Cytomegalovirus sequence variability, amplicon length, and DNase-sensitive non-encapsidated genomes are obstacles to standardization and commutability of plasma viral load results


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ABSTRACT

Background: Cytomegalovirus (CMV) management post-transplantation relies on quantification in blood, but inter-laboratory and inter-assay variability impairs commutability. An international multicenter study demonstrated that variability is mitigated by standardizing plasma volumes, automating DNA extraction and amplification, and calibration to the 1st-CMV-WHO-International-Standard as in the FDA-approved Roche-CAP/CTM-CMV. However, Roche-CAP/CTM-CMV showed under-quantification and false-negative results in a quality assurance program (UK-NEQAS-2014).

Objectives: To evaluate factors contributing to quantification variability of CMV viral load and to develop optimized CMV-UL54-QNAT.

Study design: The UL54 target of the UK-NEQAS-2014 variant was sequenced and compared to 329 available CMV GenBank sequences. Four Basel-CMV-UL54-QNAT assays of 361 bp, 254 bp, 151 bp, and 95 bp amplicons were developed that differed in reverse primer positions. The assays were validated using plasmid dilutions, UK-NEQAS-2014 sample, as well as 107 frozen and 69 prospectively collected plasma samples from transplant patients submitted for CMV QNAT, with and without DNase-digestion prior to nucleic acid extraction.

Results: Eight of 43 mutations were identified as relevant in the UK-NEQAS-2014 target. All Basel-CMV-UL54 QNATs quantified the UK-NEQAS-2014 but revealed 10-fold increasing CMV loads as amplicon size decreased. The inverse correlation of amplicon size and viral loads was confirmed using 1st-WHO-International-Standard and patient samples. DNase pre-treatment reduced plasma CMV loads by > 90% indicating the presence of unprotected CMV genomic DNA.

Conclusions: Sequence variability, amplicon length, and non-encapsidated genomes obstruct standardization and commutability of CMV loads needed to develop thresholds for clinical research and management. Besides regular sequence surveys, matrix and extraction standardization, we propose developing reference calibrators using 100 bp amplicons.

1. Background

Cytomegalovirus represents a persisting challenge to transplant patients due to its direct and indirect effects decreasing graft and patient survival [1–4]. Significant progress has been made in the clinical management of CMV replication post-transplantation through the concerted action of stratifying CMV risk according to CMV IgG serostatus of donor and recipient pairs, implementing prophylactic or preemptive antiviral strategies, and developing consensus definitions and guidelines [5–7]. Central to current medical practice is the sensitive and specific detection and quantification of CMV replication for clinical studies as well as for therapy decisions [7,8]. Despite known

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limitations, this is widely approached by determining CMV DNA loads in blood using quantitative nucleic acid testing (QNAT) [9–11]. Although CMV QNAT is now clinically available with turn-around times of less than 6 h, results from quality assurance programs and dedicated multicenter studies indicated that the CMV DNA load results may be highly variable across different centers and across different laboratory-developed and commercial assays [12–14]. Normalization to an external reference sample was shown to significantly reduce the spread of the reported viral load results, thus providing an important rationale for developing commutable calibrators such as the 1st WHO-approved CMV International Standard (1st-WHO-IS-CMV) [15–17]. Indeed, an international multicenter study demonstrated previously that CMV load variability is mitigated across all participating laboratories when all steps and variables of the procedure were standardized such as using defined plasma volumes, automated DNA extraction and target amplification, and integrating calibration to 1st-WHO-IS-CMV as in the FDA-approved COBAS AmpliPrep/COBAS TaqMan CMV (in short: Roche-CAP/CTM-CMV) [18]. Despite an excellent performance record in our laboratories since its routine introduction [19], the Roche-CAP/CTM-CMV showed under-quantification and false-negative results of two quality assurance samples providing the same viral material in a lower and a higher dilution, respectively (UK-NEQAS-2014 distribution 3521; specimen 2194 and 2195) [20]. This observation elicited considerable uncertainty about the cause and the impact on clinical management, in several transplant centers including ours.

