Comparison of Six Real-Time PCR Assays for Qualitative Detection of Cytomegalovirus in Clinical Specimens

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In this study, we compared the performance of six real-time PCR assays for the qualitative detection of cytomegalovirus (CMV) in clinical samples other than plasma. Two hundred specimens (respiratory [$n = 72$], urine [$n = 67$], cerebrospinal fluid [CSF] [$n = 25$], tissue [$n = 18$], amniotic fluid [$n = 10$], and bone marrow [$n = 8$]) submitted for routine testing by CMV real-time PCR analyte-specific reagents (ASR) (Roche Diagnostics, Indianapolis, IN) were also tested by a laboratory-developed test (LDT) and 4 commercially available PCR assays: EraGen Multicode (Luminex, Austin, TX), Focus Simplexa (Focus Diagnostics, Cypress, CA), Elitech MGB Alert CMV (Fisher Scientific, Hanover Park, IL), and Abbott CMV (Abbott Park, IL). Nucleic acid was extracted using the MagNA Pure system (Roche Diagnostics) and subsequently tested by each PCR method. Results were analyzed by comparing each assay to a “consensus result,” which was defined as the result obtained from at least 4 of the 6 assays. In addition to the prospective samples, 13 lower respiratory samples with known positive results by CMV shell vial were tested by each PCR method. Following testing of the 200 prospective specimens, the Abbott, Elitech, EraGen, and Focus PCR assays demonstrated a sensitivity of 100% (46/46), while the Roche analyte-specific reagents (ASR) and LDT showed sensitivities of 89% (41/46) and 98% (45/46), respectively. Percent specificities ranged from 97% (149/154) by Elitech to 100% (154/154) by the LDT. Among the 13 shell vial-positive lower respiratory samples, the percent sensitivities ranged from 69% (9/13) by Elitech to 92% (12/13) by the LDT. The Abbott, EraGen, Elitech, Focus, and LDT PCR assays performed similarly ($\kappa \geq 0.89$) for the detection of CMV in clinical specimens and demonstrated increased sensitivity compared to the Roche ASR.

Infection with cytomegalovirus (CMV) is extremely common, with a seroprevalence of 40 to 60% in adults and exceeding 90% in certain patient populations (1, 2). Clinical manifestations following CMV infection may range from asymptomatic to mild disease in immunocompetent hosts to severe and sometimes fatal illness in immunocompromised patients. Notably, CMV is one of the most clinically important viruses affecting transplant recipients, potentially impacting graft success and overall patient survival (3, 4). In addition, CMV is a significant cause of congenital disease, resulting in high morbidity and mortality in infected neonates (5).

Traditionally, the laboratory diagnosis of CMV infection has been made through a combination of culture-based methods, histopathology, and, more recently, molecular detection by real-time PCR. Routine viral culture demonstrates adequate sensitivity for identification of CMV; however, viral culture is labor-intensive and subjective and may require up to 14 days for the virus to be propagated and identified (6). Due to the limitations of culture-based methods, real-time PCR has become an important laboratory tool for the diagnosis and management of CMV disease.

Prior studies have evaluated real-time PCR for the detection and quantification of CMV in plasma samples (7, 8). These studies have demonstrated that PCR offers a rapid (<24-h), sensitive, and specific means of detecting this virus. Furthermore, viral loads can be used to assist in the differentiation of transient viremia and CMV disease, as well as to monitor a patient's response to therapy. Recently, several important steps have been taken to improve the standardization and accuracy of CMV real-time PCR, including (i) the first FDA-approved assay (Cobas AmpliPrep/Cobas TaqMan; Roche Diagnostics) for detection and quantification of CMV in plasma samples (7) and (ii) the release of the World Health Organization (WHO) CMV international standard (9). These accomplishments represent major milestones in the labo-

ratory diagnosis of CMV; however, there are currently no FDA-approved PCR assays for clinical samples other than plasma, despite the fact that CMV can infect a wide range of tissues and organ systems. Therefore, the rapid and qualitative detection of CMV from clinical samples such as bronchoalveolar lavage (BAL) fluid, urine, or cerebrospinal fluid (CSF) is of significant clinical importance (10, 11). In this study, we evaluated and compared the performance of six real-time PCR assays for the qualitative detection of CMV from a variety of specimen types, including respiratory specimens, CSF, urine, fresh tissue, amniotic fluid, and bone marrow.

