INTRATYPIC VARIATIONS AMONG THAI HERPES SIMPLEX VIRUS (HSV) ISOLATES DETERMINED BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

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Abstract. Whole genomic polymorphisms for 20 HSV-1 and 20 HSV-2 isolates from Thai patients were analyzed by means of Restriction Fragment Length Polymorphism (RFLP) analysis using 4 restriction endonucleases: *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI. Variations in cleavage sites among the HSV-1 and HSV-2 isolates were compared to the cleavage patterns of standard HSV-1 strain KOS and HSV-2 strain Baylor 186. Although 70% of HSV-1 isolates with *Bam*HI digestion, 50% with *Kpn*I, 75% with *Hin*dIII and 70% with *Eco*RI digestion were found to be similar to the standard HSV-1 (KOS) pattern, new *Bam*HI restriction sites were detected in some HSV-1 isolates. For HSV-2 isolates, 85% had the same pattern as the standard HSV-2 (Baylor 186) after digestion with *Bam*HI, *Hin*dIII, and *Eco*RI. No difference was observed with *Kpn*I digestion. When the patterns from the 4 enzymes were combined, HSV-1 isolates showed more divergence than the HSV-2 isolates. HSV-1 isolates found in both non-genital and genital lesions had more variety than the HSV-2 isolates. This suggests that intratypic variations in HSV-2 are fewer than in HSV-1.

INTRODUCTION

HSV-1 and HSV-2 belong to the subfamily Alphaherpesviridae, family Herpesviridae. They are enveloped double-stranded DNA virions which are comprised of 152 kilobase pairs (McGeoch *et al*, 1998) and have 50% homology with good matching (85%) of base pairs (Kieff *et al*, 1972). HSV-1 and 2 have been associated with different clinical syndromes (Roizman and Tognon, 1983). HSV-1 commonly causes infection above the waist, while HSV-2 usually infects the genital area (Hamelin *et al*, 1991). However either type can infect either site. Recent surveys have demonstrated a difference in the traditional distribution of the two types of viruses, suggesting that epidemiological patterns are changing (Coyle *et al*, 2003; Solomon *et al*, 2003). Several studies have examined genomic variation in both HSV-1 and HSV-2 by RFLP analysis (Buchman *et al*, 1978; Hamelin *et al* 1991; Sakaoka *et al*, 1994). The results have been used for various epidemiological applications, including identification of infectious routes in some clinical cases (Sakaoka *et al*, 1995). Such detailed analysis can yield information regarding the particular strains linked with more severe disease, greater transmissibility, increased frequency of disease, or with particular syndromes, such as herpes encephalitis (Reeves *et al*, 1981; Casas *et al*, 1996).

Genetic variations among HSV-2 Thai isolates using the RFLP method have been previously reported (Chantratita and Yoosook, 1990; Sirirungsi *et al*, 1995). However, no data are available regarding HSV-1 isolates. In this study, intratypic variations in 20 HSV-1 and 20 HSV-2 clinical isolates in Thai patients were determined and the discovery of a new restriction endonuclease pattern for HSV-1 was demonstrated.

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MATERIALS AND METHODS

Cell culture and viruses

African green monkey kidney (Vero cell) cells

were grown in M199 with Earle's salt (GIBCO BRL, USA) with 10% fetal bovine serum (GIBCO), 0.01 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 100 units/ml penicillin G and 100 µg/ml streptomycin.

The HSV isolates were from clinical specimens of unrelated Thai patients who visited King Chulalongkorn Memorial Hospital during the years 2000 to 2002. HSV-1 strain KOS and HSV-2 strain Baylor 186 were used as standard viruses. All the strains were propagated in Vero cells as previously described (Bhattarakosol *et al*, 1990). Typing of HSV isolates was done using PCR amplification of the conserved region in the DNA polymerase gene followed by *Bam*HI digestion as described previously (Johnson *et al*, 2000).

