CHAPTER V

RESULTS

1. Clinical Isolates

The viral culture was performed in the Virology Laboratory Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. A total of 121 positive HSV specimens (obtained during January 1998 - June 2002 from King Chulalongkorn Memorial Hospital) was recruited in this study.

The isolates were from swabs of eyes, throat, oral and genital lesions, thus they were divided into two groups according to site of infection i.e., nongenital lesion and genital lesion. The stock viruses were prepared in Vero cells at low multiplicity of infection. Several passages were done to obtain high viral titer.

Out of 121 clinical samples, 86 samples were successfully propagated to get high viral titer enough to continue the studies (Table 3).

Table 3. The number of successive propagated isolates among 121 samples.

Year	Total clinical HSV specimens	No. of successive propagated specimens	
		Yes	No
1998	10	3	7
1999	21	14	7
2000	25	14	11
2001	48	44	4
2002	17	11	6
Total	121	86	35

Among 86 isolates HSV culture positive, were 16 (18.60%) from male and 70 (81.40%) from female patients. Most of the samples (74.42%) were collected from genital lesions (Table 4).

Table 4. The number of HSV isolates distributed by sex and site of infection.

Site of infection	Male	Female	Total
Nongenital lesions	11	11	22 (25.58%)
Genital lesions	5	59	64 (74.42%)
Total	16 (18.60%)	70 (81.40)	86 (100%)

2. Sensitivity of HSV-PCR

To determine the sensitivity of PCR assay, serial 10-fold dilutions of standard HSV-1 (KOS) DNA were used. The result showed that PCR assay can detect HSV-DNA at less than or equal to 1 fg (Figure 6).

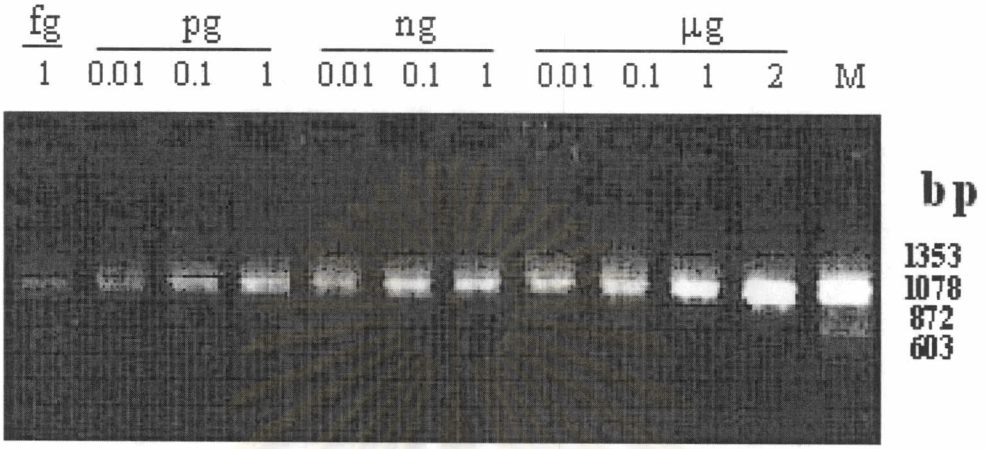

Figure 6. Sensitivity of the standard HSV-1 (KOS) DNA by PCR, M: Øx174 RE/*Hae*III marker.

3. HSV-PCR Typing

In order to identify type of HSV, the amplified product of 532 bp was further digested by *Bam*HI as previously described (Materials and Methods). Only HSV-2 amplified products will be able to be cut into 230 bp and 302 bp fragments (Figure 7).

The results of PCR typing among 86 clinical isolates, 20 of 22 nongenital isolates were HSV-1 (90.90%), two isolates were HSV-2 (9.09%) (Table 5). In genital lesions, 34 of 64 were HSV-1 (53.12%), 28 (43.75%) were HSV-2, and two isolates were mix-infection of HSV-1 and HSV-2 (3.13%). Most of isolates were obtained from female. Figures 8 and 9 demonstrated the PCR typing of some HSV isolates. The pattern of mix-infection of HSV-1 and HSV-2 was shown in Figure 9 (Lane 9, 10).

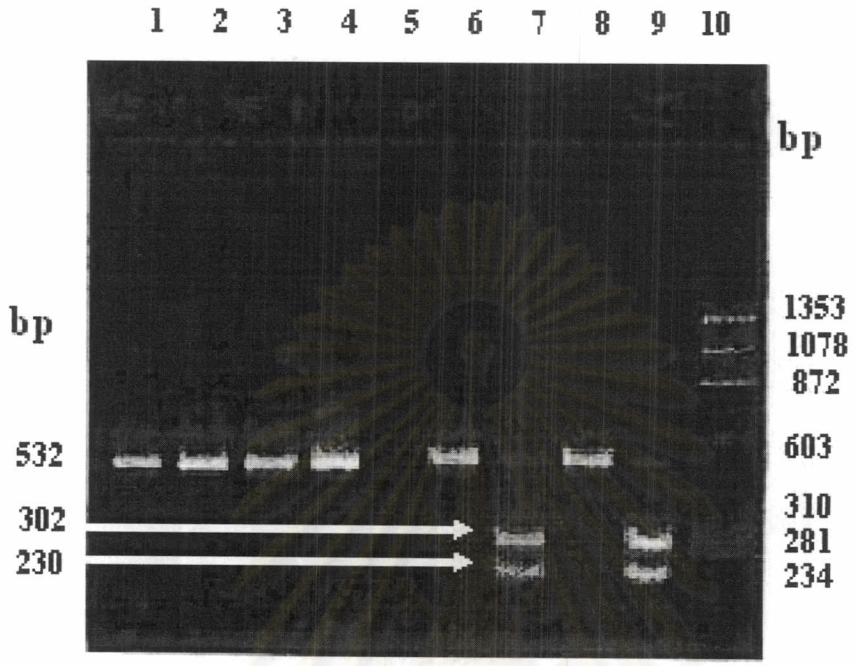

Figure 7. Determination and identification of HSV in standard strain by restriction enzyme digestion pattern for HSV PCR product. Digestion mixtures were electrophoresed on 1.5% agarose gel. Lane 1, 2: uncut HSV-1 (KOS), lane 3, 4: HSV-1 (KOS) cut with *Bam*HI, lane 5: PCR negative control (distilled water), lane 6, 8: uncut HSV-2 (Baylor 186), lane 7, 9: HSV-2 (Baylor 186) cut with *Bam*HI, lane 10: Øx174 RE/*Hae*III marker. Only fragments of HSV-2 (Baylor 186) digestion by *Bam*HI can be seen, they are readily distinguishable.

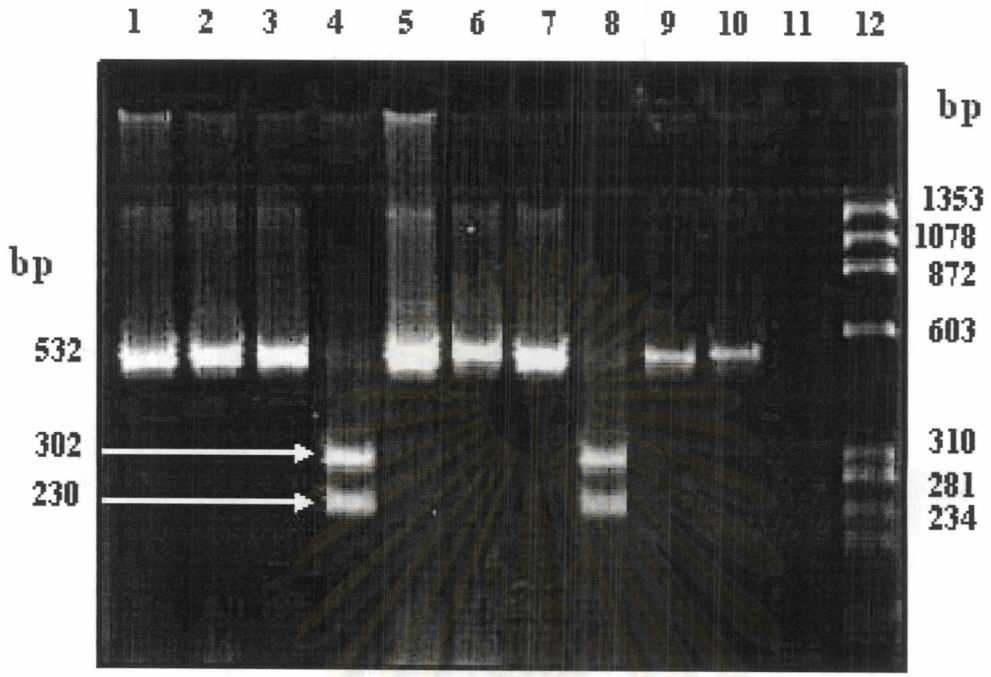

Figure 8 Detection and identification of HSV typing in five clinical isolates (I). Digestion mixtures were electrophoresed on 1.5% agarose gel. Lane 1, 3, 5, 7, 9: uncut PCR product, lane 2, 4, 6, 8, 10: *Bam*HI cut. Lane 11: PCR negative control (distilled water) and lane 12: ∅x147 RE/*Hae*III marker.

