Jpn J Ophthalmol 2004:48:328–332
DOI 10.1007/s10384-004-0081-z

LABORATORY INVESTIGATION

Study of a Polymerase Chain Reaction-based Method for Detection of Herpes Simplex Virus Type 1 DNA among Iranian Patients with Ocular Herpetic Keratitis Infection

Mohammad Ali Khodadoost[1], Farzaneh Sabahi[1], Mahmoud Jabbarvand Behroz[2], Mohammad Hassan Roustai[1], Horieh Saderi[3], Samad Amini-Bavil-Olyaee[4], and Mohsen Karimi Arzenani[4]

[1] Department of Virology, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Iran; [2] Department of Ophthalmology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran; [3] Department of Microbiology, Faculty of Medicine, Shahed University, Tehran, Iran; [4] Department of Biotechnology, Pasteur Institute of Iran, Tehran, Iran

Abstract

Purpose: To study the presence of the herpes simplex virus type 1 (HSV-1) glycoprotein D gene in tear films of Iranian patients with herpetic keratitis.

Methods: Twenty-five tear film and eye swab specimens from 25 herpetic keratitis patients and 10 specimens from 10 healthy volunteers were collected in the Farabi Eye Hospital, Tehran, Iran. HSV-1 DNA was detected by using the nested polymerase chain reaction (nPCR) method. Viral isolation was done using conventional viral techniques. A monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) was used for confirmation of positive cytopathic effect cell culture. The results of a diagnosis by an ophthalmologist team were compared with those of nPCR.

Results: HSV-1 DNA was identified in tear films of 88% (23/25) of suspected herpetic keratitis patients. All healthy controls (100%) had negative PCR results. HSV-1 was isolated in cell culture and confirmed by ELISA in 12% (3/25) of herpetic keratitis patients who had epithelial keratitis. The kappa value showed a high level of agreement between ophthalmologist team diagnosis and the PCR results (kappa $= 0.86$, $P < 0.0001$).

Conclusions: nPCR is a sensitive, rapid, and powerful tool for detection of HSV-1 DNA in tear films of ocular herpetic keratitis patients and can serve as a supplemental method for diagnosis of herpetic keratitis infection. **Jpn J Ophthalmol** 2004;48:328–332 © Japanese Ophthalmological Society 2004

Key Words: herpes simplex virus type 1, herpetic keratitis infection, human tear films, nested polymerase chain reaction, viral culture

Introduction

Human herpes virus type 1 (HSV-1) is a double-stranded DNA virus belonging to the alpha-herpesvirinae subfamily and the herpesviridae family; it is the aetiological agent of herpetic keratitis in all parts of the world and the leading cause of infectious corneal blindness in humans. The pathogenesis involves a two-step process of infection and reinfection.[1] It has been estimated that HSV-1 ocular infection occurs in all countries with an annual incidence of up to 20.7 per 100 000 population.[2–4]

The timely and rapid diagnosis of HSV keratitis has become urgent given the availability of specific antiviral drugs and appropriate patient management strategies in

Received: September 30, 2003 / Accepted: April 13, 2004
Correspondence and reprint requests to: Farzaneh Sabahi, Department of Virology, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Iran
e-mail: sabahi_f@modares.ac.ir

addition to the traditional diagnosis of HSV keratitis based on clinical evaluation. Although the conventional cell culture technique is considered the "gold standard" for diagnosis of HSV-1, it is costly, and its major drawback is the long isolation period (4–7 days). Moreover, this technique cannot be established in all routine diagnostic laboratories. A number of diagnostic assays have been developed for detection of HSV-1 keratitis in the last few years, including immunofluorescence,[5,6] in situ hybridization,[7,8] and polymerase chain reaction (PCR)-based assays.[9–15] Recently, nested PCR (nPCR), a highly sensitive method, has been found suitable for the diagnosis of ocular keratitis.[12,16,17]

In the present study, tear film specimens were used to detect HSV-1 DNA with the nPCR method in suspected keratitis patients, and the results were compared with those of the cell culture technique and diagnosis by ophthalmologists.

