PALACKY UNIVERZITY in OLOMOUC

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Study of natural history of Human Cytomegalovirus and its relationship to variability of major antigens.

Thesis

Department of Immunology
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Olomouc 2012

Declaration on word of honor

I hereby certify that all work has been performed by me and that, to my knowledge, all used literature and other sources have been cited.

Date: 3rd April 2012

Dr. Zdenek Novak

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SUMMARY

The aim of the first part of my PhD thesis is to provide an integrated overview of the natural history of human cytomegalovirus. It provides short description of all aspects of CMV such as structure, interaction with the host, clinical symptoms of CMV infection, diagnostics, and treatment and prevention options.

The second part of the thesis summarizes three directions of CMV pathogenesis on which I worked. First theme consists of a strain variation of CMV within the *gN* and *gB* genes and mixed infection in seropositive immunocompetent women and in congenitally infected babies. Second theme deals with design, preparation, and purification of recombinant antigens and their use in detection of CMV reinfection in clinical samples. The last theme deals with CMV detection in clinical samples collected from newborns and related problems.

Results of the experimental work were published in 11 papers in peer-reviewed journals with impact factor.

The third part of the thesis provides prints of author's publications.

INTRODUCTION

Human cytomegalovirus (CMV) is one of the most commonly widespread viruses infecting a majority of humans at some time in their lives. It is a β-herpesvirus, largest of the family Herpesviridae, defined by a long life cycle and restricted hosts. The virus has been shown to be a cause of opportunistic disease in normal hosts. In immunocompromised hosts such as transplant subjects or HIV-infected subjects, who are unable to build proper cellular response, it can produce acute disease. In pregnant women it is the most common congenital viral infection carried by transplacental transmission to the fetus, potentially causing it damage. CMV is usually transmitted via direct contact with infected bodily excretions such as saliva, urine and vaginal secretions, via blood transfusions, or through organ or bone marrow transplantation. The overall seroprevalence is 30-70% in developed countries. It tends to be higher in developing countries and poor socio-economic groups, exceeding 90% with transmission early in life.

NATURAL HISTORY OF HUMAN CYTOMEGALOVIRUS

CMV was accidentally discovered in 1881 by German pathologist Hugo Ribbet in a stillborn congenitally infected with syphilis. He noticed enlarged epithelial cells in the kidney with inclusion bodies resembling “owl’s eyes”. This observation was soon confirmed by Lowenstein who saw similar “cytomegaly” with nuclear inclusions resembling herpes simplex infection in parotid glands from young children. Von Glahn and Pappenheimer were the first to publish a case describing infection by a virus from a herpes group in adults in 1925. In 1950 Wyatt et al suggested the term “generalized cytomegalovirus inclusion disease (CID)” for the lethal congenital infection (214). CID was already known as an infectious disease from 1930s specified by presence of specific cytopathology in salivary glands, liver, lungs and kidneys. In 1953 Minder first visualized the virus in pancreatic cells by electron microscopy (121). It was successfully grown in tissue cell culture by 1954 and was finally isolated in three different centers independently (161, 180).

By the 1970s it was clear that only the original host species can sustain viral replication, although CMV-like viruses were isolated from different mammals.

The relationship between the infection of pregnant women, transplacental transmission (which happens more often in primary infection than in reinfection or reactivation) and occurrence of neurological damage to newborns was also established. This helped in understanding congenital disease and its sequelae, with the focus on progressive sensorineural hearing loss. CMV also remains an important pathogen for immunocopromised hosts such as solid organ and bone marrow transplant patients and those who have HIV/AIDS, resulting in CMV pneumonitis and retinitis, respectively. These adverse outcomes have resulted in focusing research efforts towards the development of therapeutic control of the disease and its possible prevention by public health measures and vaccination.

CMV, as well as other betaherpesviruses, replicate slowly and stay strictly cell associated. Due to the fact that it is very host and cell specific there are no good animal models. Currently CMV is mostly cultured in human fibroblast derived from lungs or newborn foreskin (fully matured fibroblasts are non-permissive) but it can be grown in various epithelial and endothelial cells, macrophages and dendritic cells among others. Unfortunately, fibroblast-propagated virus is no longer growing in other cell types due to relatively fast-occurring CMV genome mutations and deletions such as that seen in laboratory-adapted strains such as AD169 or Towne. Although it is interesting to note that this is not caused by a inability to attach to and penetrate the cells.

CMV STRUCTURE

CMV GENOME

The HCMV genome is GC rich ds-DNA, ranging from 196 to 260 kbp. With about 230 non-overlapping open reading frames it has the capacity to encode 166 to 200+ gene products (37 , 94). Only about 70 protein coding regions are conserved in all betaherpesvirus genomes and they cover all core functions (53). Generally all of the genes are unspliced with few exceptions. One of those exceptions are genes encoding major immediate early gene *(IE) UL122-123,* regulating gene expression, and minor *IE UL36-37* regulating cell death suppressors. The genome consists of unique short (US) and unique long (UL) regions flanked by terminal (TRL) and internal (IRL) repeat sequences and it is a

class E genome. Both regions can be inverted, giving CMV the ability to form four isomers, all of which are produced in equal amount in culture.

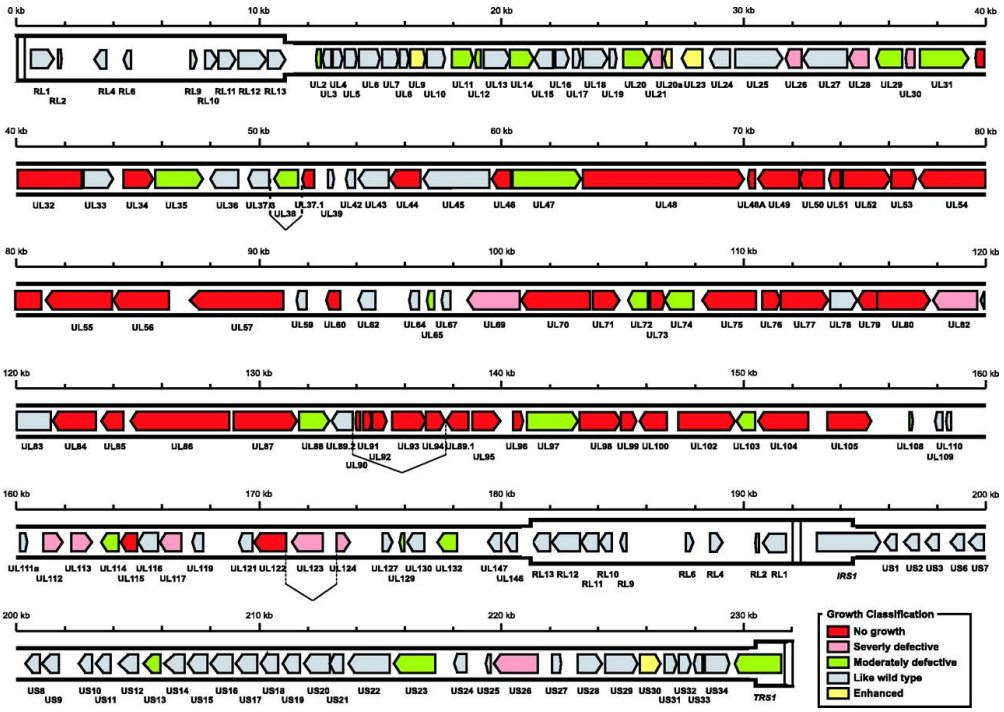

Figure 1. Genome organization and ORFs of HCMV (Towne strain) based on the genomewide shotgun sequencing of the viral sequence cloned in a BAC.

(*PNAS November 25, 2003 vol. 100 no. 24 14223-14228*)

The most characterized CMV genome is of the laboratory-adapted strain AD169 derived from adenoids and propagated in fibroblast cell culture for a prolonged period. By comparing amino acid sequences among other herpesviruses it was revealed that about 40 ORFs are highly similar to other proteins encoded by alpha- and gamma-herpesviruses (37, 94, 122). This is further proof that CMV belongs to the same family of viruses. The CMV genome is believed to encode approximately 65 glycoproteins with a wide variety of functions. The AD 169 strain has been completely sequenced and it has been found that it has 15kb deletion of at least 19 genes in the unique short region compared with clinical isolates and some other laboratory strains (35, 37). Now with the help of new sequencing techniques complete genomes of many different strains have been completely sequenced. Different clinical strains show identity of more that 95% at the nucleotide level. Most of the variability has been documented by sequence comparisons in genes encoding glycoproteins UL73, UL74, UL144 and UL146. The newer deep sequencing

techniques of low passage clinical isolates are also showing rather high variability throughout the genome at the same levels as RNA viruses. The variability is not only in the sequence but also functional variations in genome structure.

The CMV genes are expressed in a timely coordinated cascade manner and can be divided into 3 groups: immediate-early (IE), early (E), and late (L). Genes encoding these proteins are dispersed across the whole viral genome (not grouped together). Disruption of the proper gene expression is probably behind restricted growth in non-permissive cells (63).

About 15 regulatory micro RNAs (miRNA) are encoded by the HCMV genome which regulates gene expression at the post-transcriptional level of multiple viral genes involved in replication including *MIE (UL122-123).* They also regulate apoptosis, cell cycle events, immune response, and NK cell evasion (62, 101, 118).

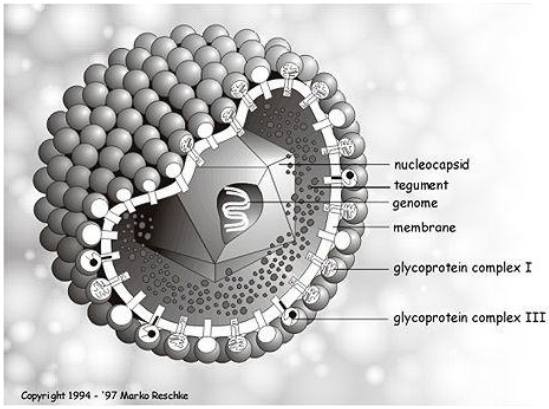

Figure 2. Cytomegalovirus structure schematics
http://www.virology.net/big_virology/bvdnaherpes.html

VIRION

The HCMV's virion has a typical herpesvirus structure and is about 200 to 300 nm in diameter. It is the most structurally complex herpesvirus, reflecting the large number of gene products encoded by this virus. It can be divided into three regions: capsid, tegument and envelope.

CAPSID

The capsid is the most inner structure that encloses CMV linear genomic DNA with a lytic origin of DNA replication (miLyt)-associated RNA (144). It has an icosahedral structure consisting of 12 pentons, 150 hexons, and 320 triplexes (38, 195) and is composed of five herpesvirus core proteins: major capsid protein (MCP, UL86) composed of hexons and most pentons; triplexes composed of two subunits-the minor capsid protein (TRL1, UL46) together with the minor capsid protein binding protein (TRL2, UL86); the smallest capsid protein (SCP, UL48A) located on MCP tips; and a portal protein (PORT, UL104) that constitutes one specialized penton used for encapsidation of viral DNA (27, 71).

Three maturation forms can be found: A, B and C. Only the C form is fully matured and contains DNA. The A and B forms can be found in the cell's nucleus and cytoplasmic or in the noninfectious enveloped particles (NIEPs). The dense bodies (DB), noninfectious defective particles consisting of tegument surrounded by an envelope and containing major antigenic determinants responsible for induction of both the humoral and the cellular immune response (139), lack DNA or capsid (70, 71).

TEGUMENT (MATRIX)

The tegument is a protein-rich region located between the nucleocapsid and the envelope and accounts for about 40% of the total virion mass. It is an amorphous structure that contains at least 27 virus-encoded as well as cell-related proteins and RNAs with the majority of the proteins being phosphorylated and highly immunogenic. It is surrounded by a lipid bilayer derived from the host cell's endoplasmatic reticulum membrane.

The most abundant teguments proteins are pp65 (lower matrix protein, UL83), pp71 (upper matrix protein, UL82), pp150 (large matrix phosphoprotein, UL32), and largest tegument protein LTP (UL48)(194).

The tegument contains major targets for host T-cell responses. One of the targets is the phosphoprotein 65 (65 kDa). The pp65 is used as a target antigen in the antigenemia assay that is currently a standard detection technique in transplanted patients (16).

ENVELOPE

The envelope is a very complex structure. The phospholipid envelope contains several virus-encoded glycoproteins, members of the herpesvirus core set, organized into three protein complexes designated glycoprotein (g) B complex, gN/gM complex and gH/gL/gO complex (gC I-III complex). All of these genes are required in CMV replication and they play an essential role in the virus-host cell interaction, cell entry and virion maturation. It has been shown that disruption of these genes open reading frames results in failure to reproduce (25, 27, 76, 77, 92, 93, 112).

CMV-infected individuals mount an immune response against these glycoproteins, producing a multitude of neutralizing antibodies. This consequentially makes these glycoproteins a potential subunit target candidate for vaccine development (72). The antibodies produced are also used in diagnostic assays (4, 83).

Three major envelope glycoproteins are gB, gN and gH. They are genetically polymorphic and elicit generation of neutralizing antibodies by the host's immune response.

Glycoprotein B (UL55) is the most abundant integral envelope membrane protein. It exists as a disulfide-linked homodimer and is one of the most highly conserved proteins. It is critical in initial interaction with heparan sulfate during cell entry and cell-to-cell spread. The mature gB membrane glycoprotein consists of a 110,000-116,000 dalton ectodomain or surface component linked by disulphide bonds to a 55,000 dalton transmembrane component. Together these are linked through interchain disulphide bonds to form the mature homodimer (9, 109). The majority of the gB-specific antibody response in serum is directed against the first antibody-binding site identified on gB, designated Antigenic Domain 1 (28, 198). Point mutations within this region tend to eliminate the antigenecity of the whole peptide (204). Furthermore, this tends to be one of the most highly conserved regions of gB, which suggests a critical role in the structure or function of the protein. In addition, antibodies recognizing Antigenic Domains can inhibit virus attachment or block fusion (130, 196).

Glycoprotein N (UL73) also binds to heparin sulfate proteoglycans and possibly contributes to initial interaction with cell surface. gN is unique by its structure and variability. The mature gN (complexed with gM) has an estimated molecular mass of

nearly 60,000 daltons and is extensively glycosylated with O-linked sugars and elicits generation of neutralizing antibodies (112, 174). The extensive carbohydrate modifications serve to shield the gN from antibody recognition similar to that observed for HIV gp120 (210).

Glycoprotein H (UL75) is a type I membrane protein with a very short cytoplasmic tail. It is expressed late during infection on nuclear and cytoplasmic membranes of infected cells. gH is anchored by a C-terminal hydrophobic region to the envelope. It exists mostly as a complex with gL as a heterodimer. It is required for the fusion of viral and plasma membranes leading to virus entry into the host cell, possibly by binding to the integrin αVβ3 (207). Membrane fusion is mediated by the fusion machinery composed of at least gB and the heterodimer gH/gL. Fusion with fibroblasts requires the additional receptor-binding protein gO, which forms a complex with gH/gL. It is also involved in the activation of gene expression via cellular transcription factors Sp1 and NF-κB (209). Host immune response makes good neutralizing antibody responses against gH (26, 29).

INTERACTION WITH HOST

CELL ENTRY AND VIRUS REPLICATION

As mentioned before, HCMV replicates slowly (whole cycle takes approximately 48 to 72 hours) in many differentiated cell types but most of the studies are carried out on cell cultures of fibroblasts of the original host organism. It has been shown that different target cell types require different genes to be activated and, consequently different viral products to be involved in the replication. This also explains the reluctance of laboratory adapted strains such as AD169 to infect cell types other than HFF (human foreskin fibroblasts) compared to fresh or low-passage clinical isolates (171, 177).

During the virus cycle we can distinguish three classes of gene expression: immediate early (IE), early (E) and late (L). The genes are activated in a regulated cascade and dependent manner during productive infection after successful entry into the permissive cells

There are at least two types of HCMV cell entry mechanisms. First is the entry into fibroblasts in a pH-independent manner not mediated through endocytosis. It is started by gB binding to PDGFR cell surface receptor resulting in fusion with the cell membrane and requiring gH/gL/gO complex (181, 208). Second is the entry into epithelial and endothelial cells in a pH-dependent manner mediated by endocytosis. It requires gH/gL complex and proteins coded by *UL128-UL131* genes, which are probably responsible for a cell tropism (these genes are lost or contain deletions in strain propagated in fibroblasts for a prolonged time) (79, 162, 206).

There is also a possible role of heparin sulphate to be a binding site for gM and other glycoproteins. Other possible candidates might be beta microglobulin, annexin, *α v β3* integrin (207), CD13 and others. None of these candidate receptors is universally required, however, which complicates possible conclusions.

Once the virus enters the cell's cytoplasm the nucleocapsid is moved by microtubules to the nucleus and viral DNA gets uncoated and released under the control of large tegument protein (UL48), as well as a binding protein (UL47) (57, 58, 216). At this moment *IE* genes expression is activated and is influenced by tegument proteins. The delay between early and late phase is about 24-36 hours. Four gene regions are expressed at this time: *UL36* and *UL37*, *IE1* and *IE2* (*UL122* and *UL123*), *TRSI* and *IRSI*, and *US3* (47,

166). IE1 and IE2 proteins are deemed to play central regulatory roles in infection as they can induce cell cycle arrest and death as well as suppress the induction of IFN activation (137). *IE1* and *IE2* genes can be methylated, thus potentially indicating continued expression during latency or reactivation (88). Also pp71 has a big role in MIE regulation as it serves as an MIEP expression stimulator; in experiments it increases viral-DNA transfection efficiency used for BAC cloning and expression (32, 85). At this phase, viral genome becomes transcriptionally active.

The IE phase is followed by the E period starting 6 hours post infection (hpi) and continuing through 18-24 hours. A minimum of 23 genes are involved in this phase. The gene *UL112* and *UL113* products (4 proteins) produced at this time contribute in initiation of DNA replication (98), a phase which follows the E period.

The presence of viral particles in the host cell stimulates mRNA and protein production as well as viral DNA synthesis. This results in complete cell cycle disregulation (82, 89, 175).

L phase starts at about 24 hpi and the gene expression is resistant to inhibition by DNA synthesis inhibitors. One of the proteins expressed is *pp28* gene product UL99. At the end of this L phase, assembly and DNA encapsidation followed by egress and release of new virions is observed. Capsid components form a procapsid around the precursor of assembly protein (originally initiated procapsid assembly by moving MCP to nucleus) and it maturates via maturational proteases next to DNA replication compartments. Then the DNA is encapsidated by packaging machinery recognizing DNA and the mature nucleocapsid is formed. This is followed by a two-step envelopment process and egress that is ended by virion release by exocytosis (24, 119). The first envelopment occurs at the inner nuclear membrane, and then the nucleocapsid is de-enveloped at the outer nuclear membrane and released into cytoplasm. The second envelopment occurs in endoplasmatic reticulum. Resulting vesicles are transported to the cell surface and released by exocytosis.

The virus is produced for several days and completely utilizes and controls the host cell's basal transcription machinery. This ability of virus to control transcription machinery at early stages of infection is probably behind cell permissiveness more than cell entry issues, as shown by replication of murine CMV in human fibroblasts with the presence of a cell death suppressor derived from HCMV (90).

Only about 1% of viral progeny are infectious because of instability of virion, production of noninfectious enveloped particles and production of dense bodies (71, 201)}.

HOST'S IMMUNE RESPONSE AND IMMUNOMODULATION

Primary HCMV infection is controlled by a combination of innate and adaptive immune responses. Innate response contributes to the immediate infection control. It is mediated by interferons and NK cell activity (114, 167). Considerable evidence suggests that the most important part of adaptive immunity is T-cell mediated response, as shown in patients with normal antibody levels but severely impaired cellular immunity such as that seen in transplant recipients or AIDS patients. Here, reconstitution of T-cells eliminates symptoms of CMV infection (3, 104, 105, 205); therefore antibody mediated protection seems less important. Nevertheless, seropositive transplant recipients control potential infection better than seronegative recipients. Similarly, the potential for transplacental mother-to-fetus CMV transmission is lower in seropositive (or passively immunized) mothers than in the CMV naïve mothers. Many of the viral proteins are highly immunogenic (gB, gH, gM/gN, pp150, pp52, Ul128, UL130 or UL131A) and interaction results in production of neutralizing antibodies (50, 69, 111). The antibodies may play a role in reducing cell-to-cell transmission of the free CMV virus but the majority of CMV virus is strictly cell-related and thus unreachable by humoral immunity.

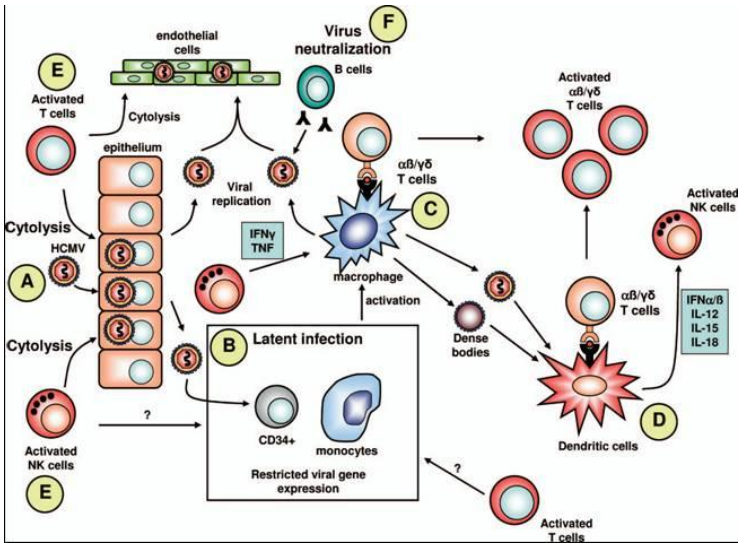

Figure 3. Immune responses against Human CMV

(*Schleiss et al, J Pediatrics, 2007)*

During evolution, HCMV developed very sophisticated strategies of modulating and evading immune control and enhancing inflammation. Both of these processes enhance spreading of the virus through the host. Many of the proteins encoded by the CMV genome are analogous to normal cellular immune effectors. They interfere with host immune effectors, thus allowing CMV evasion. The origin of respective genes is proposed to be horizontally transferred from the host genome.

Many proteins are located within infectious virion (pp65, pp71, pTRS1, pIRS1) or they are expressed early after infection (IE1, IE2, UL36, UL37). IE1 and IE2 can interfere with IFN production initiation. The proteins encoded by these genes interfere with cell-mediated immune defense by blocking apoptosis, blocking initiation of IFN production and shut down of production of other proteins (114, 117).

Other CMV proteins mimic cytokines such as CMV IL-10 analogue UL111A, which inhibits proliferation and cytokine production by mononuclear cells as well as inhibiting maturation of dendritic cells and enhancing their apoptosis. Some proteins possibly cause aberrant placental development (39, 179). Virus encoded CXC chemokines UL146, UL147 (vCXCL1 and vCXCL2) induce chemotaxis or promote neutrophil-mediated CMV dissemination (110). CC chemokine analogue pUL128 can block chemokine-driven motility, and together with UL130 it forms complexes with gH and gL allowing cell entry (190). Also, UL144 (TNF receptor superfamily analogue) upregulates NF-κB and enhances expression of CCL22, attracting Th2 cells resulting in immunoevasion. US28 is one of four chemokine receptor-like molecules encoded by the CMV genome. Its ability to bind members of different chemokine families (CC, CX3C) is unusual. It probably has a pro-inflammatory role and helps viral dissemination by enhancing migration of different cell types, but it also inhibits other cell types thus helping immunoevasion (86). US28 is also implicated in CMV vascular disease and in CMV-associated malignancy (203). CMV modulates innate immunity, for example, by disabling NK cells by binding their LIR-1 receptor through CMV UL18 glycoprotein instead of MHC I. CMV-infected cells express MHC I homologue UL142 glycoprotein on their surface hence mediating escape from lysis by NK cells (212). Other proteins such as CMV US3 can prevent MHC I from surfacing. CMV US2, US6, and US11 can cause cytoplasmic MHC I degradation, thus preventing Ag presentation and promoting evasion of $CD8^+$ T-cells. It has also been shown in animal models that respective genes are critical for reinfection/superinfection (80).

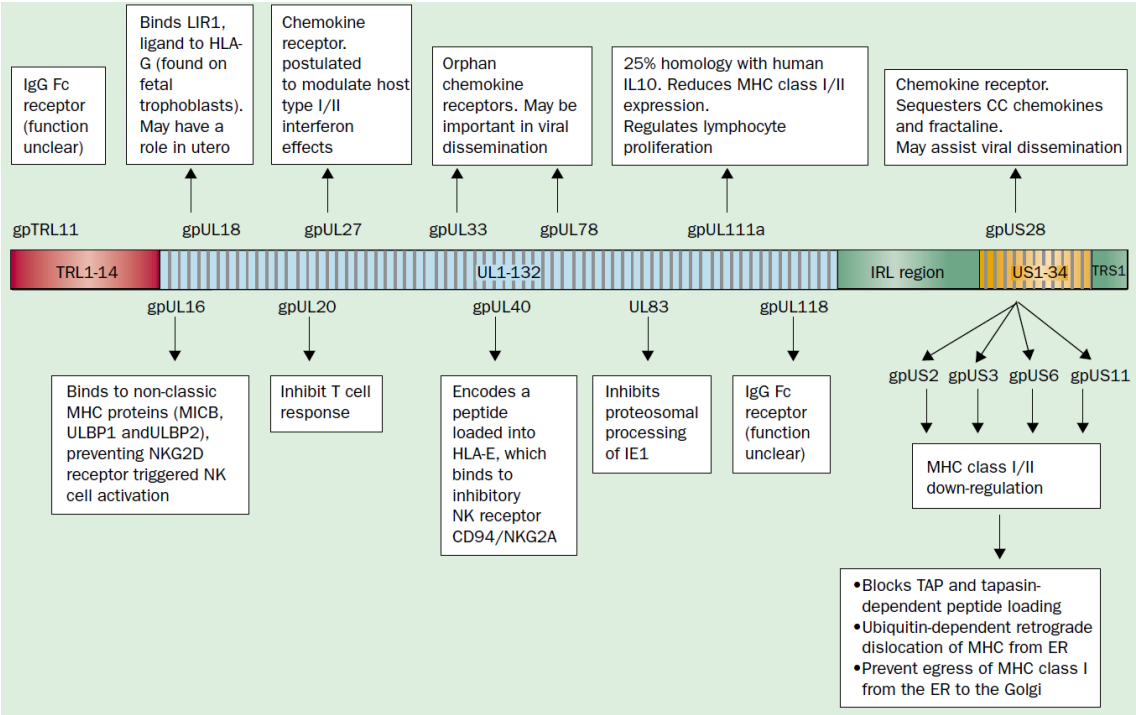

Figure 4. Examples of immunoevasion techniques of CMV

(Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments, Gandhi MK, Lancet Infectious Diseases, 2004)

LATENCY

Although the mechanisms controlling latency are not known, CMV seems to encode genes whose products helps in establishment of latent infection (with possible implication of viral ability to evade immune destruction by down regulating HLA cell surface markers) ready to be reactivated when certain conditions, such as immunosuppression or immunodeficiency, occur. Latency is likely established in anyone with primary infection (149, 150, 176). The latency is most likely present in the bone marrow in CD14+ and CD33+ and CD34+ cells. It is possible to experimentally reactivate infection in these cells (143, 163) The same genes responsible for latency might be implicated in oncogenesis since they interact with cellular p53 (CMV IE2) or c-myc (UL82) (78, 123).

CLINICAL IMPACT OF CMV INFECTION

EPIDEMIOLOGY

Cytomegaloviral infection is ubiquitous, with worldwide distribution. In general seroprevalence ranges from 30 to 100% and varies with geographical location, age and socio-economic status (highest being found in people of low socio-economic status and increasing with age) (66, 74, 182). This ”classic” picture of CMV distribution is changing in developed countries, however, due to high level of CMV infection in daycare facilities and their increased use by the higher socioeconomic groups in these countries (183). Newer studies from various populations have shown an incidence of congenital CMV infection of 0.18% in Australia, 0.47% in Italy, 2.2% in certain parts of US and up to 7% in infants born to HIV-positive mothers (95, 200).

TRANSMISSION

HORIZONTAL TRANSMISSION

CMV is generally transmitted from person to person and is easily transmitted in settings where people have contact with body fluids from people excreting virus. Direct contact with infectious material is required, since transmission time is relatively short (CMV virus viability varies from 5 minutes to 4 hours depending on surface) (189). After acquisition of CMV, infectious virus is excreted in urine, saliva, semen, and cervical secretions for months to years. Higher rates of CMV infection are seen in settings where close contact with body fluids occurs, such as between children in daycare settings, caregivers, households with children, and sex partners (1).

VERTICAL TRANSMISSION

Vertical transmission is usually from mother to baby. Unlike other herpesviruses, CMV is regularly transmitted from mother to fetus or newborn. The rate of vertical transmission was found to be 0.2% to 2.2% in previously seropositive mothers undergoing recurrent infection/reinfection during pregnancy, and 20 to 40% in pregnant women with primary infection (17, 20, 185). The rate of primary maternal infection during pregnancy

varies from 0.7% to 4.1%, with higher rates noted in unmarried women of low socioeconomic status (17).

Three different routes have been traced – transplacental (congenital), perinatal and via breast milk.

Congenital CMV infection from women who were CMV seropositive before conception has been linked to reactivation of latent virus or reinfection by a different strain of the virus. This was shown in a study of mothers who had evidence of past CMV infection and serologic evidence of re-infection who transmitted the virus to their fetus (20). The role of recurrent maternal infections is provided by a cohort study in a low-income population. The rate of congenital infection in offspring of women with CMV infection more than a year before conception (documented by virus shedding or serum antibody) was 2.2% (184).

Perinatal transmission occurs during the delivery in approximately 50% cases (155) when the virus is present in the maternal genital tract (around 2% to 28%) (184). Infants are usually CMV negative for the first 3 week of life.

Breast feeding is the most common route of CMV transmission. It has been shown, for example, that infants nursed for < 1 month do not become infected compared with almost 40% of those nursed longer. PCR studies have also demonstrated a strong relationship between the presence of viral DNA in milk and transmission to the infant (59, 202).

CLINICAL PICTURE

After acquiring sufficient infectious dose by direct contact with infected material the virus initiates its replication in mucosal epithelium at the point of entry. This is followed by systemic leukocyte-associated viremia lasting for months until the adaptive immune response is fully developed. Prolonged shedding of the virus in urine in infants is related to their poor cellular immune responses (199). At this time the virus cannot be detected in plasma since it is strictly cell-associated (but viral DNA can be used to monitor viral load in plasma). This phase is usually accompanied by a high level of viral shedding in urine, saliva and other bodily secretions (important for transmission). Epithelial cells are most likely responsible for viral production and shedding into bodily secretions

The signs and symptoms vary depending on age, route of transmission and also, very importantly, on the immune status of the patient. The infection is mostly subclinical in immunocompetent hosts. Also human CMV clinical isolates are highly diverse and variants among genes may be related to tissue tropism and different pathogenesis.

NORMAL HOST

PEDIATRIC CMV INFECTION

Congenital CMV is an important public health problem due to its capability to cause damage to CNS. It is considered to be the **leading cause of sensorineural hearing loss (SNHL)** (132). Other possible sequelae include elevated hepatic transaminases, petechia, jaundice, chorioretinitis, microcephaly and abnormalities on clinical neurologic examination and t computed tomographic scan of the brain (19, 49, 128). All newborns with congenital CMV infection shed virus in urine and other body fluids. Viremia or high viral load at birth or in early infancy has been associated with hearing loss (18, 102). Also low gestational age at the time of maternal primary infection is more likely to lead to sequelae (134).

A great majority (~90%) of infants infected peri- and post-natally by exposure to infectious cervical secretions or infected breast milk are asymptomatic or may develop minor non-specific illness within 1-3 months. Maternal antibodies do not completely prevent the infection. The rest may develop illness characterized by hepatomegaly, lymphadenopathy, poor weight gain, rash, interstitial pneumonitis and a sepsis-like syndrome. However, protracted interstitial pneumonitis has been associated with perinatally-acquired CMV infection, particularly in premature infants. Premature and ill full-term infants may have neurologic sequelae and psychomotor retardation.

ADULT CMV INFECTION

The most common clinical manifestation of CMV infection in normal hosts is a heterophil antibody-negative mononucleosis syndrome (100). This manifestation may develop spontaneously or may follow the transfusion of leukocyte-containing blood products such as transfusion of whole blood or platelets. Although the syndrome occurs at all ages, it most often involves sexually active young adults. Incubation period may

range from 20 to 60 days, and the illness generally lasts for 2 to 6 weeks. Prolonged high fevers, sometimes accompanied by chills, profound fatigue and malaise characterize this disorder. Myalgias, headache and splenomegaly are frequent, but in CMV mononucleosis (as opposed to Infectious Mononucleosis caused by EBV), exudative pharyngitis and cervical lymphadenopathy are rare. Mild and rarely symptomatic hepatitis could be associated with CMV mononucleosis. Occasionally patients develop rubelliform rashes, often after exposure to ampicillin (99). Less common manifestations include interstitial pneumonitis (51), pleuritis, arthritis, myocarditis and encephalitis. In rare cases, Guillain-Barré syndrome complicates CMV mononucleosis (129, 188). Common laboratory findings include lymphocytosis and elevated hepatic transaminases.

Sometimes CMV infection of the normal host can be associated with severe life-threatening complications involving specific organs. These would include enteritis, thrombotic disease, encephalitis, myocarditis, neuropathies and ocular disease.

Another route of CMV-related injury to the host is infection of the vasculature. The **Vascular Disease** associated with CMV includes atherosclerosis, coronary artery restenosis after angioplasty and transplant vascular stenosis. Possible mechanisms involved are mechanical immune-mediated injury followed by inflammation combined with immunomodulatory properties of CMV (US28, IE2) (191).

IMMUNOCOMPROMISED HOST

Infection with CMV is one of the most common complications of immunocompromised patients. It can result from primary infection, reinfection with a new virus strain or reactivation of the latent virus. Advances in diagnostic and therapeutic modalities have reduced the frequency of life-threatening CMV complications and improved overall survival. The severity of CMV disease varies depending on the population, type of transplantation and level of immunosupression and can range from a self-limiting febrile illness to multi-system disease. CMV also has a number of indirect effects that contribute to increased morbidity and poorer outcomes after transplantation. There are three distinct groups of immunocompromised patients potentially affected by CMV infection: those who have solid organ transplantation, hematopoetic stem cell transplant recipients, and patients with HIV/AIDS. The latter two groups can exhibit the most severe CMV disease due to severely impaired cellular immunity.

SOLID ORGAN TRANSPLANTATION (SOT)

CMV infection in SOT remains one of the major causes of extended hospitalization resulting in a significant part of the overall cost of care provided to these patients. It should be expected that 70-90% of patients undergoing SOT will be infected with CMV unless they receive antiviral therapy (214). The source of the infection can be environmental exposure to the CMV virus, the transplant recipient's latent viral infection, virus contained in the transplanted organ or virus in a blood product. Clinical manifestations of CMV infection in SOT recipients can be expressed as an acute systemic febrile illness with symptoms such as fever, malaise, arthralgia and rash. Alternatively, CMV can affect specific organs in 10-30% of patients with CMV disease. Due to the immunomodulatory properties of CMV, infection can have indirect effects resulting in opportunistic infections with fungi or bacteria, or graft rejection (91, 107). For these patients, serologic methods are of limited usefulness for identification of CMV disease in immunocompromised individuals (170) but these assays are used for pretransplant assessment of the solid organ transplant donor and recipient. CMV-seronegative transplant recipients (with no preexisting CMV-specific immunity) who receive an organ from a CMV-seropositive donor are at highest risk for CMV disease (52, 81). Serological testing is also useful to screen the donors of blood products to minimize the risk of CMV infection in seronegative recipients (84).

The expected time of onset is dependent on the recipient's pretransplant immunostatus, level of immunosuppression and presence or absence of antiviral prophylaxis. It is important to monitor the levels of CMV viremia by either the pp65 antigenemia assay or by whole blood or plasma PCR in order to begin preemptive antiviral therapy promptly.

HEMATOPOETIC STEM CELL TRANSPLANTATION (HSCT)

CMV infection continues to cause significant morbidity in HSCT despite the use of CMV prophylaxis and preemptive therapy following allogenic HSCT. CMV disease usually presents as pneumonitis with an interstitial pattern on radiographs with respiratory distress and hypoxemia, or as gastrointestinal disease with mucosal inflammation or erosion anywhere in the GI tract. The affected are mostly patients belonging to a higher risk group: seronegative recipients (R-) of bone marrow from seropositive donors (D+) or

seropositive recipients (R+) with a non-ideal histocompatibility match (173). The indirect effects of CMV can be presented via its immunomodulatory properties potentially enhancing graft versus host disease (GVHD) and opportunistic infections. Breakthrough and late-onset CMV infections in high-risk patients negatively impact outcome. It is important to screen blood products using serological techniques as well as nucleic acid amplification techniques before they are administered to HSCT patients. This allows reduction of exposure of seronegative recipients to products containing CMV.

CMV pneumonia, the most common manifestation of CMV disease in HSCT, is usually diagnosed by detection of CMV in bronchoalveolar lavage (BAL) or lung biopsy specimens in the presence of clinical findings (15, 169)

CMV INFECTION IN HIV AND AIDS PATIENTS

The occurrence of CMV disease in patients infected with HIV is closely related to the CD4 T-cell counts. About 10% of patients with CD4 counts less than 250 cells/mm^3 and more than 20% of patients with counts of less than 100 will develop CMV disease (45, 65, 165, 186). CMV disease is typically seen when HIV viral load is > 100,000 copies/ml of plasma (165) or when p24 antigen is increased along with a low CD4 count (36). CMV disease in HIV patients most often manifests as retinitis, esophagitis and enteritis. Other manifestations include peripheral neuropathy, polyradiculoneuritis, pneumonitis, gastritis or hepatitis and colitis. In HIV infected children, the presence of CMV infection was associated with more rapid progression to AIDS and death. However, with current highly active antiretroviral therapy (HAART), reconstitution of the immune function results in reduced occurrence of CMV disease in HIV-infected individuals (55, 211).

PREVENTION OF CMV INFECTION

REDUCTION OF EXPOSURE OF CHILDREN TO INFECTIOUS SECRETIONS

Pregnant women should take steps to reduce their risk of exposure to CMV and thus reduce the risk of congenital infection. These rules should also be applicable in daycare settings and hospitals (34).

- Wash hands often with soap after changing diapers, feeding young children, wiping a young child's nose, handling toys
- Do not share food, drinks or utensils with young children
- Do not put child's pacifier in your mouth
- Do not share toothbrush with a young child
- Avoid saliva when kissing a child
- Clean toys, countertops

AVOIDANCE OF CMV TRANSMISSION FROM BLOOD PRODUCTS

Patients at risk of CMV disease, mainly seronegative immunocompromised patients, seronegative newborns and pregnant mothers, should be given only irradiated pre-screened CMV-negative blood products. Blood products may also be treated by leukoreduction or leucofiltration, but efficiency might vary (106). CMV has been detected in semen so, in cases of assisted reproduction attention should be given to CMV infection status of donor and recipient (103).

PASSIVE IMMUNIZATION

Administration of hyperimmune globulin preparations is commonly used in SOT (HSCT) as part of prophylaxis (108). Clinical studies showed significant reduction in CMV disease compared to placebo in seropositive donors and seronegative renal transplant recipients, further stressing the importance of antibodies in the prevention of CMV infection.

PREVENTIVE TREATMENT

Ganciclovir is often administered to prevent life-threatening CMV pneumonia or other CMV-related diseases, especially in HSCT or SOT (heart and liver) recipients (73).

VACCINATION

Congenital infection may produce various levels of disease in newborns and can manifest itself later in life. This is the main rationale for development of a CMV vaccine. Also Institute of Medicine, the health arm of the National Academy of Sciences, recognizes the need for development of an effective CMV vaccine as a high priority with symptomatic congenital disease being the main target. It is obvious that innate, humoral and T-cell mediated immunity contribute to protection from CMV. Unfortunately this protection is not 100% effective. For example, it has been shown that previous infection of pregnant mothers reduces transplacental transmission by about 30% in subsequent pregnancies (64).