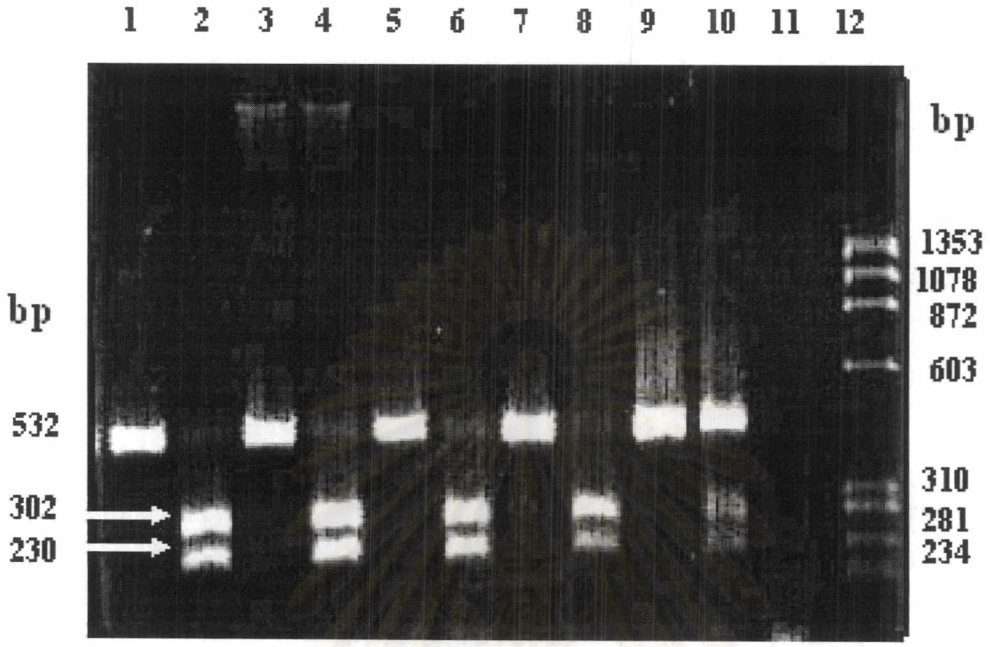

Figure 9. Detection and identification of HSV typing in five clinical specimens (II). Digestion mixture were electrophoresed on 1.5% agarose gel. Lane 1, 3, 5, 7, 9: uncut, lane 2, 4, 6, 8, 10: cut with *Bam*HI, Lane 11: PCR negative control (distilled water) and lane 12: ∅x147 RE/*Hae*III marker.

Table 5. The results of HSV-PCR typing.

Site of infection	HSV-1			HSV-2			Mix-infection		
	Male	Female	Total	Male	Female	Total	Male	Female	Total
Nongenital lesion	9	11	20 (90.90%)	2	0	2 (9.09%)	0	0	0
Genital lesion	5	29	34 (53.12%)	0	28	28 (43.75%)	0	2	2 (3.13%)
Total	14	40	54 (62.79%)	2	28	30 (34.88%)	0	2	2 (2.33%)

4. Molecular Epidemiology of Genetic Variation of HSV by Restriction Fragment Length Polymorphism (RFLP)

Restriction endonuclease (RE) cleavage of purified DNA from each of HSV-1 and HSV-2 were digested with each of four restriction enzymes. The isolates selected to study molecular epidemiology of genetic variation of HSV DNA by RFLP were randomly selected, 20 HSV isolates for each types. They were from both nongenital lesions and genital lesions (Table 6 and 7). The stock solution of virus was prepared several passages between 8^{th} to 10^{th} with low multiplicity of infection in Vero cells thus, the viral DNA prepared from passage 9^{th} to 11^{th}.

The DNA extracted from HSV-1 (KOS) and HSV-2 (Baylor 186) were digested with *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI restriction enzymes. The fragments were separated and used as standard pattern to compare with those HSV isolates from clinical specimens. The RE in this study divided into two groups according to multi-cut such as *Bam*HI and *Kpn*I and low-cut such as *Hin*dIII and *Eco*RI as shown in Figure 10. Each enzyme shows a unique restriction pattern and difference in electrophoretic mobility between HSV-1 (KOS) and HSV-2 (Baylor 186) (Figure 10).

Table 6. Characteristics of HSV-1 clinical isolates for study molecular epidemiology of genetic variation.

HSV-1 No.	Clinical specimen		
	Strain	Sex	Site of lesion
1	4/44	F	G
2	004	F	NG
3	16/44	F	G
4	37/44	F	G
5	5/44	M	NG
6	25/44	F	G
7	36/44	F	G
8	39/44	F	G
9	001	F	NG
10	9/43	M	G
11	5/45	F	G
12	19/43	M	NG
13	24/44	F	G
14	23/43	F	NG
15	38/44	F	G
16	19/44	F	G
17	28/44	F	G
18	20/43	F	NG
19	35/44	F	G
20	41/44	M	G

Symbols: M (Male), F (Female), NG (Nongenital), G (Genital)

Table 7. Characteristics of HSV-2 clinical isolates for study molecular of epidemiology genetic variation.

HSV-2 No.	Clinical specimens		
	Strain	Sex	Site of lesion
1	7/44	F	G
2	9/45	F	G
3	2/45	F	G
4	4/45	F	G
5	30/44	F	G
6	11/44	F	G
7	14/43	M	NG
8	10/44	F	G
9	22/43	F	G
10	6/44	F	G
11	29/44	F	G
12	6/45	F	G
13	17/43	F	G
14	9/44	F	G
15	34/44	F	G
16	16/43	M	NG
17	1/45	F	G
18	2/43	F	G
19	18/44	F	G
20	8/44	F	G

Symbols: M (Male), F (Female), NG (Nongenital), G (Genital)

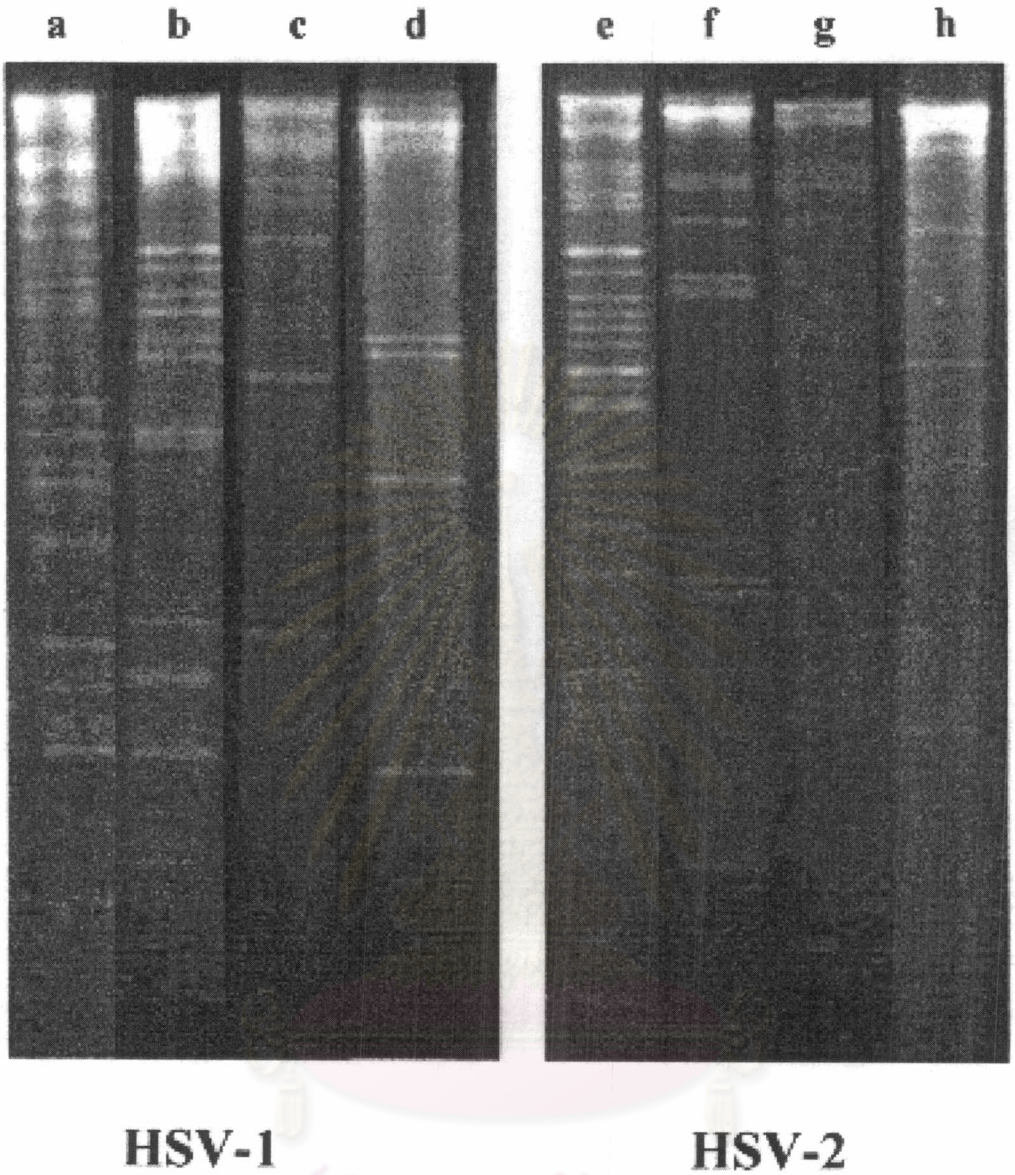

Figure 10. Cleavage patterns of HSV-1 (KOS) and HSV-2 (Baylor 186). DNAs with RE; HSV DNAs (2-3μg) were digested with *Bam*HI (a, e), *Kpn*I (b, f), *Hin*dIII (c, g), and *Eco*RI (d, h). The digestion products were electrophoresed at 60 V for 16 hours. Lane a-d; HSV-1 DNAs, e-h; HSV-2 DNAs.

4.1 HSV-1 Isolates

Differences in the HSV-1 strains as compared to HSV-1 (KOS) were reflected in different cleavage patterns. Among 20 HSV-1 isolates, several patterns were demonstrated after digesting with *Bam*HI (Figure 11), *Kpn*I (Figure 12), *Hin*dIII (Figure 13), and *Eco*RI (Figure 14). Of those, five distinct patterns were found in *Bam*HI (Figure 15), three patterns of *Kpn*I (Figure 16), three patterns of *Hin*dIII (Figure 17), and four patterns of *Eco*RI (Figure 18).

According to those distinct patterns, there were some gain and some loss of DNA bands compared to standard HSV-1 (KOS) pattern.

Fourteen isolates (70%) are identified to HSV-1 (KOS) with *Bam*HI (pattern 1). One of isolates exhibited the presence of gain fragments site between T-U due to the loss of a fragment W (pattern 2). One isolate showed the loss of a fragment N (pattern 3). Pattern 4, which occurred in three isolates, showed the presence of a gain fragment site between T-U and loss of fragments S and W. Pattern 5, which occurred in one isolate, showed the presence of gain a fragment site between O-P and T-U and loss fragments N, O and S (Figure 15, Table 8).

For *Kpn*I, the distinct cleavage patterns were shown (Figure 16, Table 8). Half of 20 isolates (50%) was similar to standard HSV-1 (KOS), pattern 1. Eight isolates showed the gain of a fragment L-M due to loss fragments P, R, and fusion of fragments namely T-U (pattern 2). Two isolates showed fusion of fragment T-U (pattern 3).