Materials and Methods

Patients and Controls

Twenty-five tear film samples from herpetic keratitis patients (8 female and 17 male) were collected in Farabi Eye Hospital (the main referral center for treating eye diseases in Iran), Tehran, Iran. An ophthalmologist team used clinical evidence to recognize herpetic keratitis in all patients. Three of the 25 cases were diagnosed as epithelial keratitis, and the remaining 22 were diagnosed as stromal keratitis. All samples were collected before the use of any indicated antiviral therapy. To obtain a tear film or swab specimen, a tear film or swab was placed on the lower temporal eyelid margin of the infected eye for 1 min. Each tear film or swab specimen was placed in 2 ml of viral transport media (VTM) (Sigma, Steinheim, Germany) and transported immediately to the viral laboratory of the Virology Department at Tarbiat Modarres University (TMU), Tehran, Iran. Half of each sample was inoculated onto cell monolayers, and the residual sample was kept frozen at −70°C until DNA extraction and nPCR were performed. As negative controls, ten tear film samples were obtained from healthy volunteers (three female and seven male), who did not show any evidence of ocular infection, as judged by the same ophthalmologist team.

Virus Isolation and Enzyme-linked Immunosorbent Assay

HeLa and Vero cell lines were obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran), with accession numbers of NCBI C115 and NCBI C101, respectively, and seeded into culture tubes (Nunc, Roskildes, Denmark). Samples were decontaminated with antibiotics, inoculated onto HeLa and Vero confluent cell lines and incubated for 1 h at 37°C for adsorption of the virus. After removal of samples, monolayers were covered with Dulbecco's modified Eaglés medium (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Karlsruhe, Germany), antibiotics, and antifungal agents. The cell cultures were incubated at 37°C in a 5% CO_2 atmosphere at 95% humidity (Memmert, Schwabach, Germany) and observed every day for 2 weeks for the presence of cytopathic effects (CPE).

Positive CPE were confirmed by HSV-1 and HSV-2 monoclonal antibodies (MAb), gifts of Dr. Jan-Ake Lilijeqvist, Virology Department, Gothenburg University, Gothenburg, Sweden, in an enzyme-linked immunosorbent assay (ELISA) described previously.[18,19] The first MAb (B1.E6) was an HSV-type common anti-glycoprotein E antibody for both types of HSV, the second MAb (B1.C1) was an HSV-1-specific anti-glycoprotein C-1 (gC-1) antibody, and the third MAb (O1.C5.B2) was an HSV-2-specific anti-glycoprotein G-2 (gG-2) antibody. Uninfected negative controls as well as HSV-1- and HSV-2-positive controls were included in each run.

HSV DNA Extraction and nPCR

To prepare HSV-1 DNA, 100 µl of freeze-thawed tear film samples was added to 100 µl of proteinase K solution [20 mM Tris-HCl, pH 8.0; 10 mM ethylenediamine tetraacetate; 1% sodium dodecyl sulfate; 20 mg/ml glycogen (Sigma); and 250 µg/ml proteinase K (Gibco)] and incubated at 65°C for 2 h. DNA was extracted from the mixture with phenol/chloroform/isoamylalcohol, precipitated with absolute ethanol, resuspended in 30 µl distilled water, and used for PCR analysis. The DNA concentration was determined by using a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany) and adjusted to a final concentration of 100 ng/µl. The primers used in this HSV-1 nPCR assay amplify a fragment belonging to the glycoprotein D-encoding gene (Table 1). nPCR was performed as previously described by Hidalgo et al.[17] with minor modification. Briefly, a PCR reaction mixture (final volume of 25 µl) containing 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 U *Taq* DNA polymerase (Fermentas AB, Vilnius, Lithuania), 0.01% TritonX100, 0.05% Tween-20, 100–150 ng DNA and 0.2 µM each of the outer primers was used for the first-round PCR. The PCR reaction mixture for the second-round PCR was like that of the first round, but contained 3 µl of first-round PCR product as the template and 0.5 µM of each of the inner primers. The PCR program consisted of 40 and 25 cycles for the first- and second-round PCR, respectively. The first-round PCR program consisted of 95°C for 5 min (first denaturation), Ten cycles of 94°C for 45 s, 55°C for 40 s, 72°C for 50 s, followed by 30 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 50 s, followed by 72°C for 5 min (final extension). The second-round PCR program consisted of 25 cycles of 95°C for 5 min (first denaturation), 94°C for 40 s, 57°C for 40 s, and 72°C for 45 s followed by 72°C for 5 min (final extension). Five microliters of second-round PCR product were elec-