As mentioned in previous sections, neutralizing antibodies are made mostly against gB, gH and gM/gN complex (28, 30, 31, 112, 174, 197). The genes encoding these glycoproteins are known for their polymorphic nature. This is probably the underlying cause of possible re-infection by different strains (126, 140, 157). Other CMV-related antigens such as pp65 or pp150 tegument proteins also induce antibody response but without neutralizing properties (68, 141). The crucial role of specific cell-mediated immunity in defense against CMV was proven by many authors in experiments involving adoptive T-cell transfers or on the examples of immunocompromised patients with impaired cellular immunity (75, 104, 205). Major targets recognized by CD4 and CD8 cells include pp65, IE1, pp50, pp150, gH and gB (16, 213).

CMV vaccine should prevent infection of pregnant mothers and/or modify virus replication in the placenta, thus preventing transmission to a fetus and/or it should modify fetal infection so CMV does not cause disease. The vaccine could also reduce potential risk for transplantation and HIV patients by generally reducing the number of CMV infections.

Despite all efforts to develop an effective CMV vaccine for prevention of maternal and congenital CMV no vaccine is currently licensed or nearing licensure.

Several approaches for the vaccine have been tested:

LIVE ATTENUATED VACCINE

The live attenuated vaccine is based on an attenuated CMV strain (Towne) grown in fibroblasts. It was administered either intranasally without antibody induction or subcutaneously (SC) leading to some local reaction at the injection side. The virus didn't establish latency and was not detectable in bodily fluids. After SC administration it induced antibody production, CD4 proliferation and CD8 cytolytic responses. This vaccine did not prevent new infection but rather modified the course of infection. The levels of protection were generally lower than those of naturally infected subjects (115, 142). The immunogenicity of attenuated CMV is less than that of the wild types. It was also attempted to combine it with IL-12 (87). Other attempts to attenuate virus by removal of evasion genes or make a recombinant Towne/Toledo strain were tested with partial success.

SUBUNIT PROTEIN VACCINES

Many experimental vaccines are based on recombinant gB since it is the target of the majority (60-70%) of all neutralizing antibodies with the prominence of AD-1 domain (28, 30). It has also been reportedly shown that it can generate CD8 CTL response and CD4 proliferation during normal infection (16). The clinical trials were conducted to test the gB vaccine with MF59 or alum adjuvants and 3 doses were administered at 0, 1, and 6 months. The vaccine was generally safe with some local or generalized mild reactions. Efficacy was around 50% based on infection rates per 100 person-years (135). Other attempts to make a vaccine were based on other glycoprotein complexes such as gM/gN or gH/gL as well as on pp65 and IE1 targeting cytotoxic T-cells (136). The future potential of vaccines based on a single protein has been met with skepticism, due to great variability found in these proteins, further stressed by new findings provided by next generation sequencing techniques (126, 152).

Another avenue of vaccine design was based on pp65-derived peptides recognized by CD8 cells. Peptides were modified by lipids at the N-terminus so no

adjuvant was required. The drawback of this approach is the HLA restriction requiring HLA-specific motifs. This could be potentially overcome by mixing several peptides. Estimates show that even two pp65 peptides might have sufficient coverage of population (5).

Further, attenuated viral vectors such as canarypox (5), vaccinia or adenovirus (6) were tested for the delivery of CMV antigens to the host. The protein/peptide is expressed within a cell and presented to the immune system. It induces cell mediated as well as humoral immunity. Unfortunately the canarypox based-vaccine failed in humans to produce antibodies against gB. In contrast, pp65-based vaccine induced detectable CTL responses, Th responses and production of antibodies in seronegative individuals. Other vectors have been tested in animal models with varying success. One interesting delivery approach is based on Venezuelan Equine Encephalitis virus (VEE, alphavirus) because of its capability to produce high amounts of heterologous proteins targeting dendritic cells for efficacious presentation. Optimally it leads to induction of humoral and cellular immune responses. Since the VEE structural proteins are removed, VEE vector is propagation defective (146). In Phase 1 clinical trials recombinant VEE expressing pp65/IE1 fusion protein were safe with some mild to moderate local reaction after two intramuscular applications. The recipients developed neutralizing antibodies and T- cell responses (IFN-gamma, TNF-alpha, IL-2) against pp65/IE1 antigens (7).

DNA VACCINES

It is known that plasmids are capable of transfecting skeletal muscles, hepatocytes, dendritic cells and macrophages and sustain protein production within those cells. In murine models, DNA vaccines were able to induce responses to gB, pp65 or gM/gN and provided some protection against lethal CMV infection. Sadly, responses seen in larger animals are weaker than in smaller animals. DNA vaccines based on gB and pp65 that have been clinically tested showed T-cell responses in 25-50% of seronegative subjects (IFN-gamma) (131).

CMV THERAPY

ANTI-CMV AGENTS

Antiviral therapy is mostly used for immunocompromised patients since immunocompetent people are rarely symptomatic and CMV infection will resolve on its own in the majority of them. Anti-CMV drugs can be used to treat CMV disease or CMV-disease-related symptoms such as CMV retinitis or CMV end organ disease. Anti –CMV drugs can also be used prophylactically and as preemptive therapy to prevent disease caused by primary and reactivated CMV infection

The most commonly used drug therapies for CMV infection are ganciclovir and its derivative valganciclovir (analogues of 2'-deoxyguanosine) that block DNA elongation. They are activated by phosphorylation by viral UL97 proteinkinase and cellular kinases (11, 187). Foscarnet (a pyrophosphate analog) and cidofovir (analogue of cytosine) don't require the presence of viral kinases and are immune to UL97 mutants (44, 164). There are also new antivirals used in treatment of CMV infection such as fomivirsen (antisense oligonucleotide blocking translation of viral mRNA). It is a potent and specific agent for first-line and second-line treatment of cytomegalovirus associated AIDS retinitis (54). Another new drug is maribavir (Benximidazole ribonucleosides), a promising inhibitor of CMV DNA synthesis and egress of nucleocapsid (12) and CMV replication targeting UL97 by a competitive inhibition of ATP binding (41). Mutations in UL27 may lead to resistance (145).

Each of these drugs has been shown to lower or eliminate viremia or CMV shedding and to prevent or control CMV. No anti-CMV drug has been approved for use in congenital CMV infection because effectiveness and impact on outcome has not been proven beyond limited shedding control resuming weeks after therapy discontinuation.

As with any other treatment of prolonged infection it is expected that drug resistance might and will develop. The first drug-resistant strains of CMV emerged in AIDS patients. The resistance develops by newly occurring mutations in the UL97 and/or UL54 genes encoding protein kinase and CMV DNA polymerase, respectively. This results in defective intracellular phosphorylation of drugs dependent on viral protein kinase and lower levels of active forms of those drugs (GCV, VGCV). In case of mutations of CMV DNA polymerase

resistance affects all of the drugs used in CMV treatment since it is the target for all of them. Mutations of UL97 occurs at higher frequencies than that of the UL54 gene. It is interesting that in the majority of GCV resistance cases it first develops in UL97 and if the drug is still used UL54 resistance follows. This might result in a generation of multiresistant strains of CMV. Location of mutations in the *UL54* gene responsible for CDV and FOS resistance are different. At this time there are some CMV strains resistant against all currently used anti-CMV drugs. They are usually isolated from AIDS patients one example is laboratory strain TR (60).

DETECTION OF THE VIRUS IN CLINICAL SAMPLES.

This is no universal technique for CMV infection detection and monitoring during the course of illness and therapy. CMV-associated disease should be first diagnosed clinically and it should then be confirmed in combination with the laboratory findings

SEROLOGY

Serological tests are determined by the presence or absence of CMV IgG and represent historical status of CMV infection. Anti-CMV IgG antibodies can be detected by complement fixation, enzyme-linked immunosorbent assay (ELISA), anticomplement immunofluorescence, radioimmunoassay, and indirect hemagglutination (133). IgM antibody is not specific for primary infection as IgM can persis for months causing false-positive results, and because IgM can be positive in reactivated CMV infections (8, 124, 148). Primary infection can be serologically detected utilizing IgG avidity assays and is based on the fact that IgG antibodies of low avidity are present during the first few months after infection and avidity increases over time along with antibody maturation. Thus, high anti-CMV IgG avidity represents longstanding infection in an individual. Avidity levels are reported as the avidity index, which is the percentage of IgG bound to the antigen following treatment with denaturing or detergent agents (153).

CELL CULTURE

Specimens are inoculated onto human fibroblast cells, incubated and observed for a period of time ranging from 2 to 21 days until CMV exhibits a typical cytopathic effect (CPE) characterized by foci of flat, swollen cells. The results can be reported as negative after 3 weeks. This lengthy assay has been replaced by a modified shell vial assay. The difference is in a spin-amplification technique (centrifugation of specimen onto the cell monolayer improving virus absorption and infectivity) designed to accelerate virus growth (43). Viral antigens are then detected by antibodies against the CMV MIE antigens by indirect immunofluorescence after 16 hours of incubation (151). This method was adapted to be performed in 96-well microtiter plates (23) allowing for screening of a larger number of samples.

ANTIGENEMIA

The antigenemia assay is the most commonly used assay in clinical settings for CMV virus **quantification** in blood specimens but it is slowly being replaced by PCR-based techniques. The antigenemia assay is based on monoclonal antibodies detecting the viral pp65 antigen. Antigenemia is measured by the counting of leukocyte nuclei positive for phosphoprotein pp65 in a cytospin preparation of 2×10^5 peripheral blood leukocytes in direct immunofluorescence assay (68, 154, 192). This test is limited to detection of the virus in leukocyte nuclei so in patients with neutropenia it tends to give false-negative results (14).The results correlate closely with viremia and clinical disease severity in immunosupressed populations (193). It is very labor intensive with low throughput and no automation, is biased by the reader and the samples have to be processed immediately (within 6 hours) (168).

POLYMERASE CHAIN REACTION AMPLIFICATION

PCR is a rapid and sensitive method of CMV detection based on amplification of viral nucleic acids. The techniques target early and late antigenic genes in their well conserved regions (56, 147), DNA can be obtained from whole blood, leucocytes, and plasma or any other tissue (tissue biopsy samples) or fluid (urine, cerebro-spinal fluid, broncho-alveolar lavage) (33, 61, 67, 96, 113). Specimen stability is generally good so delay between sample collection and processing is not critical (156). PCR for CMV DNA can be either qualitative or quantitative. With today's technological advancements and availability, quantitative (Real-time) assays are used. Real-Time PCR allows continuous monitoring of disease behavior and efficacy of treatment (97). An important part of assessment is properly set thresholds for differentiation between latent and active infection, to identify patients at risk for CMV disease for preemptive treatment (172). Also viral mRNA can be detected but it appears to be less sensitive than the antigenemia test and PCR diagnosis of CMV disease. Furthermore, the extraction of mRNA is relatively difficult (120).

NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION (NASBA)

This assay allows the specific nucleic assay sequence-based amplification of unspliced viral mRNAs (late pp67 mRNA expression) in a background of DNA using specific isothermal technique of amplification. The data suggest that NASBA may be more sensitive than the antigenemia assay for the detection of CMV infection in blood (13). Whole blood samples can be stored prior to testing, and the test can be completed in a day.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is used primarily on tissue (liver, lungs) or body fluid samples prepared as slides or cytospins. Monoclonal or polyclonal antibodies against early CMV antigens are applied and visualized by immunofluorescence or immunohistochemistry techniques. This approach is more sensitive and very specific compared to plain histological microscopy but it is very labor intensive and requires expertise for assessment (138). Also false-negative results might occur due to focal distribution of the virus (48).

HYBRID CAPTURE ASSAY

Hybrid capture assay uses RNA probes to detect and quantify viral DNA in an ELISA-type format where resulting signal is measured. Because it detects DNA without amplification, its sensitivity is questionable (116).

NEW EMERGING DETECTION TECHNIQUES

With the need to rapidly and accurately detect viral presence and the requirement to perform these tasks in large scale for screening purposes, new methods of detection are being developed and commercialized. One of the technologies used for such detection is Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). This technology is used to directly detect and identify CMV proteins or as a detection system for underlying multiplex PCR-based technique (178).

SUMMARY OF THE THESIS GOALS

The presented set of publications deals with various aspects of cytomegaloviral infection:

- Strain variations of CMV

  Sequences of various genes were examined by different techniques for their variability and the data were analyzed. Also different applications affected by strain variability were examined.

- Detection of CMV reinfection

  Recombinant gB and gH strain specific proteins were expressed in *E.coli* and purified. ELISA-based method was developed and successfully used in consequent studies.

- Viral detection in clinical samples

  A multiplex Real-time PCR-based technique was developed, tested and compared to the current gold standard rapid culture technique and used to screen newborns.

STRAIN VARIATIONS OF CMV

It has been shown by many investigators that CMV genome contains many genes with significant variability, thus allowing it to recognize and distinguish viral isolates or strains (40, 140, 152). Many different techniques were used in the past to detect this variability. The techniques include restriction fragment length polymorphism (RFLP) (40) of whole genome or single gene, single-strand conformation polymorphism (SSCP) (10), various hybridization techniques (46), and DNA sequencing (140). The most commonly examined genes are those exposed to host immune system and thought to be under selective pressure such as *UL144, gN, gB, gO, gH*. Based on these studies, genotypes among these genes were established (42, 140).

Our efforts in the field of CMV gene variability were focused on gN and gB genes as shown in presented publication #11 (126). Here we used the genotyping technique based

on cloning of the PCR *gN* or *gB* gene products from DNA extracted from blood or urine followed by DNA sequencing of the clones in order to reveal different variants of genes if present. The results of the cloning/sequencing were compared to results acquired from the traditional RFLP-based technique. It was shown that 93% and 69% of samples contained multiple variants (up to 3 per sample) of *gN* and *gB* gene, respectively. Also by comparison with RFLP results it was shown that the RFLP-based technique is less sensitive in recognition of multiple gene variants per sample and associated with technical difficulties. Such results were in contradiction with other investigators using different techniques that showed only one variant per sample in most cases. We did not observe any linkage between two genes but our conclusions are limited by sample size.

These results prove that multiple CMV strains are present during infection in the normal healthy host. Our general interest is in **congenital** infection and specifically this fact led to the next presented publication #1 (160) where we focused on determining whether multiple CMV strains can be transmitted to the fetus. The same genotyping technique was used for gN genotyping. Glycoprotein B and glycoprotein H were genotyped using Real-time PCR utilizing type-specific primers and probes. We also looked at compartmentalization of genotypes since blood, urine and saliva samples were tested. Results revealed that 21.4% of saliva samples obtained within the first 2 days of life contained >1 CMV genotypes. Multiple CMV genotypes were found in 39% of urine, saliva, and blood samples obtained within the first 3 weeks of life. Also, 4 infants demonstrated distinct CMV strains in different compartments. These results show that multiple strains can cross the placenta and cause congenital infection. The impact of this fact is currently unknown.

Gene variability has many potential consequences even for clinical settings. We deal with the same issues in our screening study, where one of the techniques used to detect CMV in clinical samples was real-time PCR as described in presented publications #4 and #6 (21, 22). After initial runs targeting previously established “well conserved” AD-1 region of gene encoding *glycoprotein B* we found rather low sensitivity of the assay caused by false-positive results, as reported later in our next presented publication #3 (125). Originally unknown mutations in the *gB* gene were found. This problem was overcome by addition of another target (*IE2* gene exon 5) in a multiplexed PCR. This reduced false-positive rates by targeting two independent conservative genes (based on current knowledge). Unfortunately, in the future new mutations might appear in the targeted region of these genes and PCR targets might need to be reworked.

Yet another significant outcome resulting from genomic variability, and consequentially protein variability, is its effect on development of an effective vaccine. As mentioned before, most of the tested vaccines are based on conserved proteins or peptides with high immunogenicity ideally resulting in development of neutralizing antibodies or T-cell mediated responses. All of the tested vaccines used a single protein variant of gB, gM/gN or pp65/IE2 without accommodating for other possible variants with the exception of vaccines targeting CD8 cells where HLA-restricted peptides are a must. It is possible that some significant (or sufficient) cross reactivity might exist but this is unpredictable.

The most recent studies using new technologies such as deep sequencing are showing much higher intrahost and interhost diversity in almost every ORF of the HCMV genome. The genes examined so far are surprisingly among the "more conserved" genes and are probably not a good measure of diversity. The reasons behind such high variability could be high mutation rates during replication of viral DNA genomes (which is unlikely since CMV encodes for proofreading DNA polymerase), high levels of replication, leading to an accumulation of mutations (only a single or very few virions cross the placenta to initiate a congenital infection, initially 10^7 copies/ml), or reinfection or co-infection with possible recombination. However, many variants exist before any selective pressure is applied (152).

DETECTION OF CMV REINFECTION

Reinfection is an important part of CMV's natural history since it has been associated with damaging congenital infection and adverse outcomes in transplant recipients (20). Unfortunately, naturally developed immune responses are not capable of preventing new infection by CMV. Although significant cross-immunity exists, as shown in studies of CMV-positive mothers rate of congenital CMV was reduced by approximately 30% compared to naïve mothers. This could be caused by high genomic variability of the CMV and its immune evasive capacity in preventing the host from being newly infected/reinfected(64).

In order to prove reinfection, we focused on developing an ELISA based assay usable in detection of reinfection. This is presented as publication #9 (127). The idea is based on the premise that reinfection of seropositive individuals will be caused by a

different variant of the CMV virus, followed by development of new antibodies with a different specificity than those originally present. The appearance of antibodies with new specificity in sequential samples is considered proof of reinfection. So far there is no way to distinguish reactivation (of dormant virus) from reinfection. To detect the appearance of new antibodies I expressed set of recombinant strain specific regions of gH and gB glycoproteins derived from 2 different laboratory- adapted strains AD169 and Towne. Glycoprotein B peptides were expressed in *E.coli* Rosetta cells as His-tag labeled peptide, using pET 21 expression vector and purified on metal affine Cobalt resin columns. Glycoprotein H peptides were expressed as β-galactosidase fusion proteins and purified by a series of detergent washes. These peptides were successfully used in ELISA assay to detect reinfection by new CMV viral strains in serial samples.

This reinfection detection technique was applied to a group of healthy seroimmune women in order to determine the frequency of and risk factors for CMV reinfections as presented in publication #7 (157). Here, we prospectively followed 205 seropositive women. Serum samples were collected on at least 2 visits together with a risk factors questionnaire (sexually transmitted infections, information on numbers of sexual partners, and information on child care) and demographic information. Reinfection was established in 29% of study participants. None of the risk factors was linked with CMV reinfection. It was also interesting to discover that the presence of antibodies against at least one of the tested antigens at baseline decreased risk of reinfection by 63%. This indirectly implies a protective role of antibodies as shown by other studies (64).

The approach presented in publication #9 (127) was used to establish CMV reinfection rates in order to determine the role of reinfection on the incidence of congenital CMV infection in a group of previously seropositive women, and is presented in publication #8 (215)

Demographics and risk factor information was collected as well as prenatal and postnatal sera from 40 mothers of congenitally infected infants and from 109 mothers of uninfected infants. These samples were examined for reinfection. The only statistically significant risk factor was the fact that more mothers of infected infants cared for children less than 3 years of age. All first samples were anti-CMV IgG positive with high avidity. Reinfection was found in a statistically significant higher number of mothers of infected infants compared to mothers of uninfected babies (17.5% vs 4.6% respectively). This finding as well as DNA sequencing of virus recovered from newborns and its comparison

to CMV genotypes found in mothers suggests that reinfection is a major source of congenital infection in this population.

Our technique successfully detected CMV reinfection; due to the large potential of CMV variability, this technique has its limitations. It is probably greatly underestimating rates of reinfection as we are only testing 2 variants of 2 glycoproteins. Also, it is capable of detecting reinfection only in subjects effectively developing detectable amounts of antibodies to react with these linear epitopes. Moreover, high CMV DNA variability has been found in many other genes not covered by this technique. A very limited number of gene changes will change the amino acid sequence, resulting in induction of different antibody specificity.

VIRAL DETECTION IN CLINICAL SAMPLES

Correct and accurate viral detection as well as its correct quantification is very important in clinical settings. This is even more vital for high-risk patients such as those with SOT, HSCT, and AIDS patients with heavily deficient cellular responses. Timely detection and accurate monitoring of responses to therapy is crucial for preemptive treatment and therapy. The multitude of detection techniques currently used is comprehensively described in our review presented as publication #2 (159).

In presented publications #6 (21) and #4 (22), we focused on comparing PCR and rapid culture CMV detection techniques applied to different clinical samples. The emphasis of these studies was to develop a mass screening technique. This was done under the auspices of The CMV and Hearing Multicenter Screening (CHIMES) study. In publication #6 (21) we compared Real-time PCR results obtained from dried blood spots (collected from newborns) to results from saliva rapid culture (here recognized as the "gold standard" technique) in 20 448 infants. Real-time PCR initially targeted *gB* gene, a second multiplexed target was added later (Exon 5 of *IE2* gene) in order to increase sensitivity that was shown to be relatively low in preliminary analyses. Unexpected gene variability resulted in failure of the PCR reaction targeting *gB.* This was elucidated publication #3 (125), emphasizing correct choice of target sequence and design of PCR. The testing revealed 92 positive newborns (incidence 0.45%). Original sensitivity was 28% which increased to 34% after addition of the second PCR target, both with a specificity of >99%. Although sensitivity increased, it was still low compared to saliva rapid culture,

rendering it unusable as a screening method. These results lead the studies documented in publication #4 (22) in which we applied the same Real-time PCR technique in a multiplex setting to liquid or dried saliva specimens. This was again compared to saliva rapid culture. The tests were performed on 34 989 infants yielding 177 positive results (incidence 0.5%). From these, 17 662 were screened using liquid saliva specimens with 85 positive infants and assay sensitivity of 100% and specificity of 99.9%. Also, 17 327 dried saliva samples were collected from newborns. Of these, 74 samples tested positive with a sensitivity of 97.4% and specificity of 99.9%. Based on these results, both techniques/specimens should be considered as a potential screening tool.

The same PCR technique was applied to different specimens collected from newborns with dramatically different results, ie, with very low sensitivity in detecting CMV in DBS specimens. Generally PCR detection of CMV in peripheral blood is used as a standard detection technique in immunocompromised patients (SOT, HCST recipients, AIDS patients) so the failure of the same technique in DBS from newborns was not anticipated. This is most likely caused by a possibly different pathogenesis of congenital CMV infection compared to immunocompromised hosts. Since the fetus could be infected months before being tested, the viremia might not be present by the time of testing. Further testing of the performance of Real-time PCR compared it to a nested PCR-based method also suggests that the sensitivity of the PCR is not the reason for failure.

The research focus of our laboratory has always been on congenital CMV infection, which is the major cause of sensorineural hearing loss in children, and on topics related to this such as transmission risks and routes. Publication #5 (2) describes CMV blood presence and CMV shedding in urine of mothers as one of the risk factors for congenital or perinatal infection. Serial blood and urine samples were collected from 205 CMV seropositive women and viruria and DNAemia were assessed. The results show that CMV DNA was detected in 83% of urine samples and 52% of blood samples at least once. Also no difference was observed in CMV positivity at baseline between the groups of women with and without evidence of reinfection. These findings led us to conclude that the presence of viruria and DNAemia are common and that naturally acquired immunity to CMV does not alter shedding patterns. This suggests that young women are generally at risk of delivering congenitally infected infants, although, as shown earlier, the risk of delivering an infected baby is even higher in previously naïve mothers (64).

The same quantitative detection technique was used in the last presented publication #10 (158) to establish existence of the relationship between CMV viral load in infants and development of sensorineural hearing loss. Here we tested blood from 16 symptomatic and 119 asymptomatic congenitally infected children for CMV burden. Analysis revealed no difference in viral load between compared groups after comparison was performed on 3 different age groups. Interestingly, a viral load <3500 copies/ml in the age group younger than 2 months had negative predictive value of 94.4%, suggesting that low viral load in asymptomatic children is associated with lower risk for development of hearing loss. These finding are somewhat in contradiction to earlier studies by Lanari et al (102). This is most likely the result of differences of study populations since Lanari et al. did not focus specifically on development of hearing loss (only 1 of 37 congenial CMV infected children in that study had HL). There is also disagreement with an older study by our group (Boppana et al) (18) in which a portion of the study subjects were shared. The numbers of subjects was increased and previous observations could not be confirmed.

OVERVIEW OF AUTHOR'S PUBLICATIONS

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PATENTS

Novel Saliva Polymerase Chain Reaction Assay for Congenital Cytomegalovirus Infection. Patent Pending #U2010-0045

AUTHOR'S LEVEL OF CONTRIBUTION IN PUBLICATIONS

1. Mixed infection and strain diversity in congenital cytomegalovirus infection.
   Second author: sample collection and processing, assays design, participation in sequence and data analysis.
2. Overview of the diagnosis of cytomegalovirus infection.
   Second author of the review publication.
3. Diagnostic consequences of cytomegalovirus glycoprotein B polymorphisms.
   First author: principal author of the study, participated in data acquisition; sequence analysis, manuscript preparation.
4. Saliva polymerase-chain-reaction assay for cytomegalovirus screening in newborns.
   Tenth author: significant role in assays design; participation in data acquisition, analysis and data interpretation as wells as critical comments, participation on preparation of methods section of manuscript.
5. Cytomegalovirus viruria and DNAemia in healthy seropositive women.
   Second author: participation in sample acquisition and processing, data collection and statistical data analysis.
6. Dried blood spot real-time polymerase chain reaction assays to screen newborns for congenital cytomegalovirus.
   Third author: crucial role in assays design, PCR assay development and comparisons; participation in data acquisition, analysis and interpretation, participation in preparation of methods section.
7. Cytomegalovirus reinfections in healthy seroimmune women.
   Third author: design, expression and purification of recombinant protein and related assays, participation in sample acquisition, data collection and data analysis.
8. Human cytomegalovirus reinfection is associated with intrauterine transmission in a highly cytomegalovirus-immune maternal population.
   Fourth author: expression and purification of recombinant proteins and design of genotyping assays.
9. Enzyme-Linked Immunosorbent Assay Method for Detection of Cytomegalovirus Strain-Specific Antibody Responses.
   First author: crucial role in design, expression and purification of recombinant proteins and assay development, participation in data acquisition and statistical analysis, participation in manuscript preparation.

10. Cytomegalovirus blood viral load and hearing loss in young children with congenital infection.

    Second author: sample collection and processing, participation in data acquisition and statistical analysis.

11. Cytomegalovirus Strain Diversity in Seropositive Women

    First author: crucial role in genotyping assay development, participation in data acquisition; sequence analysis, manuscript preparation.

I hereby certify that all statements made by me are true, accurate and complete.

Date: 3rd April, 2012

Dr. Zdenek Novak

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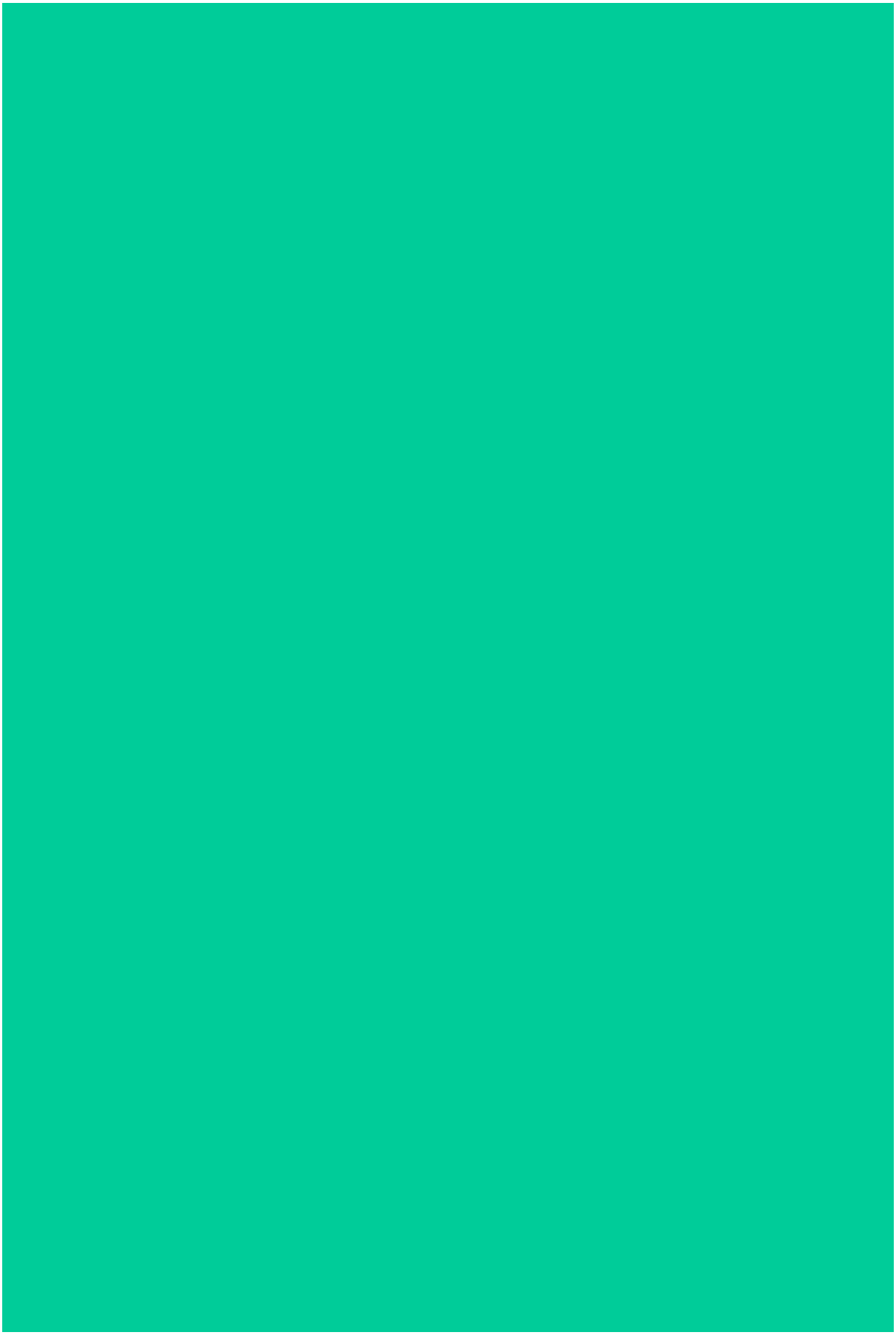

Mixed Infection and Strain Diversity in Congenital Cytomegalovirus Infection

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Background. Cytomegalovirus (CMV), the most common cause of congenital infection, exhibits extensive genetic variability. We sought to determine whether multiple CMV strains can be transmitted to the fetus and to describe the distribution of genotypes in the saliva, urine, and blood.

Methods. Study subjects consisted of a convenience sampling of 28 infants found to be CMV-positive on newborn screening as part of an ongoing study. Genotyping was performed on saliva specimens obtained during newborn screening and urine, saliva, and blood obtained at a later time point within the first 3 weeks of life.

Results. Six (21.4%) of the 28 saliva samples obtained within the first 2 days of life contained >1 CMV genotype. Multiple CMV genotypes were found in 39% (5/13) of urine, saliva, and blood samples obtained within the first 3 weeks of life from 13 of the 28 newborns. There was no predominance of a CMV genotype at a specific site; however, 4 infants demonstrated distinct CMV strains in different compartments.

Conclusions. Infection with multiple CMV strains occurs in infants with congenital CMV infection. The impact of intrauterine infection with multiple virus strains on the pathogenesis and long-term outcome remains to be elucidated.

Cytomegalovirus (CMV) is a frequent cause of congenital infection worldwide. Between 20 000 and 40 000 children are born each year in the United States with congenital CMV infection; ~15% of those will develop permanent sequelae, the most common being sensorineural hearing loss (SNHL) [1, 2]. The reason only some children develop SNHL or other sequelae after congenital CMV infection is not understood but could be related to both host and viral factors.

Human CMV (HCMV) is a large virus with >200 open reading frames. CMV isolates from infected individuals have been shown to be genetically diverse [3]. Several regions of the HCMV genome have been used to define distinct genotypes based on clustering of polymorphisms. Mixed infection with multiple CMV strains occurs in various patient populations including immunocompetent and immunocompromised subjects [4–7]. However, infection with multiple strains has rarely been described in newborns with congenital CMV. This study sought to determine if congenital infection can result from multiple CMV strains by genotyping glycoproteins gB, gN, and gH in blood, urine, and saliva specimens of newborns with congenital CMV infection. In addition, the distribution of CMV genotypes in saliva, urine, and blood was examined.

METHODS

Study Population

From May 2007 through January 2009, 201 infants were found to be CMV-positive on newborn screening at the

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7 hospitals participating in the National Institute on Deafness and Other Communication Disorders (NIDCD) CMV and Hearing Multicenter Screening Study (CHIMES Study) [8]. Screening for congenital CMV infection was performed by rapid culture of saliva specimens (screening saliva samples). Infants that screened positive for congenital CMV infection were enrolled in the follow-up component of the study and urine, saliva, and dried blood spot (DBS) samples (follow-up samples) were obtained to confirm infection. Institutional review board approval was obtained at each study site. Written informed consent was obtained from a parent for their newborn's enrollment in the study. A convenience sampling of 28 study subjects from all 7 study sites was selected based on the availability of adequate remnant saliva, urine and blood specimens for genotyping. The demographic characteristics of these 28 subjects did not differ significantly from the demographics of the entire group of 201 children found to be CMV-positive during the study period (data not shown). Laboratory personnel were blinded to demographic characteristics, clinical findings, and previous genotyping results of study subjects.

Characterization of CMV Genotypes

DNA was extracted from urine and saliva samples using commercial spin columns (Qiagen, Inc). DNA extraction from DBS samples was performed from 2 3-mm punches as described elsewhere [8]. For gN (UL73) genotyping, samples underwent polymerase chain reaction (PCR) to amplify the gN region using primers and conditions previously reported [7]. To reduce the possibility of PCR artifact, positive and negative controls were included with each PCR run. PCR products were directly cloned into the TOPO TA cloning vector pCR 2.1 (Invitrogen Inc) and up to ten individual colonies were screened for the presence of the gN insert [7]. The nucleotide sequences were compared with the published sequences of the 7 described gN genotypes (GeneBank accession numbers AF309971, AF309976, AF309980, AF390773, AF309987, AF309997, and AF310004). Genotyping of gB (UL55) and gH (UL75) was performed as described using the TaqMan platform [6, 9]. Specimens in which no gB genotype could be determined by this method underwent PCR to amplify the 961–1738 bp gB region with the following primers: gBlong-Fw (5′cac agg ttg gtg gct ttt ct) and gBlong-rev (5′gtc gtg agt agc agc gtc ct). The PCR conditions were optimized for HotStart Taq polymerase (5′) and included initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 40 seconds, extension at 68°C for 1 minute, and final extension at 68°C for 5 minutes. Nucleotide sequences of purified PCR products were compared with published sequences in the NCBI database (GeneBank accession numbers M60929, M60932, M60933, M60926, and GU180092). Different laboratory personnel performed genotyping on screening and follow-up samples (for blinding purposes).

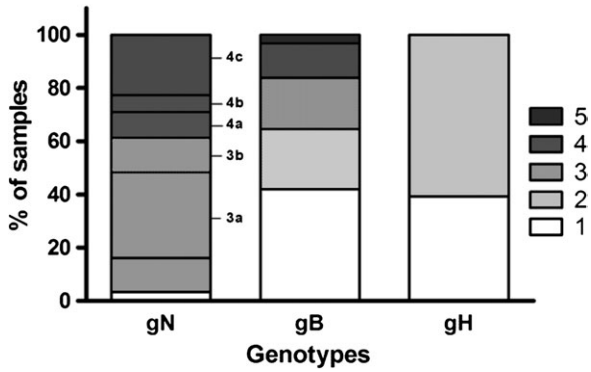

Figure 1. Frequency of CMV gN, gB, and gH genotypes in the saliva of 28 infants with congenital CMV infection. All 7 gN genotypes (gN-1, gN-2, gN-3a, gN-3b, gN-4a, gN-4b, gN-4c), all 5 gB genotypes (gB-1, gB-2, gB-3, gB-4, gB-5), and both gH genotypes (gH-1 and gH-2) were demonstrated. Saliva samples were obtained within the first 2 days of life.

RESULTS

Population Demographics

Study infants were primarily identified from the newborn nursery (27/28, 96%). Just over half were black, 5 (18%) were non-Hispanic white, 4 (14%) were Hispanic white, 1 (4%) was multiracial, and 1 (4%) was Asian. The gender of the study subjects was equally distributed (50% female).

CMV Genotyping of Samples

Screening saliva samples obtained at a median of 1 day (range, 0–2 days) of age from the 28 study subjects were analyzed for gN, gB, and gH genotypes. All 7 gN genotypes (1, 2, 3a, 3b, 4a, 4b, 4c), all 5 gB genotypes (1–5) and both gH genotypes (1, 2) were represented in the saliva samples (Figure 1). gN genotyping was performed on 26/28 screening saliva samples because the gN gene could not be amplified from 2 specimens. gN type 3a was the most commonly observed strain (32%) followed by type 4c (23%). Genotyping of gB and gH was performed on all 28 screening saliva samples. gB type 1 was the most common variant (42%) followed by type 2 (23%). In 2 subjects, gB genotypes could not be determined using the type-specific real-time PCR. However, nucleotide sequence analysis matched these to a previously described gB genotype 5 [10]. gH types 1 and 2 were distributed in 39% and 61% of specimens, respectively.

6 of the 28 (21.4%) newborn saliva samples had more than one CMV strain. 4 infants had 2 distinct CMV genotypes and 2 infants were shedding 3 genotypes in saliva specimens obtained within 2 days of birth. To further investigate CMV strain diversity, genotyping was carried out on urine, saliva, and DBS samples obtained at enrollment into the follow-up component of the study. To avoid the possibility of identifying CMV strains acquired postnatally, only samples from the group of 13 infants that were enrolled in the follow-up study

Table 1. Distribution of CMV Genotypes in the Saliva, Urine, and Dried Blood Spot (DBS) of 13 Children With Congenital Infection

		gN				gB				gH		
Subject	Screen[a] Saliva	Urine	Saliva	DBS[b]	Screen Saliva	Urine	Saliva	DBS	Screen Saliva	Urine	Saliva	DBS
a	…[c]	4c	4c		1	…	1		2	…	2	
b	4c	4c	4c		1	2	2,3		2	2	2	
c	3a	3a	3a		4	4	4		1	1	1	
d	3a	3a	3a	4a	2,3	…	…	…	1	…	…	…
e	1	4a	1	1	2	1	2	…	1	2	1	…
f	2	2	2		4	4	4		1	1	1	
g	2	2	2		3	3	3		2	2	2	
h	3a	3a	3a	4c	2,4	2,4	2,4	1,2	1	1	1	…
i	4c	4c	4c	4c	2	…	2	…	2	…	2	…
j	4a	4a	1		1	1	1		2	2	2	
k	4b,3a,4c	4c	4c		3	3	3		2	2	2	
l	3b,3a,4c	3b	3b		2	2	2		1	1	1	
m	3b	3b	3b		1	1	…		2	2	…	

NOTE. DBS, dried blood spot.

[a] Saliva samples taken within the first few days of life. Urine, saliva, and DBS samples were taken at a later time point within the first 3 weeks of life.

[b] CMV DNA was detected in only 4/13 DBS samples.

[c] Ellipses denotes sample unavailable for testing.

and had samples obtained within the first 3 weeks of life were examined. The average age at sample collection was 15.3 days (±4.3 days). Genotyping of follow-up urine, saliva, and DBS samples showed that multiple CMV genotypes were present in 5 (39%) of 13 infants (Table 1). Four infants shed 2 different CMV strains, whereas 1 infant (Subject h) shed 3 different gN and gB genotypes. When both screening and follow-up samples were considered, approximately a third of study infants (9/28, 32%) were infected with multiple CMV strains. Interestingly, in 2 infants, (subjects b and j) a new genotype was detected in the saliva specimen taken at follow-up that was not present in the screening saliva specimen. In 1 infant (subject b), gB genotype 1 was detected in the screening saliva specimen; however, gB genotypes 2 and 3 were detected in the follow-up saliva specimen. Another infant (subject j) had gN genotype 4a in screening saliva, whereas the follow-up saliva contained type 1.

CMV Genotyping by Compartment

The presence of different viruses in different compartments was determined by analyzing saliva, urine, and blood specimens from the group of 13 infants obtained at enrollment into the follow-up study (Table 1). As shown in Table 1, no genotype predominated in saliva, urine, or blood compartments. However, 5 infants (subjects b, d, e, h, and j) had different CMV genomic variants in different compartments. Of the 6 infants whose screening saliva samples contained multiple CMV genotypes, follow-up saliva, urine, and blood samples were available from 4 (subjects d, h, k, and l). Multiple CMV genotypes were found in the follow-up samples of 2 of the 4 (subjects d and h) (Table 1).

