PCR volume was 25 µl, which contained 600 nM of each primer, 400 nM probe, 12.5µl TaqMan Universal Master Mix (Applied Biosystems, Warrington UK) and 5ul sample input.

Sensitivity and specificity of PCR

Primer sequences were compared with sequences on BLAST GenBank to avoid the likelihood of non-specific amplification. 30 CMV negative control samples were analysed. The controls were produced from viral clinical isolates (Herpes simplex virus 1 & 2, VZV, Epstein Barr virus and Human herpes virus 6) and were extracted using the EasyMag semi-automated extraction system prior to analysis.

Tenfold serial dilutions of CMV (Ad169) were used to determine the assay sensitivity. Standard calibration curves were generated using ABI 7500 system SDS Software version 1.2.

UL69 Assay Validation

The UL69 PCR was validated against the QCMD 2007 CMV DBS quality assurance panel. The panel consisted of nine DBS samples in total. Seven samples were derived from whole blood, negative for CMV DNA and antibody, and spiked with cell-grown CMV strain Towne in various concentrations or clinical material (7.3×10^2 – 9.6×10^5 ge/ml). Two additional samples were CMV-negative whole blood.

Total nucleic acid was extracted from the DBS using the semi-automated Nuclisens EasyMag system (as previously described in chapter 3). Briefly, blood was eluted from the DBS in EasyMag lysis buffer with the addition of proteinase K. The mixture

was incubated at room temperature for 30 minutes on a plate rocker. The total resulting supernatant was used as sample input into the EasyMag system with a final elution volume of 60µl.

The resulting DBS extract was tested in triplicate in both the real time gB and UL69 PCR, the DBS was counted positive if ≥2 of the triplicates gave a positive signal in the real time PCR assay. The cycle threshold values of the CMV positive samples were compared.

4.3.3 Statistical analysis

The mean cycle threshold values of the CMV positive samples were compared using a Wilcoxon signed rank test.

The mean $\log_{10}$ viral load in the children with confirmed CCMV was compared to the children with normal hearing using a student's t test (not assuming equal variances).

The mean $\log_{10}$ viral load in children with bilateral SNHL was compared to those without using a student's t test (not assuming equal variances).

The relationship between $\log_{10}$ DBS viral load and SNHL the best and worst ear average hearing thresholds was investigated using a Spearman's rank correlation

Other confounding variables associated with SNHL were examined using Fisher's exact or Wilcoxon signed rank tests of significance as appropriate. Significantly associated factors (p=>0.05) were entered into a logistic regression model. Step wise logistic regression was performed for the predictive variables against increased risk of SNHL.

A four parameter logistic regression model was used to model the relationship between DBS CMV viral load and SNHL. The equation for the model was as follows

$y=A*C/A+(C-A)*exp(-B*x))+D$

Computations were performed using Regress+ available at

http://www.causascientia.org/software/Regress_plus.html

4.4 Results

4.4.1 Sensitivity of dried blood spots for retrospective diagnosis of congenital CMV

84 children took part in the study; the DBS was retrieved for 74 children (88%) in total. 52 participants were female and 22 male. The median age at DBS testing was 7 years (see figure 4-2). Of these 39 DBS were received from children with confirmed CCMV infection 5 of which had received ganciclovir therapy. In the remaining 34 untreated children nine had normal hearing and 25 had SNHL. 28 of the 39 DBS from children with confirmed CCMV infection tested positive (71.8%).DBS were received from 35 children with sensorineural hearing loss of unknown cause, 8 tested positive for CMV DNA (23%) consistent with a diagnosis of CCMV (see figure 4.1).

One false positive low level result was identified as part of the CHIC study. The false positive was identified following clinical observation that urine PCR and serum IgG from this child were negative. Following this observation a confirmatory CMV PCR was developed to detect a different region of the CMV genome to the screening gB assay. The PCR was designed to amplify the UL69 region of the CMV genome. All samples which initially tested positive in the gB assay were re-extracted and tested with the UL69 assay to confirm positive results.

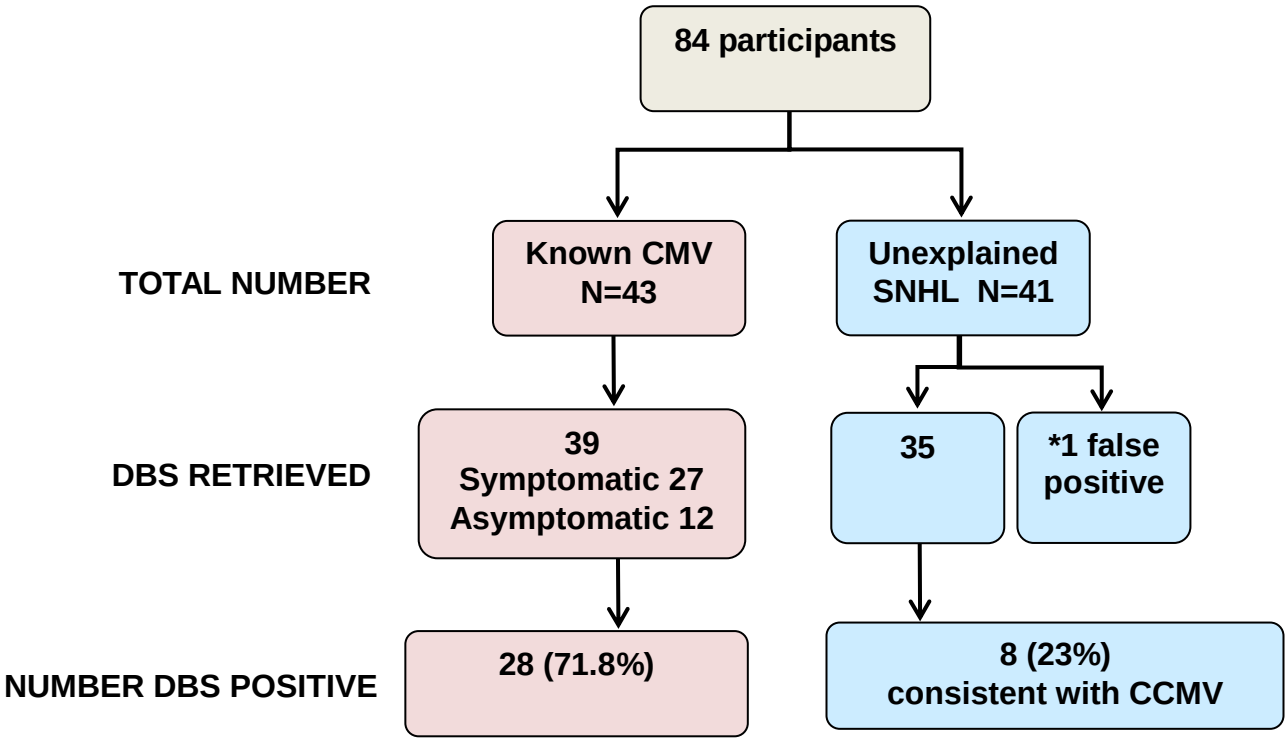
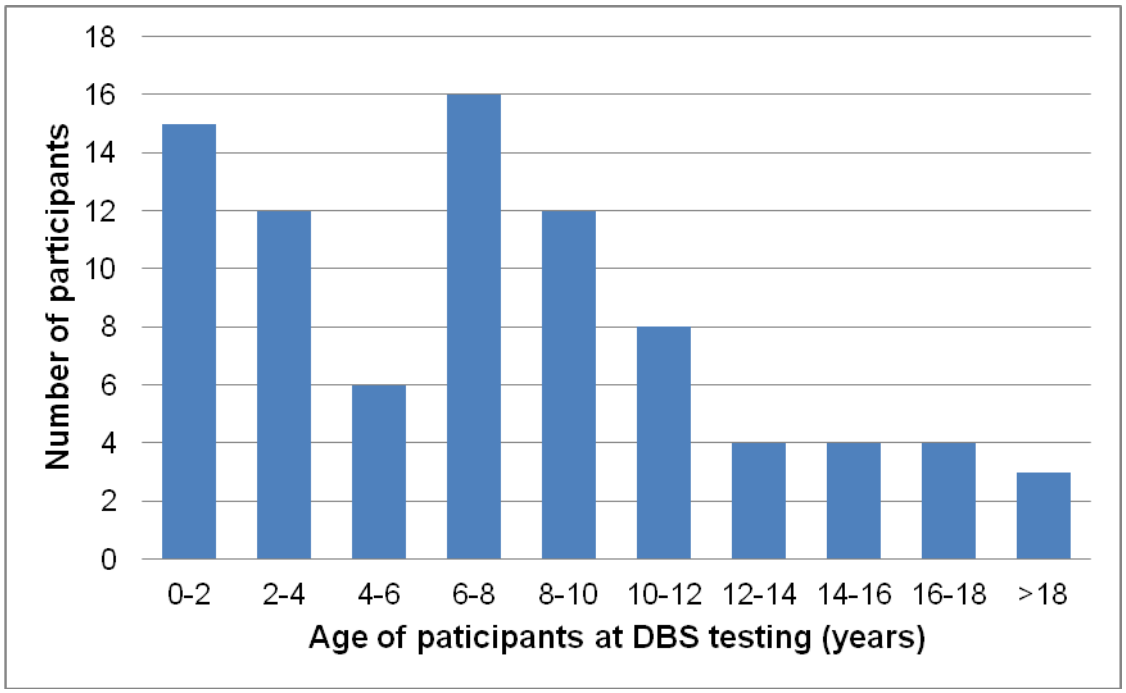


Figure 4-1: Flow chart of the dried blood spot samples received as part of CHIC study.

The number of DBS retrieved and percentage positive in each study group is shown.

* one false positive DBS result was later identified

A)



B)

Participant	Gender	Age (years)
1	Male	6.3
2	Male	10.4
3	Female	8.27
4	Female	9.25
5	Female	8.85
6	Female	0.69
7	Female	7.69
8	Male	2.72

Figure 4-2:The age of participants at time of DBS testing in the CHIC study

A) shows the number of participants and their age in years for the CHIC study (grouped into 2 year time periods).**B)** shows the age and gender of the unexplained SNHL DBS positive children.

4.4.2 Relationship between CMV viral load and hearing loss

The relationship between the CMV viral load measured in a DBS and the hearing loss was investigated. The CMV viral load per semicircle of DBS was calculated for the DBS retrieved from the CHIC study. The log DBS viral loads showed normal distribution, however hearing thresholds were not with most children being classified as normally hearing or bilaterally profoundly deaf (consistent with longitdudinal audiological data from (Dahle, Fowler et al., 2000).

In the 39 children known to have had CCMV 5 had been treated with ganciclovir so were excluded from the analysis. Of the remaining 34 children, 9 had normal hearing and 25 had SNHL. The mean log viral load was significantly higher in the 25 children with SNHL than in the 9 children with normal hearing (2.69 $\log_{10}$ genomes vs 1.64; p=0.01, 95% CI -1.84to -0.27). The mean log DBS CMV viral load was significantly higher in the 18 children with bilateral severe SNHL (>70dBHL) than in the children with milder hearing loss (2.84$\log_{10}$ genomes vs 1.93; p=0.01, 95% CI -1.6 to -0.2). When the DBS were categorised into groups based on increasing viral load a relationship was seen between the number of cards testing positive and the number of children having SNHL. When each group was expressed as a percentage of children with SNHL only 50 % of children had SNHL in the negative group compared to the viral load group >3 $\log_{10}$ genomes where 100% of children had SNHL (figure 4.3).Overall as the CMV viral load recovered from the DBS increased so did the percentage likelihood of the child having SNHL (see figure 4.3).

To examine the relationship between $\log_{10}$ DBS viral load and SNHL the best and worst ear average hearing thresholds were plotted against $\log_{10}$ DBS viral load. Negative DBS were assigned a value of 1 $\log_{10}$ for data analysis purposes. In the 34 children with SNHL there were significant positive Spearman's rank correlations

between pure tone average hearing thresholds and the CMV viral load measured in the DBS (r=0.445, p=0.008 (worst ear) and r=0.482, p=0.004 (best ear)). The scatterplot showed a non-linear relationship (R^2=0.51) with a sigmoid best fit curve suggesting a threshold effect, linking severity of SNHL with higher CMV viral load (see figure 4.4). Other confounding variables associated with SNHL were examined for all children (Jaundice, admission to neonatal intensive care unit, gentamicin treatment and gestation) and found not to be significantly associated with SNHL.

In the symptomatic group, presence of petechiae, hepatosplenomegaly, intrauterine growth retardation, birth weight, maternal antenatal symptoms, brain imaging abnormalities, visual problems and cerebral palsy were found not to significantly associated with SNHL.

A significant relationship between SNHL and increasing age, increasing log10 DBS viral load and presence of CNS involvement was found. On multivariable regression analysis the Log_{10} CMV viral load remained independently associated with increased risk of SNHL.

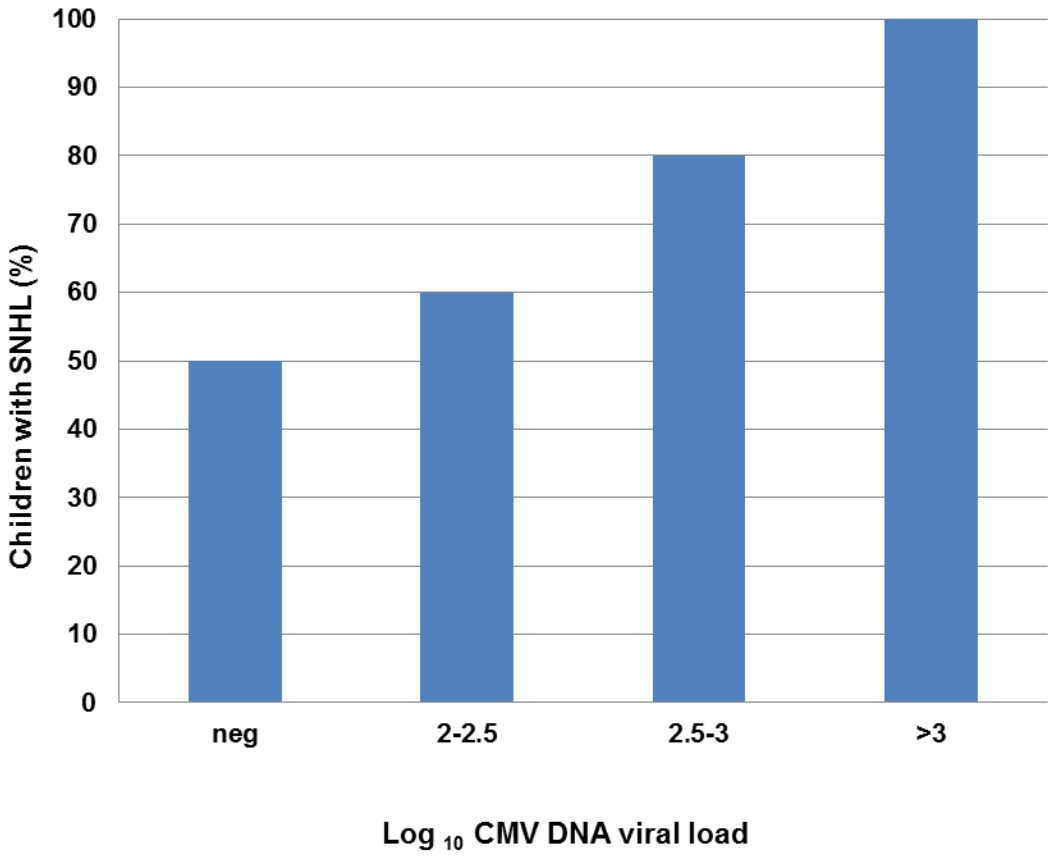


Figure 4-3: The percentage of children with SNHL in the CHIC study according to the log_{10} dried blood spot viral load.

CMV positive patients were grouped into 4 groups based on the dried blood spot result/viral load. As the CMV viral load measured in the DBS increased so did the percentage of children having SNHL

Abbreviation: DBS- dried blood spot, CMV – cytomegalovirus, SNHL- sensorineural hearing loss, CHIC - CMV in Hearing Impaired Children

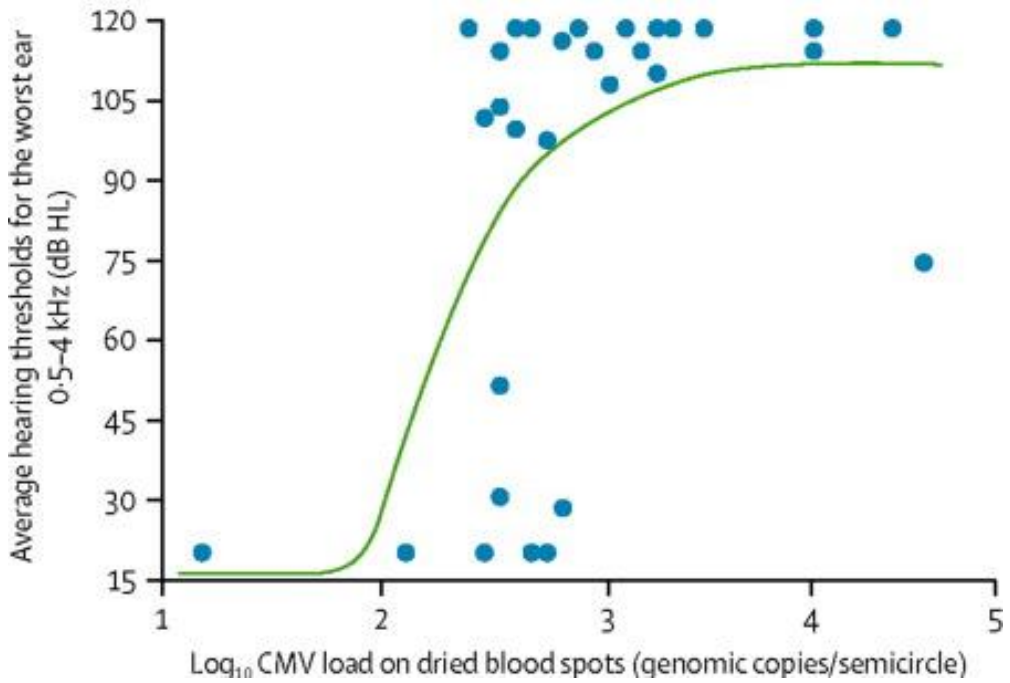


Figure 4-4 Relation between dried blood spot CMV viral load and hearing thresholds.

The best fit curve was sigmoid suggesting a threshold effect, linking severity of SNHL with higher CMV viral load (Threshold interpretation: <20 dBHL: normal hearing, >95 dBHL: profound SNHL) adapted from (Walter, Atkinson et al., 2008).

Abbreviation: DBS- dried blood spot, CMV – cytomegalovirus, dBHL – decibel hearing level, SNHL- sensorineural hearing loss

Initially, in the unexplained SNHL group 9/35 DBS tested positive for CMV DNA. However one false positive was detected in this group and was excluded from the analysis. The DBS gave a low level viral load result (310 genomes/ semicircle DBS), the child was found to be CMV IgG seronegative and not excreting CMV in urine. Her mother was also found to be CMV IgG seronegative.

Therefore for the unexplained SNHL group, 8/35 (23%) DBS tested positive for CMV DNA consistent with congenital CMV as a possible cause for their deafness. It is important to be aware of false positive laboratory results, therefore a diagnostic algorithm for the diagnosis of CCMV in SNHL was developed which limits the use of DBS testing in clinical practice to children with evidence of previous exposure to CMV either by positive serology, blood or saliva samples (see figure 4.5).

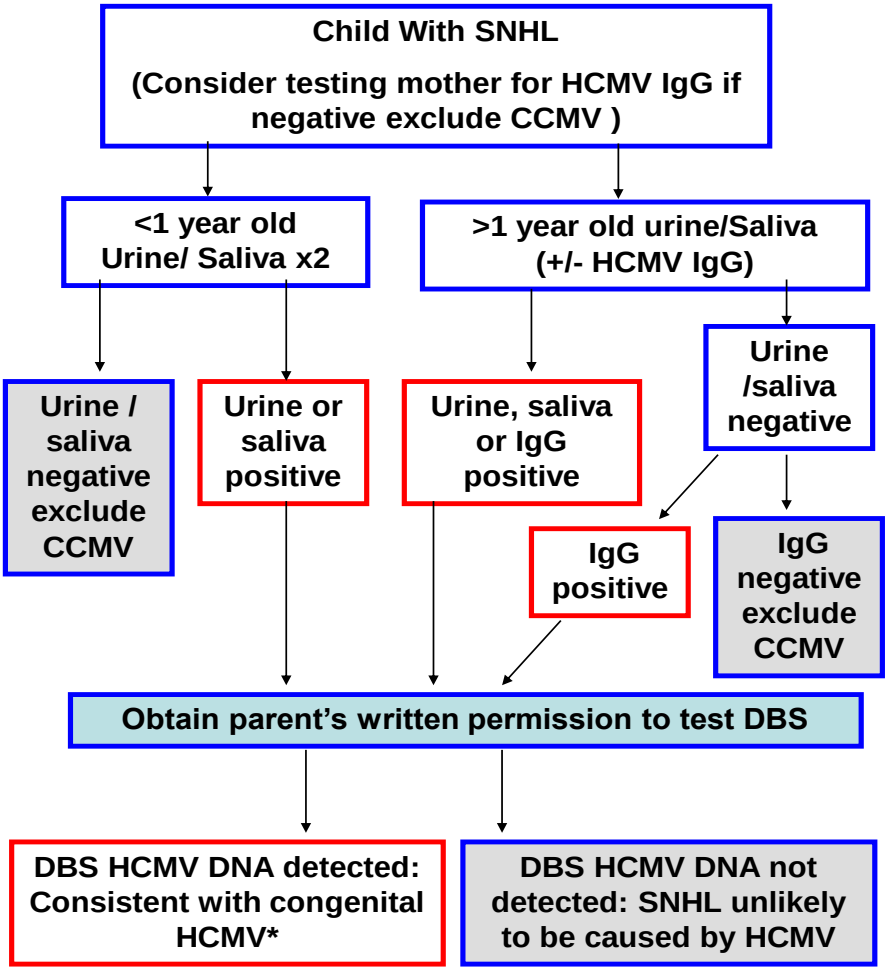


Figure 4-5: Suggested diagnostic algorithm for investigating congenital CMV infection as a cause of sensorineural hearing impairment.

