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Frequency of Human Herpesvirus 6 (HHV-6) in Pterygium Using Real-Time PCR Based on SYBR-Green I Fluorescence

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ABSTRACT

Background and objectives: Pterygium is a non-cancerous growth of conjunctival tissue that can extend onto the corneal surface. The presence of some oncogenic viruses in pterygium and the neoplastic nature of these lesions led us to the postulated involvement of the viruses in the etiology of pterygium. Given the association of human herpesvirus 6 (HHV-6) with ocular diseases, we aimed to investigate presence of this virus in pterygium.

Methods: Fifty tissue specimens were collected from patients with pterygium who underwent pterygium surgery between February 2013 and May 2015. The specimens were tested by real-time PCR using Maxima SYBR Green/ROX qPCR Master Mix (2X) kit. Demographic and clinical data were collected and analyzed using SPSS software (version 18).

Results: Six (12 %) specimens were positive for HHV-6 DNA. There was no statistically significant correlation between pterygium and presence of HHV-6.

Conclusion: Based on the results, a direct association between HHV-6 and development of pterygium seems less probable, which suggests that other etiologic agents must be involved in the multistep process of the disease.

Keywords: Human Herpesvirus 6; pterygium; Real-time PCR.

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INTRODUCTION

Pterygium is a common ocular lesion that appears as wing-shaped, vascular and fleshy growth that can extend onto the corneal surface. The disease is relatively common in the general population and thought to be caused by ultraviolet-light exposure, low humidity and dust (1).

Much attention has recently been paid to the possibility of viral oncogenes involvement in the multistage process of pterygium formation. Until now, a few viruses such as human papillomaviruses (HPV) and Epstein-Barr virus **(**EBV**)** have been investigated as possible causative agents of pterygium (2, 3).

Human herpesvirus 6 (HHV-6) belongs to the *Roseolovirus* genus of the *Betaherpesvirinae* subfamily and has a linear, double-stranded genome. The virus was first isolated in 1986 from AIDS patients with lymphoproliferative disorders (4). HHV-6 is an opportunistic pathogen with up to 90% seroprevalence in the human population and is commonly acquired during early childhood. The virus is separated into two major subgroups, HHV-6A and -6B, on the basis of genetic, immunological, epidemiological and functional features (4, 5).

Primary infection with HHV-6B can cause roseola (also known as sixth disease or exanthem subitum), a common childhood disease that resolves spontaneously. Although, HHV-6 infections are usually benign, the virus has been associated with several neurologic syndromes, such as febrile convulsions, encephalitis and multiple sclerosis. Besides roseola infantum, research on causative role of HHV-6 in other illnesses has been inconclusive (1, 6-8).

Overall, HHV-6 variant B is found more frequently than variant A in both peripheral blood mononuclear cells and solid tissues (5, 9). Variant A is predominantly present in the skin and active in nearly all Lyme disease patients. In some cases, both variants can coexist in the brain despite being localized in different brain areas (5, 9, 10). In addition, HHV-6 is reported to be associated with different cancers including Hodgkin's lymphoma (HL), T-cell leukemia, EBV-negative B-cell lymphoma, African Burkitt's lymphoma and adenomatous gastrointestinal polyps (6, 11-13). Given the association of HHV-6 with ocular diseases, we aimed to investigate presence of this virus in pterygium.

MATERIALS AND METHODS

With the aim to determine the prevalence of HHV-6 in specimens from patients with pterygium, this cross-sectional study was performed in the Department of Ophthalmology and Virology of Golestan University of Medical Sciences (Iran) between February 2013 and May 2015. The study received approval from ethics committee of the Golestan University of Medical Sciences.

Fifty human biopsy specimens of pterygium were divided into three groups based on the grade of pterygium (grade 1: up to 2 mm, grade 2: 2-4 mm, and grade 3: more than 4 mm) (14). The samples were stored at -70 °C until processing.

DNA was extracted using QIAamp DNA Mini kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. The following β-globin primers were used to analyze quality of the extracted DNA after excluding false negative results: 5'-GAAGATGGTGATGGGATTTC-3' and 5'-GAAGGTGAAGGTCGGAGTC-3'. PCR was performed in a 25 µl reaction solution containing 5 µl of the extracted DNA (500 ng/ml), 0.1 µl of each primers with 10 pmol/µl, 0.3 U of Taq DNA polymerase (Genet Bio [A type]), 0.5 µl dNTPs (10mM), 2 µl of $MgCl_2$ (25 mM) and 5 µl of 10X PCR reaction buffer. Amplification was done in a Peq Lab thermal cycler (Primus Advanced 96 thermal cycler) under the following conditions: 5 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C and 72 °C for 7 min.