2. Objectives

To determine the reasons for the Roche-CAP/CTM-CMV to fail the UK-NEQAS-2014 distribution 3521, we hypothesized that sequence variations in the CMV-UL54 target region were responsible. Since the manufacture declined to provide this information or to reveal the target region, we identified two publications through a literature search [21,22], which suggested the presumed target region of the Roche-CAP/CTM-CMV to reside in the CMV UL54 DNA polymerase gene. We determined the sequence in the presumed UL54 target of the Roche-CAP/CTM-CMV resulting in quantification failure and compared the results to 329 available CMV GenBank sequences. We developed four new in-house CMV-UL54 QNAT assays designed to accommodate UL54 sequence variations but differing in amplicon length. The assays were validated using reference and variant plasmid dilutions, 1st-WHO-IS-CMV, UK-NEQAS-2014 sample, as well as 107 frozen and 69 prospectively collected plasma samples submitted for routine CMV load testing, with and without DNase-digestion prior to nucleic acid extraction.

3. Study design

3.1. Primers, probes, and plasmids

Primers and probes used for amplification, QNAT and sequencing are indicated in Table 1. Plasmids harboring the presumed target sequence of CMV-strain AD169 and the UK-NEQAS-2014 sequence were chemically synthesized into the pUC57 plasmid (Eurogentec, Belgium) as denoted pCMV-AD169-UL54 and pCMV-UKNEQAS2014-UL54.

3.2. Plasma samples

176 plasma samples tested for CMV load quantification were available from kidney and liver transplant patients consisting of 107 frozen samples for a retrospective study, and 69 prospectively collected samples. In 111 cases (49 retrospective; 62 prospective), CMV loads were in the linear range of the Roche assay, above the lower limit of quantification (LLOQ, 150 copies/mL; 137 IU/mL) in 50 cases (all retrospective) below the limit of detection (LOD 91 IU/mL) of the CAP/CTM-CMV assay [23], and in 15 cases (8 retrospective; 7 prospective) above the LOD of 91 IU/mL, but below LLOQ of 137 IU/mL (detected, but not quantifiable).

3.3. DNase I digestion

Samples above the LLOQ of the Roche assay were compared with and without DNase I digestion prior to extraction. DNase digestion was performed with 1 μL of Deoxyribonuclease I (238 U/μL; Invitrogen, USA) and 4.5 μL of 1 M MgCl2 (Ambion, USA) per 200 μL sample volume at 37 °C for 30 min with subsequently added 11.1 μL 0.5 M EDTA (Invitrogen, USA). As DNase digestion control, normal human plasma spiked with 106 copies of pCMV-AD169-UL54 was run in parallel and then submitted to automated extraction.

3.4. Extraction and quantitative nucleic acid testing (QNAT)

Patient plasma and quality assurance samples were processed for the Roche-CAP/CTM-CMV assay as described by the manufacturer (Roche, Rotkreuz, Switzerland; see above). For in-house measurements, 200 μL of plasma without and with prior DNase I digestion,
reconstituted UK-NEQAS-2014 sample, and 1st-WHO-IS-CMV was used for extraction by the MagNA Pure 96 System (Roche, Switzerland) and eluted in 100 μL. The novel Basel-CMV-UL54-assays were performed with an ABI7500 Fast Real-Time PCR System (Thermo Fisher Scientific, USA) using a reaction volume of 25 μL and 5 μL of extracted nucleic acids. QNAT was carried out with the qPCR MasterMix Plus Low-ROX containing uridine and the uracil-N-glycosylase (UNG) (Eurogentec, Belgium), 300 nmoles end concentration of the forward primer Basel-CMV-UL54_80134_F for −95 bp, −151 bp, or −254 bp and −361 bp assay, and 300 nmoles of the indicated reverse primers, Basel-CMV-UL54-254_80387_R, Basel-CMV-UL54-151_80284_R or Basel-CMV-UL54-95_80228_R, or 600 nmoles of Basel-CMV-UL54-361_80494_R (Eurogentec, Belgium). The denaturation and cycling conditions were as follows: 50 °C for 2 min for uracil-N-glycosylase (UNG) activation; 95 °C for 10 min to inactivate UNG and activation of HotGoldStar polymerase; 45 cycles of 95 °C for 30 s and 60 °C for 90 s for annealing and extension. The threshold of 0.02 was used for all Basel assays. The Basel-CMV-UL111a-77 bp targeting another genome sequence UL111a has been described previously [18,24].

