IV. RESULT

1. Immunofluorescence assay and Weil-Felix test

The WF test showed positive seroconversion (Proteus OX-K titer $\geq$ 160) in 52 whereas IF assay gave positive 84 out of 300 patients (Table 2). The patients who showed serum antibody titer but considered as negative serconversion were shown in Table 3. Sixty eight and 32 seronegative conversion patients were observed by IF and WF test, respectively. High antibody titers (IgM > 400 and /or IgG > 400) were detected in 48 patients by IF test (Table 2). One hundred and forty eight and 216 out of 300 patients gave no antibody against *O. tsutsugamushi* by IF and WF. The indirect immunofluorescence test revealed the presence of antibody in 152 out of 300 patients (IgM> 50 and /or IgG >50) (Table 2 and 3). The patient sera with *O. tsutsugamushi* antibodies showed fluorescence staining in the cytoplasm of L 292 cell line infected by *O. tsutsugamushi* observed under fluorescence microscope by IF assay(Figure 4). The WF test revealed the presence of antibody in 104 out of 300 patients (Table 2 and 3).

2. Amplification of *O. tsutsugamushi* DNA by nested PCR

At the early stage of infection, nested PCR could detect *O. tsutsugamushi* DNA in 76 (table 4) whereas the IF and WF showed positive seroconversion in 52 and 12 out of 300 patients respectvely. Amplifications of *O. tsutsugamushi* DNA from prototype reference strain with primer a and primer b in the first PCR , then using primer c and primer d in the second PCR, resulted in amplified products with the sizes of approximately 487 bps for all *O. tsutsugamushi* serotype. The amplification of *O. tsutsugamushi* DNA from reference strains were shown in Figure 5. Among 84 *O. tsutsugamushi* positive seroconversion samples confirmed for the presence of *O. tsutsugamushi* by amplification using the universal primers, showed positive in 80 patients. The amplification of *O. tsutsugamushi* DNA from patients were shown in Figure 6.

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
200009	3	M	50	50	Positive	<40	<40	Negative
		G	100	400				
200013	4	M	50	50	Positive	<40	<40	Negative
		G	50	400				
200015	5	M	50	100	Positive	<40	40	Negative
		G	100	400				
200019	3	M	100	200	Positive	<40	<40	Negative
		G	200	800				
200023	4	M	50	50	Positive	<40	40	Negative
		G	200	800				
200031	4	M	100	50	Positive	40	<40	Negative
		G	100	400				
200038	5	M	100	50	Positive	40	160	Positive
		G	200	400				
200042	5	M	100	200	Positive	40	40	Negative
		G	200	400				
200045	6	M	200	100	Positive	40	<40	Negative
		G	400	200				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution ; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation: positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
200050	6	M	400	400	Positive	40	160	Positive
		G	400	800				
200053	5	M	400	200	Positive	40	160	Positive
		G	400	800				
200057	3	M	400	800	Positive	40	160	Positive
		G	400	1600				
200059	5	M	400	800	Positive	80	320	Positive
		G	800	1600				
200164	5	M	800	400	Positive	40	160	Positive
		G	800	800				
200168	4	M	800	400	Positive	40	160	Positive
		G	400	400				
200171	7	M	400	200	Positive	160	320	Positive
		G	800	1600				
200172	8	M	400	400	Positive	160	320	Positive
		G	1600	400				
200174	4	M	400	400	Positive	40	160	Positive
		G	800	800				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
200176	6	M	400	800	Positive	40	160	Positive
		G	800	1600				
200177	9	M	800	400	Positive	160	640	Positive
		G	1600	1600				
200180	7	M	400	400	Positive	80	320	Positive
		G	800	1600				
200190	3	M	50	50	Positive	<40	<40	Negative
		G	100	400				
200193	4	M	50	50	Positive	<40	<40	Negative
		G	50	400				
200195	5	M	50	100	Positive	<40	40	Negative
		G	100	400				
200199	3	M	100	200	Positive	<40	<40	Negative
		G	200	800				
200223	4	M	50	50	Positive	<40	40	Negative
		G	200	800				
200231	4	M	100	50	Positive	40	<40	Negative
		G	100	400				

[a]IFA: Immunofluorescent assay, [b]WF: Weil Felix test

[c]The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d]Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e]Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f]Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titerer

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
200238	5	M	100	50	Positive	40	160	Positive
		G	200	400				
200242	5	M	100	200	Positive	40	40	Negative
		G	200	400				
200245	6	M	200	100	Positive	40	<40	Negative
		G	400	200				
200250	6	M	400	400	Positive	40	160	Positive
		G	400	800				
200253	5	M	400	200	Positive	40	160	Positive
		G	400	800				
200257	3	M	400	800	Positive	40	160	Positive
		G	400	1600				
200259	5	M	400	800	Positive	80	320	Positive
		G	800	1600				
200364	5	M	800	400	Positive	40	160	Positive
		G	800	800				
200368	4	M	800	400	Positive	40	160	Positive
		G	400	400				

[a] IFA: Immunofluorescent assay. [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
200472	7	M	400	200	Positive	160	320	Positive
		G	800	1600				
200504	8	M	400	400	Positive	160	320	Positive
		G	1600	400				
200574	4	M	400	400	Positive	40	160	Positive
		G	800	800				
200576	6	M	400	800	Positive	40	160	Positive
		G	800	1600				
200577	9	M	800	400	Positive	160	640	Positive
		G	1600	1600				
200580	7	M	400	400	Positive	80	320	Positive
		G	800	1600				
200582	3	M	50	50	Positive	<40	<40	Negative
		G	100	400				
200583	4	M	50	50	Positive	<40	<40	Negative
		G	50	400				
200590	5	M	50	100	Positive	<40	40	Negative
		G	100	400				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
200592	3	M	100	200	Positive	<40	<40	Negative
		G	200	800				
200600	4	M	50	50	Positive	<40	40	Negative
		G	200	800				
210602	4	M	100	50	Positive	40	<40	Negative
		G	100	400				
210003	5	M	100	50	Positive	40	160	Positive
		G	200	400				
210005	5	M	100	200	Positive	40	40	Negative
		G	200	400				
210015	6	M	200	100	Positive	40	<40	Negative
		G	400	200				
210021	6	M	400	400	Positive	40	160	Positive
		G	400	800				
210025	5	M	400	200	Positive	40	160	Positive
		G	400	800				
210029	3	M	400	800	Positive	40	160	Positive
		G	400	1600				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
210176	6	M	400	800	Positive	40	160	Positive
		G	800	1600				
210177	9	M	800	400	Positive	160	640	Positive
		G	1600	1600				
210181	7	M	400	400	Positive	80	320	Positive
		G	800	1600				
220009	3	M	50	50	Positive	<40	<40	Negative
		G	100	400				
220013	4	M	50	50	Positive	<40	<40	Negative
		G	50	400				
220016	5	M	50	100	Positive	<40	40	Negative
		G	100	400				
220020	3	M	100	200	Positive	<40	<40	Negative
		G	200	800				
220022	4	M	50	50	Positive	<40	40	Negative
		G	200	800				
220030	4	M	100	50	Positive	40	<40	Negative
		G	100	400				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
220037	5	M	100	50	Positive	40	160	Positive
		G	200	400				
220041	5	M	100	200	Positive	40	40	Negative
		G	200	400				
220045	6	M	200	100	Positive	40	<40	Negative
		G	400	200				
220051	6	M	400	400	Positive	40	160	Positive
		G	400	800				
220053	5	M	400	200	Positive	40	160	Positive
		G	400	800				
220056	3	M	400	800	Positive	40	160	Positive
		G	400	1600				
220058	5	M	400	800	Positive	80	320	Positive
		G	800	1600				
220161	5	M	800	400	Positive	40	160	Positive
		G	800	800				
220168	4	M	800	400	Positive	40	160	Positive
		G	400	400				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 2 Serum antibody titer of positive seroconversion patients of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig Class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
220175	7	M	400	200	Positive	160	320	Positive
		G	800	1600				
230003	8	M	400	400	Positive	160	320	Positive
		G	1600	400				
230175	4	M	400	400	Positive	40	160	Positive
		G	800	800				
230176	6	M	400	800	Positive	40	160	Positive
		G	800	1600				
230178	9	M	800	400	Positive	160	640	Positive
		G	1600	1600				
230180	7	M	400	400	Positive	80	320	Positive
		G	800	1600				
No. of positive diagnosis			84			52		

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 3 Serum antibody titer of negative seroconversion patients against of *O. tsutsugamushi* by IFA and WF

Patient no.	Day of onset	Ig class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
200008	3	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
200012	4	M	<50	50	Negative	<40	40	Negative
		G	50	50				
200028	5	M	<50	50	Negative	<40	40	Negative
		G	<50	<50				
200032	1	M	<50	50	Negative	<40	<40	Negative
		G	<50	<50				
200034	5	M	<50	50	Negative	<40	40	Negative
		G	<50	<50				
200036	6	M	<50	<50	Negative	<40	<40	Negative
		G	50	100				
200040	5	M	<50	50	Negative	<40	40	Negative
		G	<50	50				
200044	2	M	<50	<50	Negative	<40	<40	Negative
		G	<50	50				
200048	3	M	<50	50	Negative	<40	40	Negative
		G	50	50				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400. WF $\geq$ 160 or four fold rising titer

Table 3 Serum antibody titer of negative seroconversion patients against of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
200051	1	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
200054	3	M	50	100	Negative	<40	40	Negative
		G	50	100				
200056	4	M	50	100	Negative	<40	40	Negative
		G	<50	50				
200162	2	M	<50	50	Negative	<40	<40	Negative
		G	50	50				
200166	3	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
200169	6	M	<50	<50	Negative	<40	80	Negative
		G	50	50				
200173	2	M	<50	<50	Negative	<40	<40	Negative
		G	<50	50				
200179	2	M	<50	50	Negative	<40	<40	Negative
		G	<50	<50				
210007	3	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 3 Serum antibody titer of negative seroconversion patients against of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
210011	4	M	<50	50	Negative	<40	40	Negative
		G	50	50				
210028	5	M	<50	50	Negative	<40	40	Negative
		G	<50	<50				
210032	1	M	<50	50	Negative	<40	<40	Negative
		G	<50	<50				
210034	5	M	<50	50	Negative	<40	40	Negative
		G	<50	<50				
210037	6	M	<50	<50	Negative	<40	<40	Negative
		G	50	100				
210041	5	M	<50	50	Negative	<40	40	Negative
		G	<50	50				
210044	2	M	<50	<50	Negative	<40	<40	Negative
		G	<50	50				
210049	3	M	<50	50	Negative	<40	40	Negative
		G	50	50				
210053	1	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 3 Serum antibody titer of negative seroconversion patients against of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
210054	3	M	50	100	Negative	<40	40	Negative
		G	50	100				
210056	4	M	50	100	Negative	<40	40	Negative
		G	<50	50				
210162	2	M	<50	50	Negative	<40	<40	Negative
		G	50	50				
210166	3	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
220169	6	M	<50	<50	Negative	<40	80	Negative
		G	50	50				
220173	2	M	<50	<50	Negative	<40	<40	Negative
		G	<50	50				
220179	2	M	<50	50	Negative	<40	<40	Negative
		G	<50	<50				
230008	3	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
230009	4	M	<50	50	Negative	<40	<40	Negative
		G	50	50				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class : M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 3 Serum antibody titer of negative seroconversion patients against of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
230028	5	M	<50	50	Negative	<40	40	Negative
		G	<50	<50				
230032	1	M	<50	50	Negative	<40	<40	Negative
		G	<50	<50				
230034	5	M	<50	50	Negative	<40	40	Negative
		G	<50	<50				
230036	6	M	<50	<50	Negative	<40	<40	Negative
		G	50	100				
230040	5	M	<50	50	Negative	<40	40	Negative
		G	<50	50				
230044	2	M	<50	<50	Negative	<40	<40	Negative
		G	<50	50				
230048	3	M	<50	50	Negative	<40	40	Negative
		G	50	50				
230051	1	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
230054	3	M	50	100	Negative	<40	40	Negative
		G	50	100				

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 3 Serum antibody titer of negative seroconversion patients against of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
230056	4	M	50	100	Negative	<40	40	Negative
		G	<50	50				
230162	2	M	<50	50	Negative	<40	<40	Negative
		G	50	50				
230166	3	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
230169	6	M	<50	<50	Negative	<40	80	Negative
		G	50	50				
230173	2	M	<50	<50	Negative	<40	<40	Negative
		G	<50	50				
230177	2	M	<50	50	Negative	<40	<40	Negative
		G	<50	<50				
230009	3	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
230011	4	M	<50	50	Negative	<40	40	Negative
		G	50	50				
230029	5	M	<50	50	Negative	<40	40	Negative
		G	<50	<50				

