

# Comparison of Droplet Digital PCR to Real-Time PCR for Quantitative Detection of Cytomegalovirus

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**Quantitative real-time PCR (QRT-PCR) has been widely implemented for clinical viral load testing, but a lack of standardization and relatively poor precision have hindered its usefulness. Digital PCR offers highly precise, direct quantification without requiring a calibration curve. Performance characteristics of real-time PCR were compared to those of droplet digital PCR (ddPCR) for cytomegalovirus (CMV) load testing. Tenfold serial dilutions of the World Health Organization (WHO) and the National Institute of Standards and Technology (NIST) CMV quantitative standards were tested, together with the AcroMetrix CMV tc panel (Life Technologies, Carlsbad, CA) and 50 human plasma specimens. Each method was evaluated using all three standards for quantitative linearity, lower limit of detection (LOD), and accuracy. Quantitative correlation, mean viral load, and variability were compared. Real-time PCR showed somewhat higher sensitivity than ddPCR (LODs, 3 log<sub>10</sub> versus 4 log<sub>10</sub> copies/ml and IU/ml for NIST and WHO standards, respectively). Both methods showed a high degree of linearity and quantitative correlation for standards ( $R^2 \geq 0.98$  in each of 6 regression models) and clinical samples ( $R^2 = 0.93$ ) across their detectable ranges. For higher concentrations, ddPCR showed less variability than QRT-PCR for the WHO standards and AcroMetrix standards ( $P < 0.05$ ). QRT-PCR showed less variability and greater sensitivity than did ddPCR in clinical samples. Both digital and real-time PCR provide accurate CMV load data over a wide linear dynamic range. Digital PCR may provide an opportunity to reduce the quantitative variability currently seen using real-time PCR, but methods need to be further optimized to match the sensitivity of real-time PCR.**

Over the past several years, viral load testing has evolved from a highly complex and labor-intensive procedure to a routine part of patient care. Such methods are now integral to a diverse range of clinical practice settings and diagnostic and treatment guidelines. These include their implementation in patients with human immunodeficiency virus (HIV) infection or hepatitis and posttransplant monitoring for cytomegalovirus (CMV), Epstein-Barr virus, adenovirus, and BK virus, among others. Quantitative values have been used to follow the efficacy of antiviral therapy and to help determine changes in that therapy. Rising viral burden has been used as a trigger for preemptive treatment, to prevent symptomatic infection. CMV testing is the archetype for the latter application; viral load testing by real-time PCR in particular has changed the epidemiology of CMV disease in transplant patients and is now a standard part of posttransplant care (1–6).

Despite these advances, challenges in viral load testing remain, relate primarily to intrinsic limitations of the current methodology, and center on the issues of accuracy, standardization, and precision (7–10). Quantitative determinations by real-time PCR are indirect, depending on the relationship of the cycle threshold ( $C_T$ ) of a test sample to a calibration curve. The latter, in turn, is typically generated by testing a series of known standards across the linear range of the assay. However, marked variation in assay performance characteristics and in materials used as calibration standards may prevent agreement between different laboratories, even when testing identical material. The use of international standards, such as those that have been made available by the World Health Organization (WHO) (11–13), has helped mitigate this issue but has not resolved the problem. Standards are available only for a few of the most common target analytes. Even when available, they may not be fully commutable and thus may behave differently, depending on the assay system utilized (14, 15).

Perhaps even more problematic is the poor reproducibility often seen among quantitative molecular tests. Within- and between-lab precision, even when using an identical methodology, can be surprisingly poor (7, 8, 16). Variations in any aspect of these complex methods are magnified by the multistep nature of the process and by the reliance on calibration curves, which themselves may vary over time. The very nature of relying on the measure of a dynamic process, such as the rate of target amplification, carries with it intrinsic fluctuations that one could not expect to fully eliminate. This suggests that a more direct method of quantification may be of value.

Droplet digital PCR (ddPCR) is such a direct method (17–22). It relies on limiting partition of the PCR volume, such that a positive result in any of a large number of microreactions (in this case, 20,000) indicates the presence of a single target molecule in a given reaction. The number of positive reactions, together with Poisson's distribution, can then be used to produce a direct, high-confidence measurement of the original target concentration. This methodology removes both the reliance on rate-based measurements ( $C_T$  values) and the need for the use of calibration curves. Studies targeting low-copy-number genes, typically in the

Received 1 October 2012 Returned for modification 22 October 2012

Accepted 26 November 2012

Published ahead of print 5 December 2012

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JCM.02620-12>.