3.5. Sequencing

The UK-NEQAS-2014 sample with the higher CMV loads was used for sequencing (specimen 2194). Briefly, following PCR using primers Basel-CMV-UL54_80133_F and Basel-CMV-UL54_80500_R (Table 1) and verifying PCR products on a 1% agarose gel, the amplicons were purified using Illustra ExoProStar 1-Step (GE Healthcare, England). The sequencing reaction was performed using the BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, MA, USA), purification with Sephadex G-50 Superfine (GE Healthcare, England) and sequencing by capillary electrophoresis on a 3500 Genetic Analyzer (Thermo Fisher Scientific, MA, USA). The sequences were analysed using the CodonCode Aligner (MA, USA) and then submitted to basic local alignment search tool (BLAST) analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
3.6. Statistical analysis

A sensitivity analysis was done by limiting dilution using the indicated plasmids at $10^6$, $10^4$, and $10^2$ copies per reaction, followed by two sets of 5-fold replicates of 2-fold dilutions starting from 100 copies/reaction down to 0.39 copies/reaction as described previously [25]. The results of the Roche-CAP/CTM-CMV and the corresponding Basel-CMV-UL54 assays were compared by linear regression and Bland-Altman analysis as described previously [25,26]. Statistical analysis was performed using ANOVA for multiple comparisons (in Figs. 3, 5) on GraphPad Prism version 7.0c (GraphPad Software, USA).

4. Results

Primers Basel-CMV-UL54_80133_F and Basel-CMV-UL54_80500_R (Table 1) were designed to amplify and sequence the presumed UL54 target region in the CMV-AD169 reference strain and in the UK-NEQAS-2014 sample. The presumed Roche CAP/CTM-CMV target is highlighted in light grey. Same forward primer (yellow) and probe (grey) were used for all four Basel QNATs, reverse primers are marked in purple for Basel-CMV-UL54-361bp, turquoise for Basel-CMV-UL54-254bp, orange for Basel-CMV-UL54-151bp and green for Basel-CMV-UL54-95bp assay. Dotted line indicates presumed CAP/CTM-CMV primer that in contrast to the Basel-CMV-UL54-361bp reverse primer, presumably has a G-to-A mismatch with UK-NEQAS-2014 at the 3′ position.

B. Influence of reverse primer and target sequence on quantification. Indicated are the copy numbers of the plasmids pCMV-AD169-UL54 and pCMV-UKNFQAS2014-UL54 quantified with reverse primers ending with a mismatch at the 3′ end. Primer sequences are listed in Table 1.
meantime under the acc. no. KJ361971 (Fig. 1).

Since no relevant variations were found in the sequence targeted by the forward primer, Basel-CMV-UL54_80134_F was combined with different reverse primers, which were either specific for CMV-AD169 or the UK-NEQAS-2014 sequence, using the option of degenerate bases to

Fig. 3. New Basel-CMV-UL54 QNATs.
A. Ct values of $10^6$, $10^5$, and $10^4$ intact plasmid pCMV-AD169-UL54 target copies per reaction, determined by the four Basel-CMV-UL54 QNATs.
B. Ct values of intended $10^4$, $10^3$, and $10^2$ IU per reaction (pre-extraction) of the 1st WHO HCMV International Standard (1st-WHO-IS-CMV, NIBSC 09/162, Merlin strain, acc. no. AY446894), determined by the four Basel-CMV-UL54 QNATs. The hatched bars indicate results above the pre-defined limit of Ct 45.
C. Viral load (log_{10} copies/mL) of UK-NEQAS-2014 sample (no.2195) that was detected, but not quantifiable using the Roche CAP/CTM-CMV assay. Mean of independent triplicates with standard deviation is indicated for each bar. The red star indicates the result of the Roche assay being detectable below LLOQ.

Fig. 4. Comparison of new Basel-CMV-UL54-95bp QNAT and the Roche-CAP/CTM-CMV assay by retrospective analysis.

A. Viral loads (log_{10} copies/mL) of patient samples determined by the Roche-CAP/CTM-CMV assay and retrospectively by the Basel-CMV-UL54-95 bp QNAT (median, 25th and 75th percentile).

B. Agreement plot with dashed line indicating 100% agreement level, linear correlation between Roche-CAP/CTM-CMV and Basel-CMV-UL54-95 bp results with 95% confidence interval (CI) and Spearman's correlation coefficient.

