CHAPTER II

LITERATURE REVIEWS

1. Virology

Human Cytomegalovirus (HCMV) is a member of the *Betaherpesvirinae*, a subfamily of the herpesviridae. The beta-herpesviruses tend to have a relatively restricted host range, long growth cycle, and slow spread in cell culture. The virus can replicate only in humans or cells of human origin. *In vivo*, the virus can infect a large variety of cells (endothelial cells, monocytes/macrophages, epithelial cells, fibroblasts etc.). *In vitro*, efficient replication is restricted to primary fibroblasts. HCMV-infected cells may become enlarged (cytomegalia) and show intranuclear inclusions. Co-survival of cell and virus is often established. After infection, the virus can never be eliminated, and may maintain latency in various tissues of the host. Reactivation of the virus is of significant concern in the immunocompromised individual, particularly when the deficiency affects cell mediated immune responses in, for instance, HIV-infected patients. Immunosuppressed individuals, including allograft recipients, are also at great risk of developing HCMV disease. Treatment (in the form of direct and indirect viral polymerase inhibitors) is available to reduce viral proliferation in patients who develop the clinical symptoms associated with HCMV disease, but such therapy cannot clear the virus completely. Subunit vaccines (immunogenic viral peptides) have also been considered and are currently under investigation. The virion of HCMV consists of an icosahedral capsid encasing a 235 kb linear double-stranded DNA genome, with a 57% G-C content, surrounded by a tegument or matrix and enveloped in a lipid bilayer carrying a large number of virus-encoded glycoproteins[6, 7]. HCMV is the only betaherpesvirus known to have a class E genome structure, a genome arrangement first characterized in HSV-1. This complex arrangement of unique and inverted repeats in HCMV, including the existence of four genome isomers, was first appreciated in the course of restriction mapping studies[8-12]. The genome of HCMV has two unique components, UL and US, flanked by inverted repeats *b* (TRL/IRL) and *c* (IRS/TRS). A directly repeated "*a*" sequence found at the genome termini is also present in inverted orientation at the L-S junction. This arrangement of

"*a*" sequences promotes genome inversion. The "*a*" sequences carry cis-signals, called *pac*-1 and *pac*-2, for cleavage and packaging the viral genome. The *pac*-1 and *pac*-2 sequence elements are conserved in all herpesvirus genomes. However, their location varies relatively to the cleavage site. In HCMV, cleavage occurs to one side of the *pac*-1 and *pac*-2 element. Inversion of the L and S genome components (Figure 1) is mediated by repeated sequences located at the genomic termini and L-S junction[4].
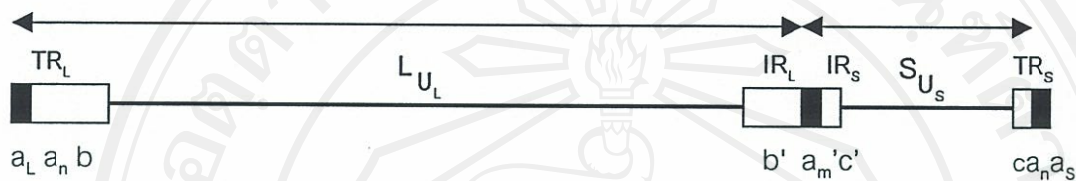

Figure 1 HCMV genome structure[4].

2. Viral growth cycle

Productive replication follows the coordinate expression of ordered sets of viral genes. Gene expression may be divided into sequentially expressed kinetic classes: α or immediate early (IE), β or early, and γ or late.

2.1 α (immediate-early) gene expression and gene product

Four regions of α gene expression have been mapped on the HCMV genome, UL36-38, *ie*1/*ie*2 (UL122-123), TRS1-IRS1, and US3[13-15], with an additional region (UL115-119) that may also fit into this class[13, 16]. The expression of "*a*" genes begins within a one hour period (hpi), peaks between 4 and 8 hours, and is independent of *de novo* protein synthesis.

The overwhelming majority of expressions, immediately after viral entry, arises from a single major locus, the so-called MIE locus, which is located between 169 and 178 kb on the HCMV genome (Figure 2)[17, 18]. Transcription from the *ie*1/*ie*2 promoter-enhancer gives rise to differentially spliced and polyadenylated transcripts encoding the two major products, $IE1_{491aa}$ and $IE2_{579aa}$, which share a common 85-amino-acid amino-terminal domain, but spliced to either

UL123 for $IE1_{491aa}$, or to UL122 for $IE2_{579aa}$[14, 15]. In addition to the important role of MIE products in viral replication, the ancillary α gene plays a part in regulation (TRS1/IRS1)[19, 20], inhibition of apoptosis (UL36 and UL37)[21], and modulation of host cell MHC class I expression (US3)[17, 22].

2.2 β (delayed early) gene expression and gene products

Two abundantly expressed β genes, encoding 1.2 kb ($\beta_{1.2}$) and 2.7 kb ($\beta_{2.7}$) transcripts, remain prototypes for studies on gene regulation despite the fact that their translation products have not been studied[14]. Additional genes, whose regulation and gene products have been studied in some detail are involved in DNA replication (UL112-UL113, UL54/DNA polymerase, UL44/DNA processivity factor), immunomodulation (US11), and an early glycoprotein expressed from multiple promoters employing transcriptional and post-transcriptional controls, UL4 (gp48).