*provided that the dried blood spot was collected within 3 weeks of date of birth and that the initial PCR positive result with gB (UL55) was confirmed by retesting with UL69 primers.

4.4.3 Development of a confirmatory CMV PCR for DBS

One false positive was detected during the CHIC study. To address this problem a confirmatory PCR to detect a different region of the CMV genome to the screening gB assay; the UL69 gene was developed and optimised.

Optimisation of UL69 primer and probe concentrations

After optimisation, the optimum primer and probe concentrations were found to be 600 nM of each primer and 400 nM probe in a total sample volume of 25µl (12.5µl TaqMan Universal Master Mix (Applied Biosystems, Warrington UK) and 5ul sample input). PCR cycling conditions were 95 °C for 10 min (Taq Activation), followed by 50 cycles at 95°C for 15 s, and at 55°C for 1 min.

Sensitivity of CMV UL69 PCR

Tenfold serial dilutions of CMV Ad169 were run in the CMV UL69 PCR. Each dilution series was run ten times and the mean Ct used to generate a standard curve. The standard curve derived showed a high R^2 value 0.998 and a slope of -3.3578 corresponding to a PCR efficiency of 98.52%. The dynamic range of the assay ranged from 10^3−10^7 copies/ml and was associated with a very high correlation coefficient (>0.985) with the coefficient of variation being <5% across the replicates.

The limit of detection was determined from serial dilution of the Ad169 strain. The dilution series was run in triplicate on ten separate UL69 PCR runs. The lowest replicate which showed amplification for 3/3 triplicates was 700 ge/ml (7 genomes/reaction). However it should be noted that PCR of low copy

number dilutions are stochastically limited and theoretically detecting less than 3 copies per PCR reaction is not possible.

Specificity of CMV UL69 PCR

Primers were aligned to UL69 sequences (n=4) published in GenBank to ensure that the selected primer and probe combinations were in conserved regions of the gene. No mismatches were found in primer and probe set selected. Nucleotide BLAST searches found no marked sequence homology between the UL69 PCR amplicon with other sequences registered on the database confirming that the amplification should be specific for CMV UL69.

None of the 30 CMV negative control samples or viral clinical isolates (HSV-1, HSV-2, VZV, EBV and human herpes virus 6) showed any reactivity in the CMV UL69 assay.

CMV UL69 assay validation using QCMD samples

The QCMD CMV DBS panel was used to validate the UL69 PCR for DBS testing. Each DBS was extracted and tested in triplicate. Qualitative results are shown in table 4-1 together with the number of triplicates testing positive (the DBS was counted positive when ≥2 triplicates tested positive). CMV DNA was detected in at least 2/3 triplicates in all DBS with CMV viral loads of 9.4 x 10^3 ge/ml or higher. The two DBS with viral loads reported to be 730 ge/ml were negative in the CMV UL69 assay (0/3 triplicates positive). Both of the CMV negative DBS showed no amplification in the UL69 assay.

Comparison of the CMV UL69 PCR to the CMV gB assay

The purpose of the UL69 PCR is to confirm the CMV positive result in all DBS which test positive in the first line screening PCR (CMV gB), therefore both the assays should show similar detection limits.

The Ct values of the UL69 and gB were compared. The mean Ct for both the UL69 and gB PCR was determined for each of the QCMD DBS. No significant difference was found between the resulting mean values when the two methods were compared p=0.4393 (paired *t* test). See figure 4.6.

Table 4-1: Qualitative results of CMV DNA detection in the QCMD DBS 2007 panel.

DBS were tested using the CMV UL69 PCR, the results, expected results and the percentage of results reported back correctly to the QCMD from participating laboratories () show the number of CMV positive wells per triplicate.

Abbreviation: DBS- dried blood spot, CMV- cytomegalovirus ge/ml - genomes/ml

QCMD CMV DBS Viral load (ge/ml)	CMV UL69 PCR result	Expected result (% correct qualitative results of all participants)
Negative	-	Negative (96)
Negative	-	Negative (96)
7.3×10^2	-	Positive (0)
7.3×10^2	-	Positive (7)
9.4×10^3	Positive (2/3)	Positive (48)
9.4×10^3	Positive (2/3)	Positive (52)
8.8×10^4	Positive (3/3)	Positive (93)
9.6×10^5	Positive (3/3)	Positive (96)
3.9×10^6	Positive (3/3)	Positive (100)

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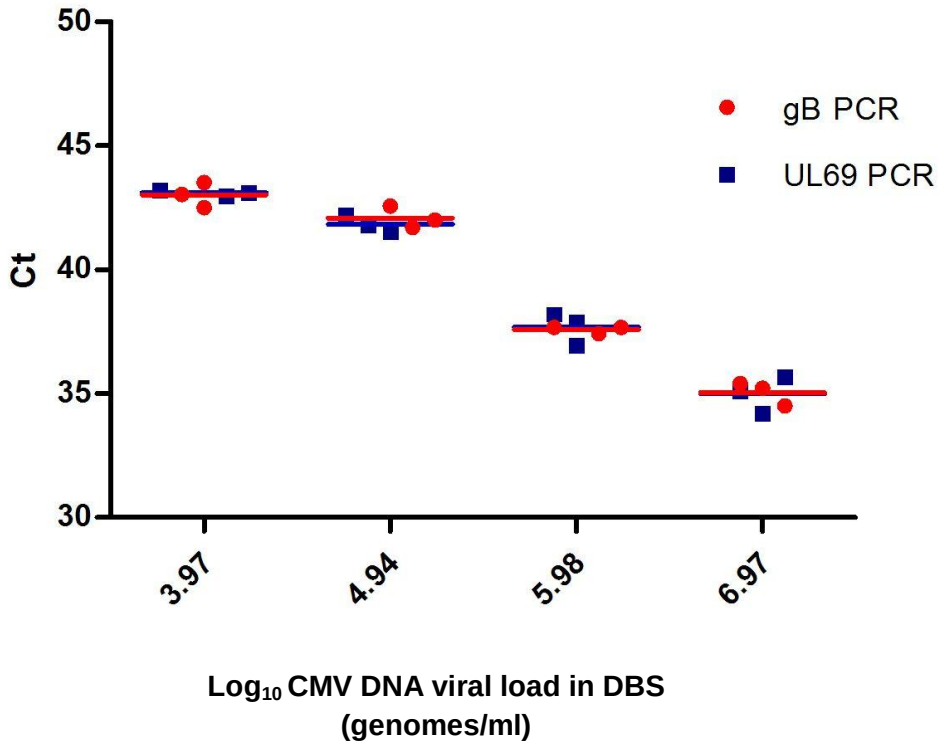


Figure 4-6: The Ct values of the 2007 QCMD dried blood spot panel after testing by either CMV gB or UL69 PCR.

DBS were tested in triplicate. Depicted are the Ct values obtained from each DBS and their corresponding stated CMV viral loads in Log_{10} genomes/ml. Bars show the mean Ct of triplicate testing of each PCR protocol. No significant difference seen between the resulting mean values when the two methods were compared p=0.4393.

Abbreviation: DBS- dried blood spot, CMV – cytomegalovirus, Ct- cycle threshold.

4.5 Discussion

CCMV is the most common cause of infant SNHL and current management of this condition is inadequate. This study aimed to establish and validate a new diagnostic approach for the use of DBS for the retrospective diagnosis of CCMV.

The percentage of confirmed congenital children testing positive in this retrospective study was found to be 72%, so providing an estimate of the sensitivity of detecting CCMV infection in children selected because they had SNHL.

Varying sensitivities have been reported in the literature (Barbi, Binda et al., 2000) with a variety of methods and varying amounts of DBS tested and different groups of patients.

Soetens *et al*, reported a sensitivity of 73% in a CCMV cohort of 53 asymptomatic and 2 symptomatic children when using a whole DBS sample (Soetens, Vauloup-Fellous et al., 2008). In this thesis the BPSU study (chapter 3) gave a similar sensitivity (74%) when using DBS to retrospectively diagnose CCMV. It should be noted that in this study a semicircle of DBS was used as there is often insufficient material left over from routine testing to use a full DBS for routine testing. Interestingly, the studies which report a sensitivity of 100% used a population of children who were diagnosed with CCMV via cell culture from urine and may reflect infants with the highest viral loads and therefore not give a true picture of the sensitivity of their assay. Two DBS studies have tested unselected samples with urine and or saliva samples tested simultaneously. They reported DBS sensitivities for CMV detection of

70% and 80% which is more consistent with the results found in this chapter (Johansson, Jonsson et al., 1997;Yamamoto, Mussi-Pinhata et al., 2001).

In the children with unexplained SNHL 8/33 (23%) DBS tested positive for CMV DNA which is consistent with previous reports. Barbi *et al,* reported a similar detection rate of 25% in children with unexplained deafness (Barbi, Binda et al., 2006).

One false positive was detected during the CHIC study. A Quality Control in Molecular Diagnostic DBS panel for CMV reported a false positivity of 9% across laboratories in 11% of datasets submitted (Barbi, MacKay et al., 2008). To address this problem, I developed a confirmatory PCR to detect a different region of the CMV genome to the screening gB assay. The PCR was designed to amplify the UL69 region of the CMV genome. This second confirmatory real time PCR removed the possibility of amplicon contamination producing false positives in the gB assay. All DBS testing positive in the gB assay were re-tested using the UL69 PCR to confirm their positivity. The assay was fully validated to ensure that the sensitivity of both the first line screening test (CMV gB PCR) and the UL69 PCR were comparable. A diagnostic algorithm for the diagnosis of CCMV in SNHL was developed for clinicians which limits the use of DBS testing in clinical practice to children with either positive serology, blood or saliva samples, thereby saving excessive testing and precious blood spots. In 2008 the testing algorithm was published and has been adopted in the UK as the standard of care for investigating CCMV in children with unexplained SNHL (Walter, Atkinson et al., 2008).

The relationship between CMV viral load and risk of disease is well established for transplant and AIDS patients.(Cope, Sweny et al., 1997;Cope, Sabin et al., 1997;Emery, Cope et al., 1999;Emery, Sabin et al., 2000;Emery, Sabin et al., 2000)) The results from the CHIC study suggest that there is also a correlation between CMV viral load from the DBS and SNHL. Studies using real time PCR quantitation have shown that viral load in urine and blood at birth is higher in symptomatic babies compared to asymptomatic babies born with CMV infection but more importantly that viral load correlates with future SNHL(Rivera, Boppana et al., 2002;Boppana, Fowler et al., 2005;Bradford, Cloud et al., 2005). Ross *et al,* studied symptomatic and asymptomatic children with CCMV and showed that peripheral blood viral load was not directly associated with hearing loss but viral loads of <3,500 copies/ml were associated with a lower risk of hearing loss and better hearing outcomes (Ross, Novak et al., 2009). This provides an important rationale for the antiviral therapy studies now underway in children born with congenital CMV disease whose objective is to reduce the future development of SNHL.

In the transplant setting the risk of CMV disease increases significantly at key CMV load thresholds with a non-linear relationship (Cope, Sweny et al., 1997). This illustrates the importance of early intervention with antiviral drugs to maintain CMV loads at relatively low levels to minimise the risk of patients progressing to CMV disease and such pre-emptive therapy has successfully reduced CMV disease in transplant patients (Atabani, Smith et al., 2012;Owers, Webster et al., 2013) This threshold effect may explain the reported beneficial effects of a 6 week course of ganciclovir on hearing outcomes in CCMV (Kimberlin, Lin et al., 2003) when children with CCMV

continue to excrete virus for months. Thus, a transient lowering of peak CMV viral load may reduce the risk of SNHL despite the fact that CMV replication continues at lower levels.

The results of the CHIC study suggesting a sigmoid relationship for the severity of SNHL versus CMV viral load may inform the design of future clinical trials designed to reduce the proportion of children with CCMV who develop SNHL. In particular, the threshold relationship supports the investigation of relatively short courses of treatment as a way of controlling disease while minimising exposure of young children to drugs with potential side effects.

Chapter 5

5 Applicability of DBS for CMV testing in clinical cohorts from resource poor countries

5.1 Introduction

Dried blood spots are widely used for newborn screening of inherited and metabolic conditions, additionally the use of DBS for the retrospective diagnosis of congenital CMV infection is well established,(reviewed by Barbi *et al* (Barbi, Binda et al., 2006).

Detection and quantitation of CMV from blood samples is routinely performed diagnostically and has many clinical uses (Atkinson & Emery, 2011). In contrast CMV DNA detection from DBS is an emerging technique consisting of a multi-step process of nucleic acid extraction and DNA amplification. The detection of CMV DNA from DBS is challenging due to the limited amount of blood available. However, this approach for sampling blood has several advantages over traditional methods of venipuncture sample collection; crucially there is no need for phlebotomy, cards are prepared from a small volume of blood (usually a single finger or heel prick), the method is relatively inexpensive and cards need no specialist storage or transport requirements. These practical benefits have made DBS an attractive alternative to fresh whole blood for the diagnosis and monitoring of viral infections in the developing world or in infant cohorts where limited blood volume may be available. However sensitivity and specificity of the DBS sample has not been systematically evaluated against 'gold standard' sample types of plasma or whole blood.

CMV was first suggested as a co-factor in HIV progression in 1989, with CMV seropositive individuals 2.5 times more likely to progress to AIDS defining

diseases compared to CMV seronegative individuals and survival analysis showed that the risk increased approximately 2 years after seroconversion (Webster, Grundy et al., 1989). Since this seminal study HIV and CMV co-infection in adults has been associated with disease progression and mortality (Kempen, Martin et al., 2003;Spector, Hsia et al., 1999;Deayton, Prof Sabin et al., 2004) and a recent large HIV cohort study of 6,111 individuals by Lichtner *et al*, has shown that CMV co-infection is associated with an increased risk of severe non-AIDS defining events (Lichtner, Cicconi et al., 2014). In addition Johnson *et al* have recently shown that HIV-1 infected mothers with ongoing or primary CMV infection have an elevated frequency of HIV-1 transmission and suggested cord blood mononuclear cells undergo increased proliferation when stimulated with CMV antigens, upregulating the T central memory cells and expression of CCR5 which may promote in utero transmission of HIV-1 (Johnson, Howard et al., 2015).

In resource limited settings children born to HIV positive mothers with maternal CMV DNAemia are associated with impaired growth and development (Gompels, Larke et al., 2012a) and a higher risk of mortality (Slyker, Lohman-Payne et al., 2009). CMV can be transmitted in utero, during delivery or postpartum via exposure to CMV in breast milk or saliva. In sub-Saharan Africa >80% of children acquire CMV within the first 12 months of life establishing a lifelong latent infection, with 90% of HIV exposed but uninfected and 89% of HIV infected children experiencing detectable DNAemia by 6 months of age (Slyker, Lohman-Payne et al., 2009).

In this chapter I identified a cohort of HIV positive mother/ baby pairs in Nairobi, Kenya where both longitudinal concomitantly collected DBS and plasma were available. These paired samples were used to investigate the sensitivity and specificity of CMV detection from DBS and to validate a higher throughput methodology for extraction of DBS suitable for large research cohorts. The results provide valuable estimates to guide the use of DBS to monitor viral acquisition and address how DBS specimen selection may influence the overall outcome of a study.

5.2 Aims

The work in this chapter aimed to systematically quantify differences in CMV DNA detection using real-time PCR in DBS compared to paired plasma as a reference sample in an HIV positive population.

Using paired plasma and DBS samples collected longitudinally, I aimed to determine ways in which using DBS may potentially misclassify results in a longitudinal study providing valuable information to guide the use of DBS in future prospective studies in this cohorts.

To achieve this I have three aims:

- to determine the sensitivity, specificity, negative predictive value and positive predicative value of the DBS sample for CMV DNA detection when compared to reference plasma results.
- to compare detection rates between DBS and plasma samples and determine if sample type affects the time to first detection.
- to determine if CMV viral load plays a role in CMV detection from DBS in this cohort.

To be able to carry out these aims I established a collaboration with the Department of Global Health, University of Washington, Seattle USA for access to paired infant and child DBS and plasma samples.

5.3 Methods

5.3.1 Participants

All studies were approved by the University of Washington Institutional Review Board and the Ethics and Research Committee of Kenyatta National Hospital, and written informed consent was obtained from all mothers on behalf of themselves and their infants. A cohort of infants born to HIV infected women were used to study acute CMV acquisition and detection. Specimens were selected from a larger perinatal HIV transmission cohort, details of which have been presented elsewhere (Lohman-Payne, Slyker et al., 2009;Lohman, Slyker et al., 2005;Obimbo, Mbori-Ngacha et al., 2004;John-Stewart, Mbori-Ngacha et al., 2009;Gichuhi, Obimbo et al., 2005;Lohman, Slyker et al., 2003). Mothers were recruited during the third trimester of pregnancy from prenatal clinics in Kenyatta National Hospital, Nairobi between 1999 and 2004. Women were provided with short-course antenatal zidovudine for the PMTCT according to contemporaneous guidelines (Shaffer, Chuachoowong et al., 1999).This study was conducted before antiretroviral therapy (ART) became widely accessible, and women received no ART other than PMTCT during study follow-up. Mother- infant pairs were followed-up in the study clinic for 1 year, with an additional year of follow-up for infants acquiring HIV infection. Infant peripheral blood and DBS were collected at birth and months 1, 3, 6, 9, 12, 15, 18, 21 and 24. Blood was separated using density-gradient centrifugation and plasma was cryopreserved at -70ºC for future virological studies.

5.3.2 Infant HIV-1-diagnosis

Infant HIV-1 infection was diagnosed by detection of either HIV-1 gag DNA (Panteleeff, John et al., 1999), or RNA from plasma (Emery, Bodrug et al., 2000) using the GenProbe assay.

5.3.3 Specimen selection

60 mother/infant pairs were selected form the larger cohort based on availability of previous CMV testing in plasma, as reported in (Slyker, Lohman-Payne et al., 2009) Because >85% of CMV transmissions occurred within the first 6 months of life. Samples from birth to 3 months were selected for comparison between plasma and DBS.

5.3.4 CMV DNA detection in plasma samples

CMV extraction and quantitative CMV PCR were determined on plasma samples in infants up to 24 months of age by the collaborators in the Department of Epidemiology, University of Washington USA. Briefly total nucleic acid was extracted from 50µl -200µl of plasma using the Qiagen UltraSens virus extraction kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol. For comparative purposes the CMV gB real time PCR protocol was used as described in chapter 2.

5.3.5 Development of high throughput methodology for extraction of DBS samples

An extraction methodology was developed for extraction of DBS samples on the QIAsymphony instrument (Qiagen, UK). This instrument has the benefit of being fully automated with barcode input and sample tracking. A selection of known positive (previously tested by EasyMag) DBS were used to evaluate the higher throughput system. Nucleic acid was extracted from DBS using the QIA|Symphony automated extraction system (Qiagen, Crawley, UK) with the QIAsymphony DNA Mini Kit following manual pre-treatment: DBS was added to 400µl Buffer ATL and 20µl proteinase K, and incubated at 56°C for 30 minutes. The resulting supernatant was transferred into a 2 ml tube, without disturbing the digested DBS, and loaded onto the QIAsymphony SP. Extraction was carried out using the "VirusBlood200_V5_DSP" protocol with an elution volume of 60µl. A total of 96 samples could be processed per batch.

5.3.6 CMV DNA detection in DBS

Real-time PCR was used to detect the CMV glycoprotein B gene (UL55) as previously described in chapter 2 and 3 and the assay used for CMV quantitation from paired plasma samples for comparison.

5.3.7 **Statistical analysis**

Sensitivity and specificity of DBS for CMV detection were performed using plasma DNA detection as a gold standard. An online calculator was used to determine sensitivity, specificity, NPV and PPV available at http://www.medcalc.org/calc/diagnostic_test.php (accessed October 2013). STATA SE version 13.0 (STATA Corp., College Station, Texas USA) was used for all other statistical analysis. Assuming that plasma would be more sensitive method for CMV detection, non parametric receiver operating characteristic (ROC) curves were used to determine the optimal cut-off of plasma CMV load for the detection of CMV from the DBS sample. The AUC was compared between HIV-infected and HIV-exposed uninfected infants using the Mann-Whitney U test. The time to first CMV DNA detection between DBS and plasma samples was investigated using a paired Wilcoxon signed-rank test, with the first CMV DNA detection used to define infection. A *t* -test was used to test the difference between the plasma CMV viral loads with CMV DNA detected in paired DBS and those with CMV DNA not detected. All *p* values reported are for two-tailed tests with *p* values ≤0.05 regarded as significant

5.4 Results

5.4.1 Patient characteristics

60 mother infant pairs were selected for this sub-study; all mothers were known to be HIV positive. 40 infants were HIV infected (HI) (67%) and 20 were HIV exposed uninfected (HEU) (33%) during the study period. Patient characteristics have been described elsewhere in detail (Slyker, Lohman-Payne et al., 2009). In both the HI and HEU infants the majority of CMV infections (plasma detection) occurred in the first 3 months of life (90%) as previously reported (Slyker, Lohman-Payne et al., 2009) 5 cases of congenital CMV (8%) were included: CMV DNA being detected in the infant plasma sample at birth, paired DBS samples were available for 4 of the CCMV infants of which 3 (75%) were positive for CMV DNA. The DBS sample without detectable CMV DNA had the lowest peak viral load of 1.76 $\log_{10}$ CMV genomes/ml, while the CMV detected viral loads were 2.25, 2.6 and 3.0 $\log_{10}$ CMV genomes/ml in the CCMV children.

5.4.2 Sensitivity and specificity of CMV DNA detection in DBS compared to plasma samples

DBS were compared to the paired 'gold standard' plasma samples to assess the sensitivity and specificity for detection of CMV DNA. The overall sensitivity of DBS compared to plasma samples for detection of CMV DNA was 45%. Specificity was 100% with a PPV of 100% and NPV of 71% (Table 5.1).