Table 1. Primer properties of HSV-1 DNA for nPCR

Designation	Sequence (5′–3′)	Position	Fragment size
Outer primers			
Forward	ATCACGGTAGCCCGGCCGTGTGACA	HSV-1 *gD*: 19–43	221 bp
Reverse	CATACCGGAACGCACCACACAA	HSV-1 *gD*: 218–239	
Inner primers			
Forward	CCAACCGACCACACCGACGA	HSV-1 *gD*: 51–71	138 bp
Reverse	GGTAGTTGGTCGTTCGCGCTGAA	HSV-1 *gD*: 166–188	

HSV-1, herpes simplex virus type 1; nPCR, nested polymerase chain reaction; *gD*, glycoprotein D gene; bp, base pair.

trophoresed in 10% polyacrylamide gel. The gel was then immersed in 0.5 μg/ml ethidium bromide solution for 30 min at room temperature and photographed by a gel documentation system (BioRad, Richmond, CA, USA). DNA of the HSV KOS strain was used as a PCR positive control and was included in each run.

Precaution Against PCR Contamination

PCR reagents were prepared before each assay in a master mixture that was then aliquoted under mineral oil. The preparation of the master mixture, extraction of HSV DNA, addition of template to the PCR mixture, and the thermal cycling were performed in three different, wall-separated rooms, each with its own dedicated set of micropipettes and gowns. General precautions against contamination, including systemic use of aerosol-barrier-protected tips, frequent changes of gloves, and frequent decontamination of surfaces with UV light and DNAaway solution (MBP, San Diego, CA, USA) were strictly adhered to. Meanwhile, the guidelines of Kwok et al.[20] were followed.

Statistical Analysis

The kappa coefficient (SPSS software, Version 11.0, Chicago, IL, USA) was computed to test agreement in the data analysis.

Results

The study population consisted of subjects with diagnosed herpetic keratitis that was confirmed by ophthalmologists using clinical evidence. Healthy persons without any herpetic clinical symptoms were selected as negative controls. All patients and controls were evaluated for the presence of virus by standard conventional cell culture techniques using HeLa and Vero cell lines. HSV-specific CPE appeared in only three samples from patients that had epithelial keratitis. HSV was isolated in 12% (3/25) of patient samples. Samples obtained from controls did not show positive CPE.

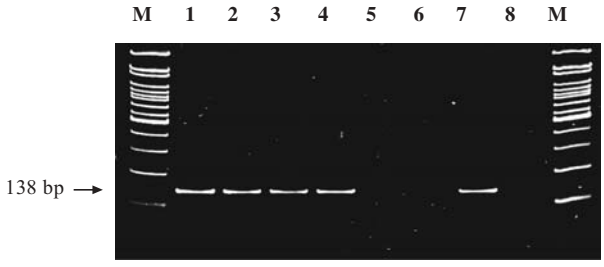

Figure 1. Detection of herpes simplex virus type 1 (HSV-1) DNA in tear film samples by nested polymerase chain reaction (nPCR) in a 10% polyacrylamide gel. *Lanes 1–4* show positive PCR results (138 bp) in tear films of four herpetic keratitis patients diagnosed by the ophthalmologist team. *Lanes 5–6* show negative PCR results of tear film specimens from two healthy controls considered as negative by the ophthalmologist team. *Lane 7* shows the PCR result of the KOS HSV strain used as a positive control. *Lane 8* shows a blank (no DNA added). *M*, 100-bp DNA ladder.

