# CHAPTER V

## DISCUSSION

Uveitis or inflammation of the inner eye can be initiated by diverse infectious and non-infectious causes and may lead to permanent visual impairment or even blindness. The early identification of infectious causes of uveitis is of extreme importance because a specific antibiotic treatment can be employed for patients with infections, whereas the non infectious uveitis is usually treated by immunosuppressive drugs. The long term treatment of steroids may cause serious adverse effects (Synyder *et al*., 1994; Jabs *et al*., 2000).

The major causes of infectious uveitis have been identified in other parts of the world. However, the spectrum of uveitis throughout the world differs according to various factors, including the geographic area, demographic group and socioeconomic status of the population studied (Chang *et al*., 2002; Wakefield *et al*., 2005; Suhler *et al*., 2008). Toxoplasmosis and herpetic infection have been documented as the common causes of infectious uveitis in western countries. However, the causes of uveitis in South East Asia are discordantly reported in different geographic areas (Rathinam SR.*et al*., 2007). In addition, the fast growth of the HIV/AIDS population in South East Asia contributes to the disparity (Terazawa *et al*., 2003). In Thailand, CMV infection was considered as the common cause of retinitis in HIV-infected patients (Ausayakhun *et al*., 2003; Pathanapitoon *et al*., 2007; Pathanapitoon *et al*., 2008). Moreover, the seroprevalence of *T.gondii* in uveitis patients was significantly

higher than in the non-uveitis group (Wongboonma, 2005; Daidee, 2006; Sirirungsi *et al*., 2009). Thus, we investigated whether herpesviruses and *T.gondii* might be the causes of infectious uveitis in the Northern Thai population.

The diagnosis of uveitis is generally based on a clinical examination. However, overlapping clinical findings may make it difficult to accurately diagnose the pathogens causing infectious uveitis. In addition, diagnostic evaluation of intraocular fluid provides useful evidence for the ophthalmologist. Real-time PCR, the molecular technique that we employed, has proven to be valuable in laboratories around the world (Wittwer *et al*., 2001; Mackay *et al*., 2002; Mackay *et al*., 2004; TIB MOLBIOL GmbH., 2009). Furthermore, the method proposed by the Department of Virology, University Medical Center Utrecht, The Netherlands has proven to be of value in intraocular inflammation diagnosis and ophthalmic management. Specific primers and probes of real-time PCR for herpesviruses and *T.gondii* detection, and efficiency of these techniques have been published (de Groot-Mijnes *et al*., 2006; Westeneng. *et al*., 2007). The PCR principle, is well suited for the detection of small amounts of pathogenic DNA in the intraocular fluid sample. In addition, we developed the duplex and mulitplex real-time PCR to minimize the cost of diagnostic tests. GWC analysis was analyzed in available paired intraocular and plasma samples. A positive GWC analysis implies that the inflammation was caused by the organism which induced the specific antibody. Furthermore, real-time PCR and GWC analyses were considered together the most suitable diagnostic tool for infectious uveitis diagnosis.

The plasmid DNA controls included PhHV-1, CMV, HSV-1, HSV-2, VZV and *T.gondii* prepared by the PCR cloning technique. With this method, a large

amount of specific gene fragment at high purity could be produced. Moreover, the possibilities of unlimited preparation of the plasmid DNA controls were beneficial for routine diagnosis. The concentrations of the purified DNA controls were determined by the DNA fluorescence assay using the Quant-iT™ dsDNA HS Assay Kits, Invitrogen, USA, which yielded direct indicators of the DNA concentration.

PhHV-1 DNA was added in all intraocular fluid samples before the DNA extraction step. The detection of amplified signal of PhHV-1 confirmed the success of the DNA extraction procedure as well as the reproducibility of real-time PCR amplification. Therefore, in the reaction that showed the amplified signal of PhHV-1, no positive signals of the focal pathogens were detected, confirming the negative result (de Groot-Mijnes *et al*., 2006; Westeneng *et al*., 2007).

Reference singleplex real-time PCR for CMV, HSV-1, HSV-2, VZV and *T.gondii* were separately determined for sensitivity. The 10-fold dilution of plasmid DNA controls at a concentration of 100 ng/reactions to 1 x $10^{-4}$ fg/reaction was tested. The reference singleplex real-time PCR for CMV, HSV-1 and VZV had the similar sensitivities. The minimal concentration that could be detected was 0.1 fg (30 copies or equal to 3 x $10^{3}$ copies/mL). Meanwhile, the concentration of 1 fg (300 copies or equal to 3 x $10^{5}$ copies /mL) and 0.01 fg (3 copies or equal to 3 x $10^{2}$ copies /mL) of HSV-2 and *T.gondii*, respectively were the minimal concentrations for detection. Stocher M, *et al*. who used LightCycler protocols reported the sensitivity of real-time PCR for detection of CMV, EBV, HSV-1, HSV-2 and VZV at 250 copies/mL. (Stocher *et al*., 2003) The detection limit of other groups (Hass *et al*., 2004) using LightCycler protocols for HSV-1 and HSV-2 detection has been reported at 600 GE/mL and 200 GE/mL. In addition, Hodgson J, *et al*. found detection limits of HSV-

