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MINIREVIEW

Persistent Challenges of Interassay Variability in Transplant Viral Load Testing

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ABSTRACT While quantification of viruses that cause important infections in transplant recipients has been the standard of care for years, important challenges related to standardization remain. The issues are wide ranging, and until they are adequately addressed, the full impact of viral load testing regarding clinical management decisions will not be realized. This review focuses on a broad array of problems, including the lack of available FDA-approved/cleared tests, limited uptake of international standards, accurate quantification of secondary standards, specific assay characteristics, and commutability. Though some of these topics are nuanced, taken together they greatly influence the clinical utility of testing. For example, it has not been possible to define thresholds that predict the risk of developing disease and determine significant changes in serial viral load values for a given patient. Moreover, the utility of international guidelines may be limited due to the lack of a standardized assay. By summarizing the issues, the hope is that commercial companies, regulatory agencies, and professional societies can come together to advance the field and solve these problems.

KEYWORDS PCR, commutability, digital PCR, quantitation, standardization, transplant viruses, viral load

Measurement of viral load (DNAemia) has become an accepted element in the care of transplant patients, with results used to determine initiation of preemptive therapy, therapeutic responsiveness, and treatment endpoints. Such methods have existed for more than 20 years and since their inception have been plagued by variability of results both within and, particularly, between laboratories and assays. Such variability has undermined the development of consensus therapeutic breakpoints, has made it difficult to interpret literature across sites, and has reduced the portability of patient results. Over time, the lack of international quantitative standards and a paucity of commercially produced assays have been identified, among other factors, as key obstacles to result harmonization. Although great strides have been made in understanding and addressing these problems, progress has been uneven at best, and recent studies have demonstrated continued discordance among test results. This minireview will focus on cytomegalovirus, BK virus, Epstein Barr virus, adenoviruses, and human herpes type 6 virus.

AVAILABILITY OF COMMERCIAL ASSAYS

Some of the biggest barriers to standardization of DNAemia assays is the lack of international standards and FDA-approved/cleared assays. Experience with human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV) has shown that the availability of FDA-approved assays calibrated to WHO international standards has led to excellent agreement of viral load values across assays from different manufacturers (1). Unfortunately, similar progress has not been made for most of the transplant viruses. Prior to the availability of commercial assays and an international standard for

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TABLE 1 Comparison of NIST and WHO standards (7, 32, 33)

NIST standard reference materials	WHO international convention calibrators
Traceable to international system of units (SI)	Not directly traceable to SI units
Concn determined by reference method (digital PCR), expressed as copies/ml	Concn (nominal value) determined by consensus, expressed as international units (IU)/ml
Whole virus, cloned into bacterial artificial chromosome (BAC)	Intact virus or clinical material
3 concentrations of nucleic acid in buffer (TE)	Single concn of intact virus, frozen and lyophilized
Dilute and use immediately	Reconstitute in 1 ml of nuclease-free water, dilute in matrix and extract prior to use

cytomegalovirus (CMV), differences in viral load values of three $\log_{10}$ or more were reported between different laboratory-developed tests (1, 2). The FDA approval of commercial assays for the quantification of CMV DNA calibrated to the WHO international standard from three different manufacturers has led to improved agreement in viral load values (3). However, the financial and time investment required by manufacturers to receive FDA approval for a CMV assay is substantial. To address this problem, in the fall of 2016, an FDA panel was convened and voted to down classify CMV viral load assays (from class III (high risk) to class II (moderate risk)). Unfortunately, 3 years later, this change has still not been implemented. The down classification of CMV would substantially lower the cost and complexity of the clearance process for commercial companies and undoubtedly lead to the clearance of additional tests. Viral load assays for the other transplant viruses have not been submitted to the FDA and, as such, remain unclassified. There is hope that these tests will have lower regulatory requirements than those used for CMV. The regulatory hurdles coupled with a relatively small market compared to many other diagnostic assays continue to limit the availability of commercial tests for transplant viruses.

Given that there are no FDA-cleared or -approved tests for the other transplant viruses, clinicians must rely on laboratory-developed tests (LDTs). The proposed regulation of LDTs will add additional burden and cost for clinical laboratories that develop and use these tests. To be clear, viral load assays for BK virus (BKV), Epstein Barr virus (EBV), adenovirus (ADV), and human herpesvirus type 6 (HHV-6) have been the standard of care for managing transplant recipients for years. There are international guidelines recommending the use of these tests to initiate preemptive therapy, diagnose disease, and monitor response to therapy (4–6). The lack of commercial tests continues to hold back standardization, as it allows for the persistence of large numbers of LDTs. Based on experience with CMV, when commercial tests become available, laboratories actively transition from LDTs to these commercial tests. This is important because commercial tests have several advantages, including ease of use, reduced burden on laboratories for verification and quality assurance, and improved harmonization due to the use of fewer tests. Reducing regulatory challenges, lowering development cost, and ensuring adequate reimbursement of these tests are important issues that need to be addressed to increase the availability of commercial approved/cleared tests.

