Interpreting Quantitative Cytomegalovirus DNA Testing: Understanding the Laboratory Perspective

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Cytomegalovirus (CMV) is an important cause of morbidity and mortality in transplant patients, and is typically monitored using laboratory-developed quantitative molecular assays. Clinicians who use quantitative CMV DNA testing should be aware of a number of aspects of testing that will aid in decision making while managing CMV disease in their patients. These include (1) the specimen type used (whole blood or plasma), (2) the limit of detection and limit of quantification chosen by the clinical laboratory, (3) the linear range of the assay, (4) the reproducibility of the assay within the institution, and (5) the wide variability of viral load values among different assays. The biologic properties of CMV, including its variability within the host and of its half-life, are also important factors in the clinical testing for this virus.

Cytomegalovirus (CMV) is an important pathogen that leads to significant morbidity and mortality. With more patients undergoing transplants, along with the expanding indications for immune-modulating agents, the number of patients at risk for developing CMV disease is increasing. CMV viral load testing is routinely used to determine when to initiate preemptive therapy, diagnose active disease, and monitor the response to therapy. Molecular assays that use real-time amplification and detection are more widely available and attractive because of their broad linear range, low limits of detection and quantification, and reduced risk of contamination, as both the amplification and detection processes occur simultaneously in a closed system. However, CMV viral load testing is not straightforward, and clinicians who interpret test results for clinical decision making should understand these limitations. Technical characteristics affect interpretation of test results and their application to clinical care; these issues are frequently unrecognized. The purpose of this minireview is to provide clinicians with an understanding of the analytical performance of CMV viral load testing and to familiarize them with the strengths and limitations of the test in order to make informed clinical management decisions.

TECHNICAL ASPECTS OF CMV VIRAL LOAD TESTING

There are currently no US Food and Drug Administration (FDA)–approved laboratory tests for the quantification of CMV DNA. Therefore, CMV viral load tests are considered laboratory-developed tests (LDTs) that are developed and validated by an individual laboratory to the standard of the laboratory-inspecting agencies [1]. This analytical validation establishes the individual test characteristics and specifically determines the lower and upper limits of quantification (LOQ), precision, accuracy, and linear range of the test. Clinicians use test results to help them understand (1) the likelihood that a symptomatic patient has active CMV disease, (2) the likelihood that an asymptomatic patient will develop active disease, (3) the patient’s response to therapy, (4) the risk of relapsed infection, and/or (5) the appropriate time.
to discontinue therapy. Without a standardized test across laboratories, each laboratory, along with their clinicians, must establish the viral load cutoff values that are considered significant or that correlate with disease in the local population. These cutoff values will vary among institutions not only because there is no standardization for comparing viral load values but also because the performance characteristics of different LDTs vary.

**Agreement of Viral Load Values**

A multicenter study conducted to assess the variability of CMV viral load testing across 33 laboratories in the United States, Europe, and Canada [2] showed that the variability in viral load values for individual samples ranged from $2.0 \log_{10}$ copies/mL to $4.3 \log_{10}$ copies/mL. For example, a CMV DNA value of 100,000 copies/mL in a laboratory may be reported as 100 copies/mL (3 $\log_{10}$ difference) with a different LDT in another laboratory. These findings reinforce the fact that clinicians cannot compare test results from 2 laboratories nor can clinically relevant cutoffs developed using 1 test be applied to results from another test, unless the 2 tests have been rigorously compared and the relationship between them is well understood. This requires defining the correlation between the tests by running a significant number of clinical specimens (40–50 specimens distributed throughout the linear range of the test) in both LDTs and developing a mathematical conversion.

In November 2010, an international standard for CMV composed of a standardized quantity of CMV was developed and approved by the World Health Organization. This international standard will allow laboratories and manufacturers to assess the accuracy of viral load values and to calibrate different LDTs. Once completed, this process, which is in the early stages, will lead to improvement in the agreement of viral load values between laboratories when results are reported as international units (IU) per milliliter rather than as copies per milliliter (as is done for hepatitis C virus). Laboratories should provide clinicians with the conversion between copies per milliliter and IU per milliliter and may choose to report both values initially, which will allow clinicians to “recalibrate” their own interpretations of the test results in their clinical decision making.

