CHAPTER VI

DISCUSSION

In this study, the investigator described assay system for detection and species identification of HSV based on consensus PCR followed by restriction endonuclease digestion. The previously developed PCR (341) which is primers are anchored in highly conserved region of the DNA polymerase gene was used. The 532 bp amplified product of both HSV-1 and HSV-2 could be detected (Figure 7), but the differentiation between two types had to be further analysis by using restriction enzyme, *Bam*HI (Figure 7).

Although the nucleotide sequence of HSV-1 and HSV-2 have 50% sequence homology (4), there is no genetic gradient between the two, and to date there have been no report of recombinant virus. It has been proposed that HSV-1 and HSV-2 are in fact distinct virus species (367). The use of these PCR assay with clinical samples illustrated that, each virus could be detected repeatedly and reproducibly.

HSV-1 normally produces lesions above the waist and HSV-2 is responsible for lesions below the waist. This association is not absolute and either virus type may, in fact, cause lesions anywhere on the body. The prevalence of HSV isolates from clinical samples of King Chulalongkorn Memorial Hospital's patients were found to be HSV-1 the most common type in nongenital lesions (90.90%) whereas both HSV-1 (53.12%) and HSV-2 (43.75%) were equally found in genital lesions (Table 5). Both nongenital lesions and genital lesions were found HSV infection in female higher than in male. Interestingly, nowadays HSV-1 was dominantly detected in genital area, corresponding to recent studies which reported an increasing prevalence of genital HSV-1 infection (21-23).

HSV-2 genital infection is frequently associated with instances of multiple sexual partners, however, a change in sexual practices and increase in oro-genital contact may be contributing to increase in patients with HSV-1 genital infection. While HSV-1 genital is becoming the major cause of primary genital infection, HSV-1 genital infection is less likely to recur (a longer time

to recurrence and fewer recurrences) than that caused by HSV-2 (173, 199, 200, 201).

RFLP analysis is a common and powerful method and this technique has been shown to be useful for molecular epidemiology of HSV (38,64). Restriction polymorphism may serve as convenient markers for identification of functional variation among HSV strains and has the potential to detect very small differences between closely related virus strains. Genomic variations are clearly demonstrated using four restriction endonucleases, *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI. By use of multi-cut endonuclease such as *Bam*HI and *Kpn*I, the analysis of base sequence variation results in more resolution. The criteria used for the classification was based on the gain or loss of RE cleavage site(s).

Digestion of the viral DNA molecules with restriction enzyme and comparison of the resultant fragments after electrophoresis were used to differentiate HSV-1 and HSV-2 isolates. The results of molecular epidemiology of genetic variation among those HSV isolates by RFLP using those four enzymes, were compared to standard HSV-1 (KOS) and HSV-2 (Baylor 186) reflecting as differences in cleavage patterns and identified based on the gain or loss of restriction enzyme maps.

The great majority of each 20 clinical isolates of HSV-1 distinguished by digestion with *Bam*HI (70%), *Kpn*I (50%), *Hin*dIII (75%), and EcoRI (70%), were found to be the same pattern as standard HSV-1 (KOS) (pattern 1) (Table 8). *Bam*HI digestion can classified into five patterns. Patterns 4, showed gain of fragment between site T-U due to loss of fragments S and W in high frequency (15%) more than patterns 2, 3, and 5 (5%). *Kpn*I cut was classified into three predominant cleave patterns. Patterns 2 showed gain of fragment L-M due to loss of fragment P and R, and fusion between site T-U, occurred very high frequency (40%) than pattern 3 (10%). *Hin*dIII cut showed three cleavage patterns. Pattern 2, loss a fragment of M, was found 20% while only 5% of pattern 3 was determined. *Eco*RI cut had classified into four patterns. Pattern 4, which showed gain of fragment between site L-M and loss of a fragment L had high frequency (20%) than pattern 2 (5%) and 3 (5%) (Table 8).

