IV. RESULTS

1. Conservation of differentiating nucleotide substitutions

During the 1980 and 1987 epidemic seasons in Bangkok, members of the subtype IIIa differed from those of subtype IIIb at six non-synonymous nucleotide substitutions (positions 421, G vs A; 607, A vs G; 922, G vs A; 1452, U vs C; 1472, U vs C; 1473, C vs U)(Sittisombut et al., 1997). For subsequent epidemic seasons, these differentiating substitutions may be useful for subtyping dengue serotype 2 viruses by hybridization analysis if they remain fixed in the viral population. To determine whether dengue serotype 2 viruses circulating in the year 1994 still retained the differentiating base substitutions, the entire envelope gene of four randomly selected viruses (strains D94-039, D94-179, D94-376 and D94-548) were amplified, gel purified and sequenced by employing a modified Sanger's sequencing method. The resulting nucleotide sequences and their deduced amino acid sequences were then compared with the published sequences of various strains of dengue serotype 2 viruses (Sistayanarain, 1993; Duangchanda et al., 1994; Lewis et al., 1995; Sittisombut et al., 1997). All six differentiating nucleotide substitutions remained in these four 1994 isolates (Fig. 2), indicating strongly that these substitutions were conserved among dengue serotype 2 viruses circulating in the 1994 epidemic season. Therefore, these nucleotide substitutions should be useful in the differential hybridization analysis for dengue serotype 2 viruses present in 1994.

```
D94-039    ATGCGTTGCA TAGGAATATC AAATAGAGAC TTTGTAGAAG GGGTTTCAGG 50
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .....A.... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      .......... .......G.. .......... .....G.... ..........

D87-1421   .......... .......... .......... .......... ..........
MK42-86    .......... .......... .......... .......... ..........
D80-141    .......... .......... .......... .......... ..........
D80-038    .......... .......... .......... .......... ..........

D94-039    AGGAAGCTGG GTTGACATAG TCTTAGAACA TGGAAGCTGT GTGACGACGA 100
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... ..C....... .......... ..........
16681      .......... .......... .......... .......... ..........

D87-1421   .......... .......... .......G.. ......T... ..........
MK42-86    .......... .......... .......G.. ......T... ..........
D80-141    .......... .......... .......... ......T..  ..........
D80-038    .......... .......... .......... .......... ..........
```

Figure 2. Nucleotide sequences of the envelope gene of D94-039, D94-179, D94-376, D94-548 and other dengue type 2 virus isolates classified as subtype IIIa (upper panel) or IIIb (lower panel). Dots represent bases identical to those of D94-039. Lines represent the oligonucleotide probe binding sites and bold bases represent the non-synonymous nucleotide substitutions that differed between the subtype IIIa and IIIb strains.

```
D94-039    TGGCAAAAAA CAAACCAACA TTGGATTTTG AACTGATAAA AACGGAAGCC 150
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ...A......
D80-100    .......... .......... .......... .......... ...A......
16681      .......... .......... .......... ..........A......

D87-1421   .......... .......... .......... ..........A......
MK42-86    .......... .......... .......... .......... ...A......
D80-141    .......... .......... .......... .......... ...A......
D80-038    .......... .......... .......... .......... ...A......

D94-039    AAACAGCCTG CCACCCTAAG GAAGTACTGC ATAGAAGCAA AGCTAACCAA 200
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... .......G..
PUO-280    .......... .......... .........T ..G....... ..........
D80-100    .......... .......... .........T .......... ..........
16681      .......... .......... .........T .....G.... ..........

D87-1421   .....A.... ....T..... ...A.....T .......... G...G.....
MK42-86    .....A.... ....T..... ...A.....T .......... ....G.....
D80-141    .....A.... ....T..... .........T .......... ....G.....
D80-038    .......... .T........ .........T ........T. ..........

D94-039    CACAACAACA GAATCTCGTT GCCCAACACA AGGGGAACCC AGCCTAAAAG 250
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    ......G..G .......... .......... .......... ........T.
D80-100    .......... .......... .......... .......... ........T.
16681      .......... ........C. .......... .......... ........T.

D87-1421   T......... .......... .......... .......... ..T.......
MK42-86    T......... .......... .......... .......... ..T.......
D80-141    T......... .......... .......... .......... ..T.....T.
D80-038    .......... .......... ........T.. ......G... ........T.
```

Figure 2. (continued).

```
D94-039    AAGAGCAGGA CAAGAGGTTC GTCTGCAAAC ACTCCATGGT AGACAGAGGA 300
D94-179    .......... T......... .......... .......... ..........
D94-376    .......... T......... .......... .......... ..........
D94-548    .......... T......... .......... .......... ..........
D87-642    .......... T......... .......... .......... ..........
D87-1040   .......... T......... .......... .......... ..........
PUO-280    .......... T......... .......... .......... ....C.....
D80-100    .......... T........G .......... .......... ..........
16681      .......... ...A...... .......... .......... ..........

D87-1421   .......... ...A...... .......... .......A.. ......G...
MK42-86    .......... ...A...... .......... .......A.. ......G...
D80-141    .......... ...A...... .......... .......... ......G...
D80-038    .......... T........G .......... .......... ......G...

D94-039    TGGGGGAATG GATGTGGATT ATTTGGAAAG GGAGGCATTG TGACCTGTGC 350
D94-179    .....A.... .......... .......... .......... .A........
D94-376    .....A.... .......... .......... .......... ..........
D94-548    .....A.... .......... .......... .......... ..........
D87-642    .....A.... .......... .......... .......... ..........
D87-1040   .....A.... .......... .......... .......... ..........
PUO-280    .....A.... .......... C......... .......... ..........
D80-100    .....A.... .......... .......... .......... ..........
16681      .....A.... ........C. .......... .......... ..........

D87-1421   .....A.... .......... .......... ..G....... ..........
MK42-86    .....A.... .......... .......... .......... ..........
D80-141    .....A.... .......... .......... .......... ..........
D80-038    .....A.... .......... .......... .......... ..........

D94-039    TATGTTCACA TGCAAAAAGA ACATGGAAGG GAAAATCGTG CAACCAGAAA 400
D94-179    .......... .......... .......... ......T... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .....G.... .......... .......... ..........
PUO-280    .......... .......... .......... A......... ..........
D80-100    C......... .......... .......... A......... ..........
16681      ........G. .......... .......... A...G.T... ..........

D87-1421   ......T... .......... .......... A......... ..........
MK42-86    ......T... .......... .......... A......... ..........
D80-141    ......T... .......... .......... A......... ..........
D80-038    ......T... .......... .......... A......... ..........
```

