CHAPTER IV

RESULTS

Patient recruitment and specimen collections

Twenty-three diagnosed DENV-infected adult patients and five non-DENV-infected patients from our previous project "Survival of dengue virus in blood, urine, saliva and buccal mucosa in complete-recovery dengue patients" were included in this study. All patients were admitted to King Chulalongkorn Memorial Hospital during 29 April 2007 to 30 November 2007. For the former group, there were 12 males (age: 19-53 years old) and 11 females (age: 18-56 years old). For the latter group, there were 2 males (age: 23-35 years old) and 3 females (age: 33-65 years old). The day of illness in DENV-infected patients were 5-9 days in male and 4-9 days in female while in non-DENV-infected patients were 7-9 days in male and 6-15 days in female. Their clinical diagnoses were based on ELISA test and WHO criteria (2009) [40]. They were classified as DF (1), DHF grade I (6), DHF grade II (13), DHF grade III (2) and DSS (DHF grade IV) (1). According to 23 DENV-infected patients, there were 22 patients diagnosed as secondary DENV infections and 1 patient diagnosed as primary DENV infection according to the ELISA results (Appendix C) of the previous project by Laosakul *et al.* [30]. The summary of enrolled patients is presented in Table 20.

Table 20: The summary of both DENV and non-DENV infected patients in this study

No.	Code	Sex	Age (years old)	DOF	Clinical diagnosis	ELISA interpretation (type of infection)
DENV-infected patients						
1	N2	F	25	4	DHF II	secondary infection
2	N3	M	20	7	DHF II	secondary infection
3	N4	F	37	5	DHF II	secondary infection
4	N5	M	19	7	DHF II	secondary infection
5	N6	F	55	6	DHF I	secondary infection

No.	Code	Sex	Age (years old)	DOF	Clinical diagnosis	ELISA interpretation (type of infection)
6	N8	M	23	5	DHF I	secondary infection
7	N9	F	22	9	DHF II	secondary infection
8	N10	M	30	5	DHF II	secondary infection
9	N12	F	35	6	DHF II	secondary infection
10	N13	F	55	5	DHF I	secondary infection
11	N17	F	56	7	DHF I	secondary infection
12	N20	M	26	8	DHF I	secondary infection
13	N21	M	22	7	DF	secondary infection
14	N22	M	24	7	DHF II	secondary infection
15	N23	M	19	8	DHF I	secondary infection
16	N24	F	18	6	DSS	secondary infection
17	N28	M	26	8	DHF II	secondary infection
18	N29	F	48	8	DHF II	secondary infection
19	N30	M	53	9	DHF III	secondary infection
29	N33	M	28	6	DHF III	secondary infection
21	N34	M	20	6	DHF II	primary infection
22	N35	F	25	5	DHF II	secondary infection
23	N40	F	28	4	DHF II	secondary infection
Non-DENV-infected patients						
24	N16	M	35	7	Graves' disease	ND
25	N27	F	65	6	Unspecified viral infection	ND
26	N37	F	33	5	Influenza virus infection	ND
27	N39	M	23	9	Unspecified viral infection	ND
28	N43	F	41	15	Influenza virus infection	ND

DOF = duration of fever. M = male, F = female. ND = not determined.

Clinical diagnosis is based on WHO criteria 2009.

Unspecified viral infection refers to the patients coming with suspected viral infection but clinical and laboratory diagnoses were not DENV infection.

The ratio of IgM: IgG is used to distinguished either primary or secondary infection. IgM: IgG $\geq$ 1.8 is interpreted as primary DENV infection whereas the value $<$ 1.8 is interpreted as secondary infection.

Plasma, PBMCs, saliva and urine were taken from both groups of patients during acute, early convalescent and late convalescent periods. Some patients lacked all or partial febrile specimen collections. Therefore, the first specimen collections were in early convalescent period. In addition, some patients had more than one specimens collected during early or late convalescent period. However, the duration time between dates of these specimens was at least 7 days, enough to be included in this project. The total number of each specimen type in each time point (except for negative controls) is presented in Figure 12 and Appendix B.

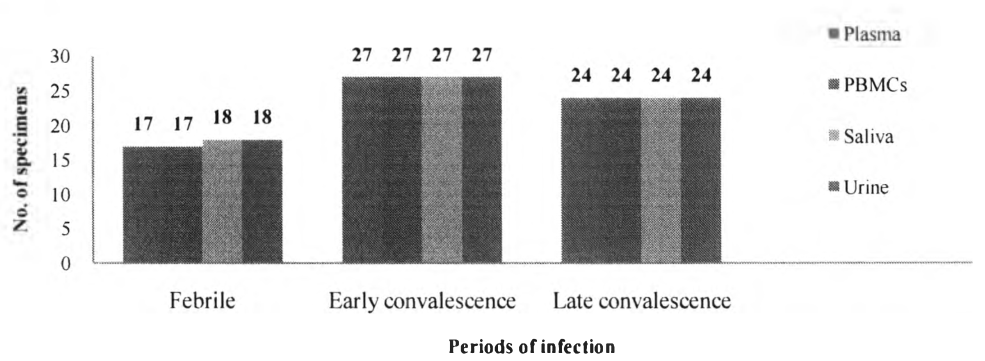


Figure 12: A number of specimens collected from 23 dengue-infected patients divided by each specimen type and period of infection (the result including double specimen collections).

Nested RT-PCR (E gene) result for DENV detection

Nested RT-PCR for E gene (partial sequence, 434 bp) was done in all specimens of 23 DENV infected patients to explore the longest day of DENV detection and generate the sequences for serotype and other molecular characterizations. Primers were taken from published article by Yenchitsomanus *et al.* and the PCR product spanned more than 95% of domain III of E gene reported as an important region for DENV pathogenesis [25, 56, 143].

All 23 DENV-infected patients were positive for nested RT-PCR (E gene primers). Dengue genome was presented in various specimen types and time points (Table 21, 22 and Figure 13). During the late convalescent period, only PBMCs and urine were positive by this method. The latest day of detectability was day 7 for plasma, day 8 for saliva, day 27 for PBMCs and day 46 for urine, respectively. Specimens from non-dengue-infected patients were all negative by nested RT-PCR using the E gene primers.

Table 21: Nested RT-PCR results of dengue and non-dengue infected patients in plasma, PBMCs, saliva and urine in different periods of infection (using E gene primers)

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N2	F	4	DHF II	3	+	+	+	+
				21	-	+	-	-
				90	-	-	-	-
N3	M	7	DHF II	7	+	+	-	-
				22	-	-	-	-
				75	-	-	-	-
N4	F	5	DHF II	5	-	+	-	-
				21	-	-	-	-
N5	M	7	DHF II	6	+	+	-	-
				23	-	-	-	+
N6	F	6	DHF I	4	-	+	-	-
				23	-	-	-	-
				80	-	-	-	-
N8	M	5	DHF I	7	+	+	+	+
				25	-	-	-	-
N9	F	9	DHF II	9	-	+	-	-
				15	-	-	-	-
				30	-	-	-	-
				90	-	-	-	-
N10	M	5	DHF II	4	+	+	-	+
				27	-	+	-	-
				90	-	-	-	-
N12	F	6	DHF II	4	+	+	-	-
				12	-	-	-	+
				26	-	-	-	+
				75	-	-	-	-
N13	F	5	DHF I	8	-	+	-	+
				15	-	-	-	+
				29	-	-	-	-
				64	-	-	-	-
N17	F	7	DHF I	7	+	+	+	+
				13	-	-	-	+
				33	-	-	-	-

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N20	M	8	DHF I	6	ND	ND	-	+
				30	-	-	-	+
N21	M	7	DF	7	-	+	-	-
				14	-	-	-	+
				24	-	-	-	-
N22	M	7	DHF II	4	-	+	-	-
				24	-	-	-	-
N23	M	8	DHF I	7	+	-	+	+
				17	-	-	-	-
				45	-	-	-	-
N24	F	6	DSS	3	+	+	+	+
				13	-	-	-	-
				32	-	-	-	-
				49	-	-	-	-
N28	M	8	DHF II	6	+	+	-	-
				14	-	-	-	+
				46	-	-	-	+
				90	-	-	-	-
N29	F	8	DHF II	5	+	+	-	+
				19	-	-	-	+
				33	-	-	-	-
N30	M	9	DHF III	7	+	+	-	+
				20	-	-	-	-
				38	-	-	-	-
N33	M	6	DHF III	7	+	+	+	+
				18	-	-	-	+
				31	-	-	-	-
N34	M	6	DHF II	8	-	+	+	+
				14	-	-	-	+
				28	-	-	-	-
N35	F	5	DHF II	7	-	-	-	-
				13	-	-	-	+
				27	-	-	-	-
N40	F	4	DHF II	4	+	+	-	+
				21	-	+	-	-

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N40	F	4	DHF II	71	-	-	-	-
N16	M	7	Graves' disease	6 13 34 64	- - - -	- - - -	- - - -	- - - -
N27	F	6	Unspecified viral infection	5 14 54	- - -	- - -	- - -	- - -
N37	F	5	Influenza virus infection	4 28	- -	- -	- -	- -
N39	M	9	Unspecified viral infection	7 15 33	- - -	- - -	- - -	- - -
N43	F	15	Influenza virus infection	4 23 33	- - -	- - -	- - -	- - -

+ = positive, - = negative and ND = not determined. M = male and F= female.

Definitions: acute period (duration of fever), early convalescence (first day of fever recovery until day 25 of illness) and late convalescence (day 26 – day 90 of illness).

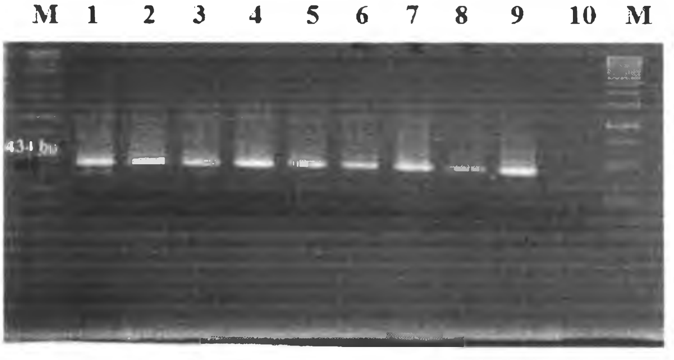


Figure 13: 1.5% agarose gel electrophoresis of nested RT-PCR product using E gene primers in specimens collected from dengue-infected patients in different time points. The expected product is 434 bp (shown in red arrow). M= 1 kb DNA marker, 1 = N5 PBMCs, 2 = N5/2 urine, 3 = N13 PBMCs, 4 = N13 urine, 5 = N13/2 urine, 6 = N12 PBMCs, 7 = N12/2 urine, 8 = N12/3 urine, 9 = positive control DENV4 and 10 = negative control (no template added).

Table 22: The summary of nested RT-PCR results (E gene primers) of plasma, PBMCs, saliva and urine collected from each patient at different time points. The results were divided by each specimen and time point.

	Plasma	PBMCs	Saliva	Urine
Febrile period	12/17 (70.59%)	16/17 (94.12)	4/18 (22.22%)	9/18 (50.00%)
Early convalescence	2/27 (7.41%)	5/27 (18.52%)	3/27 (11.11%)	15/27 (55.55%)
Late convalescence	0/24 (0.00%)	1/24 (4.16%)	0/24 (0.00%)	3/24 (12.50%)

Dengue serotypes, genotypes and strains were analyzed in all 23 patients but only 13 patients (13/23, 56.52%) with positive nested RT-PCR (in any specimen type) for at least 2 time points were continuously studied for sequential genetic variations.

Serotype classification in DENV-infected patients

To classify the serotype of DENV in all dengue-infected patients, semi-nested or nested RT-PCR using the primers of Lanciotti *el al.* and Yenchitsomanus *et al.* was used [56, 58]. Moreover, direct sequencing of partial E gene was also done to classify serotype of DENV in each patient by blasting with GenBank.

The result showed that 20 patients (20/23, 86.96%) were infected with single DENV serotype infections and 3 patients were infected with multi or mixed DENV serotypes. For single serotypic infection, more patients were infected with DENV2 (11/20, 55.00%) than with DENV1 (6/20, 30.00%) or DENV3 (3/20, 15.00%). No patient was infected with DENV4. Concurrent multi-serotype infections were found in 3 patients - N33, N34 and N40 (3/23, 13.04%) with DENV2+DENV4, DENV1+DENV3 and DENV1+DENV2, respectively (Figure 14 and Table 23). The mixed infections in 3 patients were found in different specimens and time points. In N33 patient, 1^{st} early convalescent plasma, PBMCs, saliva and 2^{nd} convalescent urine were DENV4 while 1^{st} convalescent urine was DENV2 + DENV4. In N34 patient, 1^{st} early convalescent PBMCs, saliva and urine were DENV1 but 2^{nd} early convalescent urine was DENV1 + DENV3. The febrile plasma, PBMCs and urine of N40 patient were DENV1 whereas early convalescent PBMCs was DENV1+DENV2 (Figure 15).

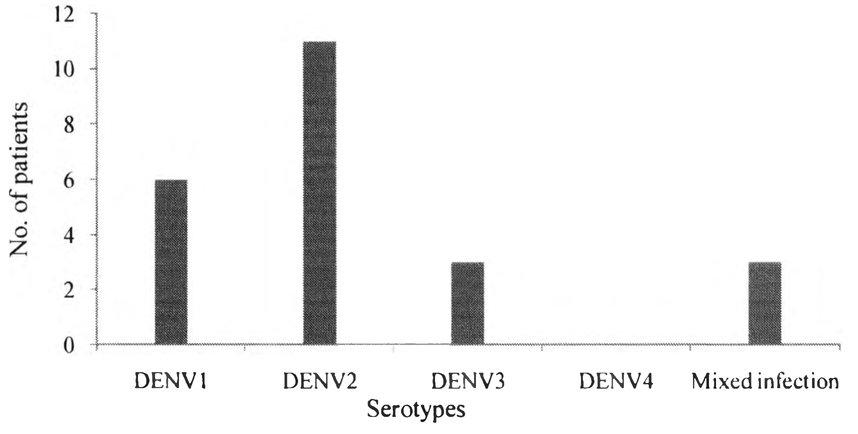


Figure 14: Serotype classification of 23 DENV-infected patients.

Table 23: DENV serotype results of 23 DENV-infected patients

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N2	F	4	DHF II	3	D2	D2	D2	D2
				21	-	D2	-	-
				90	-	-	-	-
N3	M	7	DHF II	7	D2	D2	D2	-
				22	-	-	-	-
				75	-	-	-	-
N4	F	5	DHF II	5	-	D2	-	-
				21	-	-	-	-
N5	M	7	DHF II	6	D2	D2	-	-
				23	-	-	-	D2
N6	F	6	DHF I	4	-	D2	-	-
				23	-	-	-	-
				80	-	-	-	-
N8	M	5	DHF I	7	D2	D2	D2	D2
				25	-	-	-	-
N9	F	9	DHF II	9	-	D1	-	-
				15	-	D1	-	-
				30	-	-	-	-
				90	-	-	-	-

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N10	M	5	DHF II	4	D2	D2	D2	D2
				27	-	D2	-	-
				90	-	-	-	-
N12	F	6	DHF II	4	D1	D1	-	-
				12	-	-	-	D1
				26	-	-	-	D1
				75	-	-	-	-
N13	F	5	DHF I	8	-	D2	-	D2
				15	-	-	-	D2
				29	-	-	-	-
				64	-	-	-	-
N17	F	7	DHF I	7	D2	D2	D2	D2
				13	-	-	-	D2
				33	-	-	-	-
N20	M	8	DHF I	6	ND	ND	-	D1
				30	-	-	-	D1
N21	M	7	DF	7	-	D2	-	-
				14	-	-	-	D2
				24	-	-	-	-
N22	M	7	DHF II	4	-	D2	-	-
				24	-	-	-	-
N23	M	8	DHF I	7	D3	-	D3	D3
				17	-	-	-	-
				45	-	-	-	-
N24	F	6	DSS	3	D3	D3	D3	D3
				13	-	-	-	-
				32	-	-	-	-
				49	-	-	-	-
N28	M	8	DHF II	6	D3	D3	-	-
				14	-	-	-	D3
				46	-	-	-	D3
				90	-	-	-	-
N29	F	8	DHF II	5	D1	D1	-	D1
				19	-	-	-	D1
				33	-	-	-	-

Code	Sex	DOF	Clinical Diagnosis	Days of collection	Plasma	PBMCs	Saliva	Urine
N30	M	9	DHF III	7	D1	D1	-	D1
				20	-	-	-	-
				38	-	-	-	-
N33	M	6	DHF III	7	D4	D4	D4	D4+D2
				18	-	-	-	D4
				31	-	-	-	-
N34	M	6	DHF II	8	-	D1	D1	D1
				14	-	-	-	D1+D3
				28	-	-	-	-
N35	F	5	DHF II	7	-	-	-	-
				13	-	-	-	D1
				27	-	-	-	-
N40	F	4	DHF II	4	D1	D1	-	D1
				21	-	D2+D1	-	-
				71	-	-	-	-

D1= DENV1; D2= DENV2; D3= DENV3; D4= DENV4. DOF= duration of fever.

"-"= negative and ND = not determined. M = male and F= female.

Definitions: acute period (duration of fever), early convalescence (first day of fever recovery until day 25 of illness) and late convalescence (day 26 – day 90 of illness).

All non-DENV infected specimens were negative for all RT-PCR for serotype classification (data not shown).

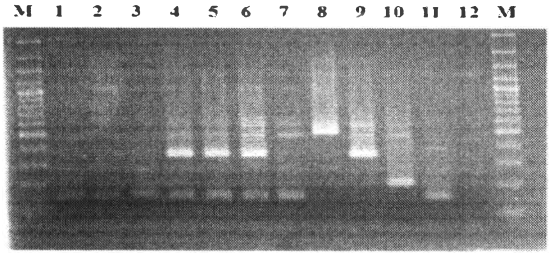


Figure 15: Serotype nested RT-PCR results using Yenchitsomanus protocol in N33 specimens. Lane 1 is N33 plasma. Lane 2 is N33 PBMCs, Lane 3 is N33 saliva. Lanes 4 & 5 are N33 urine. Lane 6 is new extraction of N33 urine. Lane 7 is N33/2 (day18 of illness) urine. Positive controls (DENV1-DENV4) are presented in lane 8-11, respectively. Lane 12 is negative control (DW). M = 100 bp (plus) DNA marker.

To find out which serotype represented major clones in each multi-serotype or mixed-serotype specimen, PCR products (E gene) from those specimens in 3 mixed serotype infected patients were taken to do cloning and sequencing techniques. Ten to fifteen colonies from each experiment were picked for sequencing. Clones representing each serotype in each specimen were counted. The results revealed that all single-serotype-infected specimens in these 3 patients showed uniform and identical serotype in all relevant clones. Three mixed serotype infected specimens (1st early convalescent urine of N33, 2nd convalescent urine of N34 and early convalescent PBMCs of N40) presented the heterogeneous clones containing 2 serotypes. The major serotype in 1st early convalescent urine (N33) was DENV4. In 2nd convalescent urine of N34, the major serotype was DENV3 and the major serotype of early convalescent PBMCs of N40 was DENV2 (Table 24).

Table 24: The major and minor serotypes of DENV in 3 mixed-serotype-infected patients

Code	DOF	Specimens	No. of clones	Serotypes	No. of major clones (serotype)	No. of minor clones (serotype)
N33	6	plasma (7)	14	4	14 (DENV4)	0
		PBMCs (7)	11	4	11(DENV4)	0
		saliva (7)	12	4	12 (DENV4)	0
		urine (7)	15	4+2	11 (DENV4)	4 (DENV2)
		urine (18)	13	4	13 (DENV4)	0
N34	6	PBMCs (8)	14	1	14(DENV1)	0
		saliva (8)	13	1	13 (DENV1)	0
		urine (8)	13	1	13 (DENV1)	0
		urine (14)	16	1+3	14 (DENV3)	2 (DENV1)
N40	4	plasma (4)	12	1	12 (DENV1)	0
		PBMCs (4)	15	1	15 (DENV1)	0
		urine (4)	12	1	12 (DENV1)	0
		PBMCs (21)	13	1+2	12 (DENV2)	1 (DENV1)

DOF = duration of fever. The number in "( )" represents the day of specimen collections.

Genotype and strain classifications of DENV in DENV-infected patients

E gene sequences (388 bp except primers) from positive specimens of 23 patients were taken to explore genotypes and strains by blasting each sequence with the Dengue database in the Viral Bioinformatics Resource Center (VBRC) and GenBank, respectively. In mixed-serotype-infected specimens, both major and minor serotypes were also investigated.

The genotype of all DENV1-infected patients were classified in genotype I. DENV2-infected patients were clustered into 2 different genotypes, Asian I (all patients, excluding N13) and cosmopolitan (N13 patient) whereas all DENV3-infected patients were classified in genotype II. DENV4 in mixed-serotype-infected patient was classified in genotype I (Table 25 and Figure 16). No mixed genotype infection was found in all single-serotype-infected patients.

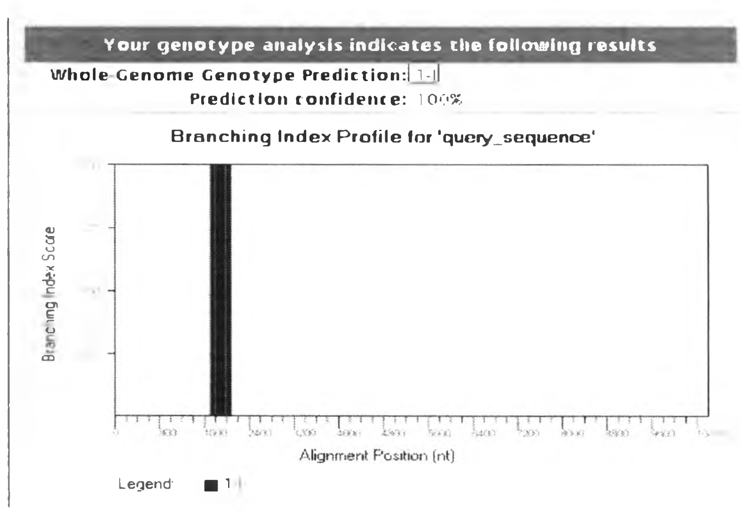
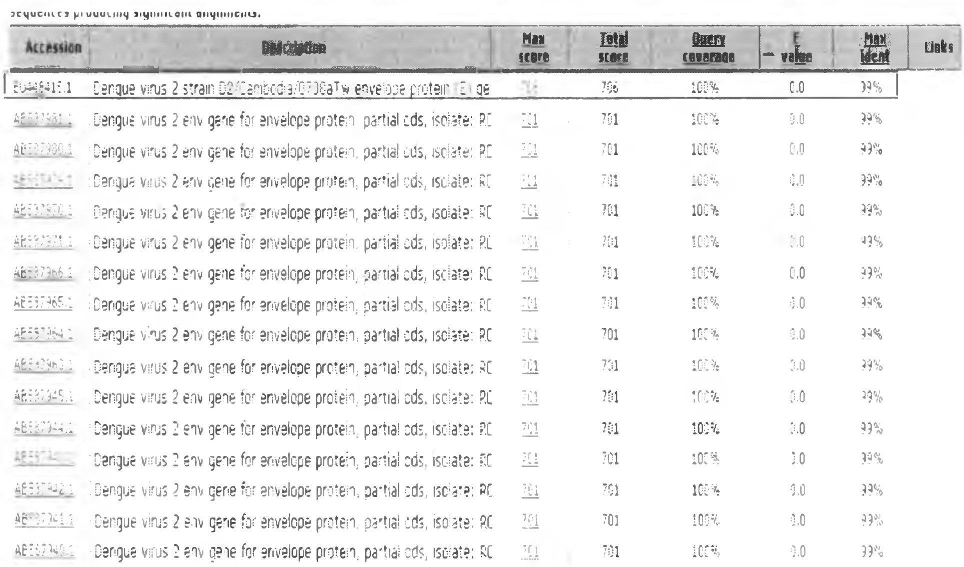


Figure 16: The example of genotype blast result of N12 specimens during febrile (N12 plasma and PBMCs), early convalescent (N12/2 urine) and late convalescent (N12/2 urine) periods. All specimens gave the same results of genotype I as marked in the red box. The result "1-I" means "serotype 1 genotype I".

In this study, there were 6 strains of DENV1 (ThD1_K0107_98, 00132/09, D1.Myanmar.059/01, 02128/07, 16007 and ThD1_0049_01), 6 strains of DENV2 (D2/Thailand/0606aTw, 16681, ThD2_0981_00, D2/Cambodia/0708aTw, D2/Singapore/0806aTw and D2/Vietnam/0804bTw), 3 strains of DENV3

(D3/Myanmar/0810aTw, Thai0308a/Tw and D3/Myanmar/0707aTw) and 1 strain of DENV4 (TN2511) circulating in our patients. The single strain infection was found in all patients with single serotype infection, excluding 2 patients (N2 and N10) presenting mixed strains of DENV2 infection. Moreover, multi strain infections of DENV1 were found in mixed-serotype-infected specimen of N34 and N40. There were strains ThD1_0049_01 and 16007 in N34 patient while strains 02128/07 and ThD1_K0107_98 were found in N40 patient (Table 25 and Figure 17).


Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
[illegible]	Dengue virus 2 strain D2/Cambodia/0708aTw envelope protein (E) ge	[illegible]	706	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	
[illegible]	Dengue virus 2 env gene for envelope protein, partial cds, isolate: RC	701	701	100%	0.0	99%	

Figure 17: The example of DENV strain analysis of N17 specimens during febrile (N17 plasma, PBMCs, saliva and urine) and early convalescent (N17/2 urine) periods. All specimens presented the same DENV strains. The blast result is noted in the red box.

Table 25: Summary results of serotypes, genotypes and strains of DENV in 23 DENV-infected patients

Code	Sex	Clinical diagnosis	Serotypes	Genotypes	Similar strains
N2	F	DHF II	2	Asian I	ThD2_0981_00 D2/Vietnam/0804bTw *
N3	M	DHF II	2	Asian I	ThD2_0981_00
N4	F	DHF II	2	Asian I	ThD2_0981_00
N5	M	DHF II	2	Asian I	ThD2_0981_00
N6	F	DHF I	2	Asian I	ThD2_0981_00
N8	M	DHF I	2	Asian I	ThD2_0981_00
N9	F	DHF II	1	I	ThD1_K0107_98

Code	Sex	Clinical diagnosis	Serotypes	Genotypes	Similar strains
N10	M	DHF II	2	Asian I	ThD2_0981_00 D2/Thailand/0606aTw*
N12	F	DHF II	1	I	ThD1_K0107_98
N13	F	DHF I	2	Cosmopolitan	D2/Singapore/0806aTw
N17	F	DHF I	2	Asian I	D2/Cambodia/0708aTw
N20	M	DHF I	1	I	00132/09
N21	M	DF	2	Asian I	ThD2_0981_00
N22	M	DHF II	1	I	D1.Myanmar.059/01
N23	M	DHF I	3	II	D3/Myanmar/0810aTw
N24	F	DSS	3	II	Thai0308a/Tw
N28	M	DHF II	3	II	D3/Myanmar/0707aTw
N29	F	DHF II	1	I	02128/07
N30	M	DHF III	1	I	02128/07
N33	M	DHF III	4 (major) 2 (minor)	I Asian I	TN2511 16681
N34	M	DHF II	1 (major) 3 (major)$ 1 (minor)$	I II I	ThD1_0049_01 ThD3_0140_84 16007
N35	F	DHF II	1	I	02128/07
N40	F	DHF II	1 (major) 2 (major)# 1 (major)#	I Asian I I	02128/07 D2/Thailand/0606aTw ThD1_K0107_98

M = male and F = female. * = different strains during convalescent period.

Major = major serotype in each specimen and the sequence was retrieved from direct sequencing.

Minor = minor serotype in each specimen and the sequence was retrieved after doing cloning and sequencing techniques.

The consensus sequence of minor serotype is generated from overall sequences of selected colonies on the plate of mixed-serotype-contained specimen.

$N34 urine (14) shows DENV3 as a major serotype and DENV1 as a minor serotype.

#N40 PBMCs (21) shows DENV2 as a major serotype and DENV1 as a minor serotype.

To explore serotypes, genotypes and strains of DENV in different time points of each patient, only 13 DENV-infected patients positive for nested RT-PCR (E gene) at least 2 time points were investigated. The results presented that serotypes, genotypes and strains of single-serotype-infected patients in different specimens and time points were the same except 2 patients (N2 and N10). In N2 and N10 patients, serotypes and genotypes in different specimens and time points were identical but the difference of DENV strains were found during early and convalescent PBMCs in both 2 patients (Table 26). In 3 mixed-serotype-infected patients, different serotypes, genotypes and strains were definitely found. Moreover, mixed strains of DENV1 were found in N34 and N40 patients although the genotype of 2 patients was the identical.

