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Comparison of two methods of PCR followed by enzymatic restriction digestion for detection and typing of herpes simplex viruses isolated from patients with mucocutaneous or cutaneous lesions

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ABSTRACT. A multitude of different polymerase chain reactions (PCRs) have been described for detection and typing of Herpes simplex virus (HSV). This paper compares two PCRs coupled to enzymatic restriction (PCR/RFLP) to detect and type HSV. A primers set was designed to amplify a HSV DNA fragment from UL30 and UL15 genes. Typing was done by restriction of the UL30 and UL15 amplicons with Ava II and Hpa II enzymes, respectively. This strategy was tested with two reference strains (HSV-1 McIntyre, and HSV-2 G), and 47 clinical HSV isolates. Both PCRs produced the expected amplicons (a 492 bp UL30, and 305 bp UL15). The restriction of both amplicons clearly differentiated HSV-1 from HSV-2, and produced equal results. Thirty one (66%) of the isolates were identified as HSV-1, and the other 16 (34%), as HSV-2. Most of the HSV-1 isolates (27/31) were from orofacial and thoracic lesions; and also, one half of the HSV-2 isolates (8/16) were from the same anatomical regions. Our results showed that either of the two PCR/RFLP could be used to detect and type HSV. Furthermore, our results of the anatomical site of HSV-1 and HSV-2 infections are consistent with previous reports which have shown changes in the classical anatomical localization of herpesvirus infections.

Key words: Polymerase chain reaction, enzyme restriction, herpes simplex viruses.

INTRODUCTION

Herpes simplex virus (HSV) infections are among the most common diseases affecting humans.⁴⁰ The common infections include mucocutaneous herpetic ulcers, encephali-

RESUMEN. Se han descrito una gran variedad de reacciones de amplificación en cadena de la polimerasa (PCR) para detectar y tipificar a los virus herpes simplex (HSV). En este trabajo se comparan dos reacciones de PCR acopladas a la restricción enzimática (PCR/RFLP) para detectar y tipificar a los HSV. Se diseñaron dos pares de iniciadores, unos para amplificar una región del gen UL30 y otros para una región del gen UL15 de los HSV. La tipificación se hizo por restricción de los amplicones de UL30 y UL15, con la enzimas AvaII y Hpa II, respectivamente. Esta estrategia se probó con dos cepas de referencia de HSV (la cepa MacIntyre de HSV-1 y la cepa G de HSV-2), así como con 47 cepas de HSV aisladas de pacientes. Las dos PCRs produjeron los amplicones esperados (uno de 492 pb para UL30 y de 305 pb para UL15). La restricción de esos dos amplicones claramente diferenció a HSV-1 de HSV-2 y con ambos genes se obtuvieron los mismos resultados. Treinta y uno (66%) de los aislados virales fueron identificados como HSV-1 y los otros 16 (34%) como HSV-2. La mayoría de los aislamientos de HSV-1 (27/31) se obtuvieron de muestras de lesiones orofaciales y torácicas; pero también la mitad de los aislamientos de HSV-2 (8/16) fueron de las mismas regiones anatómicas. Nuestros resultados mostraron que cualquiera de las dos PCR/RFLP se puede utilizar para detectar y tipificar HSV. E inclusive los resultados del sitio anatómico del aislamiento de HSV-1 y HSV-2 coinciden con los trabajos de otros autores que han demostrado el cambio en cuanto al sitio clásico de localización de las infecciones producidas por los herpesvirus.

Palabras clave: PCR, restricción enzimática, virus herpes simplex.

tis, visceral infections,^{2,5,13,36} and ocular diseases.^{1,12,43} Generally, HSV diagnosis is based on standard laboratory methods such as the immunological detection of viral antigens in direct smears, and virus isolation from clinical specimens.¹ In recent years polymerase chain reaction (PCR) has proven to be a highly sensitive and specific method for diagnosis of HSV. Therefore, PCR has made the diagnosis of HSV infections more rapid and accurate.^{5,22,34,42} A multitude of different primer pairs have been used for the detection of HSV.^{4,10,15,19,24} In general, several strategies consider the amplification of independent PCRs to detect single viruses. On the other hand, human herpesvirus DNA polymerase genes contain a highly conserved region, and degenerate primers have been described to amplify several species of human and animal herpesvirus.³⁷ Other conserved regions of

