Clinical Validation of a New Triplex Real-Time Polymerase Chain Reaction Assay for the Detection and Discrimination of *Herpes simplex* Virus Types 1 and 2

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A new triplex real-time polymerase chain reaction (PCR) assay for *Herpes simplex* virus (HSV) (artus HSV-1/2 TM PCR kit, QIAGEN) was evaluated. This assay simultaneously uses three differently labeled probes targeted to HSV-1 (FAM), HSV-2 (NED), and to the manufacturer's Internal Control (VIC). HSV-1/2 typing capability and quantitation accuracy were determined using HSV stocks and quality control panels. Performance in routine clinical testing was compared with a nested HSV-1/2 PCR assay. Dilution series and quality control panel testing revealed an approximately 10-fold higher HSV-2 sensitivity in real-time PCR compared with an in-house nested PCR assay. The sensitivity for HSV-1 was comparable in both assays. All HSV-positive proficiency panel samples ($n = 21$) and virus stocks were typed correctly as HSV-1 or HSV-2 using real-time PCR. Quantitation correlated well with reference values (HSV-1, $r = 0.98$; HSV-2, $r = 0.88$), and 95% detection limits were determined as 9.4 HSV-2 copies/reaction and 18 HSV-1 copies/reaction. Based on C_t values, the mean intra-assay coefficient of variation was 1%, whereas the interassay coefficient of variations were 2.7% and 2.5% for HSV-1 and -2, respectively. Testing of 309 clinical samples resulted in 100% specificity and 97% sensitivity. In conclusion, the artus HSV-1/2 TM PCR kit represents an excellent tool for the detection and differentiation of HSV-1 and -2 in clinical samples. (*J Mol Diagn* 2008, 10:361–367; DOI: 10.2353/jmoldx.2008.070104)

Herpes simplex virus (HSV) infections are very common; the seroprevalence in adults is about 80 to 90% for HSV-1 and varies between 10 and 25% for HSV-2 in most studies.^{1,2} HSV infection in immunocompetent individuals normally does not cause major health problems. However, HSV reactivation in the central nervous system can occur and might cause a wide range of clinical symptoms

from mild meningitis (Mollaret's) to severe encephalitis with a mortality rate of up to 70% in the absence of therapy.^{3,4} Primary infection of neonates or reactivation of HSV in immunocompromised individuals can be associated with a higher incidence of meningitis, severe encephalitis, and dangerous eye infections. In addition, patients with atopic eczema are prone to widespread cutaneous herpes simplex infection.⁵ The classical method for diagnosis of herpes simplex infections has been virus culture, but this method is time-consuming and has a low sensitivity, especially for the analysis of cerebrospinal fluid (CSF). Therefore, more rapid and sensitive polymerase chain reaction (PCR) methods have been widely accepted for diagnosis of HSV infections, as antiviral therapy is most effective when started early.^{6–9} A major advance in this respect has been the development of real-time PCR. This technology provides the opportunity to perform duplex and/or triplex PCR simultaneously, based on the use of specific targeted probes, coupled to different fluorophores. Recently, a TaqMan (ABI Prism 7000 and 7900)-based triplex real-time assay has become commercially available, using FAM-, VIC-, and NED-coupled probes in a single PCR reaction simultaneously, targeted to the HSV-1 and HSV-2 glycoprotein B and to an Internal Control (QIAGEN Hamburg GmbH, Hamburg, Germany). Since this assay is the only commercially available kit for ABI Prism TaqMan platforms, the goal of this study was to evaluate this assay for diagnostic use and compare it with an in-house nested HSV PCR validated for routine testing.

Supported by Akademie der Wissenschaften und Literatur zu Mainz project 2 1.223, by Interdisciplinary Center for Clinical Research of the University Hospital Erlangen, IZKF, project B16 (H.R.), and by the Bayerisches Staatsministerium für Kultus, Erziehung und Wissenschaft (K.K.).

Accepted for publication February 5, 2008.

J.D. and T.G. have affiliations (employment, consultancies, stock ownership, honoraria, expert testimony) with QIAGEN GmbH, which has a direct financial interest in the subject matter or materials discussed in the article.