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doi:10.1128/JCM.02620-12

field of molecular oncology, have demonstrated a high degree of sensitivity and precision of digital PCR (dPCR) compared to quantitative real-time PCR (QRT-PCR) (23–26). To date, limited data exist regarding the application of this new technology to viral load testing, but it promises to markedly improve our ability to reproducibly quantify viruses and to obviate developing a costly series of international standards.

Herein, we compared ddPCR to real-time PCR for quantitative detection of CMV in both artificially seeded samples and clinical plasma samples, with the former including recently developed international standard materials.

## MATERIALS AND METHODS

**Reference materials. (i) WHO international standard for hCMV.** WHO human CMV (hCMV) was purchased from the National Institute for Biological Standards and Control (NIBSC; Potters Bar, Hertfordshire, United Kingdom) and was prepared from whole virus of the hCMV Merlin strain. It was supplied in a lyophilized format containing a total of  $6.7 \log_{10}$  international units (IU;  $5 \times 10^6$  IU). One vial of the WHO standard was reconstituted with 1.0 ml of nuclease-free water to a concentration of  $6.7 \log_{10}$  IU/ml ( $5 \times 10^6$  IU/ml). Dilutions were made in CMV-negative human plasma to obtain a 10-fold concentration gradient from 1 to  $6 \log_{10}$  IU/ml. For each concentration, 6 aliquots of 200  $\mu$ l were extracted on a Qiagen EZ1 XL extractor using a Qiagen EZ1 virus minikit (version 2.0) (Qiagen, Inc., Valencia, CA) following the manufacturer's recommended protocol, which includes the addition of an internal control (IC; 7.2  $\mu$ l). The specimens were eluted in 60  $\mu$ l, pooled, aliquoted, and stored at  $-80^\circ\text{C}$ .

**(ii) NIST standard reference material.** NIST hCMV was purchased from the National Institute of Standards and Technology (NIST; Gaithersburg, MD) and prepared from a bacterial artificial chromosome of CMV Towne<sub>Δ147</sub> containing the genome of the Towne strain of CMV. The standard was supplied in a unit of three component DNA materials, with each component containing 150  $\mu$ l of purified DNA solution at a different concentration of CMV genome per  $\mu$ l. NIST component C (127  $\mu$ l, with a given concentration of 19,641 copies/ $\mu$ l [ $4.29 \log_{10}$  copies/ $\mu$ l]) was diluted with 373  $\mu$ l of TE (Tris-EDTA) for a concentration of  $6.7 \log_{10}$  copies/ml ( $5 \times 10^6$  copies/ml). Dilutions were made in TE to obtain a 10-fold concentration gradient from 1 to  $6 \log_{10}$  copies/ml and stored at  $-80^\circ\text{C}$ . As the NIST was received as purified nucleic acid, it was not extracted prior to amplification.

**(iii) AcroMetrix CMV tc panel.** The AcroMetrix CMV tc panel was purchased from AcroMetrix (Life Technologies, Carlsbad, CA) and contains intact, encapsulated viral particles of hCMV strain AD169. The panel consisted of a normal human plasma sample (which tested nonreactive for CMV DNA) and four members over a 10-fold concentration gradient from 2.5 to  $5.5 \log_{10}$  IU/ml ( $2.9 \times 10^2$  to  $2.9 \times 10^5$  IU/ml) in human plasma. For each concentration, 200  $\mu$ l was extracted and eluted in 60  $\mu$ l on the Qiagen EZ1 XL extractor using the Qiagen EZ1 virus minikit (version 2.0; Qiagen, Inc., Valencia, CA) following the manufacturer's recommended protocol. The IC (7.2  $\mu$ l) was added preextraction. Six aliquots of 200  $\mu$ l from each concentration were processed, and extracts were pooled, aliquoted, and stored at  $-80^\circ\text{C}$  until usage.

**Patient specimens.** Fifty deidentified human plasma specimens were used in the study. Samples were collected from residual material, originally collected for clinical testing at Emory University Hospital from June through August 2011. Samples were chosen to represent a wide spectrum of CMV concentrations, based on clinical test results. For each clinical specimen, three aliquots of 200  $\mu$ l were extracted on the Qiagen EZ1 XL extractor using the Qiagen EZ1 virus minikit (version 2.0; Qiagen, Inc., Valencia, CA) following the manufacturer's recommended protocol, which includes the addition of an IC (7.2  $\mu$ l). The specimens were eluted in 60  $\mu$ l, pooled, aliquoted, and stored at  $-80^\circ\text{C}$ . This protocol was approved by the Emory University Institutional Review Board (IRB).

