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Diagnosing Herpesvirus Infections by Real-Time Amplification and Rapid Culture

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Procedures using real-time technique were developed to demonstrate the presence of herpes simplex virus type 1 (HSV-1) and HSV-2, varicella zoster virus (VZV), and cytomegalovirus (CMV) in miscellaneous clinical specimens. The assays were compared to rapid culture using centrifugation followed by detection with monoclonal antibodies. A total of 711 consecutive samples were collected from different patient groups. Throat swabs were obtained from transplant patients; dermal or oral specimens were collected from patients suspected for VZV or HSV infection. Genital specimens were taken from patients who attended the Clinic for Sexually Transmitted Diseases at the Dijkzigt Hospital Rotterdam presenting with symptoms of a primary genital ulcer. Nucleic acid extraction was carried out using a MagnaPure LC instrument. The amplification steps were performed on the ABI Prism 7700 sequence detection system. To monitor the process of extraction and amplification, a universal control consisting of seal herpesvirus type 1 (PhHV-1) was added to the clinical specimens. By culture 127 of 668 (19%) samples were positive for HSV-1, 72 of 668 (10.8%) specimens were positive for HSV-2, and 17 of 366 (4.6%) were positive for VZV. Using real-time amplification the numbers of positive specimens were 143 of 668 (21.4%), 97 of 668 (14.5%), and 27 of 366 (7.4%), respectively. Eighty-six specimens were tested for CMV, 12 (14.0%) were positive by culture, and 17 (19.8%) were positive by real-time PCR. The clinical data of the patients with discrepant results were reviewed thoroughly. In all cases the patients with only real-time PCR-positive results could be considered as truly infected. We concluded that the real-time amplification technique is suitable for the detection of human herpesvirus infection. It offers a semiquantitative and reliable assay with a quick result that is more sensitive than rapid culture, especially for the diagnosis of HSV-2 and VZV infections.

Standard laboratory technique for the detection of herpesvirus infection is cell culture and the recognition of a cytopathic effect followed by unequivocal serological identification of the virus involved. Modifications of the cell culture technique by centrifugation of the inocula on the cell monolayers and the use of immunofluorescence techniques provide a more rapid detection. Nevertheless, sensitivity of detection of the herpes simplex viruses (HSVs) (HSV type 1 [HSV-1] and HSV-2) can be improved by implementing amplification techniques (1, 2, 3, 4, 6, 10, 11). Increased sensitivity of the laboratory diagnosis of varicella zoster virus (VZV), another member of the herpesvirus family, has also been reported, when using DNA amplification (5, 10). For the diagnosis of human cytomegalovirus (HCMV) infection, molecular techniques establishing viral loads in serum or plasma have largely replaced culture-based techniques (8). However, the clinical value of amplification techniques compared to culture using centrifugation and immunofluorescence for the detection of HCMV in other specimen's remains to be assessed.

The implementation of molecular techniques in diagnostic virology has made a step forward by the availability of semiautomated extraction systems, as well with the introduction of real-time technology. This has produced a substantial reduction in throughput time, as well as limiting the time needed to handle the samples. Furthermore, real-time technologies enable one to quantify the results. Quantification of viral sequences in the specimens can provide more insight into the clinical significance of the presence of the detected virus. In this study, our goal was to compare culture techniques for the detection of HSV, VZV, and HCMV with a real-time amplification technique, allowing (relative) quantification of the viruses in the clinical material. Furthermore, with the introduction of a universal and nonhuman viral control, i.e., seal herpesvirus type 1 (PhHV-1), an accurate control for monitoring the process from extraction through amplification became feasible (9).

