CHAPTER IV

RESULTS

1. Samples Characteristics

95 dengue cases were confirmed by the nested RT-PCR and IgM and IgG antibody.

2. Detection of dengue infection by nested RT-PCR

Nested RT-PCR amplification of dengue virus RNA using highly conserved primer pairs denouter-1 and denouter-2 was performed for all types of dengue virus. The first PCR product was used as a cDNA template and subsequently amplified in the second step using deninner-1 and deninner-2 primers. The PCR products were 104 base pairs when compared with 100 bp DNA ladder and dengue virus stock as the positive control. (Figure.8). 95 cases were positive for dengue viral RNA by nested RT-PCR. The PCR products of the predicted size of 104 bp were seen in reaction containing the RNA templates derived from blood and body fluid compartments of individual patients.

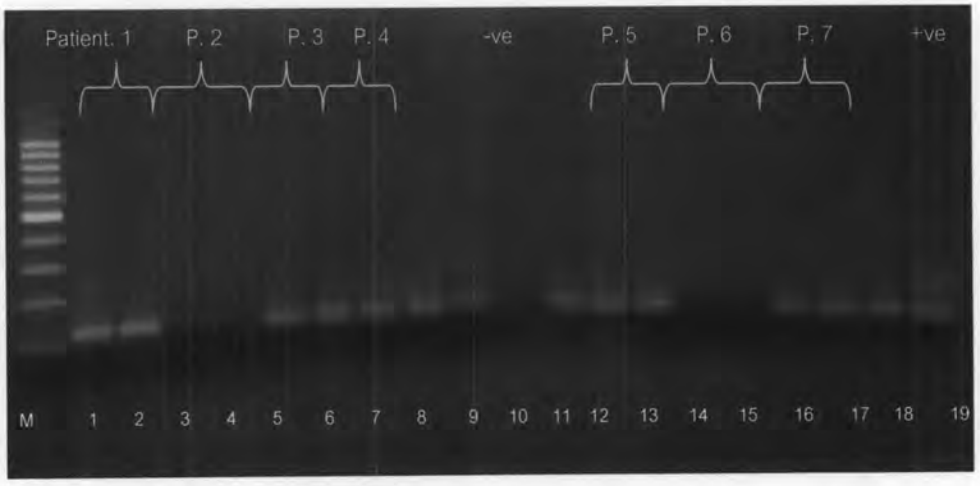

Figure 8. Agarose gel analysis of the product from blood and body fluid compartments by nested RT-PCR. Dengue virus infection have product size as 104 bp compare with 100 bp DNA ladder marker and positive control (dengue virus stock).

Lane M: 100 bp DNA ladder marker

Lane 1, 2: plasma and PBMC were positive for dengue from patient 1.

Lane 3, 4: plasma and PBMC were negative for dengue from patient 2.

Lane 5, 6: plasma and PBMC were positive for dengue from patient 3.

Lane 7, 8: plasma and PBMC were positive for dengue from patient 4.

Lane 10 was negative control.

Lane 9, 11: plasma and PBMC were positive for dengue from patient 5.

Lane 12, 13: plasma and PBMC were positive for dengue from patient 6.

Lane 14, 15: plasma and PBMC were negative for dengue from patient 7.

Lane 16, 17: plasma and PBMC were positive for dengue from patient 8.

Lane 18: plasma was positive for dengue from patient 9.

Lane 19: was positive control obtained dengue virus mix serotypes 1-4.

Nested RT-PCR detected dengue virus infection in both pediatric and adult patients, 54 pediatric cases were dengue positive (56.84%) and 41 adult cases were dengue infected (43.16%) respectively.

Table 6. The result of ELISA tests and nested RT-PCR for screening dengue virus infection.

