INVITED ARTICLE

Practical Points for Molecular Diagnostics of Cytomegalovirus Infection in Solid Organ Transplant Recipients

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Cytomegalovirus infection

Cytomegalovirus (CMV) is a prototypic opportunistic pathogen causing significant morbidity and occasional mortality among patients with immunocompromising conditions, especially those who had undergone solid organ transplantation (SOT). Depending on the interplay between the virus (e.g., viral burden or viral load), the host (net state of immune status), and transplant-related factors (e.g., antiviral prophylaxis), the clinical presentation of CMV could range from an asymptomatic illness to symptomatic, early- to late-onset, mild to severe, and localized to disseminated disease[1]. Clinical manifestations and severity could vary depending on the specific organ and concurrent immunosuppressive regimen. Those who have undergone thoracic organ transplants have a greater chance of CMV infection than intraabdominal organ recipients due to the more intense immunosuppressants needed[2.]

CMV infection has traditionally relied on tissue histopathology, which demonstrates viral cytopathic changes with intranuclear and cytoplasmic viral inclusions of CMV-infected cells resembling an “owls eye”[2,3]. Later, immunohistochemical staining could be applied to improve the sensitivity to identify the virus in CMV-infected tissue[4]. However, obtaining tissue for diagnosis through an invasive procedure may not always be feasible for vulnerable and compromised individuals. Often times, the biopsy cannot be performed due to the instability of the patient’s hemodynamic status when they present with severe disease.

Therefore, less invasive diagnostic methods such as molecular testing, particularly nucleic acid amplification testing (NAAT) using the real-time polymerase chain reaction (PCR) technique on blood samples, has been utilized preferentially in clinical practice. This more convenient tool has allowed for clinicians’ early diagnosis of CMV infection. Furthermore, this molecular test could detect viral

replication early to trigger the prompt initiation of anti-CMV therapy in a strategy called “preemptive approach”, and it has been used as a monitoring tool to assess response to treatment **(Figure 1)**[5].

Molecular diagnostics for CMV infection

Quantitative NAAT (QNAT) is clinically useful because it allows for measuring the amount of virus in a clinical sample, which has been correlated with clinical outcomes. A high absolute level or a rapid rise in CMV DNA load has been correlated with symptomatic CMV infection and its clinical progression. Additionally, a decline and clearance of CMV DNA loads in clinical samples have been used as surrogates for improvement and resolution of CMV disease **(Table 1)**. Therefore, QNAT has been widely utilized as a convenient tool for the diagnosis of active infection and for monitoring an impending or resolving disease. However, practical points for interpreting QNAT for CMV infection are essential for every transplant provider who should understand the dynamic change of this specific infection in SOT recipients **(Table 2).**

Several commercial CMV QNAT assays have been available and they have replaced laboratory-developed in-house assays[6]. However, not all QNAT are created equal, and there are variabilities in viral load reporting between these assays. Efforts to standardize reporting was made with the creation of the World Health Organization (WHO) International Standard (IS) for calibration of CMV assays. Despite this, there remains some variability due to differences in clinical samples, methods for DNA extraction and the amplification efficiency based on target genes among the various QNAT assay. While the WHO standard is allowed for a standardized reporting, by promoting the conversion of viral load reporting from copy/mL to international unit/mL[7], there remains differences between assays. Accordingly, the international