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the DNA polymerase gene have also been used to detect the major human herpesviruses HSV-1, HSV-2, CMV, VZV, and HHV-6 in cerebrospinal fluid.²⁷ Finally, several types of PCRs have been described, such as the multiplex nested-PCR for the specific detection of five human herpesviruses using as a target the viral DNA polymerase genes.^{30,35} Real-time PCR has been compared to nested PCR for detection of HSV-1, HSV-2 and VZV, however no significant difference in sensitivity was found between real-time PCR and nested PCR.³² Multiplex PCR for HSV-1 and HSV-2,²⁶ duplex quantitative real-time PCR for HSV-1 and HSV-2,¹⁷ real-time duplex PCR using the gD gene as a target³⁹ and also a PCR coupled to restriction enzyme digestion, have been described by using the viral DNA pol gene and digestion with Hinf I and Alu I,³¹ Hae III and Taq I,²³ Ava II.²⁹ Following this approach, we designed and compared a primers set for a specific region of UL30 (DNA polymerase) and UL15 (to encoding for an essential γ protein required for packing of viral DNA) genes, followed by enzymatic restriction of the amplicons to identify and type several HSV isolates obtained from ambulatory patients with different anatomical localization of mucocutaneous or cutaneous lesions.

MATERIAL AND METHODS

Cells and viruses

African green monkey kidney cells (Vero) were obtained from ATTC CCL 81 (Rockville, MD). Cells were propagated and maintained in 199 Medium supplemented with 6% newborn calf serum and 0.11% sodium bicarbonate.

McIntyre HSV-1 (ATTC VR-539), and HSV-2, G (ATTC VR-540) reference strains were propagated and the titers of the viruses were determined by TCID₅₀ and PFU assays in Vero cells.

VIRAL ISOLATES

A sample from dermal or genital lesions was obtained from 224 ambulatory patients attending the Medical Service for Transfer Factor treatment¹⁶ at the Immunology Department of Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, during the period from September 1999 to July 2001. The sample was collected in 500 μ l of newborn calf serum containing 100 IU/ml of penicillin G, 0.25 μ g/ml of amphotericin B and 0.1 mg/ml of streptomycin sulfate. Previously each patient agreed to sign an informed consent form to obtain a sample from his or her mucocutaneous or cutaneous lesion.

Vero cells grown in four-well plastic dishes were infected in duplicate with 50 μ l of the patient sample. When cells showed more than 50% of cytopathic effect (CPE), the virus

was harvested after one freeze-thaw cycle. Cell debris was removed by centrifugation and the supernatant was placed in Eppendorf tubes and frozen at -70°C until used.

REFERENCE VIRUS PURIFICATION

Vero cells grown in 40 cm² flasks were infected with HSV-1 or HSV-2 reference strains at 0.1 multiplicity of infection (MOI), when cells showed more than 90% CPE, virus was harvested by one freeze-thaw cycle. Supernatant was separated by centrifugation at 800 xg. The virus was precipitated with 7% polyethylene glycol-6,000 (PEG) and 2.3% NaCl, collected by centrifugation at 12,000 x g, and diluted in 1 ml of TES buffer.²⁵

VIRAL DNA EXTRACTION

One volume of each purified reference virus was mixed with one tenth volume of lysis buffer (10 mM Tris pH 8, 1 mM EDTA, 0.5% SDS, 100 μ g/proteinase K), 100 μ g/RNase, and viral DNA was purified by DNAzol[®] reagent (GIBCO BRL), following the manufacturer instructions. Viral DNA from the patients' isolates were obtained through the same procedure.

PCR

The amplification protocols were standardized with the viral DNAs obtained from the reference strains by using the QIAGEN[®] Taq PCR Core kit. To amplify a 492 bp fragment from UL 30 gene, the following oligonucleotide primer sequences were used, sense 5'CAGTACGGC-CCCGAGTTCGTGA3', and antisense 5'GTAGATGGT-GCGGGTGATGTT3'; and for a 305 bp fragment from the UL15 gene: 5'TGCGGTTTCAGGAGTGC GCG3', and 5'GAGGAGCACGGCGGCCAGAA3'. The protocol for the UL30 fragment was as follows: one cycle at 94°C for 5 minutes, followed by 25 cycles at 94°C for 40 seconds; 60°C for 40 seconds; 72°C for 40 seconds, and one cycle at 72°C for 7 minutes. For the UL15 amplicon we used one cycle at 94°C for 5 minutes, followed by 25 cycles at 94°C for 40 seconds, 62°C for 40 seconds, and 72°C for 40 seconds, and a final cycle at 72°C for 7 minutes. The amplicons were analyzed by 1% agarose gel electrophoresis at 100 Volts for 30 minutes, and stained with ethidium bromide.