Figure 2. (continued).

```
                          _____141_____
D94-039    ACTTAGAATA CACCATTGTG GTAACACCCC ACTCAGGGGA AGAGCATGCG 450
D94-179    ....G..... .......... ........T. .......... .........A
D94-376    ....G..... .......... ........T. .......... ..........
D94-548    .T..G..... .......... ........T. .......... ..........
D87-642    ....G..... .......... ........T. .......... ..........
D87-1040   .......... .......... ........T. .......... ..........
PUO-280    ....G..... .......... ........T. .......... ..........
D80-100    ....G..... .......... ........T. .......... ..........
16681      ....G..... .......... A.......T. .......... .........A

D87-1421   .T..G..... ......C... A.......T. ........A.. ......C..T
MK42-86    .T..G..... ......C... A.......... .......A.. G.....C..T
D80-141    .T..G..... ......C... A.......T. .......A.. ......C..T
D80-038    .T..G..... ......C... A.......T. .......... ..........

D94-039    GTCGGAAATG ACACAGGAAA ACATGGCAAG GAAATCAAAG TAACACCACA 500
D94-179    .......... .......... ......T... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .....T.... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... ...G...... ..........
PUO-280    .......... .......... .......... .....A.... ..........
D80-100    .......... .......... .......... .....A.... ..........
16681      .......... .......... .......... .........A ..........

D87-1421   ..A..T.... .......... .......... .........A ..........
MK42-86    ..A..T.... .......... .........T ..G......A ..........
D80-141    ..A..T.... .......... .......... .....A...A ..........
D80-038    .......... .......... .......... .....A.... ..........

D94-039    GAGTTCCATC ACAGAAGCAG AATTGACAGG TTATGGCACC GTCACGATGG 550
D94-179    .......... .......... .......... .........T ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..T.......
PUO-280    .......... G......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      .......... .......... .......... .........T .....A....

D87-1421   .......... .......... ..C....... C.........T A.........
MK42-86    .......... .......... ..C....... C.........T ..........
D80-141    .......... .......... ..C....... C.........T ..........
D80-038    .......... .......... .......... .........T ..........
```

Figure 2. (continued).

```
D94-039    AGTGCTTTCC GAGAACAGGC CTTGACTTCA ATGAGATGGT GTTGCTGCAG 600
D94-179    .......... ...G...... ..C.....T. .......... ..........
D94-376    .......... .......... ..C..... .......... ..........
D94-548    ......C... .......... ..C..... .......... ..........
D87-642    ......C... .......... ..C..... .......... ..........
D87-1040   ......C... .......... ..C..... .......... ..........
PUO-280    ......C... .......... ..G..... .......... ..........
D80-100    ......C... ........T. ..G..... .......... ..........
16681      ......C... A.....G... ..C..... .......... ..........

D87-1421   ......C... ......G... ..C..... .......... .C........
MK42-86    ......C... .....G.... ..C..... .......... .C........
D80-141    ......C... .....G.... ..A..... .......... .C........
D80-038    ......C... .....G.... ..A..... .......... .C........
           ________203_______
D94-039    ATGGAAAATA AAGCTTGGCT GGTGCATAGG CAATGGTTCC TAGACCTGCC 650
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... A......... .......... .......A..
D80-100    ........C. .......... A........A .......... .......A..
16681      .......... .......... ......C... .......... ..........

D87-1421   ......G.C. .......... ......C... .......... ..........
MK42-86    ......G.C. .......... ......C..A .......... ..........
D80-141    ......G.C. .......... ......C... .......... ..........
D80-038    ......G.C. .......... ......C... .......... ..........

D94-039    ATTACCATGG CTGCCCGGAG CGGATACACA AGGGTCAAAT TGGATACAGA 700
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    G......... .......... ....C..... .......... ..........
D80-100    G......... .......... .......... .......... ..........
16681      G......... T......... ....C..... .......... ..........

D87-1421   G......... ..A....... ....C..... ...A...... ..........
MK42-86    G......... ..A....... ....C..... ...A...... ..........
D80-141    G......... ..A....... ....C..... ...A...... ..........
D80-038    G......... ..A....... ....C..... ...A...... ..........
```

Figure 2. (continued).

```
D94-039    AAGAAACATT GGTCACTTTC AAAAATCCCC ATGCGAAGAA ACAGGATGTT 750
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      ....G..... .......... .......... .......... ..........

D87-1421   ....G..... ......C... .......... .C..A..... .........C
MK42-86    ....G..... ......C... .......... .C..A..A.. .........C
D80-141    ....G..... .......... .......... .C..A..... .........G
D80-038    ....G..... .......... .......... .......... .........C

D94-039    GTTGTTTTAG GATCCCAAGA AGGGGCCATG CATACAGCAC TCACAGGAGC 800
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .....C.... .......... .......... ..C....... ..........
16681      .......... .......... .......... ..C....... .T.....G..

D87-1421   .......... ....T..... .......... ..C..G.... .......G..
MK42-86    .......... ....T..... .......... ..C..G.... .......G..
D80-141    .......... .......... .......... ..C..G.... .......G..
D80-038    .......... .......... .......... ..C....... .A.......

D94-039    CACAGAAATC CAAATGTCAT CAGGAAACTT ACTCTTCACT GGACATCTCA 850
D94-179    .......... ........G. .......... G......... ..........
D94-376    .......... ........G. .......... .......... ..........
D94-548    .......... ........G. .......... .......... ..........
D87-642    .......... ........G. .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .........A ..........
D80-100    .......... .......... .......... .........A ..........
16681      .......... .......... .......... .........A ..........

D87-1421   .......... ..G....... .......... ...A.....A ..........
MK42-86    .......... ..G....... .......... ...A.....A ..........
D80-141    .......... ..G....... .......... ...A.....A ..........
D80-038    .......... .......... .......... .........A ..........
```

