V DISCUSSION

In this study, we used sequence-specific oligonucleotide hybridization for subtyping of dengue serotype 2 viruses and also for following known changes of the viral genome. Oligonucleotide hybridization has been employed successfully in the detection of viruses in infected cell cultures or clinical specimens (Ausubel et al., 1995). This method is based on the principle that a single-stranded DNA or RNA molecule of defined sequence (the probe) can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the target) (Ausubel et al., 1995). Other molecular biological method which can be used for detecting genetic variation in RNA viruses are the RNA fingerprinting technique, sequence analysis and phylogenetic analysis.

Comparatively, RNA fingerprinting technique is sensitive to small change in the genome and is particularly useful only when comparing closely related RNAs or regions of RNA that have overall base sequence homology greater than 90% (Repik et al., 1983). However, the RNA fingerprinting method is slow, expensive, labor-intensive, and limited to laboratories with well trained personnel. In addition, it requires large amounts of virus and a high level of technical expertise to perform the experiments and interpret the data (Kerschner et al., 1986). On the other hand, the oligonucleotide hybridization method requires known sequence variation for efficient probe design. The advantages of the oligonucleotide hybridization are short processing time, high throughput, low cost, and the ability to use non-radioactive detection methods (Ausubel et al., 1995). Nucleotide sequence analysis is useful in the determination of the nucleotide variation at the level of single base difference. However, this method is expensive and time

consuming when it is compared with the RNA fingerprinting and the oligonucleotide hybridization methods. It is also not practical for the comparison of large numbers of isolates or large regions of genome.

The use of oligonucleotide hybridization in this study is possible due to the fixed differences between the subtype IIIa and IIIb viruses. In a study by Sittisombut et al (1997), it was found that dengue serotype 2 viruses isolated in the 1980 and 1987 epidemic seasons in Bangkok contained five positions of single amino acid substitution that were different between the subtype IIIa and IIIb viruses. These amino acid substitutions were conserved in all subtypes IIIa and IIIb viruses tested and had been fixed in both subtypes since 1980 (Sittisombut et al., 1997). Our analysis of four dengue serotype 2 isolates derived from the 1994 Bangkok epidemic confirmed that the conserved substitutions were also present in 4 out of 4 viruses tested. Thus, these conserved nucleotide substitutions should be useful in the differentiation of subtype IIIa and IIIb in Bangkok in this study.

When using oligonucleotides as probes in the differential hybridization, the aim is to detect only the perfect match in the genome (target) of interest. Therefore, it is important to design the oligonucleotide probe that is most specific to the target sequence. In the design of the oligonucleotide probe, there were two factors that had been considered:

1. Length of the oligonucleotide probe. The hybridization rate between the oligonucleotide probe and the target sequence is dependent on the length of the oligonucleotide probe. Because the increase in the length of the oligonucleotide probe will decrease the chance that it will find the perfect match in the genome of interest. Therefore, sufficiently long oligonucleotide probe will allow the discrimination between the target sequence and other closely related sequences (Sambrook et al.,

1989). The oligonucleotide probes used in this study were chosen to be 18 bases in length; in general, probes are used at 18-28 nucleotides in length and having 50% G+C content (Innis et al., 1990).

2. Site of variation. Mismatch between the oligonucleotide probe and the target sequence affects the stability of the hybrid as the melting temperature (Tm) of the hybrid will decrease by 1-1.5°C for every 1% of mismatching of bases in a hybrid (Sambrook et al., 1989). A mismatch in the middle of the oligonucleotide probe will strongly destabilize the hybrid between the probe and the target sequence during the washing process. In contrast, the mismatch at the end of the probe may have no effect (Sambrook et al., 1989). Therefore, we placed the known nucleotide differences between the subtype IIIa and subtype IIIb of dengue serotype 2 viruses (Sittisombut et al., 1997) in the middle of the 18-base probes.