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Materials and Methods

Virus Titration

HSV-1 and -2 virus stocks, originally isolated from patient material and typed by immunofluorescence using HSV-1 and -2 specific antibodies, were cultured on Vero cells propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and gentamicin until a clear cytopathic effect was observed. To quantify the 50% tissue culture infective dose (TCID₅₀) from the respective virus stocks, serial fourfold dilutions were made in eight replicates, added to fresh Vero cells seeded in 96-well plates, and cultivated at 37°C with 5% CO₂. Wells showing cytopathic effect after 3 days were recorded, and TCID₅₀ was calculated according to the method of Reed and Muench.¹⁰

Determination of Sensitivity

Plasmids containing the HSV-1 or HSV-2 PCR amplification products were quantified by absorption spectroscopy. Standard dilution series were set up for HSV-1 from 25.7 to 0.008 HSV copy equivalents/ μ l and for HSV-2 from 35.3 to 0.012 HSV copy equivalents/ μ l. Testing was performed in eight replicates on three different days on the ABI Prism 7000 instrument. The sensitivity, defined as 95% cut-off value, was determined by probit analysis using PriProbit version 1.63 software.

Clinical Samples

Initially, 21 clinical specimens with existing nested PCR results were analyzed. These samples had been stored at -20°C as DNA extracts between 1 month and 1 year. Afterward, a total of 288 specimens (cerebrospinal fluid, oral, dermal, genital, and ocular swabs, blister fluid, bronchoalveolar lavage (BAL), and other respiratory samples, whole blood, amniotic fluid, and biopsies) were collected prospectively over 4 months. The distribution of different specimen types is shown in Table 1. These samples were processed after arrival at our diagnostic department following established standard operating procedures for nucleic acid testing. Swabs that had been placed in

sterile transport medium containing phosphate-buffered saline at pH 7.1 were vortexed to resuspend cellular content before nucleic acid isolation.

Quality Control (QC) Panels

In total, 28 lyophilized samples from three proficiency panels (QCMD HSV 2005: $n = 12$, Glasgow, UK; INSTAND HSV 4/2005: $n = 8$ and 11/2005: $n = 8$, Düsseldorf, Germany) were dissolved in 1 ml of sterile distilled water.

Nucleic Acid Extraction

A total of 200 μ l of all specimens were extracted using the DNA Large Volume kit on the MagNA Pure instrument (Roche, Mannheim, Germany) according to the manufacturer's protocol. Elution volume was 100 μ l. DNA extracts were subjected to both the in-house nested HSV PCR and the artus HSV-1/2 TM PCR kit. If possible, DNA from specimens with discordant results was extracted again using the QIAamp DNA Mini kit as indicated by the manufacturer and subjected to the artus HSV-1/2 TM PCR kit and to an additional third method, the artus HSV 1/2 LC PCR (all kits from QIAGEN, Hilden/Hamburg, Germany), performed on the LightCycler 1.2 instrument (Roche).

PCR Methods

The artus HSV1/2 TM PCR was performed on an ABI Prism 7000 instrument (Applied Biosystems, Darmstadt, Germany) in a 50- μ l reaction volume. Twenty microliters of HSV TM Master PCR mix (containing complete reaction mix, including ROX, primers, and FAM-, VIC- or NED-coupled probes, targeted to HSV-1 and -2 glycoprotein B and to an Internal Control, respectively) was combined with 10 μ l of HSV TM Mg-Sol and 2 μ l of Internal Control, all provided by the manufacturer. For each real-time PCR reaction, 30 μ l of the prepared master mix was placed in each well of a 96-optical-well plate and 20 μ l of extracted DNA was added. Thermal cycling conditions were as follows: 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 55°C for 1 minute. Optical four-color discrimination between FAM (HSV-1), NED (HSV-2), VIC (Internal Control), and ROX (passive reference control) is achieved on the ABI Prism 7000 instrument by four different filters for the fluorescence readout.