ENZYMATIC RESTRICTION

Using the Webcutter2 program (www.firstmarket.com/cutter/cut2.html), we selected the Ava II and Hpa II enzymes for the specific restriction of UL30 and UL15 ampli-

icons, respectively, to differentiate HSV-1 from HSV-2. A sample of 17 µl of each PCR was restricted using 1 IU of Ava II (GIBCO), or 1 IU of HpaII (GIBCO), according to manufacturer's instructions. The digested products were analyzed by 12% acrylamide gel electrophoresis at 80 volts, and stained with ethidium bromide.

RESULTS AND DISCUSSION

HSV Isolation

HSV is a very important human pathogen, and may cause a wide range of signs and symptoms, varying from trivial mucocutaneous lesions, ocular lesions such as uveitis and keratitis, as well as life-threatening infections in the central nervous system. Due to its clinical importance, several methods have been developed to diagnose HSV infections.^{2,15,22}

Viral culture has traditionally been regarded as the gold standard for HSV diagnosis.^{6,14,17,26,32,38} Our laboratory received 224 samples from non-hospitalized patients who suffered some type of mucocutaneous or cutaneous lesions, and these samples were used for viral isolation in Vero cells. A total of 47 (21%) samples showed a cytopathic effect characterized by syncytia formation, highly suggestive of herpes simplex virus. The isolated viruses were tested to determine their sensitivity to acyclovir (ACV), which is the election drug for herpes virus infections.^{8,9} Since the 47 viral isolates were obtained from immunocompetent patients, it was expected that all to would be highly sensitive to 1 µM acyclovir, to which we in fact found (unpublished results). It is known that the prevalence of acyclovir resistant isolates has remained stable at less than 1% among immunocompetent hosts,^{3,7,9,18,28} and this low prevalence of ACV resistant isolates is not altered during the one or several cycles of replication that occurs during the recovery of HSV in the laboratory, in absence of selection pressure.³³

To identify and type the viral isolates we selected a PCR reaction coupled to an enzymatic restriction, and used the set of primers for the DNA polymerase (UL30) gene described by Podzorki et al., (2000), and the primers for the UL15 gene described by Baron et al., (1996). First we attempted the amplification of the DNAs obtained from the reference strains HSV-1 McIntyre and HSV-2 G. Neither of the two strains produced an amplicon even after several modifications of reaction conditions. Therefore, we aligned the sequences of these two sets of primers with the HSV DNA sequences obtained from GenBank (NC_001806 and X14112 for HSV-1; and NC_001798, and Z86099, for HSV-2), and no specific alignments were found either with the UL30 or the UL15 genes from HSV-1 and HSV-2. For these reasons, we designed a new set of primers to amplify

a highly conserved segment of the UL30 (DNA pol) and UL15 (role in DNA packaging) HSV genes.

DETECTION OF HSV BY PCR

As expected from the *in silico* analysis performed with the HSV UL30 gen, a fragment of 492 bp was amplified from both HSV-1 and HSV-2 reference strain (Fig. 1, lane 2 and 4), from the position 1,366 to 1,857 in HSV-1, and 1,369 to 1,860 in HSV-2. Also, from the UL 15 gene both strains amplified a fragment of 305 bp (Fig. 2, lanes 2 and 4), from position 359 to 663 in HSV-1, and 576 to 880 in HSV-2. Similar amplified fragments were obtained for the UL30 and UL15 genes using the DNA from all 47 viral isolates. Figures 1 and 2, lanes 6, 8, 10 and 12 show four representative HSV amplicons from the viral isolates. No amplification products were obtained with other viral DNA such as adenovirus (data not shown).

TYPING OF HSV-1, AND HSV-2

The Ava II enzymatic digestion of the HSV-1 UL 30 amplicon showed the expected three fragments of 207, 198 and 87 bp; while HSV-2 had only two fragments, one of 405 bp and the other of 87 bp (Fig. 1, lanes 3 and 5, respectively). These restriction patterns clearly differentiated between HSV-1 and HSV-2, since a RFLP of three fragments was obtained for HSV-1, and a two fragment pattern for HSV-2.

On the other hand, the UL15 amplicons digested with Hpa II, differentiated HSV-1 from HSV-2. Restriction of the HSV-1 UL15 amplicon produced four fragments, 21, 72, 78 and 134 bp, while HSV-2 showed three fragments of 21, 134, and 150 bp. However in a small acrylamide gel the smallest fragments ran off the gel; and even though both viruses showed a two band pattern, the size and band position were different (72-78 and 134 bp for HSV-1; and 134 and 150 bp for HSV-2). Figure 2, lanes 3, and 5, respectively.

