Comparison of QIAGEN Automated Nucleic Acid Extraction Methods for CMV Quantitative PCR Testing

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Key Words: Cytomegalovirus; Real-time polymerase chain reaction; Nucleic acid extraction; Viremia; Transplant monitoring

DOI: 10.1309/AJCPE5VZL1ONZHFJ

Abstract

We examined the effect of nucleic acid extraction methods on the analytic characteristics of a quantitative polymerase chain reaction (PCR) assay for cytomegalovirus (CMV). Human serum samples were extracted with 2 automated instruments (BioRobot EZ1 and QIAsymphony SP, QIAGEN, Valencia, CA) and CMV PCR results compared with those of pp65 antigenemia testing. Both extraction methods yielded results that were comparably linear and precise, whereas the QIAsymphony SP had a slightly lower limit of detection (1.92 log10 copies/mL vs 2.26 log10 copies/mL). In both cases, PCR was more sensitive than CMV antigen detection, detecting CMV viremia in 12% (EZ1) and 21% (QIAsymphony) of antigen-negative specimens. This study demonstrates the feasibility of using 2 different extraction techniques to yield results within 0.5 log10 copies/mL of the mean value, a level that would allow for clinical comparison between different laboratory assays.

Cytomegalovirus (CMV) is a 230-kb member of the herpesvirus family that can infect a wide variety of host tissues. Although about 50% of the US population has been exposed to CMV by early adulthood, severe clinical illness is mostly limited to immunocompromised people, including fetuses, neonates, HIV-infected people, and transplant recipients. Recognizing and responding to primary infection or reactivation early in the course of the illness is key to limiting CMV disease severity. Serology, viral culture, antigen detection, and viral nucleic acid detection have all been used for diagnosis and monitoring of at-risk immunocompromised patients for active CMV infection. Nucleic acid methods have the advantages of high sensitivity, rapid time to results, and ability to provide quantitative viral load measurements and have become the assays of choice for most applications. However, no current US Food and Drug Administration–approved test for CMV viremia exists, and universal reference standards that would allow for result harmonization among the various test methods are not available. Each laboratory, therefore, aims to provide precise and reproducible viral load results with the expectation that clinicians will be monitoring their patients at the same laboratory over time, because results from different laboratories cannot be readily compared.

Nucleic acid extraction methods have a major impact on polymerase chain reaction (PCR) test performance. Most clinical laboratories are moving toward automated technologies, and there are a variety of instruments available with varying capacity and specimen handling protocols. Most of these rely on silica binding to DNA/RNA for removal of nonnucleic acid components. For applications requiring high sensitivity, such as viral detection in clinical samples, extracted nucleic acid cannot be measured directly postextraction but must be...
coupled to an amplification or detection system such as real-
time PCR. Therefore, there are a large number of variables
to be considered when evaluating these systems, including
sample type(s) used, virus(es) of interest, extraction technol-
yogy, fluidics automation, throughput, concentration factor,
run time, ease of use, and cost. A recent study showed some
extraction methods having 10- to 100-fold reduced sensitivity
in virus detection when analyzed by real-time PCR at a single
center.5

We performed this study to evaluate 2 automated nucleic
extraction instruments, the QIAGEN BioRobot EZ1, a small
bench-top batch processor, and the QIAGEN QIAsymphony
SP (QIAGEN, Valencia, CA), a recently available nucleic
acid extractor with high throughput capacity. We performed
real-time quantitative PCR for CMV on human plasma sam-
ple and cultured virus material and compared PCR results
with the CMV pp65 antigenemia assay. This is the first report
we are aware of that describes use of the QIAsymphony SP
extractor for a clinical assay.

Materials and Methods

Specimens

Excess specimen was used from plasma collected for
CMV analysis by antigenemia or PCR methods in accord-
ance with institutional review board–approved protocols.
Samples from adult and pediatric hematopoietic stem cell or
solid organ transplant recipients were included. Samples were
taken for routine outpatient monitoring or inpatient diagnostic
workup and subsequent monitoring for CMV disease.

Cultured CMV was prepared from American Type
Culture Collection stock (VR-538) by growing the cells in
human foreskin fibroblast (Diagnostic Hybrids, Athens, OH)
or SF human fibroblast (ViroMed Laboratories, Minnetonka,
MN) cell lines using RM-02 (refeed medium 2% serum) at
36°C until cell death. Cell culture supernatant, termed CMV
AD-169, containing viral particles was removed and stored at
−70°C until used in viral load assays.

