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Localization of Herpes Simplex Virus Type 1 DNA in Latently Infected BALB/c Mice Neurons Using *in situ* Polymerase Chain Reaction

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ABSTRACT

Background: Herpes simplex virus type-1 (HSV-1) establishes a lifelong latent infection in neurons following primary infection. The existence of latent HSV-1 DNA in the trigeminal ganglia of infected BALB/c mice was examined using a direct *in situ* PCR technique, based on Digoxigenin-11-dUTP detection system with anti-digoxigenin-peroxidase and 3,3'-diaminobenzidine (DAB) substrate. **Methods:** Eight-week-old male BALB/c mice were inoculated via the eye by 10^4 plaque forming unit of wild type Iranian isolates of HSV-1. After establishment of latency, trigeminal ganglia were removed and examined using *in situ* PCR to detect HSV-1 genome. Finally, the results of *in situ* PCR were verified by a two-round PCR method, using amplification cocktail of *in situ* reaction, as a template for a conventional gel base PCR. **Results and Conclusion**: The results suggest that a direct *in situ* PCR method using a peroxidase and DAB detection system is a useful means for detection of latent HSV-1 DNA in the latently infected ganglia. *Iran. Biomed. J. 14 (3): 83-88, 2010*

Keywords: Herpes simplex virus-1, Latency, *In situ* PCR, two-round PCR, *Trigeminal ganglia*

INTRODUCTION

HSV-1 is a neurotropic DNA virus that infects a broad range of cells. This virus causes several numbers of important human diseases including gingivostomatitis, pharyngitis, keratitis, and encephalitis [1]. One of the defining aspects of HSV-1 life cycle is the ability of the virus to establish a whole life latent state in neurons of the sensory ganglia (particularly trigeminal ganglia) after primary infection. Latency is characterized by the long-term persistence of viral DNA in latently infected neurons, and lack of replicating virus. It is shown that in latency, the HSV genome persists in the nucleus in a circular or concatemeric form [2]. The only viral transcripts in the latent cells that are abundantly expressed are the latency-associated transcripts (LAT), which are not abundant during productive infection [3, 4]. Periodic reactivation of the latent virus leads to recurrent infections in certain individuals.

PCR is a powerful tool for the amplification of the trace amounts of nucleic acids, and has rapidly become an essential analytical tool for virtually all aspects of biological research in experimental biology and medicine. In this technique by using a minute amount of extracted DNA or RNA, one can enlarge the amount of the sequence of interest, which can be analyzed, cloned, sequenced, and quantified [5]. However, until recent years, performing PCR in individual cells seemed unlikely. A few years after emergence of the PCR method, some researchers have described *in situ* PCR as a new procedure, allowing the amplification and detection of nucleic acid sequences in cultured cells or in the cells of wax-embedded tissue section [6].

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In this study, the presence of HSV-1 DNA in trigeminal ganglia of latently infected mice was detected by a direct *in situ* PCR technique, based on digoxigenin-11-dUTP detection system with anti-Digoxigenin-peroxidase and 3,3'-Diaminobenzidine (DAB) substrate. The results of *in situ* PCR were confirmed by a two-round PCR method.

MATERIAL AND METHODS

Specimens. Trigeminal ganglia containing latent HSV-1 genome were obtained from latently infected BALB/c mice. Briefly, the total numbers of 32 eight-week-old male BALB/c mice were obtained from the Pasteur Institute of Iran (Karaj, Iran). Animals were handled in accordance with the Animal Care and Use Protocol of Tarbiat Modares University (Tehran). The mice were anesthetized by an intraperitoneal injection of 100 mg/kg ketamine (Parke-Davis, Pontypool, UK) and 10 mg/kg xylazine (Bayer, Bury St, Edmunds, UK); each cornea scratched 10 times with a 26-gage needle. Ten mice (mock-inoculated) were received DMEM medium (Gibco BRL, Gaithersburg, MD, USA) containing fetal calf serum and 22 mice (test group) were received 3 µl medium containing 10^4 plaque forming unit of wild HSV-1 (Iranian isolate) on each cornea. Thirty days after infection, at a time when latency has been established, those mice with latent infection of trigeminal ganglia were screened. One ganglion of each mouse was co-cultivated with permissive Vero cells. First, the Vero cells were examined for the signs of cytopathic effect, within 14 days. Second, the existence of HSV-1 genome in the infected Vero cells was confirmed using PCR (data not shown). Third, the establishment of latency has been confirmed in 19 out of 22 mice in the test group using above methods. Finally, the other ganglia of the mice were subjected to *in situ* PCR.