2.3 γ (late) gene expression and gene products

Most late genes of human HCMV encode structural proteins. Regulation of the gene promoters, some of which appear to be β_2 and others γ_1 or γ_2, are not yet well defined, although a complex picture of the influence of both viral DNA replication and additional viral *tran*-activators is emerging. $IE1_{491aa}$ plus $IE2_{579aa}$ do not influence the expression of certain late genes, even though they are strongly activated in virus-infected cells. For example, the promoter of the pp28 gene, $\gamma IE2_{338aa}$ gene promoter, and the promoter for the ICP36 gene are *trans*-activated by viral infection in transient assays, but are unresponsive to *ie*1 plus *ie*2. Transient assays have suggested that the additional viral *tran*-activator needed for ICP36 promoter activation is encoded by the TRS1 or IRS1 ORF. Together, TRS1, $IE1_{491aa}$ and $IE2_{579aa}$ *trans*-activate each of the three independent promoters that are part of this gene. TRS1 (IRS1) does not appear to be the additional factor needed for activation of the pp28 or $\gamma IE2_{338aa}$ promoters, and this factor remains to be identified.
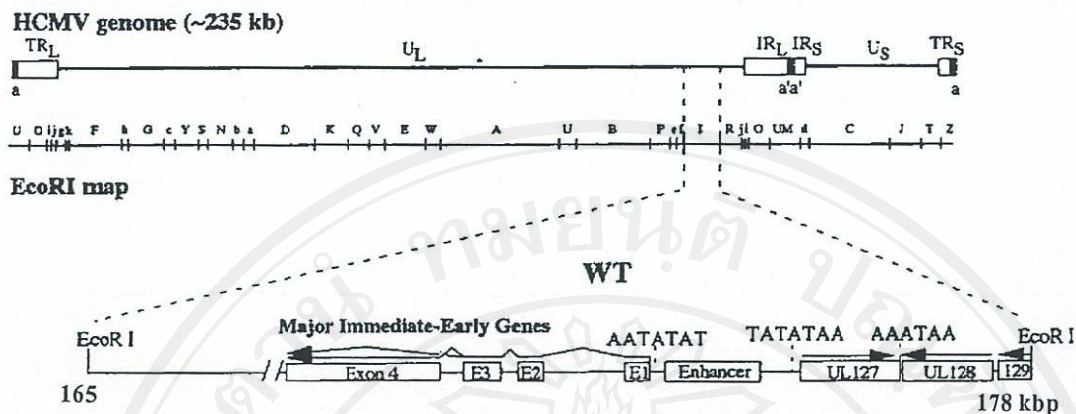
Figure 2 Human cytomegalovirus genome structure. The HCMV MIE gene is located between 165-178 kbp of the EcoRI fragment. It is composed of 4 exon regions (Exon1, Exon2, Exon3, and Exon4)[23].

3. Host immune response

The central importance of cell mediated immunity for the control of HCMV infection is reinforced by the observation that individuals with deficiencies in cellular immunity exhibit the highest risk for HCMV disease. Resistance to HCMV infection depends on successful collaboration between the innate and adaptive immune systems. Both natural killer (NK) cells[24] and CTL[25], whose presence correlates with protection from HCMV disease[26], play prominent roles in immune clearance.

Borysiewicz *et al.* studied human NK responses to HCMV *in vitro* and found that they were mediated by large granular lymphocytes, independent of interferon, and manifested at the same time as early viral proteins expression[27]. Indirect evidence for the importance of NK cells, in defense against HCMV infection, comes from the observation that the viral gene products, which appear to be conserved, can protect against NK cell-mediated lysis[28].

Infection with HCMV induces both humoral and cell-mediated immune responses to viral antigens in the normal host. The humoral immune response includes the production of IgG, IgM, and IgA antibodies, which appear together with or just after the onset of viral excretion in saliva or urine[29]. IgM antibodies persist for 2-8 months after an initial infection, whereas IgA-class

antibodies remain detectable for up to 12 months. IgG titers rise initially following infection and then gradually decline. A residual antibody is detectable by serological testing for several years. In most immune compromised patients, however, the serological response may be significantly delayed[30]. Antibodies to approximately two dozen immunogenic HCMV proteins can be detected in sera from seropositive human studies of infected cell lysates or purified virions. Mixtures of recombinant proteins have shown that immune human sera vary in their reactivity with these immunogenic proteins. The proteins to which immune human sera react most consistently are listed in Table 3. Infants with congenital HCMV infection are capable of developing IgG- and IgM-class antibodies, but the IgM response occurs in about 75% of these patients. In addition, the detection of IgM antibodies is not absolute proof of recent primary infection. This response may be detectable in organ transplant patients with recurrent HCMV infections. HCMV IgG antibodies possess neutralizing activity and have been shown to retain a direct cytolytic effect against infected cells[31]. During pregnancy, prior immunity to HCMV in the mother can reduce the viral inoculum transmitted to the fetus, resulting in a reduced level of viral replication and disease severity without entirely eliminating the infection.

Cell-mediated immunity is the major response capable of containing the dissemination of HCMV infection. In humans, lymphocyte proliferative responses to HCMV preparations are consistently present in seropositive adults. However, they appear to be markedly suppressed after HCMV mononucleosis in young children with congenital infection, immunocompromised transplant patients with HCMV disease, and even normal adolescents after asymptomatic primary infection[32]. In humans with naturally acquired HCMV infection, CD4+ helper T-lymphocyte responses to multiple structural and nonstructural HCMV proteins have been demonstrated.

Although CD4+ T-lymphocyte responses to envelope glycoproteins, gB and gH, the lower matrix protein pp65, and immediate-early proteins are most commonly detected, responses to other proteins have also been reported[33-35].

Quinnan et al. studied both lymphoprolifertative responses and human leukocytic antigen (HLA) class I restricted CTL responses of peripheral blood lymphocytes from bone marrow transplant patients. The CTL response to HCMV was associated with a clinical recovery from HCMV disease[36]. In humans, CD8+ CTL responses to a number of HCMV proteins also have been described, including pp65 (UL83), pp150 (UL32), gB (UL55), and IE1 (UL123)[37 38].