Real-time PCR was performed in an ABI system 7300 with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Ampliqon, Denmark) kit according to the manufacturer's instructions (15). The primer pair 5'-TCGAAATAAGCATTAATAGGCACACT-3' and 5'-CGGAGTTAAGGCATTGGTTGA-3' (98 bp) for the U22 open reading frame of both HHV-6A and -6B was used in a 25-µL reaction mixture (containing 12.5 µL of PCR master mix) to evaluate the presence of HHV-6 sequences (15). Thermocycling conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s and 60 °C for 30 s. An HHV-6-positive control was purchased from Vircell Microbiology Company (Figure 1). Finally, 98-bp amplicons were detected on 1% agarose gel containing 0.5 µg/ml ethidium bromide (Figure 2).

Prevalence of positive samples was calculated at 95% confidence interval (CI). Chi-square test and Fisher's exact test were used to evaluate distribution of characteristics associated with HHV-6 infection. Statistical significance was set at 0.05 for statistical analyses. Demographic and clinical data were entered and analyzed with SPSS software (version 18).

RESULTS

Of 50 specimens examined in this study, 20 (40%) were collected from males and 30 (60%) were from females. Mean age of patients was 61.1±16.9 years (range: 22-85 years). Thirteen patients had bilateral pterygia, 42 specimen were primary lesions and eight were postoperative recurrence. In the clinical examination for grading pterygia, 14 (28%) were atrophic/grade 1, 17 (34%) were intermediate/grade 2 and 19 (38%) were fleshy/grade 3. A positive family history of pterygium was noted in two cases (4%). Five positive cases (10%) had a history of outdoor activities. Seven patients (14%) had a positive smoking history. Six (12 %) patients with pterygium were positive for HHV-6 DNA (Table 1).

PCR of the *β-globin* gene was used as an internal control for DNA extraction, which showed that all samples were β-globin positive. There was no statistically significant correlation between pterygium and the presence of HHV-6.

Table 1- Comparison of variables between patients with pterygium positive and negative for HHV-6 in Gorgan, Iran

Variable / Group	Positive for HHV-6 (n=6)	Negative for HHV-6 (n=44)	P-value (Chi-square test or Fisher's exact test)
Sex			
Male	2 (33.34%)	18 (40.9%)	0.252
Female	4 (66.66%)	26 (50.09%)	
Type of pterygium			
Primary	6 (100%)	36 (81.81%)	
Recurrent	0	8 (18.18%)	
			0.512
Grade of pterygium			
Atrophic/grade1	4 (66.66%)	10 (22.72%)	
Atrophic/grade2	0	17 (38.63%)	0.253
Atrophic/grade3	2 (33. 34%)	17 (38. 63%)	
Family history of pterygium			
Positive	2 (33.33%)	0	
Negative	4 (66.66%)	44 (100%)	0.816
History of outdoor activities			
Positive	0	5 (11.36%)	0.435
Negative	6 (100%)	39 (88.63%)	

Figure 1-. (A) Dissociation curve. Violet: positive control, blue and pink: positive samples and green: negative control. (B) Amplification plot indicated Ct number in positive control, clinical samples (red line) and negative control (green line)

