CHAPTER II
LITERATURE REVIEW

1. Biology of Human cytomegalovirus (HCMV)

1.1. Taxonomy

HCMV is a member of the betaherpesvirinae subfamily, one of three herpesvirus subgroup (Table 1.), whose virion structure, kinetics of viral gene expression and persistence for the long time of their host are typical of other herpesviruses. However, its strict species specificity, salivary gland tropism, and slow growth in cell cultures differentiate it as the prototype betaherpesvirus.

1.2. Viral structure and viral genome

The mature virions of HCMV are spherical or pleomorphic and have an overall diameter of 200 nm. HCMV is an enveloped virus with includes from outside to inside (Figure 3a):

i.) An envelope with surface projections consisting of at least 25 to 30 virion-encoded proteins and glycoproteins

ii.) A tegument consisting of amorphous material,

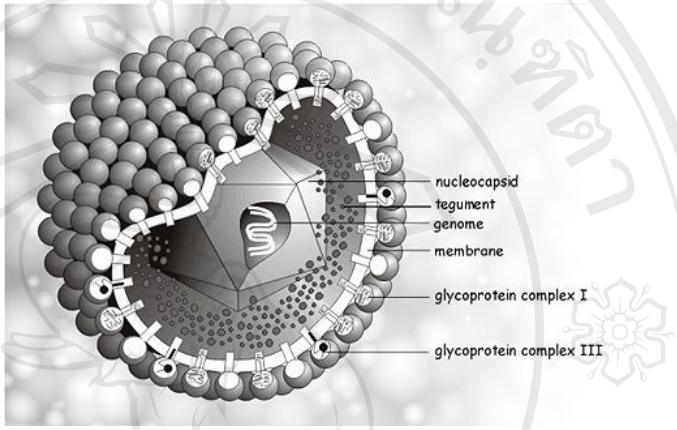
iii.) An icosahedral nucleocapsid 110 nm in diameter with 162 prismatic capsomeres.

iv.) A 64 nm core consisting fibrillar spool on which the DNA is wrapped.

The genome consists of single molecule of linear, double-stranded DNA approximately 240 kbp in size, which makes it the largest of all herpesviruses. The G + C content are high. It has unique long (UL) and short (US) sequences, both of which are bounded by inverted repetitive sequences. The repeats of L component are designated *a b* and *b'a'*; those of the S component are *a'c'* and *c a* (Figure 3b). The

number of sequence repeats at the L-S junction and at the L terminus is variable. The genome encodes about 200 open reading frames. Until now, more than 30 structural proteins, and at least as many nonstructural proteins have been described [18]. The AD169 laboratory strain is the only completely sequenced HCMV.

(a)



(b)

Figure 3. Structure of human cytomegalovirus virion (**a**) and human cytomegalovirus genome (**b**). The lettering above designate the following features: The L-terminal *a* sequence (a_L), a variable (n) number of additional *a* sequences (a_n), the L-terminal *b* sequence of the L-component (*b*), the unique sequence of the L component (*UL*), the repetitions of the *b* sequence (*b'*) and of a variable (m) number of *a* sequences (a'_m), the inverted *c* sequence (*c'*), the unique sequence of the S component (*US*), the S-terminal *c* sequence repeat (*c*), and the S-terminal *a* sequence repeat (a_S) with variable number (n) of additional copies of the *a* sequence (a'_n).

Table 1. The members of *Herpesviridae* Family [19]

Subfamily	Genus	Host category	Growth in culture	Type species	Example
Alphaherpesvirinae	*Simplexvirus*	Vertebrates	1-4 days	*Human herpesvirus 1*	Herpes simplex virus 1
	Varicellovirus	Vertebrates	5-7 days	*Human herpesvirus 3*	Varicella-zoster virus
Betaherpesvirinae	*Cytomegalovirus*	Vertebrates	1-4 weeks	*Human herpesvirus 5*	Human- cytomegalovirus
	Muromegalovirus	Vertebrates	6-8 days	*Mouse cytomegalovirus 1*	Murine- cytomegalovirus
	Roseolovirus	Vertebrates	1-3 weeks	*Human herpesvirus 6B*	Human herpesvirus 6
Gammaherpesvirinae	*Lymphocryptovirus*	Vertebrates	3-8 weeks*	*Human herpesvirus 4*	Epstein-Barr virus
	Rhadinovirus	Vertebrates	2-3 weeks	*Ateline herpesvirus 2*	Saimiri-ateles-like herpesvirus

* Do not growth in routine culture

1.3. Viral multiplication

1.3.1. Virus binding and penetration

Virus attachment and penetration are rapid. The poorly characterized receptor for HCMV is widely distributed among host cell types, and contributes to the broad viral tropism observed during natural infections. Viral entry is the result of a cascade of interactions between viral and cellular proteins that culminate in fusion of the virion envelope with the cellular plasma membrane by pH-independent mechanism. During the initial virus-cell interactions, HCMV attaches to the cell surface by low-affinity binding of gB (UL55) to heparan sulfate proteoglycans. The subsequent interaction of gB (UL55) with its nonheparin receptor then turns the weak adhesion of the viral particle into a more stable binding or docking state. However, final fusion of the viral envelope with the cell membrane to allow viral penetration has not yet been defined. Fusion of the virus and cell membrane is followed by entry into the host cytoplasm of the nucleocapsid and tegument proteins, and their translocation into nucleus is rapid [20].