Table 3 Immunogenic proteins of HCMV[4]

Name (ORF)	Location	Immune response
gB (UL55)	Envelope	Major target of neutralizing antibody
gH (UL75)	Envelope	Major target of neutralizing antibody
pp150 (UL32)	Tegument	Nonneutralizing antibody and CD8+ CTL
pp28 (UL99)	Tegument	Nonneutralizing antibody
pp71 (UL82)	Matrix	Nonneutralizing antibody
pp65 (UL83)	Matrix	Main target of CD8+ CTL and target of antibody
pp52 (UL44)	NS, DNA bunding	Nonneutralizing antibody
pp72 (UL123)	IE, NS, regulating	Target of nonneutralizing antibody and CTL

NS, nonstructural; IE, immediate; CTL, cytotoxic T lymphocyte

A precursor frequency comparison between CTL specific for gB (UL55) and the 72-kd IE protein (UL123) using vaccinia recombinants to infect target cells revealed a low frequency of gB-specific CTLs ($\leq$6%), whereas 18% to 58% of CTLs recognized the vaccinia IE-infected target[39]. An assessment of CTL precursor frequencies to five HCMV proteins, pp65 (UL83), gB (UL55), gH (UL75), pp50 (UL44), and pp28 (UL99), found that pp65 was immunodominant[38]. Kern *et al.* used multiple, overlapping pentadecamer peptides spanning the entire 491 amino acid sequence of the 72-kd IE1 protein to identify CD8+ T-cell epitopes and measure CTL frequencies[40]. They found that the frequencies of IE1-specific and pp65-specific CTLs were similar in most donors. However, some donors had responded to IE1 or to pp65, but not to both. Although it is likely that more targets for CTL will be identified among HCMV proteins, at the present time pp65 (UL83) and IE1 (UL123) appear to be the major targets.

Based on the observed association between recovery of CTL responses to HCMV and recovery from CMV pneumonia in bone marrow transplant patients[41], adoptive transfer of HCMV-specific clones of CTL was evaluated as a therapeutic intervention[42].

4. Immune evasion of HCMV

HCMV evades the immune system through a complicated multifactorial mechanism. Several viral gene products are involved in establishing immune evasion in the productively infected cell. US2 and US11 bind the major histocompatibility complex (MHC) class I heavy chains, transporting them in a retrograde fashion from the endoplasmic reticulum (ER) into the cytosol, where they are degraded by the proteasome[17, 43-45]. US3 binds and retains major histocompatibility complex class I heavy chains in the endoplasmic reticulum and prevents transportation of the MHC class 1 complexes from the ER to the golgi[17, 22]. US6 inhibits peptide translocation into the ER by the transporter associated with antigen processing (TAP) [46-48]. The HCMV–encode MHC class I homologue (UL18) was initially thought to block NK cell activity through binding with the killer inhibitory receptor, as endogenous MHC class I would[49, 50]. The HCMV glycoprotein UL40 (gpUL40) contains a nine-amino-acid sequence that is homologous to the class I MHC sequence, which up-regulates the surface expression of HLA-E. HLA-E protects potential target cells from NK cell-mediated lysis by binding to inhibitory receptors on the NK cell surface. HCMV exhibits a number of other activities that could impede viral host defense. These include binding and sequestration of β-chemokines (US27, UL28, UL33)[51], competition with the TNF receptor superfamily through a homolog (UL144)[52], interference with apoptosis[53], down-regulation of MHC class II expression on the surface of macrophages[54], and protection of infected cells from complement-mediated lysis[55, 56]. The UL37 open reading frame has recently been shown to encode a mitochondrial-localized inhibitor of apoptosis that is unrelated to bcl-2. Lastly, the US28 gene encodes a C-C chemokine G-protein-coupled receptor (GCR), which binds both CC and CX_3C chemokines, and signals in response to others. The pUS28 may function as a chemokine sink, mediate intracellular signal, and control the level of host cell activation through inducing chemokinesis or chemotaxis. The outcome in any of these scenarios would indicate the altered behavior of infected cells.

5. Persistence and latency

HCMV persists indefinitely within its host. There is growing evidence that cells in the bone marrow and peripheral blood are a key reservoir for HCMV through latent infection. A small percentage of peripheral blood monocytes from subjects with past (presumably latent) infection harbor HCMV DNA, but viral gene expression is limited to an early gene[57, 58]. However, tissue macrophages (a differentiated form of circulating monocytes) express early and late HCMV antigens[59]. These observations have led investigators to propose a model for HCMV persistence and reactivation in which bone marrow precursors of monocytic peripheral blood cells are a key site of latency. Peripheral blood monocytes provide a mean to disseminate virus. Meanwhile, differentiation of latently infected monocytes into macrophages, leads to reactivation and productive infection. The experimental evidence supports a model of HCMV persistence, in which myelomonocytic stem cells residing in bone marrow maintain latent infection, and latently infected CD14+ monocytes circulate in peripheral blood. Differentiation of latently infected monocytes into tissue macrophages from allogeneic stimulation (transplantation or transfusion), or triggering of proinflammatory cytokines, as occurs with intercurrent infections, leads to productive HCMV infection.

Using traditional virus isolation techniques, infectious viruses can be recovered from various body sites for many months after primary infection. It is likely that application of more sensitive techniques for the detection of HCMV in body fluids will reveal that the duration of viral shedding with primary infection is longer, and the frequency in episodes of shedding (reactivation of latent virus) years after primary infection is greater than current knowledge suggests. The possibility remains that some tissues maintain productive HCMV infection over many years.

6. Pathology

Studies of the pathologic features of HCMV infection have focused primarily on descriptions of the distribution of typical cytomegalic inclusion-bearing cells in various tissues. With severely disseminated disease, evidence of HCMV involvement can be found in virtually all organ systems. Viruria is a consistent feature of HCMV infection in all age groups and results

from viral replication in the genitourinary tract. In the kidneys, cytomegalic cells are most frequently observed in the proximal tubules near the cortical area. In renal transplant patients, HCMV has been proposed as playing a direct role in renal dysfunction and graft rejection. Gastrointestinal (GI) involvement, which was infrequently observed as relative to the aged and immunocompromised individuals, is now commonly reported as a manifestation of HCMV infection in patients with HIV.

In subclinical hepatitis in children and adults, the architecture of the liver is well preserved, but areas of mononuclear cells that infiltrate around the portal triads have been observed, often in the absence of typical cytomegalic cells[60]. The respiratory tract, including mucosal sites of the upper respiratory tract, is another site commonly involved in the HCMV infections of immunosuppressed individuals, especially bone marrow and heart-lung transplant recipients[61, 62]. In the lung, HCMV-infected cells are seen primarily in the alveolar and bronchial epithelium, with varying degrees of mononuclear cell inflammation.

