VII. RESULTS

1. PCR products from GAS

Full length *emm* genes of 43 GAS strains were amplified by PCR. The sizes of PCR products varied from 900 to 1600 base pairs (bp) depending on the M-types and the strains. The sizes of PCR products of M6, M24, M49 and M52 were comparable to the sizes of the published sequences of *emm* genes (Mouw *et al.*, 1988; Hollingshead *et al.*, 1986; Haanes and Cleary, 1989; Bisno, 1991) as shown in Table 9.

Table 9 Comparison of the sizes of PCR products from some M-typable GAS and those of the published *emm* genes

GAS	Sizes of the PCR products in this study	Sizes from published sequences
M6	1500 bp	1404 bp
M24	1600 bp	1571 bp
M49	1000 bp	1036 bp
M52	1250 bp	1325 bp

P6 and spf1 primers amplified all *emm* genes of the OF-negative and P49 and spf1 primers amplified all *emm* genes of the OF-positive GAS strains (Table 11). The sizes of PCR products from OF-negative GAS strains, except AK14, O20 and M 55, tended to be larger than those from OF-positive GAS strains (Figures 10-13, lanes A and Table 11).

2. Digestion of the PCR products with restriction enzymes

Eight restriction enzymes were used to digest the PCR products of M protein genes (Table 9). *Hind*III digested 9 of 21 *emm* genes tested (Figure 9). *Kpn*I and *Bgl*II digested only a few *emm* genes whereas *Sal*I, *Pst*I, *Eco*RI and *Bam*HI did not digest any *emm* genes tested. However, *Sal*I, *Pst*I, *Bam*HI, *Bgl*II, *Kpn*I, and *Hind*III digested chromosomal DNAs of GAS (Figure 21).

*Mbo*I digested almost all *emm* genes of M-typable GAS strains (19 from 22) and the patterns of digestions were different from each other (Figures 10-13 lanes B). The sizes of three *emm* genes of M-typable GAS (M5, M58, M79) which were not digested by *Mbo*I were different (Figure 14). *Mbo*I was therefore chosen for digesting the amplified PCR products.

3. Digestion patterns of M-typable and M-nontypable GAS

All PCR products from 43 GAS were digested with *Mbo*I and analyzed by electrophoresis on 1% agarose gel. The digestion patterns were shown in Tables 11 and 12 and Figures 10-13. Forty-three GAS strains yielded 33 patterns (Figures 10-13). GAS strains with more than 90% homology of the N-terminal DNA sequences (S14, S122, S142, S219 and S330; 38 and M75; H92 and M58) showed identical *Mbo*I digestion patterns (Figures15, 16 and 17). However, M22 and ARF19, and ARF3 and ARF15 gave different digestion patterns despite more than 90% homology of N-terminal nucleic acid sequences (Figures 18 and 20, lanes 1-4). Those GAS strains having less than 90% homology of the N-terminal nucleic acid sequences gave different patterns of the *Mbo*I digested fragments (Figures 10-12). Some strains gave very similar but unique *Mbo*I digestion patterns (Figures 19 and 20). Only 6 of 43 *emm* genes (3 genes derived from M-typable and the rest from M-nontypable GAS) were not digested by *Mbo*I (Table 11 and Figure 14).

4. Analysis of GAS strains by RFLP

Chromosomal DNA of two different M types of GAS, M5 and M18, were digested with *Sal*I, *Hind*III, *Kpn*I, *Bgl*II, *Bam*HI and *Pst*I. The digested fragments were visualized by staining with ethidium bromide after running on 0.8% agarose gel electrophoresis. The distinctive fragments were between 4 to 23 kb. *Pst*I digested fragments were more easily resolved than those digested by other enzymes (Figure 21). Therefore, *Pst*I was used for the digestion of GAS genomic DNAs in the analysis by RFLP. The results showed that restriction fragments from GAS strains having higher than 90% homology of N-terminal sequences of *emm* genes gave the same patterns of RFLP such as S14, S122, S142, S219 and S330 (Figure 22), 38 and M75, M22 and ARF19 (Figure 23), K16 and K3 (Figure 24), S665 and J63, H92 and M58 (Figure 25), and O20 and AK14 (Figure 26). However, M25 and 42 gave different RFLP patterns inspite of their identical N-terminal sequences (Figure 27). Other GAS strains having lower than 90% homology of N-terminal sequences of *emm* genes showed different patterns of the RFLP (Figures 28, 29 and Table 12).

5 Homology of N-terminal sequence of *emm* genes, *Mbo*I digestion patterns and RFLP fragments

*Mbo*I digestion patterns of the *emm* genes of GAS strains were correlated with the percent homology of N-terminal sequence. The percentages of homology of GAS by RFLP method were also compared as shown in Table 12.

Each of GAS strains with less than 90% homology of N-terminal sequence of *emm* genes (M1, M5, M6, M13, M18, M24, M39, M49, M52, M55, M59, M66, M73, M76, M77, M79, M80, M3R, CSF3, 104, J82, ARF3, ARF15, H140) yielded unique *Mbo*I digestion patterns (Figures 10-13, 18-20). The GAS pairs with 81-90% homology of N-terminal sequence of *emm* genes

(H140-M18, ARF3-M59, ARF15-M13, ARF3-M13, ARF15-M59) also gave low percentage of homology by the RFLP method (57.14-73.91%) (Figures 28, 29).

Almost all GAS strains with more than 90% homology of N-terminal sequence of *emm* genes (16 from 18, 88.89%) gave identical *Mbo*I digestion patterns (Tables 11, 12, Figures 12-17). The homology of GAS analyzed by RFLP patterns varied from 85.71% to 100% (Table 12, Figures 22-27). Only 2 pairs of GAS (11.11%) in this group, ARF3-ARF15 and ARF19-M22, gave different PCR product sizes (Figure 18) and different *Mbo*I digestion patterns (Figure 20). They gave 85.71 and 90% homology of chromosomal DNA respectively by the RFLP method (Figures 29 and 23, respectively).

Table 10 Number of the fragments produced by restriction enzymes digestion of the PCR products of *emm* genes

Enzyme	M 1	M 5	M 6	M 13	M 18	M 22	M 24	M 25	M 39	M 49	M 52	M 55	M 58	M 59	M 66	M 73	M 75	M 76	M 77	M 79	M 80	M 3R	digestion (%)
*Sal*I	ND	0	ND	0	0	0	ND	0	ND	0	ND	0	0	ND	0	0	0	0	ND	0	ND	0	0
*Hind*III	2	2	2	0	2	0	2	0	3	0	3	0	0	ND	0	0	0	0	2	0	2	0	42.9
*Kpn*I	0	2	ND	0	0	0	ND	0	0	0	ND	0	0	ND	0	0	0	0	ND	0	ND	0	6.25
*Bgl*II	0	0	ND	0	0	2	ND	0	ND	2	0	0	0	ND	0	0	0	2	ND	0	ND	0	18.75
*Pst*I	0	0	0	0	0	0	ND	0	ND	0	ND	0	0	ND	0	0	0	0	ND	0	ND	0	0
*Eco*RI	0	0	ND	0	0	0	ND	0	ND	0	ND	0	0	ND	0	0	0	0	ND	0	ND	0	0
*Bam*HI	0	0	ND	0	0	0	ND	0	0	0	ND	ND	0	ND	0	0	0	0	ND	0	ND	0	0
*Mbo*I	2	0	2	2	2	2	2	2	2	3	2	2	0	3	3	2	3	2	2	0	2	2	86.36

ND = not done

0 = not digested

$$\%\text{Digestion} = \frac{\text{No. of digested PCR product} \times 100}{\text{No. of tested PCR product}}$$

Table 11 Summary of the sizes of the PCR products from *emm* genes of GAS isolated from patients and standard strains and their respective restricted fragments and homology of M type

No.	GAS	Primer	Homology of M type (%)	OF	Sizes of PCR products (bp)	Sizes of *Mbo*I restricted fragments (bp)
1	M24	P6		N	1600	680,200
2	M6	P6		N	1500	1250,220
3	M39	P6		N	1500	920,500
4	M1	P6		N	1500	750,480
5	M5	P6		N	1450	1450
6	104	P6		?	1450	800,680
7	H140	P6	M18 (82)	N	1400	1000,320
8	M80	P6		N	1300	1000,280
9	S14	P6		N		
10	S122	P6		N		
11	S142	P6	M41 (95-97)	N	1250	870,250
12	S219	P6		N		
13	S330	P6		N		
14	M18	P6		N	1250	900,280
15	M52	P6		N	1250	820,350
16	M22	P49		P	1200	800,250
17	M59	P49		P	1200	580,180,130
18	M13	P49		P	1200	780,280
19	M55	P6		N	1100	700,250
20	ARF19	P49	M22 (100)	P	1100	680,300
21	42	P49	M25 (100)	P	1100	800,300
22	M25	P49		P	1100	800,300
23	ARF15	P49	M63 (98)	P	1100	700,280
24	M76	P49		P	1100	700,350
25	M58	P49		P	1100	1100
26	H92	P49	M58 (97)	P	1100	1100
27	38	P49	M75 (98)	P	1000	380,350,280
28	M75	P49		P	1000	380,350,280
29	M79	P49		P	1050	1050
30	M49	P49		P	1000	380,270,120
31	CSF3	P49	-	?	1000	700,250
32	M73	P49		P	1000	650,250
33	K16	P49	-	?	1000	650,280,100
34	K3	P49	-	?	1000	650,280,100
35	S665	P49	M44 (99)	P	1000	1000
36	J63	P49	M44 (99)	P	1000	1000
37	M77	P49		P	980	680,200
38	ARF3	P49	M63 (97)	P	980	650,300
39	M3R	P49		P	900	650,180
40	M66	P49		P	900	500,350,280
41	O20	P6	M10, 12 (99)	N	900	380,300,120
42	AK14	P6	M10, 12 (99)	N	900	380,300,120
43	J82	P49	-	?	900	280

N = OF-Negative , P = OF-Positive

- = lower than 80% homology of *emm* gene of M-nontypable to that of M-typable

Table12 Homology of N-terminal sequence of *emm* genes, *Mbo*I digestion patterns and RFLP fragments.

