CHAPTER I

INTRODUCTION

Dengue virus is the causative agent of dengue fever (DF) and dengue hemorrhagic fever (DHF). It is the most important disease in the tropical and subtropical regions of the world. There are four antigenically related serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4), by plaque reduction neutralization test (PRNT). The four dengue virus serotypes are RNA viruses belonging to the genus *Flavivirus* of the *Flaviviridae* family, and their main arthropod vectors *are Aedes aegypti* and *Aedes albopictus*. The dengue virus genome consists of a single-stranded, positive-sense RNA of approximately 11 kb in length containing a single opening reading frame. Infection with any genotype generally leads to a mild, self-limiting febrile illness (dengue fever). However, a more severe form of the disease, involving vascular and homeostatic abnormalities (dengue hemorrhagic fever-dengue shock syndrome: DHF-DSS), is responsible for a high mortality rate, primarily among children (1, 2, 3, 4). This syndrome was first recognized in outbreaks of dengue in Southeast Asia in the mid-1950s (5). The co-circulation of all four-dengue genotypes and their capacity to produce severe dengue disease was recognized in 1958 in Bangkok, Thailand (6). As each year, millions of human dengue infection occurred, and over two billion people are at risk of infections (2, 3, 6, 7, 8). In Thailand, there were moderately severe epidemics in 1984, 1985, 1989, 1990, and 1997, and severe epidemics in 1987 and 1998 (6). The incidence rate of dengue infection during January 1, 2003 — December 20, 2003 was 98/100 000 (61,806 cases) and case fatality rate as 0.12% (76 cases) (9).

At present, there are three models of severe pathogenesis of dengue infection, the antibody-dependent enhancement model, the nutritional status model and specific dengue genotype model. First model is antibody-dependent enhancement model (10, 11, 12, 13, 14), associated with prior immune sensitization by a heterotypic virus. This kind of dengue infection provides lifelong homotypic immunity, but only transient cross-protection against other genotypes is achieved, making sequential infection possible. Severe disease is postulated to be the result of heterologous, non-neutralizing antibodies facilitating virus infection of mononuclear cells via immune complex and Fc receptor. An alternative hypothesis is nutritional status, the study in Thailand by Usa Thisyakorn and Suchitra Nimmannitya (1993) reported that most patients with dengue hemorrhagic fever are not undernourished. This may be related to strong immune response in patients with good nutritional status. The last hypothesis is specific dengue genotype model. Some specific viral genotypes may be risk factors in the production of more severe disease (8). There is some virus strains that have greater epidemic potential. Phenotypic expression of genetic changes in the virus genome may increase virus replication and viremia, severity of the disease, and epidemic potential, as several studies showed an increasing of dengue disease severity with DEN-2 virus type (1, 15, 16). However, severity of the disease is also related to other dengue genotype.

There is no specific therapeutic treatment for dengue virus infection. In order to provide timely infection for the care of the patient, it is important to establish a diagnosis of dengue virus infection during the few days of clinical symptoms. Furthermore, determination of the serootype of dengue virus is also important for the surveillance of dengue fever. The current methods used by most laboratories for the diagnosis of acute dengue virus infections are the detection of virus or antibody in blood samples. Several serological techniques have been devised for dengue diagnosis but the immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA) is the most widely

used diagnostic test (17). A definitive serological diagnosis requires testing of acute- and convalescent- phase samples, usually collected at least 7 days apart, to demonstrate a fourfold or greater increase in antibody titer. However, the specific serotype of dengue virus responsible for the infection cannot be determined reliably by this method, particularly for secondary dengue virus infections. Also, virus isolation by tissue culture assays, inoculation of suckling mice or intrathoracic inoculation of mosquitoes, followed by genotype identification with immunofluorescence antibody testing using monoclonal antibodies usually takes at least a week. However, viral isolation is not always successful because of small amounts of viable virus in the inocular, virus- antibody complexes, and in appropriate handling of samples. Advance molecular diagnostic systems have led to the development of various reverse transcriptase-polymerase chain reaction (RT-PCR) for detecting dengue viral RNA in human serum or plasma samples. RT-PCR assays can provide a some-day genotype-specific laboratory diagnosis of dengue virus infection and have sensitivity similar to the viral isolation in cell culture with more rapid result (18, 19, 20, 21).