In addition to the two factors discussed above, the pattern of nucleotide variation in the dengue virus system had also been taken into consideration in the probe design. It is known that, in addition to the fixed variation between the two subtypes, any dengue serotype 2 viruses may undergo a random mutational event (Sittisombut et al., 1997). If the randomly mutated site occurred in the region that is being used as a probe, this sequence variation will affect the hybridization of such probe to the particular strain bearing the mutation. If the random mutation event occurred at a high rate, a large number of viral isolates may bear the mutation(s) at the target site and the chance that these isolates be typed accurately may be reduced. It appears, however, that the rate of random mutation in the dengue system is not very high to preclude the use of oligonucleotide hybridization for typing purpose. However, in order to reduce the effect of such random mutation on the hybridization, all known random variations of the viral sequences around the fixed mutation at the target sites were included in the

probe sequence. For example, probe 308 was designed to detect the nucleotide difference at the base position 922 (G vs A), which resulted in the substitution of valine (subtype IIIa) into isoleucine (subtype IIIb) at the amino acid position 308. The dengue virus strain D87-167, belonging to the subtype IIIa, contains additional nucleotide variations at the base positions 918 (T→C) and position 930 (G→A) which did not result in the amino acid substitution. Therefore, the probe 308-IIIa was designed to contain two bases at position 918 and 930 [5' C(CT)TTCACAACTTT(AG) AACT 3'] so that it can effectively hybridize to dengue virus strain D87-167 or its daughter strains. Using this strategy, oligonucleotide probes should be able to hybridize to all known viruses belonging to a particular subtype regardless of the variation in the surrounding nucleotides.

The problem of random sequence variation, which may occur on the viral genome at the sites selected for hybridization and, thus, affects the hybridization of the probe to a (or some) particular virus(es), may also be alleviated by employing several target sites for the typing purpose. In this way, mutation at one particular site in a virus isolate may reduce the hybridization with one probe, but it will not affect hybridization at the other sites (unless, of course, mutation occurs at a high rate and several target sites contain random mutation). This strategy, on the other hand, introduces a practical problem of different Tm for various probes due to differences in the nucleotide sequence and the G+C content of the target sites. It is known that the binding of an oligonucleotide probe to the target depends on both the length of the hybrid formed and the G+C content of the probe/target site (Wood et al., 1985). Because of this G+C content variation, hybrid with mismatch(es) which is formed by oligonucleotide probe and the target site bearing a high G+C content may be more

stable than a perfectly matched hybrid at other sites with lower overall G+C content. For this reason, we used tetramethylammonium chloride (TMAC) in the washing step to eliminate the dependence of Tm on the G+C content. TMAC binds selectively to A-T base pairs, thus displacing the association equilibrium and raising the melting temperature (Jacob et al., 1988; Sambrook et al., 1989; Wood et al., 1985). This displacement is sufficient to shift the melting temperature of A-T base pairs to that of G-C base pairs (Wood et al., 1985), therefore the hybridization rate depends only on the length of the probe (or the size of matched sequence). The use of TMAC should allow us to employ only one temperature level for the washing step for all of the 18-base probes regardless of the variation of the G+C content at target sites.

Even when we used multiple target sites for typing by oligonucleotide hybridization method, the problem of random sequence variation can still affect the outcome because of the possibility of multiple mutational event occurred at a target site. A virus strain bearing several additional random mutations may be typed as negative for a particular probe if the temperature of the washing step is too high for it. In order to try to detect viruses bearing additional mutational event, the temperature during the TMAC washing step was titrated from 47°C, 52°C, 55°C and 57°C for all probes. The lowest level of the washing temperature that can differentiate between the known subtype IIIa and subtype IIIb viruses was then selected for use against the unknown viruses of the 1994 epidemic.

