CHAPTER III

MATERIALS AND METHODS

1. Study population

Patients with uveitis were selected from those attending the Ophthalmology Clinic, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University. Uveitis patients were designated by an ophthalmologist into 4 groups of clinical classification; anterior uveitis (AU), intermediate uveitis (IU), posterior uveitis (PU) and panuveitis (PanU) according to The International Uveitis Study Group classification system (Munoz-Fernandez *et al*., 2006). All uveitis patients underwent a tailored screening protocol which included a chest X-ray and various laboratory tests, including erythrocyte sedimentation rate and complete blood counts, and serology for HIV and *Treponema pallidum*. A tuberculosis skin test was done if the patients had keratic precipitates, iris nodules or chest X-ray suggestive of tuberculosis. HLA-B27 typing was performed if the patients had an acute type of AU. Clinical diagnosis of Fuchs heterochromic uveitis syndrome (FHUS) was made in patients who had at least four of the following five signs: 1) unilateral low-grade AU without acute redness, 2) typical scattered keratic precipitates (KPs), 3) absence of synechiae, 4) diffuse iris atrophy, and 5) cataract. Intraocular fluid and plasma were collected from all uveitis patients for laboratory investigation. In addition, non-uveitis control patients from the same geographical area as the uveitis patients were included. These patients were enrolled in our study from April 2005 to February 2011. All

patients conformed to the human ethical statement for clinical research. Human ethical committee approval was obtained from the Faculty of Medicine, Chiang Mai University and Human ethic guidelines regarding clinical research followed.

2. Specimen collection and preparation

2.1 Intraocular fluid sample

Aqueous humor and/or vitreous humor were collected from 240 uveitis patients and 30 non-uveitis control subjects (30 intraocular samples). Approximately 50 to 200 microliters of intraocular fluid from each uveitis patient was collected by an ophthalmologist and kept in a sterile microcentrifuge tube. All samples were stored at -20°C until analysis.

2.2 EDTA-anticoagulated blood sample

Three milliliters of peripheral blood were drawn by venipuncture from each uveitis patient and put into ethylene diamine tetraacetic acid (EDTA) tubes. The EDTA blood sample was mixed well and kept at room temperature or in a refrigerator (for not more than 24 hours) until further processing. The whole blood was centrifuged at 2,500 rpm for 15 minutes. Plasma was separated by using a sterile pipette and put into a sterile 1.5 mL microcentrifuge tube and stored at -20°C for further investigation.

3. Methods

3.1 Investigation of the major causes of infectious uveitis in Northern-Thai population

3.1.1 Detection of causative agents using reference method: Singleplex real-time PCR

To detect causative agents of infectious uveitis in intraocular fluid sample, molecular diagnostic method based on real-time PCR proposed by the Department of Virology, University Medical Center Utrecht, The Netherlands was used as reference method. According to this method, suspected organism was detected in each single real-time PCR reaction called Singleplex real-time PCR. Primers, probes, real-time PCR method and efficiency of these techniques have been published (de Groot-Mijnes *et al*.,2006; Westeneng *et al*., 2007).

3.1.1.1 DNA extraction from the intraocular fluid

The intraocular fluid (aqueous humor and/or vitreous) were thawed then DNA were extracted by using the QIAamp DNA blood mini kit as recommended by the manufacturer. (QIAGEN, Inc., Valencia, CA, USA). In addition, a low concentration (C_T value of around 32) of a seal herpesvirus type 1 (PhHV-1) DNA was added to each sample at the beginning of the extraction procedure.

Briefly, intraocular fluid (aqueous humor and/or vitreous) was lysed by adding 25 µl of intraocular fluid and 20 µl of PhHV-1 in the solution of protease K and buffer AL. After incubation at 56°C for 10 minuets, 200 µL of ethanol (96-100%)

were added. The entire mixture was applied to the QIAamp Spin column. Then the QIAamp Spin column was centrifuged and washed with buffer AW1 and AW2 to remove any PCR inhibitory residuals and other cellular proteins. The purified nucleic acid which was trapped in the column was then eluted with 200 μL of provided elution buffer and kept at -20°C until real-time PCR testing.