DISCUSSION

This report demonstrates that there is great diversity among the strains that cause congenital CMV infections and congenitally infected neonates often harbor multiple CMV genotypic variants. Among the 28 saliva samples from infants obtained at birth and saliva, urine, and blood samples from 13 of the 28 subjects obtained at follow-up, all gN, gB and gH genotypes were found to cause congenital infection. Furthermore, mixed infection with >1 virus strain was detected in approximately one-third of the study infants. In addition, the presence of distinct virus strains in specimens from different compartments from the same child was demonstrated.

In studies of both immunocompetent and immunocompromised hosts, multiple CMV genotypes have been detected in older children and adults. Although studies have documented that these viruses are acquired over time through reinfections [4, 11–14], it is also possible that multiple viruses can be acquired at the time of primary CMV infection. It is generally believed that vertical transmission to the fetus occurs after maternal viremia. Whether this transmission occurs as a single event during pregnancy or through multiple placental transmission events over the course of the pregnancy is unknown. Studies of the guinea pig model of congenital CMV infection have shown that maternal viremia leads to placental infection, and, in some instances, a secondary viremia can occur and result in secondary seeding of the placenta. However, dissemination to the fetus is not always immediate and the placenta serves as a reservoir for the virus [15–17]. Studies of human placentas have also shown multiple CMV genotypes at the maternal-fetal interface [18]. Thus, virus transmission to the fetus resulting in

congenital infection with multiple CMV strains could occur as a single infection with codisseminating strains, or by multiple transmission events of individual viruses, or both.

Studies examining the association between virus diversity at a single polymorphic gene and outcome in children with congenital CMV infection reported conflicting results [19–22]. However, studies in the murine model and in immunocompromised patients suggest that coinfection with multiple strains of CMV could lead to enhanced pathogenicity [5, 23, 24]. In a study that examined the diversity of 3 polymorphic CMV genes in infants with congenital CMV infection, >1 virus strain was detected in 8/10 specimens obtained from stillborn infants but only a single genotype from 22 living newborns [25]. However, the interpretation of these findings is difficult because tissue-cultured viruses from living newborns were compared with paraffin-embedded tissue specimens from stillborn infants. Since propagation of virus in tissue culture often selects for a single virus strain [26–29], the inability to detect multiple virus strains in infants with congenital CMV infection in previous studies [22, 25] could be due to methodological issues.

In the current study, original saliva, urine, and DBS samples from congenitally infected infants were analyzed to avoid tissue culture selection of viruses, and real-time PCR and cloning of PCR products were used to genotype virus strains. Previous studies examining genotype distribution have used either virus from tissue cultures or samples from urine only. In addition, genotyping was performed by gene amplification and sequencing of the product [22, 25]. Thus, it is likely that only dominant or selected genotypes were detected. Although the implications of finding that congenital infection can be caused by multiple CMV strains, with respect to sequelae such as SNHL, are not yet known, the results from the ongoing NIDCD CHIMES study [8] that includes prospective follow-up of a large number of congenitally infected infants should provide a better understanding of the role of mixed infection on outcome.

In 13 study infants, urine, saliva, and blood samples were available within the first 3 weeks of life and were examined to determine the CMV strain diversity in different compartments. Although there was not a predominant genotype, unique strains in different compartments were found in 5 of these infants. Compartmentalization of CMV strains has been reported in immunocompromised populations [30, 31]. CMV has the ability to grow in certain cell types, and variable cell tropism is conferred by particular viral genes that are present in clinical CMV strains [32, 33]. This observation has raised the possibility that CMV strain variation might explain differences in the biological behavior of different virus strains. The finding that infants with congenital CMV infection can harbor multiple CMV genotypes, and that unique genotypes are found in different compartments, underscores the need for examining the relationship between strain variation and biological characteristics of viruses.

In 2 study infants, genotypes that were detected in saliva at follow-up (at 2–3 weeks of life) were different from those detected in the saliva samples obtained at birth. This finding could have multiple explanations. Although it is possible that these infants acquired new strains during the time between screening and follow-up, this is unlikely because the follow-up samples were obtained between 2 and 3 weeks of age. Alternatively, both strains may have been present at both time points but not detected in the screening samples because relatively low numbers of the minor virus populations were present. In a recent study, investigators examined plasma and bronchoalveolar samples from 9 immunocompromised patients using a highly sensitive deep sequencing method. All 9 patients had mixed infections with 1 or 2 dominant genotypes and several low-abundance genotypes. In addition, the prevalence of the individual genotypes was shown to change over time, with strains that were initially minor becoming dominant [6]. The appearance of a ''new'' genotype not detected in the initial sample in our study infants may reflect a similar change in the prevalence of the individual strains.

A limitation of this study is that only a small proportion of the infected children identified in the NIDCD CHIMES study were included, which may have led to selection bias. However, this potential selection bias was unlikely to have affected the findings since the 28 study subjects have similar demographic characteristics as all CMV positive infants during the described time period. An additional limitation of the study is the use of real time PCR to detect gB and gH genotypes. The sensitivity of this assay is dependent on the relative amounts of viral DNA; thus, minor viral populations with low abundance of type-specific viral DNA could have been missed. This reduced sensitivity of the real-time PCR may have resulted in an overall underestimation of the true viral diversity within a sample and subject. Our conclusion that infection with multiple virus strains can occur in infants with congenital CMV infection remains valid, however. CMV is a large virus with >140 genes. In this study, only 3 loci (gB, gH, and gN, all known targets of neutralizing antibody) were examined to determine genetic diversity. Since many more polymorphic CMV genes have been identified, it is likely that the true virus strain diversity in the study population was underestimated. Although the relative frequency of mixed infection might change if a larger proportion of infected infants were included in the study and if more CMV loci were examined for diversity, the finding that some congenitally infected infants harbor multiple virus strains remains an interesting observation.

In summary, the present report demonstrates that there is great diversity in the CMV strains that cause congenital infection and that infection with multiple CMV strains occurs in congenital CMV infection. However, the relationship of specific genotypes and the implications of infection with multiple viral strains for the pathogenesis and long-term outcome in children with congenital CMV infection are not yet known.

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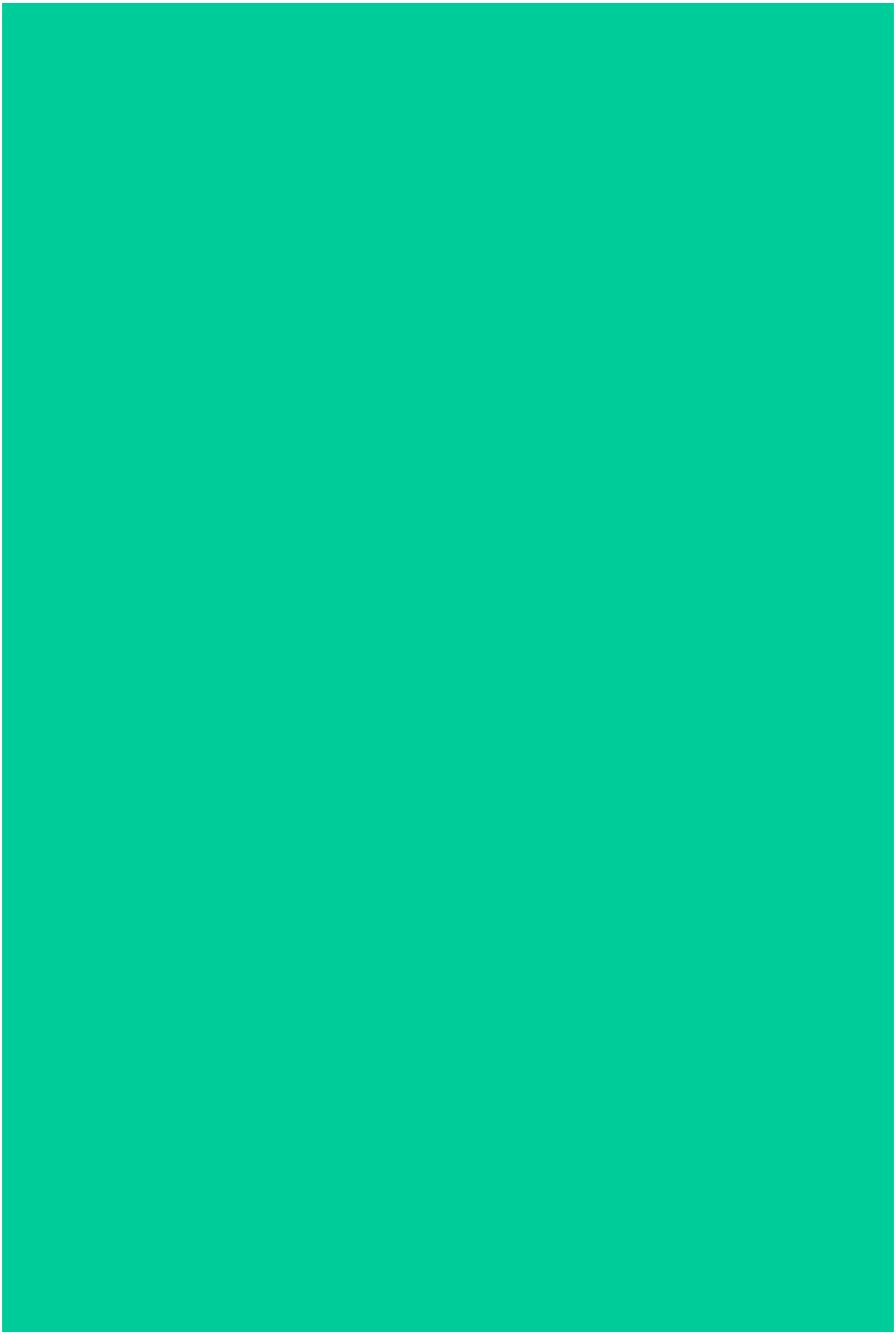

Overview of the Diagnosis of Cytomegalovirus Infection

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Abstract: Cytomegalovirus (CMV) is recognized as the most common congenital viral infection in humans and an important cause of morbidity and mortality in immunocompromised hosts. This recognition of the clinical importance of invasive CMV disease in the setting of immunodeficiency and in children with congenital CMV infection has led to the development of new diagnostic procedures for the rapid identification of CMV. Diagnosis of acute maternal CMV infection by the presence of immunoglobulin (Ig)M and low-avidity IgG requires confirmation of fetal infection, which is typically performed using polymerase chain reaction (PCR) assays for CMV on amniotic fluid. Viral culture of the urine and saliva obtained within the first two weeks of life continues to be the gold standard for diagnosis of congenitally-infected infants. PCR assays of dried blood spots from newborns have been shown to lack sufficient sensitivity for the identification of most neonates with congenital CMV infection for universal screening purposes. However, saliva PCR assays are currently being assessed as a useful screening method for congenital CMV infection. In the immunocompromised host, newer rapid diagnostic assays, such as phosphoprotein 65 antigenemia and CMV real-time PCR of blood or plasma have allowed for preemptive treatment, reducing morbidity and mortality. However, lack of standardized real-time PCR protocols hinders the comparison of data from different centers and the development of uniform guidelines for the management of invasive CMV infections in immunocompromised individuals.

Keywords: Cytomegalovirus, diagnosis, congenital infection, maternal infection, fetus, immunocompromised.

INTRODUCTION

Human cytomegalovirus (HCMV) is the largest member of the virus family *Herpesviridae* and is a ubiquitous virus that infects almost all humans at some time in their lives. The virus was first isolated by three different groups of investigators (Rowe and colleagues, Weller and colleagues, and Smith) simultaneously in 1956 [1]. It has since been recognized as the most common congenital viral infection in humans, infecting between 20,000 and 40,000 infants each year in the United States [2]. Congenital CMV infection is the leading non-genetic cause of sensorineural hearing loss (SNHL) in children [3]. In addition, CMV has been recognized as an important cause of morbidity and mortality in immunocompromised hosts [4]. This recognition of the clinical importance of invasive CMV disease in the setting of immunodeficiency and in children with congenital CMV infection has led to the development of diagnostic assays for the rapid identification of CMV.

DIAGNOSTIC METHODS FOR CMV

Serology

Serological tests are useful for determining whether a patient has had CMV infection in the past, determined by the presence or absence of CMV immunoglobulin (Ig)G. Many different assays have been described and evaluated for the detection of CMV IgG antibodies. Among these are complement fixation, enzyme-linked immunosorbent assay (ELISA), anticomplement immunofluorescence, radioimmunoassay, and indirect hemagglutination [5]. The detection of IgM antibodies has been used as an indicator of acute or recent infection. Many different assays are available, but ELISAs based on crude viral preparations as antigen sources are the most widely used. The widely-employed IgM capture assays are based on selective binding of IgM antibody to the solid phase. Recombinant IgM assays using recombinant HCMV proteins and peptides have been developed in an attempt to standardize serological assays [6]. However, studies have shown poor correlation of results obtained with different commercial kits for IgM testing [7]. In addition, assays for IgM antibody lack specificity for primary infection because IgM can persist for months after primary infection, and because IgM can be positive in reactivation of or reinfection with CMV, leading to false positive results [8-10].

Because of the limitations of the IgM assays, IgG avidity assays are utilized in some populations to help distinguish primary from non-primary CMV infection. These assays are based on the observation that IgG antibodies of low avidity are present during the first few months after the onset of infection and avidity increases over time, reflecting maturation of the immune response. Thus, the presence of high-avidity anti-CMV IgG is considered evidence of long-standing infection in an individual. Avidity levels are reported as the avidity index, which is the percentage of IgG bound to the antigen following treatment with denaturing agents [6].

Cell Culture

The traditional method for detecting CMV is conventional cell culture. Clinical specimens are inoculated onto human fibroblast cells, incubated, and observed for a period of time ranging from 2 to 21 days. In the standard tube cell culture technique, CMV exhibits a typical cytopathic effect characterized by foci of flat, swollen cells and this effect is

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directly related to the virus titer. However, this method requires 2-3 weeks until a result can be reported as negative.

The shell vial assay is a viral culture modified by a centrifugation-amplification technique designed to decrease the length of time needed for virus detection. It utilizes fibroblast cell cultures propagated on cover slips contained in flat bottom plates. Centrifugation of the specimen onto the cell monolayer greatly assists adsorption of virus, effectively increasing infectivity of the viral inoculum [11]. Viral antigens then may be detected by monoclonal antibody directed at a CMV immediate-early (IE) antigen by indirect immunofluorescence after 16 hours of incubation [12]. This method was adapted to be performed in 96-well microtiter plates [13], allowing for the screening of larger numbers of samples.

Antigenemia

The antigenemia assay has been commonly used for more than a decade for CMV virus quantification in blood specimens. This assay depends on the use of monoclonal antibodies that detect the viral phosphoprotein (pp)65 antigen, a structural late protein expressed in blood leukocytes during the early phase of the CMV replication cycle. Antigenemia is measured by the quantitation of positive leukocyte nuclei in an immunofluorescence assay for the CMV matrix pp65 in a cytospin preparation of 2×10^5 peripheral blood leukocytes (PBL) [14-18]. This test is limited to detection of the virus in leukocytes; the demonstration of positive-staining signals in the nuclei of leukocytes indicates a positive result. The test not only gives a qualitative result but is also quantitative, correlating closely with viremia and clinical disease severity in immunosuppressed populations [19-21].

The disadvantages of the antigenemia assay are that it is labor-intensive with low throughput and not amenable to automation. It is also affected by subjective bias, necessitating skilled persons for accurate test performance and interpretation of results. The samples have to be processed immediately (within 6 hours) since delay greatly reduces the assay's sensitivity [22]. Particularly in neutropenic patients, false-negative results may occur, since the antigenemia test depends on the presence of a sufficient number of polymorphonuclear leukocytes [23].

Polymerase Chain Reaction (PCR) Amplification

PCR is a widely available rapid and sensitive method of CMV DNA detection based on amplification of nucleic acids. The techniques usually target highly-conserved regions of major IE and late antigen genes [24-26], but a number of other genes have also been used as targets for detection of CMV DNA. DNA can be extracted from whole blood, leukocytes, plasma, or any other tissue (tissue biopsy samples) or fluid [urine, cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL) fluid] [27-32]. Specimen deterioration over time is not as problematic with PCR assays as with other tests for CMV [33].

PCR for CMV DNA can be either qualitative or quantitative, in which the amount of virus DNA in the sample is measured. Qualitative PCR has been largely replaced by quantitative assays due to increased sensitivity for detecting CMV and because quantitative PCR (Real-Time PCR) allows for continuous monitoring of immunocompromised individuals to identify patients at risk for CMV disease for preemptive therapy and to determine response to treatment [34-36]. This method is generally more expensive compared to the antigenemia assay, but it is rapid and can be automated. Results are usually reported as number of copies/mL of blood or plasma.

Reverse transcriptase (RT-PCR) can be used to detect viral messenger (m) RNA transcripts in PBL independent of the presence of DNA. The absence of circulating mRNA is associated with a lack of CMV-associated symptoms, irrespective of the presence or absence of CMV DNA, while its presence is detected only in the setting of disease [37]. The presence of CMV IE mRNA has been demonstrated in monocytes and polymorphonuclear leukocytes during active CMV infection [38, 39]. It appears to be less sensitive, however, than the pp65 antigen test and PCR to diagnose CMV disease [40, 41].

Immunohistochemistry

Immunohistochemistry is performed primarily on tissue or body fluid samples. Slides are made from frozen sections of biopsy tissue samples (e.g., liver, lung) or by centrifuging cells onto a slide. Monoclonal or polyclonal antibodies against early CMV antigens are applied to the slides and visualized by fluorescently-labeled antibodies or enzyme-labeled secondary antibodies which are detected by the change of color of the substrate. The stained slides are examined by fluorescent or light microscopy. This technique is more sensitive and very specific compared to plain histological microscopy, but is labor-intensive and requires experienced personnel to read the slides [42]. False negative results can also occur due to focal distribution of the virus [43].

MATERNAL AND FETAL INFECTIONS

The natural history of CMV infection during pregnancy is complex and not fully understood. Primary maternal infections are more likely to be transmitted to the fetus (in 30-40%) [2, 44-46]. However, unlike toxoplasmosis and rubella, preconceptional immunity to CMV is incomplete and intrauterine transmission and damaging fetal infection can occur in women who are CMV seroimmune prior to pregnancy [47-51]. The majority (>90%) of CMV infections in pregnant women (primary and non-primary) are subclinical [52]. No tests can reliably define transmission of infection to the fetus. In most countries, pregnant women are not routinely screened for CMV infection and testing is usually performed when CMV infection is clinically suspected in the mother or fetus, or in women considered at high risk for acquiring CMV infection (http://www.cdc.gov/cmv/clinicians.htm) [53].

Maternal Infection

The diagnosis of primary CMV infection is accomplished by documenting seroconversion through the *de novo* appearance of virus-specific IgG antibodies in the serum of a pregnant woman known previously to be seronegative. The pres-

ence of IgG antibodies indicates past infection ranging from 2 weeks' to many years' duration. Women with primary CMV infection during pregnancy are at greatest risk for having a child with congenital CMV infection with intrauterine transmission of CMV occurring in approximately 30-40% [46]. However, maternal reinfection with a different strain of CMV can occur and such reinfections have been associated with intrauterine transmission, damaging fetal infection, and long-term sequelae [47, 48, 54].

IgM assays in pregnant women have been assessed as an indicator of acute or recent infection. In addition to the methods for IgM detection listed above, an IgM immunoblot utilizing structural and nonstructural viral proteins was shown to have 100% sensitivity and 100% specificity for detecting mothers who transmitted CMV to their offspring when samples were obtained at 21-24 weeks' gestation [55]. Recently, assays utilizing protein microarray technology have been developed to detect CMV antibodies in sera. These are in the early stages of development and testing [56]. With most IgM assays, detection of IgM in the serum of a pregnant woman may indicate a primary infection. However, IgM can be produced in pregnant women with non-primary CMV infection [57] and false positive results are common in patients with other viral infections [58, 59]. In addition, anti-CMV IgM can persist for 6-9 months following primary CMV infection [6, 46, 60].

Because of the limitations of the IgM assays, IgG avidity assays are utilized to help distinguish primary from non-primary CMV infection. The sensitivity of IgG avidity and IgM by immunoblot in serum samples obtained at 6-18 weeks' gestation and at 20-23 weeks' gestation in 124 pregnant women was evaluated [61]. In early gestation, IgG avidity detected 100% of women who had an infected fetus or newborn. However, the sensitivity of IgG avidity to detect women who transmitted the virus to their fetuses was much lower (63%) at 20-23 weeks' gestation. When IgM testing in addition to IgG avidity was performed at 20-23 weeks' gestation, the sensitivity of detecting a mother who transmitted CMV to her offspring increased to 81% [61]. Other researchers have utilized microneutralization testing in combination with avidity testing for diagnosing recent primary CMV infection in the second trimester of pregnancy [62]. Based on these data, some investigators propose screening pregnant women with serum IgG and IgM. If the IgM is positive, then serum IgG avidity could be performed to help determine recent or past infection. Using this algorithm, some argue that the sensitivity is similar to documenting *de novo* seroconversion [60, 63, 64]. Lazzarotto *et al.* examined a cohort of 2477 pregnant women referred to their center because of a positive screening CMV IgM. Of these women, 1110 were confirmed to be IgM-positive by immunoblot and 514 had low/moderate IgG avidity and thus were considered at risk of transmitting CMV to their fetus. Twenty-five percent (121/514) of these infants were congenitally infected, similar to 53/183 (30%) of infants infected as a result of primary CMV infection during pregnancy documented by seroconversion. Among the 1110 women with confirmed IgM positivity, 336 had high-avidity anti-CMV IgG and six (2.0%) delivered CMV-infected infants [63].

Several studies have examined the utility of maternal virological tests to diagnose recent primary infection and to determine the risk of virus transmission to the fetus. These studies have shown that fewer than 50% of pregnant women have detectable CMV in their blood as assessed by either PCR or pp65 antigenemia at the time of serological diagnosis [59, 64-66]. Investigators from Italy tested sequential blood samples from a small group of pregnant women with primary CMV infection for CMV DNA, pp65 antigenemia, and IE mRNA. During the first month of infection, all three tests showed high sensitivity (80-100%). However, the ability to detect evidence of CMV infection in the blood dropped off rapidly after the first 30 days of infection [67].

Fetal Infection

Detection of CMV in the amniotic fluid has been the standard for diagnosis of infection of the fetus. Viral isolation in tissue culture was first utilized; however, the sensitivity was found to be moderate (70-80%) with a high rate of false-negative results [6, 68-73]. With the advent of PCR, detection of CMV DNA in amniotic fluid has been shown to improve prenatal diagnosis of congenital CMV infection [72, 74-77]. The highest sensitivity of this assay (90-100%) has been shown when amniotic fluid samples are obtained after the 21st week of gestation and at least 6 weeks after the first positive maternal serologic assay. This allows adequate time for maternal transmission of the virus to the fetus and virus shedding by the fetal kidney [72-74, 77]. However, even when PCR on amniotic fluid is performed at the optimal time, false negative results may occur. A recent study showed that among 194 women who underwent prenatal diagnosis of congenital CMV infection, 8 mothers with negative amniotic fluid PCR results for CMV delivered infants who were confirmed to be CMV-infected [65].

Recently, CMV DNA quantification in amniotic fluid samples has been proposed as a means to evaluate the risk that a fetus can develop infection or disease. Several groups of investigators have shown that higher CMV DNA viral load in the amniotic fluid [$\geq 10^5$ genome equivalents (ge)/mL] was associated with symptomatic infection in the newborn or fetus [72, 78, 79]. However, other studies have failed to confirm a correlation between CMV DNA levels and the clinical status at birth [80, 81]. Rather, CMV viral load in the amniotic fluid correlated with the time during the pregnancy when the amniocentesis was performed, with higher CMV viral loads observed later in gestation [79, 80]. However, as with qualitative PCR on amniotic fluid, even when sampling was done at the appropriate time, very low or undetectable CMV DNA by quantitative PCR was found in some infants infected with CMV [78, 80, 81].

In addition to CMV viral load, some investigators have examined the prognostic value of determining the CMV genotype in infected fetuses. Studies of the two polymorphic CMV genes, *glycoprotein (g)B* and *unique long (UL)144*, have failed to correlate a particular virus genotype with severity of fetal infection [82, 83]

Fetal blood sampling has been evaluated to determine the prognostic value of virologic assays in the diagnosis of congenital infection as well as the determination of severity of CMV disease. The utility of CMV viremia, antigenemia,

DNAemia, and IgM antibody assays on fetal blood was examined for the diagnosis of congenital infection. Although these assays were highly specific, the sensitivity was shown to be poor (41.1%-84.8%) for identifying fetuses infected with CMV [6]. The same group found in 21 fetuses/infants with congenital CMV infection that all virologic parameters as well as IgM were higher in fetuses with ultrasound or clinical/laboratory abnormalities [84]. More recently, fetal thrombocytopenia has been shown to be associated with more severe disease in the fetus/newborn [73, 85]. However, investigators from Belgium documented fetal loss after funipuncture in an uninfected child. Thus, it is important to balance the value of cordocentesis against that known risk of miscarriage [73, 85].

Fetal imaging by ultrasound can identify structural and/or growth abnormalities and, thus, can identify fetuses with congenital CMV infection that will be symptomatic at birth. The more common abnormalities on ultrasound include ascites, fetal growth restriction, microcephaly, and structural abnormalities of the brain [74]. However, the majority of infected fetuses will not have abnormalities on ultrasound examination [53]. In a recent retrospective study of 650 mothers with primary CMV infection, among the 131 infected fetuses/neonates with normal sonographic findings *in utero*, 52% were symptomatic at birth. Furthermore, when the fetal infection status was unknown, ultrasound abnormalities predicted symptomatic congenital infection in only one third of infected infants [86].

Fetal magnetic resonance imaging (MRI) has been evaluated in a few small, retrospective studies to assess its utility in detecting fetal abnormalities *in utero*. MRI appears to add to the diagnostic value of ultrasound with increased sensitivity and positive predictive value (PPV) of both studies versus ultrasound or MRI alone [87, 88]. However, more studies are needed to determine the true diagnostic and prognostic value of MRI in CMV-infected fetuses.

CONGENITAL CMV INFECTION

CMV infection has been recognized as a leading cause of congenital infection and brain disease in children in the U.S. and Northern Europe. Between 20,000 and 40,000 infants are born with congenital CMV infection each year in the U.S. Of those, only approximately 10% exhibit clinical findings at birth or during the neonatal period (symptomatic congenital CMV infection) [2, 89]. Approximately 10-15% of infants with sub-clinical or asymptomatic congenital CMV infection develop SNHL, suggesting that congenital CMV infection is the leading non-genetic cause of SNHL in the U.S. Many children with CMV-associated SNHL have normal hearing at birth and hearing loss may be static or progressive during early childhood [90-92]. Therefore, most children with CMV-associated SNHL will not be detected based on clinical examination in the nursery and newborn hearing screening. Since predictors of SNHL in children with congenital CMV infection have not been defined, in particular among children with asymptomatic infection, it is currently not possible to identify infants at risk for CMV-related SNHL early in life. Early identification of congenitally-infected infants at increased risk for SNHL is essential to provide appropriate monitoring and intervention measures during critical stages of speech and language development [93]. Therefore, detection of these at-risk infants early in life using rapid, reliable, and relatively inexpensive methods to screen newborns for congenital CMV infection was identified as a public health priority (NIDCD Workshop on Congenital Cytomegalovirus Infection and Hearing Loss: http://www.nidcd.nih.gov/funding/programs/hb/cmvwrkshop.htm).

The diagnosis of congenital CMV infection is typically made by the demonstration of the virus or viral genome in newborn urine or saliva. The detection of virus in urine or saliva within the first two weeks of life is considered the gold standard method for the diagnosis of congenital CMV infection. In contrast to symptomatic infants, most infants with asymptomatic congenital CMV infection are not identified because of the absence of clinical findings. Furthermore, identification of the virus or viral genome in samples obtained from infants after the first two to three weeks of life may represent natal or postnatal acquisition of CMV and, therefore, it is not possible to confirm congenital CMV infection in infants older than three weeks.

Serological methods are unreliable for the diagnosis of congenital infection. Detection of CMV IgG antibody is complicated by transplacental transfer of maternal antibodies. In addition, currently available tests for the detection of CMV IgM antibody do not have the high level of sensitivity and specificity of virus culture or PCR [94, 95].

Virologic Methods

Detection of CMV in the saliva and urine of infants is easily accomplished because newborns with congenital CMV infection shed large amounts of virus. Traditional tissue culture techniques or shell vial assay are considered the standard methods for the diagnosis of congenital CMV infection [5, 96-98]. Rapid culture methods have been shown to have comparable sensitivity and specificity to the standard cell culture assays and the results are available within 24 to 36 hours. A rapid method using a 96-well microtiter plate and a monoclonal antibody to the CMV IE antigen was shown to be 94.5% sensitive and 100% specific to detect CMV in the urine of congenitally-infected infants [13]. This microtiter plate assay has been adapted for use with saliva specimens with comparable sensitivity and specificity [99].

The CMV antigenemia assay is used widely to diagnose CMV infections and monitor treatment in immunocompromised patients [100]. However, the utility of this assay in the diagnosis of congenital CMV infection has not been evaluated.

Nucleic Acid Amplification Methods

PCR amplification of virus DNA is a very sensitive method for the detection of CMV in a variety of clinical specimens. The PCR assay is used routinely for the diagnosis of CMV infection in allograft recipients and other immunocompromised hosts, who are at increased risk for invasive CMV disease. Quantitative PCR has also been proven to be useful to monitor these patients for response to antiviral therapy [101-103]. However, the usefulness of PCR or other nucleic acid amplification assays to diagnose of congenital

CMV infection has not been defined. An early study by Demmler *et al*. found that PCR using primers targeting IE and late CMV genes was 93% sensitive and 100% specific when testing urine samples from newborns with congenital CMV infection [25]. In a study by Warren *et al*., PCR was found to be 89.2% sensitive and 95.8% specific when compared with standard tissue culture and rapid culture techniques on saliva from CMV-infected infants [104]. In another study, CMV was detected in the CSF of 60% (6/10) of infants with symptomatic congenital CMV infection [105]. Nelson and colleagues were able to detect CMV DNA in the serum samples of all 18 children with symptomatic congenital infection tested, in 1 of 2 children with asymptomatic infection, and in 0 of 32 controls [106]. A disadvantage of PCR on the peripheral blood is that viremia may not be present in all infants with congenital CMV infection; thus, PCR may not identify every infected infant [106-109]. A DNA hybridization assay has excellent sensitivity and specificity for the rapid diagnosis of CMV infection [110]. However, the need to concentrate virus using high-speed centrifugation and to hybridize using radio-labeled probes renders this method cumbersome and impractical for the routine diagnosis of congenital CMV infection.

Since dried blood spots (DBS) are collected for routine metabolic screening from all infants born in the U.S., there has been increasing interest in utilizing PCR-based assays for the detection of CMV in newborn DBS samples. The advantages of DBS PCR for newborn CMV screening include: 1) the specimens are already routinely collected for metabolic screening from all newborns; 2) PCR can detect viral DNA in DBS samples from CMV-infected infants; 3) PCR does not require tissue culture facilities; and 4) PCR is amenable to automation, so large numbers of specimens may be screened at relatively low cost. Most reports have studied selected infant populations and a prospective comparison of DBS PCR to a standard (i.e., tissue culture) method for identifying CMV infection in an unselected newborn population has only recently been performed [111-116]. The sensitivity of DBS PCR in the diagnosis of congenital CMV infection may vary with the amount of blood collected on the filter card, the method used for DNA extraction, and the PCR method used. Early studies have examined the utility of PCR on DBS obtained from infants in the nursery to diagnose congenital CMV infection retrospectively at the time of detection of SNHL. A study retrospectively tested DBS of 16 infants with proven congenital CMV infection and 14 were positive by a nested PCR assay [113]. A number of studies from the group of investigators in Italy examined DBS from newborns and reported a sensitivity of the DBS PCR assay approaching 100% with a specificity of 99% [111].

However, in a large multi-center study of more than 20,000 newborns, a DBS real-time PCR assay was compared with saliva rapid culture for identification of infants with congenital CMV infection and demonstrated that DBS PCR could only detect less than 40% of congenitally-infected infants [117]. The sensitivity and specificity of the DBS PCR assay when compared with the saliva rapid culture were 30.4% [95% confidence interval (CI), 21.5 – 41.0%] and 99.9% (95% CI, 99.9 – 100%), respectively. The PPV of the DBS PCR assay was 84.8% (95% CI, 67.3 – 94.3%) and the negative predictive value (NPV) was 99.6% (95% CI, 99.5 – 99.7%). These results indicate that such methods as currently performed will not be suitable for the mass screening of newborns for congenital CMV infection. The high specificity of the DBS PCR assay suggests that a positive DBS PCR result will identify infants with congenital CMV infection. However, the negative DBS PCR assay result does not exclude congenital CMV infection. These findings underscore the need for further evaluation of high throughput methods performed on saliva or other samples that can be adapted to large-scale newborn CMV screening [118].

The reasons for the low sensitivity of DBS PCR to identify infants with congenital CMV infection are not entirely clear. PCR testing of peripheral blood has been widely used as a standard diagnostic method to detect invasive CMV infections in immunocompromised individuals including allograft recipients and patients with AIDS [101, 103]. However, it is likely that the pathogenesis of congenital CMV infection is different from that in the immunocompromised host, since such patients usually experience acute CMV infection or symptomatic reactivation shortly before testing, whereas congenitally-infected infants may have acquired CMV infection months before birth and thus are no longer viremic when tested as newborns. Several previous studies that included smaller numbers of subjects examined the utility of testing saliva samples with PCR-based methods and demonstrated the feasibility and high sensitivity of these methods [49, 104, 119]. However, none of these studies has included screening of unselected newborns or a direct comparison of a saliva PCR assay to the standard rapid culture method on saliva or urine. Although a more recent study from Brazil, in which more than 8,000 newborns were screened for congenital CMV infection, demonstrated the utility of a saliva PCR assay to screen newborns for CMV, the PCR assay was not directly compared to the standard culture-based assay [50].

As part of an ongoing multicenter newborn screening study, the utility of real-time PCR of saliva samples to identify infants with congenital CMV infection is being evaluated. Thus far, the results are promising and demonstrate that real-time PCR of saliva samples has excellent sensitivity (100%; 95% CI, 95.1 – 100%) and specificity (99.9; 95% CI, 99.9 – 100%). The PPV and NPV of saliva PCR were 91.3% and 100%, respectively [120]. A major advantage of the saliva real-time PCR assay used in that study was that there was no need for processing of saliva samples for DNA extraction. Elimination of the DNA extraction step will make it easier to adapt this assay for screening large numbers of newborns in a high throughput fashion. These findings demonstrate that saliva PCR could become a useful approach to screen newborns for congenital CMV infection.

In addition, there is also growing interest in examining the feasibility of a newborn CMV screening program in conjunction with universal newborn hearing screening. It is somewhat disappointing that DBS PCR assays have been shown to have insufficient sensitivity for the identification of most infants with congenital CMV infection. Nevertheless, the development of saliva PCR assays could have the potential to adapt these methods in a high throughput approach to screen large numbers of newborns for congenital CMV infection. In addition, the ability to measure virus burden in

saliva specimens from infants with asymptomatic congenital CMV infection using saliva PCR assays could provide the means to identify at-risk infants early in life, ensuring judicious use of resources by targeting at-risk children for follow-up and monitoring.

PERINATAL CMV INFECTIONS

Perinatal infections can be acquired by three routes: 1) contact with virus in maternal genital tract secretions during delivery, 2) ingestion of breast milk containing virus, or 3) through transfusions of CMV-seropositive blood. Transmission *via* breast milk and through blood transfusion can result in severe symptoms in very low birth weight infants [121, 122]. For definitive diagnosis of perinatal CMV infection, it is important to demonstrate no viral shedding in the first two weeks of life, since CMV excretion does not begin until 3 to 12 weeks after exposure [123, 124]. There is no agreed-upon standard method for diagnosis of perinatal CMV infections, however. Viral culture and CMV DNA detection by PCR using urine or saliva are the preferred diagnostic methods [125, 126]. Recently, investigators have utilized quantitative plasma PCR assays in infants with perinatally-acquired CMV infection. However, similar to blood PCR assays to diagnose congenital infection, not all infants who shed virus in their urine or saliva as a result of perinatal infection have detectable CMV DNA in their blood [125]. In perinatal CMV infection, serological assays have the same limitations described above for infants with congenital CMV infection.

FUTURE DIRECTIONS

Continual advances are being made in our understanding of the natural history and pathogenesis of congenital CMV infection and CMV disease in the compromised host. It is hoped that ongoing work to develop and standardize molecular diagnostic methods will result in the availability of reliable, rapid, and simple methods for routine clinical use in the future.

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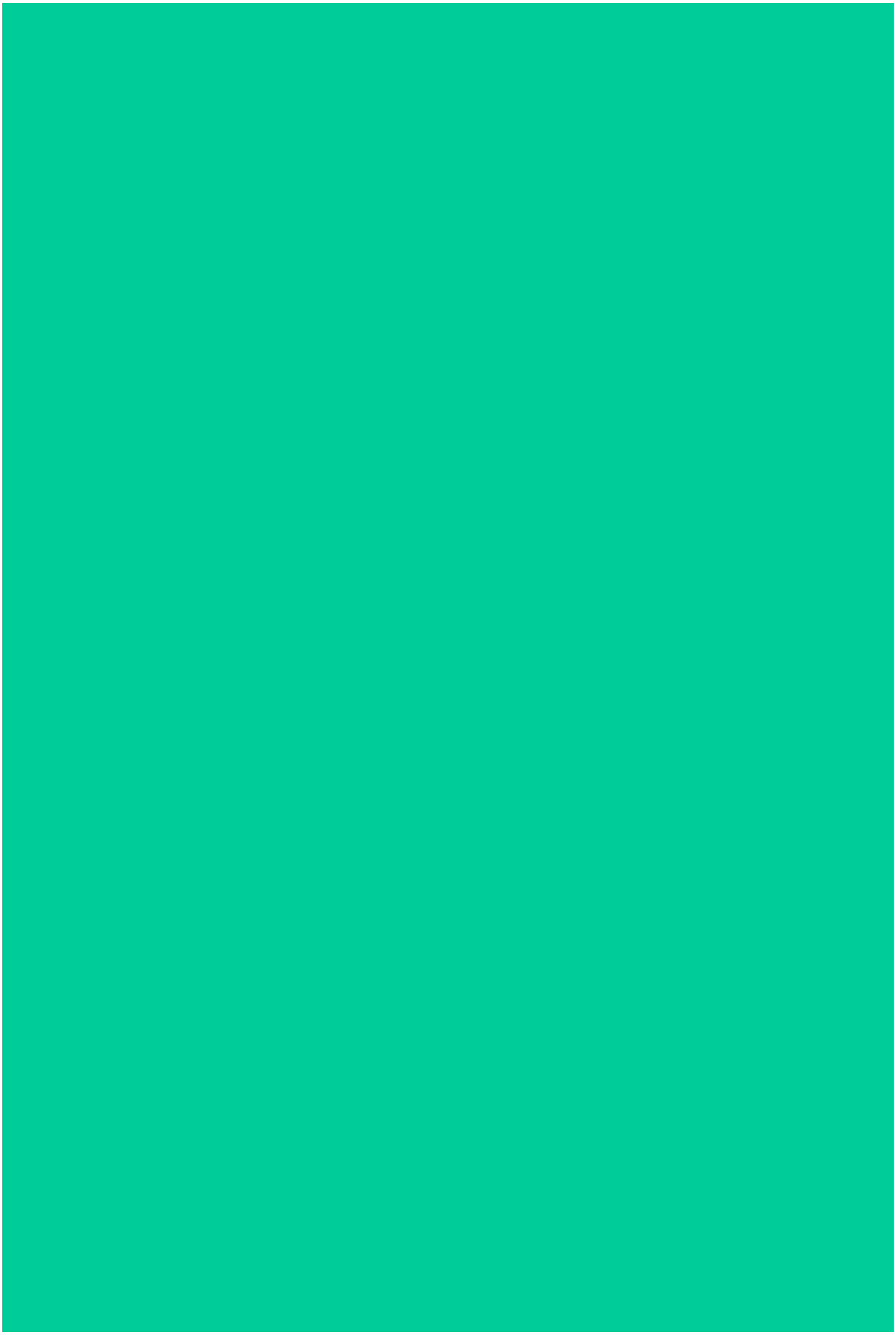

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Diagnostic Consequences of Cytomegalovirus Glycoprotein B Polymorphisms[▽]

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Failure of a cytomegalovirus (CMV) real-time PCR assay targeting glycoprotein B (gB) was investigated. A multiplex assay targeting gB and immediate-early 2 (IE2) genes showed discordant results (gB negative and IE positive or a >10-fold-higher viral load with IE primers) in saliva from 14.6% of CMV-infected newborns. Sequencing revealed 3 patterns of gB variations.