1.3.2. Viral replication

Replication takes place in the nucleus. Replication starts with circularization of viral DNA. Transcription and translation are coordinately regulated and sequentially ordered in a cascade which involved 3 groups of genes; Immediate early (IE), early (E), late (L) genes. Immediate early genes are transcribed by nuclear enzymes, and mRNAs are transported to the cytoplasm and translated. Regulate proteins are then transported to the nucleus and are involved in the synthesis of early mRNAs. Early proteins are involved in the replication of the viral DNA by a rolling circle mechanism. Late mRNAs are mostly translated into structural proteins. [19].

1.3.3. Regulation of viral gene expression

During productive infection, the HCMV genome is expressed in a temporally coordinated and regulated cascade of transcriptional events that lead to the synthesis of three categories of viral proteins described as immediate-early (IE or α), early (E or β), and late (L or γ), based on the appearance of different classes of CMV-specific proteins during each interval. The IE period is defined up to 2 to 4 hours post-infection. It is characterized by the restricted transcription of specific segments of the genome and the production of regulatory IE proteins, which are required for the further transcription of the CMV DNA and the transition into the E phase of CMV replication. The E period is defined as beginning after the IE period and persisting through the long eclipse phase of CMV replication. Much larger portions of CMV genome are transcribed continuously during this period. In addition, a distinct class of infected cell proteins are synthesized, most of them necessary for viral DNA replication. The L period occurs from 36 to 48 hours after viral infection and coincides with the production of virion structural proteins and the release of infectious virus [6].

1.3.4. Viral assembly, maturation, and release

Formation of HCMV capsids and packaging of viral DNA occur in the nucleus. Subsequently, nucleocapsids acquire a primary envelope by budding at the nuclear membrane, and then mature in the cytoplasm before leaving the cell via an exocytotic-like pathway [18].

1.4. Target cells of HCMV infection

HCMV infects a wide range of epithelial tissues *in vivo*. The ductal epithelial cell is the most commonly infected and develops a typical cytopathology with the cell enlargement and rounding. However, during natural infection, it is thought that HCMV replicates productively in epithelial cells, endothelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, granulocytes, and monocyte-derived

macrophages. Thus, viral DNA can remain latent in macrophage-granulocyte progenitors in the bone marrow and in peripheral monocytes. In contrast, the only cells fully permissive for *in vitro* replication of laboratory strains are human skin or lung fibroblasts, whereas clinical isolates replicate preferentially on endothelial cell cultures [20].

1.5. Pathogenesis of HCMV infection

HCMV is an ubiquitous virus which infects most individuals by childhood in developing countries and late adulthood in developed countries. Similar to other herpesvirus, primary HCMV infection is followed by persistent infection, and viral latency from which it may be reactivated following appropriate conditions.

Primary infection typically starts with replication in mucosal epithelium as a result of direct contact with infectious secretions from an infected individual. Then, virus disseminates in the host via leukocyte-associated viremia. This systemic phase may last for several months. Cell-free infectious virus was rarely found in blood [21]. Peripheral blood neutrophils, monocytes, and epithelial cells have all been observed to carry infectious virus in immunocompromised hosts, but the role of particular leukocyte subsets as vehicles for dissemination during primary infection in the immunocompetent host is still unclear. The systemic phase of primary infection is accompanied by persistent viral shedding in urine, saliva, breast milk, and genital secretions, which represent important sources for transmission between hosts. Viremia continues for a long period of time after adaptive immune response can first be detected [18].

Pathogenesis of HCMV infection is directly linked to the immune status of the host. HCMV infection of immunocompetent hosts may cause a mild febrile illness but is usually asymptomatic. In contrast, primary infection or virus reactivation in newborns and immunocompromised hosts, such as organ and bone marrow transplant recipients, patients on immunosuppressive drugs, and HIV-1 infected people, can cause severe symptoms or death [22, 23].

1.6. Latency and reactivation

The term latency is defined as a persistent infection in which the viral genome is present but gene expression is limited and infectious virus is not produced. It is presently unclear whether HCMV exists in some particular cells but no virus particles are produced until the cell receives some external stimulus or whether it establishes a chronic, persistent infection producing undetectable level of virus.

1.6.1. Sites of latency

There was an evidence of multiple sites for latency to occur. HCMV can be transmitted by blood transfusion and through solid organs transplant such as kidney, heart and liver. Viral DNA has been detected in uterine tissue and in arterial walls of patients with atherosclerosis. Peripheral blood monocytes, CD34+ hemopoietic progenitor cells, and endothelial cells are major sites of HCMV latency in human. Moreover, only very small percentages (0.01%) of latently infected cells are present in bone marrow-derived mononuclear cell preparation. Experiments in mice have been identified that sites of murine CMV latent infection may be whole blood, heart, lung, liver, spleen, brain, salivary glands, prostate, and kidney [22].