Conventional polymerase chain reaction. Two sets of primers were used in this study: the first set (5' TACCCGAGCCGATGACTTAC 3' and 5' GCGCTTGTCATTACCACCGC 3') amplifies a 130-bp product from thymidine kinase gene of HSV-1 that has been described by Mitchell *et al.* [7]. But, the second set (5' TTGCTGGAAAAGATGCTGTG 3' and 5' TTCAGAGACAGCTTGCAGGA 3') yields a 203-bp product in mice constant region of immunoglobulin κ light chain that used as an internal control, which have been designed by Gene Runner, primer design program (version 3.05, Hastings Software Inc., Hastings, NY) using mouse genome sequences in NCBI (NC_000072). The annealing temperatures of these two sets were optimized in conventional PCR procedure, before moving the conditions to *in situ* PCR amplification. Amplifications reactions were performed in 25-µl volumes containing extracted DNA genome, 2.5 µl of 1X PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl, 0.01% gelatin), 1.5 mM $MgCl_2$, 200 µM each deoxyribonucleotide triphosphates, 2 U of Taq DNA polymerase (MBI Fermentas, Hanover, MD) and 0.5 µM each primer. The amplification profile consisted of a single cycle of initial denaturation at 94°C for 4 min followed by 34 cycles each consisting of denaturation at 94°C for 35 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The amplified products were analyzed by gel electrophoresis on 2% agarose gel containing 0.5 µg/ml ethidium bromide and visualized by UV light.

In situ PCR. The *in situ* PCR assay was carried out as described by Bagasra and Hansen [8]. Trigeminal ganglia were removed, washed quickly in 1× PBS and placed in 4% paraformaldehyde for 20 hour and then paraffin blocks were prepared of them as described previously [9]. Five-µm sections were prepared by a microtome apparatus, using a new disposable microtome blade for each paraffin block. At least five sections of one ganglion were placed on a slide that was previously coated with 3-aminopropyltriethoxysilane (Sigma, USA). The coated slides containing adherent tissue were deparaffinized by incubation in an oven at 75°C for 1 h and subsequently, they were dipped twice in xylene for 5 min, then in 100, 90, 70, and 50% ethanol, and finally in deionized H_2O for 5 min. After heating at 105°C for 90 s and refixing in 4% Paraformaldehyde for 4 hour, the slides were washed once in 3× PBS and then twice in 1× PBS. Endogenous peroxidase activity was removed by incubation in 0.3% hydrogen peroxide solution overnight. Slides were then digested with proteinase K (Roche Diagnostics, Mannheim, Germany, 15 µg/ml at 37°C) for 14 min, placed on a 95°C heat block for 2 min, and washed thoroughly in 1× PBS for 10 s and sterile DDW for 20 s. The slides were heated at 70°C before starting the reaction. The *in situ* PCR reaction mixture contained 10 mM Tris-Cl pH 8.3, 50 mM KCl, 0.001% gelatin, 0.02% BSA, 2.5 mM $MgCl_2$, 200 µM each of dATP, dGTP, dCTP, 190 µM dTTP, 10 µM digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany), 1.25 µM each primer, and 5 units of Taq DNA polymerase

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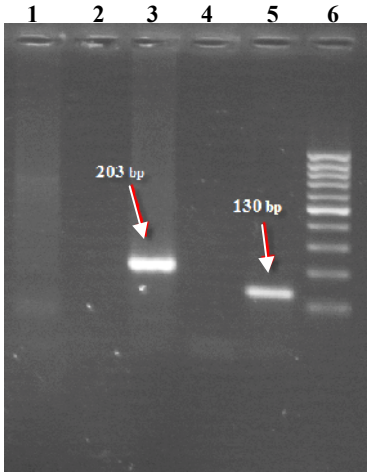

Fig. 1. PCR amplification result of desired thymidine kinase gene. Ethidium bromide stained 2% agarose gel electrophoresis shows a 130-bp product of herpes simplex virus thymidine kinase gene, and a 203-bp product of mice constant region of immunoglobulin κ light chain. Lane 6, the reaction in which BALB/c genome amplified with thymidine kinase-specific primers; lane 2, negative (water) control, and finally; lane 3, product of reaction in which BALB/c genome has been amplified with mouse specific primers; lane 4, the reaction in which HSV-1 genome amplified with mouse specific primers, shows that no amplification has occurred; lane 5, product of reaction in which HSV-1 genome (extracted from infected Vero cells) has been amplified with thymidine kinase-specific primer sets; Lane 6, DNA size marker.