ELISA assays with HSV-1 and HSV-2 monoclonal antibodies were used to confirm the type of HSV. ELISA confirmed HSV type-1 CPE in positive cell cultures.

nPCR detected HSV-1 DNA, within the the glycoprotein D (*gD*) gene in 88% (22/25) of samples, and a 138-bp fragment was shown by a polyacrylamide gel stained with ethidium bromide. Of the 22 positive nPCR subjects, 86.4% (19/22) had been diagnosed with stromal keratitis and 13.6% (3/22) with epithelial keratitis. Twelve percent (3/25) of subjects diagnosed with herpetic keratitis had negative PCR results (Fig. 1). The reliability of nPCR was confirmed by the positive results obtained from positive controls and the negative results from specimens from healthy subjects. The kappa value, used to determine the agreement between the ophthalmologist team diagnosis of herpetic keratitis and the PCR results, showed a significant and high level of agreement (kappa = 0.86, $P < 0.0001$).

Discussion

Herpes simplex virus type 1 (HSV-1) is a prevalent microbial pathogen infecting 60% to 90% of the adult world

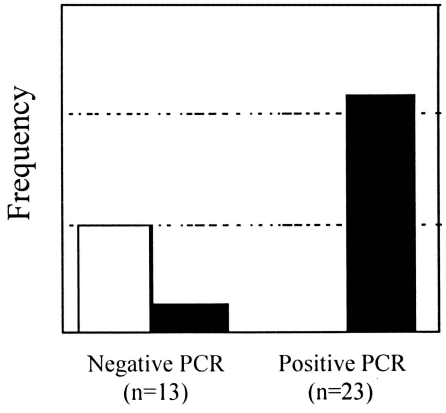

Figure 2. Frequency distribution of negative and positive HSV-1 DNA PCR results on tear film specimens of healthy controls (□) and herpetic keratitis patients (■). *n*, number of samples.

population. The coevolution of the virus with humans is due, in part, to adaptations that the virus has evolved to aid it in escaping immune surveillance, including the establishment of latent infection in its human host. Conjunctivitis and keratitis are common forms of ocular diseases seen in general practice and in eye units. HSV-1 ocular infection occurs in all countries, with an annual incidence of up to 20.7 per 100 000 population and is the most common infective cause of blindness in the world.[21] Primary epithelial keratitis may be cured by itself without any visible defects. But it establishes a latent infection, which upon activation, causes significant morbidity due to inflammation of epithelia and conjunctiva. HSV epithelial keratitis is typified by dendritic or geographical corneal ulcers, which usually have dendritic edges with terminal bulbs and epithelial infiltration. However, sometimes such factors as duration since onset, previous medication, systemic diseases (atopic dermatitis), or history of corneal transplantation can make the epithelial lesions appear different from classic HSV lesions.[22] In addition, differential diagnosis between herpetic keratitis and other fungal or parasitic infections such as acanthamoeba keratitis is important.[23–25] The above-mentioned factors along with the availability of appropriate treatment clearly justify the need for accurate laboratory investigation in the case of keratoconjunctivitis.[26]

Different diagnostic laboratory techniques such as viral culture, ELISA, immunofluorescence assay (IFA), and PCR have been used. Serologic techniques have been shown to be unsuitable for diagnosis of herpetic eye infection. Immunofluorescence assays have not shown promising results either.[17,27] Cell culture techniques, which have the highest specicifity, are considered the "gold standard" of HSV diagnosis, but they have low sensitivity, especially when certain kinds of specimens are involved, such as those obtained from herpetic stromal keratitis patients. In the most recent studies, the frequency of virus isolation from ocular specimens with cell culture technique has been low: Kaye et al.,[19] reported 1 of 10 (1991); Pramond et al.,[6] 14 of 70 (1998); Kaye et al.,[8] 1 of 51 (2000); and we found 3 of 25 (12%) in the present study. All three of our specimens were from patients who had epithelial keratitis. None of the stromal keratitis samples showed any growth of HSV on either HeLa or Vero cell lines. These data show that cell culture techniques have low sensitivity for detection of virus in patients with stromal keratitis.