%homology of N-terminal sequence of *emm* genes	*Mbo*I Digestion Pattern		RFLP			
	Identical	Different	N1	N2	NS	%homology
≤90%		M1, M5, M6, M13, M24, M39, M49, M52, M55, M66, M73, M76, M77, M79, M3R, CSF3, 104, J82	-	-	-	-
82%		H140, M18	25	20	15	66.66%
87%		ARF3, M59	19	16	10	57.14%
89%		ARF15, M13	19	20	13	66.66%
89%		ARF3, M13	19	19	11	57.89%
89%		ARF15, M59	25	21	17	73.91%
100%						57.14-73.91%
>90%						
93%	S142, S219		22	21	20	93.02%
94%	S14, S219		22	21	20	93.02%
95%	S142, S330		26	26	26	100%
96%	S14, S330		26	26	26	100%
96%	S122, S219		22	21	20	93.02%
96%	S219, S330		21	22	20	93.02%
96%	S14, S142		26	26	26	100%
97%	S665, J63		25	25	24	96%
97%	S122, S330		26	26	26	100%
97%		ARF3, ARF15	19	16	15	85.71%
98%	38, M75		27	27	27	100%
98%	H92, M58		21	21	20	95.24%
98%	S122, S142		26	26	26	100%
99%	K16, K3		24	26	24	96%
99%	O20, AK14		19	17	17	94.44%
100%	42, M25		20	19	17	87.18%
100%	S14, S122		26	26	26	100%
100%		ARF19, M22	21	19	18	90%
	16/18 = 88.89%	2/18 = 11.11%				85.71-100%

N1, N2 = Number of bands in the two prints compared

NS = Numbers of bands shared

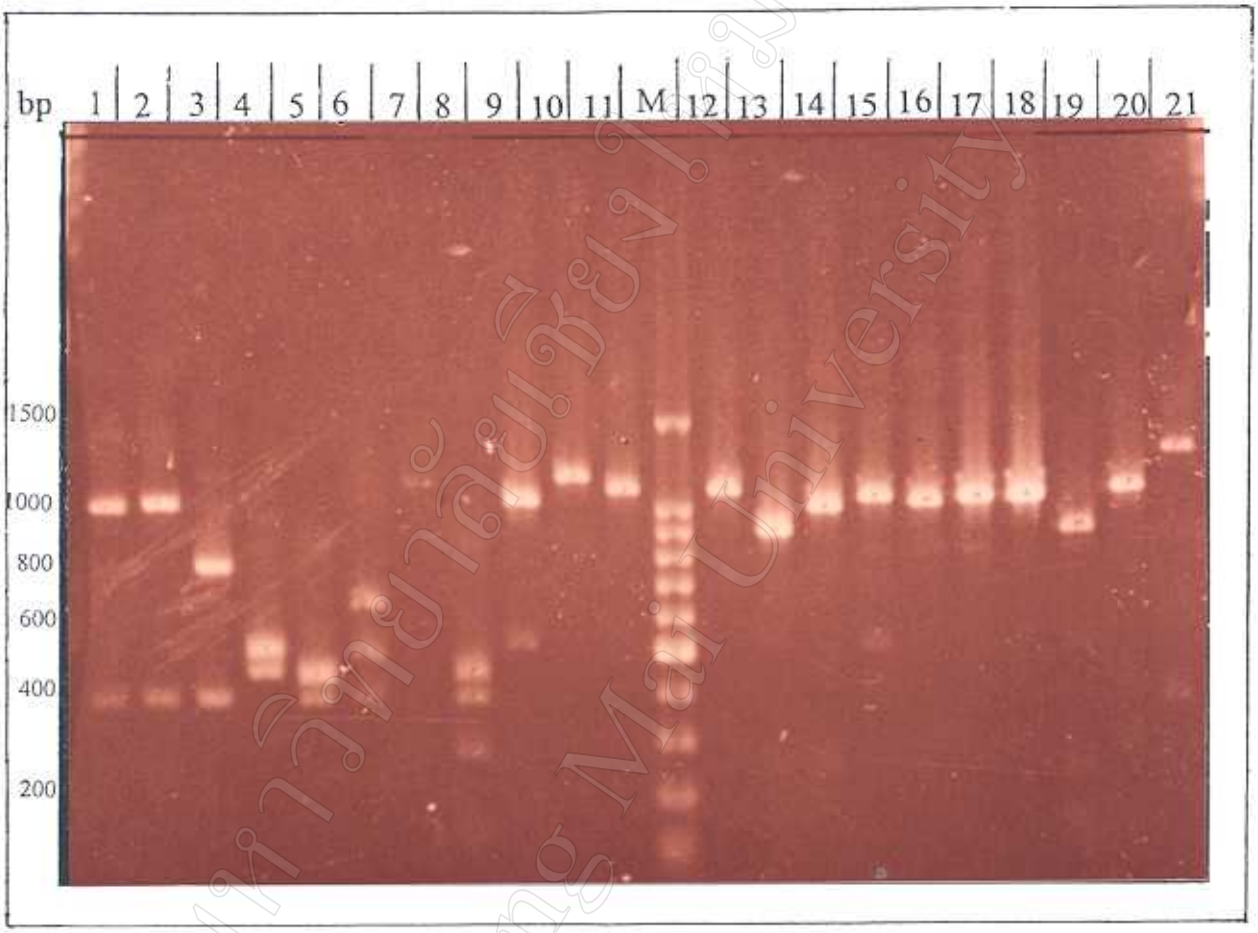

Figure 9 *Hind*III restriction of PCR products from M-typable GAS. *Hind*III digested PCR products of M-typable GAS, M5, M1, M18, M77, M80, M39, M6, M52, M49, M22, M13, M58, M66, M73, M76, M75, M55, M79, M3R, M25 and M24 are shown in lanes 1-21, respectively. Only 9 out of 21 PCR products, M1, M5, M6, M18, M24, M39, M52, M77, M80, were digested by enzyme *Hind*III. Lane M represents 100 bp ladder molecular weight markers.

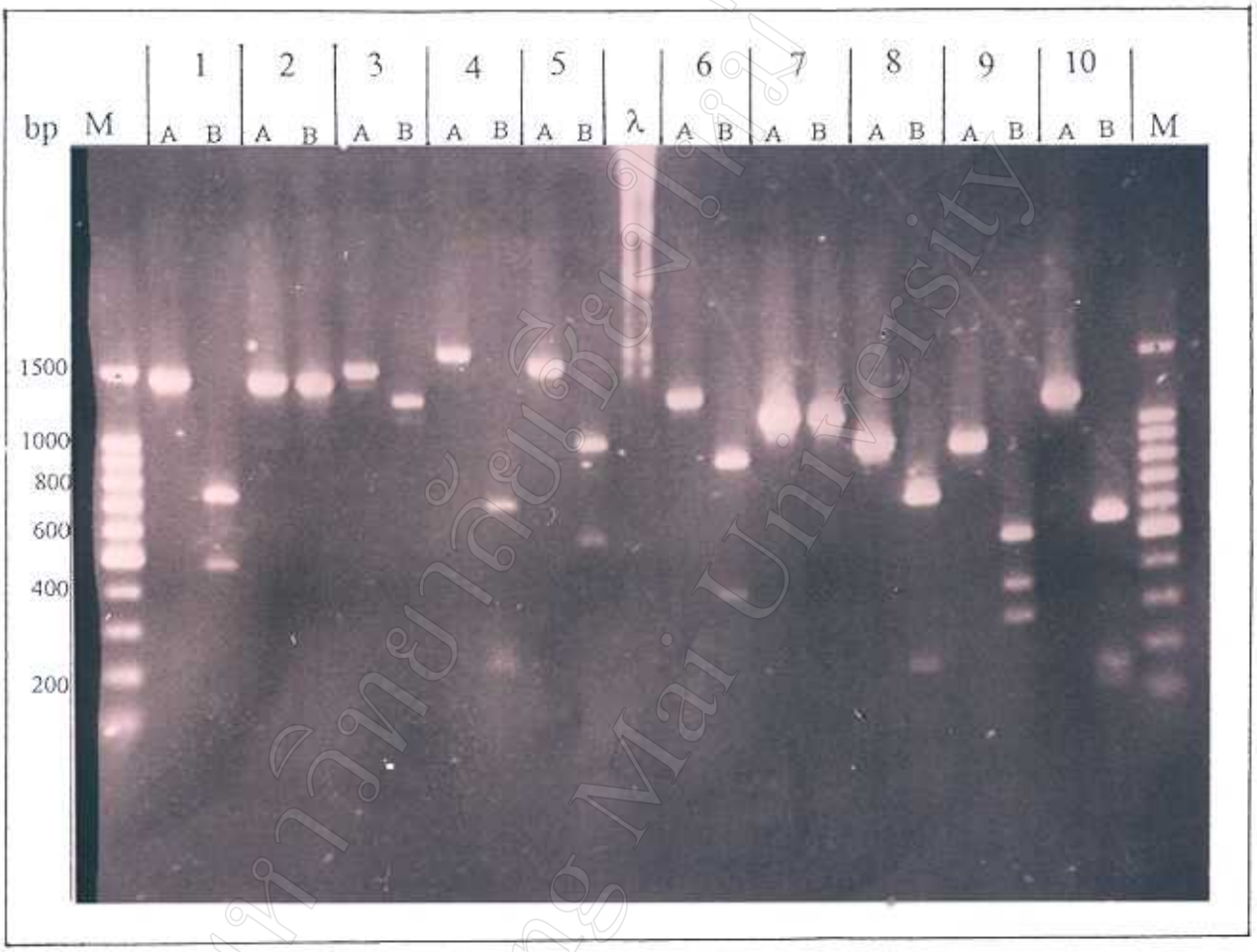

Figure 10 *Mbo*I digestion patterns of 10 M-typable GAS. The *Mbo*I digestion patterns of M1, M5, M6, M24, M39, M52, M58, M3R, M66 and M59 are shown in lanes 1-10, respectively. Lanes A and B represent the undigested and *Mbo*I digested PCR products of each M-type, respectively. Lanes M represent 100 bp ladder DNA molecular weight markers. Lane λ represents *Hind*III-digested λ DNA markers.

