Detection of Herpes Simplex Virus DNA by Real-Time PCR

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Molecular detection of herpes simplex virus (HSV) DNA is recognized as the reference standard assay method for the sensitive and specific diagnosis of central nervous system infections caused by HSV. In this study, a molecular assay based on real-time PCR on the LightCycler (LC) instrument was compared with a home-brew molecular assay. The detection limit of the LC assay was determined with 10-fold dilutions of plasmid pS4 with the SalI restriction fragment of the DNA polymerase gene and with the First European Union Concerted Action HSV Proficiency Panel. A total of 59 cerebrospinal fluid (CSF) specimens were investigated for the comparative study. With plasmid pS4, the detection limit of the LC assay was found to be 10 copies per ml, i.e., 12.5 copies per run. When samples of the First European Union Concerted Action HSV Proficiency Panel were tested, 2 x 10^3 to 5 x 10^3 HSV type 1 genome equivalents (GE) per ml, i.e., 2.5 to 6.3 GE per run, could consistently be detected. There was a correlation between the LC assay and the home-brew assay in 55 of 59 specimens. In conclusion, the LC assay allows very rapid detection of HSV DNA in CSF. It was found to be laborsaving and showed sufficient sensitivity.

Materials and Methods

Study design. In the first step, a plasmid (pS4), kindly provided by K. W. Knopf, German Cancer Research Center, Heidelberg, Germany, containing a single copy of a SalI restriction fragment of the HSV polymerase gene from the HSV type 1 (HSV-1) strain Angelotti served as a standard for the determination of the detection limit. Tenfold dilutions of the plasmid were subjected to the molecular assay based on a rapid DNA extraction protocol and to real-time PCR on the LC instrument. Experiments were repeated five times on different days.

In the second step, the First European Union Concerted Action HSV Proficiency Panel, which contained different concentrations of HSV type 1 strain Macnichtre (American Type Culture Collection), HSV type 2 strain MS (American Type Culture Collection), and negative samples, was used (Table 1). Samples were tested five times on different days by the home-brew molecular assay including real-time PCR on the LC instrument and, except for vial no. 5, two times on different days by the home-brew assay.

In the third step, a total of 59 cerebrospinal fluid (CSF) specimens were investigated in a comparative study. All specimens had been collected from patients who were admitted to University Hospital, Frankfurt am Main, Germany, and had been obtained prior to the start of therapy. CSF samples were divided into aliquots. One aliquot was routinely investigated with a home-brew molecular assay at the Institute of Medical Virology, Frankfurt am Main, Germany. Each positive result was confirmed by a second PCR run. Another aliquot was stored frozen at −70°C and later sent to the Institute of Hygiene, Graz, Austria, for blind investigation with the new molecular assay. Each sample was run twice with real-time PCR on the LC instrument.

DNA extraction. For the home-brew molecular assay, a DNA blood kit (Qiagen, Hilden, Germany) that required 200 μl of sample was used according to the manufacturer’s instructions. For the real-time PCR assay, a rapid DNA extraction protocol was used. In a 1.5-ml tube, 100 μl of sample was added to 300 μl of a suspension consisting of 20% (wt/vol) Chelex 100 resin (Bio-Rad Laboratories, Richmond, Calif.) in 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA–0.1% sodium azide. After the tube sample was vortexed for 10 s, incubated at 100°C for 10 min, and vortexed for another 10 s, the tube was allowed to cool to room temperature. Following complete settlement of the resin, 5 μl of the supernatant was directly used for amplification.

Primer design. For the home-brew molecular assay, oligonucleotides deduced from the published sequence of the glycoprotein D region of the HSV genome were used (16, 27). This set of primers has recently been used for diagnosis of herpes simplex virus encephalitis by nested PCR (20, 21). The primer and probe sequences and characteristics are shown in Table 2.

