III. MATERIALS AND METHODS

1. Dengue viruses

Twenty-four dengue serotype 2 viruses were identified from infected patients hospitalized during the 1994 epidemic season at the Bangkok Children's Hospital. Identification was based on virus isolation in Toxorhynchites mosquitoes (at the Department of Virology, Armed Forces Research Institute of the Medical Sciences [AFRIMS], Bangkok) and/or RT-PCR assays done at three institutions (AFRIMS, Siriraj Medical Molecular Biology Center, Faculty of Medicine Siriraj Hospital, Mahidol University, and Department of Microbiology, Faculty of Medicine, Chiang Mai University). Materials for actual analysis were in various physical forms: 7 were in the form of patients' sera, 1 was purified viral RNA derived from patients' sera, 12 were C6/36 culture supernatant and 4 were PCR products (Table 2). The characteristics of all dengue serotype 2 viruses were summarized in Table 3.

Twenty-nine dengue serotype 2 viruses isolated from the 1980 and 1987 epidemic seasons in Bangkok and Maha Sarakham served as positive and negative controls for the differential hybridization. Among these, 24 belonged to subtype IIIa whereas 5 (D80-141, D87-1421, MK42-86, MK116-87, and MK244-87) belonged to subtype IIIb (Table 4). The envelope gene sequence of these viruses were known (Blok et al., 1989; Duangchanda et al., 1994; Sittisombut et al., 1997).

2. Oligonucleotide primers for RT-PCR amplification

One pair of oligonucleotide primers (D_2J-134 and cD_2J-2504) was used in the amplification of dengue genomic RNA (Table 5). These primers derived from the

Table 2. Dengue viruses derived from the 1994 epidemic season in Bangkok.

Strain	Form	Size[1]	Source
D94-035	TS-1, C6-1, Apr 1995	2.4	AFRIMS
D94-039	TS-1, C6-1, Mar 1995	2.4	AFRIMS
D94-041	TS-1, C6-1, Mar 1995	2.4	AFRIMS
D94-082	TS-1, C6-1, Apr 1995	2.4	AFRIMS
D94-123	TS-1, C6-1, Aug 1995	2.4	AFRIMS
D94-173	TS-1, C6-1, Nov 1995	2.4	AFRIMS
D94-179	TS-1, C6-1, Nov 1995	2.4	AFRIMS
D94-197	TS-1, C6-1, Aug 1995	2.4	AFRIMS
D94-336	PCR product	0.35	AFRIMS through Dr. Pathai Yenchitsomanus
D94-337	PCR product	0.35	AFRIMS through Dr. Pathai Yenchitsomanus
D94-339	Patients' sera	2.4	AFRIMS through Dr. Niwat Maneekarn
D94-373	Patients' sera	2.4	AFRIMS through Dr. Niwat Maneekarn
D94-376	TS-1, C6-1, Apr 1995	2.4	AFRIMS
D94-377	PCR product	0.35	AFRIMS through Dr. Pathai Yenchitsomanus
D94-389	TS-1, C6-1, Mar 1995	2.4	AFRIMS

Table 2. (continued)

Strain	Form	Size[1]	Source
D94-411	Patients' sera	2.4	AFRIMS through Dr. Niwat Maneekarn
D94-435	PCR product	0.35	AFRIMS through Dr. Pathai Yenchitsomanus
D94-439	Patients' sera	2.4	AFRIMS through Dr. Niwat Maneekarn
D94-476	TS-1, C6-1, Apr 1995	2.4	AFRIMS
D94-529	Patients' sera	2.4	AFRIMS through Dr. Niwat Maneekarn
D94-539	Patients' sera	2.4	AFRIMS through Dr. Niwat Maneekarn
D94-548	Purified genomic RNA	2.4	AFRIMS through Dr. Boonyos Raengsakulrach
D94-568	TS-1, C6-1, Jun 1995	2.4	AFRIMS
D94-586	Patients' sera	2.4	AFRIMS through Dr. Niwat Maneekarn

1, size in kb of PCR products used in differential hybridization.

Table 3. Characteristics of dengue viruses derived from the 1994 epidemic season in Bangkok.