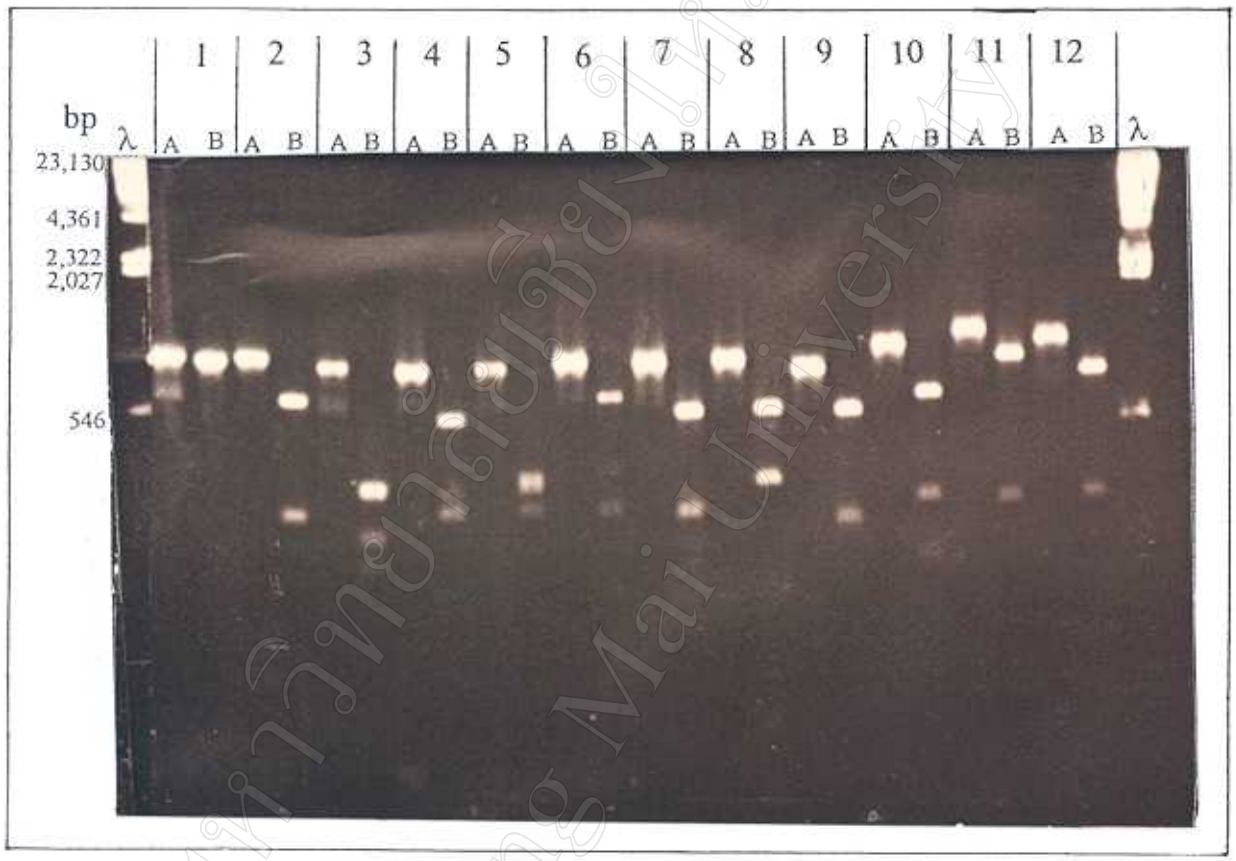

Figure 11 *Mbo*I digestion patterns of 12 M-typable GAS. The *Mbo*I digestion patterns of M79, M13, M49, M73, M75, M25, M55, M76, M77, M22, M80 and M18 are shown in lanes 1-12, respectively. Lanes A and B represent the undigested and *Mbo*I digested PCR products of each M-type. Lanes λ represent *Hind*III-digested λ DNA markers.

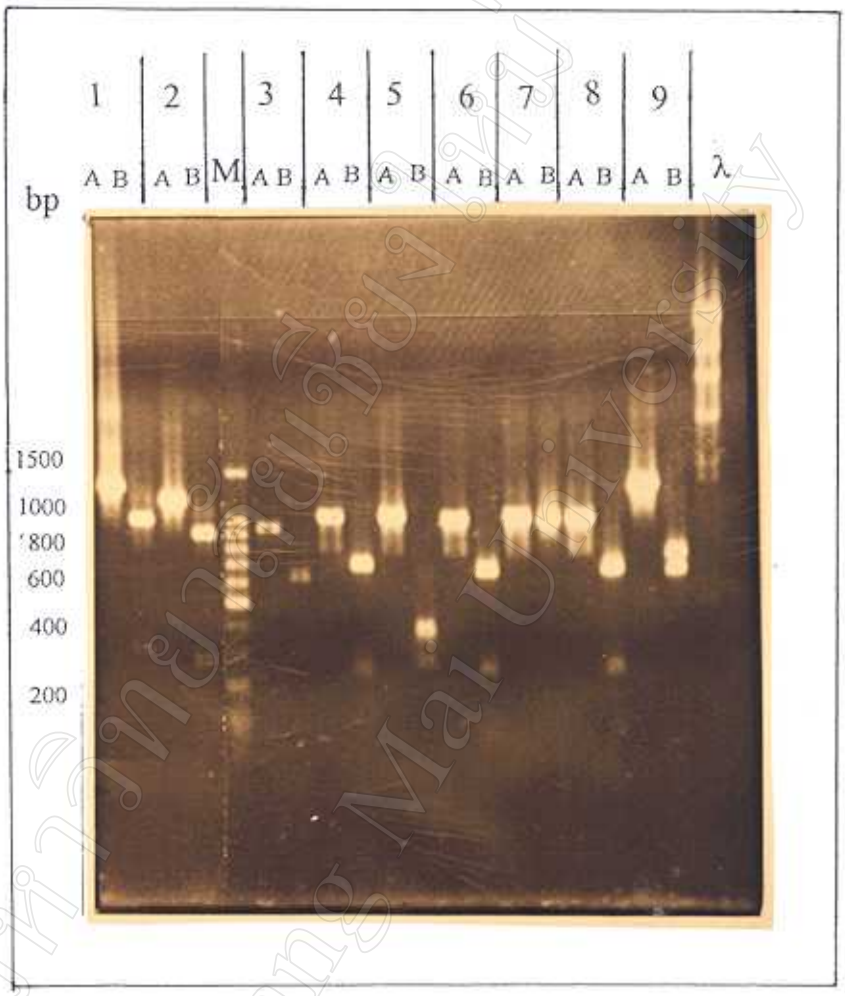

Figure 12 *Mbo*I digestion patterns of M-nontypable GAS. The *Mbo*I digestion patterns of H140, M18, ARF3, CSF3, 38, K16, J63, K3 and 104 are shown in lanes 1-9, respectively. Lanes A and B represent the undigested and *Mbo*I digested PCR products of each strain. Lane M represents 100 bp ladder DNA molecular weight markers. Lanes λ represents *Hind*III-digested λ DNA markers.

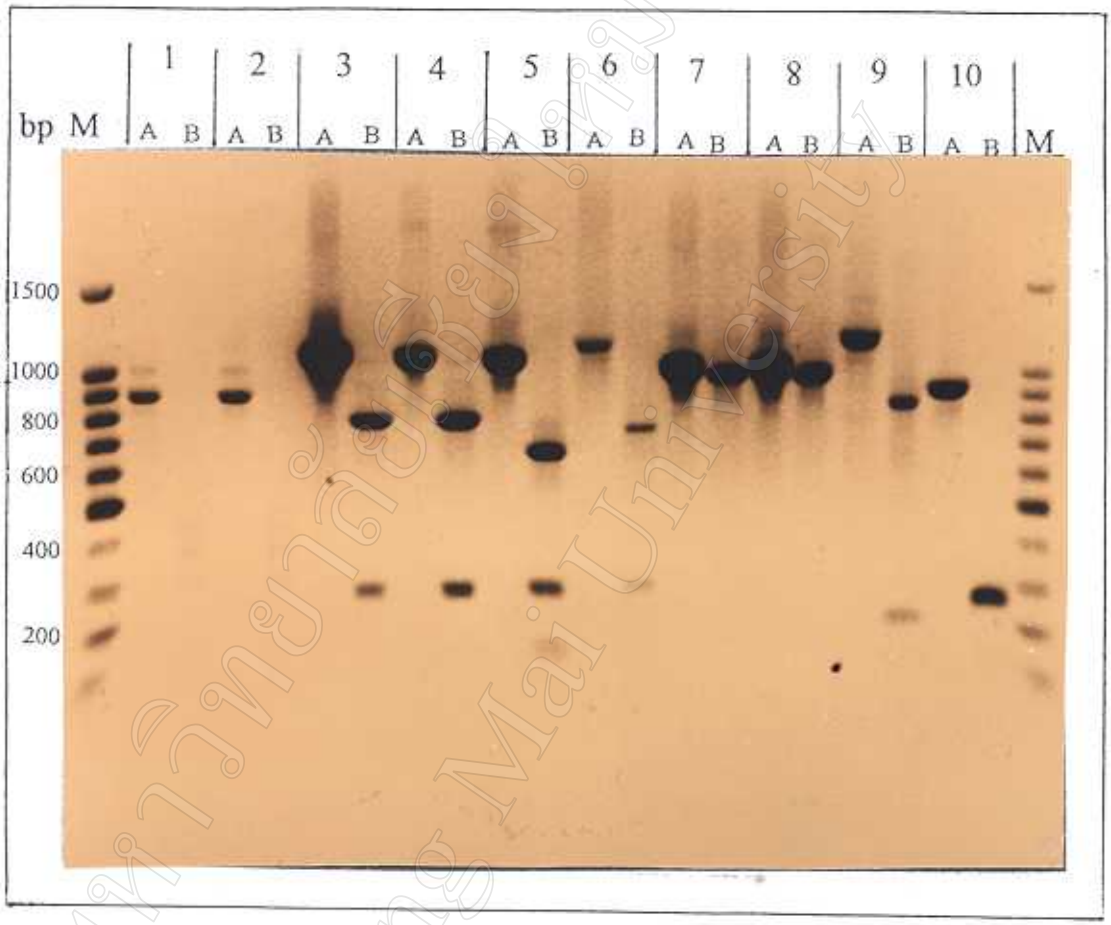

Figure 13 *Mbo*I digestion patterns of each pair of GAS with high percentage homology of N-terminal sequence of *emm* genes and the other non-homology GAS strains. The *Mbo*I digestion patterns of O20-AK14, 42-M25, ARF19-M22, S665-J63 GAS pairs, with high percentage homology of N-terminal sequence of *emm* genes, and S219 and J82 GAS strains are shown in lanes 1-10, respectively. Lanes A and B represent the undigested and *Mbo*I digested PCR product of each strain, respectively. Lanes M represent 100 bp ladder DNA molecular weight markers.

