Diagnosis of infection with human herpes viruses in routine laboratory practice

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Abstract

Background: The potential for faster detection of human herpes viruses using PCR compared to other methods is undisputed. However, because of fear of contamination, the clinical implication of nucleic amplification methods in routine laboratories is not widespread. Herpes viruses cause a wide spectrum of diseases and can cause morbidity and mortality in immune-compromised patients. Using real-time PCR, most of the problems associated with PCR (contamination, cumbersome detection, and rather expensive tests) are solved, and a rapid, economical, and – most importantly – closed system is at hand.

Methods: We evaluated work procedures in our laboratory that enable the routine diagnosis of viral infections with high accuracy and rapid turn-around time. In parallel, inherent problems usually associated with PCR testing, especially cross-contamination could be suppressed to a minimum. The start of the work flow process begins with an automated nucleic acid extraction procedure that yields high quality DNA. A common – internally and externally controlled – PCR program for all six viruses allows rapid sample turn around.

Results: In all, 7500 analyses for human herpes virus infection were performed in the last 5 years. Results for various different specimens were produced within 24 h. Contamination occurred rarely and could be ameliorated easily. The use of internal controls identified rare PCR-inhibited samples. The detection limits for our assays are markedly below the clinically relevant range.

Conclusions: Our workflow allowed rapid, cost-efficient, and labor saving routine diagnostic detection of viral infections.

Key words: real-time PCR; human herpes virus; automated sample extraction; rapid turn-around time.

Introduction

Real-time PCR enables rapid analyses of clinical samples and can be streamlined for routine use in the clinical setting. For the past 5 years, we have utilized this technology for the diagnoses of viral infections. In all, we have performed over 30,000 analyses for viral infection [herpes viruses (HVs), respiratory viruses, hepatitis C virus] using an optimized routine protocol. Here, we detail our experience with various specimens, work procedures, and results for diagnoses of six human HVs.

These viruses cause a spectrum of diseases that are self-limiting usually but can be reactivated in immune-compromised patients (1–5). Cytomegalovirus (CMV), varicella-zoster virus (VZV), and herpes simplex virus (HSV) type 1 and 2 can cause encephalitis, meningitis, myelitis, or polyradiculitis (6). The human HVs are an important cause of central nervous system (CNS) disease in human immunodeficiency virus (HIV) infected patients.

Generalized or disseminated infections may occur because of neoplasia, organ transplantation, and inherited or acquired immunodeficiency disease. These viruses can then cause life-threatening diseases including pneumonia and encephalitis. Infections caused by different HVs may be clinically indistinguishable in AIDS patients, and simultaneous CNS infections caused by different HVs have occasionally been described (2). A multiplex PCR assay with the ability to detect simultaneously different HVs in cerebrospinal fluid (CSF) might therefore be a useful and practical approach to the differential diagnosis of CNS disorders. Such a method has the advantage of rapidity and economy, particularly in cases where more than one HV may be involved. In addition, it offers the possibility of detecting co-infections with several HVs.

For the clinician, a rapid and meaningful result from the laboratory is needed (7–10). The gold standard of viral detection, i.e., virus isolation in cell culture, is too slow and cumbersome and not useful for CSF (11, 12). Other detection methods, such as enzyme linked immunosorbent assays (ELISA) and latex agglutina-
tion, nucleic acid probe, and fluorescent antibody methods generally fail when low titers of HVs are present in specimens that are inoculated into cell cultures (13).

In contrast, real-time PCR provides qualitative and quantitative results within several hours that are internally and externally controlled (14, 15). Thus, PCR has become a valuable tool for the diagnosis of viral infection because of its speed, specificity, and sensitivity.

Materials and methods

PCR

Two hundred μL of each specimen was subjected to extraction of viral nucleic acids using the Magna Pure automated extractor (Roche Diagnostics, Vienna, Austria) on the day of arrival. For HSV and VZV requested by the dermatological ward, we received microscope slides with micucotaneous smears on it. A cotton swab wetted in 400 μL of buffer was used to wipe the smear in order to absorb the specimen. The swab was then transferred back into the vial with the remaining buffer. Pressing the swab against the wall of the tube containing the remaining buffer allowed most of the adsorbed liquid with the dissolved specimen to be placed back into the tube. Next, 200 μL were used for extraction using the Magna Pure.