Among those 20 clinical isolates of HSV-2, 85% were similar to standard HSV-2 (Baylor 186) (pattern 1), after digestion with *Bam*HI, *Hin*dIII, and *Eco*RI. No difference was found with *Kpn*I digestion (Table 9). *Bam*HI digestion gave four predominant cleavage patterns but patterns 2, 3 and 4 were equal frequency (5%). For *Hin*dIII cut, the results also demonstrated two cleavage patterns. Pattern 2 showing a loss of fragment M, occurred in frequency of 15%. *Eco*RI cut was classified into three patterns. Patterns 2, and 3 were found at 5% and 10%, respectively (Table 9).

Diversity of RE cleavage patterns among 20 HSV-1 isolates by combination with two enzymes (*Bam*HI and *Kpn*I) (Table 13) were identified into seven patterns, 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 into ten patterns (Table 15). The number of enzymes can be extended the diversity of genetic variation. The more enzyme used the more diversity was detected. The pattern in $B_1K_1H_1E_1$ (35%) which is the same as standard HSV-1 (KOS) pattern showed the combination frequency higher than other patterns, followed by $B_1K_2H_1E_1$ (15%), $B_4K_2H_1E_4$ (10%), and $B_1K_1H_2E_1$ (10%) (Table 15).

All of 20 HSV-2 clinical isolates showed low diversity than those HSV-1 isolates. With combination of two enzymes (*Bam*HI and *Kpn*I), they were identified the diversity into four patterns (Table 18), combination with three enzymes (*Bam*HI, *Kpn*I, and *Hin*dIII) were identified the diversity into five patterns, and combinations with four enzymes (*Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI) were identified the diversity into seven patterns (table 19). The pattern of $B_1K_1H_1E_1$ (70%) was the highest. In this study, it was clearly indicated that HSV-2 strains were not high diversified when compared to those HSV-1 strains (7 vs 10 patterns, Table 15 and 20). Our observation was similar to the study by Maitland *et al.*, (262).

There are a number of studies about RFLP patterns of both HSV-1 and HSV-2 (269). Among those, the diversity of patterns was predominantly detected in HSV-1 isolates (198) while few of RE cleavage patterns were shown within HSV-2 isolates (198,262,271,348). Although they are the indirect evidences which may vary depend on number of samples and number of enzymes used, study on one TK gene comparing between HSV-1 and HSV-2 strains revealed that the diversity of HSV-1 strains was four-fold higher than that of HSV-2 strains (363). The different rate of variation probably specifically depended on the properties of each HSV type.

Variation in RE cleavage patterns were divided into two types, one type termed RFLP, is due to mostly a gain or loss of an RE cleavage site. The RFLP is stable serve as a physical marker for differentiation and classification of HSV strains. Another type is an irregularly of RE-cleavage fragments derived from a region of HSV genome. This common type was located in fragments containing reiterated sequences (14). Thus, the presence of four isomeric forms of HSV-DNA accounts for the unique fragment patterns produced when HSV DNA is digested. RE analyses have revealed that HSV-1 DNA exhibit a small degree of heterogeneity (microheterogenity) within the regions corresponding to the L component and S-L junction (369). Our study also indicated that the variation was observed mostly in S component in both HSV-1 and HSV-2 whereas few variations was found in L component and S-L junction (Figure 27, 28).

The positions of suspected gene variation in HSV-1 isolates observed by *Bam*HI RFLP in our study were different from those demonstrated in Saudi patients (370). In this present work, the differences were observed at T-U, W, S, O, O-P, and N cleavage sites (Table 8) while Al-Aladal *et al.*, (370) reported at A, D-H, W-K, B-L, C-K positions. However, similar variation of *Hin*dIII digestion was found (at M-N position). The distinct patterns between these studies may imply the variation of HSV-strains in different geographic area.

According to HSV-2 observation, our data on *Bam*HI restriction pattern (Table 9) showed some pattern (Gain T-U, Z-A') similar to previously reported (371,271). However the common *Bam*HI pattern, gain of site in G, which Chantratita W and Yoosook C (371) found 68.7%, was not detected in our study.

