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Short Report

Comparative Molecular PCR-RFLP Study of Native Herpes Simplex Virus Type 1 (HSV-1) with KOS Strain

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ABSTRACT

Background: Recent research on several DNA fragments covering open reading frames (ORF) 1-37 shows a new genetic marker in ORF 6 which is specific for differentiating wild-type varicella-zoster virus (VZV) strains from Oka varicella vaccine strain. On the other hand, herpes simplex virus (HSV) genome analysis by restriction enzymes is used to differentiate types one and two of the virus and even strains of each type. Previous studies using PCR-sequencing technique have shown that the thymidine kinase (TK) gene of HSV-1 is polymorphic. **Methods:** In this study, TK gene and DNA binding protein (UL29) gene of HSV-1 were selected. Both genes were analyzed with restriction endonucleases in order to identify a genetic marker for differentiating native strains of HSV-1 from the foreign strain. Three isolates of HSV-1 as well as standard strain of KOS were propagated in Vero cells. Initially, a pair of specific primers for each gene was designed to amplify UL29 and TK genes of these isolates. Subsequently, PCR products of these genes were digested separately with five restriction enzymes and subjected to polyacrylamide gel electrophoresis. **Results:** Using PCR-RFLP (Restriction fragment length polymorphisms) technique, results indicate that the patterns of restriction endonuclease digestion of UL29 and TK genes of the three isolates show no differences when compared to KOS strain. **Conclusion:** The genotypes of Iranian isolates are the same as KOS genotype and both genotypes are derived from a common ancestor. Hence, it can be postulated that in the process of random population flow among Iran, Europe and USA, the original KOS strain infected the Iranian population at some point in time. *Iran. Biomed. J. 10 (3): 157-161, 2006*

Keywords: Herpes simplex virus type 1 (HSV-1), PCR, DNA binding protein (UL29), Thymidine Kinase (TK, UL23), Polymorphism

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a large enveloped DNA virus capable of replicating in a wide variety of cell types in culture. The genome has been fully sequenced and consists of two covalently linked components designated as L (long) and S (short). The genome encodes at least 74 genes [1].

HSV-1 is an infectious agent, widespread in human populations. The analysis of HSV strain genome by restriction endonuclease (RE) cleavage patterns has been used not only to distinguish between HSV-1 and -2 but also to differentiate strains within a HSV type [2].

To prevent immunocompromised children from developing severe varicella, a live attenuated varicella vaccine (Oka strain) has been developed by Takayama M. and Takayama N. [3] in Japan, and it is now used to immunize healthy children. Research on several DNA fragments covering ORF 1-37 shows a new genetic marker in ORF 6 which is specific for differentiating wild-type varicella-zoster virus (VZV) strains from Oka vaccine strain [3]. We used the same approach to compare Iranian HSV-1 strains with KOS standard strain to study genetic

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variation of native virus and identify a specific genetic marker to differentiate those strains. Restriction fragment length polymorphisms (RFLP) of amplified regions of DNA binding protein (UL29) and TK genes of HSV-1 were then compared.

UL29 gene of HSV-1 encodes a single-stranded DNA binding protein (ICP8) which is one of seven genes essential for DNA replicating *in vivo* [4, 5]. The HSV gene layout for UL was found to be very similar to the corresponding segment of the VZV genome [6]. Since recent research on ORF 1-37 indicates mutations in VZV strains [3], we used UL29 gene to find mutations or a genetic marker in HSV-1. ICP8 contains 1196 amino acids with a molecular weight of 128351 kDa [7]. TK gene of HSV-1 is able to phosphorylate a wide range of nucleoside analogues and is one of the key enzymes in the determination of susceptibility to acyclovir, which is widely used for the treatment of HSV infections.

RFLP is due to a gain or loss of an RE cleavage site. RFLP are stable and serve as physical markers of the HSV-1 genome in molecular, epidemiological and evolutionary studies [8]. This assay is relatively difficult and troublesome, as it requires the identification of numerous DNA fragments that range in size from a few hundred bp to over 10 kbp, which must be analyzed on a single gel. We considered using these two genes instead of whole genome for RFLP assay, as Nagamine *et al.* [9] have shown that analysis of the HSV-1 TK gene is comparable to RFLP analysis of the whole genome.