[a]IFA: Immunofluorescent assay, [b]WF: Weil Felix test

[c]The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d]Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e]Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f]Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 3 Serum antibody titer of negative seroconversion patients against of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
230033	1	M	<50	50	Negative	<40	<40	Negative
		G	<50	<50				
230035	5	M	<50	50	Negative	<40	40	Negative
		G	<50	<50				
230039	6	M	<50	<50	Negative	<40	<40	Negative
		G	50	100				
230041	5	M	<50	50	Negative	<40	40	Negative
		G	<50	50				
230045	2	M	<50	<50	Negative	<40	<40	Negative
		G	<50	50				
230047	3	M	<50	50	Negative	<40	40	Negative
		G	50	50				
230050	1	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
230052	3	M	50	100	Negative	<40	40	Negative
		G	50	100				
230057	4	M	50	100	Negative	<40	40	Negative
		G	<50	50				

[a]IFA: Immunofluorescent assay, [b]WF: Weil Felix test

[c]The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d]Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e]Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f]Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

Table 3 Serum antibody titer of negative seroconversion patients against of *O. tsutsugamushi* by IFA and WF (continued)

Patient no.	Day of onset	Ig class[c]	IFA[a]			WF[b]		
			Early stage[d]	Convalescent stage[e]	Interpretation[f]	Early stage[d]	Convalescent stage[e]	Interpretation[f]
230160	2	M	<50	50	Negative	<40	<40	Negative
		G	50	50				
230167	3	M	<50	50	Negative	<40	<40	Negative
		G	<50	50				
230168	6	M	<50	<50	Negative	<40	80	Negative
		G	50	50				
230171	2	M	<50	<50	Negative	<40	<40	Negative
		G	<50	50				
230179	2	M	<50	50	Negative	<40	<40	Negative
		G	<50	<50				
No. of negative diagnosis			68			32		

[a] IFA: Immunofluorescent assay, [b] WF: Weil Felix test

[c] The letter indicate the immunoglobulin class ; M: Immunoglobulin M, G: Immuunoglobulin G

[d] Reciprocal of the maximum serum dilution; Acute serum at early stage obtained at the date of admission

[e] Reciprocal of the maximum serum dilution; Convalescence serum at convalescence stage obtained 7-10 days after the acute serum obtained.

[f] Interpretation; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160 or four fold rising titer

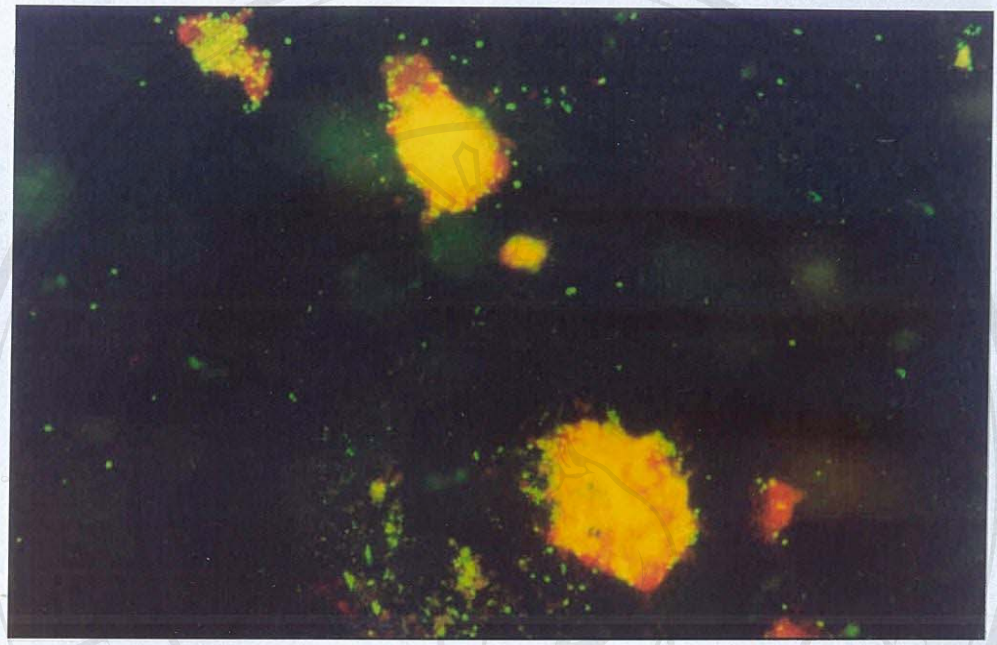

Figure 4 Indirect immunofluorescent assay of *O. tsutsugamushi* in cytoplasm of L 929 cell culture. *O. tsutugamushi* was stained with fluorescein dye and observed as tiny greenish particle. Magnification was 400X.

Table 4 Comparison of early diagnosis of scrub typhus by IFA, WF and nested PCR

Patient no.	Day of illness	Early stage		
		IFA[a]	WF[b]	PCR[c]
200004	8	+	+	+
200009	3	-	-	+
200013	4	-	-	+
200015	5	-	-	+
200019	3	-	-	+
200023	4	-	-	+
200031	4	-	-	+
200038	5	-	-	+
200042	5	-	-	+
200045	6	+	-	+
200050	6	+	-	+
200053	5	+	-	+
200054	3	-	-	+
200056	4	-	-	+
200057	3	+	-	+
200059	5	+	-	+
200164	5	+	-	+
200168	4	+	-	+
200172	7	+	+	-
200174	4	+	-	+
200176	6	+	-	-
200177	9	+	+	-
200180	7	+	-	-
200190	3	-	-	+
200193	4	-	-	+

[a]Immunofluorescent assay; [b]Weil Felix test; [c]Polymerase chain reaction

Early diagnosis; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160

Table 4 Comparison of early diagnosis of scrub typhus by IFA, WF and nested PCR

(continued)

Patient no.	Day of illness	Early stage		
		IFA[a]	WF[b]	PCR[c]
200195	5	-	-	+
200199	3	-	-	+
200223	4	-	-	+
200231	4	-	-	+
200238	5	-	-	+
200242	5	-	-	+
200245	6	+	-	+
200250	6	+	-	+
200253	5	+	-	+
200257	3	+	-	+
200259	5	+	-	+
200364	5	+	-	+
200368	4	+	-	+
200472	7	+	+	-
200504	8	+	+	+
200574	4	+	-	+
200576	6	+	-	-
200577	9	+	+	-
200580	7	+	-	-
200582	3	-	-	+
200583	4	-	-	+
200590	5	-	-	+
200592	3	-	-	+
200600	4	-	-	+
210003	4	-	-	+
210005	5	-	-	+

[a]Immunofluorescent assay; [b]Weil Felix test; [c]Polymerase chain reaction

Early diagnosis; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160

Table 4 Comparison of early diagnosis of scrub typhus by IFA, WF and nested PCR (continued)

Patient no.	Day of illness	Early stage		
		IFA[a]	WF[b]	PCR[c]
210007	3	-	-	+
210008	3	-	-	+
210010	4	-	-	+
210011	4	-	-	+
210015	5	-	-	+
210021	6	+	-	+
210022	6	+	-	+
210025	5	+	-	+
210029	3	+	-	+
210035	5	+	-	+
210164	5	+	-	+
210168	4	+	-	+
210170	7	+	+	-
210172	8	+	+	+
210174	4	+	-	+
210176	6	+	-	-
210177	9	+	+	-
210181	7	+	-	-
220020	3	-	-	+
220030	4	-	-	+
220037	4	-	-	+
220039	5	-	-	+
220041	5	-	-	+
220045	6	+	-	+
220051	6	+	-	+
220053	5	+	-	+

[a]Immunofluorescent assay; [b]Weil Felix test; [c]Polymerase chain reaction

Early diagnosis; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160

Table 4 Comparison of early diagnosis of scrub typhus by IFA, WF and nested PCR (continued)

Patient no.	Day of illness	Early stage		
		IFA[a]	WF[b]	PCR[c]
220056	3	+	-	+
220058	5	+	-	+
220161	5	+	-	+
220168	4	+	-	+
220175	7	+	+	-
230003	8	+	+	+
230008	3	-	-	+
230012	4	-	-	+
230054	3	-	-	+
230056	4	-	-	+
230028	5	-	-	+
230175	4	+	-	+
230176	6	+	-	-
230178	9	+	+	-
230180	7	+	-	-
No. of positive diagnosis		52	12	76

[a]Immunofluorescent assay; [b]Weil Felix test; [c]Polymerase chain reaction

Early diagnosis; positive seroconversion when the titer of IFA $\geq$ 400, WF $\geq$ 160

Table 5 Comparison of day of illness in early diagnosis of scrub typhus by IFA, WF and nested PCR

No. patient	Day of illness	IFA[a]	WF[b]	PCR[c]
16	3	4	-	16
24	4	8	-	24
24	5	12	-	24
12	6	12	-	8
8	7	8	4	1
4	8	4	4	3
4	9	4	4	-
Total		52	12	76

[a]Immunofluorescent assay ; [b]Weil Felix test ; [c]Polymerase chain reaction

Table 6 Comparison of laboratory diagnosis of scrub typhus by IFA, WF and nested PCR in 300 patients

Diagnosis	IFA[a]	WF[b]	PCR[c]
Early diagnosis (single specimen)	52	12	76
Serological diagnosis (paired specimen)	84	52	80

[a]Immunofluorescent assay; [b]Weil Felix test; [c]Polymerase chain reaction

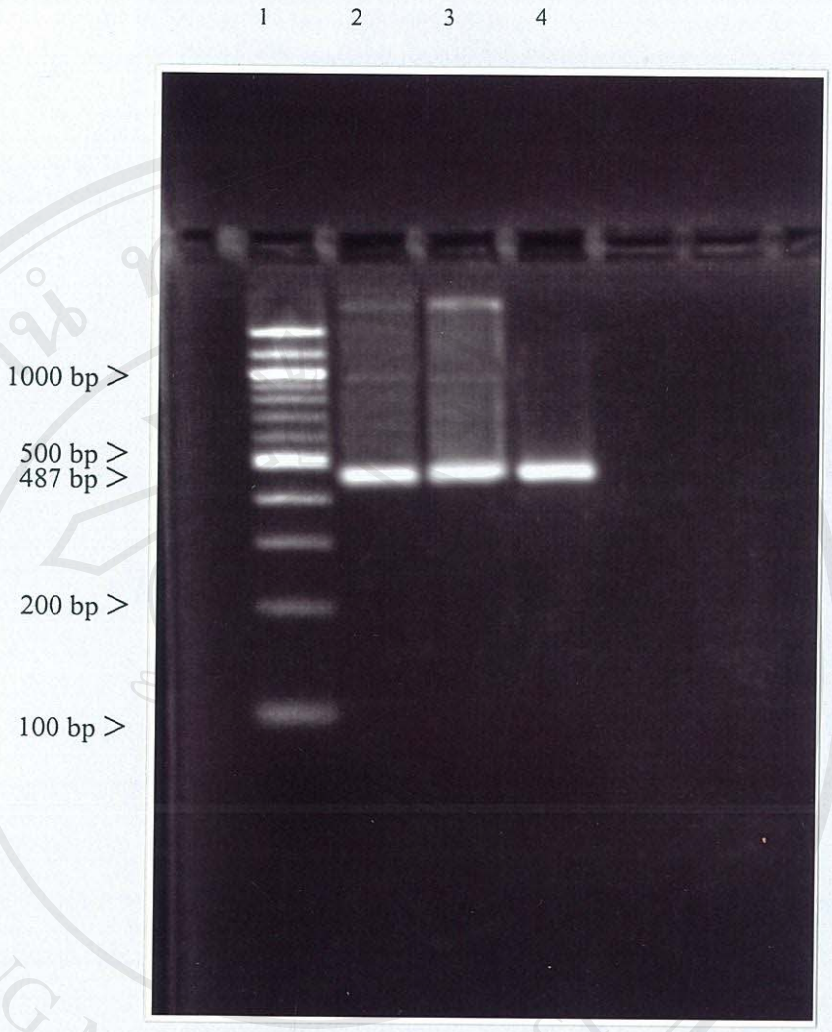

Figure 5 Agarose gel electrophoresis of amplified *O. tsutsugamushi* DNA from reference serotypes of *O. tsutsugamushi.* Detection of the 487 bp DNA encoding for 56 kDa of *O. tsutsugamushi* by nested PCR using 1.5 % agarose gel electrophoresis. Lane 1, the 1-Kb DNA ladder; Lane 2, *O. tsutsugamuhsi* Serotype Karp; Lane 3, *O. tsutsugamushi* serotype Kato; Lane 4, *O. tsutsugamuhsi* Serotype Gilliam. The numbers on the left are size of base pairs.