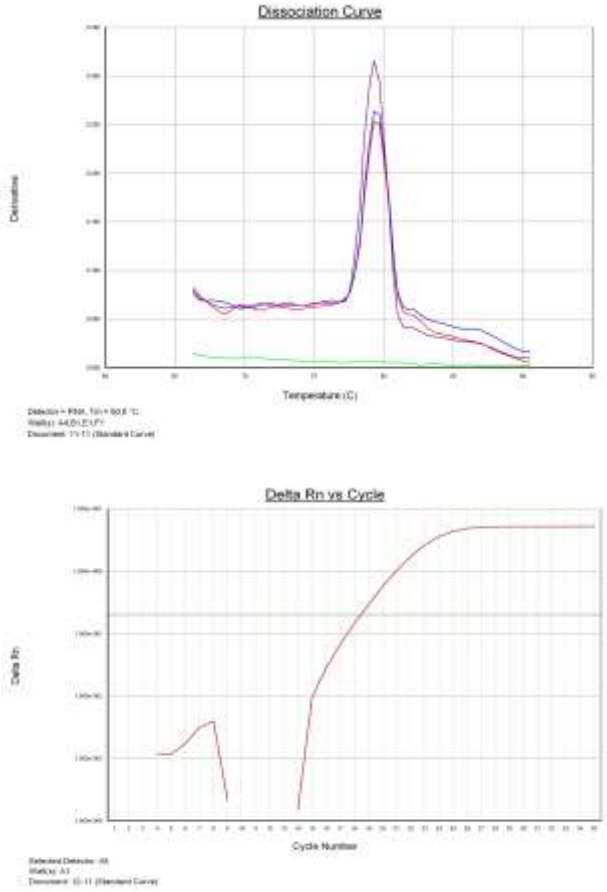
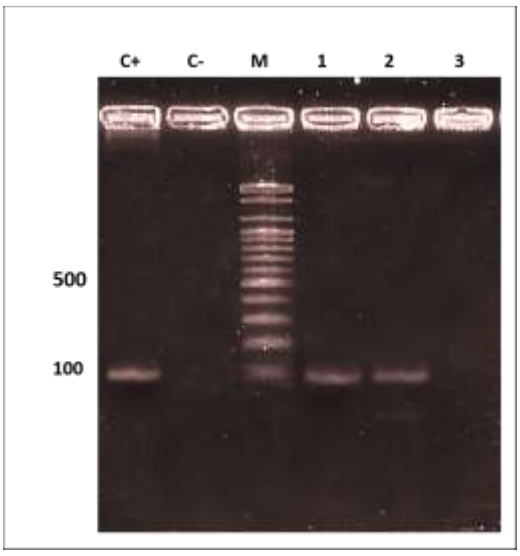


Figure 2-. Results of agarose gel electrophoresis of PCR products. C+: positive control, C-: negative control, M: DNA ladder (100 bp), lanes 1-3: HHV-6 positive specimens.



DISCUSSION

Many different genetic and environmental factors have been proposed to be involved in development of pterygium, but none has been found to be directly linked to the lesion formation.

The potential role of viral infections with HPV, EBV and herpes simplex virus as well as exogenous agents including ultraviolet irradiation in the development of pterygium remains controversial. However, since the disease creates a unique and favorable environment for viral oncogenes, viruses might have a role in progression of the lesions (2).

Cell proliferation, interference with signaling pathways, resistance to apoptosis and inducing angiogenesis are the hallmarks of pterygium, all of which support the neoplastic nature of the lesion (16). However, pterygia are benign growths and typically do not require surgical excision until manifestation of acute symptoms, but recurrence is frequent after tissue excision (as high as 46%) (17).

Some studies have suggested an association between HHV-6 and ocular disease, corneal inflammation, optic neuropathy, tonic pupil, bilateral uveitis and Bell's palsy. These results suggest that HHV-6 may be capable of invading the corneal tissue (6, 18-24).

Although HHV-6 is identified as an opportunistic virus, it has also shown transforming and trans-activating properties, thus playing a definitive role in human neoplasia (6, 9).

The detection of HHV-6 viral DNA sequences in some Hodgkin's and non-Hodgkin's lymphomas indicates the possible role of HHV-6 in human neoplasia (25,26).

The vast majority of studies utilized nested-PCR for diagnosing infection with HHV-6. Since pterygium usually grows slowly through life, even with this sensitive and specific technique, 30-60% of patients remain undiagnosed, because the HHV-6 may be chronically active in the tissue and only limited number of viruses circulate outside the tissue. Hence, real-time PCR must be sensitive-enough to detect HHV-6 infections in the tissues (24, 25, 27). In our study, the HHV-6 DNA was found in 6 (12%) patients using real-time PCR.

The limitation of this study were the small number of patients and not analyzing association between HHV-6 and variables such as grade of pterygium. To our knowledge, no study has investigated presence of HHV-6 in pterygium, but in a study by Crawford et al., 14 of 30 (46.7 %) central nervous system tumors were determined as HHV-6-positive using nested-PCR (27). A report in 2014 detected HHV-6 DNA in one of 21 (5%) cases of glioblastoma multiforme (28). Chi et al**.** reported that 17 of 40 (42.5%) glioma samples contained the HHV-6 DNA in nested-PCR (29).