TYPING OF THE ISOLATED HSV

The enzymatic restriction coupled to PCR from UL30 and UL 15 HSV genes clearly showed that both genes could differentiate HSV-1 from HSV-2. Therefore, we tested our two PCR/RFLP systems to type the 47 HSV isolates obtained from mucocutaneous or cutaneous lesions of non-hospitalized patients, 20 of these were isolated from samples of the orofacial region, 7 from genital region, 15 from thoracic lesions, and 5 without any information about the anatomical site of infection.

Restriction of the UL15 and UL30 amplicons obtained from the 47 viral isolates produced same results with both

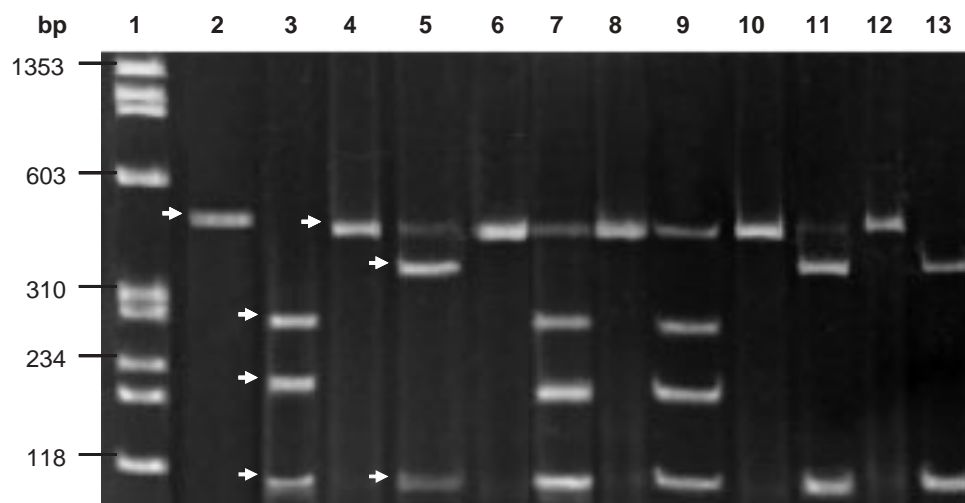


Figure 1. Detection and typing of HSV by PCR/RFLP using the UL30 gene and *Ava II* restriction. Lane 1) DNA molecular weight marker ϕ x174/*Hind III*, lane 2) HSV-1 MacIntyre strain, lane 3) HSV-1 MacIntyre strain/digested with *Ava II*, lane 4) HSV-2, G strain, lane 5) HSV-2, G strain/digested with *Ava II*. Lanes 6, 8, 10 & 12) viral isolates No. 1, 127, 138 & 173, respectively, lanes 7, 9, 11, & 13) viral isolates No. 1, 127, 138 & 173 digested with *Ava II*. Viral isolates No. 1 and No. 127 showed the RFLP of HSV-1, and the viral isolates No. 138 and No. 173, the HSV-2 pattern. The arrows indicate the specific restriction fragments to identify HSV-1 or HSV-2.