C. Bland-Altman plot of Roche-CAP/CTM-CMV compared to Basel-CMV-UL54-95bp. The mean bias was -0.60, and the 95% limit of agreement between -1.19 and 0.00.

D. Viral loads determined by Basel-CMV-UL54-95 bp before and after DNase I-digestion.

accommodate the sequence variations (Basel-CMV-UL54-361_80494_R) or were additionally moved closer to the forward primer (Basel-CMV-UL54-254_80387_R, Basel-CMV-UL54-151_80284_R, Basel-CMV-UL54-95_80228_R; Table 1). Thereby, four different amplicons were generated of 361 bp, 254 bp, 151 bp, and 95 bp, respectively (Fig. 2A). For detection, the probe targeted a sequence region close to the forward primer, which had no relevant base variations and which could be used to assay the generation of all four amplicons (Basel-CMV-UL54-361_80203_80174_P, Table 1). To evaluate the impact of the sequence differences, the reverse primer harboring degenerate bases were compared with specific primers for the CMV-AD169 and for UK-NEQAS-2014 sequences using 10^9 copies/reaction of the respective plasmids pCMV-AD169-UL54 and pCMV-UKNEQAS2014-UL54 (Fig. 2B). The results revealed that the degenerate primer Basel-CMV-UL54-361_80494_R detected both targets equally well, whereas the other reverse primers having a mismatch only at the last position of the 3’ end (but degenerate bases at the other positions) failed to quantify the mismatched plasmid target by approximately 100-fold. Thus, the sequence variations were sufficient to explain the impaired CMV DNA quantification of the UK-NEQAS-2014 variant, which could be overcome by degenerate bases at the indicated positions.

To evaluate the performance of the new Basel-CMV-UL54 assays, defined copy numbers of pCMV-AD169-UL54 of 10^4, 10^5 and 10^6 were compared. The amplification efficiency differed slightly showing slopes of –3.8 for the 95 bp amplicon, –3.9 for the 151 bp amplicon, –4.3 for the 254 bp amplicon, and –4.1 for the 361 bp amplicon, all having correlation coefficients above 0.98. However, we noted that the novel Basel-CMV-UL54 assays differed in Ct values for the same plasmid copy number (Fig. 3A). Thus, the Basel-CMV-UL54-95 bp consistently required less amplification cycles for detection than any of the other Basel-CMV-UL54 assays having longer amplicons. Review of potential primer and probe incompatibilities including primer dimers was unremarkable.

Next, we evaluated the novel Basel assays using the 1st-WHO-IS-CMV, which was diluted into negative human plasma (e.g. 10000 IU in 1 mL plasma) and extracted to contain the calculated international unit (IU) values over 4 orders of magnitude per reaction as indicated (10000 IU, 100 IU, 1 IU; Fig. 3B). The results also confirmed the inverse correlation of amplicon size and Ct values for this relevant calibrator. Moreover, the assays generating larger amplicons failed to detect CMV genomes at the lowest value of 1 IU/reaction. Finally, the quality assurance sample UK-NEQAS-2014 was re-analyzed using all available CMV assays including the previously described Basel-CMV-UL111a-77 bp assay having an amplicon length of 77 bp, but in a different CMV genome target, namely UL111a [24]. Both, the Basel-CMV-UL54-95 bp (p = 0.05) and Basel-CMV-UL111a-77 bp (p < 0.05) showed higher viral loads compared to the intended results of this QA probe (Fig. 3C), whereas the Roche-CAP/CTM-CMV failed to quantify the CMV genome load. Together, the data demonstrated that sequence variability and amplicon size contributed significantly to the variability of CMV DNA loads.

