

**DIAGNOSIS OF CYTOMEGALOVIRUS:  
CORRELATION BETWEEN VIRAL LOAD AND  
IMMUNOSTAINING IN PATIENTS  
WITH POST RENAL TRANSPLANTATION**

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**Thesis**

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**DIAGNOSIS OF CYTOMEGALOVIRUS: CORRELATION BETWEEN VIRAL LOAD AND IMMUNOSTAINING IN PATIENTS WITH POST RENAL TRANSPLANTATION****CHAMAIPORN SUWANASOPHON 4437539 TMTM/M****M.Sc. (TROPICAL MEDICINE)****THESIS ADVISORS: SURANG TANTIVANICH, M.S., PANBUBPA CHOOVICHIAN, M.D.****ABSTRACT**

Cytomegalovirus (CMV) is a major problem in renal transplantation recipients. Thus, it is important to use sensitive and specific diagnostic assays to detect CMV infection and identify the patients at risk of developing CMV disease in order to start preemptive treatment. In this study, peripheral blood was collected from 50 normal healthy subjects and 50 asymptomatic post renal transplantation recipients and tested for the presence of CMV by pp65 immunostaining, nested polymerase chain reaction (PCR), viral load quantitative PCR (Roche COBAS AMPLICOR test kit), and detection of the antibodies by ELISA.

The results showed that most of the subjects had some evidence of CMV infection (>80%). CMV infection was detected in most subjects by immunostaining and PCR assay, whereas the viral load test rarely detected CMV infection in asymptomatic people. The present data suggests that immunostaining and nested PCR could be used as the diagnostic tests of renal transplantation patients at risk of CMV infection.

**KEY WORDS: CYTOMEGALOVIRUS / IMMUNOSTAINING / VIRAL LOAD/  
COBAS AMPLICOR / RENAL TRANSPLANTATION**

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## LIST OF ABBREVIATIONS

Abbreviations	Term
C	cytosine
CMV	cytomegalovirus
°C	degree Celcius
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
<i>et al.</i>	et alii
G	guanine
hr	hour
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kbp	kilo basepair
min	minute
mL	millilitre ( $10^{-3}$ )
$\mu$ L	microlitre ( $10^{-6}$ )
NaOH	sodium hydroxide
nm	nanometer ( $10^{-9}$ )
pH	negative logarithm of hydrogen ion activity
PCR	polymerase chain reaction
rpm	rounds per minute
RPMI	Rosewell Park Memorial Institute
%	percent

## CHAPTER I

### INTRODUCTION

Cytomegalovirus (CMV) is 230 kilo basepairs (kbp) linear double-stranded deoxyribonucleic acid (DNA) virus in beta-herpesvirinae family. It can be found universally throughout all geographic locations and socioeconomic groups, and more widespread in developing countries and in area of lower socioeconomic conditions. The characteristic properties of herpesvirus group is tend to have a restricted host range, long growth cycle, slow spread in cell culture, and be able to remain dormant within the host body and can never be eliminated.

The CMV infection in human is worldwide. The routes of infection are varies such as congenitally, perinatally and early postnatally via breast milk, and horizontally through direct physical contact with bodily fluids e.g. saliva, urine, cervical and vaginal secretions, semen, tears, feces, and blood. For the immunocompetent host who acquired CMV infection after birth, there are few symptoms and no long-term health consequences. Some persons with symptoms experience a mononucleosis-like syndrome with prolonged fever and a mild hepatitis. Recurrent disease rarely occurs unless the person's immune system is suppressed due to therapeutic drugs or diseases. Therefore, for the vast majority of people, CMV infection is not a serious problem. However, CMV become the most important problem for the immunocompromised host such as the unborn baby whose mother is infected with CMV during pregnancy, the persons infected with human immunodeficiency virus (HIV), and organ transplant recipients.

Primary CMV infection in immunocompromised patient can cause serious disease. However, the more common problem is the reactivation of the dormant virus. Both reactivation from latency and increased replication are commonly observed following organ transplantation. Among the population of immunocompromised host especially organ transplant recipients, the CMV causes morbidity either directly (e.g.,

CMV syndrome, hepatitis, pneumonitis, encephalitis, and colitis) and indirectly through its immune-modulating properties (e.g., increased bacterial and fungal infections, or allograft dysfunction). Therefore, the follow up status of CMV in the host body is essential to protect the poor prognosis that will be happen to the renal transplant recipient. The methods to detect the CMV status in the patient can be either qualitative or quantitative test.

The qualitative test for CMV is probably made in order to test the result of antibody test such as enzyme linked immunosorbent assay (ELISA) or equivalent, antigenemia test by leukocyte immunostaining and qualitative PCR assay. The result of qualitative test is either positive or negative. The result of ELISA means that the host has been infected with CMV sometimes in their lives. In the case of immunostaining, the positive result means that the hosts have active CMV infection in their bodies, but it doesn't indicate the quantity of the viruses in their bodies. The main drawback of using qualitative PCR assays is the inability to differentiate between latency and higher levels of replication. Quantitation of CMV viral load is the test that can estimate the amount of CMV present in a fix volume of patient blood (usually 1 mL of plasma), which is more useful to follow up the CMV symptomatic patient especially in patient with acute graft rejection. This test has also been used to identify high risk patients and to monitor response to CMV therapy.

Therefore the aims of this study is to study the correlation of the qualitative (immunostaining and ELISA) and quantitative (viral load) test for CMV in the blood of normal healthy person and the post renal transplant patients.

## CHAPTER II

### REVIEW OF LITERATURES

#### The Cytomegalovirus (CMV)

##### Characteristics of the virus

CMV is a member of the herpesvirus group and belongs to the family herpetoviridae (Roizman, 1982; Fanner, 1975), which includes herpes simplex virus type 1 and 2, varicella-zoster virus, Epstein-Barr virus, and herpes virus type 6, 7 and 8. These viruses share a characteristic ability to remain dormant within the body over a long period. The common basic characteristics of the herpesvirus group include fairly large size, enveloped DNA, cell association and potential oncogenicity. CMV shared characteristics with these viruses are its icosahedral viral capsid, with 162 capsomers and double-stranded DNA structure of its genome. The complete virion is 150 to 200 nm in diameter. The viral capsid contains the viral genomes as its core and is surrounded by a lipid containing envelope. The enveloped is acquired as the viral particle buds through the nuclear membrane or through cytoplasmic vacuoles. Dense bodies, which contain viral polypeptides, but no viral DNA, are also produced in the course of CMV replication. The molecular weight of CMV DNA is  $130 \times 10^6$  to  $150 \times 10^6$ , with a 56% G+C composition (Ho, 1982). The analysis of CMV DNA by restriction endonuclease shows that clinical isolates share more than 80% DNA sequence homology with the laboratory strain AD-169 (Huang *et al.*, 1976). The analysis of glycoproteins, by monoclonal antibodies or by others, demonstrates that several antigenically distinct CMV glycoproteins exist and that some are targets of virus neutralizing antibody (Reynolds *et al.*, 1979; Weller, 1971; Griffiths and Grundy, 1987; Somogyi *et al.*, 1986; Pereira *et al.*, 1984; Furlini *et al.*, 1987).

CMV is a labile virus and is readily inactivated by lipid solvents (20% ether for two hours), low pH (below 5), heat 37°C for one hour or 56°C for 30 minutes, and

ultraviolet light (for 5 minutes). Preservation of infectivity can best be achieved by freezing the suspension of the infected cells in bicarbonate-free diluent. Addition of sucrose phosphate or 30% to 50% sorbital adds to the viability. The optimal temperature for storage is  $-190^{\circ}\text{C}$  (liquid nitrogen) and results in little or no loss of infectivity. The virus can be kept at  $-70^{\circ}\text{C}$  for several months, but storage at  $-20^{\circ}\text{C}$  results in incomplete loss of infectivity over a short period of time. At  $4^{\circ}\text{C}$ , CMV could be stored for several days without an appreciable decrease in the virus titer. Cell-free virus can be obtained by sonication of infected cells and can be kept indefinitely at  $-190^{\circ}\text{C}$ , CMV can survive in environmental surfaces for several hours (Reynolds *et al.*, 1979; Ruebner 1965; Naraq 1991; Mocarski and Stinski, 1979).

CMV enters the host cell by fusion of the virus envelope with the cell membrane or via phagocytosis. Infectious particles are first detected by electron microscopy one to several days after inoculation of susceptible tissue culture. During this latent period, however, viral DNA and protein can be detected in the infected cells. Virus particles are made and assembled in the nucleus, attain an envelope by budding through the inner nuclear membrane while exiting, and then migrate through the cytoplasm in vacuoles. Defective virus particles with incomplete DNA may also be formed. These defective particles (dense bodies and noninfectious enveloped particles) lack DNA, and are noninfectious. They may prove useful in development of vaccine, because they can provoke immune response without having the risk of latency or oncogenesis (Furukawa *et al.*, 1973; Smith *et al.*, 1975; Naraq 1991).

The replication of CMV is relatively slow. It required at least 24 hours to produce infectious progeny virus. The viral genome is slowly transcribed in a regulated sequence, and based on the appearance of different classes of CMV specific proteins. The replication cycle can be divided into 3 periods: immediate-early, early, and late period by using monoclonal antibodies against the various proteins produced by CMV. The immediate-early period is defined as the first 4 hours after infection. During this period restricted transcription of specific segments of the DNA genome occurs, as well as production of certain regulatory proteins that allow the virus to take control of host cell macromolecular synthesis. The early period begins after the

immediate-early phase and persists for almost 20 hours. This period is characterized by replication of viral DNA, production of infected cells proteins, and production of progeny virus. The late period usually is considered to occur 24 hours after infection. During this period the structural components of the virus are produced and infectious virus is released from the cell (Demmler *et al.*, 1988).