No different profile of *Kpn*I digestion was observed in those isolates of Sirirungsi W *et al.*, with reference to SAV strain. This result of *Kpn*I digestion profile was the same as in this present study (100% similar to standard HSV-2, Baylor 186 strain). However, Chantratita W and Yoosook C (371) reported the presence of fusion fragment D-I in *Kpn*I cleavage patterns was commonly found in Thai isolates (79.5%, 89/112). This feature was also found in other studies (271,372). The absence of this *Kpn*I D-I pattern in our study probably due to small number of HSV-2 isolates.

Since the restriction endonuclease cleavage pattern showed the distinction among HSV-1 and HSV-2 isolates. The differences in the cut site of each enzyme can reflect the difference in nucleotide itself. Fortunately, both HSV-1 and HSV-2 genome were clearly sequenced and quite well known. The HSV genome map (Figure 4) generated by McGeoch *et al.*, (3,160) is very useful and served as the basis for the interpretation of the nucleotide sequence data in association to their products. The restriction endonuclease cleavage maps of HSV-1DNA and HSV-2 DNA by *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI are shown in Figures 27 and 28. The viral gene products of HSV-1 and HSV-2 genome are also demonstrated in Figures 29 and 30.

Determination of the gain and loss of some fragments in the RE patterns can predict the related viral products. For HSV-1 RE patterns (Table 8), the variable fragments were suggested to relate with different viral proteins as indicated in Table 22 as well as HSV-2 RE patterns (Table 9) which were shown in Table 23.