Strain	Date of blood collection	Province	Clinical Diagnosis	Serodiagnosis
D94-035	23 Jan 94	Nakorn Sawan	DHF grade 3	Acute secondary dengue infection
D94-039	26 Jan 94	Bangkok	DHF grade 3	Acute secondary dengue infection
D94-041	26 Jan 94	Bangkok	DHF grade 3	Acute secondary dengue infection
D94-082	15 Feb 94	Bangkok	DHF grade 2	Acute secondary dengue infection
D94-123	11 Mar 94	Samut Prakarn	DHF grade 2	Acute primary dengue infection
D94-173	2 May 94	Bangkok	DHF grade 3	Secondary dengue infection
D94-179	14 May 94	Bangkok	DF	No serologic diagnosis
D94-197	24 May 94	Bangkok	DHF grade 3	Acute secondary dengue infection
D94-336	11 Aug 94	Bangkok	DF	Acute secondary dengue infection
D94-337	11 Aug 94	Bangkok	DHF grade 3	Acute secondary dengue infection
D94-339	13 Aug 94	Bangkok	DHF grade 2	Acute secondary dengue infection
D94-373	28 Aug 94	Bangkok	DF	Acute secondary dengue infection

Table 3. (continued)

Strain	Date of blood collection	Province	Clinical Diagnosis	Serodiagnosis
D94-376	30 Aug 94	Bangkok and Ubol	Acute diarrhea, dengue infection	No serologic diagnosis (single specimen or less than 5 days)
D94-377	30 Aug 94	Bangkok	DHF grade 2	Acute secondary dengue infection
D94-389	3 Sep 94	Bangkok	DHF grade 2	Acute secondary dengue infection
D94-411	9 Sep 94	Bangkok	DHF grade 3	Acute primary dengue infection
D94-435	24 Sep 94	Bangkok	DHF grade 1	Acute secondary dengue infection
D94-439	23 Sep 94	Samut Prakarn	DF	Acute secondary dengue infection
D94-476	16 Oct 94	Bangkok	DHF grade 4	Acute secondary dengue infection
D94-529	14 Nov 94	Bangkok	DF	Acute secondary dengue infection
D94-539	21 Nov 94	Bangkok	DHF grade 3	Acute secondary dengue infection
D94-548	28 Nov 94	Bangkok	DHF grade 3	Acute secondary dengue infection
D94-568	12 Dec 94	Bangkok	DHF grade 3	Acute secondary dengue infection
D94-586	21 Dec 94	Bangkok	DHF grade 2	Acute secondary dengue infection

Table 4. Dengue virus isolates derived before the 1994 epidemic season.

Strain	Form	Size[1]	Source
DV1 (Hawaii)	C6-5, Aug 1994	2.4	AFRIMS
DV3 (H87)	TS-1, C6-2, Nov 1994	2.4	AFRIMS
DV4 (814669)	TS-1, C6-1, Sep 1994	2.4	AFRIMS
Dengue serotype 2 virues			
D_2V NGC	C6-7, May 1995	2.4	Lot 124, Mar 1994/AFRIMS
D_2V 16681	May 1996[3]	2.4	Lot 1/38, May 1995/ AFRIMS
Subtype IIIa			
D80-100	MK2-2, C6-2, Jul 1995	2.4	MK2-2,C6-1, Sep 1986/ AFRIMS
PUO80-218	MK2-3, C6-2, Sep 1995	2.4	MK2-3, C6-1, Dec 1980/ AFRIMS
PUO80-280	MK2-3, C6-1, Feb1986	2.4	AFRIMS
D87-011	C6-2, Sep 1991	2.4	AFRIMS
D87-167	TS-1, C6-1, Feb 1993	2.4	AFRIMS
D87-199	C6-2, Oct 1991	2.4	AFRIMS
D87-279	C6-3, May 1993	2.4	AFRIMS
D87-300	TS-1, C6-2, Feb 1993	2.4	AFRIMS
D87-499	C6-1, Sep 1991	2.4	AFRIMS

Table 4. (continued).