INTERNATIONAL STANDARDS

Apart from the development of commercial assays, one of the areas where progress has been most marked is in the production of international quantitative viral standards. World Health Organization (WHO) standards, produced by the National Institute of Biologic Standards and Controls (NIBSC, UK), have now been made available for most widely recognized transplant-associated viruses, including CMV, EBV, BKV, ADV, and HHV-6 (Table 1). WHO standards are biologic controls produced from well-characterized reference strains of cultured virus, suspended in buffered matrix and assigned a value in international units/ml (IU/ml) based on consensus (mean) results from a large number of laboratories sent material during prerelease evaluation studies. Subsequently, a large number of aliquots are lyophilized for sale and distribution as primary standards, with the intention that these are largely used by manufacturers of secondary standards for broad-scale distribution to end users. Once the first lot of a given WHO

standard is exhausted, subsequent lots are prepared by attempting to match concentrations to that initial lot. The U.S. National Institute of Standards and Technology (NIST, Washington, DC) has also produced quantitative CMV and BKV standards. Unlike WHO material, NIST material is assigned an absolute value based on physical measurement with a reference standard. In the case of CMV, this was done by using a bacterial artificial chromosome (BAC) carrying the full viral genome propagated in bacteria (*Escherichia coli*) and quantified by digital PCR. The process for BKV was similar, but a bacterial plasmid vector (pUC57-AMP) was used instead of a BAC. Like the WHO standards, NIST material is a certified, primary reference material, whose primary purpose is to establish a recognized consensus standard. Materials then developed by manufacturers, whose nominal concentrations are directly traceable to those primary reference materials, are referred to as secondary standards (7).

In fact, a number of manufacturers have now produced secondary standards, largely based on the WHO material. Secondary standards can now readily be found based upon CMV, EBV, and BKV. HHV-6B and ADV, much more recently released by WHO (2017 and 2018, respectively), can be expected to follow suit. While primary standards can be used to calibrate *in vitro* diagnostic (IVD) assays to produce results in IU/ml (see below), the availability of secondary standards directly enables normalization of both IVDs and LDTs and can also be valuable for quality assurance. For example, material with known IU/ml values can be well suited for use in verification of analytical measurement ranges or as external assay controls in tests producing quantitative results in like units. As with any reference material, the value of secondary standards, however, depends on how faithfully the production of that material matches primary reference material in concentration. This cannot be assumed to be the case. Early CMV secondary standards were tested using a common reference method (digital PCR [dPCR]) and shown not to have equivalence to WHO material assayed by the same methodology. Inquiries revealed that some manufacturers relied on real-time PCR assays to assign nominal values, creating a situation where secondary standards with corresponding nominal values contained unequal and variably accurate quantities of CMV DNA based on dPCR analysis (8). While more manufacturers have subsequently adopted dPCR as a means of normalizing viral nucleic acid concentrations to primary reference materials, a systematic survey of current manufacturers has not taken place, and the original study, comparing reference materials available for CMV, has not been recently replicated, leaving it to consumers of such materials to confirm the reliability of methods used in their production.

Even given the now broad availability of primary and secondary standards, their adaptation has been slow and uneven. Recent proficiency testing results (College of American Pathologists, Northfield, IL) reveal that while all CMV results are reported in IU, roughly half of EBV, 25% of BKV, and none of the ADV and HHV-6 results were reported in IU by participants (VLS-B survey, Participant Summary Report, 2018). The causes of slow adaptation are almost certainly multifactorial. Among transplant viruses, development of international standards has been spread roughly over the past decade; those viruses for which standards have been available the longest show the highest degree of use among proficiency testing participants. One of the greatest drivers of adaption is likely the availability of commercially produced, FDA-approved/cleared, *in vitro* diagnostic assays whose results are generated in IU/ml. Unfortunately, such assays remain sparse (see above). Among LDT users, not only is the reporting of results in IU far from universal, but the heterogeneity of such assays, trueness of secondary and tertiary standards (already noted), various means of mathematically normalizing to IU, and other factors may diminish the efficacy of these materials in achieving harmonization across assays and laboratories.