Utilization of virus-specific laboratory assays including fluorescence in situ hybridization**,** loop-mediated isothermal amplification and immunohistochemistry could also be effective for clarifying virus replication in the lesion (6, 23, 24, 29). Recently, studies introduced a highly precise diagnostic tool named droplet digital–PCR, which has lower variability and outstanding quantification accuracy (26).

Our results suggest that HHV-6 infection may be just a bystander that creates an immunosuppressive milieu, which could contribute to disease severity. Elucidating whether the virus plays an oncogenic role in pterygium requires a more in-depth investigation.

CONCLUSION

Based on the results, a direct association between HHV-6 and development of pterygium seems less probable, which suggests that other etiologic agents must be involved in the multistep process of the disease.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Sjö NC, Von Buchwald C, Prause JU, Norrild B, Vinding T, Heegaard S. *Human papillomavirus and pterygium. Is the virus a risk factor?* Br J Ophthalmol. 2007; 91(8): 1016-8.

2. Kelishadi M, Moradi A, Bazouri M, Tabarraei A. *Is Epstein-Barr virus associated with pterygium?* Journal of Gorgan University of Medical Sciences. 2014; 16(3): 117-21.

3. Detorakis ET, Sourvinos G, Spandidos DA. *Detection of herpes simplex virus and human papilloma virus in ophthalmic pterygium.* Cornea. 2001; 20(2): 164-7.

4. Faten N, Agnès G-D, Nadia BF, Nabil ABS, Monia Z, Abderrahim K, et al. *Quantitative analysis of human herpesvirus-6 genome in blood and bone marrow samples from Tunisian patients with acute leukemia: a follow-up study.* Infectious agents and cancer. 2012; 7(1): 31. doi: 10.1186/1750-9378-7-31.

5. Reynaud JM, Horvat B. *Human herpesvirus 6 and neuroinflammation.* ISRN Virology. 2013; 2013.

6. Cohen JI, Fahle G, Kemp MA, Apakupakul K, Margolis TP. *Human herpesvirus 6-A, 6-B, and 7 in vitreous fluid samples.* Journal of medical virology. 2010; 82(6): 996-9.

7. Gupta S, Agrawal S, Gollapudi S. *Differential effect of human herpesvirus 6A on cell division and apoptosis among naive and central and effector memory CD4+ and CD8+ T-cell subsets.* Journal of virology. 2009; 83(11): 5442-50.

8. Pitkäranta A, Piiparinen H, Mannonen L, Vesaluoma M, Vaheri A. *Detection of human herpesvirus 6 and varicella-zoster virus in tear fluid of patients with Bell's palsy by PCR.* Journal of clinical microbiology. 2000; 38(7): 2753-5.

9. Lacroix A, Collot-Teixeira S, Mardivirin L, Jaccard A, Petit B, Piguet C, et al. *Involvement of human herpesvirus-6 variant B in classic Hodgkin's lymphoma via DR7 oncoprotein.* Clinical Cancer Research. 2010; 16(19): 4711-21.

10. Flaitz C, Hicks M. *Molecular piracy: the viral link to carcinogenesis.* Oral oncology. 1998; 34(6): 448-53.

11. Steininger C, Rassenti L, Vanura K, Eigenberger K, Jäger U, Kipps T, et al. *Relative seroprevalence of human herpes viruses in patients with chronic lymphocytic leukaemia.* European journal of clinical investigation. 2009; 39(6): 497-506.

12. Lacroix A, Jaccard A, Rouzioux C, Piguet C, Petit B, Bordessoule D, et al. *HHV-6 and EBV DNA quantitation in lymph nodes of 86 patients with Hodgkin's lymphoma.* J Med Virol.. 2007; 79(9): 1349-56. DOI:10.1002/jmv.20868.

13. Kashanchi F, Araujo J, Doniger J, Muralidhar S, Hoch R, Khleif S, et al. *Human herpesvirus 6 (HHV-6) ORF-1 transactivating gene exhibits malignant transforming activity and its protein binds to p53.* Oncogene. 1997;14(3): 359-67. DOI:10.1038/sj.onc.1200840.