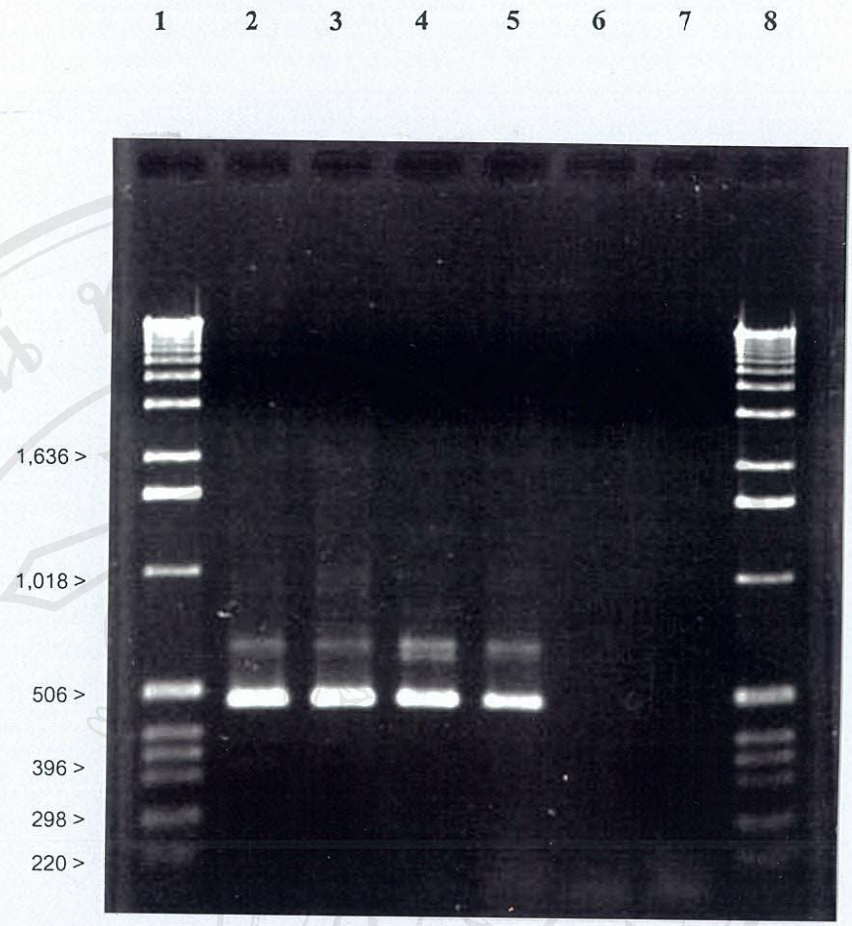

Figure 6 The amplification of *O. tsutsugamushi* DNA from clinical sample by nested PCR. Detection of the 487 bp DNA encoding for 56 kDa of *O. tsutsugamushi* by neste PCR using 1.5 % agarose gel electrophoresis. DNA extracted from the suspected scrub typhus patient No. 200054, 200056 and 200059 (Lane 2, 3 and 4), healthy blood donor (Lane 6), Control *O. tsutsugamuhsi* (Lane 5), Control *R. Typhi* (Lane 7). Lane 1 and 8 contained a 1-Kb DNA ladder as a size marker (GIBCO BRL, Inc.) The numbers on the left are size of base pairs.

3. Genotyping of *O. tsutsugamushi* by nested PCR

The amplification of *O. tsutsugamushi* DNA of reference serotype with the universal primers(primer a and primer b) in the first PCR and using primer c and serotype specific primer in the second PCR, were identified as positive for Gilliam, Karp and Kato serotype when the 410, 220 and 232 bp size specific bands were detected, respectively(Figure 7). The amplification of *O. tsutsugamushi* from patients using serotype specific primer in second PCR were shown in Figure 8. The nested PCR results are summarized in Table 7. Seventy-eight (26%)were positive for Karp serotype. Only two patients (0.6%) were positive for Kato serotype. Gilliam serotype was not detected in any of the samples. The Karp serotype was predominant and Kato serotype was found only in the southern region of Thailand (Figure 9).

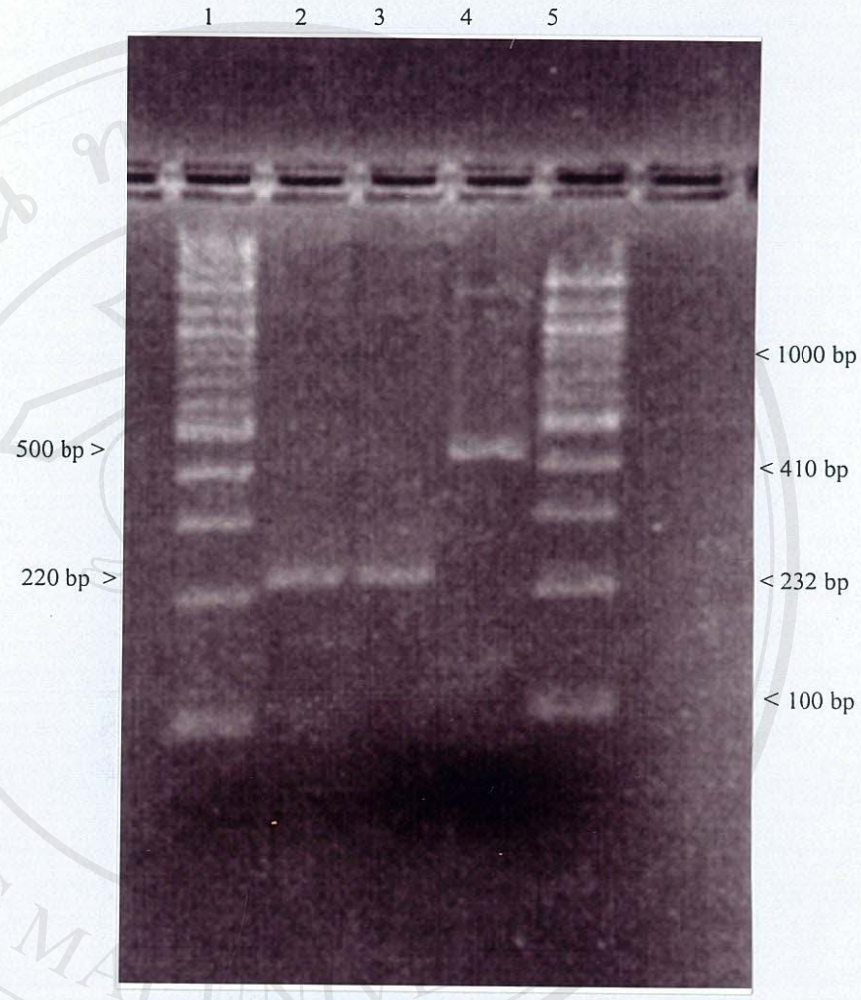

Figure 7 Genotyping of *O. tsutsugamushi* DNA from reference serotype by nested PCR. Gel electrophoresis of nested PCR amplified DNA from prototype *O. tsutsugamushi* using of primers a and b in the first PCR and primers c and serotype specific primer in second PCR Lane 1 and 5, the 100-bp DNA ladder; lane 2, *R tsutsugamushi* serotype Karp (220 bp); lane 3, *O. tsutsugamushi* serotype Kato (232 bp); lane 4 *O. tsutsugamushi* serotype Gilliam (410 bp). The numbers on the left and the right are the size of base pairs.

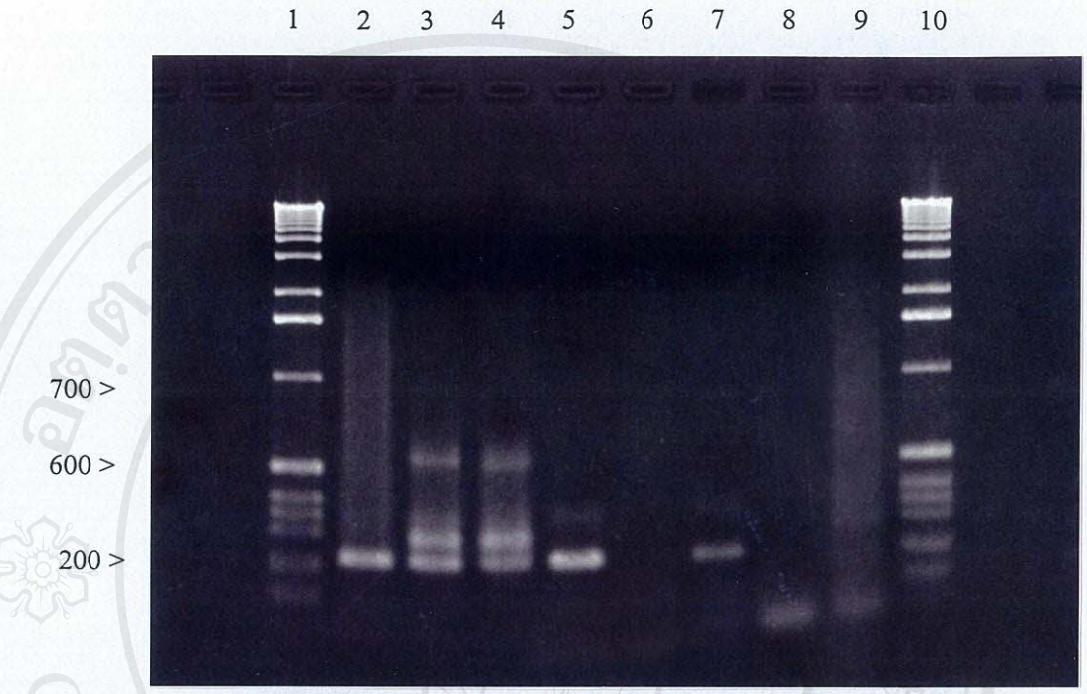

Figure 8 Agarose gel electrophoresis of nested PCR amplified DNA from patients using serotype specific primer in secound PCR. Lane 1 and 10, the 100-bp DNA ladder; lane 2, 3, 4 and 5, positive nested PCR fragment of *O. tsutsugamushi* using primer Kp (220 bp); lanne 7, positive nested PCR fragment of *O. tsutsugamushi* using primer Kt (223 bp); lane 6 and 8, negative nested PCR fragment of *O. tsutsugamushi*: lane 9, reagent control. The numbers on the left are the sizes of base pairs.

Table 7 Geographic distribution of *O. tsutsugamushi* genotype in Thailand.

RMSc. Center[1]		No. of positive patients	No. of genotype specific positive samples						
			%	Kp[2]	%	Kt[3]	%	G[4]	%
Northern	Chiang Mai	12	26.2	12	15.3	0	0	0	0
	Phitsanulok	9		9	11.5	0	0	0	0
North-Eastern	Udonthani	15	28.7	15	19.2	0	0	0	0
	Khon Kaen	8		8	10.2	0	0	0	0
Central	Cholburi	11	17.5	11	14.1	0	0	0	0
	MSc. Bangkok	3		3	3.8	0	0	0	0
Southtern	Songkhla	14	23.7	13	16.6	1	50	0	0
	Surathani	5		4	6.4	1	50	0	0
Total		80	100	78	97.5	2	2.5	0	0

[1]RMSc = Regional Medical Sciences; MSc = Medical Sciences.

[2]Kp = Karp serotype; [3]Kt = Kato serotype; [4]G = Gilliam serotype.

Table 8 Comparison of advantages of PCR, IFA and WF

	Assay /Test		
	PCR[a]	IFA[b]	WF[c]
Diagnosis time	1 day	10-14 days	10-14 days
Assay time	6 hrs	3 hrs	4 hrs
Specimen required	single	paired	paired
Cost (Baht)	300	200	100