Published data have suggested that even when international standards are utilized, discrepancies remain between quantitative values reported using common samples (9). The degree of this disharmony varies, depending on the virus being tested. In the case of CMV, recent work shows marked remaining spread of results, at least some of which appears correlated with amplicon size, with assays using larger amplicons tending to

TABLE 2 Assay characteristics affecting variability of transplant viral load values

Assay characteristic	Example
Specimen type	Whole blood vs plasma
Kinetics of viral load	May vary by sample type, whole blood vs plasma
Form of the virus in the clinical specimen	Intact virus vs fragmented DNA
Extraction method	Efficiency of nucleic acid extraction with short fragments vs genomic DNA
Genetic target	Single gene, multicopy gene, degree of genetic variability
Amplicon size	CMV: assays with smaller amplicons (≤100 base pairs) provide higher viral load values than larger amplicons (>250 base pairs)

produce lower viral loads. In contrast, the use of international standards for EBV assays appeared to have a greater degree of success in harmonizing results. Data regarding efficacy of standardization for other transplant-related viruses (BKV, HHV-6, ADV) are so far lacking and will depend upon greater study and adaptation of those respective standards. It is clear, however, that disharmony of results is due to a number of factors, and the use of a common standard alone may not address all of these issues. Standards must be well characterized and shown to be commutable across all assays for which they are used (see below). This has not yet been demonstrated to be the case for all international standards or for secondary standards. As an important example, it has been shown that the passaged BKV used for the international standard contains subpopulations with deletions in the T region (10). This has important potential implications for quantitative values and secondary standards normalized with this reference material.

ASSAY CHARACTERISTICS

There are multiple characteristics of viral load assays that contribute to variability of results across different tests, including specimen type, the form of virus in the specimen, extraction method, and genetic target (Table 2). Any attempt to define clinical thresholds for initiating antiviral therapy or predicting the risk of disease development needs to take into consideration specimen type. In general, DNA is quantified as either cell free (plasma) or cell associated (peripheral blood mononuclear cells [PBMCs] or whole blood). Though there is strong opinion, there is not consensus regarding the ideal specimen type for measuring transplant virus DNA in clinical samples. When comparing the amount of CMV DNA in plasma and whole blood, DNA levels follow similar kinetics in the two compartments (11); however, in the ascending phase of infection, CMV DNAemia is detected more frequently in whole blood than in plasma, while in the descending phase of infection, DNA is detected more often in plasma than whole blood (11, 12). Several studies have shown that DNA levels are on average ~1 $\log_{10}$ higher in whole blood compared to levels found in plasma (11, 12). Similarly, levels of EBV DNA are higher in whole blood than plasma (11), and while this study showed that measuring EBV in plasma may have an inadequate sensitivity for detecting disease in transplant recipients, another has shown that testing plasma samples has a higher specificity and sensitivity for EBV disease than measuring EBV DNA in PBMCs (13). Data on HHV-6 is less abundant; a recent study showed markedly higher sensitivity (90% versus 55%), but lower specificity (68% versus 100%), for whole blood versus plasma based on HHV-6 mRNA detection as an arbiter (14).

In general, higher levels of CMV and EBV DNA correlate with an increased risk of disease in both solid organ transplant recipients (SOTR) and hematopoietic stem cell transplant recipients (HSCTR) (5, 15–17), although clinical cutoffs for predicting disease still have not been established. Determining cutoff values for CMV will require a large multicenter clinical study, given that the widespread use of preemptive and prophylactic therapies has greatly reduced the occurrence of active disease. Even if a single test were to be used, thus removing the issues around assay variability, the complex study logistics and high cost make it unlikely that such a study will be performed.

There is less controversary surrounding testing for BK virus DNA, with both plasma and urine having predictive value in driving decisions regarding performing renal biopsies for assessing BKV nephropathy (4). The cutoff recommended in plasma is 10^4 copies/ml, although this cutoff has not been validated with a wide array of assays. Regarding ADV, testing is routinely performed on plasma, whole blood, and stool samples. Stool samples are used primarily for monitoring children post-HSCT. DNA levels in plasma and whole blood tend to be greater than those found in PBMC samples and DNA levels tracked similarly over time in all three compartments (18), leading the authors to conclude that laboratory logistics favor the use of whole blood or plasma over PBMC samples. For any specific virus, the ideal compartment for testing will likely vary based on the virus and transplant type.