Infection of the central nervous system (CNS) with HCMV was infrequently reported in the past, except when following severe fetal infection[63, 64]. In recent years, CNS involvement has been more frequently noted in adults, as a complication of HCMV infection in patients with AIDS.

7. Epidemiology

Humans are believed to be the only reservoir for HCMV. Natural transmission occurs by direct or indirect person–to–person contact. Because of the viability of HCMV following exposure to such common environmental conditions as heat and drying, close or even intimate contact is believed to be required for its horizontal spread. Sources of the virus include oropharyngeal secretion, urine, cervical and vaginal excretion, semen, breast milk, tears, feces, and blood[65]. The spread within susceptible populations is enhanced by the prolonged excretion of infectious virus following primary infection. Virus excretion persists for years after congenitally, perinatally, and early postnatally acquired infections[66]. In fact, prolonged replication of HCMV can follow primary infection in older children and adults, and recurrences are associated with

intermittent shedding of HCMV from many sites in a significant proportion of seropositive young adults.

Transmission of HCMV through sexual contact is an important means of spreading the disease. The prevalence of HCMV infection is higher among populations with other markers of sexual activity, such as greater number of partners, sexually transmitted disease and sexually active adolescents[67].

Day care workers have markedly increased rates of HCMV infection, ranging from 7% to 20% per year, compared to an expected rate in U.S. adults of around 2% per year[68, 69]. Parents of young children who attend day care centers are also at increased risk from HCMV infection, as are parents of premature babies who acquired HCMV nosocomially in the hospital nursery. Among the former group, the risk of HCMV infection is clearly related to the shedding of virus by the child. A seroconversion rate of 45% per year was noted in parents of toddlers who shed HCMV, compared to 0% among parents whose child did not shed HCMV.

Vertical transmission and transmission from mother to fetus or the newborn not only occurs, but is common and plays an important role in maintaining HCMV infection in the population. HCMV is spread from mother to baby by three routes: transplacental, intrapartum, and human milk.

The rate of primary maternal infection during pregnancy varies from 0.7% to 4.1%, with higher rates noted in young, unmarried women of low socioeconomic status[70]. The rate of transplacental HCMV transmission to the fetus with primary maternal infection during pregnancy is around 20% to 40%[70]. There is no clear evidence of a gestational age effect on the rate of transmission.

Intrapartum transmission of HCMV is related to local shedding of the virus. Approximately 10% of women shed HCMV from the vagina or cervix; near the time of delivery. Rates of 2% to 28% have been reported[71].

The most common route for mother-to-infant transmission of HCMV is human milk. Transmission of HCMV to nursing infants is related to duration of nursing prior to weaning and the formula and presence of virus in the milk. Dworsky et al. followed a cohort of infants nursed by a HCMV seropositive mother[72]. They found that no infants nursed for less than 1 month by the

seropositive mother were infected, compared to a 39% HCMV infection rate in those who were nursed by her for longer than 1 month.

A Swedish study of serial milk specimens from seropositive women reported as much as 70% HCMV isolated from samples collected from 9 days to 3 months postpartum. All of the samples collected prior to or after this interval were negative. More recent studies using PCR to detect HCMV DNA in milk have reported similar rates of HCMV transmission from seropositive mothers and a strong relationship between the presence of viral DNA in milk and transmission of HCMV to the infant[73, 74].

8. Laboratory diagnosis

8.1 Cell culture technique

Human fibroblasts are the only cells that support HCMV replication *in vitro*. Human embryo lung and human foreskin fibroblasts are commonly used for recovery of the virus in the laboratory. The time required for the development of cytopathic effects (CPE) is directly related to the titer of the virus in the sample. Typically, a mean time of 8 days is required for most strains. Because of the lengthy time required for the development and recognition of CPE by this method, the results are not always useful for the clinical management of and therapy for the patient. The time for the appearance of cytopathic effects, due to HCMV, ranges from 2-21 days.

Alternatively, in the last few years of routine laboratory practice, the rapid detection of HCMV in cell cultures has been achieved by the use of the shell vial assay[75]. In this procedure, 1-dr (3.697 ml) shell vials that contain a circular coverslip are seeded with fibroblast cells that develop into a monolayer. The growth medium is removed and specimen extracts are then inoculated into vials, which are subsequently centrifuged at 700 x g (2,000 rpm) for 45 min to enhance the infectivity of HCMV relative to the use of conventional tube cell cultures. Medium is added back to the vials, and after overnight inoculation (16 h at 36 °C), cell monolayers on the coverslip are stained with a monoclonal antibody (directed to the immediate early antigen of HCMV) by the indirect immunofluorescence test. Stained coverslips are examined at 400x magnification for the detection of intact fluorescing cells.

The interpretation of positive viral culture for HCMV from any site always depends upon the clinical circumstance (Table 4). In general, the occurrence of viremia and the isolation of the virus from multiple sites or in high titers is more likely to be associated with clinical disease.

8.2 Antigen detection methods

These methods are based on the use of monoclonal antibodies. The advent of monoclonal antibodies against the major immediate early proteins of HCMV can be used to detect the virus in a cell culture before CPE becomes apparent. Among these techniques, the shell viral assay[76] and the DEAFF test were used to detect the early antigen fluorescent foci[77]. Routinely, when the shell vial system is used, the vial contains coverslips of fibroblast cells that are inoculated with specimens, incubated for 24 to 48 hr. and stained by an immunofluorescence method using monoclonal antibodies against HCMV immediate early antigen.

The result of system evaluation showed that the HCMV early antigen could be detected as soon as 16 hr. after inoculation[76]. When this technique was compared with the standard cell culture from samples other than urine, the shell vial was found to be useful for HCMV isolation from all types of specimens[78]. However, culturing in a conventional tube as well as in shell vials was recommended to ensure optimal sensitivity of HCMV isolation from sources other than urine[79].