For the nested HSV PCR, primers corresponding to the *PoA* gene of HSV-1 and HSV-2 were used. PCR reactions were performed in a 50- μ l reaction volume, containing 5 μ l of DNA extract from the clinical specimen, 200 μ mol/L deoxynucleoside-5'-triphosphates, 300 nmol/L of each primer, Taq buffer (0.5 mol/L KCl, 0.1 mol/L Tris, 15 mmol/L MgCl₂, 0.01% gelatin, pH 8.3) and 0.25 μ l of AmpliTaq polymerase (5 U/ μ l; Applied Biosystems, Darmstadt, Germany). The outer PCR was performed with the primers described by Yamamoto et al (sense-1 5'-TC-GAACAGCTCCTGGCCGATTT-3', antisense-1 5'-CGGT-TGATAAACGCGCAGT-3') at 95°C for 2 minutes, followed by 35 cycles of 94°C for 40 seconds, 50°C for 30

Table 1. Sample Types and Real-Time PCR Results of Prospectively Tested Samples

Materials	No. of samples tested and %	No. of positive samples	HSV positivity rate (%)
CSF	105 (37)	4	3.8
BAL	45 (16)	9	20
Other respiratory samples	22 (8)	11	50
Swabs	32 (11)	15	47
Blisters	6 (2)	3	50
Whole blood	17 (6)	0	0
Biopsies	46 (16)	1	2.2
Vitreous body	7 (2)	0	0
Others	8 (3)	0	0
Total	288 (100)	43	15

seconds, and 72°C for 1 minute.¹¹ The nested PCR was performed using 2.5 µl of the outer PCR product in the second PCR reaction mix containing primers sense-2 (5'-GTGCGTTGATAAACGCGCAGT-3') and antisense-2 (5'-ATCATCTACGGGGACACGGACT-3') at 95°C for 2 minutes, followed by 35 cycles of 94°C for 40 seconds, 58°C for 30 seconds, and 72°C for 1 minute. The size of the amplification product is 331 bp for the outer PCR and 224 bp for the nested PCR, respectively. HSV-1 and HSV-2 can be differentiated by restriction enzyme digestion with *AluI*, which yields two fragments for HSV-1 and three fragments for HSV-2.

The artus HSV 1/2 LC PCR was performed on a Light-Cycler 1.2 instrument (Roche Diagnostics) in a 20-µl reaction volume (5 µl of DNA extract plus 15 µl of ready mixed master mix containing reaction buffer, including bovine serum albumin, primers, and LC640- and LC705-coupled probes targeted to the HSV-1 and -2 glycoprotein B and the Internal Control). For this assay a touch-down protocol according to the manufacturer's user manual was used, and discrimination between HSV-1 and HSV-2 was achieved by a final melting curve analysis.

Results

HSV-1 and -2 Typing, Sensitivity, and Quantification Accuracy

Our first goal was to test the artus HSV1/2 TM PCR kit (QIAGEN Hamburg GmbH) for its ability to discriminate between HSV-1 and HSV-2 using dilution series from HSV-1 and HSV-2 cell cultures with defined viral titer and several commercially available quality control panels. Tenfold dilution series of HSV containing culture medium were prepared and extracted using the Roche MagNA Pure (Roche Diagnostics) instrument. Subsequently, DNA extracts corresponding to 20 to 0.02 TCID₅₀ were analyzed by the triplex real-time HSV1/2 and nested PCR. Representative results from a single experiment are shown in Tables 2 and 3. The data reveal a correct discrimination between HSV-1 and HSV-2 as well as a comparable sensitivity for both virus types for the triplex real-time PCR. Whereas the sensitivity for HSV-1 detection seemed to be comparable for the two different PCR approaches, the in-house nested HSV PCR was about 10-fold less sensitive for HSV-2 than the triplex real-time HSV-1/2 assay. These re-

Table 2. Comparison of Real-Time and Nested PCR Results for All Clinical Samples

Real-time	Nested	
	+	-
+	58	3*
-	4 [†]	244

*Two specimens (BAL) were confirmed as positive and one (throat wash) as negative by additional testing.

[†]Three specimens (2 BAL, 1 lip swab) were confirmed as positive by additional testing; in one case (BAL) no material was available for further analysis.