Four patterns of *Hin*dIII digestion were demonstrated in HSV-1 isolates (Figure 17, Table 8). Fifteen out of 20 (75%) isolates were the same as standard HSV-1 (KOS), pattern 1. Four isolates showed a loss of fragment M (pattern 2). In pattern 3, which occurred in three of the isolates showed the loss of fragments M, and N.

*Eco*RI restriction patterns of HSV-1 isolates have four patterns (Figure 18, Table 8). Fourteen of the isolates (70%) exhibited the presence of the fragments similar to standard HSV-1 (KOS) (pattern 1). One isolate showed the presence of a gain fragment K (pattern 2). One isolate showed gain of fragments between site J-K and L-M, loss of a fragment L (pattern 3). Another isolate exhibited a gain of fragment between site L-M and loss a fragment L (pattern 4).

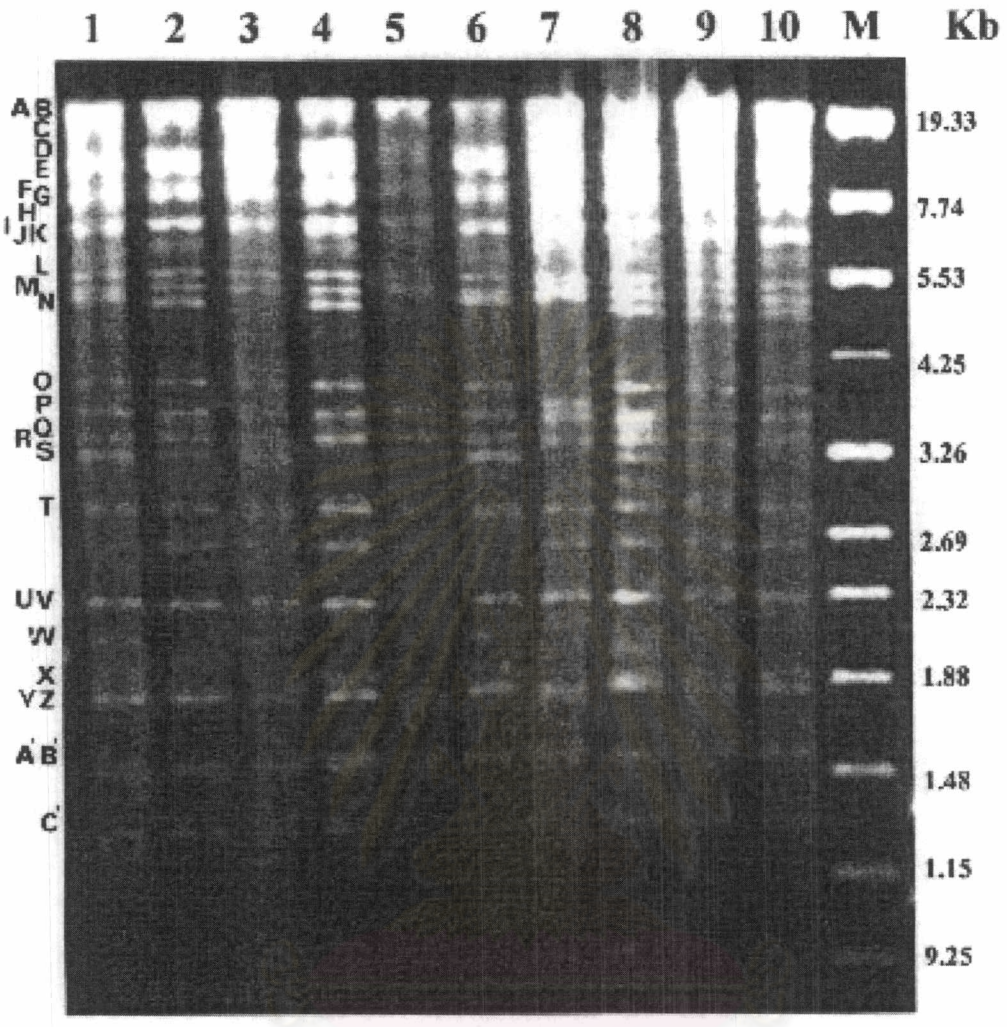

Figure 11. *Bam*HI profiles in HSV-1 clinical isolates. Lane 1 HSV-1 (KOS), lane 2 to 10 clinical isolates and lane 11 marker IV (M).

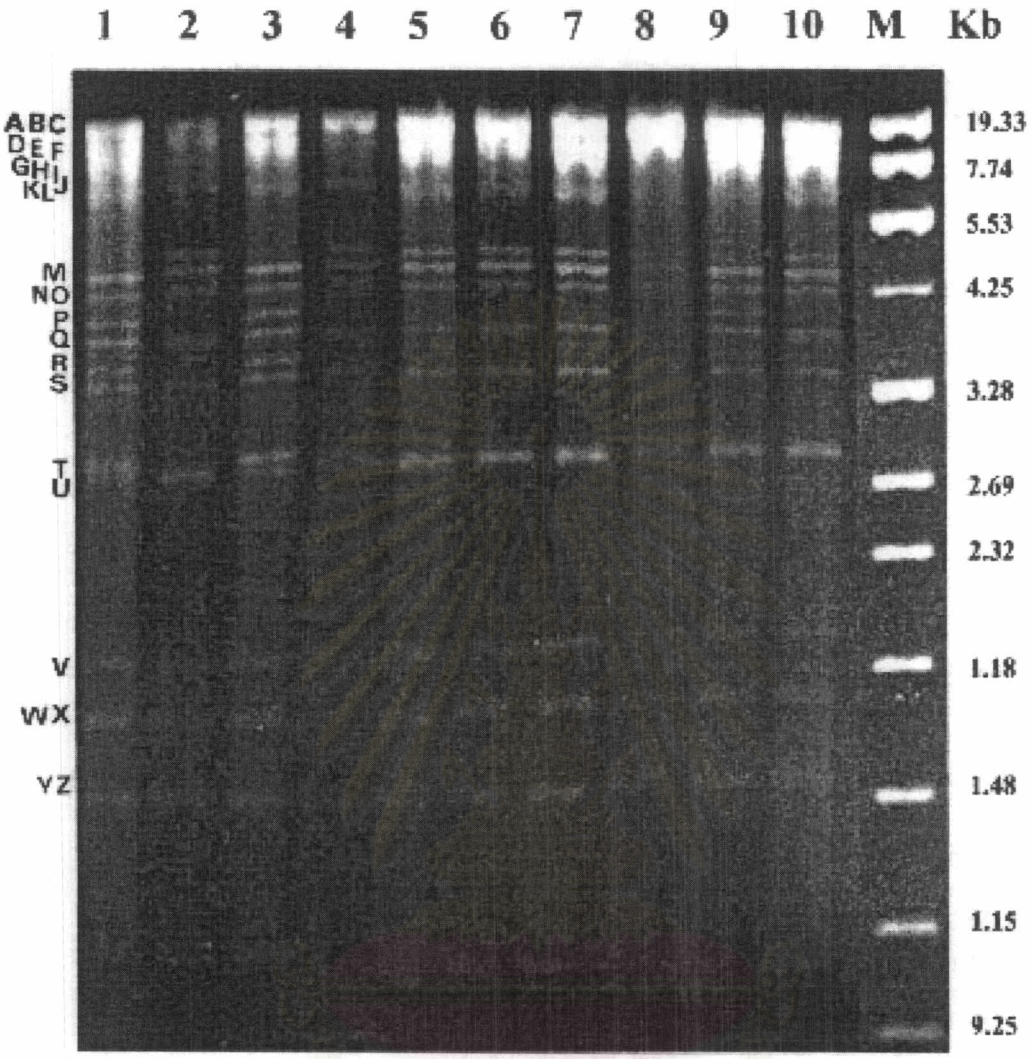

Figure 12. *Kpn*I profiles in HSV-1 clinical isolates. Lane 1 HSV-1 (KOS), lane 2 to 10 clinical isolates and lane 11 marker IV (M).

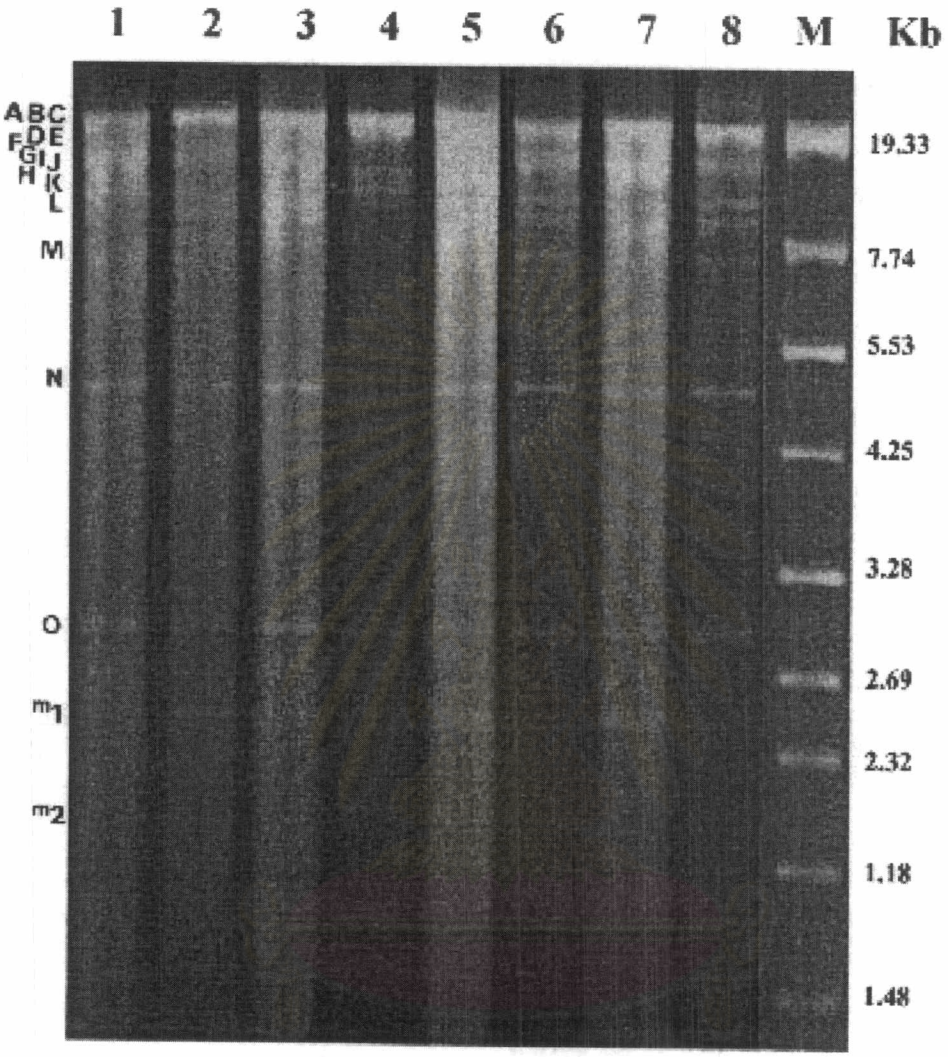

Figure 13. *Hin*dIII profiles in HSV-1 clinical isolates. Lane 1 HSV-1 (KOS), lane 2 to 8 clinical isolates and lane 9 marker IV (M).