**ddPCR.** A laboratory-developed test (LDT) for CMV detection was used, together with a QX100 droplet digital PCR system (Bio-Rad, Pleasanton, CA). The ddPCR reaction mixture consisted of 10  $\mu$ l of a  $2\times$  ddPCR master mix (Bio-Rad), 2  $\mu$ l of CMV primer/probe mix (artus CMV PCR analyte-specific reagent; Qiagen, Inc., Valencia, CA), and 5  $\mu$ l of sample nucleic acid solution in a final volume of 20  $\mu$ l. The entire reaction mixture was loaded into a disposable plastic cartridge (Bio-Rad) together with 70  $\mu$ l of droplet generation oil (Bio-Rad) and placed in the droplet generator (Bio-Rad). After processing, the droplets generated from each sample were transferred to a 96-well PCR plate (Eppendorf, Germany). PCR amplification was carried out on a T100 thermal cycler (Bio-Rad) using a thermal profile of beginning at  $95^\circ\text{C}$  for 10 min, followed by 40 cycles of  $94^\circ\text{C}$  for 30 s and  $60^\circ\text{C}$  for 60 s, 1 cycle of  $98^\circ\text{C}$  for 10 min, and ending at  $12^\circ\text{C}$ . After amplification, the plate was loaded on the droplet reader (Bio-Rad) and the droplets from each well of the plate were read automatically at a rate of 32 wells per hour. ddPCR data were analyzed with QuantaSoft analysis software (Bio-Rad), and the quantification of the target molecule was presented as the number of copies per  $\mu$ l of PCR mixture.

**Quantitative real-time PCR.** Quantitative CMV PCR testing was performed using an LDT incorporating artus CMV PCR analyte-specific reagents (Qiagen, Inc., Valencia, CA) on a Qiagen Rotor-Gene instrument. For each amplification reaction, 20  $\mu$ l of purified nucleic acid was added to 30  $\mu$ l of a reaction mixture of master mix and magnesium. The thermocycler parameters were as follows: hold for 10 min at  $95^\circ\text{C}$ , followed by 15 s at  $95^\circ\text{C}$ , 30 s at  $65^\circ\text{C}$ , and 20 s at  $72^\circ\text{C}$  for 45 cycles. A four-point standard curve as well as a positive and a negative control were included on all runs. The laboratory-determined limit of detection (LOD) in this assay using plasma was  $2.3 \log_{10}$  IU/ml.

**Statistical analysis.** For each standard's dilution series, the concentration was log transformed as  $\log_{10}(\text{concentration} + 1)$  for purposes of subsequent statistical analyses. Digital PCR measurements were transformed as  $\log_{10}(\text{number of copies/ml} + 1)$ , and real-time PCR measurements were transformed as  $\log_{10}(\text{IU}/1.38 + 1)$  for purposes of subsequent statistical analyses. The factor of 1.38 was used in the transformation of real-time PCR measurements to convert IU/ml to the number of copies/ml units reported by digital PCR. One was added prior to log transformation to avoid obtaining undefined values by taking the log of 0 for negative samples. For each technology and standard, the LOD was defined as the smallest nonzero concentration at which all replicates gave a positive qualitative result. For each standard and technology, the log-transformed measurements at or above the LOD were regressed against the log-transformed concentrations by simple least squares. Bartlett's test (27) was used to compare the standard deviations (SDs) of log-transformed results across technologies for each concentration and standard.

For clinical samples, the mean log-transformed measurement was computed for each technology and each clinical sample. Simple least-squares regression was used to evaluate the quantitative agreement of the mean values of the two technologies. For each sample, the number of repeats giving a positive result was determined for each technology. The number of samples with a difference in the number of positive results was determined. A binomial test was used to determine the significance of the number of samples with fewer positive results by digital PCR than by real-time PCR. Similarly, the standard deviation of the results was computed for each sample and technology. A binomial test was used to determine the statistical significance of the number of samples with a smaller standard deviation by real-time PCR than by digital PCR.