The recently developed LightCycler amplification technology combines rapid glass capillary thermal cycling with real-time microvolume fluorescence monitoring. The system is equipped with a 470 nm light emitting diode has detection channels at 530, 640, and 705 nm, monitoring the 32 capillaries simultaneously. Melting point analysis of the amplicons at the end of the run is used as a specificity control when the fluorochrome SYBR Green is used for detection of double stranded DNA. Alternatively, specificity can be tested during the run by using two target-specific hybridization probes, which utilize fluorescence resonance energy transfer (FRET) to generate a measurable signal. In the latter case, two oligonucleotide probes bind to immediately adjacent regions of the respective amplicon. The anchor probe is labeled at its 3' end with fluoresein, while the 5' end of the detection probe is labeled with either of the fluorochromes LC-Red 640 and

LC-Red 705. To avoid elongation by the *Taq* polymerase, the detection probe is 3' phosphorylated. When it is ensured that the anchor and detection probes are spaced no more than five nucleotides apart. Upon excitation of fluorescein by the light emitting diode, energy is transferred to LightCycler-Red. Fluorescence emitted from the fluorophores attached to the hybridization probes is measured from the tip of the capillaries once during each cycle. The increase in PCR product by each cycle is displayed on the screen as a growing amount of DNA. After a complete PCR run, there is a possibility of running a melting point analysis, whereby the temperature is lowered to below annealing temperature for the probes and then slowly increased. The fluorescence signal drops when the detection probe melts off its target. The melting temperature (Tm) of the detection probe is visualized as the first negative derivation (-dF/dT) of the melting curve. The sudden drop in fluorescence signal is thereby transformed a peak, allowing easy identification of the T_m (23, 24).

The LightCycler system is allowed PCR product detection and identification with a variety of fluorescence chemistries. The LightCycler system can provide the clinical virologist with rapid PCR results because of very fast temperature transition rates, online analysis of data, and with reduced risk of contamination because product detection is accomplished in a closed system, advantages that have also been recently described for other applications in Clinical virology (25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38).

The studies of new PCR technology based upon fluorescent probes and melting curve analysis, detecting the match of the probes with its target, is a rapid and simple method ideally suited for detection single nucleotide polymorphism (SNPs). The hybridization probe format has many applications in diagnosis and therapy. The sequence-specific hybridization probes can be designed that allow detection and analysis of PCR products on the LightCycler without the need for any post PCR sample

manipulation, allowing high throughput genotyping and product quantification. After amplification, a melting curve is generated that allows rapid genotyping (26, 30, 39, 40, 41, 42, 43, 44, 45, 46).

Matthias Schröter et al., (2002) reported that genotyping of HCV by using three different pairs of hybridization probes could be discriminated HCV genotype by measurement different of LC-Red emission and the different of melting temperature of the detection probes. They used 3 pair of hybridization probes, which one labeled with LC-Red 705 and another labeled with LC-Red 640. These data suggest that Tm analysis was performed, and the fluorescence signals were first measured in channel F2 for LC-Red 640 emission. Genotypes 1 and 3 displayed similar Tm, but genotypes 2 and 4 could be determined by their different Tm. For discrimination of type 1 and 3, T_ms were determined by using measurement of LC-Red 705 emission in F3 (38).

In this study, for dengue virus detection and genotyping simultaneously are developed by using the LightCycler instrument, two pairs of hybridization probes are designed that recognize adjacent internal sequence with in target sequences of dengue virus. The two-oligonucleotide primers that used in this study are conserved in 4 dengue genotypes. The detection probes are labeled with different fluorophore, LC-Red 640 and LC-Red 705. The hybridization probes are design by perfect match in one genotype of dengue virus and mismatch in another. This will alternatively differentiate with different of melting temperature. Hybridization probes that are bound to perfectly matching target DNA separate at a higher Tm than those that are bound to DNA containing destabilizing mismatches.