Comparison of Real-Time and Nested PCR Assays for Detection of Herpes Simplex Virus DNA

Jun-ichi Kawada¹, Hiroshi Kimura* ¹, Yoshinori Ito¹, Yo Hoshino¹, Naoko Tanaka-Kitajima², Yoshihiro Ando³, Masahide Futamura⁴, and Tsuneo Morishima⁵

¹Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Aichi 466–8550, Japan, ²Department of Pediatrics, Nagoya Memorial Hospital, Nagoya, Aichi 468–0011, Japan, ³Department of Pediatrics, Aichi Children’s Health and Medical Center, Aichi 474–0031, Japan, ⁴Department of Neonatology, Aichi Prefectural Colony Hospital, Aichi 486–0392, Japan, ⁵Department of Nursing, Nagoya University School of Health Science, Nagoya, Aichi 461–8673, Japan

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Abstract: We performed a real-time PCR assay to detect herpes simplex virus (HSV) DNA, and compared it prospectively with a nested PCR assay in 164 clinical samples (109 cerebrospinal fluid and 55 sera) from patients suspected of having neonatal HSV infection or HSV encephalitis. In 25 of 164 samples, HSV DNA was detected by the nested PCR assay. All samples positive for HSV DNA in the nested PCR assay were also positive in the real-time PCR assay, and all but two samples negative for HSV DNA in the nested assay were negative in the real-time assay. The real-time PCR assay thus had a sensitivity of 100% and a specificity of 99%, when compared with the nested assay. Sequential assays in a case of disseminated HSV showed that a decrease in HSV DNA paralleled clinical improvement. Quantification of HSV DNA by real-time PCR was useful for diagnosing and monitoring patients with HSV encephalitis and neonatal HSV infection.

Key words: Herpes simplex virus, Real-time PCR, Nested PCR, Herpes simplex virus encephalitis

The development of effective antiviral therapy has resulted in an increased need for the rapid and sensitive diagnosis of herpes simplex virus (HSV) infections (7, 8). Because of the varied clinical manifestations of HSV and its similarity to other mucocutaneous and central nervous system infections, accurate diagnosis is key to its effective clinical management. HSV DNA detection in cerebrospinal fluid (CSF) is used to define central nervous system infection (3, 9, 15), and PCR testing of CSF has become the gold standard (18). Bloodstream spread of HSV with resultant widely disseminated disease occurs mainly in the newborn (9). PCR assays of CSF and blood are critical to the diagnosis of neonatal HSV infection, especially in the absence of skin lesions (12).

Qualitative PCR assays, such as nested PCR or PCR coupled with Southern blot hybridization, have been used to diagnose infection, as they are specific and sensitive enough to detect HSV DNA in CSF. On the other hand, a quantitative system using a real-time PCR assay has recently been developed to measure the viral load of HSV (1, 4, 6, 10, 17). It is expected that the real-time PCR assay will be useful for diagnosing HSV infection and for monitoring patients during antiviral therapies. Quantification of viral load may be useful for assessing the prognosis, and may provide additional information for management of HSV infection (10). It is shown that a real-time PCR assay is more sensitive than a cell culture for the detection of HSV (17). Some studies compared the sensitivities of real-time PCR and conventional PCR (6). However, it is not clear whether real-time PCR is sensitive enough when compared with conventional PCR in clinical samples. To evaluate the usefulness of real-time PCR for diagnosing and monitoring patients with HSV encephalitis (HSE) and neonatal HSV infection, we prospectively compared results of real-time PCR and nested PCR, using clinical samples.

Materials and Methods

Patients and samples. Between January 1998 and August 2003, 101 patients (67 with suspected HSE and 34 with suspected neonatal HSV infection) were prospectively enrolled. All had been referred to the Nagoya University Graduate School of Medicine for the diagnosis of HSV infection. All the specimens were obtained after

Abbreviations: CSF, cerebrospinal fluid; HSE, herpes simplex virus encephalitis; HSV, herpes simplex virus.
Table 1. Comparison of nested PCR and real-time PCR assays for the detection of herpes simplex virus DNA

<table>
<thead>
<tr>
<th></th>
<th>Cerebrospinal fluid (n=109)</th>
<th>Serum (n=55)</th>
<th>Total (n=164)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested PCR</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>16</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>93</td>
<td>2</td>
</tr>
</tbody>
</table>