MATERIALS AND METHODS

Growth of cells and virus culture. Iranian virus isolates were obtained from Dr. Roostaee, Virology Department of Tarbiat Modares University (Tehran, Iran) [10]. For the production of large stocks of 3 Iranian HSV-1 virus isolates, Vero cells cultured as monolayers in DMEM with 5% FCS (Bahar Afshan Co., Tehran, Iran) were incubated at 37^{o}C and gassed at 5% Co_2. Working stocks of KOS strain were also prepared (0.01 PFU/cells). Confluent monolayers were rinsed twice with phosphate-buffered saline (PBS) containing trypsin (Bahar Afshan Co.) and EDTA (Merck, Germany) [1]. Harvested cells were either subcultured or kept in liquid nitrogen. Subsequently, HSV strains were propagated in these Vero cells. To evaluate the contamination of Iranian isolates, mock-infected cells were used.

DNA extraction. For DNA extraction, "Proteinase K-Phenol-Chloroform" procedure was chosen using a lysis buffer containing: Tris-HCl, 20 mM (pH 8); EDTA, 10 mM; SDS, %1; Proteinase K, 200 µg/ml, a solution containing: phenol-chloroform-isoamyl-alcohol (25:24:1), and Tris-EDTA solution containing: Tris-HCl, 10 mM (pH 8) and EDTA, 0.1 mM [11] .

Primer design, PCR and agarose gel electrophoresis. Using the computer software Generunner, a pair of specific primers for each gene was designed as follow:

UL29 gene primers. Forward Primer:

5'- AGAGGAATCACGGCCCGCCC -3'

Tm = 68^{o}C

Reverse Primer:

5'- TTATAATAGCGGCCACGCC -3'

Tm = 58^{o}C

UL23 (TK) gene primers. Forward Primer:

5'- CCTTCCGTGTTTCAGTTAGC -3'

Tm = 57.3^{o}C

Reverse Primer:

5'- GTGATATTACCGGAGACCTTC -3'

Tm = 57.9^{o}C

Accordingly, amplified region for UL29 gene and TK gene was determined to be 4071bp and 1599bp, respectively.

Fifty microliters of a reaction mixture containing 50-200 ng of template viral DNA, 0.2 µM of each primer, 1 µl of deoxynucleoside triphosphate, 0.4 µl of Taq DNA polymerase (Fermentase, Lithuania), 2 µl of $Mgcl_2$, 5 µl of PCR Buffer, was subjected to PCR. PCR conditions were five min at 94°C for one cycle, one min at 94°C, 75 s at 64.5°C (UL29) or 53.5°C (TK) and 3 min at 72°C for 35 cycle, and a final extension for 10 min at 72°C for one cycle.

Subsequently, PCR products were examined on %1 and agarose gel for UL29 gene and on %0.6 agarose gel for TK gene [12].

Restriction endonuclease digestion and polyacrylamide gel electrophoresis. PCR products of UL29 gene were digested separately with *Kpn*I, *Pst*I, *Pvu*II, *Dpn*I and *Hinf* I restriction endonucleases (Roche Diagnostic, Germany). Moreover, PCR products of TK gene were digested separately with *Hinf* I, *Dpn*I, *Apa*I, *Pst*I and *Alu*I restriction endonucleases (Roche Diagnostic, Germany). RFLP products were electrophoresed

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separately on polyacrylamide gels and stained with silver nitrate. The markers used were Smart Ladder (Eurogentec, Belgium) and 100 bp DNA Ladder Plus (Fermentase, Lithuania).

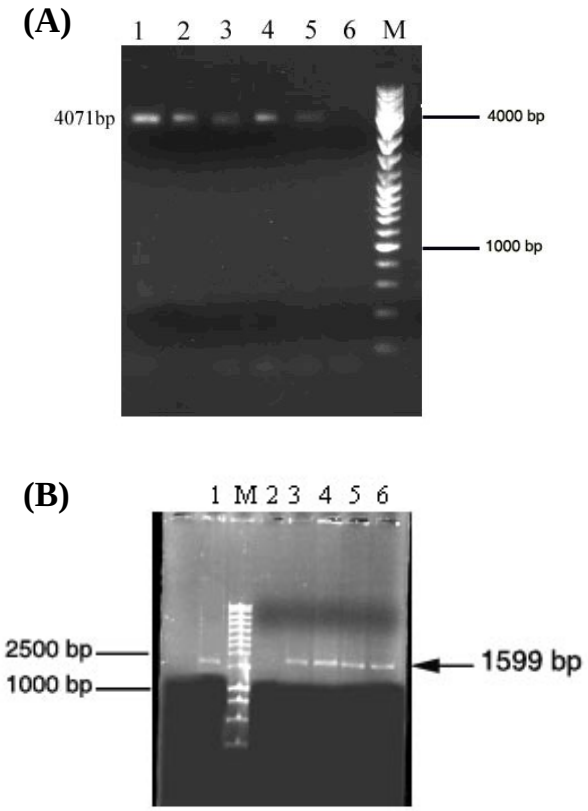

Fig. 1. PCR products of UL29 and TK genes of HSV-1. **(A)** PCR product of UL29 gene. Lane 1, Plasmid = positive control; Lane 2, KOS strain; Lanes 3, 4 and 5, Iranian isolates; M, 100 bp DNA ladder plus. **(B)** PCR product of TK gene. Lane 1, positive control; M, smart ladder marker; lane 2, negative control; lane 3, KOS strain; lanes 4, 5 and 6, Iranian isolates.