Table 3. Typing of Titrated HSV-1 and -2 Virus Stocks

HSV cell culture stocks	Nested PCR	Artus HSV-1/2 TM PCR	
		HSV-1 copies/well	HSV-2 copies/well
TCID ₅₀ HSV-1/well			
20	+	500	—
2	+	50	—
0.2	+	10	—
0.02	—	—	—
TCID ₅₀ HSV-2/well		HSV-1 copies/well	HSV-2 copies/well
20	+	—	800
2	+	—	50
0.2	—	—	3
0.02	—	—	—

+, positive; -, negative.

sults could be confirmed by testing defined quality control panels (Figure 1). All 28 samples gave correct results in the triplex real-time HSV1/2 PCR, as 21 HSV-positive samples were identified properly as HSV-1 ($n = 11$) and HSV-2 ($n = 10$), whereas in the remaining negative samples no DNA amplification was observed ($n = 7$). In comparison, the lowest HSV-1-positive sample (580 copies/ml) and the three HSV-2-positive samples with the lowest copy numbers (1500, 3000, and 12,000 copies/ml) were not detected by the in-house nested PCR. Comparison of the quantitative results obtained by the triplex real-time PCR assay and the expected reference values of the quality control panels revealed a good correlation ($r = 0.98$ for HSV-1 and $r = 0.88$ for HSV-2; Figure 1). The detection limit of the real-time assay was determined by probit analysis using photometrically quantified plasmids as standards containing PCR amplification products from the respective HSV-1 and HSV-2 primer and probe target sequences. This procedure was necessary due to the lack of available international HSV standards. The 95% cut-off value for HSV-1 was 18.4 DNA copy equivalents/reaction and 9.4 DNA copy equivalents for HSV-2. The graphical probit analysis is shown in Figure 2.

Test Evaluation with Clinical Specimens

A total of 309 clinical specimens were included into this study. To increase the number of HSV-positive samples, some specimens ($n = 21$) were selected according to existing results and analyzed retrospectively, whereas the majority of specimens ($n = 288$) were tested prospectively. All retrospective specimens (17 HSV-positive, four HSV-negative) gave concordant results in the triplex real-time and nested PCR. Interestingly, one of the positive samples (anal swab) was positive for both HSV-2 (3300 copies/ml) and for HSV-1 (1300 copies/ml) in the triplex real-time PCR assay. However, in the nested PCR, restriction enzyme digestion with *AluI* showed only the two fragments specific for HSV-1. The discrepancy between the real-time and nested PCR is probably due to the lower sensitivity for HSV-2 of the nested PCR approach.

To characterize the clinical specimens sent to our diagnostic department during the observation period, the distribution of the different clinical materials and the HSV positivity rate determined by the triplex real-time PCR are

Figure 1. Accuracy of HSV quantitation. Specimens from three different quality proficiency panels (see Materials and Methods) were extracted using the MagNA Pure instrument (Roche). DNA extracts were subjected to the triplex real-time PCR on the ABI Prism 7000 instrument. **A:** Values from HSV-1 and HSV-2 quantitation are plotted against the expected reference values of each sample. **B:** Target values and results from real-time PCR are presented as columns; results from nested PCR are shown as - or + below.

shown in Table 1. Analyzing only the prospective specimens, CSF samples constituted almost 40%, and respiratory materials like BAL or tracheal aspirates approximately 25% of all specimens. The HSV positivity rate in CSF was low (3.8%), whereas 20 of 67 (30%) of the respiratory specimens were positive. The highest positivity rate was observed in samples from skin lesions (swabs and blisters) with almost 50% (18/38 samples). Referring to the triplex real-time assay, the overall HSV detection rate was 14% for HSV-1 ($n = 40$) and 1% for HSV-2 ($n = 3$). All three HSV-2-positive samples were genital swabs. Initially, the triplex PCR assay identified four samples showing PCR inhibition of the internal control. After repeated extraction, inhibition was no longer observed and the samples turned out to be true negatives (data not shown).