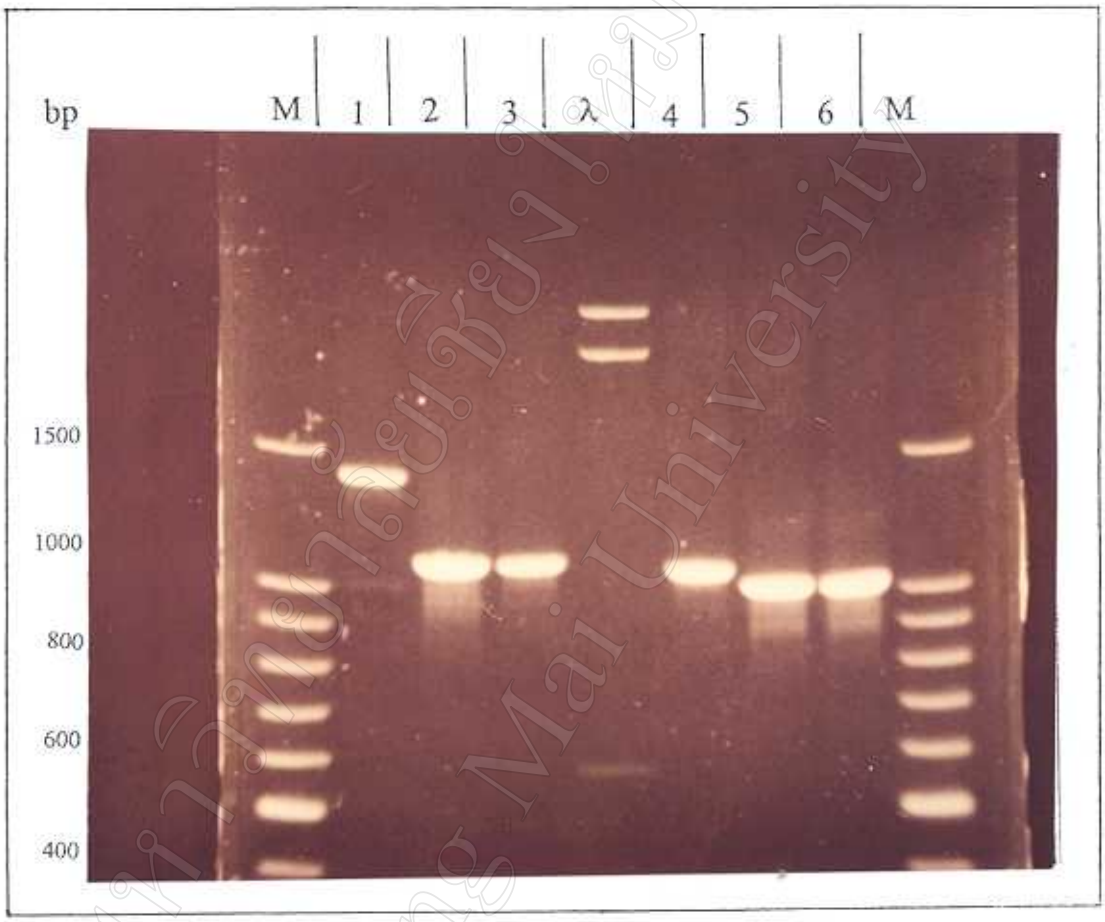

Figure 14 PCR products of 6 undigested *emm* genes. The PCR products of undigested *emm* genes of M5, M58, H92, M79, S665 and J63 are shown in lanes 1-6, respectively. Lane M represents 100 bp ladder DNA molecular weight markers. Lanes λ represents *Hind*III-digested λ DNA molecular weight markers.

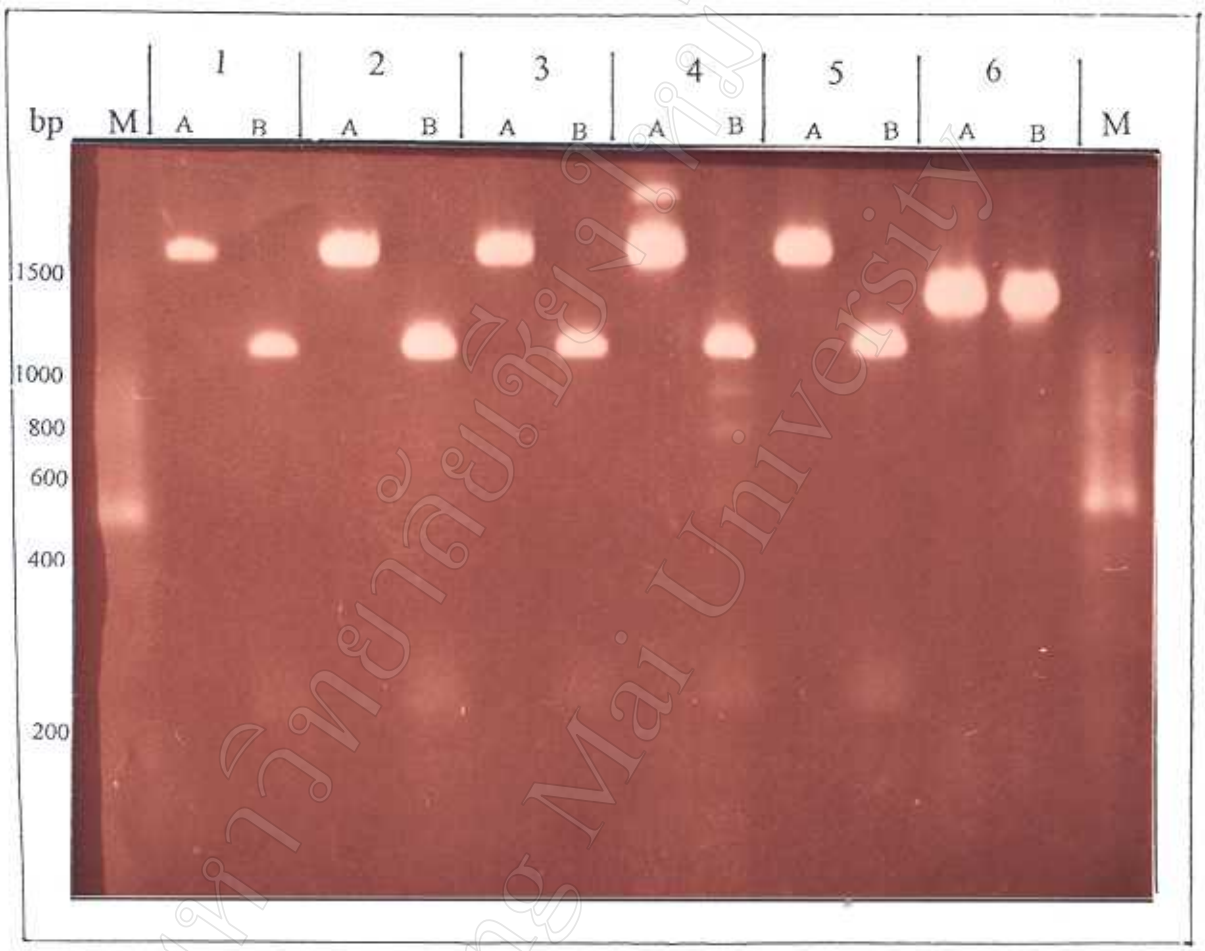

Figure 15 *Mbo*I digestion patterns of M-nontypable GAS with more than 90% homology of N-terminal sequences of *emm* genes. Lanes 1-5 are undigested (A) and MboI digested (B) PCR products of S14, S122, S142, S219, S330 GAS (93-100% homology of N-terminal sequences among themselves), respectively. The differrent digestion pattern of S665 is shown in lane 6. Lanes M represent 100 bp ladder DNA molecular weight markers.