MATERIALS AND METHODS

Clinical specimens and nucleic acid extraction. A total of 200 prospective clinical samples (respiratory [$n = 72$], urine [$n = 67$], CSF [$n = 25$], fresh tissue [$n = 18$], amniotic fluid [$n = 10$], and bone marrow [$n = 8$]) were submitted to our reference laboratory for CMV real-time PCR (Roche analyte-specific reagents [ASR]; Roche Diagnostics, Indianapolis, IN). Respiratory samples included BAL fluid ($n = 25$), bronchial washing ($n = 40$), nasal swab ($n = 4$), tracheal secretions ($n = 2$), and throat swab ($n = 1$). Tissue samples were treated and nucleic acid was extracted as previously described (12). For all other specimen types, 200 μ l of raw sample was extracted on the MagNA Pure system (Roche Diagnostics) using the total nucleic acid extraction protocol. The institutional review board at our center approved the use of all specimens included in this study.

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Real-time PCR. Six real-time PCR assays were compared for the detection of CMV nucleic acid, of which five were commercially available: EraGen Multicode (Luminex, Austin, TX), Focus Simplexa (Focus Diagnostics, Cypress, CA), Elitech MGB Alert (Fisher Scientific, Hanover Park, IL), LightCycler (LC) CMV UL54 (13) (Roche Molecular, Indianapolis, IN), and Abbott CMV (Abbott Park, IL). Of note, the Abbott PCR utilizes the Artus CMV analyte-specific reagents that consist of the same primers/probes as those in the assay distributed by Qiagen (Germantown, MD). In addition, the Roche PCR included in this evaluation is not the same test as the recently FDA-approved Cobas AmpliPrep/Cobas TaqMan CMV test (Roche), as the FDA-approved assay is only for plasma samples. Testing by each of the 5 ASR assays was performed according to the manufacturer's instructions. In brief, 5 μ l of extracted material was added to 15 μ l of master mix for each assay, with the exception of the EraGen test, which required 10 μ l of extracted material. Four assays (Abbott, Elitech, EraGen, and Roche) were run using the LightCycler (LC) 2.0 (Roche), while the Focus assay was performed on the 3M Integrated Cyclor (Focus Diagnostics).

In addition to testing by the five commercially available assays described above, each sample was analyzed by a laboratory-developed test (LDT). The LDT targets a 285-bp region of the US9 gene of CMV. Real-time PCR primers were designed as follows: sense, 5'-GGA CGT ATC CAC CTC AGG TAC ACA-3'; antisense, 5'-TAC GTT ACG AAA CTG AGC TCC CAC-3'. Fluorescent resonance energy transfer (FRET) probe sequences were as follows: 5'-CGT GTT TCA CAA ACT GCA CCA GTA CCA C-fluorescein-3' and 5'-Red 640-TAG AGG AAT GTC AGG TAG CGT CTC TCC phosphate-3'. The final master mix concentrations of the LDT were 1.0 μ M (each) forward and reverse primer, 0.2 μ M fluorescein and Red 640 probes, and 1 $\times$ FastStart DNA Master HybProbe (Roche Diagnostics). Real-time PCR by the LDT was performed on the LC 2.0, with the following cycling conditions: 1 cycle at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 15 s. Subsequently, the reaction was increased to 95°C with no hold, followed by 59°C for 20 s, 40°C for 20 s, 85°C with no hold, continuous acquisition for 1 cycle, and 40°C for 30 s for 1 cycle.