Figure 2. (continued).

```
D94-039    AGTGCAGGCT GAGAATGGAC AAGCTACAGC TCAAAGGAAT GTCATACTCT 900
D94-179    .......... ...G...... .......... .......... ..........
D94-376    ...C...... ...C...... .......... .......... .........C
D94-548    ...C...... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      .......... .......... .......... .......... ..........

D87-1421   .......... .......... ..A....... .T.......... ..........
MK42-86    .......... .......... ..A....... .......... ..........
D80-141    .......... .......... ..A....... .......... ..........
D80-038    .......... .......... ..A....... .......... ..........
                                 ________308________
D94-039    ATGTGCACAG GAAAGTTCAA AGTTGTGAAG GAAATAGCAG AAACACAACA 950
D94-179    .......... .......T.. .......... .......... ..........
D94-376    .......... .......T.. .......... .......... ..........
D94-548    .......... .......T.. .......... .......... ..........
D87-642    .......... .......T.. .......... .......... ..........
D87-1040   .......... .......T.. .......... .......... ..........
PUO-280    .......... .......T.. .......... .......... ..........
D80-100    .......... .......T.. .......... .......... ..........
16681      .......... .......T.. .......... .......... ..........

D87-1421   .....T.... .......T.. .A........ .......... ..........
MK42-86    .....T.... .......T.. .A........ .......... ..........
D80-141    .....T.... .......T.. .A........ .......... ..........
D80-038    .....T.... .......T.. .A........ .......... ..........

D94-039    TGGAACGGTA GTTATCAGAG TGCAATATGA AGGGGACGGC TCCCCAAGTA 1000
D94-179    .......... .......... .......... .......... ..T.......
D94-376    ......AA.. .......... .......... .......... ..T...T...
D94-548    ......AA.. .......... .......... .......... ..T.......
D87-642    .......A.. .......... .......... .......... ..T...T...
D87-1040   .......A.. .......... .......... .......... ..T...T...
PUO-280    .......A.. .......... .......... .......... ..T...T...
D80-100    .......A.. .......... .......... .......... ..T...T...
16681      ......AA.. .......... .......... .......... ..T...T.C.

D87-1421   ......AA.. .......... .A........ .......... ..T...T...
MK42-86    ......AA.. .......... .A........ .......... ..T...T...
D80-141    ......AA.. .......... .A........ .......... ..T...T...
D80-038    ......AA.. .......... .......... .......... ..T...T.C.
```

Figure 2. (continued).

```
                                                  ________346_________
D94-039    AAATCCCTTT TGAGATAATG GATTTGGAAA AAAGATATGT CTTAGGCCGC 1050
D94-179    .......... .......... .......... .......... ..........
D94-376    ..G....... .......... .......... .......... ..........
D94-548    ..G....... .......... .......... .......... ..........
D87-642    .......... .......... ..C....... .......... ..........
D87-1040   .......... .......... .......... .......... .........T
PUO-280    .......... ......C... .......... .....C.... ..........
D80-100    .......... .......... .......... .....C.... T.........
16681      .G........ .......... .......... .....C.... ......T...

D87-1421   .G........ .......... .......... .....C.... ......T...
MK42-86    .G........ .......... .......... .....C.... ......T...
D80-141    .G........ ......C... .......... .....C.... ......T...
D80-038    .......... .......... .......... .....C.... ......T...

D94-039    CTGATCACAG TCAACCCAAT TGTGACAGAA AAAGATAGCC CAGTCAACAT 1100
D94-179    .......... .......... .......... .......... ....T.....
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .A........ .......... .......... ..........
D80-100    .......... .A........ .......... .......... .......A..
16681      .....T.... .......... .......... .......... ..........

D87-1421   .....T.... .T.....G.. C..A...... .......... ..........
MK42-86    .....T.... .T.....G.. C..AG..... .......... ..........
D80-141    .....T.... .T.....G.. C..A...... .......... ..........
D80-038    .....T.... .T.....G.. C..C...... .......... ..........

D94-039    AGAAGCAGAA CCTCCATTCG GAGACAGCTA CATCATCATA GGAGTAGAGC 1150
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      .......... .......... .......... .......... ..........

D87-1421   .......... .......... .......... .......... ..........
MK42-86    .......... .......... .......... .......... ..........
D80-141    .......... .......... .......... .......... ..........
D80-038    .......... .......... .......... .......... ..........
```

Figure 2. (continued).

```
D94-039    CGGGACAACT GAAGCTCAAT TGGTTTAAGA AAGGAAGTTC TATCGGCCAA 1200
D94-179    .......... .........C .....C.... .........T ..........
D94-376    .......... .........C .....C.... .......... .........G
D94-548    .......... .........C .....C.... .......... .........G
D87-642    .......... .........C .....C.... .......... ..........
D87-1040   .......... .........C .......... .......... ..........
PUO-280    .......... .........C .......... .......... ..........
D80-100    .......... A........C .......... .......... ..........
16681      .......... .........C .......... .......... ..........

D87-1421   ........T. ...A.....C .......... .......... C.........
MK42-86    ........T. ...A.....C .......... .......... C.........
D80-141    ........T. A..A.....C .......... .......... C.........
D80-038    ........T. ...A.....C .......... .......... C.........

D94-039    ATGTTTGAGA CAACAATGAG GGGGGCGAAG AGAATGGCCA TTTTGGGTGA 1250
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      .......... .......... .......... .......... ....A.....

D87-1421   .......... .......... A..A...... .......... ....A.....
MK42-86    .......... .......... A..A.....A .......... ....A.....
D80-141    .......... .......... A..A...... .......... ....A.....
D80-038    .......... .......... A..A...... .......... ....A.....

D94-039    CACAGCCTGG GATTTCGGAT CCCTGGGAGG AGTGTTTACA TCTATAGGAA 1300
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..C.......
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    ..........T.... .......... .......... .......... ..........
D80-100    .....T.... .......... .......... .......... ..........
16681      .....T.... ..T....... .......... .......... ..........

D87-1421   .....T.... .......... .......... ..C.......
MK42-86    .....T.... .......... .......... ..........
D80-141    .....T.... .......... .......... ..........
D80-038    .....T.... .......... .......... ..........
```

Figure 2. (continued).

```
D94-039    AAGCTCTCCA CCAAGTCTTT GGAGCAATCT ATGGAGCGGC CTTCAGTGGG 1350
D94-179    .......... .......... .......... .......T.. ..........
D94-376    .......... .......... .......... .......T.. ..........
D94-548    .......... .......... .......... .......T.. ..........
D87-642    .......... .......... .......... .......T.. ..........
D87-1040   .......... .......... .......... .......T.. ..........
PUO-280    .......... .......... .......... .......T.. ..........
D80-100    .......... .......... .......... .......T.. ..........
16681      .G........ .......... .......... .......T.. ..........

D87-1421   .G........ ......T..C .......... ....G..T.. ...T......
MK42-86    .G........ ......T..C .......... ....G..T.. ...T......
D80-141    .G........ ......T..C .......... ....G..TA. ...T......
D80-038    .......... .......... .......... .......T.. ...T......

D94-039    GTTTCATGGA CTATGAAAAT CCTCATAGGA GTCATTATCA CATGGATAGG 1400
D94-179    .......... .A........ .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    ..C....... .......... .......... .......... ..........
16681      .......... .......... .......... .......... ..........

D87-1421   ..C....... .......... .......... .....C.... ..........
MK42-86    ..C....... .......... .......... .....C.... ..........
D80-141    ..C....... .......... ...A...... .....C.... ..........
D80-038    .......... .......... ......C... .......... ..........

D94-039    AATGAATTCA CGCAGCACCT CACTGTCTGT GTCACTAGTA TTGGTGGGAA 1450
D94-179    ......C... .......... .......... .......... ..........
D94-376    ......C... .......... .......... .........G ..........
D94-548    ......C... .......... .......... .......... ..........
D87-642    ......C... .......... .......... .......... ..........
D87-1040   .......... .....T.... .......... .........G C.........
PUO-280    .......... .......... .......... .C........ ..........
D80-100    ...A...... .......... .......... .......... ..........
16681      .......... .......... .......... .A........ ..........

D87-1421   .......... ..T....... .......... .......... .........G
MK42-86    .......... ..T....... .......... .......... .........G
D80-141    ...A...... ..T....... .......... .......... .........G
D80-038    .......... ..T....... .......... .......... .........G
```