### **Epidemiology**

CMV is found universally throughout all geographic locations and socioeconomic groups, the rate of infection was found between 50% and 85% of adults in the United States by 40 years of age. CMV is also the virus most frequently transmitted to a developing child before birth. CMV infection is more widespread in developing countries and in areas of lower socioeconomic conditions. For the mostly healthy persons who acquire CMV after birth there are few symptoms and no long-term health consequences. Some persons with symptom experience a mononucleosis-like syndrome with prolonged fever, a mild hepatitis. Once a person becomes infected, the virus remains alive, but usually dormant within that person's body for life. Recurrent disease rarely occurs unless the person's immune system is suppressed due to therapeutic drugs or disease. Therefore, for the vast majority of people, CMV infection is not a serious problem.

The incidence of primary CMV infection in pregnant women in the United States varies from 1% to 3%. Healthy pregnant women are not at special risk for disease from CMV infection. When infected with CMV, most women have no symptoms and very few have a disease resembling mononucleosis. It is their developing unborn babies that may be at risk for congenital CMV disease. Approximately 1% of newborns in the United States is infected with CMV and the percentage is higher in many less developed countries depending on the socioeconomic condition of the population.

The prevalence of CMV antibody in adults range from 30% to 97% (Griffiths and Emery, 1997). Infection is more common among lower socioeconomic groups. In Thailand, the prevalence rate of CMV-seropositive had been shown as high as 47%

among Thai population (Tantivanich and Prasertsiriroje, 1981). In addition, the prevalence of seropositivity for CMV is 95.5% of the female blood and 82.9% of the male blood donors (Kositanont *et al.*, 1985). Therefore, it might be necessary to test CMV infection in every unit of blood before giving blood to immunocompromised patients such as newborn babies, organ transplantation, and AIDS patients. In pregnant women, 95% of the 185 Thai pregnant women had CMV infection while in hospitality girls in Bangkok the infection rates of CMV were 14.7% of 190 hospitality girls and 5.3% of 75 married women (Tantivanich *et al.*, 1982; Tantivanich and Udomratana, 1985). Serological studies were done in these two groups of women and unmarried women by using ELISA. All hospitality girls and married women were seropositive for CMV antibodies while the unmarried women group had only 96%.

### **Pathology and pathogenesis**

CMV infection may be localized in the salivary glands or be generalized within involvement of many organs. For infants who are infected by their mothers before birth, generalized infection may occur in the infant. The symptoms may range from moderate enlargement of the liver and spleen (with jaundice) to fatal illness.

With supportive treatment most infants with CMV disease usually survive. However, from 80% to 90% will have complications within the first few years of life that may include hearing loss, vision impairment, and varying degree of mental retardation. Another 5% to 10% of infants who are infected but without symptoms at birth will subsequently have varying degree of hearing and mental or coordination problem.

In immunocompromised patients, primary CMV infection can cause serious disease. However, the more common problem is the reactivation of the dormant virus. Infection with CMV is a major cause of disease and death in immunocompromised patients, including organ transplant recipients, patients undergoing hemodialysis, patients with cancer, patients receiving immunosuppressive drugs, and HIV-infected patients. Pneumonia, retinitis, and gastrointestinal disease are the common manifestations of disease.

After CMV infection, the virus spreads locally to lymphoid tissues and then systemically in circulating lymphocytes and monocytes to involve the lymph nodes and spleen. The infection then localizes in the ductal epithelial cells of some of the following tissues such as salivary glands, kidney tubules, cervix, testes, and epididymis. Infected cells may be multinucleated or bear intranuclear inclusions surrounded by a clear halo. However, pathologic changes are minor in the immunocompetent host and infection is generally asymptomatic. In rare cases, a CMV mononucleosis syndrome develops, with potential complications including pneumonia, hepatitis, meningitis, and autoantibody production. Impaired cellular immunity means the effects of CMV were more serious. In renal transplant recipients, CMV can lead to renal dysfunction and graft rejection. In organ transplant recipients, CMV can lead to severe respiratory problems (pneumonia). In HIV infected patients, CMV can lead to gastrointestinal involvement (causing lesions and even perforation of the gut), central nervous system involvement (causing brain inflammation and possible damage to the retina and cochlea), and respiratory involvement (causing pneumonia).

The process of CMV replication in cells, both *in vivo* and *in vitro*, results in greatly enlarged round cells (25-40  $\mu\text{m}$  in diameter). These cells contain intranuclear as well as intracytoplasmic inclusion bodies. They are about 10  $\mu\text{m}$  in size, stain red by hematoxylin and eosin, and are separated from the nuclear membrane by a clear zone or halo (owl-eyed) (Reynolds *et al.*, 1979; Weller 1971; Smith and De Harven, 1974). Cell enlargement is visualized within 6 hours after infection. A paranuclear eosinophilic inclusion is seen within 24 hours. The basophilic nuclear inclusion appears 48 to 72 hours after infection. Margination of nucleoli is also observed. Multinucleated giant cells with intranuclear inclusion are encountered with CMV. They may be seen in epithelial or fibroblast cells (Knowles 1976; Diosi and Babusceac, 1973; Garnett 1979; Booth *et al.*, 1978). Inclusion bodies have been seen in almost every human organ, and electron microscopic studies show that they contain clusters of virions (Ruebner *et al.*, 1965; Smith and De Harven, 1973; Smith and De Harven, 1974).

CMV is a virus that possesses low pathogenicity. Clearly, CMV is cytopathic and consequently can produce tissue destruction as manifested *in vivo* by the cytomegalic inclusion-bearing cells, similar to those that undergo cytolysis in infected cell culture. *In vitro*, however, viral replication is inherently slow, more cell-associated than extracellular virus is produced, and many more defective than infectious particles are generated. If these properties are operative in humans they would likely result in reduced generalized spread of CMV and probably even reduced fusional spread within individual organs, a process that is also inherently slow *in vitro*. Recent evidence suggests that these properties are indeed a feature of infection in man. Given these circumstances, the host immune mechanisms are essential. Many structural and nonstructural viral proteins are recognized by different components of the host cellular immune system such as Class I MHC-restricted CD8<sup>+</sup> cytotoxic T lymphocytes, Class II MHC-restricted CD4<sup>+</sup> T lymphocytes, antibody-dependent cellular cytotoxicity, and nonspecific natural killer cell activity. Antibody response to CMV has been thoroughly studied and response to individual viral proteins is well known, but the importance of antiviral antibody in host protection is unclear. Preexisting seroimmunity has been associated with protection from clinically significant CMV in some population (infants with maternal anti-CMV's and transplant recipients receiving passively administered immunoglobulin specific for CMV proteins), but is not sufficient to clear infection in the absence of cellular immunity. In human, CMV is the most common congenital viral infection. Most significant risk factor for severe congenital infection with symptomatic CMV is primary CMV infection during pregnancy. Presence of maternal antiviral antibodies and cellular antiviral responses prior to conception can prevent or modulate fetal CMV infection and reduce (but not eliminate) intrauterine transmission. Overall, CMI mechanisms and antibody responses eventually control the virus, although infected cells remain in the body throughout life and can be a source of reactivation and disease when CMI defenses are impaired.

**Mode of transmission**

Transmission of CMV occurs from person to person. Infection presumably requires close, intimate contact with a person excreting the virus in their saliva, urine, or other bodily fluids.

**Sexual transmission**

CMV can be sexually transmitted by the CMV excretion in the semen of asymptomatic young men (Lang and Kummer, 1975) and in the cervical secretion of asymptomatic young women (Jordan, *et al.*, 1973). The CMV was isolated from semen and the other investigators demonstrated that the groups of women suspected of having venereal disease had both CMV excretion in cervical secretion and the serology test were positive significantly more frequent than in normal control group raising the possibility that the virus may be transferred during sexual transmission (Lang and Kummer, 1975).

**Transplacental route**

Mode of the transmission of virus from mother to fetus has been demonstrated. Morphologic evidence of placental involvement has been described and a few years thereafter, and revealed the recovery of CMV from the fetus at the 12<sup>th</sup> weeks of pregnancy. The exact mechanism of transplacental transmission of CMV is not known. Infection may occur and transmit in many stages of pregnancy (Stagno *et al.*, 1982; Reynolds *et al.*, 1973; Shearer *et al.*, 1972). Viremia seems to be the major mechanism of transmission in the prenatal period (Bale JF *et al.*, 2002). A rare cause of prenatal transmission of CMV is in intrauterine (King-Lewis and Gardner, 1969).

**Oral transmission**

Oral transmission of CMV, via the gastrointestinal or respiratory tract or both, is probably the most common way of acquiring infection under natural circumstances. Close and prolonged contact with infected individuals or infectious secretions appear to be necessary for oral transmission of CMV such as kissing in transmission by

saliva, nursing in transmission by breast milk and passage through the birth canal in transmission by cervical secretion.

### **Blood transfusion and organ transmission**

Transfusion of fresh blood and organ transplantation from a latent infected donor can result in primary CMV. CMV remains latent following primary infection by integration within the host cell chromosome or under a low level of replication controlled by an effective anti-CMV immune system. Both reactivation from latency and increased replication are commonly observed following organ transplantation.

### **Diagnosis of CMV infection**

#### **Antibody detection**

Most infections with CMV are not diagnosed because the virus usually produce few, if any, symptoms and tends to reactivate intermittently without symptoms. However, persons who have been infected with CMV develop antibodies to the virus, and these antibodies persist in the body for the life of that individual. A single positive test which measures CMV-specific IgG antibody is a good indication of present or past exposure to the virus but has no diagnostic significance. A fourfold or greater rise in antibody titer indicates active infection. Specimens should be collected immediately and after 2, 4, and 8 weeks after onset of illness. A number of laboratory tests that detect these antibodies to CMV have been developed to determine if infection has occurred and are widely available from commercial laboratories.