Cytomegalovirus (CMV) is a leading cause of congenital infection and hearing loss in children and an important pathogen in immunocompromised patients. PCR-based methods are used widely in diagnostic laboratories to detect and monitor CMV infections. Since the glycoprotein B (gB) gene of CMV is essential for the infectivity of the virus and has a largely conserved nucleotide sequence, various regions of the gB gene are commonly used as PCR assay targets (3, 5, 7, 9, 18). A real-time PCR assay used in our laboratory targets the conserved region that lies upstream of antigenic domain 1 (3) of the gB gene, between base pairs 1541 and 1612.

As part of the National Institute on Deafness and Other Communication Disorders (NIDCD) CMV and Hearing Multicenter Screening (CHIMES) study, newborns at seven medical centers were screened for congenital CMV infection by a multiplex real-time PCR with concurrent use of primers and probes targeting conserved regions of the gB gene and exon 5 of the immediate-early 2 (IE2) gene (2). During the course of this study, discordant results in the performance of primers/probes targeting the gB and IE2 genes were observed. The failure of the PCR using gB primers/probes was investigated in this study.

Newborn CMV screening was carried out using rapid culture and/or real-time PCR of saliva specimens. Between March 2007 and July 2010, 386 infants tested positive for congenital CMV infection. Of these, 246 infants (63.7%) had real-time PCR of saliva specimens completed. The real-time PCR assay protocol was previously described (2). Briefly, 5 μl of saliva samples was used as a template without DNA extraction by utilizing the ABI TaqMan 7500 system (Life Technologies Corporation, Carlsbad, CA). Primers and probes include gB fw (AGGTCTTCAAGGAACTCAGCAAGA), gB re (CGGCAATCGGTTTGTTGTAAA), gB FAM/TAMRA probe (6FAM-AACCCGTCAGCCATTCTCTCGGC-TAMRA) (where 6FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine), based on the AD169 sequence, and IE2 fw (GAGCCCGACTTTACCATCCA), IE2 re (CAGCCGGCGGTATCGA), and VIC/MGBNFQ probe (VIC-ACCGCAACAAGATT-MGBNFQ), based on the consensus CMV sequence. Quantified plasmid DNA containing target sequences of both primer sets served as calibration standards.

TABLE 1. Quantitative PCR results for the gB and IE2 target genes of discordant samples and corresponding mutation patterns from 28 sequenced samples

Identifier	No. of copies/reaction		Mutation pattern[a]
	gB	IE2	
1	0.0	25.0	B
2	0.0	31.0	A
3	0.0	31.5	ND
4	0.0	85.0	ND
5	0.0	96.0	ND
6	0.0	110.0	A
7	0.0	115.0	A
8	0.0	125.0	A
9	0.0	199.0	ND
10	0.0	350.0	A
11	0.0	430.0	A
12	0.0	560.0	ND
13	0.0	690.0	A
14	0.0	900.0	A
15	0.0	1,100.0	A
16	0.0	1,500.0	ND
17	0.0	2,000.0	A
18	0.0	2,880.0	A
19	0.0	11,700.0	A
20	0.0	15,000.0	A
21	0.0	19,000.0	A
22	0.0	28,000.0	A
23	0.0	64,000.0	A
24	0.0	98,000.0	A
25	0.0	150,000.0	ND
26	0.0	310,000.0	C
27	0.6	24,000.0	A
28	1.2	43,000.0	A
29	2.5	8,500.0	A
30	2.6	200.0	A
31	3.0	9,700.0	A
32	3.5	7,900.0	A
33	5.3	190.0	ND
34	10.0	125,000.0	A
35	20.0	65,000.0	A
36	43.5	53,000.0	A

[a] ND, sequencing not performed.

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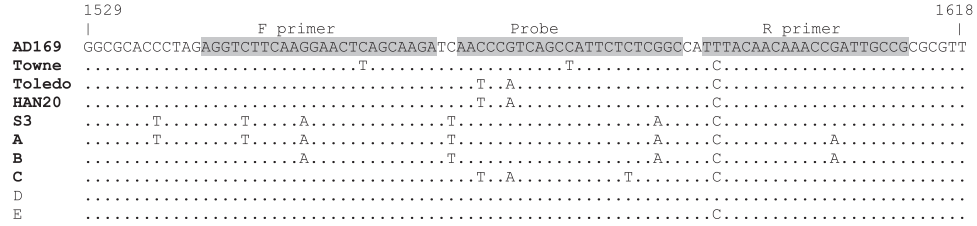

FIG. 1. Alignment of known sequences and the mutation patterns found in study samples. A, B, and C represent sequencing result of discordant samples. D and E are sequences obtained from positive concordant samples.

Samples were considered to be discordant if the IE2 gene was PCR positive and the gB gene was PCR negative or if the IE2 gene copy number was at least >10-fold higher than the gB gene copy number. Discordant samples were subjected to PCR amplification of the gB region containing the target sequences using gB forward (CACAGGTTGGTGGCTTTTCT) and reverse (GTCGTGAGTAGCAGCGTCCT) primers. PCR products were sequenced and aligned with CMV sequences retrieved from NCBI GenBank (sequences for AD169, Towne, Toledo, HAN20, and S3 [accession numbers BK000394, FJ616285, GU180092, GQ396663, and GU937742, respectively]) by using BioEdit software (Ibis Therapeutics, Carlsbad, CA). Ten random CMV-positive newborn saliva samples with concordant results between gB and IE genes were also amplified and sequenced. Informed consent was obtained from study participants, and the study was conducted in accordance with the guidelines of the Institutional Review Board of the University of Alabama at Birmingham.

Among the 246 CMV-positive newborns, discordant results were observed for 36 (14.6%) infants. Of the 36 specimens with discordant PCR results, 26 were PCR negative using the gB primers, and the remaining 10 samples showed >10-fold-higher CMV copy numbers per reaction with IE2 primers than that observed with gB primers. The viral load levels in the 36 specimens are shown in Table 1. Amplification and nucleotide sequence analysis for the gB target region were completed for 28/36 discordant samples due to sample availability. This analysis showed 3 different patterns of polymorphisms within the target region for the gB primers/probe compared to the reference AD169 sequence. All of the nucleotide substitutions were synonymous mutations (Fig. 1). Of the 10 samples with concordant results, 2 contained a silent single-nucleotide substitution in the reverse primer target region (Fig. 1, pattern E). The remaining 8 samples matched the reference AD169 sequence (Fig. 1, pattern D).

In this study, we demonstrated polymorphisms within the region of the gB gene that were previously thought to be highly conserved. Discordant results, in which IE2 primers/probes detected CMV DNA but gB primers/probes failed, were observed with real-time PCR analysis of newborn saliva specimens in 14.6% of congenitally infected infants. The gB sequence variability of the CMV present in the saliva specimens most likely resulted in a mismatch between our primers and probes, leading to significant lowering of the sensitivity of the real-time PCR assay. Similar findings were also reported by Lengerova et al., who experienced about a 5% false-negative rate with an in-house assay due to mismatches in the target region of primers/probes located within the major immediate-early exon 4 region (12).

Studies investigating the diversity within genes of CMV (4, 6, 15, 16) have demonstrated extensive variability and that multiple variants can coexist in an individual (14, 17). Falsely lower copy number readings might be obtained in cases where multiple strains are present in a sample, because minor variants may be detected while the major virus populations might not be detected due to primer/probe mismatch. In addition, infection with multiple virus strains can also lead to the generation of new CMV variants by recombination, and the newly formed variants may not be detected (8). These findings, in addition to the results of our study, emphasize the importance of selecting primers and probes from highly conserved regions of CMV among different strains in order to avoid false-negative PCR results which could significantly impact clinical care. A significant number of CMV-infected newborns would have been missed if our real-time PCR assay included primers/probes targeting gB alone. Since new polymorphisms are being described on a regular basis, it is important to maintain constant vigilance so that the molecular diagnostic assays remain highly sensitive for the detection of CMV in clinical specimens (11, 13). Although it may be difficult to completely avoid false-negative PCR results due to the high diversity of CMV strains, it may be possible to overcome imperfections in the primer/probe design by the use of degenerate primers for both qualitative and quantitative PCRs (1, 10). Alternatively, the use of multiplex assays targeting two or more independent target regions, the strategy used in our study, could be another approach to reduce the chances for false-negative results.

The following investigators participated in the study: Amina Ahmed, Carolinas Medical Center, Charlotte, NC; April L. Palmer, University of Mississippi Medical Center, Jackson, MS; David Bernstein, University of Cincinnati and Cincinnati Children's Hospital Medical Center, Cincinnati, OH; Marian G. Michaels, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA; Pablo J. Sánchez, University of Texas Southwestern Medical Center, Dallas, TX; and Robert W. Tolan, Jr., Saint Peter's University Hospital, New Brunswick, NJ.

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We report no commercial interests.

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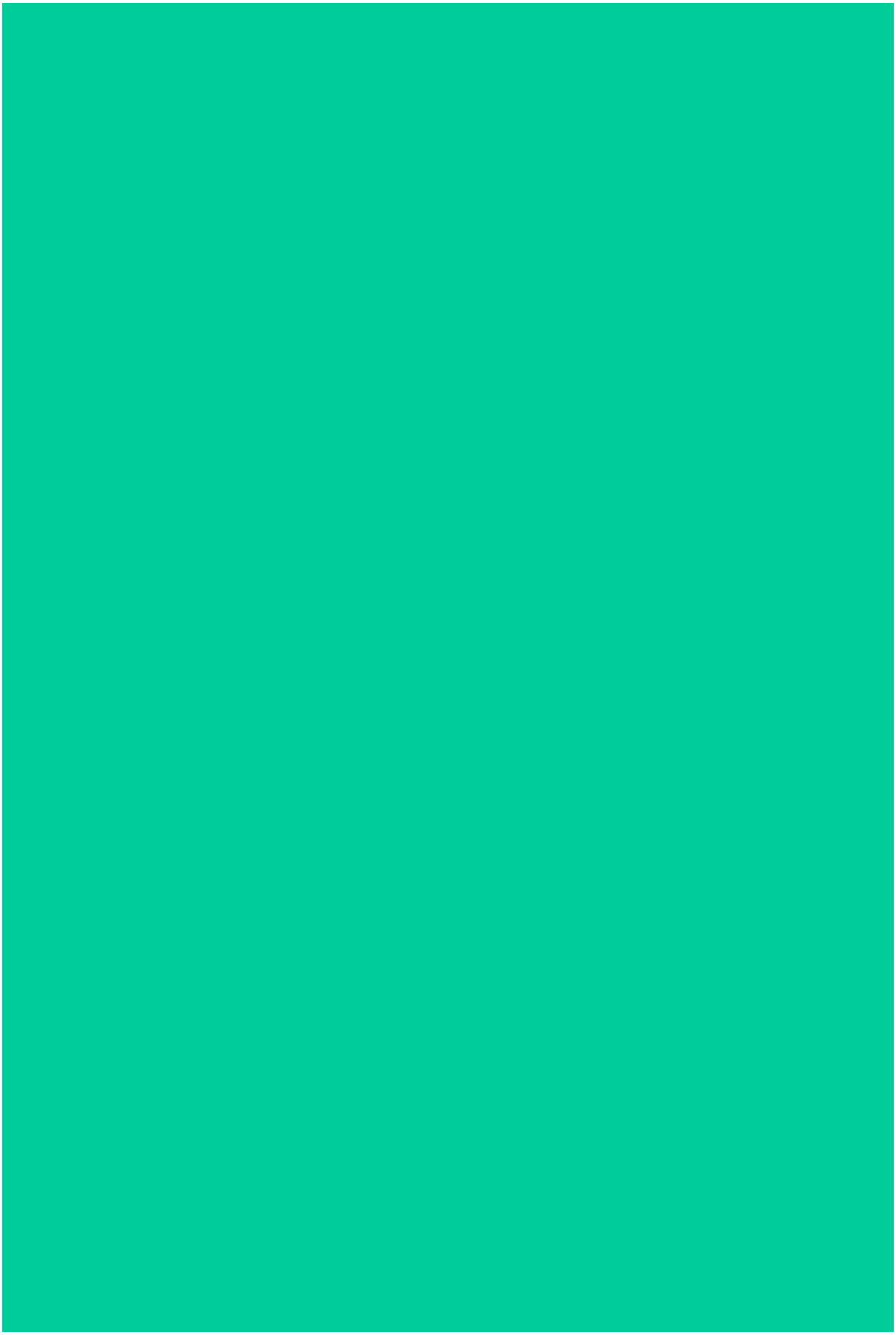

ORIGINAL ARTICLE

Saliva Polymerase-Chain-Reaction Assay for Cytomegalovirus Screening in Newborns

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ABSTRACT

BACKGROUND

Congenital cytomegalovirus (CMV) infection is an important cause of hearing loss, and most infants at risk for CMV-associated hearing loss are not identified early in life because of failure to test for the infection. The standard assay for newborn CMV screening is rapid culture performed on saliva specimens obtained at birth, but this assay cannot be automated. Two alternatives — real-time polymerase-chain-reaction (PCR)–based testing of a liquid-saliva or dried-saliva specimen obtained at birth — have been developed.

METHODS

In our prospective, multicenter screening study of newborns, we compared real-time PCR assays of liquid-saliva and dried-saliva specimens with rapid culture of saliva specimens obtained at birth.

RESULTS

A total of 177 of 34,989 infants (0.5%; 95% confidence interval [CI], 0.4 to 0.6) were positive for CMV, according to at least one of the three methods. Of 17,662 newborns screened with the use of the liquid-saliva PCR assay, 17,569 were negative for CMV, and the remaining 85 infants (0.5%; 95% CI, 0.4 to 0.6) had positive results on both culture and PCR assay. The sensitivity and specificity of the liquid-saliva PCR assay were 100% (95% CI, 95.8 to 100) and 99.9% (95% CI, 99.9 to 100), respectively, and the positive and negative predictive values were 91.4% (95% CI, 83.8 to 96.2) and 100% (95% CI, 99.9 to 100), respectively. Of 17,327 newborns screened by means of the dried-saliva PCR assay, 74 were positive for CMV, whereas 76 (0.4%; 95% CI, 0.3 to 0.5) were found to be CMV-positive on rapid culture. Sensitivity and specificity of the dried-saliva PCR assay were 97.4% (95% CI, 90.8 to 99.7) and 99.9% (95% CI, 99.9 to 100), respectively. The positive and negative predictive values were 90.2% (95% CI, 81.7 to 95.7) and 99.9% (95% CI, 99.9 to 100), respectively.

CONCLUSIONS

Real-time PCR assays of both liquid- and dried-saliva specimens showed high sensitivity and specificity for detecting CMV infection and should be considered potential screening tools for CMV in newborns. (Funded by the National Institute on Deafness and Other Communication Disorders.)

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Cytomegalovirus (CMV) is a frequent cause of congenital infection and a leading nongenetic cause of sensorineural hearing loss.[1-5] In most infants with congenital CMV infection, clinical abnormalities do not manifest at birth; rather, the infection is asymptomatic. However, sensorineural hearing loss eventually develops in approximately 10 to 15% of CMV-positive children,[3,4,6-8] in a substantial proportion who are not diagnosed by means of newborn hearing screening.[7-9] Screening of newborns for CMV infection will permit early identification of at-risk congenitally infected infants for purposes of targeted monitoring and intervention during critical stages of speech and language development.[10,11]

A variety of methods have been evaluated for use in the diagnosis of congenital CMV infection on the basis of saliva, urine, and dried-blood-spot specimens obtained from newborns.[12-17] Culture-based testing of urine and saliva specimens has been the standard method to identify infants with congenital CMV infection.[13,18,19] However, culture-based methods are not easily amenable to automation and, therefore, cannot be adapted for large-scale newborn screening.

Since dried-blood-spot specimens are obtained routinely in all infants, the usefulness of polymerase-chain-reaction (PCR) testing of dried-blood spots for the diagnosis of congenital CMV infection has been examined.[15,16,20-23] In addition, our recent large-scale newborn-screening study of a dried-blood-spot PCR assay that was prospectively compared with the standard saliva rapid culture showed that real-time dried-blood-spot PCR assay fails to identify the majority of CMV-infected newborns.[14] Therefore, challenges remain in achieving high sensitivity of dried-blood-spot testing to screen newborns for CMV infection.[24] Urine specimens collected on filter disks have also been explored as samples for CMV screening in newborns, but urine samples are harder to collect than saliva samples; this approach has not been validated by direct comparison with culture.[17,25]

Because of their ease of collection and since high titers of CMV are shed in the saliva of infected newborns, saliva specimens appear to be a better and less invasive type of sample for newborn screening.[24,26,27] The current study was designed to determine the usefulness of a real-time PCR assay of saliva specimens obtained from newborns for CMV screening. During phase 1 of the study, saliva specimens were placed in transport medium and stored at 4°C before testing. PCR testing of dried-saliva specimens (those that were not placed in transport medium and remained at ambient temperature during specimen storage and transport) was examined in phase 2 of the study, since dried specimens are easier to store and transport. Finally, all PCR assays were performed without a DNA-extraction step, to test an assay that would be more practical for screening all newborns.

Methods

Study Design

Infants born at seven hospitals in the United States from June 2008 through November 2009 were enrolled prospectively in our National Institute on Deafness and Other Communication Disorders (NIDCD) CMV and Hearing Multicenter Screening (CHIMES) study. All live-born infants were eligible for participation. Infants with positive saliva-screening results (from rapid culture or PCR assay) were enrolled in the follow-up component of the study to monitor hearing outcome. Clinical decisions about evaluation and possible treatment of the CMV-infected infants were made by the physicians at each study site.

The NIDCD was the study sponsor and provided general oversight for the design and conduct of the study. However, the NIDCD had no role in the collection, management, analysis, and interpretation of the data or in the preparation, review, or approval of the manuscript. Institutional-review-board approval was obtained at each study site, and written informed consent was obtained from a parent or parents of all participating infants. The study was conducted according to the protocol (available with the full text of this article at NEJM.org). Race or ethnic group was reported by a parent. The study was designed by the CHIMES study investigators in consultation with NIDCD project officers. All authors vouch for the integrity of the data and data analyses and made the decision to submit the manuscript for publication. Members of the CHIMES study group are listed in the Supplementary Appendix, available at NEJM.org.

Specimen Collection

A real-time PCR protocol developed in our laboratory was adapted to test saliva specimens from newborns.[14] Saliva specimens were collected by swabbing the inside of the baby's mouth using a

sterile polyester-fiber–tipped applicator (PurFybr) and transported to the central laboratory at the University of Alabama at Birmingham within 1 week after collection.[14,19]

Saliva swabs were placed in transport medium, transported to the central laboratory, and tested by means of rapid culture. During phase 1 of the study (beginning in June 2008), the specimens were also tested by means of liquid-saliva PCR assay. For phase 2 of the study (March through November 2009), an additional saliva swab collected at the same time was allowed to air-dry, placed in a sterile tube without transport medium, maintained and transported at ambient temperature to the central laboratory, and tested by means of dried-saliva PCR assay. Saliva specimens from some of the infants born between June 2008 and February 2009 were tested with the use of all three methods (rapid culture, liquid-saliva PCR assay, and dried-saliva PCR assay).

SPECIMEN PROCESSING AND TESTING

Liquid-saliva specimens were processed for rapid culture and PCR assay as described previously.[14,19] Dried-saliva specimens were processed by adding 300 μl of PCR-grade water to the tubes containing the swabs, vortexing, and incubating for 20 minutes at room temperature. Then, 5 μl of the eluate containing saliva was used, without first undergoing DNA extraction, in the real-time PCR assay.

Rapid-Culture Assay

A rapid-culture assay for the detection of early-antigen fluorescent foci, involving a monoclonal antibody against the major immediate early antigen of CMV, was used to detect CMV in saliva specimens.[14,18,19] Laboratory personnel performing the rapid culture were unaware of the results of PCR assay, and those performing the PCR assay were unaware of the results of the rapid culture.

Real-Time PCR Assay

A real-time PCR protocol described previously for dried-blood spots was performed to detect CMV DNA in saliva samples.[14] A sample was considered positive if five or more copies per reaction were detected.

Follow-up Testing

Infants with positive rapid culture, PCR assay, or both were reevaluated to determine whether the PCR results were true or false positive results. This was done by testing saliva and urine[18,19] specimens with the use of rapid culture and PCR assay (as described above).

STATISTICAL ANALYSIS

The results of the liquid- and dried-saliva real-time PCR assays were compared with those of saliva rapid culture (the standard method). Sensitivity, specificity, and predictive values for the PCR assays were calculated using standard methods for proportions and exact 95% confidence limits.

Likelihood ratios are based on the ratio of sensitivity and specificity and are independent of the prevalence of congenital CMV infection in the population; therefore, likelihood ratios can be used directly to estimate the probability of congenital CMV infection at the individual level.[28] The positive likelihood ratio was calculated as the sensitivity divided by (1−specificity), the negative likelihood ratio was calculated as (1−sensitivity) divided by the specificity, and the 95% confidence intervals were calculated according to the method described by Simel and colleagues.[24] All statistical analyses were performed using SAS software, version 9.2 (SAS Institute).

RESULTS

STUDY POPULATION AND SPECIMENS

During the study period, 34,989 infants were enrolled. The mean (±SD) age at the time of collection of saliva specimens was 1.0±1.2 days. Characteristics of the study population are shown in Table 1. Nearly all the infants (98.0%) were from well-baby nurseries. The median age at the time of collection of follow-up samples was 3.6 weeks (interquartile range, 2.6 to 6.6). Overall, 177 newborns (0.5%; 95% confidence interval [CI], 0.4 to 0.6) tested positive for CMV on screening by means of rapid culture, PCR assay, or both. No study-related adverse events were observed.

NEWBORN CMV SCREENING WITH SALIVA RAPID CULTURE AND REAL-TIME PCR ASSAY

Rapid Culture and Liquid-Saliva PCR Assay

During phase 1, liquid-saliva specimens were collected from 17,662 newborns and tested for CMV with the use of rapid culture and liquid-saliva real-time PCR assay. A total of 93 infants (0.5%; 95% CI, 0.4 to 0.6) tested positive for CMV by any

Table 1. Baseline Characteristics of the 34,989 Study Newborns.*

Characteristic	Value
Sex — no. (%)	
Female	17,278 (49.4)
Male	17,711 (50.6)
Race or ethnic group — no. (%)†	
Asian	1,358 (3.9)
Black	8,298 (23.7)
White, Hispanic	11,356 (32.5)
White, non-Hispanic	12,835 (36.7)
Other, including >1 category	1,142 (3.3)
Insurance for hospital stay — no. (%)	
Private	23,326 (66.7)
Public or no insurance	11,663 (33.3)
Hospital nursery — no. (%)	
"Well-baby" nursery	34,275 (98.0)
Neonatal intensive care	714 (2.0)
Maternal age — yr	
Mean	27.3±6.1
Median (range)	27 (12–52)

* Plus–minus values are means ±SD.
† Race or ethnic group was reported by a parent.

test (Fig. 1). All 85 infants with a positive rapid-culture result also had a positive liquid-saliva PCR assay, and the PCR assay also identified 8 additional infants as infected although their culture results were negative (Table 2). The sensitivity of liquid-saliva real-time PCR assay as compared with standard rapid culture was 100% (95% CI, 95.8 to 100) (based on 85 of 85 infants); the specificity was 99.9% (95% CI, 99.9 to 100) (based on 17,569 of 17,577 infants). The positive and negative predictive values for the saliva PCR assay were 91.4% (95% CI, 83.8 to 96.2) and 100% (95% CI, 99.9 to 100), respectively (based on 85 of 93 infants and 17,569 of 17,569 infants, respectively). The positive likelihood ratio for the liquid-saliva PCR assay was 2197 (95% CI, 1099 to 4393), and the negative likelihood ratio was 0 (95% CI, 0.0 to 0.1). Of the 93 newborns who were positive on screening, 79 (85%) were enrolled for follow-up, of whom 72 tested positive on both rapid culture and PCR assay, with 1 of the 72 found to be negative on retesting by means of rapid culture and PCR assay of both saliva and urine specimens. Of the 8 infants who tested positive on PCR assay only, 7 were enrolled in follow-up; of those, 6 were found to be negative for CMV on retesting by means of rapid culture and PCR assay of both saliva and urine specimens.

Rapid Culture and Dried-Saliva PCR Assay

During phase 2, a dried-saliva specimen was also collected from 17,327 newborns. Of the 84 (0.5%; 95% CI, 0.3 to 0.5) newborns who were positive for CMV on either type of screening assay, 76 (90%) were positive on rapid culture (Fig. 1). The dried-saliva real-time PCR assay yielded positive results for 74 of the 76 samples that were positive on rapid culture and an additional 8 samples that were negative on rapid culture (Table 2). As compared with rapid culture, the sensitivity of the dried-saliva PCR assay was 97.4% (95% CI, 90.8 to 99.7) (based on 74 of 76 infants) and the specificity was 99.9% (95% CI, 99.9 to 100) (based on 17,245 of 17,253 infants), respectively. The positive and negative predictive values for the dried-saliva PCR assay were 90.2% (95% CI, 81.7 to 95.7) and 99.9% (95% CI, 99.9 to 100), respectively (based on 74 of 82 infants and 17,243 of 17,245 infants, respectively). The positive likelihood ratio for the dried-saliva PCR assay was 2100 (95% CI, 1049 to 4202), and the negative likelihood ratio was 0.03 (95% CI, 0.0 to 0.1) (Table 2). Of the 84 infants who were positive for CMV on either test, 74 (88%) were enrolled in follow-up. All 66 infants whose specimens were positive by means of both rapid culture and PCR assay and were enrolled in follow-up were positive for CMV on retesting. The 2 infants who were positive on rapid culture but negative on PCR assay were found to still be positive for CMV on retesting with the use of rapid culture and PCR assay. Of the 8 infants who were found to be CMV-positive on PCR assay but not rapid culture, 2 were lost to follow-up and 6 underwent retesting with the use of rapid culture: 4 were found to be CMV-negative and 2 were found to still be CMV-positive.

Liquid-Saliva vs. Dried-Saliva PCR Assay

Between June 2008 and February 2009, all three screening methods (saliva rapid culture, liquid-saliva PCR assay, and dried-saliva PCR assay) were carried out on saliva specimens obtained from 5276 newborns. There was 100% agreement between the results of the liquid-saliva and the dried-saliva PCR assays (Table 3). Both types of PCR assay confirmed the CMV-positive status of all 42 infants with positive rapid-culture results and identified 1 additional infant as being CMV-positive after re-

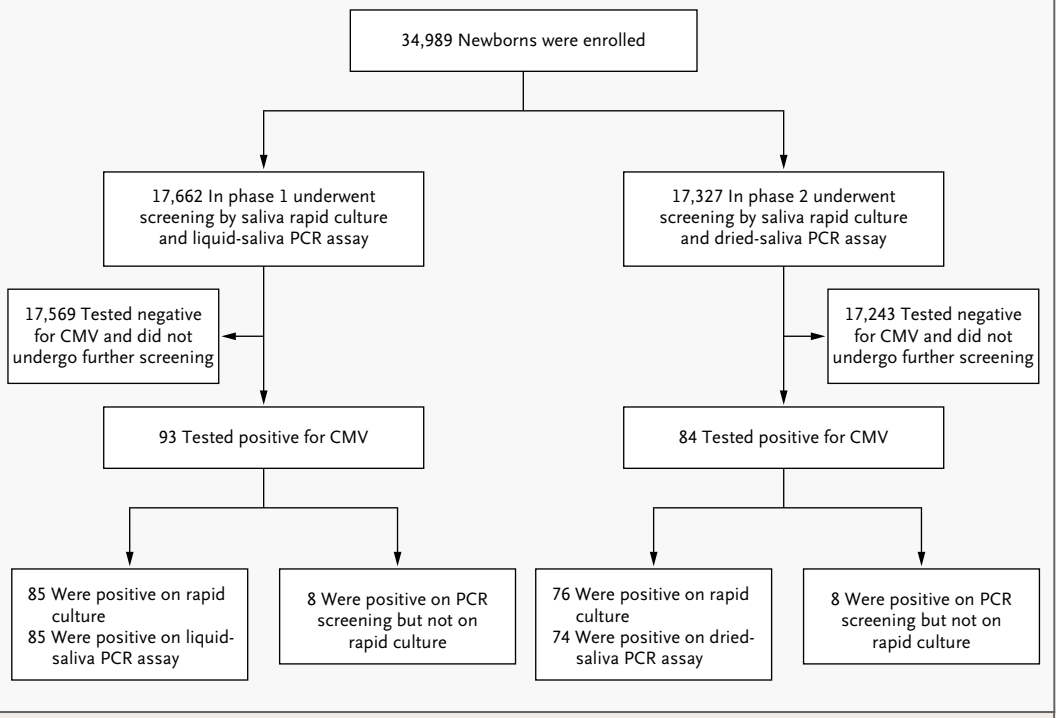

Figure 1. Enrollment and Prospective Screening of 34,989 Newborns.
CMV denotes cytomegalovirus, and PCR polymerase chain reaction.

ceiving negative results on rapid culture and positive results on retesting.

DISCUSSION

Our large, prospective study of CMV screening in newborns shows that the real-time PCR assay of both liquid-saliva and dried-saliva samples has excellent sensitivity (>97%) and specificity (99.9%) as compared with the standard saliva rapid culture. This indicates that the saliva PCR assays, which can easily be adapted for large-scale screening of newborns, will identify most infants who have congenital CMV infection.

The majority of infants with congenital CMV infection will not be identified by means of clinical examination during the newborn period. In addition, sensorineural hearing loss can develop after birth and continue to progress during early childhood in a significant proportion of children with CMV-associated sensorineural hearing loss.[1,6-8,29] Thus, the availability of rapid and reliable diagnostic methods that can be adapted for high-throughput screening is essential for early identification of children at risk for CMV-associated sensorineural hearing loss. Testing dried-blood-spot specimens with the use of PCR-based methods appeared to be a promising strategy for CMV screening in newborns, because several previous studies reported that dried-blood-spot PCR assay is highly sensitive in identifying infants with congenital CMV infection.[15,20,21,30]

However, the results of our recent multicenter study comparing dried-blood-spot real-time PCR assays with saliva rapid culture in more than 20,000 infants revealed that dried-blood-spot PCR assays identified fewer than 40% of CMV-infected newborns.[14] In addition, the performance of the dried-blood-spot PCR assay has been shown to vary according to the size of the filter-paper punch, the DNA-extraction methods, and the PCR-assay protocols used.[16,22,23,31] These findings, in addition to demonstrating the challenges in developing sensitive high-throughput assays for testing dried-blood spots, suggest that many newborns with congenital CMV infection may not have detectable CMV DNA in peripheral blood. Further advances in PCR methods might improve the sensitivity of the dried-blood-spot PCR assay, however, allowing for acceptable levels of detec-

Table 2. Real-Time Polymerase-Chain-Reaction (PCR) Assays of Liquid- and Dried-Saliva Specimens, vs. Rapid Culture, Used to Screen for Congenital Cytomegalovirus Infection.

Rapid Culture	Liquid-Saliva PCR Assay			Dried-Saliva PCR Assay		
	Positive	Negative	Total	Positive	Negative	Total
Positive	85	0	85	74	2	76
Negative	8	17,569	17,577	8	17,243	17,251
Total	93	17,569	17,662	82	17,245	17,327
Sensitivity (95% CI) — %	100 (95.8–100)			97.4 (90.8–99.7)		
Specificity (95% CI) — %	99.9 (99.9–100)			99.9 (99.9–100)		
Positive likelihood ratio (95% CI)	2197 (1099–4393)			2100 (1049–4202)		
Negative likelihood ratio (95% CI)	0 (0.0–0.1)			0.03 (0.0–0.1)		
Positive predictive value (95% CI) — %	91.4 (83.8–96.2)			90.2 (81.7–95.7)		
Negative predictive value (95% CI) — %	100 (99.9–100)			99.9 (99.9–100)		

tion of infants with congenital CMV infection in the future.

The data reported here show that the same dried-blood-spot PCR protocol applied to saliva[14] identified more than 97% of CMV-infected newborns. In addition, these findings show that saliva is a more reliable type of specimen than dried-blood spots for identifying congenital CMV infection by means of PCR assay and can be an effective tool for mass screening of newborns for CMV. Although testing of urine specimens collected on filter disks inserted into diapers of newborns was recently shown to be a promising approach for newborn CMV screening, urine specimen collection is not without challenges.[17,32] Obtaining urine specimens from infants requires additional steps and time that are not needed for collecting saliva, and validation of methods of urine collection and urine PCR assay are needed before the practicality of urine-sample screening can be evaluated for large-scale CMV screening in newborns.

In 16 infants, saliva specimens were positive on screening by means of real-time PCR assay but not rapid culture. To determine whether these PCR results were false positives, retesting was performed with the use of PCR assay of saliva and rapid culture of saliva and urine specimens obtained at the time of enrollment into the follow-up study. If these tests were negative, we considered the screening results to be false positives. Three infants who were found to be CMV-positive only at birth, one by means of liquid-saliva PCR assay and two by means of dried-saliva PCR assay, had positive results on rapid culture and PCR assay during follow-up. These findings indicate that PCR assays identified additional CMV-infected newborns missed when tested with the use of rapid culture.

In 10 infants who had negative rapid culture results but positive PCR results (6 on liquid-saliva PCR assay and 4 on dried-saliva PCR assay), retesting yielded false positive PCR results: the follow-up saliva and urine specimens were negative for CMV. As CMV is occasionally shed in the genital tract secretions of seropositive women at delivery and in the breast milk of most seropositive mothers, these false positive results could be due to CMV-containing maternal secretions present in the infants' saliva samples.[33-38] Although false positive saliva PCR results could lead to unwarranted parental anxiety and additional testing in infants to confirm or rule out congenital CMV infection, the overall frequency of false positive results for both liquid-saliva and dried-saliva PCR assays was less than 0.03%. In addition, the small negative likelihood ratios for both saliva PCR assays indicate that a negative result on these assays does rule out congenital CMV infection (Table 2).[28] Nevertheless, when saliva PCR assay is used to screen newborns, a positive screening result should be confirmed within the first 3 weeks of age to avoid false positive screening results.

The dried-saliva PCR assay failed to detect CMV infection in two newborns, leading to slightly lower sensitivity (97.4%; 95% CI, 90.8 to 99.7) than for the liquid-saliva PCR assay. Nevertheless, the simplified procedures for specimen collection, storage, and transport, combined with the high

Table 3. Real-Time Polymerase-Chain-Reaction (PCR) Assays of Liquid- and Dried-Saliva Specimens, vs. Rapid Culture, in 5276 Newborns Who Underwent All Three Assays Used to Screen for Congenital Cytomegalovirus Infection.

Rapid Culture	Liquid-Saliva PCR Assay		Dried-Saliva PCR Assay		Total
	Positive	Negative	Positive	Negative	
	number of newborns				
Positive	42	0	42	0	42
Negative	1	5233	1	5233	5234
Total	43	5233	43	5233	5276

sensitivity, support dried-saliva PCR assay as a reasonable approach to CMV screening in newborns. Although the need for collection of an additional specimen adds to the complexity of the existing newborn-screening programs, the saliva PCR assays described in this study have four main advantages for CMV screening in newborns. These are reasonable sensitivity and specificity, noninvasive specimen collection, elimination of the DNA-extraction step (which simplifies the laboratory procedures, thus providing considerable cost savings), and the fact that dried-saliva specimens can be stored and transported at room temperature, further simplifying specimen handling and transport.

A limitation of this study is that the 34,812 infants found to be CMV-negative on both rapid culture and PCR assay of saliva samples obtained at the screening visit were not enrolled in follow-up to definitively exclude congenital CMV infection (by retesting with the use of rapid culture of saliva or urine). Therefore, it is possible that CMV-infected newborns may have been missed by the rapid culture, affecting our determination of the sensitivity and specificity of saliva PCR assay. However, we believe this possibility is quite low, since the saliva rapid culture has been shown to have a sensitivity of at least 98%.[14,19] At present, although imperfect, rapid culture of saliva or urine specimens remains the most widely accepted standard method for identification of infants with congenital CMV infection.[14,19,27]

In summary, the usefulness of saliva specimens for identification of CMV by means of PCR assay was shown. The screening methods have been further simplified, with the use of dried specimens and processing that does not require a DNA-extraction step, without significant loss of sensitivity or specificity. This strategy appears to be suitable for a high-throughput assay for large-scale screening to identify newborns with congenital CMV infection.

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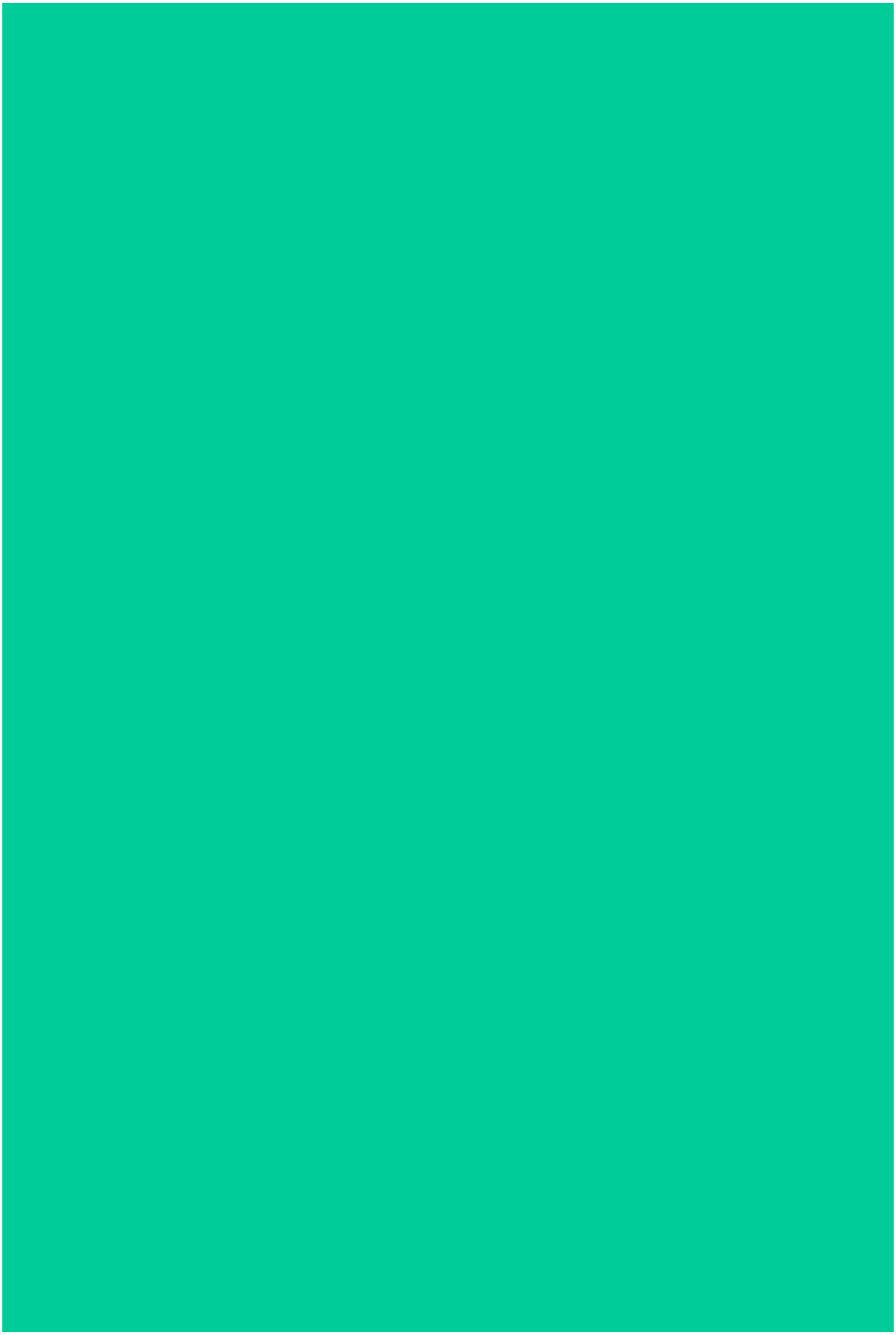

BRIEF REPORT

Cytomegalovirus Viruria and DNAemia in Healthy Seropositive Women

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Viruria and DNAemia patterns were investigated in 205 seroimmune women enrolled in a prospective cytomegalovirus (CMV) reinfection study. CMV DNA was detected at least once in urine and blood specimens from 83% and 52% of patients, respectively. At baseline, 39% of patients had viruria, and 24% had DNAemia. Intermittent viruria and viremia was observed throughout the study. There were no differences in baseline CMV positivity by polymerase chain reaction or in longitudinal DNAemia and viruria between the women with and without serological evidence of reinfection. In young seropositive women, CMV DNAemia and viruria are common, which suggests that naturally acquired immunity to CMV does not alter shedding patterns.