To investigate the impact of the new assays on CMV loads in transplant patients, we retrospectively examined 107 cryopreserved plasma samples submitted for routine CMV load testing using the Roche assay: 50 were below the LOD and found to be positive in 8 cases with ≥100 copies/mL using the Basel-CMV-UL54-95 bp assay. In another 8 samples below LLOQ, but above LOD in the Roche assay, the Basel quantified 5 above 150 copies/mL. Finally, all of the 49 samples above LLOQ in the Roche assay had detectable CMV loads using the Basel-CMV-UL54-95 bp, which were significantly higher as compared to the Roche-CAP/CTM-CMV results (Fig. 4A). Linear regression and Bland-Altman analyses revealed that this difference amounted to 0.6 log10 copies/mL on average across a range of CMV loads (Fig. 4B). To investigate the contribution of unprotected CMV genome fragments, Basel-CMV-UL54-95 bp QNAT was performed without or with DNase-I treatment prior to nucleic acid extraction. The results revealed that 35 of 49 (71.4%) samples became undetectable, and that the CMV loads were significantly decreased for the remaining 14 (28.6%) samples (p < 0.05) (Fig. 4D).

Since freezing and thawing of the plasma samples might affect these results, the Roche-CAP/CTM-CMV and the new Basel-CMV-UL54-95bp; –254bp, and –361bp were prospectively compared in 69 plasma samples submitted for CMV load testing. Seven samples had CMV loads below the LLOQ of the Roche assay, but could be quantified by the Basel-CMV-UL54-95 bp assay in 6 cases. For the larger amplicons of –254 bp and –361 bp, the number decreased to 5 and 3, respectively. Comparing the CMV loads, the results demonstrated that Basel-CMV-UL54-95 bp yielded higher CMV loads including 6 of 7 samples, for which the Roche-CAP/CTM-CMV results had been below LLOQ (Fig. 5A). Linear regression and Bland-Altman analysis indicated higher CMV loads in the Basel-CMV-UL54-95 bp across a range of CMV loads (average –1.08 log_{10} copies/mL, Fig. 5B, top panel). The 10-fold higher CMV loads of the Basel-CMV-UL54-95 bp to the Roche-CAP/CTM-CMV disappeared for the assays having the 254bp- and 361bp-amplicons showing values of –0.23 and 0.33 log_{10} copies/mL, respectively (Fig. 5B, middle and lower panel).

Finally, DNase-I treatment of the prospectively collected plasma samples before nucleic acid extraction similarly revealed loss in CMV DNA detection in 42 of 68 samples (61.8%), and a significant decline in CMV loads in the remaining 26 samples (38.2%) for Basel-CMV-UL54-95 bp (Fig. 5C). For Basel-CMV-UL54-254 bp and –361bp, similar results were obtained. The results indicated that large parts of plasma CMV genomes were not protected suggesting that naked CMV DNA fragments significantly contributed to patient plasma CMV loads.

5. Discussion

The sensitive and specific quantification of CMV replication in peripheral blood is a cornerstone of current clinical management and key to clinical studies [9,10]. Although the 1st-WHO-CMV-IS as potentially commutable calibrator has improved result comparability across different laboratories [16,27], a general agreement and definition of clinically relevant cut-offs is subject of current investigations [17]. The current study demonstrates that standardization of CMV QNAT results is challenged by sequence variability, amplicon length, and non-encapsidated CMV genome fragments, which alone or in combination represent significant obstacles to commutability of blood CMV loads.

As shown here, these challenges arise on several, partly conceptual levels and cannot be solved by solely relying on commercial assay development. In fact, the failure of a well-validated commercial assay, that had been reported by Hirsch et al. [18] to mitigate differences between different laboratory through standardized matrix and volumes, automated extraction and amplification, came about through simple sequence variability in the assay target. We demonstrate here that sequence variations can be technically handled, but even though 329 CMV genome sequences were interrogated to provide a more robust choice of primers and probes, this can only be considered the latest
state of knowledge, hence remaining error-prone, as more sequence information becomes available. Thus, regular review of sequence variation in the diagnostic target needs to be performed with corresponding updates of the assay chemistry. It remains to be debated if that can be achieved more reliably by diagnostic vendors interested in balancing cost – benefit and approval by medical agencies, or by dedicated and accredited clinical virology laboratories. Clearly, QA programs may help to identify some of these issues.