1 and HSV-2 based on LightCycler protocols, at 2 x $10^4$ copies/mL. (Hodgson *et al.*, 2007). Whereas, the reaction using Taqman protocol reported detection limits of HSV-1 and HSV-1 at 580 copies/mL and 430 copies/mL, respectively (vann Doornum *et al.*, 2003). The differences of our detection limits compared to the previous reports may be due to differing real-time PCR mixtures, specific primers and probes that were complementary to different genome sequences and also the conditions of reaction. Furthermore, the detection of approximately 5 x $10^3$ copies/mL was reported as the diagnostic level that confirmed clinically diagnosed CMV infection. In contrast, CMV DNA at a mean of 1.5 x $10^3$ copies/mL has been found in asymptomatic patients (Gerna *et al.*, 2002). Therefore, our real-time PCR was reasonable for distinguishing reactive CMV infection from the latent or asymptomatic infection.

In addition, all primers and probes amplified and detected only their specific target DNA. No cross-reaction with other DNA controls was observed. These results confirmed the specificity of each primer and probe set.

Contaminations and concerns about cross- and carry over contamination are widely recognized problems with PCR. Thus, uracil-N-glycosylase (UNG) was added to all real-time PCR reactions (Burkardt *et al.*, 2000).

Only fairly small volumes of intraocular fluid samples, ranging from 50-200 µL, can be collected. Thus, in previously reports, Danise *et al.* and Knox *et al.* proposed a sample preparation technique of heating at 100°C for 10 min. prior to PCR. They showed the reliably of this technique as a standard ocular sample preparation procedure when using an appropriate volume (Danise *et al.*, 1997; Knox *et al.*, 1998). However, we realized the problem of inhibitors that may present in the

intraocular fluid sample. This may affect DNA target amplification; therefore we concentrated on the DNA extraction method.

Using reference singleplex real-time PCR, the incidence of the focal pathogens were obtained. In our study group, 40% of infectious uveitis among all with uveitis were diagnosed and CMV was the most common etiology. Meanwhile, infectious etiology has been documented in at least 20-30% of all uveitis cases in the west (Gritz *et al*., 2004). In contrast, infectious uveitis in developing countries is more frequently found, attributed to 11.9%-50% of cases (Rathinam *et al*., 2007). Toxoplasmosis and herpetic infection are the most common infectious agents involved in intraocular inflammation in the western world (Gritz *et al*., 2004) as well as in West Africa (Ronday *et al*., 1996). However, tuberculosis was the most infectious cause of uveitis cases (10.1%) in India and Japan (6.9%) (Singh *et al*., 2004; Wakabayashi *et al*., 2003). Herpetic infection was documented as the most common cause of uveitis in China (1.5%) and North Africa (11.9%) (Yang *et al*., 2005; Khairallah *et al*. 2007). We found that CMV was the most common cause of uveitis in both HIV-infected patients (39/74, 52.7%) and non-HIV-infected patients (50/166, 30.1%). This result conformed to the prevalence of CMV retinitis in approximately 30% of Thai population with AIDS as investigated by clinical assessment together with PCR of intraocular fluid (Ausayakhun *et al*., 2003; Pathanapitoon *et al*., 2005; Pathanapitoon *et al*., 2007). The study of Pathanapitoon K., *et al*. in 2005- 2006 found that 31% (62/200) of HIV-infected uveitis patients were due to infection and CMV infection was noted in 85% (53/62) of cases. In the non-HIV infected group, 22% (30/138) were diagnosed as infectious uveitis with the most common cause being toxoplasmosis (12/138, 8.7%) (Pathanapitoon *et al*., 2008). In

that study, clinical features and serology were used to conclude the diagnosis. Results, diagnosed by real-time PCR, showed that approximately 4% were positive for *T.gondii* in uveitis patients with HIV (3/74, 4.1%) and without HIV infection (7/166, 4.2%). However, the incidence of toxoplasmosis in HIV-infected and non-HIV infected uveitis patients in both studies, were not significant different (*p=0.108*, Pearson chi-square test).