**Complement Fixation:** The complement fixation (CF) test is simple, reliable, and specific, has been used for many years in clinical laboratories before the enzyme immunosorbent antibody technique became available. Reagents for the CF test are commercially available, and detailed procedures have described elsewhere (Cremer *et al.*, 1978). Choice of antigen preparation for CF should in part depended on the purpose of the test. The CF test with glycine-extracted antigen is more sensitive for distinguishing seropositive from seronegative specimens. This method is technically demanding, requires rigid standardization, has a long turn around time and is also less

sensitive than other methods in detecting low levels of antibodies (Cremer *et al.*, 1978; Kettering *et al.*, 1971).

***Indirect Fluorescent Antibody (IFA) Test:*** Indirect fluorescent antibody (IFA) test for IgM can show its applicability to the diagnosis of congenital and acquired infection. However, the positive IFA-IgM might also be a result of varicella-zoster virus or Epstein-Barr virus infections. The IFA-IgG assay is relatively easy to perform as long as care is taken to read fluorescence of intranuclear inclusions only. The reason for the latter precaution is that cytoplasmic inclusions that carry Fc receptors for IgG molecules develop in CMV-infected cells. These receptors bind IgG nonspecifically and will give false positive fluorescence with sera not containing CMV antibody (Keller *et al.*, 1976).

***Radioimmunoassay (RIA):*** The radioimmune assay (RIA) has been applied to the detection of CMV-specific IgM and IgG antibodies. This technique is one of the specific and sensitive method for the diagnosis of CMV infection and appears to be capable of detecting primary infection with CMV early in pregnancy, but it is less reliable at demonstrating significant changes in titers as most laboratories establish binding ratio at fixed serum dilution to compare the quantities of antibodies present in two sera (Griffiths *et al.*, 1981; Griffiths *et al.*, 1980; Stagno *et al.*, 1996).

***Enzyme-linked Immunosorbent assay (ELISA):*** The ELISA is the most commonly available serologic test for measuring antibody to CMV. The result can be used to determine if acute infection, prior infection, or passively acquired maternal antibody in an infant is present. An ELISA technique for CMV-specific IgM is available, but may give false-positive results unless steps are taken to remove rheumatoid factor or most of the IgG antibody before the serum sample is tested. Because CMV-specific IgM may be produced in low levels in reactivated CMV infection, its present is not always indicative of primary infection. Only virus recovered from a target organ, such as the lung, provides unequivocal evidence that the current illness is caused by acquired CMV infection. If serologic test detects a positive or high titer of IgG, this result should not automatically be interpreted to mean

that active CMV infection is present. Detection of CMV-specific IgG antibody in neonates or adult does not mean active infection, because of passive transplacental transfer and life long persistence of specific-IgG following infection. However, if antibody test of paired serum samples show a fourfold rise in IgG antibody and a significant level of IgM antibody, meaning equal to at least 30% of the IgG value, or virus is cultured from a urine or throat specimen, the findings indicate that an active CMV infection is present. The advantages of ELISA are that it is rapid, sensitive, and specific. In addition, multiple specimens can be handled daily. An ELISA can be used effectively to determine the immune status of a patient and to detect significant rises in antibody titers (Griffiths *et al.*, 1982; Sarov *et al.*, 1984; Wreghit *et al.*, 1980).

### **Antigen detection**

Various methods had been used for diagnosis of CMV infection.

**CMV Culture:** CMV culture has been generally regarded as the definitive method for detection CMV infection and mostly used in cases when serology is unreliable, those are, in congenital infection, in the post transplant and AIDS setting. Amniotic fluid culture may be appropriate in some circumstances. Culture CMV on human embryonic fibroblasts is sensitive and specific but is not a rapid test. The characteristic cytopathic effect (CPE) may take up 2 to 5 weeks to become apparent, because the characteristic CPE develops very slowly in specimens with very slow titer of virus (Drew and Bates, 1998; Guerrant *et al.*, 1999).

**Electron Microscopy:** The technique of electron microscopy (EM) can be used to detect CMV particles in urine, body secretions and tissue specimens of congenitally CMV infected infant (Macris *et al.*, 1981). These patients have a high titer of cell-free virus in the urine. The sensitivity of this method has been reported to be as 95% as compared to virus isolation. Body secretions and tissues may also be used. This method is not specific because herpes simplex virus and other herpesviruses would be confused with CMV. The sensitivity decreases when the virus titer is lower than  $10^4$  PFU/mL (Lee KF *et al.*, 1978; Montplaisir S, *et al.*, 1972). The advantage of EM is its rapidity, also, stored specimens can be examined. The main

disadvantages are its relative insensitivity and the need for experience personal and expensive equipment (Macris *et al.*, 1981).

**Early Fluorescent Antigens:** Like other viruses, CMV produces some antigen (“early antigen”) before viral nucleic acid synthesis or indeed even in the absence of nucleic acid synthesis. Antibody to one such antigen can be demonstrated by fluorescent antibody on infected cells maintained in the presence of cytosine arabinoside, which inhibits CMV DNA synthesis (Furukawa *et al.*, 1973). The report finding antibody to early antigen only in individuals with recent infection, whereas those with past infection were seronegative.

**Immunoperoxidase staining:** The immunoperoxidase (IP) technique was reported to the diagnosis of active CMV infection by the detection of CMV immediate early antigen (IEA) in peripheral blood leukocyte (PBL) (Van der Bij *et al.*, 1988).

This antigen was detected very early during the course of an active infection even before seroconversion, positive virus culture or overt disease. Consequently, even low numbers (>5 CMV-Ag positive cells per slide), as observed in most patients at the beginning and the end of the period of antigenemia, are readily identifiable by microscopic screening with a 25x objective. The advantages of IP technique are the potential for great sensitivity, the fact that an ordinary light microscopy can be used and the turn around time takes less time than the detection of CMV early antigens in fibroblast inoculated with buffer coat cells DNA-DNA hybridization of blood leukocytes. The disadvantage includes the need for a few more manipulations and the fact that some of the earlier substrates used were subsequently shown to be mutagenic. However, nonmutagenic substrates can be substituted satisfactorily.

**Hybridization:** Isotopic and nonisotopic probes were used for DNA-DNA and RNA-RNA hybridization in the diagnosis of CMV infection. DNA-DNA and RNA-RNA hybridization have also examined clearance of CMV and applied for rapid CMV detection in specimens such as peripheral blood cells and urine (Buffon *et al.*, 1988; Chou and Marigan, 1983; Crumpacker *et al.*, 1988; Martin *et al.*, 1984; and Spector *et*

*al.*, 1984). These hybridization techniques provide a rapid diagnosis assay, but in daily practice the sensitivity is limited to 5 to 100 pg, corresponding to as many as  $10^4$  to  $2 \times 10^5$  CMV genome (Churchill *et al.*, 1987; Hilborne *et al.*, 1987, and Myerson *et al.*, 1984).

***Polymerase chain reaction (PCR) DNA amplification:*** The PCR DNA amplification is a newly introduced, sensitive and rapid method for amplifying and detecting small quantities of specific nucleic acid in clinical specimens. The sensitivity and specificity of PCR for diagnosis of active CMV infection have been evaluated (Demmler *et al.*, 1988). In several studies, the sensitivity of the assay was increased by amplifying gene fragments, from both the immediate-early and the late CMV gene by using nested primers to a single gene fragment.

***Shell vial assay:*** The shell vial assay has gained wide acceptance as a rapid method for the detection of CMV in clinical specimens and it has been shown to be more sensitive than serology in diagnosis of infection in the neonate and immunodeficient patient (Weber *et al.*, 1992). The technique is based on the amplification of virus in cell culture after low-speed centrifugation and detect viral antigens produced early in the replication of CMV before the development of CPE. Even low titers of virus present in specimens are easily amplified and rapidly detected within 24 hours.

***Branched DNA assay:*** The branched DNA assay is intended to monitor patients at risk for developing CMV disease such as AIDS patients and transplant recipients, by quantitating virus levels using the CMV bDNA assay early in the course of infection.

In this assay, peripheral blood leukocytes are lysed. CMV is denatured and hybridized with DNA probes directed against the *gb* gene (major envelope glycoprotein) of CMV. Hybrids are captured on the surface of microtiter wells. Amplified (bDNA) molecules are bound to the captured hybrids prior to detection with an alkaline-labeled probe and chemiluminescent substrate. Although a very

reproducible technique, it is unfortunately relatively insensitive compared with PCR. Improved sensitivity has been achieved by the reduction in nonspecific hybridization using modified nonnatural nucleotides in genetic sequences and the incorporation of an additional amplification step (Myerow S *et al.*, 1997). A recent study has shown 100% agreement between antigen staining results and the bDNA assay for the diagnosis of CMV polyradiculopathy (Flood J *et al.*, 1997).

### **The COBAS AMPLICOR CMV Monitor Test**

The AMPLICOR assay is a PCR-based method for the quantification of CMV DNA in plasma. Nucleic acid is extracted from the equivalent of 25  $\mu$ L of plasma per PCR by using the reagents and methodology provided by the manufacturer. An automated gene amplification and detection system (COBAS; Roche) is used for DNA amplification, and the amount of PCR product present is compared to that of an internal standard coamplified in the same reaction with the same primer set. The protocol recommended by the manufacturer is followed throughout. Results are calculated with the computer software provided with the COBAS machine, using the signal detection from each PCR product, and are expressed as DNA copies per mL of plasma. The lower detection limit is 400 copies/mL.

### **Correlation of laboratory testing and CMV disease in organ transplantation**

Cytomegalovirus (CMV) is the most important pathogen affecting transplant recipients, causing significant morbidity and mortality. The effects of CMV infection in transplant recipients may be classified as direct and indirect. The direct effects are seen clinically as manifestations of active CMV disease including, for example, fever, leukopenia, hepatitis, colitis, and retinitis. Indirect effects are more subtle and are believed to lead to allograft injury and loss (de Otero *et al.*, 1998; Rubin RH, 1998) and increased susceptibility to infections with other organisms, as well as to decreased patient survival. The highest rate of graft loss was found in the CMV D+/R- group within the first postoperative year and in the D+/R+ subgroup within long-term follow-up between years 1 to 5 after transplantation (Gerstenkorn *et al.*, 2001).