When evaluating a continuous-scale diagnostic test, specificity and sensitivity may change depending on the set point of measurement. ROC curves were used to evaluate the sensitivity and specificity of DBS to diagnose CMV across the range of observed plasma CMV loads (Figure 5.1a). The analysis was repeated but stratified by HIV status (figure 5.1b) to see if HIV co-infection had any effect on outcome. For the ROC sensitivity was plotted against 1- specificity (false positive rate) for different viral load cut off points. Each point therefore represents a sensitivity/specificity pair corresponding to a viral load threshold. For all DBS tested the ROC showed an optimal cut-off for CMV viral loads above 206 genome/ml. When infants were stratified by HIV status, similar optimal cut-offs for plasma viral load were seen (>286 CMV genomes/ml for HEU, >221 CMV genomes/ml for HI, p=0.4). The AUC of the ROC was 0.91 for detection of CMV DNA from all DBS, when stratified by HIV status the AUC were not significantly different between each group (AUC 0.981 for HEU, 0.828 for HI respectively, *p*= 0.6).

Table 5-1: ***Sensitivity, specificity, positive predictive values and negative predictive value of the Dried blood spot samples when compared to paired plasma samples for the detection of Cytomegalovirus DNA.***

Parameter	%	CI (95%)
Sensitivity	45%	29-61%
Specificity	100%	93-100%
PPV	100%	82-100%
NPV	71%	59-80%

The results are shown with the 95% confidence intervals (CI).

(PPV: positive predictive value; NPV: Negative predictive value)

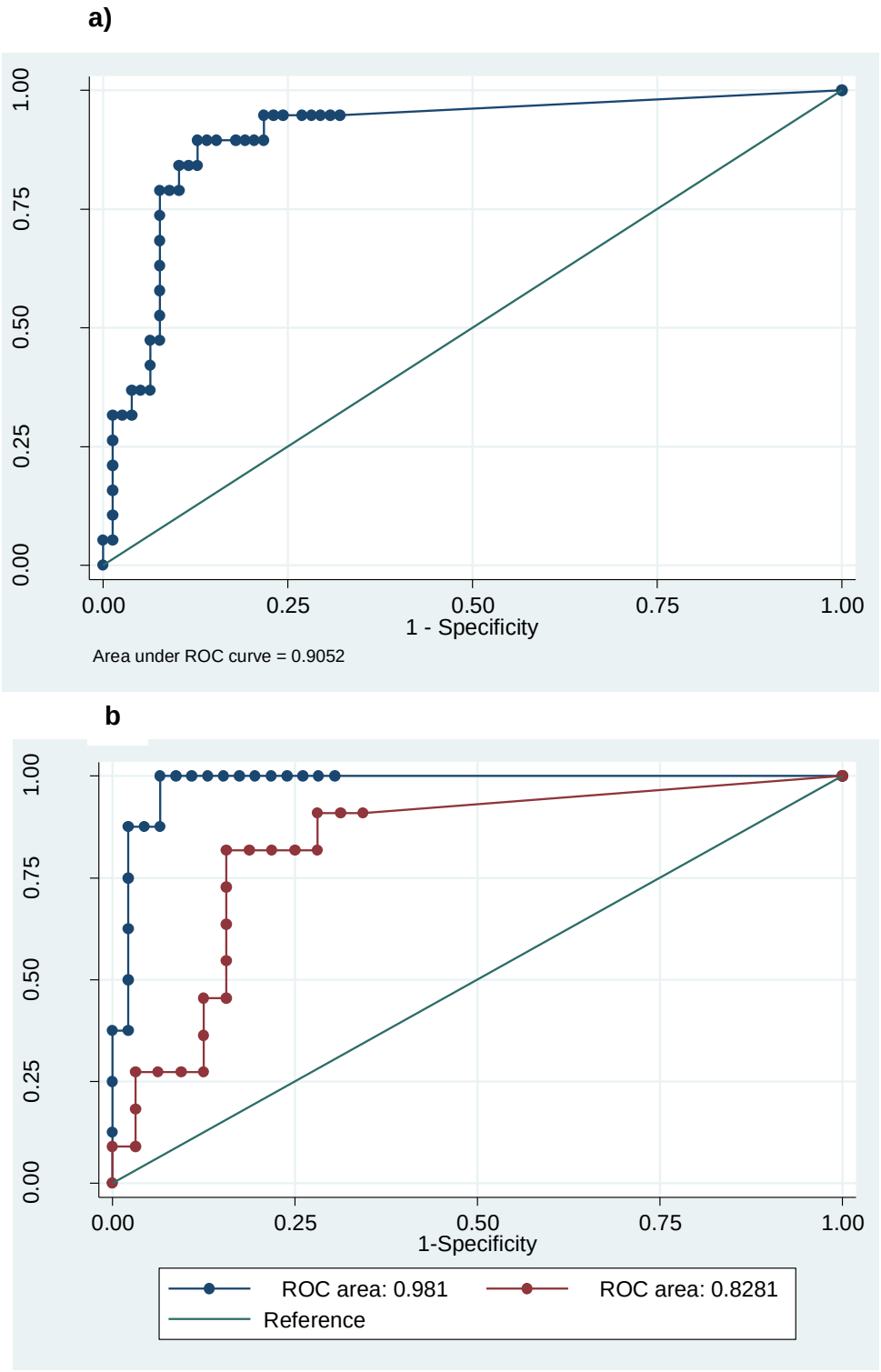


Figure 5-1*: Receiver operator curves for cytomegalovirus DNA detection from dried blood spots compared to paired plasma samples.*

Specificity was plotted against 1-specificity to determine a threshold CMV viral load of detection for CMV from the DBS sample and area under curve (AUC) for overall accuracy. **a)** represents all DBS samples showing good overall accuracy for discriminating CMV DNA in DBS samples (AUC 0.905) **b)** shows ROC stratified by HIV status, HIV exposed uninfected (blue), HIV infected (red), with no significant difference seen between groups (AUC 0.981, 0.828 respectively).

The time to first detection of CMV DNAemia was considered the time of CMV acquisition. The time to first detection of CMV DNA was compared between DBS and plasma. The incidence of CMV DNA detection in plasma samples was 90% at 3 months of life while in contrast DBS testing showed 31% overall detection of CMV DNA at 3 months.

To further compare the time to first detection in the two sample groups a paired Wilcoxin signed-rank test was performed. For all samples the Wilcoxin shows a trend for significance (p=0.051) between time to first CMV detection in plasma versus time to first detection in DBS. To investigate this trend further; to determine whether using DBS would significantly affect an estimate of timing of infection in a longitudinal study the median time to first detection of CMV DNA was compared for DBS and plasma, Overall, using the plasma specimen resulted in earlier detection of CMV DNA (median= 2months) compared to DBS (median=2.8 months; p=0.05) No difference was seen when samples were stratified by HIV status (table 5.2).

Table 5-2: Comparison of time to first Cytomegalovirus DNA detection in dried blood spots and plasma samples.

	Median time to 1st CMV detection (months)		
Sample type	**Plasma**	**DBS**	***p* value**
HIV infected	1.50	1.90	0.31
HIV exposed	2.33	2.67	0.55
All	**2.0**	**2.8**	**0.05**

The mean time to first CMV detection for DBS versus plasma for all samples and samples stratified by HIV status is shown. Overall, using the plasma specimen resulted in earlier detection of CMV DNA. No difference was seen when samples were stratified by HIV status

The viral load of the plasma samples was then investigated to see if there was a correlation between viral load and CMV DNA detection from DBS. The mean CMV viral load of the paired plasma samples in which CMV was DNA detected in DBS was 3.04 $\log_{10}$ CMV genomes/ml. In contrast the mean CMV viral load from plasma samples in which CMV DNA was not detected was 2.37 $\log_{10}$ CMV genomes/ml (p=0.0023)(figure 5.2). The mean peak viral load in plasma was 2.96 $\log_{10}$ CMV genomes/ml (range 2.6 - 4.9 $\log_{10}$ CMV genomes/ml), with the mean peak viral load in DBS being 3.7 $\log_{10}$ CMV genomes/10^6 cells (range 2.6 -5.2 $\log_{10}$ CMV genomes/10^6 cells).

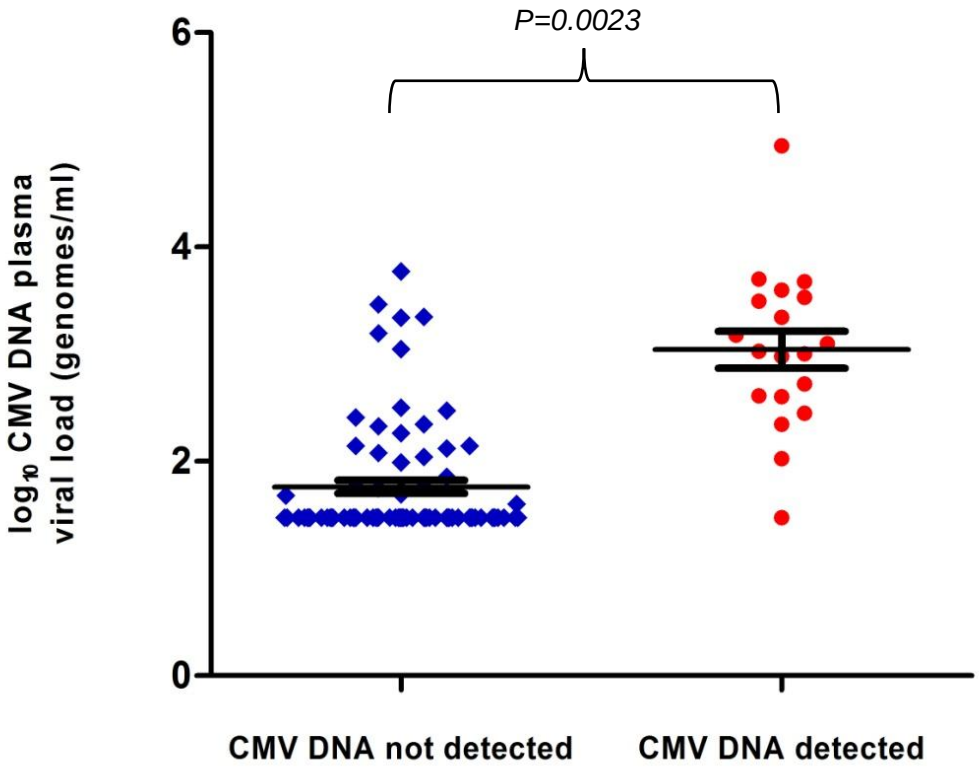


Figure 5-2: ***The Log_{10} Cytomegalovirus genomes/ml in plasma samples related to paired dried blood spot result.***

DBS samples were reported as CMV DNA not detected or detected. Error bars show the mean CMV viral load and SD. There was a significant difference between the plasma viral loads stratified by DBS detection (p=0.0023).

5.5 Discussion

The aim of this study was to systematically quantify differences in CMV DNA detection using real-time PCR in DBS compared to plasma as a reference sample in an HIV positive population. Using paired plasma and DBS samples collected longitudinally, I was able to determine ways in which using DBS may potentially misclassify results in a longitudinal study providing valuable information to guide the use of DBS in future prospective studies in these cohorts.

This sample cohort chosen was ideal to evaluate sensitivity and specificity of CMV detection from DBS for the following reasons, most plasma samples were CMV DNA positive, a wide range of viral loads were observed and finally most children remained viraemic. Although the sensitivity of DBS was low compared to plasma (45%), the PPV (100%) and NPV (71%) were high, and the tests were most comparable when CMV viral loads were >206 genomes/ml. Together, these data suggest DBS are a useful tool to identify individuals with significant levels of CMV replication in populations where CMV is highly prevalent. Clinically significant (over 1000 CMV DNA copies/ml) values are thus likely to be accurately classified as positive using DBS, although CMV viral loads measured by DBS would be expected to return values below what was measured in plasma. This sensitivity is consistent with the viral load data in which the mean CMV viral load of the paired plasma samples in which CMV was DNA detected in DBS was 3.04 $\log_{10}$ CMV genomes/ml. In contrast the mean CMV viral load from plasma samples in which CMV DNA was not detected was 2.37 $\log_{10}$ CMV genomes/ml and will

account for why many of the plasma samples with low virl loads were not detectable in the DBS assay.

These results show that using DBS for a prospective study, even with a very narrow window of CMV acquisition (0-3 months) will likely delay the detection of CMV in viraemic individuals as the lower viral loads will not be detected. loads are expected, although it should be noted that due to the monthly Detection of CMV in DBS has mainly focused on children with congenital CMV where there is a lack of comparison between concomitantly collected blood samples. Sensitivity and specificity has been ascribed to number of cases of congenital CMV or the production of DBS from known quantities of CMV spiked into whole blood or plasma prior to analysis (Atkinson, Walter et al., 2009;Binda, Caroppo et al., 2004;Barbi, Binda et al., 2006;Barbi, Binda et al., 2000;Boudewyns, Declau et al., 2009;de Vries, Barbi et al., 2012;Boppana, Ross et al., 2010). Although sensitivity of CMV DNA detection in the DBS compared to plasma was only 45%, this study suggests the sensitivity would be appropriate for monitoring cohorts at risk of CMV disease for example solid organ transplant patients and AIDS patients where the relationship between CMV viral load and risk of disease is well established (Cope, Sweny et al., 1997;Cope, Sabin et al., 1997;Emery, Cope et al., 1999;Emery, Sabin et al., 2000;Emery, Sabin et al., 2000); (Humar, Gregson et al., 1999) and significant levels of DNAemia are expected; but not for cohorts with low viral loads. Specificity was found to be 100% with a PPV of 100%. The ROC was in agreement with a good ability to differentiate patients positive for CMV DNA from those negative for CMV DNA.

Overall, plasma samples showed superior sensitivity for detection of CMV DNAemia with the detection of CMV DNA associated to plasma CMV viral load, however it must be noted that the amount of sample available from a DBS will be significantly lower than the volume of plasma tested.

Plasma collection may not be suited to every study, plasma separation requires specialist equipment and cold-chain storage for shipping, which may be lacking at low-resource facilities. In young infants, blood volumes may be very limiting. In contrast, DBS utilise very small blood volumes (~50µl), require no processing, can be stored without refrigeration prior to shipping, and can be transported for analysis at reference laboratories without the sample associated biohazard risk. In resource limited settings DBS sampling allows testing of patients for viral infections as it greatly facilitates the logistics of sample collection.

In conclusion, the use of DBS can simplify sample collection and virological monitoring for CMV infection, but has reduced sensitivity compared to plasma, which is dependent upon CMV viral load and ultimately may reflect sample volume. Clinic resources, patient origin (adult/infant), and emergent need for prospective diagnosis should be evaluated critically to determine whether DBS is an appropriate specimen for a site. A sampling strategy based on DBS should be fully validated prior to use to determine sensitivity and specificity in a study population with lower prevalence of CMV DNAemia than examined here. Despite its limitations, DBS may have the ability to improve patient care in remote clinics and are often the only viable option in resource limited settings where logistical constraints often preclude storage and transport of plasma or whole blood samples.

Chapter 6

6 The relationship between CMV detection in different maternal body compartments and acquisition on CMV by the infant in the setting of HIV-1 infection

6.1 Introduction

Mother to child transmission of infection is a major public health concern with World Health Organisation programmes in place to try to eliminate transmission of HIV. In the context of HIV infection, in developed countries transmission is prevented by a combination of interventions including elective caesarean sections, antiviral prophylaxis, HAART and avoidance of breast feeding. However vertical transmission continues to be a problem in developing countries where HIV status may not be known or interventions not possible. In the absence of antiretroviral prophylaxis, breastfeeding is a major route of transmission of HIV infection (Dunn, Newell et al., 1992).

CMV acquisition from breast milk is a common mode of transmission of CMV Premature infants are particularly at risk of CMV disease if they acquire CMV via breast feeding in the neonatal period (Kurath, Halwachs-Baumann et al., 2010). In areas with high CMV seroprevalence, breast milk transmission has been show to account for the majority of infants acquiring CMV before the age of one year (Stagno, Reynolds et al., 1980).

It has been shown that >80% of healthy women have detectable CMV in their breast milk or cell free whey (Vochem, Hamprecht et al., 1998 (Hotsubo, Nagata et al., 1994;Asanuma, Numazaki et al., 1996;Hamprecht, Maschmann et al., 2001;Yasuda, Kimura et al., 2003) with CMV being first detected at 2–3 weeks after delivery, peaking by 3–6 weeks and becoming undetectable in most individuals by 8–10 weeks after delivery (Hamprecht, Witzel et al., 2003). The CMV present in breast milk could be from multiple sources

including mammary epithelial cells, lymphocytes and migrating monocyte/macrophages.

Intrapartum transmission is thought to result from contact with CMV from the cervix or vagina during birth. Asymptomatic cervicovaginal shedding of the virus is common with CMV being detected in ~10% of non-pregnant women, and 11-35% of pregnant women in the third trimester (Stagno, Reynolds et al., 1975a;Shen, Chang et al., 1993;Chandler, Alexander et al., 1985). In women with cervicitis CMV inclusion bodies are found within glandular epithelial cells, endothelial cells, and mesenchymal stromal cells, suggesting that these cells may be involved in CMV reactivation in the cervix (McGalie, McBride et al., 2004).

Maternal immunity does not prevent CMV infection but seems to prevent overt CMV disease. Studies of healthy children in highly seroprevalent regions show that 85% of children are infected with CMV by 1 year of age (Miles, van der et al., 2007). In Kenya, in the setting of maternal or infant HIV-1, virtually all HIV infected women are co-infected with CMV (Slyker, Lohman-Payne et al., 2009b). CMV acquisition occurs early in life, with >80% of Kenyan infants infected by 3 months of age (Slyker, Lohman-Payne et al., 2009). For those infants who acquire HIV, CMV is linked to early mortality, neurologic deficit and rapid progression to AIDS (Nigro, Krzysztofiak et al., 1996;Kovacs, Schluchter et al., 1999;Slyker, Lohman-Payne et al., 2009b;Nigro, Krzysztofiak et al., 1996;Gompels, Larke et al., 2012b)). In the Zambia, in studies of both HIV-1 infected (HI) and HIV-1-exposed, uninfected infants (HEU) early CMV acquisition has been associated with numerous detrimental

outcomes including poorer growth and lower psychomotor scores (Gompels, Larke et al., 2012b).

These observations emphasise that vertical transmission of CMV in African infants with HIV coinfection can be harmful, however efforts to address this problem are hampered by the lack of a CMV vaccine or antivirals with a good safety profile. Preventing or delaying CMV acquisition may represent a novel strategy to improve the health of infants in areas with high seroprevalence and HIV co-infection but requires a better understanding CMV replication and transmission in the setting of maternal HIV-1.

6.2 Aims

This chapter aims to bring together and build on the DBS methods developed and validated in the previous chapters of this thesis and apply them practically to investigate a large cohort of maternal and infant samples.

In this chapter I have worked collaboratively with the University of Washington USA to study a large cohort of HIV-1 positive women and their infants. Using specimens and data from a randomised trial of valaciclovir suppressive therapy in Nairobi, Kenya, I have studied the incidence of CMV detection in different maternal compartments (blood, breast milk and cervical secretions) to investigate potential relationships between HIV viral load, CD4 count and CMV detection in these compartments, with the ultimate aim of identifying maternal correlates of vertical transmission of CMV.

Specific aims of this chapter are summarised below.

- To set up a collaborative study with the University of Washington USA to study a large mother/infant cohort.
- To use the methods developed in this thesis to analyse both maternal and infant samples for the detection and quantitation of CMV in the context of a maternal HIV infection and high CMV seroprevalence.
- To investigate potential relationships between maternal CMV DNA viral load in different compartments, immunosuppression (CD4 count) and HIV viral load in MTCT of CMV infection.

6.3 Materials and methods

6.3.1 Study collaboration

The work in this chapter was a collaborative study with the University of Washington, Seattle USA. All work undertaken by them is acknowledged in this chapter, all other work is my own. This study was added as an additional arm into a clinical trial which evaluated the efficacy of valaciclovir to reduce maternal HIV-1 RNA levels (NCT00530777) the results of which were published in 2012 (Drake, Roxby et al., 2012a).

6.3.2 Participants and Sampling

All studies were approved by the University of Washington Institutional Review Board and the Ethics and Research Committee of Kenyatta National Hospital Kenya, and written informed consent was obtained from all mothers on behalf of themselves and their infants.

All women were HIV/HSV-2 co-infected and were recruited during 28-32 weeks of pregnancy. All were treated with antivirals for the prevention of HIV transmission. The antiviral regime consisted of twice daily zidovudine from 28 weeks and every 3 hours during labour until delivery and single dose nevirapine at the onset of labour. From June 2009, maternal lamivudine and twice daily zidovudine for 1 week postpartum were also offered. Women were randomised to valaciclovir 500 mg b.i.d. or placebo at 34 weeks gestation (74 to each group), and continued to 1 year postpartum. Participants were evaluated at 34 and 38 weeks gestation, delivery, and at 2, 6, 10, and 14 weeks and 6, 9, and 12 months *postpartum*. Venous blood was collected from

mothers and DBS were collected from infants at all visits. Cervical swabs were collected at 34 weeks (pre-randomisation) and 38 weeks gestation, and breast milk was collected at all postpartum visits.

6.3.3 HIV-1 detection and CD4 counts

Both the HIV-1 load and CD4 counts were previously determined as part of the original study by the Epidemiology Department in the University of Washington as described (Drake, Roxby et al., 2012a)

Briefly, CD4 counts were measured at 32 weeks gestation. HIV-1 RNA viral load was measured using the GenProbe assay (Gen-Probe Inc, San Diego, CA). The limit of detection for this study was 150 copies/ml for plasma and 100 copies/ml for cervical secretions and breast milk.