Although the availability of an international standard will improve interlaboratory test result agreement, other test characteristics affect the results such that samples run in different laboratories may still have different viral load values. Test methods differ in the following ways: (1) nucleic acid extraction methods, (2) the selection of primers and probes targeting different genes with variable amplification efficiencies, and (3) test chemistry and instrumentation. Based on experience with human immunodeficiency virus type 1 (HIV-1) RNA testing, the availability and widespread use of FDA-approved tests will further improve interlaboratory agreement. Ultimately, these steps toward standardization will allow the development of meaningful multicenter clinical studies to identify viral load values that predict active disease, risk of relapse, and appropriate duration of therapy. Variations in both patient populations and immunosuppressive regimens may influence these cutoffs; that is, recommendations may differ among solid and stem cell transplant recipients, recipients of different types of solid organ transplants, HIV-infected patients, and those with other disorders including immune-mediated disease and malignancies.

**Analytical Performance Characteristics**

In addition to the issues described above, actual test performance relies on preanalytical (specimen collection, transport, processing), analytical (testing), and postanalytical (reporting, interpretation of results) characteristics.

**Preanalytical Considerations**

The way in which a specimen is handled before the assay is performed can affect the test results. As part of a clinical laboratory’s test validation, storage conditions should be evaluated, especially when samples are sent for testing to referral labs or testing is delayed for batch testing. Current recommendations are to keep the sample refrigerated for no longer than 72 hours [3]. Sample type is another important preanalytical variable. Quantitative CMV testing is typically performed on whole blood or plasma. Each specimen type has strengths and limitations. CMV DNA is detected more frequently and viral load values are often higher in whole blood compared with plasma because both cell-free and intracellular viruses are detected in the former. A recent study showed that viral load values in most patients are about $1 \log_{10}$ (10-fold) higher in whole blood compared with plasma [4]. However, in some patients the difference was as great as $2 \log_{10}$ copies/mL (100-fold), and occasionally plasma viral load was found to be higher than whole blood viral load [4]. Because latent DNA persists in the cellular fraction of blood, the detection of CMV in plasma is thought to more likely reflect active infection [5]. The use of plasma instead of whole blood in these tests requires rapid separation to keep the DNA from degrading with resultant fragmentation [6]. A recent study has shown that once plasma is separated from whole blood, the plasma DNA is stable for 14 days at 4°C [7]. Clinicians should familiarize themselves with the strengths and limitations of testing different specimen types and use a single specimen type to monitor patients longitudinally.

**Analytical Test Characteristics**

There are performance characteristics of viral load tests that must be understood in order to properly use and interpret test results. These include the limit of detection (LOD),
lower and upper LOQ, linear range, precision (reproducibility), and accuracy. The LOD, defined as the lowest concentration of DNA that can be detected in 95% of replicates [8], is typically used for qualitative molecular tests; however, the term can also be applied to quantitative viral load tests. The upper and lower LOQs are the highest and lowest concentrations of DNA that can be quantified with "acceptable precision" [8]. Currently, there is no agreed-upon definition for "acceptable precision"; therefore, individual laboratories define this locally. The way in which "acceptable precision" is defined can greatly influence the LOQ. The LOD may be equivalent to the lower LOQ or it may be lower than the lower LOQ. In the latter situation, the test may detect CMV DNA, but the value cannot be accurately quantified. In these cases, the laboratory could report the result as "detected but below the LOQ"; some laboratories report this as "low positive." The laboratory should be able to provide both the LOD and the LOQ; for example, in our laboratory the LOD of the CMV viral load test is 100 copies/mL, while the LOQ is 300 copies/mL. After consultation with clinicians, the decision was made to report values of 100–300 copies/mL as "low positive" results, while those >300 copies/mL are reported as an integer value.

