CHAPTER V

DISCUSSION AND CONCLUSION

It is generally believed that concurrent infections by different serotypes of dengue virus occur during epidemics in which multiple dengue virus serotypes are being transmitted. Although co-circulation of multiple serotypes of dengue virus in the same region has been recognized in several countries for decades, it was not until 1982 that the first case of concurrent infections with 2 dengue virus serotypes was reported (82).

Our study demonstrated that 76.47% (65 of 85) of patients were single infection cases and 23.53% (20 of 85) were multiple infections. 49.23% (31 of 65) were typed as DEN-4, 24.61% (16 of 65) were typed as DEN-1, 16.93% (11 of 65) were typed as DEN-3, and 9.23% (6 of 65) were typed as DEN-2 respectively. DEN-4 was commonly detected as single infection, followed by DEN-1, DEN-3, and DEN-2, respectively.

DEN-1 and DEN-4 were identified to be the most frequent dengue virus combinations. This was followed by DEN-2 and DEN-4, and then DEN-2 and DEN-3 combination. Although DEN-2 was not commonly detected as single infection, it was seen in three cases of multi-serotype infections. The number of DEN-2 infections seen as single serotype was less as compared to DEN-4 or DEN-1.

The pattern of muti-serotype infections in this study is worth mentioning. In all cases of multi-serotype infections, two or more serotypes were identified in a single compartment, sometimes more. One or more serotypes found in such compartment could be detected in the other compartments as well. These findings reflect the fact that oral fluid and urine are parts of "plasma filtrates" and thus are expected to contain most microbes present in plasma or blood. The fact that not all serotypes in individual patients are not detected in all compartments is not surprising. This is explained by

several factors, including variable numbers and concentration of the virus in various compartments and the lower limit of detection of the assay.

Although dengue virus replicates in a variety of cell *in vitro*, including cell of myeloid, lymphoid, epithelial, endothelial, and fibroblastic lineage, the cell types supporting dengue virus replication *in vivo* are thought to be the cell of a mononuclear phagocyte (20). This helps explain why we are able to detect the virus in various compartments. Non-blood samples have a potential to be utilized as a tool to understand dengue dynamics in humans as well as as potential specimens for diagnosis.

The frequency of multi-infections in this study, 23.53% (20 of 85) is high, compared with previous reports. Wei Kung Wang et al. found concurrent infection rate at 9.5% in Taiwan (82). Preeti Bharaj et al. found 19% in India (84), Lorono Pino et al. found 11% in Indonesia and 5.5% in Mexico (78). Phaisan Khasak et al. found 5% in Thailand (81). The high percentage of multi-serotypes in our study were partly from extensive typing of various samples such as plasma or serum, PBMC, urine pellets, saliva, and oral brush, and partly from a possibility that the virus might have a more tendency to infect concurrently nowadays than in the past.

It has been postulated that infections by multiple dengue virus serotypes may influence the clinical manifestations of the disease (84). Dar L. et al. studied co-circulation of dengue serotypes in India, demonstrating a somewhat higher percentage of cases with multi-infections with severe diseases. DHF were found in 6 of 9 (66.6%) in multiple infections (90). Our study found multi-serotypes in 20 cases, 5 (25%) of which were DF, 14 (70%) of which were DHF and 1 had no clinical data. We will need more patients to see any possible statistical significance in this aspect.

In our study, no statistically significant association was found between the proportions of case with multi-serotypes dengue virus infection and clinical outcomes. However, the numbers are small and this aspect requires further study before a definitive conclusion can be drawn. Also, the virology and epidemiologic significance of dual infections remain to be determined.

The co-circulation of multiple virus serotypes in a community, opportunities for dual infections of human are also increased by the feeding behavior of *Ae. Aegypti.* This mosquito frequently feeds multiple times during a single gonotropic cycle (94). *Aedes aegypti* mosquitoes experimentally infected with dengue viruses spend a longer time probing to acquire a blood meal compared with uninfected mosquitoes (95). Longer feeding periods increase the chance of host defensive behavior against blood-seeking mosquitoes, and increase the possibility that mosquitoes will feed on more hosts to complete their blood meal. These types of *Ae. Aegypti* feeding behavior may thus increase the chance that they will become dually infected and subsequently transmit multiple viruses to a single host.

RSS-PCR was initially used to determine dengue genotypes from blood and body fluid compartments of individual patients. We, unfortunately, experienced a problem with this technique. Known dengue viral strains have different pattern bands in our hand, compared with which were previous described by Eva Harris et al. (85, 86). Dengue virus exhibits a high degree of sequence variation not only among isolates from different individuals but also among viruses within the same individual because of non-proofreading and thus error-prone nature of viral RNA dependent RNA polymerase. One genetic mutation occurs in nearly every cycle of the virus replication (25, 26). During their replication, RNA genomes mutate at average rates of 10^{-3} to 10^{-5} substitutions per nucleotide copied (91) and a single infectious particle can produce on average 100,000 copies in 10 hours (92). Many reports to study about sequence variation on the Capsid, Envelope, and NS2B genes indicate that dengue virus also exhibits substantial sequence diversity in humans. This suggests that intra-host sequence variation of the virus reflects genetic drift. These findings would add to understanding of the evolution of dengue virus (79) and to a lesser extent, in mosquitoes (89). These demonstrate that dengue virus have intrahost sequence variation. Drifting in sequences with time may partly explain the discrepancy in some selected RSS-PCR of ours compared to previous reports. Another factor may be that our RSS-PCR has still not been fully optimized, even after several attempts.

Nucleotide sequence analysis of dengue virus can be used to define genetic variations between strains of the same serotype, follow geographical movement of the strains, and thereby determine the evolutionary origin of epidemic dengue virus (93). Our study of the capsid/prM or 3'UTR of the dengue virus in circulation during 2003-2008 in King Chulalongkorn Memorial Hospital show that sample of each year had very similar nucleotide sequences. The sequence shows that the same dengue serotype from an individual have the same genotype or strain. Interestingly, our study found the nucleotide sequences from various host compartments in the same day of collection are identical. Subsequent samples a few days later, however, contain significant differences in nucleotide sequence compared with the first ones. One explanation might be that RNA virus replication, RNA genomes mutate at average rates of 10^{-3} to 10^{-5} substitutions per nucleotide copied (91) and a single infectious particle can produce on average 100,000 copies in 10 hours (92).

In summary, we succeed in detecting and serotyping dengue virus from not only serum or plasma specimens but also from urine, saliva, and oral brush. DEN-4 was commonly detected as single infection, followed by DEN-1, DEN-3, and DEN-2. DEN-1 and DEN-4 were identified to be the most frequent dengue virus combination in individuals. Multi-serotype infections may be associated with clinical severity, although additional studies are needed to address this question. Dengue virus from blood and body fluids of the same patient is of the same serotype and genotype.