(MBI Fermentas, Hanover, Germany). Amplification reactions were performed in 67-µl volumes and the mixture was added to tissues and covered with Frame-Seal incubation chamber (Bio-Rad, Hercules, CA, USA) as described by the manufacturer. I*n situ* PCR reactions were performed for 30 cycles of 45 s at 94°C, 35 s at 58°C, and 35 s at 72°C in a Master cycler gradient, which armed with a slide-holding block (Eppendorf, Hamburg, Germany).

Signal detection. Following amplification, the plastic cover slips were removed and the slides were placed on a heat-block at 92°C for 1 min, and they were washed two times with 2X SSC for 5 min. The *in situ* PCR products were visualized by using peroxidase-conjugated antidigoxigenin antibody (Roche Diagnostics, Mannheim, Germany, 1:100 dilution in 1X PBS) and DAB substrate (Sigma, USA) as described by the manufacturers. The reactions were observed by a light microscope, looking for brownish-red granular staining in the cells of tissues.

Two-round PCR. When the *in situ* PCR reaction was completed, the cover slips were removed and a 2-µl sample of amplification cocktail was used as template to two-round PCR amplification, in which the product of the first round of PCR served as template to second round reaction. This assay was also used as control to confirm *in situ* PCR results.

RESULTS

Specificity of primers. In the experiment shown in Figure 1, the primers used for detection of HSV-1 and mouse endogenous sequence were highly specific with no detectable artificial products that were deemed acceptable for use in an *in situ* PCR reaction (Fig. 1).

Localization of target nucleic acids by in situ PCR. The amplification of HSV-1 and BALB/c specific sequences within trigeminal ganglia cells of both mock and latently infected mice are shown in Figure 2. Through 12 separate experiments, the negative controls have consistently been free from any positive signal, but the latently infected ganglia always show positive signals (Fig. 2).

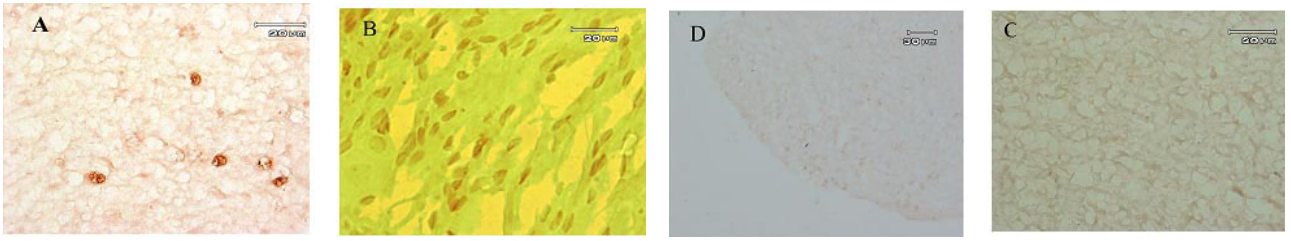

Fig. 2. *In situ* PCR labeling of HSV-1 and internal control in sections of latently infected ganglion. **(A)** The results of typical *in situ* PCR analysis of HSV-1 sequences in trigeminal ganglion from latently infected BALB/c mice, by HSV-1 specific primers. At least 4 positive cells are shown. **(B)** The amplification results of the BALB/c endogenous region, using BALB/c specific primers, within cells of trigeminal ganglia. More than 50% of the cells examined yield positive reactions. Because the gene is present in every cell in the section, this ability suggests the amplification system had a good efficiency. **(C)** Section of a ganglion from mock-infected (uninfected) mouse amplified with HSV-1 specific primers. No positive cells are evidenced in this picture. **(D)** The BALB/c specific primers have been omitted and no positive reactions are evidenced.

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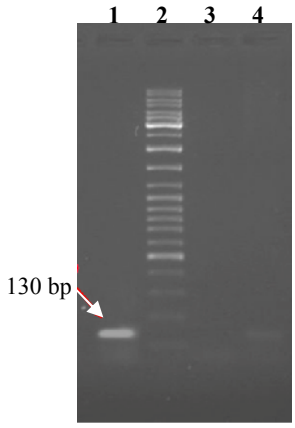

Fig. 3. Confirmation of *in situ* PCR results by two-round PCR. PCR products were loaded on 2% agarose. Lane 1 represents second round of the same reaction in which 1 µl of first round PCR product served as template in second round. Lane 2, DNA size marker; lane 3, negative control; lane 4 represents the first round of the reaction in which a 2-µl sample of *in situ* PCR cocktail for HSV-1 positive section was amplified with thymidine kinase primers during 30 cycles.