Table 26: Serotypes, genotypes and strains of DENV in 13 prolonged dengue-infected patients

Code	Specimens	Serotype	genotype	Similar strain
N2	plasma (3)	2	Asian I	ThD2_0981_00
DOF=4	PBMCs (3)	2	Asian I	ThD2_0981_00
	saliva (3)	2	Asian I	ThD2_0981_00
	urine (3)	2	Asian I	ThD2_0981_00
	PBMCs (21)	2	Asian I	D2/Vietnam/0804bTw
N5	plasma (6)	2	Asian I	ThD2_0981_00
DOF=7	PBMCs (6)	2	Asian I	ThD2_0981_00
	urine (23)	2	Asian I	ThD2_0981_00
N10	plasma (4)	2	Asian I	ThD2_0981_00
DOF=5	PBMCs (4)	2	Asian I	ThD2_0981_00
	urine (4)	2	Asian I	ThD2_0981_00
	PBMCs (27)	2	Asian I	D2/Thailand/0606aTw
N12	plasma (4)	1	I	ThD1_K0107_98
DOF=6	PBMCs (4)	1	I	ThD1_K0107_98
	urine (12)	1	I	ThD1_K0107_98
	urine (26)	1	I	ThD1_K0107_98
N13	PBMCs (8)	2	Cosmopolitan	D2/Singapore/0806aTw
DOF=5	urine (8)	2	Cosmopolitan	D2/Singapore/0806aTw
	urine (15)	2	Cosmopolitan	D2/Singapore/0806aTw
N17	plasma (7)	2	Asian I	D2/Cambodia/0708aTw
DOF=7	PBMCs (7)	2	Asian I	D2/Cambodia/0708aTw
	saliva (7)	2	Asian I	D2/Cambodia/0708aTw
	urine (7)	2	Asian I	D2/Cambodia/0708aTw
	urine (13)	2	Asian I	D2/Cambodia/0708aTw
N20	urine (6)	1	I	00132/09
DOF=8	urine (30)	1	I	00132/09

Code	Specimens	Serotype	genotype	Similar strain
N21	PBMCs (7)	2	Asian I	ThD2_0981_00
DOF=7	urine (14)	2	Asian I	ThD2_0981_00
N28	plasma (6)	3	II	D3/Myanmar/0707aTw
DOF=8	PBMCs (6)	3	II	D3/Myanmar/0707aTw
	urine (14)	3	II	D3/Myanmar/0707aTw
	urine (46)	3	II	D3/Myanmar/0707aTw
N29	plasma (5)	1	I	02128/07
DOF=8	PBMCs (5)	1	I	02128/07
	urine (5)	1	I	02128/07
	urine (19)	1	I	02128/07
N33	plasma (7)	4	I	TN2511
DOF=6	PBMCs (7)	4	I	TN2511
	saliva (7)	4	I	TN2511
	urine (7)	4	I	TN2511
	urine (18)	4 (major)	I	TN2511
		2 (minor)	Asian I	16681
N34	PBMCs (8)	1	I	ThD1_0049_01
DOF=6	saliva (8)	1	I	ThD1_0049_01
	urine (8)	1	I	ThD1_0049_01
	urine (14)	3 (major)	II	ThD3_0140_84
		1 (minor)	I	16007
N40	plasma (4)	1	I	02128/07
DOF=4	PBMCs (4)	1	I	02128/07
	urine (4)	1	I	02128/07
	PBMCs (21)	2 (major)	Asian I	D2/Thailand/0606aTw
		1 (minor)	I	ThD1_K0107_98

Definitions: febrile period (duration of fever), early convalescence (first day of fever recovery until day 25 of illness) and late convalescence (day 26 – day 90 of illness).

DOF = duration of fever. The number in "( )" indicates the day of specimen collections.

Major = a major serotype in each specimen and the sequence was retrieved from direct sequencing.

Minor = a minor serotype in each specimen and the sequence was retrieved after doing cloning and sequencing techniques.

DENV detection and viral load in different time points of infection

Real time SYBR Green I RT-PCR (qRT-PCR) was used to detect, monitor and explore the viral load of DENV in plasma, PBMCs, saliva and urine in different time points of 23 patients. The protocol and primers were adapted from the previous study of dos Santos *et al.* [61]. Primers were designed to detect all 4 serotypes of DENV at 5' UTR region. Stock DENV of 4 serotypes were firstly used as positive controls and for construction the standard curve for viral quantification. All positive controls were firstly done to validate the limit of detection and melting temperature (Tm) for the expected PCR product. The results showed that melting temperature of expected PCR products of 4 serotypes was in the range of 80.70°C - 81.86 °C (Figure 18). The limits of detection in this study were 5 x 10^{-3} PFU/ml (DENV1), 4.75 x 10^{-2} PFU/ml (DENV2), 2.75 x 10^{-3} PFU/ml (DENV3) and 2.5 x 10^{-3} PFU/ml (DENV4), respectively (data not shown).

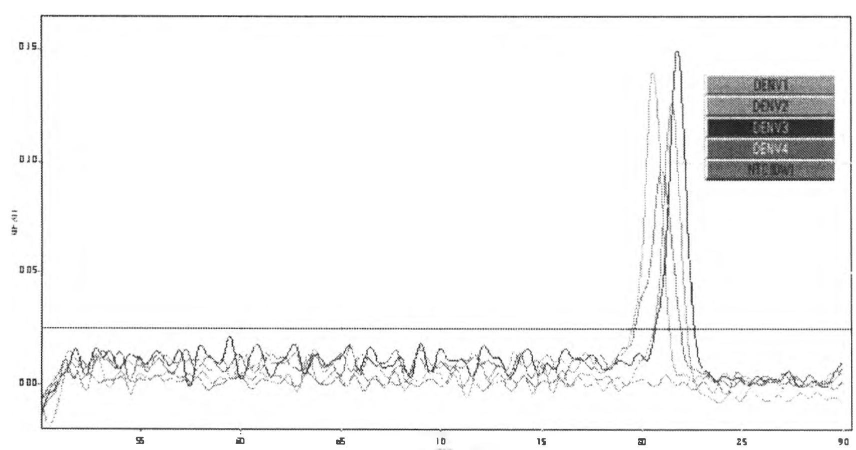
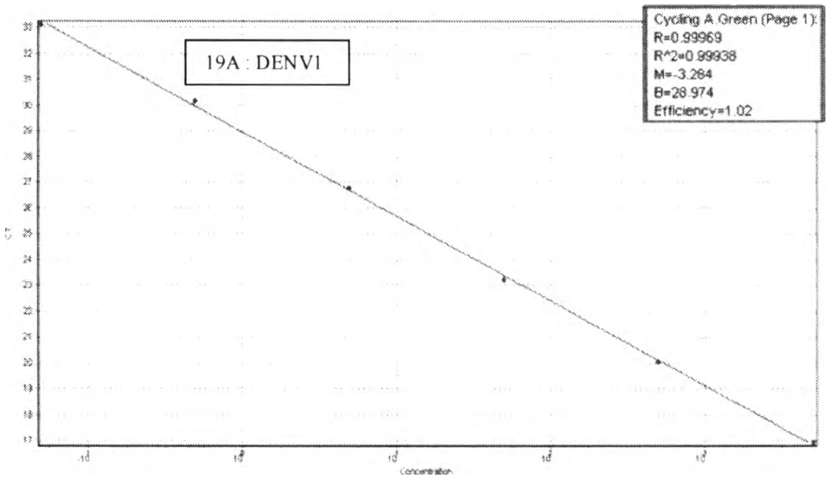
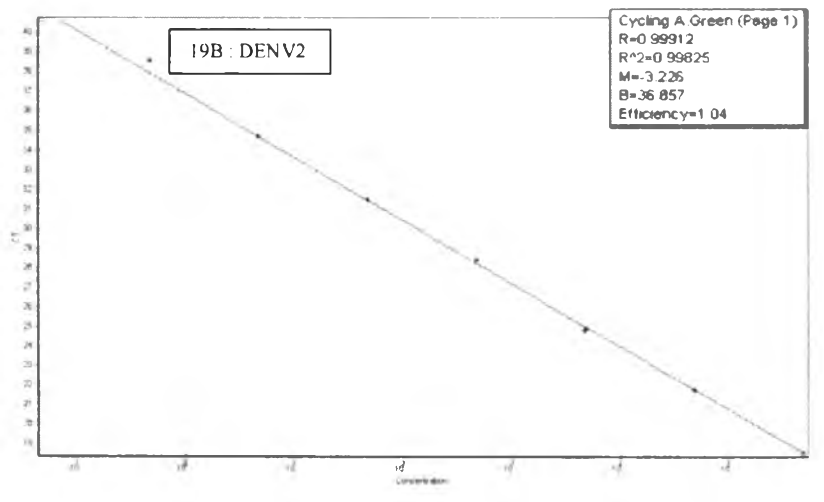
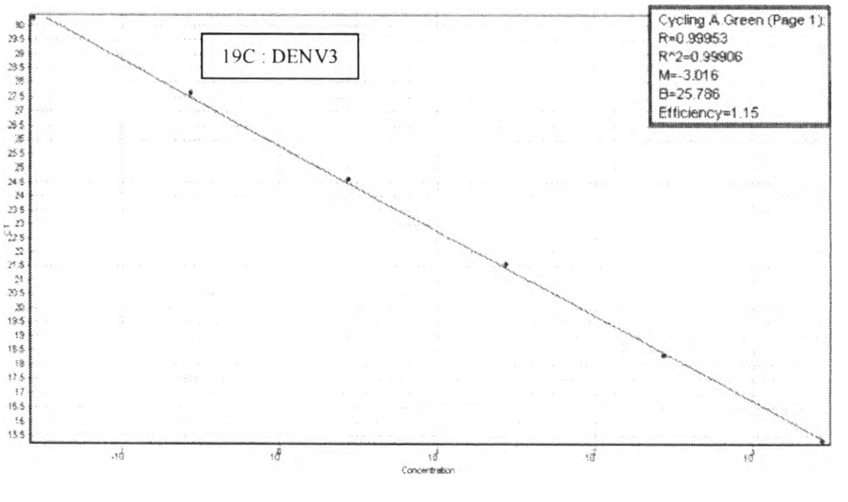


Figure 18: The melting curve analysis of positive controls (DENV1-DENV4).

To construct the standard curve for determining dengue viral load in clinical specimens, stock DENV1 was diluted from 5 x 10^{3} PFU/ml to 5 x 10^{-2} PFU/ml, DENV2 from 4.75 x 10^{5} PFU/ml to 4.75 x 10^{-2} PFU/ml, DENV3 from 2.75 x 10^{3} PFU/ml to 2.75 x 10^{-2} PFU/ml and DENV4 from 2.5 x 10^{4} PFU/ml to 2.5 x 10^{-3} PFU/ml. The standard curve of all 4 serotypes is presented in Figure 19.


19A : DENV1
Cycling A.Green (Page 1):
R=0.99969
R^2=0.99938
M=-3.284
B=28.974
Efficiency=1.02
Concentration


19B : DENV2
Cycling A.Green (Page 1)
R=0.99912
R^2=0.99825
M=-3.226
B=36.857
Efficiency=1.04
Concentration


19C : DENV3
Cycling A.Green (Page 1):
R=0.99953
R^2=0.99906
M=-3.016
B=25.786
Efficiency=1.15
Concentration

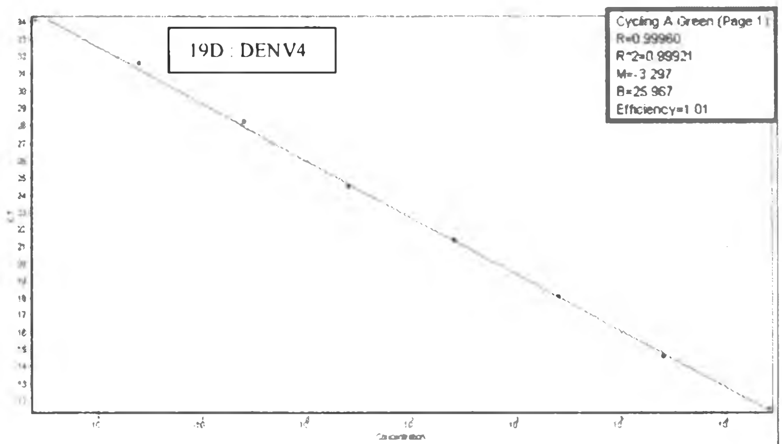


Figure 19: The standard curve of all 4 serotypes using DENV stocks (Figure 19A-19D).

The qRT-PCR was continuously used (after validation of the assay) to detect and quantification of DENV in plasma, PBMCs, saliva and urine of all patients during febrile, early convalescent and late convalescent periods. Starting templates for standard viral concentration and specimens were equal as 4 μl per reaction (25 μl). Results were presented in a cycle threshold (Ct), a melting curve analysis (Tm) and a viral load (PFU/ml). If the Ct of some specimens were out of the length of standard curve, viral RNA was either diluted and repeated or reported as "less than the last point of standard concentration" (PFU/ml). In mixed serotype infections, standard curve was based on the major serotype of infected specimens.

DENV genome could be detected in all DENV-infected patients. The positive results varied in each specimen and time point similar to nested RT-PCR results (Table 27, Figure 20 and 21). Plasma, PBMCs, saliva or urine was positive during febrile and early convalescent periods. However, the positive results were only found in PBMCs and urine during late convalescent period (Table 28). All non-dengue-infected patients (negative control group) were completely negative for qRT-PCR.

After determining the longest time of DENV detection by qRT-PCR, the result showed that 17 of 23 (73.92%) DENV-infected patients were positive for DENV detection at least 2 times points of specimen collections. DENV genome could be detected as late as day 8 of illness in saliva, day 21 of illness in PBMCs, day 22 of illness in plasma and day 46 of illness in urine, respectively.

Table 27: qRT-PCR results of 23 DENV-infected patients

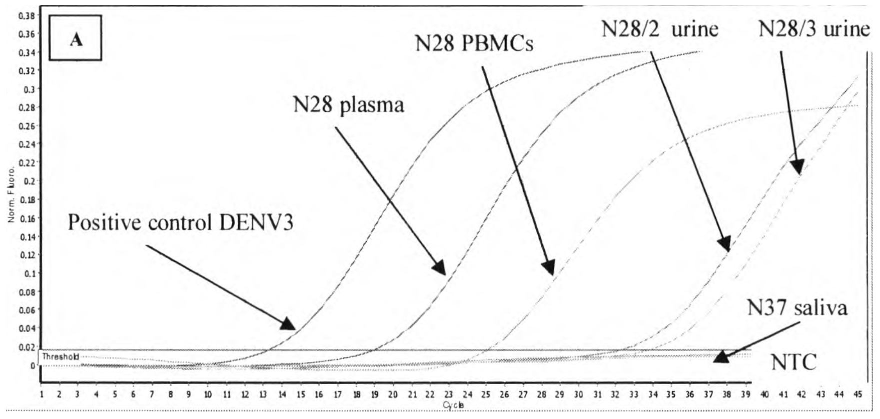
Code	Sex	DOF	Clinical Diagnosis	Day of collection	Plasma	PBMCs	Saliva	Urine
N2	F	4	DHF II	3	+	+	+	+
				21	-	+	-	-
				90	-	-	-	-
N3	M	7	DHF II	7	+	+	+	+
				22	+	-	-	+
				75	-	-	-	-
N4	F	5	DHF II	5	+	+	+	+
				21	-	-	-	-
N5	M	7	DHF II	6	+	+	+	+
				23	-	-	-	+
N6	F	6	DHF I	4	+	+	-	-
				23	-	-	-	-
				80	-	-	-	-
N8	M	5	DHF I	7	+	+	+	+
				25	-	-	-	+
N9	F	9	DHF II	9	-	+	-	-
				15	-	+	-	-
				30	-	-	-	-
				90	-	-	-	-
N10	M	5	DHF II	4	+	+	+	+
				27	-	-	-	-
				90	-	-	-	-
N12	F	6	DHF II	4	+	+	-	+
				12	-	-	-	+
				26	-	-	-	+
				75	-	-	-	-
N13	F	5	DHF I	8	-	+	-	+
				15	-	-	-	+
				29	-	-	-	-
				64	-	-	-	-
N17	F	7	DHF I	7	+	+	+	+

Code	Sex	DOF	Clinical Diagnosis	Day of collection	Plasma	PBMCs	Saliva	Urine
N17	F	7	DHF I	13	-	-	-	+
				33	-	-	-	-
N20	M	8	DHF I	6	ND	ND	+	+
				30	-	-	-	+
N21	M	7	DF	7	-	+	-	-
				14	-	-	-	+
				24	-	-	-	-
N22	M	7	DHF II	4	+	+	+	+
				24	-	-	-	+
N23	M	8	DHF I	7	+	-	-	+
				17	-	-	-	-
				45	-	-	-	-
N24	F	6	DSS	3	+	+	+	+
				13	-	-	-	-
				32	-	-	-	-
				49	-	-	-	-
N28	M	8	DHF II	6	+	+	-	-
				14	-	-	-	+
				46	-	-	-	+
				90	-	-	-	-
N29	F	8	DHF II	5	+	+	-	+
				19	-	-	-	+
				33	-	-	-	+
N30	M	9	DHF III	7	+	+	-	+
				20	-	-	-	-
				38	-	-	-	-
N33	M	6	DHF III	7	+	+	+	+
				18	+	-	-	+
				31	-	-	-	-
N34	M	6	DHF II	8	+	+	+	+
				14	-	-	-	+
				28	-	-	-	-
N35	F	5	DHF II	7	+	+	+	+
				13	-	-	-	-
				27	-	-	-	-

Code	Sex	DOF	Clinical Diagnosis	Day of collection	Plasma	PBMCs	Saliva	Urine
N40	F	4	DHF II	4	+	+	-	+
				21	-	+	-	-
				71	-	-	-	-
N16	M	7	Graves' disease	6	-	-	-	-
				13	-	-	-	-
				34	-	-	-	-
				64	-	-	-	-
N27	F	6	Unspecified viral infection	5	-	-	-	-
				14	-	-	-	-
				54	-	-	-	-
N37	F	5	Influenza virus infection	4	-	-	-	-
				28	-	-	-	-
N39	M	9	Unspecified viral infection	7	-	-	-	-
				15	-	-	-	-
				33	-	-	-	-
N43	F	15	Influenza virus infection	4	-	-	-	-
				23	-	-	-	-
				33	-	-	-	-

M = male and F= female. + = positive, - = negative and ND = not determined. DOF = duration of fever

Definitions: febrile period (duration of fever), early convalescence (first day of fever recovery until day 25 of illness) and late convalescence (day 26 – day 90 of illness).



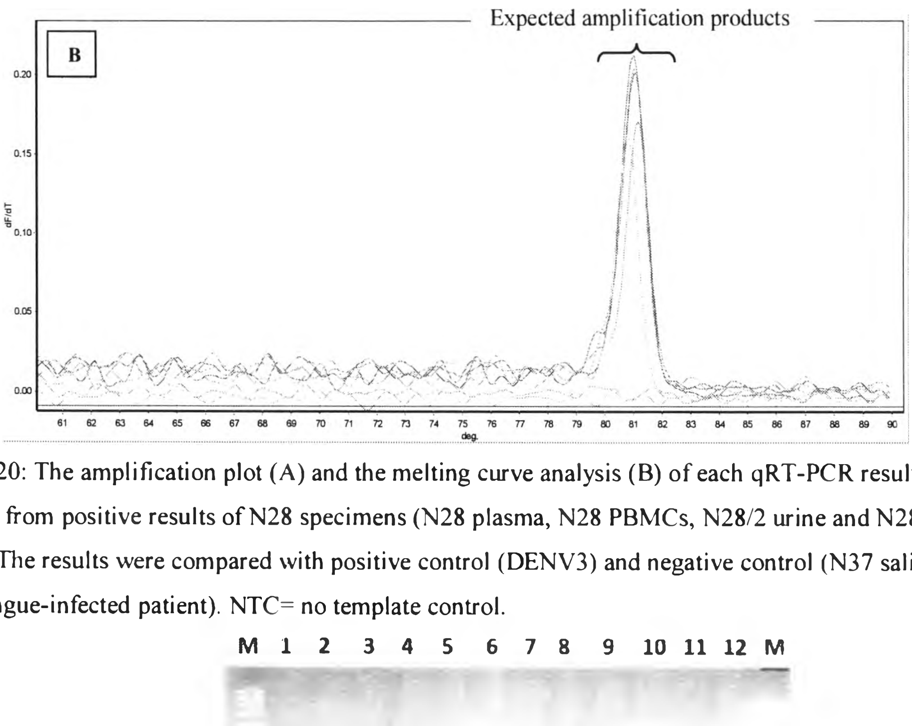


Figure 20: The amplification plot (A) and the melting curve analysis (B) of each qRT-PCR result derived from positive results of N28 specimens (N28 plasma, N28 PBMCs, N28/2 urine and N28/3 urine). The results were compared with positive control (DENV3) and negative control (N37 saliva: non dengue-infected patient). NTC= no template control.

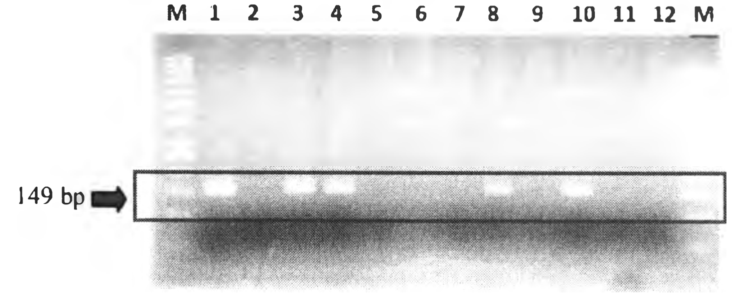


Figure 21: 2% gel electrophoresis of qRT-PCR products of N28 specimens. The expected band is noted as the red box. M= 50 bp DNA marker, 1= positive control DENV3 (2.75 x 10^4 PFU/ml), 2= positive control DENV3 (2.75 x 10^{-3} PFU/ml), 3= N28 plasma, 4= N28 PBMCs, 5= N28 urine, 6= N28 saliva, 7= N28/2 PBMCs, 8= N28/2 urine, 9= N28/3 plasma, 10= N28/3 urine, 11= NTC (no template control) and 12 = N37 saliva (negative control, non dengue-infected patient).

Table 28: qRT-PCR results of DENV-infected patients

(The results are presented in each specimen and time point)

	Plasma	PBMCs	Saliva	Urine
Febrile period	15/17 (88.23%)	16/17 (94.12%)	9/18 (50.00%)	14/18 (77.78%)
Early convalescence	7/27 (25.92%)	8/27 (29.63%)	4/27 (14.81%)	19/27 (70.37%)
Late convalescence	0/24 (0.00%)	0/24 (0.00%)	0/24 (0.00%)	4/24 (16.67%)

The viral load of each patient varied in different specimens and time points (Table 29 and Figure 22). In some specimens, the viral load was reported "less than the last standard point of standard concentration" such as < 0.05 PFU/ml in febrile PBMCs of N12 (DENV1) because the Ct was out of the last point of DENV1 standard curve (0.05 PFU/ml). During febrile period, dengue viral load in plasma or PBMCs was mostly higher than in saliva and urine, except 2 patients showing the high dengue viral load in urine (N4, N17). The high viral load was found in urine instead of plasma and PBMCs in early convalescent urine of some patient (N12, N13, and N35). In late convalescent period, DENV was detected in urine only and the viral load was lower than all specimens during febrile and early convalescent periods. In two patients (N9 and N23), the different viral load in all specimens could not be investigated because the Ct values were out of the range of standard curve. Therefore, the comparison of viral load among different time points was not explored.

The viral load in different specimens and time points of each patient depended on the day of specimen collections (Table 29). If the first specimens were collected during febrile period, viral load was higher in plasma or PBMCs than in saliva and urine as well as than in all convalescent specimens such as in N2 patient (Figure 22A). In addition, when all convalescent specimens were compared such as in N13 and N35, the high viral load was mostly found in the first urine during that period. Moreover, although the high viral load was found in plasma or PBMCs during febrile period, the viral load in convalescent specimen of some patients was higher than all febrile specimens such as in N12 patient. The high viral load in convalescent specimen was stable in a short period of time, then the viral load decreased until undetectable when monitoring for a long period (Figure 22B). In other cases, if the first specimens were collected at the lasted day of fever or early convalescent period, the viral load was higher in urine than in all specimens during the first collection. Subsequently, the viral load in secondly collected specimens during convalescent period became decreased and lower than first specimen collections such as in N17 patient (Figure 22C). The comparison of viral load in N9 patient between 2 time points could not be investigated because the values were out of the range of standard curve. Nevertheless, if the results were monitored by the Ct values, the viral load early convalescent PBMCs was higher than in febrile PBMCs.

Table 29: The demonstration of dengue viral load in different specimens and time points (All specimens were started at the same volume of 4 μl RNA)

Code	DOF	Positive specimen (day of collection)	Serotype	Tm (°C)	Ct	Viral load (PFU/ml)
N2	4	plasma (3)	2	79.90	21.99	46,200
		PBMCs (3)	2	79.90	24.53	7,310
		saliva (3)	2	80.10	31.50	45.70
		urine (3)	2	80.06	36.82	0.93
		plasma (21)	ND	80.10	38.65	0.24
		PBMCs (21)	2	80.30	40.52	0.06
		urine (21)	ND	80.70	38.80	0.21
N3	7	plasma (7)	2	79.94	34.64	1.07
		PBMCs (7)	2	79.94	37.06	0.216
		saliva (7)	2	80.00	36.52	0.311
		urine (7)	ND	79.86	37.41	0.17
		plasma (22)	ND	79.90	39.08	0.0555
		urine (22)	ND	79.86	36.20	0.386
N4	5	plasma (5)	ND	79.84	37.07	0.215
		PBMCs (5)	2	79.86	36.20	1.91
		saliva (5)	ND	80.14	36.94	0.235
		urine (5)	ND	79.96	36.63	0.288
N5	7	plasma (6)	2	79.90	21.99	2.64
		PBMCs (6)	2	79.92	29.37	22.60

Code	DOF	Positive specimen (day of collection)	Serotype	Tm (°C)	Ct	Viral load (PFU/ml)
N5	7	saliva (6)	ND	80.37	35.00	0.62
		urine (6)	ND	80.30	37.13	0.16
		urine (23)	2	80.62	26.85	113.50
N6	6	plasma (4)	2	80.20	35.83	0.496
		PBMCs (4)	2	80.34	34.27	1.41
N8	5	plasma (7)	ND	80.04	28.25	1.80
		PBMCs (7)	2	79.90	28.34	1.69
		saliva (7)	ND	79.80	28.69	1.30
		urine (7)	ND	80.00	22.47	122
		urine (25)	ND	80.20	32.53	0.0795
N9	9	PBMCs (9)	1	80.40	35.58	< 0.05
		PBMCs (15)	1	80.20	32.53	< 0.05
N10	5	plasma (4)	2	80.00	8.10	2,130,000
		PBMCs (4)	2	79.98	16.04	34,900
		saliva (4)	ND	80.47	30.15	23.45
		urine (4)	2	80.25	28.30	61.05
N12	6	plasma (4)	1	80.26	29.37	0.052
		PBMCs (4)	1	80.20	31.15	< 0.05
		urine (4)	ND	80.10	34.19	< 0.05
		urine (12)	1	80.30	22.16	7.11
		urine (26)	1	80.56	27.80	0.152
N13	5	PBMCs (8)	2	78.88	34.82	1.71
		urine (8)	2	78.88	28.37	95.85
		urine (15)	2	79.55	35.90	0.86
N17	7	plasma (7)	2	80.25	40.79 (diluted 1:10)	15.50
		PBMCs (7)	2	79.92	34.77	38.75
		saliva (7)	2	80.02	37.81	7.64
		urine (7)	2	80.02	28.37	1,160
		urine (13)	2	80.47	30.15	449
N20	8	saliva (6)	ND	81.14	29.35	<0.05
		urine (6)	1	80.30	26.35	0.301
		urine (30)	1	80.44	29.97	< 0.05
N21	7	PBMCs (7)	2	80.54	32.13	0.342
		urine (14)	2	80.84	25.05	42.80

Code	DOF	Positive specimen (day of collection)	Serotype	Tm (°C)	Ct	Viral load (PFU/ml)
N22	7	plasma (4)	ND	80.84	30.65	0.313
		PBMCs (4)	1	80.86	28.37	1.65
		saliva (4)	ND	80.60	27.71	2.68
		urine (4)	ND	80.94	31.62	0.155
		urine (24)	ND	80.64	30.29	0.405
N23	8	plasma (7)	3	81.00	39.53	< 0.0275
		urine (7)	3	80.80	39.16	< 0.0275
N24	6	plasma (3)	3	80.76	22.24 (diluted 1:2)	786
		PBMCs (3)	3	80.66	22.74 (diluted 1:2)	134
		saliva (3)	3	80.94	36.51	< 0.0275
		urine (3)	3	80.46	43.67	< 0.0275
N28	8	plasma (6)	3	81.00	18.97	267
		PBMCs (6)	3	81.04	25.10	2.41
		urine (14)	3	81.16	32.28	< 0.0275
		urine (46)	3	80.80	33.78	< 0.0275
N29	8	plasma (5)	1	80.70	18.18 (diluted 1:2)	3,580
		PBMCs (5)	1	80.80	21.44	197
		urine (5)	1	80.80	31.30	0.248
		urine (19)	1	81.16	30.39	0.461
		urine (33)	ND	81.24	38.07	< 0.05
N30	9	plasma (7)	1	80.56	21.17	61.4
		PBMCs (7)	1	80.60	24.79	4.92
		urine (7)	1	80.60	37.67	< 0.05
N33	6	plasma (7)	4	80.54	30.17	0.0533
		PBMCs (7)	4	80.54	31.42	0.0222
		saliva (7)	4	80.90	33.79	0.00425
		urine (7)	4+2	80.54	29.64	0.077
		plasma (18)	ND	80.60	33.77	0.00429
		urine (18)	4	80.44	28.25	0.203
N34	6	plasma (8)	ND	80.50	35.62	< 0.05
		PBMCs (8)	1	80.10	36.05	< 0.05
		saliva (8)	1	80.66	32.79	0.0908
		urine (8)	1	80.46	30.16	0.537

Code	DOF	Positive specimen (day of collection)	Serotype	Tm (°C)	Ct	Viral load (PFU/ml)
N34	6	urine (14)	1+**3**	81.06	31.10	< 0.0275
N35	5	plasma (7)	ND	80.90	33.17	< 0.05
		PBMCs (7)	ND	80.90	32.92	< 0.05
		saliva (7)	ND	80.70	34.40	< 0.05
		urine (7)	ND	80.80	32.99	< 0.05
		urine (13)	1	80.87	25.99	2.19
N40	4	plasma (4)	1	80.86	22.07 (diluted 1:2)	15.08
		PBMCs (4)	1	81.06	22.90	4.30
		urine (4)	1	80.94	28.79	0.0775
		PBMCs (21)	1+**2**	79.76	29.92	< 0.0475

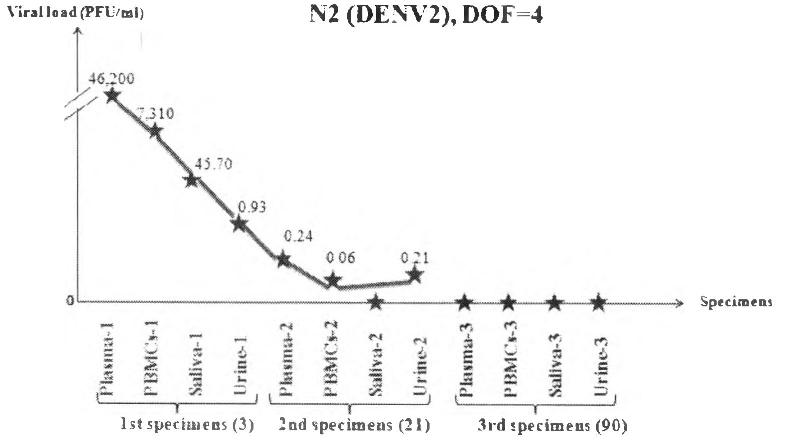
DOF = duration of fever. Tm = Melting temperature (°C). Ct = cycle threshold.