The comparison of both PCR methods showed concordant results for 302 of 309 specimens (98%), with 244 samples identified as negative and 58 as positive. Of the seven samples (5 BAL, 1 throat wash, 1 lip swab) with discrepant results, three were positive only in the nested PCR and four only in the triplex real-time PCR, respectively (Table 2). Three of the four samples that were positive in the triplex real-time PCR had threshold cycles greater than 40, indicating very low virus titers. If enough material was available ($n = 4$), samples were subjected to a manual extraction method (QIAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany) and were retested by the triplex real-time PCR and addi-

tionally by another real-time method, the artus HSV 1/2 LC PCR (QIAGEN Hamburg GmbH, Hamburg, Germany), performed on the LightCycler 1.2 instrument (Roche Diagnostics). Otherwise, the original DNA extracts were used for further PCR tests ($n = 2$). From one specimen, no further testing was possible at all. This sample was therefore excluded from the sensitivity and specificity determinations. Retesting of the remaining six samples revealed that one sample was consistently positive, four were intermittently positive, and one sample was negative in all additional tests. The latter was defined as true negative, whereas the other five samples were considered as true positives. Thus, there was one false positive and three false negative results in the nested PCR, equivalent to a specificity of 99.6% (244/245 negative samples) and a sensitivity of 95% (60/63 positive samples). For the triplex real-time PCR, the specificity was 100% (no false positives) and the sensitivity was 97% (61/63 positive samples). When calculated on the prospective samples only, the sensitivity decreases to 93% and 96% for the nested and the real-time-PCR, respectively, due to the smaller number of positive samples (45 versus 63).

Characterization of Positive Samples

The virus load of the HSV-positive samples from all prospective and retrospective clinical specimens varied

Figure 2. Probit analysis HSV-1/HSV-2. Dilution series have been set up for each HSV-1 and HSV-2 plasmid standard DNA, which was quantified by absorption spectroscopy. Different copy numbers ranging from 25.7 to 0.008 for HSV-1 and from 35.3 to 0.012 HSV copy equivalents/ μ l for HSV-2 were quantified in eight replicates on three different days on the ABI Prism 7000 instrument. The sensitivity was determined as 0.47 HSV-2 copies/ μ l, equivalent to 9.4 copies/reaction and 0.9 HSV-1 copies/ μ l, equivalent to 18 copies/reaction, defined as 95% cut-off value, by probit analysis using PriProbit version 1.63 software.

over a wide range, showing threshold cycles between 16 and 44 (corresponding to more than 10^9 to less than 10^2 HSV copies/ml), with a maximum occurrence between C_t 26 and 30 (Figure 3A). Copy numbers were calculated from C_t values according to the standard curve shown in Figure 4. Comparing different types of samples, it is noteworthy that most HSV-positive CSF specimens had threshold cycles of 31 or higher, corresponding to a moderate virus load of less than 10^5 HSV copies/ml.

Next, the robustness of the Internal Control was monitored in all HSV-positive specimens. For this purpose, the threshold cycle values for HSV and for the Internal Control of each individual run were plotted against each other. It could be shown that the Internal Control was stably amplified in HSV samples showing a threshold cycle of 22 or higher, corresponding to a virus load of 5×10^7 HSV copies/ml or less. In these cases the threshold cycle values of the Internal Control varied between C_t 25 and 29. Only in cases where more than 5×10^7 HSV copies/ml were present, corresponding to HSV C_t values <22 , the amplification of the Internal Control was suppressed due to competition effects (Figure 3B).

Figure 3. C_t value distribution of HSV-positive clinical samples. **A:** C_t values achieved in triplex real-time PCR for CSF, BAL, and materials other than CSF and BAL (Table 1) positive for HSV-DNA are shown. **B:** C_t values of HSV and the Internal Control in all HSV-positive PCR reactions were plotted against each other. The dotted line depicts the HSV C_t value (C_t 22, corresponding to approximately 5×10^7 HSV copies/ml), where PCR competition between the amplification of HSV and the Internal Control begins to occur. When PCR competition is not relevant, the C_t value of the Internal Control vary between C_t 25 and 30 (arrows).