14. Pereira CCdL, Vieira SA, Leite EP, Felix GAdL, Torquato JA. *A descriptive study of primary pterygium surgery with fibrin glue.* Rev bras oftalmol. 2013; 72(4): 227-31.

15. Yavarian J, Gavvami N, Mamishi S. *Detection of human herpesvirus 6 in cerebrospinal fluid of children with possible encephalitis.* Jundishapur J Microbiol. 2014; 7(9); e11821. doi: 10.5812/jjm.11821.

16. Rodrigues F, Arruda J, Silva R, Moura K. *TP53 gene expression, codon 72 polymorphism and human papillomavirus DNA associated with pterygium.* Genet Mol Res. 2008; 7(4): 1251-8.

17. Yaisawang S, Piyapattanakorn P. *Role of post-operative topical corticosteroids in recurrence rate after pterygium excision with conjunctival autograft.* Journal of the Medical Association of Thailand= Chotmaihet thangphaet. 2003; 86: S215-23.

18. Van Gelder RN. *Ocular pathogens for the twenty-first century.* Am J Ophthalmol. 2010; 150(5): 595-7. doi: 10.1016/j.ajo.

19. Oberacher-Velten IM, Jonas JB, Jünemann A, Schmidt B. *Bilateral optic neuropathy and unilateral tonic pupil associated with acute human herpesvirus 6 infection: a case report.* Graefes Arch Clin Exp Ophthalmol. 2005; 243(2): 175-7.

20. Boto-de-los-Bueis A, Gómez MPR, del Hierro Zarzuelo A, Sanchez EG, Mediero S, Noval S. *Recurrent ocular surface inflammation associated with human herpesvirus 6 infection.* Eye & contact lens. 2015;41(3):e11-e3. doi: 10.1097/ICL.0b013e3182a70a1b.

21. Sugita S, Shimizu N, Watanabe K, Ogawa M, Maruyama K, Usui N, et al. *Virological Analysis in Patients with Human Herpes Virus 6–Associated Ocular Inflammatory DisordersVirological Analysis for HHV-6 in the Eye.* Invest Ophthalmol Vis Sci. 2012; 53(8): 4692-8. doi: 10.1167/iovs.12-10095.

22. Bajric J, Smith WM. *A case of bilateral human herpes virus 6 panuveitis with genomic viral DNA integration.* J Ophthalmic Inflamm Infect. 2014; 4(1):1. doi: 10.1186/s12348-014-0016-x.

23. Fillet AM, Reux I, Joberty C, Fournier JG, Hauw JJ, Hoang PL, et al. *Detection of human herpes virus 6 in AIDS-associated retinitis by means of in situ hybridization, polymerase chain reaction and immunohistochemistry.* Journal of medical virology. 1996; 49(4): 289-95.

24. Qavi HB, Green MT, Segall GK, Hollinger FB, Lewis DE. *The incidence of HIV-1 and HHV-6 in corneal buttons.* Current eye research. 1991; 10(sup1): 97-103.

25. Chen M, Wang H, Woodworth CD, Lusso P, Berneman Z, Kingma D, et al. *Detection of human herpesvirus 6 and human papillomavirus 16 in cervical carcinoma.* The American journal of pathology. 1994; 145(6): 1509-16.

26. Leibovitch EC, Almira-Suarez MI, Jacobson S. *Human herpesvirus multiplex ddPCR detection in brain tissue from low-and high-grade astrocytoma cases and controls.* Infectious Agents and Cancer. 2016; 11(1): 32. doi: 10.1186/s13027-016-0081-x.

27. Crawford JR, Santi MR, Cornelison R, Sallinen S-L, Haapasalo H, MacDonald TJ. *Detection of human herpesvirus-6 in adult central nervous system tumors: predominance of early and late viral antigens in glial tumors.* Journal of neuro-oncology. 2009; 95(1): 49-60.

28. Chi J, Gu B, Zhang C, Peng G, Zhou F, Chen Y, et al. *Human herpesvirus 6 latent infection in patients with glioma.* Journal of Infectious Diseases. 2012; 206(9): 1394-8. doi: 10.1093/infdis/jis513.

29. Ablashi D, Agut H, Alvarez-Lafuente R, Clark DA, Dewhurst S, DiLuca D, et al. *Classification of HHV-6A and HHV-6B as distinct viruses.* Archives of virology. 2014; 159(5): 863-70.