Patient	ELISA test		Total	Nested RT-PCR	Total
	Primary	secondary			
pediatric	9 (16.66%)	45(83.34%)	54	54	56.84%
adults	1(2.44%)	40(97.56%)	41	41	43.16%
Total	10 (10.41%)	86 (89.58%)	95	95	100%

3. Serologic Study by IgM and IgG.

Of the 95 dengue-confirmed cases by molecular and serology methods, 85 cases and 10 cases determined to be primary and secondary infection respectively. 9 pediatric cases were of primary infection and 45 cases were of secondary infection. Similarly, 1 adult case was primary infection and 40 cases were secondary infection.

4. Detection of dengue serotypes by multiplex RT-PCR

RT-PCR was using a highly conserved primer pair DV1 and DV2, and a step of second round PCR using the primer DV1 and four serotype-specific primers TS1, TS2, TS3, and TS4. An aliquot of RNA elutes derived from stock viruses of four dengue serotypes, Hawaii (DEN-1), New Guinea (DEN-2), H-87 (DEN-3), and H-241 (DEN-4) as the positive control. An aliquot of the diluted first PCR products was subjected to the second round PCR. Amplified products of the expected size of 482 bp, 119 bp, 290 bp, and 392 bp were seen in the reaction of DEN-1, DEN-2, DEN-3, and DEN-4 viruses respectively.

85 patients were suspected dengue cases were subjected to detect and type dengue virus serotypes. Among 95 patients, 85 (89.47%) were confirmed dengue cases base on the presence of amplified products of predicted sizes. The results of some samples of single infection and/or double infections of dengue virus are shown in Figure 9. Patient 2 and patient 5 infected by two serotypes of dengue virus which patient 2 infected with DEN-2 and DEN-4 in plasma sample, patient 5 infected with DEN-1 and DEN-3 in plasma sample. Moreover, infection by three serotypes of dengue virus found in some patient. Patient 1 infected by three serotypes of dengue as DEN-1, DEN-3, and DEN-4 in plasma, PBMC, and serum of the first sample collection. Similarly, second sample collection of patient 1 typed as DEN-1, DEN-3, and DEN-4 in plasma, PBMC, saliva, and serum (Figure 10).

Figure 9. Agarose gel analysis of the product from clinical specimens by multiplex RT-PCR to detect dengue serotype, DEN-1 (482 bp), DEN-2 (119 bp), DEN-3 (290 bp), and DEN-4 (392 bp).

Lane M: 100 bp DNA ladder marker.

Lane 1, 2: plasma and PBMC positive for DEN-2 (119 bp) from patient 1.

Lane 3, 4: plasma sample with concurrent infection of DEN-2 and DEN-4 and PBMC was negative for dengue from patient 2.

Lane 5, 6: plasma and PBMC positive for DEN-1 (482 bp) from patient 3.

Lane 7, 8: plasma sample with concurrent infection of DEN-1 and DEN-3 (482 bp) and 290 bp) and PBMC sample positive for DEN-1 (482 bp) from patient 4.

Lane 9, 10: plasma and PBMC positive for DEN-4 (392 bp) from patient 5.

Lane 11, 12: plasma and PBMC positive for DEN-1 (482 bp) from patient 6.

Lane 13, 14: plasma negative for dengue and PBMC sample positive DEN-3 (290 bp) from patient 7.

Lane 15, 16, 17, 18 DEN1-4 virus stocks as positive control.

Lane 19: negative control.