Accuracy of the Triplex Real-Time PCR

To monitor the interassay precision, the reproducibility of the isolation efficiency between different extraction runs and the PCR performance over time has been determined. For this purpose, supernatants from HSV-1- and HSV-2-infected Vero cells were mixed, stored in aliquots

Figure 4. HSV-1 standard curve. Illustrated is a HSV-1 standard curve that was generated with five standards ranging from 5×10^3 to 5×10^6 HSV-1 copies/ml provided by the manufacturer. Data are mean values of five different runs performed on five different days. Standard deviation as indicated by error bars.

Figure 5. Interassay precision. HSV-1 and HSV-2 virus stocks were mixed, stored in aliquots at -20°C , and used as a control for each PCR run. C_t values of all three reporters (FAM for HSV-1; NED for HSV-2; and VIC for the Internal Control, IC) from 26 individual runs are documented. The mean C_t values were 28.5 ± 0.78 for HSV-1, 26.5 ± 0.65 for HSV-2, and 25.5 ± 0.45 for IC, corresponding to a mean coefficient of variation of 2.7%, 2.5%, and 1.8% respectively.

at -20°C , and used as a control for each run. For 26 different runs, the mean C_t values were 28.5 ± 0.78 for HSV-1, 26.5 ± 0.65 for HSV-2, and 25.5 ± 0.45 for the Internal Control, corresponding to a mean coefficient of variation of 2.7% for HSV 1, 2.5% for HSV 2 and 1.8% for the Internal Control (Figure 5). To estimate the intra-assay precision, four clinical samples were divided into five aliquots each, extracted in parallel and quantified by the triplex real-time PCR in quintuplets. Here, the coefficient of variation for the threshold cycle value was lower and varied only between 0.7 and 1.3%, which corresponds to coefficient of variation values of 16% (range, 8 to 23%) for the calculated HSV copy numbers (data not shown).

Discussion

Simultaneous detection of at least two different DNA molecules in single-tube PCR assays is becoming standard of care in the management of molecular nucleic acid testing for infectious agents to ensure the detection of possible PCR failures caused by extraction dropouts or PCR inhibitors. Whereas duplex PCR assays using DNA probes coupled to reporter dyes with different emission wavelength maxima have been frequently used, the development of triplex real-time PCR is more complex, due to potential cross-talk caused by emission spectrum overlap. As a result of the improvement of cyclers platforms and new reporter dyes like NED (575 nm) or Cy5 (667 nm) with distinct emission maxima from, eg, FAM (518 nm) or VIC (554 nm), the development and validation of triplex real-time PCR assays for pathogens or mRNA have been reported recently.^{12–14} In the present study, a triplex real-time assay for the simultaneous detection of HSV-1, HSV-2, and Internal Control DNA, based on the detection of three different probe-coupled dyes (FAM, NED, and VIC) in a single PCR reaction was evaluated on the ABI Prism 7000. To our knowledge, this assay is the only European Community-approved commercially available kit for HSV-DNA detection and differ-

entiation on the ABI Prism platforms ABI 7900 and 7000 using the triplex PCR technology.

In this study, more than one-third of a total of 288 prospectively tested clinical samples were CSF. For these 105 samples the positivity rate was 3.8%. This is in agreement with other studies that reported positivity rates from 2.5 to 5% for CSF in a clinical setting.^{15,16} In contrast, the positivity rate was much higher for samples from skin and genital lesions (almost 50%) and also for respiratory samples (30%). When respiratory samples were further differentiated, BAL specimens as samples from the lower respiratory tract had less than half of the positivity rate of other respiratory samples, which originated from the upper respiratory tract (20% versus 50%). Whereas PCR has been accepted as the diagnostic method of choice for detection of HSV in CSF for more than 10 years, many diagnostic laboratories have relied on virus culture for testing samples from mucocutaneous lesions. However, recent reports have shown that also for this type of specimens and for BAL, detection rates by PCR were substantially higher than by virus culture.¹⁷ In this study, the detection rates achieved by real-time PCR were very similar to ours, whereas detection rates by virus culture were substantially lower with 33% for skin and mucous membrane lesions and 18% for respiratory samples. The difference between virus culture and PCR was mainly due to samples with low viral loads, but other factors such as inadequate transport and storage conditions may also play a role. In our analysis, we also observed a wide range of HSV C_t values, corresponding to virus loads from less than 10^2 to more than 10^9 copies/ml. Although the median viral load in CSF was lower than in other types of specimens, BAL or other respiratory samples with very low copy numbers were also observed. One of the major advantages of real-time PCR is to fulfill the demand for such a broad dynamic range.