The extraction quality for the diverse specimens was evaluated by adding standards directly into negative specimens and comparing the results with standards added directly to the PCR mixes. External quality assessment programs (German Institute for Standardization and Documented in the Medical Laboratory – INSTAND) for all the viruses examined were performed continuously. With this program, four samples each of the viruses to be tested (all HVs) are sent to laboratories for quantitative analyses of viral loads. For the past 5 years, we have successfully fulfilled the criteria for this assessment program and received accreditation to conduct these analyses.

Five μL aliquots of the extracted nucleic acids were subjected to real-time PCR on a LightCycler (Roche Diagnostics, Vienna, Austria) by mixing with the respective virus master mix reagents according to the manufacturer’s instructions. Reagent kits utilized for the virus specific PCRs were: ARTUS EBV LC PCR kit, ARTUS CMV LC PCR kit, ARTUS HSV LC PCR kit, ARTUS EBV LC PCR kit, ARTUS VZV LC PCR kit (QIAGEN, Berlin, Germany), and HHV6 LightMix kit (TIB MolBio, IPC mix (TIB MolBio GmbH, Berlin, Germany), each containing reagents and enzymes for the specific amplification of the genome and for the direct detection of the specific amplicon with the LightCycler.

A denaturation step (10 min at 95°C) was followed by 5 s at 95°C, 5 s at 65°C down to 55°C with a step size of 1°C cycle, and 15 s at 72°C, for 10 cycles. Forty cycles of 5 s at 95°C, 5 s at 55°C, and 15 s at 72°C followed with detection at 55°C. All ramp rates were 20°C/s. All reactions included internal controls; standards and external positive and negative controls were run in parallel. A calibration curve for quantitation was created using the standards that were supplied. Because external quantification standards with international units are not available for the viruses assayed, we utilized the standards of the detection reagents from QIAGEN for quantitative results expressed as viral copies/mL.

Results

Over the past 5 years, we have performed 7500 analyses for HVs, ~2200 for HSV, 1800 for Epstein-Barr virus (EBV), 1700 for VZV, and lesser amounts for CMV (1200) and human herpes virus 6 (HHV6) (500). Various specimens were sent from the wards according to their needs (see Table 1). The gender distribution of patients was nearly equal, and patient ages ranged from 1 day to 90 years. All analyses (including further 25,000 respiratory and hepatitis C viral detections) were performed by two laboratory technicians. For HSV (type 1 and 2), most analyses were performed for patients from the dermatological ward. Most were performed using material from blisters, with less from urine and serum. Likewise, most requests for the VZV diagnosis came from this ward.

The percentages of positive results were 15% for HSV 1/2, and 12% for VZV.

The other viral diagnoses were requested primarily by our children’s department. Historically, for EBV, the laboratory performed a quick test for anti EBV IgG and IgM. Following the introduction of the PCR test, the number of serologic diagnoses did not vanish completely, allowing comparison between the two test formats. Of the 270 PCR positive sera, ~120 were also tested for antibodies. Half of these came at times when overt symptoms could be seen but no immunological response had started. Clearly, the results of direct antigen detection were more informative in these cases.

For EBV, 15% of specimens were positive and for CMV, 7% of specimens were positive. The least amount of positive results (2%) was for HHV6 infection, we received requests for this test 500 times only.

Table 1 Percentage of positive specimens with diagnosed human herpes viruses infections.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>CMV, %</th>
<th>HHV6, %</th>
<th>HSV, %</th>
<th>VZV, %</th>
<th>EBV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>60</td>
<td>60</td>
<td>2</td>
<td>3</td>
<td>57</td>
</tr>
<tr>
<td>Urine</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF</td>
<td>19</td>
<td></td>
<td>1</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Liquor</td>
<td>40</td>
<td></td>
<td>3</td>
<td>3</td>
<td></td>
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<tr>
<td>Blister</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28</td>
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<tr>
<td>Sputum</td>
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The percentage of the various specimens with positively diagnosed human herpes viruses infections are shown. CMV, cytomegalovirus; HHV6, human herpes virus 6; HSV, herpes simplex virus; VZV, varicella-zoster virus; EBV, Epstein-Barr virus; NF, nasal wash fluid.
We did not find co-infection with two or more viruses. However, because the majority of patients had more than one request for viral detection, the overall positive rate related to the number of patients was higher (21%) (Table 2).

