Are We There Yet? Impact of the First International Standard for Cytomegalovirus DNA on the Harmonization of Results Reported on Plasma Samples

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(See the Editorial Commentary by Valsamakis on pages 590–3.)

Background. Interassay harmonization of cytomegalovirus (CMV) DNA measurement is important for infection management. Uncertainty exists regarding the result harmonization achievable in patient plasma samples using quantitative polymerase chain reaction (qPCR) assays with calibrators now traceable to the First World Health Organization International Standard (IS) for CMV DNA.

Method. Serial dilutions of the IS and a blinded panel of 40 genotyped CMV DNA–positive pooled plasma samples and 10 negative plasma samples were tested by 6 laboratories using 10 qPCR assays calibrated to the IS. Each clinical sample was constructed using plasma from a single unique transplant recipient.

Results. The variance for individual CMV DNA–positive samples was greater for clinical samples (median, 1.50 [range, 1.22–2.82] log10 IU/mL) than for IS dilutions (median, 0.94 [range, 0.69–1.35] log10 IU/mL) (P < .001); 58.9% of all clinical sample results and 93.6% of IS dilution results fell within ±0.5 log10 IU/mL of the mean viral load of each sample. Result variability was not impacted by either genotype or quantitative levels of CMV DNA. Testing procedure differences can significantly influence results, even when analyte-specific reagents are identical. For clinical samples, all assays demonstrated result bias (P < .008). Assays with amplicon sizes ≤86 bp had significantly higher results compared to assays with larger amplicon sizes (≥105 bp) (P < .001).

Conclusions. The variability in CMV DNA results reported on individual samples has been reduced by the IS, but ongoing clinically relevant variability persists, preventing meaningful interassay result comparison.

Keywords. cytomegalovirus; international standard; result harmonization; viral load.

Measurement of human cytomegalovirus (CMV) DNA in peripheral blood samples of solid organ transplant (SOT) and hematopoietic stem cell (HSCT) recipients using quantitative polymerase chain reaction (PCR) assays has become standard practice in many transplant centers. These assays are used to direct preemptive strategies for disease prevention, to diagnose disease, to monitor response to therapy, and to detect relapse, as surrogate markers of CMV antiviral drug resistance and as safety markers in clinical trials of new immunosuppressive agents [1–3]. However, recent studies have demonstrated significant interassay variability in results reported on individual samples. This makes interinstitutional result comparison and the development of international clinical practice guidelines that reference CMV viral loads difficult [4–7].

Human CMV strains exhibit significant genotypic diversity, the clinical significance of which is uncertain; multiple strains may also coexist in individual patients [8]. Several typing systems, including a system based on diversity in a major glycoprotein gene (gB) have been used to group CMV strains [9]. When testing clinical samples, false-negative results and changes in the quantitative accuracy of CMV DNA measurement have been reported, resulting from polymorphisms in genes targeted by CMV assays [10–12].

There are many additional potential reasons for variability in CMV DNA results, including interassay differences in input volumes, extraction methods, detection chemistry and reagents, and instrumentation as well as operator-induced variability [2, 6]. However, interlaboratory result variability significantly exceeds intralaboratory variability, suggesting that a common assay calibrator may be an important first step in improving result harmonization [4]. In response to this need, the First World Health Organization (WHO) International Standard (IS) for CMV for nucleic acid amplification techniques was developed by the National Institute for Biological Standards and Control (NIBSC; United Kingdom) and endorsed by the WHO Expert
Committee on Biological Standardization in October 2010 [13]. However, one cannot assume that assays with calibrators traceable to this IS will improve result harmonization among assays or to estimate the extent of improvement without formal evaluation [14, 15].

We designed a study to determine current CMV DNA result variability by simulating conditions required for meaningful interinstitutional result comparison. These conditions included the testing of a significant number of the same clinical samples at several laboratory testing sites using many different assays with calibrators now traceable to the IS. Potential causes of ongoing result variability were identified, and clinical implications of our observations are discussed.