1.6.2. The latent state

The mechanisms by which the CMV genome enters and is maintained in the latent state are poorly understood. Entire HCMV genome is present in latently infected cells. However, whether the DNA is integrated or exists in the form of either circular or concatenated molecules not integrated into host DNA genome is still unknown. In addition, the number of copies of CMV DNA in latently infected tissue does not reflect the amount of preceding virus replication in that tissue [22].

Productive infection required regulation of IE gene expression to sufficient levels. During latent infection, DNA replication does not occur and the L genes are not produced due to limited expression of IE gene.

1.6.3. Reactivation from latency

Reactivation of infection can be defined as indefinite, but intermittent, excretion of the virus from single or multiple sites, such as saliva, urine, tears, cervicovaginal fluid, breast milk, and semen. Reactivation is thought to occur as a result of at least three collaborating events;

i.) Cytokine stimulation of latently infected cells causing differentiation into permissive cell type that support viral replication.

ii.) Viral amplification caused by reduced immune surveillance that leads to a widespread systemic infection with a readily detectable viremia and virus in many tissues.

iii.) Disease states developing as a result of a poorly understood collaboration of immune-, organ-, and virus-specific determinants.

1.7. HCMV transmission

HCMV infection is life long. Following initial acquisition of HCMV, infectious virus is present in urine, saliva, tears, semen, and cervical secretions for months to years. Latent HCMV may activate and produce infectious virions that are shed in the saliva, urine, and other body fluids. Thus, HCMV transmission occurs through very close contact, including;

i.) ***Vertical transmission*** Infants are infected from their mothers via placenta, during delivery, or by breast feeding. Intrauterine infection occurs in only one-third of pregnant women with primary infection. Although macrophages in the placenta may constitute a barrier, but it is still unknown how the fetus escapes infection. Ingestion of infected maternal cells in genital secretions during delivery or of breast milk are the main perinatal route of infection. Virus titers are usually low in breast milk, but long-term feeding results in the build-up of effective inoculums. Once ingested, the virus infects the mucosa of the oropharynx, esophagus, or the upper airways [20].

ii.) ***Horizontal transmission*** occur through person-to-person contact, blood transfusion, transplantation, nursing, sexual contact, and aerosol droplets. High rates of HMCV infection occur in settings where close contact with body fluids is expected,

such as in day care centers, or between preschool-aged children and their care giver. Therefore, these modes of transmission can explain the increasing HCMV prevalence with age and sexual activity.

2. Host responses to HCMV infection

HCMV infections are kept under control by the immune system. However, total clearance is rarely achieved, and the viral genome remains at selected sites in a latent state. Initiation of productive replication results in transient virus shedding and recrudescent disease, especially in individuals with impaired immune function.

2.1. Cell-mediated immunity

Several studies [24, 25] demonstrated that both natural killer (NK) cells and CMV-specific CD8+ cytotoxic T lymphocyte (CTL) response are required for protection of CMV recurrence. Suppression of CTLs caused reactivation and dissemination of natural infection. The virus-encoded proteins that are target of CTLs include structural and non structural forms. The high frequencies for pp65, major tegument component, and the 72-kDa IE1 proteins show that they are the main target of the CTL-mediated immune response [26].

2.2. Humoral immunity

During primary infection, immunocompetent individuals produce anti-HCMV immunoglobulin (Ig) M class antibodies that persist for 3-4 months, followed a few weeks later by IgG class antibodies that persist for life. The humoral response is importance for protection against a lethal challenge in mice after murine CMV gB immunization [27]. Intrauterine infection is less severe when transmitted by means of recurrent rather than primary maternal infection. Because during primary infection, woman who transmit the virus *in utero* have total IgG with low avidity and neutralizing activity, even though it still has high level [23]. Many CMV proteins are recognized by humoral immune system. The envelop glycoproteins, mainly gB and

gH, are the targets of virus-neutralizing antibodies in both human and mouse models [26]. The predominance of gB as a target is best explained by its dominant immunogenicity and abundance compared with other components of envelope. Anti-gH antibodies have a potent, complement-independent, but minor neutralizing activity.