MATERIALS AND METHODS

Patients and clinical samples. A total of 711 specimens were included from November 2000 through June 2001. Anogenital samples ($n = 161$) were collected from patients attending the Clinic for Sexually Transmitted Diseases at the Dijkzigt Hospital Rotterdam. Dermal specimens ($n = 116$), ocular swabs ($n = 27$) and specimens from the oral region ($n = 403$), and bronchoalveolar lavage samples ($n = 4$) were collected from different patient groups. The specimens from the oral region consisted of 275 throat swabs that were taken from transplant patients (heart, liver, kidney, or bone marrow) who were screened for HCMV disease; and in case of ulcerative lesions specimens of the mouth or lips ($n = 128$) were collected. The dermal specimens were taken from clinical lesions suspected for HSV or VZV infection. Lastly, the 27 ocular swabs were taken in case of possible herpes keratitis or conjunctivitis. All specimens but the bronchoalveolar lavage fluid specimens were collected in virus transport medium consisting of minimal essential medium-HEPES balanced salt solution (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml).

Culture technique. Monolayers of human embryonic lung fibroblasts were cultured in 24-well microtiter plates. For each virus to be detected, 200 μl of the

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sample was inoculated in duplicate, and plates were centrifuged at 3,500 × *g* for 15 min at room temperature. After 48 h, one part of the cells was incubated with monoclonal antibodies against HSV-1 or HSV-2 (De Beer Medicals, Uden, The Netherlands, and Imagen, Dako Diagnostics, Cambridgeshire, United Kingdom) or VZV or CMV (Argene-Biosoft, Varilhes, France) separately. The other part of the cell culture was maintained for another 14 days. When a cytopathic effect was observed, cells were incubated again with the monoclonal antibody specific for the agent related to the typical cytopathic effect.

Nucleic acid extraction. For the isolation of nucleic acids from the original clinical material, the MagnaPure LC Isolation station (Roche Applied Science, Penzberg, Germany) was used. Briefly, 200 μl of material was isolated in duplicate using the Total Nucleic Acid isolation kit (Roche Applied Science) exactly as described by the manufacturer. The nucleic acid was resuspended in a final volume of 100 μl. To monitor the whole process from isolation of nucleic acids until real-time detection, a universal internal control was used. This internal control sample consisted of a whole-virus preparation of a seal herpesvirus (PhHV-1), which was added to the original clinical sample at a final concentration of approximately 5,000 to 10,000 DNA copies per ml, equivalent to a threshold cycle (Ct) value of approximately 30 in the real-time detection system used (9).

Real-time TaqMan assay. The amplification tests were performed as individual assays for each virus. Multiplex amplification was not performed, as this resulted in a loss of sensitivity (data not shown). Primers for the detection of HSV-1 and VZV were published before (10, 11). The sequences for HSV-2, CMV, and PhHV-1 were designed using Primer Express software (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). The sequences of the primers and the probes used, and the location of the genes from which they were derived, are listed in Table 1. All PCR amplification reactions were performed in a 50-μl volume containing 2× TaqMan Universal Mastermix (Applied Biosystems), forward primer (45 pmol/μl), 2.5 pmol of reverse primer, 5 pmol of TaqMan probe, and 20 μl of isolated DNA. The reactions were carried out in a 96-well plate, which was centrifuged for 1 min at 1,000 × *g* at room temperature in a swing-out rotor (Rotina 48R; Hettich, Tuttlingen, Germany) to remove small air bubbles in the reaction vessels. The amplification and detection was performed with an ABI Prism 7700 sequence detection system (Applied Biosystems). After incubation for 2 min at 50°C with uracil *N′*-glycosylase to inactivate possible PCR contaminants from former reactions, the reaction tube was incubated for 10 min at 95°C to inactivate the uracil *N′*-glycosylase and to release the activity of the AmpliTaq Gold DNA polymerase. The PCR cycling program consisted of 42 two-step cycles of 15 s at 95°C and 60 s at 60°C. Real-time measurements were taken, and a Ct value for each sample was calculated by determining the point at which the fluorescence exceeded a background limit of 0.04. Each run contained several negative controls (no template), and a positive control containing a known viral copy number based on a standard counted by electron microscopy (EM) when available. Each specimen was isolated and amplified in duplicate and only considered positive if both replications were above the threshold limit.