METHODS

Sample Panels
Residual plasma samples remaining and normally discarded after routine sample testing for CMV DNA at the Alberta Provincial Public Health Laboratory were collected and stored at −70°C. To minimize matrix effects and obtain sufficient specimen volume for testing by multiple assays, samples serially collected from the same transplant recipient were pooled to create individual "clinical samples" for the panel. The blinded sample panel included 40 anonymized human plasma samples positive for CMV DNA collected from 29 SOT (10 kidney, 10 lung, 6 liver, 2 heart and 1 pancreas) and 9 HSCT recipients as well as 10 samples negative for CMV DNA collected from 2 SOT (1 kidney, 1 heart) and 8 HSCT recipients. Concentrations of CMV DNA in the final positive sample panels spanned the dynamic range of 10^2–10^6 IU/mL. Two CMV DNA–positive samples were tested twice in the panel as duplicate samples. Initial designation as CMV DNA positive or negative was based on the results of a laboratory-developed test (LDT) [16]. Genotyping (gB) of the CMV isolates in the CMV DNA–positive samples was performed using methods previously described [9]. Approval for the University of Alberta ethics review board was obtained for creation of the clinical sample panel (study number Pro00035373).

Lyophilized vials of the WHO IS for CMV (5 × 10^6 IU/mL) provided by Dr Jacqueline Fryer (NIBSC code 09/162) along with CMV IgG– and CMV DNA–negative EDTA plasma from a single healthy donor was used to create the WHO IS sample panel. Each laboratory reconstituted the vial of lyophilized IS in 1 mL of deionized nuclease-free molecular-grade water and then used the plasma provided to create 4 additional samples (10-fold dilutions), resulting in the 5 samples in the IS panel (5 × 10^2–5 × 10^6 IU/mL). All plasma samples in the panel were frozen at −70°C and shipped overnight on dry ice to the testing laboratory.

Testing Procedures and Testing Laboratories
The clinical sample and IS panels were tested at 6 different laboratory sites using 2 laboratory-developed assays [10, 16] and 6 commercial assays/analyte-specific reagents (ASRs), resulting in 10 unique assays already calibrated relative to the IS (Table 1). Although for some assays components such as reagents used may have been identical, all 10 of the assays studied had some component that differed. Two different laboratories tested the panels using assays that had major identical components (RealStar CMV PCR Kit 1.0, Altona Diagnostics, Hamburg, Germany or artus CMV RG PCR Kit, Qiagen, Hilden, Germany) but had one or more steps within the procedure that differed. All samples in the panel were thawed once, extracted, and tested without replicates. The laboratory’s standard operating procedures based on the manufacturer’s instructions were followed, with results reported as international units per milliliter. Details of the testing procedures were obtained for each assay.

Statistical Analyses
All CMV DNA results were converted to logarithm-transformed values (log_{10}) prior to analysis. Results reported as CMV DNA detected but not quantifiable were assigned a value of 10 IU/mL less than the self-reported limit of quantitation (LOQ) for the testing assays; the reported value was used when results were reported quantitatively, even when less than the assay’s LOQ. The mean and standard deviation (SD) were calculated. Levene test, analysis of variance (ANOVA), and the Tukey honest significant difference (HSD) test were used to compare results among groups. SPSS 21 and R software were used for the analysis. The paired-samples 2-tailed test at a significance level of .05 was used to assess assays for significant result bias relative to the mean of all log_{10} results for each sample.

RESULTS

Assay Characteristics
Details of human CMV DNA measurement procedures for each assay including nucleic acid extraction technique used, plasma input volume, gene target, probe chemistry, detection method, limit of detection, and LOQ are summarized in Table 1.

WHO IS Panel and Clinical Panel Qualitative Testing Results
There were 3 missing results for the undiluted IS sample, 2 because of insufficient volume and 1 because of an invalid result. For the clinical sample panel, there was 1 missing result due to a process error. Two false-negative results and 1 false-positive result were observed using 2 of the assays. All assays were able to detect CMV DNA in all 6 of the positive clinical and IS samples with mean viral loads <3.08 log_{10} IU/mL.

WHO IS Panel Quantitative Testing Results
The results reported for the WHO IS panel relative to the expected result (based on value assigned to the WHO IS [5 × 10^6 IU/mL]) are summarized in Figure 1. Among reported results, 44 of 47 (93.6%) fell within ±0.5 log_{10} IU/mL range of the mean of all assay results, and 45 of 47 (95.7%) fell within ±0.5 log_{10} IU/mL of the expected result. The variance for
<table>
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<tr>
<th>Test</th>
<th>Lab1</th>
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<td>CMV LC-PCR artus CMV RG PCR Kit&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Quantitative TaqMan PCR (UL55/UL123-exon 4)</td>
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<td>105</td>
<td>64 (UL55), 76 (UL123)</td>
<td>95 (UL80.5)</td>
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<td>1.64</td>
<td>1.76</td>
<td>0.78</td>
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Abbreviations: ASR, analyte-specific reagents; CAP/CTM, COBAS AmpliPrep/COBAS TaqMan CMV Test; CE, Conformité Européenne; CMV, cytomegalovirus; FAM, fluorescein; FDA, US Food and Drug Administration; FRET, fluorescence resonance energy transfer; LD, laboratory developed; LDT, laboratory-developed test; LOD, limit of detection; LOQ, limit of quantitation; MIE, major immediate early; MP, manufacturer provided; NA, not available; PCR, polymerase chain reaction.