2.3 Immune evasion by human cytomegalovirus

A characteristic feature of HCMV infection in the normal host is persistence, for months or even years, by the viral genome in a nonproductive form at specific anatomical sites. HCMV escapes the immune system through different mechanisms.

i.) Latency state the simplest way to avoid recognition by the immune system is to express no viral antigens. HCMV successfully employs this technique. It establishes itself in latency in circulating monocytes, bone-marrow progenitor populations including CD34+ cells and CD14 +cells, and probably endothelial cells.

ii.) Exploitation of immunologically privileged tissues for replication (i.e., epithelial cells of the salivary glands express an insufficient number of MHC Class I molecules to trigger clearance by CD8+ cell.)

iii.) Expression of genes that interfere with the immune response, for instance,

a) HCMV avoids the stringent control of MHC-I class restricted immunity by the expression of multiple genes that affect antigen presentation by MHC class I molecules. Several genes are identified as involved in this process, such as, US2, US3, US11, US6, and UL83 expressed during the IE phase of infection

b) HCMV induces the disruption of the microtubule network in macrophages, which reduces the surface MHC class II molecules reduce thus the recognition of infected cells by HCMV specific CD4+ T-cells. This mechanism leads to immune evasion and intracellular persistence.

c) HCMV effectively interferes with innate immunity. By interfering with MHC class I expression, HCMV infected cells become a target for NK cell killing. To avoid this lysis, HCMV infected cells can code their own

analogues of MHC I molecules which are recognized as inhibitory signal by NK cell receptors.

d) HCMV interferes with the chemokine-driven inflammation. HCMV-infected cells express viral G-protein coupled receptor (GPCR) homologues, encoded by US27, US28, UL33, and UL78, which are needed for virus replication *in vitro*. The US28-encoded GPCR binds to and sequesters several β-chemokines and, thus, enables the virus to evade the immune response.

3. HCMV infection in non-HIV infected population

3.1. HCMV infection in general population

Acquisition of HCMV arises progressively from an early age. A large percentage (50–90%) of healthy adults worldwide is chronically infected with HCMV [28]. The prevalence of antibodies to HCMV varies upon populations and geography. In developed countries, the overall seroprevalence is 30 – 70%. In particular groups such as homosexual men, poor socioeconomic groups and in developing countries the HCMV seroprevalence rates can exceed 90% [7]. In Thailand population, HCMV infection is common in general population. Among adult blood donors aged between 17 – 50 years, the seroprevalence of HCMV ranged from 70 – 94% [29-32], and did not statistically differ upon sex or age of sample population [30-32].

3.2. HCMV infection in pregnant women and infants

In industrialized countries, 50-70% of pregnant women are seropositive for HCMV [6]. Since most women are infected before puberty, reactivation is a more frequent cause of congenital infection than primary maternal infection, the latter presenting a greater risk to the fetus than the recurrent infection. Primary HCMV infection during pregnancy occurs in approximately 2% of women. In general, over 90% of primary infections in pregnant women are asymptomatic and are likely undetected. After primary infection, the transmission rate from mother to fetus has been estimated to be around 40%. The incidence of congenital infection is the highest

in poor communities. Congenital HCMV infection is the leading infectious cause of brain damage and hearing loss in children. When mother acquires the infection within the first trimester, the most severe sequels including intrauterine death of the fetus may develop. If the infection is acquired before the secondary trimester, severe brain damage, congenital heart defects and intrauterine growth retardation or even intrauterine death may occur. The consequences of infection acquired in the third trimester are not as severe; probably due to fetus formed almost completely organs or the beginning of maternal antibodies transplacental transfer.

The prevalence of HCMV infection in pregnant women was higher than non pregnant women [32]. In Thailand, 91 – 100% pregnant women are seropositive for HCMV IgG antibodies due to acquisition of HCMV before pregnancy [32-35].

In general worldwide, about 10% of infants are infected by the age of 6 months [20]. Among Thai children less than sixteen years old, seroprevalence of HCMV antibodies was 62 – 84% [29, 35, 36]. Diagnosis of congenital HCMV infection using IgM ELISA was confirmed in 1.6% among newborns aged of 1 day to 3 weeks, whereas it increased to 21% among infant aged 3 weeks to 1 year. The main clinical manifestations were hepatomegaly (94.3%) and splenomegaly (72.6%) [37]. Correlation between HCMV seroconversion and age, sex, number of children in family, family income and place of child growing factors were analysed. Only low family income was associated with HCMV infection in infants [36].

4. HCMV infection in HIV infected population

4.1. HCMV infection in HIV infected adults

Human cytomegalovirus is one of the most common opportunistic pathogens in immunocompromised patients. HCMV infection can result from reactivation of latent virus, reinfection in patients with past infection, or primary infection. HIV infected adults were more likely to have evidence of HCMV infection than HIV uninfected adults [38-40]. The severity of HCMV infection roughly parallels with the degree of immunosuppression [41]. The most severe infections are seen in AIDS patients with very low CD4+T cells count [42]. Prior to the use of HAART, it was

estimated that around 40% of adults would develop disease due to HCMV. Among adults with advanced HIV-1 infection, approximately 10% to 30% per year develop CMV disease [43]. Its severity ranges from self-limited febrile illness to multisystem disease that can be life-threatening or debilitating. The most common clinical manifestations among HIV infected patients with CMV disease is retinitis, esophagitis, and colitis. Less common clinical presentations of CMV disease in AIDS patients include encephalitis, peripheral neurophathy, pneumonitis, gastritis, and hepatitis [41]. Since the introduction of HAART, more than a fivefold decrease in the incidence of CMV disease has been reported [44].