Fig. 5. Comparison of the Basel-CMV-UL54 QNATs to Roche-CAP/CTM-CMV assay by prospective analysis. A. Viral loads (log10 copies/mL) of patient samples determined prospectively by the Roche-CAP/CTM-CMV assay and three of the new Basel-CMV-UL54 QNATs (median, 25th and 75th percentile). B. Agreement plots (left panels) with dashed line indicating 100% agreement level, linear correlation between Roche-CAP/CTM-CMV and Basel-CMV-UL54 QNAT results with 95% confidence interval (CI) and Spearman’s correlation coefficient. Bland-Altman plots (right panels) of Roche-CAP/CTM-CMV compared to each of the three Basel-CMV-UL54 assays. C. Viral loads determined by the three QNATs Basel-CMV-UL54–361 bp, –254 bp, and –95 bp before and after DNase I digestion.
Second, the amplicon length emerged as an important variable in QNAT determined CMV load. Although initially driven by the desire to identify a more robust, hence reliable QNAT assay, our results demonstrate that the Ct values required to detect the same target DNA load were inversely correlated to amplicon length. This observation was made for plasmid targets, an international calibrator, as well as for patient plasma. The impact of amplicon length was more pronounced for low target copy numbers, where more rounds of enzymatic duplication were required suggesting a cumulative effect of the lower efficiency. This effect led to substantial under-quantification and even false-negative results in the Roche CAP/CTM-CMV as compared to the novel Basel assays. The differences in Ct values between the indicated Basel assays using plasmid or WHO dilutions were most pronounced, when using the ~95 bp assay as comparator (Fig. 3). Comparison with our previously established Basel-CMV-UL111a-77 bp, which is based on 77 bp amplicon, revealed only little difference to the Basel-CMV-UL54-95 bp, but an about 10-fold difference to the Basel-CMV-UL54-361 bp assay, that has the same amplicon length as the presumed Roche-CAP/CTM-CMV. Indeed, this difference between the Basel-CMV-UL111a-77 bp and the Roche-CAP/CTM-CMV has been observed previously in the multicenter study by Hirsch et al. in Fig. 3 (site 4) [18], but was not yet understood in this way. Together, the data independently strengthen the role of amplicon size across different CMV gene targets.

Notably, as this work was in progress, a seminal study of six North American centers using serial dilutions of the 1st-WHO-CMV-International Standard and 40 plasma samples from patients indicated that the median inter-laboratory variation of CMV DNA loads remained in the order of 10-fold [17]. Also in that study, amplicon length appeared to be the most important determinant across different commercial and laboratory-developed CMV QNAT assays. Moreover, Preiksaitis and colleagues demonstrated that large parts of the plasma CMV loads were fragmented and susceptible to DNase digestion suggesting the presence of non-encapsidated CMV genome fragments [28]. These results are of utmost importance as they change the hitherto uncritical extrapolation from plasma viral loads of RNA viruses such as HIV representing mostly encapsidated (infectious) viral particles to quantification of plasma CMV loads, which detects partly unprotected CMV DNA from virus replication and cell lysis elsewhere in the body [29,30].

Our results are in line with this observation in retrospectively and prospectively studied plasma samples reducing CMV loads both qualitatively (by 82% and 76%, respectively), as well as quantitively. Thus, the presence of unprotected CMV genomic fragments may potentially limit the value of retrospective studies on frozen samples, especially when using large amplicons that intrinsically underestimate patient viral loads. In how far this affects the strategy of dual-target assays advocated by some centers [31] is presently little studied [24], but given the goal of detecting the wild-type CMV isolate, dual detection leading to at least 2-fold differences may have an impact on management decisions.

Finally, differences in viral loads attributable to viral DNA fragmentation may become less apparent if intact plasmid targets or virion genomes are used. The approach of defining international units by using mixed or heterogeneous preparations and simply averaging the quantification results of so-called reference laboratories that apply differently sized amplicons in commercial or laboratory-developed assays becomes questionable [34]. Since the results are likely to affect other DNA viruses as well, and since internationally commutable calibrators for viral load determination are key to progress in clinical research and management, the question arises how to best achieve this goal. Given the constraints on primer and probe design arising from sequence variability, GC-content, primer dimers, secondary structure, and melting temperatures, it may be reasonable to develop generic baseline calibrators of 100 bp in size, which could be referenced against probit and digital droplet analyses.

In summary, our data together with the results of other researchers indicate that CMV sequence variability, amplicon size, and unprotected fragmented CMV DNA are obstacles to commutability of CMV load assays. Thus, besides standardization of sample matrix, volume, concentration and extraction procedure, we propose to standardize CMV amplicon size in QNAT assays to a reference length of 100 bp.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcv.2018.04.013.

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