<sup>a</sup> Adapted from Hayden et al [18].

<sup>b</sup> The product has been renamed artus CMV RGQ MDx Kit.
individual samples is defined as the difference between the highest and lowest result (both expressed as \(\log_{10}\) IU/mL). The median variance for the dilutions in the WHO IS panel was 0.94 \(\log_{10}\) IU/mL (range, 0.69–1.35 \(\log_{10}\) IU/mL). For the IS panel, 6 of the 10 assays (RealStar CMV-D-Lab2, RealStar CMV-D-Lab3, RealTime CMV-C-Lab5, COBAS AmpliPrep/COBAS TaqMan CMV Test [CAP/CTM]-G-Lab1, Multi-Code-RTx CMV-E-Lab1, LDT-B-Lab4) had result bias relative to the expected value \(P < .05\), paired-samples 2-tailed \(t\)-test).

Clinical Sample Panel Quantitative Testing Results

The dynamic range covered by the CMV DNA–positive clinical samples based on the mean of all \(\log_{10}\)-transformed results for each sample was 2.62–5.71 \(\log_{10}\) IU/mL. All 40 CMV DNA–positive samples were genotyped with result distribution as follows: 45% (18/40) gB1, 12.5% (5/40) gB2, 12.5% (5/40) mixed genotype (gB1 predominant), 20% (8/40) gB3, and 10% (4/40) gB4. The quantitative CMV DNA results for each sample tested by each assay are summarized in Figure 2 and Supplementary Figure 1. False-negative results were excluded from the analyses. Among reported results for all CMV DNA–positive samples, 234 of 397 (58.9%) fell within ±0.5 \(\log_{10}\) IU/mL of the mean for all results of the sample tested. The variance for individual CMV DNA–positive clinical samples was higher (median, 1.50 [range, 1.22–2.82] \(\log_{10}\) IU/mL) than that observed for IS dilutions \(P < .001\), Levene test). The results were not significantly different when detectable but not quantifiable results were excluded from the analysis. For the clinical sample panel, all assays demonstrated result bias relative to the mean result for each sample in the panel \(P < .008\), paired-samples 2-tailed \(t\)-test) (Figure 2).

Factors Influencing Variability in Reported Results

For the 2 sets of duplicated samples in the clinical sample panel, significantly less variation in results was reported within a laboratory (intralaboratory) than among laboratories (interlaboratory) \((P < .001; \text{Figure 3})\).

The SD of reported results was not significantly impacted by either the quantity of CMV DNA in the sample \((P = .52)\) or gB genotype \((P = .50\), Levene test) \((\text{Supplementary Figure 2A and 2B})\). However, a trend toward greater variability in reported results was observed for the gB3 genotype, with significantly lower loads reported using the CAP/CTM assay for 4 of the 8 gB3 samples (>1.4 \(\log_{10}\) IU/mL difference from the mean viral load for each sample; Figure 4).

In clinical samples, results from assays with amplicon sizes \(\leq 86\) bp were significantly different when compared to assays with amplicons \(\geq 105\) bp \((P < .001)\) and \(\geq 254\) bp \((P < .001)\) \((\text{Tukey HSD test})\). In the clinical sample panel, a trend toward higher quantitative CMV DNA results was observed when using assays with progressively smaller amplicon sizes (Figure 2); this was not seen in the IS panel testing results.

The impact on results reported from 2 different laboratories when components of test procedures are changed, even when...
the same commercial reagents are used, are summarized for the RealStar CMV PCR Kit 1.0 (Figure 5A) and the artus CMV RG PCR Kit (Figure 5B).