Standardization and quality control (QC). For the standardization of the HCMV and HSV-1 and HSV-2 real-time detection assays, an EM-counted standard (Advanced Biotechnologies Incorporated) was used. Dilutions ranging from 10 million down to 10 copies per ml were made to characterize linearity, precision, specificity, and sensitivity of the TaqMan assay. For VZV and PhHV-1, such a standard is not available. For these viruses, the standard curves generated were extrapolated on the basis of the curves made for HSV-1 and -2.

To determine the performance of detection for HSV-1 and -2, we participated in the European Union (EU) Concerted Action of QC of Nucleic Acid Amplification program.

Statistics. Epi Info 6.02 (Centers for Disease Control and Prevention, Atlanta, Ga.) was used to calculate the characteristics of the different assays compared. The means of the TaqMan test results and their differences with 95% confidence intervals were calculated using confidence interval analysis (7).

RESULTS

QC. To enable accurate monitoring of the amplification steps combined with automated extractions, we included a universal control in each clinical sample before the extraction was started. This consisted of a seal herpesvirus (PhHV-1) which was added at a low concentration to each sample. This internal viral control agent was isolated and amplified in each sample, and this standard provided a measurement for the

TABLE 1. Sequences and region of primers and probes used for real-time detection according to virus

Virus	Region (length of amplification product [bp])	Sequence(s) of	
		Primers	Probe[c]
HSV-1[a]	gG gene (166)	Forward, 5′-TCCTSGTTCCTMACKGCCTCCC; reverse, 5′-GCAGICAYACGTAACGCACGCT	5′-FAM-CGTCTGGACCAACCGCCACACAGGT
HSV-2	gD gene (71)	Forward, 5′-CGCCAAATACGCCTTAGCA; reverse, 5′-GAGGTTCTTCCCGCGAAAT	5′-FAM-CTCGCTTAAGATGGCCGATCCCAATC
HCMV	DNA polymerase (133)	Forward, 5′-GCCGATCGTAAAGAGATGAAGAC; reverse, 5′-CTCGTGCGTGTGCTACGAGA	5′-VIC-AGTGCAGCCCCGACCATCGTTC
VZV[b]	Gene 38 (89)	Forward, 5′-AAGTTCCCCCCGTTCGC; reverse, 5′-TGGACTTGAAGATGAACTTAATGAAGC	5′-FAM-CCGCAACTGCAGTATATATCGTCTCA
PhHV-1	gB polymerase (89)	Forward, 5′-GGGCGAATCACAGATTGAATC; reverse, 5′-GCGGTTCCAAACGTACCAA	5′-TET-TTTTTATGTGTCCGCCACCATCTGGATC

[a] Data from reference 11.
[b] Data from reference 10.
[c] Abbreviations: FAM, 6-carboxyfluorescein; TET, tetramethylrhodamine.

TABLE 2. Detection of HSV, CMV, and VZV by rapid culture and real-time PCR

Virus	Real-time PCR result	No. of specimens with result by rapid culture using immunofluorescence		Total no. of specimens
		Positive	Negative	
HSV-1	Positive	123	20	366
	Negative	4	521	525
	Total	127	541	668
HSV-2	Positive	71	26	97
	Negative	1	570	571
	Total	72	596	668
CMV	Positive	11	6	17
	Negative	1	68	69
	Total	12	74	86
VZV	Positive	16	11	27
	Negative	1	338	339
	Total	17	349	366

precision and reproducibility of the assays (mean Ct value, 30.7; coefficient of variation, 3.2% [data not shown]). In case the Ct value of the internal control virus exceeded 32.7 (mean + 2 standard deviations), it was assumed that inhibition or loss of sample had occurred. The whole procedure was then repeated, and in most cases, the results were within the expected range. It was established that in 0.52% of the clinical samples, no amplification product could be detected in duplicate aliquots. These samples were therefore classified under the heading "no amplification possible" and excluded from the analysis. The cause of these undetectable results was not determined.