Strain	Form	Size[1]	Source
D87-642	TS-1, C6-5, May 1995	2.4	TS-1, C6-4, Nov 1994/ AFRIMS
D87-704	TS-1, C6-4, Mar 1996	2.4	TS-1, C6-3, Oct 1991/ AFRIMS
D87-738	C6-2, Apr 1995	2.4	Lot 37, Aug 1991/AFRIMS
D87-881	TS-1, C6-3, Nov 1991	2.4	AFRIMS
D87-1040	C6-2, Jul 1991	2.4	AFRIMS
D87-1051	TS-1, C6-1, Feb 1993	2.4	AFRIMS
D87-1077	C6-2, Apr 1996	2.4	Lot 36, Aug 1991/AFRIMS
D87-1113	TS-1, C6-4, May 1995	2.4	Lot 72, Oct 1991/AFRIMS
D87-1121	TS-1, C6-5, Nov 1991	2.4	AFRIMS
D87-1152	C6-2, Sep 1991	2.4	AFRIMS
D87-1283	C6-2, Apr 1995	2.4	Lot 35, Aug 1991/AFRIMS
D87-1372	TS-1, C6-3, Jul 1996	2.4	TS-1, C6-2, Aug 1991/ AFRIMS
D87-1418	TS-1, C6-3, Aug 1991	2.4	AFRIMS
D87-1441	TS-1, C6-4, Dec 1991	2.4	AFRIMS
DST87-60	TS-1, C6-2, Mar 1993	2.4	AFRIMS

Table 4. (continued).

Strain	Form	Size[1]	Source
Subtype IIIb			
D80-141	MK2-3, C6-2, Jul 1995	2.4	MK2-3, C6-1, Feb 1986/ AFRIMS
D87-1421	C6-3, May 1995	2.4	Lot 49, Sep 1991/AFRIMS
MK42-86	C6-1, May 1995	2.4	DF, Sep 1993/Virus Res. Institute[2]
MK116-87	C6-1, May 1995	2.4	DSS, Sep 1993/Virus Res. Institute[2]
MK244-87	C6-1, May 1995	2.4	C6 (Japan), Jan 1994/Virus Res. Institute[2]

1, size in kb of PCR products used in differential hybridization. 2, Virus Research Institute, Department of Medical Sciences, Ministry of Public Health, through Khun Sumlee Duangchanda and Khun Suntharee Rojanasuphot. 3, passage history unknown.

Table 5. Oligodeoxyribonucleotide primers for reverse transcription-polymerase chain reaction and nucleotide sequence analysis.

Designation	Sequence (5' to 3')	Position
For RT-PCR		
D_2J-134	TCAATATGCTGAAACGCGAGAGAAACCG	134-161
cD_2J-2504	GGGGATTCTGGTTGGAACTTATATTGTTCTGTCC	2504-2537
Expected size = 2,400 base pairs		
D_2L^1	ATCCAGATGTCATCAGGAAAC	808-828
D_2R^1	CCGGCTCTACTCCTATGATG	1153-1134
Expected size = 350 base pairs		
For nucleotide sequence analysis		
C1204	GGAGTGTTTGCAGACGAA	1221-1204
C1518	CATCTGCAGCAACACCATCTC	1539-1519
C1763	GAGATGTCCTGTGAAGAGTAAGT	1785-1763
C1976	TGTAATCAGGCGACCTAAGA	1995-1976
C2260	AAAGGCAGCTCCATAGATTGC	2280-2260
C2496	GGGCTGCAGTTGTTCTGTCCATGTGTGC	2514-2496
S1699	TCTCAAGAAGGGGCCATGCA	1699-1718

1, primer pairs for nested PCR of Dr. Pathai Yenchitsomanus (Yenchitsomanus et al., 1996).

study of Lewis et al (1992) and based on the nucleotide sequence of dengue serotype 2 Jamaica 1409 sequence (Lewis et al., 1992). In dengue genome, the target sequence of the primer pair is conserved among all 4 dengue serotypes and the primers specifically amplified the pre-membrane (prM), membrane (M) and envelope glycoprotein (E) genes. Nucleotide sequence of the primers, the expected size of amplified products and the corresponding locations in dengue genome are shown in Table 5.