DISCUSSION

When used for CMV disease prevention and management, “acceptable limits” for assay-related variance in CMV DNA results are uncertain. In the human immunodeficiency virus (HIV) setting, HIV viral load results differing by >0.5 log_{10} copies/mL are considered clinically significant [17]. Extrapolating from this, it has been suggested that quantitative CMV DNA results reported on individual samples should not differ by more than ±0.5 log_{10} IU/mL [1].

The WHO IS is a lyophilized form of cell-free virus pelleted from the supernatant of the clinical strain Merlin (gB genotype 1) grown in MRC-5 cells [13]. The result variance in the IS dilution panel observed in the current study represents a dramatic improvement in result harmonization compared with historical studies that reported testing results using assays without a common calibrator and samples constructed using CMV strains grown in tissue culture. In these historical studies, the result variance for individual constructed samples was sometimes >4.0 log_{10} copies/mL and seldom <2.0 log_{10} copies/mL [4–7, 15].

As previously reported by others [11, 15], we observed a larger result variance in clinical samples than in samples containing tissue culture–derived CMV DNA (IS dilutions). Few studies had been performed prior to the implementation of the IS that included all or most of the variables present in the current study, including the testing of a significant number of the same clinical samples using multiple assays, many of which were commercial assays at different laboratory testing sites. This makes the impact of the IS on result variance in clinical sample testing more difficult to assess [4, 7, 15]. However, comparison of our study results to the observations from these limited number of historical studies [4, 7, 15] suggests that use of the IS as a common assay calibrator has also improved result harmonization for clinical samples. However, the result variance for individual positive clinical samples that persists (median, 1.50 log_{10} IU/mL and maximum variances as high as 2.82 log_{10} IU/mL) is significant enough that it may impact patient management if results from different assays, even US Food and Drug Administration–approved assays, are used.

Although our study was not designed to test factors other than calibration that might contribute to result variability [1, 6], we made several observations that highlight other variables that warrant future study. An intriguing observation was that primer designs that resulted in small amplicon sizes appear to result in higher viral loads than those that yielded larger amplicons. Our observations support those of Boom et al [18], who studied plasma samples from 3 renal transplant patients and reported that CMV DNA in plasma is predominantly composed of free DNA and may be highly fragmented. This would explain our results; smaller fragments may not be measured by assays that use larger amplicons. If the biologic form of CMV DNA plasma is confirmed to be predominantly or entirely highly fragmented free DNA rather than virion-associated DNA, this has significant clinical implications. Human plasma, even if positive for CMV DNA, is likely to be noninfectious in the setting of blood transfusion. Clinicians and researchers would also need to change assumptions that one is directly measuring virion replication when interpreting dynamic changes in plasma CMV DNA loads for patient management or for mathematical modeling of CMV infection.
Figure 5. Examples of results from 2 testing sites using the RealStar cytomegalovirus (CMV) polymerase chain reaction (PCR) Kit 1.0 (A) but with different nucleic acid extraction techniques, plasma input volume, and detection system. The median difference in the 2 results reported for each individual sample was 0.74 (range, 0.20–2.44) log_{10} IU/mL (Tukey honest significant difference [HSD] test, P < .001). B. The artus CMV RG PCR Kit when only plasma input volumes differed. The median difference in the 2 results reported for each individual sample was only 0.01 (range, −0.93 to 0.70) log_{10} IU/mL (Tukey HSD test, P > .05).

Why result harmonization for clinical samples differs from samples constructed using tissue culture–derived CMV DNA is uncertain. The raw data from our study’s 90 testing procedure pairs was used to compare linear regression and correspondence analysis methods for the evaluation of the CMV DNA IS for a property important for result harmonization known as commutability [19, 20]; this has been reported separately [21]. In that analysis, we found that the WHO IS demonstrated poor or absent commutability in up to 50% of the assays used in our study. In the analysis described here, we observed that an assay’s amplicon size impacts CMV DNA measurement in clinical plasma samples but not in IS dilutions. This suggests that the biologic form of CMV DNA in plasma (fragmented DNA) may differ from the longer CMV DNA fragments and concatamers found in tissue culture–derived material such as the WHO IS, providing a possible explanation for the lack of commutability of the IS for some assays.