3.1.1.2 Preparation of the DNA controls (Plasmid DNA controls)

From the available data on seroprevalence and the association with uveitis from the literature review, five focal pathogens were selected for real-time PCR setting. All of five pathogenic DNA were prepared for use as the positive controls by the PCR Cloning technique.

- HSV-1 and HSV-2 DNA were kindly provided by Asst. Prof. Dr. Wasna Sirirungsi of the Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand.

- CMV and VZV DNA were kindly provided by Dr. Krauvan Balachandra of the Department of Medical Sciences, National Institute of Health, Ministry of Public Health, Thailand.

- *T.gondii* and PhHV-1 DNA were kindly provided by Dr. Jolanda D.F. de Groot-Mijnes, Department of Virology, Eijkman-Winkler Institute, University Medical Center Utrecht, Utrecht, The Netherlands.

All plasmid DNA controls were prepared via the following steps;

1) Amplification of focal gene by PCR

The focal gene of each organism was amplified by conventional PCR using the specific primers (de Groot-Mijnes *et al*., 2006; Table 5). The conventional PCR mixture comprised 0.2 mM dNTP (dNTP: dATP, dTTP, dGTP and dCTP), 1.25 units (U) of Taq DNA Polymerase (BioLabs, USA), PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 at 25°C), 0.4 μM of primer, DNA sample and distilled water to a final volume of 50 μL.

The amplification was performed in an automated thermal cycler (GreenAmp PCR system 2700, Applied Biosystems, USA). The initial step, a single incubation cycle of 95°C for 5 min. was followed by 35 cycles of 95°C for 1 min., 60°C for 1 min. and 72°C for 90 seconds. These were followed by a final cycle of 72°C for 5 min.

To check the reproducibility of PCR product, five microliters of the amplification products were electrophoreses on 2.5% agarose gel in 0.5x TAE (Tris-Aceteate-EDTA, pH 8.0) buffer at 100 volts for 45 min. The amplified fragments were stained with 2 μg/mL of ethidium bromide for 10 min. then destained with distilled water for 10 min. Then the amplified fragments were photographed under ultraviolet light using an ultraviolet transilluminator. The DNA fragment size was determined by using the standard DNA size marker (GeneRuler^{TM}100 bp DNA Ladder Plus, Fermentas Life Sciences).

2) Precipitation of the focal gene fragment

The amplified products of the gene fragments were precipitated before being used in the ligation step. Briefly, the amplified products which showed positive for fragments of each gene were then pooled. One in ten volume of 5M potassium acetate and two volumes of cold absolute ethanol were added. The mixture was kept at -70°C overnight. After that the mixture was centrifuged at 4°C with at 14,000 rpm for 15 min. The supernatant was then discarded and the DNA pellet was washed with 70% ethanol. The DNA pellet was dried for 10 min at room temperature. Twenty microliters of distilled water were added to resuspend the DNA. Finally, the DNA was kept at -20°C for further analysis.

3) Ligation of the gene fragment into a vector

The precipitated product of the focal gene fragment was cloned into a vector using the pGEM®-T Easy Vector kit (Promega, USA). The map of pGEM®-T Easy Vector is shown in Figure 4. The ligation was performed according to the manufacturer's instructions. Briefly, one μL of the precipitated DNA was added in the ligation reaction mixture which contained the Rapid Ligation buffer (60mM Tris-HCl, pH 7.8 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% Polyethylene glycol), 50 ng of the pGEM®-T Easy Vector and 3 Weiss units of T4 DNA ligase was added at a final volume of 10 μL. After mixing, the ligation reaction was incubated at 4°C for overnight.