For some viruses, the amplified products (0.35 kb) were obtained from Dr. Pathai Yenchitsomanus, Siriraj Medical School, Bangkok because viruses could not be isolated by the mosquito system and attempts to amplify the larger region from purified genomic RNA by RT-PCR were unsuccessful. The primers employed by Dr. Pathai Yenchitsomanus (D_2L and D_2R) (Yenchitsomanus et al., 1996) are listed in Table 5.

3. Preparation of genomic RNA

Viral genomic RNA was prepared directly from the culture fluids of infected C6/36 cells or acute serum of dengue infected patients by guanidine isothiocyanate-phenol-chloroform extraction (Bagnarelli et al., 1991). One hundred microliters of serum or culture fluids were mixed with 400 µl of solution D (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol). The mixture was shaken vigorously and then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25 : 24 : 1 v/v). The solution was mixed vigorously for 10 seconds and cooled on ice for 15 min. It was then centrifuged at 13,000 rpm in a high speed refrigerated centrifuge (Model 4239R, ALC, Milano, Italy) for 20 min at 4°C. The aqueous layer phase containing the viral genomic RNA was transferred to a fresh tube. Sodium acetate pH 5.2 was added to a

final concentration of 0.3 M and the viral RNA was precipitated with 2.5-3 volume of 95% ethanol for 1 h at -20°C. The RNA was then collected by centrifugation at 13,000 rpm for 30 min at 4°C. After washing once with 70% ethanol, RNA pellet was allowed to dry at room temperature and then resuspended in diethyl pyrocarbonate-treated water. Viral genomic RNA was stored at -70°C.

4. Synthesis of complementary DNA

Viral genomic RNA was reverse transcribed into complementary DNA (cDNA) by using reverse transcriptase as follows (Lewis et al., 1992). Viral genomic RNA was heated to 70°C for 10 min, then quickly chilled on ice. Reverse transcription was carried out in a reaction mixture containing 10 mM Tris-HCl, pH 8.3 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 10 mM dithiothreitol, 0.5 mM each of the four dNTPs, 100 pmol of antisense primer and 20 units of Superscript II RNase H^- Reverse Transcriptase (GIBCO/BRL, Grand Island, New York, USA). Reverse transcription was allowed to proceed at 56°C for 1 h and stopped by heating at 94°C for 4 min. The cDNA products were used directly in the amplification reactions.

5. Amplification of dengue envelope gene

The cDNA was amplified in 100 µl volume of the mixture containing 10 mM Tris-HCl, pH 8.3 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 200 µM each of the four dNTPs, 100 pmol of sense primer, and 2.5 units of *Taq* DNA polymerase (Promega Corporation, Madison. Wisconsin, USA) (Lewis et al., 1992). After overlaying the mixture with two drops of mineral oil, the amplification was carried out in the thermal cycler (Perkin Elmer, Forster City, California, USA) for 30 cycles as

follows: denaturation, 94°C for 1 min, annealing, 65°C for 1 min, and extension, 72° C for 10 min. The last extension step was at 72°C for 20 min. To detect the contamination of the extracted RNA by the PCR products, each RNA samples were tested as a PCR control for amplified the E gene without the synthesis of cDNA.

Five µl of the PCR products were analyzed by electrophoresis through a 0.8% agarose gel in Tris/acetate/EDTA (TAE) buffer at 110 volts for 20 min. The gel was stained with ethidium bromide (1 µg/ml) for 10 min and destained, and DNA bands were visualized with an ultraviolet light source.

6. Oligonucleotides and labeling

Five pairs of oligonucleotide probes were employed for the differentiation of the subtype IIIa and subtype IIIb of dengue serotype 2 viruses by hybridization (Table 6). These probes were based on six locations of nucleotide difference between the two subtypes identified between 1980 and 1987 epidemic seasons (Sittisombut et al., 1997). In order to maximize the sensitivity of the probes, sequence variation surrounding the differentiating nucleotides was taken into consideration: the differentiating nucleotide was placed in the middle of the 18-base probe and all known variations were included into the probe so that the probes could hybridize to all known viruses belonging to a particular subtype regardless of the variation in the surrounding nucleotides.