The antigenemia test is based on the use of monoclonal antibodies to detect HCMV antigens directly in leukocytes from the blood of immunocompromised patients. The monoclonal antibody reacts with HCMV lower matrix early structural protein (pp65), the product of UL83, and does not react with the MIE transactivating proteins[80]. In this technique, peripheral blood leukocytes and endothelial cells are separated from whole blood specimens, placed on a microscope slide, and stained with monoclonal antibodies directed against the HCMV antigen. After incubation and washing steps, horseradish peroxidase-labeled anti-mouse antibodies are added, followed by a substrate solution. The cells are then examined by light microscopy for dark or red-brown nuclear staining. This technique provides several advantages, such as a rapid diagnosis, and does not require facilities for cell culture or PCR. In one typical study, an

antigenemia assay was compared with a shell vial and conventional culture, and it was found to have a higher sensitivity for rapid detection of HCMV[81]. The antigenemia assay has been evaluated for early diagnosis of HCMV infection in heart transplant recipients, with results indicating a sensitivity of 83% for future development of HCMV disease[82].

8.3 Nucleic acid detection

8.3.1 Polymerase chain reaction

The detection of HCMV by DNA amplification techniques provides the potential for rapid and early diagnosis. PCR can selectively amplify and detect specific HCMV DNA. A variety of amplification targets have been used for the detection of HCMV DNA by use of the oligonucleotide primers that are complementary to specific sequences flanking the target segment of the DNA to be amplified. Sequence variation of the viral genome found in different populations of patients, including the immunocompromised, has been shown to affect the performance for detecting HCMV DNA targets.

PCR is a highly sensitive technique and offers an earlier detection of HCMV when compared with other methods such as the shell vial assay, conventional tube cell culture, and antigenemia test[83]. However, its main disadvantage is its specificity and predictive value for the diagnosis of HCMV disease[84]. However, PCR quantitation techniques have recently been evaluated showing an enhanced specificity and predictive value for HCMV disease when HCMV DNA is quantitated[85].

This has allowed a better interpretation of the diagnostic data at a molecular level, improving the follow-up of disease progression and monitoring of HCMV therapy[86].

8.3.2 Branched DNA assay

This new technique is intended to monitor patients at risk of developing HCMV disease (AIDS patients, transplant recipients), by quantitating virus levels using the HCMV bDNA assay early in the course of infection. In this assay, peripheral blood leukocytes are lysed.

HCMV is denatured and hybridized with DNA probes directed against its *gb* gene (major envelope glycoprotein). Hybrids are captured on the surface of microtiter wells. Amplified (bDNA) molecules are bound to the captured hybrids prior to detection with an alkaline phosphatase-labeled probe and chemiluminescent substrate. Although a very reproducible technique, unfortunately, it is relatively insensitive compared to PCR. Improved sensitivity has been achieved by the reduction of nonspecific hybridization using modified non-natural nucleotides in generic sequences and the incorporation of an additional amplification step. A recent study has shown 100% agreement between antigen staining results and the bDNA assay for the diagnosis of HCMV polyradiculopathy[87].

8.3.3 Hybrid capture assay

This is a signal-amplified solution hybridization assay that utilizes unlabeled RNA probes, antibodies to RNA:DNA hybrids, and a chemiluminescent detection system, which is similar to that of the enzyme-linked immunosorbent assay (ELISA). This is technically easy to perform. However, the main disadvantage is relative time consumption, with results taking about 2 days to finalize. A second-generation assay has been developed providing a higher sensitivity, but no change in the assay procedure or turn-around time is achieved.

8.4 Serologic testing

The serological diagnosis is used to document the susceptibility to HCMV or HCMV immunity resulting from infection at some time in the past. It can also identify individuals with recent primary HCMV infection. The documentation of immunity resulting from previous infections requires a serological procedure that is specific and sensitive for the detection of low titers of IgG antibody to HCMV.

Several procedures have been formatted for detecting antibodies to HCMV. Methods such as complement fixation, indirect hemagglutination, immune adherence hemagglutination, neutralization, indirect immunoperoxidase, and radioimmunoassay have the disadvantages in laboratory practice of being insensitive, poorly standardized, and labor-intensive. Indirect and

anti-complement immunofluorescence tests and several modifications of ELISA methods are most commonly used in clinical laboratories today. In general, these quantitative assays detect antibodies to HCMV with a high level of sensitivity compared to complement fixation. One criterion for optimal matching of donor to recipient for organ transplantation is the serostatus of these individuals to HCMV. The highest risk for HCMV infection in a seronegative recipient is transplantation of an organ from a donor who has evidence of previous HCMV infection, which is denoted by antibodies to the virus. In contrast, HCMV seronegative patients are optimally matched with a donor who also does not have detectable antibodies to the virus.

The presence of IgG antibodies against HCMV by any of these assays is indicative of past infection. Since seropositive individuals experience reactivation of latent HCMV, the presence of IgG antibodies against HCMV is also a marker of potential infectivity. The diagnosis of primary HCMV infection can be made serologically by using any of these assays for the HCMV IgG antibody in order to document seroconversion. The interpretation of a single high titer of antibody to HCMV is difficult, because normal HCMV immune subjects without recent infection have a broad range of antibody titers to the virus with all these assays. Similarly, the interpretation of an increase in HCMV IgG titer from an initial positive serum to one that is four times higher over a period of weeks (seroconversion) is difficult, since normal immune subjects may have some variation in HCMV antibody titer over time. The effect of the reactivation of latent HCMV upon IgG titer is also unknown. Serodiagnosis based on a single high titer or rise in titer from an initially positive serum depends upon detailed knowledge of the usual variation range of the assay being used in the laboratory performing the test. If a presumptive diagnosis of recent primary HCMV infection is made, it should be confirmed by other methods. Several methods including the indirect immunofluorescence, immunoperoxidase, ELISA, and rapid immunoassay can be adapted to measure IgM antibody to HCMV[51]. Other assays, such as indirect hemagglutination and neutralization, detect a combination of IgG and IgM antibodies in unfractionated sera and can be used to measure HCMV-specific IgM antibody if IgG is removed from the sample before testing. These methods demonstrate that HCMV IgM antibodies are produced by most individuals who have primary HCMV infections. The detection of IgM antibodies can be used to detect infection in immunocompromised patients. However, it should not be recommended for immunocompromised individuals. The HCMV IgM antibody is