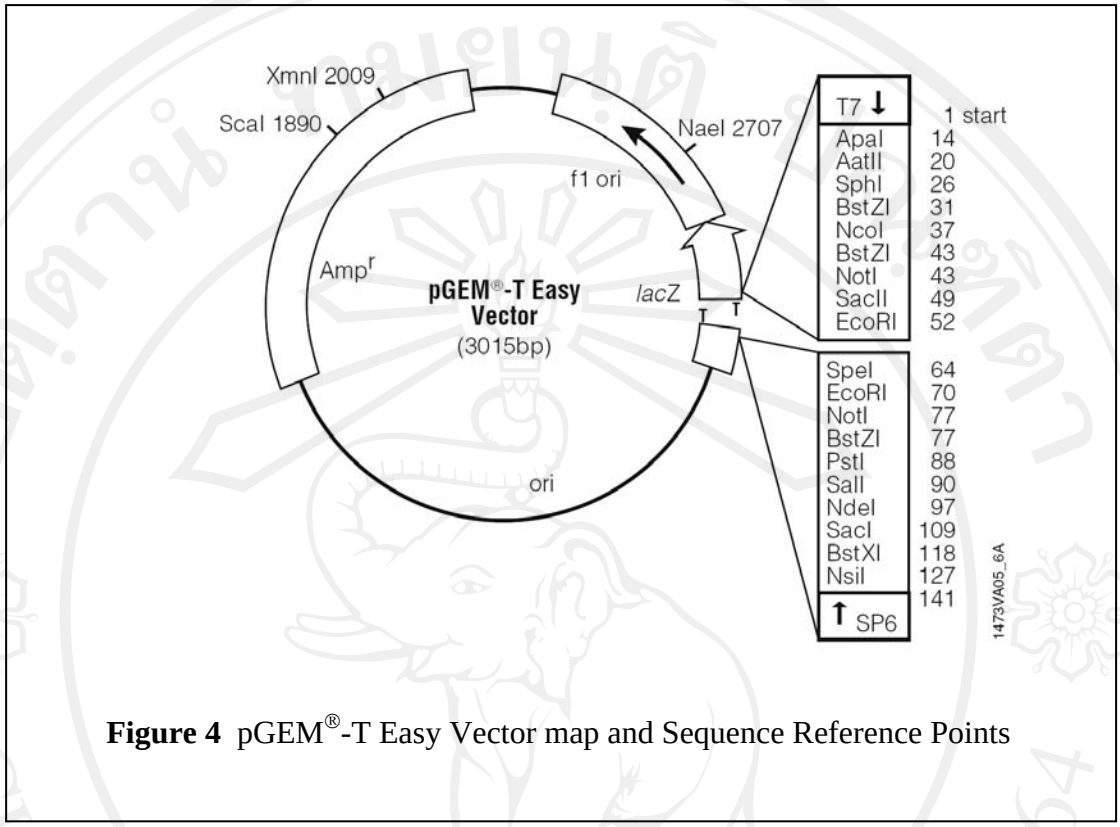

Figure 4 pGEM®-T Easy Vector map and Sequence Reference Points

4) Transformation of the recombinant plasmid DNA

The recombinant plasmid from the previous step was transformed into the competent *E.coli* cells (JM 109 strain; Promega, USA). The transformation was carried out in accordance with the protocol recommended by the manufacturer. Briefly, 2 μL of each ligation reaction mixture were added into 50 μL of competent cells in a sterile 1.5 mL microcentrifuge tube on ice. The mixture was flicked to mix and then placed on ice for 20 min. The mixture was then heat shocked at exactly 42°C in a water bath for 50 seconds, and the mixture was returned immediately to the ice box for 2 min. Nine hundred and fifty μL of SOC medium were added into the mixture tube which contained transformed cells with ligation reaction mixture, and incubated for 90 min at 37 °C while slowly shaking (around 150 rpm). One hundred

microliters of transformation culture were placed onto a Luria-Bertani (LB) plate containing 100 μg/mL of amplicillin and incubated at 37°C overnight. After incubation, the bacterial colonies were screened for the presence of the recombinant plasmid.

5) Screening for the recombinant plasmid DNA in the transformed bacteria.

The transformed colonies were screened for the presence of recombinant plasmid DNA. The colonies grown on LB plate were randomly selected and were then either subjected directly to the conventional PCR as described above or processed through the plasmid DNA preparation. Only colonies that showed PCR products specific to each gene were confirmed as successfully transformed.