Duplex real-time PCR was developed to minimize the costs of diagnostic tests. Since CMV was found as the most frequently diagnosed pathogen in our population, we established duplex real-time PCR mainly for CMV diagnosis and additionally for HSV-1, HSV-2, VZV or *T. gondii* detection. However, as *T.gondii* is mainly diagnosed by the ophthalmologist, so singleplex real-time PCR is a satisfactory test. The successful developments were in CMV/HSV-1 and CMV/VZV duplex real-time PCR. Both sets had comparable detection limits to singleplex real-time PCR of each pathogen. CMV/HSV-2 and CMV/T.*gondii* had the lowest efficiency in detection under the conditions of other pathogenic DNA being present. CMV/VZV duplex real-time PCR was further investigated for diagnostic efficiency in the known positive samples for CMV and VZV. These true positives were detected at 85% (66/76) and 67% (6/9) for CMV and VZV, respectively. However, positive results from the same samples were equal for both CMV/VZV duplex real-time PCR and singleplex real-time PCR of each pathogen. The samples that showed as false negatives in the reanalyzed reaction were those previously presenting a weakly positive result. This result may due to degradation of DNA with storage.

The advantages of multiplex real-time PCR are reduced cost of detection, amelioration of amplification variation with separate singleplex real-time PCR testing,

small amount sample needed and increased throughput for each run. Accordingly, the multiplex real-time PCR was set up so that CMV, HSV and VZV could be identified in the same test tube. However, CMV/HSV-2 duplex real-time PCR was not successfully developed. Thus, we synthesized new primers and probes targeting the *UL30* gene of HSV. *UL30* gene, coding for DNA polymerase, was proven as the conserved region among HSV-1 and HSV-2 (Burrel *et al.*, 2010). The sensitivity of the newly designed primers and probes for HSV was determined. Our results, revealed the detection limit at 0.1 fg (or equal of 3 x $10^3$ copies/mL) for both HSV-1 and HSV-2. The primers and probes that were used in the study of Burrel S, *et al* had detection limits at 3 x $10^4$ copies/mL and 3 x $10^5$ copies/mL for HSV-1 and HSV-2, respectively (Burrel *et al.*, 2010). However, when our new primers and probes for HSV were mixed in the multiplex real-time PCR and evaluated, only 4/11 (36%) of known positives for HSV were detected. As well as in singleplex real-time PCR amplification using the new primers and probe set only 36% were identified. From these results, all known positive HSV-1 and HSV-2 samples (4 samples and 7 samples, respectively) were re-diagnosed by reference singlexplex real-time PCR (de Groot-Mijnes *et al.*, 2006) to confirm the amplification efficiency of the primers and probe set in clinical sample diagnosis. The results of reference singleplex real-time PCR for HSV-1 and HSV-2 were positive for all samples. Therefore, the new primers and probe for *UL30* gene that we used would not sound suitable for clinical diagnosis. This problem may be related to the conditions of multiplex real-time PCR, thus multiplex real-time PCR mixtures and conditions should be carefully optimized for HSV detection (Biosearch Technologies, Inc., 2000-2011; Wittwer *et al.*, 2001). For CMV and VZV detection, 100% of true positives (40/40 and 8/8, respectively) were

detected in multiplex real-time PCR testing. Our finding is supported by the report from Japan on the successful use of multiplex PCR for herpesviruses genome detection in ocular fluid samples of uveitis patients.. Sixty-five of 100 uveitis patients (65%) showed positive results in that study. The most frequently diagnosed pathogen was VZV (29%) followed by EBV (19%), HSV-1 (7%), CMV (6%) and HSV-2 (3%), respectively (Sugita *et al.*, 2008). Therefore, the developed duplex real-time PCR for CMV/VZV and CMV/HSV-1 detection can provide a rapid and reliable diagnosis, even when only a small amount of intraocular fluid is available.

Paired GWC analysis and real-time PCR in 66 intraocular and plasma samples of non-HIV patients with uveitis provided a 22% (5/23) increase in infectious uveitis identification of anterior uveitis patients and an 8% (5/66) increased diagnosis overall. These findings support previous reports from France (Fekkar *et al.*, 2008) and The Netherlands (de Groot-Mijnes *et al*., 2006) which showed that combination of GWC analysis and PCR technique can improve the positive rate of viral diagnosis. The additional herpesviruses positive results obtained from GWC analysis in our group were found in AU patients solely and were not observed in other anatomical uveitis entities. In addition, *T.gondii* was identified solely by real-time PCR testing. These findings may be influenced by the type of clinical manifestation, duration of infection and immune status of patients, since specific antibodies and genomes of pathogens are present in plasma and intraocular fluid at different times during infection. A correct diagnosis depends also on the biological technique and the immunosuppressive status (Davis *et al*., 2005; Westeneng *et al*., 2007). However, Errera MH, *et al*. reported greater sensitivity and specificity of GWC compared to real-time PCR in posterior uveitis patients. Especially for *T.gondii* detection, GWC

showed higher diagnostic efficiency than real-time PCR, even when the test was carried out later in the disease course, up to 15 months. In contrast, the sensitivity and positive predictive value (PPV) of PCR assessment was higher than that of GWC when the study was performed with the clinically collected samples (Errera *et al.*, 2011). Due to the limited amount of positive results and the high cost of GWC analysis, GWC should be used as an additional method in patients suspected from infectious uveitis that had discordant clinical features and real-time PCR results.