Confirmation of in situ PCR results by two-round PCR reaction. The total number of 6 positive and 6 negative samples was evaluated randomly using two-round PCR reaction. In all cases, the positive and negative results of *in situ* PCR were confirmed by this method (Fig. 3).

DISCUSSION

The diversity of pathogenesis presents a spectrum of challenges to a researcher who is required to utilize a wide variety of tools and techniques with those histological, immunological, virological and molecular natures. Within the larger context of pathogenesis, determination of viral latent infections is particularly provoking.

There are some standard virological and molecular methods used for detection of latent HSV-1 in latent ganglia including co-cultivation of the ganglia with permissive cell lines (e.g. CV-1 or Vero) [10, 11], PCR based techniques, and *in situ* techniques (*in situ* hybridization [ISH] and *in situ* PCR). Although development of PCR specific for HSV-1 has dramatically improved the accuracy of diagnosis of this virus in clinical samples, in this method, the cells are destroyed during the viral genome extraction process and consequently, it is impossible to know which type of the cells are infected in a particular tissue [12, 13]. Identification of the viral infected target cells or understanding the percentage of the viral infected cells is very important in some studies. Therefore, only ISH or *in situ* PCR can be used for such studies. ISH is the specific annealing of labeled probes to the target sequence in fixed tissue or cells, followed by visualization of the location [14]. This technique has proven to be valuable to localize cellular and also viral DNA or RNA nucleic acid sequences in tissue sections or individual cells. Unfortunately, the sensitivity of this procedure is limited when the small amounts of target have to be detected [8, 15, 16]. This is the most sensitive ISH protocols that need 50 to 100 copies of the target molecule per cell for the signal to be distinguished [6, 8]. Most DNA targets of the cells and also many latent viral genomes do not exist in that high titer. Therefore, the ISH technique becomes insensitive in case of some occult or latent viral infections [17]. With HSV-1, the number of viral genomes per latently infected cell has been calculated to be in the range of 10 to 100 copies [18, 19]. Hence, those groups of cells in a ganglion that contains few numbers of latent viral genomes cannot be detected by ISH method. Although the LAT accumulates to a high copy number per latent cells that can be recognized by ISH [20, 21], the number of LAT-expressing neurons ranges between 5 to 30% of the total latently infected neurons containing viral genomes [22-24]. Perhaps latently infected neurons, which express LAT, represent only a small subset of the true number of latently infected cells [8,10, 25]. Moreover, the ability to detect HSV-1 DNA sequences without a requirement for LAT will allow for detection of LAT negative mutants and will be of great value in the determination of the true nature of HSV-1 latency. *In situ* PCR is a relatively new technique that amplifies a target sequence within cells that can be visualized microscopically with increased sensitivity compared to detection by ISH [10, 26].

The number of neurons in a ganglion that will ultimately harbor latent HSV-1 genomes following primary infection depends upon the host, the strain of virus, the concentration of infecting viruses, and the conditions at the time of infection. HSV-1 is possible to infect as few as 1% to as many as 50% of the neurons in a ganglion [27], correspondingly, about 5% of neurons have been estimated as latently infected cells in this study.

Moreover, we have shown here a direct *in situ* PCR method which has been developed to detect latent HSV-1 DNA in trigeminal ganglia of latently infected mice. The validity of *in situ* PCR was examined in every reaction through two sets of

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negative controls and one positive (or internal) control simultaneously. Finally, the results of *in situ* PCR were examined with a two-round PCR. It has been shown that even with proper proteinase K digestion, there is usually a minute amount of leakage from the cells into the amplification cocktail, but there is little or no leakage into other cells of the tissue [9]. Therefore, these products can be amplified in a subsequent conventional PCR reaction. It was found that the leakage of the products into reaction mixture is probably weak (because of proper proteinase K digestion). Therefore, a two-round PCR was used to verify the results of the *in situ* PCR. It can be concluded from the experiments described in this study that a direct *in situ* PCR method using a DAB detection system is an effective means for detection of latent HSV-1 DNA in the latently infected ganglia. Therefore, it is possible to find the true number of HSV-1 containing cells in a latent tissue.

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