Although very sensitive molecular tests are often important for the detection of infectious pathogens, this may not be the case for CMV DNA tests. The clinical significance of low levels of CMV DNA (<100–500 copies/mL) may be difficult to interpret, particularly if whole blood samples are tested, because low levels of CMV DNA in whole blood or plasma do not always correlate with the development of disease. Some studies have been done to determine the threshold for treatment, including a natural history study of CMV disease in liver transplant recipients [9]. Using the COBAS Amplicor Monitor test (Roche Molecular Diagnostics), the authors showed that a viral load value of 2000–5000 copies/mL correlated with the development of end organ disease. This cutoff value is test dependent and may be higher or lower for other LDTs. Studies have also shown that both the viral load value and the rate of change in viral load are important predictors of the development of active disease [10]. The faster the rate of increase in viral load value, the greater the risk of developing CMV disease. Once tests are recalibrated with the international standard, a universal clinically relevant LOD and lower LOQ can be identified. Based on currently available data and experience, 250–500 IU/mL may be an appropriate LOD and/or lower LOQ.

Given that most LDTs use real-time polymerase chain reaction technologies, the linear range of CMV viral load tests is usually at least 6 log_{10}; for example, the test may detect from 2.0 log_{10} copies/mL (100 copies/mL) to 7.0 log_{10} copies/mL (10 million copies/mL). A review of CMV viral load testing performed in 2009 at Emory Medical Laboratories showed that of the approximately 8500 CMV DNA viral load tests performed on plasma samples, 671 (7.9%) were positive. Of these, 394 (58.7%) were <1000 copies/mL, 169 (25.2%) were between 1000 and 10 000 copies/mL, and 63 (9.4%) were between 10 000 and 100 000 copies/mL. In addition, 45 (6.7%) had a CMV DNA viral load of >100 000 copies/mL. Although low viral load values are common, their clinical significance is unclear. Very high viral load values are uncommon and, when they do occur, are usually seen in immunosuppressed patients with primary CMV infection or in those with very severe disease. Because viral load values as high as 10 million may be present in some individuals, the upper limit of quantification of a test should be in this range.

The interpretation of important differences in longitudinal viral load values in an individual patient requires an understanding of the precision or reproducibility of the test. Typically the variability of viral load tests is greatest near the lower and upper LOQ and lowest in the middle range of the assay. As a result, small changes in low viral load values are not significant; for example, no clear difference exists between results in a patient in whom the viral load value has increased from 200 copies/mL to 500 copies/mL because this difference is within the variability of the assay. The most useful data regarding clinically important changes in viral load values come from experience with HIV-1. For these FDA-approved tests, the variability of the test ranges from 0.1 log_{10} copies/mL to 0.2 log_{10} copies/mL, as compared with the biological variability observed in chronically infected untreated individuals, which is about 0.3 log_{10} copies/mL. Therefore changes in viral load of >0.5 log_{10} copies/mL are thought to represent biologically important changes in viral replication. Although the 0.5-log_{10} copies/mL value has been widely adopted for use with many other viral pathogens, it may not be accurate for CMV. Because the biological variation of CMV is not known, important changes in viral load values have historically been assessed based only on the test variability. Further work with HIV-1 showed that the SD of the test needed to be =0.15 log_{10} copies/mL to discern 5-fold changes in viral load values [11]. For CMV LDTs the variability of the assay is expected to be no better than what is seen for the FDA-approved HIV-1 viral load tests and it may well be higher, particularly near the lower LOQ [12]. Therefore, when detecting CMV viral load values of <1000 copies/mL, changes of <5-fold (0.7 log_{10}) will rarely reflect clinically important changes in viral replication. For values >1000 copies/mL, 3-fold (0.5 log_{10}) changes in viral load may be significant, although this may differ among LDTs. Some centers report viral loads as copies/mL and as log_{10} copies/mL because clinicians may be less likely to overinterpret small changes in viral load values when expressed as log_{10} values.
A baseline sample for viral load testing should be collected on the day CMV treatment is initiated, even if the CMV viral load testing was performed a few days earlier. Rapid increases in CMV viral loads may occur over a few days in patients with untreated disease, making it important to have the CMV viral load value at the time of treatment. If viral load values exceed the upper LOQ, it is recommended that the laboratory dilute the specimen to determine the actual viral load value. Once therapy is initiated, the ideal interval for monitoring is weekly because the half-life of CMV DNA in plasma is 3–8 days [13]. Another reason for using this interval is that it is not uncommon for the CMV DNA viral load to increase in a patient a few days after initiation of therapy, and this may be misinterpreted as treatment failure. Because patients with persistent DNA in plasma or whole blood are at increased risk of relapse [4, 13, 15–17], current guidelines [14, 15] recommend continuing antiviral therapy until viral load values are undetectable. Some patients have persistent low levels of CMV DNA in plasma or whole blood and never develop CMV disease or relapse. In these individuals, it is more useful to follow trends in viral load values over time than to assess the significance of any given viral load value. Viral load patterns that are worrisome for drug-resistant virus include those that do not decrease after 2 weeks of adequate therapy [14, 18], those with a plateau in the rate of viral load decline, and those that have an initial drop and then a subsequent increase in viral load while on therapy. These patients should be evaluated for resistant virus by sequencing the UL97 and/or UL54 (polymerase) genes directly from a plasma specimen.

