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Detection of Herpes Simplex Virus in Corneal Buttons with Clinical Diagnosis of Corneal Scarring

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Abstract

***Purpose*:** The evaluation of the presence of Herpes Simplex Virus (HSV) in corneal scars by real-time polymerase chain reaction (PCR) and comparison of the results with histopathologic findings and clinical diagnoses

***Methods*:** Eighty-seven corneal scar samples obtained after penetrating keratoplasty (PK), were selected after reviewing the records from 2006 to 2010. Nucleic acid was extracted from paraffin embedded corneal samples and PCR-amplified for HSV DNA.

***Results*:** Among 87 samples, four samples were excluded because internal controls were negative. HSV infection was established in 9.6% (8/83) of all patients. The prevalence of HSV infection in patients with no clinical suspicion of herpetic keratitis was 7.3%. Histopathologic evaluation revealed that among samples with positive PCR results, 100% had evidence of inflammation, 62.5% had giant cells, 37% had necrosis, 62.5% had vascularization, 62.5 % had ulcer and all of them had inclusion bodies.

***Conclusion*:** Because some of the patients with no clinical suspicion of herpes infection were found positive, we suggest that HSV keratitis to be considered as one of the underlying etiologies in any patient with corneal scar. Therefore, performing further diagnostic methods, including PCR and histopathology, are mandatory to rule out the infection.

***Keywords*:** Herpes Simplex Virus, Keratitis, Corneal Scar, Polymerase Chain Reaction

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Introduction

Herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) belong to the same herpes virus family, Herpesviridae, and cause various ocular diseases which are considered as one of the main causes of blindness worldwide.[1,2] Herpetic stromal keratitis (HSK), as one of the major forms of ocular involvement by herpes viruses, is still one of the major causes of corneal blindness and visual acuity (VA) loss rated just second to trauma in most developed countries.[3]

HSV-1 becomes latent in neural ganglions including trigeminal and superior cervical ganglions in which the virus intermittently reactivates from latency with or without explicit stimuli.[4-6]

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Ultraviolet light irradiation, trauma, immune deficiency, fever, and emotional stress are among the stimuli that can reactivate the virus. Reactivation leads to viral replication and translocation down through a nerve axon to the cornea and subsequently causes herpetic keratitis.[7] Patients who have frequent episodes of reactivation eventually develop irreversible corneal stromal swelling, neovascularization, and scarring with severe visual loss. Diagnosis of herpetic disease of cornea is made by using clinical history and can be confirmed by pathologic evaluation of the obtained corneal samples. Pathologic features showing corneal viral infection is not limited to stromal edema but other symptoms such as acute and chronic inflammation, vascularization, necrosis, scar can be seen.[8] Chronic cases presenting for surgery with corneal scar and insufficient clinical history pose a diagnostic challenge. In such cases inattention to herpetic etiology of the scar can lead to keratitis, relapse and treatment failure.

Some of the corneal scars are caused by latent HSV infection that has been detected by various methods such as immunohistochemistry and polymerase chain reaction (PCR). These methods are used to detect HSV-1 and HSV-2 antigens or DNA in latent and reactivated HSV infection. Furthermore, HSV-1 DNA in the cornea is quantified by real-time PCR, and the copy number in the cornea is correlated with the frequency of reactivation of herpetic keratitis.[9-11]

Because the prevalence and causes of corneal scars are different in different countries, the aim of this study was to evaluate the presence of HSV virus in corneal scars in correlation with histopathologic and clinical findings.