HSV genomic variation by RFLP

Twenty HSV-1 and 20 HSV-2 strains were obtained for the study. Fourteen HSV-1 isolates were from patients with herpes dermatitis and 6 isolates were from patients with genital herpes. Eighteen HSV-2 isolates were from patients with genital herpes and 2 isolates were from patients with herpes dermatitis. They were all grown in Vero cells for 8-10 passages before RFLP analysis. The restriction endonucleases *Bam*HI, *Kpn*I, *Hin*dIII and *Eco*RI were used for analysis. Viral DNA samples were extracted by a conventional phenol-chloroform extraction technique (Chantratita and Yoosook, 1990). Samples containing approximately 3-5 µg of viral DNA were digested with 20 to 30 units of each enzyme for 3 to 4 hours at 37ºC. The digested products were loaded onto 0.8% agarose gel (14x20 cm) for electrophoresis. The gel was electrophoresed at 60 volts for 15 hours with *Bam*HI, 17 hours with *Kpn*I, *Hin*dIII, and *Eco*RI, in TBE (0.1 M Tris base, 0.1 M boric acid, 2 mM EDTA, at a pH of 8.3). After electrophoresis, the gel was visualized under a UV transilluminator and photographed.

The RFLP patterns of each isolate after cutting with each restriction endonuclease were determined and compared to the standard HSV-1 (KOS) or HSV-2 (Baylor 186). The percentages of the frequencies of each pattern were determined.

RESULTS

Intratypic variation in the HSV-1 isolates

Of the 20 HSV-1 isolates, several patterns were apparent after digestion with *Bam*HI (5 patterns), *Kpn*I (3 patterns), *Hin*dIII (3 patterns), and *Eco*RI (4 patterns) (Fig 1). When the patterns of these isolates were compared to the

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

Enzyme	Pattern of variable restriction endonuclease cleavage sites	Total (20)	Frequency (%)
*Bam*HI	(B1) *Bam*HI, HSV-1 (KOS)	14	70
	(B2) Gain: T-U, loss: W	1	5
	(B3) Loss: N	1	5
	(B4) Gain: T-U, loss: S, W	3	15
	(B5) Gain: O-P, T-U, loss: N, O, S	1	5
*Kpn*I	(K1) *Kpn*I, HSV-1 (KOS)	10	50
	(K2) Gain: L-M, loss: P, R, Loss: U	8	40
	(K3) Loss:U	2	10
*Hin*dIII	(H1) *Hin*dIII, HSV-1 (KOS)	15	75
	(H2) Loss: M	4	20
	(H3) Loss: M, N	1	5
*Eco*RI	E1) *Eco*RI, HSV-1 (KOS)	14	70
	(E2) Loss: K	1	5
	(E3) Gain: J-K, L-M, loss: L	1	5
	(E4) Gain: L-M, loss: L	4	20

standard HSV-1 strain KOS (pattern 1: B1K1H1E1), the majority were found to have the same pattern as the standard HSV-1 (KOS), in 70, 50, 75 and 70% after digestion with *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI, respectively (Table 1). Pattern B4 with *Bam*HI digestion showed a gain of a fragment at site T-U due to the loss of fragments S and W at a higher frequency (15%) than in patterns B2, B3, and B5 (5%). *Kpn*I digestion gave a K2 pattern which showed the gain of fragment L-M due to the loss of fragments P and R, and the loss of fragment U, occurred in higher frequencies (40%) than the pattern K3 (10%). Twenty percent of pattern H2 of the *Hin*dIII cut showed a loss of fragment M, while only 5% of pattern H3 was determined. Pattern E4 of the *Eco*RI cut, which showed a gain of a fragment between sites L-M and a loss of fragment L had a higher frequency (20%) than patterns E2 (5%) and E3 (5%) (Table 1).

