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Diagnosis and Quantitative Detection of Herpes Simplex Virus DNA in Corneal Ulcers

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ABSTRACT

Herpes simplex keratitis is one of the important causes of blindness and its early diagnosis is essential to the treatment of the respective patients accordingly. The aim of this study was to diagnose and analyze quantitatively the herpes simplex DNA in patients with suspected Herpes keratitis using TaqMan real-time PCR method. Corneal swabs from HSV keratitis suspected patients were collected from September 2005 to December 2009. Upon DNA extraction, the samples were analyzed by quantitative TaqMan real-time PCR assay. In this method, a set of primers amplified a common sequence of HSV-1 and HSV-2 glycoprotein B gene. The copy number of unknown samples were expressed via standard curve drawn with known amount of amplified cloned plasmid containing target sequences. Of the 296 samples, 178 (60.1%) belonged to males and 118 (39.9%) to females. The HSV DNA was detected in 111 (37.5%) of the cases by PCR, consisting of 69 males and 42 females. The drawn standard curve was linear in 10 to 10^6 copies of virus (R2 = 0.982). The ranges of the HSV DNA copy numbers in the collected samples were detected from 2.7×10^3 to 6.1×10^5 copies/samples. Present results suggest that Herpes simplex keratitis remains as an epidemiologically important eye disease and improvement in the monitoring and detecting of ocular herpetic disease by quantitative PCR method is informative and helpful to the clinical diagnosis, decision for treatment and follow up.

Key words: Herpes keratitis, TaqMan real-time PCR, quantitative analysis, viral load

INTRODUCTION

Herpes Simplex Virus (HSV) keratitis is an important cause of ocular morbidity. Corneal HSV infections cause number of clinical manifestations ranging from blepharitis, acute infectious epithelial keratitis to the potentially blinding chronic inflammatory disease herpetic stromal keratitis (Kaye and Choudhary, 2006). Following primary infection through the mouth, nasal cavity and other parts of the facial area including the eyes, HSV may become latent for life in trigeminal ganglion. Depending on the individuals physiologic status and environmental conditions including exposure to the sunlight, presence of stressful and trauma and finally being immuno-compromised, the recurrence of HSV infection may happen to the individuals. The trigeminal ganglion innervates the eye that is, the reactivation may also reach the corneal tissues through anterograde axonal spreading from the trigeminal ganglion (Toma *et al.*, 2008; Ziyaeyan *et al.*, 2007a). Frequent recurrences followed by immunological responses may lead to corneal scarring, thinning, neovascularization (Suryawanshi *et al.*, 2011) and consequently the patient may need to undergo keratoplasty (Knickelbein *et al.*, 2009; Inoue, 2008; Carr and Tomanek, 2006).

Herpes Simplex Keratitis (HSK) can be diagnosed by multiple lab studies, of which the gold standard is the isolation of HSV from the cornea; however, this technique is time consuming and possesses low level of sensitivity (El-Aal *et al*., 2006). Another technique is cytology and detection of intranuclear inclusions and multinucleated giant cells in the slides of the corneal scrapping. Although it is faster, it is not sensitive enough (Subhan *et al*., 2004). Enzyme or fluorescence based immuno-histochemical techniques have acceptable sensitivity, but the interpretation of their consequently results is challenging in most cases and yields false positive results. Polymerase Chain Reaction (PCR) is among the techniques quickly replacing the previous ones. It possesses adequate levels of sensitivity and specificity (Soleimanjahi, *et al*., 2007; El-Aal *et al*., 2006; Amir and Paul, 2006; Subhan *et al*., 2004). Quantitative real-time PCR has a high level of sensitivity and specificity and is time saving, compared to other PCR methods (Fukuda *et al*., 2003). In addition, since it reports the DNA copy number in the involved tissues, it can serve as the direct evidence of infection severity in corneal tissues (Remeijer *et al*., 2009). Clinicians can also monitor the consumption and efficacy of the treatment with anti-viral drug.

In the present study, corneal scraping specimens were used to detect and quantify HSV DNA by the TaqMan quantitative real-time PCR method in the suspected HSK patients.

MATERIALS AND METHODS

Patients and samples collection: Two hundred ninety-six patients clinically suspected with herpetic corneal infection were enrolled in this study. One hundred seventy-eight of the patients were male and 118 were female and their ages ranged from 11 months to 89 years, with a mean of 57.2 years.