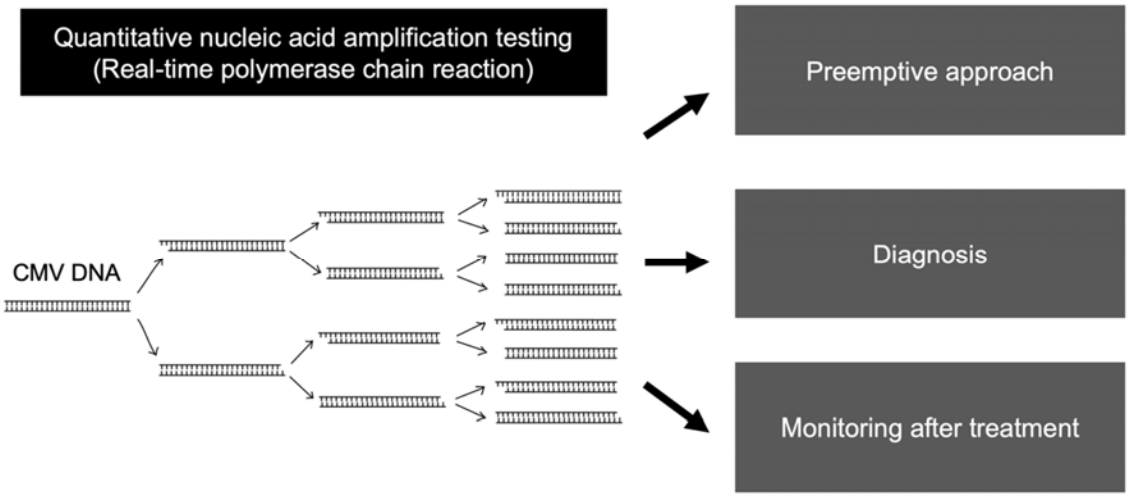

Figure 1 Roles for molecular diagnostics of cytomegalovirus infection in solid organ transplant Recipients

Table 1 Cytomegalovirus DNA load as an appropriate surrogate marker for CMV infection (adapted from Kraft CS et al.) [8]

	Explanation
CMV DNA load in CMV disease is significantly greater than in asymptomatic DNAemia	Solid organ transplant recipients with symptomatic CMV infection, called "CMV disease," tend to have higher CMV DNA in their blood when compared to those with asymptomatic CMV DNAemia.
Kinetics of CMV DNA replications are strongly associated with progression to disease	A more rapid rise or an accelerating rate of CMV DNA in the blood is well correlated with progression of CMV disease.
Treatment of CMV DNAemia prevented disease	Solid organ transplant recipients with low level asymptomatic CMV load that is treated with anti-CMV drugs could have regression of viral replication and prevent further progression of CMV infection.
CMV DNA load decline correlated with symptom resolution	A reduction and clearance of CMV DNA load in the blood are correlated with improvement and resolution of clinical symptoms.

society guidelines have continued to recommend the use of the same assay and specimen when caring for a transplant patient with CMV[2]. Monitoring of the viral load of a patient should not be performed using multiple assays, because their results will not be directly comparable.

1. Preemptive approach

CMV viral load could be used to routinely monitor patients after transplant for evidence of early virus replication[9]. This will allow for a preemptive approach to prevention of CMV disease. Using this strategy, transplant recipients are monitored closely by CMV DNA load testing of whole blood or plasma on a once weekly schedule for 12 weeks after transplant, especially among CMV-seropositive recipients. However, this stringent strategy requiring frequent (weekly) testing and its associated cost may not be practical in resource-limited countries where transplant patients are predominantly CMV seropositive[10-12]. Hence, a selective and stratified method has been suggested, focusing on CMV-seropositive recipients with a high risk of CMV reactivation, such as those receiving anti-thymocyte globulin for induction therapy[13]. A less frequent interval, such as every 2-4 weeks, which coincides with an outpatient visit, has also been suggested to be another logical approach, although this will need to be subjected to more studies.

Another caveat is that a viral load threshold to initiate preemptive therapy is not well defined.