## RESULTS

**Evaluation using quantitative standards.** The three quantitative standards (WHO, NIST, AcroMetrix) were tested as unknowns by both real-time PCR and ddPCR to generate analytical operating characteristics for each method. Each concentration of each standard was run in triplicate on three separate runs (total of nine results per sample). Analysis was performed to determine the limit

TABLE 1 Results for standards<sup>a</sup>

Standard	Nominal log <sub>10</sub> concn <sup>b</sup>	Real-time PCR				Digital PCR				Variability (P value)
		No. of positive specimens/total no. of attempts	Mean viral load (log <sub>10</sub> no. of copies/ml)	SD	% CV	No. of positive specimens/total no. of attempts	Mean viral load (log <sub>10</sub> no. of copies/ml)	SD	% CV	
AcroMetrix	2.48	9/9	2.11	0.34	16.11	7/7 <sup>c</sup>	2.23	0.24	10.76	0.409
AcroMetrix	3.48	9/9	3.15	0.12	3.81	8/8 <sup>c</sup>	3.06	0.08	2.61	0.248
AcroMetrix	4.48	9/9	4.19	0.12	2.86	8/8 <sup>c</sup>	4.16	0.03	0.72	0.003
AcroMetrix	5.48	9/9	5.25	0.07	1.33	9/9	5.19	0.03	0.58	0.048
NIST	1	0/9	0	0	NA	1/9	0.27	0.82	303.7	NA
NIST	2	7/9	1.09	0.67	61.47	3/9	0.83	1.24	149.4	0.104
NIST	3	9/9	2.35	0.23	9.79	7/9	2.1	1.2	57.14	<0.001
NIST	4	9/9	3.29	0.09	2.74	9/9	3.66	0.1	2.73	0.637
NIST	5	9/9	4.34	0.05	1.15	9/9	4.63	0.07	1.51	0.348
NIST	6	9/9	5.35	0.06	1.12	9/9	5.6	0.05	0.89	0.394
NIST	6.7	9/9	6.1	0.11	1.8	9/9	6.21	0.08	1.29	0.345
WHO	1	6/9	1.17	0.91	77.78	1/7 <sup>c</sup>	0.29	0.76	262.07	0.660
WHO	2	9/9	2.18	0.38	17.43	3/7 <sup>c</sup>	0.91	1.14	125.27	0.008
WHO	3	9/9	3.16	0.11	3.48	9/9	2.71	0.1	3.69	0.727
WHO	4	9/9	4.14	0.11	2.66	8/8 <sup>c</sup>	3.77	0.04	1.06	0.007
WHO	5	9/9	5.13	0.07	1.36	9/9	4.75	0.02	0.42	0.004
WHO	6	9/9	5.99	0.05	0.83	8/8 <sup>c</sup>	5.72	0.02	0.35	0.020

<sup>a</sup> The number of positive specimens/number of attempts, the mean log-transformed measurements, and standard deviation of the log-transformed measurements are given for each technology at each concentration of each standard. Bartlett's test was used to compare the variability of the two technologies, based on the SD. A small-variability *P* value indicates that there is significant evidence that the two technologies do not have equal standard deviation in their measurements. CV, coefficient of variation; NA, not available.

<sup>b</sup> Nominal concentrations in IU/ml for AcroMetrix and WHO standards and number of copies/ml for NIST standards.

<sup>c</sup> Decreased replicates due to failure of the instrument (either failed to generate or read the droplet).

of detection, quantitative linearity, quantitative agreement compared both to the nominal concentration and to results from real-time PCR, and reproducibility of results using each methodology.

As shown in Table 1, each method had the same LOD when using AcroMetrix standards (2.48 log IU/ml). However, real-time PCR demonstrated a lower LOD by 1-log-unit dilution for both the WHO and NIST standards (10-fold greater sensitivity than ddPCR). dPCR detected 100% of samples at log 3 IU/ml using WHO material and at log 4 copies/ml using NIST material.

Both methods showed excellent linearity above the LOD. For each technology and standard, the estimated slope coefficients were close to 1 (0.93 to 1.04) and *R*<sup>2</sup> values were very close to 1 ( $\geq 0.98$ ), indicating both linearity and correlation between nominal and measured values (Fig. 1). Quantitative correlation was also shown between methods (Fig. 2). Correlation was reduced, particularly for ddPCR, at the low end of each dilution series, corresponding to analyte concentrations with less than 100% detection. Regression model equations for Fig. 1 and 2 are shown in Tables S1 and S2 in the supplemental material, respectively.