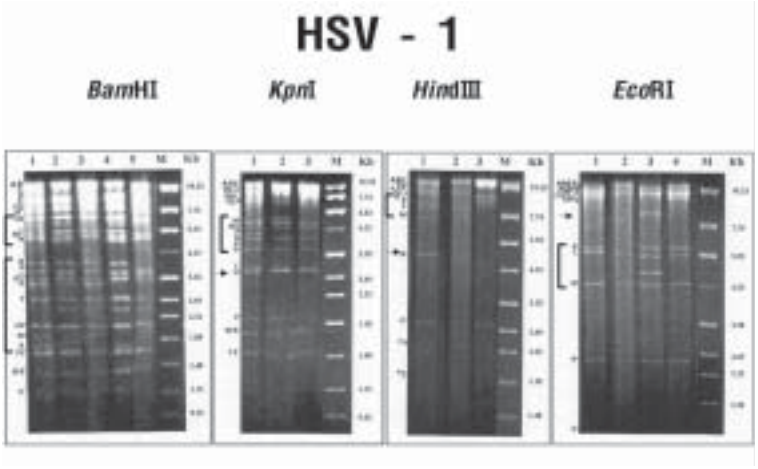

Fig 1–The distinct patterns of HSV-1 DNA after *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI digestion. Lane 1 HSV-1 (KOS) (Pattern 1), lane 2 (Pattern 2), lane 3 (Pattern 3), lane 4 (Pattern 4), lane 5 (Pattern 5), and DNA molecular weight marker IV 0.07-19.3 Kbp from Roche Diagnostics GmbH, Germany (M). The arrow and blanket indicate the location of genomic variation.

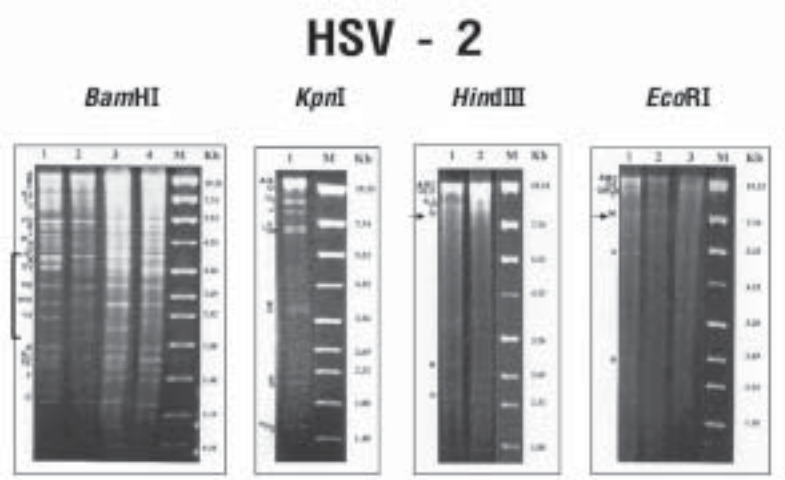

Fig 2–The distinct patterns of HSV-2 DNA after *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI digestion. Lane 1 HSV-2 (Baylor 186) (Pattern 1), lane 2 (Pattern 2), lane 3 (Pattern 3), lane 4 (Pattern 4), and DNA molecular weight marker IV 0.07-19.3 Kbp from Roche Diagnostics GmbH, Germany (M). The arrow and blanket indicated the location of genomic variation.

Intratypic variation among HSV-2 isolates

Similar observations were seen with the HSV-2 isolates (Fig 2 and Table 2). Of the 20 clinical isolates of HSV-2, 85% were similar to the standard HSV-2 Baylor 186 (pattern 1: B1K1H1E1), after digestion with *Bam*HI, *Hin*dIII, and *Eco*RI. No differences were found with *Kpn*I digestion. *Bam*HI digestion gave 4 cleavage patterns, of which patterns B2, B3 and B4 had equal frequencies (5%). The results of the *Hin*dIII cut demonstrated 2 cleavage patterns. Pattern H2 showing a loss of fragment M, occurred in 15%. *Eco*RI digestion was classified into 3 patterns, of which patterns E2 and E3 were found in 5% and 10%, respectively (Table 2).

Diversity of RFLP patterns in HSV isolates

The diversity of RFLP patterns was identified by a combination of the patterns from the 4 enzymes. The HSV-1 isolates after combination with 2 enzymes (*Bam*HI and *Kpn*I) were divided

into 7 patterns, after 3 enzymes (*Bam*HI, *Kpn*I, and *Hin*dIII) there were 9 patterns, and after 4 enzymes (*Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI) there were 10 patterns. For HSV-2 isolates, 4 patterns were identified after combining the patterns of 2 enzymes (*Bam*HI and *Kpn*I), 5 patterns with 3 enzymes (*Bam*HI, *Kpn*I, and *Hin*dIII) and 7 patterns with 4 enzymes (*Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI). The more enzymes used the more diversity was detected.