Table 2 Practical points for interpretation of CMV DNA load for preemptive approach, treatment, and monitoring

	Explanation
Use the same assay	-Use the same assay, either in-house assay or commercial assay, to avoid variability of the viral quantification.
Use same specimen type	-Collect the same type of sample, e.g. plasma, whole blood, bronchoalveolar lavage fluid, to avoid variability of viral quantification.
Use quantitative NAAT	-Using quantitative NAAT rather than qualitative since the presence of DNA alone (without quantification) may not be considered clinically significant compared to the quantifiable high level viral load.
Trends in CMV DNA load is more useful than any single value	-Consider the dynamic change of CMV DNA load between two-time points rather than the single time point value. -No universal viral load cut-off value for initiation of preemptive therapy or diagnosis.
Follow-up CMV DNA load testing at 5- to 7-day intervals	-Due to the doubling time of CMV replication being approximately 3-8 days. Therefore, the regular time interval for viral load follow-up is approximately one week. -Twice a week may be only considered in an exceptional situation when active severe CMV disease is cautiously being monitored, or there is a high risk of severe disease progression.
A more remarkable change of CMV DNA load is required for low-level compared to high-level CMV DNAemia to be considered significant.	-Clinical significance is considered for >5-fold (0.7 log10) changes in CMV DNA load < 1000 copies/mL >3-fold (0.5 log10) changes in CMV DNA load > 1000 copies/mL
Histopathological confirmation is the gold standard for a diagnosis of tissue-invasive disease, although NAAT has been more practical.	-Quantification of CMV DNA load may not always be possible, especially those developed compartmentalized diseases such as GI disease, and retinitis

	-A highly sensitive QNAT (very low limit of quantification <200 IU/mL) has improved the chance of quantification and assisted in an earlier diagnosis. -Tissue diagnosis remains essential, especially when a co-pathogen or condition exists.
CMV DNA load values can increase at very early after initiation of therapy	-A slight increase of DNA load in the blood is commonly seen within a week after initiating an anti-CMV drug, and does not necessarily indicate refractory or resistant CMV infection. -Consider the possibility of refractory/resistant CMV infection if there is no significant decline in viral load at two-three weeks of an appropriately dosed anti-CMV drug

This is due to differences in viral load reports among different assays. Thus, the international guidelines still recommend that this viral load threshold should be determined for every center, type of organ transplant, and individual's immune status. A nationwide survey among transplant infectious disease physicians and transplant nephrologists in a setting where the majority of recipients were CMV-seropositive, identified a cut-off of 2,000-3,000 cps/mL (or 1,820 and 2,730 IU/mL) as a trigger to start preemptive treatment[14]. This data is compatible with one study reporting the threshold value of 2,275 IU/mL that distinguished those who spontaneously resolved and those who required treatment for viral clearance, and a viral load threshold of 3,893 IU/mL, which distinguished those with and without symptoms in CMV-seropositive SOT recipients[15,16]. However, a universal cut-off value remains elusive and may not be available for widespread application. Additionally, dynamic changes in CMV DNA load between two different timepoints may be more important than a single absolute viral load value. Moreover, a clinically relevant viral load threshold may be individualized according to a transplant patient's immune status. For example, CMV seromismatch (D+/R-) SOT recipients, lung transplant recipients, and those who recently received augmented immunosuppressants with T-cell depleting agents are at high risk of CMV disease progression and should be treated even at a lower viral load cut-off value.

2. Diagnosis

The gold standard of diagnosis of tissue-invasive CMV disease still requires a biopsy of tissue for histopathology. However, demonstrating CMV in the blood using QNAT is more easily accessible for primary clinicians. Interestingly, there is increasing data demonstrating the role of NAAT in confirming the diagnosis of CMV, while still awaiting tissue biopsy procedure and results[17].

Taksinwarajarn T et al. reported a potential use of a negative plasma CMV DNA load to exclude CMV GI disease in CMV-seropositive kidney transplant recipients. This approach is reasonable due to a highly sensitive PCR assay, defined as a lower limit of detection of 200 IU/mL[18]. However, one has to consider compartmentalized CMV

diseases that lack any detectable CMV viremia, especially among CMV-seropositive recipients with CMV gastrointestinal diseases and CMV retinitis[19-20]. In these situations, a negative CMV DNA load in plasma does not exclude the diagnosis.