[a]Polymerase chain reaction; [b]Immunofluorescent assay ; [c]Weil Felix test

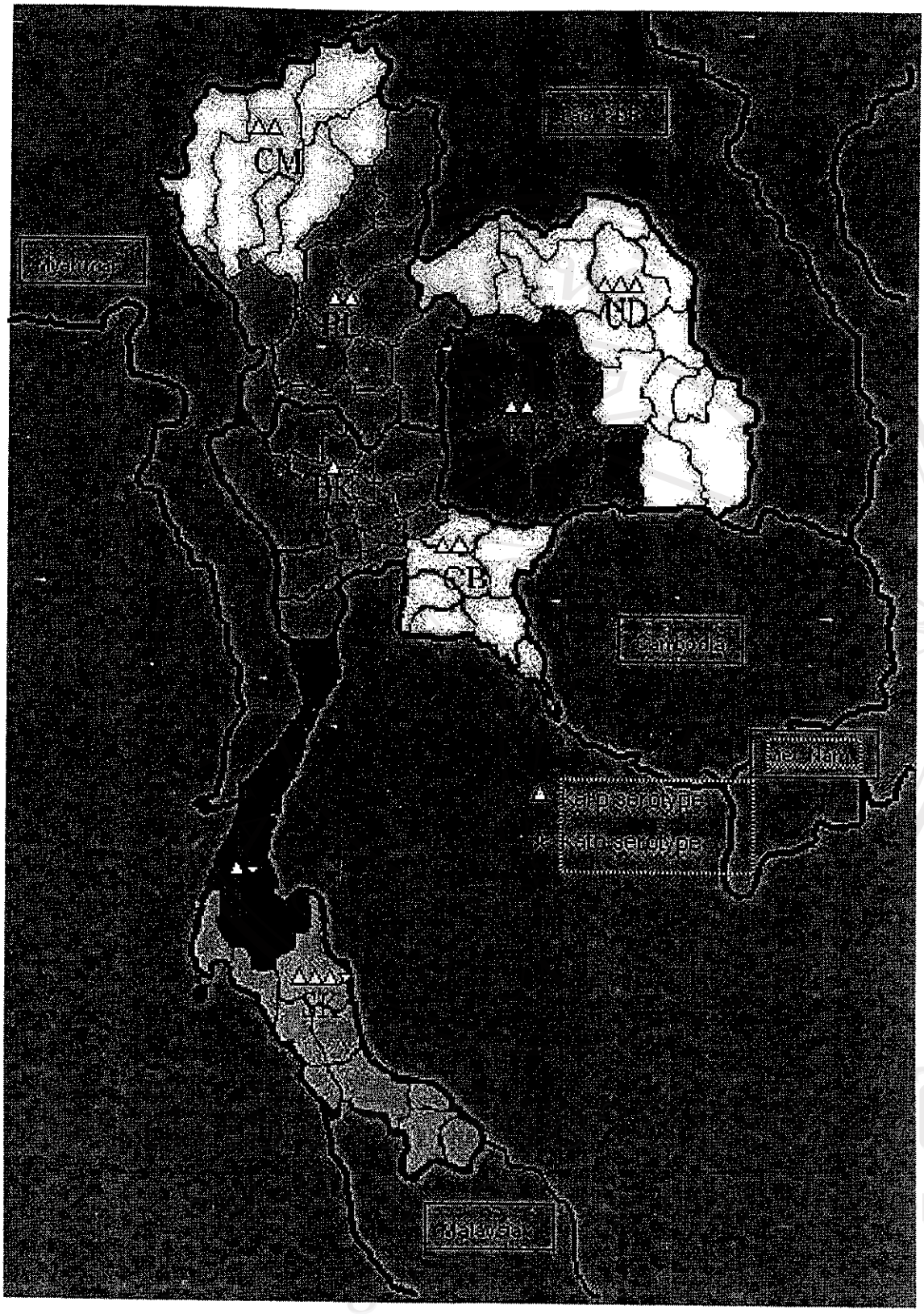

Figure 9 Geographical distribution of Karp and Kato serotypes of *O. tsutsugamushi* in various RMSc in Thailand.

CM = Chiang Mai; PL = Phitsanuloke; SK = Songkhla; ST = Surathani; UD = Udonthani; KK = Khon Kaen; CB = Cholburi; BK = Bangkok.

4. Genotyping of *O. tsutsugamushi* by nucleotide sequence analysis

The genotyping of *O. tsutsugamushi* using serotype specific primers by nested PCR could not always differentiate the type variants, especially some variants had a few nucleotide substitution or deletion in one or two nucleotides which represented point mutation. To ensure that the genotypes were classified correctly from nested PCR, the nucleotide sequencing of samples were determined. The electrophoregram of nucleotide sequences in the 56 kDa protein gene of sample were shown in Figure 10-21. In the electrophoregram, each of the four different colored curves indicated the fluorescence intensity of particular dye that was linked to specific ddNTP involved in the termination of the primer extension reaction (green, red, black and blue were linked with ddATP, ddTTP, ddGTP and ddCTP, respectively). The data were analyzed by computer programmes ABI 310 data collection version 3 and ABI 310 DNA sequencing version 2.2.

In analysis of genotype, the resulting nucleotide sequences were compared to the prototype sequences obtained from the GenBank reported by Stover and Kawamura (5, 6). The genotype of *O. tsutsugamushi* resulting from nucleotide sequencing of the 56 kDa protein gene of nested PCR were almost identical to the nucleotide sequence from GenBank. There were 78 samples shown as Karp genotype and 2 samples as Kato genotype. The Karp genotype was more prevalent than Kato genotype and accounted for 97.5% of those identified in this study. The Karp and Kato genotype,as identified by nested PCR, all agreed with nucleotide sequencing.

Indeed, all Karp and Kato genotypes identified in this study had identical 56 kDa protein gene sequences to the reference Karp and Kato serotype respectively. There were 3 samples shown as Karp genotype in the North (Figure 10-12), 3 as Karp genotype in the North East (Figure 13-15), 3 as Karp genotype in the central (Figure 16-18), 1 as Karp genotype in the south (Figure 19-21) and 2 as Kato genotype in the south (Figure 16).

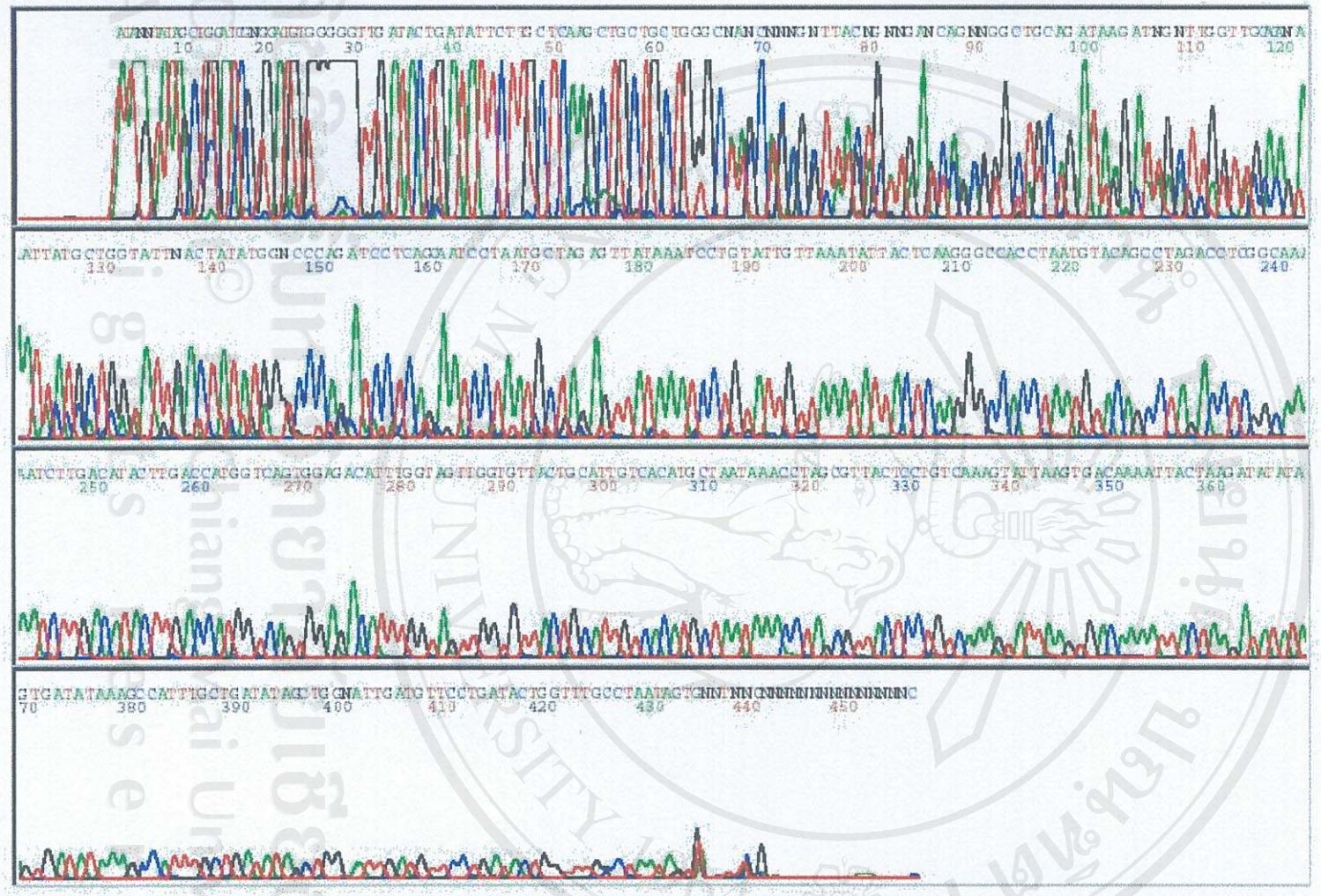

Figure 10 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 200054 collected from the northern region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

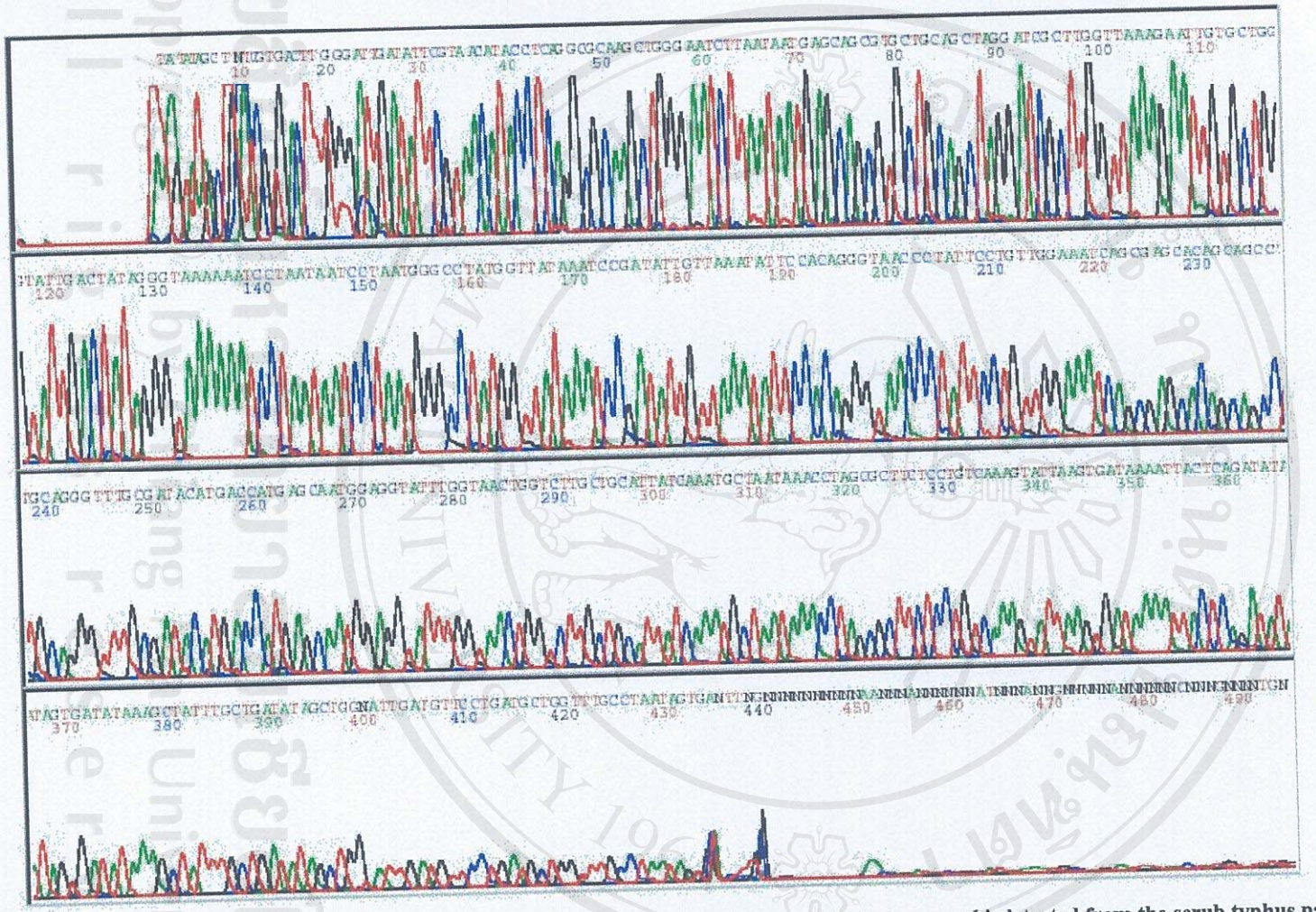

Figure 11 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 210050 collected from the northern region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

