Real-time PCR and multiplex real-time PCR for ocular infections by human herpes viruses

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Abstract

Currently, there are 9 human herpes viruses known that remain latent at various sites of the human body, and some of them could become reactivated and trigger various diseases. We have been applying the real-time polymerase chain reaction (PCR) method to help clinicians diagnose ocular surface infection, especially herpetic keratitis. In the current study, we applied multiplex real-time PCR to quantify herpes simplex virus 1 (HSV-1), Human herpesvirus 6 (HHV-6), and Human herpesvirus 7 (HHV-7) DNA in tears taken before and after cataract surgery and penetrating keratoplasty (PKP), and aqueous humor collected at the beginning of cataract surgery. This is the first report in the field of ophthalmology on multiplex real-time PCR carried out using ocular samples to quantify HSV-1, HHV-6, and HHV-7 DNA.

Key words:

There are presently 9 human herpes viruses known to remain latent at various sites of the human body, and some of them could become reactivated and trigger various diseases. In the field of ophthalmology, herpes simplex virus (HSV-1) has long been studied as a pathogen that causes herpetic keratitis, conjunctivitis, and endothelitis. Some reports stated that cytomegalovirus (CMV) and human herpesvirus 7 (HHV-7) also caused endothelitis. Okuno et al. reported that HHV-6 DNA was detected by polymerase chain reaction (PCR) in tears of patients with dry eye, allergic conjunctivitis, and keratitis. Sugita et al. reported that HHV-6 DNA was detected in the vitreous humor of 2 out of 500 patients with uveitis or endophthalmitis, and that HHV-7 DNA was not detected in the 500 patients by multiplex PCR. Thus, molecular biological techniques have been used to investigate the relationship between human herpes viruses and ocular infections.

PCR was devised by Mullis et al., for which they were awarded the Nobel Prize. It is now widely performed, even in college students' training laboratories. The PCR method can selectively amplify a specific DNA fragment from a small amount of DNA. The time required for amplification is only about 2 hours. In general, electrophoresis is necessary after DNA amplification. PCR is a qualitative examination, not quantitative one.

Generally speaking, real-time PCR should be used for DNA quantification. Electrophoresis is not required when real-time PCR is used. Real-time PCR is classified into two methods: the fluorescent-labeled probe method and intercalator method. The fluorescent-labeled probe method includes the Linear Probe (such as TaqMan probe) method, Structured probe (such as the Molecular Beacon probe) method, and Cycling probe method. On the other hand, SYBR® Green I etc. are added to the PCR reaction system in the intercalator method.

We performed real-time PCR by the Linear Probe method using a TaqMan probe for herpetic keratitis cases. The subjects were 56 patients with herpetic keratitis: epithelial keratitis in 27 eyes; persistent epithelial defect in 6; active disciform stromal keratitis in 14; silent stromal keratitis in 6; and endothelitis in 3.
All 27 epithelial keratitis samples were positive for HSV DNA (6.4±4.4×10⁶ copies/sample). In those with a persistent epithelial defect, HSV DNA was detected in all 6 samples (8.5±3.3×10⁴ copies/sample). In active disciform stromal keratitis, HSV DNA was positive in 8 of the 14 affected eyes (1.4±1.1×10⁷ copies/sample). Although it has been regarded as extremely difficult to detect HSV-1 by viral culture in disciform keratitis patients, HSV DNA was quantified in 8 of the 14 eyes. However, patients in whom HSV was not suspected to be involved may show a small amount of positive HSV DNA. In such cases, quantitative results of HSV DNA can be helpful in diagnosing whether or not the patients have herpetic keratitis.

In recent years, an increasing number of research laboratories perform real-time PCR for identifying a causative virus of uveitis. Our group has performed real-time PCR using a cycling probe to adjunctively diagnose bacterial, fungal, or Acanthamoeba keratitis. Cycling PCR uses a chimeric DNA-DNA probe with a strand length of 10 to 14 base pairs. As compared with a linear or structured probe, a cycling probe is shorter. When a mismatch is present in the vicinity of the RNA in the cycling probe, the cleavage by RNaseH does not occur. This PCR can detect the difference of a single nucleotide.

One of the advantages of real-time PCR is its ability to quantify. However, when multiple viruses, bacteria, or fungi are quantified, the same number or more reaction tubes are necessary. On the other hand, multiplex PCR can detect more than one kind of DNA in a single test tube. Sugita et al. analyzed ocular samples firstly by multiplex PCR and secondly by quantitative real-time PCR for HHV-1 through HHV-8. If multiplex PCR results were positive, then real-time PCR was conducted. Strictly speaking, these methods were not multiplex real-time PCR.