Nucleic Acid Extraction

BioRobot EZ1 extractions were performed using 400
µL of plasma with an elution volume of 90 µL (concentra-
tion factor, 4.44) using the EZ1 Virus 2.0 Kit (QIAGEN).
QIAsymphony SP extractions were performed using 1,000
µL of plasma with an elution volume of 85 µL (concentra-
tion factor, 11.76) using the QIAsymphony Virus/Bacteria Midi
Kit (QIAGEN). An internal control plasmid (Roche CMV
UL54 Recovery Template, Roche Diagnostics, Indianapolis,
IN) was added to each sample before extraction. All results
were corrected by the extraction concentration factor to yield
the quantity of virus present in the original plasma specimen
in log_{10} copies/mL.

Real-Time PCR

Real-time PCR was performed using CMV analyte-spe-
cific reagents (Roche) targeting the UL54 DNA polymerase
gene, as previously described,6 with the following modifica-
tions: uracil-DNA glycosylase (Roche) was added at 0.25 µL
per 20 µL reaction, and samples were held at 40°C for 10 min-
utes to degrade uracil-containing DNA before PCR amplifica-
tion. Each run contained a single standard whose value was
used to anchor the assay standard curve determined in a separate
run. The “f” maximum, or “second derivative maximum,”
method was used to determine the amplification result. Runs
were considered valid if a negative control yielded no ampli-
fication and the low-positive and high-positive control values
were within 2 SD of the expected mean. Samples without
amplification were considered to be true negatives only if the
internal control amplified within 3 SD of the mean value. All
instrument-determined values were reviewed and adjusted, if
necessary, by manual review of the data plots. Amplification
was performed on the LightCycler 2.0 instrument (Roche),
and results were analyzed with Roche software, version 4.05.

CMV Antigenemia Assay

CMV antigenemia testing was performed using EDTA
whole blood following the test procedure described in the
package insert of the CMV Brite Turbo kit (IQ Products,
Groningen, Netherlands). Cytocentrifuged preparations
(Cytospin 3, Shandon Scientific, Cheshire, England) of a
known quantity of peripheral blood leukocytes (200,000 cells)
were stained using indirect immunofluorescence with a cock-
tail of 2 fluorescein isothiocyanate–labeled monoclonal anti-
odies (C10/C11) directed against CMV lower matrix protein
pp65. The slides were immediately read using a fluorescence
microscope looking for leukocytes exhibiting homogeneous
bright green polylobate nuclear staining. The CMV antigen-
positive cells were counted and reported per 200,000 WBCs.

Study Design

Linearity was assessed by using CMV AD-169 viral
supernatant serially diluted with normal human plasma
that did not contain detectable CMV DNA. Dilutions were
assessed in duplicate and results averaged. Results were
considered linear when the R² value was more than 0.99 and
actual results were within 0.3 log_{10} copies/mL of the expected
value. The limit of detection was determined by using serially
diluted CMV AD-169 in duplicate during the course of 3 sep-
perate runs and was defined as the lowest dilution having more
than a 95% detection rate. Precision was determined by using
2 levels of CMV AD-169 tested in duplicate during 10 days
for a total of 20 data points for each level. Agreement between
Miller et al / CMV PCR Nucleic Extraction

the 2 extraction methods was determined by using 29 patient plasma samples extracted by both methods and amplified during the same run, with the overall bias for samples positive in both methods determined by using difference plot analysis (Bland-Altman). Comparison with CMV antigenemia was made by using whole blood patient samples that were split and analyzed for antigenemia or centrifuged with plasma extracted and CMV DNA amplified by PCR. Limited sample volume precluded simultaneous analysis of some specimens by CMV antigenemia and both extraction methods.

Results

Extraction Time and Sample Throughput

The BioRobot EZ1 has a capacity of 6 samples, whereas the QIAsymphony SP accepts up to 4 racks containing 24 samples each. Each run on the EZ1 takes approximately 30 minutes to set up and 45 minutes of run time on the instrument. The QIAsymphony takes approximately 2.5 hours to set up and has a run time of 1 hour and 20 minutes.

Linearity and Limit of Detection

Both extraction methods were linear for approximately 4 orders of magnitude, up to the upper limit tested. With a criterion of being within 0.3 log_{10} units of expected values, the BioRobot EZ1 had a linear range of 6.76 to 2.86 log_{10} copies/mL. The QIAsymphony SP had a linear range of 6.95 to 2.83 log_{10} copies/mL. EZ1 extracted material was detected in at least 95% of samples down to a level of 2.26 log_{10} copies/mL, while the QIAsymphony-extracted material was detected to a level of 1.92 log_{10} copies/mL.