After the design, the sensitivity and specificity of all five pairs of the oligonucleotide probe were determined by testing them against dengue serotype 2 viruses subtype IIIa and IIIb with known envelope gene sequence from the 1980 and 1987 epidemic seasons. Using the appropriate cutoff points that we selected, it was found that all five pairs of the oligonucleotide probe hybridized strongly with viruses

belonging to the corresponding subtype (100% sensitivity). However, the specificity of three oligonucleotide probes was less than 100%; that is, they hybridized with some of the isolates that belonged to a non-corresponding subtype. For example, probe 141-IIIa has a cross-reaction with one (out of five) subtype IIIb strains (MK42-86). This cross-reactivity might be due to the large amount of the PCR product being spotted onto the nylon membrane; low level of cross reactivity with highly abundant target may raise the signal upto the level of the corresponding subtype. This possibility, however, is quite unlikely because we divided the hybridization value (derived by densitometric analysis of the exposed X-ray film) of one differentiating probe with those from a common probe for every dots. Using this normalization procedure, the amount of signal derived from different quantity of the spotted PCR products were equalized. Alternatively, the observed cross-reaction may be due to the peculiar nature of the sequence surrounding the target site of the MK42-86 strain which resulted in enhanced binding of this particular probe to the target sequence (even in the presence of mismatch between the probe and target). Another possible explanation is the mutation in the strain MK42-86, either within the target site (reversion), outside of the target site but within the envelope gene, or even outside of the envelope gene, which created 100% matched sequence with the probe. Although it is quite unlikely that reversion has happened during the propagation of MK42-86 in vitro as sequence changes during viral propagation in *Toxorhynchites amboinensis* or in C6/36 cell line are generally minimal (Rico-Hesse, 1990): it is not yet possible to distinguish formally between these possibilities.

When tested against dengue type 2 isolates from the years 1980 and 1987, the specificity of some of the oligonucleotide probes was not at the level of 100%. This observation raised the question whether we should use only selected probes which gave

the sensitivity and specificity of 100%, or use all of the probes we had in hand, for the study of viruses from the 1994 epidemic season. Because the 1994 viruses may have additional changes in their nucleotide sequences that were unknown to us, the sensitivity and specificity of each probe (against the 1994 viruses) may actually differ from those derived by testing with the 1980 and 1987 viruses. The use of all five pairs of probe, thus, may help in the determination of the subtype when one/or some of the probes gave problematic results for certain viruses. Additional advantage of using all five pairs of probe is the possibility of detecting non-subtype III viruses; these viruses will give hybridization signals/patterns which are not typical of the subtype IIIa and subtype IIIb viruses.

For the determination of the proportion of subtype IIIa and subtype IIIb among dengue serotype 2 viruses circulating in Bangkok in 1994, 20 viral genomic RNAs and 4 PCR product samples were tested. For 12 viral samples that were derived in the form of infected C6/36 culture supernatant, a negative control was included for each RT-PCR reaction (i.e., by directly using the extracted RNA for the PCR amplification without prior cDNA synthesis step). The purpose of this control is to detect the contamination of extracted RNA by the PCR products during the RNA extraction process. In contrast, there were eight RNA samples which were extracted directly from sera specimen; the volume of the samples was very small (only 10 μl). Since we did not know whether there would be adequate viral RNA for the amplification of the 2.4 kb target, all 10 μl of the RNA were used in the RT-PCR and there was no negative control for these samples. Although it is possible that these RNA samples are contaminated by the PCR products, this is considered unlikely because these RNA samples were tested (by Dr. Niwat Maneekarn and Dr. Boonyos Raengsakulrach) for the contamination with three other serotypes of dengue viruses. Moreover, the chances

that these RNA samples are contaminated by the PCR products of the other dengue 2 subtypes can be eliminated by our use of probes specific for the subtype IIIa and subtype IIIb viruses in the hybridization to all unknown samples. This would allow us to detect the contamination that occurred in the RNA extraction process or in the RT-PCR reaction by PCR products of the opposite subtype. For example, when an RNA sample which belonged to subtype IIIa was contaminated with PCR product of the subtype IIIb, this sample would react with both subtype IIIa and subtype IIIb probes, and vice versa. None of the 1994 viral samples gave a dual reactivity pattern. Because the objective of this study is to determine the proportion between dengue serotype 2 virus subtype IIIa and subtype IIIb that circulated in Bangkok in 1994, contamination of a subtype IIIa RNA sample with the PCR product belonging to the same subtype will not affect our results.