Figure 1A–D shows the copy numbers per mL of specimen for the viruses (except HHV6) tested. These amounts are informative and show the relative magnitude of the various specimens. The highest viral load can be found in material from blisters of HSV and VZV. Up to $10^{10}$ viral copies could be found. In serum, the viral copy numbers usually were in the range of $10^4$–$10^5$/mL, whereas in CSF, copy numbers up to $10^9$/mL were found. These data show that the largest proportion of specimens from our hospital have copy numbers many orders of magnitude higher than the test limits (HSV: 1 copy/μL, CMV: 0.5 copies/μL, EBV: 5 copies/μL, VZV: 0.8 copies/μL).

The efficiency of our extraction apparatus was evaluated using positive samples that were extracted in duplicate. After an initial extraction step using several of the same samples, the resulting liquid was pooled and aliquoted. One aliquot was again extracted. The supernatants of the first and second extraction were quantified using PCR. The first extraction was used as the efficiency test standard.

The second extract was compared with it and the efficiency was calculated. The average efficiency of extraction was found to be 60%, which is excellent according to expected results of automated extraction procedures (16, 17).

**Discussion**

The potential for faster detection of HVs by PCR as compared to other methods is undisputed. Quantitative PCR assays allow monitoring of the kinetics of the viral infection in order to distinguish between acute and persistent infections and to estimate the success of antiviral therapy (18–20). These viruses can cause
life-threatening diseases including pneumonia and encephalitis in immune-compromised patients. The human HVs are important causes of CNS disease in HIV-infected patients. CMV, VZV, and HSV types 1 and 2 can cause encephalitis, meningitis, myelitis, or polyradiculitis (6).

Such neurological complications are common in HIV-infected patients. Unfortunately, clinical, CSF, and neuroimaging findings are frequently non-specific. Furthermore, routine CSF tests such as viral culture have a very low diagnostic yield.

In recent years, PCR analysis of CSF has contributed substantially to improving the diagnosis and clinical management of the CNS complications in HIV-infected patients (1, 21).

However, because of fear of contamination, the clinical implication of nucleic amplification methods in routine laboratories is not yet widespread. Using real-time PCR, most of the problems associated with PCR (contamination, cumbersome detection, and rather expensive tests) are solved, and a rapid, economical (considering the reduced workload), and – most importantly – closed system is available. The use of internal controls simplified the assay protocol and allowed monitoring of sample adequacy (10, 22, 23).

The remaining areas of cross contamination need to be monitored carefully. Separate rooms for specimen extraction and target loading need to be in place and must be separated from the master mix preparation room (24).

With the vast number of analyses executed and the known inherent problems of PCR, it is of no surprise that we did have problems with contamination. However, questionable results were ameliorated within 2–3 days. The use of negative controls gives a clear indication of the quality of results. Of further help is the fact that many tests for a given virus were done simultaneously and contamination showed up as increased numbers of positive samples at a same level of target concentration. To restore the quality of the diagnoses, reagents for extraction and/or PCR were discarded immediately.

We only employ reagents for these analyses that are certified for in vitro diagnosis (IVD) (CE marked). These pose a slight drawback to reagents with hybridization probes that can be examined further using melting curve analysis. Using this, another degree of certainty would be introduced in the assay because probe sequences could be uncovered. Unfortunately, inherent problems of PCR, it is of no surprise that primary vials will be handled by the automated extraction with a sample list provided directly by the laboratory data system. Using automated sample pipetting, the extracted nucleic acids will be prepared for PCR, and resultant PCR data will be directed back to the laboratory data system. The separate building blocks for such an integrated work flow are already on the market. Clinical chemistry laboratories like ours will implement such solutions in the near future.

We can provide test results for the clinicians within 24 h using two laboratory technicians only. This is due to automated sample extraction and a common temperature cycling program on the real-time PCR machine for all six viruses. Within a short time, an online apparatus will be available. This means that primary vials will be handled by the automated extraction with a sample list provided directly by the laboratory data system. Using automated sample pipetting, the extracted nucleic acids will be prepared for PCR, and resultant PCR data will be directed back to the laboratory data system. The separate building blocks for such an integrated work flow are already on the market. Clinical chemistry laboratories like ours will implement such solutions in the near future.

References