Figure 2. (continued).

```
          484                    491
D94-039   TTGTGACACT GTATTTGGGA GTCATGGTGC AGGCC          1485
D94-179   .......... .......... .......... .....
D94-376   .......... .......... .......... .....
D94-548   .......... .......... .......... .....
D87-642   .......... .......... .......... .....
D87-1040  .......... .......... .......... .....
PUO-280   .......... .......... .......... .....
D80-100   .......... .......... .......... .....
16681     .......... .......... .......... .....

D87-1421  .C..A..... ...C..... .CT....... ....T
MK42-86   .C..A..... ...C..... .CT....... ....T
D80-141   .C........ ...C..... .CT....... ....A
D80-038   .C..A..... ...C..... .CT....... T...T
```


Figure 2. (continued).

Comparison of the envelope gene sequences also revealed that new nucleotide substitutions occurred in all four 1994 isolates (Figure 2). The majority of mutations occurred at the third codon position resulting mostly in synonymous substitutions. No other types of mutation such as deletion, insertion and inversion was detected.

Comparison of the deduced amino acid sequences of the four 1994 isolates and the previously isolates indicated that the short segment of the E protein sequence from amino acid position 98-111 was completely conserved among dengue 2 viruses including all four 1994 isolates (Figure 3). These residues are thought to be involved in the fusion process (Thant et al., 1996). Two potential glycosylation sites in the E gene region (NTT at amino acid position 67-69 and NDT at amino acid position 153-155) and twelve cysteine residues in the E region were also conserved in our four 1994 isolates.

The four 1994 viruses differed from each other by 8-24 bases (Figure 4). Similarly, they varied from the subtype IIIa viruses of the 1980 and 1987 epidemic seasons by 14-44 bases. In contrast, the differences between the four 1994 viruses and the subtype IIIb viruses of the 1980 and 1987 were from 66 to 107 nucleotides whereas the subtype IIIb viruses varied from each others by 13-56 bases. Using direct comparison, these four 1994 serotype 2 viruses can be grouped together with the subtype IIIa viruses of the five-subtype nomenclature system (Lewis et al., 1993).

2. Sensitivity and specificity of oligonucleotide probes

In order to examine the sensitivity and the specificity of each oligonucleotide probe, the envelope gene of 24 subtype IIIa viruses and 5 subtype IIIb viruses derived

```
D94-039    MRCIGISNRD FVEGVSGGSW VDIVLEHGSC VTTMAKNKPT LDFELIKTEA 50
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      .....M.... .......... .......... .......... ..........

D87-1421   .......... .......... .......... .......... ..........
MK42-86    .......... .......... .......... .......... ..........
D80-141    .......... .......... .......... .......... ..........
D80-038    .......... .......... .......... .......... ..........

D94-039    KQPATLRKYC IEAKLTNTTT ESRCPTQGEP SLKEEQDKRF VCKHSMVDRG 100
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... M......... .......... ..N....... ..........
D80-100    .......... .......... .......... ..N......L ..........
16681      .......... .......... .......... ..N....... ..........

D87-1421   .......... ...R...... .......... .......... .....I....
MK42-86    .......... .......... .......... .......... .....I....
D80-141    .......... .......... .......... ..N....... ..........
D80-038    .......... .......... .......... ..N......L ..........
```

Figure 3. Deduced amino acid sequence of envelope protein of D94-039, D94-179, D94-376, D94-548 and other dengue type 2 virus isolates classified as subtype IIIa (upper panel) or IIIb (lower panel). Dots represent amino acids identical to those of D94-039.

```
D94-039   WGNGCGLFGK GGIVTCAMFT CKKNMEGKIV QPENLEYTIV VTPHSGEEHA 150
D94-179   .......... .......... .......... .......... ..........
D94-376   .......... .......... .......... .......... ..........
D94-548   .......... .......... .......... .......... ..........
D87-642   .......... .......... .......... .......... ..........
D87-1040  .......... .......... .......... .......... ..........
PUO-280   ......F... .......... .......... .......... ..........
D80-100   .......... .......... .......... .......... ..........
16681     .......... .........R .........V. .......... I.........

D87-1421  .......... .......... .......... .......... I.........
MK42-86   .......... .......... .......... .......... I.........
D80-141   .......... .......... .......... .......... I.........
D80-038   .......... .......... .......... .......... I.........

D94-039   VGNDTGKHGK EIKVTPQSSI TEAELTGYGT VTMECFPRTG LDFNEMVLLQ 200
D94-179   .......... .......... .......... .......... ..........
D94-376   .......... .......... .......... .......... ..........
D94-548   .......... .......... .......... .....S.... ..........
D87-642   .......... .......... .......... .....S.... ..........
D87-1040  .......... .V........ .......... .....S.... ..........
PUO-280   .......... .......... A......... .....S.... ..........
D80-100   .......... .......... .......... .....S...V ..........
16681     .......... ...I...... .......... .....S.... ..........

D87-1421  .......... ...I...... .......... I....S.... ..........
MK42-86   .........N ...I...... .......... .....S.... ..........
D80-141   .......... ...I...... .......... .....S.... ..........
D80-038   .......... .......... .......... .....S .... ..........

D94-039   MENKAWLVHR QWFLDLPLPW LPGADTQGSN WIQKETLVTF KNPHAKKQDV 250
D94-179   .......... .......... .......... .......... ..........
D94-376   .......... .......... .......... .......... ..........
D94-548   .......... .......... .......... .......... ..........
D87-642   .......... .......... .......... .......... ..........
D87-1040  .......... .......... .......... .......... ..........
PUO-280   .......... .......... .......... .......... ..........
D80-100   .......... .......... .......... .......... ..........
16681     .......... .......... .......... .......... ..........

D87-1421  ..D....... .......... .......... .......... ..........
MK42-86   ..D....... .......... .......... .......... ..........
D80-141   ..D....... .......... .......... .......... ..........
D80-038   ..D....... .......... .......... .......... ..........
```