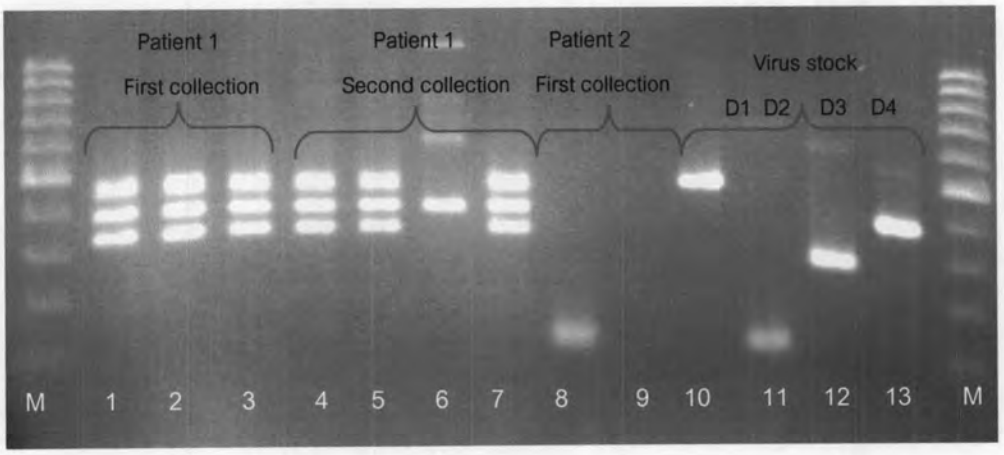

Figure 10. Agarose gel analysis of the product from clinical specimens by multiplex RT-PCR for detecting dengue serotype, DEN-1 (482 bp), DEN-2 (119 bp), DEN-3 (290 bp), and DEN-4 (392 bp).

Lane M: 100 bp DNA ladder.

Lane 1, 2, and 3 were plasma, PBMC, and serum infected with combination of DEN-1 (482 bp), DEN-3 (290 bp), and DEN-4 (392 bp) from first samples collection in patient 1.

Lane 4, 5, 6, and 7 were plasma, PBMC, saliva, and serum which plasma, PBMC, and serum infected with combination of DEN-1 (482 bp), DEN-3 (290 bp), and DEN-4 (392 bp) excepted saliva infected with one serotype as DEN-4 from second samples collection in patient 1 which period of first collection and second as 2 days.

Lane 8, 9: plasma positive foe DEN-2 (119 bp) and PBMC negative for dengue

Lane 10, 11, 12, 13 were positive control as DEN-1, DEN-2, DEN-3, and DEN-4 respectively.

In 2003-2007, 95 cases were dengue infections, 76.47% (65 of 85) of patients were single infection cases and 23.53% (20 of 85) were multiple infections. Single infection cases as 49.23% (32 of 65) were typed as DEN-4, 24.61% (16 of 65) were typed as DEN-1, 16.93% (11 of 65) were typed as DEN-3, and 9.23% (6 of 65) were typed as DEN-2 respectively. (Table.7)

In 2003, 2 cases of dengue infection, one was typed as DEN-1 in a pediatric DHF patient, and similarly, the other was typed as DEN-4 an adult DHF patient.

In 2004, 46 cases of dengue infection, 78.26% (36 of 46) of patients were single infection and 21.74% (10 of 46) of patients were multi-serotype infections. Single infection as 13.04% (6 of 46) were typed as DEN-1, 5 cases from pediatric patients which 1 case as DF, 4 cases as DHF and 1 case from adult DHF patient and , 4.35% (2 of 46) were typed as DEN-2 from pediatric DHF patient, 13.04% (6 of 46) were typed as DEN-3 from 2 cases pediatric DF patients and 2 cases pediatric DHF patient, and 2 cases from adult DHF patient, 47.83% (22 of 46) cases were typed as DEN-4, 9 cases from pediatric DF patients, 11 cases from pediatric DHF patients and 2 cases from adult DHF patient. Multi-serotype infections were 23.91% (11 of 46). These were of multiple combinations. DEN-1 and DEN-2 were found to constitute 2 cases, co-infection with DEN-1 and DEN-3 were found in 2 cases, co-infections with DEN-1 and DEN-4 were found in 4 cases. Co-infection with DEN-2 and DEN-4 were found in 2 cases, and co-infections with DEN-1, DEN-2 and DEN-4 were found in only one case. 11 cases of multi-serotypes from pediatric patients which 8 cases were found as DF patients, 2 cases as DHF patient and 1 case no clinical data.