LoD studies. To evaluate the analytical sensitivity of each PCR assay, whole-virus control (Acrometrix; Life Technologies) at a starting concentration of 500,000 copies/ml was used to generate a dilution panel. In brief, samples were diluted 1:2 in Tris-EDTA (TE) buffer to a final concentration of 8 copies/ml. Each member of the dilution panel was then tested in triplicate by each assay, and the limit of detection (LoD) was defined as the highest dilution at which all replicates tested positive.

Shell vial analysis of lower respiratory samples. Thirteen lower respiratory tract samples (BAL fluid [$n = 6$] and bronchial washing [$n = 7$]) that were determined to be positive by shell vial were also tested by each PCR method. For shell vial analysis, an 0.2-ml aliquot of each clinical sample was inoculated onto each of two shell vials containing MRC-5 cells (Diagnostic Hybrid, Athens, OH) and subsequently centrifuged at 2,000 rpm for 40 min. After 16 to 18 h of incubation at 37°C, the coverslips were washed twice using 1 ml phosphate-buffered saline (PBS) and fixed with acetone for 10 min. The cells were then stained for CMV using monoclonal antibody and a fluorescein isothiocyanate (FITC)-labeled, anti-mouse IgG secondary antibody (Light Diagnostics-Millipore, Temecula, CA). Slides were then reviewed by fluorescence microscopy and interpreted as positive if characteristic nuclear fluorescence was observed.

Statistical analysis. For the prospective study, the reference standard was established as a "consensus result," which was defined as the result obtained by at least 4 of the 6 real-time PCR assays. Percent sensitivity, specificity, and 95% confidence intervals were calculated using GraphPad software for categorical data (<http://graphpad.com/quickcalcs/>). Kappa values (κ) were also calculated as a measure of overall agreement, with values categorized as near-perfect (0.81 to 1.0), substantial (0.61 to 0.8), moderate (0.41 to 0.6), fair (0.21 to 0.4), slight (0 to 0.2), or poor (<0) (14).

TABLE 1 Limit of detection studies for six CMV real-time PCR methods

No. of copies/ml	No. of replicates (%) positive at each dilution by ^a :					
	Roche ASR	LDT	Elitech	EraGen	Abbott	Focus
1,953	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
977	2 (67)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)
488	1 (33)	2 (67)	3 (100)	3 (100)	2 (67)	2 (67)
244	1 (33)	3 (100)	3 (100)	2 (67)	1 (33)	2 (67)
122	0 (0)	3 (100)	0 (0)	1 (33)	0 (0)	0 (0)
61	1 (33)	0 (0)	0 (0)	1 (33)	0 (0)	0 (0)
30	0 (0)	0 (0)	0 (0)	1 (33)	0 (0)	0 (0)
16	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

^a The limit of detection for each assay is highlighted in bold.

RESULTS

Limit of detection. Each member of a 2-fold dilution panel (3,906 copies/ml to 8 copies/ml) was tested in triplicate by the six real-time PCR assays. The limit of detection (highest dilution at which all replicates tested positive) ranged from 1,953 copies/ml by the Roche ASR assay to 122 copies/ml by the LDT assay (Table 1).

Clinical performance of six CMV real-time PCR assays. Following testing of 200 prospective clinical specimens, the Abbott, Elitech, EraGen, and Focus PCR assays demonstrated a sensitivity of 100% (46/46), while the Roche analyte-specific reagents (ASR) and LDT showed sensitivities of 89% (41/46) and 98% (45/46), respectively. Percent specificities ranged from 97% (149/154) by Elitech to 100% (154/154) by the LDT (Table 2). Overall, the highest rate of detection was observed in respiratory samples (41.7% [30/72]), with urine (20.9%), fresh tissue (33.3%), and bone marrow (37.5%) also demonstrating substantial rates of detection for CMV (Table 3). Among the 13 shell vial-positive lower respiratory samples, the Elitech PCR assay detected 9 (69.2%), Abbott and Roche assays each detected 10 (76.9%), EraGen and Focus detected 11 (84.6%), and the LDT detected 12 (92.3%).