Using titrated virus stocks of HSV-1 and HSV-2 and QC samples, we could observe an excellent performance of the triplex HSV PCR in terms of typing capability, sensitivity, and accuracy of quantitation. Comparing the triplex real-time PCR and an in-house nested PCR with titrated viral stocks and QC samples, we found that the sensitivity of the nested PCR for HSV-2 was about 10-fold lower than for the triplex real-time PCR, whereas the sensitivity for HSV-1 was comparable. No such discrepancy was observed when clinical samples were tested. In this case, the agreement between the two methods was good, as only seven of 309 samples gave discrepant results. Additional testing of these samples revealed similar rates of false negative samples (2/63 versus 3/63 for real-time and nested PCR, respectively). This good agreement may be due to the fact that among the clinical samples, only five of the positives were HSV-2 (three from prospective and two from retrospective testing), whereas the vast majority of positive samples including all samples with discrepant results were HSV-1-positive.

Looking at the specimen type of the samples with discrepant results, it is interesting to note that five of the seven were BAL specimens. Four of the discrepant samples had only borderline results (weak bands in nested PCR or threshold cycle >40 in real-time PCR), assuming very low

HSV copy numbers in these samples. The distribution of C_t values in Figure 3 reveals for BAL, that very high, but also extremely low copy numbers equivalent to C_t values higher than 40 can occur. These very low copy numbers may be due to high dilution during the lavage procedure.

The possibility of the real-time PCR to quantify HSV virus load and to distinguish between different HSV types can be important for prognosis or for monitoring treatment success. It could thus be shown that severe encephalitis is mainly caused by HSV-1, whereas HSV-2 is predominantly diagnosed in patients with self-limiting recurrent aseptic meningitis.^{9,18,19} HSV virus load in CSF could be correlated with morbidity and mortality in some studies.^{20,21} Domingues and colleagues reported that patients with a high HSV copy number ($>10^5$ /ml) had a poorer prognosis than those with lower copy numbers,^{5,20} whereas others did not see such a correlation.^{22,23} However, quantitation of HSV DNA may also be important to monitor treatment success, as HSV DNA can be detected in CSF for up to 40 days after onset of symptoms, a time period normally treatment is not continued.²³ However, more clinical studies using quantitative HSV DNA assays will be necessary for determining their actual value in establishing the prognosis and improving treatment results in herpes simplex encephalitis. Another advantage of the triplex real-time PCR is the incorporation of an Internal Control that allows monitoring each single reaction for failures of extraction and/or PCR. Noteworthy is that using the triplex PCR we could detect four PCR failures within 309 specimens (1.3%; data not shown). After repeated extraction and PCR, all four samples showed a valid amplification of the Internal Control, but were still negative for HSV. Due to the advantages of the real-time PCR discussed above, we replaced the nested HSV assay by the triplex real-time PCR kit in our routine testing. To reduce costs per sample, the test is routinely run without standard curves, as interpretation of C_t values is sufficient for almost all clinical situations. To monitor test-to-test variation, a single positive control consisting of a mixture of HSV-1 and HSV-2 from cell culture turned out to be sufficient (Figure 5). Analysis of the workflow revealed that the turnaround time from receipt of samples to availability of results was reduced by 40% after the introduction of the triplex real-time PCR from a median of 1.8 days to 1.1 day. Taken together, the artus HSV1/2 TM PCR kit is an excellent and reliable tool for diagnosis of herpes simplex virus infections.

Acknowledgments

We thank Thomas Laue (QIAGEN, Hamburg, Germany) for constructive discussions and B. Fleckenstein (University Hospital, Erlangen, Germany) for continuous support.

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