The hybridization analysis of dengue serotype 2 viruses derived from the 1994 epidemic season revealed that 24 out of 24 of the dengue serotype 2 viruses were subtype IIIa. No subtype IIIb virus was detected in this group of samples. Data from previous studies indicate that among dengue serotype 2 viruses that circulated in Bangkok, the subtype IIIa viruses were more commonly found than subtype IIIb since 1980 (Walker et al., 1988; Blok et al., 1989; Sittisombut et al., 1997). In the 1980 epidemic season, the subtype IIIb viruses were present at about 20% (2 out of 10 by RNA fingerprinting study; 2 out of 5 by nucleotide sequence and phylogenetic analyses) of serotype 2 viruses, whereas in the year 1987 the proportion decreased to about 5% (1 out of 19). Our finding that this proportion decreases to 0% (0 out of 24) in the 1994 epidemic season may reflect the actual decreasing trend of the subtype IIIb transmission in Bangkok. The underlying basis for such decrease may include: the poorer replicative ability of the subtype IIIb viruses in mosquitoes, the greater

interference of viruses on the longevity of mosquitoes and the lower (or shorter) level of viremia in infected human hosts.

Alternatively, the subtype IIIb viruses may still exist in the year 1994 at the former level of 5-20%, but we failed to detect them appropriately. Several reasons for the failure may include:

1. Too small sample size. In this study, the samples from infected patients who came to the Bangkok Children's Hospital were limited to only 24 samples. These samples were diagnosed as having dengue infection, and were confirmed by the PCR analyses. Although, our sample size is somewhat larger than the ones reported previously (10 for the year 1980 and 19 for the year 1987). There is a good statistical chance that with only 24 samples we still cannot detect the subtype IIIb viruses when they exist at the level of 5-20%.

2. Comparatively lower level of the viral RNA/virion of the subtype IIIb in the sera samples. If the amount of the subtype IIIb viruses in the patients' sera was too low, it will not be possible to amplify the viral genome by the PCR analysis and we will miss such patients. However, our samples were derived from a larger group of specimens of which PCR analysis was performed concurrent with the analysis for anti-dengue antibody. The overall sensitivity for the detection of the viral genome by PCR analysis when compared with significant dengue specific antibody rise was about 90% (Raengsakulrach et al., manuscript in preparation). This observation indicated that if we are losing some patients because of the low level of subtype IIIb viremia, only about 10% of the dengue infections are missed.

3. Inability to isolate the subtype IIIb viruses. It is possible that the subtype IIIb viruses were transmitted in Bangkok but they cannot be isolated by using mosquito inoculation procedure as efficiently as the subtype IIIa viruses. However, 12

out of 24 samples employed in this study were RNA prepared directly from the sera samples. Therefore, if the subtype IIIb viruses were present in this group of patients, we should be able to detect them at least in these 12 serum samples.

4. Lower diseasê severity following subtype IIIb infection. If the subtype IIIb viruses did not cause severe diseases (as compared with the subtype IIIa viruses), patients infected the subtype IIIb viruses may not seek medical helps and may not be included in the study samples. This possibility can be excluded formally by performing a prospective study of all possibly infected person in the community. However, this possibility does not agree with the observation that the subtype IIIb viruses can establish themselves well in Cuba and the Caribbean islands and have caused (together with the other serotypes) a major epidemic of dengue hemorrhagic fever in this area since 1980 (Gubler and Trent, 1994).