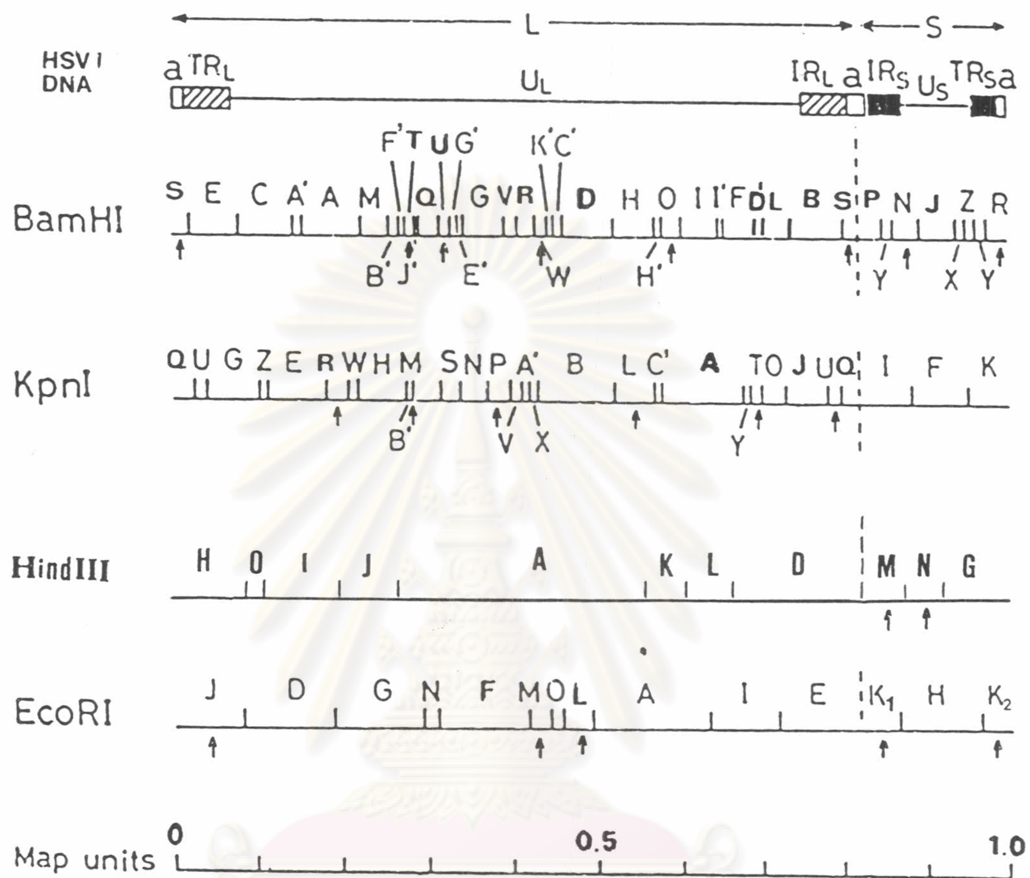

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Figure 27. Map position of restriction site variation in the genome of HSV-1 DNA used for comparison among isolates. *Bam*HI, *Kpn*I, *Hin*dIII (Locker & Frenkel, 1979; Roizman & Buchman, 1979) and *Eco*RI (Umene K et al, 1984) maps are based on HSV-1 strain F, under each map, (↑) indicates the position of RE sites and fragments that show variation among strains, corresponding to the sites previously described.

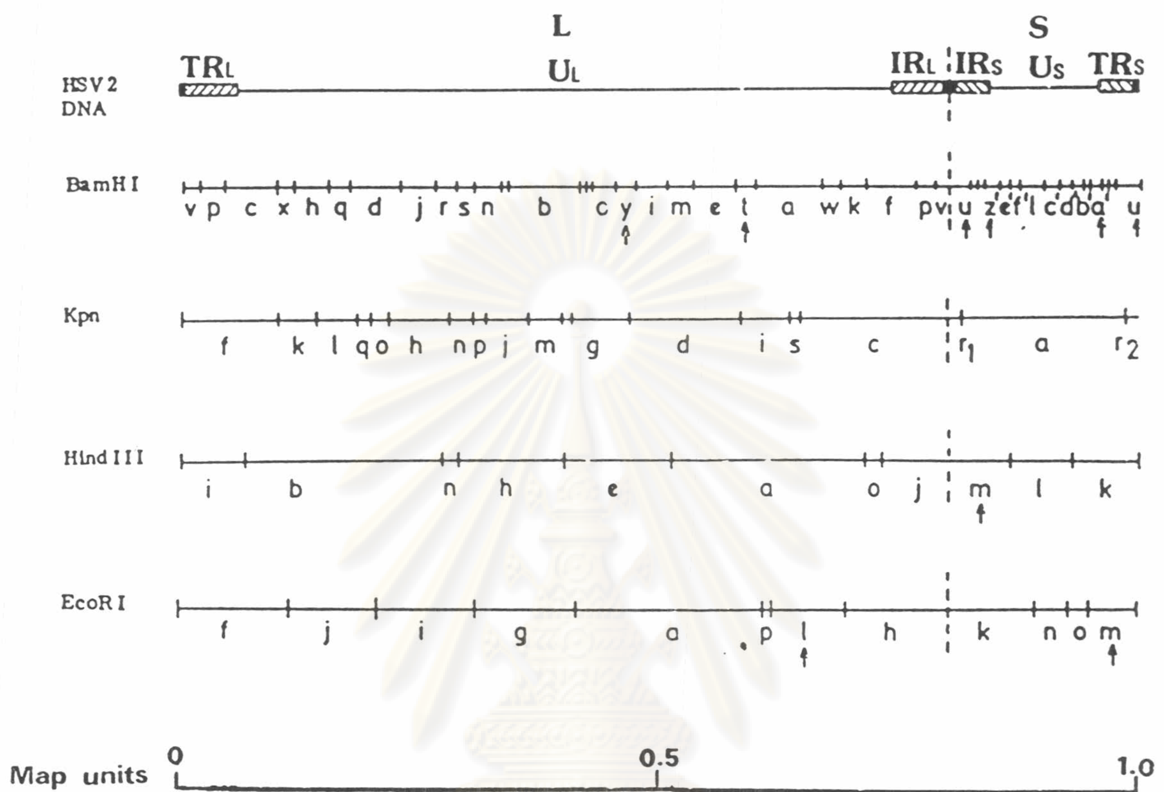

Figure 28. Map position of restriction site variation in the genome of HSV-2 DNA used for comparison among isolates. *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI (Wilkie et al, 1978 & Roizman B, 1979) map are based on HSV-2 strain G, under each map (↑) indicates the position of RE sites and fragments that show variation among strains, corresponding to the sites described.