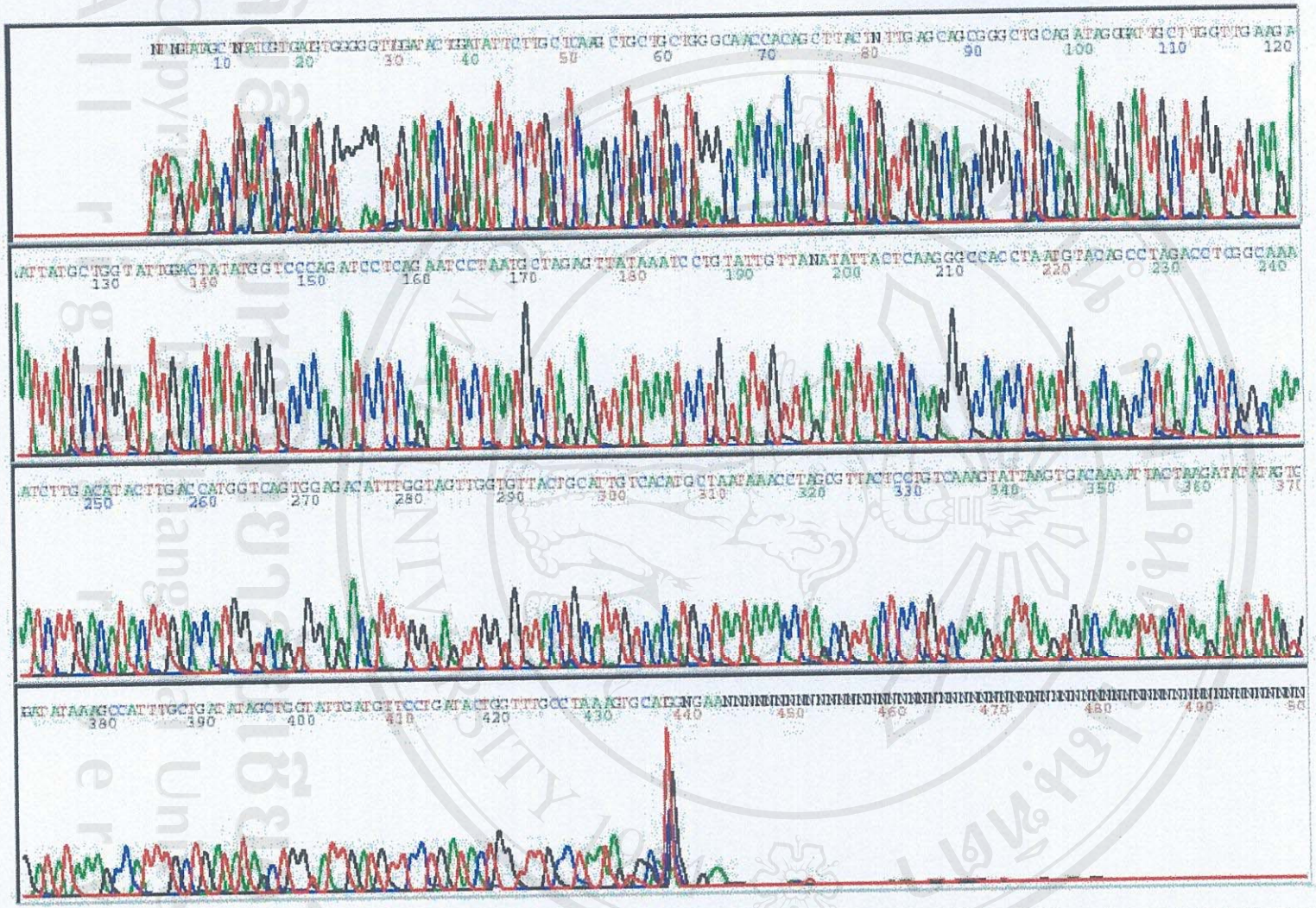

Figure 12 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 220054 collected from the northern region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

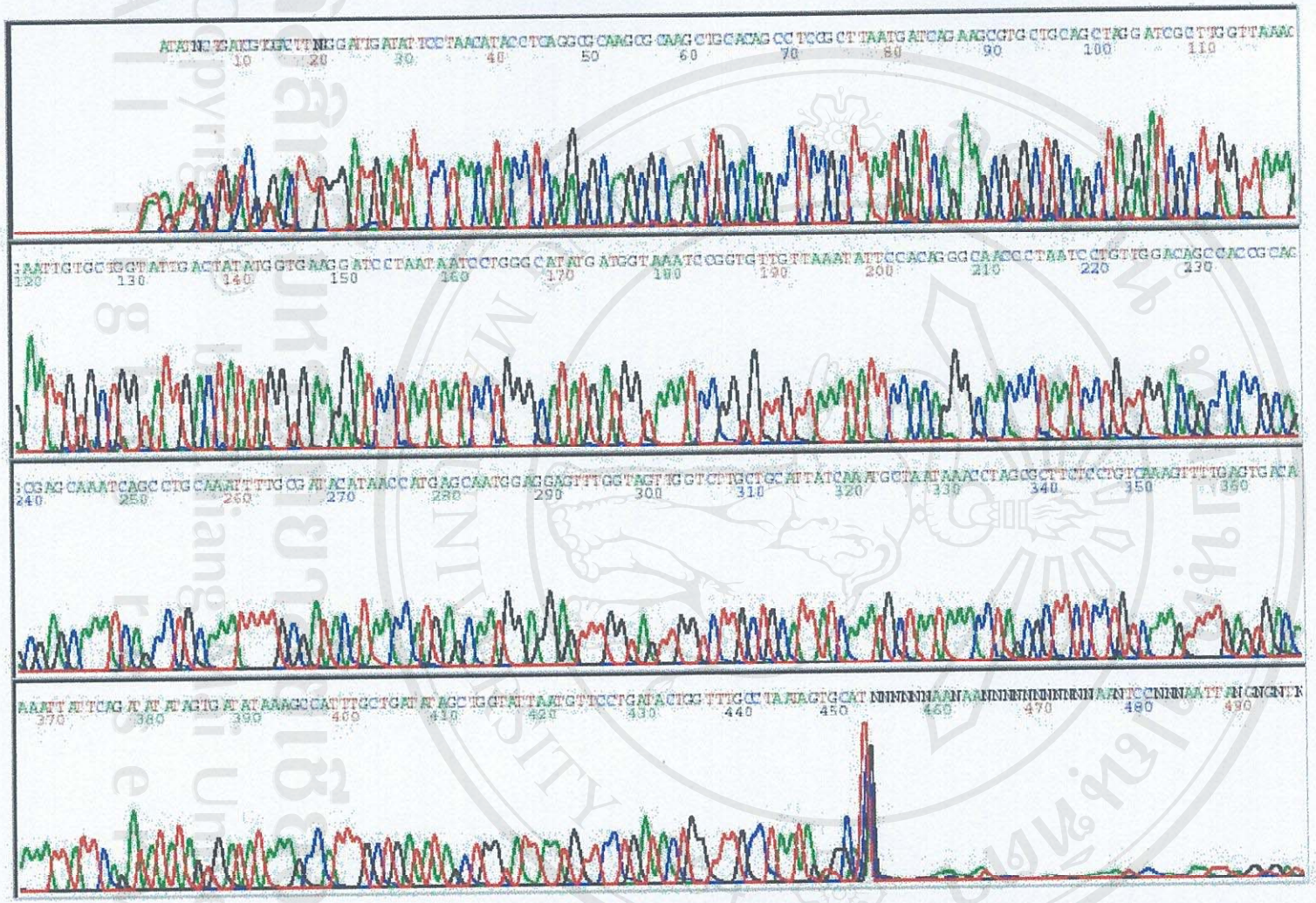

Figure 13 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 200056 collected from the North East region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

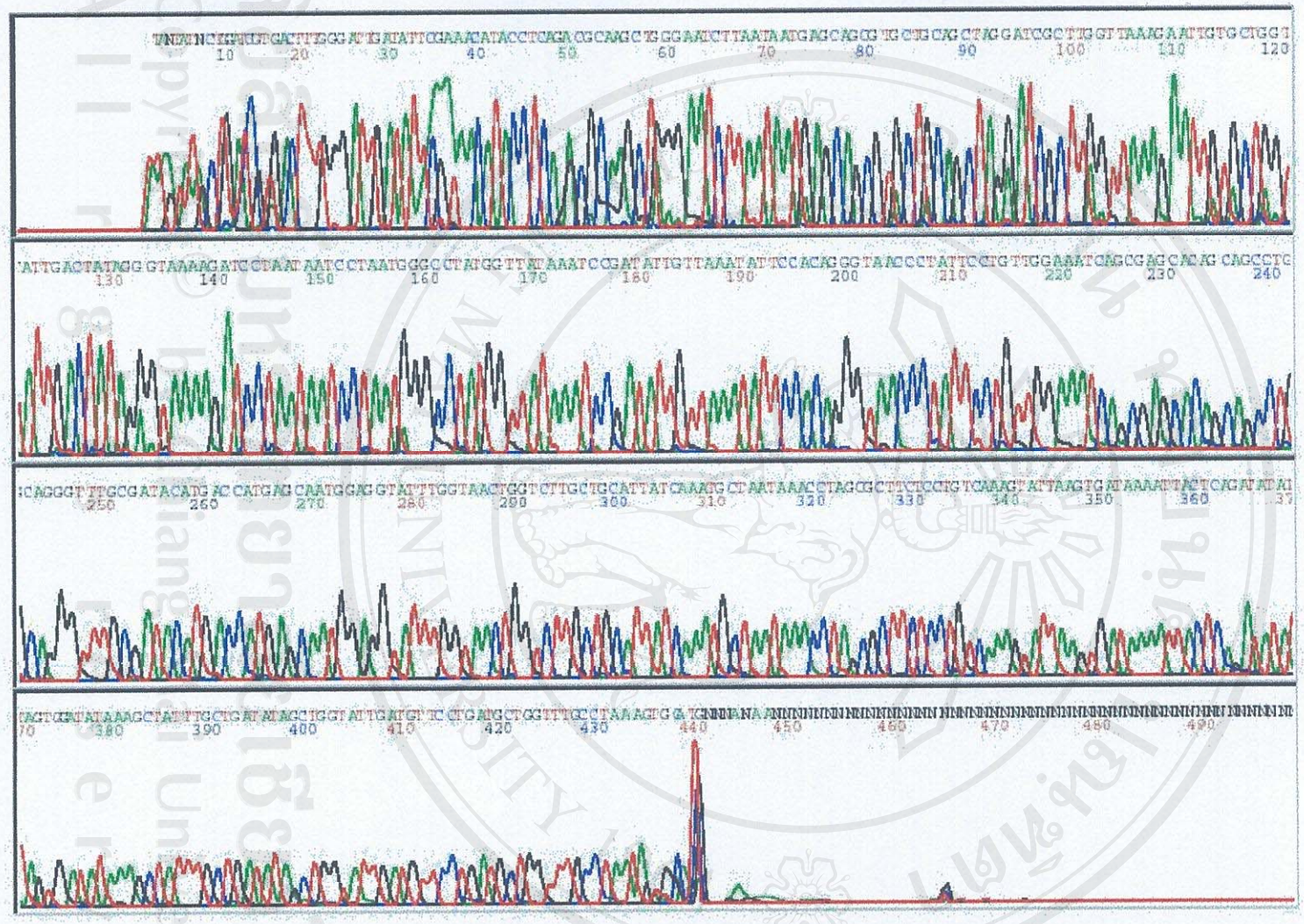

Figure 14 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 210053 collected from the North East region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

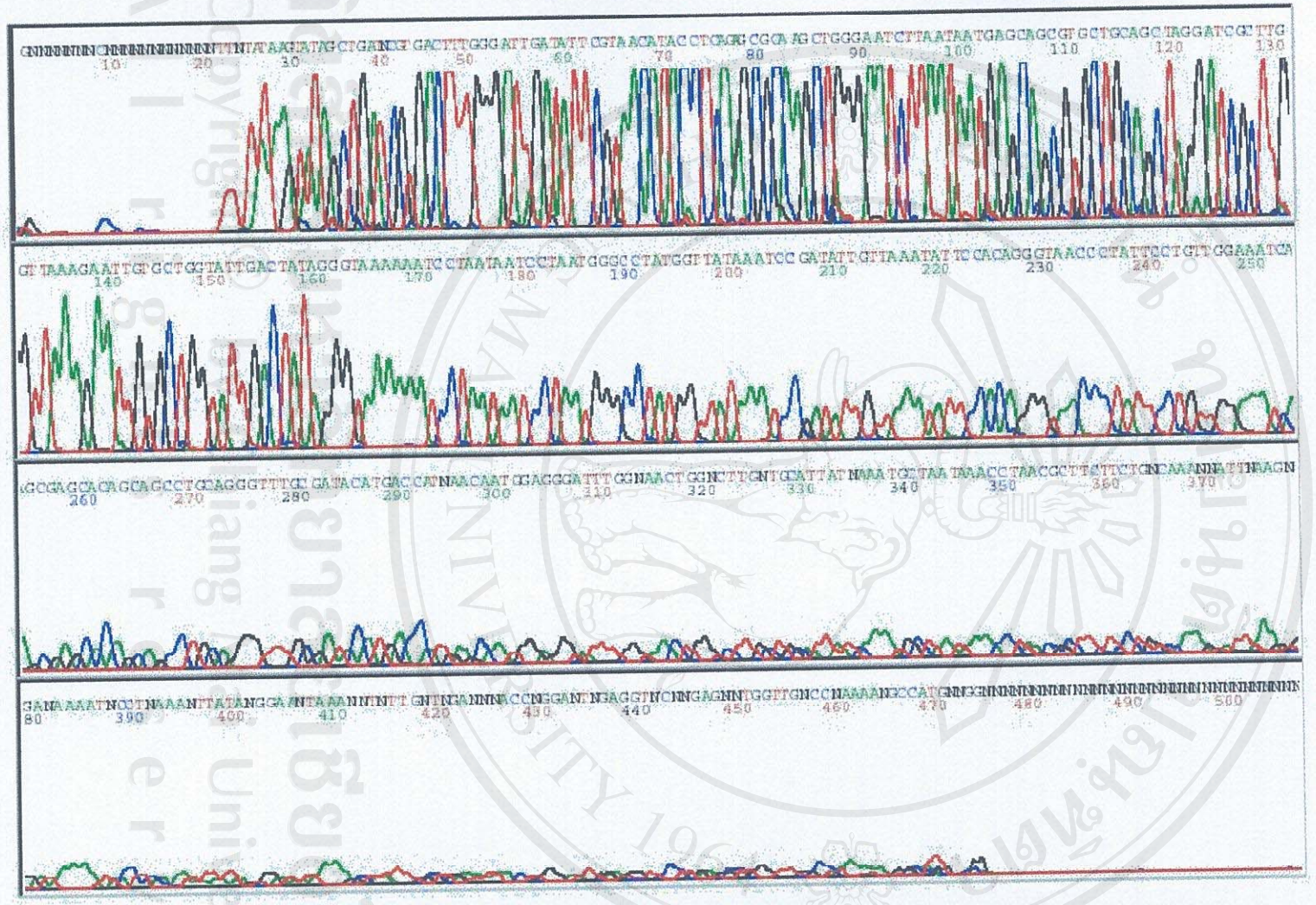

Figure 15 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 220056 collected from the North East region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