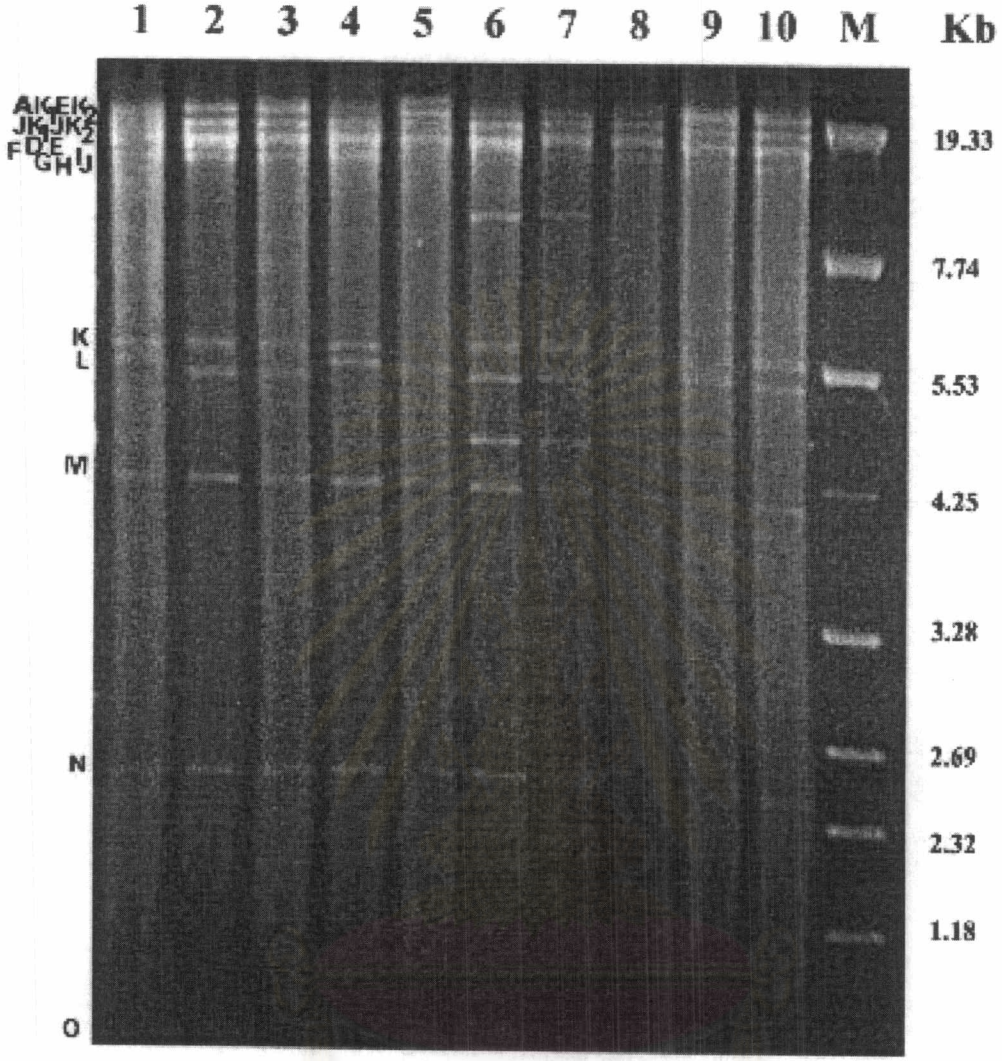

Figure 14. *Eco*RI profiles in HSV-1 clinical isolates. Lane 1 HSV-1 (KOS), lane 2 to 10 clinical isolates and lane 11 marker IV (M).

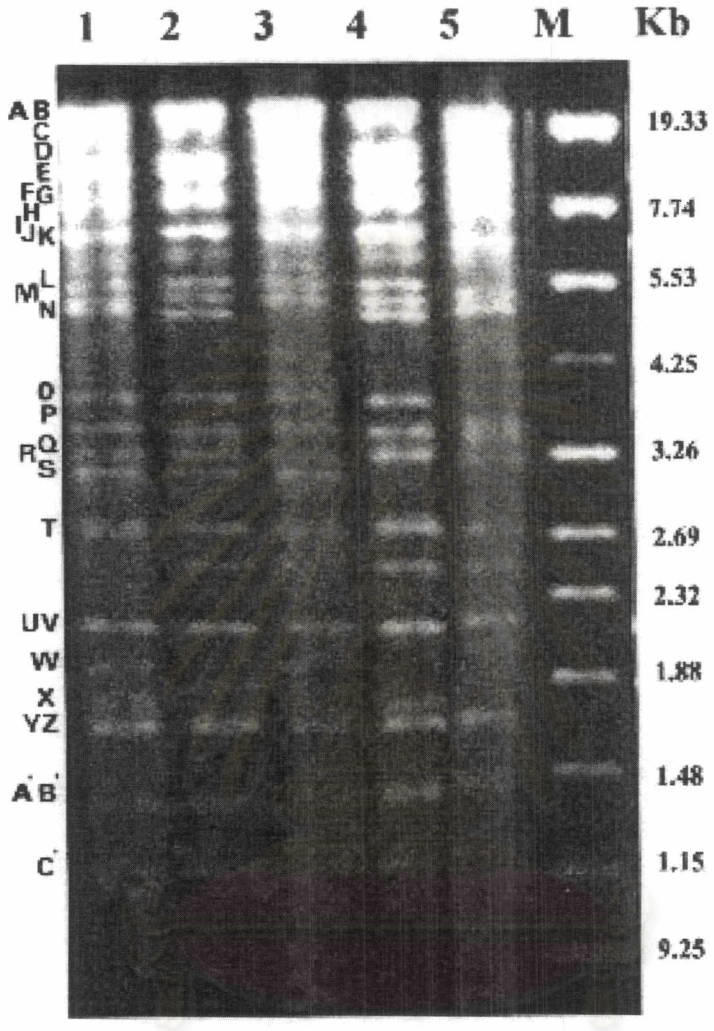

Figure 15. Five distinct patterns of HSV-1 DNA after *Bam*HI digestion. Lane 1 HSV-1 (KOS) (Pattern 1), lane 2 (Pattern 2), lane 3 (Pattern 3), lane 4 (Pattern 4), lane 5 (Pattern 5), and lane 6 marker IV (M).

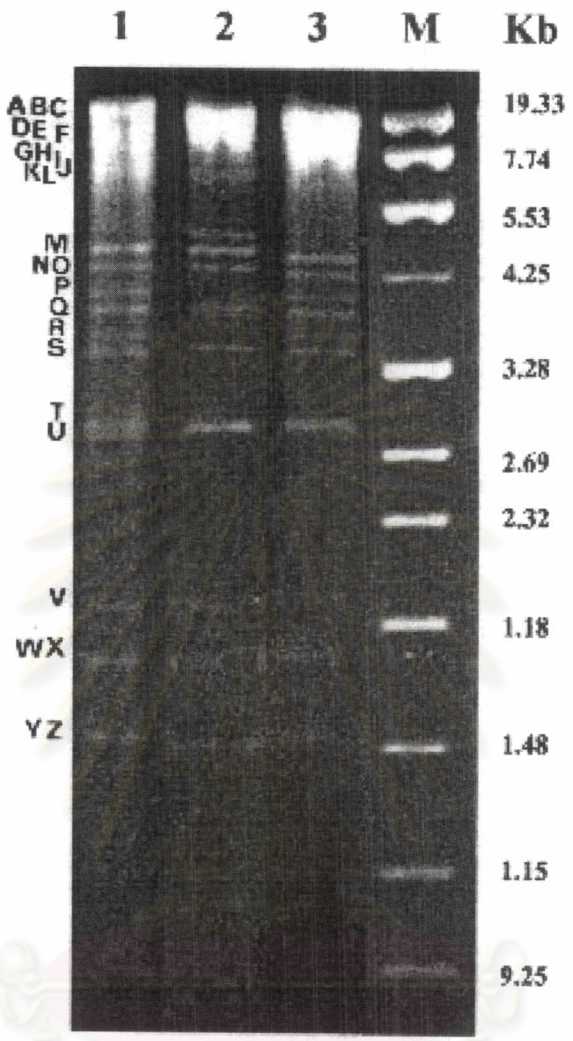

Figure 16. Three distinct patterns of HSV-1 DNA after *Kpn*I digestion. Lane 1 HSV-1 (KOS) (Pattern 1), lane 2 (Pattern 2), lane 3 (Pattern 3), lane 4 marker IV (M).