In 2005, 25 cases of dengue infections, 12% (3 of 25) were typed as DEN-1, which 1 case from pediatric DHF patients and 2 cases from adult DHF patient, 24% (6 of 25) were typed as DEN-3, 1 case from pediatric patients was no clinical data and 5 cases from adult patients, 1 case was from DF patient and 4 cases were DHF patients, 44% (11 of 25) were typed as DEN-4, 2 cases from pediatric patients which 1 case was DHF and another one was no clinical data and 9 cases from adult patients which 1 case was DF and 6 cases were DHF patients and 2 cases no clinical data, 20%

(5 of 25) were multi-serotypes infection from adult DHF patients, DEN-1 and DEN-4 combination were found 1 case, co-infection with DEN-2 and DEN-4 were found 3 cases, and co-infection with DEN-1, DEN-2 and DEN-4 were found 1 case.

In 2006, 4 cases were co-infection, 3 cases were co-infection with DEN-2 and DEN-3, 1 was co-infection with DEN-1, DEN-3 and DEN-4 both co-infection from adult DHF patients.

In 2007, 5 cases, 3 cases were typed as DEN-1 which 2 cases from pediatric DF and another one as DHF patients, 1 case was type as DEN-2 from adult DF patient and 1 case was typed DEN-3 from child DF patient.

Of the 95 dengue patients, DF was seen in 17 cases with single serotype infection and in 5 cases with multi-serotypes (all pediatric), whereas DHF was present in 40 cases with single serotype infection and 14 cases with multiple serotypes.

Table 7. Serotypes of dengue patients determined by multiplex RT-PCR were detected with single infection (DEN-1, DEN-2, DEN-3, and DEN-4), and multiple infections (two or more dengue serotypes).

patient	Multiplex RT-PCR					No detected	Total
	DEN-1	DEN-2	DEN-3	DEN-4	Multi-serotypes		
Pediatric	10 (19.23%)	3 (5.77%)	6 (11.53%)	17 (32.7%)	11 (21.15%)	2	52
Adult	6 (18.18%)	3 (9.1%)	5 (15.15%)	15 (45.45%)	9 (27.27%)	8	33
Total	16 (18.82%)	6 (7.06%)	11 (12.94%)	32 (37.65%)	20 (23.53%)	10	85

Table 8. Distribution of multi-infections with 2 or more dengue serotypes within the same compartment as well as plasma or serum, PBMC, urine pellet, oral brush, and saliva.

Dengue infection	Plasma	PBMC	Pellet	Oral brush	Saliva	Serum
Multi-infection	10/19 (52.63%)	12/18 (66.66)	3/5 (60%)	4/5 (80%)	2/6 (33.33%)	2/4 (50%)

Multi-serotypes infections were found in various compartments as well as plasma or serum, PBMC, pellet, oral brush, and saliva.

5. Detection of dengue genotypes by RSS-PCR

PCR amplification of dengue virus RNA was used 4 primers of restriction site in the E gene region of dengue serotype1-4. An aliquot of RNA elution derived from stock viruses of 13 dengue strains, whose geographic source and year of isolation matched those of previously classified strains were analyzed to optimize primers and PCR conditions. RSS-PCR patterns were compared with the subtype designation of the matching strain, distinct patterns were found to correlate with known subtypes (Figure.11)

Figure 11. RSS-PCR patterns of DEN-2 strain of blood and body fluid compartments from individual patient compare with known DEN-2 (16681) strain.

Lane 1, 2, 3, 4, 5: plasma, PBMC, pellet, oral brush, serum from patient 1 who infected with DEN-2.

Lane 6, 7: saliva and serum from patient 2 (multi-serotypes DEN-2+DEN-3) detected with DEN-2 primer.

Lane 8: DEN-2 as positive control.

Lane 9, 10: saliva and serum from patient 2 (multi-serotypes DEN-2+DEN-3) detected with DEN-3 primer.

Lane 11: DEN-3 as positive control

Lane 12, 13: saliva and serum (multi-serotypes DEN-2+DEN-3) detected with D2+D3 primer.