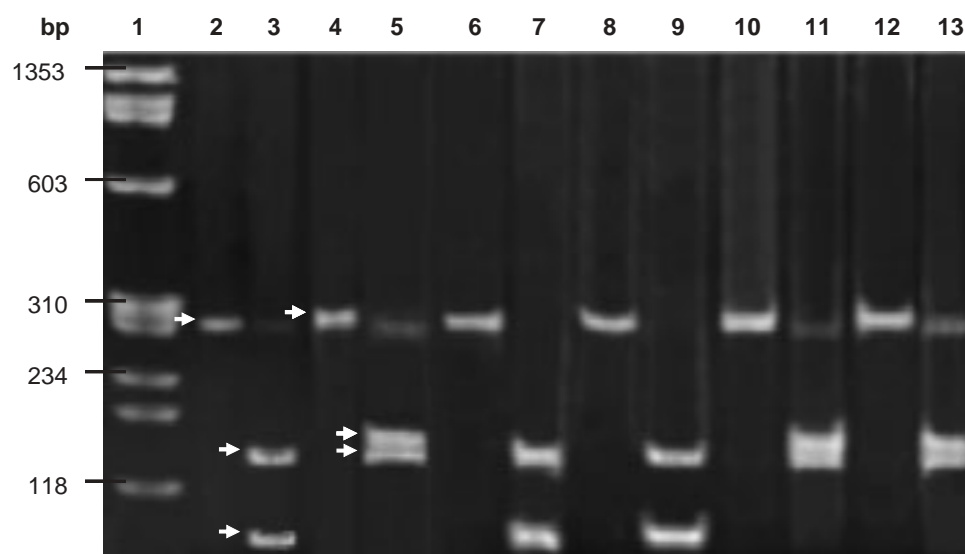


Figure 2. Detection and typing of HSV by PCR/RFLP using the UL15 gene and *Hpa II* restriction. Lane 1) DNA molecular weight marker ϕ x174/*Hind III*, lane 2) HSV-1 MacIntyre strain, lane 3) HSV-1 MacIntyre strain/digested with *Hpa II*, lane 4) HSV-2, G strain, lane 5) HSV-2, G strain/digested with *Hpa II*. Lanes 6, 8, 10 & 12) viral isolates No. 1, 127, 138 & 173, respectively, lanes 7, 9, 11, & 13) viral isolates No. 1, 127, 138 & 173 digested with *Hpa II*. Viral isolates No. 1 and No. 127 showed the RFLP of HSV-1, and the viral isolates No. 138 and No. 173, the HSV-2 pattern. The arrows indicate the specific restriction fragments to identify HSV-1 or HSV-2.

amplicons. And 31 (66%) of the 47 HSV isolates were identified as HSV-1 and 16 (34%) as HSV-2. Figures 1 and 2, lanes 7, 9, 11, and 13 show the same viral isolates, two of them typed as HSV-1 (lane 7, viral isolate No. 1 and lane 9, viral isolate No. 127), and the other two as HSV-2 (lane 11, viral isolate No. 138 and lane 13, viral isolate No. 173).

From the 20 viral isolates obtained from orofacial lesions, 16 (80%) were typed as HSV-1. The other four viral isolates produced the digestion pattern of HSV-2. The viral isolates obtained from thoracic lesions showed that 11 of the 15 samples produced the digestion pattern of HSV-1, and the other four, the HSV-2 pattern; while five of seven viral isolates obtained from genital lesions were identified as HSV-2, and the other two as HSV-1 (Table 1).

In summary we found that 87% (27/31) of the HSV-1 isolates were obtained from orofacial and thoracic lesions. However, half of the HSV-2 isolates were also isolated from similar anatomical regions (orofacial and thoracic lesions). Furthermore, two of the seven isolates from genital lesions were identified as HSV-1. These results showed that HSV-1 and HSV-2 were isolated from lesions found in anatomical regions other than their typical anatomical localization. HSV-1 has been associated with orofacial infections and HSV-2 with genital infections. Our results are in substantial agreement with the change in HSV-1 and HSV-2 classical tropism, since, in recent years, several studies from industrialized countries all around the world have described that HSV-2 is as common as HSV-1 in extragenital infections and HSV-1 is involved in genital infections.^{6,11,20,21,38,41}

Table 1. Anatomical distribution of herpes simplex viruses type 1 and 2 (HSV-1, HSV-2) isolated from samples of non-hospitalized patients with mucocutaneous or cutaneous lesions.

Anatomical site for sampling	Identified virus*				Number of total isolates
	HSV-1		HSV-2		
	UL30 (DNA pol)	UL15 (packing protein)	UL30 (DNA pol)	UL15 (packing protein)	
Orofacial	80% (16/20)	80% (16/20)	20% (4/20)	20% (4/20)	42% (20/47)
Genital	29% (2/7)	29% (2/7)	71% (5/7)	71% (5/7)	15% (7/47)
Thoracic	73% (11/15)	73% (11/15)	27% (4/15)	27% (4/15)	32% (15/47)
Unknown	40% (2/5)	40% (2/5)	60% (3/5)	60% (3/5)	11% (5/47)
Total	31 (66%)		16 (34%)		47 (100%)

* HSV was detected by PCR using the UL30 and UL15 primers, and typified by enzymatic restriction of the amplicons.

This paper has shown that herpesviruses isolated from ambulatory patients with cutaneous or mucocutaneous lesions could be typed and differentiated by a PCR/RFLP system using as a target for the DNA amplification a highly conserved region of UL30 or UL15 genes. The PCR/RFLP procedure we described either for UL30 or UL15 genes is a simple and fast method to identify and type HSV, since only a pair of primers is required to obtain the amplicon for both types of HSV, and by a single restriction reaction is possible to distinguish HSV-1 from HSV-2. This approach helped us confirm that the anatomical localization of HSV-1 and HSV-2 lesions is wider than it was originally established. The primers designed could be very useful for detection and typing of herpes simplex viruses directly from the patient's samples.

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