Serology and shell vial assays were not effective for the early diagnosis of CMV infection. Serology seems to be useful for detecting any previous CMV infection and for a retrospective confirmation of CMV infection in renal transplant recipients. However, it cannot be used for early diagnosis and does not provide clinically relevant information. Obviously, immunosuppression causes a poor immune response, and may also result in a poor rise in anti-CMV IgG or IgM titers (Tanabe *et al.*, 1997). Both CMV antigenemia and CMV PCR were extremely useful for the early diagnosis of CMV disease when such testing was carried at least once or twice per week during the first 3 to 4 months after renal transplantation. Although the CMV antigenemia assay reflected the clinical course closely, the CMV-PCR assay, serology and shell vial assay did not. Overall, the CMV antigenemia assay, when it is monitored in a surveillance program. It much more useful than the CMV PCR assay, serology, and the shell vial assay for the early diagnosis and monitoring of CMV infection (Kazunari *et al.*, 1997).

The utility of antigenemia testing is limited by the leukocyte preparation (Lipson and Della-Latta, 2000) and by the rapid loss of signal during storage because specimens have to be processed within 6 hours of collection (Weinberg *et al.*, 2000; Boeckh *et al.*, 1994). In contrast, the result of quantitative CMV PCR is stable for at least 1 week at refrigerator temperature.

On treatment of CMV disease in lung transplant recipients, antigenemia test results became negative after 1 month of therapy for all patients, including those who later experienced a recurrence of the disease. Unlike PCR, antigenemia testing did not identify the patient at high risk of recurrent CMV.

The quantitative plasma CMV PCR might be a useful tool to monitor the efficacy of anti-CMV therapy in lung transplant recipients (Razonable RR, 2001). In addition, patients who developed recurrent CMV disease will still positive by PCR at the time therapy for the preceding episode was discontinued whereas patients who did not develop recurrent disease tended to clear the CMV DNA from their blood faster.

The result suggests that continuing anti-CMV therapy until the plasma CMV PCR becomes negative might prevent recurrent CMV disease (Weinberg *et al.*, 2000).



## CHAPTER III

### MATERIALS AND METHODS

#### **Patients**

Fifty patients with more than 3 months post renal transplantation were collected from the Nephrology Unit, Department of Medicine, Phramongkutklao Hospital. Their ages varied from 6 to 67 years. Their immunosuppressive regimen included cyclosporine A, tacrolimus (or Prograf®) and mycophenolate mofetil (or Cellcept®) in sometimes.

The 50 normal controlled subjects, the healthy individuals who were symptomatic in CMV infection were collected from normal healthy blood donors and laboratory workers at Phramongkutklao Hospital. Their ages varied from 21 to 57 years.

#### **Blood collections**

Ten millilitres of blood were taken from each individual. Five millilitres were collected in EDTA for further white blood cells and plasma. The another five millilitre were left to clot and serum were collected after clot reaction. The EDTA blood was layered at the surface of one volume of Isoprep® and centrifuge at 2500 round per minute (rpm) for 30 minutes. The upper layer, the plasma was collected and the white blood cell (WBC) layer which appeared as the ring in the middle of the tube was separated, and washed three times with RPMI 1640 (Gibco, USA) at 1500 rpm for 10 minutes. Ten microlitres of the WBC pellet were smeared duplicate at the glass slide and left it dry. The WBC slide was immunostained for antigenemia assay at once or wrapped in aluminium foil and kept it at  $-70^{\circ}\text{C}$  until used. Plasma and serum were aliquoted and kept at  $-70^{\circ}\text{C}$  for further examinations.

### **CMV pp65 antigenemia assay**

The CMV pp65 antigenemia assay, to detect the pp65 lower matrix protein of CMV, was performed on WBC that was applied to glass slide. Fix the WBC on the slide with 1% nonidet P-40 solution in phosphate buffer saline pH 7.2 for 10 minutes and then permeabilized with 1% formaldehyde for 10 minutes at room temperature. After fixing the cells, applied 10  $\mu$ L of 1:10 dilution of first monoclonal antibody in PBS (mouse anti-CMV AD169 clone AAC10 (Dako, Denmark)), onto the WBC dot and incubated for 45 minutes in moist chamber. Washing the excess antibody 3 times with PBS. Subsequently the slides were incubated with 10  $\mu$ L of horseradish peroxidase (HRP) conjugate rabbit anti-mouse immunoglobulin (Dako, Denmark) as second antibody, 1:20 dilution for 45 minutes in moist chamber and followed by washing step again. The 20  $\mu$ L of substrate of HRP, 3-amino-9-ethylcarbazone in N, N-dimethyl formamide solution and H<sub>2</sub>O<sub>2</sub> in sodium acetate solution, were incubated for 45 minutes in moist chamber for color development. The color developing reaction was stopped by sodium acetate for 5 minutes and followed by washing the slide with water. And lastly, counterstained the slides with 1 drop of hematoxylin dye for a few seconds, washed out the excess dye by water and then mounted the slide in glycerol-gelatin for further microscopic examination. A positive staining result required the presence of at least five dark brown color cells, localized mostly in the nucleus of WBC, per slide.

### **ELISA of CMV serology in serum**

The 96-well polystyrene flat bottom plate was used for ELISA to determine Immunoglobulin M (IgM) and IgG to CMV in serum. The CMV antigen was prepared by infecting human embryonic lung fibroblast cells with CMV strain AD169 which showed 3+ CPE within seven days. The infected cells were subjected to freezing and thawing 3 times in dry-ice alcohol bath, then centrifuged at 1000 rpm for 10 minutes to remove the cell debris. The supernatant was sonicated at 10 KH/sec for 10 minutes by using ultrasonic liquid processor (Heat Systems Inc., USA), then centrifuged at 2000 rpm for 10 minutes at 4°C and kept in a small aliquot at -70°C.

For coating the ELISA plate, 50  $\mu\text{L}$  of 2.5% of fetal bovine serum (FBS; GIBCO, USA) in normal saline were added into each well and let it dry in 37°C incubator oven overnight. Then 50  $\mu\text{L}$  of 0.25% glutaraldehyde in PBS pH 7.0 were added into each well in order to fix the FBS to the plate surface and incubate for 30 minutes at room temperature. The plate was washed 3 times with distilled water and 50  $\mu\text{L}$  of 1:500 CMV antigen were added to the well and air dry in 37°C incubator oven overnight and kept it in a dry environment for IgM and IgG determination.

For detection of IgG antibody, 10  $\mu\text{L}$  of serum were diluted with 190  $\mu\text{L}$  of PBS in the first well, and diluted the sample in serial to 2-fold dilution to the next well until the last well and discarded 100  $\mu\text{L}$  of the sample from the last well. Therefore the dilutions of the sample were 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560. The last two columns of the well were used for positive and negative control. The antigen-antibody reaction was incubated at 37°C for 30 minutes. The plate was washed 3 times with PBS, then add 100  $\mu\text{L}$  of 1:400 peroxidase-conjugated rabbit anti-human IgG in PBS in each well. After incubation at 37°C for 30 minutes, the plate was washed 3 times with PBS. Then 100  $\mu\text{L}$  of freshly prepared substrate, 1,4-para-phenylene diamine dihydrochloride (PPD) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in citrate buffer, was added and incubated in dark chamber for 10 minutes. The reaction was stopped by 50  $\mu\text{L}$  of 1 normal (N) sodium hydroxide (NaOH). The plate was determined the optical density (O.D.) at wavelength ( $\lambda$ ) of 492 nanometre (nm) with a Multiskan Ex Spectrophotometer (Labsystems, Finland). The antibody titre  $\geq$  1:160 was considered as positive.

For detection of IgM antibody, 10  $\mu\text{L}$  of serum were diluted with 190  $\mu\text{L}$  of PBS in the Eppendorf tube, and diluted the sample in serial two fold dilution to the next seven tubes and discarded 100  $\mu\text{L}$  of the sample from the last tube. The diluted samples were incubated with 100  $\mu\text{L}$  of human IgG, 1:1000 dilution in PBS, in order to block the non-specific reaction at 37°C for overnight. The human IgG was prepared by adding 5 mL of normal human serum into 0.9 g of  $\text{Na}_2\text{SO}_4$  by slowly stirring in room temperature for 40 minutes until dissolved completely and centrifuged to collect

the precipitate. The precipitate was dissolved in 17.5 mM phosphate buffer, pH 6.3, dialyzed at 4°C for overnight. After that, centrifuged and collected the supernatant to run through DEAE cellulose column (length about 10 – 12 cm) with the flow rate about 12.5 mL/hour, measured O.D. at  $\lambda$  280 nm which was the part of IgG fraction. After overnight incubation, 100  $\mu$ L of each serial 2-fold diluted sample were incubated in CMV coated plate for 30 minutes at 37°C, so the dilutions of the serum were 1:40 to 1:5120. The last two columns of the well were used for positive and negative control. The following steps were the same as IgG detection except that peroxidase-conjugated rabbit anti-human IgM was used instead of peroxidase-conjugated rabbit anti-human IgG.

#### **Quantitation of CMV viral load in plasma**

The COBAS AMPLICOR CMV MONITOR Test was used to quantify CMV DNA in human plasma on the COBAS AMPLICOR™ Analyzer. These test kits were kindly supported by Roche Diagnostics, U.S.A. The principle of COBAS AMPLICOR CMV MONITOR Test was based on four major processes: specimen preparation, PCR amplification of target DNA using CMV specific complimentary primers, hybridization of the amplified DNA to oligonucleotide probes specific for the targets, and detection of the probe-bound amplified DNA by colorimetric determination.