For the real-time PCR assay, oligonucleotides deduced from the published sequence of the DNA polymerase gene-coding region from HSV were used (15, 25). This set of primers, which was chosen within a highly conserved region of the DNA polymerase gene from the herpesvirus group, allows amplification of a 92-bp fragment from each of the HSV-1 and HSV-2 DNA polymerase genes in clinical samples (2, 3). The TaqMan probe (TIB MOLBIOL, Berlin, Germany)
was labeled with 6FAM at the 5' end and with TAMRA at the 3' end. The primer and probe sequences and characteristics are shown in Table 3.

**Real-time PCR on the LC instrument.** The real-time PCR was performed on the specially designed LC instrument (Roche Diagnostics, Mannheim, Germany). Evaluation of the different assay formats has been described in detail elsewhere (11). For the present study, all samples were tested by the LC- DNA Master Hybridization Probes assay (Roche Diagnostics) using a TaqMan probe (Table 3). Additionally, the hot start technique was used. TaqStart antibody (Clontech, Palo Alto, California) was added directly to the 10× DNA Master solutions, and the mixtures were incubated at room temperature for 5 min. Then, MgCl₂ primers, TaqMan probe, and water were added. Fifteen microliters of master mix and 5 μl of DNA template were added in each capillary. Sealed capillaries were centrifuged in a microcentrifuge and placed into the LC rotor. After denaturation for 2 min at 95°C, 55 PCR cycles were run.

**RESULTS**

When tenfold dilutions of plasmid pS4 were tested by real-time PCR on the LC instrument, the detection limit was found to be 10³ copies per ml, i.e., 12.5 copies per LC PCR run. With the dilution containing 10⁴ copies per ml, i.e., approx. 1 copy per LC PCR run, the assay employed produced inconsistent negative and positive results.

When samples of the First European Union Concerted Action HSV Proficiency Panel were tested with the real-time PCR assay, 2 × 10³ to 5 × 10⁵ HSV-1 genome equivalents (GE) per ml, i.e., 2.5 to 6.3 GE per LC PCR run, could consistently be detected. With the dilution containing 0.7 × 10³ to 1.7 × 10⁵ HSV-1 GE per ml (vial no. 12), i.e., 1 to 2 GE per LC-PCR run, the assay produced inconsistent (negative and positive) results. When HSV-2 samples from the same panel were tested, 2 × 10⁴ to 5 × 10⁵ GE per ml, i.e., 25 to 62.5 GE per LC PCR run, could consistently be detected, whereas 2 × 10³ to 5 × 10³ HSV-2 GE per ml (vial no. 3), i.e., 2.5 to 6.3 GE per LC PCR run, were not detected at all. With the home-brew assay, 2 × 10⁸ to 5 × 10⁹ and 2 × 10⁹ HSV-2 GE per ml, i.e., all the positive samples of the First European Union Concerted Action HSV Proficiency Panel, could be detected.

From a total of 59 CSF samples, 20 were repeatedly found to be positive by real-time PCR on the LC instrument and by the home-brew PCR assay and 35 were found to be negative by both PCR assays (Fig. 1). Four samples yielded discrepant results: two of them were positive with the home-brew PCR assay and negative with the real-time PCR assay, and the other two were positive with the real-time PCR assay and negative with the home-brew PCR assay (Table 4). Upon repetition, one of the samples that had been positive with the real-time PCR assay and another one that had been positive with the home-brew PCR assay both yielded negative results. The remaining two samples were repeatedly positive or negative.

The real-time PCR assay on the LC instrument proved to be very quick and laborsaving. The whole procedure could be finished within less than 1 h. The time required for DNA isolation was 15 min, followed by 10 min for pipetting and another 30 min for cycling and detection combined. In contrast, the home-brew PCR assay took about 5 h. Thirty minutes were required for DNA extraction, 4 h were required for nested amplification, and another 30 min were required for detection by gel electrophoresis.
DISCUSSION

In the present study, a real-time PCR assay on the LC instrument was evaluated and compared to a conventional home-brew PCR assay. Compared with the home-brew assay, the detection limit of the LC assay was found to be one log unit higher. For the LC assay, 100 μl of sample was used for DNA extraction and the theoretical detection limit of the LC assay could almost be achieved, i.e., 2 to 3 GE could be detected. For the home-brew PCR, a sample of double the above-mentioned volume was taken. Increasing the extract volume may improve the sensitivity of the LC assay. This is presently impossible, however, because of the LC capillary format, which allows only a small total reaction mixture volume (20 μl). For detection of pathogens, larger capillaries would be useful in the future.