Cytomegalovirus (CMV) is a frequent cause of congenital infection and an important opportunistic pathogen in immunocompromised individuals. The virologic characteristics of primary CMV infection have been described in a small number of healthy individuals. CMV shedding in urine, saliva, and vaginal secretions and CMV DNA (DNAemia) in peripheral blood, as assessed by qualitative polymerase chain reaction (PCR), have been observed in most individuals after CMV seroconversion. However, the DNAemia became undetectable within a few months after primary infection when patients were followed up for at least 1 year [1, 2]. CMV is shed in the urine for ⩾6 months after seroconversion; thereafter, viruria becomes intermittent. However, the virologic characteristics of CMV infection in seroimmune women (ie, nonprimary infection), especially in those with frequent CMV reinfections, are not known.

Most sequelae associated with congenital CMV infection are thought to result from primary maternal CMV infection during pregnancy. Early reports by Ahlfors et al [3, 4] suggested that congenitally infected children born to women with preexisting CMV immunity are also at significant risk of adverse neurodevelopmental sequelae. More recent studies have confirmed these observations and shown that congenital CMV infection after nonprimary maternal infection contributes significantly to CMV-associated morbidity [5–7]. Therefore, vaccine strategies aimed at prevention of primary maternal infection to reduce the morbidity associated with congenital CMV infection will be of limited value, especially in highly seropositive populations. Although the mechanisms and the pathogenesis of intrauterine transmission and severe fetal infection in the presence of preexisting maternal immunity are unknown, an analysis of CMV strain–specific antibody responses revealed an association between intrauterine transmission of CMV and reinfection with new or different virus strains in seroimmune women [8, 9]. Knowledge of the virologic characteristics in women seroimmune to CMV infection is important not only for a better understanding of the natural history and pathogenesis of this chronic viral infection but also for designing strategies to prevent or reduce sequelae associated with congenital CMV infection. In the present study, we examined viruria and peripheral blood DNAemia in a cohort of seropositive women enrolled in a prospective study of CMV reinfection.

Methods. The study population consisted of 205 healthy CMV-seropositive women who participated in a longitudinal study of CMV reinfection. Women were recruited from the postpartum ward at the University of Alabama Hospital (Birmingham) and were derived from a predominantly urban, low-income, black population. The mean age of the study women was 18 years, and the majority of women were unmarried and had 1 previous pregnancy [10]. Study participants were followed up at 6-month intervals with a goal follow-up period of 3 years. At each study visit, urine and blood samples were obtained. The first urine and/or blood specimen was obtained from the study women at a mean (± standard deviation) of 81 ± 48.7 days after delivery. The study specimens consisted

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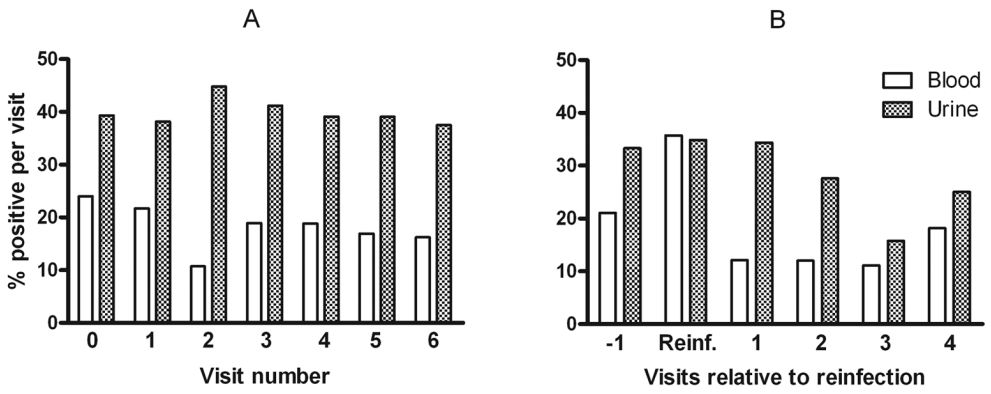

Fig. 1. *A*, Proportion of healthy, seropositive women ($n = 205$) with cytomegalovirus (CMV)–positive blood and urine samples by polymerase chain reaction (PCR) over the study period ("0" corresponds to baseline visit). *B*, Proportion of participants reinfected with a new virus strain ($n = 59$) with CMV-positive blood and urine samples by PCR relative to the visit of reinfection. "−1" corresponds to visit before serological determination of reinfection; "Reinf" corresponds to visit with serological evidence of reinfection.

of 814 urine and 800 peripheral blood samples. Approximately one-third (59 [29%] of 205) of study participants were noted to have CMV reinfection on the basis of the appearance of strain-specific antibody responses during follow-up [10]. Informed consent was obtained from all study participants, and the study was conducted in accordance with the guidelines of the Institutional Review Board for Human Use of the University of Alabama at Birmingham.

Urine and peripheral blood specimens were processed within 24 h after collection, and DNA was extracted using a commercial spin column kit (Qiagen). Each extraction run included a negative control. The presence and the amount of CMV DNA was assessed using a real-time PCR assay with an ABI 7500 Sequence Detection System (Applied Biosystems) and Absolute Low ROX QPCR mix (ABGene), as described elsewhere [11]. Each PCR run included plasmid standards incorporating the target regions of CMV gB and IE-2 to generate standard curves. CMV burden in whole blood was expressed as CMV genomic equivalents (ge) per milliliter. The sensitivity of the assay was determined using 10-fold serial dilution of known quantities of the AD169 strain DNA to be ~250 ge per 1 mL of blood [11].

Results. The study women were followed up for a median duration of 30.3 months (range, 6–58 months), and the median number of follow-up visits was 5 (range, 2–7 visits). The median number of study visits during which urine and blood specimens were positive by PCR was 2 (range, 1–5 visits) and 1 (range, 1–5 visits), respectively. Figure 1*A* shows the proportion of patients with urine and blood samples positive by PCR at each study visit. An analysis of serial specimens collected from the participants during the study showed that most study women (171 [83%] of 205) had at least 1 CMV-positive urine specimen, and approximately half (105 [52%] of 204) had at least 1 CMV-positive blood sample (Table 1). At study entry, 59 (39%) of 150 and 36 (24%) of 150 participants had CMV-positive urine and blood specimens by PCR, respectively. During subsequent visits (visits 2–6), results of PCR of blood and urine specimens were positive for 11%–19% and 39%–45% of participants, respectively (Figure 1*A*). Among women who completed at least 2 years of follow-up (5 study visits), the frequency of positive PCR results during visits 1–5 was 10%-43% and 49%–69% for blood and urine specimens, respectively. The median peak viral load was 3.9×10^3 ge/mL (range, 4.4×10^3 to 8.8×10^5 ge/mL) in urine samples and 5.2×10^3 ge/mL (range, 1.0×10^3 to 2.3×10^6 ge/mL) in blood samples.

Of the 205 participants, 59 (29%) of 205 had serological evidence of reinfection during the course of the study [10]. In the group with reinfection, 49 (83%) of 59 and 32 (54%) of 59 had CMV-positive urine and blood samples, respectively, by PCR at ⩾1 visit; these findings were similar to those observed in women without reinfection. An analysis of specimens collected during the visit immediately before reinfection showed that approximately one-fifth of blood specimens were positive by PCR, and one-third of urine specimens were positive by PCR. The rate of blood PCR positivity steadily decreased to ~10% for the subsequent visits, and viruria was present in a

Table 1. Cytomegalovirus Polymerase Chain Reaction (PCR) Positivity and Visit Distribution in Blood and Urine Compartments of 205 Healthy, Seropositive Women

Characteristic	Blood	Urine
PCR positivity	105 (51.5)[a]	171 (83.4)
No. of positive visits, mean ± SD	1.4 ± 0.7	1.9 ± 1.0
Positive at >1 visit	34/105 (32.3)	96/171 (56.1)

NOTE. Data are no. (%) of participants, unless otherwise indicated. SD, standard deviation.

[a] 204 women.

higher proportion of patient during the remainder of the follow-up period (Figure 1*B*). Virologic characteristics in the group of 146 women without reinfection were similar to those in the 59 women with documented reinfection (data not shown).

Discussion. The virologic characteristics of primary CMV infection in healthy adults have been examined and have typically been described as several weeks of viremia with virus shedding in the urine for months intermittently [1, 2]. In the present study of young seropositive women, a similar pattern of intermittent virus shedding and DNAemia was seen. The majority of our study population had detectable CMV DNA in their urine at least once during the study, and approximately half of the women had detectable CMV DNA in peripheral blood at 1 of the study visits. No differences in the presence of CMV DNA in blood or urine samples were found between the group of women with and without serologic evidence of reinfection. These findings demonstrate that, in healthy young women known to be at increased risk of delivering a congenitally infected infant, CMV can be frequently detected in urine and blood.

Although intrauterine CMV transmission can occur even in the presence of preconceptional immunity, most of the newborn disease and CMV-related sequelae were thought to occur in infected infants born to mothers with primary CMV infection. Therefore, strategies to prevent morbidity associated with congenital CMV infection have been focused on prevention of primary maternal infection. However, the accumulation of data demonstrates that CMV-specific immunity resulting from naturally acquired infection does not prevent reinfection with new or different virus strains, and such reinfections have been associated with intrauterine transmission in seropositive women and with symptomatic congenital infection [6,8–10,12]. In addition, similar rates of hearing loss were observed in children with congenital CMV infection born to women with primary CMV infection and in those born to seroimmune mothers [6,7]. It has been suggested that CMV vaccines, although unlikely to prevent reinfection, could change the natural history of infection, resulting in shorter duration of viremia or viral shedding [1]. In the present study, 59 of the 205 seropositive women had serological evidence of reinfection with a new virus strain. There were no differences in baseline CMV positivity by PCR, timing of DNAemia and viruria, and peak viral load between study women with and without reinfection. At study entry, CMV DNAemia and viruria were present in approximately one-fifth and one-third of all study women, respectively. In addition, 8 (21%) of 38 study women had CMV DNA in peripheral blood samples obtained during the visit immediately before the documentation of reinfection. This was followed by a peak of 36% (15 of 42 women) during the visit at which serological evidence of reinfection was captured. Subsequently, there was a decrease in DNAemia; however, intermittent CMV shedding in urine samples was observed throughout the study. This pattern of viral shedding is very similar to that in reports of virologic findings in primary CMV infections in populations with similar demographic characteristics [1]. Although it is not known whether intermittent DNAemia and viruria in the study population represent reactivation of previously acquired CMV or reinfection with new virus strains, our findings suggest that preexisting immunity to CMV neither prevents reinfection nor shortens the duration of viremia or viruria. This information could have important implications for CMV vaccine development and, in addition, provides baseline virologic data for an evaluation of interventional strategies to prevent or reduce morbidity associated with congenital CMV infection in highly seropositive populations.

Previous studies in populations with demographic characteristics similar to those of the current study participants showed that acquisition of CMV infection (primary infection or reinfection) occurs frequently and that these young women are at increased risk of delivering a congenitally infected infant [1, 10, 13]. A recent study showed that in this young, urban population, caring for young children and recent onset of sexual activity greatly increased the risk of having a child with congenital CMV infection [14]. In our recent study of CMV reinfection, we were unable to identify an association between exposures and the acquisition of a new strain of CMV [10]. However, more than two-thirds of the study population was involved in the direct care of young children, and the majority of women had multiple sexual partners—factors known to be associated with acquisition of CMV infection [14]. Our findings reveal that, in this population of young, low-income, black women, CMV circulates frequently, resulting in the high rate of viremia and viruria. Although the mechanisms for the increased risk of delivering an infant with congenital CMV infection are not known, the high rate of virus shedding could be an explanation for the higher prevalence of congenital CMV infection in this population. Our findings reinforce the suggestion for an urgent need for public health interventions in young, low-income, black populations because of the disproportionately higher burden of congenital CMV infection and disabilities associated with it [15]. However, the persistent virus shedding in seropositive women reveals the challenges in designing prevention strategies.

Because the study population is known to have an increased risk of primary CMV infection and reinfection, the results of this study may not be applicable to the general population of women. Another possible limitation of our study is that the study participants were followed up at 6-month intervals. Although CMV shedding in the urine has been shown to last $\geq$6 months in the majority of women with primary CMV infection, DNAemia is expected to last for a much shorter duration;

therefore, the number of women with viremia could have been underestimated. Because not all study participants completed 3 years of follow-up, the overall frequency of reinfection could have been underestimated in our study. The number of PCR-positive specimens in women who completed at least 2 years of follow-up was similar to that in the overall study group, arguing against the possibility that variable follow-up may have influenced the virologic findings.

In summary, this study of the virologic characteristics in young, seropositive women shows that viremia and viruria are common and that naturally acquired immunity to CMV, whether recently acquired or not, does not appear to alter shedding patterns, even when CMV infection due to a new virus strain has occurred.

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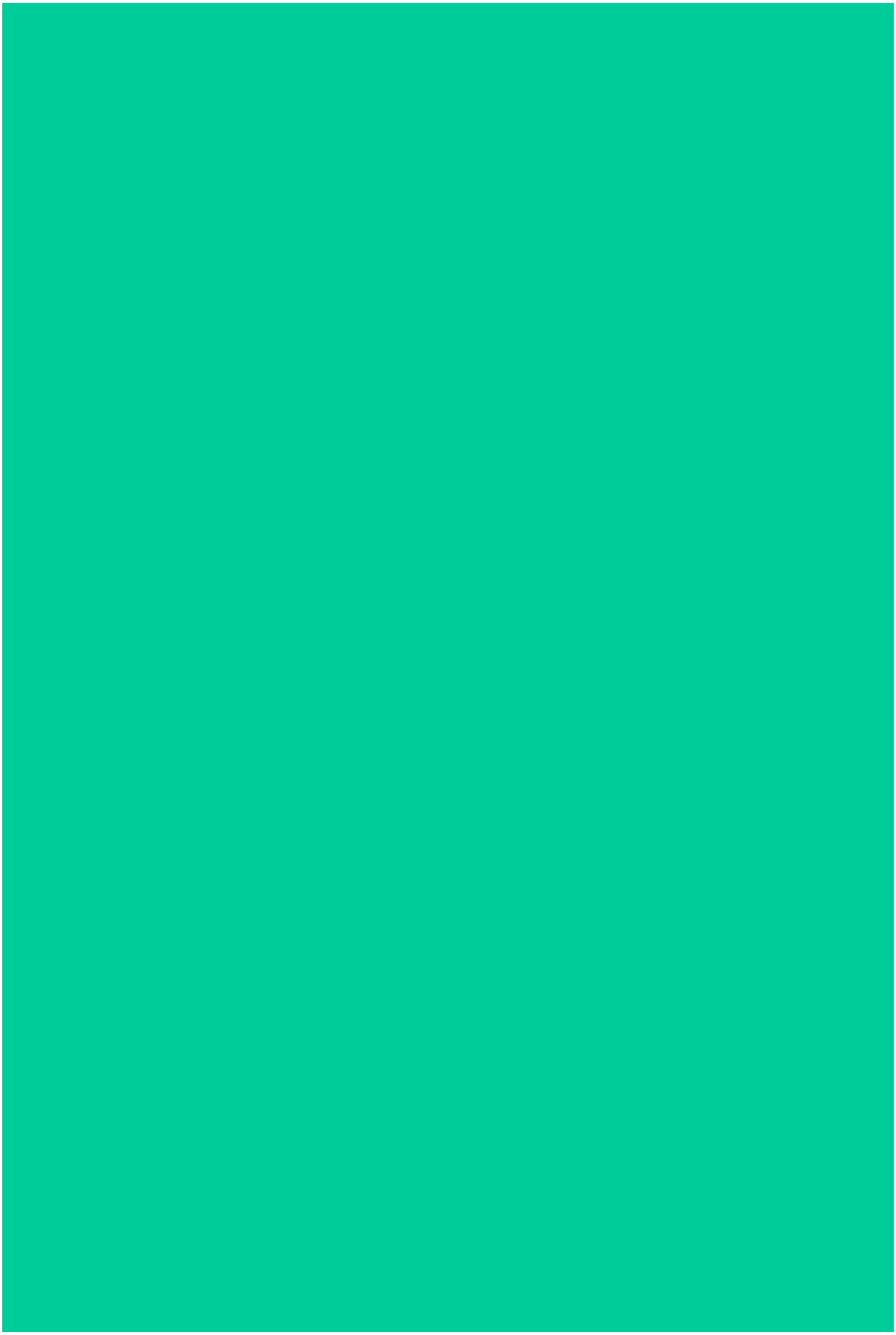

Dried Blood Spot Real-time Polymerase Chain Reaction Assays to Screen Newborns for Congenital Cytomegalovirus Infection

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Context Reliable methods to screen newborns for congenital cytomegalovirus (CMV) infection are needed for identification of infants at increased risk of hearing loss. Since dried blood spots (DBS) are routinely collected for metabolic screening from all newborns in the United States, there has been interest in using DBS polymerase chain reaction (PCR)–based methods for newborn CMV screening.

Objective To determine the diagnostic accuracy of DBS real-time PCR assays for newborn CMV screening.

Design, Setting, and Participants Between March 2007 and May 2008, infants born at 7 US medical centers had saliva specimens tested by rapid culture for early antigen fluorescent foci. Results of saliva rapid culture were compared with a single-primer (March 2007-December 2007) and a 2-primer DBS real-time PCR (January 2008-May 2008). Infants whose specimens screened positive on rapid culture or PCR had congenital infection confirmed by the reference standard method with rapid culture testing on saliva or urine.

Main Outcome Measures Sensitivity, specificity, and positive and negative likelihood ratios (LRs) of single-primer and 2-primer DBS real-time PCR assays for identifying infants with confirmed congenital CMV infection.

Results Congenital CMV infection was confirmed in 92 of 20 448 (0.45%; 95% confidence interval [CI], 0.36%-0.55%) infants. Ninety-one of 92 infants had positive results on saliva rapid culture. Of the 11 422 infants screened using the single-primer DBS PCR, 17 of 60 (28%) infants had positive results with this assay, whereas, among the 9026 infants screened using the 2-primer DBS PCR, 11 of 32 (34%) screened positive. The single-primer DBS PCR identified congenital CMV infection with a sensitivity of 28.3% (95% CI, 17.4%-41.4%), specificity of 99.9% (95% CI, 99.9%-100%), positive LR of 803.7 (95% CI, 278.7-2317.9), and negative LR of 0.7 (95% CI, 0.6-0.8). The positive and negative predictive values of the single-primer DBS PCR were 80.9% (95% CI, 58.1%-94.5%) and 99.6% (95% CI, 99.5%-99.7%), respectively. The 2-primer DBS PCR assay identified infants with congenital CMV infection with a sensitivity of 34.4% (95% CI, 18.6%-53.2%), specificity of 99.9% (95% CI, 99.9%-100.0%), positive LR of 3088.9 (95% CI, 410.8-23 226.7), and negative LR of 0.7 (95% CI, 0.5-0.8). The positive and negative predictive values of the 2-primer DBS PCR were 91.7% (95% CI, 61.5%-99.8%) and 99.8% (95% CI, 99.6%-99.9%), respectively.

Conclusion Among newborns, CMV testing with DBS real-time PCR compared with saliva rapid culture had low sensitivity, limiting its value as a screening test.

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CYTOMEGALOVIRUS (CMV) IS an important cause of congenital infection and a leading cause of sensorineural hearing loss (SNHL) in children.[1-5] Of the estimated 20 000 to 40 000 infants born each year with congenital CMV infection in the United States, most (90% to 95%) have no detectable clinical abnormalities at birth and thus will not be identified by routine clinical examination.[2,6,7] Furthermore, SNHL occurs in approximately 10% to 15% of infants with clinically inapparent congenital CMV infection and the majority of children with CMV-related SNHL will have late-onset losses, progressive losses, or both.[1,8,9] Therefore, both routine physical

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examination and newborn hearing screening will miss potential diagnosis in many children who develop SNHL secondary to congenital CMV infection. To identify these at-risk infants early in life, rapid, reliable, and relatively inexpensive methods to screen newborns for congenital CMV infection are needed.[10] Identification of children at increased risk of CMV-associated SNHL early in life will allow targeted monitoring of these children in order to intervene at critical stages of acquiring speech and language skills.[11]

Although traditional virus isolation from saliva or urine specimens in tissue culture is considered the standard method for identification of infants with congenital CMV infection, it is not amenable to mass screening (even when modified to produce rapid results) because it is labor- and resource-intensive and requires tissue culture facilities. Real-time polymerase chain reaction (PCR) technology, in contrast, is well-suited for mass screening because it does not require tissue culture facilities and is amenable to automation with the screening of large numbers of specimens at low cost. A variety of newborn specimens including saliva, urine, and dried blood spots (DBS) can be tested with PCR-based methods for the diagnosis of congenital CMV infection.[12-19] Since DBS are collected routinely for newborn metabolic screenings from all infants born in the United States, there has been considerable interest in using PCR assays for detecting CMV in newborn DBS specimens. Despite the benefits of DBS PCR-based methods, the sensitivity and specificity of these assays for universal newborn CMV screening have not been determined. Most reports have studied selected infant populations and none have prospectively compared the results of a DBS PCR assay with a standard (ie, tissue culture) method for identifying CMV infection in an unselected newborn population.[13-16,20-22] This study examined the diagnostic accuracy of real-time PCR analysis of DBS as an approach for mass screening of newborns for congenital CMV infection.

METHODS

Study Population

Between March 2007 and May 2008, infants born at 7 US medical centers (University of Alabama at Birmingham Hospital; The University of Mississippi Medical Center, Jackson; Carolinas Medical Center, Charlotte, North Carolina; Saint Peter's University Hospital, New Brunswick, New Jersey; Good Samaritan Hospital, Cincinnati, Ohio; Magee Women's Hospital, Pittsburgh, Pennsylvania; and Parkland Memorial Hospital, Dallas, Texas) were enrolled prospectively in the National Institute on Deafness and Other Communication Disorders CMV and Hearing Multicenter Screening (CHIMES) study. Institutional review board approval was obtained at each site. Mothers were approached postpartum to obtain written informed consent for their newborn's enrollment in the study. Upon enrollment, saliva specimens were collected from participating infants along with an additional blood spot obtained at the time of newborn metabolic screening. The DBS specimen for the study was collected only after the completion of metabolic screening and infants were not subjected to additional heel sticks for the CHIMES study. Infants with positive saliva or DBS screening specimens were enrolled in the follow-up component of the study to confirm congenital CMV infection, as well as evaluate hearing outcomes during the first 4 years of life (ongoing). Race and ethnicity data were collected as self-reported by parents because the prevalence of congenital CMV infection has been shown to vary according to racial and ethnic composition of the delivery population.[23,24]

Specimen Collection

Saliva specimens were collected from the enrolled newborns at a mean (SD) age of 0.9 (0.6) days and before nursery discharge. Collection was made by swabbing inside the infant's mouth using a sterile polyester fiber-tipped applicator (PurFybr Inc, Munster, Indiana) and placed in 1.0 mL of transport medium containing sucrose phosphate.[25] The specimens were stored at 4°C until they were transported, on ice, within 1 week of collection. A temperature-monitoring device was included in shipments to monitor for temperature variation during transport (TL20, 3M, St Paul, Minnesota).

DBS specimens were collected at the time of newborn metabolic screening and the mean (SD) age at collection was 1.9 (1.8) days. The additional blood spots were collected on a separate filter paper (Whatman 903, Florham Park, New Jersey), placed in individual envelopes, and stored in plastic resealable bags containing desiccant. DBS specimens were maintained at room temperature and shipped once weekly. Saliva and DBS specimens were transported to the University of Alabama at Birmingham central laboratory.

Detection of CMV in Saliva Specimens

The mean (SD) interval between the collection of initial saliva specimens and testing at the University of Alabama at Birmingham central laboratory was 7.4 (4.0) days. The presence of CMV in saliva specimens was detected by a rapid culture method for detecting early antigen fluorescent foci using a monoclonal antibody against the CMV major immediate early antigen in duplicate wells of a 96-well microtiter plate.[25,26] Each run included 2 positive control wells inoculated with the AD169 strain of CMV at a titer producing approximately 100 infectious foci per well. A specimen was considered positive if at least 1 focus of distinct nuclear fluorescence was detected in at least 1 well. Individuals ascertaining the results of the saliva rapid culture assay or the DBS PCR were blinded to the results of the other test.

DNA Extraction From DBS Specimens

From each DBS, two 3-mm disks were punched into 1.5-mL sample tubes

using the BSD600 automated filter paper puncher (BSD Robotics, Acacia Ridge, Queensland, Australia). The punched filter paper disks were processed to extract DNA using the Qiagen M48 robotic system with MagAttract technology according to the manufacturer's instructions (Qiagen Inc, Valencia, California). The extracted DNA specimens were stored at –20°C. A blank filter card was punched and included in each extraction run to serve as a negative control for DNA extraction and to monitor for cross contamination. In addition, a filter paper spotted with 10 000 copies of AD169 strain of CMV was punched and included in the extraction run to serve as a positive control and to monitor for consistency and reliability of the extraction protocol.

Real-time PCR

The mean (SD) interval between DBS specimen collection and PCR analysis was 14.6 (9.6) days. The detection of CMV DNA was performed using the ABI 7500 Real-time PCR System (Applied Biosystems Inc, Foster City, California) and ABsolute QPCR Low ROX Mix (ABgene USA, Rockford, Illinois). The reaction mixture contained primers at a concentration of 900 nM and the probe at 250 nM. Each specimen was run in duplicate using 25 µL of reaction mixture containing 20 µL of master mix and 5 µL of test specimen. To generate standard curves, each plate contained plasmid standards incorporating the target sequences in 10-fold dilutions ranging between 100 000 and 10 genomic equivalents per reaction. The real-time PCR amplification conditions have been previously described.[27,28] During the first 10 months of the study, the real-time PCR assay included primers to detect the highly conserved AD-1 region of the major envelope glycoprotein B.[27-29] During the final 5 months of the study, the PCR method was modified to include a second primer set from the highly conserved immediate early 2 exon 5 region (forward primer, GAG CCC GAC TTT ACC ATC CA; reverse primer, CAG CCG GCG GTA TCG A; and probe, VIC-ACC GCA ACA AGA TT-MGBNFQ) in an effort to improve the sensitivity of the assay (GeneBank accession numbers GU179001, AY446871, AY446870, FJ616285, AY446868). The real-time PCR was repeated on all specimens with a positive signal in either well and a specimen was considered positive if 1 or more genomic equivalents per reaction were detected on both PCR runs. In addition, real-time PCR was repeated on DBS specimens from infants with saliva specimens positive by rapid culture assay that were negative on the first PCR run. The detection limit of our real-time PCR assay, as determined by the sensitivity titration analysis, was 250 genomic equivalents per milliliter for the single-primer assay and 50 genomic equivalents per milliliter for the 2-primer assay (eAppendix available at http://www.jama.com).

Efficiency of DNA Extraction and DBS PCR Performance Characteristics

To determine whether the sensitivity of DBS real-time PCR for CMV DNA detection was influenced by the extraction method, detection of CMV DNA by the 2-primer real-time PCR protocol was compared between a commercial column-extraction method (Qiagen Inc, Valencia, California) and the robot-extraction protocol used in this study (eAppendix). In addition, the amount of genomic DNA as determined by real-time PCR amplification of RNase P (TaqMan RNase P control reagents kit, Applied Biosystems Inc, Foster City, California) in 185 randomly selected DBS specimens from CMV-negative infants was compared between the robot- and column-extraction methods (eAppendix). A comparison of the 2-primer real-time PCR assay and a previously described nested PCR protocol was undertaken to assess whether our real-time PCR method would be as sensitive or more sensitive for detecting CMV DNA than a standard nested-PCR method (eAppendix).[13]

Confirmation of Screening Results

To account for the possibility that saliva rapid culture assay may be less than 100% sensitive in identifying CMV-infected newborns, infants with positive saliva specimens or DBS screening specimens were enrolled in the follow-up component of the CHIMES study to confirm congenital CMV infection.[25] Urine and repeat saliva specimens were obtained from these infants at the enrollment visit for the follow-up study and were tested for CMV with the rapid culture assay (previously described). The rapid culture assay on the follow-up saliva or urine specimen was considered the reference standard for this study and therefore, a confirmed congenital CMV infection was defined as identification of CMV in either saliva or urine obtained at enrollment into the follow-up study. Infants were considered to be uninfected if both the saliva and the urine specimens tested negative by rapid culture assay. Newborns who were negative for CMV by both screening assays (saliva rapid culture and DBS PCR) were not enrolled in follow-up and not retested with the reference standard assay.

Data Analysis

Only infants enrolled in the follow-up component of the study for confirmation of congenital CMV infection status were included in determining the diagnostic ability of the DBS real-time PCR assays. Sensitivity, specificity, and predictive values for both the single-primer and the 2-primer DBS real-time PCR assays were calculated using standard methods for proportions and exact 95% confidence limits. The positive predictive value was the ratio of true positives to all positive DBS PCR results and the negative predictive value was the ratio of true negatives to all negative DBS test results. Likelihood ratios (LRs) were calculated to summarize the diagnostic accuracy of the DBS PCR assays. Positive LR was sensitivity/(1–specificity) and

the negative LR was (1–sensitivity)/specificity. Confidence intervals (CIs) for LRs were determined using the method described by Simel et al.[30] Statistical differences between nested and real-time PCR methods were calculated using the χ^2 test. All statistical analyses were performed using SAS software version 9.2 (SAS Institute Inc, Cary, North Carolina).

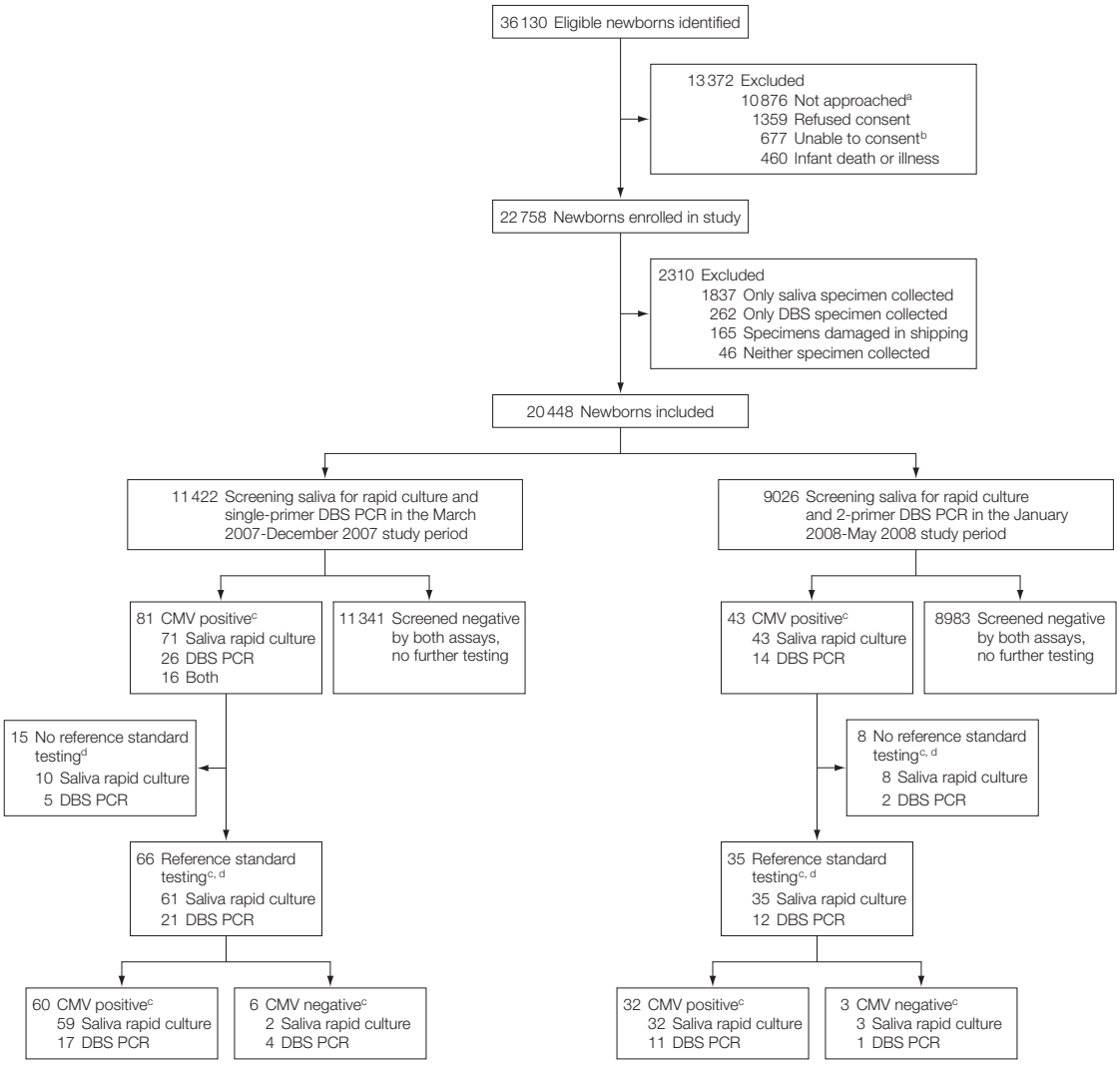
RESULTS

Study Population and Specimens

Of the 36 130 eligible infants, 22 758 (63%) infants were enrolled in the study. Although all live-born infants were eligible for participation, some of the infants born over holidays or weekends and those discharged prior to obtaining consent for participation in the study (n = 10 876) were not enrolled. Additional reasons for nonenrollment included refusal to participate (n = 1359); unable to obtain consent due to maternal factors such as illness, mental capacity, age, or language (n = 677); and infant death or illness (n = 460).

Both saliva and DBS specimens were collected from 20 613 (91%) infants, only saliva specimens were collected from 1837 infants, only DBS specimens were collected from 262 infants, and 46 infants had neither specimen collected (FIGURE).

Figure. Evaluation of DBS Real-time PCR Assays for Identifying Infants With Congenital CMV Infection



DBS indicates dried blood spots; PCR, polymerase chain reaction; and CMV, cytomegalovirus.
[a] Infants born over holidays or weekends or discharged before consent could be obtained.
[b] Unable to obtain consent due to illness, mental capacity, maternal age, or language.
[c] Will not sum because some participants were counted multicategorically.
[d] Rapid culture on saliva and urine samples collected at enrollment into follow-up to confirm congenital CMV infection was considered the reference standard for the study.

The reasons that both specimens were not available from these newborns included (1) the infants were unavailable or discharged from the nursery prior to collection (n=1214); (2) the newborn metabolic screening was completed before infants were enrolled in the study or there was insufficient blood left for the study DBS specimen (n=731); or (3) the specimens were mislabeled or misplaced (n=200). The infants (n=2145) who did not have both specimens collected were more likely to be in the neonatal intensive care unit than infants who had both specimens collected (14.7% vs 2.9%; χ^2 test=707.2; $P<.001$). Of the 20 613 infants who had both specimens collected, saliva specimens from 165 infants could not be tested due to leakage or temperature variations during shipment (Figure). Thus, the study population comprises the 20 448 infants who had both saliva and DBS specimens collected and tested.

Most of the study infants (19 858 [97.1%]) were from the well-baby nurseries (TABLE 1). The infants were evenly distributed by sex (male, 51.0% vs female, 49.0%). Mean (SD) maternal age was 27.3 (6.1) years. The mean (SD) age at enrollment into the follow-up study for confirmation of congenital CMV infection in infants positive by screening saliva rapid culture or DBS PCR was 6.4 (6.1) weeks of age. Overall, 92 of the 20 448 (0.45%; 95% CI, 0.36%-0.55%) infants had confirmed congenital CMV infection.

Newborn CMV Screening With Saliva Rapid Culture and the Single-Primer DBS PCR Assay

Between March 2007 and December 2007, 11 422 newborns were screened for congenital CMV infection using saliva rapid culture and the single-primer DBS PCR assay (Figure). Eighty-one newborns tested positive for CMV infection by either saliva rapid culture assay (n=71), the DBS PCR assay (n=26), or both methods (n=16). Sixty-six of the 81 infants (81%) who tested positive by either screening method were enrolled in the follow-up study and of those, 60 children were confirmed to have congenital CMV infection based on the positive reference standard assay. Congenital CMV infection status could not be determined in 15 infants because they were not enrolled in the follow-up study. Reasons for not enrolling in the follow-up study included refusing participation (n=8), loss to follow-up (n=6), and relocation (n=1).

Screening saliva rapid culture correctly identified 59 of the 60 infants (98%) with confirmed congenital CMV infection, whereas the single-primer DBS PCR only identified 17 of the 60 infants (28%) confirmed to have congenital CMV infection (TABLE 2). Congenital CMV infection was not confirmed in 2 of 61 infants (3%) with saliva specimens positive by rapid culture assay and in 4 of 21 infants (19%) who were DBS PCR-positive because of the negative reference standard assay. The sensitivity and specificity of the single-primer DBS PCR assay in identifying infants with confirmed congenital CMV infection were 28.3% (95% CI, 17.4%-41.4%) and 99.9% (95% CI, 99.9%-100%), respectively. The positive LR for the single-primer DBS PCR assay was 803.7 (95% CI, 278.7-2317.9) and the negative LR was 0.7 (95% CI, 0.6-0.8). The positive predictive value of the single-primer PCR assay was 80.9% (95% CI, 58.1%-94.5%) and the negative predictive value was 99.6% (95% CI, 99.5%-99.7%).

Newborn Screening With Saliva Rapid Culture and the 2-Primer DBS PCR Assay

During the study period between January 2008 and May 2008, there were 9026 newborns screened for congenital CMV infection using saliva rapid culture and the 2-primer DBS PCR assay (Figure). Forty-three newborns tested positive for CMV infection by either saliva rapid culture assay (n=43) or the DBS PCR assay (n=14). Thirty-five of the 43 infants (81%) who screened positive were enrolled in the follow-up study and of those, 32 children were confirmed to have congenital CMV infection based on a positive reference standard assay (Figure). Congenital infection status could not be determined in 8 infants since they did not enroll in the follow-up study. Reasons for not enrolling in the follow-up study included refusing participation (n=4), loss to follow-up (n=2), death (n=1), and relocation (n=1).

Screening saliva rapid culture correctly identified all 32 infants (100%) who were confirmed to have congenital CMV infection, whereas the 2-primer DBS PCR identified only 11 of the 32 infants (34%) confirmed to have congenital CMV infection (Table 2). Congenital CMV infection was not confirmed in 3 of 35 infants with saliva rapid culture (8%) and 1 of 12 screening DBS PCR-positive infants (8%) because the reference standard assay was negative. The sensitivity and specificity of the 2-primer DBS PCR assay for detecting infants with confirmed congenital CMV infection were 34.4% (95% CI, 18.6%-53.2%) and 99.9% (95% CI, 99.9%-100%), respectively. The positive LR for the 2-primer DBS PCR assay was 3088.9 (95% CI, 410.8-23 226.7) and the negative LR was 0.7 (95% CI, 0.5-0.8). The positive predictive value of the 2-primer as-

Table 1. Study Characteristics of 20 448 Newborns Who Underwent Saliva Rapid Culture and DBS PCR Assays for CMV Infection

Characteristic	No. (%)
Sex	
Male	10 422 (51.0)
Female	10 026 (49.0)
Race/ethnicity	
Asian	1409 (6.9)
African American	5526 (27.0)
White, Hispanic	4765 (23.3)
White, non-Hispanic	7850 (38.4)
Other including biracial or multiracial	898 (4.4)
Maternal age, mean (SD), y	27.3 (6.1)
Median (range), y	27 (12-52)
Hospital nursery	
Well-baby	19 858 (97.1)
Neonatal intensive care	590 (2.9)
Infants with confirmed congenital CMV infection	92 (0.45)

Abbreviations: CMV, cytomegalovirus; DBS, dried blood spots; PCR, polymerase chain reaction.

