CHAPTER V

DISCUSSION AND CONCLUSION

The diagnosis of viral retinitis is generally based on clinical examination. However, overlapping clinical findings may make it difficult to diagnose HCMV retinitis from varicella zoster virus, or herpes simplex virus retinitis, particularly in the early stages of the disease. Necrotizing retinitis, caused by the herpesviruses, may also be difficult to distinguish from ocular diseases inflicted by other pathogens, such as *Toxoplasmosis gondii* or *Treponema pallidum*. In the case of retinitis, in which the diagnosis cannot be established by clinical examination alone, diagnostic evaluation of ocular fluid becomes a useful adjunct for the clinician. Because of difficulties concerning the HCMV culture, and poor diagnostic efficiency in serology techniques, the diagnostic methods based on the PCR principle are well suited for the detection of small amounts of HCMV DNA in ocular samples.

HCMV infection is a major cause of mortality and morbidity in organ transplant patients, causing severe complications such as HCMV syndrome, hepatitis, pneumonitis, encephalitis, and colitis. Antiviral agents have clinical benefits by either preventing or curing HCMV disease after organ transplantation. Nevertheless, drug therapeutic agents are associated with toxicity and excess cost and must therefore be used to treat only those patients at high risk of HCMV disease. Thus, rapid and sensitive tests for the diagnosis of HCMV infection are important and should ideally be predictive on the occurrence of HCMV disease. Serology tests for specific immunoglobulin G or M antibodies are not reliable as markers of HCMV infection in organ transplant patients. Therefore, the detection of HCMV by PCR provides a potential tool for rapid and early diagnosis in pre- and post-organ transplant patient.

According to these reasons, the In-House HCMV PCR assay was set up in this study. The nested PCR conditions were optimized based on a published method by Arai *et al.*[96] and a duplex PCR reaction for detection of the beta-globin gene was added. Optimal conditions of the duplex nested PCR were established. In the meantime, a variety of amplification targets have been used to detect HCMV DNA by the use of specific oligonucleotide primers. Examples of HCMV amplification targets are UL123 region Limaye *et al.*[99], US17 region[100], the unspliced UL21.5 gene

Boriskin *et al.*[101], *Hin*dIII-X fragment[3], *Eco*RI fragment D[3], IEA1 gene[3] and MIE gene[85]. The primer pairs used in this study corresponded to the major immediate-early (MIE) gene that satisfied several criteria: (a) approximately 50% GC content, (b) lack of self-homology (c) exact alignment in only a single portion of the HCMV genome, (d) sequence conservation between AD169, Towne, and Davis strains, and (e) lack of complement to available HSV, VZV, EBV, HHV-6, and human DNA sequence information.

Oligonucleotides were also synthesized to complement the internal 20 bases as a hybridization probe. The specificity of amplified products was confirmed by Southern blot analysis with this internal probe. However, this probe did not provide the advantage of increasing the sensitivity of PCR product detection. Therefore, it was reasonable to use the easy and low-cost method of ethidium bromide staining.

The HCMV standard positive control was prepared by the gene cloning technique. By using this method, a high purity and large amount of specific HCMV MIE gene fragment could be produced. Furthermore, the possibility of unlimited preparation of the HCMV DNA control was beneficial for the routine diagnostic laboratory.

During the colony selection step, a part of the *E. coli* colony was added directly into a PCR mixture. By this technique, the HCMV MIE gene fragment was successfully amplified. It was effectively proved as a short-cut technique that could avoid plasmid DNA preparation. This method was easy and several colonies could be screened at the same time. However, the exact amount of colony that should be added into a PCR needs to be verified for further study.

The concentration of the purified plasmid DNA and human genomic DNA were determined by different methods. The purified human genomic DNA was determined by the DNA fluorescence assay, which was a direct indicator of the DNA concentration. The RNA did not interfere significantly with the DNA assay because RNA does not generally bind to fluorescence dye. The HCMV plasmid DNA was prepared by using the cloning technique and the recombinant plasmid was purified during the plasmid DNA preparation step. Therefore, the amount of plasmid DNA was measured by the simple spectrophotometric method.