The lower sensitivity of ddPCR may be largely attributed to use of a 4-fold smaller input volume. When we plotted the probability of detection against the nominal total number of input viral copies (number of copies/ml  $\times$  input volume, in ml), the two technologies showed very similar performance (see Fig. S1 in the supplemental material). This indicated that the sensitivity difference was largely attributable to the input volume differences.

Table 1 shows the standard deviation and mean log<sub>10</sub>-transformed number of copies for each technology, standard, and concentration. Only matched concentrations of each standard run with both methods are reported. Overall, the methods produced similar results. The maximum differences between mean measurement values of the

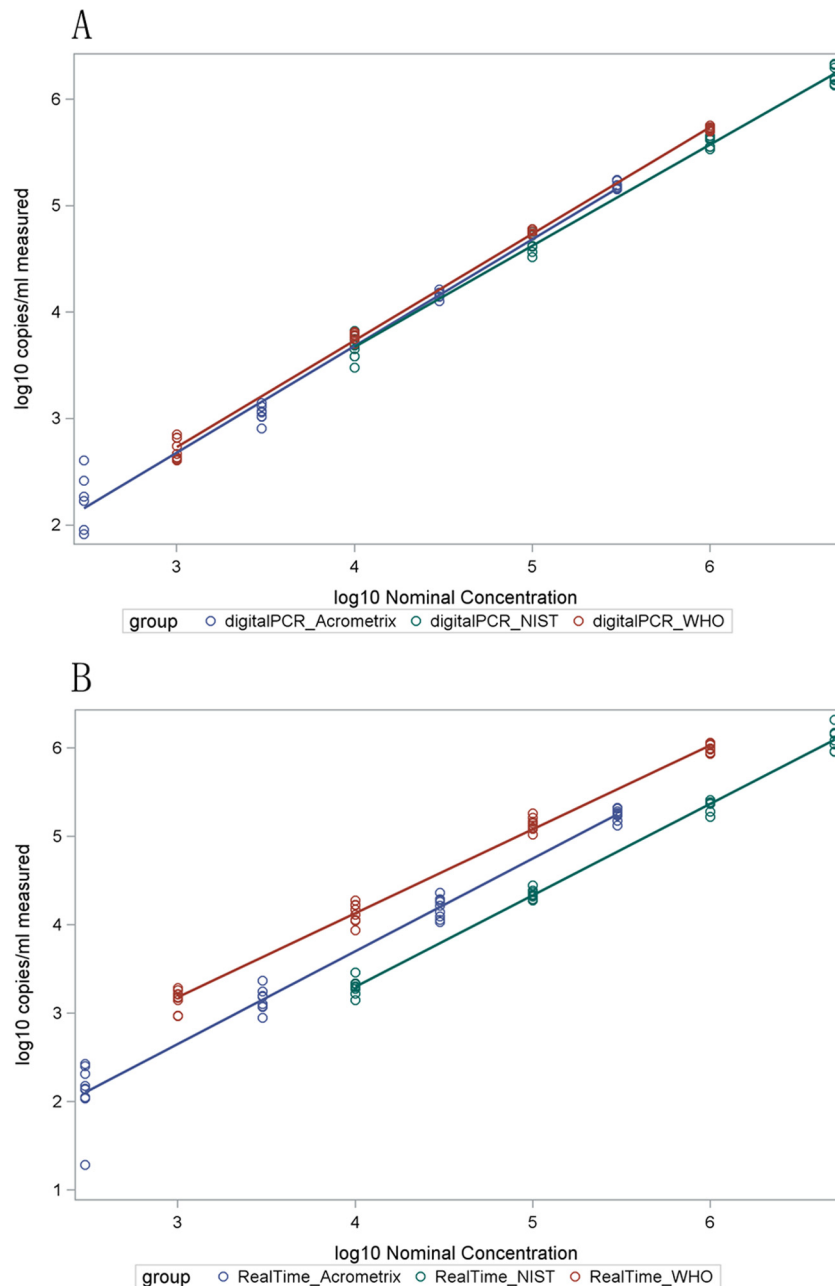
two technologies for all concentrations tested were 0.12, 0.37, and 1.27 log<sub>10</sub> copies/ml for the AcroMetrix, NIST, and WHO standards, respectively. Among concentrations above the LOD for both methods, the maximum differences were 0.12, 0.37, and 0.45, respectively. Digital PCR showed significantly less variability than real-time PCR for AcroMetrix log<sub>10</sub> concentrations of  $\geq 4.48$  and for WHO log<sub>10</sub> concentrations of  $\geq 4$ . Real-time PCR showed significantly less variability than digital PCR at an NIST log<sub>10</sub> concentration of 3 and a WHO log<sub>10</sub> concentration of 2. The two technologies did not show significantly different variability for the other concentrations of the standards.