Another test characteristic that contributes to variability in viral load values is amplicon size. For CMV, it has been shown that tests with small amplicon sizes ($\leq$100 bp) tend to give higher viral load values than tests with larger amplicon sizes ($>$250 bp); these differences can be as great as 1 $\log_{10}$ (3, 19). Recent studies have provided insight into the role that the form of CMV in plasma plays in assay variability. In plasma, the vast majority of CMV DNA is cell free (98 to 100%), highly fragmented, and not virion associated. Moreover, the size of the fragments is quite small and likely accounts for the lower viral load values seen using assays with larger amplicon sizes (19, 20). Further studies are needed to assess the range of amplicon size and the percentage of DNA of various sizes. These findings are restricted to CMV in plasma; additional studies are needed to determine the form of DNA in whole blood and for viruses other than CMV in plasma and whole blood.

An additional variable to consider in viral load assay variability is the efficiency of the extraction method. This is particularly important for CMV (and possibly other viruses) that are highly fragmented in plasma. A recent study evaluated the efficiency of DNA yield using oligonucleotide fragments varying in size from 50 to 1,500 bp with 11 commercial extraction methods, including 4 newer methods designed to isolate cell-free DNA (21). There were inconsistent quantitative results across the various extraction methods. This was particularly evident with the small fragments (50 and 100 bp), where there was poor yield across all standard methods. Improvement in DNA yield was noted for some but not all of the cell-free DNA extraction kits. The FDA-approved CMV viral load assays were developed and validated prior to our current understanding of the extent of DNA fragmentation in plasma, so the extraction methods may not have been optimized for small fragments. These findings coupled with the delayed clearance of DNA from plasma compared to whole blood need to be considered when choosing the specimen type for testing and interpreting changes in viral load values during treatment.

COMMUTABILITY

Commutability is a key characteristic of reference materials that has helped to describe and explain differences in assay performance and result disparities. Originally described in the clinical chemistry literature (22), commutability describes the extent to which reference material behaves in a like manner to patient samples. This is crucially important, because if this is not the case, and if the relationship of those measures is not consistent across different assays, then reference materials with the same target concentration may produce differing final measures of DNA concentration when used to calibrate patient sample results, potentially defeating the entire purpose of standardization. Over the past decade, the applicability and importance of commutability to quantitative viral assays have become increasingly apparent, commutability of reference material having been shown to be a necessary element for improved interassay agreement (23).

The determination of commutability has largely been based on relative behavior of two or more assays. This can be done by linear regression analysis showing whether pairs of assays regress to the same line irrespective of whether reference material or patient samples are examined. Alternatively, entire groups of assays can be compared

through principle component analysis, again demonstrating relative relationships of reference material and patient samples when tested as unknowns by different assays (24). In both cases, a relative, qualitative result (commutable or not commutable) is produced, answering the question of whether the quantitative relation between patient samples and reference material is the same across assays. This methodology does not give an absolute assessment of commutability for a single assay and requires an assessment of at least two assays simultaneously. The relative nature of the assessment means that the results of the evaluation are dependent on which assays are included, potentially giving a markedly variable picture of commutability for a given assay. It also does not give a quantitative result, leaving unanswered the question of what degree of commutability is sufficient to attain a desired degree of interassay agreement. Recent work has utilized dPCR as a reference standard method, producing absolute, quantitative measures of commutability, allowing development of minimal commutability thresholds based on the desired degree of interassay agreement, and potentially allowing assessment of commutability on an individual assay basis (see below) (25, 26).

The concept of commutability has proven useful both in describing the behavior of assay-reference material systems and in suggesting ways in which interassay harmonization can be achieved. It must be realized that commutability cannot be viewed as an isolated characteristic, but one that is interrelated to all aspects of assay design, including elements such as nucleic acid extraction, primer/probe design, amplification, and calibration. If any of these (or other) elements create differential performance depending on whether reference material or patients are tested, the entire system can be viewed as noncommutable. Going back to the example of CMV, noncommutability or reduced commutability, as well as divergence of viral load results, was associated with increased amplicon size (3). This, combined with findings that circulating CMV is often found in a highly fragmented state, led to the hypothesis that larger amplicon assays were probably underquantifying circulating patient sample CMV compared to intact, whole-genome, reference material CMV. This resulted both in decreased commutability and in divergent DNAemia results compared to those from small-amplicon ($<$100 bp) assays. This hypothesis was later confirmed in part by data showing that fragmentation of reference material resulted in improved commutability for large-amplicon assays (27).

As already noted, commutability has not yet been demonstrated or adequately explored for more recently developed WHO material or for secondary, commercially available standards. The deletions already demonstrated in the BKV reference material could result in noncommutable results if incorporated into quantitative assays targeting the T region. Initial characterization of the ADV standard was limited to the use of only three clinical samples, leaving open the question of its commutability across the full spectrum of ADV species and serotypes. In fact, the development of reference materials that are commutable across all assays in use, in all sample matrices and all viral serotypes remains a significant challenge, particularly for those viruses with a wide variety of circulating genotypes and those routinely tested in more than one matrix.