The result that the subtype IIIa viruses were the predominant viruses that circulated in the 1994 epidemic season correlates well with a few previous studies of dengue serotype 2 viruses found outside of Bangkok. The subtype IIIa viruses represent: 1/1 dengue serotype 2 viruses isolated from Kanchanaburi in 1987 (Sittisombut et al., 1997), 3/3 viruses isolated from Chiang Mai in 1991 (Sistayanarain et al., 1996) and 4/4 viruses isolated from Nakhon Phanom province in 1993 (Thant et al., 1995). On the other hand, our observation that the subtype IIIb viruses were not detected in the 1994 epidemic season was in contrast to the report of Duangchanda et al (1994) that 3/3 dengue serotype 2 viruses isolated from patients with different disease severities in Maha Sarakham province during the 1986-1987 epidemic seasons were subtype IIIb viruses (Duangchanda et al., 1994). These data reinforce the notion that there is an uneven distribution of dengue serotype 2 subtypes in various localities of Thailand (Sittisombut et al., 1997).

For the last 40 years, there has been a lot of migration into and out of Bangkok. Some of the migrants may carry dengue type 2 viruses from their hometowns and may serve as a primary source for disseminating the new variant in Bangkok. It is possible that these subtype IIIa viruses that we detected in Bangkok in the 1994 epidemic season descended directly from their Bangkok precursors of 1987 or, on the other hand, at least some of them might be introduced from other places, such as Malaysia or China where subtype IIIa viruses were also known to circulate. In order to differentiate between these two possibilities, we employed the observation that, during the 1987 epidemic season in Bangkok, all circulating subtype IIIa viruses shared a non-synonymous nucleotide substitution (base position 1036, T; amino acid position 346, tyrosine) which was absent in the subtype IIIa viruses found in 1980 in Bangkok, or any dengue serotypes which have been sequenced so far (Sittisombut et al., 1997). When an oligonucleotide probe, 346-IIIa-87, was designed and employed in the differential hybridization, it was found that, even with the low level of sensitivity, the probe 346-IIIa-87 hybridized strongly to all dengue serotype 2 subtype IIIa strains found in Bangkok in 1994. Thus, the subtype IIIa strains found in Bangkok in 1994 might be direct descendants of the 1987 Bangkok precursors.

A study of the nucleotide and deduced amino acid sequences of the structural genes and the NS1 gene of four dengue 2 strains which were isolated from Nakhon Phanom province, northeastern Thailand, in 1993 revealed that amino acid position 346 of these four strains contained tyrosine (TAT) (Thant et al., 1996). Similarly, the analysis of three other dengue serotype 2 viruses which were isolated from Chiang Mai in 1991 also disclosed tyrosine at the position 346 (Sistayanarain et al., 1996). The mutation His346$\rightarrow$Tyr was first observed among 18/18 of the subtype IIIa viruses in

Bangkok during the 1987 epidemic season and its presence was interpreted to represent the occurrence of genetic (population) bottleneck occurring between the 1980 and 1987 epidemic seasons in Bangkok (Sittisombut et al., 1997). The other possibility which may result in the same observation is the extinction of subtype IIIa viruses (carrying His346) from Bangkok and the re-introduction of a new variant (carrying Tyr346) from somewhere else in Thailand. Our finding that all 24/24 subtype IIIa viruses in Bangkok in 1994 carry the Tyr346 mutation appears to indicate that once population bottleneck took place between 1980 and 1987, the Tyr346 variant could spread rapidly out of Bangkok to many areas of the country. On the other hand, the Tyr-346 variant may possibly be the predominant variant of the subtype IIIa viruses for areas outside of Bangkok for a long time, but it is never studied until recently.

Additional analyses of the nucleotide and deduced amino acid sequences of the structural and NS1 genes of the dengue serotype 2 virus (strain D2-04) which was isolated from a patient in Hainan, China in 1985 revealed histidine at position 346 (base position 1036, C) (Yang et al., 1994). The analysis of the nucleotide sequence of the envelope gene of three Malaysian dengue serotype 2 virus which were identified during the 1986-1987 epidemic season also detected the histidine residue at position 346 (base position 1036, C) (Samuel et al., 1989). Up to now, it is evident that dengue serotype 2 viruses containing His346 were generally found outside of Thailand and also in Bangkok up until 1980. Therefore, the mutation Tyr346 may be useful as a genetic marker for the subtype IIIa viruses which originated in Thailand at least after the 1987 epidemic season. Because of its stability, it will be of value in the epidemiological investigation of the movement of subtype IIIa viruses into or out of Thailand in the recent years.