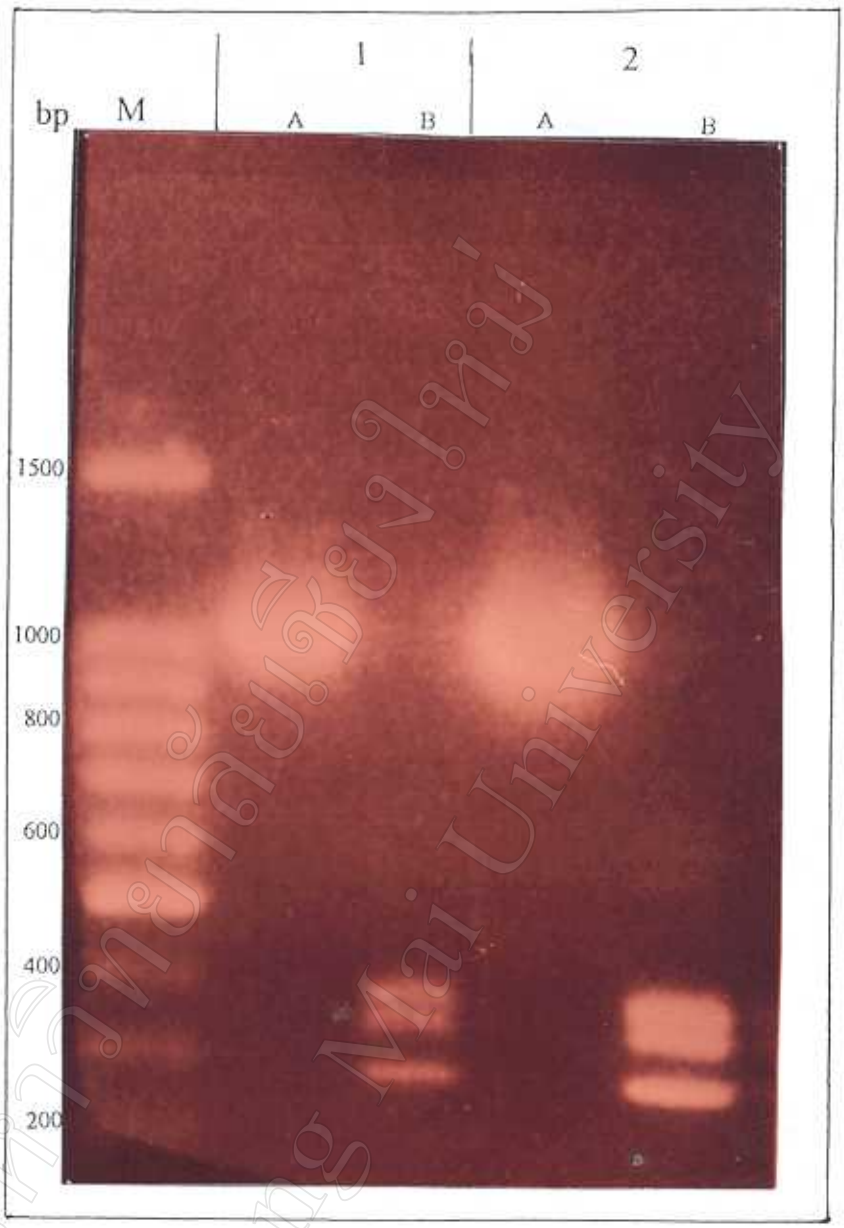

Figure 16 Identical *Mbo*I digestion patterns of 38 and M75. The *Mbo*I digestion patterns of 38 and M75 with 98 % homology of N-terminal sequence of *emm* genes are shown in lanes 1 and 2, respectively. Lanes A and B represent the undigested and the *Mbo*I digested PCR products of each strain, respectively. Lane M represents 100 bp ladder molecular weight markers.

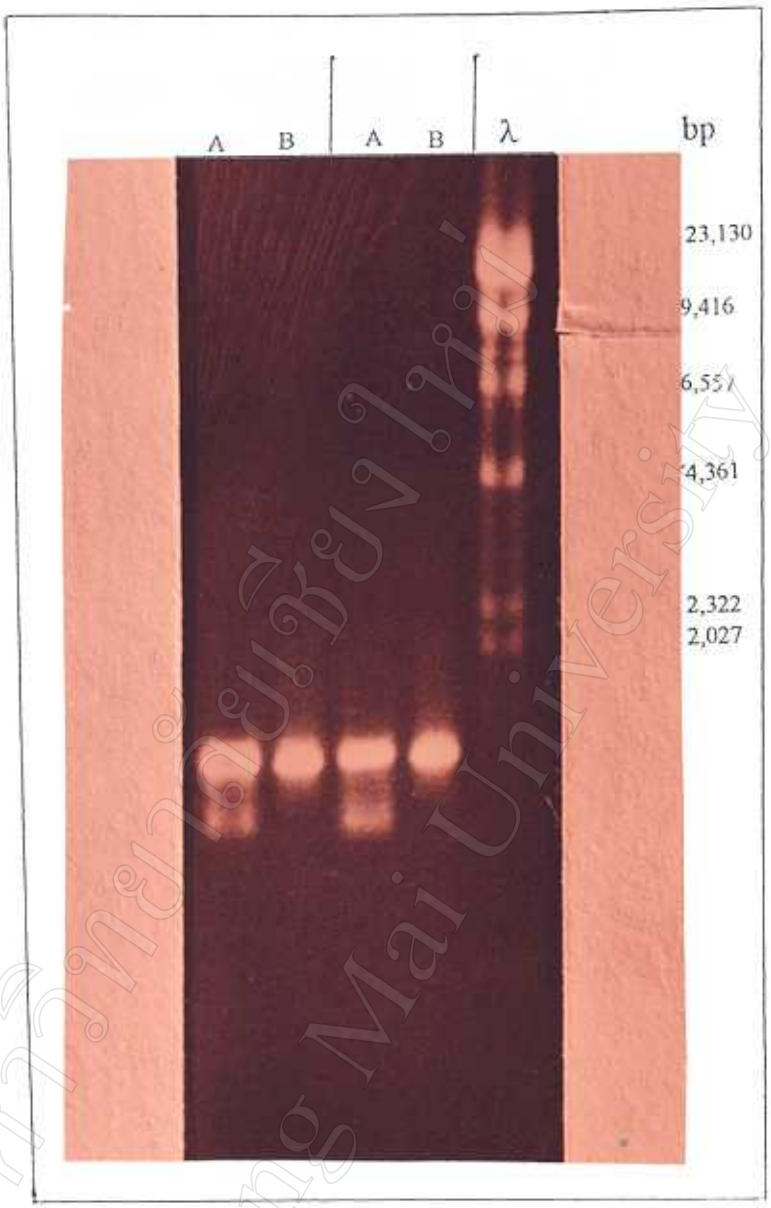

Figure 17 Identical *Mbo*I digestion patterns of H92 and M58. The *Mbo*I digestion patterns of H92 and M58 with 98 % homology of N-terminal sequence of *emm* genes are shown in lanes 1 and 2, respectively. Lanes A and B represent the undigested and *Mbo*I digested PCR products of each strain, respectively. Lane λ represents *Hind*III-digested λ DNA molecular weight markers.

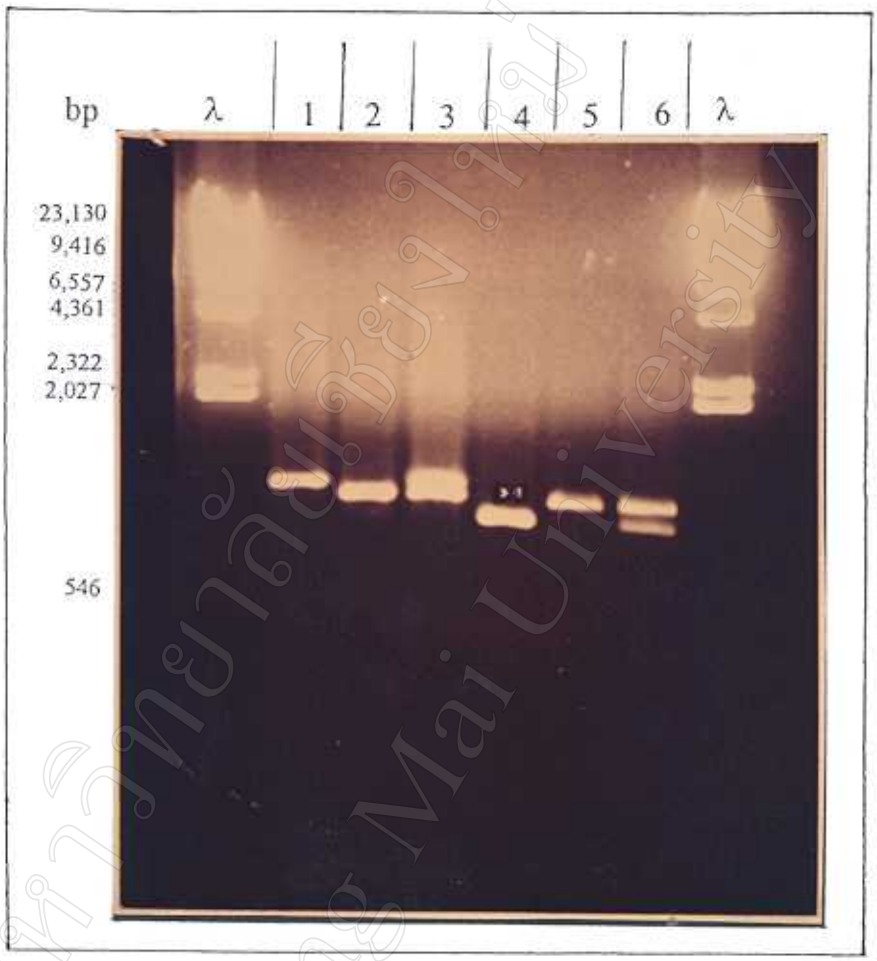

Figure 18 The size differences of PCR products of M22-ARF19 and ARF3-ARF15 GAS pairs. The PCR products of M22, ARF19, ARF19 plus M22, ARF3, ARF15 and ARF3 plus ARF15 are shown in lanes 1-6, respectively. Lane λ represents *Hind*III-digested λ DNA molecular weight markers.

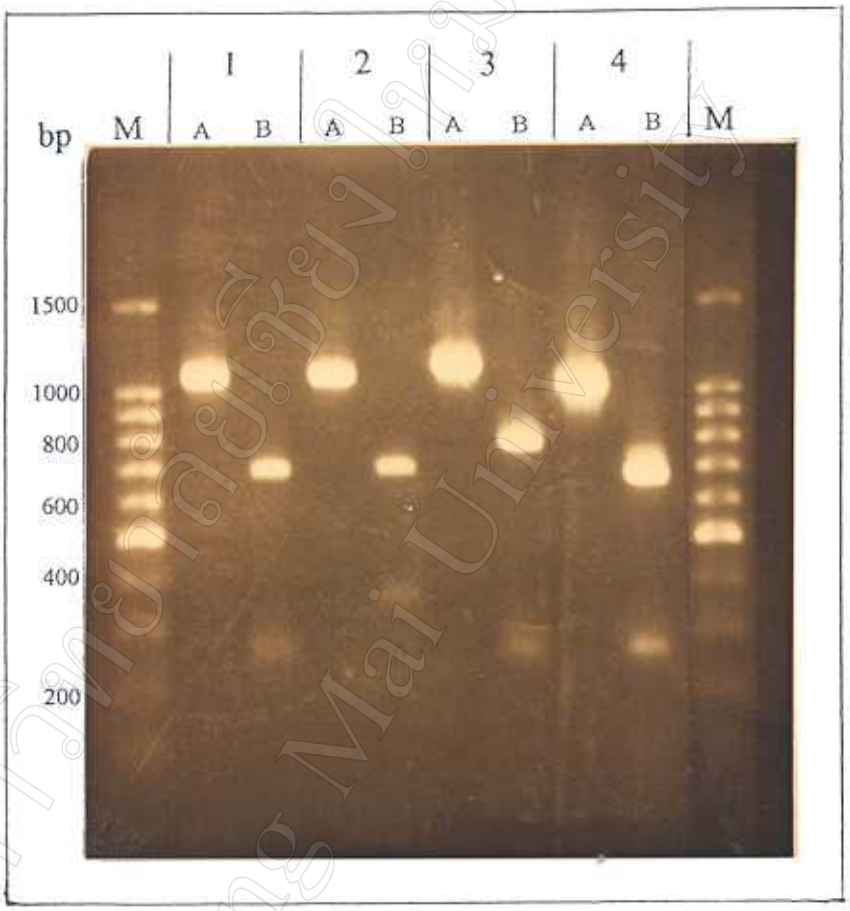

Figure 19 Similar *Mbo*I digestion patterns of some GAS strains. Lanes 1-4 are undigested (A) and *Mbo*I digested (B) PCR products of M55, M76, M13 and K16, respectively. Lanes M represent 100 bp ladder DNA molecular weight markers.