6) Purification of the recombinant plasmid DNA

For plasmid DNA preparation, the plasmid DNA was extracted from the bacterial colony by a mini-preparation of the alkaline lysis method (Samdroof, *et al*., 1987). Briefly, a single bacterial colony was cultured in 3 mL of LB medium containing 100 μg/mL of ampicillin in a loosely capped 15 mL tube, and then incubated at 37°C for 18-24 hours with vigorous shaking (150 rpm). After incubation, 1 mL of the bacterial culture was transferred into a 1.5 mL microcentrifuge tube and centrifuged at 8,000 x g for 3-5 min. at 4°C. Meanwhile the left bacterial culture was stored in 20% glycerol-LB medium containing 10 μg/mL amplicillin at -20°C. After centrifugation, the supernatant was discarded and then the bacterial pellet suspended with 100 μL of Solution I. The mixture was vortexed quickly and incubated on ice for

5 min. Then, 200 μL of Solution II were added and the contents mixed by inverting the tube and incubating on ice for 5 min. Fifty μL of ice-cold Solution III were added and mixed by gently vortexing. The mixture was then incubated on ice for 5 min. After being centrifuged at 12,000 x g for 5 min. at 4°C, 250 μL of the supernatant were collected. 0.5 volumes of absolute Phenol and 0.5 volume of Chloroform/Isoamyl alcohol were added and then the mixture was re-centrifuged as above. The upper layer supernatant was transferred by gentle aspiration into the microcentrifuge tube. One volume of Solution III and 2 volumes of 95% ethanol were added to precipitate the DNA. Then the mixture was stored at -70°C for 10 min, and centrifuged at 12,000 x g for 15 min. at 4°C. After the supernatant was discarded, the DNA pellet was rinsed with 1 mL of 70% ethanol and centrifuged at 12,000 x g for10 min. at 4°C. The supernatant was removed and the DNA pellet was allowed to dry in air for 30 min. Finally, the DNA was dissolved in 50 μl of distilled water and stored at -20°C until further examination.

7) Quantitation of plasmid DNA controls by the fluorescence assay.

The concentration of plasmid DNA control was measured by the DNA fluorescence assay using the Quant-iT™ dsDNA HS Assay Kits, Invitrogen, USA. For measurement of the concentration of dsDNA, the dye which was provided in the kit will be changed in fluorescence characteristics in the presence of DNA allowing accurate DNA quantification. Ten microliters of the standard (10 ng/ μL) were diluted in 190 μL of Quant-iT™ dsDNA HS working buffer. The 1-20 μL of each DNA

sample were diluted in 180-199 µL of Quant-iT™ dsDNA HS working buffer. Then, the concentration was read using a Qubit™ fluorometer. The quantity of DNA was calculated by using the following equation 1):

1) Quantity of DNA sample (ng/ µL) = $\frac{O.D._{485/530} \text{ of DNA sample} \times \text{Standard conc. (10 ng/ µL)}}{O.D._{485/530} \text{ of Standard}}$

The Quant-iT™ fluorometer gives values of the Quant-iT™ dsDNA HS working assay in ng/mL. This value corresponds to the concentration after each DNA sample was diluted into the assay tube. The concentration of the DNA sample was calculated by using the following equation 2):

2) Concentration of the DNA sample = QF value $\times \left(\frac{200}{X} \right)$

Note: QF value = the value (ng/mL) given by the Quant-iT™ fluorometer

X = the number of microliters of the DNA sample that was added to the assay tube.

The plasmid DNA concentration (ng/µL; from equation 2) was calculated to convert into the number of DNA copies by using the correlation formula (Hammond *et al*., 2005):

$$\frac{6 \times 10^{23} \text{ (copies/mol)} \times \text{concentration (g/µL)}}{\text{MW (g/mol)}} = \text{amount (copies/µL)}$$

Note: MW = (number of base pairs) x (660 daltons/base pairs)

1 mol = 6 x 10^{23} molecules (copies)

3.1.1.3 Detection of interested DNA by reference singleplex real-time PCR

- **Real-time PCR primer and probe**

The specific primers and probes for the focal pathogens used in this study are shown in Table 5. All specific primers and probes except that for HSV were reproduced according to the work of de Groot-Mijnes JD and college (Groot-Mijnes *et a*l., 2006). Specific primer and probe for non-typed HSV targeting the *UL30* gene were designed by using the Primer premier 5 software. (PREMEIR Biosoft International). The *UL30* gene encodes for DNA polymerase that is in the conserved region of HSV. This region shows high identity among HSV-1 and HSV-2 (Burrel *et a*l., 2010).