To determine the detection limits of the amplification reactions, data generated using dilution series of available EM standards were used. However, for the detection of CMV it was shown that the amount counted by EM must be an underestimation, since dilutions down to 0.1 copy per reaction were always detected (results not shown). More-accurate data were observed using the EM-quantified viral stocks of HSV-1 and -2. Participation in the EU QC program determined the lower detection level for these two viruses: 580 copies per ml for HSV-1 and 430 copies per ml for HSV-2.

Clinical samples. A total of 711 samples were included in the study. Of these, 668 samples were processed for the detection of HSV. For the presence of HCMV, 86 specimens were screened. Detection of VZV was carried out on clinical indication in 366 specimens.

By culture 127 of 668 (19%) samples were positive for HSV-1 and 72 of 668 (10.8%) specimens were positive for HSV-2. HCMV was found by culture in 12 of 86 (14.0%) specimens, and 17 of 366 (4.6%) were positive for VZV. By real-time amplification the number of specimens positive for HSV-1 was 143 of 668 (21.4%) and the number positive for HSV-2 was 97 of 668 (14.5%). By real-time PCR 17 of 86 (19.7%) specimens were positive for CMV and 27 of 366 (7.4%) were positive for VZV. These results are summarized in Table 2.

The samples showing discrepant results were retested in both assays when sufficient material was available. A total of 24 (20 only PCR positive) remained discrepant for HSV-1, 27 (26 only PCR positive) remained discrepant for HSV-2, 7 (6 only PCR positive) remained discrepant for CMV, and 12 (11 only PCR positive) remained discrepant for VZV. Subsequently, the clinical data of the patients with discrepant results were reviewed. Based on results from previously or subsequently collected samples the majority of the PCR-positive but culture-negative specimens could be considered to be true positives. Of the 20 patients whose specimens were HSV-1 cell culture negative and positive by PCR, 12 were known to be latently infected with HSV-1, and 3 of the 4 patients whose specimens were HSV-1 cell culture positive and negative by PCR were known to be latently infected with HSV-1. With regard to HSV-2, 14 of the 26 patients whose specimens were HSV-2 cell culture negative and positive by PCR were patients with documented recurrences of genital herpes. One patient suffered from a herpes keratitis. With regard to CMV, six samples were positive for CMV only by real-time PCR. Two specimens were collected from patients for whom samples taken from other sites were also positive for CMV; three samples were double infected with HSV. It is conceivable that in cell culture of specimens containing both HSV and CMV, the latter virus may not be detected due to the more rapidly growing HSV. Lastly, all 11 specimens positive for VZV only by PCR were obtained from patients with clinical symptoms of a primary infection or a reactivation of VZV, including two patients with neurological symptoms such as facial paresis and a vesicle on the tympanic membrane. The one discrepant specimen shown to contain HSV-2 by PCR, and in which cell culture identified VZV, was collected from an anal lesion. The clinical presentation of the lesion was typical of anogenital herpes rather than of a solitary VZV eruption.

As we did not define an extended "gold standard," we calculated the test characteristics of the real-time PCR using the cell culture as standard and vice versa. The results are shown in Tables 3 and 4. Compared to the cell culture as the standard, the real-time PCR is sensitive but also less specific for detecting HSV-1, HSV-2, HCMV, and VZV. When the real-time PCR is taken as the standard, the cell culture is much less sensitive for the detection of VZV, HCMV, HSV-2, and, to a lesser extent, HSV-1.

TABLE 3. Test characteristics of real-time PCR according to virus culture with immunofluorescence as standard

Virus	No. of specimens with test result/no. of specimens with result by standard (%)			
	Sensitivity	Specificity	PVPT[a]	PVNT[b]
CMV	11/12 (91.7)	68/74 (91.1)	11/17 (64.7)	68/69 (98.6)
VZV	16/17 (94.1)	338/349 (96.8)	16/27 (59.3)	338/339 (99.7)
HSV-1	123/127 (96.9)	521/541 (96.3)	123/143 (86.0)	521/525 (99.2)
HSV-2	71/72 (98.6)	570/596 (95.6)	71/26 (73.2)	570/571 (99.8)

[a] PVPT, predictive value positive test.
[b] PVNT, predictive value negative test.