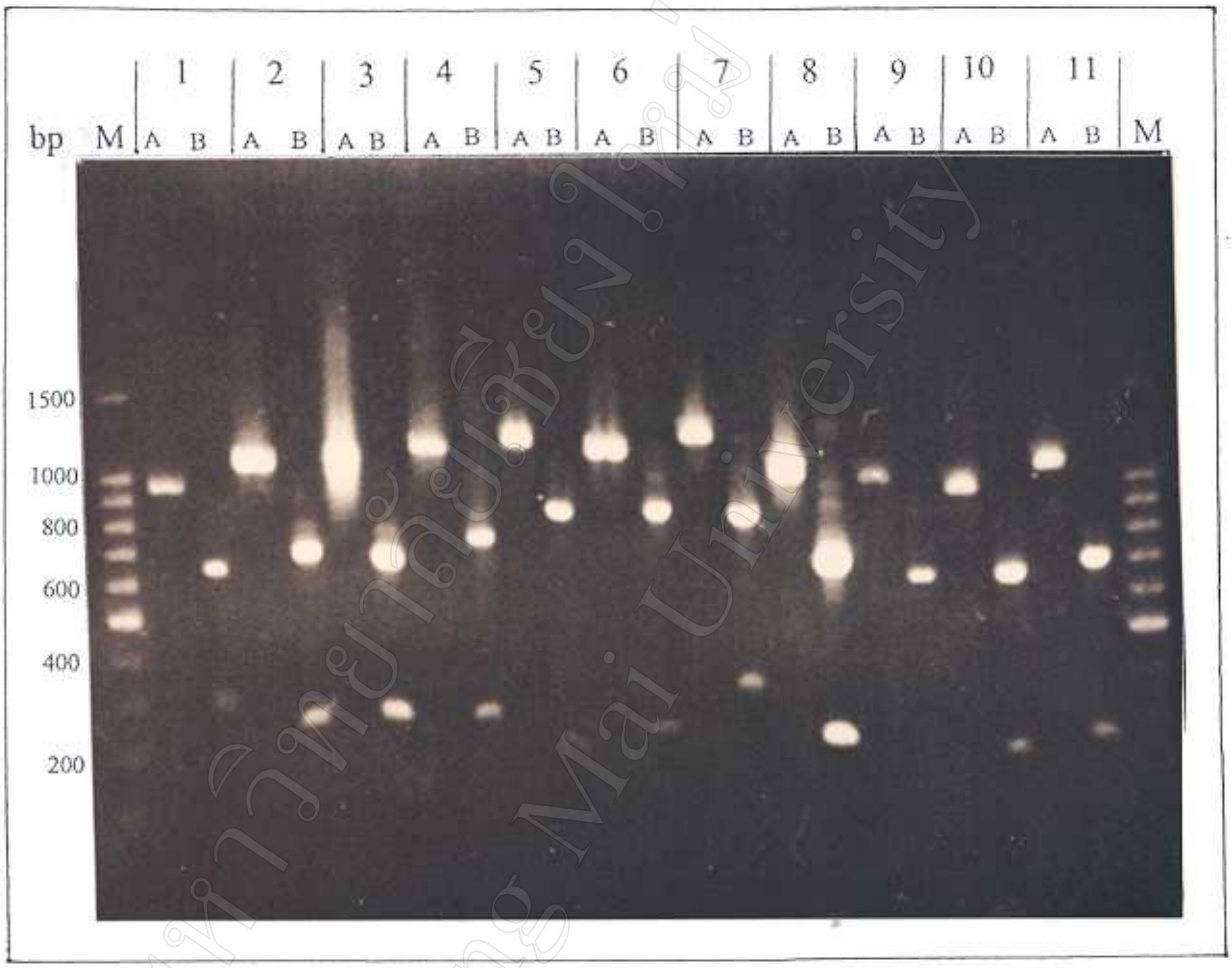

Figure 20 The *Mbo*I digestion patterns of some GAS strains. Lanes 1-11 are undigested (A) and *Mbo*I digested (B) PCR products of ARF3, ARF15, ARF19, M22, S330, M18, M52, CSF3, M73, M77 and M55, respectively. Lanes M represent 100 bp ladder DNA molecular weight markers.

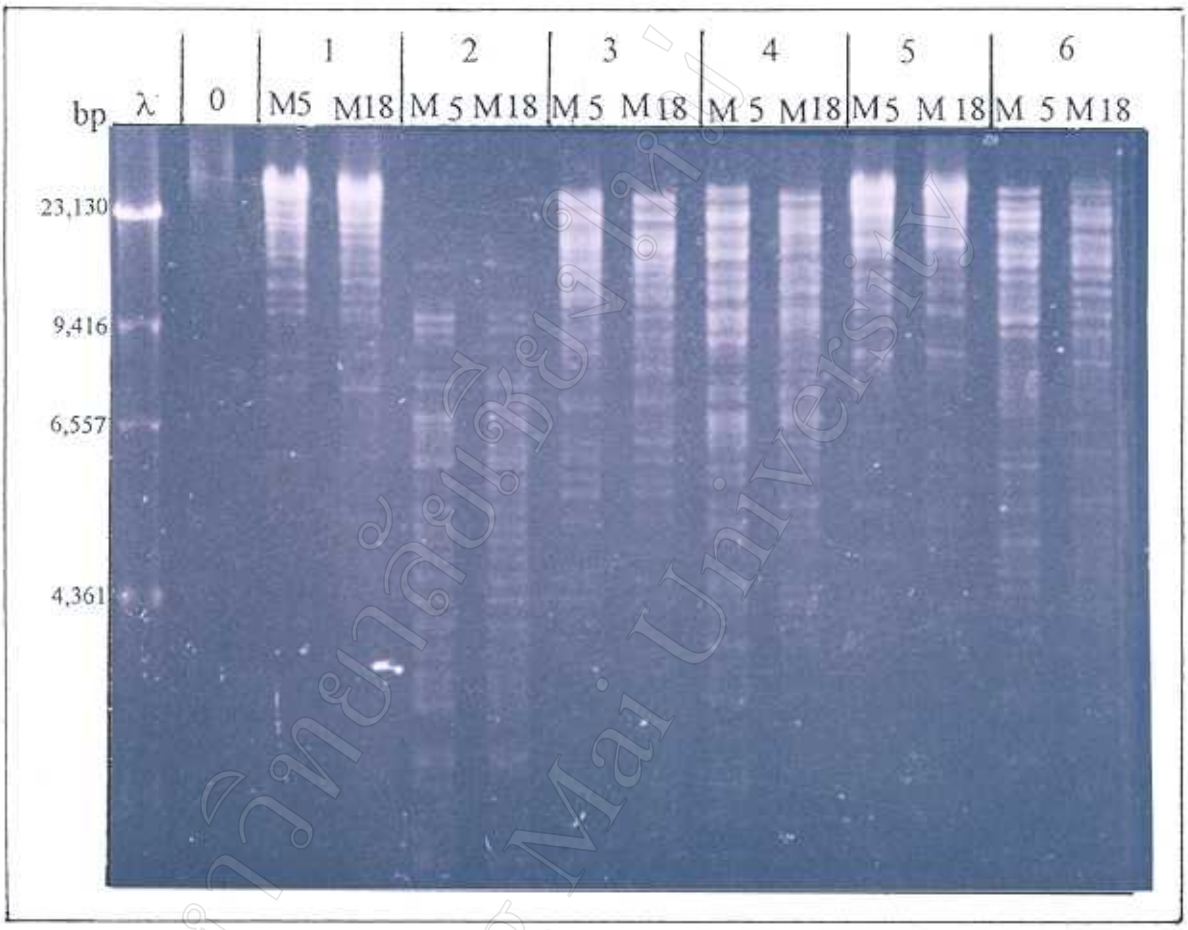

Figure 21 Restriction enzyme digestion of GAS chromosomal DNAs. Chromosomal DNA of M5 and M18 were digested with *Sal*I (lane 1), *Hind*III (lane 2), *Kpn*I (lane 3), *Bgl*II (lane 4), *Bam*HI (lane 5), and *Pst*I (lane 6) and analyzed by 0.8% agarose gel electrophoresis. Lane 0 is undigested DNA. Lane λ represents *Hind*III-diested λ DNA molecular weight markers.