Figure 3. (continued).

```
D94-039    VVLGSQEGAM HTALTGATEI QMSSGNLLFT GHLKCRLRMD KLQLKGMSYS 300
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... ....S..S.. ..........
D94-548    .......... .......... .......... ....S..... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      .......... .......... .......... .......... ..........

D87-1421   .......... .......... .......... .......... ..........
MK42-86    .......... .......... .......... .......... ..........
D80-141    .......... .......... .......... .......... ..........
D80-038    .......... .......... .......... .......... ..........

D94-039    MCTGKFKVVK EIAETQHGTV VIRVQYEGDG SPSKIDFEIM DLEKRYVLGR 350
D94-179    ..........I......... .......... .......... ..........
D94-376    .......... .........I .......... ...C.V..... ..........
D94-548    .......... .........I .......... ....V..... ..........
D87-642    .......... .........I .......... ..C....... ..........
D87-1040   .......... .........I .......... ..C....... ..........
PUO-280    .......... .........I .......... ..C....... .....H....
D80-100    .......... .........I .......... ..C....... .....H....
16681      .......... .........I .......... ..C....... .....H....

D87-1421   .......I.. .........I .......... ..C....... .....H....
MK42-86    .......I.. .........I .......... ..C....... .....H....
D80-141    .......I.. .........I .......... ..C....... .....H....
D80-038    .......I.. .........I .......... ..C....... .....H....

D94-039    LITVNPIVTE KDSPVNIEAE PPFGDSYIII GVEPGQLKLN WFKKGSSIGQ 400
D94-179    .......... .......... .......... .......... ......F...
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .....K.... .......... .......... ..........
16681      .......... .......... .......... .......... ..........

D87-1421   .......... .......... .......... .......... ..........
MK42-86    .........A. .......... .......... .......... ..........
D80-141    .......... .......... .......... .......... ..........
D80-038    .......... .......... .......... .......... ..........
```

Figure 3. (continued).

```
D94-039    MFETTMRGAK RMAILGDTAW DFGSLGGVFT SIGKALHQVF GAIYGAAFSG 450
D94-179    .......... .......... .......... .......... ..........
D94-376    .......... .......... .......... .......... ..........
D94-548    .......... .......... .......... .......... ..........
D87-642    .......... .......... .......... .......... ..........
D87-1040   .......... .......... .......... .......... ..........
PUO-280    .......... .......... .......... .......... ..........
D80-100    .......... .......... .......... .......... ..........
16681      .......... .......... .......... .......... ..........

D87-1421   .......... .......... .......... .......... ..........
MK42-86    .......... .......... .......... .......... ..........
D80-141    .......... .......... .......... .......... ......T...
D80-038    .......... .......... .......... .......... ..........

D94-039    VSWTMKILIG VIITWIGMNS RSTSLSVSLV LVGIVTLYLG VMVQA      495
D94-179    .......... .......... .......... .......... .....
D94-376    .......... .......... .......... .......... .....
D94-548    .......... .......... .......... .......... .....
D87-642    .......... .......... .......... .......... .....
D87-1040   .......... .......... .......... .......... .....
PUO-280    .......... .......... .......P.. .......... .....
D80-100    .......... .......I.. .......... .......... .....
16681      .......... .......... .......T.. .......... .....

D87-1421   .......... .......... .......... ...V...... A....
MK42-86    .......... .......... .......... ...V...... A....
D80-141    .......... .......I.. .......... ...V...... A....
D80-038    .......... .......... .......... ...V...... A..L.
```

Figure 3. (continued).

Figure 4. Number of nucleotide differences (above diagonal axis) and amino acid differences (below diagonal axis) of envelope gene/protein among selected dengue serotype 2 viruses isolated from 1980, 1987 and 1994 epidemic seasons.

	039	179	376	548	642	1040	280	100	16681	1421	42-86	141	038
D94-039	-	24	22	20	16	20	33	37	51	99	100	95	72
D94-179	1	-	21	20	16	28	40	44	53	104	107	102	77
D94-376	5	6	-	8	10	20	34	38	51	97	102	96	71
D94-548	4	5	3	-	8	20	31	35	49	95	97	91	66
D87-642	3	4	4	3	-	14	26	30	44	91	95	89	63
D87-1040	4	5	5	4	1	-	32	36	50	98	101	95	69
PUO-280	9	10	10	9	6	7	-	22	44	94	97	84	61
D80-100	9	10	10	9	6	7	8	-	49	95	96	82	63
16681	11	12	12	11	8	9	10	10	-	79	83	76	62
D87-1421	13	14	14	13	10	11	13	14	12	-	13	21	53
MK42-86	13	14	14	13	10	11	13	14	12	4	-	24	56
D80-141	13	14	14	13	10	11	13	10	10	6	6	-	48
D80-038	12	13	13	12	9	10	13	9	11	7	7	5	-

from the 1980 and 1987 epidemic seasons were amplified and hybridized with the two sets of probes designed to detect specifically the subtype IIIa and subtype IIIb sets, respectively. For each oligonucleotide probe the temperature during the TMAC washing step was titrated from 47°C, 52°C, 55°C and 57°C. The lowest temperature levels that appeared to result in the best contrast between the two groups of viruses are shown in Table 6. Following the hybridization and washing at the selected temperatures, the hybridization signal derived from the hybridization with each of the five pairs of oligonucleotide probes was quantified by scanning with a densitometer and the resulting adjusted volume and maximum intensity values were then divided by the corresponding values derived from a reference probe (C1204 or C1763). An example of the hybridization signals when reacted with probe C1204, 141-IIIa, 141-IIIb, and 346-IIIa-87 was shown in Figure 5, 6, 7, and 8, respectively. The adjusted volume ratio and the maximum optical density ratio for each hybridization experiment are listed in Appendices A-E. The range, mean and standard deviation of the adjusted volume ratio and maximum optical density ratio of each probe are shown in Table 7 and Table 8, respectively.

When the lowest levels of adjusted volume ratio against the corresponding set of viruses were employed as the cutoff points, the sensitivity of the probes 141-IIIa, 203-IIIa, 308-IIIa, 484-IIIa and 491-IIIa for detecting the subtype IIIa viruses was all 100%. Similarly, the sensitivity of the probes 141-IIIb, 203-IIIb, 308-IIIb, 484-IIIb and 491-IIIb for the subtype IIIb viruses was also 100% (Table 9). At this high level of sensitivity, the specificity of most probes was 100%. An exception was in the case of 141-IIIa (specificity, 80%) which appeared to cross-react with one of the non-corresponding strain, MK42-86.