Role of HCMV infection in disease progression is unclear. In one hand, HCMV infection is arguably the frequent opportunistic infection among adults with advanced AIDS. In one hand, HCMV infection has been associated with a more rapid progression to HIV disease [11-15, 45]. The age-adjusted relative risk of developing AIDS in HCMV-seropositive patients was 2-2.5 times higher than in HCMV seronegatives [11, 12, 14]. High IgG titer against HCMV have been correlated with disease progression in HIV infected patients [46]. HCMV/HIV-1 co-infected patients were also more likely to have detectable HIV p24 antigenaemia [11] and higher HIV-1 load than the patients who were infected with HIV-1 only [15], suggesting that HCMV may enhance HIV replication. HCMV viremia was associated with a decline in the HIV patient's clinical state, whereas HCMV negative patient did not show a progression to HIV-associated disease [47]. Moreover, the presence of multiple HCMV strains in HIV-1-positive patients is associated with progression to AIDS [48].

In contrast, other studies [49-51] did not find any relationship between progression to AIDS and HCMV status, but rather with age and CD4+T cell count. Also no difference in survival was observed between HCMV/HIV coinfected patients and HIV infected patients with HCMV seronegative [43]. Moreover, HCMV can reduce the cell surface expression of CCR5, which is the main co-receptor for HIV-1 [52], thus possibly limit the entry of HIV into target cells.

In Thailand, Tantivanich, et al [32] showed HIV infected patients had a lower HCMV seroprevalence and lower median of IgG titer than HIV negative patients (32.6% versus 53.4% and 80 versus 160, respectively). These results were contradictory with reports from Enzenberger, et al [39] and Quesnel, et al [40]., which

could be due to the number of patients included in the study and the number of CD4+T cell in HIV infected patients

CMV retinitis and central nervous system are the most frequent manifestations in northern Thai AIDS patients [53]. Additionally, HCMV can cause pneumonitis [54], chronic diarrhea [55], and hepatitis [56, 57]. More recently, as the result of CD4+T cell count recovery following initiation of HAART, immune recovery vitritis associated with posterior segment inflammation has been observed.

Whether HCMV infection and disease are simply markers of the immune dysfunction that follows HIV replication or whether HCMV infection itself promotes HIV progression, is debatable. However, the reduction of HIV viral load following the initiation of HAART has greatly reduced the HCMV load and HIV disease progression [7].

4.2. HCMV infection in HIV infected infants and children

Approximately 50% of children with HIV infection have serologic evidence of HCMV infection. Doyle M et al reported the significantly higher rate of *in utero* HCMV infection in HIV-infected infants than uninfected infants (21% vs. 3.8%, P = 0.008) [58].

In another study, among children born to HIV-infected woman, the rate of congenital HCMV infection in both groups was similarity (4.3% vs. 4.5%, respectively), but the rate of acquisition of HCMV during birth and infancy increased in HIV-infected infants compared to the uninfected infants, even though they were not breast-fed [13].

The mean survival of HIV/HCMV co-infected infants was about 25 months, which is shorten than HIV infected alone [58, 59]. Moreover, among HCMV/HIV-1 coinfected children with HCMV DNAemia had significant shorter mean survival time (42.5 versus 60 months; $P < 0.01$) and lower CD4+T cell count (218 versus 499 cells/μl; $P < 0.01$) than those without HCMV DNAemia [60]. Thus, HCMV DNAemia may represent a good prognostic indicator of severe outcome of HIV disease in HIV-1-positive children [21].

Children infected with HIV only had better clinical outcome than HCMV/HIV coinfected children [61]. Symptomatic CMV disease occurred more often in young HCMV/HIV co-infected children with low CD4+ lymphocytes count and elevated HIV p24 antigen concentrations [62].

Common manifestations in children are central nervous system (CNS) disease, pneumonitis, and gastrointestinal disease [8]. HCMV retinitis is less common in HIV-1 infected children than in adults [63]. HIV/HCMV co-infected infants develop more frequently progressive CNS disease than those with only HIV infection.

In Thailand, HCMV infection is more commonly found in HIV-infected infants whether congenitally or early infected until 18 months [64]. The prevalence of IgG antibodies reached 100% by the age of 3 years, indicating that primary infection must occur either during early infancy. e.g. by herizontal transmission or breast feeding [65].

Infection with HCMV in early life is associated with greater immunosuppression and may be associated with a more rapid progression of HIV infection in infants. Whether progressive immunodeficiency allows the emergence of CMV disease or HCMV infection causes more rapidly progressive HIV-1 disease or whether there is a more complex relationship remains to be determined. Moreover, the timing of acquiring HCMV infection also requires to be investigated.

5. Interactions between HCMV and HIV-1

Mechanisms through which HCMV and HIV interact on replication are still not clearly understood. General mechanisms regarding pathogenesis of CMV/HIV co-infection have been proposed as following:

5.1. Role of co-infection

In human sarcoma (HOS) cell line co-infected with CMV and HIV-1, the expression of LTR gene is increased 20 fold compared to HOS infected with HIV only, thus enhancing production of HIV-1 p24 antigen [66]. Also, HCMV could enhance HIV-1 replication via a mechanism involving both TNF-α and TGF-β [67].