Lane 14: DEN-2 and DEN-3 virus stock detected with DEN-2 and DEN-3 primers.

Dengue virus strain 16681 in previous reported by Eva Harris et al (85, 86) have different 2 bands at 150 bp and 582 bp, but in this experiment it had 2 bands at 582 bp and 754 bp, repectively. Due to the lack of similarity between our experiment and previous report in selected standard strains, we decided to switch to another method to study genotypes and strains. DNA sequencing was chosen to analyze nucleotide sequences of Capsid/prM gene and/or 3'UTR.

6. Sequencing of 3' UTR and/or Capsid/prM gene

The sequences of 3'UTR and/or Capsid/prM genes were analyzed, using clinical dengue strains available in our laboratory (Figure 12.)

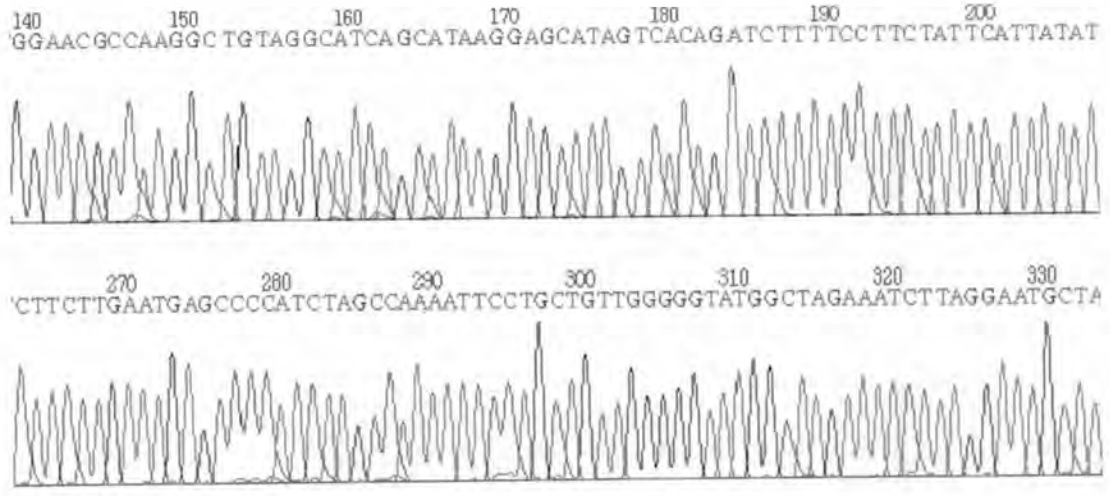

Figure 12. Chromatogram nucleotide sequencing of Capsid/prM gene using TS2 type specific primer for dengue-2 from PBMC sample.

The Chromatograms were chosen of major and minor peak for blast with database may be having 1 or more dengue strain. The results from database showed sequence identity and strain of dengue virus. The result showed that DEN-1, DEN-2, DEN-3, and DEN-4 from all specimens from the same day, 1-3 days of individual patients had the same genotype or strain (Table 9).

Table 9. Blast results of various specimen compartments from individual patients. The strains and genotypes defined by the number of nucleotide base and percentage of identity compare with database (GENBANK) and multiplex RT-PCR.