The bold characters represent major serotype in mixed infected specimens.

The number in "( )" represents the day of specimen collections. This table presents only positive results by illustrating the melting curve analysis (Tm) and cycle threshold of amplification curve (Ct). The expected melting curve analysis of PCR product is approximately 79.90-81.40 °C for all 4 serotypes (the data from ten-fold dilution of each stock DENV). Melting curve data out of this range was acceptable after confirming by 2% agarose gel electrophoresis and comparing with positive control.

The last standard viral concentrations of DENV1 to DENV4 were 5 x 10^{-2} PFU/ml (DENV1), 4.75 x 10^{-2} PFU/ml (DENV2), 2.75 x 10^{-2} PFU/ml (DENV3) and 2.5 x 10^{-3} FPU/ml (DENV4).

22A



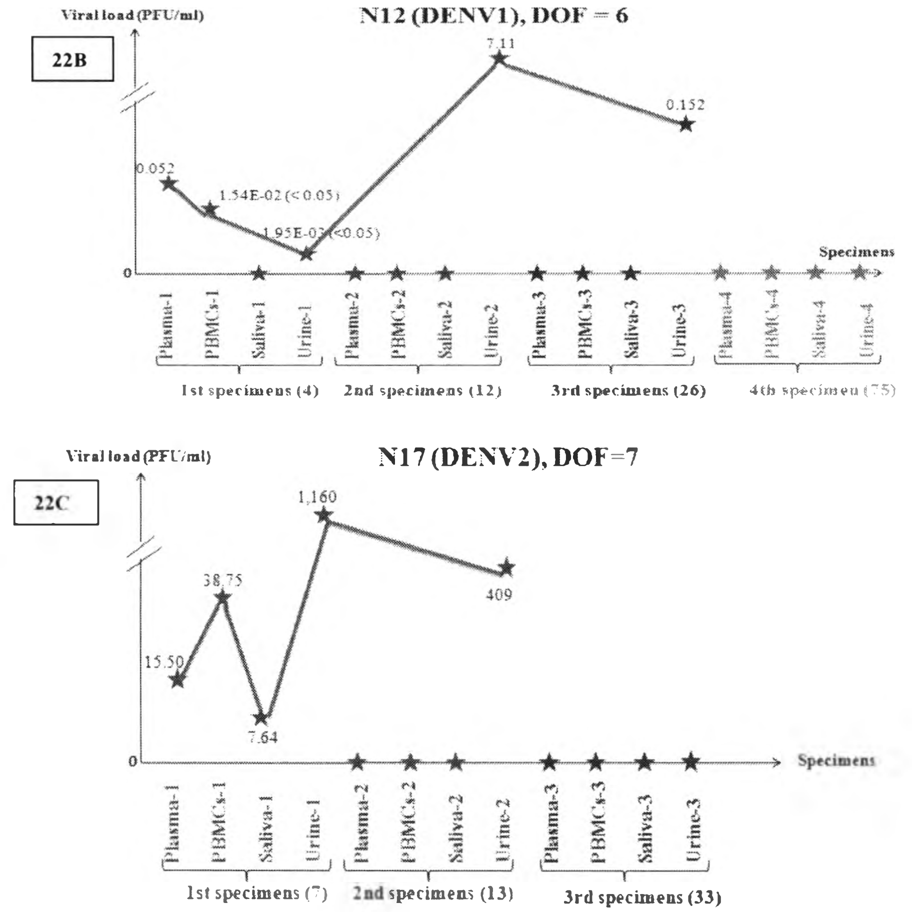


Figure 22: The viral load of DEN- infected patients (N10, 12 and N17). N10 (figure 22A) presented the viral loads in plasma, PBMCs, saliva and urine collected during day 4, 27 and 90 of illness. N12 (figure 22B) presented the viral loads in plasma, PBMCs, saliva and urine collected during day 4, 12, 26 and 75 of illness. N17 (figure 22C) presented the viral loads in plasma, PBMCs, saliva and urine collected during day 7, 13 and 33 of illness. The viral load scales are separately in each figure that can not be used to compare the finding among each patient.

When comparing the results between qRT-PCR and nested RT-PCR by using different primer sets (5'UTR and E gene, respectively) in 23 patients, the tendency of positive results after using qRT-PCR was higher than using nested RT-PCR. Additionally, the number of patients positive for DENV at least 2 time points were also higher when the results of qRT-PCR were analyzed (See Appendix C). The qRT-PCR results were mostly correlated with the results of nested RT-PCR while some specimens were positive only in either qRT-PCR or nested RT-PCR. The longest time of DENV detection in each specimen was varied in each RT-PCR technique. After examining the results of both protocols, the longest times of DENV detection in each specimen were day 7 of illness in saliva, day 22 of illness in plasma, day 27 of illness in PBMCs, and day 46 of illness in urine (Table 30).

Table 30: The longest time of positive DENV detection in each specimen by comparing two methods of RT-PCR (The bold type presents the longest time of DENV detection in each specimen)

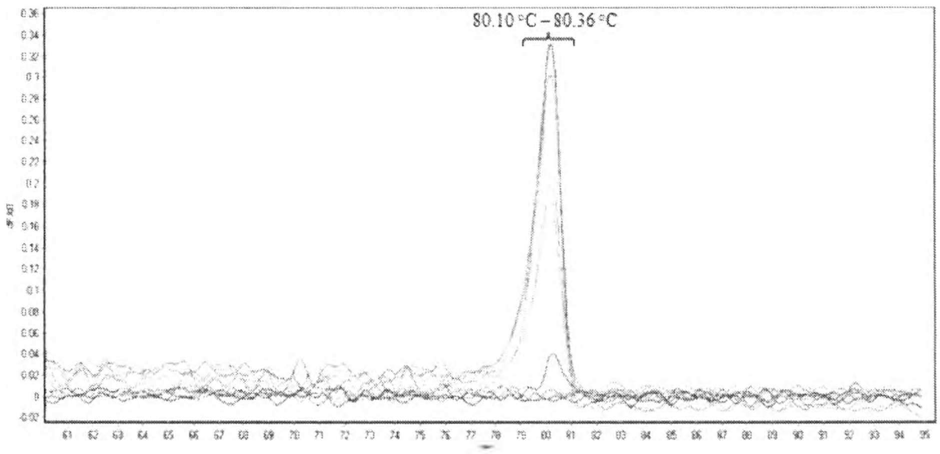
	Plasma	PBMCs	Saliva	Urine
qRT-PCR	**day 22 of illness**	day 21 of illness	day 7 of illness	**day 46 of illness**
Nested RT-PCR	day 7 of illness	**day 27 of illness**	**day 8 of illness**	**day 46 of illness**

Negative strand detection of DENV in different specimens and time points using tagged real time RT-PCR (tagged qRT-PCR)

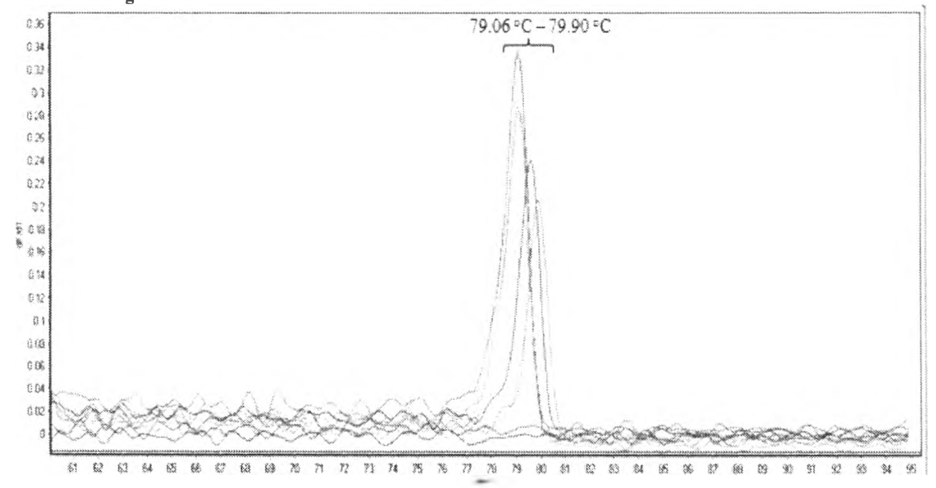
Tagged real time RT-PCR (tagged qRT-PCR) was used to detect the negative strand of DENV in all positive qRT-PCR specimens during febrile, early convalescent and late convalescent periods. The results were reported as "detected" or "not detected" after confirming by melting temperature (Tm) and gel electrophoresis (when the Tm results were inconclusive).

This assay was firstly validated to determine the limit of detection using recombinant plasmids containing negative strand PCR product of positive control DENV1-DENV4. The limits of detection were 2.39 copies/μl (DENV1), 5.60 copies/μl (DENV2), 3.37 copies/μl (DENV3) and 4.29 copies/μl (DENV4) (data not shown). The melting curve analysis of negative strand DENV using diluted positive controls from 1:2 to 1:512 (cDNA of each stock DENV1-DENV4) was in the range of 79.06°C – 80.70 °C (Figure 23).

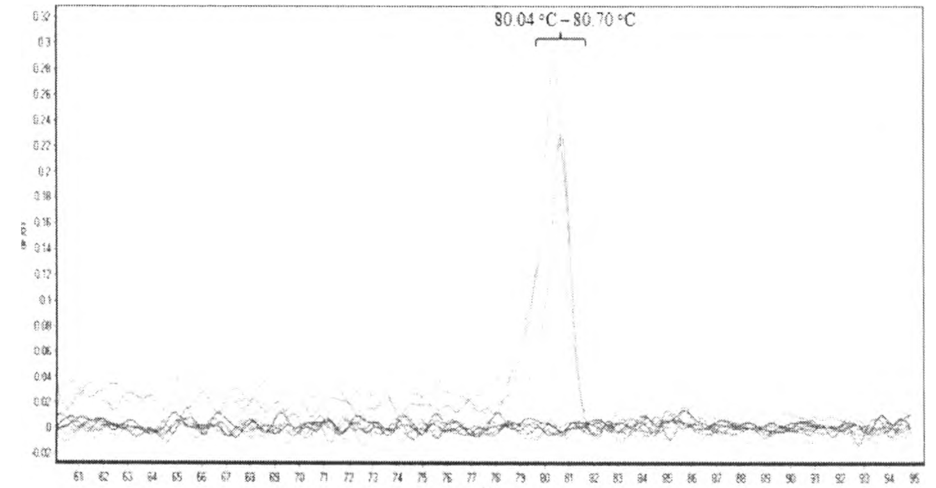
23A: Negative strand of DENV1



23B: Negative strand of DENV2



23C: Negative strand of DENV3



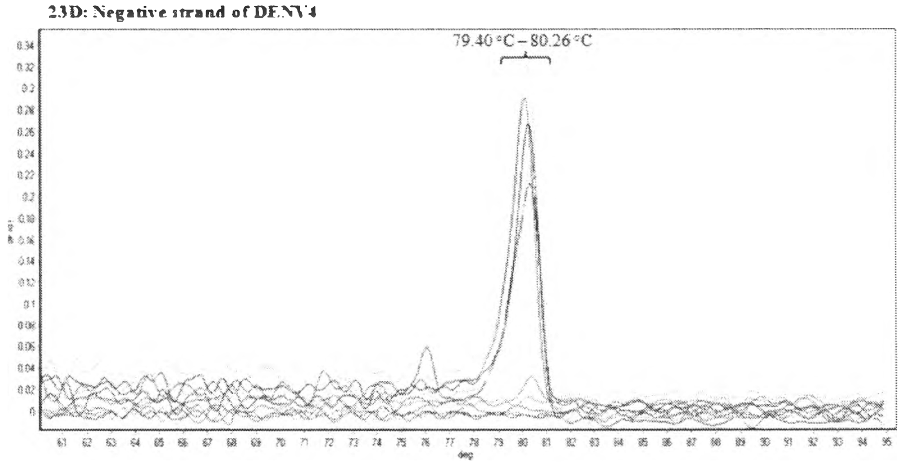


Figure 23: The melting curve analysis of negative strand DENV1-DENV4 (23A – 23D). The cDNA of each stock DENV was diluted from 1:2 to 1:512. All 4 serotypes gave the same length of PCR products. The melting temperatures of each DENV ranged from 79.06°C – 80.70 °C (80.10 °C – 80.36°C (DENV1), 79.06 °C – 79.90 °C (DENV2), 80.04 °C – 80.70°C (DENV3) and 79.40 °C – 80.26°C (DENV4)).

Of 96 positive qRT-PCR specimens in 23 patients in different time points, there were 16 specimens in 9 patients positive for negative strand detection (16.67%) suggesting the evidence of viral replication (Table 31, Figure 24 and 25). During febrile period, the negative strand of DENV was found in plasma (6/15), PBMCs (5/16) and urine (1/14). In early and late convalescent periods, the negative strand of DENV was detected in urine (3/19 and 1/4, respectively) (Figure 26). No negative strand detection of DENV was found in saliva. The longest times of negative strand detection were day 7 of illness in plasma and PBMCs and day 26 of illness in urine.

Table 31: Tagged qRT-PCR results in positive q RT-PCR specimens

Code	DOF	Positive specimen	Serotype	Negative strand detection result	Tm (°C)
N2	4	plasma (3)	2	detected	79.84
		PBMCs (3)	2	detected	79.84
		saliva (3)	2	not detected	ND
		urine (3)	2	not detected	ND
		plasma (21)	ND	not detected	ND
		PBMCs (21)	2	not detected	ND

Code	DOF	Positive specimen	Serotype	Negative strand detection result	Tm (°C)
N2	4	urine (21)	ND	not detected	ND
N3	7	plasma (7)	2	not detected	ND
		PBMCs (7)	2	not detected	ND
		saliva (7)	2	not detected	ND
		urine (7)	ND	not detected	ND
		plasma (22)	ND	not detected	ND
		urine (22)	ND	not detected	ND
N4	5	plasma (5)	ND	not detected	ND
		PBMCs (5)	2	not detected	ND
		saliva (5)	ND	not detected	ND
		urine (5)	ND	not detected	ND
N5	7	plasma (6)	2	not detected	ND
		PBMCs (6)	2	not detected	ND
		saliva (6)	ND	not detected	ND
		urine (6)	ND	not detected	ND
		urine (23)	2	not detected	ND
N6	6	plasma (4)	ND	not detected	ND
		PBMCs (4)	2	not detected	ND
N8	5	plasma (7)	2	not detected	ND
		PBMCs (7)	2	not detected	ND
		saliva (7)	2	not detected	ND
		urine (7)	2	not detected	ND
		urine (25)	ND	not detected	ND
N9	9	PBMCs (9)	1	not detected	ND
		PBMCs (15)	1	not detected	ND
N10	5	plasma (4)	2	detected	79.40
		PBMCs (4)	2	detected	79.20
		saliva (4)	2	not detected	ND
		urine (4)	2	not detected	ND
N12	6	plasma (4)	1	not detected	ND
		PBMCs (4)	1	not detected	ND
		urine (4)	ND	not detected	ND
		urine (12)	1	detected	80.30
		urine (26)	1	detected	80.20
N13	5	PBMCs (8)	2	not detected	ND

Code	DOF	Positive specimen	Serotype	Negative strand detection result	Tm (°C)
N13	5	urine (8)	2	not detected	ND
		urine (15)	2	not detected	ND
N17	7	plasma (7)	2	not detected	ND
		PBMCs (7)	2	not detected	ND
		saliva (7)	2	not detected	ND
		urine (7)	2	not detected	ND
		urine (13)	2	not detected	ND
N20	8	saliva (6)	ND	not detected	ND
		urine (6)	1	not detected	ND
		urine (30)	1	not detected	ND
N21	7	PBMCs (7)	2	not detected	ND
		urine (14)	2	not detected	ND
N22	7	plasma (4)	ND	not detected	ND
		PBMCs (4)	1	not detected	ND
		saliva (4)	ND	not detected	ND
		urine (4)	ND	not detected	ND
		urine (24)	ND	not detected	ND
N23	8	plasma (7)	3	not detected	ND
		urine (7)	3	not detected	ND
N24	6	plasma (3)	3	detected	80.14
		PBMCs (3)	3	detected	80.50
		saliva (3)	3	not detected	ND
		urine (3)	3	detected	80.54
N28	8	plasma (6)	3	detected	80.54
		PBMCs (6)	3	not detected	ND
		urine (14)	3	not detected	ND
		urine (46)	3	not detected	ND
N29	8	plasma (5)	1	detected	79.80
		PBMCs (5)	1	not detected	ND
		urine (5)	1	not detected	ND
		urine (19)	1	detected	80.10
		urine (33)	ND	not detected	ND
N30	9	plasma (7)	1	detected	80.16
		PBMCs (7)	1	detected	80.34
		urine (7)	1	not detected	ND

Code	DOF	Positive specimen	Serotype	Negative strand detection result	Tm (°C)
N33	6	plasma (7)	4	not detected	ND
		PBMCs (7)	4	not detected	ND
		saliva (7)	4	not detected	ND
		urine (7)	4+2	not detected	ND
		plasma (18)	ND	not detected	ND
		urine (18)	4	not detected	ND
N34	6	plasma (8)	ND	not detected	ND
		PBMCs (8)	1	not detected	ND
		saliva (8)	1	not detected	ND
		urine (8)	1	not detected	ND
		urine (14)	1+3	not detected	ND
N35	5	plasma (7)	1	not detected	ND
		PBMCs (7)	1	not detected	ND
		saliva (7)	1	not detected	ND
		urine (7)	1	not detected	ND
		urine (13)	1	detected	80.00
N40	4	plasma (4)	1	not detected	ND
		PBMCs (4)	1	detected	79.80
		urine (4)	1	not detected	ND
		PBMCs (21)	1+2	not detected	ND
		Stock DENV1	1	detected	80.10-80.36*
		stock DENV2	2	detected	79.06-79.90*
		stock DENV3	3	detected	80.04-80.70*
		stock DENV4	4	detected	79.40-80.26*

ND = not determined. DOF = duration of fever.

The number in "( )" represents the day of specimen collections.

The interpretation of positive result is based on the melting curve analysis and gel electrophoresis.

*Tm results of stock DENV1-DENV4 were derived from the diluted 1:2 until 1:512 of cDNA in each serotype.

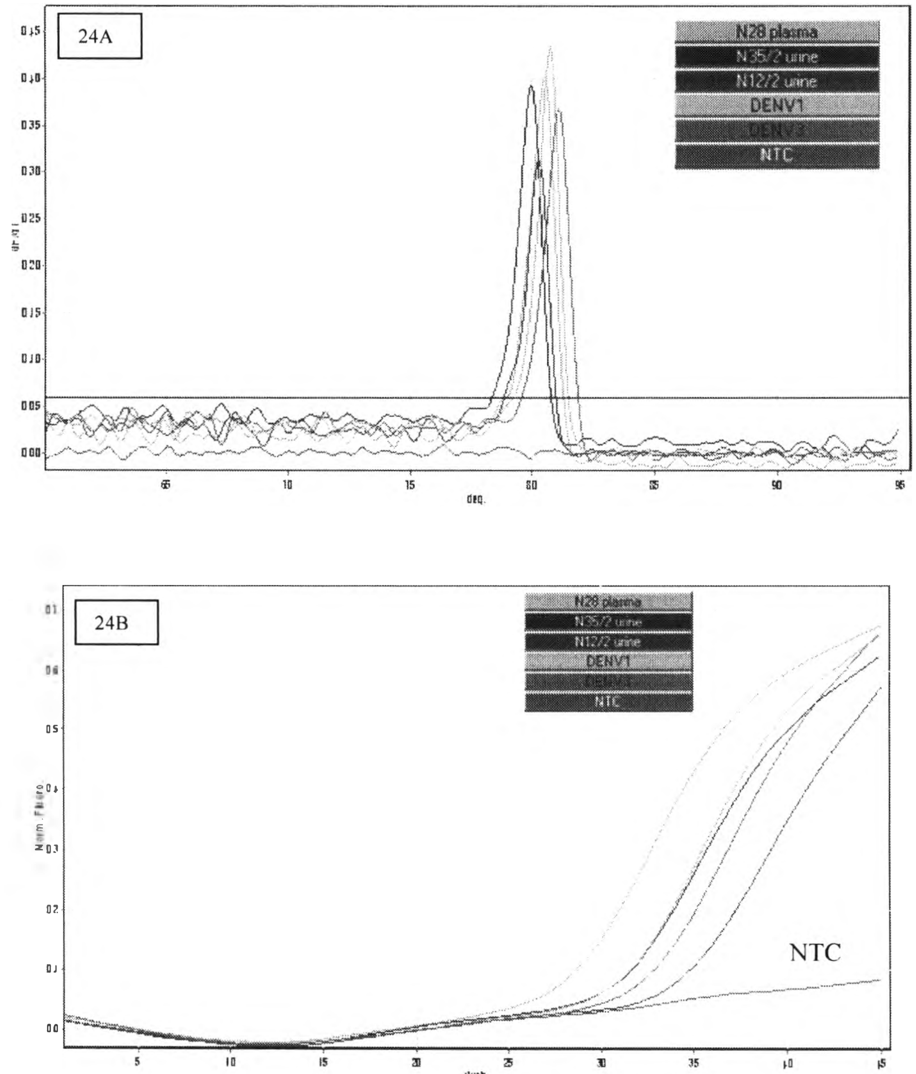


Figure 24: The melting curve analysis (24A) and the amplification plot (24B) of negative strand detection results in positive qRT-PCR specimens (N35/2 urine , 2[nd] convalescent urine, N28 plasma, febrile plasma and N12/2 urine, early convalescent urine) compared with positive control of DENV1 and DENV3negative strands.

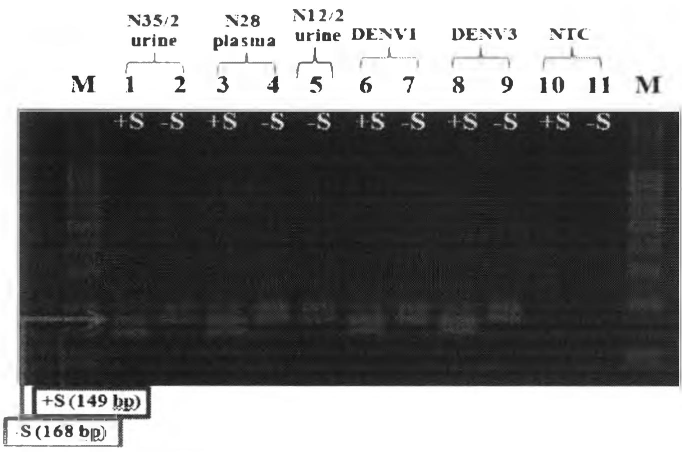
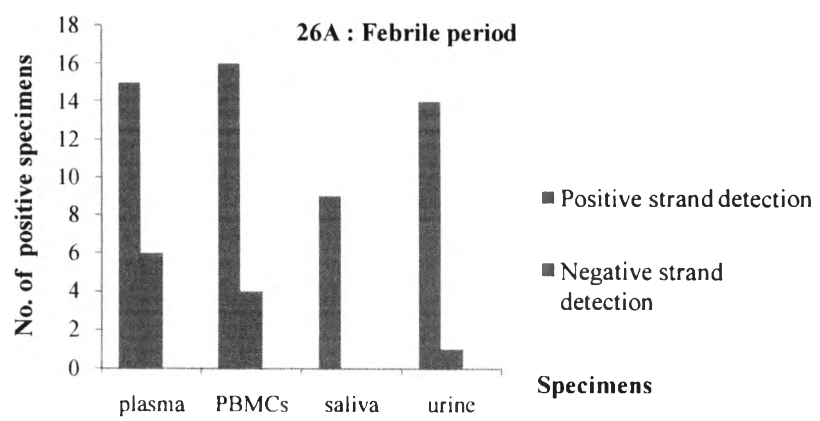
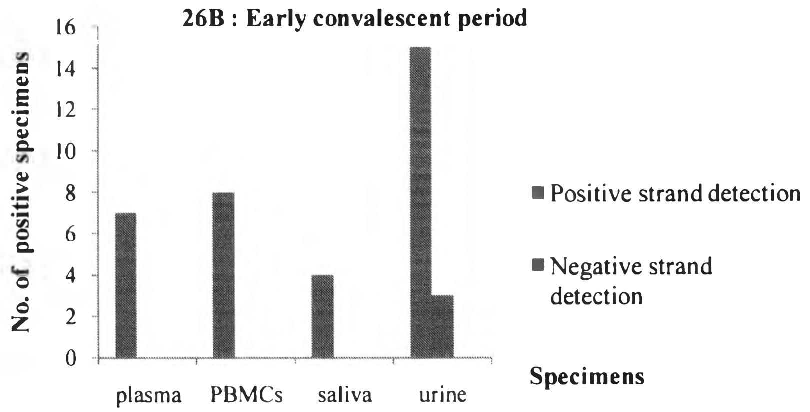


Figure 25: The 2% agarose gel electrophoresis of negative strand (168 bp) detections by tagged qRT-PCR of N35/2 urine (2nd convalescent urine), N28 plasma (febrile plasma), N12/2 urine (early convalescent urine), positive control DENV1 and positive control DENV3. The results were compared with qRT-PCR results (positive strand detection, 149 bp) except N35/2 urine. "+S" refers positive strand detection whereas "-S" refers negative strand detection. M = 100 bp DNA marker. NTC = no template control.