Saksirisampant G et al. also reported a quite similar finding; however, they focused their study on CMV pneumonitis in the immunocompromised (including SOT recipients). There was a significant correlation between plasma and bronchoalveolar lavage (BAL) fluid CMV DNA load, which suggests that a plasma sample could potentially be used as a surrogate measure. Accordingly, an undetectable plasma CMV DNA could almost entirely exclude CMV pneumonitis when a highly sensitive PCR was utilized[18].

Tissue diagnosis remains essential to confirm a definite diagnosis and to explore for possible co-infections or coexisting pathology such as rejection. Furthermore, histopathological investigation should be considered especially when there is concern for compartmentalized, refractory, or recurrent CMV infection[21,22].

3. Treatment monitoring

The cornerstone of CMV management is comprised of two essential components 1) reduction of immunosuppressants in order to allow for CMV-specific immunity to recover and control viral replication, and 2) anti-viral agents to halt further viral replication. [2]

Monitoring CMV viral loads among SOT recipients during treatment of CMV infection is another essential aspect of management. Apart from assessing for improvement of clinical symptoms, QNAT has been utilized as a marker for regression of infection. Therefore, the recommendation to follow plasma CMV DNA load once a week until two consecutive undetectable viral loads for a total duration of at least two (asymptomatic infection) to three weeks (CMV disease) is recommended[20]. Lately, maintenance therapy or secondary prophylaxis after viral clearance has been proposed, although this is not mandatory particularly when the profound immunosuppression has been resolved. Recently, CMV-specific T cell immune monitoring has been investigated for its role in assisting clinicians on the decision whether maintenance antiviral therapy is necessary at the end of induction therapy[23]. Those with reconstituted CMV-specific immunity, defined by a reactive QuantiFERON-CMV assay, are at a lower risk of recurrent infection when compared to those with non-reactive (inadequate CMV-specific immunity) or indeterminate (profoundly immunosuppressed) results[24].

A lower plasma CMV DNA load at the diagnosis (less than 18,200 IU/mL) or clearance of CMV viremia (less than 31 IU/mL) at the end of a 3-week induction therapy has been correlated with a favorable response to treatment[25]. More interestingly, using different QNAAT assays, with different sensitivities and performance characteristics, may result in different times to clearance of CMV DNAemia[26]. A more sensitive assay is more likely to extend the duration of DNAemia (because it detects virus at lower levels), which could result in prolongation of antiviral therapy. A clinical significance of low-level CMV DNAemia (less than 3log10 copies/mL) has been investigated. However, 44% of those could achieve a viral clearance[27]. More importantly, a low level of CMV DNA may persist in a whole blood sample even if it was no longer detected in plasma; this is commonly encountered in clinical practice since plasma is 1log10 less than whole blood. This difference in viral load values across blood

compartments highlight the need to use the same type of specimen during follow-up[25].

At 2-3 weeks of induction antiviral therapy, NAAT could be used to identify those with refractory or resistant CMV. Persistent symptoms or sustained CMV DNAemia without an appropriate decrease in viral load or an increased viral load at 2-3 weeks of appropriate antiviral therapy should be suspected as refractory or resistant CMV infection/disease[29]. Gene sequencing should be performed to determine the presence of specific gene mutations associated with drug-refractory or resistant CMV infection[29-30]. Additionally, whole genome sequencing has been available to determine emerging clinically relevant mutations that may influence treatment of CMV in real-world settings[31].

In clinical practice, QNAT could serve as a tool for diagnosing and monitoring CMV infection after SOT. In addition, QNAT can assist clinicians in understanding the dynamic changes in CMV load, thereby allowing them to interpret the results appropriately in the context of variability in immune function after transplantation.