DISCUSSION

To our knowledge, this is the first large-scale evaluation of commercially available tests for the qualitative detection of CMV in clinical samples other than plasma. The ability to rapidly detect CMV in specimens such as BAL fluid, CSF, bone marrow, and fresh tissue is important for the timely diagnosis and management of patients with CMV disease. Furthermore, the quantitation of CMV from some specimen types may not be needed, or feasible, due to (i) the potential clinical significance of a positive result, regardless of the viral load, and (ii) the inability to accurately standardize testing/quantitation from certain specimen types because of inherent variability in sample collection (e.g., volume of sample obtained) and specimen processing (e.g., diluted versus nondiluted). Our data suggest that all of the 6 PCR methods yielded comparable results ($\kappa \geq 0.89$); however, we did observe a notable difference in the sensitivity of the methods during our prospective study, with the Roche ASR demonstrating a sensitivity of only 89.1% (41/46), while all other assays showed a sensitivity of $\geq 97.8\%$. These findings were supported by our limit of detection (LoD) studies, which demonstrated that the Roche ASR assay had the poorest LoD (1,953 copies/ml) of the six real-time PCR assays (Table 1).

Although we observed a high level of correlation between the six PCR assays, there were discrepancies that deserve discussion.

TABLE 2 Comparison of six real-time PCR assays for the detection of CMV in prospective clinical samples ($n = 200$)

Test and result	No. with consensus result ^a		Kappa (κ)	% (95% CI) ^b	
	Positive	Negative		Sensitivity	Specificity
Roche ASR					
Positive	41	3 ^b	0.89	89.1 (76.5–95.7)	98.1 (94.2–99.6)
Negative	5	151			
Abbott					
Positive	46	2 ^c	0.97	100 (90.8–100)	98.7 (95.1–99.9)
Negative	0	152			
EraGen					
Positive	46	3 ^d	0.96	100 (90.8–100)	98.1 (94.2–99.6)
Negative	0	151			
Elitech					
Positive	46	5 ^e	0.93	100 (90.8–100)	96.8 (92.4–98.8)
Negative	0	149			
Focus					
Positive	46	2 ^f	0.97	100 (90.8–100)	98.7 (95.1–99.9)
Negative	0	152			
LDT					
Positive	45	0	0.99	97.8 (87.6–99.9)	100 (97.1–100)
Negative	1	154			

^a The reference standard was defined as the result obtained from at least 4 of the 6 PCR assays.

^b These three samples had a crossing point (C_p) of 37.2, 38.5, and 40 cycles by the Roche ASR assay.

^c These two samples had a crossing point (C_p) of 32 and 36 cycles by the Abbott assay.

^d These three samples had a crossing point (C_p) of 38.1, 38.6, and 36.4 cycles by the EraGen assay.

^e These five samples had a crossing point (C_p) of 40, 38.5, 37.6, 38.7, and 40 cycles by the Elitech assay.

^f These two samples had a crossing point (C_p) of 37.5 and 38.4 cycles by the Focus assay.

^g CI, confidence interval.

We identified that the majority of discordant results (71.4% [15/21]) were due to samples with a positive result by an individual PCR assay and negative result by the “consensus” reference standard (≥ 4 of the 6 PCR assays). We reviewed the PCR crossing point (C_p) for these samples, and most C_p values were high (C_p range, 37 to 40 cycles), suggesting that the discrepancies might have been due to a small amount of target nucleic acid present in the clinical sample. However, we were unable to definitively conclude whether this was the case or if these discrepancies represented false-positive results by the individual PCR assays (Table 2).