Results

Results of the nested and real-time PCR assays are shown in Table 1. All CSF and serum samples positive for HSV DNA in the nested assay were also positive in the real-time assay, and all but two samples negative for HSV DNA in the nested assay were also negative in the real-time assay. Real-time PCR had a sensitivity of 100% (25/25) and a specificity of 99% (137/139), as compared to nested PCR. The copy number of HSV DNA, measured by the real-time PCR assay, ranged from 1.0×10^2 to 3.1×10^3 copies/ml in CSF and from 1.0×10^2 to 2.5×10^3 copies/ml in serum samples. The copy number of two serum samples in which HSV DNA was positive by the real-time PCR assay but negative by the nested assay were 1.0×10^2 and 1.3×10^2 copies/ml, respectively. From the results of these PCR assays, 10 patients were diagnosed with neonatal HSV infection (4 were disseminated form, 4 were central nervous system form, and 2 were skin-localized form) and 5 patients were diagnosed with HSE.

Sequential samples from a neonate with the disseminated form of HSV infection were obtained, and tested

informed consent from the parents. A total of 164 samples (109 CSF and 55 sera) were obtained. DNA was extracted from 200 µl of CSF or serum, using a QIAamp Blood Kit (QIAGEN, Hilden, Germany), and eluted in 50 µl of distilled water.

Nested PCR assay. The nested PCR assay was performed using the following primers: 5′-outer primer, 5′-CAGTACGGCCCCAGTTCTGACC5′; 3′-outer primer, 5′-GGCGTATGGCAGGCTGCGC-3′; 5′-inner primer, 5′-TACAACATCTCAACTCTG-3′; 3′-inner primer, 5′-CTCTCTCTGT5′-C-CTTCAGGACGG-3′. These HSV sequences are located in the UL30 gene, which encodes the viral DNA polymerase. Ten µl of the DNA solution was added to a reaction mixture consisting of PCR Buffer (TaKaRa, Ohtsu, Japan), 200 µM dATP, dCTP, dGTP, dTTP, 1 µM each of the outer primers, and 1.25 U Taq polymerase (TaKaRa). Following 5 cycles of 20 sec at 97°C and 1 min at 58°C, 45 cycles of 20 sec at 96°C and 1 min at 58°C were carried out using a Model 7700 Sequence Detector (Applied Biosystems). Real-time fluorescence measurements were made, and the threshold cycle value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation for the base line) (11). Standard curves were constructed using the values obtained from serially diluted positive control plasmids that contained UL 30 gene. The values from clinical samples were plotted on the standard curve, and the copy number was calculated by automatically using the Sequence Detector v1.6 (Applied Biosystems). A minimum of five copies of either HSV-1 or HSV-2 DNA was detected by the real-time PCR assay.

Real-time PCR assay. The primers and fluorogenic probe for this assay are also from the UL30 gene (5). The upstream and downstream primer sequences were 5′-ACATCATCAACTCTGACCG-3′ and 5′-CTCGAGTC-CTTTTCTTGTC5′-CCTTCAGGACGG-3′, respectively. A fluorogenic probe consisted of an oligonucleotide sequence 5′-ATG-GTGAACATCGACATGTACG-3′ located between the PCR primers. The real-time quantitative PCR assay was carried out using a Taq Man PCR kit (Applied Biosystems, Foster City, U.S.A.), as described previously (5). Briefly, 10 µl of DNA extraction solution from the samples was added to a PCR mixture containing Buffer A (Applied Biosystems), 5 mM MgCl₂, 200 µM dATP, dCTP, dGTP, dTTP, 200 nM each primer, 100 nM fluorogenic probe, and 1.25 U of AmpliTaq Gold (Applied Biosystems). Following 5 cycles of 20 sec at 97°C and 1 min at 58°C, 45 cycles of 20 sec at 96°C and 1 min at 58°C were carried out using a Model 7700 Sequence Detector (Applied Biosystems). Real-time fluorescence measurements were made, and the threshold cycle value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation for the base line) (11). Standard curves were constructed using the values obtained from serially diluted positive control plasmids that contained UL 30 gene. The values from clinical samples were plotted on the standard curve, and the copy number was calculated by automatically using the Sequence Detector v1.6 (Applied Biosystems). A minimum of five copies of either HSV-1 or HSV-2 DNA was detected by the real-time PCR assay.
using real-time PCR (Fig. 1). The patient was a nine-
day-old boy, admitted to hospital because of fever and hepatic dysfunction. HSV DNA was detected in serum (1.6×10^5 copies/ml) and CSF (1.0×10^5 copies/ml). He was treated with acyclovir (45 mg/kg/day) and the viral load decreased as symptoms resolved. He was discharged from the hospital on day 32, and no sequelae were noted.