Genomic variation of HSV isolates from different sites of infection

Table 3 shows the frequency of RFLP patterns of HSV isolates from different sites of infection. Since the number of HSV-2 isolates from

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

Enzyme	Pattern of variable restriction endonuclease cleavage sites	Total (20)	Frequency (%)
*Bam*HI	(B1) *Bam*HI, HSV-2 (Baylor 186)	17	85
	(B2) Gain: Z-A', loss: YZ	1	5
	(B3) Gain: T-U, Z-A', loss YZ	1	5
	(B4) Gain: T-U	1	5
*Kpn*I	(K1) *Kpn*I, HSV-2 (Baylor 186)	20	100
*Hin*dIII	(H1) *Hin*dIII, HSV-2 (Baylor 186)	17	85
	(H2) Loss: M	3	15
*Eco*RI	(E1) *Eco*RI, HSV-2 (Baylor 186)	17	85
	(E2) Gain: L-M	1	5
	(E3) Loss: M	2	10

Table 3
The frequency of RFLP patterns among HSV-1 and HSV-2 isolates distributing to site of infection.

Site of infection	HSV type (No. of sample)	Pattern(s)	Frequency (%)
Herpes dermatitis	HSV-1 (14)	B1K1H1E1	5 (35.71)
		B1K1H2E1	2 (14.29)
		B1K2H1E1	2 (14.29)
		B1K3H1E1	1 (7.14)
		B3K3H2E4	1 (7.14)
		B4K2H1E2	1 (7.14)
		B4K2H1E4	1 (7.14)
		B5K2H1E4	1 (7.14)
	HSV-2 (2)	B1K1H1E1	2 (100)
Herpes genitalia	HSV-1 (6)	B1K1H1E1	2 (33.33)
		B1K1H3E1	1 (16.67)
		B1K2H1E1	1 (16.67)
		B2K2H1E3	1 (16.67)
		B4K2H1E4	1 (16.67)
	HSV-2 (18)	B1K1H1E1	12 (66.67)
		B1K1H1E3	1 (5.56)
		B1K1H2E2	1 (5.56)
		B1K1H2E3	1 (5.56)
		B2K1H1E1	1 (5.56)
		B3K1H1E1	1 (5.56)
		B4K1H2E1	1 (5.56)

non-genital lesions was only 2, intratypic variation could not be demonstrated. However, HSV-1 isolates from non-genital lesions showed great diversity (8 patterns from 14 isolates). For samples collected from genital lesions, 5 patterns with 6 HSV-1 isolates and 7 patterns with 18 HSV-2 isolates were detected.

DISCUSSION

RFLP analysis may help identify functional variation among HSV strains and has the potential to detect very small differences between closely related virus strains. HSV genomic variations are clearly demonstrated using the 4 restriction endonucleases, *Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI. The criteria used for classification was based on the gain or loss of restriction endonuclease cleavage site(s). In this study, RFLP patterns for HSV isolates using these 4 enzymes were compared to the RFLP patterns for standard HSV-1 (KOS) and HSV-2 (Baylor 186) (Figs 1 and 2, Tables 1 and 2). Our study indicates the variations were observed mostly in the S components of both HSV-1 and HSV-2, whereas few variations were found in the L component and at the S-L junction. This observation is similar to previously reports which revealed that HSV-1 DNA exhibited a small degree of heterogeneity (micro-heterogeneity) within the regions corresponding to the L component and the S-L junction (Wagner and Summers, 1978; Poffenberger *et al*, 1983). The restriction site may commonly be located in fragments containing reiterated sequences, but these sites do not affect the phenotypic characteristics of the virus (Roizman, 1979; Umene and Yoshida, 1993).