**Evaluation using clinical samples.** Clinical samples were each tested in duplicate on three separate PCR runs (total of six results per sample; see Table S3 in the supplemental material). Mean viral load and result variability was compared for each method. Figure 3 shows linear regression and Bland-Altman plots comparing the mean quantitative values for ddPCR with those for real-time PCR, demonstrating close agreement between the two systems (mean difference =  $-0.247$  log<sub>10</sub> copies/ml; SD =  $0.336$  log<sub>10</sub> copies/ml).

Digital PCR had a significantly lower positivity rate than real-time PCR. Digital PCR obtained fewer positive results among its six repeated replicates than did real-time PCR for 14 samples, but the converse was not true for any samples (*P* = 0.0001). Additionally, the variability of real-time results was less than that of digital PCR results for 34 of 50 (68%) samples (95% confidence interval = 53% to 81%; *P* = 0.015).

## DISCUSSION

This study offers one of the earliest published assessments of digital PCR as a viable means for quantitative detection of DNA viremia in clinical samples. Previous work with this relatively new



**FIG 1** Scatter plots with regression lines between nominal and measured concentrations of each reference material using ddPCR (A) and real-time PCR (B). All values are  $\log_{10}$  transformed.  $\log_{10}$  transformation was defined as  $y = \log_{10}(x + 1)$ , as described in Materials and Methods. Table S1 in the supplemental material provides the regression model equations for the fitted lines.

technology has focused on its application in molecular oncology (21, 23). However, the utility of a direct (rather than relative) measure of quantification for the field of clinical molecular virology cannot be overstated, particularly if demonstrated to be accurate, sensitive, and precise. Findings here show ddPCR to have such potential for the measurement of CMV. While the current assay showed somewhat lower sensitivity than the real-time methodology, other operating characteristics were comparable. Quantitative accuracy was high, and the assay generated viral loads that matched well with nominal values of international and commercial quantitative standards. Linearity and quantitative correlation

with real-time PCR results were high, and precision matched or exceeded that of real-time PCR, when assessed within the analytical measurement range of the test. Furthermore, our data suggest that the lower sensitivity and greater variability of this ddPCR may be attributed to a lower input volume; however, our experiment was not designed to definitively determine the impact of input volume on sensitivity. Another experiment designed explicitly for that purpose may be necessary to confirm our interpretation. Clinical samples showed greater variability with ddPCR than with real-time PCR, while the converse was true for reference materials. Samples with values near the LOD of either method showed

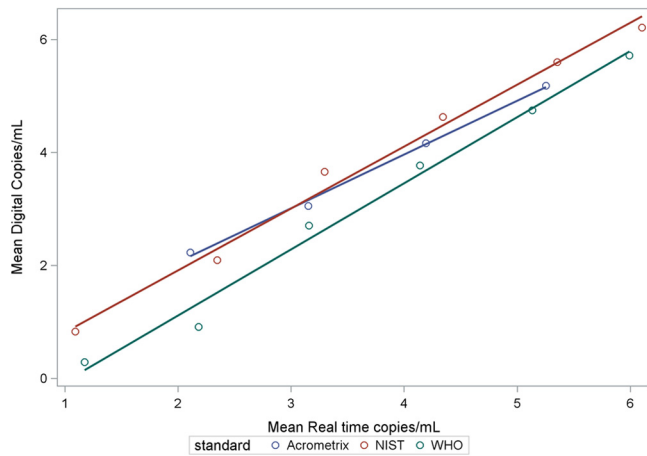


FIG 2 Quantitative correlation of mean values for each concentration of standard between droplet digital PCR and real-time PCR methods. Table S2 in the supplemental material provides the model equations for the fitted lines.

greater variability. Because the LOD of ddPCR was somewhat higher than that of real-time PCR, the results of clinical samples disproportionately fell close to the LOD of ddPCR, potentially accounting for the increased variability (since clinical samples tended to have a lower viral load overall, while reference materials had their concentrations evenly distributed along the linear range of the assays).