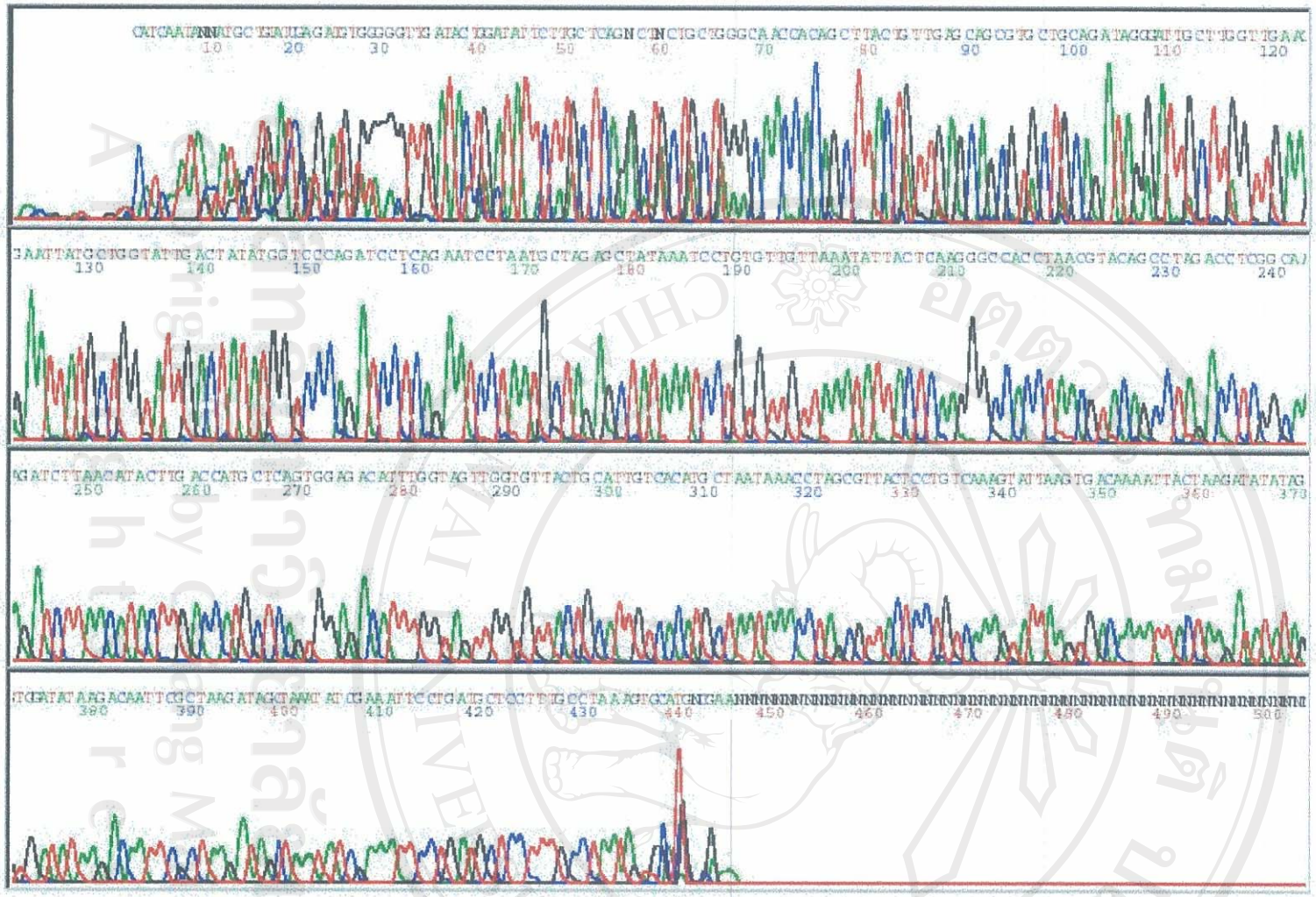

Figure 16 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 200019 collected from the Central region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

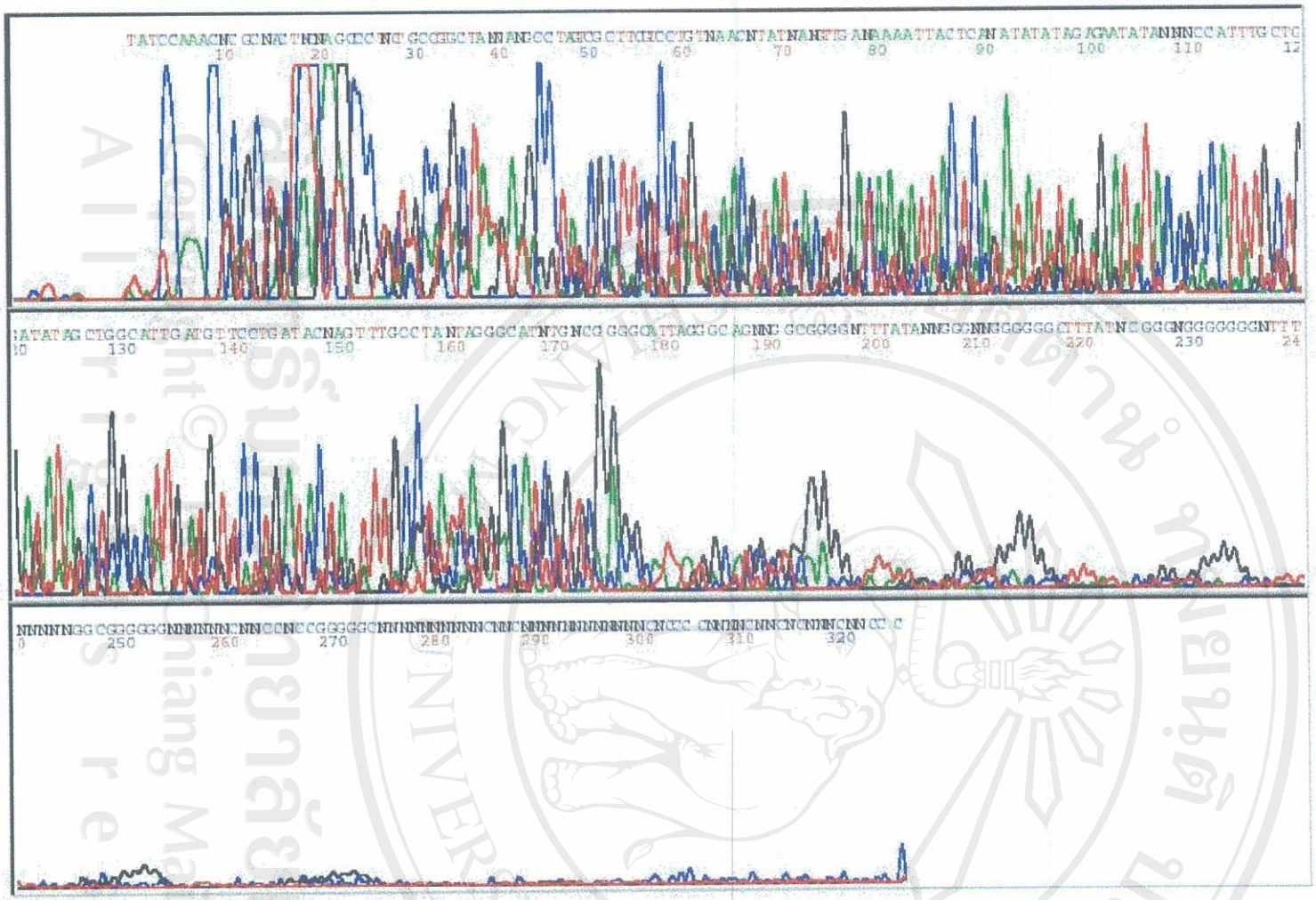

Figure 17 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 210008 collected from the Central region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

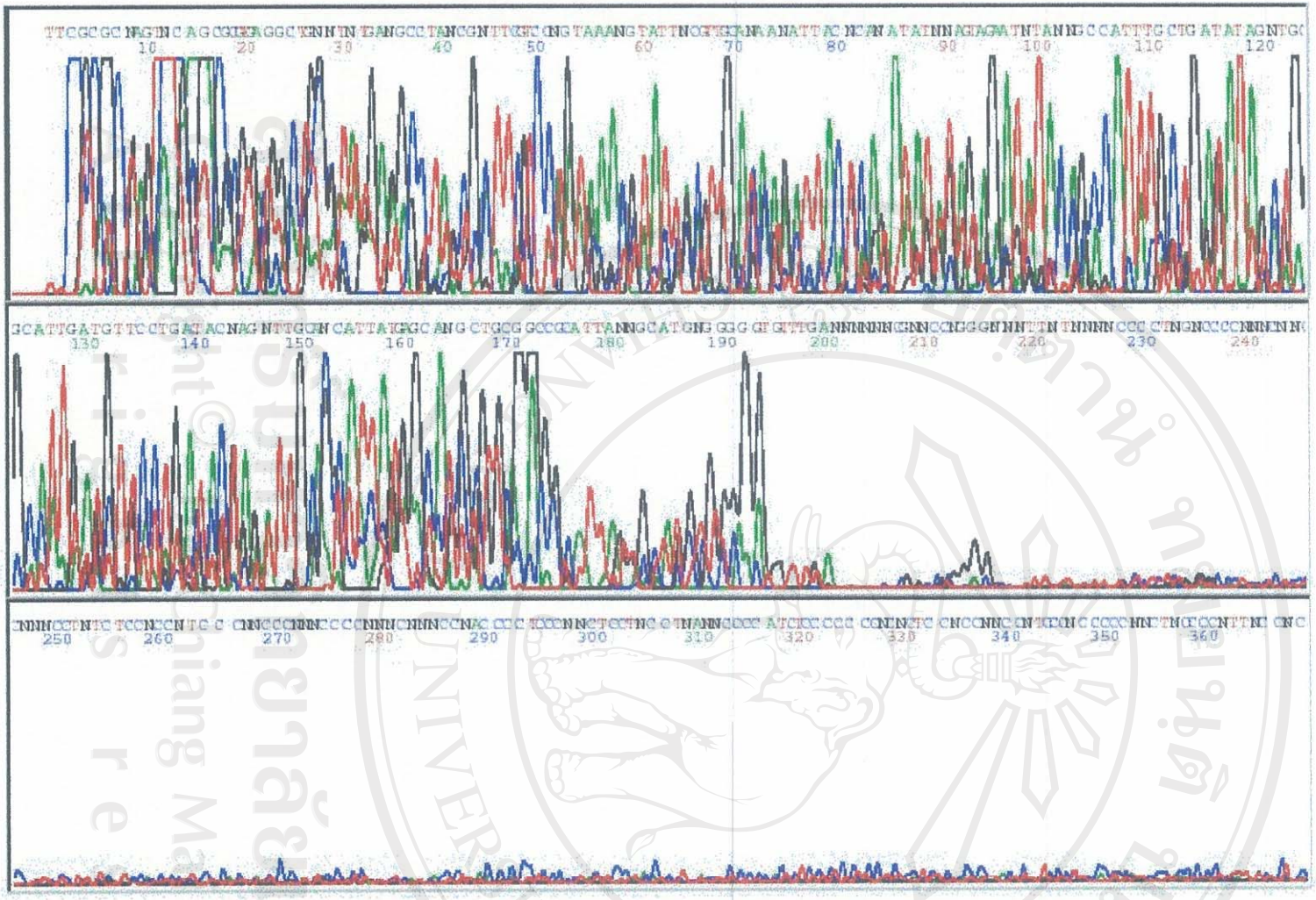

Figure 18 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 220009 collected from the Central region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