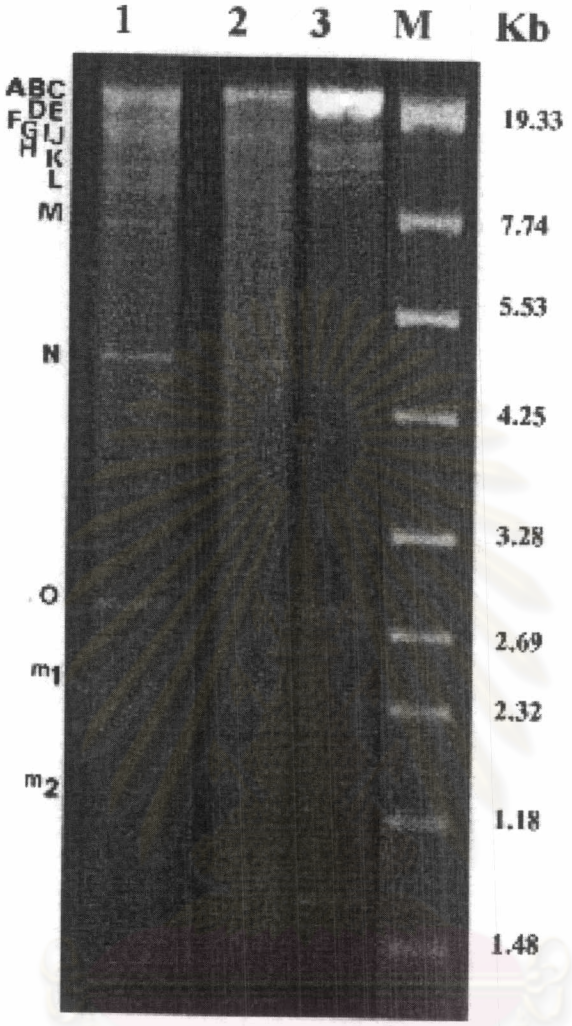

Figure 17. Three distinct patterns of HSV-1 DNA after *HInd*III digestion. Lane 1 HSV-1 (KOS) (Pattern 1) and lane 2 (Pattern 2), lane 3 (Pattern 3) and lane 4 marker IV (M).

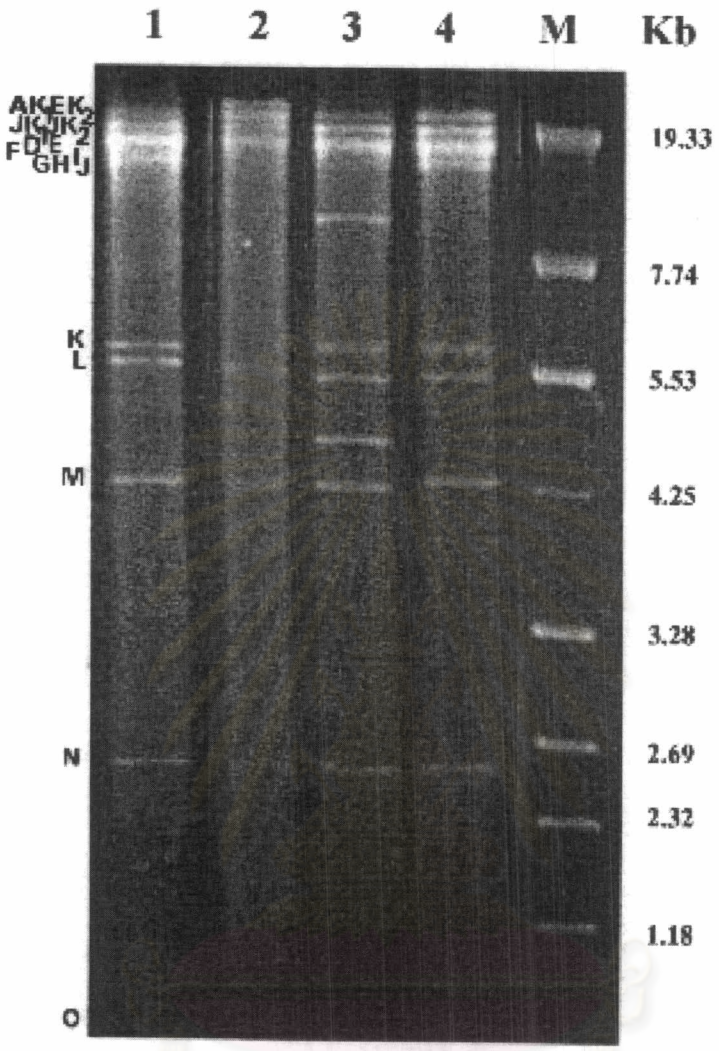

Figure 18. Four distinct patterns of HSV-1 DNA after *Eco*RI digestion. Lane 1 HSV-1 (KOS) (Pattern 1) and lane 2 (Pattern 2), lane 3 (Pattern 3), lane 4 (Pattern 4) and lane 5 marker IV (M).

Table 8. The variable restriction endonuclease cleavage sites of HSV-1 clinical isolates.

Enzyme	Pattern of variable restriction endonuclease cleavage sites	Total	Frequency (%)
***Bam*HI**	(1) *Bam*HI, HSV-1 (KOS)	14/20	70%
	(2) Gain: T-U, loss: W	1/20	5%
	(3) Loss: N	1/20	5%
	(4) Gain: T-U, loss: S, W	3/20	15%
	(5) Gain: O-P, T-U, loss: N, O, S	1/20	5%
***Kpn*I**	(1) *Kpn*I, HSV-1 (KOS)	10/20	50%
	(2) Gain: L-M, loss: P, R, fusion: T-U	8/20	40%
	(3) Fusion: T-U	2/20	10%
***Hin*dIII**	(1) *Hin*dIII, HSV-1 (KOS)	15/20	75%
	(2) Loss: M	4/20	20%
	(3) Loss: M, N	1/20	5%
***Eco*RI**	(1) *Eco*RI, HSV-1 (KOS)	14/20	70%
	(2) Loss: K	1/20	5%
	(3) Gain: J-K, L-M, loss: L	1/20	5%
	(4) Gain: L-M, loss: L	4/20	20%

4.2 HSV-2 Isolates

Similar observation was shown in HSV-2 isolates after using four restriction enzymes i.e, *Bam*HI (Figure 19), *Kpn*I (Figure 20), *Hin*dIII (Figure 21), and *Eco*RI (Figure 22).

When all patterns of HSV-2 isolates were analysed and compared to standard HSV-2 (Baylor 186), there were four, one, two, and three distinct patterns found in *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI digestion, respectively (Figure 23, 24, 25, 26, Table 9).

Analysis of *Bam*HI digested HSV-2 DNA revealed four distinct cleavage patterns (Figure 23, Table 9). Seventeen isolates (85%) were not distinguished from strain Baylor 186 (pattern 1). The variation in the presence of a gain fragment site between Z-A' due to a loss of cleavage sites Y and Z, occurred in one isolate (pattern 2). One showed gain of fragments site between T-U, and Z-A', loss of fragments Y and Z (pattern 3). One of the isolates showed gain of a fragment site between T-U (pattern 4).

Using *Kpn*I, all 20 isolates (100%) were shown similar pattern to standard HSV-2 (Baylor 186), pattern 1 (Figure 24, Table 9).

Two cleavage patterns were shown in *Hin*dIII digestion. Seventeen (85%) had pattern not different from HSV-2 (Baylor 186) (pattern 1). Three isolates showed gain of a fragment M (pattern 2) (Figure 25, Table 9).

*Eco*RI restriction in the HSV-2 clinical isolates was classified into three distinct cleavage patterns (Figure 26, Table 9). Seventeen of the clinical isolates (85%) showed the cleavage fragment liked HSV-2 (Baylor 186) (pattern 1). One of the clinical isolates exhibited a gain fragment between site L-M (pattern 2). In patttern 3, which occurred in two of the isolates, showed a loss of fragments M (pattern 3).

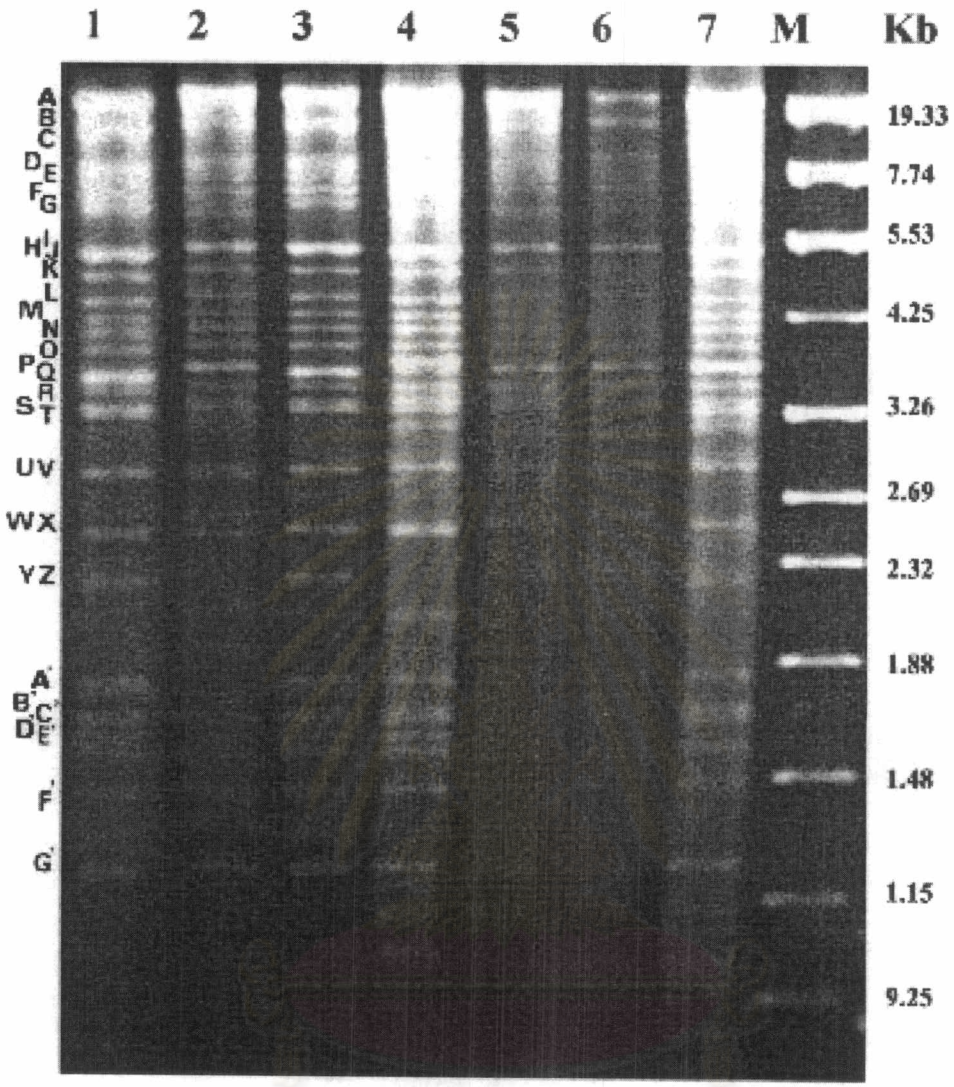

Figure 19. *Bam*HI profiles in HSV-2 clinical isolates. Lane 1 HSV-2 (Baylor 186), lane 2 to 7 clinical isolates and lane 8 marker IV (M).