Specimens with undetecctable values were assigned a load at half the lower limit of detection. At 6 weeks, infant DBS were tested for HIV-1 RNA using the Amplicor assay (Roche Molecular Systems, Inc., Branchburg, NJ) and confirmed by detection of HIV-1 gag and pol DNA by an in-house PCR as described by (Panteleeff, John et al., 1999). Infant testing at subsequent visits was performed using the in-house HIV-1 gag and pol PCR.

6.3.4 CMV DNA detection and quantitation in maternal samples

Extraction of maternal plasma samples, breast milk and cervical secretions was performed in the Epidemiology Department, University of Washington USA by Kristjana Ásbjörnsdóttir. Briefly total nucleic acid was extracted from 50µl -200µl of sample using the Qiagen UltraSens virus extraction kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol. Samples were then shipped to me for quantitative CMV PCR analysis. CMV PCR detecting the glycoprotein B gene (UL55) was used as previously described in chapter 2. The limit of detection was 100 genomes/ml for virus extracted from maternal plasma, cervical secretions and breast milk.

6.3.5 CMV DNA detection in infant DBS

Nucleic acid was extracted by me from DBS using the QIAsymphony automated extraction system (Qiagen, Crawley, UK) with the QIAsymphony DNA Mini Kit following manual pre-treatment: DBS was added to 400µl Buffer ATL and 20µl proteinase K (>600 mAU/ml, Qiagen, Crawley UK), and incubated at 56°C for 30 minutes. The resulting supernatant was transferred into a 2 ml tube, without disturbing the digested DBS, and loaded onto the QIAsymphony SP. Extraction was carried out using the "VirusBlood200_V5_DSP" protocol with an elution volume of 60 µl.

CMV PCR detecting the glycoprotein B gene (UL55) was used as previously described in chapters 2 & 3. Viral loads from infant DBS were normalised to genomes/million cells against a β-globin standard as described in chapter 5 with a lower limit of detection of 100 genomes/million cells.

6.3.6 **Statistical analysis**

STATA SE version 13.0 (STATA Corp., College Station, Texas USA) was used for statistical analysis in association with Barbra Richardson and Katie Odem-Davis in the department of Biostatistics, University of Washington, Seattle, Washington, USA. All *p* values reported are for two-tailed tests with *p* values ≤0.05 regarded as significant.

HIV-1 and CMV viral loads were $\log_{10}$-transformed to normalise distributions. For specimens where >30% of values were undetectable, data were dichotomised as detectable/undetectable (CMV in blood and cervix; HIV-1 in cervix and breast milk). Plasma HIV-1 and breast milk CMV were treated as continuous variables. CD4 was dichotomised based on the cohort median at baseline (450cells/µl).

GEE were used to measure associations where there were repeated measurements within a maternal compartment. The binomial link function was used to analyse virus detection as a dichotomised (detected/not detected) outcome. Linear models with Gaussian distribution were used to analyse CMV levels as a continuous outcome. All GEE models utilised robust standard errors and an exchangeable correlation matrix.

Correlates of infant acquisition of CMV were identified using Cox proportional hazards regression, and were restricted to the infants who remained HIV-uninfected throughout follow-up. The first positive DBS specimen was taken to indicate the timing of infant CMV acquisition.

Since HIV-1 levels were reduced by valaciclvoir treatment (Drake, Roxby et al., 2012a) all GEE models included adjustment for study treatment allocation.

Models examining CMV-CMV correlations between different maternal compartments additionally adjusted for plasma HIV-1 viral load.

Logistic regression was used to predict the probability of CMV transmission by 1 year with varying maternal baseline CD4 counts and 2 week breast milk CMV viral load. The model was first fit with infant CMV detection as outcome and with CD4 and breast milk viral load as predictors. Parameter estimates from this model were then used to predict infection rates at differing CD4 counts (350, 450, 750 and 1000 cells per µl) and breast milk viral loads. The confidence intervals were then computed using a delta method standard error for each predicted probability.

6.4 Results

6.4.1 Study participants

Study participation is summarised in figure 6.1, a total of 148 women were randomised to the study. After exclusions, a total of 131 infant/mother pairs in which the infant remained HIV- exposed but uninfected during follow-up were studied.

6.4.2 Samples for CMV analysis

For the study period, CMV DNA detection and viral loads were measured in 146 maternal plasma specimens, 246 cervical swabs, 542 breast milk specimens, and 964 infant DBS.

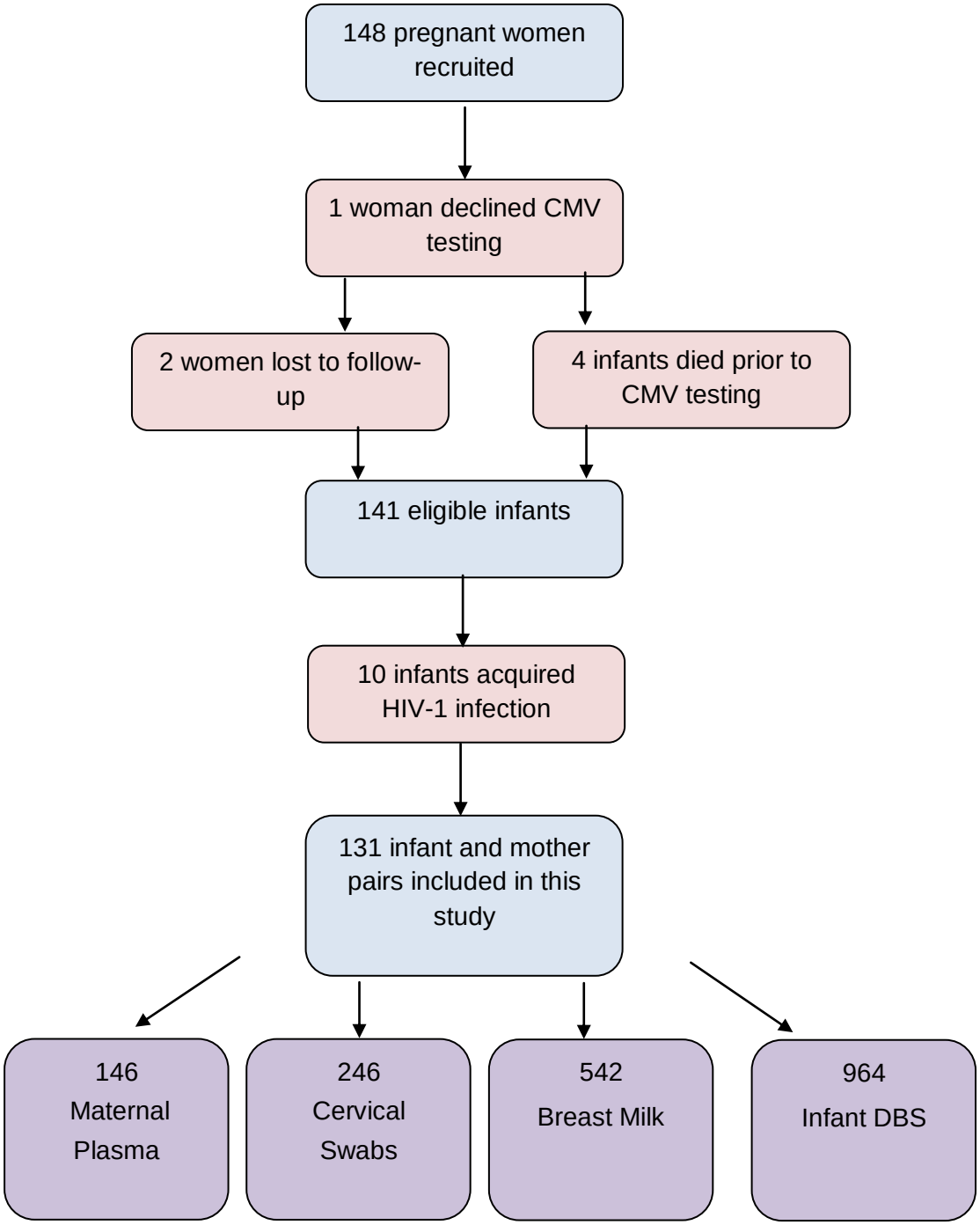


Figure 6-1: **Study participants, recruitment and samples tested for a cohort of HIV-1 positive women and their infants**

6.4.3 **Maternal CMV detection and quantitation**

In maternal plasma samples, 4.8% of women had detectable CMV DNA at 34 weeks gestation (figure 6.2a), CMV viral loads ranged from 1.6 - 4.1 $\log_{10}$ CMV ge/ml with a median viral load of 1.8 $\log_{10}$ CMV ge/ml (IQR=1.8-2.0 $\log_{10}$ CMV ge/ml; figure 6.2b).

In cervical swabs (34 and 38 weeks gestation) CMV was detected frequently with 66% of women having CMV DNA detected (figure 6.2a), cervical viral loads ranged from 1.5-5.4 $\log_{10}$ CMV ge/ml in positive specimens, with a median viral load of 2.6 $\log_{10}$ CMV ge/ml (IQR=1.7-3.5 $\log_{10}$ CMV ge/ml; figure 6.2b). Both the rate of cervical CMV detection and the level of CMV detected increased significantly over time (p=0.001) and (p=0.01) respectively.

In breast milk 99% of women had CMV DNA detected at one or more study visit with viral loads ranging from 2.1-7.5 $\log_{10}$ CMV genomes/ml, with a median of 5.5 $\log_{10}$ CMV genomes/ml (IQR=4.9-6.4 $\log_{10}$ CMV ge/ml; figure 6.2b). Breast milk CMV viral loads were highest early postpartum and declined slowly over time at a rate of approximately -0.076 $\log_{10}$ CMV ge/ml per month (p=0.002) figure 6.2b.

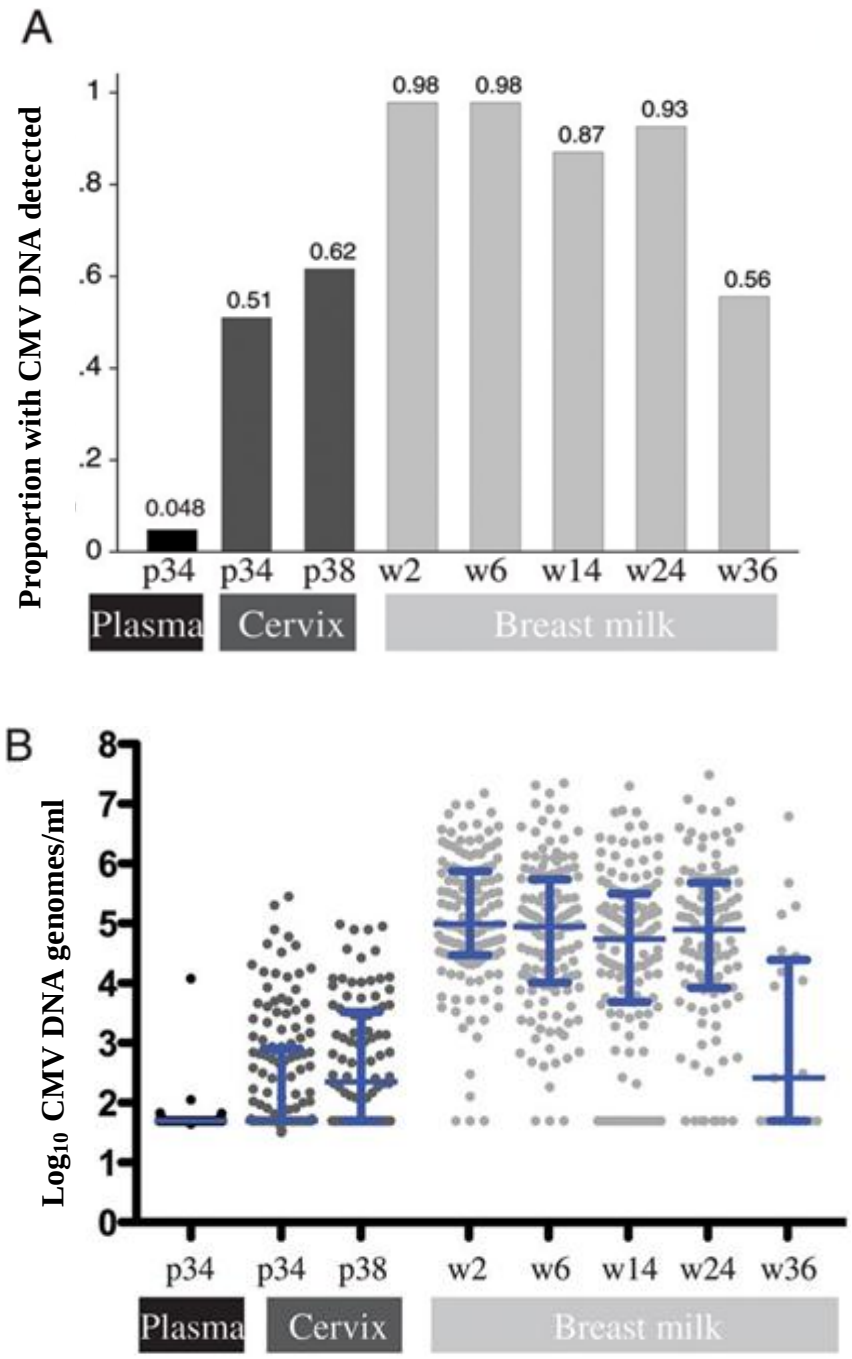


Figure 6-2: Cytomegalovirus (CMV) detection in different compartments in HIV-1 positive women.

A: *shows the proportion of women with CMV DNA detected at different study visits in plasma, cervix and breast milk samples;* ***B:*** *shows the CMV DNA level (log_{10} CMV ge/ml) at each visit for plasma, cervix and breast milk samples. Blue midlines depicit median viral load with bars depiciting interquartile range adpated from, (Slyker, Farquhar et al., 2014)*

p= number of weeks gestation of pregnancy and w = weeks postpartum

6.4.4 Relationships between CMV detection in multiple maternal compartments

142 women had samples from all three compartments analysed.

Data analysis resulted in four patterns of CMV DNA detection across the three compartments (Figure 6.3A). The majority of women had CMV detected in both cervix and breast milk (62%). Only 1 woman had no detectable CMV in any compartment tested (0.7%), and although her CMV IgG status was unknown her infant was CMV DNA positive on the DBS at the 2 week visit, suggesting that she was seropositive. The 7 women with detectable CMV DNA in the plasma also had CMV DNA detected in the other compartments (both cervix and breast milk).

Regression models were used to measure the relationship between either the detection of CMV (blood, cervix) and CMV viral load (breast milk) between the different compartments, adjusting for both plasma HIV-1 viral load and valaciclovir allocation. CMV DNA detection in plasma at 34 weeks gestation was associated with CMV DNA detection in the cervix (OR=7.2, p=0.04), and CMV viral load in breast milk (β=0.65, p=0.03; Figure 6.3B). There was also a strong association between CMV DNA detection at 34 weeks gestation in the cervix and later CMV viral load in breast milk with women with CMV DNA detected in the cervix having approximately half a $\log_{10}$ higher CMV viral load in their breast milk, when compared to women without CMV detected in the cervix (β=0.47, p=0.005).

A)

Plasma	Cervix	Breast milk	% of women CMV DNA detected (number)
-	-	-	0.7% (1)
-	-	+	32% (46)
-	+	+	62% (88)
+	+	+	4.9% (7)

B)

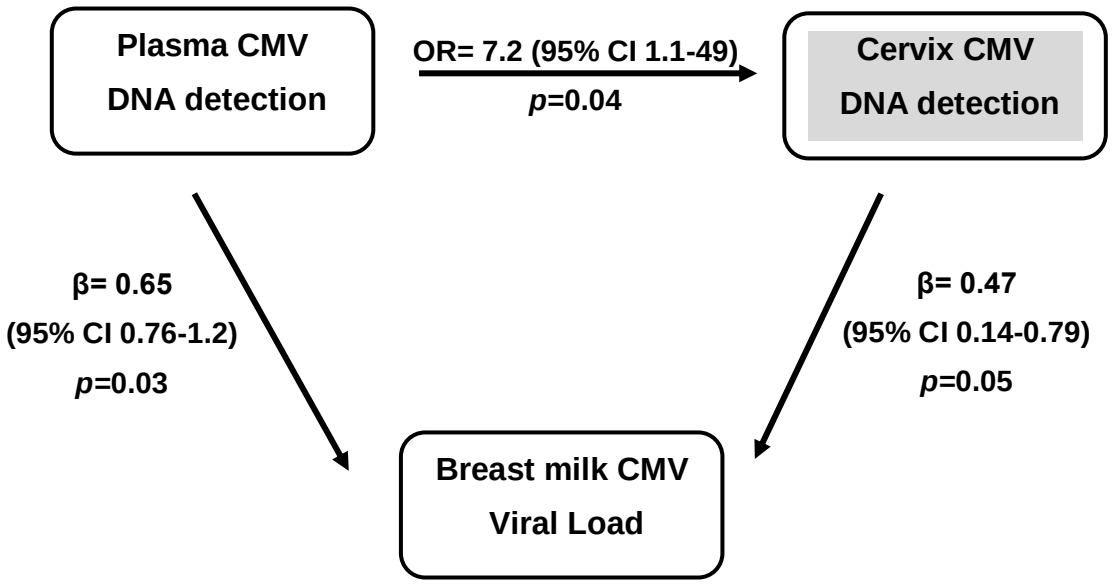


Figure 6-3: Pattern of cytomegalovirus DNA detection and relationship between different maternal compartments.

A: *shows the 4 patterns of CMV DNA detection and percentage of women in each category from 142 women with sampling in all compartments.* ***B:*** *shows the relationship between CMV DNA detection in different maternal compartments. Odds ratios (OR) and β coefficients from regression models showed; i) detection CMV DNA in plasma at 34 weeks gestation was a predictor of CMV DNA detection in the cervix and breast milk ii) detection of CMV DNA in the cervix at 34 weeks gestation was a predictor of CMV viral load in the breast milk (adapted from (Slyker, Farquhar et al., 2014). 95% confidence levels (CI) are shown.*

6.4.5 Relationship between HIV-1, CD4 count, and maternal CMV DNA detection

Maternal plasma HIV-1 viral load was strongly correlated with both cervical CMV DNA detection and breast milk CMV levels (Figure 6.4). Each $\log_{10}$ increase in plasma HIV-1 viral load was associated with a >2-fold increased likelihood of detecting CMV in the cervix (OR=2.3, p<0.001) and with a 0.15 $\log_{10}$ higher CMV DNA level in breast milk (p=0.004). No association was found between plasma HIV-1 viral load and the detection of CMV DNA in the plasma (p=0.2).

The relationships between maternal CD4 count and CMV DNA detection in the various compartments were assessed. Women were assigned to either a high or low CD4 count category, based on the cohort median at baseline (450 cells/µl). No association between CD4 category and CMV DNA detection was found in the plasma (p=0.2) or cervix (p=0.5). However, having a CD4 count above the cohort median was associated with approximately 0.5 $\log_{10}$ reduction in CMV levels in the breast milk (β=-0.45, p=0.008).

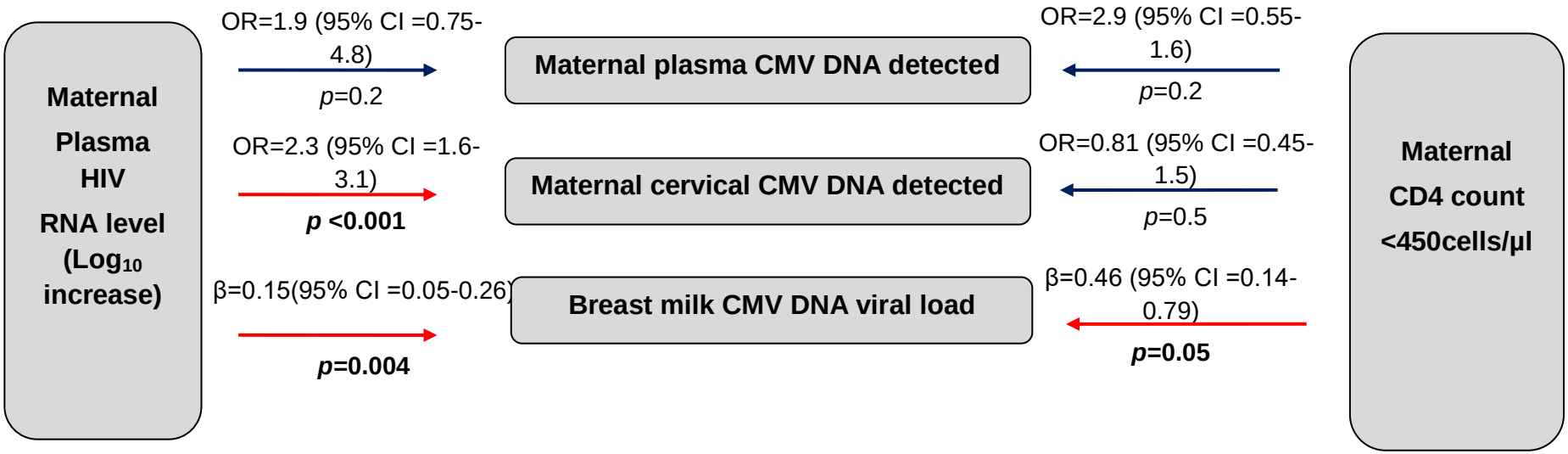


Figure 6-4: **Relationships between HIV-1 plasma viral load or CD4 count and CMV DNA detection in different maternal compartments.**

The odds ratios (OR) and beta coefficients (β) with 95% confidence intervals (CI) derived from regression models are shown. CD4 was dichotomised at the median count of 450 cells/µl at 34 weeks gestation. Models including breast milk were exclude time points after 6 months as very few women were still breastfeeding. Significant relationships are indicated by red arrows (adapted from, (Slyker, Farquhar et al., 2014)

Abbreviations HIV- Human immunodeficiency virus, CMV- Cytomegalovirus

6.4.6 Predictors of acquisition of CMV by infants

In total 66% (87/131) of infants had detectable CMV DNA in DBS during the first year of life, 3% (4/131) had detectable CMV at birth indicating congenital infection, 6% (8/131) of infants became CMV positive before 2 weeks of age, indicating congenital or early acquisition from intrapartum infection; and another 3% (4/131) became positive at their 2-week visit. The bulk of remaining infections (47%) were detected between 6 weeks and 14 weeks of age (62/131).