The amplification of the beta-globin gene was selected and added to the first round PCR. Myerson *et al.*[102] reported that the monitoring of DNA extraction using the beta-globin primer was essential in preventing a false-negative PCR result. Thus, the beta-globin gene primers were

added to the first round PCR to ensure the presence of DNA in the extracted sample. Unfortunately, the beta-globin gene primers used did not provide enough sensitivity to detect a minimal DNA in ocular specimens and extracted plasma, although they worked successfully in PBMCs extracted samples. An improvement in their sensitivity may be possible if the nested primers against the PCR product are used. Therefore, further development of the duplex nested PCR is needed.

The sensitivity of the HCMV MIE gene fragment amplified by the optimized second round PCR was increased ten fold when compared to the conventional PCR. A minimum of 10 HCMV DNA copies was successfully detected. This result was achieved when the concentration of $MgCl_2$ and dNTP was increased while reducing the annealing temperature and time. These conditions should be critically balanced because $MgCl_2$ form a complex with dNTP, and $MgCl_2$ can also act as a co-factor for polymerase. If the dNTP concentration used is too high, mismatch incorporation may occur. However, the optimal ratio between $MgCl_2$ and dNTP concentration found in this study made the duplex amplification possible. Moreover, reduction of annealing time could minimize nonspecific amplification in the nested PCR. Finally, the optimized PCR could reduce turnover time by approximately 1 hour/ round.

Generally, primer concentrations between 0.1 and 1.0 μM were optimal[103] for PCR amplification, with 0.7 μM considered optimal for amplification of both the MIE gene fragment and beta-globin gene, as reported by Ando *et al.*[104] In this study, an 0.4 μM concentration of each primer gave the highest sensitivity of both the MIE gene fragment and beta-globin gene.

Bachmann *et al.*[105], and Sarkar *et al.*[106] demonstrated that some cosolvents enhanced PCR amplification and improved its specificity. Arai *et al.*[96] found that 2.5% and 5.0% formamide enhanced the yield of the PCR product, whereas 0.25-2.0% DMSO and 5-10% glycerol had no effect. In contrast to the previous study, 2.5% formamide did not enhance the yield of the PCR product and 5.0% formamide inhibited DNA amplification in this study. The 10% glycerol also provided no benefit for the PCR.

In the standard nested PCR, the second round PCR is set up in the presence of the first round PCR amplification products, which increases the risks of assay contamination by product DNA. Contamination is a widely recognized problem with PCR. The introduction of this standard nested PCR into routine testing has been hampered by its frequent tendency to create

false positive results because of carry-over contamination. However, this problem can be solved by strict separation of the pre- and post- PCR area, a one way workflow, one-tube nested PCR and uracil-N-glycosylase (UNG) anti-contamination technology[107]. With concern about cross- and carry-over contamination in this study, all the experiments were performed by strict separation of the equipment and pre- and post- PCR area, a one way workflow, good laboratory practice and negative control setting.

To diagnose HCMV retinitis, ocular fluid needs to be collected. The vitreous humor is contained inside the eye between the lens and the retina. A membrane around the vitreous humor holds it in place while attached to the retina. Thus, the vitreous humor may be the first site where HCMV can be detected. However, collection of the vitreous humor is an invasive procedure. To replace the vitreous humor, aqueous humor and cojunctival scraping are considered.

The aqueous humor is an ocular fluid that fills the chamber between the lens and cornea. It contains glucose, proteins and minerals, vital for nourishment of the lens and cornea. Hence, the aqueous humor may contain an appropriate condition for HCMV replication. However, in the first stage of disease, the aqueous humor may contain fewer viruses than the vitreous humor.

The conjunctiva is a thin protective layer that covers the aqueous humor. Conjunctival scraping is a minor invasive procedure that is considered to harbor some viruses, as it has close contact with the aqueous humor.

According to this study, the aqueous humor could replace the vitreous humor, thus providing a less invasive method of ocular specimen collection, by using the optimized nested PCR only. However, this finding was concluded from a minimal number of patients. Therefore, further study of an increased number of patients should be performed. Hence, consideration concerning specimen selection should be taken into account if a less sensitive PCR assay is used.