Oligonucleotides were labeled at the 3' terminus with a digoxigenin-conjugated 2',3'-dideoxyuridine triphosphate (DIG-11-ddUTP) by using DIG-oligonucleotide 3' end labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany). One hundred picomoles of each oligonucleotide were mixed with the tailing buffer (0.14 mM sodium cacodylate, 30 mM Tris-HCl, pH 7.2, 10 mM $CoCl_2$), 1 mM DIG-11-ddUTP, 1 mM

Table 6. Oligodeoxyribonucleotide probes for differential hybridization.

Designation	Position		Sequence	Final
	Codon	Base		Wash (°C)
C1204		1221-1204	GGAGTGTTTGCAGACGAA	47°C
C1763		1785-1763	GAGATGTCCTGTGAAGAGTAAGT	47°C
141-IIIa	141	429-413	G(AG)GTGT(CT)ACCACAATGG[1]	57°C
141-IIIb			G(AG)GGTGTTATCACGATGG	52°C
203-IIIa	203	618-601	CCAAGCTTT(AG)TTTTCCAT	57°C
203-IIIb			CCAAGCTTTGTCTTCCAT	47°C
308-IIIa	308	931-914	C(CT)TTCACAACTTT(AG)AACT	57°C
308-IIIb			CCTTCACAATTTTAAACT	55°C
484-IIIa	484	1461-1444	CAGTGTCACAATTCCCAC	47°C
484-IIIb			CAGTGTTACGACTCCCAC	47°C
491-IIIa	491	1482-1465	CTGCACCATGACTCCCAA	52°C
491-IIIb			C(AT)GCACCATAGCTCCCAA	52°C
346-IIIa-87	346	1046-1029	CCTAAGACATAT(CT)TTTTT	52°C

1, underlined bases represent differences between the subtype IIIa and IIIb strains; base in parenthesis represent nucleotide variation in the dengue virus system.

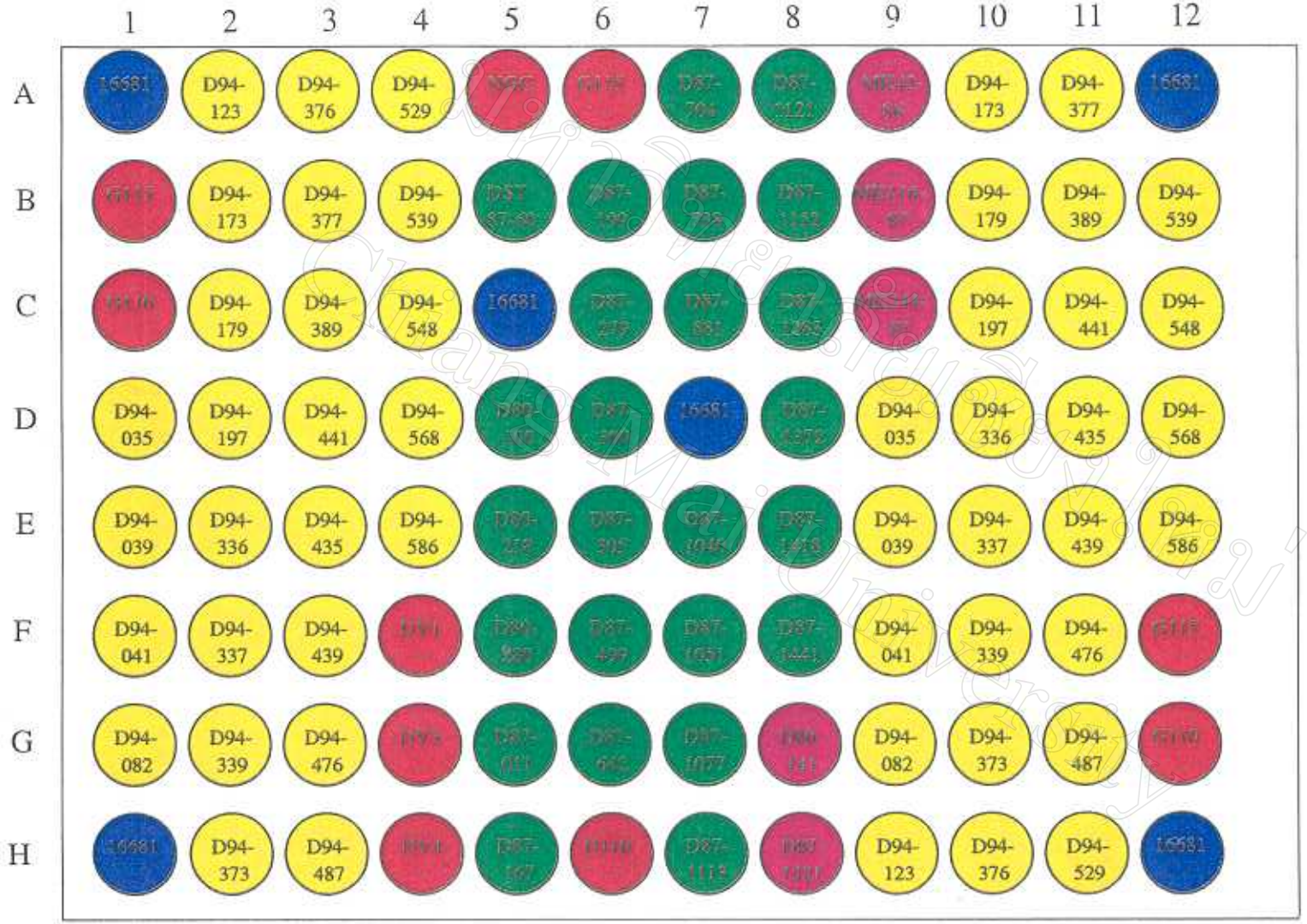
DTT and 20 units of terminal deoxynucleotide transferase. The mixture was incubated at 37°C for 15 min and stored at -20°C until use.