Patient	Blast result	No. base	Identity	Multiplex RT-PCR
CH-21 plasma	DEN-1, D1SG, Cambodia, Myanmar, Th	436/441	98%	DEN-1
CH-21 PBMC	DEN-1 Myanmar	433/441	98%	DEN-1
CH-21 oral brush	DEN-1 BID, Myanmar	435/441	98%	DEN-1
C-27 plasma	DEN-1 BID, D1SG	436/441	99%	DEN-1
C-27 PBMC	DEN-1 BID, D1SG, ThD1, D1SG, Myanmar, Cambodia	440/441	99%	DEN-1
C-27 saliva	DEN-1 BID, D1SG, Cambodia, Myanmar, Th	422/441	95%	DEN-1
C-83 plasma	DEN-1 Myanmar, D1SG, Cambodia, Th	435/441	98%	DEN-1
C-83 PBMC	DEN-1 BID, Th	436/441	98%	DEN-1
A-13 plasma	DEN-2 ThD, GD, ThNH, bra, cuba	68/73	93%	DEN-2
A-13 PBMC	DEN-2 ThD, GD, ThNH, 16681	69/73	94%	DEN-2
A-13 pellet	DEN-2 Th, GD, BID	69/73	94%	DEN-2
A-13 oral brush	DEN-2 ThD, GD, ThNH, 16681	69/73	94%	DEN-2
D-22 PBMC	DEN-2 Th, BID, GWL, GD	70/73	95%	DEN-2
D-22 pellet	Den-2 Th, BID, GWL, GD, 166841	69/73	94%	DEN-2
A-2 plasma	DEN-4 ThD	202/214	94%	DEN-4
A-2 PBMC	DEN-4 ThD	189/214	89%	DEN-4
A-2 Serum	DEN-4 ThD	202/214	94%	DEN-4

Patient	Blast result	No. base	Identity	Multiplex RT-PCR
A-2/2 plasma	DEN-4 ThD	190/214	88%	DEN-4
A-2/2 PBMC	DEN-4 ThD, Srilanka	213/214	99%	DEN-4
A-2/2 saliva	DEN-4 ThD,	212/214	99%	DEN-4
A-2/2 serum	DEN-4 ThD,	212/214	99%	DEN-4

Interestingly, the blast results of plasma, PBMC, serum, and saliva from one patient (A-2) in the 1st collection samples had sequence identity (Fig 13A-13B) but, sequence variation in the same sample of the 2nd collection samples (A-2/2) (Fig 13C-13D).

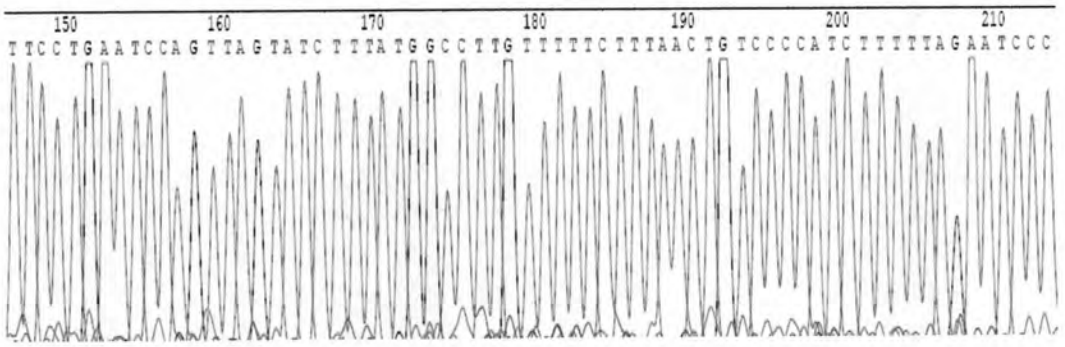

Figure 13A. Chromatogram nucleotide sequencing from A-2 Plasma 1st sample collection