Table 5 Primers and probes for detection of interested pathogens**.**

Primers/Probes	**Primer sequence (5'->3')**	**Specific region**
1. CMV		*UL 54 gene*: DNA polymerase
- FWD primer	GCC GAT CGT AAA GAG ATG AAG AC	
- RWD primer	CTC GTG CGT GTG CTA CGA GA	
probe	(5HEX)AGTGCAGCCCCGACCATCGTTC(BHQ1a~5HEX)	
2. HSV-1		*RL2 gene*: immediate early protein
- FWD primer	TCC AC**C** AGG ACC ACG TAC G	
- RWD primer	CTG **T**CG CCT TAC GTG AAC AAG AC	
probe	(6~FAM)CCCGTCTCCATGTCCAGGATGGG(TAMRA~6~FAM)	
3. HSV-2		*RL2 gene*: immediate early protein
- FWD primer	TCC AC**G** AGG ACC ACG TAG G	
- RWD primer	CTG **G**CG CCT TAC GTG AAC AAG AC	
probe	(6~FAM)CCCGTCTCCATGTCCAGGACGGG(TAMRA~6~FAM)	
4. HSV		*UL30 gene*: DNA polymerase
- FWD primer	GGGACATCCAGGACTTTG	
- RWD primer	GCTTGTAATACACCGTCAG	
probe	(5'ROX) CACCGCCGAACTGAGCAGAC (3'BHQ)	

Table 5: Primers and probes for detection of interested pathogens (continued).

Primers/Probes	Primer sequence (5'->3')	Specific region
5. Toxoplasma		*B1 gene*: serum reactive protein
- FWD primer	TCC CCT CTG CTG GCG AAA AGT	
- RWD primer	AGC GTT CGT GGT CAA CTA TCG ATT G	
probe	(6~FAM)TCTGTGCAACTTTGGTGTATTCGCAG (TAMRA~6~FAM)	
6. VZV		*ORF10 gene*: Tegument protein
- FWD primer	AAG TTC CCC CCG TTC GC	
- RWD primer	TGG ACT TGA AGA TGA ACT TAA TGA AGC	
probe	(6~FAM)CCGCAACAACTGCAGTATATATCGT C (TAMRA~6~FAM)	
7. PhHV		*Glycoprotein B gene*: glycoprotein B
- FWD primer	GGG CGA ATC ACA GAT TGA ATC	
- RWD primer	GCG GTT CCA AAC GTA CCA A	
probe	(5HEX)TTTTTATGTGTCCGCCACCATCTGGAT C (BHQ2a~5HEX)	

- **Reference singleplex real-time PCR reaction**

Five pathogens including CMV, HSV-1, HSV-2, VZV and *T.gondii* were analyzed by reference singleplex real-time PCR. Briefly, after nucleic acid extraction, each sample (and each interested pathogenic DNA control) was separately tested for five focal pathogens. PhHV DNA was added to every sample before extraction to act as an extraction control. For reference singleplex real-time PCR, 10

μL of the extracted nucleic acid was added in 15 μL of the real-time PCR reaction mixture (DyNAmo^{TM} Probe qPCR kit, New England Biolabs Inc.) which contained specific primers and probes. Real-time PCR was performed in a Chromo4-Real-time PCR detector machine (DNA Engine, Pelter thermal cycle; BIO-RAD) with a repeated cycling program as shown in Table 6

Table 6 Real-time PCR cycle.

Step	Purpose	Temperature	Time
1	UNG incubation (optional)	50°C	2 minutes
2	Initial denaturation	95°C	15 minutes
3	Denaturation	95°C	15 seconds
4	Annealing and Extension	60°C	60 seconds
5	Data acquisition		Fluorescence data collection
6	Number of cycles	45 cycles for step 3-5	

3.1.2 Determination of specificity of reference singleplex real-time PCR

The sequences of the tested organisms were blasted on the database in GenBank using the nucleotide blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The specificity of primers and probes were tested by amplifying target and non-target DNA. Amplification of target DNA alone confirmed the specificity of each assay.

3.1.3 Determination of sensitivity of reference singleplex real-time PCR

The serial 10-fold concentrations of each plasmid DNA control was tested individually. The lowest concentration of DNA that could be successfully amplified was considered as the end point of this sensitivity test. In addition, the reproducibility of PCR amplification was controlled by observing the threshold cycles (C_T) value of DNA control after each run. Only C_T values which ranged within standard deviations of the repeated runs were accepted.