References

1. Nuansri S, Kantachuvesiri S, Watcharananan SP, Thongprayoon C, Cheungpasitporn W, Bruminhent J. Clinical characteristics of late-onset cytomegalovirus infection after kidney transplantation. Transplant Proc 2021;53:2267-71.
2. Razonable RR, Humar A. Cytomegalovirus in solid organ transplant recipients-Guidelines of the American Society of Transplantation Infectious Diseases Community of Practice. Clin Transplant 2019;33:e13512.
3. Bruminhent J, Watcharananan SP. Cytomegalovirus Infection in Non-HIV Patients: A Return of the Monster. J Infect Dis Antimicrob Agents 2018;35:111-4.
4. Juric-Sekhar G, Upton MP, Swanson PE, Westerhoff M. Cytomegalovirus (CMV) in gastrointestinal mucosal biopsies: should a pathologist perform CMV immunohistochemistry if the clinician requests it? Hum Pathol 2017;60:11-5.
5. Natori Y, Alghamdi A, Tazari M, Miller V, Husain S, Komatsu T, et al. Use of viral load as a surrogate marker in clinical studies of cytomegalovirus in solid organ transplantation: A systematic review and meta-analysis. Clin Infect Dis 2018;66:617-31.
6. Pang XL, Fox JD, Fenton JM, Miller GG, Caliendo AM, Preiksaitis JK. Interlaboratory comparison of cytomegalovirus viral load assays. Am J Transplant 2009;9:258-68.
7. Fryer JF, Heath AB, Minor PD. A collaborative study to establish the 1st WHO International Standard for human cytomegalovirus for nucleic acid amplification technology. Biologicals 2016; 44:242-51.
8. Kraft CS, Armstrong WS, Caliendo AM. Interpreting quantitative cytomegalovirus DNA testing: understanding the laboratory perspective. Clin Infect Dis 2012;54:1793-7.
9. Dioverti MV, Lahr BD, Germer JJ, Yao JD, Gartner ML, Razonable RR. Comparison of standardized cytomegalovirus (CMV) viral load thresholds in whole blood and plasma of solid organ and hematopoietic stem cell transplant recipients with CMV infection and disease. Open Forum Infect Dis 2017;4:ofx143.
10. Bruminhent J, Dajsakdipon T, Ingsathit A, Supaporn T, Prommool S, Watcharananan SP. Impact of cytomegalovirus serostatus on allograft loss and mortality within the first year after kidney transplantation: an analysis of the National Transplant Registry. Transplant Proc 2020;52:829-35.