Moreover, in chimpanzee model study [68], the chimpanzee CMV was documented that it could act as cofactor in enhancing HIV-1 replication and which possibly enhance the progression to HIV disease.

In contrast, *in vitro* study of Boccuni MC, et al [69] showed that CMV ppUL44, an early DNA-binding protein, can down regulates HIV-1 LTR transactivation and may slow down HIV-1 replication.

5.2. Receptor

5.2.1. Mimicking of HIV co-receptor

HCMV encodes a β-chemokine receptor (US28) that is distantly related to the human chemokine receptors CCR5 and CXCR4, the main receptors for the HIV-1 entry into CD4+ cells. It has been shown that CD4+T cells resistant to HIV entry, can be infected by HIV after the transfer of CMV US28 gene [70]. Thus, HCMV can expand the spectrum of cells that can be infected by HIV.

5.2.2. Induction of alternative HIV receptors

Immune complexes formed by HIV and non-neutralizing antibodies can also infect monocytes and macrophages through the receptors for the Fc portion of immunoglobulins (FcRs). McKeating JA, et al [71] demonstrated that FcRs induced by HCMV allow HIV-immune complexes to infect fibroblasts which are not permissive to HIV infection. Once HIV had entered CMV-infected cells through the FcR, its replication could be enhanced by CMV transactivating factors. If existing *in vivo*, this synergism between HIV and CMV could permit the spread of HIV to cells not expressing CD4 (CD4 negative cell), and potentially enhance immunosuppression.

5.2.3. Pseudotype formation

Pseudotype is a virion containing the RNA of HIV, enveloped with the surface glycoproteins derived from a co-factor virus. This pseudotype can infect cells using

the receptor for co-factor virus instead of the CD4 molecule. If CMV infects the same cell which is producing HIV particles, then HIV could potentially form pseudotypes which cell entry would no longer be restricted by the CD4 molecule and so would be able to bind to cells which contain receptors for CMV. Thus, CMV infection may facilitate HIV-1 RNA entry into ordinarily non-permissive cells [72].

5.3. Cytokine release

Cytokines, such as IL-8, could serve as mediators for enhancing HIV-1 replication [73]. HCMV can reside in bystander cells and release several cytokines [74], including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8, which could activate latent HIV proviral DNA through signal transduction [75]. Lurain, et al [15] reported that patients with detectable HCMV DNA were more likely to have higher interleukin-1β (IL-1β) and IL-8 levels than patients with undetectable HCMV DNA. This finding supports the hypothesis that HCMV can induce proinflammatory state, then activates HIV replication.

6. Laboratory diagnosis for HCMV infection

The laboratory diagnostic methods for evaluating HCMV infection in general can be divided into virological and serological techniques.

6.1. Virological techniques

HCMV can be isolated from several clinical samples, such as urine, saliva, vaginal secretions, amniotic fluid, and blood. Moreover, HCMV infected cells can be identified in biopsy samples (e.g. liver, lung) [20]. Virological detection of HCMV infection can be subdivided into non-molecular and molecular methods.

6.1.1. Non-molecular methods

6.1.1.1. HCMV culture assay

This technique is the gold standard method for diagnosis of HCMV infection. HCMV culture using allows the detection and quantification of infectious viral particles in body fluid including blood, saliva, or urine. Conventional methods consist in inoculation of low passage (<25) human embryonic lung or foreskin fibroblasts monolayer with patient's peripheral blood leukocytes (PBL) and then identification of the characteristic focal cytopathic effect (CPE). In which the cells of the monolayer will become swollen and retractile, this CPE can easily be detected by light microscopy. This CPE usually develops very slowly, and the cultures must be carefully observed for at least 21 days before being reported as negative. [20]. This method is expensive and time-consuming. However, HCMV culture assay is the needed for phenotypic detection of the emergence of a drug resistant HCMV strain. In the past, this technique was generally used for diagnosis for congenital HCMV infection in newborns.

6.1.1.2. Spin-amplification shell vial assay

This technique has successfully replaced the culture method, as it retains the specificity and sensitivity of viral isolation, but provides results within 24 hours. A human fibroblast monolayer is inoculated with 2 x 10^5 PBL and fixed 18 hours later. The nuclei of the fibroblasts are stained by immunofluorescence using monoclonal antibodies reactive with the major immediate early (MIE) protein p72. The assay is based on the assumption that each stained cell was infected by a single PBL carrying an infectious virus. However, this method still requires cell culture and feeder cells, and the cytotoxic nature of some specimens is unavoidable [76].

The accepted gold standard for diagnosis of congenital CMV infection is the detection of HCMV in urine or saliva within the first 2 weeks of newborn life. Viral excretion after that time may represent natal or postnatal acquisition of HCMV.