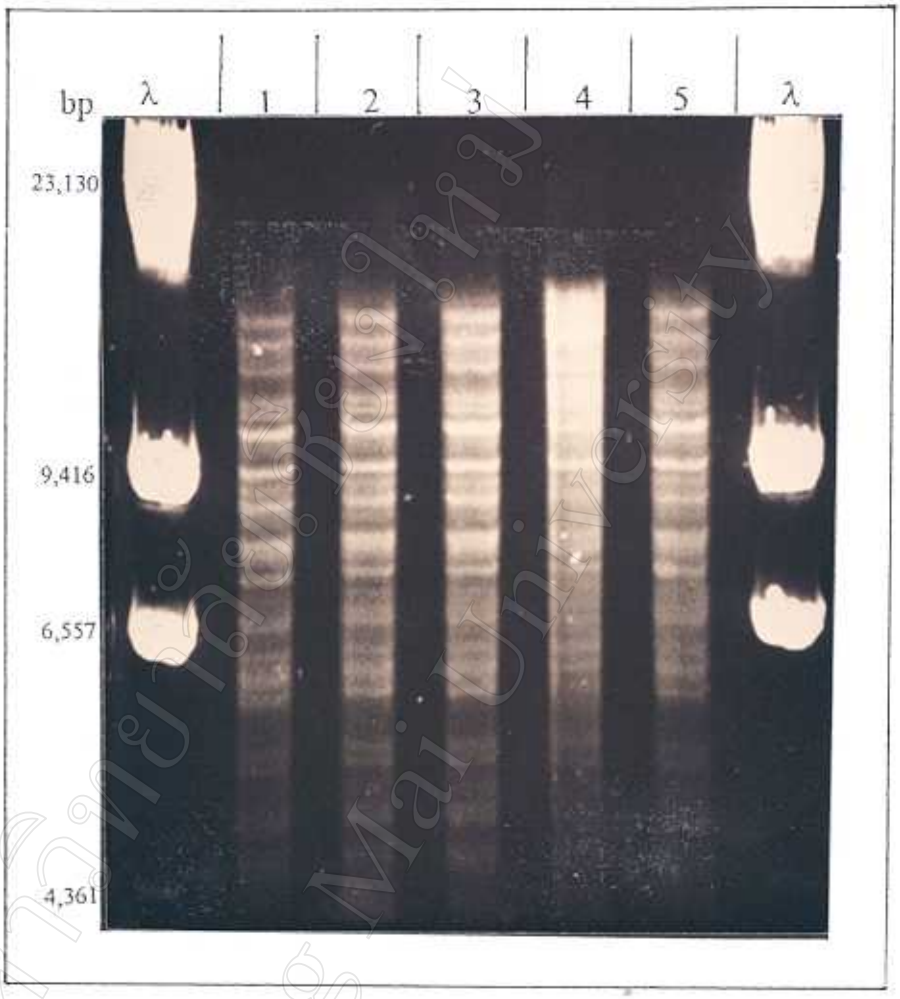

Figure 22 RFLP of S14, S122, S142, S219 and S330. Chromosomal DNA of S14, S122, S142, S219 and S330 digested with *Pst*I were analyzed by agarose gel electrophoresis in lanes 1-5, respectively. Lanes λ represent *Hind*III-digested λ DNA molecular weight markers.

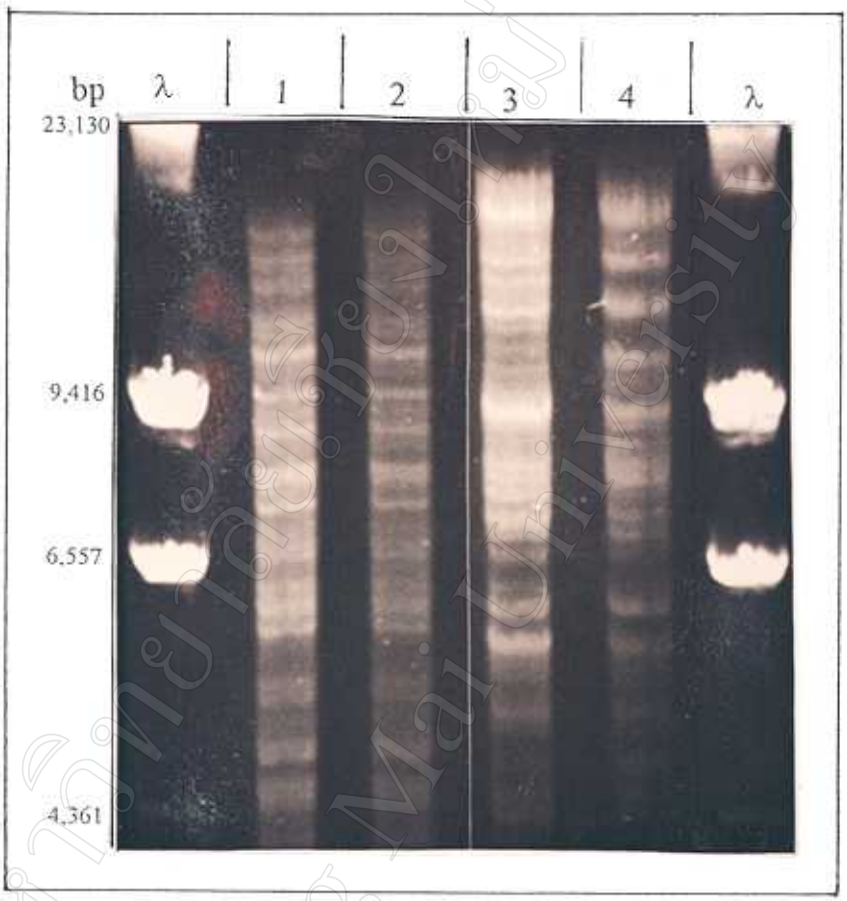

Figure 23 RFLP of 38-M75 and M22-ARF19 GAS pairs. Chromosomal DNA of 38, M75, M22, ARF 19 digested with *Pst*I were analyzed by agarose gel electrophoresis in lanes 1-4, respectively . Lanes λ represent *Hind*III-digested λ DNA molecular weight markers.

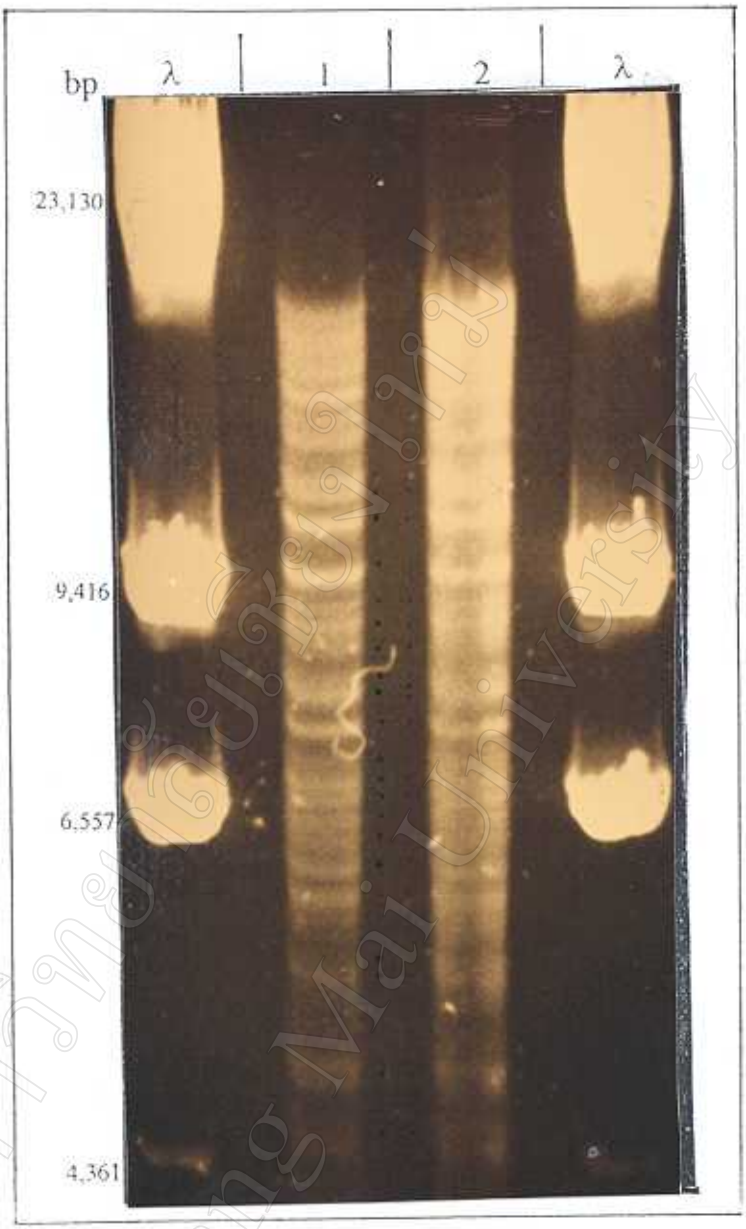

Figure 24 RFLP of K16 and K3. Chromosomal DNA of K3 and K16 digested with *Pst*I were analyzed by agarose gel electrophoresis in lanes 1and 2, respectively . Lanes λ represent *Hind*III-digested λ DNA molecular weight markers.

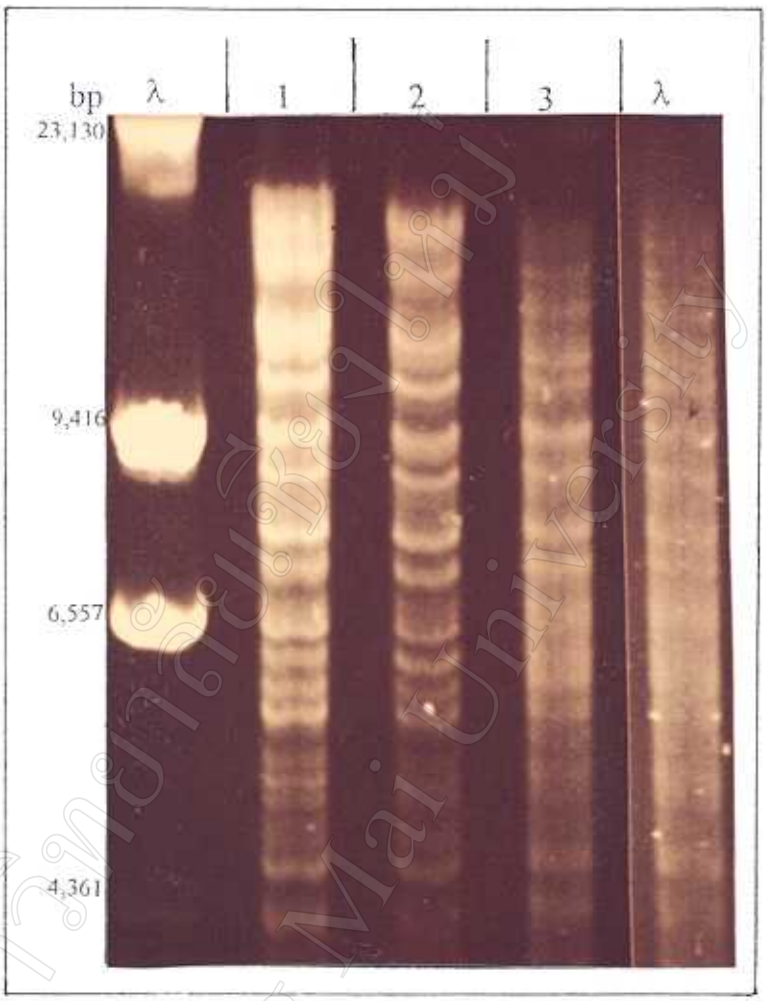

Figure 25 RFLP of H92, M58, S665 and J63. Chromosomal DNA of H92, M58, S665 and J63 digested with *Pst*I were analyzed by agarose gel electrophoresis in lanes 1-4, respectively . Lane λ represents *Hind*III-digested λ DNA molecular weight markers.