In addition to our prospective study, we tested 13 shell vial-positive lower respiratory samples (e.g., BAL fluid and bronchial washing) by each of the 6 real-time PCR assays. This was completed due to prior data in our laboratory suggesting that shell vial may demonstrate increased sensitivity for the detection of CMV in lower respiratory samples compared to real-time PCR (15). These preliminary findings were supported in this study, in which the percent sensitivity of real-time PCR compared to shell vial ranged from 69.2% (9/13) by the Elitech PCR assay to 92.3% (12/13) by the LDT. Although we cannot definitively explain the reduced sensitivity of PCR for lower respiratory tract samples, several possibilities exist, including (i) sample volume differences tested by PCR and shell vial (200 μ l versus 400 μ l, respectively), (ii) greater abundance of immediate early antigen (detected by shell vial) than of viral DNA (detected by real-time PCR), and (iii) partial PCR

TABLE 3 Detection rate of CMV by specimen type

Specimen type	Total no. tested	No. (%) with PCR result:	
		Positive	Negative
Amniotic fluid	10	1 (10)	9 (90)
Bone marrow	8	3 (37.5)	5 (62.5)
CSF	25	2 (8)	23 (92)
Fresh tissue	18	6 (33.3)	12 (66.6)
Respiratory			
BAL	25	13 (52)	12 (48)
Bronchial wash	40	14 (35)	26 (65)
Nasal swab	4	0 (0)	4 (100)
Throat swab	1	1 (100)	0 (0)
Tracheal secretion	2	2 (100)	0 (0)
Urine	67	14 (20.9)	53 (79.1)

inhibition in lower respiratory samples. Future studies should be aimed at better characterizing this difference, but until those studies have been completed, we recommend that both PCR and shell vial be performed on lower respiratory samples (BAL fluid and bronchial washing) to maximize sensitivity and provide the most rapid turnaround time in the setting of a positive PCR.

This study has several limitations that should be discussed. First, the vast majority of samples that we tested were submitted through our reference laboratory, and therefore, we were not able to correlate results with other clinical (e.g., diagnosis and treatment decisions) or laboratory (e.g., radiology and viral culture) findings. Second, it is important to emphasize that the performance of these assays may vary depending on the type of extraction and PCR platform that is used. Third, we established the reference standard as a “consensus” result, which was defined as the result obtained by at least 4 of the 6 real-time PCR assays. It is possible that the consensus result was not accurate in all cases and may have misclassified the true result of some samples in this study. However, there is no true gold standard for the diagnosis of CMV, and most studies use a single assay to help resolve discrepancies between a new method and a gold standard test. In this study, we used the collective data from six real-time PCR assays to generate a reference result to which each of the individual PCR tests was compared. A fourth limitation of this study is that we were unable to determine whether positive PCR results (even those detected by all six assays) correlated with CMV disease in all cases. It is possible for CMV nucleic acid to be present in some clinical samples (e.g., urine or BAL fluid) in the absence of CMV-associated disease. Therefore, in certain cases, a “positive” PCR result from specimen types such as urine or BAL fluid should prompt follow-up testing, either by monitoring quantitative viral loads in plasma or by using more invasive diagnostic methods, such as histopathology.

In summary, we have evaluated six real-time PCR assays for the qualitative detection of CMV from a variety of clinical samples other than plasma. Each of the PCR methods was performed on the LightCycler 2.0 instrument, with the exception of the Focus test, which was run on the Integrated Cyclor. All the assays required ~ 10 min of hands-on time, and the turnaround time following extraction on the MagNa Pure system ranged from 55 min by the Focus assay to 89 min by Elitech. The data from our evaluation suggest that the Abbott, Elitech, EraGen, Focus, and LDT

assays performed similarly ($\kappa \geq 0.89$), although the LDT and Elitech assays showed superior LoDs (122 and 244 copies/ml, respectively). In contrast, the Roche ASR demonstrated the poorest sensitivity (89.1%) and highest LoD (1,953 copies/ml). These data may assist clinical laboratories in identifying a qualitative CMV real-time PCR assay, which plays a significant role in the rapid and sensitive diagnosis of patients infected with this important viral pathogen.

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