The samples consisting of corneal epithelial scrapings in 500 µL of Viral Transport Media (VTM) and were submitted to the Professor Alborzi Clinical Microbiology Research Center, Namazi Hospital, Shiraz, Iran between September 2005 and December 2009 for HSV keratitis diagnosis. The in-house prepared VTM contained Hanks BSS with 1% bovine serum albumin, sodium bicarbonate, phenol red, penicillin and gentamicin. Swab samples were stored at -20°C until DNA extraction. The study was performed based on Declaration of Helsinki protocol and approved by the local Ethics Committee, with informed consents obtained from all the patients.

Nucleic acid extraction: DNA was extracted from 200 µL of VTM using spin-column based QIAamp Mini Kit (Quiagen, Hilden, Germany), according to the manufacture`s instructors.

Real-time PCR: The real-time quantitative PCR was performed with oligonucleotide primer pairs and probe specific for the type-common region of HSV-1 and HSV-2 glycoprotein B (gB), as reported previously (Ryncarz *et al*., 1999). The primers used were HSV-FP (5´-TCC CGG TAC GAA GAC CAG-3´) and HSV-RP (5´-AGC AGG CCG CTG TCC TTG-3´) and the probe was HSV-TCP (5´-FAM-TGG TCC TCC AGC ATG GTG ATG TTG/C AGG TCG-TAMRA-3´). Amplification was carried out in an Applied Biosystem Sequence Detector 7500 machine, programmed for a four-step protocol: 2 min of incubation at 50°C for AmpErase activation, 10 min at 95°C for polymerase activation and for 45 cycles: 15 sec at 94°C for denaturation, 60 sec at 58°C for annealing, extension and data collection. Each 50 µL-PCR mixture contained 10 µL of purified DNA, 840 nM concentrations of each primer and 100 nM probe in 1x TaqMan universal PCR master mix (Applied Biosystems, Branchburg, New Jersey USA). Negative controls were included in the extraction process between every 20 clinical samples. All the negative samples were tested twice (Ziyaeyan *et al*., 2011).

Target amplicons were produced based on a standard PCR protocol by the above-mentioned primers set and separated on agarose gel, then extracted and purified from gel and cloned into the pTZ57R vector (Fermentas UAB, Vilnius, Lithuania) (Sambrook and Russell, 2001). The plasmid containing target sequences was purified using a commercial kit (Fermentas UAB, Vilnius, Lithuania) and its concentration was determined spectrophotometrically. Quantification was carried out by drawing standard curves using the serially diluted pTZ57R plasmid that contained the target amplification product. Six positive control standards at 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 copies/reaction were used. The drawn standard curve was linear in 10 to 10^6 copies/reaction of the virus (R^2 = 0.98). The copy numbers of clinical samples were calculated automatically by applied biosystem sequence detection software v 1.3.1.

Statistical analysis: Descriptive statistics and laboratory data were analyzed using the SPSS statistical software package (version 15). Pearson correlation test was used to detect the association between viral quantity and the age. Fisher's exact test was used to analyze the relationship between PCR results and sex and t-test for that between PCR status and age.

RESULTS

From a total of 296 clinical specimens, one hundred and eleven (37.5%) corneal scraping samples tested positive for HSV DNA by real-time PCR. These positive patients consist of 69 males and 42 females. The copy number of HSV DNA, measured by the real-time PCR assay, ranged from 2.7×10^3 to 6.1×10^5 copies/500 μL of VTM, Mean±SD = $1.1\times10^5\pm1.5\times10^5$.

However, as shown in Fig. 1, a relationship exists between age and copy number .There was no relationship between gender and PCR status (p = 0.581, Fisher's exact test). There were significant differences in age among patients with PCR positive results and those with negative

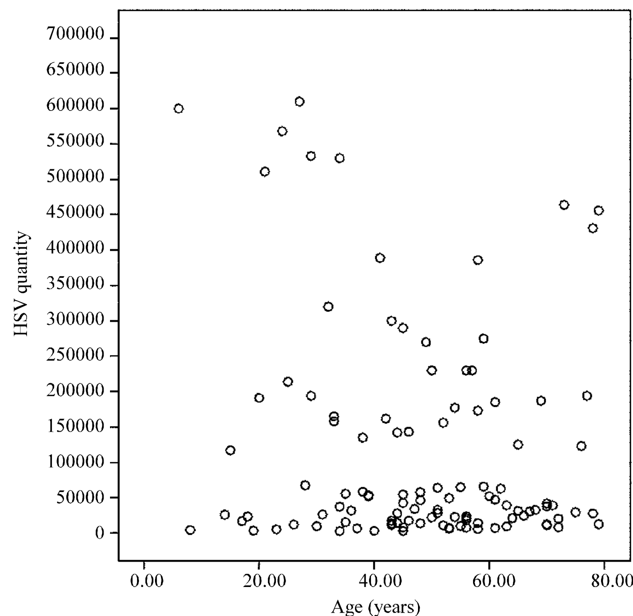

Fig. 1: Age distribution of 111 patient with positive real-time PCR result and their HSV DNA copy numbers/500 μL viral transport medium