Methods

Review of pathological records

We reviewed the records of all corneal specimens submitted from 2006 to 2010 to the Farabi Eye Hospital, affiliated to Tehran University of Medical Science (TUMS), Tehran, Iran. Totally, 87 paraffin embedded corneal scar samples obtained after penetrating keratoplasty (PK), were collected. The inclusion criteria were: 1) Presence of the corneal scar according to clinical presentation; 2) Scar caused by herpetic infection; 3) Scar not related to other known infectious causes, trauma, genetic, or degenerative diseases according to the clinical suspicion. Then the selected samples were reevaluated for histopathologic changes by an expert pathologist (FA). Samples were categorized in two groups: group A) cases suspicious of herpetic disease; group B) cases clinically unsuspicious of herpetic disease which were not caused by other known etiology. Moreover, cases in group B were subdivided in two groups: group B1: histologically suspicious of herpes; group B2: others. Histopathologic findings in group B1 included: presence of giant cells and/or intranuclear inclusions in epithelial cells. For all selected specimens the following data were also included: age, sex, date of surgery, the presence or absence of multinucleated cells, vascularization, ulcer, inflammatory cells, necrosis and intranuclear inclusion bodies in epithelial cells. TUMS committee on human ethics and research approved this study.

Nucleic acid extraction

Neucleic acid was extracted from paraffin embedded corneal samples using Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN) according to manufacturer's protocol. Briefly, two 5 µm sections of each paraffin block were prepared using disposable blades, and then the sections were transferred to DNAse/RNAse free 1.5-mL Eppendorff tubes using a disposable applicator. Following deparaffination by xylene and ethanol, the sections were digested by proteinase K and brought to extraction column. Subsequently, after three rounds of washing by inhibitor removal and wash buffers, nucleic acid was eluted and stored in −20°C until PCR was performed.

Polymerase chain reaction

To assure the quality of extracted DNA, amplification of Homo-sapiens hydroxymethylbilane synthase (HMBS) gene was performed as internal control as previously described.[12] The PCR was performed in a 20 µL reaction containing 0.2 µM of each forward and reverse HMBS primers, and 10 µL SYBR Premix Ex Taq II (Takara Bio, Ostu, Shiga, Japan). The PCR conditions were as follows: initial denaturation

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at 95°C for 1 min, and then 40 cycles of 95°C for 10 s and 60°C for 30 s.

The PCR of HSV was performed in duplicate for each sample using AmpliSens HSV I, II-FRT (Moscow, Russia) as instructed by the manufacturer. Briefly, the PCR was performed in 25 µL containing 10 µL extracted DNA. The PCR conditions were as follows: initial denaturation at 95°C for 15 mins; and then 45 cycles of 95°C for 5 s, 60°C for 20 s and 72°C for 15 s; and finally 72°C for 15 s. Detection limit of the kit, according to manufacturer was 1 X 10^3 GE/ml (genome equivalents of the microorganism per 1 ml of a clinical sample places in the transport medium specified). The results were evaluated qualitatively and CT (cycle of threshold) values greater than positive control CT were considered positive. Thermocycling was performed by a Rotor Gene 3000 (Corbett Research Mortlake, Australia).

Results

Eighty-seven patients with corneal scars enrolled in this cross-sectional study. Four patients were excluded because internal-control PCR of their samples were negative. Among the remaining 83 patients, 28 patients (33%) were included in group A, and 55 patients (67%) were included in group B. The mean±SD age was 53.3±13.3 years (range, 16-88). Fifty-eight (69.9%) patients were male and 25 (30.1%) were female. HSV infection was established in 9.6% (8/83) of all patients. The prevalence of HSV in groups A and B were 14.3% (4/28) and 7.3% (4/55), respectively and the difference between two groups was not statistically significant (p>0.05). Group B1 contained seven patients; and group B2 included 48 patients. In these two subgroups, PCR results showed significant difference (57% positive vs. 0%, p=0.01). In this study, we also evaluated the histopathologic changes and compared the findings with PCR results. Among the histopathologic findings, inflammatory process with inclusion body was highly sensitive for HSV viral infection (Figures 1 and 2). The histologic findings are summarized in table 1.