6.1.1.3. Antigenemia assay

The HCMV antigenemia test is based on the direct detection of an early structural HCMV antigen, a 65-kDa lower matrix phosphoprotein (pp65), which associated with DNA replication in leukocytes. Pp65 is not expressed in latently infected leukocytes [77]. HCMV antigen detection is measured by the quantitation of leukocyte nuclei positive for the pp65 in a cytospin preparation of 2 x 10^5 PBL using an immunofluorescence assay. This rapid assay provides a result in a few hours. Antigenaemia is an indirect marker of disseminated infection, since pp65 is transferred to uninfected leukocytes by infected cells after transient fusion of the plasma membranes and exchange of cytoplasmic material. By comparison with the test for infectious virus, the test for viral antigen gives a positive result earlier after the onset of infection and becomes negative later in the late phase of a systemic infection. The high levels of antigen are frequently found in patients with HCMV disease and low levels correlate with asymptomatic infections [20]. However, this method has not been evaluated for the diagnosis of congenital CMV infection.

6.1.2. Molecular methods

6.1.2.1 PCR conventional method

The polymerase chain reaction (PCR) has been used as the new gold standard for detecting a wide variety of templates across a range of scientific specialties, including virology. The method utilizes a pair of synthetic oligonucleotides of primers, each hybridizing to one strand of a double-stranded DNA (dsDNA) target, with the pair spanning a region that will be exponentially reproduced. The hybridized primer acts as a substrate for a DNA polymerase (most commonly derived from the thermophilic bacterium *Thermus aquaticus* and called *Taq*), which creates a complementary strand via sequential addition of deoxynucleotides. The process can be summarized in three steps; i) dsDNA separation at temperatures >90 ^oC, ii) primer annealing at 50-75 ^oC, and iii) optimal extension at 72-78 ^oC [78].

PCR was first used for HCMV DNA detection in the urine of congenitally infected newborns at the end of the 1980s [79]. When compared with the standard tissue culture isolation procedure, the PCR assay followed by dot blot hybridization showed

a sensitivity and specificity of 100%. Obvious advantages of PCR over culture were the small amount of sample required, the short time required for test results (24 to 48 hours versus 2 to 28 days), the ability to use frozen specimens with noninfectious virus, and no need for extensive DNA purification measures.

These results prompted clinical virologists to test for the presence of viral DNA in the blood of congenitally infected newborns. First, in 1992, Brytting et al [80] reported detection of HCMV DNA in the serum of 5 out of 5 congenitally infected infants tested within 2 weeks after birth, while two of these five newborns were negative for HCMV-specific IgM. In 1995, Nelson et al [81] reported detection of HCMV DNA in the serum of 18 of 18 (100%) infants with symptomatic congenital HCMV infection, 1 of 2 infants with asymptomatic congenital HCMV infection, and 0 of 32 controls.

In 1999, Revello et al [82] investigated the diagnostic and prognostic value of HCMV load as determined by different assays in the blood of 41 newborns with congenital infection and 34 uninfected newborns with respect to conventional virus isolation from urine. Sensitivities of HCMV DNAemia (by PCR), antigenemia, viremia, and IgM determination were 100%, 42.5%, 28.2%, and 70.7%, respectively, while specificity was 100% for all assays [82]. HCMV DNA was detected more frequently in whole blood than in plasma.

A further simplification of the procedure for detection of HCMV DNA in the blood of congenitally infected infants with dried blood spots (DBS) stored on filter paper was proposed [83], as originally suggested for human immunodeficiency virus type 1 [84]. This method showed 100% sensitivity and specificity with respect to virus recovery by culture.

6.1.2.2. Real time PCR assay

Recently, Real-time PCR has already proven itself valuable in laboratories around the world. In contrast to conventional assays, the amplicon detection could be visualized as the amplification progressed. The monitoring of accumulating amplicon in real time PCR has been made possible by the labeling of primers, probes or amplicon with fluorogenic molecules. This chemistry has clear benefices over radiogenic oligoprobes that include an avoidance of radioactive emission, ease of

disposal and extended shelf life. There are several major chemistries currently in used, and they can be classified into amplicon sequence specific or non-specific methods of real-time PCR detection. In this study, we will focus only the amplicon detection by 5'nuclease oligoprobes (Figure 4), which is classified into amplicon sequence specific real time PCR detection.