Table 2. Use of the 2 DBS Real-time PCR Assays to Identify Infants With Confirmed Congenital CMV Infection

	Single-Primer DBS PCR			2-Primer DBS PCR		
Congenital CMV Infection	Positive	Negative	Total	Positive	Negative	Total
Positive	17	43	60	11	21	32
Negative	4	11 343	11 347	1	8985	8986
Total	21	11 386	11 407	12	9006	9018
Other analyses, % (95% confidence interval)						
Sensitivity	28.3 (17.4-41.4)			34.4 (18.6-53.2)		
Specificity	99.9 (99.9-100)			99.9 (99.9-100)		
Positive likelihood ratio	803.7 (278.7-2317.9)			3088.9 (410.8-23 226.7)		
Negative likelihood ratio	0.7 (0.6-0.8)			0.7 (0.5-0.8)		
Positive predictive value	80.9 (58.1-94.5)			91.7 (61.5-99.8)		
Negative predictive value	99.6 (99.5-99.7)			99.8 (99.6-99.9)		

Abbreviations: CMV, cytomegalovirus; DBS, dried blood spots; PCR, polymerase chain reaction.

say was calculated to be 91.7% (95% CI, 61.5%-99.8%) and the negative predictive value was 99.8% (95% CI, 99.6%-99.9%).

Extraction Methods

Of the 71 DBS specimens from infants with positive saliva specimens, 29 robot-extracted specimens (41%) were positive for CMV DNA, whereas only 19 column-extracted specimens (29%) were positive (χ^2 test, 3.14; $P=.08$) (eTable 1 available at http://www.jama.com). In addition, in 185 randomly selected DBS specimens from infants testing negative for CMV, the mean (SD) amount of genomic DNA obtained using robotic extraction (0.86 [0.46] µg/mL) and using a commercial column kit (0.78 [0.44] µg/mL) was similar (t [368] = –1.58; $P=.11$) as measured by amplifying the RNase P gene (TaqMan Gene Expression Assays Protocol, PN 4333458) (eAppendix).

In 86 infants with confirmed congenital CMV infection, 40 (47%) were positive on the 2-primer PCR and 30 (35%) were positive by the nested PCR assay (χ^2 test = 2.41; $P=.12$). Both methods failed to identify 48% (41/86) who were confirmed CMV-positive (eTable 2 available at http://www.jama.com).

COMMENT

This study demonstrates that real-time PCR analysis of DBS has low sensitivity for correctly identifying infants with congenital CMV infection. These results have major public health implications because they indicate that such methods, as currently performed, will not be suitable for the mass screening of newborns for congenital CMV infection—the most common nongenetic cause of deafness in the United States. Our data indicate that as many as 80% of infants with congenital CMV infections could be missed, even when using 2-primer DBS real-time PCR assays. The high positive LRs for the single-primer and the 2-primer PCR assays provide strong evidence that a positive DBS PCR result using these assays will identify infants with congenital CMV infection. However, the negative LRs for both PCR assays are not sufficiently small enough to rule out congenital CMV infection in newborns with a negative DBS PCR result.

PCR testing of peripheral blood has been widely used as a standard diagnostic method to detect invasive CMV infections in immunocompromised individuals including allograft recipients and patients with AIDS.[31,32] These results, together with those of several studies that reported successful identification of infants with congenital CMV infection by DBS PCR, has led to anticipation that DBS PCR methods would become valuable tools in newborn CMV screening.[13-16,20-22] However, the pathogenesis of congenital CMV infection is likely to be different from that in immunocompromised hosts. Immunocompromised patients usually experience acute CMV infection or symptomatic reactivation shortly before blood CMV PCR testing, whereas congenitally infected infants may have acquired CMV infection months before birth and thus are no longer viremic when tested as newborns.

This study, in which the 2 DBS real-time PCR assays were directly and prospectively compared with a reference standard for identification of infants with congenital CMV infection, provides important test measures of the use of DBS PCR. Several previous reports have demonstrated that newborns with congenital CMV infection can be identified with varying degrees of success by testing DBS using different PCR methods.[13,16,33,34] However, the prospective studies that confirmed CMV infection after identifying CMV DNA in DBS did not determine the number of false negatives (infants with congenital CMV infection who tested negative on DBS PCR). Having the complete denominator, as provided by this study, is essential to determine the use of DBS PCR for newborn CMV screening.

The low sensitivity of the DBS PCR method could possibly be explained by several factors: (1) the method used for DNA extraction; (2) the real-time PCR techniques; or (3) the possibility that not all infants with congenital CMV infection have detectable CMV DNA in their blood at birth. To evaluate extraction methods, we compared the ability of the 2-primer DBS real-time PCR to detect CMV DNA in DBS specimens processed with the robot-extraction protocol used in this study

and the column-extraction method and found no difference.

A number of amplification methods including qualitative, quantitative, and real-time PCR protocols with different primers, probes, and cycling parameters have been reported with varying performance characteristics.[12-15,21,22,34,35] The single-primer real-time PCR assay used in this study was developed in the University of Alabama at Birmingham central laboratory.[27,28] A number of newborn CMV screening studies in which DBS specimens were tested using a nested PCR protocol report a sensitivity of the DBS PCR assay approaching 100% in some populations.[13,20,21] However, these studies did not include a direct comparison of the DBS PCR results with a standard culture-based assay. Further, in a more recent study in which different laboratories were given similar sample panels, the sensitivity of the CMV PCR method has been shown to vary from laboratory to laboratory.[36] A comparison of our 2-primer real-time PCR assay with a nested PCR protocol demonstrated that the 2-primer PCR had a higher sensitivity than the nested PCR but neither method identified most of the infants with congenital CMV infection.

Previous studies observed that some infants with clinically apparent or symptomatic congenital CMV infection had no detectable CMV DNA in whole-blood specimens obtained during the neonatal period.[27,28,37] These findings argue strongly that the low sensitivity of our DBS PCR methods is most likely not due to our assay performance, but to the absence of detectable CMV DNA in the peripheral blood of some newborns with congenital CMV infection. Since about 10% to 15% of infants with asymptomatic or clinically inapparent congenital CMV infection develop hearing loss, it is critical that an ideal CMV screening method identify most newborns with asymptomatic congenital CMV infection.

A limitation of our study is that the 20 324 infants who had negative results on both screening assays, saliva rapid culture and DBS PCR, did not have urine and repeat saliva specimens collected and tested with the rapid culture, resulting in the possibility that some CMV-infected newborns may have been missed by the saliva rapid culture. However, it is unlikely that the screening saliva rapid culture missed significant numbers of infants with congenital CMV infections. The saliva rapid culture assay used in our study was adapted from the shell vial assay, which has been shown to be as sensitive and specific as the conventional tube culture method and, thus, considered a standard method for the diagnosis of CMV infections in a variety of clinical settings.[38,39] In addition, the saliva rapid culture assay we used has been demonstrated to be at least 98% sensitive in identifying infants with congenital CMV infection.[25] Finally, the results of our study showed that 99% (91 of 92) of infants with confirmed congenital CMV infection were identified on screening saliva rapid culture assay.

Another possible limitation is the relative overrepresentation of African Americans in our study population, which could make the findings of this study less generalizable to other populations. Although African American infants have a greater risk of infection, there is no scientific evidence that the clinical course or the sensitivity of diagnostic assays differs by race or ethnicity.[23,24] However, the overrepresentation of African Americans may have influenced the prevalence of congenital CMV infection in our study. For populations with differing prevalences of congenital CMV infection than we found in this study, the predictive values calculated for the DBS PCR assays would not be appropriate since predictive values are dependent on the underlying prevalence of disease in the population.

In summary, the results of this large, prospective newborn CMV screening study that included a direct comparison of the DBS real-time PCR assays with the culture-based method on saliva specimens demonstrated that real-time DBS PCR assays are not suitable for screening newborns for congenital CMV infection since they miss approximately two-thirds of the infections. As the disease burden from congenital CMV infection remains a significant public health problem, there continues to be a need to identify the large number of infants with clinically inapparent congenital CMV infection early in life. The results of our study underscore the need for further evaluation of high-throughput methods performed on saliva or other specimens that can be adapted to large-scale newborn CMV screening.

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Author Contributions: Drs Boppana and Fowler had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
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Acquisition of data: Boppana, Ross, Novak, Shimamura, Tolan, Palmer, Ahmed, Michaels, Sánchez, Bernstein, Fowler.
Analysis and interpretation of data: Boppana, Ross, Novak, Shimamura, Tolan, Sánchez, Bernstein, Britt, Fowler.
Drafting of the manuscript: Boppana, Ross, Sánchez, Bernstein, Fowler.
Critical revision of the manuscript for important intellectual content: Boppana, Ross, Novak, Shimamura, Tolan, Palmer, Ahmed, Michaels, Sánchez, Bernstein, Britt, Fowler.
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Previous Presentation: This study was presented in part at the Pediatric Academic Societies Annual Meeting; May 4, 2009; Baltimore, Maryland (abstract 4115.1).
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Online-Only Material: eAppendix and eTables 1 and 2 are available at http://www.jama.com.

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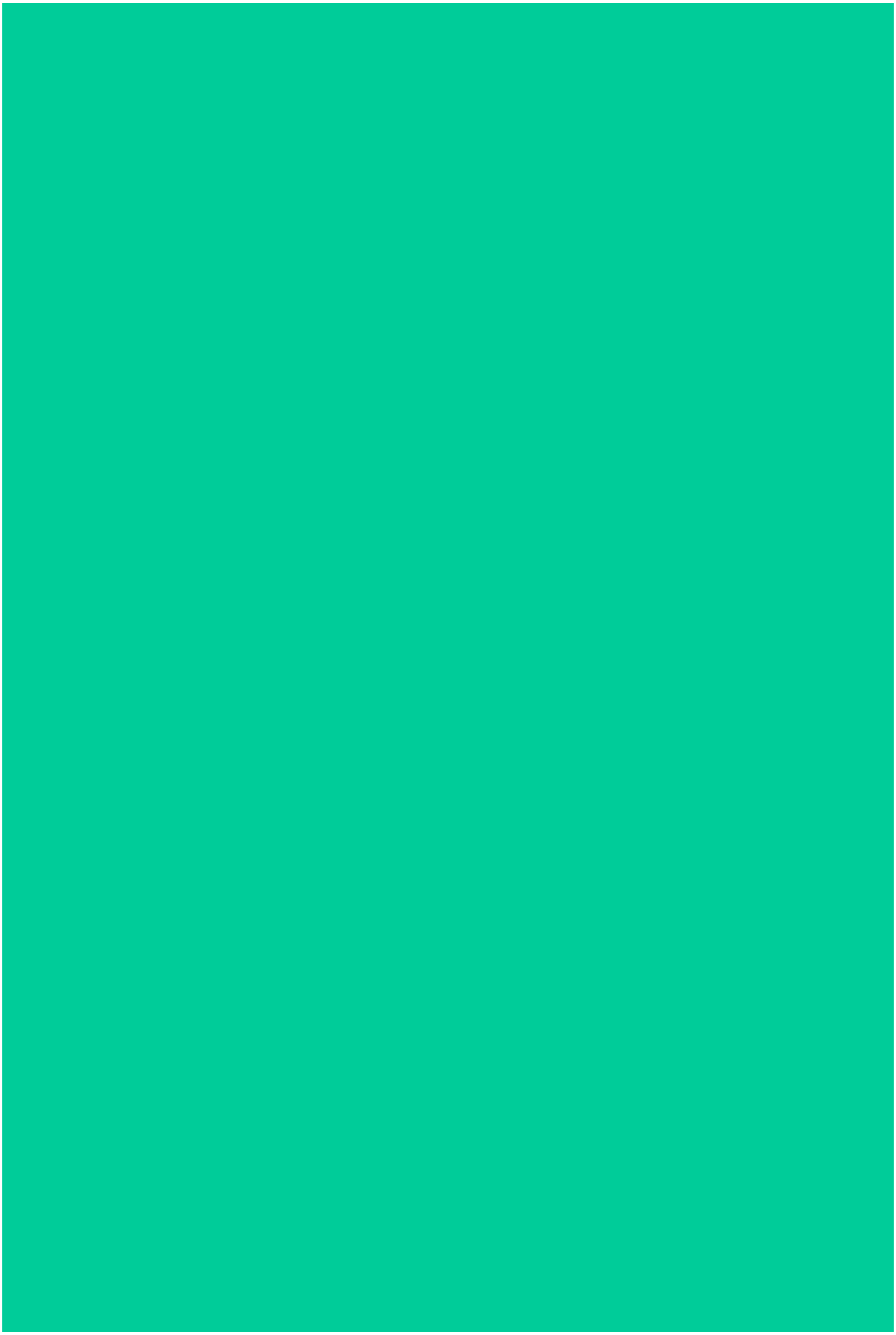

BRIEF REPORT

Cytomegalovirus Reinfections in Healthy Seroimmune Women

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Cytomegalovirus (CMV) reinfections have been associated with damaging congenital infection and adverse outcomes in transplant recipients. To determine the frequency of and risk factors for CMV reinfections, 205 seropositive women were followed up prospectively. The appearance of new antibody specificity against 1 of 4 polymorphic epitopes was considered as evidence of CMV reinfection. Approximately one-third of the study participants (59 [29%] of 205) were noted to have CMV reinfection during follow-up. None of the exposure factors were associated with CMV reinfection. Women with antibodies against at least 1 of the 4 antigens at baseline had a 63% decreased risk of reinfection, suggesting a protective role for strain-specific immunity.

Cytomegalovirus (CMV) is a frequent cause of congenital infection and an important cause of sensorineural hearing loss in children worldwide [1, 2]. Preconceptional immunity against CMV provides only incomplete protection against intrauterine transmission, and adverse outcomes can occur in infected children born to women who were seropositive prior to pregnancy [2–6]. CMV reinfections have also been associated with adverse outcomes in renal transplant recipients [7].

It is not clear whether transplacental transmission of CMV in women with preexisting seroimmunity is secondary to virus reactivation or to infection with a new or different CMV strain (reinfection) during pregnancy. We undertook a prospective study to determine the frequency of CMV reinfections in healthy seropositive women and to understand the various factors associated with such reinfections. Serial serum specimens from the study participants were analyzed for strain-specific immunoglobulin G (IgG) antibodies against the polymorphic determinants on the envelope glycoproteins gH and gB of CMV by means of an enzyme-linked immunosorbent assay (ELISA) method [3, 8].

Methods. Of the 258 CMV IgG–seropositive postpartum women enrolled in the study from February 2000 through June 2004, 205 participants had serum samples from at least 2 visits, and these women constituted the study population. A standardized interview was administered at baseline to obtain demographic characteristics and exposure factors. Standardized prenatal summary information was abstracted onto standard case report forms. The study participants were followed up at 6-month intervals for up to 3 years; at each visit, serum samples were obtained, and a standard questionnaire was administered to obtain an interval history of sexually transmitted infections (STIs), information on sexual partners, and information on child care. Serum specimens obtained at each visit were stored at −20°C until analysis. The study was approved by the institutional review board of the University of Alabama at Birmingham, and informed consent was obtained from the participants prior to study enrollment.

CMV strain–specific antibody responses were determined on the basis of polymorphisms in antibody binding sites within envelope glycoproteins gH and gB, between the 2 prototypical laboratory strains of CMV, AD169 and Towne [8–10]. The detection of new antibody specificities to either epitope (AD169 or Towne) on gH or gB in follow-up serum samples was considered evidence of infection with a new virus strain (reinfection) during the study. One of the 258 women had antibodies to all 4 antigens at enrollment in the study and was excluded from the analysis. To approximate the mean time from study entry until reinfection with a new virus strain, we measured the time from the baseline study visit to the visit during which new antibody specificities were detected.

Recombinant peptides containing antibody-combining sites within the amino terminal regions of the gH and gB genes present in the AD169 and Towne strains of CMV were synthesized and used as antigens to determine strain-specific IgG reactivity, as described elsewhere [3, 8]. Strain-specific antibodies against the polymorphic gH and gB regions of CMV were determined using an ELISA method that has been validated elsewhere [8].

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Table 1. Selected Exposure and Demographic Characteristics for Cytomegalovirus (CMV)–Seropositive Women

Variable	No. (%) of participants with CMV reinfection (n = 59)	No. (%) of participants without CMV reinfection (n = 146)	Unadjusted OR (95% CI)
White race	10 (16.9)	9 (6.2)	3.11 (1.05–9.15)
Maternal age <19 years	42 (71.2)	103 (70.6)	1.03 (0.51–2.15)
More than 1 sexual partner	57 (96.6)	143 (98.6)[a]	0.39 (0.03–5.65)
Sexually transmitted infection	13 (22.0)	31 (21.2)	1.05 (0.46–2.28)
Bacterial vaginosis	6 (10.2)	24 (16.4)	0.58 (0.18–1.56)
Chlamydia	3 (5.1)	6 (4.1)	1.25 (0.20–6.09)
Herpes	1 (1.7)	3 (2.1)	0.82 (0.01–10.49)
Trichomoniasis	3 (5.1)	3 (2.1)	2.55 (0.33–19.54)
Syphilis	0 (0)	0 (0)	…
Gonorrhea	0 (0)	4 (2.7)	0 (0–2.75)
Direct care of children	41 (69.5)	95 (65.1)	1.22 (0.61–2.50)
More than 2 children <6 years old living in household	16 (27.1)	34 (23.3)	1.23 (0.57–2.56)

NOTE. CMV reinfection was defined as the development of new antibody specificity against the polymorphic CMV gH and/or gB epitopes. CI, confidence interval; OR, odds ratio.

[a] Data were available for 145 participants.

The demographic and exposure characteristics were compared between women with CMV reinfection and those without CMV reinfection. Statistical significance was determined using the χ^2, Fisher exact, or Wilcoxon rank sum test where appropriate. Univariate odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using the exact method. Multivariate unconditional logistic regression using backward stepwise selection with $P < .10$ was used as a cutoff for retention in the model to assess whether exposure factors were associated with CMV reinfection. All data analyses were performed using SAS software (version 9.1; SAS Institute).

Results. The demographic characteristics of women with CMV reinfection were not different from those of women without evidence of reinfection. In both groups, the participants were predominantly unmarried, black women and had had 1 previous pregnancy. The mean age of the women in both groups was 18 years, and the study participants had a mean of 11 years of education. None of the study participants tested positive for human immunodeficiency virus (HIV). Twenty-nine percent of the study participants (59 of 205 participants) acquired new antibody specificities against gH or gB epitopes and thus were considered to be reinfected. The mean ($\pm$ standard deviation) time until the appearance of new strain-specific antibodies was 17.8 $\pm$ 10.3 months. The median follow-up duration was 35.4 months (range, 11–50 months) for women with reinfection and 30.6 months (range, 6–58 months) for those without reinfection ($P = .15$). Forty-nine percent of the reinfection group completed 6 study visits, compared with 34% of those without reinfection ($P = .05$). A higher proportion of white women (10 [53%] of 19) than of black women (48 [26%] of 185) had serological evidence of reinfection ($P = .02$).

Baseline exposure characteristics for women with and women without CMV reinfection were similar. The median number of persons living in the household was 5 and 4 for women with and women without reinfection, respectively ($P = .72$). The median age of sexual debut was 15 years in both groups. There were no differences between the 2 groups with regard to the number of lifetime sexual partners (median, 3 partners) or the number of sexual partners in the year prior to study enrollment (median, 1 partner). Approximately one-half of the women in each group had a history of STI. The frequency of gonococcal infection was higher in the group of women without CMV reinfection (25%), compared with the group of women with CMV reinfection (12%; $P = .04$). Sixty-eight percent of the women with reinfection and 60% of those without reinfection were involved in the direct care of young children.

The data on various exposure factors encountered during the study were compared between the group of women identified to have been reinfected with new CMV strains and the group of those without reinfection (Table 1). Only white race was significantly associated with CMV reinfection. To further examine the association between potential risk factors and CMV reinfection, we evaluated exposure factors during the 12-month period prior to the detection of new antibody specificities in women with reinfection and in the year prior to the final study visit for those without reinfection. Again, none of the exposure factors were found to be associated with reinfection (data not shown).

Race, age, new sexual partners, and direct care of children in the year prior to reinfection or in the year prior to the final study visit were entered into a logistic regression model. Race remained the only factor associated with reinfection (adjusted OR, 3.11 [95% CI, 1.1–9.2]).

The association between serological responses to the 4 an-

tigens (AP86, TO86, AP55, and TO55) at baseline and the likelihood of CMV reinfection during the study period was determined. Reactivity to strain-specific epitopes was shown to persist for a mean of 21 months. As illustrated in Figure 1, women with antibodies against 1 or more antigens at baseline were less likely to be reinfected with a new CMV strain during the study period (OR, 0.37 [95% CI, 0.19–0.73]).

Discussion. The results of this prospective study demonstrate that approximately one-third of CMV seroimmune women (59 of 205) were infected with a new or different CMV strain during the study period, as evidenced by the appearance of new antibody specificities against the linear polymorphic epitopes on gB and gH of CMV. The study participants were followed up for almost 3 years, and therefore the annualized rate of CMV reinfection was ~10%, a rate similar to the frequency of primary CMV infection in the general population [11]. The ELISA was adapted from a previously described radioimmunoassay to detect strain-specific antibodies against the envelope glycoprotein gH [3] and was validated in a recent study involving 96 seropositive and 51 seronegative individuals [8]. A similar strain-specific ELISA was employed in a recent study of CMV reinfections in renal transplant recipients [7]. However, approximately one-third of CMV-seropositive individuals in the previous study [8] and 46 of 204 women in the present study did not have detectable serum antibodies against any of the 4 antigens tested using this assay. This finding suggests that these women were infected with viruses containing gH and/or gB epitope variants that were not represented in the ELISA used in this study. Alternatively, our ELISA might lack the sensitivity to detect low levels of strain-specific antibodies in these women. Therefore, it could be argued that our study may have underestimated the actual frequency of CMV reinfections in the population. It is also possible that the appearance of new antibody specificities could be due to reactivation of endogenous virus. However, this is unlikely, because there are no data in the literature in support of this phenomenon and because the stability of CMV hypervariable genes has been shown in vitro in renal transplant recipients [12].

We were unable to identify an association between CMV reinfection and any of the known exposure factors for acquisition of CMV, including STIs, sexual practices, and caring for young children [13]. The demographic and baseline exposure characteristics were similar between the groups of women with and women without reinfection. Although more women without CMV reinfection had a history of gonorrhea at enrollment than did those with CMV reinfection, the number of women with gonorrhea was small, and thus this finding should be interpreted with caution. This study may have underestimated the number of STIs and sexual partners of participants in the population, because this information was obtained through interval questionnaires that relied on participant recall. To minimize recall bias, the study participants were interviewed individually at each visit, using a standardized questionnaire.

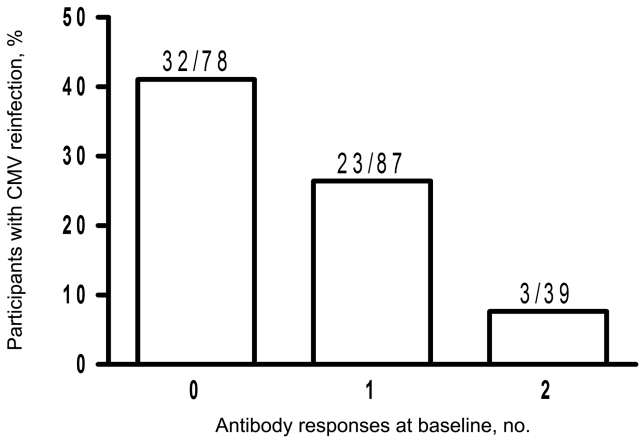

Figure 1. Frequency of cytomegalovirus (CMV) reinfection in 205 CMV-seropositive women versus the number of strain-specific antibodies to the 4 antigens (AP86, TO86, AP55, and TO55) present at study enrollment. Women with at least 1 antibody present at baseline were less likely to undergo CMV reinfection during the study period than were women with no antibodies at baseline (odds ratio, 0.37 [95% confidence interval, 0.19–0.73]).

Prenatal medical records were reviewed at enrollment for the results of laboratory studies and the dates of STIs. The smaller sample size and the fact that both groups in the study population had similar demographic and exposure characteristics may have led to our inability to identify an association between any of the exposure factors and CMV reinfection.

We did observe that women with a more broadly reactive antibody response at baseline were less likely to be reinfected during the study. Women with strain-specific antibodies to at least 1 antigen at baseline had a 63% decreased risk of CMV reinfection during the study (OR, 0.37 [95% CI, 0.19–0.73]), compared with participants who had no antibodies against any of the 4 antigens. This reduced risk of reinfection in women with antibodies to at least 1 antigen indicates that individuals infected with multiple CMV strains prior to study entry were less likely to be reinfected and that strain-specific immunity may play an important protective role against infection with new virus strains in seroimmune individuals. A recent study of a recombinant CMV gB vaccine suggested that prevention of maternal infection and of intrauterine transmission to offspring of previously nonimmune women could represent a feasible approach [14]. However, other studies have revealed that serum samples from individuals with natural infection produce higher neutralizing antibody titers and higher titers against epithelial cell entry than do serum samples from recipients of Towne or gB/MF59 vaccine [15]. This could be because individuals with natural infection are more likely to develop an antibody response against multiple CMV strains, whereas vaccines may induce antibody responses with only narrow specificity. Therefore, in populations with high maternal seroprevalence, the success of traditional vaccination approaches in

reducing intrauterine CMV transmission and CMV disease in congenitally infected children may be limited.

In the present study, we observed a higher prevalence of reinfection among white women (10 of 20 white women experienced reinfection). However, the number of white participants in our study was small, and therefore this association could be due to sampling bias. In addition, when the exposure factors were examined for white and black women independently, we did not detect differences between women with CMV reinfection and those without reinfection in either racial group. Because the group of women with CMV reinfection was followed up longer than was the group of women without serological evidence of reinfection, it is possible that more women in the group without reinfection could have been observed to acquire new antibody specificities if they had been monitored for a longer duration. However, this is unlikely to have had an effect on the lack of an association between various exposure factors and CMV reinfection, because the demographic and exposure characteristics of the 2 groups were similar.

In summary, the results of this study demonstrate that CMV reinfections are frequent in young, low-income, black seroimmune women. Our findings also suggest that, in addition to exposure, strain-specific immunity and possibly other as-yet undefined factors may play an important role in providing protection from infection with new CMV strains in seroimmune individuals.

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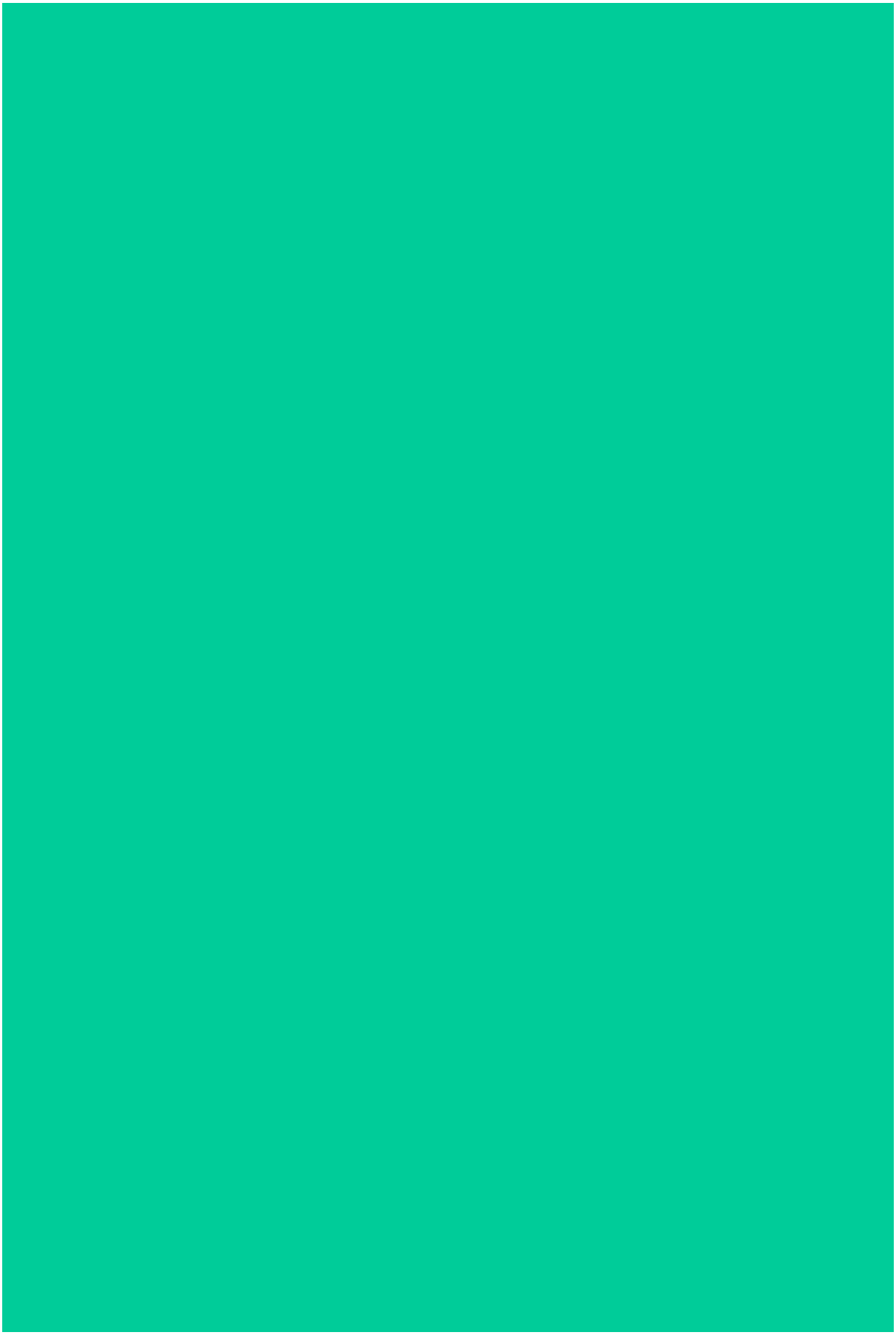

BASIC SCIENCE: OBSTETRICS

Human cytomegalovirus reinfection is associated with intrauterine transmission in a highly cytomegalovirus-immune maternal population

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OBJECTIVE: To determine contribution of reinfection with new strains of cytomegalovirus in cytomegalovirus seromimmune women to incidence of congenital cytomegalovirus infection.

STUDY DESIGN: In 7848 women studied prospectively for congenital cytomegalovirus infection from a population with near universal cytomegalovirus seroimmunity, sera from 40 mothers of congenitally infected infants and 109 mothers of uninfected newborns were analyzed for strain-specific anticytomegalovirus antibodies.

RESULTS: All women were cytomegalovirus seroimmune at first prenatal visit. Reactivity for 2 cytomegalovirus strains was found in 14 of 40 study mothers and in 17 of 109 control mothers at first prenatal visit ($P = .009$). Seven of 40 (17.5%) study women and 5 of 109 (4.6%) controls ($P = .002$) acquired antibodies reactive with new cytomegalovirus strains during pregnancy. Evidence of infection with more than 1 strain of cytomegalovirus before or during current pregnancy occurred in 21 of 40 study mothers and 22 of 109 controls ($P < .0001$).

CONCLUSION: Maternal reinfection by new strains of cytomegalovirus is a major source of congenital infection in this population.

Key words: congenital cytomegalovirus infection, cytomegalovirus reinfection, maternal reinfection with cytomegalovirus

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Human cytomegalovirus (CMV) is the most common viral infection transmitted to the developing fetus with rates of infection ranging from 0.2-2.0% of live births.[1,2] Importantly, congenital CMV infection is a major cause of sensorineural hearing loss in infants and children.[3-8] Studies of prophylactic vaccines have suggested that prevention of transmission to offspring of previously nonimmune women could be effective.[9] However, findings from studies in maternal population with high CMV seroprevalence have demonstrated that intrauterine infection and disease occurs not infrequently in the offspring of women with existing immunity, so called nonprimary infections.[2,7,8,10-12] Thus, preconceptional immunity against CMV provides only partial protection against congenital infection[12] and in maternal populations with high CMV seroprevalence, most congenital CMV infections follow nonprimary maternal infections.[10,12-14] Studies from Brazil, the Ivory Coast, India, as well as urban African American populations in the United States, have demonstrated a direct relationship between maternal CMV seroprevalence and the incidence of congenital CMV infection.[7,8,11,15-17] Proposed mechanisms for nonprimary maternal infections include reactivation of an existing persistent infection or reinfection with new strain of CMV. Only inferential evidence supports the first mechanism; however, this mechanism is consistent with lifelong persistence of CMV infection. Thus, reactivations from latency or a chronic infection could result in recurrent infections in previously infected women. Alternatively, reinfections with new strains of CMV have been documented in immunocompetent and immunocompromised patients.[18-20] Mechanisms leading to intrauterine CMV transmission and congenital infection remain undefined in maternal populations in the developing world with seroprevalences approaching 100%. Because infection with more than 1 CMV strain in immunocompetent pregnant women can lead to fetal damage, reinfection could contribute significantly to the natural history of congenital CMV infections.[21]

In the current study, we analyzed serum samples obtained at the initiation of prenatal care and at delivery from women prospectively enrolled in a study of congenital CMV infections in a highly seroimmune maternal population.[17,22] Women delivering congenitally infected infants and control women delivering uninfected infants

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from the same population were studied for CMV strain-specific serological responses to determine the contribution of maternal reinfection during pregnancy to congenital CMV infection in this population with near universal preconceptional CMV seroimmunity.

MATERIALS AND METHODS

Study population and design

Forty mothers of infants with congenital CMV infection and 109 mothers of uninfected infants were enrolled in the study. These subjects were selected from 7848 mothers of 8047 infants born at 2 maternity hospitals in the municipality of Ribeirão Preto, Brazil, whose infants were screened (85% all live births) for congenital CMV infection (1.1% rate of congenital CMV infection).[8,17] Among 84 mothers of 87 infants (3 twins) who were identified with congenital CMV infection, 58 (69%) were residents and received prenatal care in Ribeirão Preto. Of these 58 women, 40 (74%) had prenatal serum specimens stored in a central repository and represented the study population. The control mothers were selected from women delivering uninfected infants at the same hospital, residents of Ribeirão Preto, matched for gestational age of their newborn infants, and had prenatal serum specimen stored in the central repository. The study and control population were derived from a maternal population with an overall CMV seroprevalence of 96%, thus it was not unexpected that all the women in this study were seropositive for CMV at entry into the study. The study protocol was approved by the Research Ethics Committee of the University Hospital (processes no. 4782/2002 and 9145/2003).

Diagnosis of congenital infection was based on the detection of CMV DNA in saliva and/or urine samples by polymerase chain reaction (PCR) and confirmed by virus isolation from 2 urine and/or saliva samples collected before 3 weeks of life.[23,24] Infants with clinical findings, including petechiae, purpura, jaundice with direct bilirubin >2 mg/dL, hepatosplenomegaly, microcephaly, and chorioretinitis within the first 15 days of life were classified as having a symptomatic congenital CMV infection.[24]

Determination of maternal CMV serostatus

Sequential serum specimens (first prenatal visit and at delivery) from mothers were assayed for anti-CMV IgG antibodies by a conventional ELISA and anti-CMV IgG avidity indicies were determined in all prenatal serum specimens (VIDAS CMV IgG Avidity, Biomérieux, France).[25-27] An IgG avidity index of >80% is strongly suggestive of an infection that occurred at least 12 weeks earlier; however, the original data indicated that an avidity index of as low as 73% excluded 93% of CMV infections of <12 weeks' duration.[27]

Maternal CMV strain-specific serologic responses

Sequentially obtained maternal samples were tested for CMV strain-specific serologic responses based on the polymorphism within an antibody binding site on glycoprotein H (gH) between 2 prototypic laboratory strains of CMV, AD169 (gH-AP86) and Towne (gH-TO86), and a second polymorphic site for antibody reactivity on glycoprotein B (gB) that has been defined on AD169 (gB-AD54) and Towne (gB-TO54) virus strains.[21,28] Both antibody binding sites are defined by a linear sequence of amino acids.[21,29] As there is no known linkage between serologic reactivity against linear epitopes on gH and gB, 7 different patterns of antibody reactivities are possible for each study participant, including lack of recognition of the gH or gB-specific serologic determinants (Figure). Reactivity for both polymorphic antigenic sites on gH or gB indicated exposure to >1 strain of virus. The detection of new antibody reactivity to either epitope on gH or gB in delivery serum samples of seropositive women was considered as seroconversion and infection with a new virus strain (reinfection) during pregnancy.

CMV strain-specific ELISA

This assay is described in a recent report and uses recombinant peptides encoding the AD169 gH or the Towne gH and by the AD169 gB or the Towne gB antigens.[28,30] The N-terminal region of gH was expressed as beta-galactosidase fusion protein in *Escherichia coli*.[21,29] A 106 amino acid fragment from the amino-terminal region of gB was his-tagged by cloning into pET21a(+) (EMD, Gibbstown, NJ) vector, expressed in *E. coli*, and purified using TALON Superflow columns (Clonetech, Mountain View, CA). A positive control used the highly conserved and dominant antigenic domain (AD-1) from gB cloned into both vectors.[31-34] Reactivity against empty vectors expressing fusion protein alone or unrelated proteins of mouse origin were used as negative controls. A positive result was defined as 3 standard deviations (SD) above the OD value obtained from serum from a CMV seronegative individual.

Sequence analysis of viruses recovered from infected infants

CMV DNA was extracted from peripheral blood, saliva, and urine from infected infants as described.[23,35] Viral DNA was amplified (Fusion; New England Biolabs, Beverly, MA) using primers to amplify a 460 base pair (bp) product from the 5′ end of the UL75 orf (gH) (nucleotides 110,603-111,063) or a 300-bp product from the UL55 orf (gB) (nucleotides 84,117-84,423, AD169). Gel-purified amplimers were sequenced directly or in some cases cloned into the pCRBlunt vector (Invitrogen, Carlsbad, CA) and propagated in *E. coli*. Approximately 10-12 clones were selected and plasmid DNA sequenced. Nucleotide sequences were analyzed using Vector NTi software (Invitrogen).

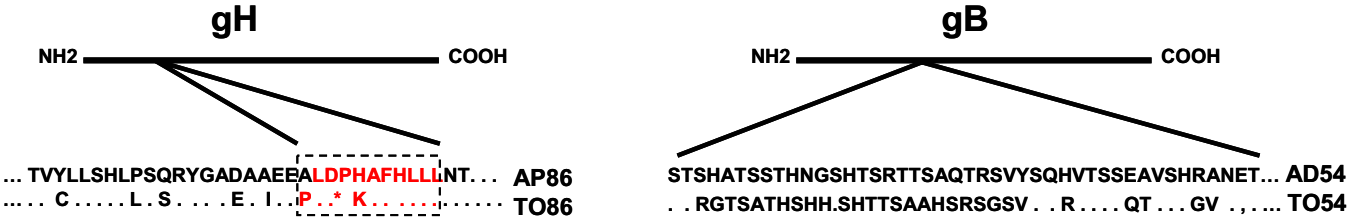
Statistical analysis

Statistical analysis was performed using the EPI INFO software program, v. 6.4 (Center for Disease Prevention and Control). The proportion of strain-specific serologic responses to different epitopes in study and control groups were compared using χ^2 test or Fisher's exact test.

RESULTS

Mothers of infected and uninfected infants did not differ in age (median, 20 vs 22 years), years of formal education (median, 8 years vs 9 years), exposure to children <2 years of age (14/40 vs 23/109),

FIGURE
Patterns of reactivity for polymorphic linear epitopes on CMV glycoproteins gH and gB



gH polymorphism reactivity		gB polymorphism reactivity		Pattern of Serologic Reactivity
AP86	TO86	AD54	TO54	
Neg	Neg	Neg	Neg	No reactivity for AD or TO gH, gB polymorphic epitopes
Pos	Pos	Neg Pos	Neg Pos	Reactivity with 2 strains of virus based on gH reactivity
Neg Pos	Neg Pos	Pos Pos	Pos Pos	Reactivity with 2 strains of virus based on gB reactivity
Pos	Pos	Pos	Pos	Reactivity with 2 strains of virus based on gH, gB reactivity
Pos Neg	Neg Pos	Neg Neg	Neg Neg	Reactivity with 1 strain of virus based on gH
Neg Neg	Neg Neg	Pos Neg	Neg Pos	Reactivity with 1 strain of virus based on gB
Neg Pos Neg Pos	Pos Neg Pos Neg	Neg Neg Pos Pos	Pos Pos Neg Neg	Reactivity with 1 strain of virus based on gH, gB

Schematic representation of primary amino acid sequence of CMV strain-specific antibody-binding sites present on envelope gH and gB. Possible patterns of antibody reactivity shown on far left with the interpretation of reactivity for number of viral strains that have infected a single individual.