Using univariate Cox regression, CMV DNA viral load in breast milk at 2 weeks postpartum was associated with earlier infant CMV detection (HR=1.5, 95% CI 1.2-1.9; p=0.001). However breast milk HIV-1 RNA detection was not associated with infant CMV acquisition. Breast milk CMV levels remained associated with infant CMV infection when adjusting for maternal CD4 category (HR=1.4, 95% CI 1.1-1.8; p=0.003) but not when adjusting for HIV-1 RNA viral load (HR=1.2, 95% CI 0.95-1.5; p=0.1). Plasma HIV-1 viral load in pregnancy and at 2 weeks postpartum was also significantly associated with infant CMV detection (HR1.5, 95% CI 1.2-1.9; p<0.001 and HR1.4, 95% CI 1.1-1.7; P=0.005 respectively).

A CD4 count below the cohort median (450 cells/µl) at 32 weeks gestation was associated with ~80% increased risk of infant CMV acquisition (p=0.008).

There was also a trend for an association between the detection of CMV in the cervix at 38 weeks gestation and the risk of infant CMV (HR=1.5 95% CI 0.88-2.7, p=0.1). However cervical HIV-1 RNA detection was not associated

with infant CMV. No association was found between maternal CMV DNA detection in plasma at 34 weeks gestation and CMV acquisition.

A logistic regression model was used to estimate the probability of CMV transmission according to maternal CD4 count and breast milk CMV viral load. The model estimated the probability of CMV infant acquisition by 1 year of age as a function of maternal breast milk CMV viral load at 2 weeks postpartum for a range of maternal CD4 counts (350 cells/µl, 450 cells/µl, 750 cells/µl and 1000 cells/µl). The probability of CMV acquisition increased as the CD4 count decreased. At a CD4 count of 350 cells/µl the 50% probability of CMV transmission occurred at a DNA load in breast milk of 3.55 $\log_{10}$ CMV ge/ml. This increased to 3.85 $\log_{10}$ CMV ge/ml at 450 cells/µl, 4.75 $\log_{10}$ CMV ge/ml at 750 cells/µl and 5.5 $\log_{10}$ CMV ge/ml at 1000 cells/µl (figure 6.5). These results suggest that maternal immune reconstitution is directly related to the CMV viral load and that mothers showing immune control of virus require a larger infectious dose of virus to transmit to their infant. It suggests that higher CD4 levels in breast milk could potentially impact local containment of CMV reactivation by supporting adaptive immune responses in this compartment.

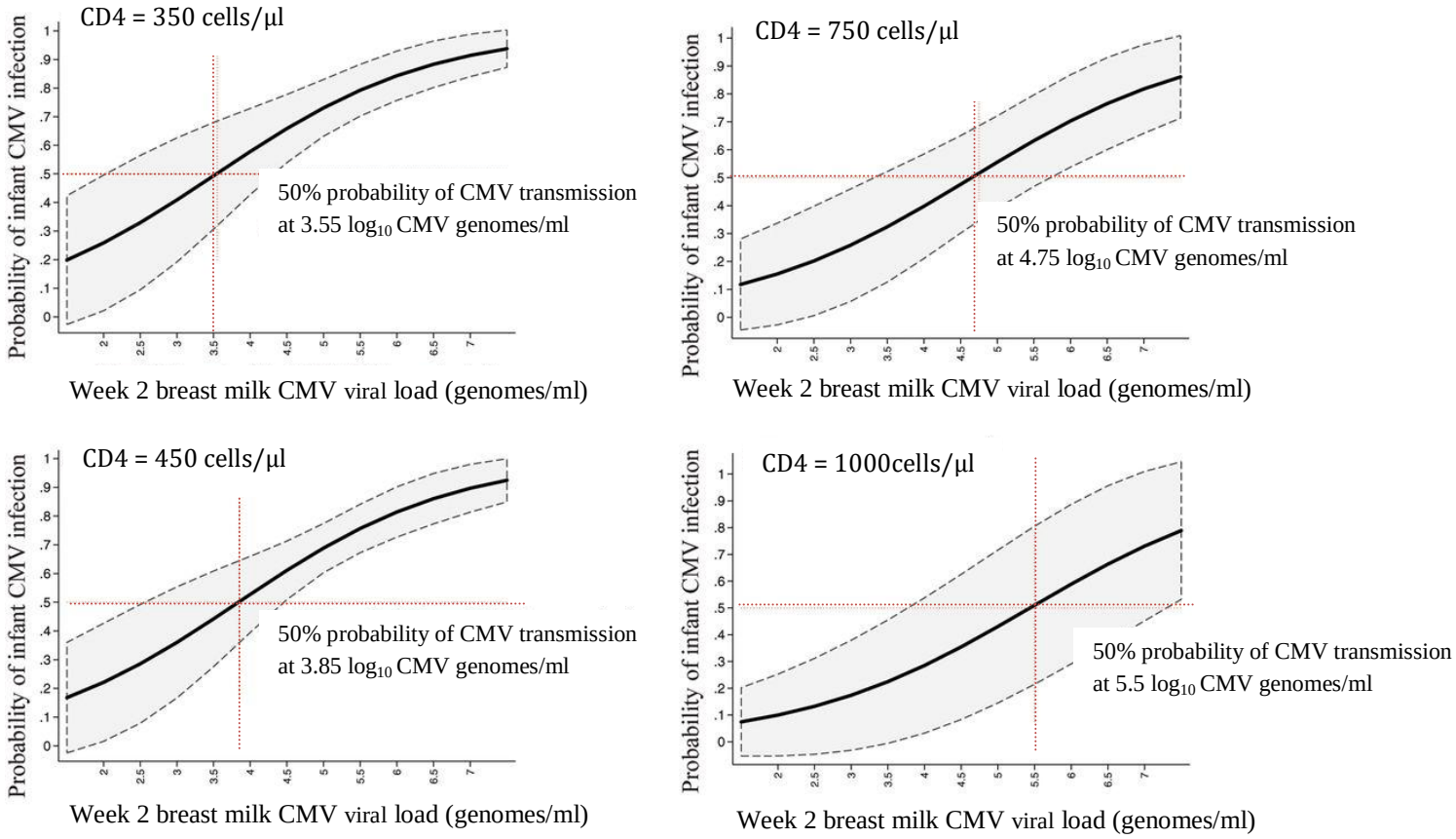


Figure 6-5: Estimated probability of CMV transmission according to maternal CD4 count and breast milk CMV DNA level.

Bold black curves, model probability of infant CMV transmission by 1 year of age as a function of breast milk CMV viral load at 2 weeks postpartum (log_{10} CMV DNA genomes/ml). Each of the 4 graphs represents a fixed maternal CD4 count at 32 weeks gestation. 95% confidence intervals are shown by a grey dashed line, with the red dashed line depicting the breast milk viral load at which there is a 50% probability of CMV trasnsmission (adapted from (Slyker, Farquhar et al., 2014).

6.5 Discussion

CMV is known to cause perinatal infection with acquisition from breast milk a major source of virus. In areas with high CMV seroprevalence breast milk transmission has been show to account for the majority of infants acquiring CMV before the age of one year (Stagno, Reynolds et al., 1980). In Kenya virtually all HIV infected women are co-infected with CMV (Slyker, Lohman-Payne et al., 2009b) with CMV acquisition occurring early so that >80% of Kenyan infants are CMV infected by 3 months of age (Slyker, Lohman-Payne et al., 2009). Perinatal acquisition of CMV infection in the setting of maternal or infant HIV-1 infection has been shown to be a predictor of morbidity and mortality (Slyker, Lohman-Payne et al., 2009b) with HEU infants showing numerous detrimental outcome from perinatal CMV acquisition (Gompels, Larke et al., 2012b).

This study is the first to examine the incidence of CMV detection in different maternal compartments (blood, breast milk and cervical secretions) during the antenatal/postpartum period and to probe the relationship between CMV DNA detection in these compartments in the HIV-1 infected woman and the relationship between maternal CMV DNA detection and infant CMV acquisition. In the majority of women, CMV DNA detection was independently associated with each compartment, and could be ascribed to 4 distinct patterns of detection with only a small subset of women having systemic CMV reactivation with virus detected in all compartments (4.8%).

A correlation between CMV detection in the cervix and later breast milk CMV DNA viral load was found suggesting that similar factors may be affecting CMV reactivation at these two sites. Plasma HIV-1 viral load during pregnancy was

associated with CMV detection in the different compartments, but local HIV-1 levels within breast milk and cervix were not.

Breast milk CMV viral load at 2 weeks postpartum and CD4 count were strongly and independently associated with infant CMV acquisition, with the predicted CMV viral load required for 50% CMV transmission increasing with higher maternal CD4 counts. This suggests that restoring cell mediated immunity may offer some protection against MTCT of CMV, with CD4 cells contributing to protection by secreting cytokines (Gamadia, Remmerswaal et al., 2003)] and by providing help for CTL activation (Khanolkar, Fuller et al., 2004).

Distribution of host cells and localised immunity may play a role in the variable CMV DNA and HIV-1 detection between maternal compartments. Consistent with previous work, we observed an increase in cervical CMV detection over time (Stagno, Reynolds et al., 1975a), and a decrease in breast milk CMV viral load (van der Strate, Harmsen et al., 2001).

Previous studies in HIV negative subjects have shown no correlation between CMV in the genital tract and in the urine, nor between breast milk and saliva. This suggests that the CMV DNA detected was due to localised reactivation in each compartment (Stagno, Reynolds et al., 1975a;Vochem, Hamprecht et al., 1998;Jim, Shu et al., 2009). In this study CMV viral load was highly variable across the different maternal sites; however longitudinal analyses enabled us to probe relationships between compartments.

Plasma CMV DNA detection was rare, being found in only 7 of the women tested and was accompanied by concurrent detection of CMV DNA in the cervix, and later in breast milk, suggesting systemic reactivation. However it should be noted that

CMV DNA detection in plasma may have a lower sensitivity of detection when compared to whole blood so some low level DNAemia may have been missed. CMV DNA detection in the cervix was strongly associated with breast milk CMV viral load. This relationship could possibly be explained by CMV tropism for epithelial and endothelial cells, which are present in both breast and cervical tissue. Additionally, mucosal immunity could result in similar selection pressure on virus in both the cervix and breast milk. CMV-specific effector memory T cells have been detected in breast milk (Ehlinger, Webster et al., 2011), gut associated lymphoid tissue (Shacklett, Cox et al., 2003) and although no data exist for the cervix, CMV specific T cells would be expected to migrate to the cervical mucosa. No relationship between CMV specific cellular immune responses and breast milk was found in a previous study by Ehlinger *et al;* supporting the data presented here and suggesting that mucosal CMV responses may be tissue specific and limited to tissues which are reactivating CMV (Ehlinger, Webster et al., 2011).

HIV-1 viral load in maternal plasma was strongly associated with CMV DNA detection in the cervix and breast milk and maternal CD4 count at 32 weeks was associated to later breast milk CMV viral load. However, HIV-1 and CMV detection in each compartment studied were not correlated.

Previous studies have demonstrated a relationship between CMV and HIV-1 in blood, cervical secretions, semen, and breast milk (Slyker, Lohman-Payne et al., 2009b;Gantt, Carlsson et al., 2008;Sheth, Danesh et al., 2006;Lurain, Robert et al., 2004) which is thought to result from immunosuppression and/or direct HIV-CMV interactions within cells or tissues.

All women in this study had antiretroviral prophylaxis for the PMTCT of HIV. Additionally, the women were randomised to valaciclovir versus placebo for

suppression of HSV 2. Maternal antiviral prophylaxis consisted of twice daily oral zidovudine from 28 weeks and every 3 hours during labour until delivery and single dose nevirapine at the onset of labour. From June 2009, maternal lamivudine and twice daily zidovudine for 1 week postpartum were additionally offered for prophylaxis. The introduction of these antiretrovirals caused a rapid fall in maternal HIV-1 viral load, and valaciclovir was also found to contribute to the reduction of HIV-1 load in plasma and in breast milk (Drake, Roxby et al., 2012b) which may have disrupted the relationship between HIV-1 and CMV in each compartment studied. This in turn may have affected the ability to detect an association between HIV-1 and CMV in the breast milk and cervix. However when data were stratified by treatment allocation, no differences were seen; suggesting valaciclovir had no effect on the association.

CMV shedding in breast milk of seropositive women is common with transmission rates of up to 70% reported (Hamprecht, Maschmann et al., 2001;Dworsky, Yow et al., 1983;Jim, Shu et al., 2004) and CMV DNA load independently associated with risk of CMV transmission to the infant (Jim, Shu et al., 2009;Hamprecht, Maschmann et al., 2008). A direct association between HIV-1 and CMV breast milk viral loads has been described in maternal HIV-1 infection (Gantt, Carlsson et al., 2008). Additionally in this study CD4 count was also directly associated with infant CMV acquisition, independently of maternal breast milk CMV viral load.

In measles infection, reduced placental transfer of measles antibodies from immunosuppressed HIV-1 infected women results in increased susceptibility of their infants to measles infection (Farquhar, Nduati et al., 2005). This reduction of maternal antibody could also occur for CMV antibodies resulting in increased susceptibility of the infants to CMV infection. Transmission of CMV via saliva may

also be an important, yet unmeasured route of CMV transmission in this cohort; immunosuppressed mothers are more likely to have CMV shedding in saliva (Fidouh-Houhou, Duval et al., 2001) and have a higher likelihood of CMV transmission. Also, due to the early acquisition of CMV, other children who may be shedding CMV in their urine and/or saliva could be additional sources of horizontal transmission in the study group (Bello, 1992).

In this study, infant DBS were used to diagnose infant CMV acquisition (no infant plasma samples were taken as part of the original study protocol). As shown in chapter 6, DBS are less sensitive than plasma samples for diagnosis of acute CMV infection and therefore it is likely that the true number of infant infections has been underestimated, as well as the relationships with maternal correlates of CMV transmission. Finally, because the focus for this study was on CMV acquisition, HIV-1 was used in the models as a predictor and CMV as the outcome and so the relationships observed could be bidirectional in nature.

An important observation in this study was that 4 infants (3%) had no detectable CMV DNA on their week 0 sample, but were CMV DNA positive in their week 2 DBS. This would be consistent with the findings of Hamprecht *et al* who detected viraeima in a breast fed child at 12 days of age (Hamprecht, Maschmann et al., 2001) and may have consequences for both retrospective diagnosis of CCMV and screening when DBS are taken at greater than 14 days after birth.

Overall these data suggest that presence of CMV DNA is different between systemic and mucosal compartments and suggests that a relationship may exist between mucosal compartments (cervix and breast). It also highlights the important role for immunosuppression in MTCT of CMV from breast milk. In the absence of a licensed CMV vaccine, these data suggest that early initiation of antiretroviral therapy for the

prevention of CMV acquisition could potentially reduce perinatal transmission of CMV and improve infant outcomes by restoring maternal immunity and reducing HIV-1 levels. This may offer an alternative approach to reducing or delaying CMV acquisition in infanthood.

Chapter 7

7 Diagnosis of congenital CMV infection: potential for newborn screening

7.1 Introduction

The diagnosis of CCMV is challenging because most infected infants are asymptomatic or have nonspecific symptoms. Early postnatal acquisition of infection is common (Peckham, Johnson et al., 1987). Perinatal CMV infection is commonly acquired at birth or through breast feeding which is not associated with CNS damage or disease. Therefore, the timing of sampling is pivotal because a diagnosis of CCMV cannot be made with certainty in children unless samples are available within a few days of birth. With the evolution of molecular techniques (namely PCR) perinatal infection has been detected in blood as early as day 12 of life by PCR, narrowing the sampling time frame to very short period for CCMV investigation (Hamprecht, Maschmann et al., 2001) as demonstrated in chapter 6 in this thesis.

DBS are taken routinely after birth in many countries for biochemical and genetic analysis and are stored for prolonged periods of time. DBS testing is a safe, simple and universally accepted screening tool for commonly inherited and metabolic conditions. The UK newborn screening programme currently screens for phenylketonuria, congenital hypothyroidism , sickle cell , cystic fibrosis (and medium-chain acyl-CoA dehydrogenase deficiency (http://newbornbloodspot.screening.nhs.uk/) (see figure 7.1). Due to the timing of sample acquisition (UK guidelines 5-8 days old), DBS have been shown to be useful to retrospectively diagnose CCMV in a child presenting with compatible symptoms later in infancy or childhood (Barbi, Binda et al., 2006;Vauloup-Fellous, Ducroux et al., 2007;Walter, Atkinson et al., 2008).

NHS

Screening Programmes

Newborn Blood Spot

Table of conditions screened in the UK

Country	Screening condition	Full implementation	Screening ended
Scotland	PKU CHT SCD CF MCADD	1970 1979 2010 2003 2010	Screening for galactosaemia ceased in 2003
England	PKU CHT SCD CF MCADD	1969 1981 2006 2007 Feb 2009	
N Ireland	PKU CHT SCD CF MCADD Homocystinuria Tyrosinaemia	1969 1980 Mar 2012 1984 Aug 2009 1969 1969	
Wales	PKU CHT SCD CF MCADD Duchenne's muscular dystrophy	1970 1981 2013 Dec 1996 Jun 2012 Jul 1990	Nov 2011

PKU: phenylketonuria; CHT: congenital hypothyroidism; SCD: sickle cell disease; CF: cystic fibrosis; MCADD: medium-chain acyl-CoA dehydrogenase deficiency

NHS Newborn Blood Spot Screening Programme – 12/08/13 v1.2

Figure 7-1:The genetic and biochemical conditions screened for as part of the UK Newborn Screening Programme (reproduced with permission)

Screening is defined as, ‘a systematic application of a test to asymptomatic individuals at risk of a specific disorder to trigger further investigation or preventative action’ (Wald, 2008) and should be performed if the benefits of identification outweigh the costs and potential harms. General criteria for screening have been proposed by Wilson and Jungner on behalf of the World Health Organisation (see table 7.1) (Wilson J JG, 1968). In a recent review Dollard *et al.* (Dollard, Schleiss et al., 2010) showed that CCMV now easily satisfies most of the Wilson and Junger criteria for screening. One exception is that antiviral treatment has not been proven to benefit asymptomatic CCMV children. However, it does reduce the incidence of future disease amongst those identified because they have CNS symptoms (Kimberlin, Lin et al., 2003) and in those with symptoms not necessarily involving the CNS (Kimberlin, Jester et al., 2015).

Overall there is a general consensus that newborn screening would identify many of asymptomatic congenitally infected infants at risk of developing late onset disease. This prompt recognition could lead to early intervention such as antiviral treatment to prevent the onset or progression of hearing loss and need for cochlear implants. Thus, early diagnosis could allow interventions to compensate for hearing loss. Despite this, no country currently screens for CCMV.

Table 7-1: Criteria for screening as proposed by Wilson and Jungner

Disease	*The condition being screened should be an important health problem* *The natural history of the condition should be well understood* *There should be a detectable early stage*
Test	*A suitable test should be devised for the early stage* *The test should be acceptable* *Intervals for repeating the test should be determined*
Treatment	*Treatment at early stage should be more beneficial than at a late stage* *Adequate health service provision should be made for the extra clinical workload resulting from screening* *The risks, both physical and psychological, should be less than the benefits* *The costs should be balanced against the benefits*

Newborn screening for CMV has been suggested using saliva or urine samples due to their reported high sensitivities (Boppana, Ross et al., 2011). However DBS have the advantage that newborn screening programmes using DBS (Guthrie cards) are already in place in many countries. Thus DBS samples are available on every child born without any additional sample requirements. Dollard *et al*. have reviewed several aspects of laboratory techniques associated with newborn screening and have concluded that in view of the existing programmes, DBS would be the most practical specimen of choice (Dollard, Schleiss et al., 2010).

A newborn screening test requires high throughput capacity. However the standard nested PCR assays with the highest reported sensitivities for CMV DNA detection from DBS are not suitable for high throughput due to inherent potential for contamination and/or labour intensive methods.

The CMV and Hearing Multicentre Screening study (CHIMES) is being conducted in the United States. This seven year study aims to develop a diagnostic assay for the detection of CCMV, with the overall aim of using the assay for universal screening. The study aims to recruit over 100,000 infants to determine whether universal screening should become health policy and thereby run in conjunction with already established newborn hearing screening programmes. Preliminary results have found a sensitivity of DBS testing of only 34% in 92 infants testing positive for CCMV from over 20,000 infants tested when compared to saliva testing. (Boppana, Ross et al., 2010) However it is not clear whether their extraction and PCR methodology has contributed to the low sensitivity. More studies are required to determine whether other high throughput methods can produce acceptable testing sensitivities.

7.2 Aims

Overall, I hope to adapt the laboratory methods developed in chapter 4 to help introduce a national diagnostic service for the retrospective diagnosis of CCMV from DBS.Once this is established I will then assess the feasibility of screening newborns for CCMV by the laboratory analysis of the newborn DBS sample by assessing the applicability of the high throughput nucleic acid extraction and one step nested protocol for high throughput testing of DBS for CMV DNA.

To achieve this I have the following aims:

- To introduce an internal control into the DBS assay. This will serve a dual purpose; firstly as an internal control to ensure that negative DBS results are true negatives and are not due to inhibition of the PCR. Secondly it will act as a standard for quantitation, meaning that a ratio of CMV genomes/ cells can be determined and results can be normalised for comparative studies.

- To develop an easy but sensitive assay with high analytical performance for detection of CMV DNA from DBS that is suitable for high throughput screening.

7.3 Materials and methods

7.3.1 Routine testing for the retrospective diagnosis of CCMV

The Department of Virology introduced routine diagnostic testing of DBS for the retrospective diagnosis of CCMV in 2005 following full assay validation (see chapters 3 and 4). A review of DBS testing was performed for all samples received between 1st January 2005 and 31st December 2012.