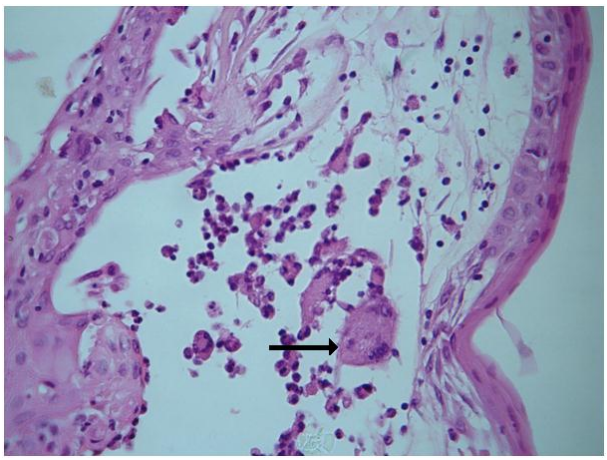

Figure 1. Corneal sample showing inflammation and giant cell formation (arrow)

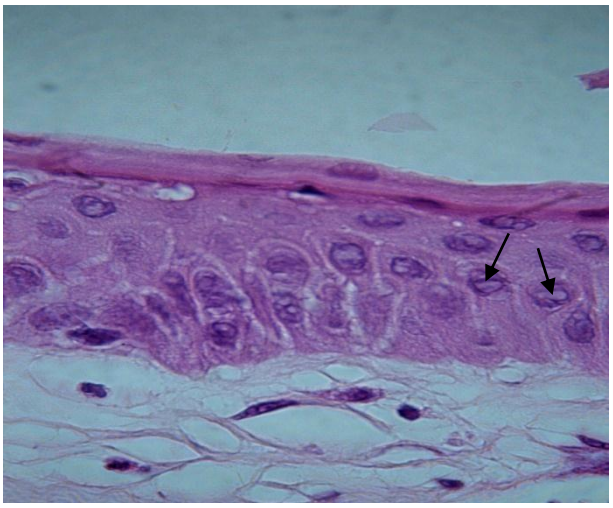

Figure 2. Corneal sample showing intranuclear inclusion (arrow)

Table 1. Histolopathologic findings upon Herpes Simplex Virus infection

Histolopathologic findings	HSV positive N (%)	HSV negative N (%)	p
Inflammation	8 (100)	20 (26)	0.01
Giant cell	5 (62.5)	3 (6.7)	0.001
Necrosis	3 (37)	6 (8)	0.005
Inclusion body	8(100)	10 (13)	0.002
Vascularization	5 (62.5)	29 (38)	0.2

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Discussion

Previous studies have shown the presence of HSV virus in corneal scars as well as cervical ganglions.[13,14] Our results revealed an overall prevalence of 9.8% (14.3% in group A and 7.3% in patients in group B) among patients who were referred to our hospital with corneal scar. These findings are in line with previous studies that showed the presence of HSV virus in corneal scars. The prevalence rate of HSV in corneal samples ranged from 7.8% to 40% in different studies.[9,15,16] This difference could be due to different detection methods used in different studies or small size of the specimens. As mentioned elsewhere, PCR is the most sensitive method for detection of HSV virus.[8] However, detection limit of a PCR method depends on primer design, optimization and extraction method. The small size of examined samples can be a cause of sampling error and consequently affect the prevalence rate. Nevertheless, in order to avoid false negative results, caused by sampling error, two more 5 µm cut sections were performed to increase sample volume on clinically HSV-suspected group. PCR tests were also repeated on all samples. However, the same results were attained.

Chronicity of the scars and different latencies of the disease from last HSV attack can be another probable reason for different detection rates for HSV virus.[14,17] These findings show the difficulty of HSV detection in corneal samples confirming the need of combining multiple detection methods in order to increase the detection rate. Also, some corneal scars are related to other viral agents that could have led to negative results in this study. Histopathologic evaluation of harvested samples can be helpful in further detection of infected samples. Although not definitive, some histopathologic features are in favor of viral infection.[8,15,18]

Conclusion

The importance of HSV detection is that patients with corneal scar, caused by HSV and treated by corneal graft, are at risk of recurrence and treatment failure.[19] Because the prevalence of HSV infection in our patients with no clinical suspicion of herpetic keratitis was 7.3%, we suggest that HSV keratitis be considered as a probable underlying etiology in any patient with corneal scar. Therefore, performing further diagnostic methods, including PCR and Histopathology, may be mandatory for management of patients with HSV viral infection.

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