3.1.4 Incidence of infectious agents in intraocular fluid samples

The incidence of each infectious agent was investigated in 240 intraocular fluid samples. Briefly, 10 μL of intraocular extracted DNA was mixed into 15 μL of the real-time PCR reaction mixture (DyNAmo^{TM} Probe qPCR kit, New England Biolabs Inc.). Then, separate analysis by reference singleplex real-time PCR for each pathogen was done as described above. In addition, the plasmid DNA controls were performed in parallel to intraocular fluid samples testing. The reproducibility of PCR amplification was controlled by observing the threshold cycles (C_T) value of plasmid DNA control after each run. Only the C_T values which ranged within 2SD of the repeated runs were accepted.

3.2 Development of multiplex real-time PCR technique to identify organisms involved in uveitis.

3.2.1 Setting up the Multiplex real-time PCR reaction panel.

Regarding, the limitation in the volume of intraocular fluid and the cost of laboratory investigation motivated the search for methods that can identify a panel of pathogenic organisms using a minimal volume of intraocular fluid. In this study, multiplex real-time PCR was developed. Based on the seroprevalence, clinical manifestation and the results from reference singleplex real-time PCR, the focal viruses including CMV, HSV and VZV were grouped and optimized to derive a multiplex real-time PCR which had similar sensitivity and specificity compared to the reference singleplex real-time PCR.

- **Specific fluorescence-quenched probe labeling**

This study purposed to set up multiplex real-time PCR capable of detecting herpesviuses, including CMV, HSV and VZV in the same reaction tube. Thus, specific probes for CMV, HSV and VZV should be labeled with different reporter fluorophore dye which will generate the fluorescence signal at different wavelengths. In addition, black hole quencher (BHQ) was recommended for use as the quencher. BHQ dyes are a true dark quencher with no native emission due to their polyaromatic-azo backbone. Substituting electron-donating and –withdrawing groups on the aromatic ring produced a complete series of quenchers with broad absorption curves that span the visible spectrum: BHQ-0 (493 nm), BHQ-1 (534 nm), BHQ-2 (579 nm) and BHQ-3 (672 nm). These quenchers can be paired with all common fluorophore

reporter dyes to construct efficiently quenched qPCR probes for multiplexing assays (Biosearch Technologies, Inc.: 2000-2011. Multiplex real-time PCR., 2010). In this study, the specific probe for CMV was labeled at the 5' end with 5-hexachloro-fluirescein CE (HEX) as the reporter dye and at the 3' end with BHQ-1a as the quencher dye. For the HSV-specific probe, ROX was labeled at the 5' end as the reporter dye and BHQ was labeled at the 3' end as the quencher dye. Specific probe for VZV was labeled at the 5' end with 6-carboxyfluorescein (FAM) as the reporter dye and at the 3' end with 6-carboxytetramethylrhodamine (TAMRA) as the quencher dye. (Table 5)

- **Optimization of multiplex real-time PCR**

The concentration of specific primers and probes of each virus were optimized to put in the Multiplex real-time PCR reaction for detecting herpesviruses (CMV, HSV and VZV) in one tube. The CMV, HSV and VZV DNA controls were prepared in 10-fold serial dilutions containing 1 μg/mL to 0.1 fg/mL. Each of CMV, HSV and VZV DNA controls that contained certain amounts of DNA were amplified in a mixture of corresponding primers and probes. The positive signal represented by C_T value of each DNA control was observed. Then, the C_T values obtained from reference singleplex real-time PCR and multiplex real-time PCR were compared and used to justify the suitable effective concentration of primers and probes mixture. The concentration of primers and probes that showed the positive signal greater or comparable to reference singleplex real-time PCR was selected to use in a suitable panel for herspesviruses multiplex real-time PCR.