7.3.2 Development of an internal control for the DBS assay

A β globin quantitative real-time PCR was developed (as described in chapter 2). The real time PCR was designed to amplify a conserved sequence in the human β-globin gene. The primers and probe sequences were taken from a previously published method (Lo, Tein et al., 1998) and adapted for use in the DBS assay.

7.3.3 Generation of a quantitation plasmid standard

To accurately quantify the amount of cellular DNA, a plasmid containing the β globin PCR target region was cloned and quantified (see chapter 2).

Generation of the β globin PCR standard curve

The standard curve was determined using a known copy number plasmid generated in the cloning experiments. A serial dilution was run from $1x10^7$ to $1x10^2$ ge/ml in triplicate on 10 different runs. The mean cycle threshold value was taken for each dilution and was plotted onto a standard curve.

Amplification plots were visualised and calibration curves were constructed using ABI 7500 system SDS Software version 1.3 (Applied Biosystems, Warrington, UK)

Linear regression was used to determine the slope of best fit and intercept for the standard curve.

7.3.4 One Step Nested PCR development

A one step nested PCR was developed to amplify a target in exon 4 of the major immediate early region of CMV (UL123).

Two sets of primers were modified from a previously published method (Taylor-Wiedeman, Sissons et al., 1991) and an internal FAM/ZEN double quenched probe was designed to allow detection of amplified products in the same tube with minimum background florescence. The published external primers (outer 1, 2) were modified to give an annealing temperature at least 10°C higher than the internal primers (inner 1,2). Theoretically this would allow first round PCR amplification to be carried out at 68°C where only the external primers would bind and amplify target sequence. The extension temperature was then reduced to 55°C to allow amplification of the target with both the external and internal primers. During this extension the probe will bind to target sequence and be degraded; resulting in fluorescence which can be monitored in real time. Double quenching was used to reduce background and improve reporter signal. Primers and probe were purchased from Integrated DNA Technologies (Leuven, Belgium). A schematic diagram of the nested PCR (figure 7.2) and the primer and probe sequences for the one step nested PCR are summarised in Table 7.2.

Optimisation of assay condition

The real time PCR assay was developed and evaluated on ABI PRISM 7500 with Sequence Detection System software Version 1.3 (Applied Biosystems).

The PCR cycling conditions were fixed at a set temperature. Therefore the primer and probe concentrations were optimised by a 'chessboard' titration. Each primer set was tested from 0.2µM to 1.2µM (in 0.2µM increments) with a set probe concentration of 0.3µM. Following primer optimisation, serial dilutions of the probe (0.2µM to 1µM) were run to determine optimal probe concentration that gave the minimum Ct for 100 genomes/reaction.

One step nested PCR amplification

DNA amplification was performed in 30µl total reaction volume.

Each reaction contained 10µl of DNA extract 15µl QuantiFAST mastermix (Qiagen, UK), 1µM of each primer and 0.2µM of probe. Real time PCR was carried out in a TaqMan 7500 system (Applied Biosystems, UK). PCR conditions were: template denaturation and activation of Taq polymerase for 10 minutes at 95°C was first followed by 15 cycles of 95°C for 15 seconds and 68°C for 45 seconds. This was followed by a second cycling step of 95°C and 55°C for 30 cycles. The TaqMan was set to acquire data during the second cycling step at the 55°C extension

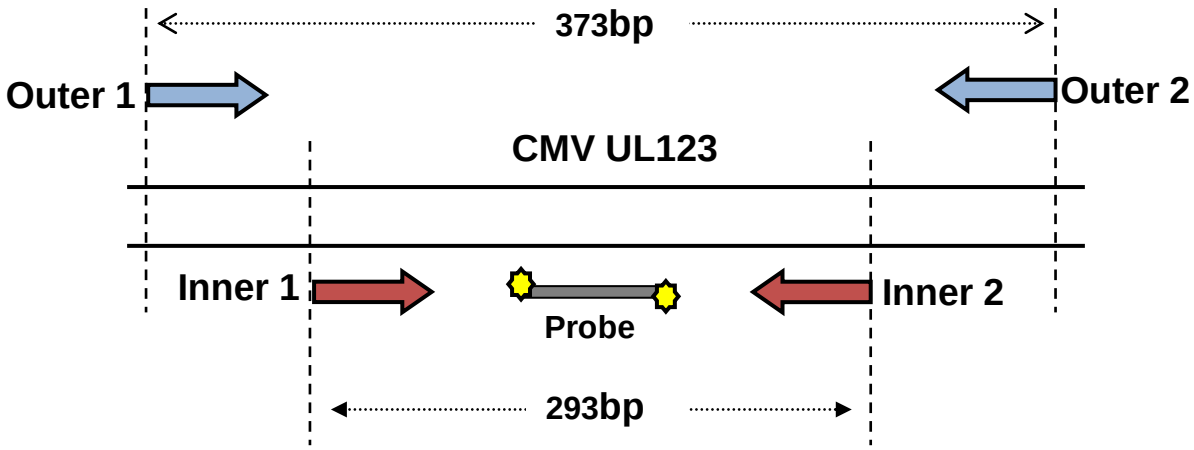


Figure 7-2: Schematic diagram of one step nested PCR
(not to scale)
A one step nested PCR was developed to amplify a target in exon 4 of the major immediate early region of CMV (UL123). The thermodynamic profile was adapted so that initial 15 cycles of PCR yielded a 373bp product from the outer primers (Outer1,2). A 2nd PCR cycle incorporated the inner primers (inner 1,2) and yielded a 293bp product. An internal double quenched probe allowed simultaneous amplification and detection of the amplicon in real time (Atkinson, Emery et al., 2014).

Table 7-2*: Primers and probe used for the CMV UL123 one step nested PCR.*

Primer probe name	***(Sequence 5'-3')***	***Tm***
Reverse Primer 1st round	**GATAGTCGCGGGTACAGGGGACTCTG**	**71**
Forward Primer 1st Round	**GGTCACTAGTGACGCTTGTATGATGACCATGTACGG**	**74**
Reverse Primer Nested	**GTGACACCAGAGAATCAGAGGA**	**58**
Forward Primer Nested	**AGTGAGTTCTGTCGGGTGCT**	**58**
Probe	**6 FAM-AGG AGA CTA /ZEN/GTG TGA TGCTGG CCA A-IB®FQ**	

FAM, 6-carboxyfluorescein; ZEN, internal quencher; IB®FQ Iowa Black dark quencher

Tm= melting temperature

7.3.5 CMV UL123 One Step nested PCR Validation

Sensitivity of one step nested assay

DBS samples were tested from 3 sample sets. Each set was extracted and tested in parallel by the two different detection methods (UL123 one step nested PCR and CMV gB real time PCR).

Sample set 3, the 2011 QCMD CMV DBS panel (CMV/DBS11) was also tested in the CMV UL69 PCR to confirm the effect of the UL123 one step nested on assay sensitivity.

Samples

1. CMV negative and positive DBS prepared from the World Health Organisation first International standard for CMV (who/35/10.2138 report). Serial dilutions from 500,000IU/ml to 100 IU/ml were prepared in a whole blood matrix and 50µl adsorbed onto standard Whatman 903™ DBS cards obtained from the Royal Free Hospital neonatal unit. The prepared cards were allowed to dry for a minimum of 48 hours prior to testing.

2. 20 DBS samples from newborns with CCMV infection were obtained from an earlier published study (17th BPSU Annual Report 2002). The cases were diagnosed by PCR or isolation of CMV from blood, urine, saliva or tissue taken via biopsy within 3 weeks of birth, or maternal serology consistent with CMV seroconversion. In addition, 6 DBS were received from infants identified through the BEST study from failed newborn hearing screening programme (NHSP).

3. The 2011 CMV DBS panel (ref CMV/DBS11) obtained from Quality Control for Molecular Diagnostics (QCMD, Glasgow UK) the panel comprised of 10 DBS. Each spot was prepared from a whole blood matrix with varying concentrations of CMV (AD169 strain). The CMV concentration ranged from 625-20,000 copies/ml. It should be noted that the DBS received as part of the panel are larger than the diagnostic newborn screening cards, with one DBS stated to be equivalent to 50µl of whole blood.

7.3.6 Assay specificity

In addition, to determine the specificity of the assay, 200 blood samples from donor/recipient CMV negative solid organ transplant patients were analysed. These samples had been received diagnostically for routine CMV screening post transplant and found to be CMV negative in our diagnostic RT PCR assay (Atabani, Smith et al., 2012).

7.3.7 Assay reproducibility

In addition, to determine the reproducibility of the assay, a set input of 100 genomes/reaction of CMV laboratory strain Ad169 was tested on 20 separate runs.

DNA extraction from DBS

An area of 100mm² of DBS was used for extraction which equates to a semicircle of DBS. To prevent contamination the scissors used to cut the DBS were cleaned with 0.1M hydrochloric acid between cards. Each DBS extract was analysed in triplicate. Nucleic acid was extracted using the QIAsymphony automated extraction system

with the QIAsymphony DNA Mini Kit (Qiagen, Crawley, UK) as previously described in chapter 5.

DNA extraction from whole blood

Nucleic acid was extracted using the QIAsymphony automated extraction system with the QIAsymphony DNA Mini Kit (Qiagen, Crawley, UK) as per manufacturer's instructions. Extraction was carried out using the "VirusBlood200_V5_DSP" protocol with an elution volume of 60µl. Each blood sample was analysed in triplicate.

CMV gB and UL69 PCR

The single round real time CMV gB and UL69 PCRs were used as previously described in Chapters 3 & 4.

7.3.8 Throughput characteristics and methodology

The applicability of the high throughput methods were evaluated by testing a large cohort of DBS samples. DBS were received as part of the maternal Valacyclovir and Infant Cytomegalovirus Acquistion: A randomised Controlled Trial among HIV Infected women. The cohort was selected to test the high throughput methods due to the large number of DBS and known high rate of CMV acquisition in infants in the first year of life. Applicability was determined by the maximum number of samples per run of the QIAsymphony automated system and the current methodology; the EasyMag extraction system.

DBS samples

Infant blood was collected by heel prick onto filter paper at birth, 2, 6, 10, 14 weeks of age and 6, 9, and 12 months of age. The resulting DBS were stored at room temperature. A total of 141 babies took part in the study with 1027 DBS received for testing. Blood from 3x6 mm DBS was extracted using the QIAsymphony DNA mini kit as previously described in chapter 5.

CMV DNA detection

Real-time quantitative PCR was used to detect the CMV glycoprotein B gene as previously described in chapter 3. CMV viral loads from DBS were normalised to copies/10^6 cells against a β-globin standard with a lower limit of detection of 100 copies/million cells as described in chapter 2.

7.4 Results

7.4.1 Routine diagnostic testing

Using the developed methodology, the Department of Virology introduced routine diagnostic testing of DBS for the retrospective diagnosis of CCMV in 2005 following full assay validation. An audit of all DBS testing performed between 2005 and 2012 has been performed.

The department received 1522 requests for DBS testing for the time period 1st January 2005 to 31st December 2012. Of these requests 74 were received as part of an ethically approved study (CHIC study) whose results were published in 2008 and discussed in chapter 4 (Walter, Atkinson et al., 2008). Excluding these study DBS, the department received 1448 diagnostic requests. The age of the DBS at testing ranged from 1 month to 20.2 years with a median age of 2.1 years.

An increase in sample numbers was seen over the 7 year period (see figure 7.3) but the overall rate of positivity remained fairly constant 9.8-14.5% for the period 2007 to 2012 (see Table 7.3). A total of 177 of the DBS received tested positive for CMV DNA (12.2%) age range 1 month to 16.8 years during the study period. A mean positive rate of 12.3% was seen between 2007 and 2012 (9.8-13.8%) (see Table 7.3).

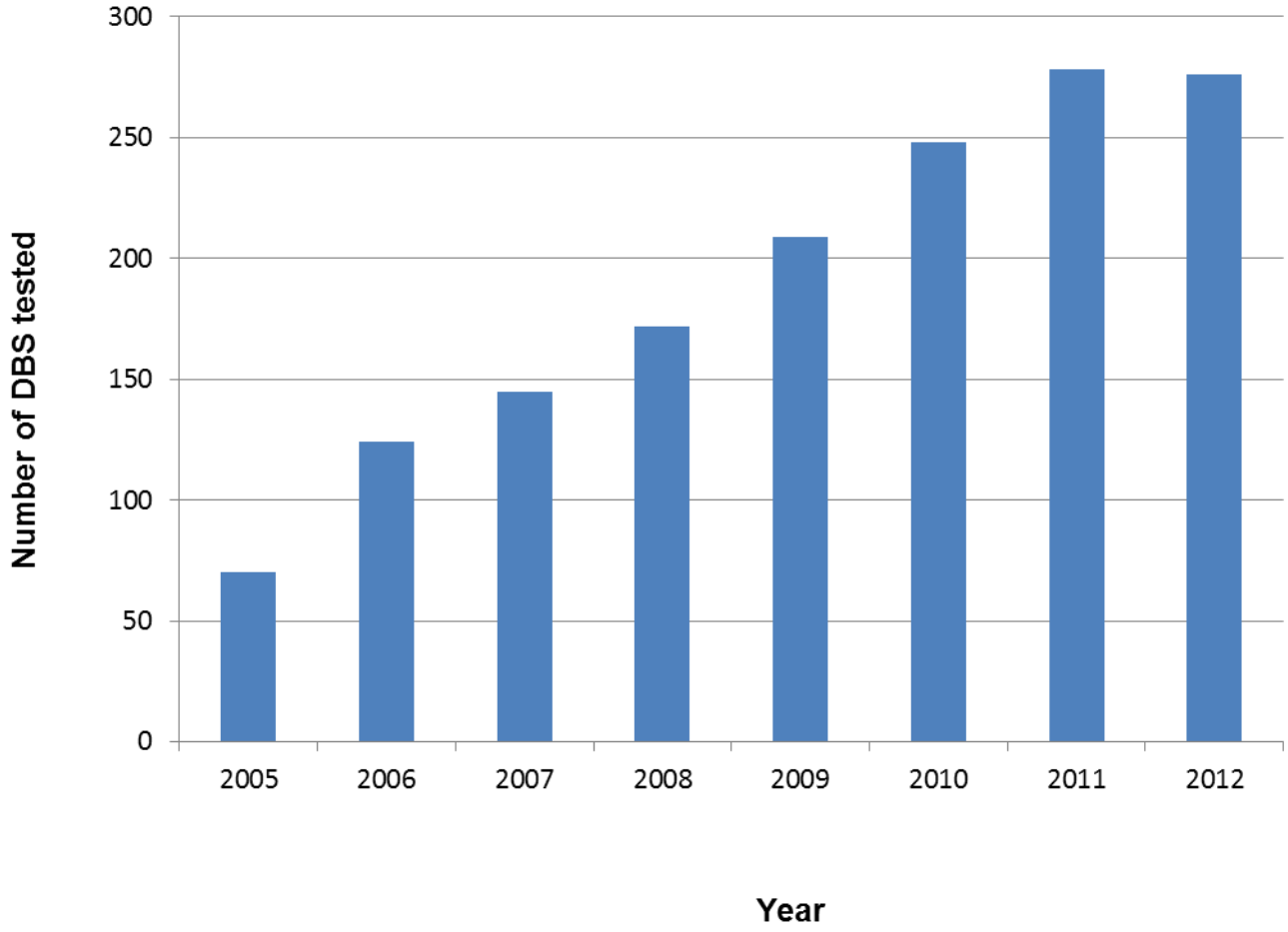


Figure 7-3: ***The number of dried blood spots received each year for retrospective testing for congenital cytomegalovirus in the Department of Virology Royal Free Hospital between 2005 and 2012.***

Table 7-3: ***The breakdown of the number of dried blood spots tested, median age of child at testing, percentage testing positive for cytomegalovirus DNA, and median age of the child testing positive for the samples received for retrospective diagnosis of congenital CMV between 2005 and 2012 in the Department of Virology, Royal Free Hospital***

Year	*Number of DBS*	*Median age at testing (years)*	*Proportion of CMV positive DBS (%)*	*Median age of positive DBS (years)*	*IQR (years)*
2005	**70**	**5**	**29**	**2.8**	**0.25-6.5**
2006	**124**	**2.5**	**30.6**	**2**	**0.16-6**
2007	**145**	**2**	**14.5**	**3**	**0.4-7**
2008	**172**	**1.92**	**11**	**4**	**1-7**
2009	**209**	**1.08**	**11**	**0.75**	**0.25-2.83**
2010	**248**	**1.83**	**13.8**	**1.5**	**0.58-4.08**
2011	**278**	**0.92**	**13.67**	**0.5**	**0.08-2.67**
2012	**276**	**1**	**9.8**	**1.2**	**0.25-4.05**

A decrease in the age of cards tested has been shown over the time period, with the median age of the DBS at time of testing reducing annually from 5 years in 2005 to 1 year in 2012. This was also reflected in the median age of the cards testing positive for CMV DNA with the median dropping from 4 years in 2008 to 1 year in 2012 (Table 7.3).

The clinical details of the 155 DBS requests for the time period June 2012- Dec 2012 were analysed. The majority of requests were received from children with unexplained SNHL (47%). However a large proportion of cards tested were received without any clinical information (35%). During this period 8 DBS tested positive for CMV DNA. 4 of which were received for investigations of SNHL, 3 had no clinical details provided and 1 DBS had both prematurity and SNHL listed as clinical indicators for testing.

7.4.2 β globin validation results

A standard curve (Ct values/copies β globin) was generated using the plasmid standard. A dilution series from $1x10^7$ to $1x10^2$ genomes was run in triplicate on 10 different runs with the mean cycle threshold obtained used to obtain the standard curve.

The standard curve derived showed a high R^2 value 0.996 across the dynamic range 10^3−10^7 ge/ml.

To determine the $\log_{10}$ β globin load the following equation was used.

$$y = -0.3216x + 12.40$$

Where *y* = Ct value of the unknown sample.

Once the absolute β globin load was determined this could be used to normalise the CMV results to the number of CMV genomes/10^6 cells.

7.4.3 One step nested PCR results

Optimisation of assay condition

Following titration an optimum concentration of 1µM for each primer and 0.2µM of probe was found to give the lowest Ct with the highest normalised fluorescent signal at a set input of 100 genomes/reaction.

Detection limit of one step nested PCR

The sensitivity of the one step nested PCR assay was determined using triplicate testing of DBS spotted with whole blood serial dilutions of the WHO CMV international standard. The analytical sensitivity per well was found to correlate to an original blood sample which contained 500 IU/ml (2.08 IU/input) figure 7.4.

The QCMD samples showed a similar detection limit with the lowest CMV concentration DBS sample (625 ge/ml) giving a positive result in the nested assay, using a previously determined conversion factor of 1.25 genomes/IU.

When the Ct value was plotted over the $\log_{10}$ CMV viral load the coefficient of determination (R^2) value was 0.972. This shows a good linear relationship between Ct and the CMV viral loads tested, suggesting that the Ct is related to CMV concentration in the linear phase of the one step nested PCR (see figure 7.4 inset).

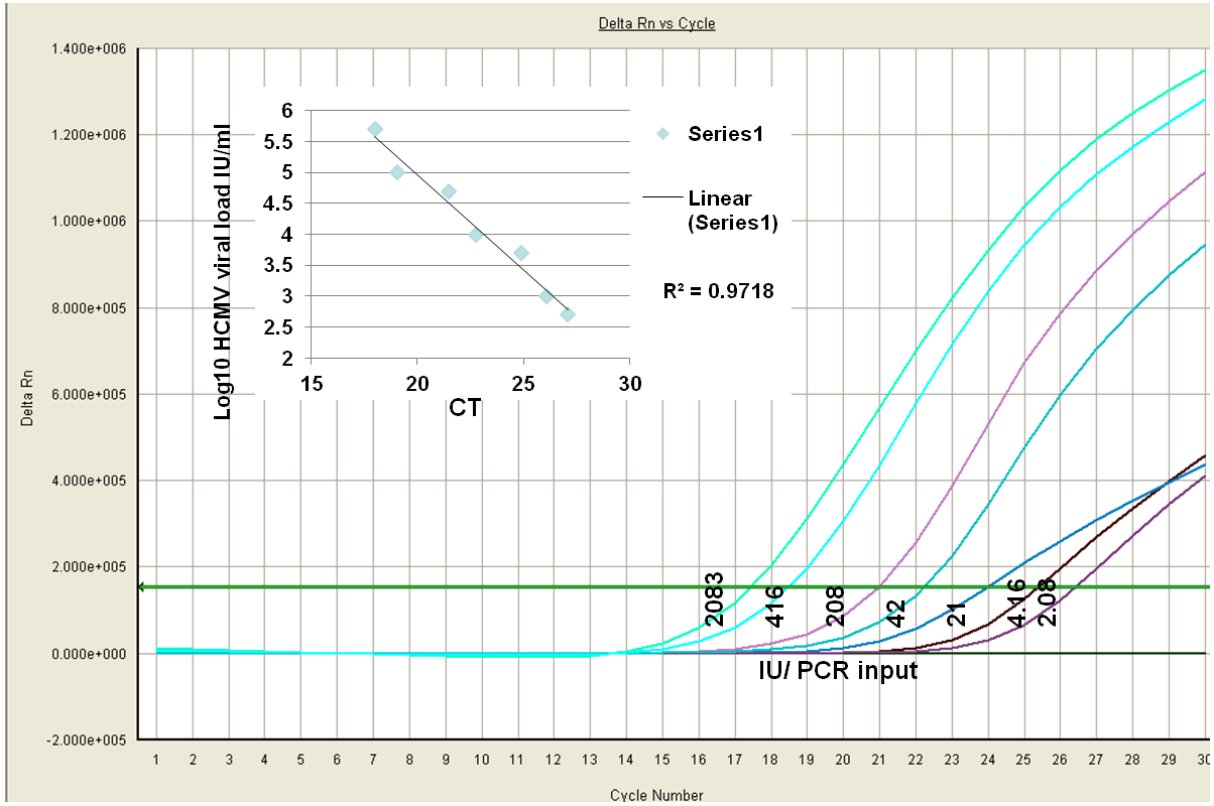


Figure 7-4: ***Real Time amplification of dried blood spots prepared from World Health Organisation international standard using one step nested UL123 assay***

Main figure shows amplification curves obtained from DBS spiked with serial dilutions of the 1st WHO international CMV standard and IU/PCR input.