As an alternative to cell culture for the demonstration of the presence of HSV DNA in the tear film or eye swab of keratitis patients, PCR is considered to be a new standard for the diagnosis of this infection.[8–17] In this study, a part of the HSV *gD* gene was amplified successfully by using an nPCR method. Twenty-two of 25 herpetic keratitis patients diagnosed by a single ophthalmologist team had positive PCR results. Only in three patients was the ophthalmologist team diagnosis not in agreement with nPCR results. The clinical diagnosis of HSV, as a history of recurrent dendritic or geographical corneal ulceration and the development of stromal scarring, is well defined. However, a variety of microbial pathogens may cause keratitis, and differential diagnosis in cases with a less typical clinical picture is of importance.[28] In addition, ocular acanthamoeba infections, a more common cause of ocular infections among contact lens users, may present itself with pseudodendritic appearance in infected eyes and may need to be ruled out, especially in these groups of patients. The results of this and other studies[8–17,29] show that PCR is a sensitive method for detecting HSV-1 DNA in cornea. However, as with other types of infections, PCR can only supplement clinical diagnosis by an ophthalmologist. Clinical diagnosis and follow-up of patients is especially important considering that in the case of herpes infections, other microbial infections or certain diseases may cause reactivation of HSV.

Our findings support that stromal keratitis can be detected by using nPCR and that conventional cell culture is not a sensitive test for detecting HSV in tear films from stromal keratitis patients. Further investigations based on quantitative measurement of HSV-1 DNA and its correlation with the pathogenesis of this kind of herpetic eye disease is suggested; this measurement could be performed by new and advanced molecular techniques such as real-time PCR. In conclusion, nPCR can be used for sensitive and rapid detection of HSV-1 DNA and is a powerful confirmatory and supplemental tool for diagnosis of ocular herpetic infections in addition to clinical diagnosis.

Acknowledgment. This work was supported by a grant from Tarbiat Modarres University (TMU), Tehran, Iran.

References

1. Whitley RJ. Herpes simplex viruses, In: Fields BN, editor. Fields virology. New York: Raven; 2001. p. 2297–2342.
2. Leisegang TJ, Melton J, Daly PJ, et al. Epidemiology of ocular herpes simplex. Arch Ophthalmol 1989;107:1155–1159.
3. Darougar S, Monnickendam MA, Woodland RM. Management and prevention of ocular viral and Chlamydia infection. CRC Crit Rev Microbiol 1989;16:369–417.