results (The mean ages were 48.4±17.1 years and 39.5±20.8 years among PCR positive and PCR negative patients, respectively $p = 0.0001$, t-Test). No significant correlation between age and load of HSV DNA was found ($p = 0.079$, Pearson's correlation test) (Fig. 1).

DISCUSSION

Thanks to its efficacy, high accuracy and early detection, PCR has replaced most previous conventional methods of HSV ocular infection diagnosis (Kakimaru-Hasegawa *et al*., 2008). It seems that the results of quantitative analysis of HSV DNA in herpetic ulcers, along with monitoring of HSV DNA levels could be used to evaluate the efficacy of antiviral therapy. The patients enrolled in the present study, were all with suspected HSK and referred to the clinics for HSV DNA diagnosis in their eye lesions. Of the 296 cases, 111 (37.5%) were found to be with HSV DNA. There have been very few reports on highly frequent HSV asymptomatic shedding in ocular surfaces of healthy people with no corneal HSV involvement, but many reports on shedding in saliva (Kaufman *et al*., 2005). However, the use of well standardized PCRs can prevent the effects of false positive results on the PCR results to a large extent (Leigh *et al*., 2008). The fact that quantitative real-time-PCR can both detect HSV DNA and evaluate the respective load can help follow the ACV treatment course. Although resistance to ACV is reported in HSK (Choong *et al*., 2010), the studies indicate that HSV resistant strains are very limited in the region and in immunocompetent individuals (Ziyaeyan *et al*., 2007b). Yet, some recent studies have demonstrated the possibility of isolating resistant and sensitive strains from herpes-caused corneal lesions at different intervals (Duan *et al*., 2009). Fortunately, HSK is usually unilateral and is responsive to the conventional medications with low toxicity (Hill and Clement, 2009; Tabbara and Al Balushi, 2010).

The results of the present study indicate that HSV DNA diagnosis in corneal ulcers is related to the patient's age, i.e., the older the age of those suspected with herpetic corneal ulcers, the greater the chance of HSV DNA diagnosis in them. Although primary HSV infection in the region occur mainly during childhood, the majority of HSK cases are due to the recurrence of latent infections which aggravate the respective sufferer's conditions at older age, as demonstrated in the current study.

On the other hand, it was observed that the increased HSV copy numbers were not significantly related to age in the patients with positive HSV DNA PCR. However, as shown in Fig. 1, a relationship exists between age and copy number. On the contrary, the HSV copy numbers in 6 patients aged sequentially as follows, were significantly high; (1) male 34 years, HSV copies: 5.3×10^5; (2) male 29 years, HSV copies: 5.3×10^5; (3) male 27 years, HSV copies: 6.1×10^5; (4) male 24 years, HSV copies: 5.6×10^5; (5) male 21 years, HSV copies: 1.1×10^5 and (6) female 6 years, HSV copies: 6×10^5. Therefore, the relationship between the older age and higher copy numbers in HSV patients is fading. We can then suggest that the reason for the higher titer of HSV in these 6 patients might be initial herpetic infection. High levels of HSV DNA in patient's samples may indicate active HSV reproduction in lesions resulting in corneal disease. Although the damage to the cornea is not remarkable in cases with primary herpetic infections in the eyes, it seems that the titer of the virus is high. In the present study, the quantitative R-T PCR, used with fluorescent probe, possessed optimal sensitivity and specificity and could enhance HSK diagnosis. As shown, the early and on time ACV therapy can remarkably reduce the viral titer of the herpetic ulcers (Deai *et al*., 2004) whcih is very helpful to HSK treatment course. Our results suggest that HSK remains as an epidemiologically important eye disease and improvement in the monitoring and

detecting of ocular herpetic disease by quantitative PCR method is informative and helpful to the clinical diagnosis, decision for treatment and follow up.

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