Intratypic variation of HSV-1 isolates in Thailand using RFLP has never been reported. In this study, the positions of suspected gene variation in the HSV-1 isolates observed with *Bam*HI RFLP were different from those previously reported (Hamelin *et al*, 1991; Al-Ahdal *et al*, 1992; Sakaoka *et al* 1994). The differences were observed at the T-U, W, S, O, O-P, and N cleavage sites (Table 1) while Al-Ahdal *et al* (1992) and Sakaoka *et al* (1994) reported at the A, D-H, W-K, B-L, C-K positions. However, similar variations with *Hin*dIII digestion were found (at the M-N position). The distinct patterns in these studies may imply variation in the HSV-strains in different geographic areas. Regarding HSV-2 observations, our data on the *Bam*HI restriction pattern (Table 2) showed a pattern (Gain T-U, Z-A') similar to that previously reported (Chantratita and Yoosook, 1990; Hamelin *et al*, 1991). However, the common *Bam*HI pattern, gain of a site at G, which Chantratita and Yoosook (1990) found in Thailand in 68.7%, was not detected in our study. With *Kpn*I digestion, no different profiles were observed. Our results are similar to the observations of Sirirungsi *et al* (1995). Although the presence of fusion fragments D-I with *Kpn*I cleavage have been commonly found in clinical isolates (Hamelin *et al*, 1991; Sakaoka *et al*, 1995) including in Thai isolates (79.5%, Chantratita and Yoosook, 1990), we did not find such a pattern.

The restriction endonuclease cleavage patterns of the 20 HSV-1 isolates cleaved by the 4 enzymes (*Bam*HI, *Kpn*I, *Hin*dIII, and *Eco*RI) were divided into 10 patterns. The pattern B1K1H1E1 (7/20, 35%) which is the same as the standard HSV-1 (KOS) pattern showed a combination frequency higher than in the other patterns (Table 3). All 20 HSV-2 clinical isolates had less diversity than the HSV-1 isolates. The pattern B1K1H1E1, which is similar to the standard HSV-2 (Baylor 186) pattern was the most common (14/20, 70%). The HSV-2 strains were not highly diversified compared to the HSV-1 strains (7 *vs* 10 patterns, Table 3). Our observations are similar to a study by Maitland *et al* (1982). This suggests that HSV-2 may have a lower capacity for genetic variation compared to HSV-1.

We attempted to find whether the diversity of HSV isolates was affected by the site of infection. HSV-1 normally produces lesions above the waist and HSV-2 is responsible for lesions below the waist. HSV-2 genital infection is frequently associated with multiple sexual partners, however, a change in sexual practices and an increase in oral-genital contact may have contributed to the increase number of patients with HSV-1 genital infections. HSV-1 was detected commonly in the genital area, corresponding to recent studies which report an increasing prevalence of genital HSV-1 infection (Smith *et al*, 1976; Solomon *et al*, 2003). While HSV-1 infec-

tion 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 (Kinghorn 1993, 1994). Therefore, HSV-2 variation was expected to be higher than HSV-1 variation. Our data did not support this theory. Five patterns were found in 6 HSV-1 isolates, but only 7 patterns were found in 18 HSV-2 isolates (Table 3). There were 3 patterns (B1K1H1E1, B1K2H1E1 and B4K2H1E4) of HSV-1 found both in non-genital and genital lesions (Table 3). From our data, HSV-1 isolates from non-genital lesions had more variation than HSV-2, whereas HSV-1 and HSV-2 isolates from genital lesions were not different. We suggest that the variation in HSV-1 occurrs in high frequencies in non-genital areas, and transfers this characteristic to genital lesions thereafter. In contrast to HSV-1, HSV-2 may have lower variation in non-genital areas.

Molecular epidemiological studies of HSV strains using analyses of RFLP can be classified into genotypes, while the number of enzymes can extend the diversity of the genetic variation. The prediction of biological properties within intratypic variations of restriction endonuclease sites can indicate the relationship between the HSV gene products and their functions. It remains to be elucidated whether genotypes of HSV are associated with biological properties and clinical manifestation (Roizman and Tognon, 1983; Umene and Kawana, 2000).

In conclusion, we demonstrated the similarity between the HSV-1 and HSV-2 isolates and their standard strains was high. We also discovered new *Bam*HI cleavage sites in HSV-1 isolates from Thai patients which have never been reported. The genetic variation was possibly due to viral replication in each tissue, which is affected by site of infection. The genomic variation of viruses changes from generation to generation. Thus, the restriction endonuclease patterns from HSV isolates studied at different periods of time may not be the same.

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