Figure 5. An example of the hybridization signal when reacted with probe C1204.

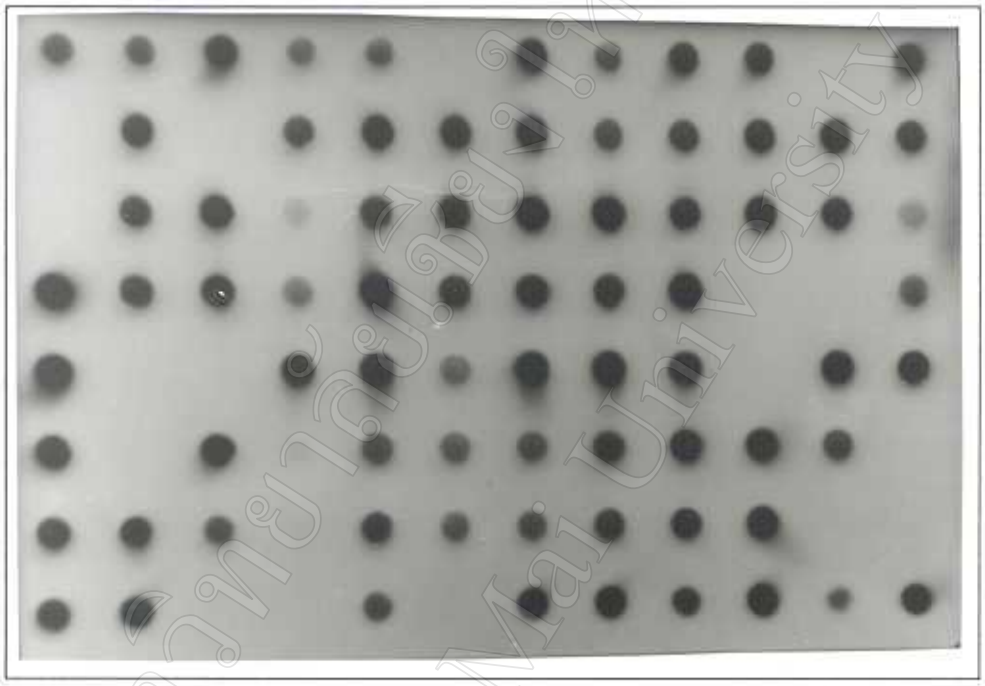

Figure 6. An example of the hybridization signal when reacted with probe 141-IIIa.

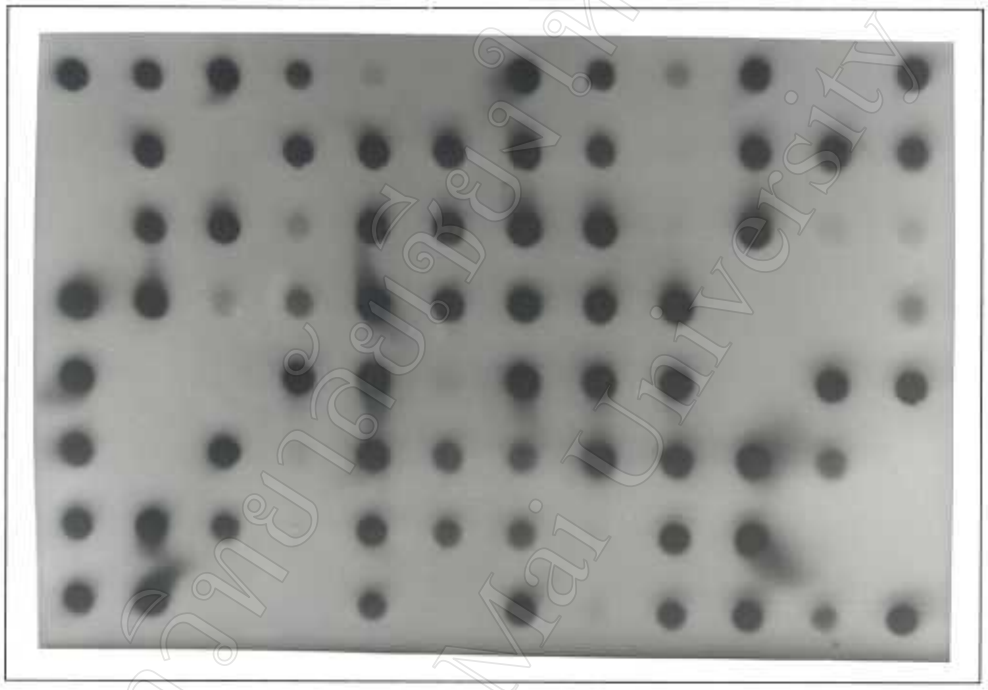

Figure 7. An example of the hybridization signal when reacted with probe 141-IIIb.

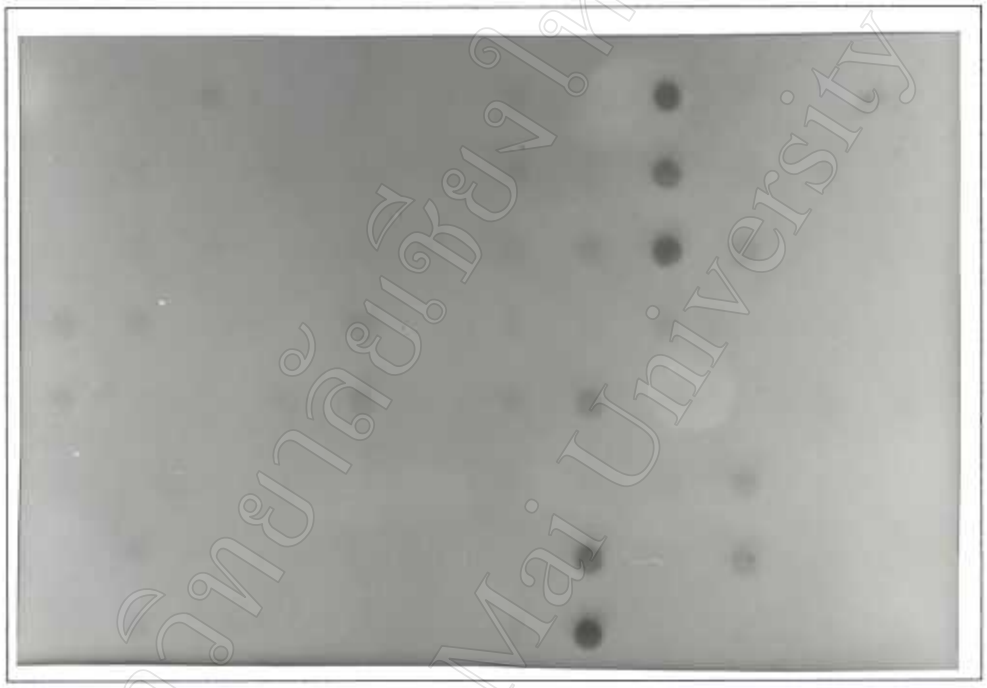

Figure 8. An example of the hybridization signal when reacted with probe 346-IIIa-87.