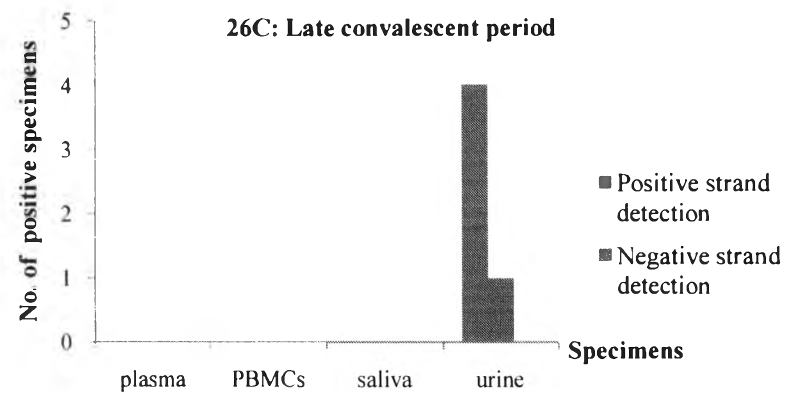
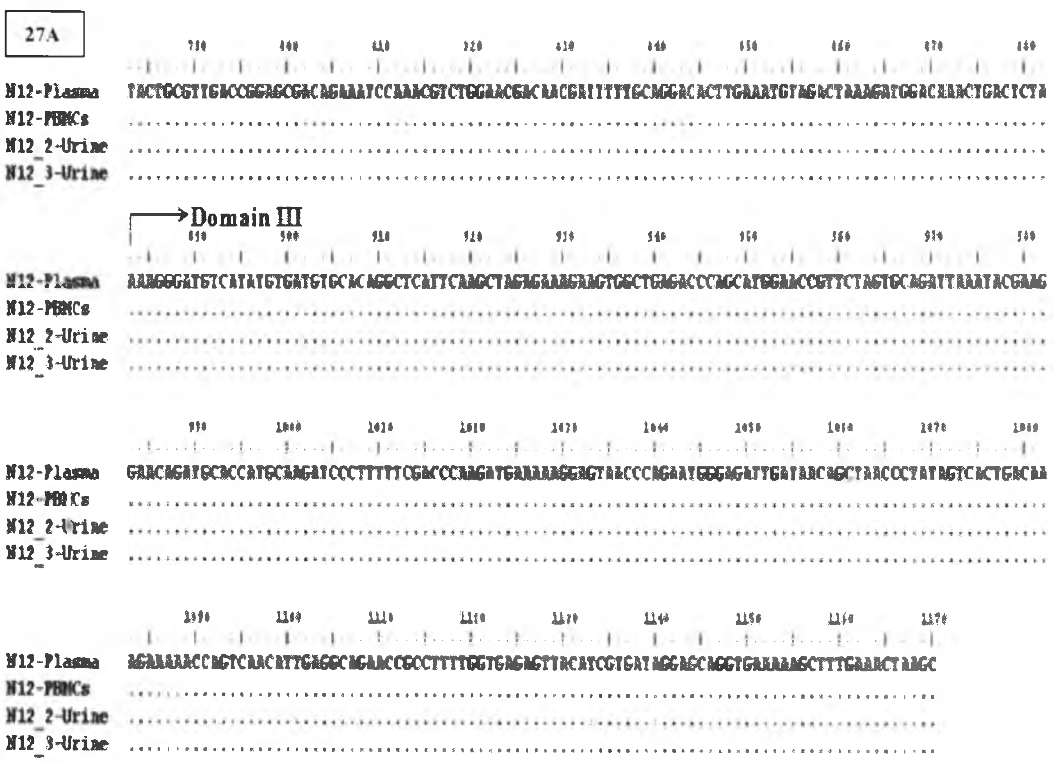


Figure 26: The comparison of positive and negative strand detection results of DENV-infected patients by qRT-PCR and tagged qRT-PCR during febrile period (26A), early convalescent period (26B) and late convalescent period (26C).

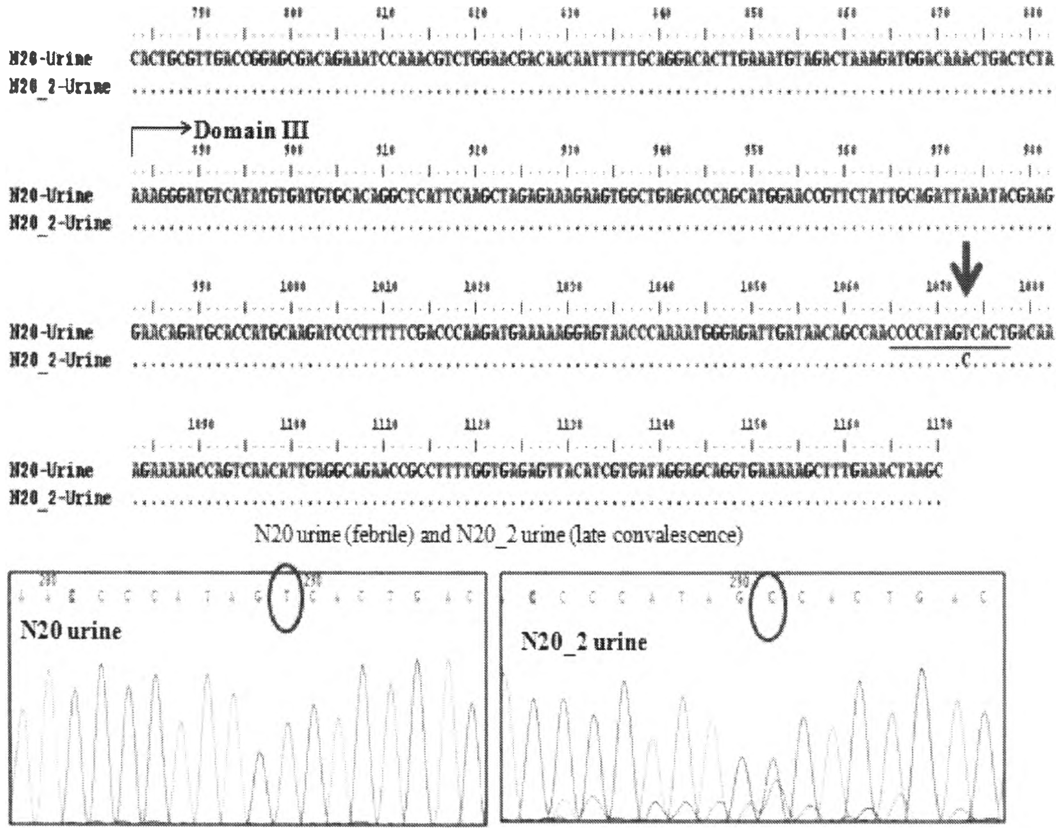
Genetic variation of DENV in different periods of infection

To determine whether genetic variations of DENV in different specimens and time points were identical, direct sequencing of partial E gene sequences (> 95% of nucleotide sequences locating at domain III) of 13 patients, except primers (388 bp) were compared. This sequence length was selected because the domain III region has been reported that it correlates with viral pathogenesis, evolution and adaptation [25]. Nucleotide and deduced amino sequence (using translation tool in BioEdit program) alignments were done using ClustalW algorithm in BioEdit program (version 7.0).

In DENV1-infected patients (N12, N20 and N29), nucleotide and amino acid sequence alignments from direct sequencing among specimens in different time points of each patient were identical, except N20 patient. In N20 patient, the result showed that the nucleotide variation between febrile and late convalescent urine was found at position 1073 (T→C) accounting for 0.25%. This variation resulted in the amino acid change at position 358 (V358A) locating on the domain III of E gene (Figure 27 and Table 32).



N12 plasma and N12 PBMCs (febrile), N12_2 urine (early convalescence) and N12_3 urine (late convalescence)


N20-Urine
N20_2-Urine
Domain III
N20 urine (febrile) and N20_2 urine (late convalescence)
N20 urine
N20_2 urine

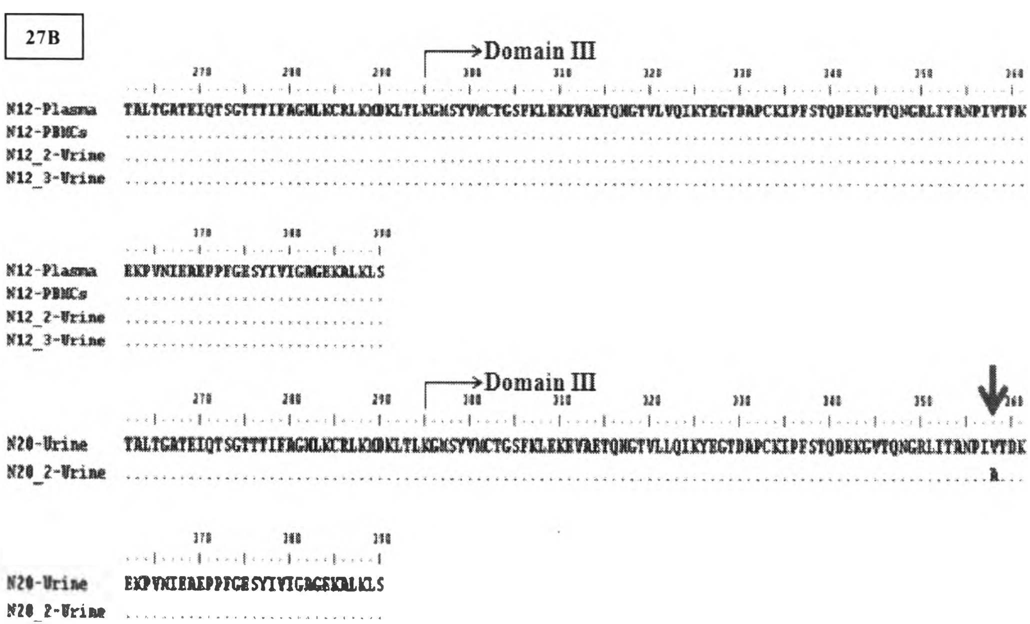
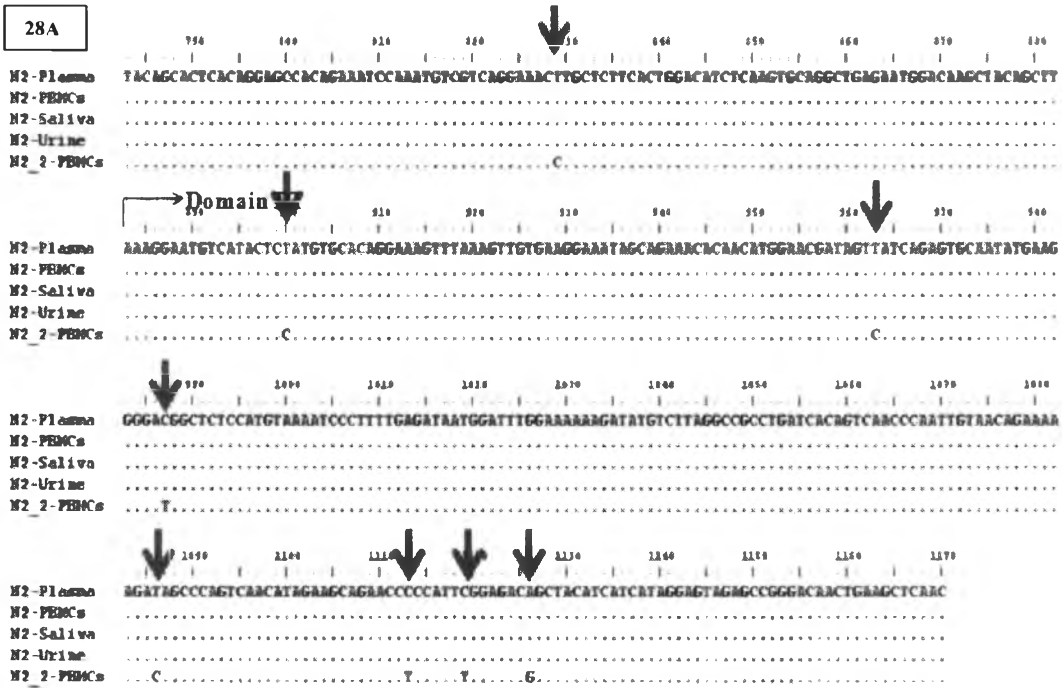

27B
Domain III
N12-Plasma
N12-PBMCs
N12_2-Urine
N12_3-Urine
N20-Urine
N20_2-Urine

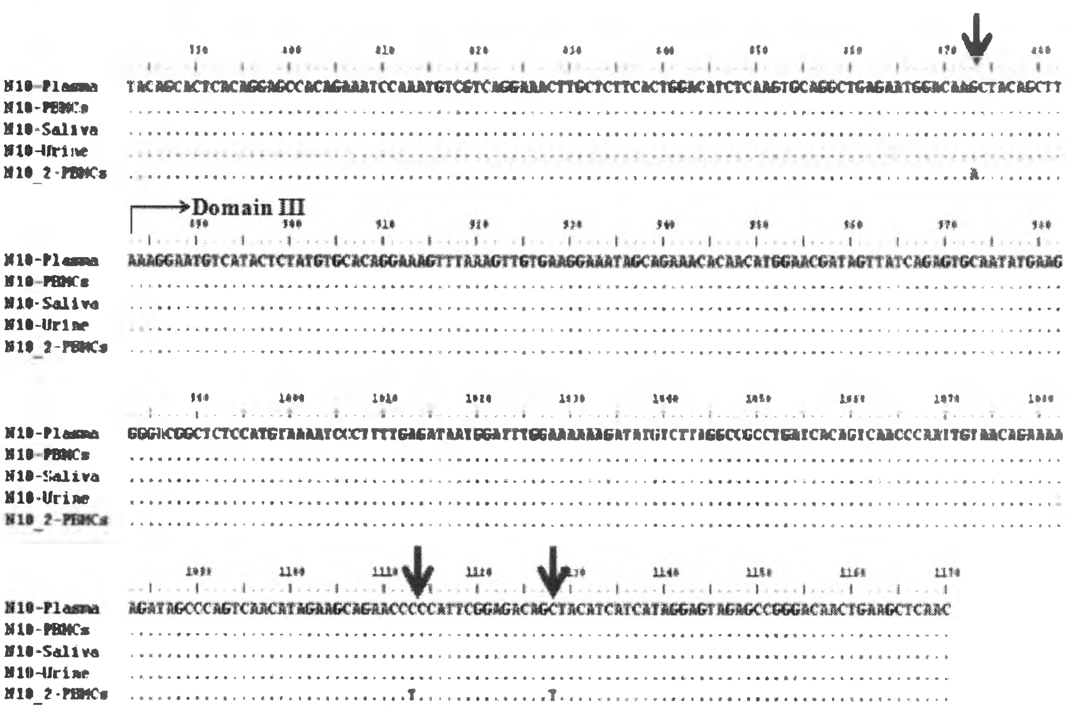
Figure 27: Nucleotide (27A) and amino acid (27B) sequence alignments of direct partial E gene sequences (388 bp and 129 aa, respectively) derived from positive specimens of DENV1-infected patients (N12 and N20) in different time points. This alignment is based on ClustalW algorithm and translation tool in BioEdit program (version 7.0). Nucleotide and amino acid positions are based on the alignment results with complete E gene sequence (1,485 bp and 495 aa) in GenBank accession no. AY732472.1 starting from the position 783 to 1170 (nucleotides) and accession no. ABB70708.1 starting from the position 262 to 390 (amino acids), respectively. Domain III of E gene locates at the nucleotide position 883 to 1180 and the amino acid position 295 to 395, respectively [143, 144]. N20 specimens showed the one position of nucleotide variation at position 1073 (T→C) (marked as the red arrow at figure 25A). This variation resulted in V358A (marked as the red arrow at figure 27B) on the domain III of E gene.

In DENV2-infected patients (N2, N5 N10, N13, N17 and N21), nucleotide and amino acid sequence alignments among specimens in different time points of each patient were the same if early or late convalescent specimen was urine. Interestingly, nucleotide variations between febrile and convalescent PBMCs were found in 2 patients (N2 and N10). The variations were 2.06% in N2 and 0.77% in N10 compared with all similar sequences in each patient, respectively. In N2 patients, the variant positions of N2_2-PBMCs (early convalescence) were found at positions 829 (T→C), 900 (T→C), 963 (T→C), 987 (C→T), 1086 (T→C), 1113 (C→T), 1119 (C→T) and 1126 (A→G). All variations resulted in silent mutations, excluding the position 1126 (A→G) causing the amino acid change at position 376 (S376G). In N10 patient, variant positions of N10_2-PBMCs (late convalescence) were found at positions 873 (G→A), 1113 (C→T) and 1128 (C→T). However, nucleotide changes in N10_2-PBMCs did not cause amino acid change (silent mutation) (Figure 28 and Table 32). Variations of both patients mostly occurred in domain III of E gene similar to DENV1-infected patients. Additionally, these results presented 2 convalescent PBMCs sharing the same position of nucleotide variation at 1113 (C→T) but this position did not cause amino acid change.

28A



N2-plasma, N2-PBMCs, N2-saliva and N2-urine (febrile) and N2_2 PBMCs (early convalescence)



N10-plasma, N10-PBMCs, N10-saliva and N10-urine (febrile) and N10_2 PBMCs (late convalescence)

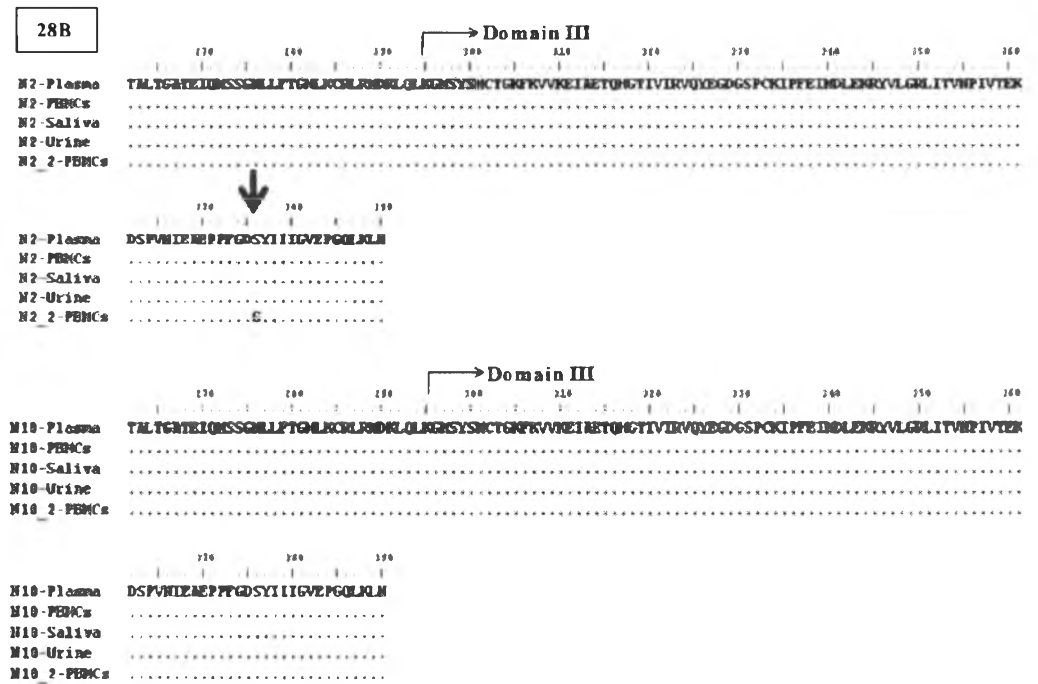


Figure 28: Nucleotide (28A) and amino acid (28B) sequence alignments of direct partial E gene sequences (388 bp and 129 aa, respectively) derived from positive specimens of DENV2-infected patients (N2, and N10) in different time points. This alignment is based on ClustalW algorithm and translation tool in BioEdit program (version 7.0). The nucleotide and amino acid positions are based on the alignment results with complete E gene sequence (1,485 bp and 495 aa) in GenBank accession no. DQ181872.1 starting from the position 783 to1170 (nucleotides) and accession no. AFN87732.1 starting from the position 262-390 (amino acids), respectively. Domain III of E gene locates at the nucleotide position 883 to 1180 and amino acid position 295 to 395, respectively [143, 144]. There were 6 positions of N2_2-PBMCs difference from all specimens during febrile period. The variation were at positions 829 (T→C), 900 (T→C), 963 (T→C), 987 (C→T), 1086 (T→C), 1113 (C→T), 1119 (C→T) and 1126 (A→G) marked as the red arrows. All nucleotide variations did not affect the amino acid change (silent mutation) except the mutation at position 1126 (A→G) caused S376G in N2_2-PBMCs (marked as the red arrow). Additionally, there were 3 positions of N10_2-PBMCs difference from all specimens during febrile period. The variations marked as the red arrows are positions 873 (G→A), 1113 (C→T) and 1128 (C→T) that did not affect the amino acid change (silent mutation).

The nucleotide and amino acid sequence variations in different specimens and time points of DENV3-infected patient (N28) were identical (Figure 29).

29A

N28 plasma, N28 PBMCs (febrile), N28_2-urine (early convalescence) and N28_3-urine (late convalescence)

29B

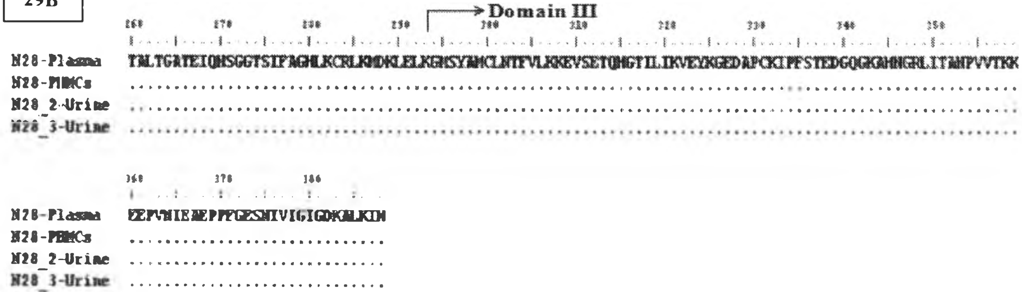
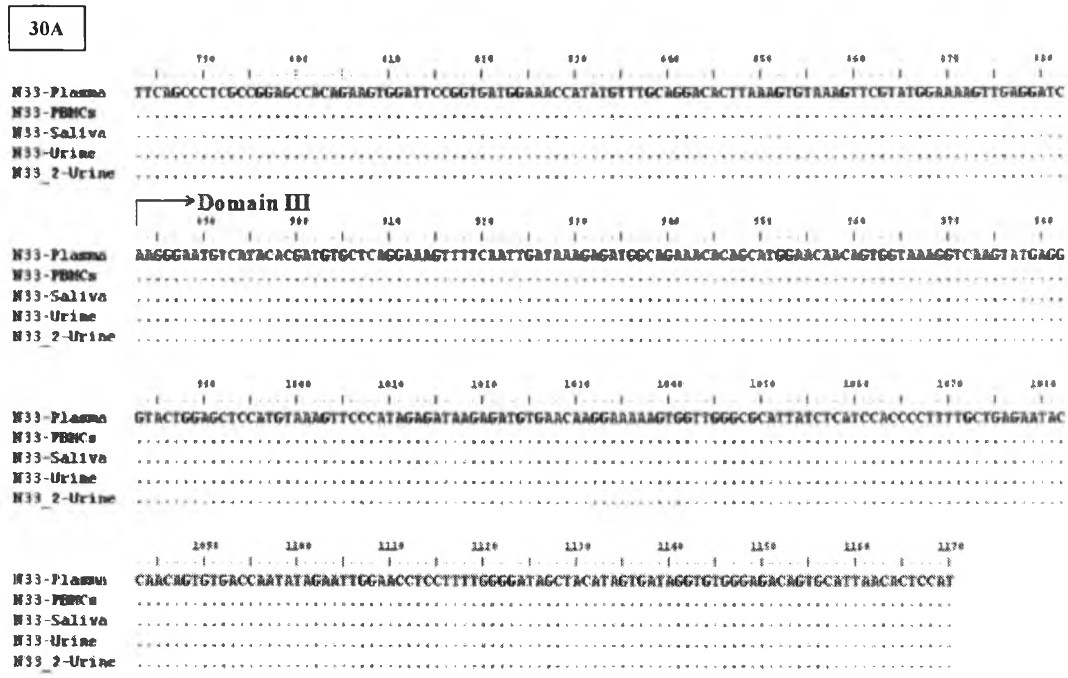


Figure 29: Nucleotide (29A) and amino acid (29B) sequence alignments of direct partial E gene sequences (388 bp and 129 aa) derived from positive specimens of DENV3-infected patient (N28) in different time points. This alignment is based on ClustalW algorithm and translation tool in BioEdit program (version 7.0). The nucleotide and amino acid positions are based on the alignment results with complete E gene sequence (1,479 bp and 493 amino acids) in GenBank accession no. JF968066.1 starting from position 777 to 1164 (nucleotides) and accession no. AF171764.1 starting from position 260 to 388 (amino acids), respectively. Domain III of E gene locates at the nucleotide position 877 to 1171 and amino acid position 293 to 393, respectively [25, 143]. The nucleotide and amino acid sequences among specimens collected during febrile, early convalescent and late convalescent periods were the same.

In 3 mixed-serotype-infected patients, the nucleotide and amino acid sequence alignments of N33 specimens were not changed. Although 1st convalescent urine of N33 presented mixed serotype infections (DENV2 + DENV4) by serotype RT-PCR, the direct sequencing result presented the clear chromatogram of single serotype (DENV4) to compare with other N33 specimens (DENV4) (Figure 30).



N33 plasma, N33 PBMCs, N33saliva, N33 urine and N33_2-urine (early convalescence but in different time points)

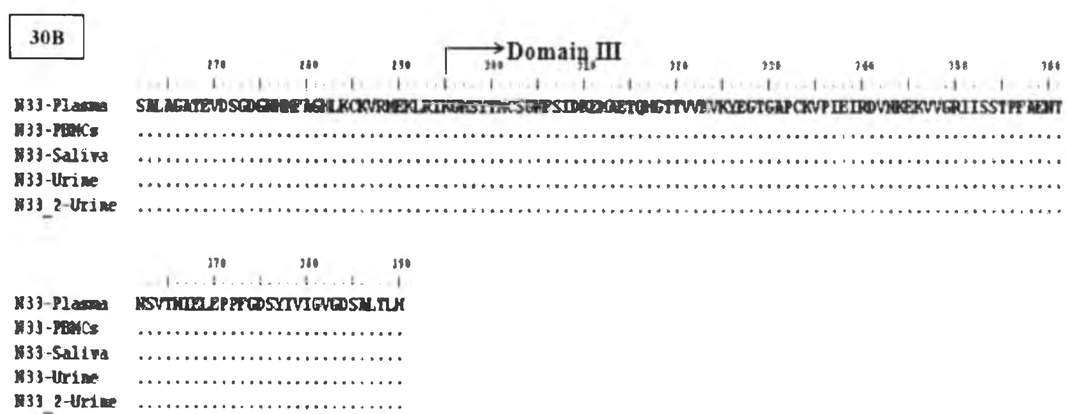


Figure 30: Nucleotide (30A) and amino acid (30B) sequence alignments of direct partial E gene sequences (388 bp and 129 aa, respectively) derived from positive specimens of DENV4-infected patient (N33) in different time points. This alignment is based on ClustalW algorithm and translation tool in BioEdit program (version 7.0). The nucleotide and amino acid positions are based on the alignment results with complete E gene sequence (1,485 bp and 495 amino acids) in GenBank

accession no. AY786197.1 starting from position 783 to 1170 (nucleotides) and accession no. AAU89351.1 starting from position 262 to 390 (amino acids), respectively. Domain III of E gene locates at the nucleotide position 883 to 1180 and amino acid position 295 to 395, respectively [25, 143]. The nucleotide and amino acid sequences among specimens collected during different time points of early convalescent periods were the same.

The nucleotide and amino acid sequences alignments in different specimens and time points were not done in both N34 and N40 patients due to clearly different serotypes of E gene sequences resulting from the mixed-serotype infection in different specimens and time points.