Another reason for decreased sensitivity may be insufficient sample preparation. For molecular assays to be applicable in the routine diagnostic laboratory, sample preparation should be as simple as possible. In this study, the cation exchanger Chelex 100, which allows DNA extraction within less than half an hour, was employed. In comparison with classic nucleic acid extraction protocols, Chelex 100 protocols have been shown to increase sensitivity of molecular assays for detection of cytomegalovirus in cultures and clinical samples, for detection of human immunodeficiency virus type 1 proviral DNA in blood, and for detection of Legionella pneumophila in bronchoalveolar lavage fluids (6, 8, 30). A sample concentration step prior to the start of the Chelex 100 protocol may further increase the yield of DNA in the future.

The formation of primer dimers during PCR may also contribute to decreased sensitivity of a molecular assay. Especially in targets with low copy number, a relatively high primer dimer concentration has to be considered. To inhibit the formation of primer dimers, the LC assay included a hot start (11).

Because high viral titers should be present in CSF during herpes simplex virus encephalitis, lack of sensitivity appears to be only the minor problem. Contamination may be the major problem: despite special efforts to prevent contamination, all discrepant results turned out to be false-positives in this study. Because of the increased number of manipulations, it had originally been supposed that the home-brew assay would be

<table>
<thead>
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<th>Primer, product, or probe (sequence)</th>
<th>Virus and positions amplifieda</th>
<th>Length (nucleotides)</th>
<th>G+C content (%)</th>
<th>Melting temp (°C)</th>
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<td>Primers</td>
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<td>Forward (5′-CATCACCGACCCGGAGAGGAC)</td>
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<tr>
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<tr>
<td>TaqMan probe (5′-6FAM-CCCGCAACTGACGACAGCACCCGCGC-GC-TAMRA)</td>
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a GenBank accession numbers: X14112 (HSV type 1 complete genome) and Z86099 (HSV type 2 complete genome).

FIG. 1. LC PCR of clinical samples. Fluorescence curves from TaqMan probe detection with hot start technique are shown. ●, sample 1 (negative); □, sample 2 (positive); ■, sample 3 (positive); ○, positive control; line without symbol, negative control.
more prone to contamination than the LC assay. In this study, however, contaminations may have occurred with both assays. The major weaknesses in assay design that could lead to false-positives include sample preparation and pipetting steps between the first-round PCR and the second-round PCR. To reduce the danger of contamination during sample preparation, techniques that required only a few manipulation steps were used in this study. Automation of sample preparation, however, is most desirable to minimize danger of contamination. The hot start technique, applied for the LC assay, requires an additional pipetting step after the addition of the extracted DNA. In the future, this step can be avoided by using the LC Fast Start assay, which will include a polymerase specially designed to remain inactive until heated during the PCR before thermal cycling and which will be brought on the market soon.

Detection formats of the LC technology include general detection of double-stranded DNA (SYBR Green technology), which corresponds to gel electrophoresis of home-brew assays, and specific detection of the target sequence by using a TaqMan probe or hybridization probes (14). Probes prevent false-positive results due to nonspecific amplification products and guarantee the specificity of results. Therefore, probes should always be employed for molecular assays to be applicable in the routine diagnostic laboratory.

False-negatives, which may result from PCR inhibitors, did not occur in this study. Removal of inhibitors, however, is crucial in molecular assays and may be better achieved by the use of classic extraction protocols. It has, however, been reported that even Chelex 100 may decrease false-negative results due to PCR inhibitors (26). Nevertheless, introduction of an internal control, which should be extracted and coamplified with the target DNA from the clinical specimen, would be helpful in the future.

In summary, the LC assay proved to be suitable for the routine diagnostic laboratory. Compared to a conventional home-brew assay, it was found to be very quick and very easy to use. Because of the significantly lower number of manipulations, there may be a lower probability of getting results that are due to false-positive contamination.

REFERENCES