detectable for a variable period after the onset of infection and, therefore, its presence may indicate infection that occurred as long as 6 months before the serum was obtained. Some individuals with recurrent HCMV infection may develop the HCMV IgM antibody. The frequency of this has not been definitely established for different assay procedures. This may be a common finding in organ transplant recipients. At present, HCMV IgM results should be interpreted in conjunction with IgG titers and viral cultures and in the context of the clinical findings. In general, a diagnosis of presumptive primary HCMV infection should not be based on a positive HCMV IgM titer alone, but should also be substantiated by viral cultures or other techniques. Table 4 shows the interpretation of laboratory tests for HCMV infection and immunity.

Table 4 Interpretation of laboratory tests for HCMV infection and immunity[3]

Antibody titers		Viral culture of urine/saliva	Interpretation	Clinical correlates
IgG	IgM			
-	-	-	No evidence of infection	Susceptible individual
+	-	-	Infection at some time, probably not recent if culture negative	Immune individual or passive antibody (e.g., from transfusion, transplacental acquisition)
+	+	+	Probable recent primary infection, reactivation not excluded	HCMV mononucleosis, reactivation in transplant patients, congenital HCMV[a]
+	-	+	Probable recurrent infection, possible recent primary infection	Reactivation in normal or immunocompromised patients, congenital HCMV[a] primary infection diagnosed after decline of IgM

[a] If culture obtained in first month of life.

9. Trends in laboratory diagnosis of cytomegalovirus infection

Recent advances in the laboratory diagnosis of CMV have focused on newer molecular based methodologies. The CMV pp65 antigenemia assay is a non-molecular method that is popular as a semi-quantitative method, and it remains the gold standard to which the new molecular methods are compared. The Digene CMV DNA hybrid capture assay is a non-amplification molecular method, which has a use equivalent to the pp65 antigenemia assay. Nucleic acid amplification by PCR methods has become the most widely used diagnostic tool in viral detection. For HCMV, PCR assays are becoming increasingly popular in the field of diagnosis, supplementing and/or replacing conventional techniques such as the time-consuming HCMV antigenemia or cell culture assays.

There are numerous versions of in-house DNA PCR methods employed by different laboratories as well as several commercially available systems. Since HCMV DNA can be detected from latent viruses in clinical samples, the results from qualitative HCMV PCR must be correlated with clinical presentations and other laboratory tests in order to guide physicians in their patient care practice. In recent years, the availability of quantitative PCR using the Roche AMPLICOR CMV MONITOR (RACM) test (Roche Molecular Systems, Pleasanton, CA), and/or in-house real-time CMV DNA PCR using the LightCycler (Roche Molecular Biochemicals, Indianapolis, IN) provides promising diagnostic tools for CMV infection. Other forms of equipment (such as TaqMan from Applied Biosystems, Foster City, CA) capable of performing real-time PCR are also available[88, 89]. With these quantitative PCR assays, the correlation of the unknown quantity of target DNA in a clinical sample can be determined from a built-in standard curve and expressed as copy number(s) per unit volume of sample. The RACM test has the advantage of uniformity compared to many in-house quantitative CMV PCR assays that may differ between laboratories in multiple aspects such as primers, target, standards and controls, reaction conditions, signal materials, methods of calculation, etc[90]. The real-time quantitative PCR methods have a promising future in clinical virology laboratories because the published data indicate that these methods are comparable to or slightly more sensitive than the CMV pp65 antigenemia assay. However, the real-time quantitative PCR method is an expensive test for HCMV detection. The goal for new diagnostic methods is to improve their sensitivity, specificity

and clinical relevance so that acute CMV infections of either new or reactivation form can be differentiated from latent infections. Quantitative DNA-based assays, and RNA-based amplification such as the NASBA assay, have the potential to provide data that can allow this distinction to be made, but more data concerning clinical correlation is required. Laboratories with a low volume of requests for CMV quantitation can handle the labor-intensive CMV pp65 antigenemia assay, but high volume laboratories should consider one of the new molecular methods to accommodate these requests.

10. Development of the PCR technique for HCMV detection[91]

PCR assay for the diagnosis of HCMV infection is becoming increasingly popular in the field of diagnosis, supplementing and/or replacing conventional techniques. PCR is a simple and rapid method for the detection of HCMV DNA. It provides an early diagnosis of HCMV infection. Despite the high cost and need for more specimens to perform the quantitative PCR, the qualitative PCR remains an important tool for the diagnosis of HCMV infection.

The PCR technique for HCMV detection was first developed by Demmler GJ et al.[31] and Shibata D[32] in 1988. Initial development of the PCR technique for HCMV detection was deemed successful when able to selectively amplify and detect specific HCMV DNA. A variety of amplification targets has been used so far for the detection of this viral nucleic acid[1, 2]. Both optimal specimens in different patient groups[92-95] and the optimized component of PCR reaction were selected for HCMV detection[96].

10.1 Specimen selection from different patient groups

The majority of clinical specimens used for the diagnosis of human HCMV are urine, saliva, throat swab, blood, bronchoalveolar lavage fluid, and tissue specimen. Urine and saliva samples are usually used to diagnose congenital or perinatal infection due to HCMV. For adults with hepatitis or infectious mononucleosis, blood (preferably) and urine are acceptable samples. Blood and urine specimens should be obtained weekly from organ transplant patients to monitor the presence of HCMV infection in these patients. PCR assay of conjunctiva scraping, aqueous or

vitreous fluids has been shown as potentially useful for the diagnosis of infectious retinitis in both HIV-infected and immunocompetent patients. By PCR assay of conjunctiva scraping, aqueous or vitreous humor, HCMV DNA has been detected in most patients with clinically diagnosed HCMV retinitis.