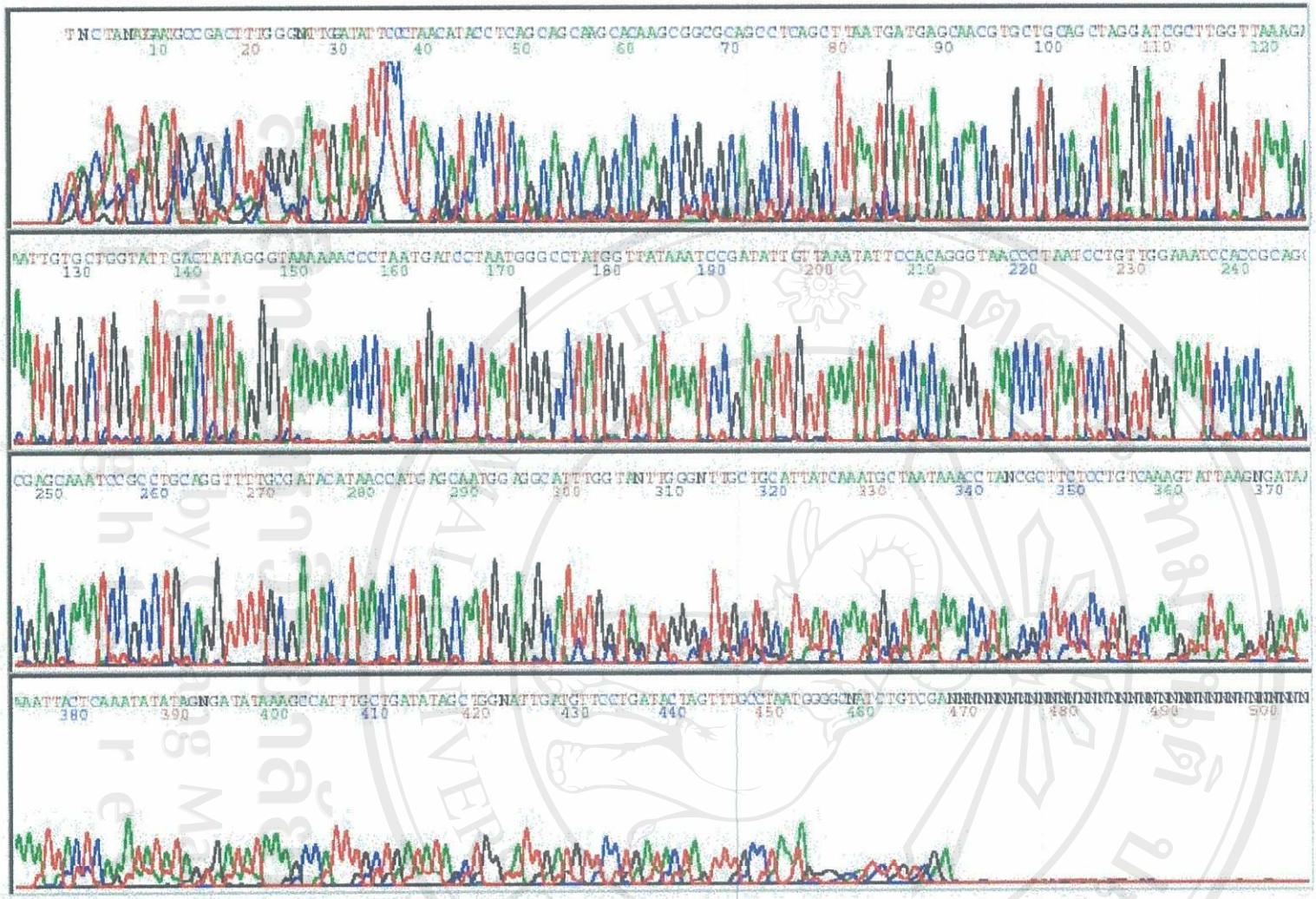

Figure 19 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 200013 collected from the Southern region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

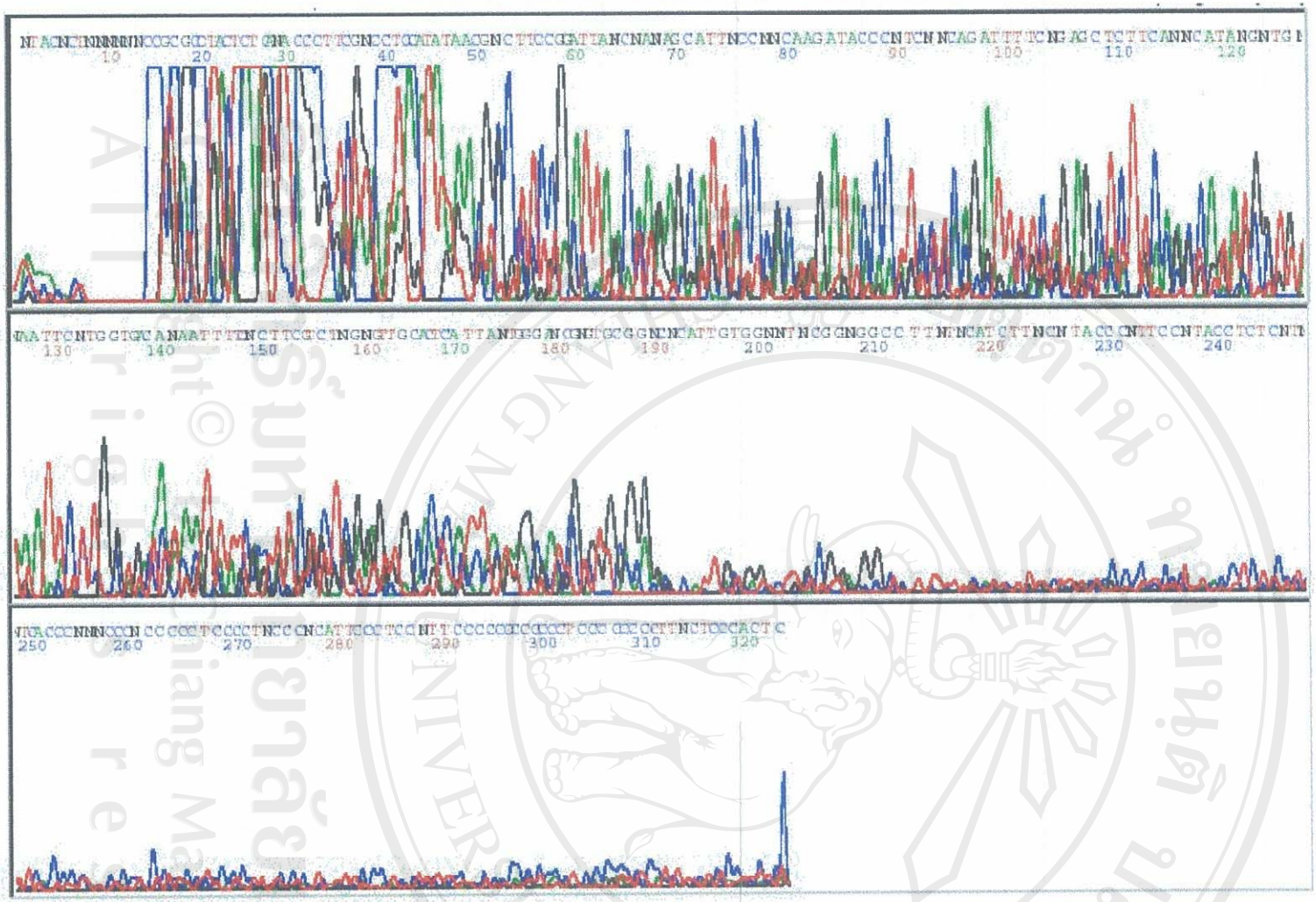

Figure 20 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 210014 collected from the Southern region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

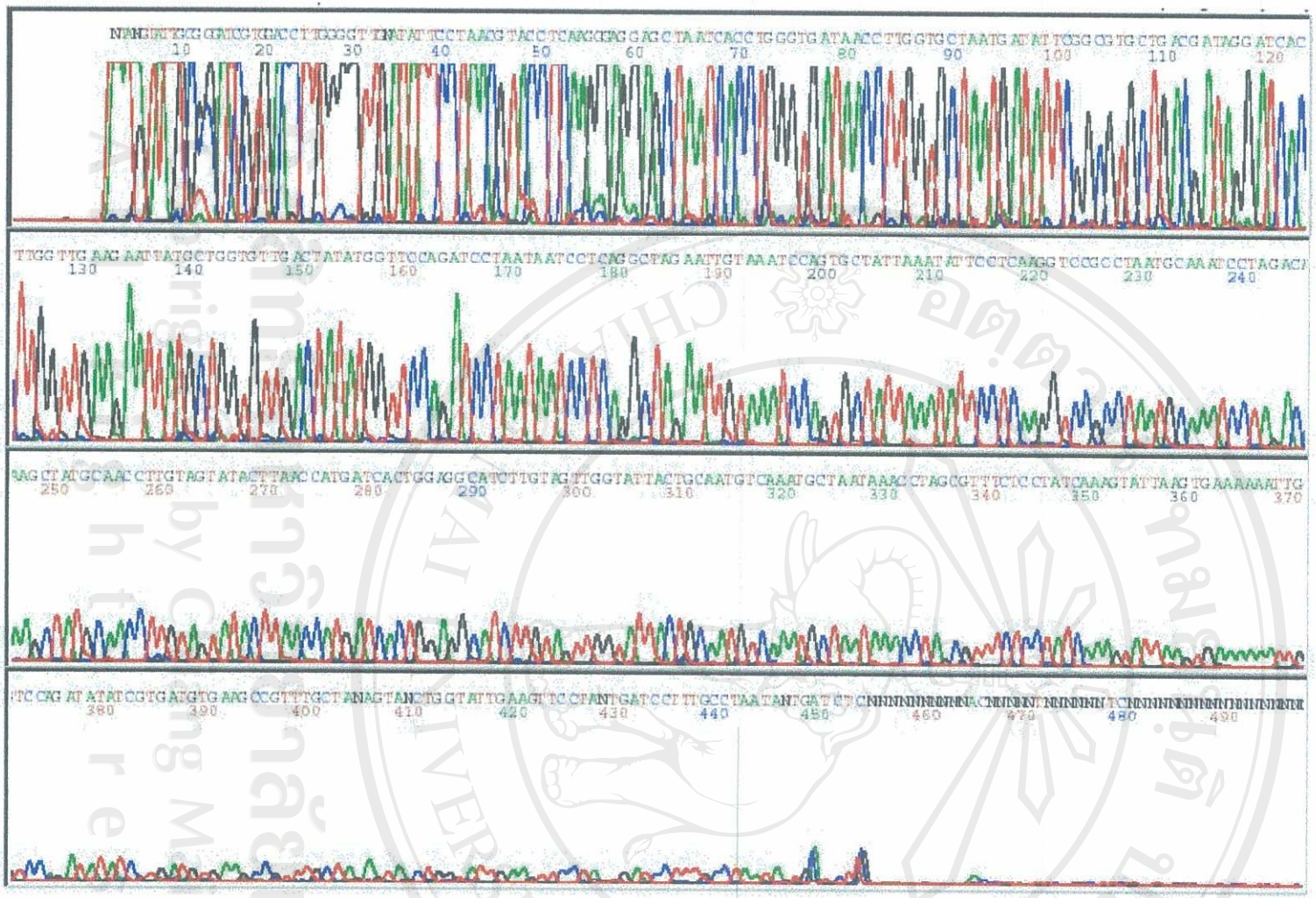

Figure 21 The electrophoregram of the nucleotide sequence of 56 kDa protein gene of *O. tsutsugamushi* detected from the scrub typhus patient number 220180 collected from the Southern region. A; Adenine (green), T; Thymine (red), G; Guanine (black), C; Cytosine (blue)

5. Analysis of nucleotide sequence polymorphism of 56-kDa protein gene

To determine the extent of nucleotide sequence polymorphism in the 56 kDa protein gene of PCR products, the nucleotide sequences of derived nucleotide sequence were analyzed in comparision to the reference prototype sequences in GenBank. The database sequence comparison confirmed that the sequence from the patients were *O. tsutsugamushi* serotype Karp and Kato.The sequence alignment of PCR product obtained by using Karp and Kato primers were 93-97 % homologous with the reference sequence of Karp and Kato serotypes, respectively (Figure 22-33). However, no antigenic variants were detected in this study.

6. Geographic distribution *O. tsutsugamushi*

In our study, the nested PCR and DNA sequencing were combined for genotype identification. The genotypic identification on this report was based on the 56-kDa protein gene which showed *O. tsutsugamushi* serotype specificity. Eighty of the 300 (26.7%) febrile patients from 8 centers were positive for *O. tsutsugamushi* with the highest prevalence (28.7%) in the North-Eastern region. In all study location, *O. tsutsugamushi* Karp serotype were found. Seventy eight patients (97.5%) were positive for Karp. Only two patients (2.5%) were positive for Kato. Gilliam serotype was not detected in any of the samples. The Karp serotype was predominant throughout Thailand whereas Kato was found only in the Southern region (Figure 9). No Gilliam serotype was found (Table 7).

