CHAPTER VI

DISCUSSION

The advantages of a new rapid PCR method for the clinical virology laboratory were developed to detection and quantification for diagnosis of virus infection using the LightCycler technology. The several studies used application of the Real-time technology to diagnosis and monitor therapy in many diseases from the virus infection, genetic diseases and the oncology studies.

In the present study, real-time PCR method was develop and evaluate for diagnosis the dengue virus genotype by using LightCycler instrument (hybridization probe format). Sera from 100 patients admitted in King Chulalongkorn Memorial Hospital with dengue infection based on clinical and laboratory data were collected. All sera, which are positive by RT-PCR method, were used in this study. The first PCR product were diluted with sterile distill water 1:50 for use in the real-time nested PCR. Nested PCR by LightCycler instrument was used because the sensitivity of assays performed with the LightCycler system may be compromised by the small volume of extracted nucleic acid that can be added to the glass capillary reaction vessels used in the instrument. The secondary amplification, product detection, and identification steps were performed with the LightCycler system using a template from the convention thermal cycler RT-PCR.

The severe pathogenesis of dengue virus is caused by antibody-dependent enhancement or by other mechanism; tools for rapid and specific laboratory diagnosis, including virus serotyping are need. Such diagnosis is necessary so that appropriate prevention, treatment, and control measures can be initiated and accurate epidemiologic data can be maintained (24). For typing dengue RNA by conventional thermal cycler,

Nested RT-PCR is a very useful method for early diagnosis of dengue virus genotype. By using a genotype specific primer dengue virus can be discriminated by the different size of the PCR product. Nested-PCR is a highly sensitive method that has been shown to be an effective tool and the most sensitive method for the detection of dengue genotype. However, analysis of PCR products is for the most part tedious and at many times ambiguous. Conventional methods such as ethidium bromide stained agarose gel electrophoresis may show complicated patterns (multiple bands or smear) making it difficult to interpret gel data and because of nested protocol are proned to contamination by PCR carry over. We have developed a method that uses a real-time PCR technique that amplifies and detects dengue virus genotypes in one reaction capillary. The primary goal in the design of the real-time assay was rapid identify genotype of dengue virus by melting temperature of the hybridization probes. The simultaneously rapid detection and classification of dengue genotype will help the clinician in provide appropriate treatment. The genotype will be useful in epidemiology for dengue infection.

A main problem of this approach is the need for highly conserved regions for routine PCR testing on the one hand and regions with sufficient variability for serotyping discrimination on the other hand. The use of primers homologous to conserved dengue virus RNA sequences ensures that all genotype of dengue virus will be amplified in the PCR and real-time PCR. The hybridization probes were designed to recognize the internal sequence of the dengue virus. We took the advantage of the ability of melting analysis to detect sequence variations (from the probe sequence). The hybridization probes are design by perfect match in one genotype and mismatch in another type. The probes that are bound to perfectly matching target DNA separate at higher temperature than DNA containing destabilizing mismatches. The detection probes are labeled with different fluorophore, LC-Red 640 and LC-Red 705. Dengue genotype 1 and 3 are measured fluorescence signal of LC-Red 640 in the F2 channel and dengue genotype 2 and 4 are

measured LC-Red 705 in F3. The method was used to discriminate the dengue virus by fluorescence signal and melting temperature of hybridization probes.

Development of the in-house master mix that used in LightCycler system was compared with commercial kit, LightCycler — Faststart Master DNA Hybridization Probe Kits (Roche Diagnotic, Germany). The melting temperature of dengue virus genotype 1-4 from both reactions gave a similar melting peak of each genotype. In this study, we used the in-house master mix instead commercial kit. Thus, we can be decrease cost and enables high throughput examinations in routine settings. Magnesium concentration is a crucial factor affecting the performance of *Taq* DNA polymerase and efficiency of primer and probe capture. Mg^{2+} titration experiments were done in real-time PCR with melting curve analysis to determine the optimal cation concentration for detection and segregation of dengue genotype-specific melting peak. The reaction with 3 mM $MgCl_2$ provided the greatest discrimination among the melting peaks for the genotypes tested. The excess free Mg^{2+} reduces the enzyme fidelity and may increase the level of nonspecific amplification. For these reasons, it is important to empirically determine the optimal $MgCl_2$ concentration for each reaction.

Using the in-house reaction in LightCycler instrument, detection of amplification can be performed by using hybridization probe format. Differentiation of dengue virus genotype can be accomplished using different hybridization probes and distinguished by different of melting temperature. Five oligonucleotide hybridization probes were design to identify the dengue virus serotyping. First probe pair the detection probe labeled with LC-Red 640 was used to detect dengue genotype 1 and 3. The melting temperature of DEN-3 is higher than DEN-1. On the other hand, dengue genotype 2 and 4 were used three oligonucleotide hybridization probes for typing the dengue virus. Because of the sequence of dengue genotype 2 and 4 has a high variation in the anchor probe region, seperate anchor probe-labeled Fluorescein was design for each genotype. As describe

above, probe for dengue genotype 2 and 4 must be clearly discrimination but the result of this study found that dengue genotype 2 and 4 had very close melting temperature and cannot discriminate the genotype. The reason of close melting temperature of dengue genotype 2 and 4 may be the individual sequence variation. The hybridization probes that have a discrimination power for typing dengue genotype 2 and 4 are need in order to serotyping dengue 2 and 4.

To analyze reproducibility of the LightCycler typing method, the variation of melting temperature were tested by within- and between-run of all four genotypes. With difference samples showing different amplification efficiencies, the derivative melting curves were highly reproducible with melting peaks differing by less than $0.9^{\circ}C$ in genotype 1, $0.6^{\circ}C$ in genotype 2 and 3, and $0.4^{\circ}C$ in genotype 4, allowing easy and unambiguous assignment of genotypes to the respective melting curve. We wanted to test sensitivity of LightCycler assay by compared with convention RT-PCR assay. The tenfold dilutions of plasmid, pTT4700 were used in both assays. All samples were tested as replicates of ten. The 75 molecules of plasmid were positive by LightCtycler assay and conventional assay by ten times repeat. The 7.5 molecules of samples were inconsistently positive in both assays. The sensitivity was evaluated only amplification step because of the method that develop in this study are nested real-time PCR. The sensitivity of real-time PCR is similar to the conventional nested PCR.

The advantages of this study are rapid detection and identification of dengue genotype for virus-infected patients that have clinical symptom like dengue infection, easy to interpretation the genotype by using the different of melting temperature and color of fluorescence signal, and epidemiological study. Another advantage of typing the dengue genotype, epidemiologic of dengue virus in Thailand was found in children, but recently, adult infected with dengue virus have a severe symptom. The severity of dengue infection in adult may be caused by the circulation of all four dengue virus genotype. In which

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patients have an antibody to one dengue genotype when they were infected with another genotype, the severity of adult infection may due to antibody dependent enhancement. The rapidity of diagnosis will help the clinician to provide care for patient in proper time and adequate treatment.

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