10.2 Primer design

One critical parameter for successful amplification in a PCR is the correct design of the PCR product, as well as define the T_m of the amplified region, a physical parameter that has been shown as important in product yield. Well-designed primers can help avoid the generation of background and nonspecific products. Primer design also affects the yield of the product greatly. When poorly designed primers are used, no or very little product is obtained. Whereas, correctly designed primers generate an amount of product close to theoretical values of product accumulation in the exponential phase of the reaction. Optimization of reaction conditions, such as adjusting the magnesium concentration and using specific cosolvents such as dimethylsulfoxide (DMSO), formamide, and glycerol, may be required, even with a good primer pair. However, primers that do not follow the basic rules of primer design may not benefit from changes in the reaction conditions. In this case, designing a new primer set might save both time and money.

Currently, there is a wide range of computer programs for performing primer selection. They vary significantly in selection criteria, comprehensiveness, interactive design, and user-friendliness. Specialty primer design software programs that offer enhanced user interfaces, additional features, and updated selection criteria are also available, as are primer design options that have been added to larger, more general software packages.

10.3 Optimization of PCR conditions

PCR has several qualitative characteristics such as specificity, efficiency (yield of product), sensitivity and fidelity (error rate). First of all it is very important to evaluate the main characteristic for each PCR case. For the detection of rare copy sequences, genetic analysis or forensic testing, the sensitivity and specificity of PCR are very important. In the case of

preparative PCR for the synthesis of probes for hybridization or sequencing, specificity and high yield of a product are highly important. For the synthesis of DNA fragments, specificity of reaction and fidelity (error rate) are of greater importance. If the product obtained under standard conditions is not satisfactory, it is recommended to change the components of the mix and reaction conditions, which have an impact on most PCR characteristics.

10.3.1 MgCl$_2$ concentration

The Mg^{++} ions concentration may affect all the following procedures: primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity. High MgCl$_2$ concentration increases the yield of non-specific products and lowers the fidelity of synthesis (and increases the incidence of primer-dimers). Meanwhile, a low concentration decreases the yield of PCR product. Mg^{++} ions form complexes with dNTPs, primer and DNA templates. Thus, the optimal concentration of MgCl$_2$ has to be selected for each given experiment. If the DNA samples contain EDTA or other chelators, the MgCl$_2$ concentration should be raised proportionally in the reaction mixture.

10.3.2 pH of PCR buffer

The higher pH of buffer gives the DNA strands greater protection against depurination and nicking during thermal cycling. Therefore, buffers with a higher pH value are recommended for PCR of long DNA fragments. At a low pH of reaction mixture, fidelity of DNA synthesis, catalyzed by *Taq* DNA polymerase, increases.

10.3.3 Adjuncts and cosolvents

Bovine serum albumin (BSA) in PCR mixture can stabilize *Taq* DNA polymerase and be helpful when a DNA template is not pure. Glycerol stabilizes *Taq* DNA polymerase during PCR and improves the yield of the product. Glycerol may also serve as a

cosolvent in PCR[4]. Cosolvents, formamide and dimethyl sulfoxide (DMSO), can be used at low concentrations in PCR and may help to amplify a GC-rich DNA template. If cosolvents are used, the amount of *Taq* DNA polymerase in the reaction mix should be increased, because the DMSO and formamide concentrations, above approximately 50%, inhibit enzyme activity. The addition of cosolvents decreases the melting temperature of the primer-template DNA duplex. In such cases, the annealing temperature should be adjusted experimentally. DMSO is especially useful in amplification of long DNA fragments. DMSO may also be used in PCR to reverse the inhibitory effects of polysaccharides.

10.3.4 Template DNA

Usually, the amount of template DNA is in the range of 0.01-1.0 ng for plasmid or phage DNA and 0.1-1.0 μg for genomic DNA - a total reaction mixture of 100 μl. Higher amounts of template DNA usually increase the yield of nonspecific PCR products. However, if the fidelity of synthesis is crucial, maximal allowable template DNA quantities should be used to increase the percentage of "correct" PCR products. Nearly all routine methods are suitable for template DNA purification. One has, however, to keep in mind the fact that even traces of agents used in DNA purification procedures (phenol, EDTA, proteinase K, etc.) strongly inhibit *Taq* DNA polymerase. Ethanol precipitation of DNA and repetitive treatment of DNA pellets with 70% ethanol is usually effective in removing traces of contaminants from the DNA sample.

10.3.5 Primers

Optimal composition of PCR primers is 18-25 nucleotides in length, with a GC content of around 40-60%. The C and G nucleotides should be uniformly distributed within the full length of the primer. More than three G or C nucleotides at the 3'-end of the primer should be avoided, as nonspecific priming might be increased in such cases. The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimers and hair-pin formation. The melting temperature of flanking primers

should not differ by more than 5 °C, therefore, the GC content and length must be chosen accordingly. It is recommended to check for all possible complementary sites between primers and the template DNA. If primers are degenerate, at least 3 conservative nucleotides must be located at the 3'-end of the primer.

For PCR of long DNA fragments, it is recommended that primers of up to 21-34 nucleotides in length, with a melting temperature near 63-68 °C, should be used. The higher annealing temperature enhances reaction specificity.

The estimated melting and annealing temperatures of the primer is based on primer length. If the primer is shorter than 25 nucleotides, the melting temperature (T_m) is calculated by using the following formula: $T_m = 4 (G+C) + 2 (A+T)$. G, C, A, and T represent the numbers of respective nucleotides in the primer. Annealing temperature should be approximate 5 °C lower than the melting temperature. If the primer is longer than 25 nucleotides, it is recommended to calculate the melting temperature using special computer programs, where the interactions of adjacent bases, the influence of salt concentration etc., are evaluated.

10.3.6 Primer to template concentration ratio

One of the most important concepts in PCR is the optimal primer to template ratio. If the ratio is too high, primer-dimers are formed, as occurs in conditions of very diluted template or excess primer. If the ratio is too low, the product will not accumulate exponentially, since newly synthesized strands will renature after denaturation. Primers are in a 10^7 molar excess with respect to template. For most applications, regardless of template concentration, the primer concentration cannot be raised much higher than 0.5 µM (12.5 pmol per 25 µl reaction) because of primer-dimer formation.