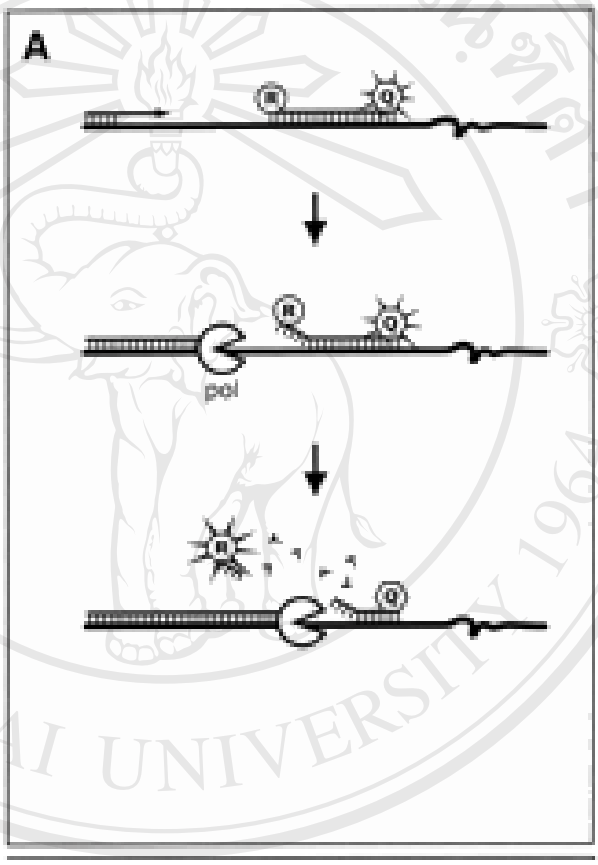


Figure 4. Amplicon detection by 5' nuclease oligoprobes. As the DNA polymerase (pol) progresses along the relevant strand, it displaces and then hydrolyses the oligoprobes via its 5'→3' endonuclease activity. Once the reporter (R) is removed from the extinguishing influence of the quencher (Q), it is able to release excitation energy at a wavelength that is monitored by the instrument and different from the emissions of the quencher.

Nuclease oligoprobes have design requirement that are applicable to the other linear oligoprobes chemistries to ensure the oligoprobes has bound to the template before extension of the primers can occur, it including;

i) A length of 20-40 nucleotides.

ii) A GC content of 40-60%.

iii) No repeats of a single nucleotide, particularly G.

iv) No repeated sequence motif.

v) An absence of hybridization or overlap with the forward or reverse primers.

vi) A T_M at least 5°C higher than that of the primers.

The fluorescence signal mirrors progression of the reaction above the background noise was used as an indicator of successful target implication. This threshold cycle (C_T) is defined as the PCR cycle at which the gain in fluorescence generated by the accumulating amplicon exceeds 10 standard deviations of the mean baseline fluorescence, using data taken from cycle 3 to 15. The C_T is proportional to the number of target copies present in the sample. HCMV quantitation by real time PCR in whole blood and plasma were highly correlated, when whole blood HCMV load $\geq$3.6 $\log_{10}$ copies/mL [85].

Schalasta et al [86] reported highly specificity and sensitivity of congenital HCMV detection in newborn, comparing to conventional PCR-ELISA and the rapid shell vial assay for detection of HCMV early antigen. Quantitation of HCMV DNA in blood or viremia by real time PCR during early infancy is associated with hearing loss and systemic CMV disease [87]. Moreover, real-time PCR could detect congenital HCMV infection in dried blood spots (DBS) collected up to 20 months earlier. This method is effective and less laborious for epidemiological studies and for identifying asymptomatic infected babies [88].

6.1.2.3 Others Nucleic acid amplification method

Detection of HCMV mRNAs in blood is a good marker for monitoring active HCMV replication and disease, since it detects mRNAs transcribed from IE or L gene in PBL or whole blood. Reverse transcription PCR was the first technique employed to detect viral transcripts.

More recently, determination of HCMV immediate-early mRNA in the blood with nucleic acid sequence-based amplification (NASBA) has been used to diagnose congenital HCMV infection in newborns. The immediate-early mRNA NASBA assay had 100% sensitivity in detecting 12 congenitally infected newborns examined during the first week of life and confirmed positive results of HCMV DNAemia and virus

recovery from urine. However, immediate-early mRNA was detected for a significantly shorter period of time (median 37 days) than DNAemia (median, 87.5 days; P = 0.04). Immediate-early mRNA detection in blood may represent a more reliable marker of active HCMV infection as no immediate-early mRNA was ever found in healthy newborns [89].

6.2. Serological technique

Serological determination of a recent or past HCMV infection in individuals bases on detection of IgM or IgG viral specific antibodies. A specific IgM response is a serologic evidence of recent primary infection or reinfection. Detection of IgM antibody by capture ELISA method with enzyme-labeled monoclonal antibody test had shown the specificity was nearly 95% and the sensitivity approximately 70% when congenitally infected infants were tested [82, 90]. Detection of anti-HCMV IgG antibodies is a useful way of documenting a past infection, and also represents a marker of potential infectivity, since reactivation of a latent infection may occur. Although serological methods for diagnosis of HCMV are readily available and inexpensive, they are unreliable for establishing the diagnosis of congenital infection. Detection of HCMV IgM antibody has a limited sensitivity in diagnosis of congenital HCMV infection. It may not be reliable as false positive may be induced by rheumatoid factor, antinuclear antibodies, and other cross-reactive factors not yet identified [20]. The presence of HCMV IgG antibodies in newborn aged less than 12 months may not be attend to determine HCMV infection, due to maternal antibodies.