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Figure 29. The functional of HSV-1 genome; Accession: NC_001806 Total Bases Sequenced: 152261 bp Completed: Apr 3, 1990.
http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=genome&gi=12187

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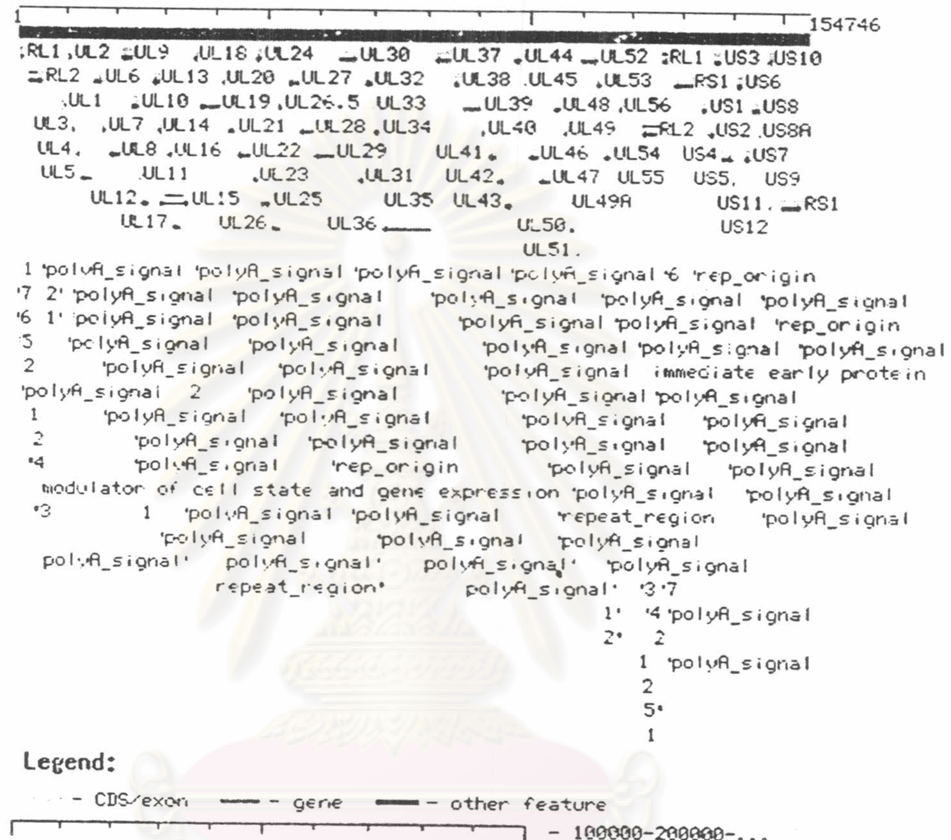

Figure 30. The functional of HSV-2 genome; Accession: NC 001798 total Base Sequenced: 154746 bp Completed: Feb 27, 1997.
http:/www.ncbi.mlm.nih.gov/cgi-bin/Entrez/framik?db=genome&gi=12163

According to the RE patterns, the intratypic variation among HSV-1 isolates was higher than that among HSV-2 isolates. A number of predicted gene products of HSV-1 was also greater than those of HSV-2 (Table 22, 23). Interestingly, the variation of viral products was found mainly in structural part of virus especially envelope glycoproteins i.e. HSV-1: gG, gL, gH, gE and HSV-2: gG, gJ, gI, gE, and gK. Few non-structural proteins for example ICP22, protein kinase and DNA polymerase were affected by these variations (Table 22, 23).

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Table 22. Identification of the variation sites on RE map of HSV-1 related to the gene products and functions.