10.3.7 dNTP concentration

It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP, dTTP) as an inaccuracy of concentration in at least one dNTP dramatically increases the misincorporation level. When the maximum fidelity of the PCR process is absolutely required,

the final dNTP concentration should be 10-50 mM, since the fidelity of DNA synthesis is maximal in this concentration range. In addition, the concentration of MgCl$_2$ should be selected empirically, starting from 1 mM, and increasing in 0.1 mM steps, until a sufficient yield of PCR product is obtained. For long PCR amplifications, it is recommended to increase the concentration of each dNTP in the reaction mixture up to 350-500 µM.

10.3.8 *Taq* DNA polymerase

Usually, 2-3 U of *Taq* DNA polymerase are used in 100 µl of reaction mixture. Higher *Taq* DNA polymerase concentrations may result in nonspecific products. However, if inhibitors are present in the reaction mixture (e.g. the template DNA used is not highly purified), higher amounts of *Taq* DNA polymerase (4-5 U) are helpful in obtaining a better yield of amplification products.

10.3.9 Temperature cycling

Initial denaturation step. The complete denaturation of the DNA template at the start of PCR is of key importance. Incomplete denaturation of DNA results in an inefficient utilization of template in the first amplification cycles and a poor yield of PCR product. It is recommended to perform the initial denaturation over an interval of 1-3 min at 95 °C if the CG content is 50% or less. This interval should be extended up to 10 min for GC-rich templates. If the initial denaturation is 3 min or shorter at 95 °C, *Taq* DNA polymerase can be added into the reaction mixture before the process. If longer initial denaturation or a higher temperature is necessary, *Taq* DNA polymerase should be added only after the initial denaturation, as the stability of enzyme dramatically decreases over 95 °C.

Denaturation step. Usually, 1-2 min denaturation at a temperature of 94-95 °C is sufficient, since the PCR product synthesized in the first amplification cycle is significantly shorter than the template DNA, and is completely denatured under these conditions. If the amplified DNA has a very high GC content, the denaturation time may be increased up to 3-4 min. Alternatively, additives facilitating DNA denaturation such as glycerol, DMSO, or formamide

should be used. In the presence of such additives, the annealing temperature should be adjusted experimentally.

Primer annealing step. Usually, the optimal annealing temperature is 5 °C lower than the melting temperature of the primer-template DNA duplex. Incubation for 1-2 min is usually sufficient. However, if non-specific PCR products are obtained in addition to the expected product, increasing stepwise by 1-2 °C should optimize the annealing temperature.

Extending step. Usually, the extending step is performed at 70-75 °C. The DNA synthetic rate of *Taq* DNA polymerase is highest at this temperature (2-4 kb/min), and 1 min extending time is sufficient for the synthesis of PCR fragments up to 2 kb. When larger DNA fragments are amplified, the extending time is usually increased by 1 min for each 1,000 bp. If the primer annealing temperature is more than 55 °C, it is possible to perform annealing and extending at the same temperature because the activity of *Taq* DNA polymerase at this temperature is sufficient for the synthesis of PCR products up to 1-1.5 kb.

Number of cycles. The choice of the number of PCR cycles depends on the amount of template DNA in the reaction mixture and the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25-35 cycles are usually sufficient.

Final extending step. After the last cycle, the samples are usually incubated at 72 °C for 5-15 min to fill-in the protruding ends of newly synthesized PCR products. Also, during this step the terminal transferase activity of *Taq* DNA polymerase adds extra A nucleotides to the 3'-ends of PCR products. Therefore, if PCR fragments are to be cloned into T/A vectors, this step can be extended up to 30 min.

11. Prevention of contamination in the PCR process

The PCR laboratory should be separated from other laboratories. All equipment is used solely for the purpose of PCR and no PCR products are allowed within the confine of this sanctuary. Also, with the sliding glass door shut, it is completely quiet inside, providing the perfect meditative area for PCR.

Avoidance of contamination during the preparation of genomic DNA should be strictly carried out. Great care should be taken to avoid contamination from any other source of DNA. The PCR technique is a powerful method that can amplify contamination in excess of the detection level. Therefore, DNA should be purified by a standard method that has been reported to the scientific literature and validated in the laboratory. DNA should be suitably stored to protect the integrity of the material.

The laboratory should have documented procedures, which have been constructed to eliminate potential causes of contamination, including training of the operator. If contamination does occur, all procedures should be reviewed and appropriate corrective action taken. Proposed changes to procedures should be validated prior to their introduction. In order to avoid contamination, the use of separate working stations or clearly defined work areas is beneficial for each stage of the PCR process, for example:

- One to prepare reagents. This is particularly important in avoiding contamination of primers.

- One dedicated to pre-PCR manipulation, e.g. DNA isolation. A Class II Laminar Flow cabinet should be able to prevent contamination of the sample with DNA from the operator.

- One dedicated to setting up PCR.

- One for manipulation of PCR amplified DNA. PCR amplified products should be kept away from areas used for pre-amplification manipulation and reagent preparation.

Each working station should be adequately and independently equipped. However, the use of such working stations should not absolve the laboratory from procedures constructed to eliminate contamination.

Examples of measures, which contribute to minimizing contamination, include:

- The use of new sterile, disposable plastic tubes or glassware for handling DNA.

- The use of freshly prepared and sterile materials and reagents when preparing solutions for DNA samples, particularly dH_2O and Tris buffers.

- Aliquoting reagents in small amounts to minimize the number of repeated samplings.

- A change of gloves and coats when moving between the areas dedicated for pre- and post-PCR manipulations.

- The use of positive displacement dedicated to pipettes or plugged tips to carry out PCR preparations.

Routine wipe-tests of pre-amplification work areas should be performed. If an amplified product is detected, the area must be cleaned to eliminate contamination and retested, and measures should be taken to prevent future contamination.