RESULTS

In order to obtain large amount of viruses, Vero cells were infected with KOS strain and incubated at 37°C for 24 hours until cytopathic effect (c.p.e) was developed. The Iranian strains developed c.p.e. on this cell line after 72 hours. To amplify UL29 and TK genes of HSV-1, DNA extraction was carried out and PCR products of UL29 and TK genes were regularly electrophoresed on %1 and %0.6 agarose gel, respectively (Fig. 1). As the Figure shows, designed primers for UL29 gene were able to amplify a 4071 bp region of HSV-1 genome and no additional bands were seen. Also, designed primers for TK gene were able to amplify a 1599-bp region of HSV-1 genome and no additional bands were observed.

In order to compare these two amplified regions in the Iranian isolates and KOS strain, each gene was digested separately using five restriction endonucleases. The cleavage site of each enzyme, number and length of fragments for each amplified region, were identified using Generunner software. Computer-generated results are shown in Table 1. Subsequently, the PCR products of each amplified gene from both the Iranian isolates and KOS strain were digested with five restriction endonucleases. Two restriction endonucleases were used for each gene and they are randomly shown in Figure 2.

The pattern of restriction fragments were similar, so probably the genotype of Iranian isolates are the same as KOS genotype and both genotypes may derive from a common ancestor. This was a first step molecular comparison for Iranian HSV-1 isolates and certainly it needs more isolates for better evaluation.

Table 1. Restriction enzyme, cleavage site of each enzyme, number and length of fragments for amplified region of UL29 and TK genes of HSV-1 using Generunner software.

Gene	Restriction enzyme	Number of fragments	Length of fragments	Cleavage site
UL29	KpnI	2	3481, 590	GGTAC/C
UL29	PstI	3	2123, 1482, 466	CTGCA/G
UL29	PvuII	5	1290, 1004, 958, 467, 352	CAG/CTG
UL29	DpnI	8	1015, 689, 539, 460, 336, 260, 168, 4	GA/TC
UL29	Hinf I	9	1008, 852, 639, 587, 362, 271, 217, 130, 5	G/ANTC
TK	Hinf I	2	1162, 437	G/ANTC
TK	DpnI	3	1132, 402, 61	GA/TC
TK	ApaI	4	1071, 264, 217, 44	GGGCC/C
TK	PstI	4	840, 397, 237, 125	CTGCA/G
TK	AluI	5	752, 359, 337, 133, 18	AC/CT

TK, thymidine kinase; UL29, DNA binding protein

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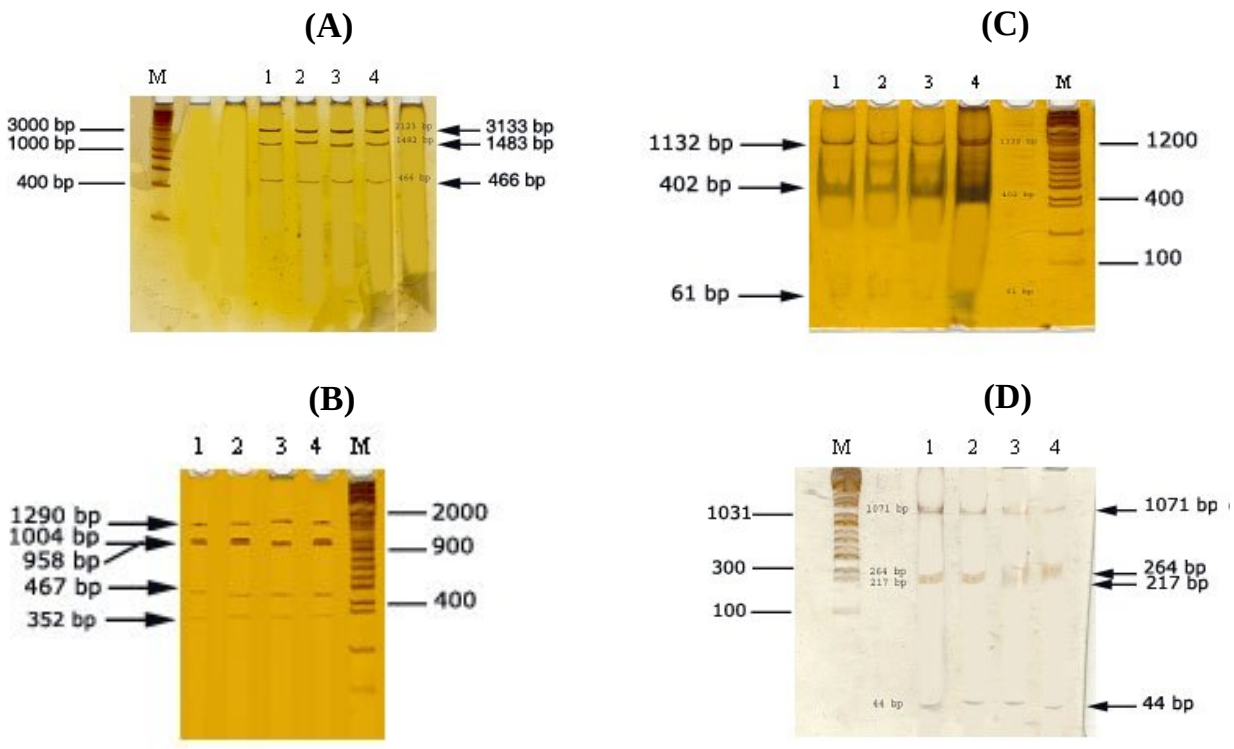