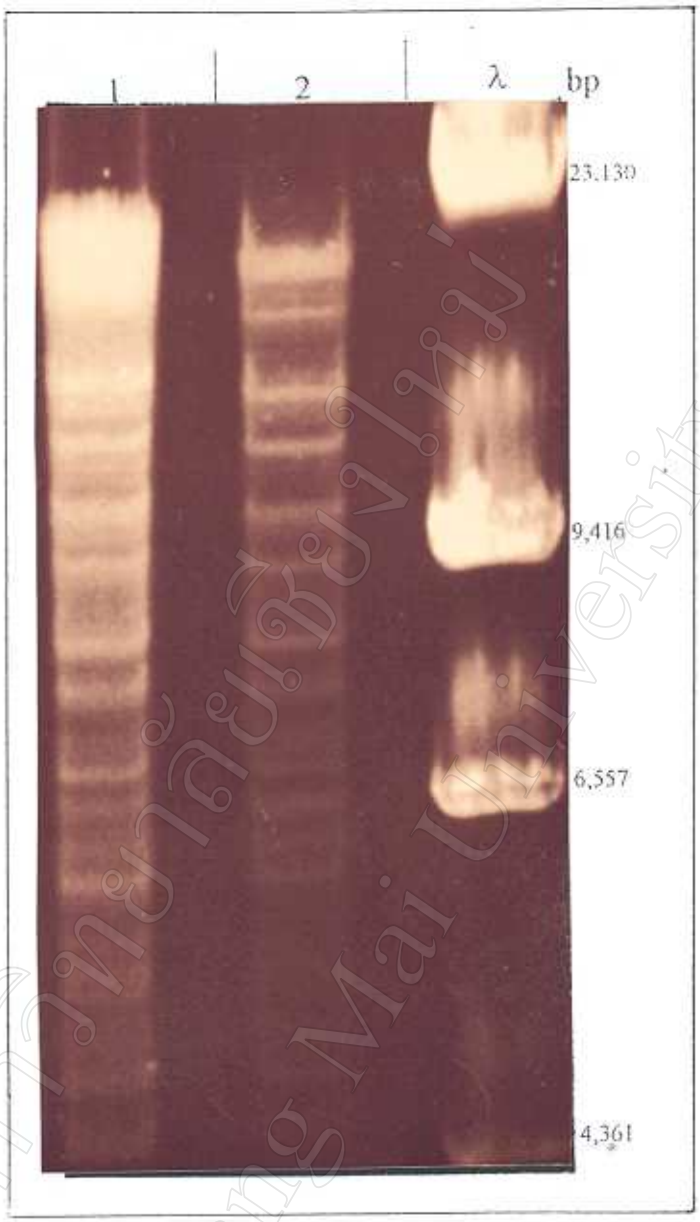

Figure 26 RFLP of O20 and AK14. Chromosomal DNA of O20 and AK14 digested with *Pst*I were analyzed by agarose gel electrophoresis in lanes 1 and 2, respectively . Lane λ represents *Hind*III-digested λ DNA molecular weight markers.

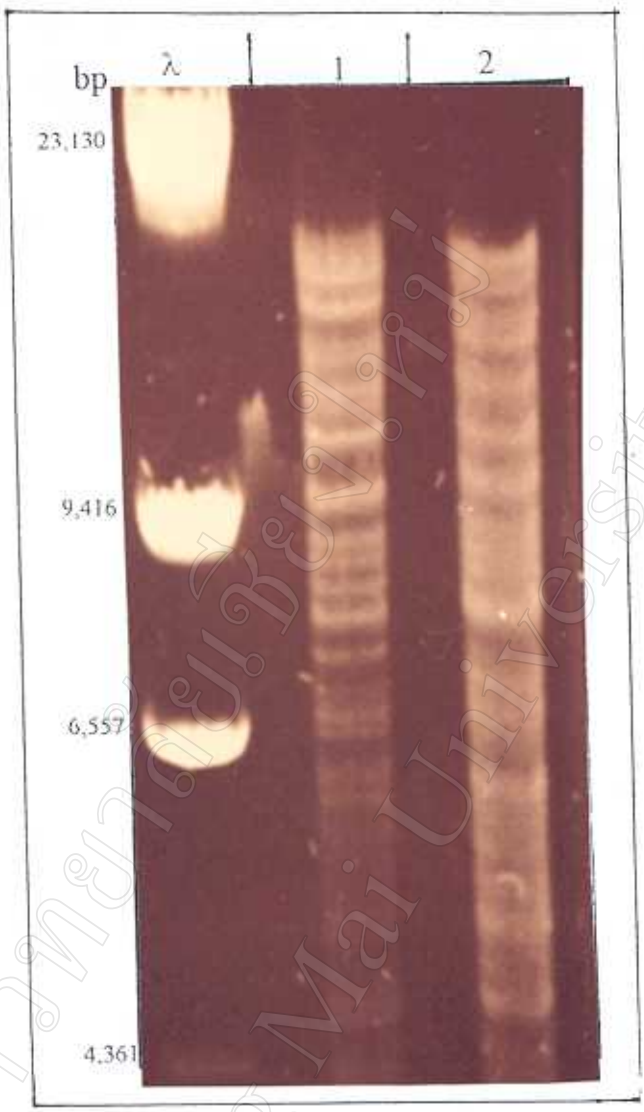

Figure 27 RFLP of M25 and 42. Chromosomal DNA of M25 and 42 digested with *Pst*I were analyzed by agarose gel electrophoresis in lanes 1 and 2, respectively. Lane λ represents *Hind*III-digested λ DNA molecular weight markers.

Figure 28 RFLP of M80, H140 and M18. Chromosomal DNA of M80, H140 and M18 digested with *Pst*I were analyzed by agarose gel electrophoresis in lanes 1-3, respectively. Lanes λ represent *Hind*III-digested λ DNA molecular weight markers.

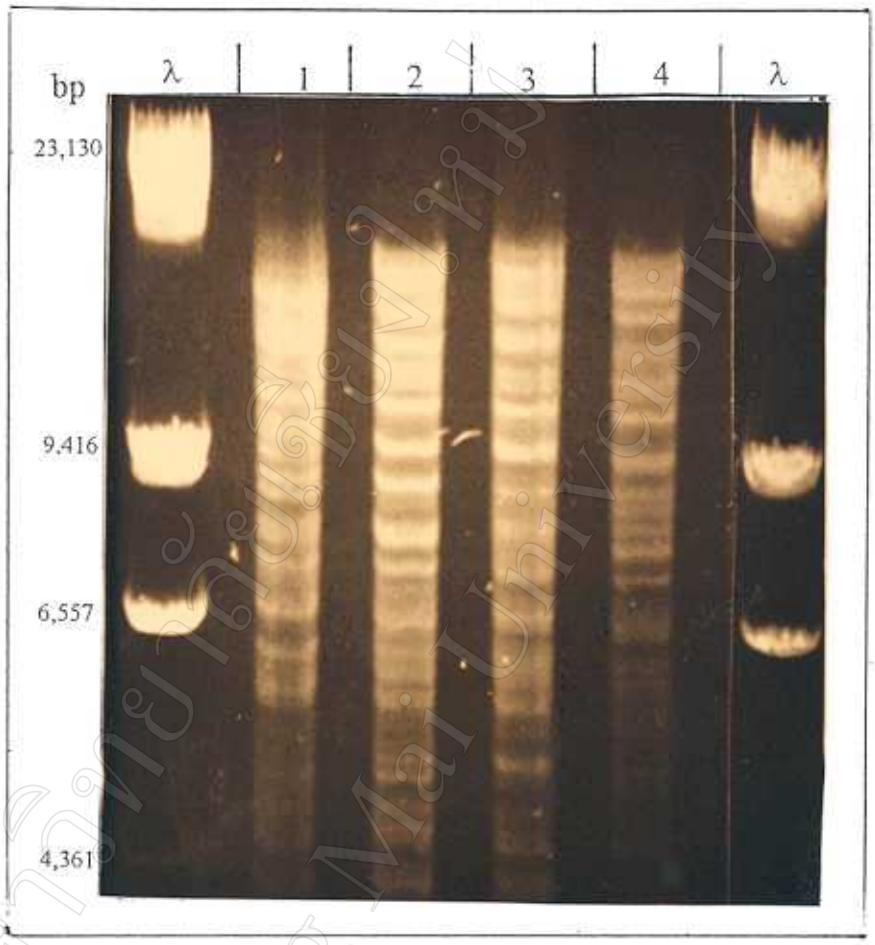

Figure 29 RFLP of ARF 3, ARF15, M59 and M13. Chromosomal DNA of ARF 3, ARF15, M59 and M13 digested with *Pst*I were analyzed by agarose gel electrophoresis in lanes 1-4, respectively. Lanes λ represent *Hind*III-digested λ DNA molecular weight markers.