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age of sexual debut (median, 15 vs 16 years), or number of sexual partners (median, 2). When exposure to young children was extended to include children ≤3 years, significantly more mothers of infected infants cared for young children (23/40 vs 37/109; $P = .01$).

The median gestational age at which the prenatal sample was obtained for study and control women was 13 weeks (range, 4–27 weeks). The median interval between prenatal and delivery serum specimens was 24 weeks (range, 8–32 weeks) in both groups. Serum from the first prenatal visit from all 40 mothers of infected offspring and 109 control mothers contained CMV IgG antibodies, a finding consistent with the CMV seroprevalence of this population.[8,17] Anti-CMV IgG antibodies of high avidity index could be demonstrated in serum specimens from women in the study group (median, 96%; range, 74–100%) and the control group (median, 94%; range, 76–100%).

CMV strain-specific antibody responses to gH and gB epitopes in the serum samples obtained during pregnancy

The strain-specific response to each CMV epitope on gH, gB, and combinations of reactivity at first prenatal visit and at delivery of mothers of infected infants and control mothers are shown in Table 1. Reactivity to at least 1 CMV polymorphic site on gH or gB was observed in the serum specimens obtained during pregnancy in all but 1 of the 40 women who delivered congenitally infected infants (97.5%) but in only 84 of 109 (77%) mothers of non-infected infants ($P = .003$).

Analysis of prenatal sera revealed that infection with 2 or more CMV strains was more frequent in mothers of infected infants than in controls (35% vs 15.6%; $P = .009$; Table 2). Similarly, reinfection during pregnancy as evidenced by acquisition of antibody reactivity at delivery was more frequent in mothers of infected infants (7/40; 17.5%) as compared with control mothers (5/109; 4.6%; $P = .02$; Table 2). Because the median interval of observation in these women was 24 weeks, these rates represented an annualized rate of reinfection

TABLE 1
Maternal CMV strain-specific serologic responses

Study population (n = 40)				Control population (n = 109)			
Prenatal serum							
gH reactivity[a]		**gB reactivity[a]**		**gH reactivity[a]**		**gB reactivity[a]**	
AP86,T086	**Negative**	**AD54,T054**	**Negative**	**AP86, T086**	**Negative**	**AP86,T086**	**Negative**
34 (85%)	6 (15%)	29 (72.5%)	11 (27.5%)	71 (65%)	38 (35%)	71 (65%)	38 (35%)
Acquisition of new serotypic reactivity during pregnancy[b]							
gH reactivity[c]		**gB reactivity[c]**		**gH reactivity[c]**		**gB reactivity**	
AP86↔T086	**Neg→pos**	**AD54↔T054**	**Neg→pos**	**AP86↔T086**	**Neg→pos**	**AD54↔T054**	**Neg→pos**
2 (5%)	3 (7.5%)	2 (5.0%)	2 (5.0%)	1 (1.4%)	2 (5.3%)	0 (0%)	2 (3.1%)
Total 4 (10.0%)[3]		Total 3 (7.5%)[3]		Total 3 (2.7%)		Total 2 (1.8%)	

CMV, cytomegalovirus; *gB*, glycoprotein B; *gH*, glycoprotein H; *neg*, negative; *pos*, positive.

[a] Reactivity of CMV antibody positive serum specimens in ELISA-based assay for linear antibody binding sites on gH or gB as described in Materials and Methods. Reactivity shown is number of positive specimens with percentage of total number in parentheses; [b] Seroconversion during pregnancy represents acquisition of reactivity in delivery serum specimen against previously unrecognized antibody- binding site on gH or gB antigens that was not present in prenatal specimen, including detection of antibody reactivity against either antigen in delivery serum specimens from women with non-reactive prenatal serum specimens. Results are shown as number with acquisition of antibody reactive with gH or gB linear epitopes with percentage of responders in parentheses; [c] Total represents the number of women in population who exhibited acquisition of antibody reactivity against previously unrecognized antibody-binding sites on gH or gB antigens in their delivery serum when compared to the reactivity of their prenatal serum. Two women in the study population developed antibodies to new serotypes of both gH and gB, thus the total number of women seroconverting was 4 for gH and 3 for gB.

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of 35% and 9% in the study and control groups, respectively. When the results from prenatal and delivery serum were combined, a higher proportion of mothers of infected infants had evidence of infection with >1 CMV strain in the past or in the current pregnancy than controls (52.5% vs 20%; $P < .0001$; Table 2). All infected infants of mothers with serologic evidence of reinfection during pregnancy were asymptomatic at birth. Among the infants born to 21 mothers with serologic evidence of infection with more than 1 CMV strain before pregnancy, 1 infant (1/21; 5%) had symptomatic congenital CMV infection.[24]

Sequence analysis of viruses from infants with congenital CMV infection

CMV DNA from blood, saliva, or urine collected from infected infants during the perinatal period was analyzed for the polymorphic regions of gH and gB by nucleotide sequencing of the respective viral genes (UL75, UL55). Of the 7 infants born to seroimmune women with evidence of reinfection by a new CMV strain during pregnancy, viral DNAs isolated from 6 (6/7; 86%) infants were shown to contain sequences encoding antigenic determinant detected by antibody reactivity that followed seroconversion during pregnancy (Table 3). In a single case (infant 7), sequence analysis of plasmids from 10 different colonies derived from the cloned PCR products resembled the sequence of AD169 gB (data not shown). Thus, seroconversion in the mother of this infant during pregnancy following reinfection with a virus encoding Towne like gB sequences was not associated with transmission of this new virus to the offspring (Table 3). Alternatively, it was also possible that in this limited sampling we failed to isolate an amplimer from a virus encoding a Towne-like gB.

TABLE 2
Infection with multiple CMV strains in mothers according to serologic responses to 2 polymorphic determinants

Variable	Mothers of infected infants, n (%) (n = 40)	Mothers of uninfected infants, n (%) (n = 109)	*P* value
Antibody reactivity against ≥2 CMV strains at first prenatal visit[a]	14 (35.0)	17 (15.6)	.009
Seroconversion to new CMV strain during pregnancy	7 (17.5)	5 (4.6)	.02
Infection with ≥2 CMV strains before and/or seroconversion during pregnancy	21 (52.5)	22 (20.2)	< .0001

CMV, cytomegalovirus; *gB*, glycoprotein B; *gH*, glycoprotein H.

[a] Antibody reactivity determined as described in Materials and Methods against polymorphic linear epitopes on gH and gB.

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COMMENT

Women from this region of Brazil with evidence of infection with multiple CMV strains, including women acquiring new virus strains during pregnancy, were more likely to deliver congenitally infected infants than women who lacked serologic evidence of infection with multiple CMV strains. These findings provided support for the hypothesis that reinfections with new virus strains were

responsible for a significant number of congenital CMV infections in offspring of women from this highly seroimmune population. It has been argued that congenital CMV infections after nonprimary maternal infections results from reactivation of existing persistent infections (recurrent maternal infection). Although this is a possible explanation for congenital infections after nonprimary maternal infections, our findings that seroconversion to a new virus-encoded determinant was observed in 17.5% of women delivering infected infants as compared with only 4.6% in control mothers of uninfected infants from the same populations argued against recurrent maternal infection as the sole cause of congenital CMV infections in this population. Furthermore, 52.5% of women who delivered congenitally infected infants exhibited evidence of infection with multiple strains of CMV as compared with only 20% of women in the control group suggesting that maternal infection after exposure to new strains of virus was a risk factor for the delivery of a congenitally infected infant. Although CMV-specific serologic responses have not been used conventionally to identify reinfection with a new strain of CMV, the finding of new antibody specificities in sequential blood samples from seropositive mothers was taken as evidence of an infection with a new virus strain (serotype), consistent with observations in other virus infections.[36-42] Alternatively, new antibody specificites in sequential serum specimens in these women could be explained by mutations in the coding sequence of CMVs persisting in the host, leading to production of new antibody specificities. However, there is little evidence for instability of the sequence encoding these specific CMV glycoproteins even after prolonged in vitro virus passage. Stanton et al,[43] have reported the stability of CMV hypervariable genes over time in vivo during the course of a persistent infection in renal transplant recipients, a finding arguing against genome instability as an explanation for expression of new antigenic determinants on CMV in seropositive individuals. A recent analysis of the coding sequences of several CMV genes indicated extensive variation between viral strains and suggested that a large number of CMV strains circulate within human populations.[44]

TABLE 3
Predicted amino acid sequence of viruses isolated from congenitally infected infants

Infant	Seroconversion	Source of Viral DNA	Sequence of amplified viral DNA
1	AD169 gH[a]	Blood	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
1	AD169 gH	Saliva	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
2	AD169 gH	Blood	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
2	AD169 gH	Saliva	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
3	AD169 gH	Urine	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
3	AD169 gH	Saliva	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
4	AD169 gH	Urine	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
4	AD169 gH	Blood	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
5	AD169 gH	Blood	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
5	AD169 gH	Saliva	YLLSHLPSQRYGADAASE***ALDPHAFHLLL*** (**AD169 gH**)
6	TogB[b]	Urine	HGTSATHSHHSSHTTSAAHSRSGSVSSQRVT SSEAVSHRANET (**Towne-like gB**)
7	TogB	Saliva	HATSSTHNGSHTSRTTSAQTRSVYSQHVTSS EAVSHRANE (**AD169 gB**)

gB, glycoprotein B; *gH*, glycoprotein H.

[a] Viral DNA amplified from sample obtained from congenitally infected offspring of women undergoing seroconversion during pregnancy as detected by acquisition of antibody reactivity for AP86 epitope of AD169 gH. AP86 epitope listed in ***bold italics***. DNA sequence obtained directly from amplified PCR product; [b] Viral DNA from infants 6 and 7 were amplified and PCR products cloned into plasmid pCRBlunt. Plasmids from 10-12 colonies were isolated and sequenced. A mixture of viral gB genotypes were identified. In the case of patient 6, the epitope associated with seroconversion during pregnancy in this mother, Towne-like gB (TO54), was demonstrated. In the case of infant 7, all sequenced plasmids expressed AD169-like gB (A54).

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Considering the assays used in this study identified only women who generated antibody responses against linear peptides expressed by the laboratory CMV strains AD169 and Towne gH and gB, the frequency of reinfection is almost certainly higher. A number of CMV genes have been shown to exhibit considerable DNA sequence variability, but our studies have suggested that only a very limited number of these changes have resulted in differences in amino acid sequences that induce viral strain-specific antibody responses. Thus, we are limited in our capacity to distinguish between specific strains of CMV within the multitude of genetically unique strains that circulate in the human population by serologic assays such as described in this report. Yet, even with this limitation in our assays, the annualized reinfection rate in women transmitting virus to their offspring was 35%, a rate approximately 5-7 times higher than the maternal seroconversion rates in populations of women with lower CMV seroprevalence but similar to rates of primary CMV infections (approximately 13%) observed in mothers of young children (<3 years of ages) excreting CMV and in day care.[45-47] When these results are viewed together, the incidence of congenital in-

fection associated with maternal reinfection in this Brazilian population reflected the phenomena of increasing incidence of congenital CMV infection with increasing maternal seroprevalence of CMV. Frequent exposure of these populations to CMV could also be expected to limit the protective activity of vaccine-induced immunity. Thus, caution must be applied to generalized estimates of vaccine efficacy and results from vaccine trials may be interpretable only in terms of the seroprevalence of a specific population.

In our study, 1 mother of an infected infant and 22 control mothers with preconceptional immunity did not have reactivity against AD169- specific gH or gB antigens at the first prenatal visit and failed to produce antibodies against these antigenic sites during pregnancy. This finding raised the possibility that additional polymorphic antibody sites are present on these 2 CMV envelope glycoproteins and that identification of these epitopes could increase the sensitivity of our assays for detection of reinfection with new strains of virus. A recent report demonstrated that serological reactivity to the AD169 and Towne gH linear antibody-binding sites in CMV seropositive blood donors was 48% and 16% respectively, and 19% had no reactivity to either epitope.[48] Increasing age in this population was correlated with increasing seroreactivity for both linear epitopes, perhaps secondary to increasing exposure to serologically distinct CMV strains through reinfection.[48] As other CMV glycoproteins can also be targets of antibody responses, polymorphic sites for antibody reactivity on other envelope glycoproteins such as gN, a glycoprotein that exhibits considerably more sequence variation than either gH or gB, could be useful in this assay.[49-51]

It is well established that previous immunity induced after primary CMV infection does not protect against infection with different strains of the virus.[18,52] We have previously demonstrated that maternal CMV reinfection can lead to fetal damage and symptomatic infection.[53] Ishibashi et al[48] demonstrated an increased frequency of adverse outcomes in transplant recipients with serologic responses consistent with reinfection with different CMV strains, a finding similar to those reported by Grundy and Chou.[19,20] Congenital CMV infections after nonprimary maternal infections can lead to symptomatic congenital CMV infection and long-term sequeale.[54,55] In fact, recent evidence suggested that the incidence of hearing loss in infants infected after nonprimary maternal infection was similar to the incidence of hearing loss in infected infants born to women with primary infection.[56] Thus, the consequences of reinfection with a new and immunologically unrecognized strain of CMV could be similar to those after primary infection in immunologically naive women. Although such a mechanism is attractive, based on the failure of immune responses such as antiviral antibodies to protect against infection and disease in viral infection such as influenza and other respiratory viruses, the pathogenesis of congenital CMV disease is complex and likely multifactorial.

Exposure to young children is a well-established risk factor for acquisition of CMV and our findings suggested that exposure to young children represented a risk for reinfection by a new strain of CMV in women with seroimmunity to CMV. Reinfections with new strains of virus have been reported in children attending group child care facilities and in individuals attending STD clinics.[18,52-57] Although mechanisms responsible for acquisition of new strains of CMV are unknown, strain-specific virus neutralizing antibodies have been suggested as an explanation for infection in previously infected host after exposure to new strains of virus.[58,59] Studies in women with primary CMV infections during pregnancy have demonstrated an association between virus transmission and levels of virus neutralizing antibodies, suggesting a threshold of seroimmunity could be required to limit intrauterine transmission in seroimmune women reinfected with a new strain of virus during pregnancy.[60]

In conclusion, results from this study demonstrated that reinfection with a new CMV strain is a risk factor for delivery of a congenitally infected infant. In this study, infection with a new strain of CMV is not an infrequent event in women in this region of Brazil. The increased rates of congenital CMV infections in highly seroimmune populations may be associated with exposure to multiple viruses leading to maternal reinfection. Strain-specific immune responses during primary CMV infection could be a major challenge for vaccine development for preventing congenital CMV infections in such populations. ■

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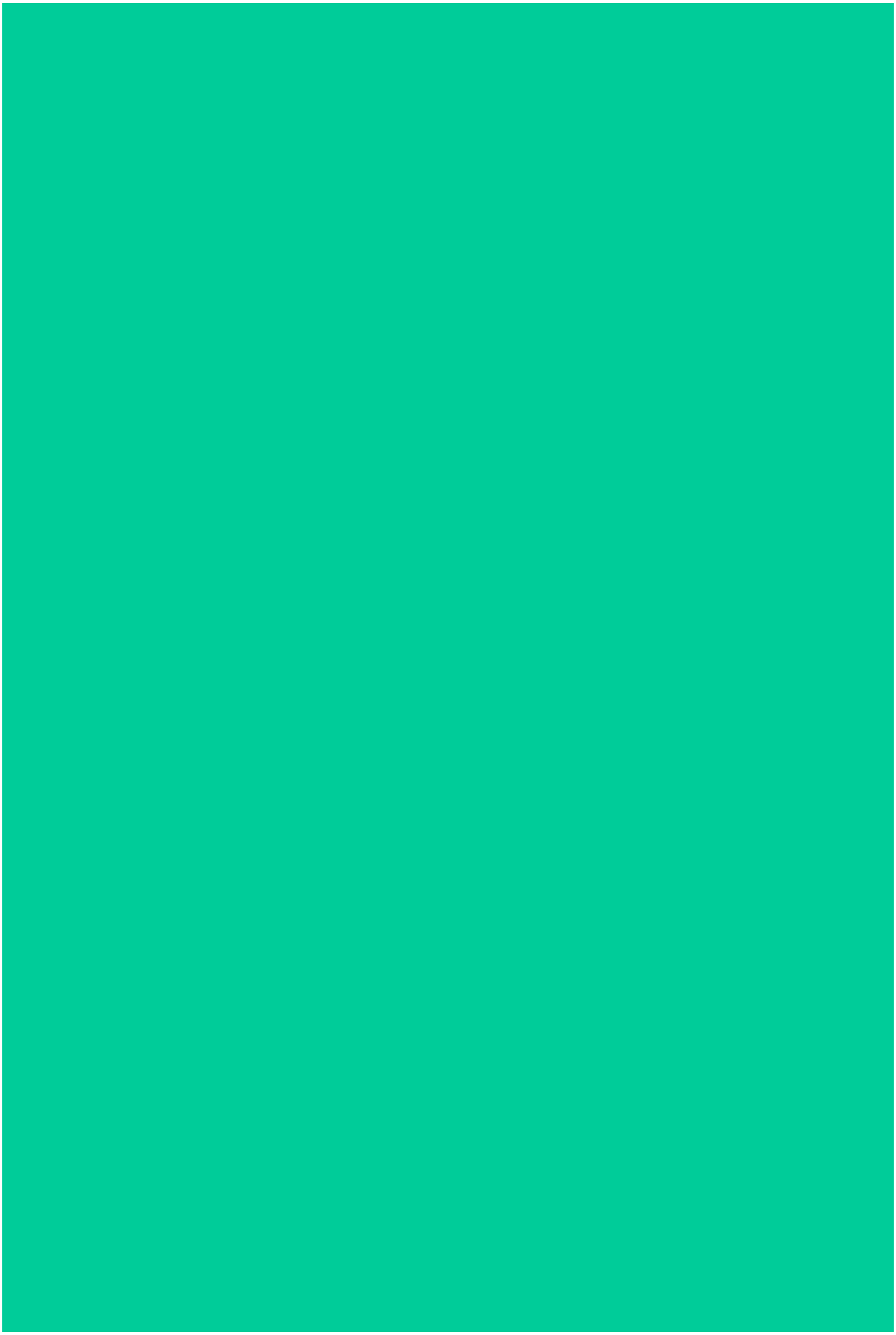

Clinical and Vaccine Immunology

Enzyme-Linked Immunosorbent Assay Method for Detection of Cytomegalovirus Strain-Specific Antibody Responses

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Enzyme-Linked Immunosorbent Assay Method for Detection of Cytomegalovirus Strain-Specific Antibody Responses▽

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Reliable methods for the detection of cytomegalovirus (CMV) strain-specific serological responses are lacking. We describe a simple and reliable enzyme-linked immunosorbent assay method developed to detect antibodies against the polymorphic epitopes within the two envelope glycoproteins of CMV, glycoproteins H and B. This assay is useful for the detection of serologic responses to CMV strains and the identification of CMV reinfections.

Cytomegalovirus (CMV) is an important pathogen in immunocompromised hosts and a frequent cause of congenital infection. CMV isolated from clinical samples exhibits extensive genetic variation (7, 12, 13), and CMV reinfections have been demonstrated to occur in seropositive individuals. However, it is thought that these reinfections have little untoward consequences with respect to congenital infections. Recent studies documenting higher rates of congenital CMV infection in populations with nearly universal seroreactivity to CMV suggest that infection with new or different virus strains could be responsible for the intrauterine transmission of CMV in immune mothers (5, 17, 18). The frequency and consequences of infection with multiple CMV strains are unclear because of the lack of reliable methods for the accurate identification of CMV strain-specific antibody responses. By utilizing the defined heterogeneity within the antibody binding epitopes on envelope glycoprotein H (gH) and gB of the AD169 and Towne strains of CMV, an enzyme-linked immunosorbent assay (ELISA) method was developed to distinguish serological responses against infection with different CMV strains.

Serum samples from 96 CMV-seropositive women participating in an ongoing study and 51 seronegative individuals were tested for anti-CMV strain-specific antibodies. Informed consent was obtained from the study participants, and the study was conducted in accordance with the guidelines of the Institutional Review Board for Human Use of the University of Alabama at Birmingham.

Purified recombinant antigens based on polymorphic antibody binding sites defined on gH (antigen gpUL75) and gB (antigen gpUL55) were used as antigens (Fig. 1). The gH antigens were constructed as β-galactosidase fusion proteins containing the coding region for amino acids (aa) 15 to 142 of gpUL75 from the AD169 strain (the AP86 antigen) and aa 14 to 42 of the Towne strain (the TO86 antigen) of CMV (16). The recombinant peptides were expressed in *Escherichia coli* and were purified as described previously (8). gB antigens were prepared as six-His-tag-labeled peptides by cloning the coding region (aa 1 to 116) from strains AD169 (the AD55 antigen) and Towne (the TO55 antigen) (9) into expression vector pET21a (EMD, Gibbstown, NJ) by using the HindIII and BamHI endonuclease restriction sites. The peptides were expressed in *E. coli* Rosetta cells and were purified by using Talon Superflow metal affinity columns (Clonetech, Mountain View, CA). A positive control antigen was constructed by cloning the antigen domain 1 (AD-1) region of the gene coding gB, which has been shown to be highly conserved among clinical isolates of CMV, as described previously (2), into each vector (AD-1). The reactivity against an empty vector expressing fusion protein alone or nonantigenic proteins of mouse origin was used as a negative control.

Strain-specific ELISA was performed on PolySorp microtiter plates (Nunc, Roskilde, Denmark). The wells of the plates were coated overnight with 50 μl of purified gH antigens (antigens AP86 and TO86) (1) or gB antigens (antigens AD55 and TO55) diluted in carbonate buffer and blocked with 3% goat serum in borate buffer (BB) for 2 h at 37°C. Serum samples diluted 1:100 in BB were added to the wells, and the plates were incubated at 37°C for 1 h. After the plates were washed three times with BB containing 0.05% Tween 20, goat anti-human immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibody (Pierce, Rockford, IL) diluted 1:10,000 was added and the plates were incubated for 1 h at 37°C. The plates were developed by the addition of 50 μl of one-step Ultra TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Pierce) for 10 min at room temperature (RT), and the reaction was stopped by the addition of 2 N sulfuric acid. The optical density (OD) values were determined with a spectrophotometer. A positive result was defined as an OD value more than three times the mean result obtained for each antigen with seronegative samples.

Western blot assays were performed with a subset of 12 samples. Appropriate antigens were run on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and then blotted onto a polyvinylidene difluoride membrane (Immobilon P; Millipore,

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                    10        20        30        40        50
                ....|....|....|....|....|....|....|....|....|....|.
UL75 AD169 MRPGLPPYLTVFTVYLLSHLPSQRYGADAASEALDPHAFHLLLNTYGRPIR
UL75 TOWNE ......S..I.LA.C.....L.S....E.I..P..*K..............

                    20        30        40        50        60
                |....|....|....|....|....|....|....|....|....|....|
UL55 AD169 SSSTSHATSSTHNGSHTSRTTSAQTRSVYSQHVTSSEAVSHRANETIYNTT
UL55 TOWNE ....RGTSATHSHH.SHTTSAAHSRSGSV..R....QT...GV........
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FIG. 1. Amino acid sequence alignment of the amino-terminal regions of gpUL75 (gH) and gpUL55 (gB) from the AD169 and Towne strains of CMV depicting the differences betweens the two strains.

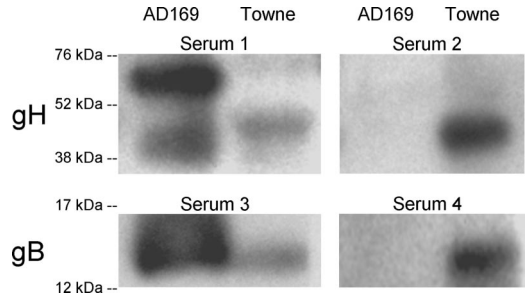

FIG. 2. Recognition of the gH antigens (antigens AP86 and TO86) and gB antigens (antigens AD55 and TO55) from the AD169 and Towne CMV strains by four representative serum samples. The band sizes shown are in agreement with the sizes predicted on the basis of the amino acid compositions of the recombinant peptides. Each of the positive serum specimens was predominantly reactive with one of the glycoprotein (gB or gH) antigens. Sizes are indicated on the left.

Billerica, MA), according to the manufacturer's recommendations. The membranes were blocked for 2 h in 3% goat serum in SuperBlock buffer (Pierce, Rockford, IL) and 0.05% Tween 20 at RT. Human sera were diluted 1:5,000 in blocking buffer and applied to the membrane, and the membrane was shaken at RT for 2 h. The membranes were washed four times in wash buffer (BB with 0.05% Tween 20), and goat anti-human IgG HRP-conjugated antibody (Pierce) diluted 1:100,000 in blocking buffer was added. After incubation at RT for 2 h, the membranes were washed four times in wash buffer and soaked into the substrate Luminol West Femmto (Pierce) for 10 min. The membranes were placed on X-ray film, and images were developed and acquired by using a VersaDoc imaging system (Bio-Rad, Hercules, CA).

Of the 96 baseline serum samples from CMV-seropositive women participating in an ongoing study testing for strain-specific antibodies, 58 (60%) samples were positive for at least one of the four antigens and 18 samples were positive for two or more antigens. The OD values (mean ± standard deviation) for each antigen for the group of 51 CMV-seronegative individuals and the samples that were considered positive and negative from the 96 CMV IgG antibody-positive women are shown in the Table 1. Forty-five percent (43/96) of the samples had reactivity to the AP86 antigen, with a mean OD value of 1.240 ± 0.498, whereas the OD values were 0.291 ± 0.134 and 0.196 ± 0.052 for the negative samples and the CMV IgG antibody-negative control serum samples, respectively. Fifteen percent (14/96) of the study samples were positive for the TO86 antigen, with an average OD reading of 1.069 ± 0.317. Responses against the AD55 and TO55 antigens were seen in 8% and 20% of the samples, respectively, with corresponding OD values of 0.681 ± 0.103 and 0.708 ± 0.278 (Table 1).

To verify the reproducibility of the assay results, two positive control samples with known reactivity to the gH and gB antigens were tested on 10 different occasions and consistently yielded similar results. Serum sample 1 was reactive against AP86 (OD = 1.442 ± 0.569) and TO55 (OD = 0.809 ± 0.438), while serum sample 2 contained antibodies against the TO86 antigen (OD = 1.132 ± 0.485). The OD values of CMV IgG antibody-positive samples against the positive (AD-1) and the negative control antigens were 1.21 ± 0.45 and 0.28 ± 0.17, respectively.

The strain specificities of the antibody responses were confirmed with a subset of 12 samples by Western blot assay. Figure 2 demonstrates the recognition of the antigens by four serum samples. The sizes of the reactive bands by the Western blot assay were similar to the predicted sizes of the recombinant peptides (16).

The seroepidemiologic study of CMV strain diversity has been hampered thus far by the lack of simple and reliable methods that can be used to accurately identify infection with multiple strains of CMV. In this study, we report the findings of an ELISA method that was used to identify the presence of strain-specific antibodies in sera from 96 seropositive women against the polymorphic epitopes on CMV gH and gB from the prototypic laboratory strains of CMV, strains AD169 and Towne. Using this method, we could demonstrate the presence of strain-specific antibodies against the antigenic determinants

TABLE 1. Reactivities against the gH (AP86 and TO86) and gB (AD55 and TO55) polymorphic epitopes from CMV strains AD169 and Towne of serum samples from 96 CMV-seropositive women and 51 seronegative individuals and two serum samples with known reactivity against AP86, TO55, or TO86

Antigen	OD values (mean ± SD)[c]				
	CMV IgG antibody-positive samples[a] (n = 96)		CMV IgG antibody negative (n = 51)	Positive control sera[b]	
	Strain-specific antibody positive	Strain-specific antibody negative		Serum sample 1	Serum sample 2
AP86	1.240 ± 0.498 (43)	0.291 ± 0.134 (53)	0.196 ± 0.052	1.464 ± 0.578	0.458 ± 0.143
TO86	1.069 ± 0.317 (14)	0.301 ± 0.158 (82)	0.238 ± 0.066	0.443 ± 0.119	1.155 ± 0.495
AD55	0.681 ± 0.103 (8)	0.238 ± 0.120 (88)	0.198 ± 0.068	0.348 ± 0.115	0.368 ± 0.125
TO55	0.708 ± 0.094 (19)	0.170 ± 0.094 (77)	0.132 ± 0.032	0.803 ± 0.434	0.191 ± 0.053

[a] Fifty-eight of 96 samples were positive for at least one antigen.
[b] Serum sample 1 had known reactivity with AP86 and TO55, and serum sample 2 had known reactivity with TO86.
[c] Numbers in parentheses represent numbers of samples.

on envelope glycoproteins gH and gB. The reliability of this assay for the identification of CMV strain-specific antibodies was documented by comparing the serological reactivities to the antigens tested between CMV-seropositive and -seronegative individuals. As can be seen in Table 1, the mean OD values for each antigen were similar between the group of CMV IgG antibody-negative individuals and the group of CMV IgG antibody-positive women who were categorized as negative for antibodies against specific gH or gB antigenic determinants. The reproducibility of the assay was demonstrated by repeated testing of two serum specimens reactive with three of the four antigens (antigens AP86, TO86, and TO55) tested. The strain-specific serological responses were confirmed in a Western blot assay with a subset of 12 serum specimens (Fig. 2).

Clinical strains of CMV exhibit extensive genetic polymorphisms in their envelope glycoproteins (10), and no two clinical isolates have been documented to be identical (7), even when they are examined by restriction fragment length polymorphism analysis (11). Studies with populations with increased exposure to CMV, such as sexually transmitted disease clinic attendees (4) and human immunodeficiency virus-infected individuals, have shown that infection with new CMV strains occurs frequently (3, 14, 15). However, the impact of infection with multiple CMV strains and/or reinfection with new virus strains with respect to the severity of CMV disease among immunocompromised hosts and intrauterine transmission of CMV are unclear. In a recent study, we documented the occurrence of infection with new strains of CMV in seropositive women between pregnancies and identified an association between reinfections and intrauterine transmission and severe fetal infection (1). Reinfection with different CMV virus strains in organ donors has been associated with an increased incidence of transplant rejection and CMV disease, as shown by a more recent study of renal transplant recipients (6).

The CMV strain-specific ELISA method described in this report could be a useful tool for determination of the CMV strain diversity in populations and, therefore, could provide a better understanding of the implications of infection with multiple CMV strains. In addition, the ability to identify the appearance of new antibody specificities over time will make it possible to document CMV reinfections in seroimmune individuals and allow the study of the factors associated with reinfections and the impact of reinfections in different populations. One of the limitations of this assay is that not all CMV-specific IgG-positive individuals can be identified by use of the four antigens used. Specimens from more than a third of the seropositive individuals (38/96) did not contain antibodies against any of the four antigenic determinants tested, suggesting the presence of additional polymorphic epitopes on glycoproteins gH and gB as well other envelope glycoproteins of CMV, such as gN. Identification of these additional epitopes could further extend the sensitivity of our assay for the detection of infection with multiple CMV strains and to determine the rates of reinfection with new virus strains in seroimmune individuals. In addition, with a clearer understanding of the frequency of CMV reinfections in seroimmune individuals and the CMV strain diversity in different populations, one could begin to address the role of the strain-specific antibody response in protective immune responses against CMV.

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We have no commercial interest to report.

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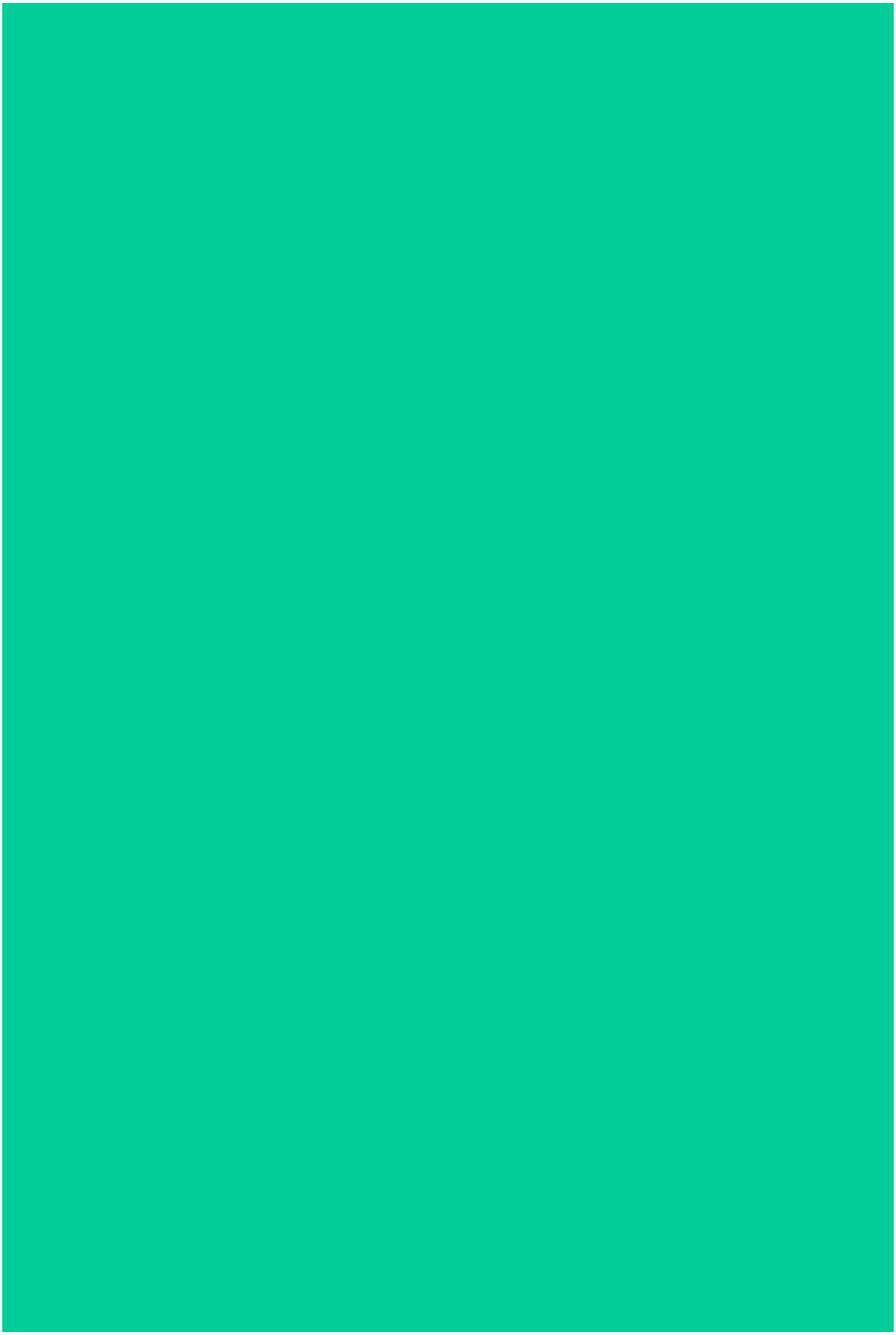

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Cytomegalovirus Blood Viral Load and Hearing Loss in Young Children With Congenital Infection

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Abstract

Background—This study was designed to determine whether elevated viral load in infants and young children is associated with congenital cytomegalovirus (CMV)-related hearing loss.

Methods—Blood samples were obtained from 135 children with congenital CMV infection. CMV DNA in the peripheral blood was quantitated with a real-time polymerase chain reaction assay. Viral load measurements were analyzed in 3 different age groups (<2 months, 2–12 months, 12–36 months).

Results—In children with symptomatic and asymptomatic infection, CMV DNA levels were not different between children with hearing deficit and those with normal hearing in all 3 age groups. In children with asymptomatic infection, the positive predictive value of a peripheral blood viral load <3500 genomic equivalents per milliliter (ge/mL) at <2 months and 2 to 12 months of age is 8%, and at 12 to 36 months of age is 11.8%. However, the negative predictive value of a viral load <3500 ge/mL is 94.4% at <2 months of age, and 100% at 2 to 36 months of age.

Conclusions—Peripheral blood viral load is not associated with hearing loss in children with congenital CMV infection. However, a viral load of <3500 ge/mL is associated with a lower risk of hearing loss in children born with asymptomatic congenital infection.

Keywords

CMV; viral load; hearing loss

Cytomegalovirus (CMV) is a common cause of congenital infection and a leading cause of sensorineural hearing loss (SNHL) in children worldwide.[1,2] Of the 20,000 to 40,000 infants born each year in the United States with congenital CMV infection, about 5% to 10% of children with asymptomatic infection and 40% to 50% of those with symptomatic congenital infection will develop hearing loss.[3,4] Although some children with CMV-related SNHL are born with a hearing deficit, the majority will experience delayed-onset loss and continued deterioration of hearing function (progressive hearing loss) during childhood.[3,5–8]

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The pathogenesis of SNHL in children with congenital CMV infection is poorly understood. Limited human temporal bone studies as well as studies in the guinea pig model have demonstrated that viral infection in the inner ear structures is important to the development of SNHL.[9–11] Recent studies from our laboratory and others have suggested that higher systemic virus burden in early infancy was associated with CMV-related hearing loss.[12–14] To determine whether elevated viral load beyond early infancy is associated with CMV-related SNHL, we examined the value of the peripheral blood (PB) CMV viral load in the prediction of SNHL in a cohort of infants and young children with congenital CMV infection.

MATERIALS AND METHODS

Study Population and Specimens

One hundred ninety-six children with congenital CMV infection born between January 1994 and February 2005 at 3 hospitals in Birmingham, AL, were monitored for hearing loss as part of a natural history study. Congenital CMV infection was identified by the presence of the virus in saliva specimens obtained during the first week of life.[15,16] Of the 196 children found to have congenital CMV infection, 61 had insufficient blood samples. The remaining 135 children with available blood samples for testing constituted the study population. The demographic characteristics and hearing outcomes were not different between the study children and children enrolled in the follow-up study with unavailable PB samples (data not shown). Two hundred two PB samples from the 135 study children were available for quantification of CMV DNA and analysis. The results of the PB viral load in samples obtained during the first month of life from 75 of these children were included in a previous report.[12] Eighty-five children (73 with normal hearing and 12 with SNHL) had only one blood sample, whereas 50 children (46 with normal hearing and 4 with SNHL) had more than one blood sample available for analysis. For children with hearing loss, only PB samples obtained before or at the time of detection of SNHL, and in children with progressive hearing loss, only samples obtained before documented progression were included in the analysis. The PB samples were processed immediately after collection to obtain DNA preparations from 200 μL of whole blood with commercial spin columns (Qiagen, Valencia, CA) and stored at −20°C.

Infants were classified as having symptomatic infection when they shed CMV during the first week of life and had any of the clinical findings suggestive of congenital infection at birth, including jaundice, petechiae, hepatosplenomegaly, purpura, microcephaly, seizures, lethargy/hypotonia, and/or poor suck.[17] One study subject received ganciclovir. This child had progression of hearing loss detected at 6 months of age, received 6 weeks of ganciclovir at the discretion of the clinician, and only PB samples obtained before ganciclovir treatment were included in the analysis.

The study was approved by the University of Alabama at Birmingham Institutional Review Board for Human Use, and informed consent was obtained from the parents or guardians of the children enrolled in the study.

Follow-Up of Children

Study participants were monitored according to a standard protocol and received age-appropriate audiologic evaluations.[3] A child was considered to have SNHL when air conduction thresholds at one or more frequencies were greater than 20 dB in one or both ears in conjunction with normal tympanograms, normal otoscopic findings, and/or normal bone conduction thresholds. Progressive hearing loss was defined as sensorineural decrease in hearing of ≥10 dB at any one frequency or auditory brainstem response threshold

documented on 2 separate evaluations. Delayed-onset hearing loss was defined as one or more hearing evaluations with a normal threshold documented for each ear before the onset of SNHL.[3,6]

Real-Time Polymerase Chain Reaction

The investigators who performed the real-time polymerase chain reaction (PCR) were blinded to the results of the audiologic follow-up. CMV viral load was assessed by real-time PCR technique with an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) utilizing Absolute Low ROX QPCR mix (ABgene, Rockford, IL). CMV primers were selected from the highly conserved AD-1 region of the major envelope glycoprotein B.[18–20] Amplification was performed under the following conditions: 1 cycle at 95°C for 15 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All samples were run with plasmid standards that were constructed from the target sequence. Plasmid standards and samples were run in duplicate and the average values were used to determine the CMV viral load. CMV virus burden in whole blood was expressed as CMV genomic equivalents per milliliter of blood (ge/mL).[12] The sensitivity of the assay has been determined to be approximately 200 ge per 1 mL of blood.