VARIABILITY

The aforementioned examples demonstrate the interrelated nature of commutability with aspects of interassay variability. It is difficult to assess commutability without accounting for variability, as highly variable assays can mask poor commutability, when using relativistic methods (regression or principle component analysis) to measure commutability (26). Numerous elements have been shown to affect differences in assay performance (interassay variability) (1). These include primer design and primer/probe target, nucleic acid extraction method, sample matrix, and the selected quantitative calibrator. Any of these can "change the result." In theory, one could normalize for many differences in assay design by using common calibrators. This should cause convergence of results from different methods, as has been shown by several investigators. However, it does not hold true if reference material shows differing commutability among the assays one is attempting to harmonize (as shown in the CMV

example discussed above), where it is seen that disparate commutability is associated with disharmony of results. The latter can also be regarded as differences in assay accuracy and interassay agreement. Other aspects of variability are related to assay precision. When this metric differs between assays, it may cause differences in clinical prediction intervals (28). Looked at another way, it may reduce confidence in being able to tell true versus nonsignificant changes in DNAemia when looking at serial samples from a given patient or from single time point measures from different patients. Minimizing all forms of interassay variability is fundamental to the earlier-mentioned goals of result portability, the setting of consensus quantitative diagnostic and treatment thresholds, and the interpretation of studies across platforms and assays.

One approach to minimizing such variability is to obviate the use of relative methods of quantitation (such as real-time amplification detection) and therefore the need for quantitative standards. Digital PCR is an endpoint amplification method that effectively splits the PCR into a large number of microreactions (limiting partition), such that each microreaction contains either zero or one target sequence molecule. Absolute quantitation can then be achieved by counting the number of positive reactions at the endpoint and dividing by the total volume (29, 30). While still requiring validation, DNA-based quantitative assays using this methodology have been highly accurate, typically with a high degree of concordance across reagents (31). The use of dPCR as a primary testing modality is therefore a potential remedy to issues of assay disharmony.

However, digital platforms remain at a relatively early stage of development, with few defined clinical, quantitative viral assays. Much has already been invested in quantitative real-time methods, both in terms of assay development and in the advent of international standards. In this context, however, dPCR can play an important role in developing and in ensuring lot-to-lot consistency of international and secondary standards, in demonstrating concordance of secondary standards and other control materials with nominal target concentrations, and in troubleshooting real-time methods when unexpected or out-of-range results are generated.

Finally, dPCR can serve as a reference method for independent, objective, and quantitative determination not only of commutability, but also of other aspects of assay variability (25). This can be used in concert with predefined acceptability thresholds for interassay agreement and for clinical prediction intervals. That is, one can predict the level of agreement to be expected from two different assays when each is independently evaluated. Alternatively, thresholds of acceptability can be set for interassay agreement (or for intra-assay precision), and what degree of commutability from a reference material is required to meet those criteria can be determined. It can be envisioned that such data will in the future be determined as a routine part of premarket evaluation for commercial assays and standards, such that the behavior of individual methods and relative agreement of different methods can be well defined prior to introduction into routine clinical use.

CLINICAL IMPLICATIONS

As we expand our knowledge of sources of variability for viral load tests, it is important to reflect on how this information impacts clinical care. In order to optimally use viral load tests, clinicians need a basic understanding of the limitations of current assays, including issues concerning interassay agreement, the inability to define thresholds that predict the risk of developing disease, and factors that impact defining significant changes in serial viral load values from a given patient. Some points are clear, such as using the same viral load assay and specimen type when monitoring viral load values over time. Given that trends in viral load over time are more useful than a viral load result at a given time, using a single assay system and specimen type is an easy way to eliminate sources of variability. Our understanding of the differences in the rate of clearance of DNA from whole blood and plasma underscore the importance of using the same specimen type. Regardless of the specimen type used, knowledge of the dynamics of viral DNA in each compartment is critical to properly interpreting

trends and assessing disease risk; unfortunately, this information is largely lacking for viruses other than CMV and EBV.