11. Bruminhent J, Razonable RR. Advances in drug therapies for cytomegalovirus in transplantation: a focus on maribavir and letermovir. Expert Opinion on Orphan Drugs 2020;8:393-401.
12. Bruminhent J, Vanichanan J, Watcharananan SP. Cytomegalovirus infection among Thai kidney transplant recipients: What is known and what should be known? J Infect Dis Antimicrob Agents 2020;37:105-10.
13. Chiasakul T, Townamchai N, Jutivorakool K, Chancharoenthana W, Thongprayoon C, Watanatorn S, et al. Risk factors of cytomegalovirus disease in kidney transplant recipients: A single-center study in Thailand. Transplant Proc 2015;47:2460-4.
14. Bruminhent J, Bushyakanist A, Kantachuvesiri S, Kiertiburanakul S. A nationwide survey of cytomegalovirus prevention strategies in kidney transplant recipients in a resource-limited setting. Open Forum Infect Dis 2019;6:ofz322.
15. Boaretti M, Sorrentino A, Zantedeschi C, Forni A, Boschiero L, Fontana R. Quantification of cytomegalovirus DNA by a fully automated real-time PCR for early diagnosis and monitoring of active viral infection in solid organ transplant recipients. J Clin Virol 2013;56:124-8.
16. Martín-Gandul C, Pérez-Romero P, Sánchez M, Bernal G, Suárez G, Sobrino M, et al. Determination, validation and standardization of a CMV DNA cut-off value in plasma for preemptive treatment of CMV infection in solid organ transplant recipients at lower risk for CMV infection. J Clin Virol 2013;56:13-8.
17. Ljungman P, Boeckh M, Hirsch HH, Josephson F, Lundgren J, Nichols G, et al. Definitions of cytomegalovirus infection and disease in transplant patients for use in clinical trials. Clin Infect Dis 2017;64:87-91.
18. Saksirisampant G, Kawamatawong T, Promsombat K, Sukkasem W, Liamsombut S, Pasomsub E, et al. A prospective study of plasma and bronchoalveolar lavage fluid CMV DNA load quantification for the diagnosis and outcome of CMV pneumonitis in immunocompromised hosts. J Clin Virol 2022;155:105243.
19. Taksinwarajarn T, Sobhonslidsuk A, Kantachuvesiri S, Thongprayoon C, Cheungpasitporn W, Bruminhent J. Role of highly sensitive nucleic acid amplification testing for plasma cytomegalovirus DNA load in diagnosis of cytomegalovirus gastrointestinal disease among kidney transplant recipients. Transpl Infect Dis 2021;23:e13635.
20. Fisher CE, Alexander J, Bhattacharya R, Rakita RM, Kirby KA, Boeckh M, et al. Sensitivity of blood and tissue diagnostics for gastrointestinal cytomegalovirus disease in solid organ transplant recipients. Transpl Infect Dis 2016;18:372-80.
21. Bruminhent J, Rotjanapan P, Watcharananan SP. Epidemiology and outcome of ganciclovir-resistant cytomegalovirus infection after solid organ transplantation: A single transplant center experience in Thailand. Transplant Proc 2017; 49:1048-52.
22. Boonsathorn S, Pasomsub E, Techasaensiri C, Apiwattanakul N. Analysis of ganciclovir-resistant cytomegalovirus infection caused by the UL97 gene mutation in codons 460 and 520 in pediatric patients: a case series. Open Forum Infect Dis 2019;6:ofz480.
23. Sester M, Leboeuf C, Schmidt T, Hirsch HH. The “ABC” of virus-specific T cell immunity in solid organ transplantation. Am J Transplant 2016; 16:1697-706.
24. Kumar D, Mian M, Singer L, Humar A. An interventional study using cell-mediated immunity to personalize therapy for cytomegalovirus infection after transplantation. Am J Transplant

2017;17:2468-73.

25. Razonable RR, Åsberg A, Rollag H, Duncan J, Boisvert D, Yao JD, et al. Virologic suppression measured by a cytomegalovirus (CMV) DNA test calibrated to the World Health Organization international standard is predictive of CMV disease resolution in transplant recipients. Clin Infect Dis 2013;56:1546-53.
26. Meesing A, Germer JJ, Yao JD, Gartner ML, Digmann BJ, Razonable RR. Differences in duration and degree of cytomegalovirus DNAemia observed with two standardized quantitative nucleic acid tests and implications for clinical care. J Infect Dis 2020;221:251-5.
27. Natori Y, Alghamdi A, Husain S, Rotstein C, Selzner N, Tikkanen J, et al. Clinical predictors of progression and clearance of low-level CMV DNAemia in solid organ transplant recipients. Transpl Infect Dis 2020;22:e13207.
28. Costa C, Sidoti F, Mantovani S, Gregori G, Proietti A, Ghisetti V, et al. Comparison of two molecular assays for detection of cytomegalovirus DNA in whole blood and plasma samples from transplant recipients. New Microbiol 2016;39:186-91.
29. Chemaly RF, Chou S, Einsele H, Griffiths P, Avery R, Razonable RR, et al. Definitions of resistant and refractory cytomegalovirus infection and disease in transplant recipients for use in clinical trials. Clin Infect Dis 2019;68:1420-6.
30. Ramanan P, Razonable RR. Cytomegalovirus infections in solid organ transplantation: a review. Infect Chemother 2013;45:260-71.
31. Yong MK, Shigle TL, Kim YJ, Carpenter PA, Chemaly RF, Papanicolaou GA. American Society for Transplantation and Cellular Therapy Series: #4 - Cytomegalovirus treatment and management of resistant or refractory infections after hematopoietic cell transplantation. Transplant Cell Ther 2021;27:957-67.