Here we report the outcomes of multiplex real-time PCR applied to quantify HSV-1, HHV-6, and HHV-7 DNA in tears and aqueous humor. This is the first report in the field of ophthalmology of multiplex real-time PCR carried out using ocular samples to quantify HSV-1, HHV-6, and HHV-7 DNA.

Fig. 1 The standard curve plot of HHV-6 with multiplex real-time PCR.

Fig. 2 The amplification plot of HHV-6 with multiplex real-time PCR.
PCR for ocular infections by HHV

Table 1  DNA copy numbers detected in the tears before and after PEA+IOL

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Pre-Op</th>
<th>Post-Op</th>
<th>HSV-1</th>
<th>HHV-6</th>
<th>HHV-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62, F</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>3.1×10^1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>81, M</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>2.0×10^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>70, F</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>3.2×10^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>70, F</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>90, M</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>73, M</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>2.5×10^1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>75, M</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>72, F</td>
<td>Pre-Op</td>
<td>3.5×10^6</td>
<td>1.6×10^2</td>
<td>(−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>85, F</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>65, M</td>
<td>Pre-Op</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Subjects were 23 patients who underwent phacoemulsification, aspiration, and intraocular lens implantation (PEA+IOL) or penetrating keratoplasty (PKP). Twenty tear samples of 10 eyes receiving PEA, 8 samples of aqueous humor from another 8 eyes receiving PEA, and 10 tear samples of 5 eyes receiving PKP were used. None of the patients had a history of herpetic eye diseases.

The sequences of the primers and TAMRA® probes used in the multiplex real-time PCR assay were the same as those designed by Wada et al.24 In the current study, probes were quenched with TET, FAM, or VIC. ABI PRISM® 7900HT Sequence Detector (Applied Biosystems, Foster City, CA, USA) was used for DNA quantification.

Figures show the standard curve plot (Figure 1) and representative amplification plot (Figure 2) of HHV-6 with multiplex real-time PCR.

Table 1 shows the DNA copy number detected in the tears before and after PEA+IOL. The maximum amounts of DNA detected were 1.6×10^2 copies/sample for HHV-6 and 2.0×10^3 copies/sample for HHV-7 (Table 1). No DNA of HSV-1, HHV-6, or HHV-7 was detected in any aqueous humor collected from another 8 eyes receiving PEA (data not shown). The DNA copy numbers before and after PKP in the tears are shown in Table 2.

The results indicated that HSV-1, HHV-6, and HHV-7 DNA were present in the ocular surface (Tables 1, 2) before and after eye surgery. However, the amount of DNA detected was small. There is a possibility that viral DNA reactivated from a latent state was quantified.25

Human herpes viruses are known to remain latent at various sites of the human body.26 We suggest that multiplex real-time PCR is convenient and useful to clarify the clinical pathogenesis of HHV in the eye.

Acknowledgements

We thank Ms. Yukiko Mimuro for editing the manuscript and Ms. Mayumi Mizuno for technical assistance.

References

2. Holland EJ, Schwartz GS (1999) Classification of
Table 2  DNA copy numbers detected in the tears from PKP

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Disease</th>
<th>Pre-Op</th>
<th>HSV-1</th>
<th>HHV-6</th>
<th>HHV-7</th>
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<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>F</td>
<td>BK</td>
<td>Pre-Op</td>
<td>(—)</td>
<td>(—)</td>
<td>(—)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post-Op</td>
<td>(—)</td>
<td>9.3×10^6</td>
<td>(—)</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>M</td>
<td>KC</td>
<td>Pre-Op</td>
<td>(—)</td>
<td>(—)</td>
<td>(—)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post-Op</td>
<td>(—)</td>
<td>(—)</td>
<td>(—)</td>
</tr>
<tr>
<td>3</td>
<td>79</td>
<td>M</td>
<td>BK</td>
<td>Pre-Op</td>
<td>(—)</td>
<td>(—)</td>
<td>(—)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post-Op</td>
<td>(—)</td>
<td>(—)</td>
<td>(—)</td>
</tr>
<tr>
<td>4</td>
<td>78</td>
<td>F</td>
<td>BK</td>
<td>Pre-Op</td>
<td>(—)</td>
<td>(—)</td>
<td>9.1×10^9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post-Op</td>
<td>(—)</td>
<td>(—)</td>
<td>(—)</td>
</tr>
<tr>
<td>5</td>
<td>61</td>
<td>M</td>
<td>BK</td>
<td>Pre-Op</td>
<td>(—)</td>
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<td>3.1×10^9</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post-Op</td>
<td>(—)</td>
<td>(—)</td>
<td>(—)</td>
</tr>
</tbody>
</table>

BK : Bullous keratopathy, KC : Keratoconus
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herpes simplex virus keratitis. Cornea 18: 144–154
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