Table 32: Summary results of nucleotide and amino acid variations of dengue-infected patients

Patients	Specimen	Time of collection	Nucleotide changes (positions)*	Amino acid changes (positions)*
N2 (DENV2)	PBMCs	early convalescence (21)	829 (T→C)	-
			900 (T→C)	-
			963 (T→C)	-
			987 (C→T)	-
			1086 (T→C)	-
			1113 (C→T)	-
			1119 (C→T)	-
			1126 (A→G)	S376G
N10 (DENV2)	PBMCs	late convalescence (27)	873 (G→A)	-
			1113 (C→T)	-
			1128 (C→T)	-
N20 (DENV1)	urine	late convalescence (30)	1073 (T→C)	V358A

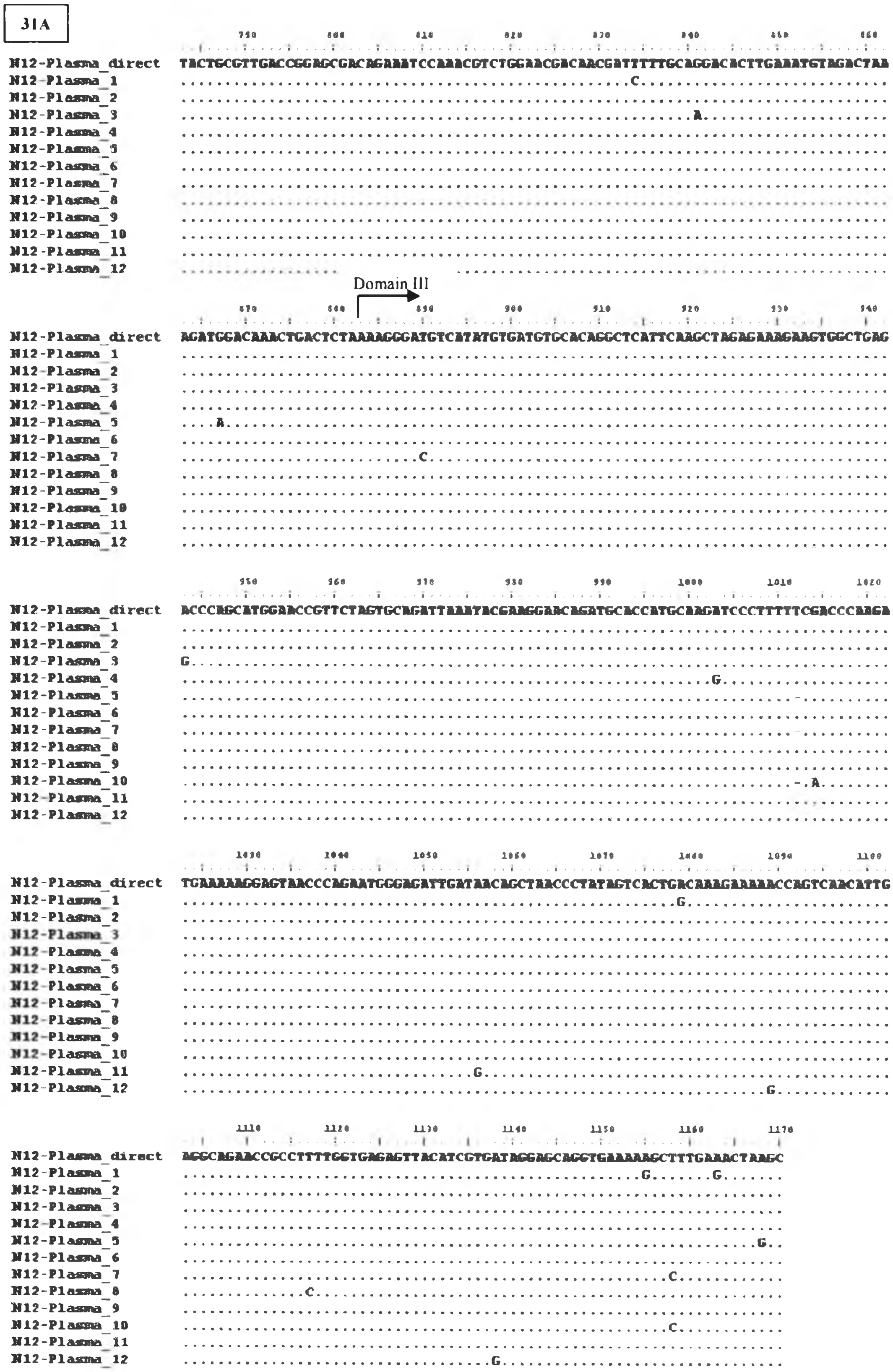
* The nucleotide and amino acid positions are based on the E gene sequence of each serotype after blasting with GenBank. The nucleotide and amino acid changes were compared with febrile specimens. The number in "( )" represents the day of specimen collection.

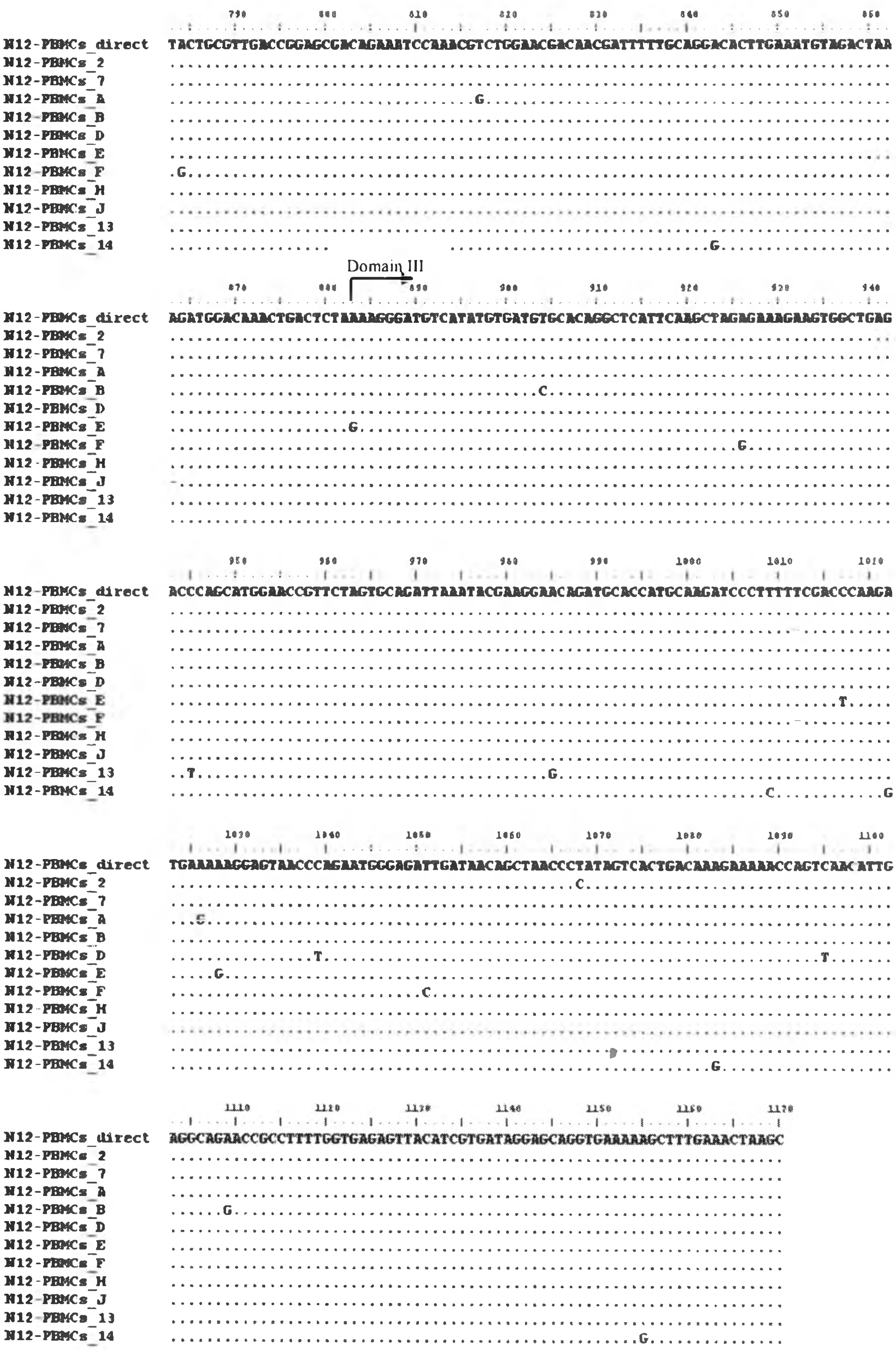
Genetic diversity of DENV in each specimen and time points in DENV-infected patients

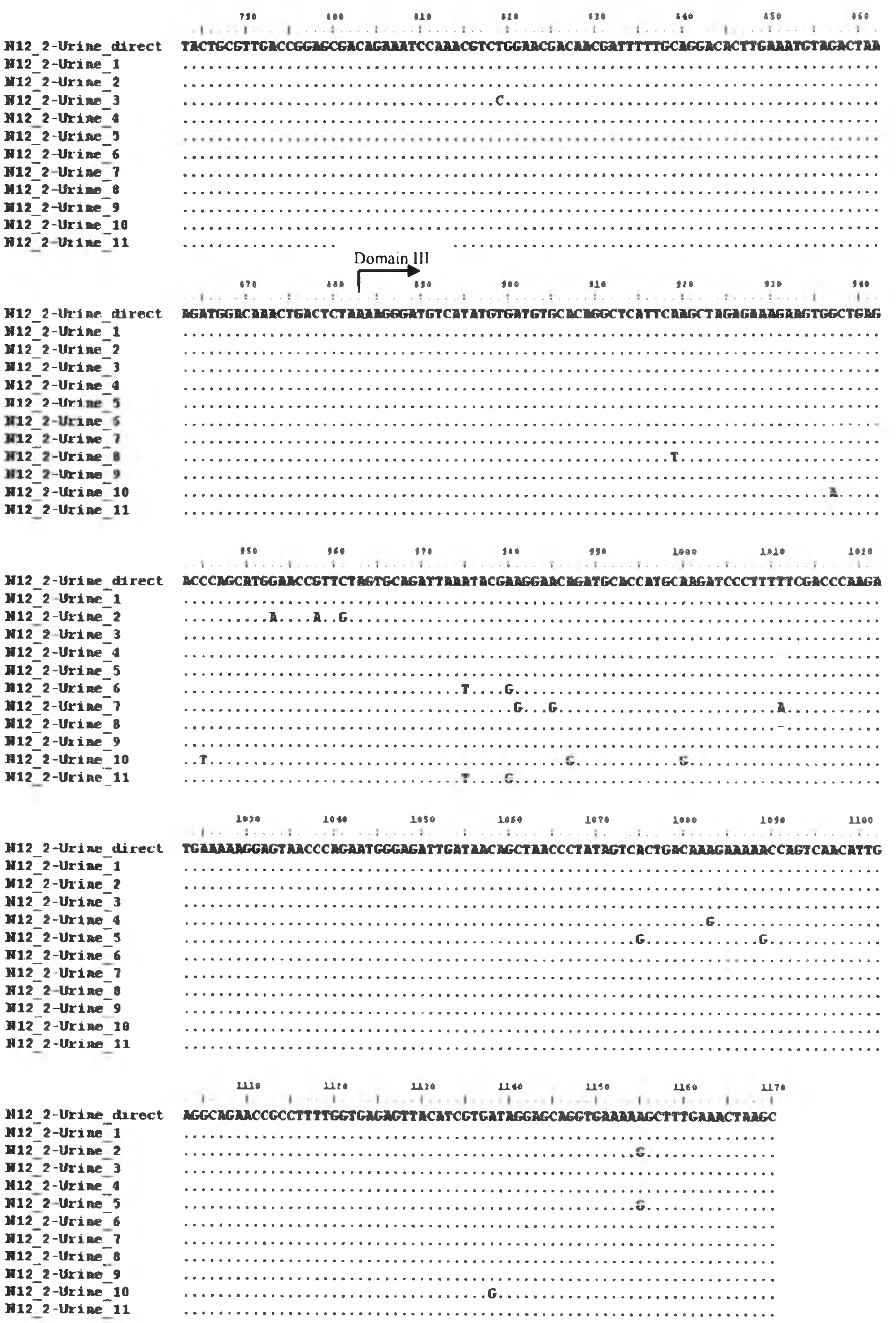
The genetic diversity of DENV in different time points were explored by sequencing 10-15 colonies derived from each specimen of individual patient. Nucleotide and amino acid sequence variations of all clones in each specimen were analyzed by aligning with direct sequencing result using BioEdit program (version 7.0).

Nucleotide sequence alignments showed that all specimens of each patient contained both major sequences (similar to direct sequencing result) and minor sequences or variants (Figure 31A and 32A) suggesting the occurrence of heterogeneous DENV population or quasispecies. The different of nucleotide variations in each clone comparing with consensus sequence (direct sequencing) varied from one to twenty-eight positions composing of single nucleotide mutation and deletion. Moreover, these mutations were frequently found at domain III of E gene.

Amino acid sequence variations were found among clones in each specimen of individual patient in the presence of both major and minor (variant) sequences confirming the presence of heterogeneous population or quasispecies similar to nucleotide alignment results (Figure 31B and 32B). The frequency of different amino acid mutation types varied in each specimen composing of missense, nonsense, silent to frame-shift mutation (resulting from one single nucleotide mutation), which depended on each type of nucleotide variations. Most amino acid sequence variations of each clone in the same specimen were found at the domain III of E gene suggesting the relationship between amino acid variations and host selection pressure.

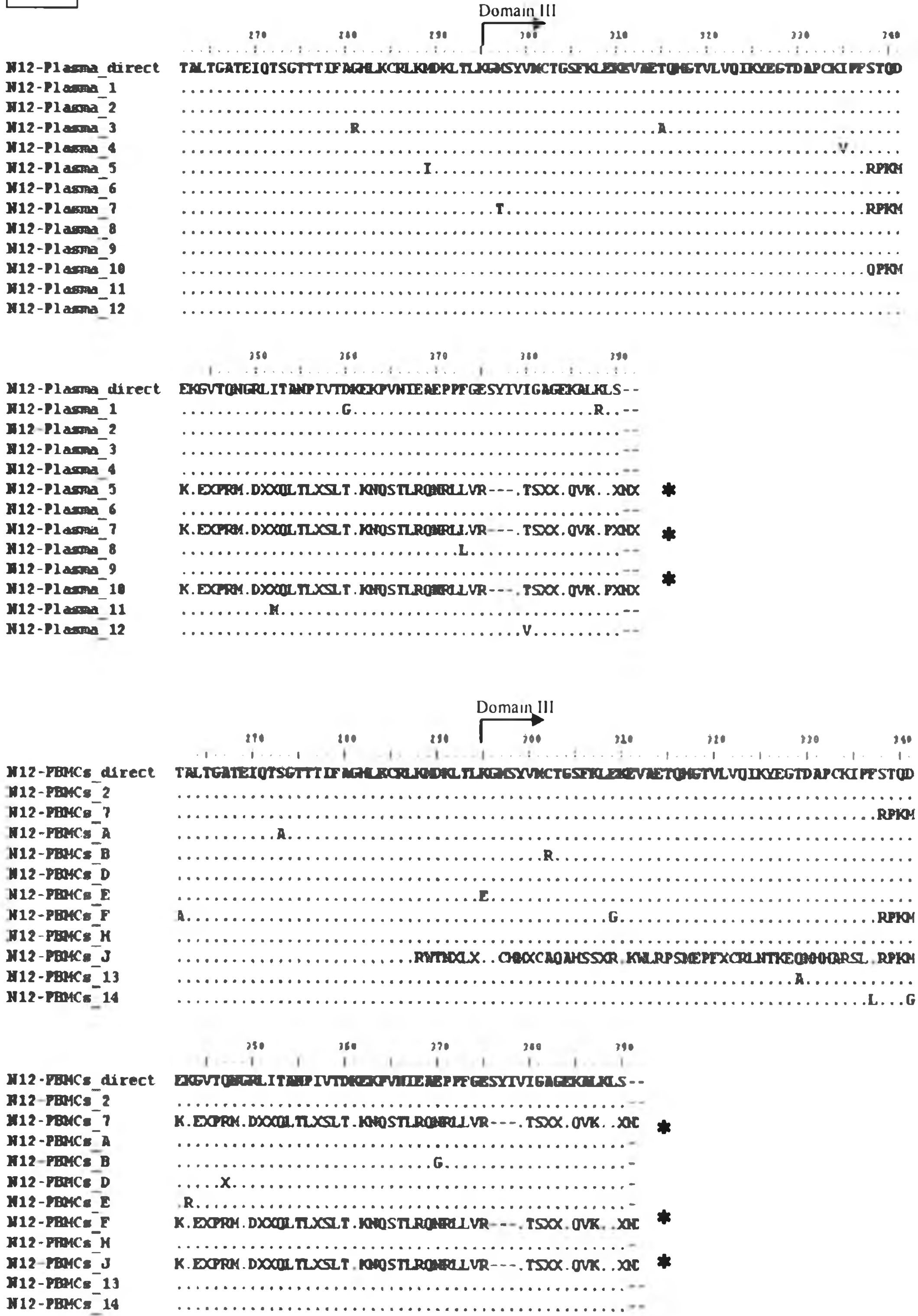

31A
Domain III
N12-Plasma_direct
N12-Plasma_1
N12-Plasma_2
N12-Plasma_3
N12-Plasma_4
N12-Plasma_5
N12-Plasma_6
N12-Plasma_7
N12-Plasma_8
N12-Plasma_9
N12-Plasma_10
N12-Plasma_11
N12-Plasma_12


Domain III


N12_2-Urine_direct
N12_2-Urine_1
N12_2-Urine_2
N12_2-Urine_3
N12_2-Urine_4
N12_2-Urine_5
N12_2-Urine_6
N12_2-Urine_7
N12_2-Urine_8
N12_2-Urine_9
N12_2-Urine_10
N12_2-Urine_11
Domain III

```
                          790       800       810       820       830       840       850       860
N12_3-Urine_direct    TACTGCGTTGACCGGAGCGACAGAAATCCAAACGTCTGGAACGACAACGATTTTTGCAGGACACTTGAAATGTAGACTAA
N12_3-Urine_1         ................................................................................
N12_3-Urine_2         ................................................................................
N12_3-Urine_5         ................................................................................
N12_3-Urine_6         ................................................................................
N12_3-Urine_8         ................................................................................
N12_3-Urine_9         ................................................................................
N12_3-Urine_13        ..........................................................................C.......
N12_3-Urine_14        ................................................................................
N12_3-Urine_15        ......................A.....................................G...................
N12_3-Urine_20        ................................................................................
N12_3-Urine_21        ................................................................................
N12_3-Urine_22        ..................             .................................................

                                           Domain III
                          870       880       890       900       910       920       930       940
N12_3-Urine_direct    AGATGGACAAACTGACTCTAAAAGGGATGTCATATGTGATGTGCACAGGCTCATTCAAGCTAGAGAAAGAAGTGGCTGAG
N12_3-Urine_1         ................................................................................
N12_3-Urine_2         ................................................................................
N12_3-Urine_5         -...............................................................................
N12_3-Urine_6         ................................................................................
N12_3-Urine_8         .....................................................C..........................
N12_3-Urine_9         ......................-.........................................................
N12_3-Urine_13        ................................................................................
N12_3-Urine_14        ................................................................................
N12_3-Urine_15        ............................A..................................G................
N12_3-Urine_20        ...................................................................G............
N12_3-Urine_21        ......................G.........................................................
N12_3-Urine_22        ................................................................A...............

                          950       960       970       980       990      1000      1010      1020
N12_3-Urine_direct    ACCCAGCATGGAACCGTTCTAGTGCAGATTAAATACGAAGGAACAGATGCACCATGCAAGATCCCTTTTTCGACCCAAGA
N12_3-Urine_1         ................................................................................
N12_3-Urine_2         ...T............................................................................
N12_3-Urine_5         ................................................................................
N12_3-Urine_6         ................................................................................
N12_3-Urine_8         ................................................................................
N12_3-Urine_9         ................................................................................
N12_3-Urine_13        ................................................................................
N12_3-Urine_14        ..............................T.................................................
N12_3-Urine_15        ................................................................................
N12_3-Urine_20        .....................................................................-..........
N12_3-Urine_21        ..........................................................................T.....
N12_3-Urine_22        ................................................................................

                         1030      1040      1050      1060      1070      1080      1090      1100
N12_3-Urine_direct    TGAAAAAGGAGTAACCCAGAATGGGAGATTGATAACAGCTAACCCTATAGTCACTGACAAAGAAAAACCAGTCAACATTG
N12_3-Urine_1         ................................C...............................................
N12_3-Urine_2         ................................................................................
N12_3-Urine_5         ................................C...............................................
N12_3-Urine_6         ................................................................................
N12_3-Urine_8         ................................................................................
N12_3-Urine_9         ................................................................................
N12_3-Urine_13        ................................C...............................................
N12_3-Urine_14        ................................................................................
N12_3-Urine_15        ................................................................................
N12_3-Urine_20        ................................................................................
N12_3-Urine_21        ................................C...............................................
N12_3-Urine_22        ................................................................................

                         1110      1120      1130      1140      1150      1160      1170
N12_3-Urine_direct    AGGCAGAACCGCCTTTTGGTGAGAGTTACATCGTGATAGGAGCAGGTGAAAAAGCTTTGAAACTAAGC
N12_3-Urine_1         ....................................................................
N12_3-Urine_2         ....................................................................
N12_3-Urine_5         ......G.............................................................
N12_3-Urine_6         ....................................................................
N12_3-Urine_8         ..................................................G.................
N12_3-Urine_9         ........................................................C...........
N12_3-Urine_13        ....G...............................................................
N12_3-Urine_14        ....................................................................
N12_3-Urine_15        ....................................................................
N12_3-Urine_20        ....................................................................
N12_3-Urine_21        ....................................................................
N12_3-Urine_22        ....................................................................
```

31B


Domain III
N12-Plasma_direct
N12-Plasma_1
N12-Plasma_2
N12-Plasma_3
N12-Plasma_4
N12-Plasma_5
N12-Plasma_6
N12-Plasma_7
N12-Plasma_8
N12-Plasma_9
N12-Plasma_10
N12-Plasma_11
N12-Plasma_12
Domain III
N12-PBMCs_direct
N12-PBMCs_2
N12-PBMCs_7
N12-PBMCs_A
N12-PBMCs_B
N12-PBMCs_D
N12-PBMCs_E
N12-PBMCs_F
N12-PBMCs_H
N12-PBMCs_J
N12-PBMCs_13
N12-PBMCs_14

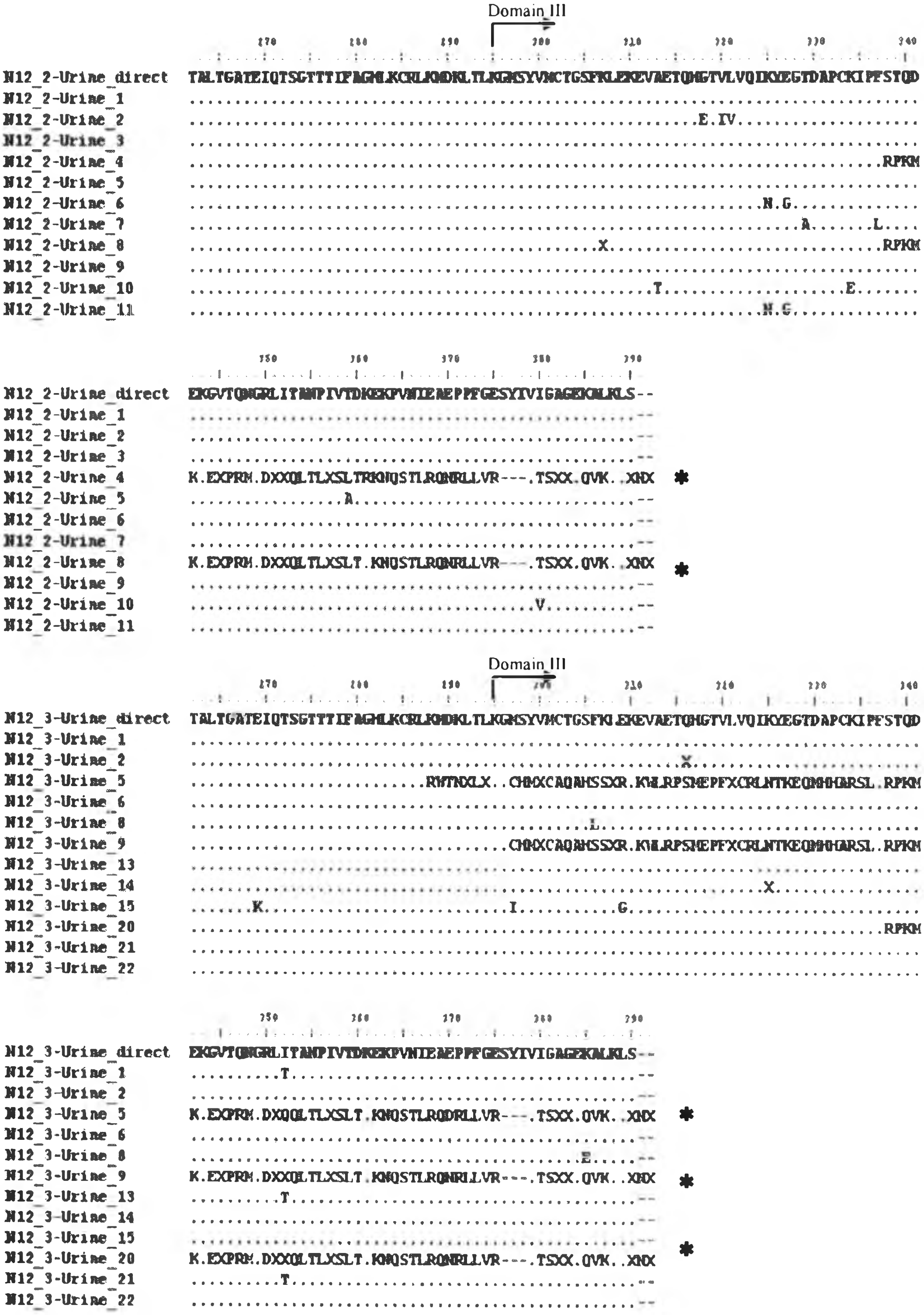
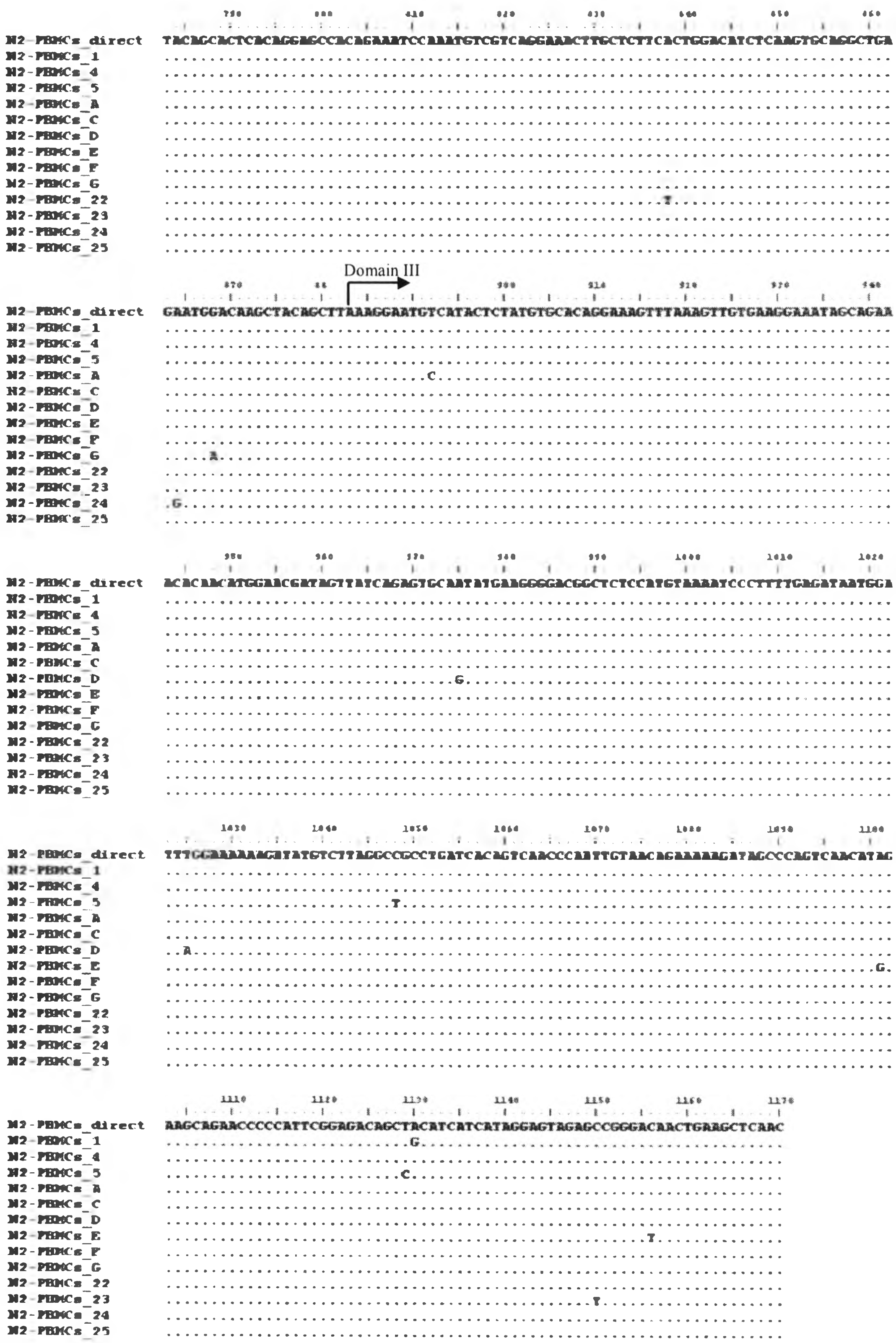
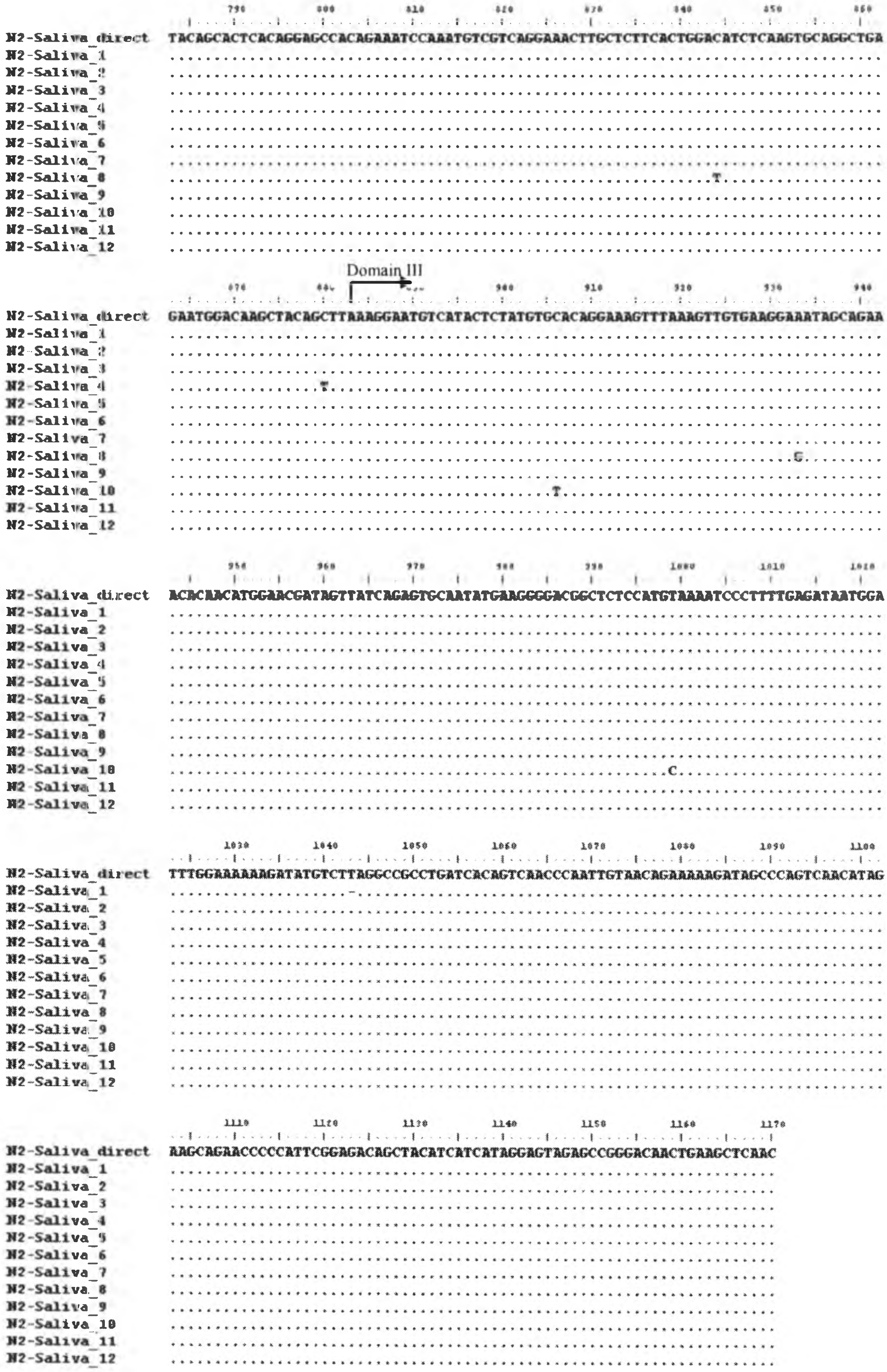