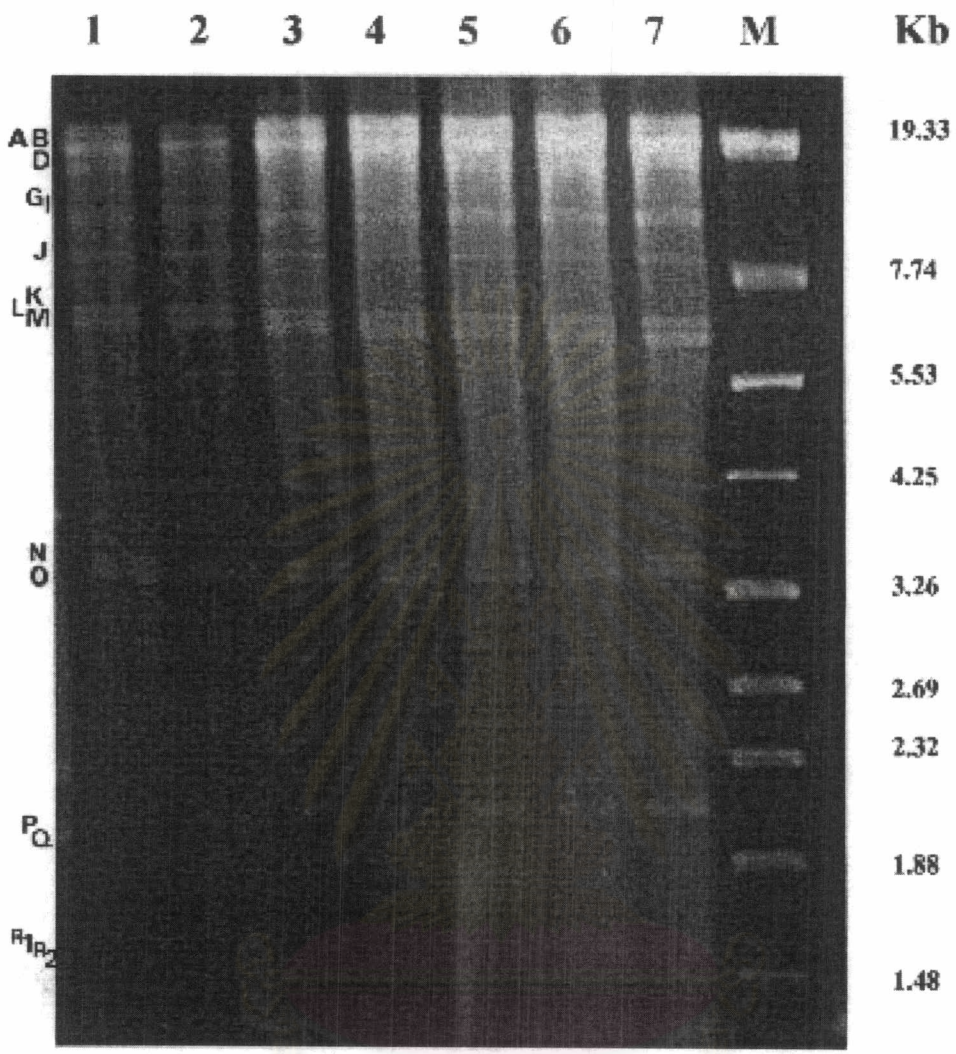

Figure 20. *Kpn*I profiles in HSV-2 clinical isolates. Lane 1 HSV-2 (Baylor 186), lane 2 to 7 clinical isolates and lane 8 marker IV (M).

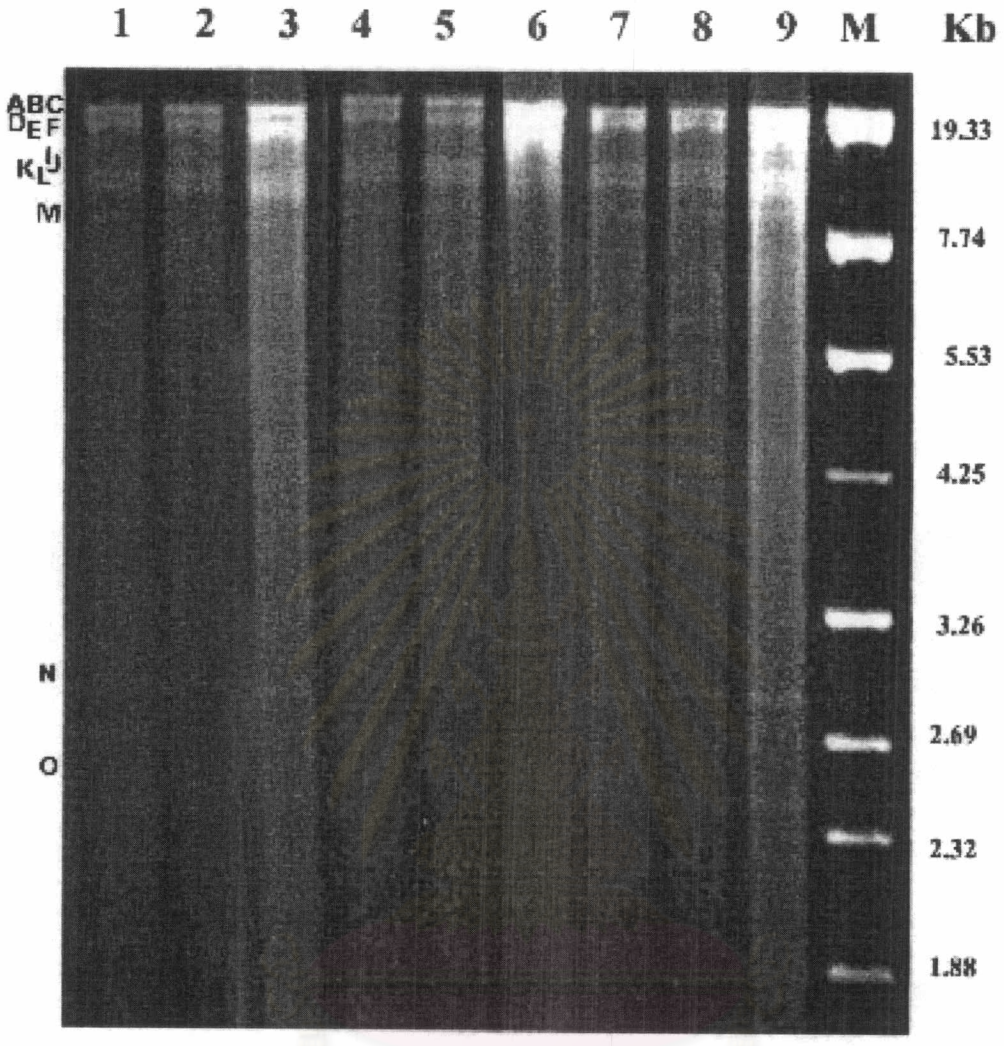

Figure 21. *Hin*dIII profiles in HSV-2 clinical isolates. Lane 1 HSV-2 (Baylor 186), lane 2 to 9 clinical isolates and lane 10 marker IV (M).

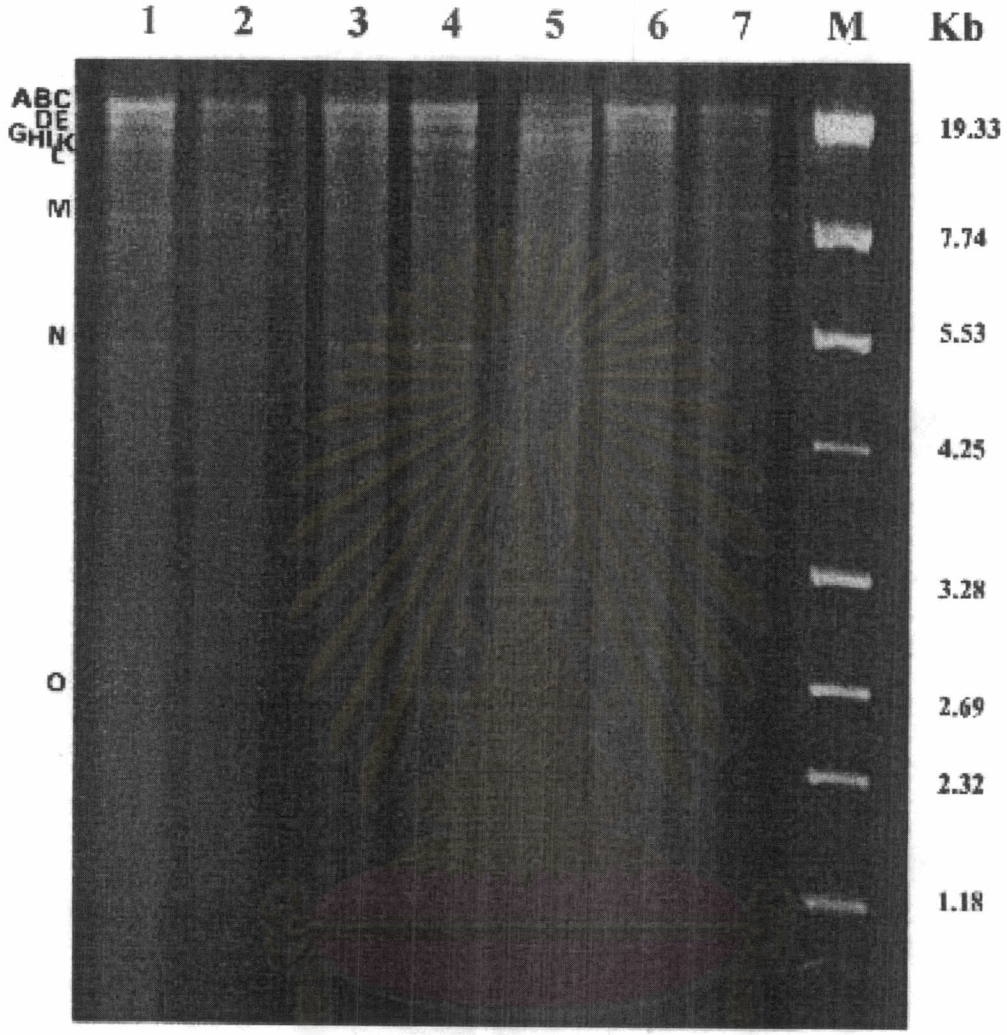

Figure 22. *Eco*RI profiles in HSV-2 clinical isolates. Lane 1 HSV-2 (Baylor 186), lane 2 to 7 clinical isolates and lane 8 marker IV (M).