7. Dot blot hybridization

Amplified DNA was spotted under negative pressure onto prewetted nylon membrane filters (Micron Separation Inc., West Borough, Massachusette, USA) by using the dot blotter (Schleicher and Schuell, Keene, New Hampshire, USA) according to the spotting order shown in Figure 1. Yellow circles represent dengue serotype 2 viruses derived from the 1994 epidemic season in Bangkok; green circles represent subtype IIIa dengue serotype 2 viruses; purple circles represent subtype IIIb dengue serotype 2 viruses; blue circles represent dengue virus type 2 strain 16681, which was used as a control for the hybridization; red circles represent the negative control that should not hybridize with any of the oligonucleotide probes. The negative controls used in the hybridization included the PCR products of the HLA-DQA gene from patients # G110 and G115 in the human DNA repository of the Virology Unit, Department of Microbiology (Viputtigul, 1996); dengue serotypes 1, 3, 4 (DV1, DV3 and DV4, respectively) and strain NGC, a subtype I dengue serotype 2 virus. The amount of the DNA to be spotted was adjusted by direct visual comparison of the fluorescence intensity of the stained DNA bands on the agarose gel.

Before dot-spotting, the DNA samples were diluted in distilled water to equalize the total volume of DNA being spotted. Following dot-spotting, the membrane was left to dry at room temperature, denatured by soaking in 0.4 N NaOH for 5 min and neutralized in 10X SSPE (1.5 M NaCl, 0.1 M NaH_2PO_4, pH 7.4, 10 mM EDTA) for 10 min. The DNA was fixed to the membrane by UV irradiation at 0.51 Joule/cm^2 with

Figure 1. Dot spotting of PCR products on nylon membrane.



Bio-Rad GS Gene linker UV chamber (Bio-Rad, Hercules, California, USA) and dried at room temperature. Membranes were kept at 4°C until use.