Other factors, such as defining a meaningful change in viral load value when serially monitoring patients and determining viral load values that correlate with the development of active infection have been more difficult to establish. Establishing a meaningful change in viral load requires consideration of both the biological variability of the virus and the analytical variability of the test. Given that little is understood about the biological variation of transplant viruses, we rely on analytical variability to define important changes in viral load. A difference of greater than 0.5 $\log_{10}$ IU/ml between two assays or between two viral load values over time is considered to be clinically important, but this threshold was established for HIV-1, where there was an understanding of both biological and analytical variability. So, applying this threshold to transplant viruses can be problematic. From an analytical perspective, the variability of values is greatest near the lower limit of quantification for an assay, so for patients with viral load values under 3.0 $\log_{10}$ IU/ml, the variability will be substantially greater than that seen with values above this threshold. We now have the tools to define "real" differences between assays and the degree of reproducibility of any given assay, but without an understanding of the biological variability, we do not know what difference is clinically important. However, an understanding of analytical variability can reduce the risk of overinterpreting small changes in viral load values.

As stated earlier, we have little information regarding viral load thresholds that predict the development of disease. For example, years of clinical experience have shown that for CMV-seropositive individuals, low levels of viral load do not always progress to clinically important disease. Defining "low level" involves consideration of clinical factors such as the organ transplanted (e.g., kidney versus lung) and degree of immunosuppression. For BKV, there are data supporting thresholds for assessing the viral load values that predict the need for kidney biopsy to assess for BKV nephropathy in renal transplant recipients. However, standardization of this cutoff requires an assessment of agreement among a wide array of laboratory-developed tests, given that there are no commercially available assay systems available for widespread clinical use. The same holds true for ADV, where we do not have a good understanding of interassay variability to help define values that predict the risk of developing disseminated disease.

Clearly, advances have been made, but challenges remain. The lack of commercially available assay systems continues to hold back progress. As these become available, it is critical that the manufacturers assess interassay reproducibility and commutability in addition to intra-assay reproductivity.

LOOKING FORWARD

While great strides in the development of international standards and the widespread adoption of transplant viral load testing have been made over the past decade, much remains to be done. Adoption of international standards has been slow, there are limited data on commutability for most assays and viruses, and LDTs make up the vast majority of assays used to quantify these viruses. Moving forward, it is essential that standardized, sample to answer, commercial tests become more widely available for the common transplant viruses. This will assist in minimizing the impact of many of the factors discussed in this minireview. As such commercial products are rolled out, it will be equally important to characterize them with respect to variability, commutability and interassay agreement. In addition, we need to define why viral load values between assays do not agree and explain why underlying issues such as poor commutability continue to be problematic. Gaps in our current knowledge that need to be filled include the biological form of circulating virus in different compartments (plasma, whole blood, PBMCs), standardizing an approach to how secondary standards are quantified (e.g., dPCR), and determining what level of viral load is predictive of active disease and what change in viral load is clinically important. Funding agencies and diagnostic companies need to devote resources to addressing these challenges.