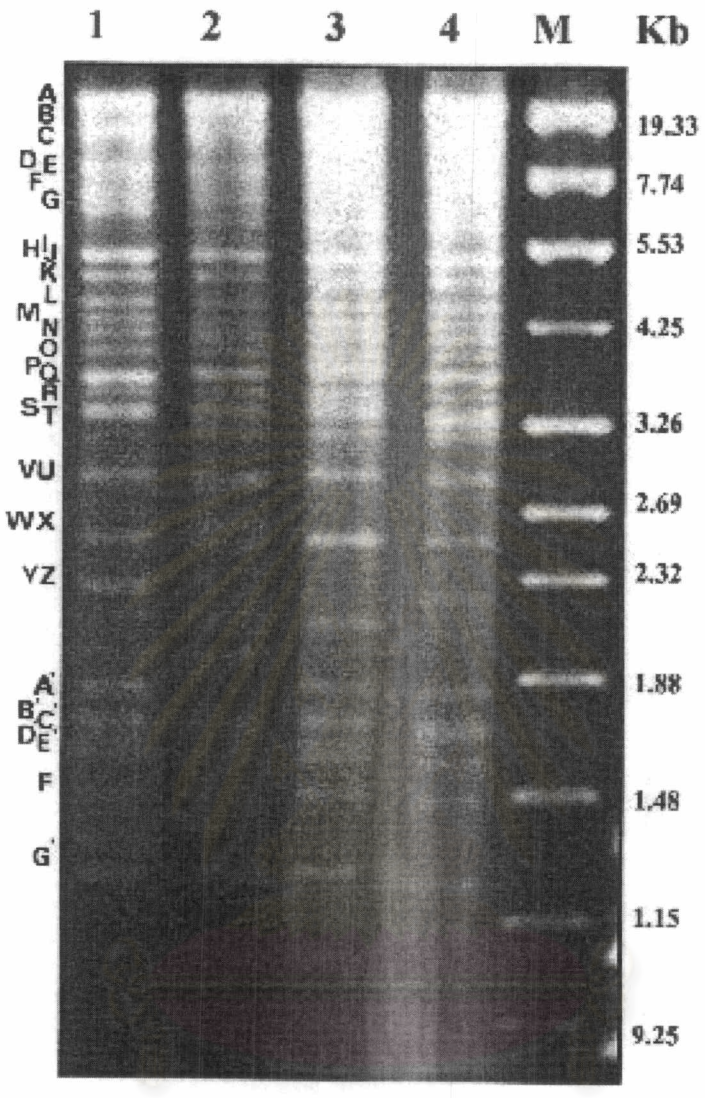

Figure 23. Four distinct patterns of HSV-2 DNA after *Bam*HI digestion. Lane 1 HSV-2 (Baylor 186) (Pattern 1), lane 2 (Pattern 2), lane 3 (Pattern 3), lane 4 (Pattern 4), and lane 5 marker IV (M).

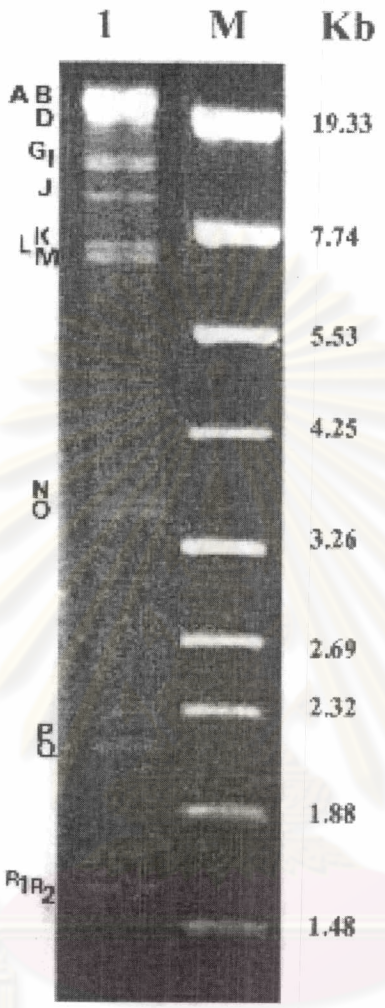

Figure 24. One distinct pattern of HSV-2 DNA after *Kpn*I digestion. Lane 1 HSV-2 (Baylor 186) (Pattern 1) and lane 2 marker IV (M).

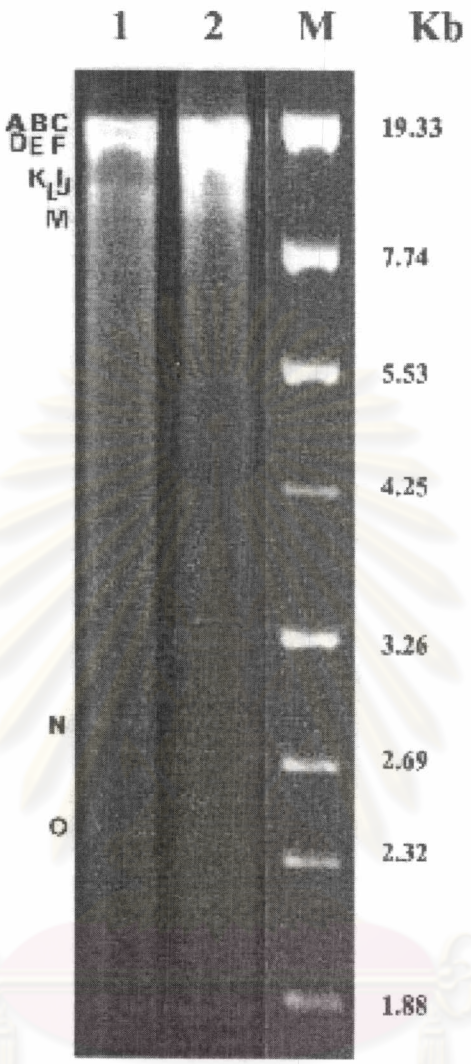

Figure 25. Two distinct patterns of HSV-2 DNA after *Hin*dIII digestion. Lane 1 HSV-2 (Baylor 186) (Pattern 1) and lane 2 (Pattern 2), lane 3 marker IV (M).

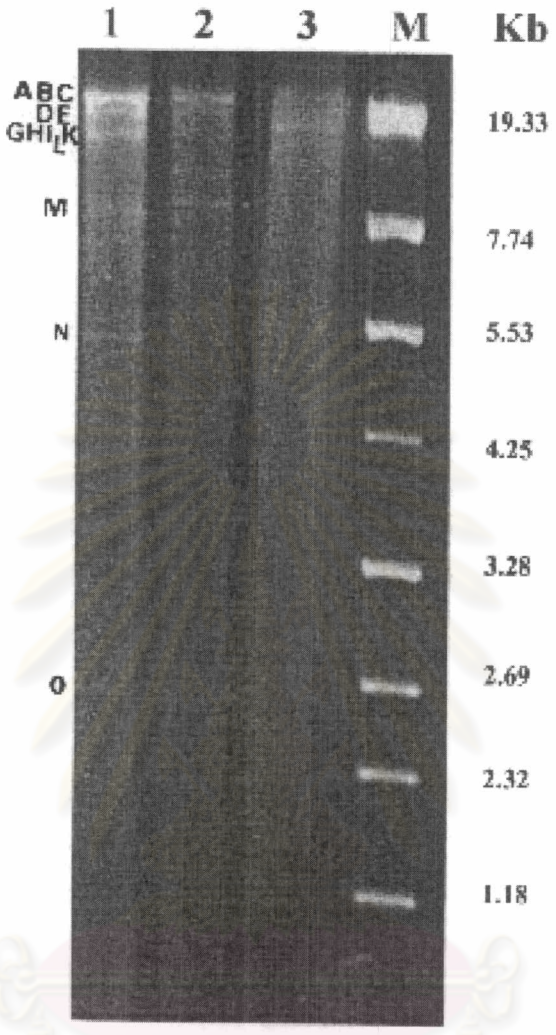

Figure 26. Three distinct patterns of HSV-2 DNA after *Eco*RI digestion. Lane 1 HSV-2 (Baylor 186) (Pattern 1) and lane 2 (Pattern 2), lane 3 (Pattern 3), lane 4 marker IV (M).

Table 9. The variable restriction endonuclease cleavage sites of HSV-2 clinical isolates.

Enzyme	Pattern of variable restriction endonuclease cleavage sites	Total	Frequency (%)
***Bam*HI**	(1) *Bam*HI, HSV-2 (Baylor 186)	17/20	85%
	(2) Gain: Z-A', loss: YZ	1/20	5%
	(3) Gain: T-U, Z-A', loss YZ	1/20	5%
	(4) Gain: T-U	1/20	5%
***Kpn*I**	(1) *Kpn*I, HSV-2 (Baylor 186)	20/20	100%
***Hind*III**	(1) *Hind*III, HSV-2 (Baylor 186)	17/20	85%
	(2) Loss: M	3/20	15%
***Eco*RI**	(1) *Eco*RI, HSV-2 (Baylor 186)	17/20	85%
	(2) Gain: L-M	1/20	5%
	(3) Loss: M	2/20	10%

Table 10. The variable RE cleavage patterns of HSV-1 clinical isolates.