The disadvantages of real-time PCR for such measurements have been well documented. Several authors have now shown a high degree of quantitative variability among such assays, not only for those targeting CMV but also for those targeting other blood-borne viruses (7–9, 28). Laboratory-developed assays are particularly prone to such variability, and a wide range of factors has been shown to affect both the accuracy and precision of these tests (7). The development of international standards for CMV and other such viruses promises to mitigate such problems (particularly that of accuracy), but numerous issues remain. These include the difficulty of generating widely commutable standards and other aspects of assay design that impinge on amplification efficiency, all integrally related to the generation of reproducible results when measurement depends on relative rates of amplification, as with real-time methods. In addition, gains seen in the development of international standards and automated, FDA-cleared assays are unlikely to be duplicated for the wide range of viruses currently of clinical interest and may be limited only to those with a strong commercial market.

The clinical import of improved assay characteristics is multi-fold. Present methods for measuring CMV load may show quantitative variability among laboratories exceeding 4 orders of magnitude (7, 8). Quantitative accuracy and precision are crucial to assay interpretation and to the ability to set meaningful, universal treatment thresholds, both for clinical disease attribution and for preemptive therapeutic strategies. In addition, the portability of results can have direct implications for patient care. Currently, ongoing treatment of patients who move from one institution to another may be challenging and require new baseline viral loads. Comparability of clinical study results that rely on such measurements is a final issue of importance fundamental to advancing patient care. While the recent availability of an FDA-cleared, com-

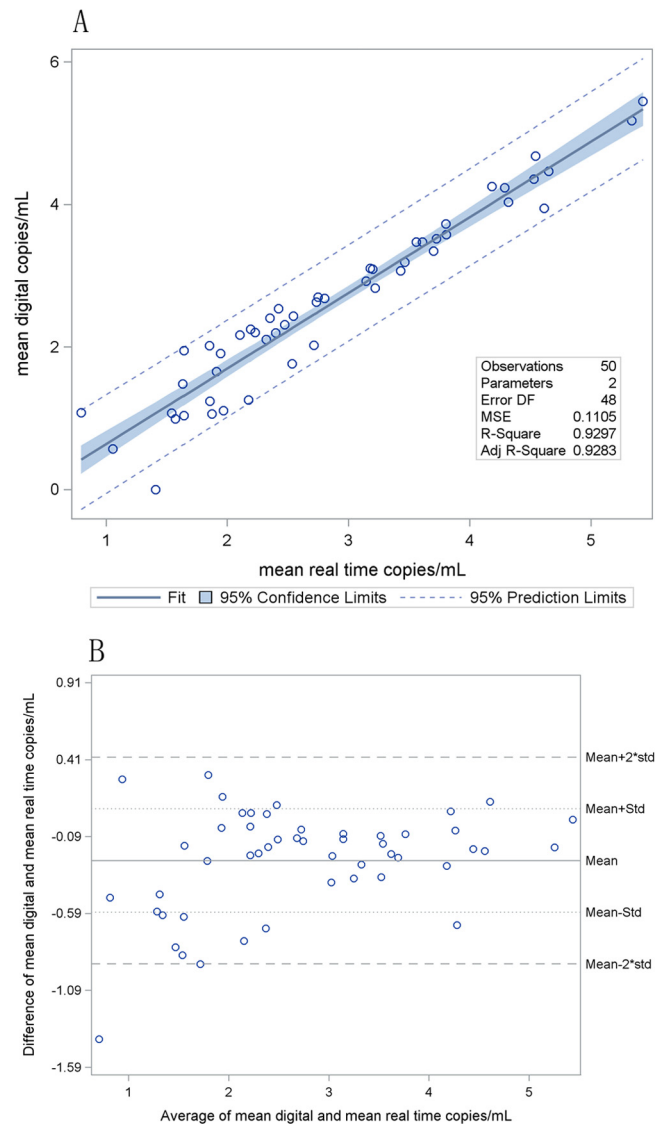


FIG 3 (A) Linear regression for 50 clinical samples (intercept =  $-0.43$ , slope =  $1.06$ ). The confidence limits are interval estimates for the regression line, and the prediction limits provide intervals that will include approximately 95% of all observations. The mean squared error is the vertical variance of the observed points from the fitted line.  $R^2$  is the proportion of variability in  $y$  that is attributable to  $x$ , and the adjusted  $R^2$  adjusts for the number of model parameters. DF, degrees of freedom; MSE, mean squared error; Adj, adjusted. (B) Bland-Altman plots comparing the quantitative values of ddPCR with those of real-time PCR for 50 clinical samples (mean difference =  $-0.247$   $\log_{10}$  copies/ml; SD [Std] =  $0.336$   $\log_{10}$  copies/ml).

mercial assay may improve the situation for CMV testing, its impact remains uncertain. Digital PCR, utilizing endpoint PCR for direct measurement of viral loads and potentially improving accuracy and precision over those of current, real-time methods, may address all of these issues.