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REFERENCES

1. Hayden RT, Yan X, Wick MT, Rodriguez AB, Xiong X, Ginocchio CC, Mitchell MJ, Caliendo AM, College of American Pathologists Microbiology Resource Committee. 2012. Factors contributing to variability of quantitative viral PCR results in proficiency testing samples: a multivariate analysis. J Clin Microbiol 50:337–345. https://doi.org/10.1128/JCM.01287-11.
2. Pang XL, Fox JD, Fenton JM, Miller GG, Caliendo AM, Preiksaitis JK, for the American Society of Transplantation Infectious Diseases Community of Practice and the Canadian Society of Transplantation. 2009. Interlaboratory comparison of cytomegalovirus viral load assays. Am J Transplant 9:258–268. https://doi.org/10.1111/j.1600-6143.2008.02513.x.
3. Preiksaitis JK, Hayden RT, Tong Y, Pang XL, Fryer JF, Heath AB, Cook L, Petrich AK, Yu B, Caliendo AM. 2016. Are we there yet? Impact of the first international standard for cytomegalovirus DNA on the harmonization of results reported on plasma samples. Clin Infect Dis 63:583–589. https://doi.org/10.1093/cid/ciw370.
4. Hirsch HH, Randhawa PS, AST Infectious Diseases Community of Practice. 2019. BK polyomavirus in solid organ transplantation: guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. Clin Transplant 33:e13528. https://doi.org/10.1111/ctr.13528.
5. Kotton CN, Kumar D, Caliendo AM, Huprikar S, Chou S, Danziger-Isakov L, Humar A, The Transplantation Society International CMV Consensus Group. 2018. The third international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation. Transplantation 102:900–931. https://doi.org/10.1097/TP.0000000000002191.
6. Florescu DF, Schaenman JM, AST Infectious Diseases Community of Practice. 2019. Adenovirus in solid organ transplant recipients: guidelines from the American Society of Transplantation Infectious Diseases Community of Practice. Clin Transplant 33:e13527. https://doi.org/10.1111/ctr.13527.
7. Holden MJ, Madej RM, Minor P, Kalman LV. 2011. Molecular diagnostics: harmonization through reference materials, documentary standards and proficiency testing. Expert Rev Mol Diagn 11:741–755. https://doi.org/10.1586/erm.11.50.
8. Hayden RT, Gu Z, Sam SS, Sun Y, Tang L, Pounds S, Caliendo AM. 2015. Comparative evaluation of three commercial quantitative cytomegalovirus standards using digital and real-time PCR. J Clin Microbiol 53:1500–1503. https://doi.org/10.1128/JCM.03375-14.
9. Hayden RT, Sun Y, Tang L, Procop GW, Hillyard DR, Pinsky BA, Young SA, Caliendo AM. 2017. Progress in quantitative viral load testing: variability and impact of the WHO quantitative international standards. J Clin Microbiol 55:423–430. https://doi.org/10.1128/JCM.02044-16.
10. Bateman AC, Greninger AL, Atienza EE, Limaye AP, Jerome KR, Cook L. 2017. Quantification of BK virus standards by quantitative real-time PCR and droplet digital PCR is confounded by multiple virus populations in the WHO BKV international standard. Clin Chem 63:761–769. https://doi.org/10.1373/clinchem.2016.265512.
11. Lazzarotto T, Chiereghin A, Piralla A, Piccirilli G, Girello A, Campanini G, Gabrielli L, Costa C, Prete A, Bonifazi F, Busca A, Cairoli R, Colombo AA, Zecca M, Sidoti F, Bianco G, Paba P, Perno CF, Cavallo R, Baldanti F, on behalf of the AMCLI-GLaIT working group. 2018. Cytomegalovirus and Epstein-Barr virus DNA kinetics in whole blood and plasma of allogeneic hematopoietic stem cell transplantation recipients. Biol Blood Marrow Transplant 24:1699–1706. https://doi.org/10.1016/j.bbmt.2018.03.005.
12. Lisboa LF, Asberg A, Kumar D, Pang X, Hartmann A, Preiksaitis JK, Pescovitz MD, Rollag H, Jardine AG, Humar A. 2011. The clinical utility of whole blood versus plasma cytomegalovirus viral load assays for monitoring therapeutic response. Transplantation 91:231–236. https://doi.org/10.1097/TP.0b013e3181ff8719.
13. Kanakry JA, Hegde AM, Durand CM, Massie AB, Greer AE, Ambinder RF, Valsamakis A. 2016. The clinical significance of EBV DNA in the plasma and peripheral blood mononuclear cells of patients with or without EBV diseases. Blood 127:2007–2017. https://doi.org/10.1182/blood-2015-09-672030.
14. Takano K, Ogata M, Kawano R, Satou T, Nashimoto Y, Shirao K. 2018. Comparison of HHV-6 DNA detection in plasma and whole blood in allogeneic hematopoietic stem cell transplant recipients: frequent false-positive results for active HHV-6 infection using whole blood samples. Int J Hematol 108:535–542. https://doi.org/10.1007/s12185-018-2498-z.
15. Gartner BC, Schafer H, Marggraff K, Eisele G, Schafer M, Dilloo D, Roemer K, Laws HJ, Sester M, Sester U, Einsele H, Mueller-Lantzsch N. 2002. Evaluation of use of Epstein-Barr viral load in patients after allogeneic stem cell transplantation to diagnose and monitor posttransplant lymphoproliferative disease. J Clin Microbiol 40:351–358. https://doi.org/10.1128/JCM.40.2.351-358.2002.
16. McBride JM, Sheinson D, Jiang J, Lewin-Koh N, Werner BG, Chow JKL, Wu X, Tavel JA, Snydman DR. 2019. Correlation of cytomegalovirus (CMV) disease severity and mortality with CMV viral burden in CMV-seropositive donor and CMV-seronegative solid organ transplant recipients. Open Forum Infect Dis 6:ofz003. https://doi.org/10.1093/ofid/ofz003.
17. van Esser JW, van der Holt B, Meijer E, Niesters HG, Trenschel R, Thijsen SF, van Loon AM, Frassoni F, Bacigalupo A, Schaefer UW, Osterhaus AD, Gratama JW, Löwenberg B, Verdonck LF, Cornelissen JJ. 2001. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-depleted SCT. Blood 98:972–978. https://doi.org/10.1182/blood.v98.4.972.
18. Perlman J, Gibson C, Pounds SB, Gu Z, Bankowski MJ, Hayden RT. 2007. Quantitative real-time PCR detection of adenovirus in clinical blood specimens: a comparison of plasma, whole blood and peripheral blood mononuclear cells. J Clin Virol 40:295–300. https://doi.org/10.1016/j.jcv.2007.09.003.
19. Naegele K, Lautenschlager I, Gosert R, Loginov R, Bir K, Helantera I, Schaub S, Khanna N, Hirsch HH. 2018. Cytomegalovirus sequence variability, amplicon length, and DNase-sensitive non-encapsidated genomes are obstacles to standardization and commutability of plasma viral load results. J Clin Virol 104:39–47. https://doi.org/10.1016/j.jcv.2018.04.013.
20. Tong Y, Pang XL, Mabilangan C, Preiksaitis JK. 2017. Determination of the biological form of human cytomegalovirus DNA in the plasma of solid-organ transplant recipients. J Infect Dis 215:1094–1101. https://doi.org/10.1093/infdis/jix06.
21. Cook L, Starr K, Boonyaratanakornkit J, Hayden R, Sam SS, Caliendo AM. 2018. Does size matter? Comparison of extraction yields for different-sized DNA fragments by seven different routine and four new circulating cell-free extraction methods. J Clin Microbiol 56:e01061-18. https://doi.org/10.1128/JCM.01061-18.
22. Cattozzo G, Franzini C, Melzi d'Eril GM. 2001. Commutability of calibration and control materials for serum lipase. Clin Chem 47:2108–2113. https://doi.org/10.1093/clinchem/47.12.2108.
23. Caliendo AM, Shahbazian MD, Schaper C, Ingersoll J, Abdul-Ali D, Boonyaratanakornkit J, Pang X-L, Fox J, Preiksaitis J, Schönbrunner ER. 2009. A commutable cytomegalovirus calibrator is required to improve the agreement of viral load values between laboratories. Clin Chem 55:1701–1710. https://doi.org/10.1373/clinchem.2009.124743.
24. Clinical and Laboratory Standards Institute. 2010. Characterization and qualification of commutable reference materials for laboratory medicine; approved guideline, vol EP30-A. Clinical and Laboratory Standards Institute, Wayne, PA.
25. Tang L, Su Y, Gu Z, Caliendo AM, Pounds S, Hayden RT. 2018. A comprehensive statistical framework for determination of commutability, accuracy, and agreement in clinical DNAemia assays. J Clin Microbiol 57:170. https://doi.org/10.1128/JCM.00963-18.
26. Tang L, Sun Y, Buelow D, Gu Z, Caliendo AM, Pounds S, Hayden RT. 2016. Quantitative assessment of commutability for clinical viral load testing using a digital PCR-based reference standard. J Clin Microbiol 54:1616–1623. https://doi.org/10.1128/JCM.03346-15.
27. Hayden RT, Tang L, Su Y, Cook L, Gu Z, Jerome KR, Boonyaratanakornkit J, Sam S, Pounds S, Caliendo AM. 2019. Impact of fragmentation on commutability of Epstein-Barr virus and cytomegalovirus quantitative standards. J Clin Microbiol 58. https://doi.org/10.1128/JCM.00888-19.