4. Elnifro EM, Cooper RJ, Klapper PE, et al. Diagnosis of viral and Chlamydia keratoconjunctivitis: which laboratory test? Br J Ophthalmol 1999;83:622–627.
5. Sharma A, Shimeld C. In vivo immunofluorescence to diagnosis herpes simplex virus keratitis in mice. Br J Ophthalmol 1997;81:785–788.
6. Pramod NP, Gopalakrishnan V, Mohan R, et al. Enhanced detection of herpes simplex virus from ocular specimens of herpetic keratitis patients. Indian J Pathol Microbiol 1998;41:49–53.
7. Laycock KA, Lee SF, Stulting RD, et al. Herpes simplex virus type 1 transcription is not detectable in quiescent human stromal keratitis by in situ hybridization. Invest Ophthalmol Vis Sci 1993;34: 285–292.
8. Kaye SB, Baker K, Bonshek R, et al. Human herpesviruses in the cornea. Br J Ophthalmol 2000;84:563–571.
9. Kowalski RP, Gordon YJ, Romanowski EG, et al. A comparison of enzyme immunoassay and polymerase chain reaction with the clinical examination for diagnosing ocular herpetic disease. Ophthalmology 1993;100:530–533.
10. Cleator GM, Klapper PE, Dennett C, et al. Cornea donor infection by herpes simplex virus: herpes simplex virus DNA in donor corneas. Cornea 1994;13:294–304.
11. Rong BL, Pavan-Langston D, Weng QP, et al. Detection of herpes simplex virus thymidine kinase and latency-associated transcript gene sequences in human herpetic corneas by polymerase chain reaction amplification. Invest Ophthalmol Vis Sci 1991;32: 1808–1815.
12. Kudo E, Shiota H, Kinouchi Y, et al. Detection of herpes simplex virus DNA in tear fluid of stromal herpetic keratitis patients by nested polymerase chain reaction. Jpn J Ophthalmol 1996;40: 390–396.
13. Pramod NP, Thyagarajan SP, Mohan KV, et al. Polymerase chain reaction in the diagnosis of herpetic keratitis: experience in a developing country. Can J Ophthalmol 2000;35:134–140.
14. Tei M, Nishida K, Kinoshita S. Polymerase chain reaction detection of herpes simplex virus in tear fluid from atypical herpetic epithelial keratitis after penetrating keratoplasty. Am J Ophthalmol 1996;122:732–735.
15. Mori Y, Inoue Y, Shimomura Y, et al. Detection of HSV mRNA using reverse transcription-polymerase chain reaction for diagnosis in murine herpetic keratitis model. Jpn J Ophthalmol 1998;42: 8–11.
16. Elske van Gelderen B, Van der Lelij A, Treffers WF, et al. Detection of herpes simplex virus type 1, 2 and varicella zoster virus DNA in recipient corneal buttons. Br J Ophthalmol 2000;84:1238–1243.
17. Hidalgo F, Melon S, De Ona M, et al. Diagnosis of herpetic keratoconjunctivitis by nested polymerase chain reaction in human tear film. Eur J Clin Microbiol Infect Dis 1998;17:120–123.
18. Nilheden E, Jeansson S, Vahlne A. Typing of herpes simplex virus by an enzyme-linked immunosorbant assay with monoclonal antibodies. J Clin Microbiol 1983;17:677–680.
19. Liljeqvist JA, Svennerholm B, Bergstrom T. Typing of clinical herpes simplex virus type 1 and type 2 isolates with monoclonal antibodies. J Clin Microbiol 1999;37:2717–2718.
20. Kwok S, Higuchi R. Avoiding false positive results with PCR. Nature 1989;339:237–238.
21. Ford E, Nelson KE, Warren D. Epidemiology of epidemic keratoconjunctivitis. Epidemiol Rev 1987;9:244–261.
22. Koizumi N, Nishida K, Adachi W, et al. Detection of herpes simplex virus DNA in atypical epithelial keratitis using polymerase chain reaction. Br J Ophthalmol 1999;83:957–960.
23. Klotz SA, Penn CC, Negvesky GJ, et al. Fungal and parasitic infections of the eye. Clin Microbiol Rev 2000;13:662–685.
24. Perez-Santonja JJ, Kilvington S, Hughes R, et al. Persistently culture positive acanthamoeba keratitis: in vivo resistance and in vitro sensitivity. Ophthalmology 2003;110:1593–1600.
25. Moreira AT, Prajna NV. Acanthamoeba as a cause of peripheral ulcerative keratitis. Cornea 2003;22:576–577.
26. Green MT, Dunkel EC. Herpes simplex virus infections: latency and reactivation, In: Darrell RW, editor. Viral diseases of the eye. Philadelphia: Lea and Febiger; 1985. p. 9–28.
27. Coyle PV, Desai A, Wyatt D, et al. A comparison of virus isolation, indirect immunofluorescence and nested multiplex PCR for the diagnosis of primary and recurrent HSV-1 and HSV-2. J Virol Methods 1999;83:75–82.
28. Helbert L. The eye: conjunctivitis, keratitis and infections of periorbital structures. In: Armstrong D, Cohen J, editors. Infectious disease, 2nd edition, Vol. 1. London: Harcourt; 2000. p. 11.3–11.6.
29. Kaye SB, Lines C, Patterson A, et al. Evidence for herpes simplex virus latency in human cornea. Br J Ophthalmol 1991;75:195–200.