The vitreous humor was found to be the best sample for HCMV DNA detection in retinitis patients, whereas conjunctival scraping showed the lowest sensitivity. From this study, the HCMV DNA could be detected from the conjunctival specimens of 7/12 (58.3%) retinitis patients. This finding correlated with a report by Lee-Wing *et al.*[94], which stated that HCMV DNA was detected in only 40% (2 of 5) of patients with clinical evidence of HCMV retinitis and 16% (4 of 25) of those with no clinical evidence of ocular HCMV infection. Therefore, the

sensitivity and specificity, when using conjunctival scraping would be too low to provide a clinical utility for predicting HCMV retinitis.

Concerning the nonspecific amplification of the latent HCMV in ocular fluid, the HCMV DNA detection in control patients was used to define the test specificity. It was found that this optimized nested PCR provided 100% specificity, as none of the control patients showed a positive result.

Beta-globin gene from only 1 vitrous humor could be amplified by the optimized duplex nested PCR. This might be due to the low number of cells contained in the specimen and the low sensitivity of the beta-globin gene primers. Ando Y *et al.*[104] reported the copy number of the beta-globin gene and HCMV genome in the aqueous humor of retinitis patients. It was found that the copy number of the beta-globin gene ranged from an undetectable level to 208 copies/μl, whereas the HCMV genome was detected in a range of $10\text{-}10^4$ copies/μl. In the present study, 3 μl of the ocular specimen was used. Therefore, this 3 μl specimen is sufficient for HCMV DNA detection by the optimized nested PCR (minimal detectable concentration = 10 copies), but it may not contain enough beta-globin gene to be demonstrated by this PCR.

The mainly small volume of ocular specimens, ranging from 50-200 μl, could be collected. Thus, the ocular samples were prepared at a heat of 100 °C for 10 min prior to PCR. This technique could be reliably used as a standard ocular sample preparation procedure when using an appropriate volume. DNA extraction could be used, when possible, to repeat PCR analysis in those cases in which a false-negative PCR result is suspected. This sample preparation technique by heating was successfully performed by Danise *et al.*[108] and Knox *et al.*[93] who detected the HCMV DNA in aqueous humor and vitreous humor by PCR assay, respectively. On the other hand, DNA extraction prior to PCR is a time-consuming procedure and is subject to the risk of nucleic acid loss and sample contamination during the different steps of the process. Furthermore, DNA extraction from ocular samples is not always possible because of the limited volume available.

PCR assays for HCMV DNA detection in ocular fluid have previously been shown as reliable for the diagnosis of HCMV retinitis in HIV-infection patients [109-115]. Furthermore, PCR was used to diagnose patients with HCMV retinopathy, for whom prompt diagnosis and treatment appear particularly important in aiming to prevent involvement of the other eye.

Serological testing is useless for the diagnosis of HCMV reactivation episodes in renal transplantation recipients because most of the general population have been exposed to HCMV and have developed HCMV antibodies. This was confirmed by the report of Preiser *et al.*[116], which revealed that the serological assays for the detection of HCMV-specific IgG, IgM, and IgA antibodies did not yield any clinically relevant information.

The finding of this present study also confirmed this situation, as most of the recipients contained anti-HCMV IgG at pre-transplantation. The anti-HCMV, IgM, was detected in only one recipient, who experienced a primary infection after transplantation. The HCMV DNA in PBMCs could be detected as soon as 2 weeks after transplantation, with the appearance of the HCMV DNA in plasma following 2 weeks later. Most of recipients appeared to harbor HCMV DNA in plasma by 8 weeks after transplantation. The use of the beta-globin gene primer was absolutely successful in PBMCs extracted samples but no plasma extracted samples showed positive results for the beta-globin gene amplification although they showed positive results for HCMV DNA. Therefore, the improvement of the test sensitivity should be considered. The appearance of HCMV DNA in plasma indicates a higher activation stage of the virus. Taylor-Wiedeman *et al.*[58] described that only the presence of HCMV DNA in plasma can be indicative of active viral replication, which is of higher clinical relevance than latent infection. Other authors, however, have found a considerable loss of sensitivity with the use of plasmas when comparing tests of leukocytes for HCMV DNA[117]. However, the present finding revealed that the detection of HCMV DNA in the PBMCs of donors correlated with the earlier development of HCMV reactivation in the recipient. Therefore, this technique may be useful for screening the qualified donor. Nevertheless, during 8 weeks of post-transplant monitoring, none of patients developed a symptom of HCMV infection, even with positive HCMV DNA in their plasma. Therefore, the HCMV DNA load detected in plasma would provide a greater advantage for predicting the onset of HCMV disease.