Data Analysis

The demographic characteristics, newborn findings, outcome data, and the results of PB real-time PCR were collected on case report forms and entered into SAS V9.1 data sets (SAS Institute, Cary, NC). Viral load measurements were analyzed in 3 different age groups by nonparametric methods: <2 months, 2 to 12 months, and 12 to 36 months, and statistical significance was determined with the Wilcoxon rank sum test. Positive predictive values (PPV) and negative predictive values (NPV) and exact 95% confidence intervals were assessed where appropriate.

RESULTS

The demographic and outcome characteristics of the study children according to their hearing status are shown in Table 1. Twelve percent (16/135) of the children in the study had SNHL. The majority of the study children are African-American and born to single mothers who received their prenatal care at public health clinics. Significantly more children with CMV–related hearing loss were born to mothers younger than 20 years of age (88%) compared with only 47% of the children with normal hearing. More children in the hearing loss group (56%) had symptomatic congenital CMV infection than those with normal hearing (13%, $P < 0.001$). The mean duration of follow-up for the children with CMV-related hearing loss was 45.0 ± 20.8 months and they underwent a median number of 9 hearing evaluations (range, 1–17), whereas those with normal hearing were monitored for 33.3 ± 22.3 months and had 6 hearing tests (range, 1–12) (Table 1). Progressive hearing loss was observed in 6 of 16 (38%) of the children with hearing deficit and delayed-onset hearing loss was seen in approximately half (7/16) of the children. The median age of onset of hearing loss was 1 month (range, 0–76 months), with SNHL in 2 study children detected at 40 and 76 months of age. Bilateral SNHL was detected in 5 of 16 (31%) of study participants (Table 2). Seventy-five of 76 children from a previous study[12] were included in this study and the demographic characteristics were similar between the 75 children from the previous report and the additional 60 children.

The median PB CMV DNA concentration was higher among children with symptomatic infection (2.93×10^4, 0–5.90×10^6 ge/mL) compared with children who had asymptomatic infection (4.17×10^3, 0–3.40×10^6 ge/mL) in the first 2 months of life, and this difference was statistically significant ($P = 0.006$). However, there was no difference in PB virus

burden between children with asymptomatic and symptomatic congenital infection in samples obtained after the first 2 months of life.

To determine the pattern of change in viral load with time, we analyzed the data from 50 children from whom more than one blood sample was available. The viral load values fluctuated by at least one log in 20 children, increased in 5, decreased in 18, and 7 children had no change in viral load values.

Viral load data were analyzed independently among children with symptomatic infection and those with asymptomatic infection; in each age group, the levels were compared between children with hearing loss and those with normal hearing (Fig. 1). Seven children had undetectable viral concentrations (<200 ge/mL) <2 months of age. Six of these children were born with asymptomatic infection and had normal hearing, whereas one child had symptomatic infection with hearing loss which was detected within the first month of life. When more than 1 PB sample was available from a child in a given age group, only the viral load level in the earliest sample was included in the analysis. Among children less than 2 months of age with asymptomatic infection, median virus burden was not significantly different between the 5 children with hearing loss (1.70×10^4, range: 1.0×10^3–3.40×10^6 ge/mL) and the group of 69 children with normal hearing (3.98×10^3, range: 0–1.36×10^6 ge/mL, $P = 0.301$). In children less than 2 months of age with symptomatic infection, CMV DNA levels were not different between the group with hearing deficit and those with normal hearing (3.2×10^4, range: 0–5.6×10^5 ge/mL and 1.9×10^4, range: 9.5×10^2–5.9×10^6 ge/mL, respectively, $P = 0.847$). PB viral load measurements were not significantly different in older infants and children with hearing loss and normal hearing in both symptomatic and asymptomatic infection. In the 2- to 12-month age group, median virus burden in asymptomatic infection was similar for the children with SNHL and those with normal hearing (3.17×10^4, range: 1.08×10^4–5.27×10^4 ge/mL vs. 2.71×10^3, range: 0–6.40×10^8 ge/mL; $P = 0.140$). Similarly, in symptomatic children between 2–12 months of age, and in both symptomatic and asymptomatic children 12–36 months of age, the amount of viral load was not different between children with hearing loss and those with normal hearing (Figure).

To determine the usefulness of PB viral load measurements in predicting SNHL in children with congenital CMV infection, we calculated the PPV and NPV of PB viral load ≤3500 and >3500 ge/mL in children with asymptomatic and those with symptomatic infection (Tables, Supplemental Digital Content 1, http://links.lww.com/A1114 and Supplemental Digital Content 2, http://links.lww.com/A1115). Among children with asymptomatic infection, only 2 of 36 children with a PB viral load measurement lower than 3500 ge/mL at <2 months of age had hearing deficit, resulting in a NPV of 94.4% (Table, Supplemental Digital Content 1, http://links.lww.com/A1114). However, the PPV for a viral load measurement >3500 ge/mL at <2 months of age was only 7.9%. None of the 26 children at 2 to 12 months of age and 0 of 25 children 12 to 36 months of age with PB virus burden ≤3500 ge/mL had SNHL resulting in a NPV of 100% (Table, Supplemental Digital Content 2, http://links.lww.com/A1115). However, PPVs for SNHL were poor in both of these age groups for a PB virus burden >3500 ge/mL.

In children with symptomatic infection, the PPV and NPV were poor in all age groups (Table, Supplemental Digital Content 2, http://links.lww.com/A1115). At <2 months of age, the PPV of a PB virus load >3500 ge/mL for SNHL is 50% and the NPV of a viral load ≤3500 ge/mL is 66.7%. Five of 6 children with a PB virus burden of ≤3500 ge/mL at 2 to 12 months of age had normal hearing resulting in a NPV of 83.3%. The PPV in this age range for a viral load >3500 ge/mL was 60%. In children aged 12 to 36 months with symptomatic infection, neither of the 2 children with PB viral load >3500 ge/mL had SNHL resulting in a PPV of 0%. The NPV in symptomatic children 12 to 36 months of age with normal hearing

with a PB virus load of ≤3500 ge/mL was 75.0.0% (Table, Supplemental Digital Content 2, http://links.lww.com/A1115).

DISCUSSION

Previous studies examining the relationship between virus burden and the risk for hearing loss in children with congenital CMV infection demonstrated that higher viral load during early infancy was associated with an increased risk of SNHL.[12–14] In the majority of children with CMV-related hearing loss, the impairment occurs beyond early infancy and in more than half of those, the hearing deficit continues to progress. In the present study, we explored whether PB viral load during infancy and early childhood can be used to predict hearing loss. As the frequency and natural history of SNHL in children with asymptomatic congenital CMV infection is different from that in symptomatic children,[3] data in the 2 groups of children were analyzed independently. This analysis showed no association between viral load and hearing loss in all 3 age groups examined. Furthermore, our results showed that PB viral burden has a poor positive predictive value for CMV-related hearing loss. On the other hand, asymptomatic children with a PB viral load level of ≤3500 ge/mL appeared to be at lower risk for SNHL as only 2 of 36 children in the <2 months age group, 0 of 26 children in the 2 to 12 months age group, and 0 of 25 children in the ≥12 months age group with PB viral load ≤3500 ge/mL had SNHL. Together, these findings indicate that in individual children with congenital CMV infection, an elevated viral load measurement may not be useful in identifying a child at risk for CMV-related hearing loss. However, this data suggests that a low viral load in children with asymptomatic infection is associated with a lower risk for hearing deficit.

We could not confirm the association between systemic virus burden during early infancy and SNHL in children with asymptomatic congenital CMV infection that was observed in our previous study.[12] The real-time PCR assay protocol used to determine the viral load in both studies was identical. Although most of the study infants in the <2 months age group were included in the previous report, the addition of fifteen asymptomatic children with normal hearing and one child with SNHL has discounted our previous findings. The association between viral load in early infancy and hearing loss was not observed in this study. There was considerable overlap in the amount of viral load between the groups with and without hearing loss and this overlap could explain the lack of an association between PB viral load and hearing loss in early infancy. Similar findings were reported in an earlier study that examined the relationship between CMV viremia and hearing loss in 50 infants with symptomatic congenital CMV infection with CNS involvement participating in phase II and phase III ganciclovir treatment trials.[21] The findings of that study showed that although baseline viremia correlated with SNHL, an increase in viral load was not predictive of hearing loss.

The data from the current study are not consistent with studies by other investigators that have reported an association between higher PB viral load and SNHL. Lanari et al[13] examined a cohort of 37 infants with congenital CMV with clinical follow-up of more than 12 months. They reported that mean blood viral loads were higher in newborns that developed sequelae than those who did not. However, their study only included 1 child with hearing loss resulting from asymptomatic infection. A group of investigators from London examined the association between dried blood spot viral load and hearing loss in a group of 34 children with confirmed congenital CMV infection that did not receive ganciclovir therapy. They reported that CMV DNA viral load in the newborn period was significantly correlated with SNHL. However, the study population contained mainly children born with symptomatic infection at birth (22/34), and only 9 of 34 (26%) of the study cohort had normal hearing.[14] Our current study includes the largest cohort of children with

asymptomatic congenital CMV infection that has been examined for the association between virus burden and SNHL. In addition, the number of hearing impaired children (16/135, 12%) in our study is similar to the reported rates of CMV-related SNHL and thus, is more reflective of the overall group of children with congenital CMV infection. This argues against a selection bias which may have influenced the results of the other reports in the literature.

Although the natural history of CMV-related hearing loss has been well documented in large cohort studies, the pathogenesis of CMV-related hearing loss is poorly understood. The data from a limited number of human temporal bone studies and the guinea pig model of congenital CMV infection suggest that CMV can infect both the epithelium and neural tissue of the inner ear and that damage can occur as a result of direct viral mediated injury to the neural tissue or secondary to host derived inflammatory responses.[9–11,22,23] Systemic virus burden has been shown to correlate with CMV disease in immunocompromised hosts including allograft recipients and HIV-infected individuals.[24–28] These findings, together with recent reports suggesting an association between high systemic virus burden and SNHL has lead to the hope that PB viral load measurements could be useful to predict CMV-related hearing loss as well as a surrogate marker to assess the effectiveness of antiviral therapy in preventing or reducing the incidence and severity of CMV-related SNHL.[12–14] However, the lack of an association between virus burden and hearing loss in children with congenital CMV infection in the present study suggests that it is premature to use systemic virus burden as a reliable surrogate marker of the amount of virus replication in the inner ear. Therefore, the results of our study argue against the use of PB viral load to predict SNHL in individual children with congenital CMV infection and to monitor the effectiveness of antiviral therapy.

The current study does have limitations. The study children were followed for varying durations and PB samples were not available at all of the follow-up visits. Among the 50 children with more than one blood sample, there was no clear pattern to the change in viral load in individual children and the virus burden levels varied greatly over time. The absence of a predictable pattern in longitudinal virus burden measurements suggests that the lack of an association between viral load and hearing loss in our study is not likely due to the arbitrary categorization of the study children into 3 age groups. However, future studies with a more consistent patient follow-up are needed to carefully assess the dynamics of PB DNAemia in children with congenital infection. An additional limitation of the present study is that the hearing loss group was followed longer (47.6 ± 22.8 months) than those with normal hearing (33.3 ± 22.7 months). To reduce the bias from the differential follow-up, only samples obtained in the first 36 months of follow-up were included in the analysis. Because of the small numbers of children with delayed-onset and/or progressive SNHL, we were unable to determine if PB CMV DNA levels are associated with hearing loss. Studies with larger numbers of children with these types of SNHL are needed. Finally, the quantitative PCR assays to measure CMV viral loads have not been standardized across the various laboratories and the assay used in the present study was developed in our laboratory. Therefore, the results shown in this study may not be directly extrapolated to other populations in which different PCR techniques with different primers, probes, and detection systems are used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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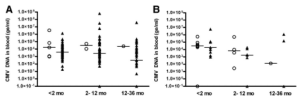


FIGURE 1.

Results of tests measuring levels of CMV DNA in PB at 3 different age ranges from children enrolled in the study with congenital CMV infection with asymptomatic (A) and symptomatic (B) infection at birth that had hearing loss (○) and normal hearing (▲). The results are expressed as genomic equivalents per mL of blood (ge/mL). The horizontal bars represent median values. In children with asymptomatic and symptomatic infection, median CMV DNA levels were not different between children with SNHL and those with normal hearing in all 3 age groups, analyzed by the Wilcoxon rank sum test. Note: the median VL of symptomatic children with normal hearing in the 12 to 36 month age group is 0 ge/mL.

TABLE 1

Demographic Characteristics, Clinical Findings and Follow-up Parameters for the Study Children With Congenital Cytomegalovirus Infection According to their Hearing Status

	Hearing Loss (n = 16)	Normal Hearing (n = 119)
Race		
African American	14 (88%)	104 (87%)
White	2 (12%)	15 (13%)
Male gender	9 (56%)	62 (52%)
Maternal marital status		
Single	15 (94%)	103 (87%)
Married	1 (6%)	16 (13%)
Maternal prenatal care		
Public health clinics	15 (94%)	103 (87%)*
Private provider	1 (6%)	7 (6%)
None	0 (0%)	3 (3%)
Maternal age		
<20 yr	14 (88%)†	56 (47%)
≥20 yr	2 (12%)	63 (53%)
Symptomatic at birth	9 (56%)	15 (13%)‡
Mean duration of follow-up (months ± SD)	45.0 ± 20.8	33.3 ± 22.3
Median number of hearing Evaluations (range)	9 (1–17)§	6 (1–12)

*Data available for 113 subjects.

†$P = 0.02$.

‡$P < 0.001$.

§$P = 0.011$.

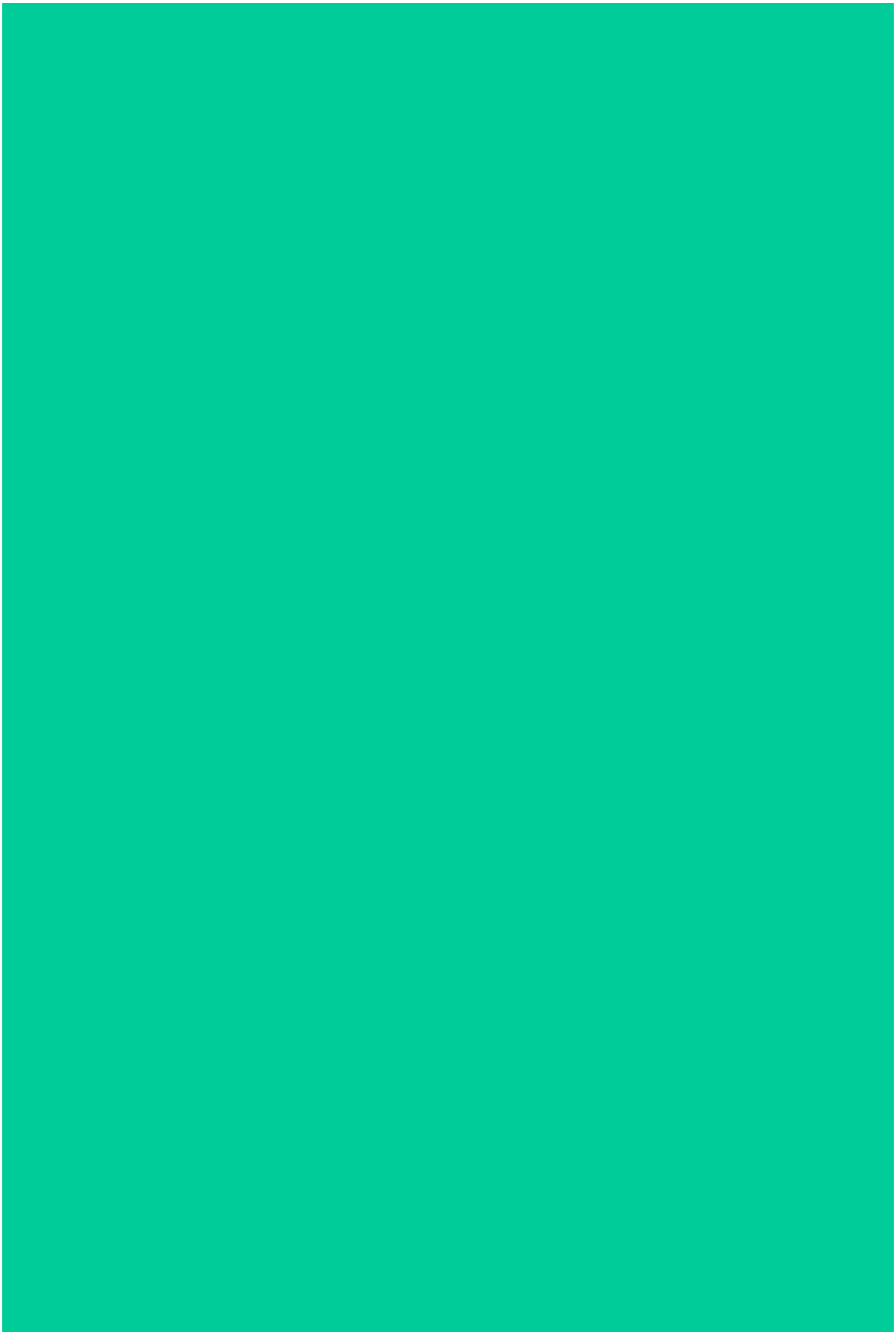
TABLE 2

Hearing loss Characteristics of the 16 Children With Cytomegalovirus-Related Hearing Loss

Delayed onset SNHL*	7/16 (44%)
Age at SNHL detection	
<2 mo	7/16 (44%)
2–12 mo	5/16 (31%)
>12 mo†	4/16 (25%)
Progressive SNHL	6/16 (38%)
Bilateral SNHL	5/16 (31%)

*Delayed onset SNHL defined as one or more hearing evaluations with a normal threshold documented for each ear before the onset of SNHL.

†Two children with SNHL detected after 36 month.



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Cytomegalovirus Strain Diversity in Seropositive Women▽

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Infection and reinfection with multiple cytomegalovirus (CMV) strains have been shown to occur in immunocompromised individuals, sexually transmitted disease clinic attendees, and children attending day care centers. To characterize the CMV diversity in healthy seropositive individuals, 16 CMV PCR-positive specimens from 113 seropositive women were analyzed for glycoprotein gN and gB genotypes by cloning, followed by nucleotide sequencing of the plasmid DNA and/or restriction fragment length polymorphism (RFLP). The results showed that most (93.7%) of the PCR-positive specimens contained multiple gN and/or gB genomic variants, suggesting that the majority of women were infected with more than one virus strain. The results also showed that the RFLP technique might not be sufficiently sensitive to detect all of the genomic variants present in a sample.

Cytomegalovirus (CMV) species are important opportunistic agents in infection of immunocompromised individuals and a frequent cause of congenital infection. Infection with multiple strains of CMV has been shown to occur frequently in immunocompromised individuals and sexually transmitted disease (STD) clinic attendees (8, 10). In addition, reinfection with different CMV strains was documented to occur in children attending day care centers (2). More recently, CMV reinfections were demonstrated in seropositive women, and such reinfections can result in intrauterine transmission and damaging fetal infection (5).

Extensive genetic polymorphisms in envelope glycoproteins of CMV, including glycoprotein B (gpUL55), glycoprotein O (gpUL74), and glycoprotein N (gpUL73), have been demonstrated among clinical CMV isolates. Major envelope glycoprotein B (gB) of CMV has been demonstrated to elicit a strong neutralizing antibody response (6), and on the basis of restriction fragment length polymorphism (RFLP) analysis of clinical samples, four unique genomic variants, gB types 1 to 4, have been identified (9). Glycoprotein N has been shown to be highly polymorphic at the amino-terminal region, and most clinical CMV isolates have been shown to cluster into four distinct genomic variants, gN-1, gN-2, gN-3a, gN-3b, gN-4a, gN-4b, and gN-4c (11). Recent studies have shown that a significant proportion of the virus-neutralizing response was also directed against the gM/gN complex (17). No linkage between gN genotypes and gB genotypes has been observed (11).

Published studies using RFLP analyses to determine the gN genotypes have identified only a single gN type in a given sample (11, 13). Studies of glycoprotein B based on RFLP analyses showed the presence of a single genotype or a limited number of samples containing mixtures of two gB genotypes (3, 7). However, a recent study using hybridization with type-specific probes (10) showed mixtures of all genotypes. To determine the CMV strain diversity in healthy seropositive women, the presence of multiple gN and gB genomic variants in urine or peripheral blood was examined by two different methods, RFLP and cloning followed by nucleotide sequence analysis of the plasmid DNA.

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MATERIALS AND METHODS

Specimens and subjects. The specimens studied consisted of 306 urine and 248 peripheral blood samples from 113 healthy, CMV-seropositive women who were tested for the presence of CMV immunoglobulin G antibodies in the postpartum period between February 2000 and June 2004. The women in the study were derived from a predominantly urban, low-income, African American population. Informed consent was obtained from all study participants, and the study was conducted in accordance with the guidelines of the Institutional Review Board for Human Use of the University of Alabama at Birmingham.

Detection of CMV DNA. DNA was extracted from urine and peripheral blood specimens with a commercial spin column kit (Qiagen Inc., Chatsworth, CA). The samples were initially tested for the presence of CMV DNA by PCR with primers from the antigen domain 1 region of the gene encoding glycoprotein B as described previously (4). The antigen domain 1 region of gB has been shown to be highly conserved among clinic isolates of CMV (9). The PCR-positive samples were further analyzed to determine gN and gB genotypes.

Characterization of gN genomic variants. (i) Nucleotide sequence analysis following cloning of the PCR-amplified gN products. The samples that were CMV PCR positive were further subjected to PCR to amplify the gN region with primers gN-Fw (5′ GGC GGT GGT GTG ATG GAG TG) and gN-Rev (5′ AAT AGC CTT TGG TGG TGG TTG C). After an initial 2-min denaturation at 96°C, the samples underwent seven cycles of denaturation at 96°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 40 s and the annealing temperature was decreased by 1°C each cycle. The samples were further subjected to 28 cycles with an annealing temperature of 58°C and a final extension step at 72°C for 7 min. The PCR products were directly cloned into TOPO TA cloning vector pCR 2.1 (Invitrogen Inc., Carlsbad, CA). The colonies were screened for the presence of the gN gene insert by direct PCR amplification and then grown to an appropriate volume of culture medium. Initially, the plasmid DNA from five individual colonies was sequenced with the gN-Fw primer at the University of Alabama at Birmingham sequencing core facility. If two or more

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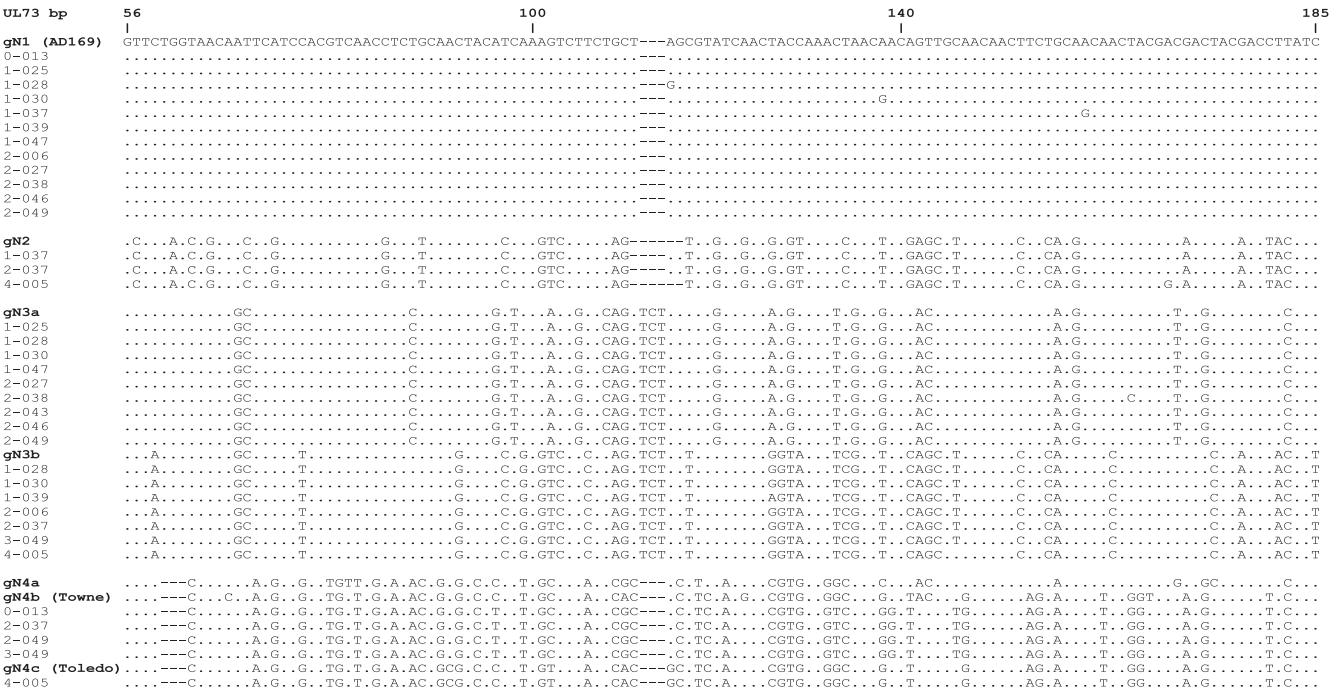

FIG. 1. Alignment of gN (UL73) nucleotide sequences with only part of the variable region shown. Dots indicate identity, and dashes indicate deletions. Strains are grouped by comparing the sequences of the recombinants with the prototypic gN genotypes previously described (11). The sequences are listed with the unique study subject identifiers. Prototypical laboratory-adapted strains are in parentheses. The nucleotide sequences were aligned with that of AD169 (gN1 prototype) with the Vector NTI Advance software package V.10 (Invitrogen, Carlsbad, CA) and displayed as a printable output by the BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html).

variants were found, no more colonies were screened. However, in the event that the first five colonies contained a single genotype, five additional colonies were sequenced. The nucleotide sequences were compared to the published gN subtype sequences of the four major gN genotypes (GenBank accession numbers AF309971, AF309976, AF309980, AF390773, AF309987, AF309997, and AF310004). A limited number of colonies from each recombinant were sequenced in both directions to confirm the sequence diversity and the genotype assignment (Fig. 1).

(ii) RFLP analysis. The PCR products amplified with the gN-Fw and gN-Rev primers described above were digested in four separate reactions with the restriction endonucleases SalI, SacI, BsaXI, and MmeI. The resulting restriction fragments were resolved by agarose gel electrophoresis, and the band patterns were analyzed in accordance with the restriction sites contained in different gN subtypes (12).

Characterization of gB genotypes by nested PCR followed by cloning. The DNA samples were initially subjected to PCR to amplify the target gB region located in the variable region between bp 1138 and 1638 with gB primers gB1138 (5′ CAA GAR GTG AAC ATG TCC GA) and gB1638 (5′ GTC ACG CAG CTG GCC AG). The PCR products were diluted 1:10 and subjected to nested PCR with primers gB1319 (5′ TGG AAC TGG AAC GTT TGG C) and gB1604 (5′ GAA ACG CGC GGC AAT CGG), yielding a 285-bp product (3). The PCR products were gel purified and cloned into the pCR 2.1 TOPO TA cloning vector (Invitrogen Inc., Carlsbad, CA). The colonies were screened by direct PCR, and those containing the insert were grown in culture medium. Plasmid DNA from five individual colonies from each recombinant was sequenced with the M13 forward primer at the University of Alabama at Birmingham sequencing core facility, and if two or more variants were found, no more colonies were screened. However, in the event that the first five colonies contained a single genotype, five additional colonies were sequenced. The nucleotide sequences were compared to the published gB genotype sequences (GenBank accession numbers M60928, M60930, M609931, and M609933). As described above for gN genotype characterization, a limited number of colonies were sequenced in both directions to confirm the genotype assignment (Fig. 2).

RESULTS

Of the 554 samples examined for the presence of CMV DNA, 16 (2.9%) were CMV PCR positive (9/306 urine samples and 7/248 blood samples). The positive samples were collected from 16 different study subjects. To determine the sensitivity of the PCR assay, the positive samples were subjected to a real-time PCR assay to estimate the amount of CMV DNA (4). This analysis showed that the sensitivity of the PCR assay was 300 copies/ml (data not shown).

The gpUL73 (gN) and gpUL55 (gB) diversity was examined in all 16 samples by RFLP and cloning of the gN gene and by cloning of the gB gene. Of the 16 samples, 15 (93%) were found to have more than one gN genomic variant (Table 1). The only sample that was found to contain a single gN genotype on cloning and nucleotide sequence analysis was also found to have a single gN genomic variant on RFLP examination (Table 2). Although the RFLP examination revealed that four additional samples contained a single gN genotype, nucleotide sequence analysis documented the presence of multiple gN genomic variants in the samples (Table 1).

Of the 16 samples analyzed for gB genotypes, 69% (11/16) were found to contain more than one gB genotype; eight samples had two genotypes, and three samples had three genotypes (Table 2). Among the 16 samples that underwent gN and gB genotype analysis by cloning, 15 (93.7%) samples contained multiple genotypes and only one sample had a single gN and gB genotype. The sample with only one gN genotype was from a blood specimen. Of the samples with a single gB genotype,

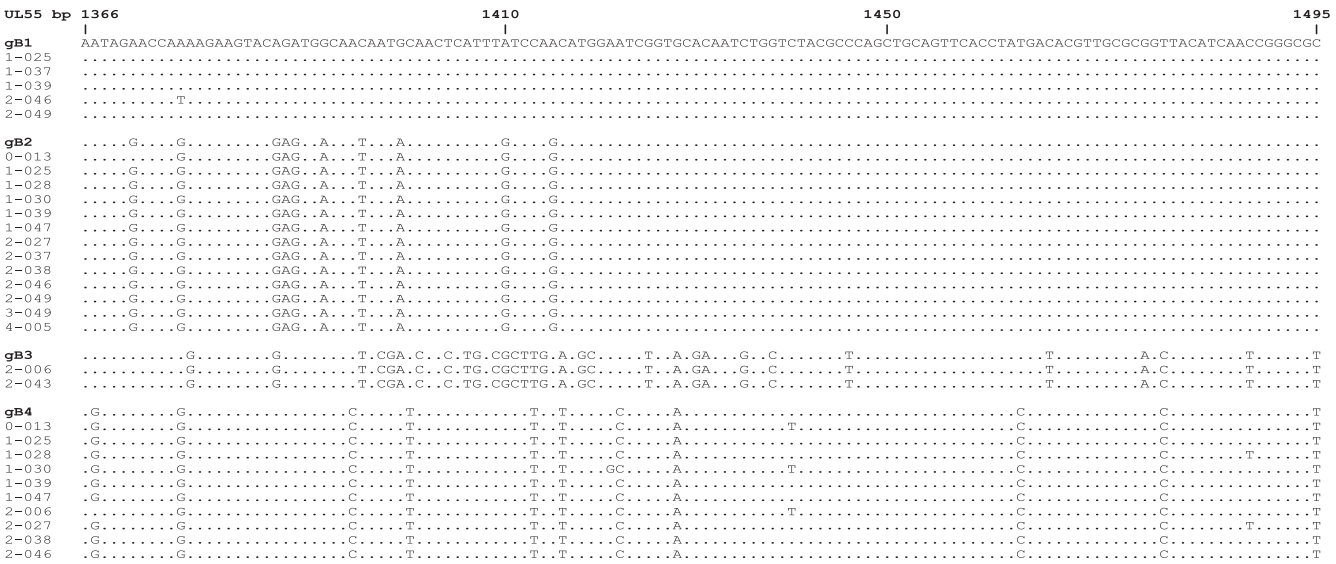

FIG. 2. Alignment of representative gB (UL55) sequences with the prototypes (only part of the variable region of gB is shown). Dots indicate identity, and dashes indicate deletions. Strains are grouped by comparing the sequences of the recombinants with the prototypic gB genotypes previously described (9). The sequences are listed with the unique study subject identifiers. The nucleotide sequences were aligned with the gB1 prototype with the Vector NTI Advance software package V.10 (Invitrogen, Carlsbad, CA) and displayed as a printable output by the BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html).

two (40%) were from blood and three (60%) were from urine specimens.

The most common glycoprotein N genotype in our study group was type 3 (41%), followed by type 1 (35%). Glycoprotein B genotype 2 (43%) was the most frequent, followed by type 4 (33%) (Fig. 3).

DISCUSSION

The findings of the present study clearly document the presence of multiple CMV strains in the majority of the CMV PCR-positive urine and peripheral blood specimens from the healthy seropositive women studied. The existence of multiple virus strains in the specimens was demonstrated by the presence of different and multiple gN and/or gB genomic variants. Similar findings were reported in a recent study in our laboratory in which multiple gN genotypes were detected in genital tract specimens from women who attended an STD clinic (15). In an earlier report, we documented the occurrence of CMV reinfection between pregnancies in seropositive women, and such reinfection could lead to intrauterine transmission and symptomatic congenital CMV infection (5). Several other studies have also documented CMV infection with multiple virus strains in a variety of population groups, including children attending day care centers, human immunodeficiency virus-infected individuals, allograft recipients, and infants with congenital CMV infection (1, 2, 10, 14). Together, the findings of these studies of different population groups suggest that infections with multiple CMV strains occur frequently.

It is possible that the observed strain diversity in our study population of healthy, seropositive women is due to an increased exposure to CMV, resulting in CMV reinfections. However, we could not determine the timing of reinfection(s) in our study. Previous studies have reported reinfection with multiple strains of CMV in immunocompetent hosts. Chandler et al. reported that four of eight women attending an STD clinic were found to be reinfected with a new strain of CMV

TABLE 1. Comparison of gN genotyping results by RFLP and the cloning techniques for each of the 16 PCR-positive samples

Sample	Observed genotype(s)	
	RFLP	Cloning
0-013	4	1, 4
1-025	1, 3	1, 3
1-028	1, 3	1, 3
1-030	1, 3	1, 3
1-037	1, 2	1, 2
1-039	1, 3	1, 3
1-047	1	1, 3
2-006	1, 3	1, 3
2-027	1, 3	1, 3
2-037	2, 3, 4	2, 3, 4
2-038	1, 3	1, 3
2-043	3	3
2-046	1	1, 3
2-049	1, 3	1, 3, 4
3-049	1, 3, 4	3, 4
4-005	4	2, 3, 4

TABLE 2. Results of gN and gB genotyping of CMV in the urine and blood of CMV-seropositive women

Glycoprotein	Technique	% of samples with indicated no. of genotypes (no.[a] of samples):		
		1	2	3
gN	RFLP	31.2 (5)	56.3 (9)	12.5 (2)
gN	Cloning	6.3 (1)	75 (12)	18.7 (3)
gB	Cloning	31.3 (5)	50 (8)	18.7 (3)

[a] The total number of samples tested was 16.

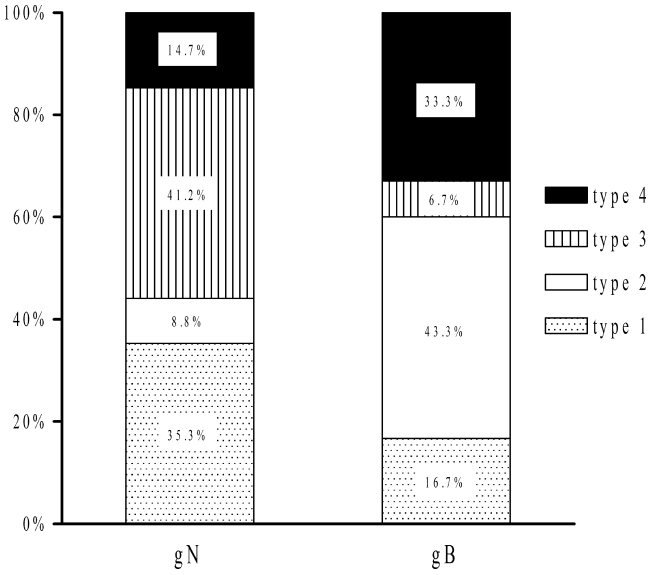

FIG. 3. Relative frequencies of CMV gN and gB genotypes in urine and blood samples from CMV-seropositive women.

(8). Bale et al. examined serial samples from 37 children attending day care centers and found that 19% had evidence of infection with more than one CMV strain (2). Although exposure to CMV through sexual activity or contact with young children could explain the strain diversity observed in our study, the association between increased CMV exposure and CMV strain diversity has not been documented.

Several previous studies that examined the genetic diversity of CMV glycoprotein gN only reported the presence of a single gN genotype in the specimens tested. Pignatelli et al. examined 223 viral isolates from a variety of patient populations, including allograft recipients, AIDS patients, congenitally infected children, and children with postnatally acquired CMV infection from four different geographic regions. The virus isolates examined in that study were obtained from urine, saliva, blood, amniotic fluid, and biopsy specimens (12). Although all four gN genotypes were present among the specimens tested, only one gN type was detected in a given specimen. In contrast, we could document the presence of multiple gN genomic variants in the majority of CMV PCR-positive specimens in our study. In the present study, we used two different techniques, RFLP and cloning of the amplified gN product, followed by nucleotide sequence analysis, to determine the gN genotypes, whereas the studies by Pignatelli et al. and others only used RFLP (16). It is likely that the addition of the cloning method may have improved our ability to distinguish different genomic variants present in a specimen. Additional factors, including the populations studied and the geographic origin of the specimens, may account for the differences between the present study and previously published reports. Furthermore, Pignatelli et al. studied virus isolates that had been propagated in cell culture rather than DNA extracted from the original specimen. The propagation of virus isolates in cell culture could have selected for a single virus strain.

Identification of gN and gB genotypes by RFLP is based on the ability to clearly visualize the restriction fragment band pattern, and therefore the amount of DNA in the samples tested is critical. Multiple gN genomic variants present in a sample may not be easily distinguished because the intensity of the fragments on gel electrophoresis depends on the amount and ratio of the type-specific DNA. Furthermore, because of overlapping and sometimes minute differences in band size, it may be difficult to properly identify all of the bands necessary for precise genotype identification, even on high-concentration agarose gel electrophoresis. The restriction sites could be affected by a single nucleotide change, which in turn makes it difficult to recognize correct genotypes. However, such a change in the restriction sites is unlikely to affect the genotype assignment because the grouping of genotypes is based on multiple nucleotide changes. The cloning and sequence analysis method is more accurate in determining the number of gN or gB genotypes because it is not limited by the relative amount of type-specific DNA contained in the sample. However, there are limitations to the use of cloning and sequence analysis in the detection of multiple strains in a sample. These include the sensitivity and specificity of the PCR assay used to amplify CMV DNA in the samples and the number of bacterial colonies that can be screened.

Like glycoprotein N, genotyping of glycoprotein B by RFLP has only shown one or two types present in a clinical sample. In a study based on samples from liver transplant patients, 87% (53/61) of the samples contained only one gB genotype (3). The remaining 13% of the samples contained two genotypes. This could be due to the fact that the use of the enzymes HinfI and RsaI produced indistinguishable restriction patterns in certain combinations of three and four different gB genotypes. Additionally, the above-described limitations of the ability of RFLP to detect multiple gN genotypes are also relevant to the detection of gB genotypes. However, mixtures of three or four different gB genotypes per sample were detected in a study using non-RFLP-based techniques. A recent study by Coaquette et al. used PCR amplification followed by hybridization with a single-stranded DNA probe specific for each CMV gB genotype detected by DNA enzyme immunoassay. In that study, of the 97 CMV isolates collected from 92 immunocompromised patients (transplant recipients and lymphoma and leukemia patients), 74% (72/97) contained a single gB genotype, 22% (21/97) contained two genotypes, 3% (3/97) contained three genotypes, and 1% (1/97) contained all four genotypes (10).

Similar to the results of previous studies, we did not observe a linkage between the gN and gB genotypes (11). However, the number of samples examined was small and we found only one sample that contained a single gN genotype and five samples containing a single variant of gB. Only one study sample contained a single genotype of both the gN and gB glycoproteins. Therefore, our study clearly did not have a sufficient sample size to examine the possible linkage between the gN and gB genotypes. In addition, our study was based on a predominantly African American, low-income population and therefore the findings of our study may not be representative of the general population.

Using RFLP and cloning followed by nucleotide sequence analysis, we could demonstrate infection with multiple CMV strains in most healthy seropositive women who had CMV DNA in their urine and/or peripheral blood. These findings

need to be confirmed in a study with a larger number of samples and more diverse population groups.

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