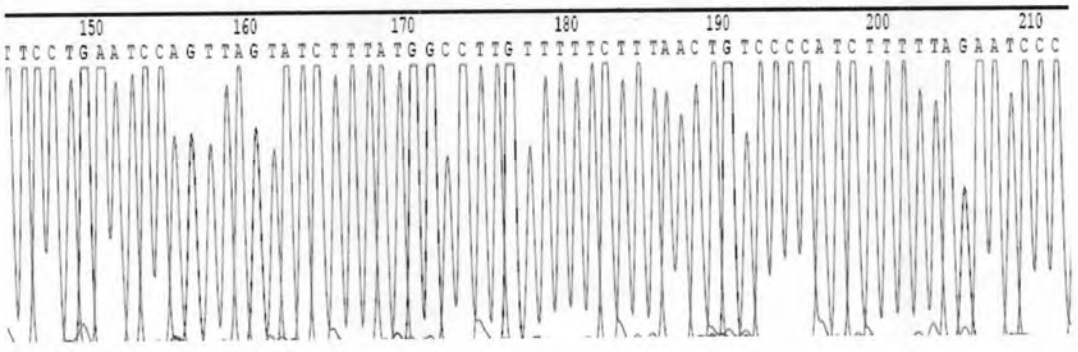

Figure 13B. Chromatogram nucleotide sequencing from A-2 Serum 1st sample collection

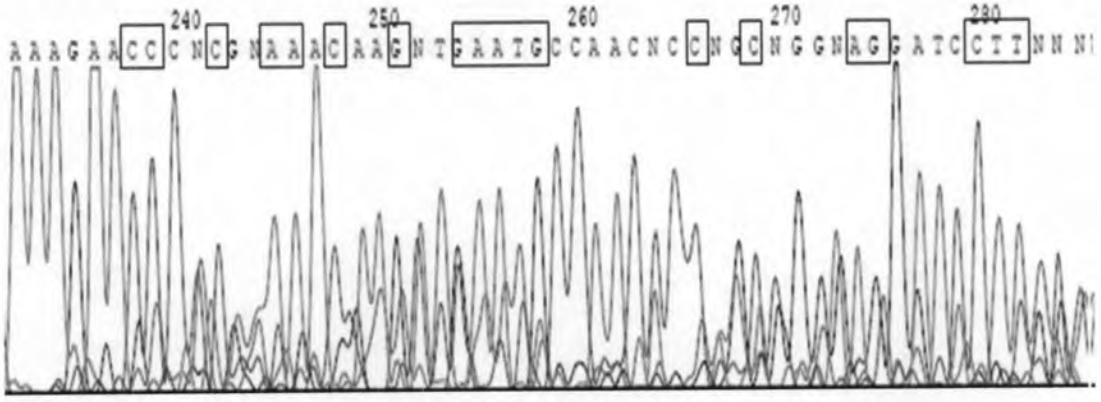

Figure 13C. Chromatogram nucleotide sequencing from A-2 PBMC 1st sample collection

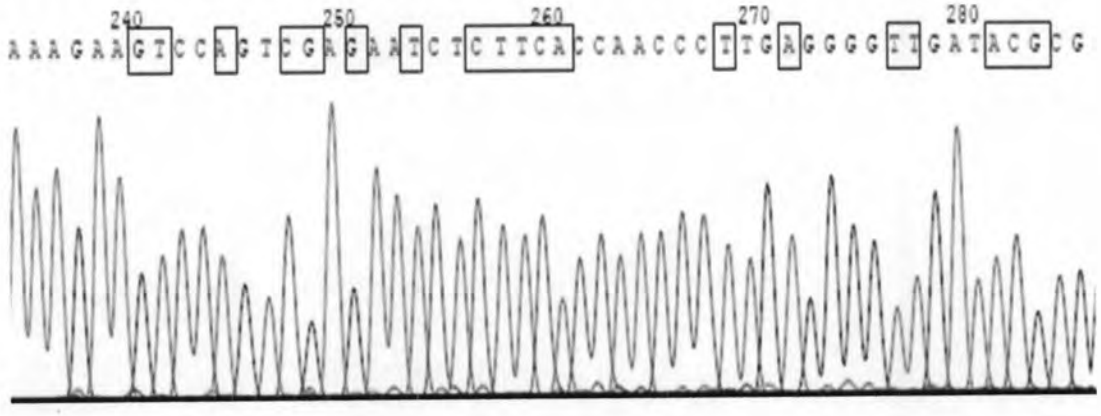

Figure 13D. Chromatogram nucleotide sequencing from A-2 PBMC 2nd sample collection