Precision

Control samples processed with BioRobot EZ1 extraction had a coefficient of variation of 4.0% for low-level control material with 3.70 log_{10} copies/mL and 1.6% for high-level control material with 6.15 log_{10} copies/mL. Control samples processed with QIAsymphony had a coefficient of variation of 3.7% for low-level control material with 3.86 log_{10} copies/mL and 1.6% for high-level control material with 6.44 log_{10} copies/mL.

Agreement

Of 29 samples tested by both EZ1 and QIAsymphony extractions, 18 were positive by both methods. Four samples were positive after QIAsymphony extraction at 2.59, 2.88, 2.97, and 3.60 log_{10} copies/mL that were not detected after EZ1 extraction. No samples that were detected after EZ1 extraction were negative with QIAsymphony extraction. The average difference between the 2 extraction methods was 0.08 log_{10} copies/mL, with the EZ1 extracted material yielding results that were 62% higher on average when expressed in copies/mL of virus particles. This positive bias was present primarily for samples with average viral loads of less than 4.0 log_{10} copies/mL, whereas samples with viral loads greater than 4.0 log_{10} copies/mL showed a slight negative bias.

Figure 2

Difference plot of 18 matched patient samples positive for cytomegalovirus viremia using EZ1 and QIAsymphony extracted material. The dotted line represents the overall bias of +0.08 log_{10} copies/mL, and the solid lines indicate ±2 SD from the mean bias.
Comparison With CMV Antigenemia

Both extraction methods showed increased sensitivity over direct CMV antigen detection in peripheral blood neutrophils. Viral DNA was detected in 12% of EZ1-extracted samples and 21% of QIASymphony-extracted samples that were negative by CMV antigen testing. CMV was not detected by PCR in 2% of EZ1-extracted samples and 3% of QIASymphony-extracted samples that were positive for the CMV antigen. In each case, there was 1 positive cell detected with the CMV antigen. Review of patient records showed that all 3 involved patients had negative CMV antigen results before and after the positive test, indicating the possibility that the CMV antigenemia result was a false-positive. The samples extracted using QIASymphony had better quantitative agreement with CMV antigenemia results, as all samples with more than 3 cells positive by antigen testing were quantified with viral loads above 3.0 log_{10} copies/mL by PCR. In comparison, 3 of 41 samples extracted on the EZ1 instrument with CMV antigenemia showing more than 5 positive cells had viral loads less than 3.0 log_{10} copies/mL by PCR analysis.

Discussion

Quantification of CMV viral load in peripheral blood is a well-established tool for monitoring the risk of developing CMV disease in solid organ and hematopoietic stem cell transplant recipients, but significant method-specific differences limit the effectiveness of universal practice guidelines and therapeutic decision limits. In a recent comparison of 33 laboratories, only 57.5% of results fell within 0.5 log_{10} copies/mL of expected values, which was thought to be an acceptable range. Development of a universal reference standard would enable laboratories to directly compare various assays and provide interpretation of their results relative to an accepted consensus value, but this material is not yet available.

Several variables in testing methods make direct comparisons difficult, including sample type (plasma, whole blood, leukocytes), nucleic acid purification (lysis, purification technique, elution), reagents used (primers, probes, enzymes, buffer), and amplification and detection instruments. Laboratory-developed tests may use unique combinations of each of these variables, while more recently developed commercial analyte-specific reagents and kits use a set combination of PCR reagents and detection chemistries. Assuming that a single specimen type is used, the major differences between assays are described by the reagent kit, extraction protocol, and real-time PCR instrument used. Most studies describe comparisons of assays that differ by combinations of 2 or 3 of these major variables, making it difficult to identify specific sources of bias. In particular, the nucleic extraction method has been relatively understudied as a potential source of assay variability.

Our results demonstrate generally good agreement between 2 automated extraction systems using similar chemistries but with subtle differences that could impact clinical interpretation. The EZ1 method was somewhat less sensitive than the QIASymphony, requiring 0.34 log_{10} copies/mL additional virus for detection of 95% of positive samples. In addition, QIASymphony-extracted specimens were more often positive for CMV than EZ1 extracted samples (21% vs 12%) when compared with CMV pp65 antigenemia, possibly owing to the larger sample input volume of the QIASymphony. Overall correlation showed that each extraction method yielded results within 0.5 log_{10} copies/mL of the average value for all patient specimens tested with a slight positive bias for EZ1-extracted specimens that appeared more pronounced for samples with viral loads less than 4.0 log_{10} copies/mL.