The quantitation of CMV viral DNA was performed using the CMV Quantitation Standard. The CMV Quantitation Standard was non-infectious plasmid DNA that contained the identical primer binding sites as the CMV DNA target and a unique probe binding region that allowed Quantitation Standard amplicon to be distinguished from CMV amplicon. The Quantitation Standard was incorporated into each individual specimen at a known copy number and was carried through the specimen preparation, PCR amplification, hybridization and detection steps along with the CMV target and was amplified together with the CMV target. The COBAS AMPLICOR Analyzer calculated the CMV DNA levels in the test specimens by comparing the CMV signal to the Quantitation Standard signal for each specimen.

### **Specimen preparation**

CMV DNA was isolated from plasma by lysis of virus particles with a chaotropic agent followed by precipitation of the DNA with alcohol. A known number of Quantitation Standard DNA molecules were introduced into each specimen with the lysis reagent. The Quantitation Standard was carried through the specimen preparation, amplification and detection steps and was used for the quantitation of CMV DNA in the test specimen. The Quantitation Standard compensated for effects of inhibition and controlled for the amplification process to permit the accurate quantitation of CMV DNA in each specimen.

The frozen plasma was thawed at room temperature, then added 200  $\mu$ L of plasma into 600  $\mu$ L working lysis reagent, vortex 5-10 seconds. In every batch of the experiment CMV negative control (CMV(-) C), CMV low positive control (CMV L (+) C) and CMV high positive control (CMV H (+) C) were used as the unknown specimen. Eight hundreds  $\mu$ L of isopropanol were added to each tube and vortex vigorously for 5-10 seconds, then centrifuged at the maximum speed for 15 minutes to pellet the DNA. Discard the supernatant by carefully and washed the DNA pellet with 1.0 mL of 70% ethanol, centrifuged and removed supernatant as much as possible. Then added 400  $\mu$ L of Tris-EDTA buffer to the DNA, vortex vigorously for 5-10 seconds. After that, the processed specimens and controls were amplified within 4 hours of preparation or store  $-20^{\circ}\text{C}$  for up to one week, with no more than one freeze-thaw. The whole next steps such as amplification, dilution of amplicon, and detection were automatically performed by the COBAS AMPLICOR Analyzer. Results were expressed as CMV DNA copies/mL

### **PCR amplification**

#### **Target selection**

Selection of the target DNA sequence for CMV depended on identification of regions within the CMV genome that showed maximum sequence conservation. Accordingly, the appropriate selection of primers and probe were critical to the ability of the test to detect the CMV genotype. The DNA target sequence, located within the human CMV DNA polymerase gene, was specific for CMV and was not homologous

to other members of the Herpesvirus family. The COBAS AMPLICOR CMV Test used the biotinylated primer LC342 and LC 383 to define a sequence of 365 nucleotides located in the amino terminus of the CMV DNA polymerase gene.

### **Target amplification**

Processed specimens were added to the amplification mixture in amplification tubes (A-tube) in which PCR amplification occurs. The reaction mixture was heated in the A-tube to denature the DNA and exposed the CMV and CMV Quantitation Standard target sequences. As the mixture cools, the biotinylated primer LC342 and LC832 annealed to the target DNA. In the presence of  $Mg^{2+}$  and excess deoxynucleoside triphosphate (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, and deoxyuridine (in place of thymidine) triphosphates, the thermostable *Thermus aquaticus* DNA Polymerase (*Taq* pol) extended the annealed primers along the target templates to produce a 365-base pair double-stranded DNA molecule termed as amplicon. The COBAS AMPLICOR Analyzer automatically repeated this process for a designated number of cycles, each cycle effectively doubling the amount of amplicon DNA. Amplification occurred only in the region of the CMV genome between the primers; the entire CMV genome was not amplified.

### **Selective amplification**

Selective amplification of target nucleic acid from the clinical specimen was achieved in the COBAS AMPLICOR CMV MONITOR Test by the use of AmpErase® and deoxyuridine triphosphate (dUTP). AmpErase (Uracil-N-glycosylase, UNG) recognized and catalyzed the destruction of DNA strands containing deoxyuridine, but not DNA containing thymidine. Deoxyuridine was not present in naturally occurring DNA, but was always present in amplicon due to the use of deoxyuridine triphosphate in place of thymidine triphosphate as one of the dNTPs in the master mix reagent; therefore, only amplicon contain deoxyuridine. Deoxyuridine rendered contaminating amplicon susceptible to destruction by AmpErase prior to amplification of the target DNA. AmpErase, which was included in the master mix reagent, catalyzed the cleavage of deoxyuridine containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first

thermal cycling step at the alkaline pH of the master mix, the amplicon DNA chain broke at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. AmpErase was inactive at temperature above 55°C, i.e., throughout the thermal cycling steps, and therefore did not destroy target amplicon. Following amplification, any residual enzyme was denatured by the addition of denaturing solution, thereby preventing degradation of any target amplicon. AmpErase in the COBAS AMPLICOR CMV MONITOR Test had been demonstrated to inactivate at least the 10<sup>3</sup> copies of deoxyuridine-containing CMV amplicon per PCR.

### **Hybridization reaction**

Following PCR amplification, the COBAS AMPLICOR Analyzer automatically added denaturing solution to the A-tubes to chemically denature the CMV amplicon and the CMV Quantitation Standard amplicon to form single-stranded DNA. To achieve quantitative results over a large dynamic range, the COBAS AMPLICOR Analyzer serially diluted the denatured amplicon in the detection cups (D-cups). A suspension of magnetic particles coated with an oligonucleotide probe specific for CMV amplicon (LC359) or CMV Quantitation Standard amplicon (SK535) was added to each of four CMV amplicon dilutions and to each of two CMV Quantitation Standard amplicon dilutions. The biotin-labeled amplicons were hybridized to the target specific oligonucleotide probes bound to the magnetic particles. Each quantitative determination required four independent reagent absorbance measurements using the CMV Probe Suspension and two absorbance measurements using the CMV Quantitation Standard Probe Suspension.

### **Detection reaction**

Following the hybridization reaction, the COBAS AMPLICOR Analyzer washed the magnetic particles in the D-cup to remove unbound material and then added avidin-Horseradish peroxidase conjugate. The avidin-Horseradish peroxidase conjugate bound to the biotin-labeled amplicon hybridized to the target-specific oligonucleotide probes bound to the magnetic particles. The COBAS AMPLICOR Analyzer removed unbound conjugate by washing the magnetic particles and then added a substrate solution containing hydrogen peroxide and 3,3',5,5' -

tetramethylbenzidine (TMB) to each D-cup. In the presence of hydrogen peroxide catalyzes the oxidation of TMB to form a colored complex, the absorbance of which was measured by the COBAS AMPLICOR Analyzer at a wavelength of 660 nanometre (nm).

### CMV DNA quantitation

Within the linear range of the assay, the absorbance (A) for each D-cup was proportional to the amount of CMV amplicon or CMV Quantitation Standard amplicon in the D-cup. The COBAS AMPLICOR Analyzer calculated total absorbance by multiplying the absorbance of D-cup by the amplicon dilution factor for that D-cup. The calculated total absorbance was proportional to the amount of CMV DNA or CMV Quantitation Standard DNA present in each PCR amplification reaction. The amount of CMV DNA in each specimen was calculated from the ratio of the total CMV absorbance to the total CMV Quantitation Standard absorbance and the input number of CMV Quantitation Standard DNA molecules using the following equation:

$$(\text{Total CMV A} / \text{Total QS A}) \times \text{Input CMV QS copies/PCR} \times 40 = \text{CMV DNA copies/mL}$$

where:

Total CMV A = calculated total CMV absorbance

Total QS A = calculated total Quantitation Standard absorbance

Input CMV QS copies PCR = number of copies of Quantitation Standard in each reaction; this information is lot specific and is entered by user

40 = factor to convert copies/PCR to copies/mL

**PCR for detection of CMV** were performed as previously described (Tantivanich *et al.*, 2002) briefly genomic DNA was extracted from peripheral blood leukocytes by standard method. The PCR was carried out in 50  $\mu$ L reactions containing PCR buffer, 25 mM  $\text{MgCl}_2$ , 25 mM of each dNTPs, 2.5 units of Taq polymerase, 0.4  $\mu$ M of primers, 5  $\mu$ L of DNA template, and distilled water. The sequences of first primers were ACATCTTCTCGGGGTTCTCGTTGC and

GTCCTCTGCCAAGAGAAAGATGGAC, and the sequences of nested PCR primers were TTGAGGGATTCTTCGGCCA ACTCTG and TCTCCTGTATGTGACCCATG TGCTT. Amplification was performed with an initial step of 5 min at 94°C, 30 sec at 65°C, and 30 sec at 72°C followed by 35 cycles of 1 min at 94°C, 2 min 65°C, and 2 min at 72°C and the product was 351 and 170 bp for the first and nested PCR respectively.



## CHAPTER IV

### RESULTS

Fifty patients with post renal transplantation and 50 normal healthy individual were studied for the status of CMV infection. All cases were asymptomatic in CMV disease at the time of venipuncture, and were detected CMV infection in peripheral blood by antigenemia assay, PCR, IgG and IgM antibodies, and viral load.

#### **The antigenemia assay of CMV**

The pp65 antigen of CMV was detected in peripheral blood leukocytes of all subjects by immunostaining method. The positive results were seen like the dark brown nucleus of leukocyte after immunostaining (Figure 1), and negative results were colorless (Figure 2). The positive results of antigenemia assay of patients and normal controls were 14% (7/50) and 48% (24/50) respectively (Table 1). There were significantly different in patients or control group and positive or negative result (Chi-square test,  $p < 0.05$ ).