Enzyme	Variation site on REs map	Arrangement of DNA sequence	Gene or transcriptional unit	Designation of protein	Regulation: kinetic group	Function of gene product
*Bam*HI	N	IR_S	U_S1	α22, ICP22	α	α Regulatory protein
			U_S3	-	β	Protein kinase
	P	IR_S	U_S1	α22, ICP22	α	α Regulatory protein
			U_S3	-	β	Protein kinase
		TR_S	U_S8	gE	γ2	Glycoprotein E
			U_S10	-	-	Virion protein
	S	TR_L	U_L1	gL	γ	Glycoprotein L
		IR_L	$U_L49.5$	-	γ2	Membrane protein
			U_L53	gK	γ	Glycoprotein K
	T	U_L	U_L30	-	β	DNA polymerase
	U		U_L31	-	γ2	Nucleotide, matrix protein
	W		U_L33	-	-	Assembly protein
	O	U_L	U_L37	ICP32	γ	Virion protein
			U_L38	VP19C	γ2	Virion protein
*Kpn*I	L	U_L	U_L35	VP26	γ2	Virion protein
			U_L36	ICP1-2	γ2	Virion protein
	M	U_L	U_L24	-	γ	Membrane fussion
			U_L25	-	γ	Virion protein
	P	U_L	U_L28	ICP18.5	γ	Assembly protein
			U_L29	ICP8	β	SS DNA binding protein
	R	U_L	U_L18	VP23	γ	Capsid protein
			U_L20	-	γ	Membrane protein
			UL22	gH	γ2	Glycoprotein H
	T-U	IR_L	$U_L49.5$	-	γ2	Membrane protein
			U_L53	gK	γ	Glycoprotein K

Table 22. Identification of variation sites on RE map of HSV-1 related to the gene products and functions. (Continued)

Enzyme	Variation site on REs map	Arrangement of DNA sequence	Gene or transcriptional unit	Designation of protein	Regulation: kinetic group	Function of gene product
*Hind*III	M	IR_S	U_S1	α22, ICP22	α	α Regulatory protein
			U_S2	-	-	-
	N	U_S	U_S3	-	β	Protein kinase
			U_S4	gG	γ	Glycoprotein G
*Eco*RI	J	TR_L	U_L1	gL	γ	Glycoprotein L
	L	U_L	U_L30	-	β	DNA polymerase
			U_L31	-	γ2	Nucleotide, matrix protein
			U_l32	-	γ2	Membrane protein
	M	U_L	U_L33	-	-	Assembly protein
			U_L34	-	-	Membrane protein
			U_L35	VP26	γ2	Virion protein
	K	IR_S	U_S2	-	-	-
			U_S3	-	β	Protein kinase
		TR_S	U_S8	gE	γ2	Glycoprotein E
			U_S10	-	-	Virion protein

Table 23. Identification of variation sites on RE map of HSV-2 related to the gene products and functions.

Enzyme	Variation site on REs map	Arrangement of DNA sequence	Gene or transcriptional unit	Designation of protein	Regulation: kinetic group	Function of gene product
*Bam*HI	Z	IR_S	U_S1	α22, ICP22	α	α Regulatory protein
			U_S2	-	-	-
			U_S3	-	β	Protein kinase
			U_S4	gG	γ	Glycoprotein G
			U_S5	gJ	-	Glycoprotein J
	A'	TR_S	U_S7	VP17/18, gI	γ	Glycoprotein I
			U_S8	gE	γ2	Glycoprotein E
	Y	U_L	U_S37	ICP32	γ	Virion protein
			U_S38	VP19C	γ2	Virion protein
	T	U_L	U_L44	gC, VP8	γ2	Glycoprotein C
			U_L45	-	γ2	-
	U	IR_S	U_S1	α22, ICP22	α	α regulatory protein
			U_S2	-	-	-
			U_S3	-	β	protein kinase
		TR_S	U_S7	VP17/18, gI	γ	Glycoprotein I
			U_S8	gE	γ2	Glycoprotein E
*Kpn*I	-	-	-	-	-	-

Table 23. Identification of variation sites on RE map of HSV-2 related to the gene products and functions. (Continued)