CMV DNA viral load tests are designed so that they do not cross-react with other herpesviruses. In an immunocompromised patient, detection of 2 herpesviruses, for example, CMV and Epstein-Barr virus, reflects reactivation of both viruses and is not due to cross reactivity.

**CLINICAL INTERPRETATION**

CMV DNA viral load testing is an important aid for assessing risk of disease, diagnosing disease, and monitoring response to therapy. The interpretation of CMV DNA viral load values can be complex, and numerous virus and host factors should be considered when assessing the risk of CMV disease. An understanding of the performance characteristics of the viral load test is necessary for appropriate interpretation of test results. Below are some clinical pearls regarding interpretation of CMV viral load testing:

1. CMV viral load values are not comparable among LDTs; when monitoring patients, use a single test and specimen type (either whole blood or plasma).

2. The availability of an international standard will improve agreement among tests and should allow for multicenter studies to determine important clinical cutoffs that predict the risk of developing CMV disease and/or relapsed disease. Before adopting a clinical cutoff from another laboratory, it is critical to fully understand the relationship of viral load values between the 2 laboratories.

3. If LOD and lower LOQ for the viral load test do not agree, discuss how to report values that are greater than the LOD and less than the lower LOQ with your laboratory.

4. Understand the precision of the viral load test used in your laboratory, particularly at or near the lower LOQ. Tests have more variability near the lower LOQ. Assume that changes in viral load must exceed 3-fold (for values in the midrange of the test) to 5-fold (for values in the lower range of the test) to represent meaningful changes in viral replication.

5. Low viral load values are common and their clinical significance can be difficult to assess. Factors to consider when interpreting these results include specimen type, type of transplant, degree of immune suppression, and donor/recipient CMV immune status, as well as the LOD and precision of the test.

6. When monitoring patients posttransplant, trends in viral load may be more useful than any single viral load value, unless the viral load is very high (>10 000 copies/mL) or the patient is at very high risk (eg, donor/recipient mismatch). Both the rate of change in viral load and the absolute viral load are important in predicting risk of developing disease.

7. Once the decision is made to initiate treatment, viral load testing should be performed the day therapy is begun, even if a recent value is available, because viral load levels may increase rapidly in patients with active disease. An accurate starting viral load is critical when monitoring response to therapy.

8. Follow-up viral load testing should be performed at 5- to 7-day intervals because the half-life of CMV DNA in the plasma ranges from 3 to 8 days [13]. Avoid obtaining viral load values more frequently, particularly very early after initiation of therapy, because viral load values can increase and be misinterpreted as treatment failure. After initiating therapy, the viral load should be followed to document clearance of CMV from the plasma or whole blood. Depending on the initial viral load, this may take weeks or longer. Persistently elevated CMV viral load values or viral load values that increase after an initial decline are concerning for resistant virus [18]. Genotypic resistance testing can be performed directly using plasma specimens when viral loads are at least several thousand copies per milliliter.
Notes

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