The use of oligonucleotide probes for the subtyping of dengue serotype 2 viruses belonging to the 1980, 1987 and 1994 epidemic seasons in Bangkok in this study appears to be quite successful. However, this set of oligonucleotide probes may be of a limited value in the subtyping of dengue serotype 2 viruses that were isolated before the 1980 epidemic season. This limitation was evident with strain 16681 which was isolated in 1964. The hybridization pattern of strain 16681 with five pairs of probes did not conform to the patterns of the subtype IIIa strains of later epidemic seasons. For example, 16681 hybridized strongly with the 141-IIIb probe, but not with the IIIa probe; this unusual hybridization of 16681 was not caused by technical problems but corresponded well with the known sequence of 16681 (Blok et al., 1992). Therefore, the oligonucleotide probes appear to be of best value for subtyping dengue viruses in a limited time period in which sequence variations of the target viral population are well characterized.

The amino acid variations distinguishing between the subtype IIIa and IIIb dengue serotype 2 viruses of the 1980-1994 epidemic seasons are distributed in various domains of dengue envelope protein. Whether these variations cause phenotypic difference(s) in the envelope protein of dengue viruses is not yet known. According to the crystal structure of the envelope protein of the tick-borne encephalitis virus (Rey et al., 1995), these amino acid variations are found at the following positions:

1. The valine vs isoleucine (V vs I) at position 141. This position is located on strand E_0 of the central domain I. Because of the hydrophobic nature of this amino acid, the side chain is likely to be buried in the internal portion of the protein.

2. The asparagine vs aspartic acid (N vs D) at position 203. This position is located within a loop between strands f→g of the dimerization domain II.

These strands are positioned within a region that has hinge-like characteristic. It is believed that this region, which contains several hydrophilic amino acids, is important in the elevation of the tip of the domain above the viral membrane and bringing the fusion peptide into prominence (Rey et al., 1995). This variation results in changing an uncharged amino acid (N in subtype IIIa) to a negatively-charged (D in subtype IIIb) amino acid. However, this variation is quite commonly found among dengue type 2 viruses; a comparison of 17 dengue serotype 2 viruses revealed that there are only two amino acids at this position: asparagine (frequency 12/17) and aspartic acid (frequency 5/17). Because of the high frequency of this asparagine ↔ aspartic acid variation, it is quite unlikely that the function of envelope protein will be drastically affected.

3. The valine vs isoleucine (V vs I) at position 308. This position is located on strand A of domain III; this hydrophobic amino acid may be located in the internal portion of the domain.

4. The isoleucine vs valine (I vs V) at position 484. This position is located in the membrane-spanning domain of the envelope protein.

5. The valine vs alanine (V vs A) at position 491. Similar to the I 484 vs V listed above, this position is located in the transmembrane domain of the envelope protein.

Four out of five variations that differentiate the envelope protein of the subtype IIIa and IIIb dengue serotype 2 viruses do not change the hydrophobic nature of the amino acids, and are unlikely to affect the antigenic property and the functioning of the envelope protein. The other variation is more or less commonly distributed among various dengue serotype 2 strains. Such possible preservation of the structure and

function of these variations may explain the conservation of these variations during the 15-year period.

Currently, the biological difference, if there is any, between the subtype IIIa and subtype IIIb of dengue serotype 2 viruses, such as the kinetics and efficiency of replication in mosquito, human PBMC and cultured cells, is still unknown. Apart from the fact that the subtype IIIb viruses can be isolated from patients with DF, DHF and DSS in Maha Sarakham (Duangchanda et al., 1994) and that this subtype can cause epidemic of DHF in the Caribbean region (Gubler and Trent, 1993), there has been no other clinical/experimental data on the comparison of the biological properties of dengue subtypes. This remains an area of research which needs more interest and input from virologists.