Inset shows the linear range of the one step nested PCR by plotting cycle threshold over log10 CMV viral load

Specificity of one step nested PCR

None of the 200 CMV negative blood samples showed amplification when tested in triplicate in the one step nested PCR assay.

Reproducibility of one step nested PCR

For a set input of 100 genomes/reaction (CMV Ad169) the assay showed good inter-assay reproducibility. For 20 separate runs the mean Ct was 21.29 with a standard deviation of 0.94 and a variance coefficient of 0.04.

Performance and evaluation of one step nested PCR with DBS sample

Results obtained with the one step nested PCR assay were compared to those generated using the CMV gB assay. All samples were tested in parallel using the equivalent input.

In the QCMD panel all 9 CMV positive samples were correctly identified (100%) in the one step nested PCR assay. In comparison only 6/9 (67%) positive samples were identified with the CMV gB assay. The one true CMV negative DBS showed no amplification in both assays. The DBS with discordant results corresponded to the 3 samples with the lowest viral loads 625, 1250 and 2500 ge/ml (viral loads stated in the QCMD final report). To confirm that enhanced detection was not due to the target region or primer design, the QCMD panel was also tested using a real time PCR CMV UL69 assay. The same 6/9 samples tested positive in the assay for the UL69 region as the gB assay confirming these results with an independent primer set.

DBS samples obtained from 20 children with laboratory confirmed CCMV gave positive results in 18/20 (90%) samples with the one step nested assay. 16/20

samples tested positive in the CMV gB real time assay (80%), again showing enhanced detection with the one step nested PCR (figure 7.5). The two negative samples in the one step assay were also negative in the gB assay. In addition 6 DBS were received from infants identified through the BEST study from failed newborn hearing screening programme (NHSP) 3/6 cards tested positive by the one step assay (50%) compared to 2/6 with the single round PCR (33%).

Overall sensitivity of the one step nested PCR assay in identifying neonates with confirmed CCMV was 21/26 (81%; 95% CI, 60.6% to 93.4%). In contrast the single step gB real time PCR had a sensitivity of 18/26 (69%;95% CI, 48.2%-85.6%) giving an increased detection rate of 12% in children with laboratory diagnosed CCMV infection. After analysis of the clinical data from the 20 confirmed CCMV children the two negative samples came from babies with asymptomatic presentation with a normal clinical outcome (no problems repo rted, apparently normal development) at follow up (20.8 and 20.5 months after birth; (table 7.4). On further investigation, the additional positive DBS in the CCMV failed NHSP identified through the BEST study had a sample of whole blood tested in the neonatal period (prior to the DBS being taken) with a viral load of 7,700 ge/ml. The c0hild presented with unilateral SNHL, subependymal cysts on cranial imaging and received 6 weeks' treatment with valganciclovir.

Overall the outcome was known in 25/26 CCMV children. The mean follow up period was 19.9 months (SD 4.6 months). The one step nested PCR detected CMV DNA in 20/25 samples compared to the gB assay with 17/25 testing positive. On further investigation of the 3 samples positive only with the nested PCR, one DBS was from a symptomatic infant with mild SNHL at follow up. The two other DBS samples were from symptomatic children with bilateral hearing loss at follow-up (table 7.4).

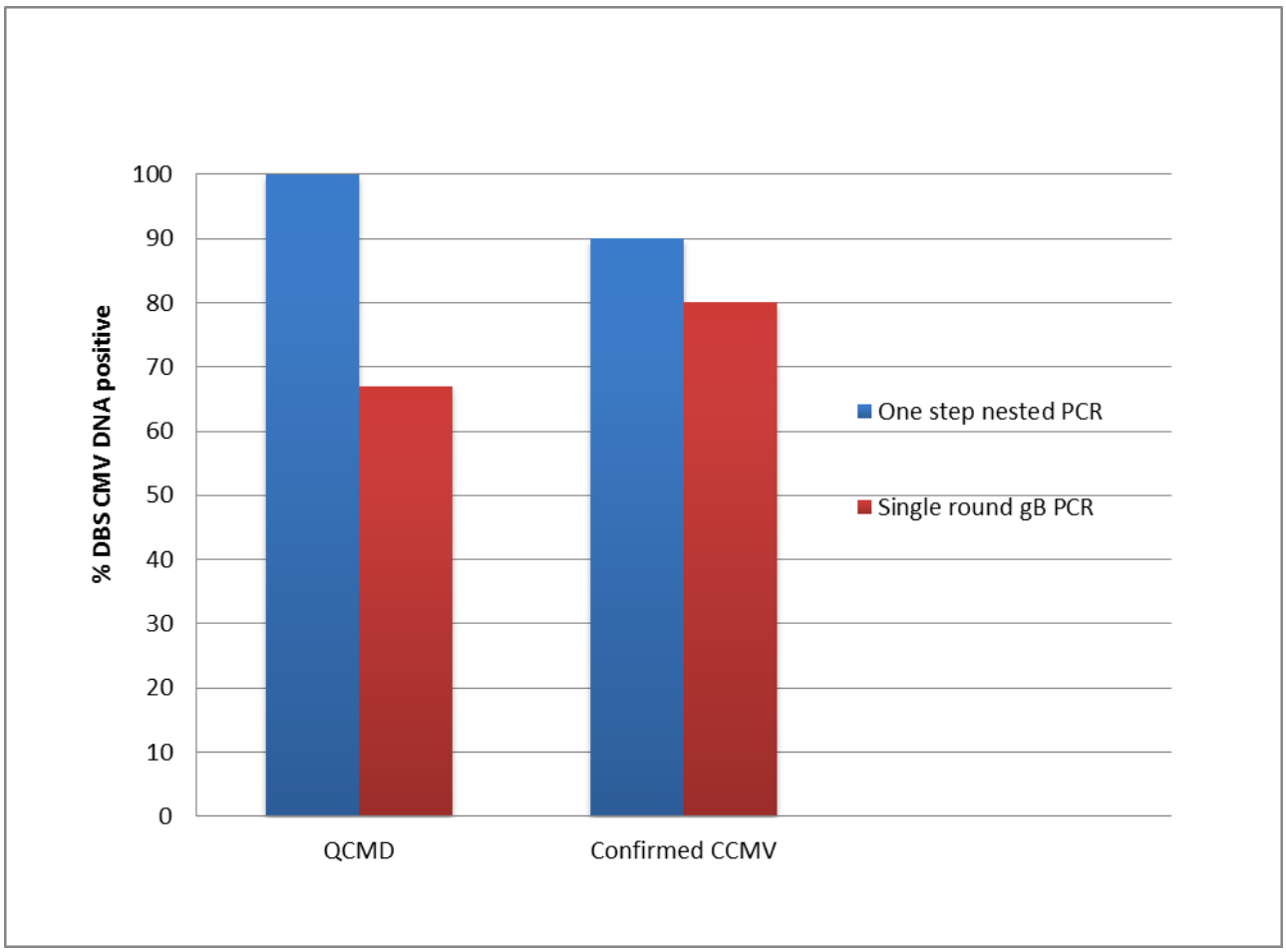


Figure 7-5: ***Sensitivity of one step nested and single round PCR for detecting Cytomegalovirus from dried blood spots in two clinical sample sets.***

Table 7-4: ***Correlation between neonatal presentation of congenital cytomegalovirus, clinical outcome and the proportion of dried blood spots testing positive for CMV DNA in the one step nested PCR.***

Neonatal Presentation	Outcome*	Number of DBS testing positive with single round PCR	Number of DBS testing positive with nested one step PCR
Asymptomatic	**Normal**	**3/7**	**3/7**
Symptomatic	**Normal**	**2/2**	**2/2**
Symptomatic	**Mild**	**5/6**	**6/6**
Symptomatic	**Moderate**	**5/7**	**7/7**
Symptomatic	**Severe**	**3/3**	**3/3**

***Outcome: Normal**- No reported problems **Mild**: Unilateral hearing loss, mild cerebral palsy, mild language delay, **Moderate:** Bi-lateral profound deafness, deafness and other problem **Severe**: Multiple serious problems e.g. Severe global delay. Taken from (Atkinson, Emery et al., 2014).

High throughput results to detect perinatal infection

A total of 1027 infant DBS were tested from 141 infants. 198 DBS samples tested positive for CMV DNA (19.3%). CMV DNA was detected in 93 infants, giving a rate of CMV acquisition of 66% in the first year of life. Among infants who acquired CMV the range of viral loads measured from DBS samples was 154 to 12,224,673 genomes/10^6 cells (2.2 to 7.1 $\log_{10}$ genomes/10^6 cells). The median viral load detected was 44,505 genomes/10^6 cells. The results of the study were published in 2014 (Roxby, Atkinson et al., 2014).

Extraction Time and Sample Throughput

At present all automated extraction systems require a manual pre-treatment step for DBS samples, there is no capacity for using DBS as a primary input.

The QIAsymphony has a capacity of 96 samples (4 racks containing 24 samples each). Each run of 24 takes approximately 1 hour to set up (including pre-treatment) and 1hr 10 minutes on the instrument. The QIAsymphony can be continuously loaded and 96 DBS can be extracted in 6 hours.

Once extracted, DNA extracts can be eluted into a 96 well plate format which can be used directly for PCR-set up (either manual or automated). Depending on detection methods (one step nested or gB PCR) the total processing time to result for a single DBS is 4hrs 30 minutes. For 96 samples total processing time is 8hrs and 30 minutes with a manual (hands on) processing time of 1hour 30 minutes (figure 6.6).

In contrast the EasyMag has a capacity of 24 samples. Each run of 24 takes approximately 1 hour to set up (including pre-treatment) and 40 minutes on the

instrument. The extraction method is more labour intensive as samples need to be loaded and resulting nucleic acid extracts unloaded. There is no continuous loading function. Depending on detection methods (one step nested or gB PCR) the total processing time to result for a single DBS is 4hrs. For 96 samples the total processing time would be is 8hrs, but the hands on time would be much greater at 4hours (figure 7.6).

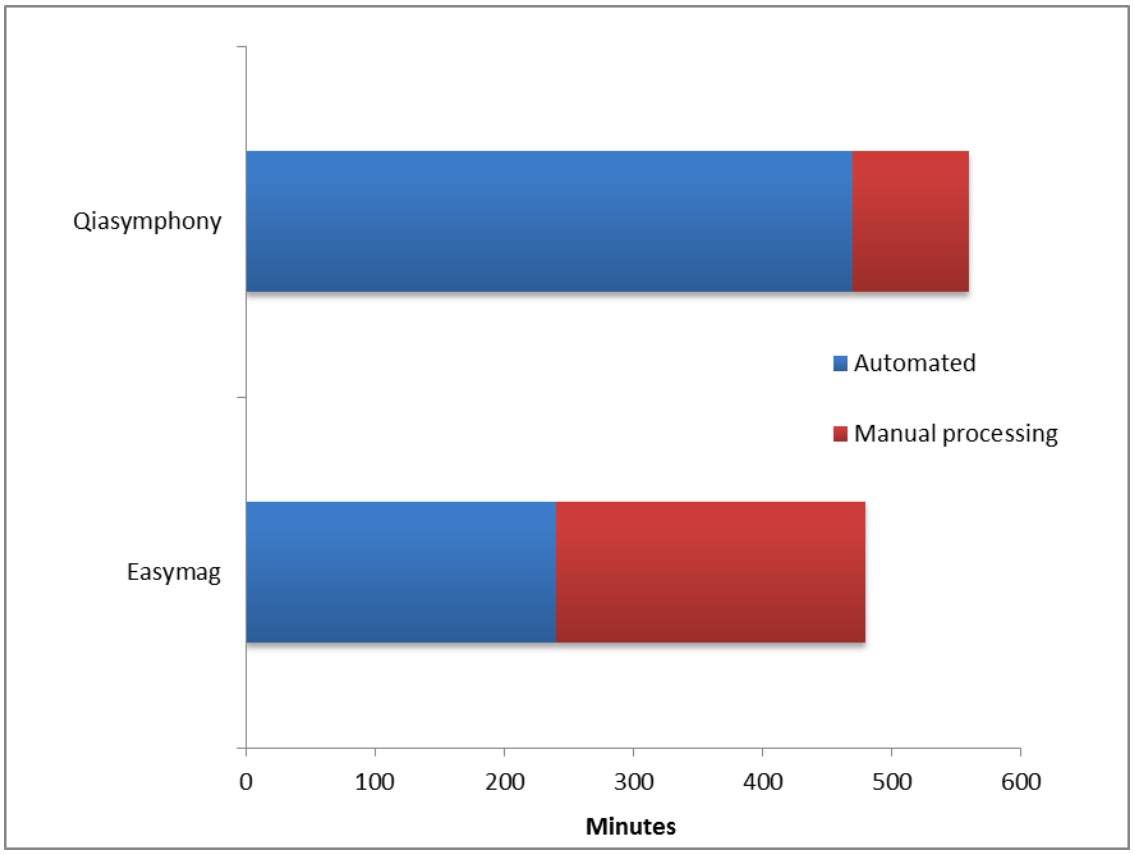


Figure 7-6: The total processing time to result for 96 dried blood spots for both the QIAsymphony and EasyMag extraction system.

The automated (blue) and manual processing time (red) for each system is shown. The QIAsymphony shows the longest processing time but a much reduced manual processing requirement.

7.5 Discussion

The Department of Virology introduced routine retrospective testing for CMV DNA from DBS in 2005. During the audit period a total of 1448 diagnostic requests were received with an increase in test requests over the 7 year period. This highlights both the number of children with compatible symptoms presenting after 21 days of age and the demand for an accurate test.

A decrease in the age of infants tested has been shown over the time period, with the median age of the DBS at time of testing reducing annually to 1 year in 2012. This age at testing is significant for two reasons. Firstly, the CMV DNA has been shown to be stable for at least 24 months on simulated DBS samples (Chapter 3) so clinical samples should ideally be submitted within this time. Secondly, provision of an earlier diagnosis may allow infected children to be recruited into clinical trials to determine if hearing can be preserved during the critical period of speech development or improve language and developmental outcomes through intervention for example, speech therapy, sound amplification and early fitting of cochlear implants in children with hearing loss. At present, evidence from randomised controlled trials requires treatment to be initiated within the first month of life but a new study, starting in 2015, will address whether treatment starting up to the fourth birthday can also provide clinical benefits.

CCMV has long been identified as a potential candidate for inclusion in newborn screening programs because it satisfies several of the criteria for screening as proposed by Wilson and Jungner (Wilson J JG, 1968) as adopted by the WHO to qualify for screening.

The incidence of CMV infection in West London is high in 0.32% of births (Townsend, Forsgren et al., 2013) compared with the incidence of the metabolic and

endocrinological disorders tested for in the core neonatal screening panel (0.01-0.08%).

Universal newborn hearing screening programmes have been introduced by use of otoacoustic emission (OAE) in the UK and most European countries. However, hearing loss with CCMV can be progressive and may develop months after birth in asymptomatic children, establishing that screening for CMV would identify these at-risk children at birth (Dollard, Grosse et al., 2007)

Current methodologies for DBS testing for CCMV have shown poor sensitivity providing no encouragement to begin screening (Boppana, Ross et al., 2010). The work in this chapter demonstrates enhanced detection of CMV DNA from DBS using a novel one step nested protocol when compared with a single round PCR. PCR based testing for viral targets often report high sensitivities but use a much larger sample volume. For example whilst an HIV RNA viral load assay may detect a sensitivity of 50 copies/ml an original volume of sample of 1ml would be required. I hypothesised that the low sensitivity reported from DBS is due to the small sample volume (typically equivalent to between 10 and 80ul of whole blood) and that a highly sensitive detection method would be required in order to detect these very small quantities of CMV, such as nested PCR.

Proof of concept studies show that CMV can be detected in DBS. However the methods reporting the highest sensitivities for CMV DNA detection from DBS are not suitable for high throughput screening because of labour intensive methodologies and/or detection. My study is the first to report a sensitive method which has the potential to be used as a high throughput screening tool. The single tube format should minimise cross contamination compared with conventional nested PCR

methods.

Newborn screening for CMV has been suggested using saliva or urine samples due to their reported high sensitivities (Boppana, Ross et al., 2011). However DBS have the advantage that newborn screening programmes using DBS (Guthrie cards) are already in place in many countries. Thus DBS samples are available on every child born without any additional sample requirements.

A study by Boppana et al recently reported DBS to have low sensitivity when compared to saliva rapid culture. (Boppana, Ross et al., 2010) It has been shown that DNA extraction and CMV detection methods can have significant effects on the reported assay sensitivity. (de Vries, Claas et al., 2009);(Soetens, Vauloup-Fellous et al., 2008) highlighting the importance of assay methodology.

In this chapter, the overall sensitivity of the one step nested PCR was 81%. Interestingly the four CMV negative samples from confirmed CCMV cases with known outcome (table 7.4) were asymptomatic infants with normal outcome at follow up. Studies using PCR quantitation have shown that viral load in urine and blood at birth is higher in symptomatic babies compared to asymptomatic babies, but more importantly that level of viraemia correlates with future sensorineural hearing loss (Boppana, Fowler et al., 2005;Bradford, Cloud et al., 2005). In solid organ transplant patients quantitative studies have likewise shown that risk of disease is associated with high viral load (Cope, Sweny et al., 1997). We proposed a sigmoid relationship for the severity of SNHL versus CMV viral load at birth in chapter 4 (Walter, Atkinson et al., 2008). However this ‘threshold effect’ is novel in the context of newborn screening and denotes that a test without 100% analytical sensitivity that nevertheless detected all children at risk of developing disease would be deemed acceptable for the purposes of screening. My data support this because the DBS

from the asymptomatic children with normal outcome are CMV PCR negative in contrast to the symptomatic children with severe outcome who had CMV detected in all DBS screened. This possibility should now be evaluated in much larger numbers of cases and controls to determine whether an assay with less than 100% sensitivity for infection could nevertheless identify all cases destined to develop disease**.** We should also review at this point the definition of screening: 'a systematic application of a test to asymptomatic individuals at risk of a specific disorder to trigger further investigation or preventative action' (Wald, 2008). The main benefit of screening for CCMV is to identify children at risk of late-onset or progressive hearing loss (not detectable at birth) and other CMV-associated impairments or disabilities and to provide the opportunity for early intervention and antiviral treatment to prevent further damage.

The development of a β globin quantitative real-time PCR allows the amount of human DNA present of the DBS after extraction to be determined. This provides the unique opportunity to normalise viral loads for comparison to the number of cells present (in this study CMV genomes/10^6 cells). This normalisation could also prove useful in determining threshold levels if a sigmoid relationship between disease and viral load is confirmed.

Traditionally, newborn screening for genetic and biochemical disorders was based on mass spectrometry. The first DNA based newborn screen for severe combined immunodeficiency (SCID) was included in the USA screening core panel in 2010.The assay is based on the detection and quantitation of T cell receptor excision circles (TREC) from DBS by real time PCR (Kwan, Church et al., 2013). The UK is currently running a pilot study for SCID screening and, if implemented, the TREC assay will be the first DNA based test to be added to the UK screening panel. This will provide the

expertise and instrumentation required for DNA extraction and real time PCR. Once these are in place it will provide the ideal opportunity for the addition of new molecular based tests into the newborn screening repertoire including those for CMV.

When considering universal neonatal screening a cost effective assay with high sensitivity and high throughput (minimum 96 well format) is required. In this chapter the applicability of the high throughput methods developed were evaluated by testing a large cohort of DBS samples from Kenya. The cohort was selected to test the high throughput methods due to the large number of DBS, high maternal seroprevelence and known high rate of CMV acquisition in infants in the first year of life. It has been previously shown that >80% of infants acquire CMV in sub-Saharan Africa before the age of 1 year (Kaye, Miles et al., 2008). The rate of CMV acquisition in the study was 66% which was lower than expected. This underestimation may be due to differences in the sensitivity of detection methods for CMV between studies, with the higher detection rate testing plasma samples (as demonstrated in chapter 5). It could also be due to short duration viraemia which may have been missed by the sampling approach. No plasma samples were collected in our cohort and we were unable to confirm CMV infection using serology or compare DBS and plasma results.

In England and Wales there are seventeen newborn screening laboratories (http://www.newbornscreening.org/laboratories.asp). The last reported birth rate for the same region was 698,512 (live births) in 2013 (Office for National Statistics, 2014). Assuming each laboratory performs an equal number of tests this would equate to 41,000 DBS tests for CMV per laboratory per annum. In this chapter I tested 1,027 DBS specimens for CMV DNA in a short period of time (10 days). Therefore with adequate laboratory resources my data shows that detection of CMV

from DBS is possible and that inclusion of CMV screening in the newborn programme is feasible. However, further studies are required to develop extraction methodologies for example using DBS sample as primary input and removing the need for pre-treatment.

Retrospective testing for CCMV from DBS is needed and demand has increased on a yearly basis since its introduction in 2005. However current methodologies for DBS testing for CCMV have shown poor sensitivity providing a barrier to neonatal CCMV screening. To address this a novel real time one step nested PCR was developed which showed enhanced sensitivity for detection of CMV DNA from DBS. This rapid one step nested PCR allows detection of CMV in 1.5hrs and removes the associated contamination risk of a two-step nested PCR and/or gel based detection method.

Overall newborn screening will allow a greater number of infected infants to be identified and provide the opportunity for early intervention and to fully assess the relevance of the 'threshold effect' in context of newborn screening. Additionally, these studies should also aim to recruit asymptomatic children identified into a randomised controlled trial to assess the efficacy and safety of antiviral treatment in babies born without symptoms.

Chapter 8

8 General Discussion

The work in this PhD thesis aimed to investigate several aspects of the use of DBS for both the retrospective diagnosis of CCMV infection and their potential as a screening tool. Furthermore it addressed the use of DBS for the detection of viral DNA in studies in resource limited settings where logistical constraints often preclude storage and transport of plasma of whole blood samples.