The hybridization was performed according to the 11th International Histocompatibility Workshop Reference Protocol (Kimura, 1991). Membranes were prehybridized with 0.1 ml/cm^2 of hybridization buffer (6X SSPE, 5X Denhardt's solution, 0.1% sarcosine natrium, 0.02% SDS) at 42°C for 1 h to reduce non-specific binding. Labeled oligonucleotides were then added to the final concentration of 1 pmol/ml. The hybridization reaction was allowed to proceed for 2 hours. The hybridization solution was next poured off and the membranes were washed in 2X SSPE twice at room temperature for 10 min each. The membranes were then washed in tetramethylammonium chloride (TMAC) washing solution (50 mM Tris-HCl, pH 8.0, 3 M TMAC, 2 mM EDTA, 0.1% SDS) once at room temperature for 10 min and twice at appropriate temperature for 30 min each. TMAC was used in the washing step to reduce the influence of GC content on the hybridization behavior (Jacob et al., 1988). In the final step, the membranes were washed with 2X SSPE at room temperature for 10 min.

Following hybridization the probes were detected by using the DIG-chemiluminescent detection kit (Boehringer Mannheim Biochemica). The membranes were equilibrated in buffer 1 (100 mM Tris-HCl, pH 7.2, 150 mM NaCl) for 5 min. The buffer 2 (buffer 1 containing 0.5% blocking reagent) was next added to block non-specific binding of anti-DIG antibody to the membrane. After 30 minutes, alkaline phosphatase-conjugated anti-DIG antibody diluted in buffer 2 (1 : 20,000) was added to the membrane and agitated constantly for 30 min. Unreacted antibody was washed off twice with washing buffer (buffer 1 with 0.3% Tween 20) for 15 min each. The membranes were equilibrated in buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl,

50 mM $MgCl_2$) for 5 min and added with a chemiluminescent substrate, disodium 2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'- (5'-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl) -1-phenyl phosphate (CDP-Star, Boehringer Mannheim Biochemica), diluted to 0.25 mM in buffer 3. The reaction was allowed to proceed for 5 min after which the substrate solution was drained off. Membranes were put inside the plastic development folder (PhotoGene, GIBCO/BRL) in such a way that there was no air bubble inside the heat-sealed folder. Exposure to a X-ray film (Curix XP, Agfa-Gevaert N.V., Mortsel, Belgium) took from 2 hours to overnight at room temperature depending on the specific activity of individual probe.

To re-use the membrane, bound oligonucleotide-antibody-enzyme complexes were removed by immersing the membrane in dehybridization solution (0.2 N NaOH, 0.01% SDS) at 42°C for 30 min with agitation. Then, the membrane was neutralized in 2X SSC, pH 7.0 and stored in 2X SSPE at 4°C until use.

8. Densitometric analysis of hybridization signal

The intensity of hybridization signal captured on the X-ray film was quantified by scanning the whole film at high resolution (1200 dpi) with a densitometer (GS-700 Imaging Densitometer, Bio-Rad) employing the transmittance mode, a gray filter and the 8-bit pixel depth. Employing the Molecular Analyst software (Bio-Rad), the digital information derived from the image on the film was reproduced on the computer screen in such a way that the size and shape of the image of each DNA spots could be specified by the experimenter for further analysis. To reduce a variation of the area of the spots being analyzed, only one fixed circle, which was large enough to contain the largest visible spot, was then used to specify the area for all DNA spots displayed on

the computer screen. For each of such specified DNA spots the software determined the intensity and surface area and then reported the following values:

- Maximum O.D.: the highest O.D. within the spot.
- Minimum O.D.: the lowest O.D. within the spot.
- Mean O.D.: the average O.D. within the spot.
- Area: the area of each spot, in mm^2 (specified by experimenter).
- Volume: the integrated volume of the spot, is equal to mean O.D. x area.
- % volume: the percentage of volume of a spot in relation to the total volume of all spots identified.

In order to correct for the background level of light captured on the film during the exposure and development of films, 4-6 circles were also assigned within the film image outside of the DNA spots. The volume values of these background spots was then subtracted from the volume values of each DNA spots resulting in an additional value:

- Adjusted volume: the integrated volume of the DNA spot adjusted for background O.D.