We compared viral loads in CSF of neonates with central nervous system HSV infection and in older patients with HSE using the real-time PCR assay. In addition to 9 CSF samples that were positive in this prospective study, we assayed 10 samples that had tested positive by nested PCR in our laboratory between 1987 and 1998. A total of 10 neonates (7–22 days old, median age of 10 days) and 9 older patients with HSE (4 months–35 years old, median age of 3 years) were examined. The real-time PCR assay showed that viral loads were significantly higher in the neonates with central nervous system HSV infection than in the older patients with HSE (10^{4.4±0.3} vs. 10^{2.8±0.2} copies/ml, mean ± standard error) (Fig. 2).

Discussion

To diagnose HSE and neonatal HSV infection, conventional PCR assays such as nested PCR or PCR coupled with Southern blot hybridization have been used (3, 9, 13–16). However conventional PCR techniques are relatively cumbersome, prone to contamination, and not suited to quantification. Recently, real-time PCR has begun to demonstrate its potential utility in the field of clinical virology, specifically in the detection of herpesvirus DNA. In comparisons with conventional PCR, the main advantages of real-time PCR are speed and quantitativeness. Furthermore, a real-time PCR assay can reduce the likelihood of contamination, because no post-amplification analysis (using amplified products) is required. On the other hand, a real-time PCR assay can be slightly less sensitive than a nested PCR assay (6, 11). In this study, the real-time PCR assay was as sensitive as the nested PCR assay when control plasmids were used as standards (five vs. five copies/tube).

The real-time PCR assay showed no false-negative results in this study, indicating that it can be a valuable tool in the diagnosis of HSE and of neonatal HSV infection. The two serum samples in which HSV DNA were detected from neonates with the skin-localized form of neonatal HSV infection, with a vesicle that was positive in an HSV antigen test using a fluorescent antibody (Microtrak HSV-1/HSV-2; Syva, Palo Alto, U.S.A.). Seroconversion of HSV-specific IgM antibody for HSV was confirmed. The other neonate was diagnosed as having a skin-localized form of neonatal HSV infection because HSV DNA was detected in CSF by both real-time and nested PCR assays. It has been reported that HSV DNA could be detected from sera in 27% and 86% of neonates with the skin-localized form of
and central nervous system form of HSV infection, respectively (10). Our data may thus represent a false-negative result of the nested PCR assay, rather than a false-positive result of the real-time PCR assay.

We previously quantified viral loads in CSF by conventional PCR methods, and showed that viral loads were significantly higher in neonates with central nervous system HSV infection than in older patients with HSE (2). To confirm this, we compared viral loads in CSF using the real-time PCR assay. Again, the real-time PCR assay showed that viral loads were significantly higher in the neonates with central nervous system HSV infection than in the older patients with HSE. Differences may be due to the permissiveness of immature brain cells for replication or to an immature immune response. The pathogenesis of HSE differs from that of neonatal HSV infection. Most cases of HSE are caused by the reactivation of HSV from trigeminal ganglia, and HSE is usually a focal encephalitis, with localization of infection to the temporal lobe (18). On the other hand, neonatal HSV infection leads to meningoencephalitis, with diffuse swelling of the brain. HSV may be spread via viremia in neonates with a central nervous system HSV infection. The differences in viral load may reflect the differences in pathogenesis. We believe that quantification of viral loads will be useful in clarifying the pathogenesis of HSV infection. Furthermore, we showed the case of neonatal HSV infection whose sequential samples were obtained and viral loads were measured using the real-time PCR assay. In this case, viral load decreased as symptoms resolved. It shows that the real-time PCR assay is useful for both the diagnosis and the evaluation of antiviral therapy in neonatal HSV infection.

In conclusion, real-time PCR is a reliable method for detecting HSV DNA in CSF and serum, with high sensitivity and specificity, comparable to that of conventional PCR assays. Monitoring viral load by real-time PCR is useful for the evaluation of antiviral therapy, and may contribute to the clarification of the pathogenesis of HSV infection. With its convenience and rapidness, real-time PCR might be more suitable for clinical use than conventional PCR assays.

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