In this chapter, the implications of the main findings are discussed, overall conclusions are formulated and recommendations for future studies are made.

8.1 Disease burden and diagnostic conundrum of CCMV

CCMV is an important public health problem. It is estimated that approximately 6,000 children per annum born in the USA will have neurological disabilities due to CCMV infection, this disease burden is greater than that of either Down's syndrome or spina bifida for which prenatal screening is standard care (Cannon & Davis, 2005). The diagnosis of CCMV is challenging; maternal CMV infection is often asymptomatic or presents with non CMV specific 'flu like' symptoms and existing maternal immunity is not protective to virus reactivation or re-infection. Disease presentation in the congenitally infected child can vary and symptoms are not exclusive to CCMV alone. The biggest obstacle in the diagnosis of CCMV is that approximately 90% of congenitally infected children are asymptomatic at birth, however these children face a significant risk of developing late onset disease most commonly SNHL (Dollard, Grosse et al., 2007). In the absence of a newborn screening programme CCMV diagnosis cannot be made with certainty in children presenting after 14 days of life (unless early samples are available) due to confounding results from perinatal CMV infection (as shown in this thesis). Infants who acquire CMV infection intrapartum or

postpartum usually have no acute illness (with the exception of some low birth weight premature infants) and infections are not associated with the neurological disease associated to CCMV. This complex presentation presents a diagnostic conundrum.

8.2 Use of DBS for retrospective diagnosis of CCMV

The concept of using newborn DBS for the diagnosis of congenital CMV was first reported in 1994. Shibata *et al;* retrospectively analysed DBS for 661 newborns and found 25% of healthy babies and 33% of low birth weight infants to be positive for CMV DNA from their newborn DBS sample (Shibata, Takano et al., 1994b). Following this observation Barbi *et al;* reported 100% sensitivity and 99% specificity of the DBS sample for the diagnosis of CCMV compared to virus isolation in urine (Barbi, Binda et al., 1996;Barbi, Binda et al., 2000). In 1997 Johansson *et al,* reported 81% sensitivity and 100% specificity of DBS samples for CCMV diagnosis in children aged 12-18 years (Johansson, Jonsson et al., 1997). In another study, the median age of delayed onset SNHL in asymptomatic children was found to be 27 months of age (Fowler, McCollister et al., 1997) showing that CMV DNA could be found on DBS older than the median age of onset of SNHL. However the use of DBS for retrospective diagnosis raised some critical questions surrounding cross contamination and stability of CMV DNA because the reported results could also be dependent on other factors such as storage conditions or initial blood viral load, so stability under 'routine storage conditions' was important to establish.

The first part of this thesis aimed to address these questions. DBS are essentially small volume blood samples, it was therefore important to determine the sensitivity of the DBS assay in relation to a whole blood sample taken at the same time. The

mean CMV viral load in cord blood of 18 congenitally infected neonates has been reported as 2,300 copies/ml (3.4 $\log_{10}$) (Halwachs-Baumann, Genser et al., 2002). Another study reported a mean peripheral blood CMV DNA viral load of 400,000 (5.6 $\log_{10}$) copies/ml in symptomatic infants, 82,000 (4.9 $\log_{10}$) copies/ml in asymptomatic infants and higher viral loads in infants with hearing loss versus those with normal hearing (Boppana, Fowler et al., 2005). In my study the whole blood with the lowest viral load that still tested positive for CMV DNA when spotted onto a DBS was 1,700 genomes/ml. Importantly, this result was lower than the median viral loads reported by the two studies suggesting that the DBS assay should be capable of detecting CMV viraemia in the congenitally infected child. These observations, alongside the stability and cross contamination data, led to the introduction of a national routine diagnostic service for the retrospective diagnosis of CCMV in 2005. Which in turn produced more clinical samples for me to evaluate.

8.3 DBS as a newborn screening tool for CCMV

Screening for CCMV from DBS has been suggested (Dollard, Schleiss et al., 2010;de Vries, Vossen et al., 2011). Previous studies have focused on the detection of CMV from DBS in cohorts of congenitally infected infants with a range of sensitivities (64-100%) being reported (reviewed by (Barbi, Binda et al., 2006). These results suggested that newborn screening for CMV from DBS samples could be possible through the use of optimised methods to provide a highly sensitive platform for CMV DNA detection.

In 2010 the CMV and Hearing Multicentre Screening (CHIMES) study (a population based multicentre screening study using high throughput automated sample preparation to detect CCMV from paired saliva and DBS) published their results on the diagnostic accuracy of DBS for newborn CMV screening in comparison to rapid

culture of saliva samples. The results showed poor sensitivity with only 28% of CCMV infection being detected with a one primer assay and 34% being detected with two primer assay and led the authors to conclude that DBS real time PCR assays are not suitable for CCMV newborn screening (Boppana, Ross et al., 2010). The data presented in this thesis would dispute this finding and suggest this conclusion was premature as the methodology may have contributed to the low reported sensitivity. This thesis has highlighted the key role of nucleic acid extraction and amplification techniques in the detection of CMV DNA from DBS. In chapter 3 extraction methodology was shown to play a critical role in the recovery of CMV DNA from the DBS with sensitivities of 29-71% for the same sample set dependent on extraction method used. Other studies have also shown large differences in reported sensitivities dependent on extraction methodology, region of CMV being amplified and the amplification method itself (de Vries, Claas et al., 2009;Barbi, Binda et al., 2006;Vauloup-Fellous, Dubreuil et al., 2006;de Vries, Claas et al., 2009;Scanga, Chaing et al., 2006;Yamamoto, Mussi-Pinhata et al., 2001); (Soetens, Vauloup-Fellous et al., 2008). These factors together could have a major impact on the reported sensitivity of the CHIMES study so further investigation into DBS PCR assays should continue. The most recent study to investigate DBS sensitivity agreed with this conclusion as the prevalence measured with DBS was similar to reports using standard viral culture methods when screening 3972 newborns using established and optimised DBS methods (Kharrazi, Hyde et al., 2010).

In this thesis high throughput extraction technology was evaluated on two large cohorts of DBS samples. Additionally a one-tube nested real time PCR suitable for high throughput screening was developed which showed enhanced detection of CMV DNA from DBS. However this 90% sensitivity is novel in the context of newborn

screening and denotes that a test without 100% analytical sensitivity for infection that nevertheless detected all children at risk of developing disease would be deemed acceptable for the purposes of screening. If the reported threshold association between viral load and outcome was proven it would suggest that the 'missed' cases would be the children with the absent or lowest viral load who would therefore have the lowest risk of CMV disease (Boppana, Fowler et al., 2005;Lanari, Lazzarotto et al., 2006;Arav-Boger & Pass, 2007). This possibility should now be evaluated in much larger numbers of cases and controls.

8.4 Relationship between CMV viral load and CCMV disease

Throughout this thesis CMV viral load has been shown to be a factor in the detection of CMV DNA from DBS. This is an important consideration in determining whether DBS will perform adequately for CCMV screening and retrospective diagnosis. The pathogenesis of CCMV disease is unknown, however the severity of the disease is likely to be multifactorial, and include host and viral genetics as well as host immunological response. CMV viral load is a reflection on the 'fitness' of viral replication, or conversely, how the immune system is able to keep the CMV infection in check. Therefore the severity of disease is likely to be related to the inoculum of virus and/or the inability of the immature immune system to control replication. Recent studies have shown an impaired interferon-gamma secretion by fetal CD8-T cells and defective expansion of the pp65 specific cytotoxic T lymphocytes in response to CMV antigen in infants with CCMV (Elbou Ould, Luton et al., 2004;Pedron, Guerin et al., 2007) and that functional exhaustion limits effector CD4 and CD8 T lymphocyte responses during fetal life (Huygens, Lecomte et al., 2015). Termination of CMV excretion has also been correlated to the development of CMV specific cellular immunity (Pass, Stagno et al., 1983).

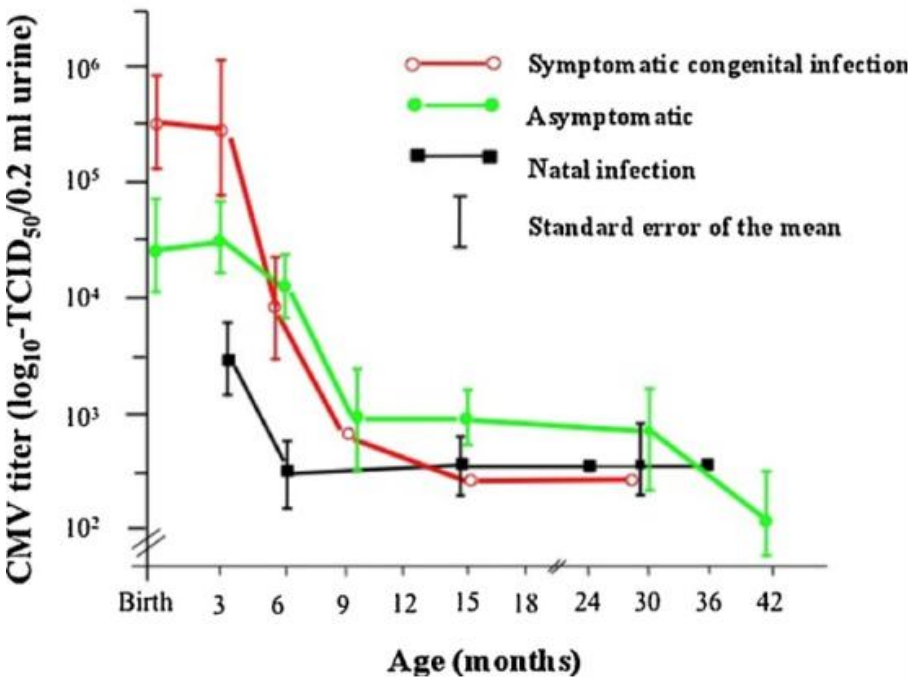
In 1975 Stagno *et al,* were the first to report that there was a significant difference between the CMV viral load in urine of symptomatic CCMV children versus asymptomatic and that the urine viral load in asymptomatic CCMV was greater than the urine viral load of perinatally infected children (figure 8.1) (Stagno, Reynolds et al., 1975b). These results were the first to imply the possibility of a threshold (non-linear) relationship between CMV disease and CMV viral load. More recently, increasing urine CMV viral load has been positively associated with SNHL by Rivera et al (Rivera, Boppana et al., 2002) and real time PCR quantitation has shown that CMV viral load in blood correlates with future sensorineural hearing loss (SNHL) (Ross, Novak et al., 2009). This result was replicated by Boppana *et al*; who have also shown higher CMV loads in both urine and blood of symptomatic CCMV children compared to asymptomatic in a prospective study (Boppana, Fowler et al., 2005).

In this thesis a statistically significant association, between viral load on DBS and the presence and degree of SNHL in both symptomatic and asymptomatic CCMV was found in 34 children, suggesting an association between CMV blood viral load and risk of hearing deficit. This relationship was reminiscent of the threshold concept of CMV disease in solid organ transplant patients and AIDS patients where the relationship between CMV viral load and risk of disease is well established (Cope, Sweny et al., 1997;Cope, Sabin et al., 1997;Emery, Cope et al., 1999;Emery, Sabin et al., 2000;Emery, Sabin et al., 2000); (Humar, Gregson et al., 1999). An observation supported by Ross *et al;* who showed that peripheral blood viral load was not directly associated with hearing loss but viral loads of <3,500 copies/ml were associated with a lower risk of hearing loss and better hearing outcomes in both symptomatic and asymptomatic CCMV (Ross, Novak et al., 2009). However, this

observation was not found in another study by Bradford *et al*; who found no association between blood CMV viral load and SNHL when compared to the presence of CMV DNA alone. But this study only recruited symptomatic CCMV children with CNS involvement which may have affected the outcome (Bradford, Cloud et al., 2005).

An ultimate goal would be to define a prognostic marker which could predict the outcome of CCMV infection. From the results generated in this thesis It was not possible to define a prognostic ‘threshold’ viral load, but the relationship itself warrants further investigation and could also explain the continued detection of CMV DNA in the DBS of older children with late onset hearing loss, as the children who are more likely to be symptomatic may have had higher viral loads.

A)



B)

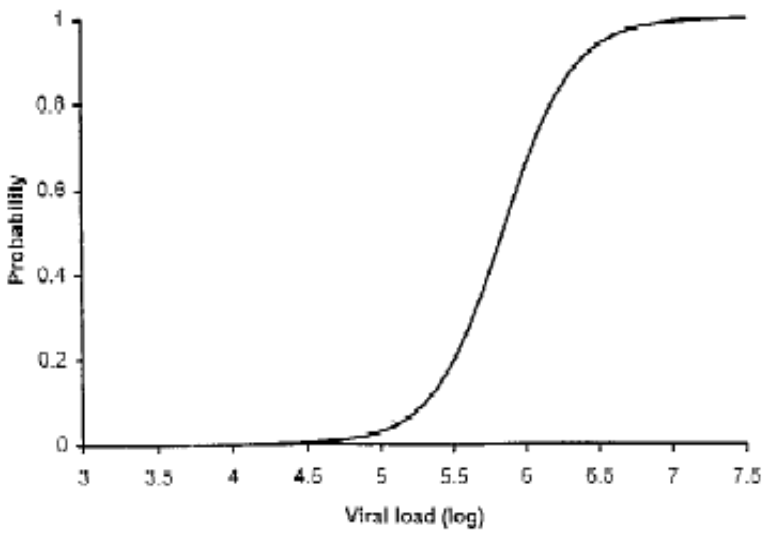


Figure 8.1 ***A)*** *relationship between viral load in the urine of newborns infected with CMV congenitally (asymptomatic or symptomatic at birth) and infected post-natally using TCID50 assessment in fibroblast cell culture over the initial 42 months of life (re-drawn from (Stagno, Reynolds et al., 1975b).* ***B)*** *Non-linear relationship between CMV viral load in renal transplant patients urine and probability of CMV disease reproduced with permission from (Cope, Sweny et al., 1997).*

Prompt antiviral treatment has the potential to prevent or delay the onset or progression of hearing loss in CCMV (Kimberlin, Lin et al., 2003;Kimberlin, Jester et al., 2015). In transplant patients CMV viral load is used to initiate and monitor pre-emptive anti-CMV therapy with the therapeutic effects of antivirals measured by the resulting decrease in viral load. Studies suggest that ongoing viral replication may be responsible for the SNHL associated with CMV DNA being detected in the perilymph of children with CCMV (Sugiura, Yoshikawa et al., 2004;Bauer, Parizi-Robinson et al., 2005;de Vries, Vesseur et al., 2013) and CMV labyrinthitis being shown in animal and human models (Woolf, Koehrn et al., 1989); (Sugiura, Yoshikawa et al., 2004;Li, Kosugi et al., 2008) Therefore it is reasonable to predict that lowering the viral load may be beneficial to a child with CCMV. This could explain the reported beneficial effects of the CASG randomised controlled trial of 6 weeks intravenous ganciclovir on CMV replication and hearing deterioration in infants with symptomatic CCMV (Kimberlin, Lin et al., 2003). Thus, a transient lowering of CMV viral load may reduce the risk of SNHL, providing an important rationale for antiviral therapy studies in children born with CCMV disease. Support for this has concept was also found in the recent six weeks versus six month CASG treatment trial where four times the drug exposure did not give four times the clinical benefit (Kimberlin, Jester et al., 2015).

While this study has been in progress the use of saliva and urine samples for screening purposes has gained popularity, this in part is due to the high viral loads associated with these sample types (Boppana, Ross et al., 2011;Yamamoto, Mussi-Pinhata et al., 2006;Paixao, Almeida et al., 2012;Koyano, Inoue et al., 2011;Williams, Kadambari et al., 2014). However, saliva sampling can be prone to false positive PCR results due to oropharyngeal contamination during birth and or breast milk CMV contamination, so timing of the newborn saliva sample is critical to outcome and the

logistics of setting up a new screening programme using a different sample type may prove to be problematic to implement.

An intrinsic part of newborn screening for CCMV will be the requirement for high throughput methodologies, an assay will be required to be sensitive, specific and suitable for 96 or 384 format testing. Classically newborn screening was based on mass spectrometry technologies, however the first DNA based newborn screen for severe combined immunodeficiency (SCID) was included in the US screening core panel in 2010 and is being piloted in the UK at present. The assay is based on the detection and quantitation of T cell receptor excision circles (TREC) from DBS by real time PCR (Kwan, Church et al., 2013) and if implemented, will be the first DNA based screen to be added to the UK screening panel. This will provide the expertise and instrumentation required for DNA extraction and real time PCR. This is the ideal opportunity for the addition of new molecular based tests into the newborn screening repertoire and based on the data in this thesis and commitment to use an iterative approach to improve the assay based on new technologies a pilot study screening for CCMV from DBS is feasible and should remain under investigation alongside screening programmes based on saliva and urine. Additionally the established technique of mass spectrometry for the detection of CMV-specific proteins could be investigated.

Prevention of CMV infection is an ultimate goal, the Institute of Medicine listed prevention of CCMV as a top priority based on cost effectiveness and improvement in quality adjusted life years (Stratton, Durch et al., 2001) and the CDC has published guidelines for the prevention of maternal acquisition of CMV whilst pregnant based on hand washing and the avoidance of exposure to bodily fluids.

A universal vaccination programme would show the largest benefits (Griffiths, 2012). CMV vaccine development is currently underway; however a licensed CMV vaccine for the prevention of CMV infection is not likely in the next few years. In the absence of a CMV vaccine, prevention of MTCT of CMV is being investigated

Maternal treatment with CMV hyperimmune globulin has been studied in Italy in a non-randomised trial to prevent vertical transmission of CMV infection. A decrease in fetal infection was reported with the infection rate in the hyperimmune globulin arm 16% compared to 40% in the non-treated arm (Nigro, Adler et al., 2005). However a recent randomised, placebo controlled blinded trial of the same preparation of hyperimmune globulin used at the same dose did not shown any beneficial effect of the hyperimmune globulin treatment and reported an increased trend for premature delivery in women who received the treatment (Revello, Lazzarotto et al., 2014). A large randomised, placebo-controlled, clinical trial of CMV immune globulin for prevention of maternal-fetal transmission of CMV infection is currently underway (http://clinicaltrials.gov/ct2/show/NCT01376778).

8.5 Maternal CMV detection and infant acquisition in resource limited settings

In resource limited settings early acquisition of CMV in infants exposed or infected with HIV has been proven to be detrimental to the infant (Slyker, Lohman-Payne et al., 2009b). In the absence of an effective vaccine or treatment, preventing or delaying rates of CMV acquisition may represent a novel strategy to improve the health of infants in areas with high seroprevalence and HIV co-infection. Chapters 6 and 7 of this thesis validated and utilised DBS methodologies developed to study

both maternal CMV detection and infant acquisition in resource limited settings where logistical constraints often preclude storage and transport of plasma or whole blood samples. The presence of CMV in distinct maternal compartments, HIV viral load and maternal CD4 count was investigated with the aim of identifying biomarkers which could predict perinatal transmission of CMV. The results presented are the first analysis of CMV natural history in different maternal body compartments and show that CMV reactivation in mucosal compartments is common during late pregnancy and breastfeeding and is relevant for transmission. It is interesting to note the relationship between CMV detection in the different maternal compartments and detection of CMV DNA in the plasma suggested that blood viral load is an overall indicator of systemic viral reactivation or infection. Breast milk CMV viral load and maternal CD4 count are major determinants of MTCT of CMV; these data suggest that restoring maternal immunity or reducing CMV breast milk levels may prevent MTCT of CMV infection and support the concept that CMV disease is likely to be related to the inoculum of the virus and/or the inability of the immune system to control replication. Therefore, expanding maternal access to highly active antiretroviral therapy in pregnancy could improve infant outcomes by directly reducing HIV replication and indirectly controlling CMV.

8.6 Overall conclusions

The burden of disease caused by CCMV is finally being recognised and the foundations for newborn screening for CCMV are being laid with several publications and reviews evaluating newborn screening for CCMV (Grosse, Dollard et al., 2009;Dollard, Schleiss et al., 2010;Din, Brown et al., 2011;Boppana, Ross et al., 2010;de Vries, Vossen et al., 2011;Kharrazi, Hyde et al., 2010). The potential to

identify asymptomatic children at risk of late onset SNHL or other sequelae and benefit of early interventions to protect hearing should not be underestimated. The benefit of early antiviral treatment to prevent hearing deterioration has been shown in symptomatic infants and a large scale study on the benefits of antiviral treatment in asymptomatic children is now required. However, the potential harms from CCMV screening must be investigated. 80% of children with CCMV do not develop permanent sequelae and therefore a positive screening result could lead to parental anxiety in a large number of cases. Maternal anxiety when screening for CCMV was recently addressed in the UK in a study by Williams *et al.,* no additional anxiety was reported in mothers of infants screened (Williams, Kadambari et al., 2014)

CCMV infection now satisfies the majority of criteria of Wilson and Jungner (Dollard, Schleiss et al., 2010) and is being recognised and acknowledged in the USA with Utah signing the first house bill (HB0081), which mandates CMV education for pregnant women, as well as CMV screening for infants who fail their newborn hearing test. The scene is now set for a large scale study on screening and intervention in CCMV. I hope that my work in producing a nested PCR might help the ultimate goal of controlling SNHL caused by CCMV.

8.7 Future studies

To address remaining analytic and logistic issues large-scale newborn screening for CCMV should be investigated using optimised methods specific for DBS. The use of commercial partners and /or commercial assays should also be investigated as new mass throughput analysers are currently in development for clinical use.

Additionally, since current metabolic screening is mainly performed using mass

Spectrometry assays, it would be logistically advantageous to investigate the detection of CMV specific proteins for the detection of CMV on DBS.

Recent advances in whole genome sequencing in clinical samples alongside human genomes may provide insight into transmission patterns of CMV from mother to fetus and their potential role in severity and outcome of CCMV infection. Future studies analysing a large number of newborns and their mothers could address this and reveal important biomarkers for CMV transmission.

Chapter 9

9 References

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