In order to normalize for possible differential binding of the PCR products to the nylon membrane, an oligonucleotide, C1204, was used to hybridize to all spots. The envelope gene sequence covered by C1204 was known to be conserved among all dengue serotype 2 viruses derived from the 1980 and 1987 epidemic seasons. Because this probe was expected to hybridize equally well to all spots, variation in the hybridization intensity (obtained with C1204) among the spots should reflect differences in actual DNA binding to the membrane at each spot location. For each spot, the maximum optical density and adjusted volume derived from other oligonucleotide probes were then divided by the corresponding values derived with

C1204 to derive at the maximum O.D. ratio and the adjusted volume ratio. These two ratios, then, served as the basis for comparison between dengue serotype 2 subtype IIIa and subtype IIIb strains. In some experiments, C1763 was used in place of C1204.

9. Determination of sensitivity and specificity

In order to determine the sensitivity and specificity of the oligonucleotide probes, all five pairs of probes were reacted with 24 subtype IIIa and 5 subtype IIIb dengue serotype 2 viruses from the 1980 and 1987 epidemic seasons. For each probe the maximum optical density ratio and the adjusted volume ratio were derived from all 29 viruses. Examination of these two ratios revealed that there generally was a clear difference between the ratios derived from the corresponding viruses and non-corresponding viruses; i.e. the 484-IIIa probe elicited a higher maximum optical density ratio values (range 2.325-0.760) from known subtype IIIa viruses than known subtype IIIb viruses (range 0.427-0.251) (Appendix D). Thus, the lowest ratio values of the maximum O.D. and the adjusted volume derived from the corresponding subtype were employed as the cutoff point for a particular probe (i.e. 0.760 in the above example); this resulted in the sensitivity of 100% against known viruses for all probes. From these cut-off points, the number of known subtype IIIa and subtype IIIb viruses that reacted with a probe was determined and used in the calculation of specificity of the oligonucleotide probes.

At a given cutoff point, the sensitivity of a subtype IIIa probe was equal to the number of the subtype IIIa viruses that reacted (had the higher ratio value than the cutoff point) when hybridized with the probe divided by the total number of the subtype IIIa viruses tested. The specificity of a subtype IIIa probe was equal to the

number of the subtype IIIb viruses that did not react (had the lower ratio value than the cutoff point) when hybridized with this particular probe divided by the total number of the subtype IIIb viruses tested.

10. Nucleotide sequence analysis

The amplified DNA products derived from strains D94-039, D94-179, D94-376 and D94-548 were purified by elution from low melting point agarose gel. Following ethanol precipitation and washing with 70% ethanol, the product was electrophoresed through a 1% low melting agarose gel (Promega) containing 1 μg/ml ethidium bromide in TAE buffer at 50 volts for 1 hour. The gel was visualized under an ultraviolet light source and the 2,400 bp DNA band was excised with a sterile scalpel. Elution was accomplished by using the QIAquick Gel Extraction kit (QIAGEN, Chatsworth, California, USA). Briefly, the weight of a gel slice was determined and 3 volumes of buffer QX1 was added to dissolve the gel. After incubation at 50°C for 10 min, 1 gel volume of isopropanol was added. Next, the sample was loaded into a QIAquick spin column placed inside a 2-ml collection tube and centrifuged at 13,000 rpm (10,000 x g) for 1 min. The flow-through was then discarded and 0.5 ml of buffer QX1 and 0.75 ml of buffer PE were added successively. The amplified DNA product bound to the column was eluted with 30 μl of water. The amount of eluted DNA was estimated by comparing with a known amount of Lambda DNA HindIII digested fragments (New England Biolabs, Beverly, Massachusetts, USA) in an ethidium bromide-stained agarose gel.

Nucleotide sequence analysis was performed by using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer). Seven

oligonucleotide primers were employed in the analysis of dengue envelope gene (Table 5).

The cycle sequencing reaction was performed in a 20-µl volume of the mixture containing 8 µl of terminator ready reaction mix, 200 ng of amplified DNA product and 3.2 pmol of individual primer. After overlaying the mixture with one drop of light mineral oil, the cycle sequencing reaction was carried out in a DNA thermal cycler (Model 480, Perkin Elmer) for 25 cycles according to the following parameters: 94°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 min. The sample was kept at 4° C after completion and purified by ethanol precipitation. For loading the sample, the sample was resuspended in 25 µl of template suppression reagent and denatured by heating at 95°C for 2 min. The sample was then analyzed by using an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer, Perkin Elmer).