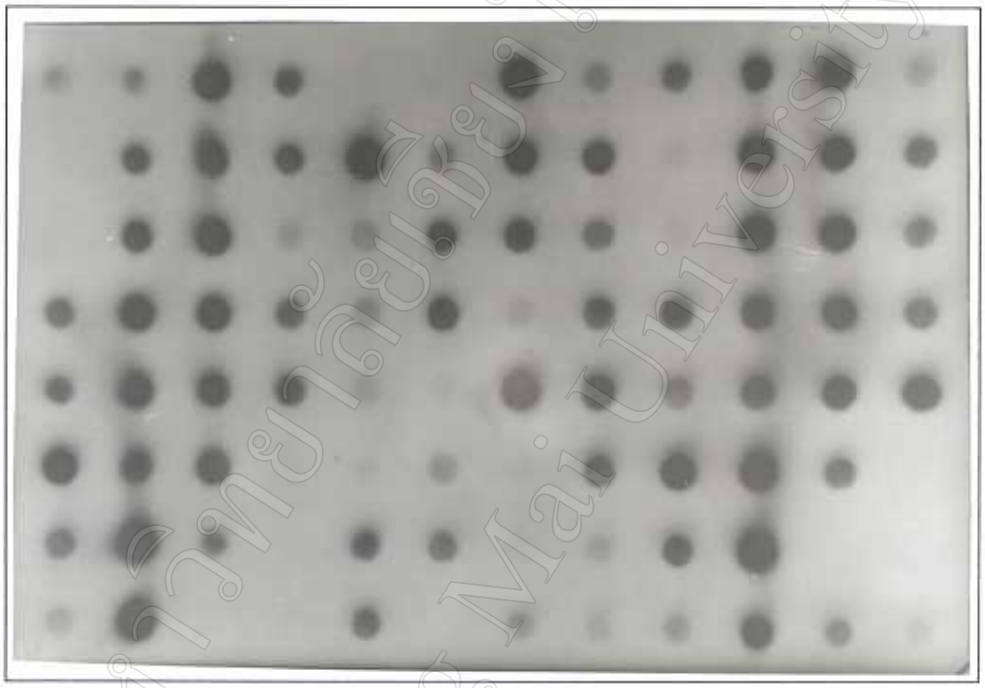

Table 7. Ratio of adjusted volume between various probes and probe C1204.

Subtype of viruses[1]	Ratio									
	141-IIIa	141-IIIb	203-IIIa	203-IIIb	308-IIIa	308-IIIb	484-IIIa	484-IIIb	491-IIIa	491-IIIb
IIIa										
range	0.043-0.569	0.070-0.188	0.179-0.561	0	0.481-1.425	-0.049-0.334	0.470-3.498	0.005-1.788	1.740-4.624	0
mean	0.198	0.117	0.384	0	0.895	0.058	1.860	0.115	2.710	0
SD	0.142	0.028	0.135	ND	0.218	0.090	0.664	0.357	0.967	ND
IIIb										
range	0.010-0.123	0.783-0.865	0.003-0.056	1.023-2.164	0.024-0.431	0.617-0.819	0.018-0.183	1.844-2.685	0.055-0.281	0.426-0.643
mean	0.039	0.823	0.032	1.679	0.146	0.732	0.060	2.240	0.132	0.495
SD	0.047	0.038	0.025	0.453	0.164	0.086	0.071	0.342	0.087	0.086

1, only dengue virus isolates derived from the 1980 and 1987 epidemic seasons were included.

Table 8. Ratio of maximum optical density between various probes and probe C1204.

Subtype of viruses[1]	Ratio									
	141-IIIa	141-IIIb	203-IIIa	203-IIIb	308-IIIa	308-IIIb	484-IIIa	484-IIIb	491-IIIa	491-IIIb
IIIa										
range	0.016-0.895	0.142-0.707	0.310-0.780	0	0.702-1.248	0.214-0.681	0.760-2.325	0.238-1.389	1.286-2.436	0
mean	0.442	0.257	0.592	0	0.953	0.410	1.452	0.468	1.721	0
SD	0.214	0.107	0.109	ND	0.130	0.172	0.341	0.250	0.335	ND
IIIb										
range	0.127-0.370	0.581-1.000	0.207-0.320	1.088-1.890	0.273-0.676	0.812-0.951	0.251-0.427	1.499-1.890	0.253-0.487	0.530-0.735
mean	0.180	0.919	0.266	1.423	0.381	0.895	0.328	1.686	0.377	0.601
SD	0.106	0.058	0.053	0.295	0.171	0.059	0.064	0.173	0.094	0.080

1, only dengue virus isolates derived from the 1980 and 1987 epidemic seasons were included.

Table 9. Sensitivity and specificity of various probes as determined by using the lowest adjusted volume ratio value as the cutoff point.

Probe	Sensitivity	Specificty
141-IIIa	24/24 (100)	4/5 (80)
203-IIIa	24/24 (100)	5/5 (100)
308-IIIa	24/24 (100)	5/5 (100)
484-IIIa	24/24 (100)	5/5 (100)
491-IIIa	24/24 (100)	5/5 (100)
141-IIIb	5/5 (100)	24/24(100)
203-IIIb	5/5 (100)	24/24 (100)
308-IIIb	5/5 (100)	24/24 (100)
484-IIIb	5/5 (100)	24/24 (100)
491-IIIb	5/5 (100)	24/24 (100)

When the lowest levels of maximum optical density ratio against the corresponding set of viruses were employed as the cutoff points, all probes gave the sensitivity of 100% (Table 10). In contrast, the specificity was detected at the 100% level only with seven probes. The probes 141-IIIa (specificity, 80%), 203-IIIa (specificity, 60%), 308-IIIa (specificity, 80%) cross-reacted with one; MK42-86, two; MK42-86 and MK116-87 and one; MK116-87, respectively, of the subtype IIIb viruses. Thus, two pairs of the oligonucleotide probes (484-IIIa, 484-IIIb, 491-IIIa and 491-IIIb) appeared to give the highest levels of sensitivity and specificity for detecting the subtype IIIa and IIIb viruses by the hybridization analysis. However, these high levels of sensitivity and specificity were achieved only by designing the probes to accommodate other known changes surrounding the differentiating nucleotides; when this set of probes were used against other viruses of which additional changes were unknown, the sensitivity and specificity of each probes might differ from those derived by testing with 1980 and 1987 viruses. Because of this consideration, all five pairs of oligonucleotide probe were employed in the subtyping of the dengue serotype 2 viruses circulating in the 1994 epidemic season.