M330004 (1255) ATACATAACCATGAGCAATGGAGGCATTTGGTAGTTGGGCTTGCTGCATTATCAAATGCT(1314)

(252) ....T.....GT..A.....AT..G.....G.... (311)

M330004 (1315) AATAAACCTAGCGCTTCTCCTGCTAAAGTAGTAAGTGATAAAATTACTCAGATATATAGT(1374)

(312) .....A.T.....T.....G..... (371)

M330004 (1375) GATATAAAAGCATTTGGCTGATATAGCTGGTATTGATGTTCCTGATACTAGTTTGCCTAAT (1434)

(372) .....G..T.....T.....A..... (431)

M330004 (1435) AGTG (1438)

(432) .... (435)

Figure 22 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 200054.

M330004 (74) CTTAATGATGAGCAACGTGCTGCAGCTAGGATCACTTGGTTAAAGAATTGTGCTGGTATT (1119)

(62) .....A.....G..... (121)

M330004 (1060) GACTATAGGGTAAAAAACCCTAATGATCCTAATGGGCCTATGGTTATAAATDDGATATTG (1179)

(122) .....T.....A..... (181)

M330004 (1120) TTAAATTATTCCACAGGGTAACCCTAATCCTGTTGGAAATCCACCGCAGCGAGCAAATCCG (1239)

(182) .....T.....-.....C.G.C.A....(235)

M330004 (1240) CCTGCAGGTTTTGCGATACATAACCATGAGCAATGGAGGCATTTGGTAGTTGGGCTTGCT (1299)

(236) .....G..... (295)

M330004 (1300) GCATTATCAAATGCTAATAAACCTAGCAGTTCTCTTGCTAAAGTATTAAGTGATAAAATT (1259)

(296) ..... (355)

M330004 (1360) ACTCATATATAGTGATATAAAGCATTTGGCTGATATAGCTGGTATTGATGTTCCTGCT (1419)

(356) .....C..G.....A..... (415)

Figure 23 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 210050

M330004 (1255) ATACATAACCATGAGCAATGAGGCATTTGGTAGTTGGGCTTGCTGCATTATCAAATGCT (1314)

(252) .....T.G.....G..T.....G.....T..A.....G...C..... (311)

M330004 (1315) AATAAACCTAGCGCTTCTCCTGTCAAAGTATTAAGTGATAAAATTACTCAGATATATAGT (1374)

(312) .....G.A.....C..... (371)

M330004 (1375) GATATAAAGCATTTGGCTGATTATAGCTGGTATTGATGATGATACTAGTTTGAATAAT (1434)

(372) .....A..A.....A..... (431)

Figure 24 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 220054

M330004 (1000) GACTTTGGGATTGATATTCCTAACCAGACCTCAGCAGCAAGCACAAGCCCGCA--GCCTCA (1057)

(15) ....T.....A.G.....GT.....A.....T...GT..... (73)

M330004 (1058) GGCTTAATGATGAGCAACGTGCTGCAGCTAGGATCGCTTGTAAATTCTAGTA (117)

(74) G-.....A..T..T.....(132)

M330004 (1118) TTGACTATAGGGTAAAAAACCCTAAATGATCCTAATGGGGCCTATGGTTATAAATCCGATA (1177)

(133) .....TG.....TG.A.....T.....TG....A.G.....A. (189)

M330004 (1187) TGTTTAAAATATCCAGAGGGGTAACCCTAATCCTGTTGGAAATCCACCGAGCGAGCAAATC (1237)

(190).....T.....T.....(249)

M330004 (1238) CGCCTGCAGTATTTTTCAATAACCCATGAGCAATGGAGGCATTAGGTAGTAGGGCTTG (1297)

(250) .....A.....T.....(309)

M330004 (1298) CTGCATTATCAAATGCATATAAAACTTAGCGCTCCTTGTCAAAGTATTAAGTGAATAAAA (1357)

(310) .....C....T....G.....(369)

M330004 (1358) TTACTCAGATATATAGTGATAATAAAAGCATTTGGCTGATATGCTGGTATTGATGTTCCTG (1417)

(370) ...G.....GA..T.....A.....(429)

M330004 (1418) ATACTAGTTTGCCTAATAGTGCAT (1441)

(430) ....A.....(453)

Figure 25 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 200056

M330004 (1060) CTTAATGATGAGCAACGTGCTGCTAGGATCGCTTTAAAGAATTGTGCTGCTATT (1119)

(65) .....T..... (124)

M330004 (1120) GACTATAGGGGTAAAACCCTAATGATCCTAATGGGCCTATGTTTATAAATCCGTAATG (1179)

(125) .....A.T.....G..... (184)

M330004 (1180) TTAAATATTCCACAGGGTAACCCTAATCCTGTTGGAAATCCACCTCGGCAGACAAATCG (1239)

(185) .....T.....AAAA.....T. (238)

M330004 (1240) CCTGCAGTTTTTGCGATACATAACCATGAGCAATGGAGGCTATGGGGCTGCTTGCT (1299)

(239) .....A.....T.....T.....T...A..... (298)

M330004 (1300) GCATTATCAAATGCTAATAAACTAGCGTCTCGTCAAAGTATTAGTATAAGTGATAAAATT (1359)

(299) ..... (358)

M330004 (1360) ACTCAGTATTATAGT-AGTATAAAGCATTTGGCTGATATAGCTGGTATTGATGTCCTGA (1418)

(359) .....T.....GT..A..... (418)

M330004 (1419) TACTAGTTTGCCTAA (1433)

(419) .G..T..... (433)

Figure 26 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 210053

M330004 (1060) CTTAATGTAGAGCAACGTCGTGCAGCTAGGATCGCTTGGTTAAAGAATTGTTGCTGGTATT (1199)

(94) .....A.....T..... (153)

M330004 (1120) GACTATAGGGTTAAAAAACCCTAATAGTCCTAATGGGCCTATGTTATAAAATCCGATATTG (1179)

(154) .....A.....T..... (213)

M330004 (1180) TTAAATATTCCACAGGGTAACCTTAAATCCTGTTGGAAATCCACCGGAGCGAGCAAATCCG (1239)

(214) .....A.....-- ..... (267)

M330004 (1240) CCTGCAGGTTTTGCGATACATAACCCATGAGCAATGGAGGCAGCTTGGTAGTTGGGCTTGCT (1299)

(268).....T.....A....T.G.....A..G.....A. (327)

M330004 (1300) GCATTATCAAATGCTAATAAACCTAGCGCTTTCTCCTGTCAAAAGTATT-AAGTGATAAAAT (1358)

(328) .....A.....T.....G...T....A...G...T..A..... (387)

Figure 27 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 220056

M330004 (1255) ATACATAACCATGAGCAATGGAGGCATTTGGTAGTTGGGCTTGCTGCATTATCAAATGCT (1314)

(253) .....AT..G....T.....T..... (312)

M330004 (1315) AATAAACCTAGCGCTTCTCCTGCTAAAGTATTAAAGTAGTAAAATTACTCAGATATATAGT (1374)

(313) .....A.T..... (372)

M330004 (1375) -GATATAAAAGCATTTGGCTGATATAGCT-GGTATTGATGTTCCTGATACTAGTATGTTGCTA (1432)

(373).....AT..A..T...G.T.....GT...T..T.....A..A..... (432)

Figure 28 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 200019

M330004 (1350) TGATAAAAATTACTCAGATATATAGTGA-TATAAAGCATTTGGCTGATATAGCTGGTTATTG (1408)

(75) ...A.....T...T.A..... (135)

M330004 (1409) ATGTTCCTGATACTAGTTGCCTAATAGTGCATCTGTCG (1447)

(136) .....T...G....T..A... (174)

Figure 29 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 210008

M330004 (1390) GCTGATATAGCTGGTATTGATGTTCCTGAAACGTCTACCGTATGGGACTTGTTTACTACTT (1450)

(111) .....A...G..... ..... (171)

M330004 (1451) GATGATATAGCTGGTATTGATGTTCCTGAAACGTCTACCGTATGGGACTTGTTTACTACGG(1551)

(172) .....T...C.....- ..... (232)

Figure 30 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 220009

M330004 (1012) GATATTCCTAACCAGACCTCAGCAGCAAGCACAAGC—CGCAGCCTCAGGCTTAATGATTG (1069)

(30) ……..A.T..G……..T……..T……. (88)

M330004 (1070) AGCAACGTGCTGCAGCTAGGATCGCTTGGTTAAAGAATTGTGCTGGTATTGACTATAGGG (1129)

(89) …… (148)

M330004 (1130) TAAAAAACCTCCCTAATGATCCTAATGGGCCTATGGTTATAAATCCGATATTGTTAAATATC (1189)

(149) …… (208)

M330004 (1190) CACAGGGTAACCCTAATCCTGTTGGAAATCCACCGCAGCGAGCAAATCCGCCTGCATGTT (1249)

(209)…… (268)

M330004 (1250) TTGCAGTACATAACCTATAGCAATGGAGGCCATTTGGTAGTTGGGCTTGTTGCATTATCA (1309)

(269)……..A…..T……... (328)

M330004 (1310) ATGCTAATAAACCTAGCGCTTCTCCTGTCAAAGTATTAAGTGATAAAATTACTCAGATAT (1369)

(329) ……..A……..T……..G…. (388)

M330004 (1370) ATAGTGTATTAAAGCCATTTGGGCTGATATAGCCTGGTATTGATGTTCCTGATACTAGTGC (1429)

(389) ….A……..GA..A……..T…… (448)

M330004 (1430) CTAATAGTGC-ATCTGTCGA (1448)

(449) …..A.C..G…… (468)

Figure 31 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Karp and clinical sample no. 200013

M63382 (969) TAAGTATTGCGG-ATCGTG-ACCTTGGGGTTG-ATATTCCTAACGTACCTCAAAG-AGA (1024)

(2) ..A.....T.....C.....T.....A..... (61)

M63382 (1025) GCTAATCTCCTGGGTGTATAATCCCTTGGTGCTAATGATATTCGGCGTGCTGACGATAGC (1084)

(62) ..... (121)

M63382 (1085) ACTTGGTTGAAGTTATATGCTGGTGTTGACTATATGGTCCTAGATCCTATAATCCTCAG (1144)

(122) ..... (181)

M63382 (1145) GCTAGAATTGTAAATCCAGTGCTATTAAAATATTCCTCAAGGTCCGCCTAATGCAAATC (1204)

(182) ..... (241)

M63382 (1205) AGACAAGCTATGCCACCTTGTAGTTATTCTTAACCATGATCACTGGAGGCATCTTGTGTT (1264)

(242) ..... (301)

M63382 (1265) GGTATTACTGCAATGTCAAAAGCTAATAAACCTAGCGTTTCTCTATCAAAGTATTAAGT (1324)

(302) ..... (361)

M63382 (1325) GAAAAAATTGTCCAGATATATCGTGATGTGAAGCCGTTTGCTAGAGTAGCTGGTATTGAA (1384)

(362) .....A....T..... (421)

M63382 (1385) GTTCCTAGTGATCCTTTGCCTAATAGTG (1412)

(422) .....A.....T.. (449)

Figure 32 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Kato and clinical sample no. 210014

M63382 (969) TAAGTATTGCCG-ATCGTG-ACCTTGGGGTTGATATTCCTAAGCTATCTCAAGG-AGGA (1024)

(2) ..T.....A.....T.....G.....T....(61)

M63382 (1025) GCTAATCACCTGGGTGATAACCTTGGTGCTAATGATATTCGGCGTGCTGACGATAAGATC (1084)

(62) .....(121)

M63382 (1085) ACTTTGTTGAAGAATTATGCTGGTGTTGACTATATGGTTCCAGATCCTAATAATCCTCAG (1144)

(122) .....(181)

M63382 (1145) GCTAGAATTGTAAATCCAGTGCTATTAAATATTCCTCAAGGTCCGCCTAATGCAAATCCT (1204)

(182) .....(241)

M63382 (1205) AGACAAGCTATGCAACTTTGTAGTATACTTAACCATGATCACTGGAGGCATCTTGTAGTT (1264)

(242) .....(301)

M63382 (1265) GGTATTACTGCAATGTCAAATGCTAATAAACCTAGCGTTCTTCTTACTAAAGTATTAAGT (1324)

(302) .....(361)

M63382 (1325) GAAAAAATTGTTCAGATATATCGTGATGTGAAGCCCGTTTGCTAGAGTAGCTGGTATTGAA (1384)

(362) .....(421)

M63382 (1385) GTTCCTAGTGATCCTTTGCCTAATAGTG (1412)

(422) .....C.....A.. (421)

Figure 33 Nucleotide sequence comparison of the 56 kDa protein gene of the prototype Kato and clinical sample no. 220180