Fig. 2. Digestion of UL29 and TK amplified genes. **(A)** digestion of UL29 amplified gene with *Pst*I restriction enzyme. M, smart ladder marker; Lane 1, KOS strain; Lane 2, 3 and 4, Iranian isolates; **(B)** Digestion of UL29 amplified gene with *Pvu*II restriction enzyme. Lane 1, KOS strain; Lanes 2, 3 and 4, Iranian isolates; M, 100 bp DNA ladder plus. **(C)**, digestion of TK amplified gene with *Dpn*I restriction enzyme. Lane 1, 2 and 3, Iranian isolates; lane 4, KOS strain; M, 100 bp DNA ladder plus. **(D)** digestion of TK amplified gene with *Apa*I restriction enzyme. M, 100 bp DNA ladder plus; lane 1, KOS strain; lanes 2, 3 and 4, Iranian isolates.

DISCUSSION

Previous studies have examined genomic variation in HSV-1 species using RFLP analysis in various countries but not in Iran. We considered the comparison of the Iranian HSV-1 isolates with KOS standard strain to study the genomic variation of the Iranian strains. To produce DNA vaccines from the local HSV strains, investigations are under way and therefore, it is imperative that the identity of these strains be known. RFLP analysis of HSV-1 isolates in Japan [2] revealed the presence of two predominant genotypes of F1 and F35. Also, studies conducted in South Korea found that F1 genotype predominated in this country [13]. The KOS strain [14] used in this work is mostly prevalent in both Europe and U.S.A. and was obtained from Ville Juif Hospital in Paris and we try to find a predominant HSV-1 genotype in Iran. The HSV gene layout for UL was found to be very similar to that of the corresponding part of the VZV genome [6]. As recent research on ORF 1-37 shows mutations in VZV strains [3], we used UL29 gene to find mutations. Also, Nagamine *et al.* [9] have shown that the analysis of the HSV-1 TK gene is comparable to RFLP analysis of the whole genome so we proceeded to use TK gene instead of whole genome.

Molecular techniques, such as PCR [15], randomly amplified polymorphic DNA fingerprinting [16], DNA hybridization [17] and gene sequencing have been tried for identification. Each of these methods has their own limitations. PCR-RFLP of the gene is highly repeatable, cheaper and quicker than the methods cited above [18]. Although, DNA sequencing and analysis is accurate and authentic, it is costly, time consuming and not suitable for routine species identification studies. PCR-RFLP has been proven to be a practical, simple and rapid technique [18, 19].

In this study, we used enzymes which are more economical, available and are able to produce separate fragments. This was the first molecular comparison of Iranian HSV-1 isolates and for more

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set of enzymes and more Iranian isolates can be used for a better and more isolates are needed to study.

After using 5 restriction endonucleases for the digestion of PCR products of each UL29 and TK genes for three different Iranian isolates and KOS standard strain, we found the pattern of restriction fragments to be similar. So, probably the genotype of Iranian isolates is the same as KOS genotype and both genotypes may derived from a common ancestor. In order to be confident that Iranian isolates were not contaminated with KOS strain, two mock-infected flasks of cultured cells were kept among flasks of infected cells to evaluate contamination. Besides, if flasks of Iranian isolates were contaminated with KOS strain, it must show cytopathic effect after 24 hours instead of 72 hours but this was not observed due to the low multiplicity of Iranian isolates.

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