Michaelides *et al.*[118] showed that the relative change in HCMV viral load is an important measure for the prediction of HCMV disease when using the In-House quantitative PCR assay in PBMCs, and the COBAS Amplicor HCMV monitor kit to detect HCMV load in plasma. A greater than 10-fold increase in HCMC DNA level was seen in more than half of the patients with HCMV disease.

Other methods of defining the episode of HCMV disease in solid organ transplantations were described by Blok *et al.*[119], who demonstrated that the NASBA kit could detect the onset of HCMV infection simultaneously with cell culture and the antigenemia assay. Nevertheless, the disadvantage of the NASBA technique, which is based on HCMV mRNA detection, is its possible inability to improve HCMV diagnosis in a routine setting. One of the reasons for this might be the short period of HCMV mRNA positivity. Since it is not possible to investigate patients every day, the actual time of appearance of HCMV mRNA might be easily missed.

However, the cost of diagnostic tests should be taken into consideration. The cost per test of the anti-HCMV IgM ELISA test is 300 Baht, the present In-House nested PCR is calculated to be 500 Baht, whereas the commercial available HCMV DNA detection is 800 Baht, HCMV mRNA detection is 2,000 Baht and the quantitative real-time PCR is 2,800 Baht.

In conclusion, the optimization of duplex nested PCR assay for amplification of the HCMV MIE gene fragment and beta-globin gene was established in this study. The critical conditions were adjusted. Those consisted of concentration of the primers, dNTP, and $MgCl_2$, annealing time, annealing temperature, and DNA target ratio. The optimized PCR condition provided 10 times more sensitivity than the conventional assay in the detection of HCMV DNA. To skip the initial PCR step, minimized annealing time, shortened the time taken for the optimized PCR assay by approximately 1 hour / round. Furthermore, the beta-globin gene primers were included in the optimized duplex nested PCR to monitor the DNA extraction process. This system could work well in the PBMCs extracted specimen. The optimized duplex nested PCR provided from this study consisted of 0.3 mM of dNTP, 3.0 mM of $MgCl_2$, 0.4 μM of each primer, PCR buffer (10 mM Tris-HCl, pH 9.0 at 25 °C, 50 mM KCl and 0.1% Triton X-100), 1.25 U of *Taq* DNA polymerase, DNA template, and distilled water that was added to make a final volume of 50 μl. The DNA amplification was performed in 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C, followed by a final cycle of 5 min at 72 °C.

The HCMV MIE gene fragment control was prepared by the gene cloning technique, and the human genomic DNA control was prepared from human PBMCs by the PureGene DNA extraction kit. These DNA control were used throughout this study.

The optimized duplex nested PCR was applied to detect HCMV retinitis and monitor the HCMV reactivation in pre- and post- renal transplant patients. It was found that this optimized

PCR method was highly sensitive, with a minimal HCMV DNA detection at 10 copies. By using this optimized PCR method, the aqueous humor was proved to be as effective as ($p>0.05$) the more invasive specimen, vitreous humor, in the detection of HCMV DNA. The specificity of the optimized PCR was also excellent, as none of the control patients were found to be positive for HCMV DNA. In renal transplantation, the detection of HCMV DNA appeared to be useful in screening for a qualified donor. The detection of anti-HCMV IgM was found to be negative in all the recipients except the one who showed a primary infection after renal transplantation. However, even with positive HCMV DNA in their plasma, PBMCs or positive anti-HCMV IgM, all patients did not showed any symptom related to HCMV disease. Therefore, relative changes and investigations in HCMV viral load are suggested in order to monitor the episode of HCMV disease.