Enzyme	Pattern	Number of clinical specimen																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
***Bam*HI, HSV-1 (KOS)**	1		+	+			+	+	+	+	+		+	+	+		+	+		+	+
Gain: T-U, loss: W	2																		+		
Loss: N	3															+					
Gain: T-U, loss: S, W	4	+				+						+									
Gain: P-Q,T-U, loss: N, O, S	5				+																
***Kpn*I, HSV-1 (KOS)**	1		+				+	+	+	+				+	+		+	+			+
Gain: L-M, loss: P, R Fusion: T-U	2	+		+	+	+					+	+	+						+		
Fusion: T-U	3															+				+	
***Hind*III, HSV-1 (KOS)**	1	+	+	+	+	+			+	+	+	+	+				+	+	+	+	+
Loss: M	2						+	+						+		+					
Loss: M, N	3														+						
***Eco*RI, HSV-1 (KOS)**	1		+	+			+	+	+	+	+		+	+	+		+	+		+	+
Loss: K	2	+																			
Gain: J-K, L-M, loss: L	3																		+		
Gain: L-M, loss: L	4				+	+						+				+					

Table 11. The variable RE cleavage patterns of HSV-2 clinical isolates.

Enzyme	Pattern	Number of clinical specimen																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
***Bam*HI, HSV-2 (Baylor 186)**	1	+		+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gain: Z-A', loss: YZ	2		+																		
Gain: T-U, Z-A', Loss: YZ	3					+															
Gain: T-U	4				+																
***Kpn*I, HSV-2 (Baylor 186)**	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
***Hind*III, HSV-2 (Baylor 186)**	1		+	+		+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
Loss: M	2	+			+						+										
***Eco*RI, HSV-2 (Baylor 186)**	1		+	+	+	+	+	+	+	+		+	+	+	+	+	+	+		+	+
Gain: L-M	2										+										
Loss: M	3	+																	+		

5. Diversity of RE Cleavage Patterns of HSV Isolates.

Analysis of the cleavage patterns of HSV DNA from 20 isolates of each HSV-1 and HSV-2 by using four endonucleases, i.e., *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI were done. The diversity of RE cleavage patterns were identified by combination with four enzymes.

Twenty clinical isolates of HSV-1 were distinguished with one enzyme (*Bam*HI) and were classified into five patterns (Table 12). The combination with two enzymes (*Bam*HI and *Kpn*I) were identified the diversity into seven patterns (Table 13), combination with three enzymes (*Bam*HI, *Kpn*I, and *Hin*dIII) were identified the diversity into nine patterns (Table 14), and combination with four enzymes (*Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI) were identified the diversity into ten patterns (Table 15). The summarized frequency of occurrences of each isolates was shown in Table 16.

Table 12. The frequency of *Bam*HI patterns in HSV-1 clinical isolates.

Enzyme(s)	Pattern(s)	Frequency of variation / percent (%)
*Bam*HI	B_1	14 (70%)
	B_2	1 (5%)
	B_3	1 (5%)
	B_4	3 (15%)
	B_5	1 (5%)

Table 13. The frequency of *Bam*HI + *Kpn*I patterns in HSV-1 clinical isolates.

Enzyme(s)	Pattern(s)	Frequency of variation / percent (%)
*Bam*HI+*Kpn*I	B_1K_1	10 (50%)
	B_4K_2	3 (15%)
	B_1K_2	3 (15%)
	B_5K_2	1 (5%)
	B_3K_3	1 (5%)
	B_2K_2	1 (5%)
	B_1K_3	1 (5%)

Table 14. The frequency of *Bam*HI+*Kpn*I+*Hin*dIII patterns in HSV-1 clinical isolates.

Enzyme(s)	Pattern(s)	Frequency of variation / percent (%)
*Bam*HI+*Kpn*I+*Hin*dIII	$B_1K_1H_1$	7 (35%)
	$B_4K_2H_1$	3 (15%)
	$B_1K_2H_1$	3 (15%)
	$B_1K_1H_2$	2 (10%)
	$B_5K_2H_1$	1 (5%)
	$B_1K_1H_3$	1 (5%)
	$B_3K_3H_2$	1 (5%)
	$B_2K_2H_1$	1 (5%)
	$B_1K_3H_1$	1 (5%)

Table 15. The frequency of *Bam*HI+*Kpn*I+*Hin*dIII+*Eco*RI patterns in HSV-1 clinical isolates.

Enzyme(s)	Pattern(s)	Frequency of variation / percent (%)
*Bam*HI+*Kpn*I+*Hin*dIII+*Eco*RI	$B_1K_1H_1E_1$	7 (35%)
	$B_1K_2H_1E_1$	3 (15%)
	$B_4K_2H_1E_4$	2 (10%)
	$B_4K_2H_1E_2$	1 (5%)
	$B_5K_2H_1E_4$	1 (5%)
	$B_1K_1H_2E_1$	2 (10%)
	$B_1K_1H_3E_1$	1 (5%)
	$B_3K_3H_2E_4$	1 (5%)
	$B_2K_2H_1E_3$	1 (5%)
	$B_1K_3H_1E_1$	1 (5%)

Table 16. The variation patterns of HSV-1 isolates by restriction endonuclease cleavage (*Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI) patterns.

Clinical No.	Endonuclease cleavage patterns			
	***Bam*HI**	***Bam*HI+*Kpn*I**	***Bam*HI+*Kpn*I+*Hin*dIII**	***Bam*HI+*Kpn*I+*Hin*dIII+*Ecp*RI**
1	B_4	B_4K_2	$B_4K_2H_1$	$B_4K_2H_1E_2$
2	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
3	B_1	B_1K_2	$B_1K_2H_1$	$B_1K_2H_1E_1$
4	B_5	B_5K_2	$B_5K_2H_1$	$B_5K_2H_1E_4$
5	B_4	B_4K_2	$B_4K_2H_1$	$B_4K_2H_1E_4$
6	B_1	B_1K_1	$B_1K_1H_2$	$B_1K_1H_2E_1$
7	B_1	B_1K_1	$B_1K_1H_2$	$B_1K_1H_2E_1$
8	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
9	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
10	B_1	B_1K_2	$B_1K_2H_1$	$B_1K_2H_1E_1$
11	B_4	B_4K_2	$B_4K_2H_1$	$B_4K_2H_1E_4$
12	B_1	B_1K_2	$B_1K_2H_1$	$B_1K_2H_1E_1$
13	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
14	B_1	B_1K_1	$B_1K_1H_3$	$B_1K_1H_3E_1$
15	B_3	B_3K_3	$B_3K_3H_2$	$B_3K_3H_2E_4$
16	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
17	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
18	B_2	B_2K_2	$B_2K_2H_1$	$B_2K_2H_1E_3$
19	B_1	B_1K_3	$B_1K_3H_1$	$B_1K_3H_1E_1$
20	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$

All of 20 clinical isolates of HSV-2 were distinguished with one enzyme (*Bam*HI) and were classified into four patterns (Table 17). The combination with two enzymes (*Bam*HI and *Kpn*I) were also identified the diversity into four patterns (Table 18), due to only one pattern of *Kpn*I, combination with three enzymes (*Bam*HI, *Kpn*I, and *Hin*dIII) were identified the diversity into five patterns (Table 19), and combination with four enzymes (*Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI) were identified the diversity into seven patterns (Table 20). The summarized frequency of occurrences of each virus was shown in Table 21.

Table 17. The frequency of *Bam*HI patterns in HSV-2 clinical isolates.

Enzyme(s)	Pattern(s)	Frequency of variation / percent (%)
*Bam*HI	B_1	17 (85%)
	B_2	1 (5%)
	B_3	1 (5%)
	B_4	1 (5%)

Table 18. The frequency of *Bam*HI + *Kpn*I patterns in HSV-2 clinical isolates.

Enzyme(s)	Pattern(s)	Frequency of variation / percent (%)
*Bam*HI+*Kpn*I	B_1K_1	17 (85%)
	B_2K_1	1 (5%)
	B_3K_1	1 (5%)
	B_4K_1	1 (5%)

Table 19. The frequency of *Bam*HI+*Kpn*I+*Hin*dIII patterns in HSV-2 clinical isolates.

Enzyme(s)	Pattern(s)	Frequency of variation / percent (%)
*Bam*HI+*Kpn*I+*Hin*dIII	$B_1K_1H_1$	15 (75%)
	$B_1K_1H_2$	2 (10%)
	$B_2K_1H_1$	1 (5%)
	$B_4K_1H_2$	1 (5%)
	$B_3K_1H_1$	1 (5%)

Table 20. The frequency of *Bam*HI+*Kpn*I+*Hin*dIII+*Eco*RI patterns in HSV-2 clinical isolates.

Enzyme(s)	Pattern(s)	Frequency of variation / percent (%)
*Bam*HI+*Kpn*I+*Hin*dIII+*Eco*RI	$B_1K_1H_1E_1$	14 (70%)
	$B_1K_1H_2E_3$	1 (5%)
	$B_2K_1H_1E_1$	1 (5%)
	$B_4K_1H_2E_1$	1 (5%)
	$B_3K_1H_1E_1$	1 (5%)
	$B_1K_1H_2E_2$	1 (5%)
	$B_1K_1H_1E_3$	1 (5%)

Table 21. The variation patterns of HSV-2 isolates by restriction endonuclease cleavage (*Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI) patterns.

Clinical No.	Endonuclease cleavage patterns			
	***Bam*HI**	***Bam*HI+*Kpn*I**	***Bam*HI+*Kpn*I+*Hin*dIII**	***Bam*HI+*Kpn*I+*Hin*dIII+*Eco*RI**
1	B_1	B_1K_1	$B_1K_1H_2$	$B_1K_1H_2E_3$
2	B_2	B_2K_1	$B_2K_1H_1$	$B_2K_1H_1E_1$
3	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
4	B_4	B_4K_1	$B_4K_1H_2$	$B_4K_1H_2E_1$
5	B_3	B_3K_1	$B_3K_1H_1$	$B_3K_1H_1E_1$
6	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
7	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
8	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
9	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
10	B_1	B_1K_1	$B_1K_1H_2$	$B_1K_1H_2E_2$
11	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
12	B_1	B_1K_1	B_1K1H_1	$B_1K_1H_1E_1$
13	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
14	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
15	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
16	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
17	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
18	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_3$
19	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$
20	B_1	B_1K_1	$B_1K_1H_1$	$B_1K_1H_1E_1$