TABLE 4. Test characteristics of virus culture-immunofluorescence with real-time PCR as standard

Virus	No. of specimens with test result/no. of specimens with result by standard (%)			
	Sensitivity	Specificity	PVPT[a]	PVNT[b]
CMV	11/17 (64.7)	68/69 (98.6)	11/12 (91.7)	68/74 (91.9)
VZV	16/27 (59.3)	338/339 (99.7)	16/17 (94.1)	338/349 (96.8)
HSV-1	123/143 (86.0)	521/525 (99.2)	123/127 (96.9)	521/541 (96.3)
HSV-2	71/97 (73.2)	570/571 (99.8)	71/72 (98.6)	570/596 (95.6)

[a] PVPT, predictive value positive test.
[b] PVNT, predictive value negative test.

Viral loads. We determined whether the viral load in the clinical sample could explain the differences between the results of the real-time PCR and cell culture, assuming that the viral load is correlated with the amount of infectious virus. Since the viral load in the sample can be relatively assessed by the Ct value, we compared these values for the samples that were cell culture positive with values for the samples that were cell culture negative. High Ct values are representative of a low viral load, while a low Ct value reflects a high viral load. In all cases the culture-positive samples had a lower Ct value than the culture negative samples. The differences for the mean Ct values were statistically significant for VZV (−4.57; 95% confidence interval, −9.00 to −1.29), HSV-1 (−7.77; 95% confidence interval, −10.7 to −4.79), and HSV-2 (−10.5; 95% confidence interval, −12.6 to −8.39). The results are shown in Table 5.

Double infection. Using real-time PCR, 11 double infections could be detected, while cell culture yielded only 1 of these double infections. The following combinations were found: HSV-1–HSV-2 (four times), HSV-1–CMV (three times), HSV-2–CMV (one time), and HSV-1–VZV (three times).

DISCUSSION

In this prospective study, real-time amplification techniques were compared with the rapid cell culture technique for the detection of herpesviruses in samples collected from genital, dermal, and oropharyngeal lesions. The real-time PCR appeared to be a more sensitive assay than cell culture for the detection of all four herpesviruses studied.

Whenever a new technique is more sensitive than a previously used one, it is important to look in detail at the clinical relevance of the specimens that are solely positive by this new technology, in order to assess the specificity of the newer assay. Definition of an expanded gold standard using a third technique seems to have advantages. However, then the question arises whether this technique has been validated and why this assay can be considered to be a reliable test to be used as a decisive judge. Detailed analysis of the patients whose samples yield discrepant results revealed that the majority of the PCR-only-positive results were probably truly positive results. The specificity of the real-time PCR assays was also tested by applying the tests on cell cultures of other viruses that can be expected to be present in the samples that were collected. All primer sets were specific for the intended target (data not shown).

The predictive value of a test is dependent on the prevalence of the disease in the population. In fact, all specimens except the throat swabs that were collected to screen for CMV were obtained from patients with lesions. Therefore, the samples with test results solely positive by real-time PCR were obtained from patients with relevant symptomatic disease; consequently, the predictive value of a positive test result relative to disease was high. It must be noted that the predictive value of a positive PCR test in comparison with the cell culture as gold standard was rather low, only because the cell culture was more often negative for samples collected from patients with symptomatic disease. As few patients without lesions were included in the study, the predictive values of a negative test result excluding disease might be different when determined in populations with differing prevalences of the conditions in question. An exception can be made for the throat swabs collected in this study, because these swabs were taken from transplant patients in order to monitor CMV activity regardless of the presence of symptoms. In fact, monitoring CMV disease in these patients can be carried out preferably by testing plasma specimens or peripheral blood mononuclear cells (8).

In other studies detection of HSV by amplification techniques turned out to be more sensitive than cell culture, especially for HSV-2 and VZV (2, 4, 5, 6). In particular for VZV, the viral load of the culture-negative specimens as represented by the low Ct value was relatively high, indicating the presence of high levels of VZV DNA and the instability of the virus and/or the presence of incomplete, noninfectious virus.