Domain III
N12_2-Urine_direct
N12_2-Urine_1
N12_2-Urine_2
N12_2-Urine_3
N12_2-Urine_4
N12_2-Urine_5
N12_2-Urine_6
N12_2-Urine_7
N12_2-Urine_8
N12_2-Urine_9
N12_2-Urine_10
N12_2-Urine_11
Domain III
N12_3-Urine_direct
N12_3-Urine_1
N12_3-Urine_2
N12_3-Urine_5
N12_3-Urine_6
N12_3-Urine_8
N12_3-Urine_9
N12_3-Urine_13
N12_3-Urine_14
N12_3-Urine_15
N12_3-Urine_20
N12_3-Urine_21
N12_3-Urine_22

Figure 31: Nucleotide (31A) and amino (31B) sequence alignments of all clones derived from each N12 specimens, N12 plasma (4), N12 PBMCs (4), N12 urine (12) and N12 urine (26), (DENV1 infected patients) present heterogeneous population or quasispecies in different time points. Direct sequencing of each specimen is used as a reference sequence (major population) to compare with all variant sequences or minor populations (10-15 sequences per specimen). The positions of nucleotide and amino acid are based on direct sequencing alignments in figure 27A and 27B. Domain III of E gene is starting from position 883 to 1,180 of nucleotide and position 295-395 of amino acid sequences (complete E gene). Deletion position is presented as "-". In-frame stop codon is presented as " *". The presence of frame-shift mutations is marked as "✱". The number in "( )" represents the day of specimen collection.

32A

```
                       790       800       810       820       830       840       850       860
N2-Plasma_direct TACAGCACTCACAGGAGCCACAGAAATCCAAATGTCGTCAGGAAACTTGCTCTTCACTGGACATCTCAAGTGCAGGCTGA
N2-Plasma_5      ................................................................................
N2-Plasma_8      ................................................................................
N2-Plasma_M      ................................................................................
N2-Plasma_N      ................................................................................
N2-Plasma_O      ................................................................................
N2-Plasma_Q      ................................................................................
N2-Plasma_S      ................................................................................
N2-Plasma_S1     ................................................................................
N2-Plasma_U      ................................................................................
N2-Plasma_V      ................................................................................
N2-Plasma_W      ................................................................................

                                          Domain III
                       870       880       890       900       910       920       930       940
N2-Plasma_direct GAATGGACAAGCTACAGCTTAAAGGAATGTCATACTCTATGTGCACAGGAAAGTTTAAAGTTGTGAAGGAAATAGCAGAA
N2-Plasma_5      ................................................................................
N2-Plasma_8      .............................................................C..................
N2-Plasma_M      ................................................................................
N2-Plasma_N      ................................................................................
N2-Plasma_O      ........G.......................................................................
N2-Plasma_Q      ................................................................................
N2-Plasma_S      ................................................................................
N2-Plasma_S1     ................................................................................
N2-Plasma_U      .................................................................G..............
N2-Plasma_V      ................................................................................
N2-Plasma_W      ................................................................................

                       950       960       970       980       990      1000      1010      1020
N2-Plasma_direct ACACAACATGGAACGATAGTTATCAGAGTGCAATATGAAGGGGACGGCTCTCCATGTAAAATCCCTTTTGAGATAATGGA
N2-Plasma_5      ................................................................................
N2-Plasma_8      ................................................................................
N2-Plasma_M      ................................................................................
N2-Plasma_N      ................................................................................
N2-Plasma_O      ................................................................................
N2-Plasma_Q      ................................................................................
N2-Plasma_S      ................................................................................
N2-Plasma_S1     ................................................................................
N2-Plasma_U      ................................................................................
N2-Plasma_V      ................................................................................
N2-Plasma_W      ................................................................................

                      1030      1040      1050      1060      1070      1080      1090      1100
N2-Plasma_direct TTTGGAAAAAAGATATGTCTTAGGCCGCCTGATCACAGTCAACCCAATTGTAACAGAAAAAGATAGCCCAGTCAACATAG
N2-Plasma_5      ................................................................................
N2-Plasma_8      ................................................................................
N2-Plasma_M      ................................................................................
N2-Plasma_N      ................................................................................
N2-Plasma_O      ................................................................................
N2-Plasma_Q      ..................................T.............................................
N2-Plasma_S      ................................................................................
N2-Plasma_S1     ................................................................................
N2-Plasma_U      ................................................................................
N2-Plasma_V      ................................................................................
N2-Plasma_W      ................................................................................

                      1110      1120      1130      1140      1150      1160      1170
N2-Plasma_direct AAGCAGAACCCCCATTCGGAGACAGCTACATCATCATAGGAGTAGAGCCGGGACAACTGAAGCTCAAC
N2-Plasma_5      ....................................................................
N2-Plasma_8      ....................................................................
N2-Plasma_M      ....................................................................
N2-Plasma_N      ....................................................................
N2-Plasma_O      ...................................G................................
N2-Plasma_Q      ....................................................................
N2-Plasma_S      ....................................................................
N2-Plasma_S1     ....................................................................
N2-Plasma_U      ....................................................................
N2-Plasma_V      ....................................................................
N2-Plasma_W      ....................................................................
```

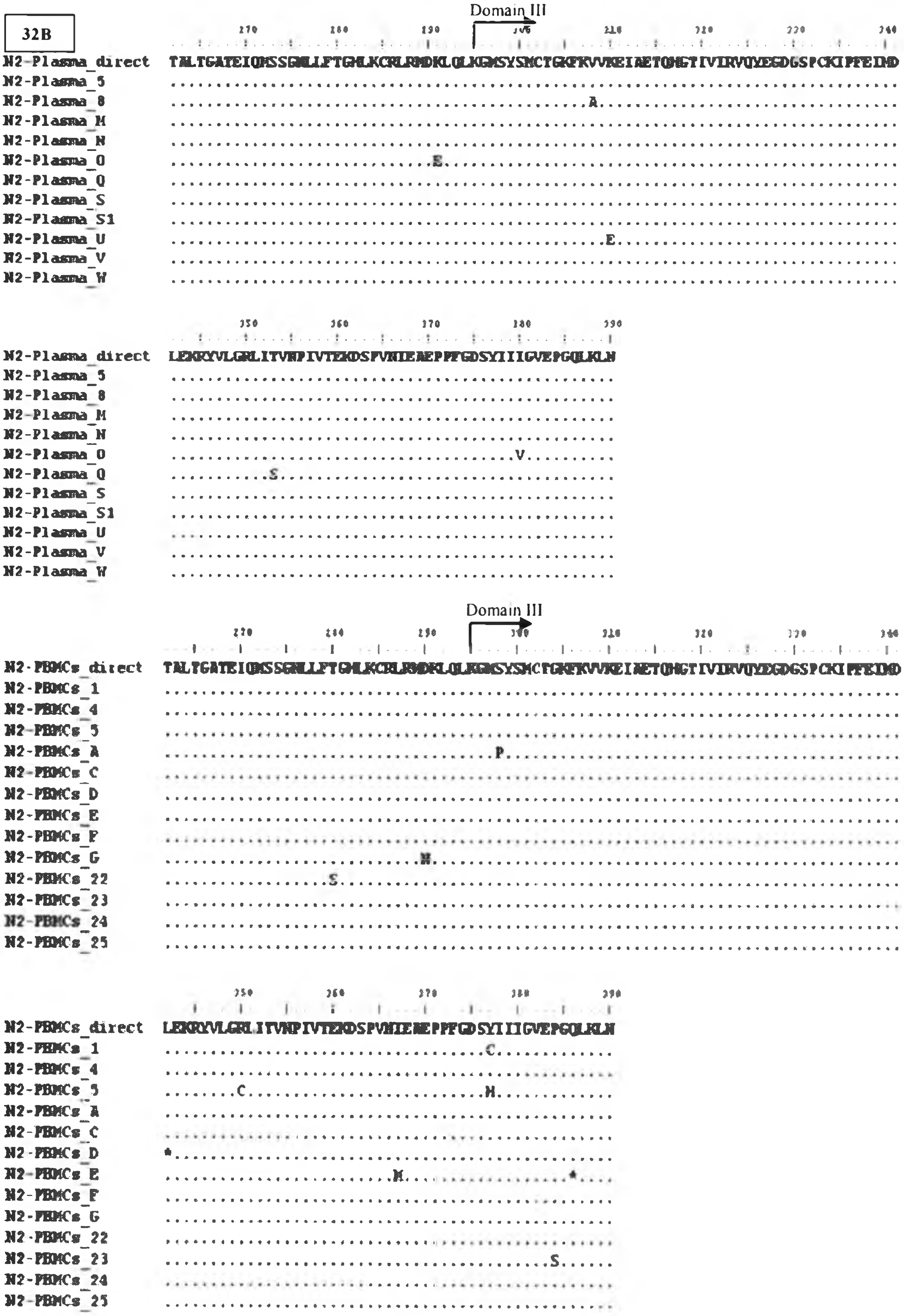

Domain III

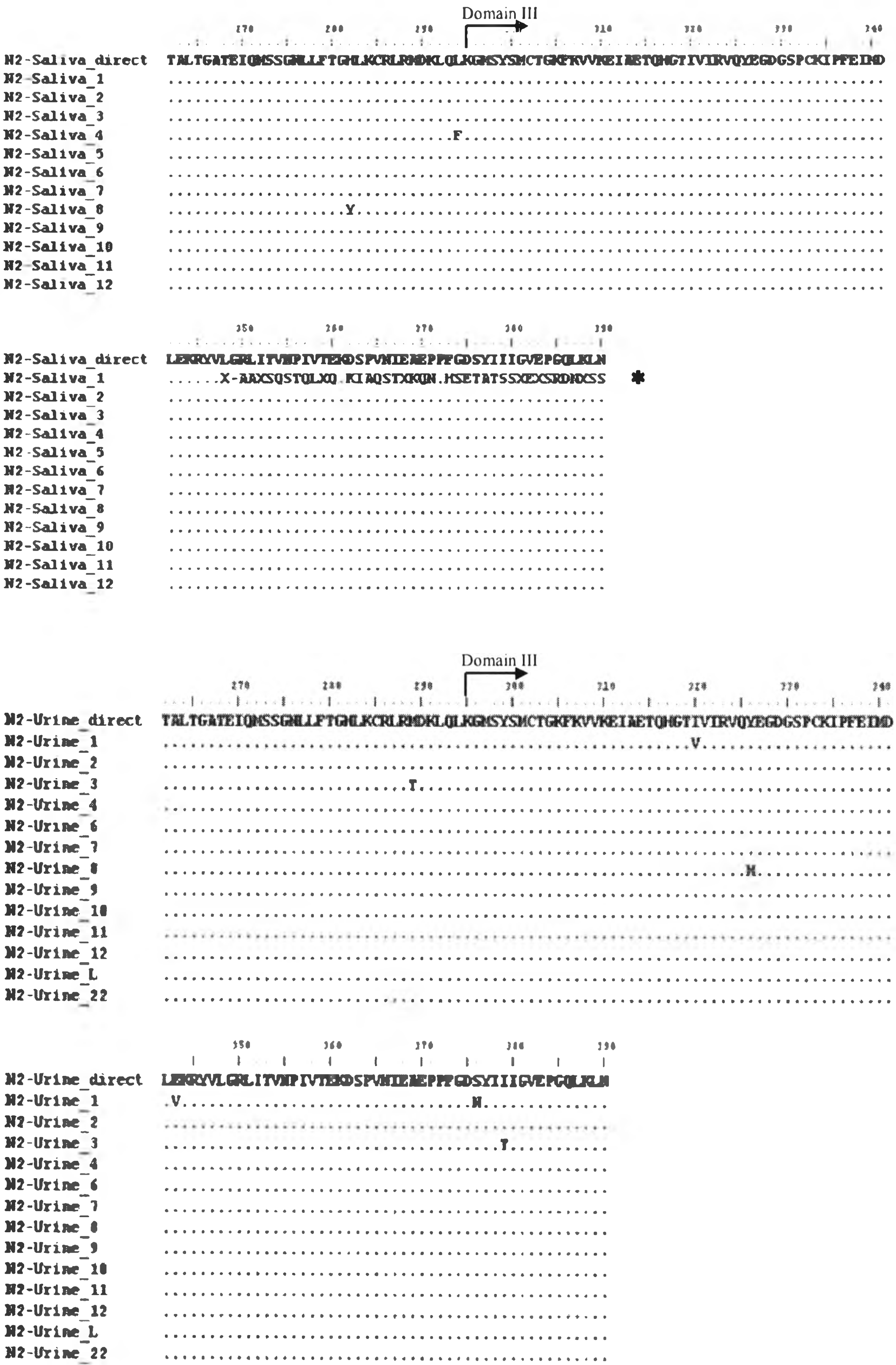


N2-Saliva_direct
TACAGCACTCACAGGAGCCACAGAAATCCAAATGTCGTCAGGAAACTTGCTCTTCACTGGACATCTCAAGTGCAGGCTGA
N2-Saliva_1
N2-Saliva_2
N2-Saliva_3
N2-Saliva_4
N2-Saliva_5
N2-Saliva_6
N2-Saliva_7
N2-Saliva_8
T
N2-Saliva_9
N2-Saliva_10
N2-Saliva_11
N2-Saliva_12
Domain III
910
920
940
N2-Saliva_direct
GAATGGACAAGCTACAGCTTAAAGGAATGTCATACTCTATGTGCACAGGAAAGTTTAAAGTTGTGAAGGAAATAGCAGAA
N2-Saliva_1
N2-Saliva_2
N2-Saliva_3
N2-Saliva_4
T
N2-Saliva_5
N2-Saliva_6
N2-Saliva_7
N2-Saliva_8
G
N2-Saliva_9
N2-Saliva_10
T
N2-Saliva_11
N2-Saliva_12
960
970
1010
1020
N2-Saliva_direct
ACACAACATGGAACGATAGTTATCAGAGTGCAATATGAAGGGGACGGCTCTCCATGTAAAATCCCTTTTGAGATAATGGA
N2-Saliva_1
N2-Saliva_2
N2-Saliva_3
N2-Saliva_4
N2-Saliva_5
N2-Saliva_6
N2-Saliva_7
N2-Saliva_8
N2-Saliva_9
N2-Saliva_10
C
N2-Saliva_11
N2-Saliva_12
1030
1040
1050
1060
1070
1080
1090
1100
N2-Saliva_direct
TTTGGAAAAAAGATATGTCTTAGGCCGCCTGATCACAGTCAACCCAATTGTAACAGAAAAAGATAGCCCAGTCAACATAG
N2-Saliva_1
-
N2-Saliva_2
N2-Saliva_3
N2-Saliva_4
N2-Saliva_5
N2-Saliva_6
N2-Saliva_7
N2-Saliva_8
N2-Saliva_9
N2-Saliva_10
N2-Saliva_11
N2-Saliva_12
1110
1120
1130
1140
1150
1160
1170
N2-Saliva_direct
AAGCAGAACCCCCATTCGGAGACAGCTACATCATCATAGGAGTAGAGCCGGGACAACTGAAGCTCAAC
N2-Saliva_1
N2-Saliva_2
N2-Saliva_3
N2-Saliva_4
N2-Saliva_5
N2-Saliva_6
N2-Saliva_7
N2-Saliva_8
N2-Saliva_9
N2-Saliva_10
N2-Saliva_11
N2-Saliva_12

```
                             790       800       810       820       830       840       850       860
N2-Urine_direct     TACAGCACTCACAGGAGCCACAGAAATCCAAATGTCGTCAGGAAACTTGCTCTTCACTGGACATCTCAAGTGCAGGCTGA
N2-Urine_1          ................................................................................
N2-Urine_2          ................................................................................
N2-Urine_3          ................................................................................
N2-Urine_4          ................................................................................
N2-Urine_6          ................................................................................
N2-Urine_7          ................................................................................
N2-Urine_8          ................................................................................
N2-Urine_9          ................................................................................
N2-Urine_10         ................................................................................
N2-Urine_11         ................................................................................
N2-Urine_12         ................................................................................
N2-Urine_L          ................................................................................
N2-Urine_22         ................................................................................

                                         Domain III
                             870       880       890       900       910       920       930       940
N2-Urine_direct     GAATGGACAAGCTACAGCTTAAAGGAATGTCATACTCTATGTGCACAGGAAAGTTTAAAGTTGTGAAGGAAATAGCAGAA
N2-Urine_1          ................................................................................
N2-Urine_2          ................................................................................
N2-Urine_3          ...C............................................................................
N2-Urine_4          ................................................................................
N2-Urine_6          ................................................................................
N2-Urine_7          ................................................................................
N2-Urine_8          ................................................................................
N2-Urine_9          ................................................................................
N2-Urine_10         ................................................................................
N2-Urine_11         ................................................................................
N2-Urine_12         ................................................................................
N2-Urine_L          ................................................................................
N2-Urine_22         ................................................................................

                             950       960       970       980       990      1000      1010      1020
N2-Urine_direct     ACACAACATGGAACGATAGTTATCAGAGTGCAATATGAAGGGGACGGCTCTCCATGTAAAATCCCTTTTGAGATAATGGA
N2-Urine_1          ...............G................................................................
N2-Urine_2          ................................................................................
N2-Urine_3          ................................................................................
N2-Urine_4          ................................................................................
N2-Urine_6          ................................................................................
N2-Urine_7          ................................................................................
N2-Urine_8          .................................C..............................................
N2-Urine_9          ................................................................................
N2-Urine_10         ................................................................................
N2-Urine_11         ................................................................................
N2-Urine_12         ................................................................................
N2-Urine_L          ................................................................................
N2-Urine_22         ................................................................................

                            1030      1040      1050      1060      1070      1080      1090      1100
N2-Urine_direct     TTTGGAAAAAAGATATGTCTTAGGCCGCCTGATCACAGTCAACCCAATTGTAACAGAAAAAGATAGCCCAGTCAACATAG
N2-Urine_1          .....T..........................................................................
N2-Urine_2          ................................................................................
N2-Urine_3          ................................................................................
N2-Urine_4          ................................................................................
N2-Urine_6          ................................................................................
N2-Urine_7          ................................................................................
N2-Urine_8          ................................................................................
N2-Urine_9          ................................................................................
N2-Urine_10         ................................................................................
N2-Urine_11         ................................................................................
N2-Urine_12         ................................................................................
N2-Urine_L          ................................................................................
N2-Urine_22         ............................................................G...................

                            1110      1120      1130      1140      1150      1160      1170
N2-Urine_direct     AAGCAGAACCCCCATTCGGAGACAGCTACATCATCATAGGAGTAGAGCCGGGACAACTGAAGCTCAAC
N2-Urine_1          ........................A...........................................
N2-Urine_2          ....................................................................
N2-Urine_3          .................................C..................................
N2-Urine_4          ....................................................................
N2-Urine_6          ....................................................................
N2-Urine_7          ....................................................................
N2-Urine_8          ....................................................................
N2-Urine_9          ....................................................................
N2-Urine_10         ....................................................................
N2-Urine_11         ....................................................................
N2-Urine_12         ....................................................................
N2-Urine_L          ....................................................................
N2-Urine_22         ....................................................................
```

```
                       790       800       810       820       830       840       850       860
N2_2-PBMCs_direct  TACAGCACTCACAGGAGCCACAGAAATCCAAATGTCGTCAGGAAACCTGCTCTTCACTGGACATCTCAAGTGCAGGCTGA
N2_2-PBMCs_1       ..............................................T.................................
N2_2-PBMCs_2       ................................................................................
N2_2-PBMCs_3       ................................................................................
N2_2-PBMCs_4       ............................................................G...................
N2_2-PBMCs_5       ................................................................................
N2_2-PBMCs_6       ............................................................G...................
N2_2-PBMCs_7       ...G........................................................G...................
N2_2-PBMCs_8       ................................................................................
N2_2-PBMCs_10      ................................................................................
N2_2-PBMCs_11      ................................................................................
N2_2-PBMCs_12      ................................................................................
N2_2-PBMCs_21      ................................................................................
N2_2-PBMCs_22      .....................................................C......G...................

                                     Domain III
                       870       88  |-->      900       910       920       930       940
N2_2-PBMCs_direct  GAATGGACAAGCTACAGCTTAAAGGAATGTCATACTCCATGTGCACAGGAAAGTTTAAAGTTGTGAAGGAAATAGCAGAA
N2_2-PBMCs_1       .....................................T..........................................
N2_2-PBMCs_2       ................................................................................
N2_2-PBMCs_3       ................................................................................
N2_2-PBMCs_4       ..................................................................G.............
N2_2-PBMCs_5       ................................................................................
N2_2-PBMCs_6       ................................................................................
N2_2-PBMCs_7       ................................................................................
N2_2-PBMCs_8       ................................................................................
N2_2-PBMCs_10      ................................................................................
N2_2-PBMCs_11      ................................................................................
N2_2-PBMCs_12      ................................................................................
N2_2-PBMCs_21      ................................................................................
N2_2-PBMCs_22      ................................................................................

                       950       960       970       980       990       1000      1010      1020
N2_2-PBMCs_direct  ACACAACATGGAACGATAGTCATCAGAGTGCAATATGAAGGGGATGGCTCTCCATGTAAAATCCCTTTTGAGATAATGGA
N2_2-PBMCs_1       ....................T.......................C...................................
N2_2-PBMCs_2       ................................................................................
N2_2-PBMCs_3       ................................................................................
N2_2-PBMCs_4       ................................................................................
N2_2-PBMCs_5       ................................................................................
N2_2-PBMCs_6       ................................................................................
N2_2-PBMCs_7       ................................................................................
N2_2-PBMCs_8       ................................................................................
N2_2-PBMCs_10      ................................................................................
N2_2-PBMCs_11      ................................................................................
N2_2-PBMCs_12      ................................................................................
N2_2-PBMCs_21      ................................................................................
N2_2-PBMCs_22      ................................................................................

                       1030      1040      1050      1060      1070      1080      1090      1100
N2_2-PBMCs_direct  TTTGGAAAAAAGATATGTCTTAGGCCGCCTGATCACAGTCAACCCAATTGTAACAGAAAAAGACAGCCCAGTCAACATAG
N2_2-PBMCs_1       ...............................................................T................
N2_2-PBMCs_2       ................................................................................
N2_2-PBMCs_3       ................................................................................
N2_2-PBMCs_4       ................................................................................
N2_2-PBMCs_5       ................................................................................
N2_2-PBMCs_6       ................................................................................
N2_2-PBMCs_7       ................................................................................
N2_2-PBMCs_8       ................................................................................
N2_2-PBMCs_10      ................................................................................
N2_2-PBMCs_11      ................................................................................
N2_2-PBMCs_12      ................................................................................
N2_2-PBMCs_21      ................................................................................
N2_2-PBMCs_22      ................................................................................

                       1110      1120      1130      1140      1150      1160      1170
N2_2-PBMCs_direct  AAGCAGAACCTCCATTTGGAGACGGCTACATCATCATAGGAGTAGAGCCGGGACAACTGAAGCTCAAC
N2_2-PBMCs_1       ................C......A............................................
N2_2-PBMCs_2       ....................................................................
N2_2-PBMCs_3       ....................................................................
N2_2-PBMCs_4       .......................A............................................
N2_2-PBMCs_5       ....................................................................
N2_2-PBMCs_6       .......................A............................................
N2_2-PBMCs_7       .......................A............................................
N2_2-PBMCs_8       ..T.................................................................
N2_2-PBMCs_10      ....................................................................
N2_2-PBMCs_11      ....................................................................
N2_2-PBMCs_12      ....................................................................
N2_2-PBMCs_21      ....................................................................
N2_2-PBMCs_22      .......................A............................................
```


32B
Domain III
N2-Plasma_direct
N2-Plasma_5
N2-Plasma_8
N2-Plasma_M
N2-Plasma_N
N2-Plasma_O
N2-Plasma_Q
N2-Plasma_S
N2-Plasma_S1
N2-Plasma_U
N2-Plasma_V
N2-Plasma_W
N2-PBMCs_direct
N2-PBMCs_1
N2-PBMCs_4
N2-PBMCs_5
N2-PBMCs_A
N2-PBMCs_C
N2-PBMCs_D
N2-PBMCs_E
N2-PBMCs_F
N2-PBMCs_G
N2-PBMCs_22
N2-PBMCs_23
N2-PBMCs_24
N2-PBMCs_25