Table 1: The results of antigenemia assay in patients and normal control subjects

	Antigenemia assay		Percentage of positive result
	Positive	Negative	
Patients	7	43	14%
Controls	24	26	48%

### The viral load of CMV

The viral load of CMV in plasma was performed by COBAS AMPLICOR CMV Test kit. The results showed that CMV viral load in all of normal healthy control were too low to be detected, which similar to the post renal transplantation, except in 1 patient who had 605 copies/mL. There was no significant difference between healthy normal individual and post renal transplantation ( $p>0.05$ , Table 2).

Table 2: The results of viral load in patients and normal control subjects

	Viral load		Percentage of positive result
	Positive	Negative	
Patients	1	49	2%
Controls	0	50	0%

### The IgG and IgM antibodies for CMV

The titer of IgG or IgM antibodies more than or equal 1:160 were considered to be as the positive results. The IgG positive results in patient and normal control were 58% (29 in 50) and 82% (41 in 50), while the IgM positive results were 26% (13 in 50) and 24% (12 in 50) respectively. The results were divided into 4 patterns as demonstrated in Table 3.

Table 3: The results of IgG and IgM in patients and normal control subjects

IgG	IgM	Patients	Controls
Negative	Negative	15	8
Positive	Negative	22	30
Negative	Positive	6	1
Positive	Positive	7	11

### The nested PCR for CMV

The nested PCR which detected viral DNA in leukocytes showed that the patients and normal controls were 18% and 50% positive by PCR respectively (Table 4). This data showed that there were significantly different in patients and control group (Chi-square test,  $p < 0.05$ ).

Table 4: The results of PCR in patients and normal control subjects

	PCR		Percentage of positive result
	Positive	Negative	
Patients	9	41	18%
Controls	25	25	50%

### The relationship between the laboratory diagnosis and the status of CMV infection in post renal transplantation and normal healthy controls

The CMV infection was defined as either CMV viremia, antigenemia, DNAmia, or the appearance of anti-CMV IgG and/or IgM antibodies (Tanabe *et al.*, 1997; Amorim *et al.*, 2001). The total numbers of the post renal transplant patients who had CMV infection and no CMV infection were almost the same when using antigenemia assay, PCR, and viral load for diagnosis of CMV infection (Table 5). Therefore the relationship between the various assays and the status of CMV infection in the patients and controls, there were found not significant different in antigenemia assay and PCR in both normal and post renal transplantation group ( $p > 0.05$ ). But the viral load and antigenemia or viral load and PCR were significant different, both in renal transplant and control groups ( $p < 0.05$ , Table 5 and 6).

Table 5. The results of diagnosis for CMV infection by antigenemia assay, PCR, and viral load in 50 post renal transplantation patients

Methods	Results	CMV infection	Total No.	No CMV infection	Total No.
Antigenemia	Positive	7	37	0	13
	Negative	30		13	
PCR	Positive	9	37	0	13
	Negative	28		13	
Viral load	Positive	1	37	0	13
	Negative	36		13	

Table 6. The results of diagnosis for CMV infection by antigenemia assay, PCR, and viral load in 50 normal healthy controls

Methods	Results	CMV infection	Total No.	No CMV infection	Total No.
Antigenemia	Positive	24	47	0	3
	Negative	23		3	
PCR	Positive	25	47	0	3
	Negative	22		3	
Viral load	Positive	0	47	0	3
	Negative	47		3	

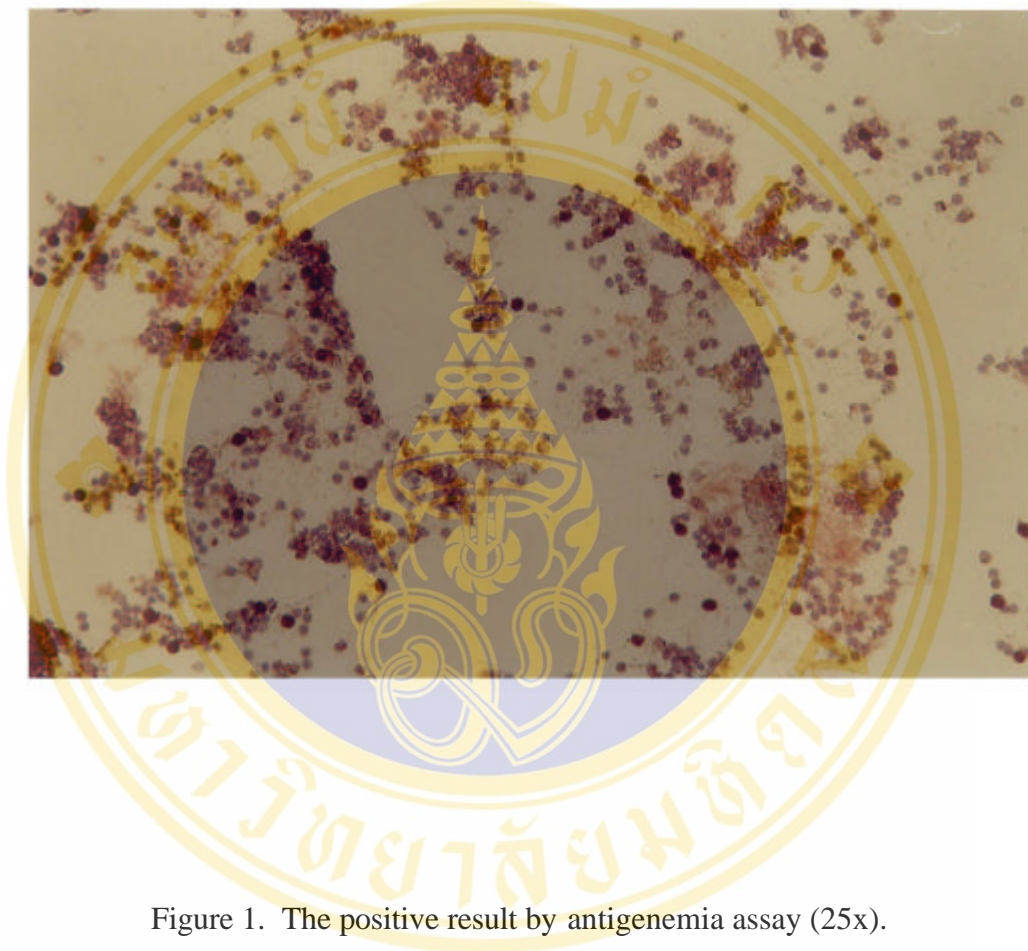


Figure 1. The positive result by antigenemia assay (25x).

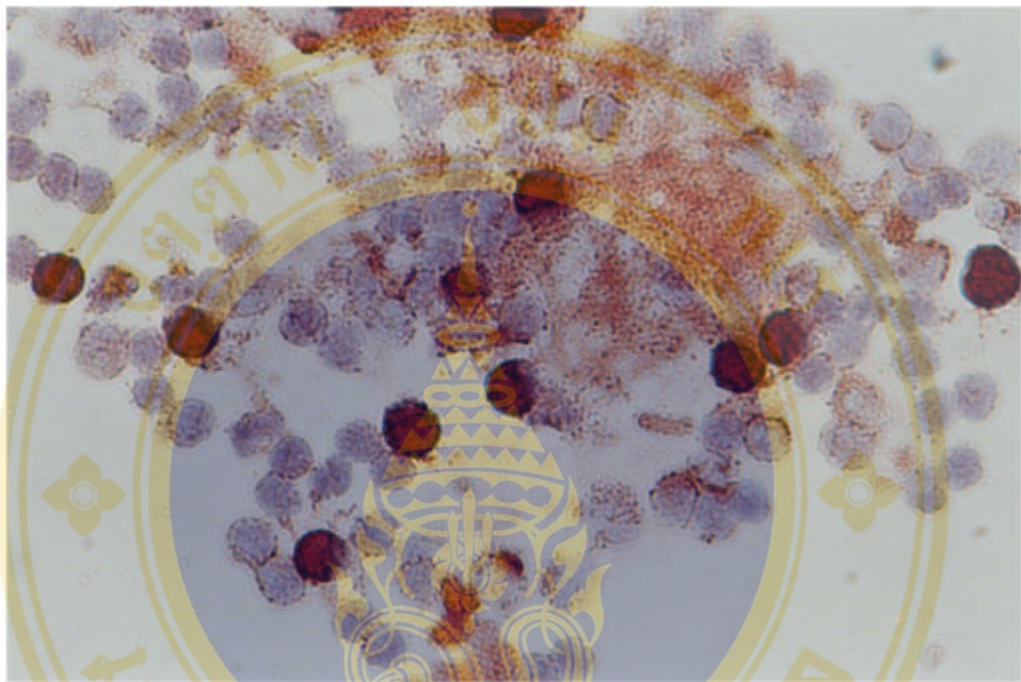


Figure 2. The positive result by antigenemia assay (100x).