Since it was initially described in the 1990s, other authors have shown digital PCR to have advantages in several early reports of studies focused primarily on research applications in molecular genetics and oncology. Specifically, it has been used for mutational analysis, assessment of allelic imbalance, cancer detection, and allelic expression analysis (17, 21, 23). Its ability to detect and

accurately quantify minority mutations in a predominating background of normal sequence has proven advantageous over other PCR methodologies. This capability has also been exploited in the area of prenatal diagnostics, where its use for the detection of fetal aneuploidies and single-gene Mendelian disorders using maternal plasma samples has also been described (29). Few authors, however, have yet investigated the application of this technology to infectious disease diagnostics. Experimental systems have demonstrated proof of principle for quantification of adenoviral genome copies, GB virus in transfected cell culture lines, serial dilutions of hepatitis C virus and HIV RNA, and purified, serially diluted HIV RNA from two clinical samples (19, 30, 31). The latter studies demonstrated a 4- $\log_{10}$ -unit linear range and variable agreement with real-time PCR methods. None of these studies examined a commercially available digital PCR system amenable to routine clinical use, and none utilized large numbers of clinical samples in a clinical laboratory setting. This report supports the clinical applicability of ddPCR for routine use in clinical diagnostic molecular virology. The system used here has a relatively small footprint (requiring less than 2 linear feet of bench space for both the droplet generator and reader together). It can be used at roughly the same cost as real-time PCR (as most reagents used here were identical in the two systems) and has scalable, rapid throughput (6.5 h for 96 reactions and 2.2 h for 8 reactions, compared to 4 h for 33 real-time PCRs). Hands-on time for ddPCR was 25 min for 8 reactions and 120 min for 96 reactions, while for real-time PCR it was 85 min for 33 reactions. This now makes digital PCR practical for routine use in clinical laboratories, adding relevance to the results shown above.

The conclusions here are, of course, limited on the basis of the number and genetic heterogeneity of clinical samples available. In addition, the conclusions presented here are drawn on the basis of a comparison using only one assay design targeting only one of a number of viruses for which such a method may have clinical utility. Indeed, ongoing work by our group seeks to extend the present study by looking at other comparator assays and other viruses, particularly those with RNA genomes. In addition, the development and use of other digital platforms will be crucial to building upon this work. The somewhat higher LOD, compared to that of real-time PCR, seen can be accounted for entirely by the small total sample volume compared to that for the real-time system used (5  $\mu$ l for ddPCR compared to 20  $\mu$ l for real-time PCR; statistical analysis not shown). An increased reaction volume for ddPCR, currently limited by system design, might also lead to an improved quantitative correlation between methods at low concentrations of analyte and might extend the precision advantage of ddPCR already seen at higher concentrations. Finally, future studies comparing ddPCR to the recently released FDA-approved test for CMV load testing (and others, as they become available) may prove valuable in assessing the future roles for each technology.

The data shown here confirm earlier work in experimental oncology and show proof of principle for the application of ddPCR for routine use in clinical, molecular virology. The advantages of direct quantification by endpoint, limiting-partition PCR could obviate development and purchase of costly quantitative calibrators. This paradigm shift could reduce testing costs and improve the accuracy and precision of quantitative viral load assays. Such improvements will have direct clinical utility, particularly in diagnostic microbiology and virology laboratories serving immunocompromised patients, where the number of viruses of impor-

tance continues to outstrip our ability to develop methods for quantitative detection and where our present diagnostic tools remain severely limited in utility due to suboptimal operating characteristics. These findings support both the continued development of this technology and further work to explore its value for routine use in clinical diagnostic testing for infectious pathogens.

## ACKNOWLEDGMENTS

Artus CMV RG PCR reagents were graciously provided by Qiagen, Inc. (Valencia, CA).

This work was supported in part by the Anderson Charitable Foundation, the American Lebanese Syrian Associated Charities (ALSAC), and the Emory Center for AIDS Research (P30 AI050409).

A. M. Caliendo is a coinvestigator on a CMV load clinical trial that is funded by Qiagen.

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