Domain III
N2-Saliva_direct
N2-Saliva_1
N2-Saliva_2
N2-Saliva_3
N2-Saliva_4
N2-Saliva_5
N2-Saliva_6
N2-Saliva_7
N2-Saliva_8
N2-Saliva_9
N2-Saliva_10
N2-Saliva_11
N2-Saliva_12
Domain III
N2-Urine_direct
N2-Urine_1
N2-Urine_2
N2-Urine_3
N2-Urine_4
N2-Urine_6
N2-Urine_7
N2-Urine_8
N2-Urine_9
N2-Urine_10
N2-Urine_11
N2-Urine_12
N2-Urine_L
N2-Urine_22

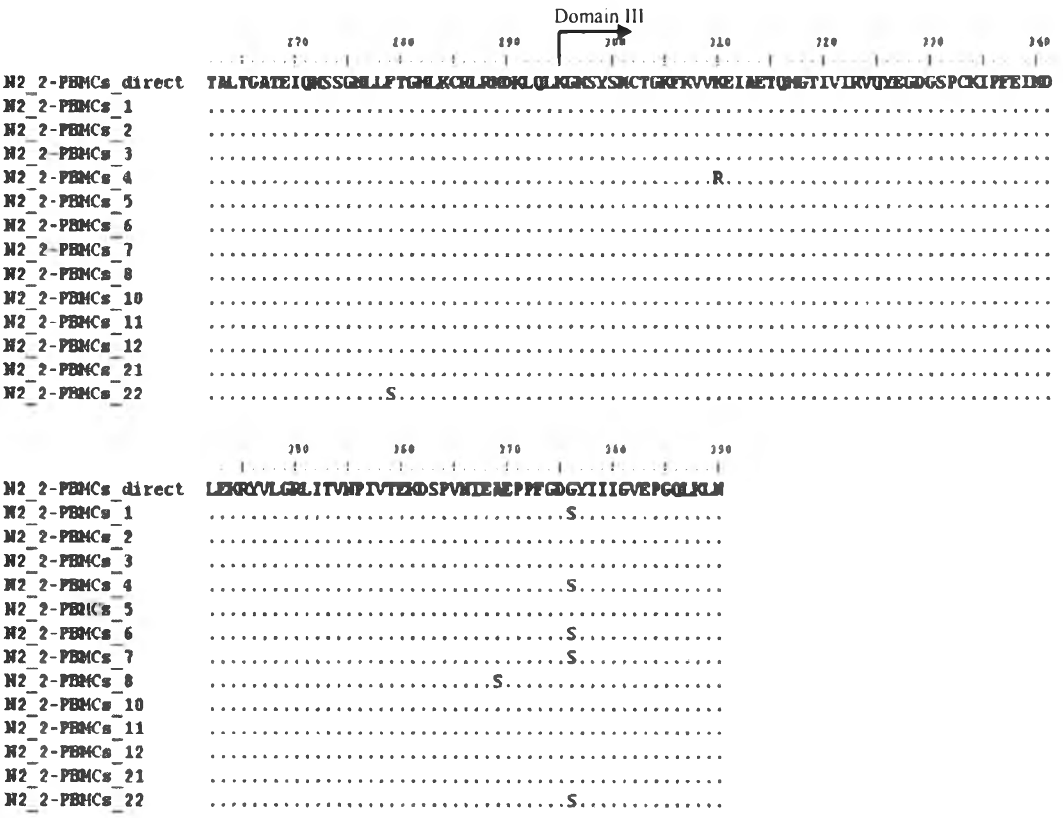


Figure 32: Nucleotide (32A) and amino (32B) sequence alignments of all clones derived from each N2 specimens, N2 plasma (3), N2 PBMCs (3), N2 saliva (3), N2 urine (3) and N2 PBMCs (21), (DENV2 infected patients) present heterogeneous population or quasispecies in different time points. Direct sequencing of each specimen is used as a reference sequence (major population) to compare with all variant sequences or minor populations (10-15 sequences per specimen). The positions of nucleotide and amino acid are based on direct sequencing alignments in figure 28A and 28B. Domain III of E gene is starting from position 883 to 1,180 of nucleotide and position 295-395 of amino acid sequences (complete E gene). Deletion position is presented as "-". In-frame stop codon is presented as " * ". The presence of frame-shift mutations were marked as "✱".The number in "( )" represents the day of specimen collection.

Moreover, some clones contained deletion and in-frame stop codon suggesting the presence of defective viral genome as totally 9.54% of all clones analyzed (in all patients). In single-nucleotide-deleted sequences, frame-shift mutations were found such as in some clones of N12 specimens and febrile saliva of N2 (Figure 31B and 32B). A number of defective clones were different among specimens and time points. Defective clones were commonly found in plasma, PBMCs, saliva and urine of DENV-infected patients. Nevertheless, specimens in some patients such as N20 and

N33 did not contain defective viral genome (data not shown). During febrile period, defective viral genome was mostly found in plasma or PBMCs than in urine and saliva while it was found in PBMCs and urine during convalescent period. Moreover, the presence of defective viral genome randomly occurred and did not depend on serotypes, genotypes and strains of DENV.

Normalized Shannon entropy (S_n) was used as a tool for determining the diversity and complexity of DENV quasispecies in both nucleotide and amino acid levels. The reference range was 0 (no diversity) to 1 (maximum diversity). In mixed-serotype-infected specimens, S_n value was calculated in both major serotypes and in minor serotype if there were sufficient clones to investigate. In this study, the S_n value ranged from 0.08 to 0.207 and 0.07 to 0.207 at nucleotide and amino acid levels, respectively in all patients (Table 33). The S_n value did not depend on each serotype. Moreover, the S_n value varied in specimens and time points suggesting the presence of different diversity (quasispecies) of DENV population. In DENV1-infected patients, the high S_n value of nucleotide level was found in febrile plasma and PBMCs indicating the occurrence of high quasispecies complexity during febrile period. Interestingly, the high S_n value was also found in early and late convalescent urine (N12 and N20). In DENV2-infected patients, the high S_n value was mostly found in febrile PBMCs or plasma. However, some patient such as N13 presented the high S_n value in 2nd convalescent urine. DENV3-infected patient (N28) presented the high S_n value in early and late convalescent urine. In mixed-serotype-infected patients (N33, N34 and N40), the high S_n value was found in multi-serotype-infected specimens (analyzed in the major serotype only).

The different S_n values at amino acid level in each specimen and time point confirmed the presence of heterogeneous population or quasispecies and indicated the diversity of E protein, especially on domain III of DENV which was the major antigenic site for host immune recognition. The S_n value at amino acid level was mostly consistence with the results of nucleotide level and varied in each specimen and time point (Table 33). In some specimens, the results were contradicted with the results of nucleotide level suggesting the presence of silently mutated clones resulting in the conservation of E protein among DENV populations such as in early convalescent urine of N12, febrile plasma of N29, 2nd early convalescent urine of

N13, late convalescent urine of N28, 2nd early convalescent urine of N34 and early convalescent PBMCs of N40.

According to previous studies mentioning that the variations of E gene, particularly at domain III affect pathogenesis of DENV infection and are under selection pressure of host immune response, the estimation of dN/dS ratio was investigated using SLAC (single likelihood ancestor counting) method in the web-based Datamonkey (www.datamonkey.org) to explore whether amino acid variations, especially on the domain III of E gene were under the selection pressure [139, 140]. In mixed-serotype-infected specimens, only major serotype populations were examined as well. Moreover, some nucleotide clones containing deletion or in-frame stop codon and minor DENV serotype populations in mixed-serotype-infected specimens were omitted because of the limitation of program requirement.

The results demonstrated that amino acid variations at domain III in all specimens of each patient during febrile, early and late convalescent periods were under either positive (dN/dS > 1), purifying (dN/dS <1) or neutral selection (dN/dS=1) suggesting that host immune pressure plays a role in genetic variations of DENV populations (Table 33). The range of dN/dS was from 0.20-2.73 in all specimens and time points. Most specimens in all time points were under purifying selection whereas some specimens were under either positive or neutral selection. The patterns of selection pressure in each specimen of individual patient were different depending on each specimen type and time point. For example, amino acid sequence variations of most specimens in N2 patient were under neutral selection whereas the amino acid variations of all specimens in N20 patient were under positive selection. Moreover, mutations of amino acid sequences in all specimens of some patients (N17 and N34) were under purifying selection. The positive selection affecting amino acid changes were mostly found in febrile plasma (N12, N29, N28 and N40) and PBMCs (N5, N10 and N28) as well as in febrile urine (N2, N20 and N40). During early and late convalescent periods, the positive selection was found in PBMCs (N13) and urine (N5, N13, N20, N28 and N33). This finding indicates that host immune response may drive amino acid variations and involve in persistent DENV population. Interestingly, this study demonstrated the purifying selection codon in febrile plasma of N17 at amino acid position 380. The dN/dS ratio could not be investigated in all minor serotype population of mixed-serotype-infected specimens because there were not enough unique sequences to calculate by this algorithm.

Table 33: Diversity (complexity) parameters of nucleotide and amino acid sequences of 13 DENV-infected patients in different specimens and time points

Code	DOF	Specimens	No. of clones	Nucleotide level			Amino acid level			No. of defective clones	dN/dS
				SS	DS	S_n	SS	DS	S_n		
DENV1-infected patients											
N12	6	plasma (4)	12	3	9	0.184	3	9	0.184	3	1.60
		PBMCs (4)	11	2	9	0.207	2	9	0.207	4	0.53
		urine (12)	11	2	9	0.207	3	7	0.179	2	0.90
		urine (26)	12	2	10	0.197	2	8	0.175	5	0.57
N20	8	urine (6)	12	7	5	0.112	7	5	0.112	0	1.19
		urine (30)	12	5	7	0.151	6	6	0.132	0	2.07
N29	8	plasma (5)	12	4	8	0.169	6	6	0.132	0	1.80
		PBMCs (5)	12	4	8	0.169	5	7	0.151	0	0.83
		urine (5)	12	5	7	0.151	5	7	0.151	1	0.84
		urine (19)	12	5	7	0.151	8	4	0.095	1	0.41
DENV2-infected patients											
N2	4	plasma (3)	11	7	4	0.105	7	4	0.105	0	1.00
		PBMCs (3)	13	4	9	0.164	6	7	0.134	2	1.00
		saliva (3)	12	9	3	0.070	9	3	0.070	1	1.00
		urine (3)	13	9	4	0.080	10	3	0.061	0	1.81
		PBMCs (21)	13	7	6	0.117	7	4	0.103	0	0.21
N5	7	plasma (6)	12	7	5	0.112	7	5	0.112	3	1.00
		PBMCs (6)	12	5	7	0.151	6	6	0.132	1	2.73
		urine (23)	13	5	8	0.150	6	5	0.117	1	1.40
N10	5	plasma (4)	13	6	7	0.134	8	5	0.099	4	0.20
		PBMCs (4)	12	10	2	0.047	10	2	0.047	0	1.01
		urine (4)	12	11	1	0.024	11	1	0.024	0	1.00
		PBMCs (27)	13	8	5	0.099	10	3	0.061	0	0.56
N13	5	PBMCs (8)	15	3	12	0.166	4	11	0.161	3	1.44
		urine (8)	12	5	7	0.151	5	7	0.151	3	1.00
		urine (15)	12	2	10	0.197	6	6	0.132	3	0.56
N17	7	plasma (7)	12	5	7	0.151	6	6	0.132	2	0.47[#]
		PBMCs (7)	13	4	9	0.164	5	8	0.150	0	0.67
		saliva (7)	12	10	2	0.047	11	1	0.024	0	0.39
		urine (7)	12	7	5	0.112	7	5	0.112	1	0.60
		urine (13)	12	5	7	0.151	7	5	0.112	1	0.40
N21	7	PBMCs (7)	12	4	8	0.169	8	4	0.092	0	0.38
		urine (14)	13	9	3	0.072	9	3	0.072	0	1.80
DENV3-infected patient											
N28	8	plasma (6)	13	3	10	0.178	6	7	0.134	0	1.85
		PBMCs (6)	12	5	7	0.151	8	4	0.092	1	1.14

Code	DOF	Specimens	No of clones	Nucleotide level			Amino acid level			No of defective clones	dN/dS
				SS	DS	S_n	SS	DS	S_n		
N28	8	urine (14)	12	3	9	0.184	5	7	0.134	1	0.85
		urine (46)	12	3	9	0.184	6	6	0.132	0	1.17
All N33 specimens were DENV4 except N33 urine (7) was DENV4+DENV2 (major serotype was DENV4)											
N33	6	plasma (7)	14	12	2	0.036	12	2	0.036	0	0.20
		PBMCs (7)	11	9	2	0.036	10	1	0.028	0	1.00
		saliva (7)	12	6	6	0.132	7	5	0.112	0	0.80
		urine (7)†	11	5	6	0.151	6	5	0.112	0	1.76
		urine (7)•	4	3	1	0.141	3	1	0.141	0	ND
		urine (18)	13	8	4	0.091	9	3	0.072	0	0.26
All N34 specimens were DENV1 except N34 urine (14) was DENV3+DENV1 (major serotype was DENV3)											
N34	6	PBMCs (8)	14	3	11	0.172	5	9	0.147	4	0.46
		saliva (8)	13	4	9	0.164	8	5	0.099	0	0.65
		urine (8)	13	4	9	0.164	6	7	0.134	1	0.53
		urine (14)†	14	3	11	0.172	9	5	0.088	0	0.18
		urine (14)•	2	ND	ND	ND	ND	ND	ND	ND	ND
All N40 specimens were DENV1 except N40 PBMCs (21) was DENV1+DENV2 (major serotype was DENV2)											
N40	4	plasma (4)	12	7	5	0.112	7	5	0.112	3	1.80
		PBMCs(4)	15	3	12	0.166	5	10	0.145	3	0.31
		urine (4)	12	7	5	0.112	7	5	0.112	2	1.35
		PBMCs (21)†	12	2	10	0.197	5	6	0.142	2	0.11
		PBMCs (21)•	1	ND	ND	ND	ND	ND	ND	ND	ND

DOF= duration of fever. ND = not determined.

The number in "( )" presents the day of specimen collection.

S_n = Normalized Shannon entropy.

SS = No. of similar sequences to direct sequencing.

DS= No. of different sequence patterns to direct sequencing.

Defective clone refers to the clone containing nucleotide deletion or in-frame stop codon.

†=Major population in mixed serotype infections. •=Minor population in mixed serotype infections.

dN/dS >1 suggests under positive selection and dN/dS < 1 suggests under purifying selection.

#Negative selection site was found in this specimen (p<0.1) at amino acid position 380 (codon 119 of 129 amino acid, Ile).

Phylogenetic tree analysis to demonstrate the association of DENV population in different specimens and time points

To characterize the association of each DENV population in different specimens and time points in each patient, phylogenetic tree of nucleotide sequences was done using MEGA program (version 5.0) based on neighbor-joining (NJ) method with Kimura 2-parameter model (1,000 replicates) [141, 142].

The phylogenetic trees of nucleotide sequences from all clones showed the intra- and inter- genetic variations among specimens in each patient (Figure 33-45). Sequences in all patients were similar but not absolutely identical confirming the presence of quasispecies or heterogeneous population, which supported nucleotide and amino acid sequence alignments in the previous experiment. The association among sequences randomly occurred in each patient and there was no specific pattern of this phenomenon. Nucleotide sequences of all clones in most patients were similarity without identical such as in N2, N10, N17, N20, N28, N29, and N40 patients. However, results of some patients showed the identical sequences in the same specimen such as in early convalescent urine of N5, N21 and N33. Moreover, nucleotide sequences of some patients composed of similar and identical sequences in different specimens at the same time point such as in N13 patient (1^{st} early convalescent PBMCs and urine, marked as the red box in Figure 37) and in N34 patient (1^{st} early convalescent PBMCs and saliva, marked as the red box in Figure 44). Nucleotide sequences of one patient (N12) illustrated similar and identical sequences in different specimens and time points (febrile PBMCs and early convalescent urine, marked as the red box in Figure 36) implying the occurrence of persistent DENV population in different time points. Interestingly, the unique DENV sequence was found in early convalescent PBMCs of N2 locating in different clusters of all sequences from N2 specimens (marked as the red box in Figure 33) and one sequence of early convalescent PBMCs was related to most febrile sequences (marked as the red arrow in Figure 33) indicating either coinfection or superinfection and persistent DENV population in PBMCs.

The nucleotide sequences of 3 mixed-serotype-infected patients clearly presented the distinguished serotype (major and minor serotypes) and each serotype composed of both related and identical sequences indicating DENV quasispecies as well (Figure 43-45).

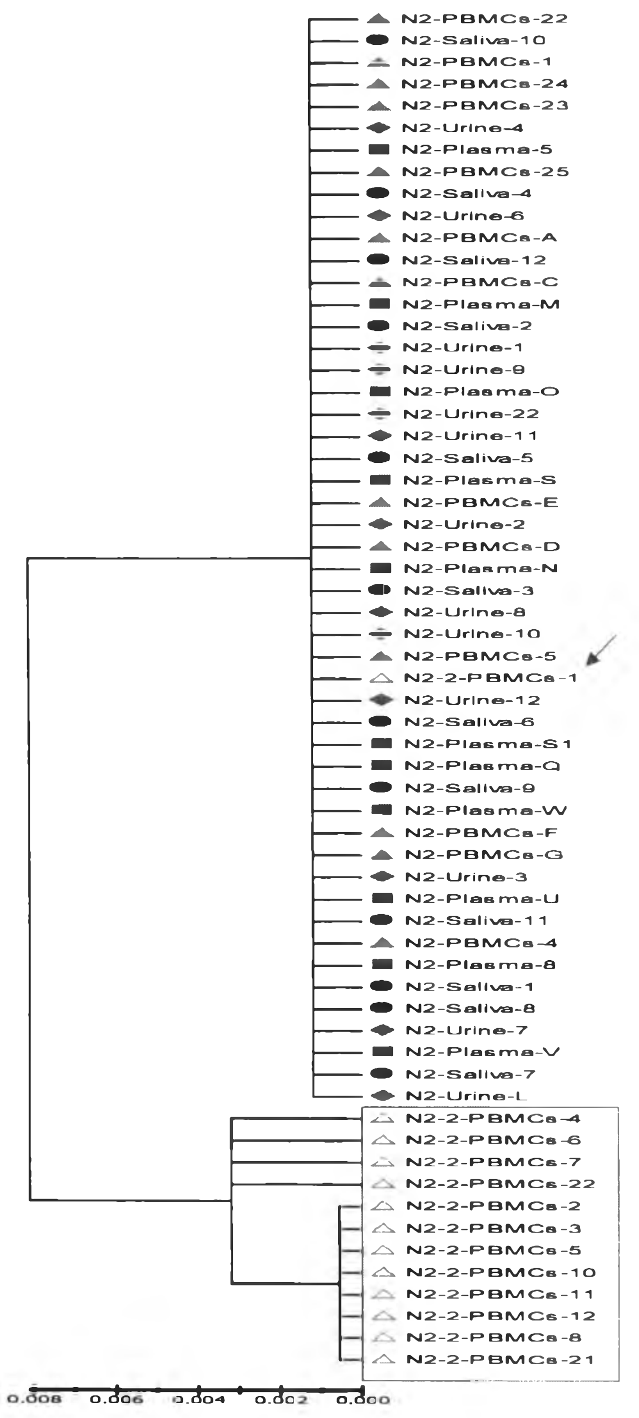


Figure 33: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N2 (DENV2-infected patient). There are febrile (N2 plasma, PBMCs, saliva and urine) and early convalescent (N2-2PBMCs) specimens. The unique DENV population in different time points is marked as the red box.

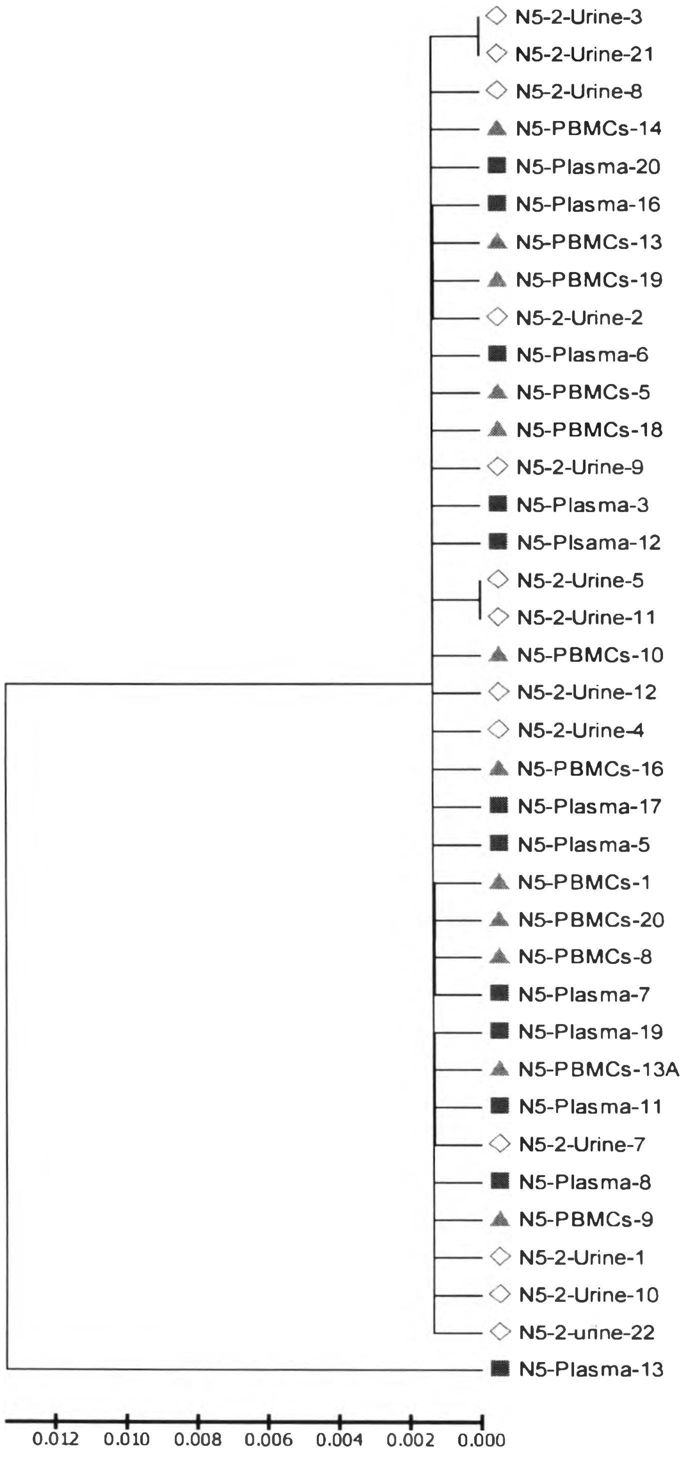


Figure 34: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N5 (DENV2-infected patient). There are febrile (N5 plasma and PBMCs) and early convalescent (N5-2 urine) specimens.

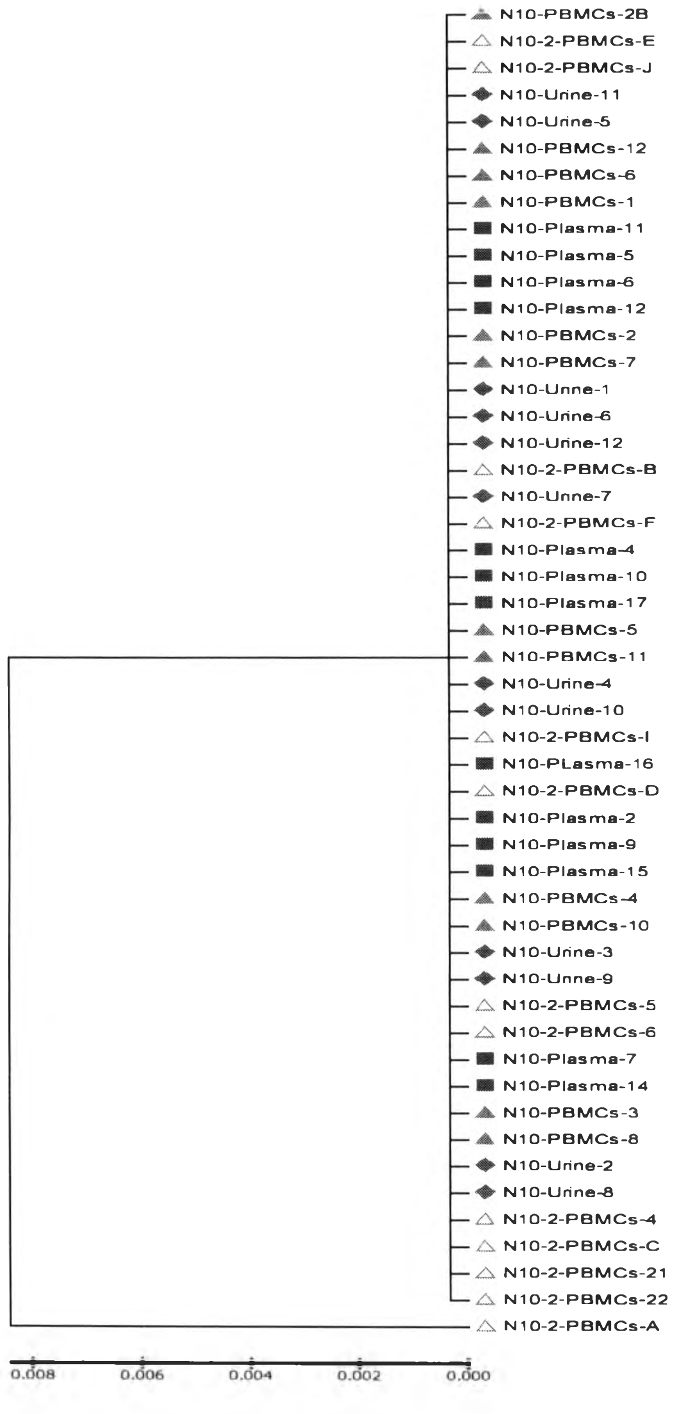


Figure 35: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N10 (DENV2-infected patient). There are febrile (N10 plasma, PBMCs and urine) and late convalescent (N10-2 PBMCs) specimens.

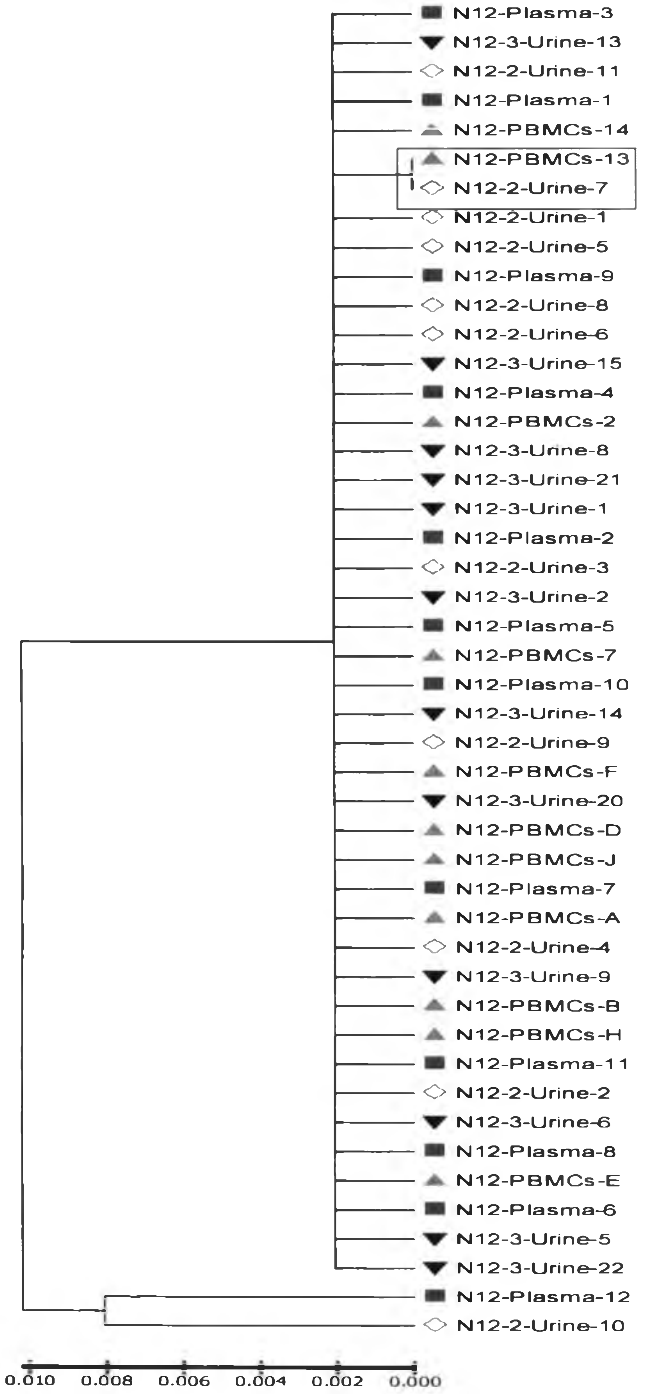


Figure 36: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N12 (DENV1-infected patient). There are febrile (N12 plasma, PBMCs), early convalescent (N12-2 urine) and late convalescent (N12-3 urine) specimens. The same DENV population in different specimens and time points is marked as the red box.