28. Kraft CS, Armstrong WS, Caliendo AM. 2012. Interpreting quantitative cytomegalovirus DNA testing: understanding the laboratory perspective. Clin Infect Dis 54:1793–1797. https://doi.org/10.1093/cid/cis212.
29. Vogelstein B, Kinzler KW. 1999. Digital PCR. Proc Natl Acad Sci U S A 96:9236–9241. https://doi.org/10.1073/pnas.96.16.9236.
30. White RA, III, Quake SR, Curr K. 2012. Digital PCR provides absolute quantitation of viral load for an occult RNA virus. J Virol Methods 179:45–50. https://doi.org/10.1016/j.jviromet.2011.09.017.
31. Hayden RT, Gu Z, Sam SS, Sun Y, Tang L, Pounds S, Caliendo AM. 2016. Comparative performance of reagents and platforms for quantitation of cytomegalovirus DNA by digital PCR. J Clin Microbiol 54:2602–2608. https://doi.org/10.1128/JCM.01474-16.
32. Anonymous. 2014. 1st WHO international standard for human cytomegalovirus for nucleic acid amplification techniques: instructions for use. National Institute for Biological Standards and Control, Potters Bar, UK. https://nibsc.org/documents/ifu/09-162.pdf.
33. Anonymous. 2020. Certificate of analysis standard reference material 2366a: cytomegalovirus DNA (TowneΔ147 BAC) for DNA measurements. National Institute for Biological Standards and Control, Potters Bar, UK. https://www-s.nist.gov/srmors/certificates/2366a.pdf.