The clinical significance of these differences in test characteristics for each nucleic acid extraction method is not completely clear because there are no universal criteria for CMV viral load interpretation, although some authors have attempted to provide guidelines. It is generally accepted that numeric values and limits of detection do not correlate

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**Table 1**
CMV PCR vs CMV Antigenemia Analysis

<table>
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<tr>
<th>Antigenemia (No. of Cells)</th>
<th>PCR (log_{10} copies/mL)</th>
<th>Not Detected</th>
<th>&lt;3.0</th>
<th>≥3.0</th>
<th>Total</th>
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CMV, cytomegalovirus; PCR, polymerase chain reaction.
* BioRobot EZ1 400-μL extractions. Discrepant results: antigen negative/PCR positive, 12%; antigen positive/PCR negative, 2%.

**Table 2**
CMV PCR vs CMV Antigenemia Analysis

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<tr>
<th>Antigenemia (No. of Cells)</th>
<th>PCR (log_{10} copies/mL)</th>
<th>Not Detected</th>
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CMV, cytomegalovirus; PCR, polymerase chain reaction.
* QIASymphony SP 1-mL extractions. Discrepant results: antigen negative/PCR positive, 21%; antigen positive/PCR negative, 3%.
well between various assay methods, although linearity and precision are acceptable. Therefore, clinicians are advised to compare viral loads over time using the same laboratory assay and determine trends that warrant treatment changes, such as increasing titers. At the same time, a single high value would be of concern in a patient at high risk for development of CMV disease, such as a recent bone marrow transplant recipient. In these cases, a decision to immediately treat could be warranted while waiting to establish a trend in CMV viral load. Each of these clinical scenarios requires an answer to the questions “What change in viral titer is considered significant?” and “What level of viremia is concerning in a high-risk patient?” At present, there is no definitive answer to these questions, but reasonable responses can be given based on experience and knowledge of each PCR assay’s attributes along with the patient’s clinical manifestations.

An analytically significant difference in viral load would be a change greater than 2 SD as determined through precision experiments. For our assay, this would require a change of approximately $0.3 \log_{10}$ copies/mL, regardless of the extraction method used. When considering clinical variation, a change in titer greater than $0.5 \log_{10}$ copies/mL would likely represent a significant change, as suggested by Pang and colleagues. For this reason, a goal to harmonize various methods to within $0.5 \log_{10}$ copies/mL of expected reference values would likely be adequate for interlaboratory comparisons of results for clinical purposes. Current CMV PCR assay comparisons show that this goal is not near being met. Our results demonstrate that a change in methods of nucleic acid extraction, using similar chemistry, can meet this goal when applied to a single amplification and detection technique.

There are limitations to both extraction methods studied here that should be considered by laboratories before their implementation. The QIASymphony method used here requires 1 mL of patient plasma, which can be difficult to obtain from pediatric patients with acute manifestations of leukemia who also require many other tests before transplantation. The QIASymphony instrument is more suited to high-volume laboratories because it takes more time to set up but can extract more specimens in a batch than can the EZ1 machine.

Significant levels of CMV viremia have been difficult to define because of assay variability between studies and differences in patient characteristics, such as endogenous and exogenous immunosuppression, previous antiviral therapy, evidence of end-organ disease, and comorbid conditions. A single numeric value for viremia is unlikely to provide a treatment threshold that will apply to all patients. Several studies have correlated pp65 antigenemia with PCR detection of CMV and proposed actionable levels of viremia based on their patient population of interest. In a similar vein, common practice at our institution has been to consider any detectable antigenemia clinically significant in a highly immunosuppressed hematopoietic stem cell transplant recipient and trigger treatment of infection, whereas a solid organ transplant recipient might be treated only for antigenemia levels of more than 5 cells per 200,000 cells. Therefore, using QIASymphony-extracted material would provide a reliable indicator for more than 5 cells by antigenemia when the viral load is greater than $3.0 \log_{10}$ copies/mL, whereas EZ1-extracted specimens would not provide this assurance. Conversely, both extraction methods are considerably more sensitive than CMV antigen detection and will provide for earlier detection of infection, allowing for earlier treatment or increased frequency of monitoring with potential clinical benefit.

Once CMV viremia has been detected, it may be present for prolonged periods, with low-level viremia that can confuse treatment decisions. Eradication of virus from the bloodstream may not be possible for some patients, so the length of treatment should not be based on detection of CMV alone, but in conjunction with other clinical factors. More data will be needed to determine optimal treatment lengths and regimens for the various patient populations currently monitored for development of CMV disease by quantitative PCR.

References