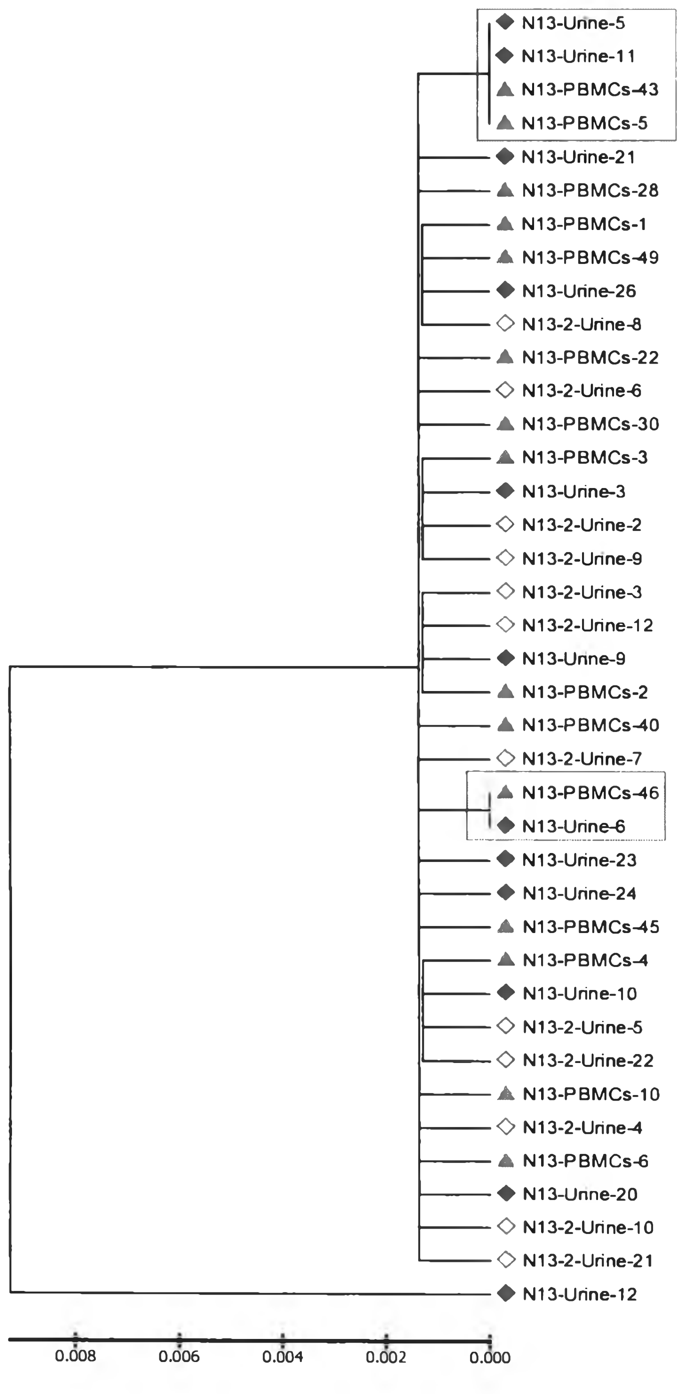


Figure 37: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N13 (DENV2-infected patient). There are 1st early convalescent (N13 PBMCs and urine) and 2nd early convalescent (N13-2 urine) specimens. The same DENV population in different specimens is marked as the red box.

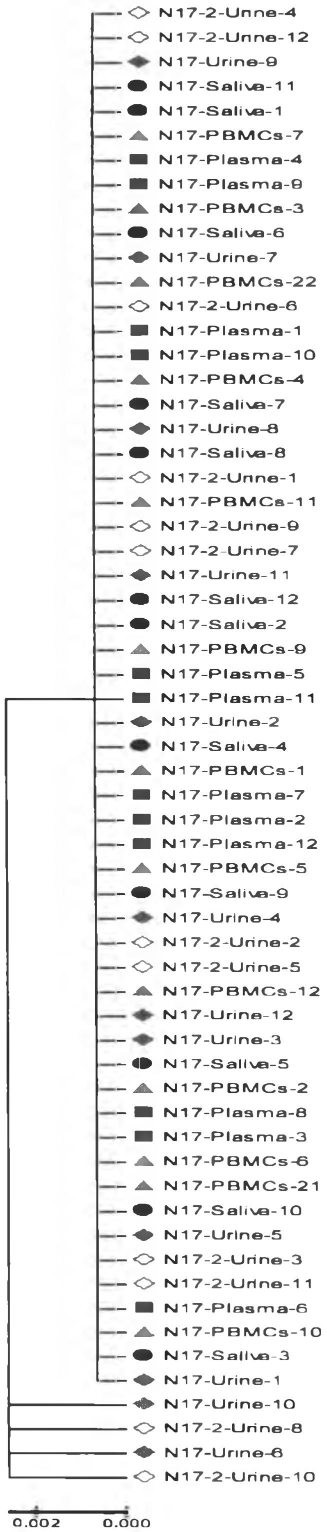


Figure 38: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N17 (DENV2-infected patient). There are febrile (N17 plasma, PBMCs, saliva and urine) and early convalescent (N17-2 urine) specimens.

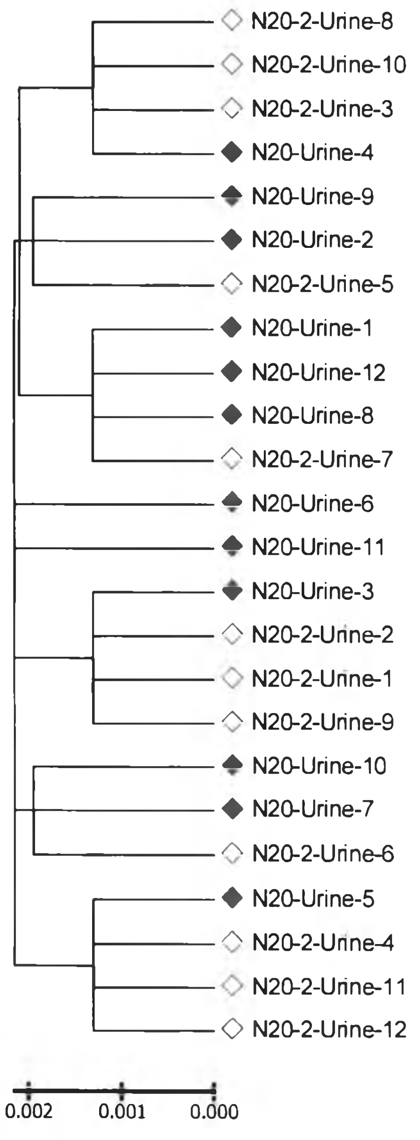


Figure 39: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N20 (DENV- infected patient). There are febrile (N20 urine) and late convalescent (N20-2 urine) specimens.

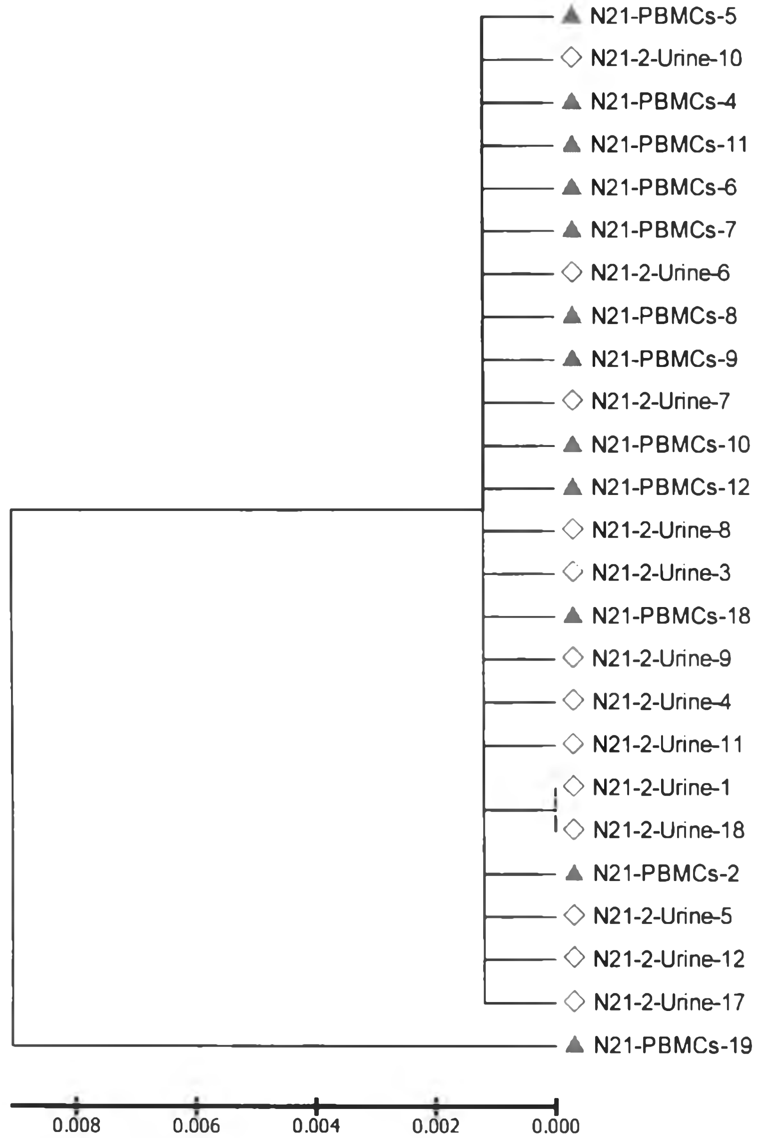


Figure 40: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N21 (DENV2-infected patient). There are febrile (N2 PBMCs) and early convalescent (N21-2 urine) specimens.

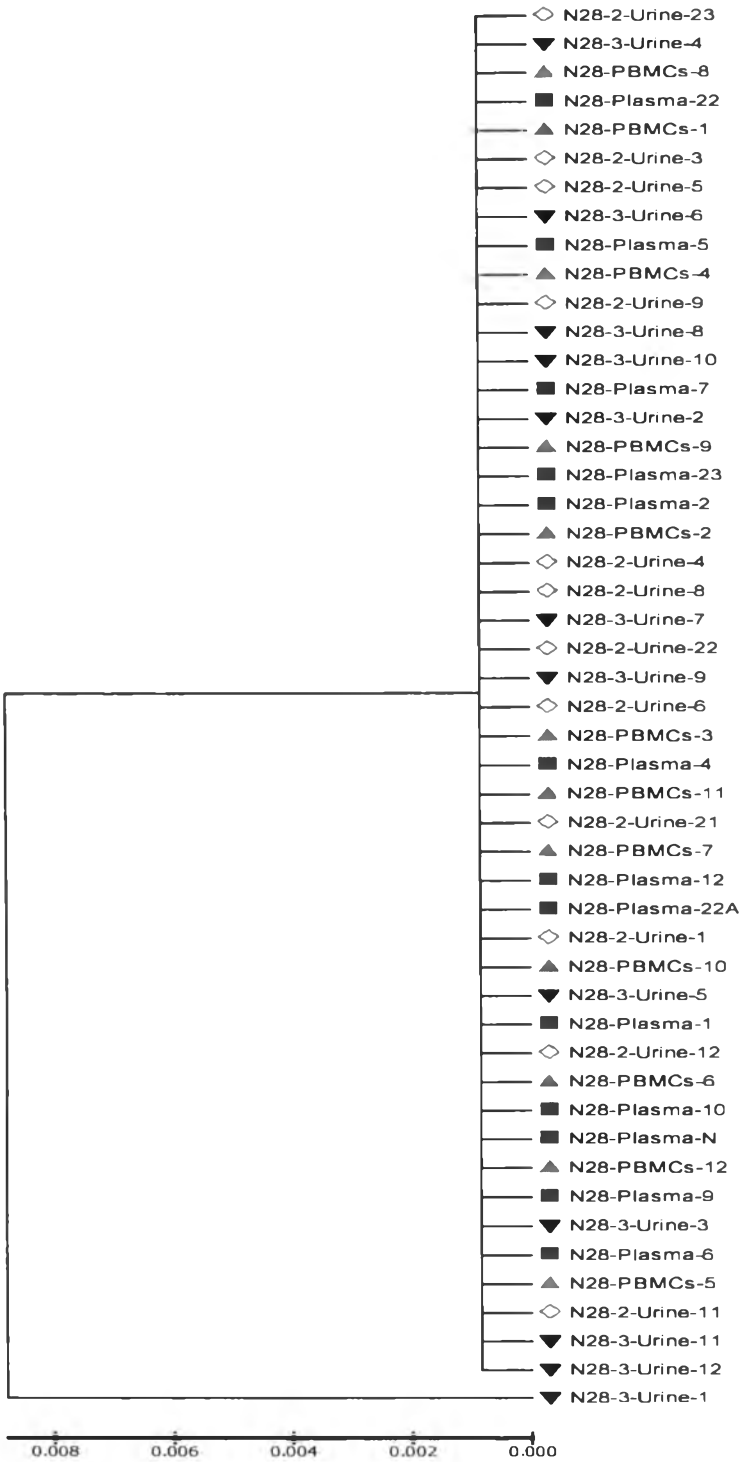


Figure 41: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N28 (DENV3-infected patient). There are febrile (N28 plasma and PBMCs), early convalescent (N28-2 urine) and late convalescent (N28-3 urine) specimens.

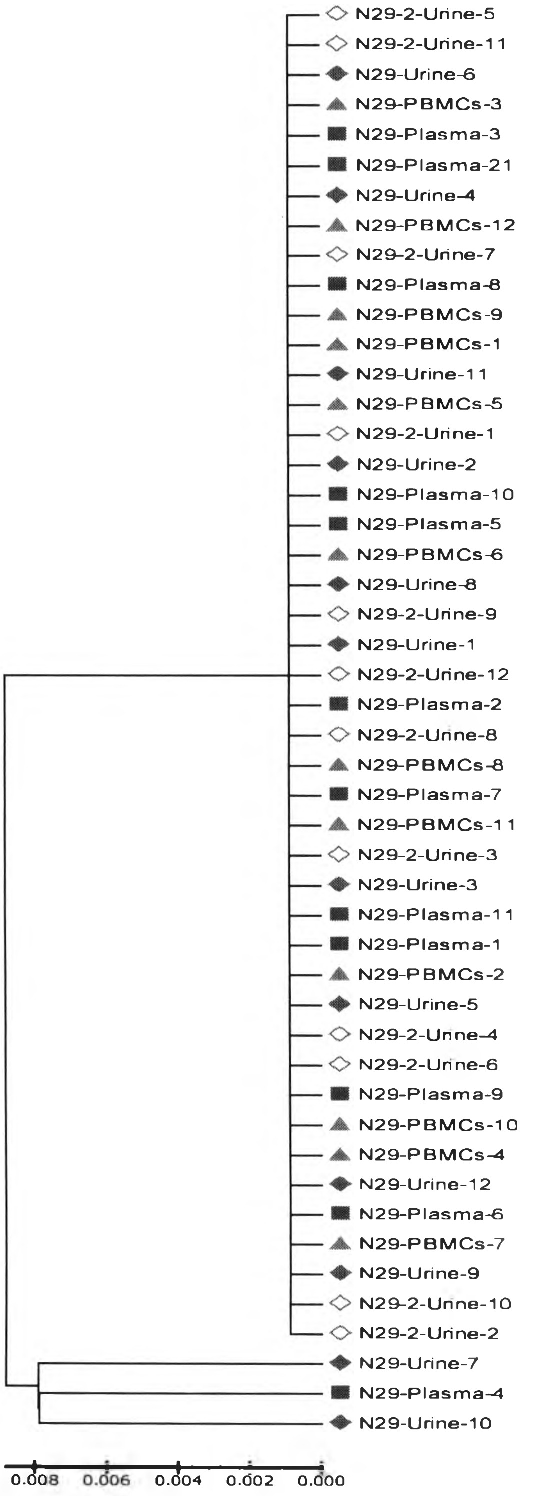


Figure 42: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N29 (DENV1-infected patient). There are febrile (N29 plasma, PBMCs and urine) and early convalescent (N29-2 urine) specimens.

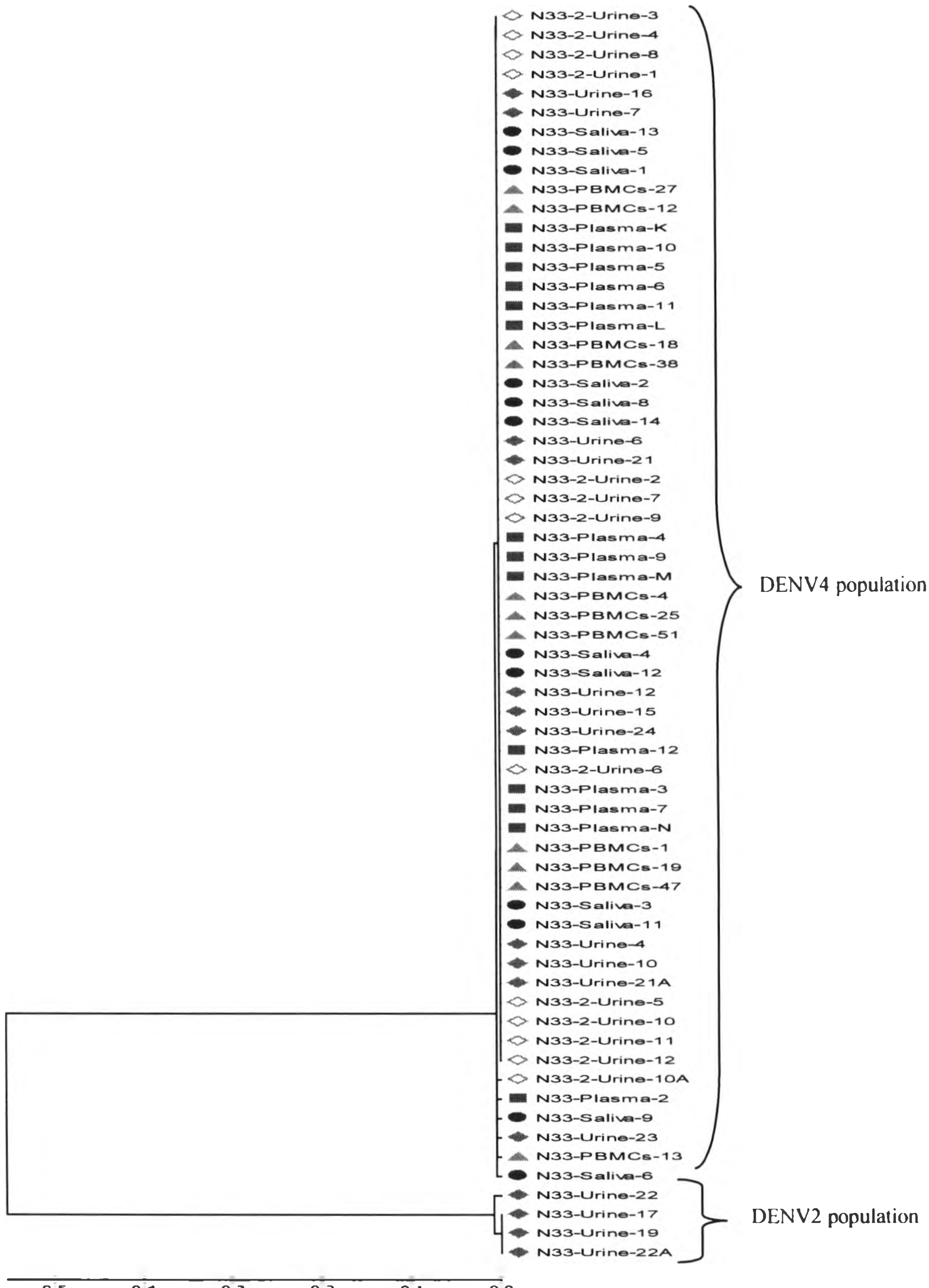


Figure 43: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N33 (DENV4+DENV2-infected patient). There are 1st early convalescent (N33 plasma, PBMCs and saliva: DENV4 and urine: DENV4+DENV2) and 2nd early convalescent (N33-2 urine: DENV4).

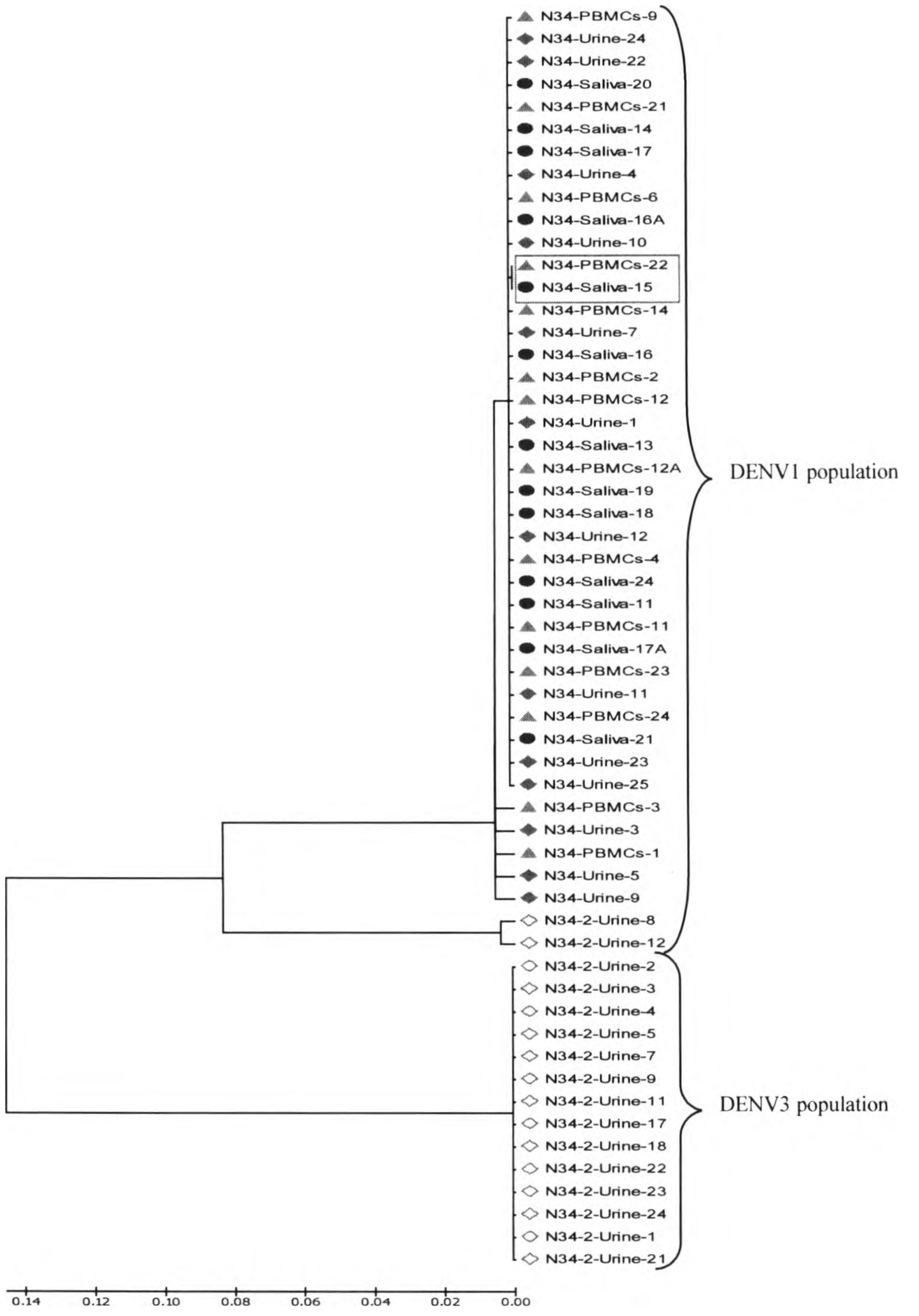


Figure 44: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N34 (DENV1+DENV3-infected patient). There are 1st early convalescent (N34 PBMCs, saliva and urine: DENV1) and 2nd early convalescent (N34-2 urine: DENV3+DENV1). The same DENV population in different specimens is marked as the red box.

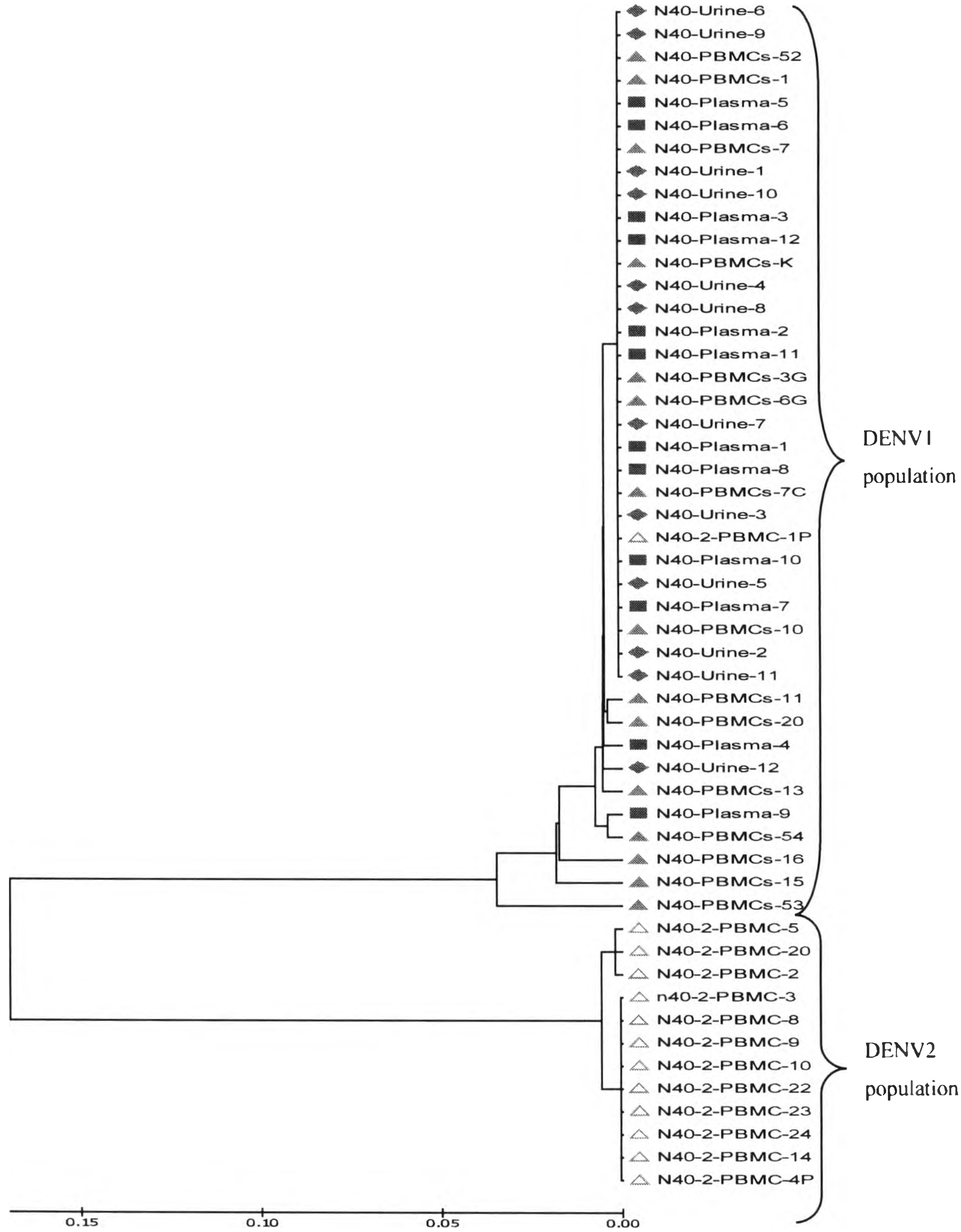


Figure 45: The phylogenetic tree of nucleotide sequences (E gene, 388 bp) derived from all clones in each specimen of N40 (DENV1+DENV2-infected patient). There are febrile (N40 plasma, PBMCs and urine: DENV1) and early convalescent (N40-2 PBMCs: DENV2+DENV1).