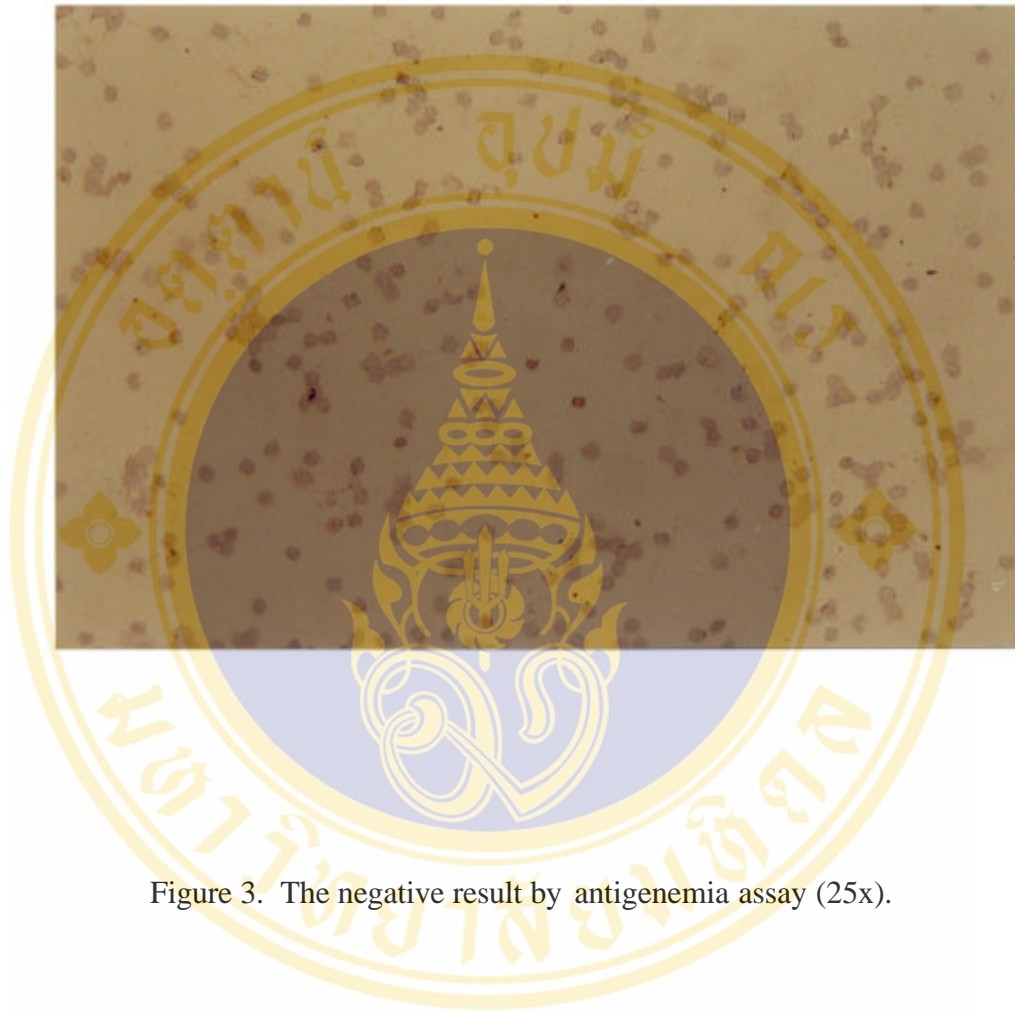


Figure 3. The negative result by antigenemia assay (25x).

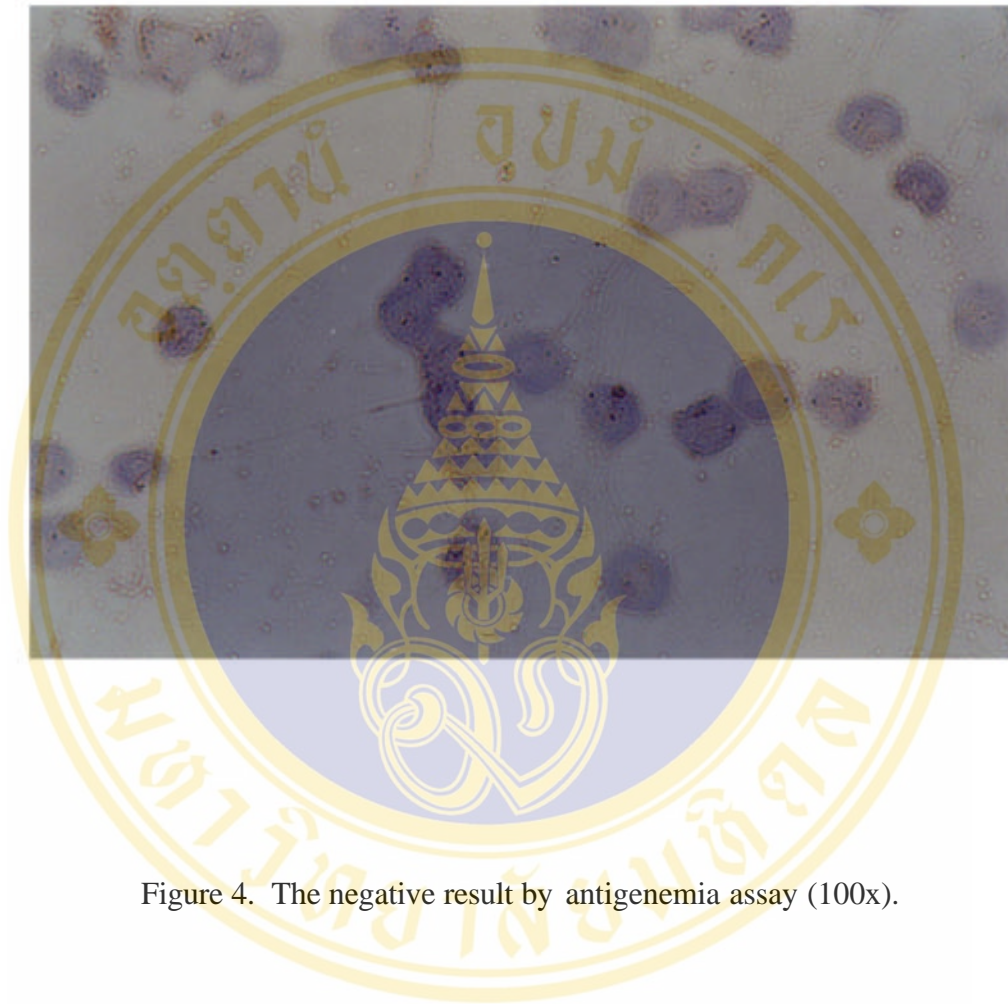


Figure 4. The negative result by antigenemia assay (100x).



Figure 5. The 170-bp PCR products of CMV by nested PCR. Lane 1 showed the 100 bp ladder marker. Lane 2, 4 and 5 showed the positive results. Lane 3, 6 and 7 showed the negative results. And positive and negative controls were shown in lane 8 and 9 respectively.

## CHAPTER V

### DISCUSSION

CMV has been recognized as the most important viral pathogens in persons undergoing transplantation. CMV infection, either primary infection or latent reactivation is a significant cause of morbidity and mortality in renal transplant, especially the seronegative recipient who received seropositive donor (Abbott *et al.*, 2002). The two major strategies to treat CMV infection in transplantation patients are prophylaxis and preemptive treatment. The antiviral agents, such as ganciclovir, can be used in prophylactic treatment in the immediate post transplantation to reduce the risk of viral acquisition or reactivation. But the adverse side effect should be considered (Anglicheau *et al.*, 2002; Sund *et al.*, 2001). The alternative, preemptive treatment, is based on the first virologic detection of CMV infection, before clinical disease has developed. Therefore the earliest CMV detection is the most valuable to predict the subsequent development of the disease. Diagnosis of CMV disease may be difficult in the absence of the characteristic intranuclear inclusions in tissue. Recently, more sensitive detection methods have been developed to identify CMV reactivation before the onset of disease. These methods include direct detection of CMV antigenemia in peripheral blood leukocytes (PBL) and detection of CMV DNA by the polymerase chain reaction (PCR) in PBL and in plasma and serum (Boeckh and Boivin, 1998; Yan and Fedorko, 2002).

The percentage of positive antigenemia assay in renal transplantation is lower than normal healthy in this study may due to the result of universal prophylactic treatment after transplantation and immunosuppressive drugs. In the past, all renal transplant recipients received conventional immunosuppression consisting of prednisolone and cyclosporin A, that designed to prevent graft rejection, but enhance both susceptibility to new infection and reactivation of latent herpesvirus, and then often in graft loss in the final. But in these studied, the immunosuppressive therapy

was replaced by the combining of cyclosporinA, mycophenolate mofetil (or Cellcept®), and tacrolimus (or Prograf®), even though the cost of these drugs are very high, but the efficacy of them may benefit, especially in reduction of CMV infection and reactivation. These results were similar to some previous reports which showed that immunosuppression with tacrolimus and mycophenolate mofetil result in a significant reduction in acute rejection compared with cyclosporin leading (Knoll and Bell, 1999; Neyts, *et al.*, 1998; Neyts, *et al.*, 1998).

About half of normal healthy individual showed the presence of pp65 antigen in the high population of PBL but they usually were asymptomatic for CMV infection. This finding is important because these asymptomatic blood donors can pass on CMV to the recipients by innocent. This adverse effect is seriously problems especially in the primary infection or in immunosuppressive transplant recipients, the leukocyte filter set is suggested to eliminate or reduce the number of donor leukocytes prior to transfuse to the recipients (Bowden, 1991; Preiksaitis 1991; Gilbert *et al.*, 1989).

Detection for the presence of CMV in the blood leukocytes by both antigenemia and PCR in this study indicated that there was CMV infection in these patients with no sign of CMV disease. Comparison of the results by antigenemia assay and PCR showed good correlation in both groups of subject because the same source of specimens i.e. PBL, were used for determination. In contrast, the plasma, were used for viral load. The data showed that almost all of them have CMV DNA copy below the lower limit of testing. These results are in accordance with the results of many reports (Boivin *et al.*, 2000; Scharma *et al.*, 1997; Amorim *et al.*, 2001; Wattanamano *et al.*, 2000; Tong *et al.*, 2000; Sia *et al.*, 2000) in that antigenemia assay and PCR could be used to safely monitor CMV viremia in transplanted recipients. The diagnostic value of the antigenemia was found to be a good diagnostic tool for the prediction of symptomatic CMV infection compared to quantitative assays in solid organ transplantation (Halwachs-Baumann *et al.*, 2001). This finding may explain by many facts that both methods can be used to detect CMV infection in different compartment of the peripheral blood, leukocytes versus plasma. Theoretically, CMV usually resides as latent viruses in leukocytes so the leukocytes

harbor the CMV in higher amount than plasma. There are many previous reports that can be detected CMV by antigenemia earlier than by other molecular biology assays (Tong *et al.*, 1998; Razonable *et al.*, 2002). The probable reasons of these findings may be due to: i) a lower detection limit in antigenemia when compared with quantitative PCR and ii) antigenemia are assigned to detect an immediate early antigen of CMV within infected WBC whereas quantitative PCR detect the polymerase gene of CMV in plasma, and gave result positive only if viral particles were circulating in the blood stream. Therefore this method should be expected to be the last to detect CMV infection. These reasons may be explained our findings that these asymptomatic subjects showed the positive results by antigenemia but negative for viral load. But the viral load has many advantages in the practical manner, commercially and good standardization.