3.2.2 Determination of sensitivity and specificity of Multiplex real-time PCR

Each of the CMV, HSV and VZV DNA controls that contained given amounts of DNA was amplified in a mixture of corresponding primers and probes developed in the previous step. The positive signal represented by C_T value of each DNA control was observed. Then, the C_T values obtained from singleplex real-time PCR and multiplex real-time PCR were compared and used to justify the suitable effective amount of primers and probes mixture. Sensitivity of multiplex real-time PCR for each virus was determined by successfully amplification of the minimal amount of DNA control concentrations. Specificity of multiplex real-time PCR was confirmed since each of the specific primers and probes showed positive signals after amplification only of its target DNA.

3.2.3 Diagnostic efficiency determination of Multiplex real-time PCR

Intraocular fluid samples which were tested positive for CMV, HSV and VZV by referenced singleplex real-time PCR were tested by using the multiplex real-time PCR. All known positive samples were simultaneously reanalyzed by reference singleplex and multiplex real-time PCR. Then, the results of each sample obtained from reference singleplex and multiplex real-time PCR were compared and evaluated.

3.3 Evaluation of the diagnostic efficiency of Goldmann-Witmer coefficient analysis and Real-time PCR technique in identifying the causes of uveitis.

In addition to real-time PCR analysis, the Goldmann-Witmer coefficient (GWC) was determined to assess the active intraocular production of specific antibodies in the eye to each of the tested organisms.

3.3.1. Analysis of infectious uveitis by Goldmann-Witmer coefficient (GWC): Determination of plasma and intraocular antibody production (de Groot-Mijnes *et al.*, 2006; Westeneng *et al.*, 2007; de Boer *et al.*, 1996)

The amount of specific immunoglobulin G (IgG) titers against HSV, VZV, CMV and *T.gondii* in plasma and intraocular fluid were determined using the Enzygnost® Anti-HSV/IgG, Anti-VZV/IgG, Anti-CMV/IgG and Toxoplasmosis/IgG ELISA kits (Dade Behring), respectively. The assays were performed according to the manufacturer's instructions. However, instead of a single 1/231 dilution, four four-fold dilutions (1/231 to 1/14,784) and four two-fold dilutions (1/115.5 to 1/924) were tested for the plasma and intraocular fluid, respectively.

Total IgG was determined by the in-house ELISA using commercially available reagents. For the plasma, five steps of serial three-fold dilutions (1/50,000 to 1/4,050,000) and for intraocular fluid, five steps of three-fold serial dilutions (1/5,000 to 1/405,000) were prepared in phosphate-buffered saline (PBS), supplemented with 0.05% Tween 20 (PBS-Tw20) and 2% skim milk. For the determination of the total

IgG concentration, seven steps of serial two-fold dilutions (50,000 to 3,200,000) of a nephelometer standardized human serum (Dade Behring) were included.

Briefly, flat-bottom microtiter wells (Nunc) were coated by incubating with 100 µL of PBS containing 1µg/mL goat $F(ab')_2$ antihuman IgG (Southern Biotechnology Associates) at 4°C for 16-24 hours. The wells were washed four times with 400 µL of PBS-Tw20; 100 µL of the diluted plasma and intraocular fluid were added and incubated for 1 hour at 37°C. Then the wells were washed four times with 400 µL of PBS-Tw20. One hundred microliters of horseradish peroxidase-conjugated goat anti-human IgG (Southern Biotechnology Associates) were added to each well at a dilution of 1/5,000 to 1/15,000, depending on optimal dilution calculated for each batch. After 1 hour incubation at 37°C, the wells were washed four times with 400 µL of PBS-Tw20. Finally, 100 µL of TMB substrate (Dade Behring) was added to each well. After 10 minutes, reaction was stopped by adding 100 µL of 0.5 mol/L of sulfuric acid and the absorption was measured at the optical density of 450 nm. Intraocular antibody production was determined by calculating the GWC. GWC analysis is considered positive when the value exceeds 3 (de Groot-Mijnes *et al.*, 2006; Westeneng *et al.*, 2007; de Boer JH *et al.*, 1996). In the case of multiple positive GWCs, the highest GWC indicates the causative pathogen (Westeneng *et al.*, 2007).

3.3.2. Determination of diagnostic value of GWC analysis and real-time PCR analysis

Paired plasma and intraocular fluid samples from non-HIV patients with uveitis were investigated by both real-time PCR and GWC analysis. Positive results obtained from referenced singleplex, multiplex real-time PCR and GWC analysis were determined.