Others have highlighted the impact of the variable efficiency of nucleic acid extraction techniques on CMV DNA quantitation in plasma samples [6, 15, 22–24]. This may have contributed significantly to the clinical panel result differences we observed when 2 laboratories used the same ASR (RealStar CMV PCR Kit) but different nucleic acid extraction techniques. However, the 2 testing procedures also used different plasma input volume and detection systems. Our experience with clinical sample testing using the artus CMV RG PCR Kit at 2 laboratory sites suggest that small differences in plasma input volume may not significantly impact CMV DNA quantitation if all other steps in the procedure are identical. However, at extremes of input volume, both the sensitivity of the assay and accuracy of quantitation at low viral loads may be impacted.

Using the gB typing system, we could observe no significant differences in result variability across genotypes. The use of dual gene targets in 2 of the assays in our study represents a strategy to mitigate this risk. Our data do suggest that the CAP/CTM assay may be underquantifying some gB3 isolates. However, the number of isolates in any gB subgroup was small, and our study lacks the power required to accurately explore genotype as a cause of result variability.

Our study results have implications for infectious disease, laboratory, and transplant physicians, assay manufacturers, and regulators. Blinded samples in proficiency panels used by laboratories for quality assurance are almost always samples artificially constructed using isolates grown in tissue culture. Our study suggests that this approach may significantly underestimate result variability when clinical samples are tested. Ideally, proficiency panels should include material sourced directly from patients, although in the case of CMV, obtaining a sufficient volume of material for this purpose may be challenging. Although laboratory-developed assays can perform very well, at an international level, more commercial assay use would facilitate ongoing evaluation of interassay result harmonization. Transplant physicians monitoring individual patients or participating in clinical trials should use the same assay performed in the same laboratory to ensure appropriate interpretation of changes in viral load. Our study and others [7] suggest that in clinical trials or patient management settings, result exchange among laboratories may be possible if the same commercial assay is used and all processing and testing steps are identical; this requires further validation. Ongoing interassay result variability continues to prohibit the establishment of trigger points for preemptive therapy, treatment endpoints, and comparisons of other parameters of viral loads that can be universally applied; suggested values for these parameters remain assay specific. Assay manufacturers should standardize and optimize all steps in their assay’s procedures. Although use of ASRs combined with a variety of nucleic acid extraction techniques and detection systems provides laboratories with flexibility and better use of available resources, this approach will complicate achieving the goal of global result harmonization. Regulatory bodies should encourage ongoing studies of result harmonization and promote continuous quality improvement with the goal of international result concordance.

The results of our study are specific for CMV DNA measured in plasma and should not be extrapolated to other matrices such as whole blood, cerebrospinal fluid, bronchoalveolar lavage fluid, urine, or amniotic fluid where both matrix effects and
the biologic form of CMV DNA may differ. Limitations of our study include our inability to control for operator-induced variability and small specimen volumes that prevented replicate testing that would have improved result precision.

In summary, we are not there yet. We have not achieved clinically acceptable limits of result harmonization for CMV DNA measurement in plasma among the assays studied that used calibrators traceable to the WHO IS. However, this calibration has moved us significantly closer to this goal and will allow us to more effectively study additional factors contributing to ongoing result variability.

Supplementary Data
Supplementary materials are available at http://cid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes
CMV Result Harmonization Study Group. Dr Randall Hayden, Jeanne Carr, Sri Suganda (Department of Pathology, St Jude Children’s Research Hospital, Memphis, Tennessee); Dr Astrid Petrich, Nurserin Dewsi (Department of Paediatric Laboratory Medicine, Hospital for Sick Children, Toronto, Canada); Linda Cook (Department of Laboratory Medicine, University of Washington, Seattle); Dr Angela Caliendo, Jessica Ingersoll (Emory University, Atlanta, Georgia); Brian Yu, Heather Gregson, (Focus Diagnostics Inc, Cypress, California); and Dr Jutta Preiksaitis, Dr Xiao-Li Pang, Dr Yupin Tong (University of Alberta, Edmonton).

Acknowledgments. Additional thanks go to Min Cao and the Provincial Laboratory for Public Health for processing and providing samples; Curtis Mabilangan and Michael Akiwumi (University of Alberta) for assistance with data analysis; the National Institute for Biological Standards and Control of the First World Health Organization International Standard for human cytomegalovirus (HCMV) DNA; and Roche Diagnostics, Altona Diagnostics, Abbott Molecular, and Qiagen for providing reagents for the tests.

Potential conflicts of interest. J. F. F. and A. B. H. were employed by the NIHSC, which produced and provided the International Standard for HCMV. B. Y. is an employee of Focus Diagnostics. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References