When compared to the antigenemia, plasma is a simpler specimen to process and is independent of a patient's leukocyte count, but there are some evidences suggested that CMV DNA-PCR performed with plasma yields false-positive results with significant frequency when specimen are prepared with delay, regardless of the storage temperature, probably due to leukocytes lysis (Schafer *et al.*, 2000). The viral load also has an advantage of uniformity compared to many in-house PCR that may differ between laboratories in multiple aspects such as primers, target, standards and controls, reaction condition, signal calculation (Caliendo *et al.*, 2001). Although the diagnostic value of the COBAS AMPLICOR assay are significantly lower than that of the antigenemia in solid organ transplantation, but it is very useful for monitor the response of preemptive treatment in CMV disease (Hiyoshi M, 1997). There was previous report that bone marrow transplantation recipients tested based on molecular techniques appeared to be superior compared to antigenemia, may be due to the low amount and poor qualitative functions of white blood cells in BMT groups (Halwachs-Baumann *et al.*, 2001, Nolte FS, 1995, Solano C, 2001).

The number of CMV viral load in the patients usually correlates with the presence of the disease. In this study, the number of CMV DNA start to rise above the

detection levels (605 copies/mL) in 1 patients without any symptom of CMV disease. This patient may develop CMV disease in the near future.

The results of antigenemia assay and PCR did not correlate with the CMV DNA viral load may indicate that both antigenemia assay and PCR are sensitive enough to be used as the standard diagnostic method. Since they can detect for the presence of CMV in the blood leukocytes prior to the presence of the clinical symptoms. These results were similar to the previous reports (Weinberg *et al.*, 2000; Van der Berg *et al.*, 1989, and Tantivanich *et al.* 2001) in that antigenemia assay can be detected from several days to one week before the onset of CMV disease. It is interesting to note that the total numbers of the post renal transplantation patients with active CMV infection and no CMV infection were almost the same when using antigenemia assay, PCR, and viral load for diagnosis methods. These results may indicated that antigenemia assay and PCR can be used for early diagnosis of CMV infection in post renal transplantation patients without waiting for the presence of the high numbers of CMV copies in the blood to be detected by viral load (de la Hoz *et al.*, 2002). In addition, the patients in this study had no clinical CMV symptoms with low level of CMV DNA viral load. These results are in accordance with the many reported in that CMV viremia is frequently used as an adjacent for the diagnosis of CMV disease when the clinical picture is compatible (Meyers *et al.* 1990).

Serology in this study did not show any good correlation with clinical symptoms. The presence of IgM antibody in some of these patients did not provide any clinically relevant information and it also cannot be used for early diagnosis. Even though CMV specific IgM antibody usually develops during primary infection but it may also appear during reactivation of latent infection. The present study demonstrated that the prevalence of CMV infection in Thai population was about 80%, which is similar to the previously report (Bhattarakosol and Sithidajporn, 1998).

It was not surprised that most of the normal healthy control in this study had positive CMV antigenemia and PCR since most of the Thai populations had been infected with CMV and had CMV antibodies (Tantivanich *et al.*, 2001). The virus

may reactive any times of their life. The total numbers of the healthy control with active CMV infection and no infection by antigenemia assay were similar but difference from the total number by viral load. The reasons for these are the same as the post renal transplantation patients in that both antigenemia assay and PCR are the best methods for early diagnosis methods for CMV infection. The low total number in the healthy control group by viral load indicated that these people have strong immunity so that the number of CMV copies in the blood are too low to be detected by viral load. Further study should be done to follow the subjects by various assays in order to confirm the exact time when it is necessary to detect by viral load and when they turn from asymptomatic to CMV disease.

In summary, immunostaining or antigenemia assay and PCR are extremely useful for early diagnosis of CMV infection in renal transplantation patients to start preemptive treatment. It is not necessary for the physician to wait until the patients had high number of CMV copies in the blood to be detected by viral load. But the viral load for CMV is postulated that it is valuable method for the symptomatic CMV infection or CMV disease, in order to follow the using of antiviral treatment.

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## APPENDIX A

### REAGENTS FOR IMMUNOSTAINING

#### 1. Complete RPMI medium

RPMI 1640 (GIBCO, USA)	10.4000	g
HEPES	5.9525	g
NaHCO <sub>3</sub>	2.0160	g
D-glucose	3.6031	g
Sodium pyruvate	1.1005	g
L-glutamine	0.2923	g

All components were dissolved in DDW. The total volume was adjusted to 1 litre and sterilized by filtration through 0.2 µm millipore membrane filter.

#### 2. Monoclonal antibody

Monoclonal mouse anti-cytomegalovirus clone AAC10 code No. M7065 (DAKO, Denmark), stored at 4°C.

#### 3. Secondary antibody

Peroxidase-conjugated goat anti-mouse immunoglobulins Code No. P0447 (DAKO, Denmark), stored at 4°C.

#### 4. Phosphate buffered saline (PBS)

The solution was prepared by dissolved all components in following steps:

NaCl	8.0	g
KH <sub>2</sub> PO <sub>4</sub>	0.2	g
Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	2.9	g or
Na <sub>2</sub> HPO <sub>4</sub>	1.17	g
KCl	0.2	g

All components were dissolved in DDW. The total volume was adjusted to 1 litre and sterilized by autoclaving. The solution was stored at 4°C or room temperature.

### 5. Substrate solution

The solution was freshly prepared by dissolving all components in the following steps:

**Solution A:** 20 mg of 3-amino-9-ethylcarbazone mixed with 5 mL of N,N dimethyl formamide.

**Solution B** (0.05 M of sodium acetate pH5.0). This solution was prepared by dissolving 0.41 g of sodium acetate in 80 mL of DDW and adjusted the pH to 5.0 and brought up to 100 mL with DDW and stored at 4°C.

**Solution C:** 30% H<sub>2</sub>O<sub>2</sub>



## APPENDIX B

### REAGENTS FOR NESTED PCR

**1. Ethidium bromide stock solution (10 mg/mL)**

One gram of ethidium bromide (Sigma) was dissolved in 100 mL of H<sub>2</sub>O and diluted to 0.5 mg/mL when using. The solution was light protected and kept at 4°C.

**2. Disodium ethylenediaminetetraacetate (EDTA, pH 8.0)**

EDTA.H<sub>2</sub>O (93.05 g) was added and dissolved in 400 mL of triple distilled water by stirring vigorously. The pH was adjusted to 8.0 with NaOH before dispensing into aliquots and sterilized by autoclaving.

**3. Tris acetate EDTA buffer (TAE)**

50xTAE stock solution was prepared by adding 242 grams of Tris base, 57.1 mL glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0) to 1 litre of triple distilled water and mix well. The solution was stored at room temperature.

**4. Proteinase digestion buffer (DB)**

DB was prepared by adding 20mM Tris Cl, pH 7.4 (prepare from autoclave, 1M stock), 20 mM EDTA, pH 8.0 (prepare from autoclave, 0.5 M stock) and 0.5% sodium dodecylsulfate (SDS), total 200 mL. The solution was stored at room temperature.



**APPENDIX C**  
**REAGENTS FOR IgM AND IgG ANTIBODIES**  
**BY ELISA METHOD**

1. **2.5% Fetal bovine serum in normal saline (FBS; GIBCO, USA)**
2. **0.25% glutaraldehyde in PBS pH 7.0 (adjust pH with 0.1 M K<sub>2</sub>HPO<sub>4</sub>)**
3. **Phosphate buffered saline-Tween 20 pH 7.4 (PBS-Tween 20)**

The solution was prepared by dissolved all components in following steps:

NaCl	8.0	g
KH <sub>2</sub> PO <sub>4</sub>	0.2	g
Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	2.9	g or
Na <sub>2</sub> HPO <sub>4</sub>	1.17	g
KCl	0.2	g
DW	1000	mL
Tween-20	0.5	mL

The solution was stored at 4°C or room temperature.

**4. Conjugate**

Peroxidase-conjugated rabbit anti-human IgM specific for Mu-chains (DAKO, Denmark), stored at 4°C for IgM detection.

Peroxidase-conjugated rabbit anti-human IgG specific for Gamma-chains (DAKO, Denmark), stored at 4°C for IgG detection.

### 5. Physiological saline-Tween 20 solution

The solution was prepared by dissolved all components in following steps:

NaCl	90	g
Tween-20	5	mL
H <sub>2</sub> O	10	L

The solution was stored at 4°C or room temperature.

### 6. Substrate solution

The solution was freshly prepared by dissolving all components in the following steps:

1. 1,4 para-phenylene diamine dihydrochloride(PPD)
2. Citrate buffer pH 4.5

Trisodium citrate (Na<sub>3</sub>C<sub>5</sub>O<sub>7</sub>·2H<sub>2</sub>O) 14.7 g was mixed with DDW 500 mL and adjusted the pH to 4.5 with 0.1 N HCl.

3. 30% H<sub>2</sub>O<sub>2</sub>

All solution were combined by using: PPD 0.0156 g in 11 mL of citrate buffer, pH 4.5 and mixed with 7 μL of 30% H<sub>2</sub>O<sub>2</sub>.

### 7. 1 N NaOH

Stopping solution was prepared from 40 g of NaOH in 1000 mL of DDW. The solution was stored at room temperature.

## BIOGRAPHY



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