3. Predominance of subtype IIIa among dengue serotype 2 viruses in 1994

In order to determine the proportion of subtype IIIa and subtype IIIb among dengue serotype 2 viruses circulating in Bangkok in 1994, the envelope region of 24 dengue serotype 2 viruses derived from patients at the Bangkok Children's Hospital during 1994 was amplified (Table 2). From 20 viruses the amplified products covered all of the envelope region allowing the analysis with all five pairs of probes. From the

Table 10. Sensitivity and specificity of various probes as determined by using the lowest maximum optical density ratio value as the cutoff point.

Probe	Sensitivity	Specificity
141-IIIa	24/24 (100)	4/5 (80)
203-IIIa	24/24 (100)	3/5 (60)
308-IIIa	24/24 (100)	4/5 (80)
484-IIIa	24/24 (100)	5/5 (100)
491-IIIa	24/24 (100)	5/5 (100)
141-IIIb	5/5 (100)	24/24 (100)
203-IIIb	5/5 (100)	24/24 (100)
308-IIIb	5/5 (100)	24/24 (100)
484-IIIb	5/5 (100)	24/24 (100)
491-IIIb	5/5 (100)	24/24 (100)

other four, only a small part of the envelope region was successfully amplified by the nested PCR procedure (the PCR products courtesy of Dr. Pathai Yenchitsomanus, Siriraj Medical School); in the latter group, only the pair 308-IIIa and 308-IIIb could be used. As before, the amplified products were hybridized with each of the five pairs of probe and the hybridization signal from each probe was then divided by the corresponding values derived with either the probe C1204 or probe C1763 to obtain the adjusted volume ratio and the maximum optical density ratio (Appendices A-E).

When the lowest adjusted volume ratio values obtained by hybridizing probes with viruses derived from the 1980 and 1987 epidemic seasons were employed as the cutoff points, four pairs of probes typed all of the dengue serotype 2 viruses from 1994 as subtype IIIa (Table 11). Only one probe, 491-IIIa, failed to identify one (out of 20) strain (D94-411) as the subtype IIIa strain. Identical results were obtained when the lowest maximum optical density ratio values were employed as cutoff points. For four viruses (D94-039, D94-179, D94-376 and D94-548) of which nucleotide sequences of the envelope region were available, the hybridization pattern matched perfectly with the sequence data. Thus, 24 out of 24 dengue serotype 2 viruses which circulated in Bangkok in 1994 belonged to the subtype IIIa.

4. Descent of subtype IIIa viruses

During the 1987 epidemic season in Bangkok, circulating subtype IIIa viruses (19 out of 19) shared a non-synonymous nucleotide substitution (base position 1036, T; amino acid position 346, tyrosine) which was absent in all subtype IIIb isolates and the subtype IIIa viruses found in 1980 (Sittisombut et al., 1997). Because most, if not all,

Table 11. Reactivity of dengue type 2 viruses derived from the 1994 epidemic season with various probes using the lowest ratio value of various probes as the cutoff points.

Cutoff point	Proportion (%)				
	141-IIIa	**203-IIIa**	**308-IIIa**	**484-IIIa**	**491-IIIa**
Lowest adjusted volume ratio	20/20 (100)	20/20 (100)	24/24 (100)	20/20 (100)	19/20 (95)
Lowest maximun O.D. ratio	20/20 (100)	20/20 (100)	24/24 (100)	20/20 (100)	19/20 (95)

Cutoff point	Proportion (%)				
	141-IIIb	**203-IIIb**	**308-IIIb**	**484-IIIb**	**491-IIIb**
Lowest adjusted volume ratio	0/20 (0)	0/20 (0)	0/24 (0)	0/20 (0)	0/20 (0)
Lowest maximun O.D. ratio	0/20 (0)	0/20 (0)	0/24 (0)	0/20 (0)	0/20 (0)

dengue serotype 2 viruses circulating in the 1994 epidemic season belonged to subtype IIIa, this base substitution may be useful for determining whether the subtype IIIa viruses of the 1994 epidemic season in Bangkok were direct descendants of the 1987 ancestral counterparts. An oligonucleotide probe (346-IIIa-87) was designed and employed in the hybridization analysis. The adjusted volume ratio and the maximum optical density ratio were derived by employing signals from the probe C1763 for adjusting differences in DNA binding to nylon membrane. The adjusted volume ratio and the maximum optical density ratio for the probe 346-IIIa-87 were listed in Appendix F. The range, mean and standard deviation of adjusted volume ratio and maximum optical density ratio of the 1980 and 1987 isolates were shown in Table 12.

In order to maximize the specificity of this probe, the highest adjusted volume ratio and the highest maximum optical density ratio derived from hybridization with the subtype IIIb strains and the subtype IIIa strains of the 1980 epidemic season were used as cutoff points. By using these cutoff points, the sensitivity of probe 346-IIIa-87 for detecting the T(1036) substitution among the subtype IIIa strains from 1987 was only 85% and 90%, respectively (Table 13). Even with these low levels 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 precursors rather than being introduced from other places.

Table 12. Ratio of adjusted volume and maximum optical density between probe 346-IIIa-87 and probe C1763.

Subtype of viruses	**Ratio**	
	Adjusted volume	Maximum optical density
IIIa (1987 epidemic season)		
range	0.100-1.046	0.334-1.194
mean	0.617	0.830
SD	0.256	0.194
IIIa and IIIb (1980 epidemic season), **IIIb** (1987 epidemic season)		
range	-0.010-0.262	0.261-0.500
mean	0.058	0.361
SD	0.094	0.071

Table 13. Sensitivity and specificity of probe 346-IIIa-87 as determined by using the highest adjusted volume ratio and maximum optical density ratio values of subtype IIIb strains and subtype IIIa strains of the 1980 epidemic season as the cutoff point.

	Sensitivity	Specificity
Adjusted volume ratio	18/21 (85)	9/9 (100)
Maximum O.D. ratio	19/21 (90)	9/9 (100)