Enzyme	Variation site on REs map	Arrangement of DNA sequence	Gene or transcriptional unit	Designation of protein	Regulation: kinetic group	Function of gene product
*Hind*III	M	IR_S	U_S1	α 22, ICP22	α	α regulatory protein
			U_S2	-	-	-
			U_S3	-	β	protein kinase
			U_S4	gG	γ	Glycoprotein G
			U_S5	gJ	-	Glycoprotein J
*Eco*RI	L	U_L	U_L52	-	β	Helicase primase
			U_L53	gK	γ	Glycoprotein K
			U_L54	α 27, ICP27	α	α regulatory protein
	M	TR_S	U_S8	gE	γ2	Glycoprotein E
			$U_S8.5$	-	β,γ1	-
			U_S9	-	-	Tegument protein
			U_S10	-	-	Virion protein

Spontaneous mutation is a phenomenon occur in all kinds of viruses during replication without any exception to HSV. Changing viral genetics affect the phenotypic properties. HSV is an enveloped virus consisting at least 12 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN) (373). Of those gB, gD, gH and gL are essential to the process of infection in cell culture, while the others contribute to viral infectivity and spread in host (374). Glycoprotein D is an important glycoprotein for both HSV-1 and HSV-2 because it is HSV-receptor-binding protein (375,376).

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Although intratypic variation of gD had been previously shown by functional assay (377), genotypic variation observed by RFLP was not found in this study. Glycoprotein H and gL are known to play role in association with gD during entry step. Changing of these two proteins might inhibit the entry step or lower efficiency of viral penetration (378,379). Glycoprotein E and gI could form a complex that function as Fc receptor for IgG. This molecule help HSV to achieve the ability to evade host immunity by blocking their specific Fc interaction (380). Absent of this molecule may increase the virulence of HSV. Glycoprotein K is required for production of infectious virions but it has shown to be absent from envelope of some viral strains (381,382). Glycoprotein B is involved in the initial phase of attachment to heparan sulphate. However, gC can also act similar to gB. Our study indicated that some envelope glycoproteins supposed to have genetic variation such as HSV-1; gG, gL, gH and gE, HSV2; gG, gJ, gI, gE, gC, and gK (Table 22 and 23). These variations may effect to their functions as previously discussed. The viruses are still able to maintain infectivity but the mechanism of pathogenesis may change. Not only glycoproteins, but also changing of virion proteins and other structural proteins may have influence on biological properties of the viruses.

The effect on nonstructural proteins such as ICP22, regulatory protein, protein kinase and DNA polymerase was also predicted. Basicly, functional enzymes of viruses mostly genetic sequences are conserved. However, spontaneous mutation of HSV DNA polymerase has been reported (383). The mutation can induce HSV resistance to antiviral drug, acyclovir (384). ICP22 is a regulatory protein involve in control of viral gene expression (385-387). Thus, it seemed that the genetic variations observed among HSV isolates did not mainly effect viral infectivity. They may be responsible for differences in pathogenesis of the disease.

Molecular epidemiological analyses aid in studies on the infection of viruses and it is possible to determine whether a second episode of HSV infection is due to reactivation of a latent virus or to re-infection with an exogenous virus. These molecular analyses are based on the heterogeneity of viral genome found among HSV isolates, and individual HSV isolates can be distinguished using REs (38,64). Molecular epidemiological studies depended mainly on such differences in RE-digestion profiles of HSV DNAs. HSV strains are differentiated by analyses of RFLPs, and HSV strains can also be classified into genotypes, which the number of enzymes can extended the diversity of genetic variation. The prediction of biological properties of the intratypic variation on REs sites can indicate the relation of HSV genes products and functions. It remains to be elucidated whether genotypes of HSV are associated with biological properties and clinical manifestation (144). This genotypic difference may possibly influence clinical manifestation (14). To determine whether genotype are associated with particular clinical presentations, determination of the biological function of HSV genotype is required. Genomic marker to sort out strains of a genotype will facilitate determination of the genotype. In the present study, we demonstrated the usefulness of RFLP in characterization of HSV isolates. The RE pattern can provide unequivocal information on a wide extent of intratypic genetic variation among HSV strains.

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