The MagnaPure LC system proved to be a reliable and standardized system for extracting nucleic acids from the dermal, genital, and throat specimens collected in virus transport medium, as has been described previously by Espy et al. (6). An advantage of the closed real-time PCR is the lack of contamination during processing, since the reaction tubes do not need to be opened for the detection step (10). The addition of the PhHV DNA as an exogenous internal control provided the evidence that negative results were real negatives and not caused by inhibition. For a low proportion of the specimens the

TABLE 5. Ct values of real-time PCR according to virus and culture-immunofluorescence result

Virus and culture result	No. of specimens[a]	Ct value		Difference between sample means (95% confidence interval)	*P*
		Mean	SD		
CMV					
Positive	10	33.1	6.25	−2.14 (−8.39–4.12)	NS[b]
Negative	6	36.3	4.35		
VZV					
Positive	15	19.5	4.75	−4.57 (−9.00–1.29)	>0.01, <0.05
Negative	11	24.1	5.95		
HSV-1					
Positive	125	27.0	6.33	−7.77 (−10.7–−4.79)	<0.01
Negative	19	34.8	4.25		
HSV-2					
Positive	72	23.2	4.35	−10.5 (−12.6–−8.39)	<0.01
Negative	25	33.7	5.16		

[a] Some specimens are missing Ct data.
[b] NS, not significant.

nucleic acid extraction had to be repeated to remove inhibiting substances. Of importance is the determination of the lower detection level of the real-time amplification test developed. The introduction of internationally active QC programs for viral diagnostic assays is important to validate the assays that laboratories develop in-house. Initiatives from the EU QC program (now taken over by the QC for Molecular Diagnostics program), endorsed by organizations such as the European Society for Clinical Virology and the European Society for Clinical Microbiology and Infectious Diseases, are instrumental to implement better-defined standard assays. Furthermore, the introduction of internal controls into each clinical sample increases the true value of the results generated. Technologies that fulfill these requirements are currently available and should be mandatorily introduced with each in-house developed assay that is used in clinical diagnostics.

In our laboratory setting, the rapid cell culture inoculation of the monolayers is performed each working day shortly after arrival of the specimens; after 48 h the detection of viral antigens is executed, and positive test results are reported to the clinician. The automated nucleic acid extraction is also commenced every working day and carried out in the afternoon or overnight. The amplification step is performed the next morning. All real-time PCR results are available within 24 h after arrival at the laboratory whether they are positive or negative, except during weekends. The mean time to produce a positive result by cell culture was 5.9 days (range, 1 to 20 days), including weekends and holidays, while the mean time to release a negative result to the clinician was 15.7 days (range, 13 to 24 day). The advantage of the real-time PCR relative to the speed of reporting both a negative as well as a positive result to the clinician is therefore obvious.

We did not execute an extensive cost comparison. In The Netherlands, a fixed price can be charged for laboratory tests. The price for a rapid cell culture using three different monoclonal antibodies is approximately 47.00 euros (without doctors' fee), and the price for four PCR tests (three diagnostic targets and one for the internal control) using an automated system is about 83.00 euros. However, the prices for the commercially available components of nucleic acid extraction and for the real-time PCR are decreasing rapidly. At the time of writing, in our situation the price for the reagents of one nucleic acid extraction with the MagnaPure instrument together with the reagents needed for four real-time PCRs is approximately 15.00 euros. We expect that in the near future the total costs for nucleic acid extraction and the subsequent real-time PCR tests will be comparable with the costs for rapid cell culture.

In conclusion, real-time amplification technique in combination with automated nucleic acid extraction is suitable for the detection of human herpesviruses HSV-1, HSV-2, VZV, and HCMV in specimens collected from genital, dermal, oral, and oropharyngeal lesions. In comparison to rapid culture, this real-time method is a rapid, more sensitive, semiquantitative, and reliable assay.

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