Comparison of two nucleic acid extraction and testing systems for HCMV-DNA detection and quantitation on whole blood specimens from transplant patients.

COMPARISON OF TWO NUCLEIC ACID EXTRACTION AND TESTING SYSTEMS FOR HCMV-DNA DETECTION AND QUANTITATION ON WHOLE BLOOD SPECIMENS FROM TRANSPLANT PATIENTS

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Abstract

Quantitative detection of human cytomegalovirus (HCMV) DNA on whole blood is currently the primary choice for virological monitoring in transplant patients and for determining the appropriate antiviral strategy, however specific issues of variability remain in terms of extraction methods, amplification efficiency, and variability. This study compared the performance characteristics of two nucleic acid extraction and testing systems for HCMV-DNA quantitation, the artus® CMV QS-RGQ kit, associated with a fully automated DNA extraction and assay set up by Qiagen (system 1) and the Q-CMV Real Time Complete kit by Nanogen, associated with a semiautomated nucleic acid extraction system by Biomérieux (system 2) in 189 specimens from transplant patients and 10 from 2012 HCMV Quality Control for Molecular Diagnostics (QCMD). The two systems exhibited a 80.4% concordance. Differences between the two systems were within ±1 $\log_{10}$ copies/ml of the averaged $\log_{10}$ results for 88.9% of the tested specimens. For all qualitatively discordant specimens, mean viral load was ≤3 $\log_{10}$ copies/ml. Considering viral load measurement, system 1 gave earlier positives that system 2, with a 14.8% of specimens resulted positive at low viral loads with system 1 and negative with system 2. In QCMD specimens, difference was below 0.7 log10 copies/ml for both the systems.

In conclusion, the two systems provided reliable and comparable results. Some specific performance characteristic and automation could be taken into account in terms of less hands of time, fewer errors and reliability.

Keywords: human cytomegalovirus; nucleic acid; extraction; quantitative PCR; efficiency; variability

1. Introduction

Human cytomegalovirus (HCMV) is an important viral pathogen in both solid organ transplant and hematopoietic stem cell transplant recipients in terms of morbidity and mortality. The frequency of HCMV infection and disease in the post-transplant period depends on the interaction of factors related to the host, virus and transplantation practices, with HCMV serostatus being considered the single most important predictor of disease. Among virus-related factors, the development of HCMV disease has been directly related to the degree of viral replication. Management of HCMV disease varies considerably among transplant centres. A panel of experts on HCMV and solid organ/hematopoietic stem cell transplant was convened to develop international guidelines for the management of HCMV including diagnostics, immunology, prevention, and treatment (Kotton et al., 2010; Zaia et al., 2009). Currently, the diagnosis of HCMV replication and disease in solid organ and hematopoietic stem cell transplant patients can be performed using different methods, including histopathological analysis, quantitative pp65-antigenaemia in peripheral blood leukocytes and quantitative nucleic acid testing. Quantitative detection of HCMV-DNAemia by real-time PCR is currently the primary choice for virological monitoring of HCMV infection in transplant patients; in fact, HCMV-DNA quantitation provides sensitive and specific data for viral detection as well as monitoring the development and course of infection, thus being fundamental for determining the appropriate antiviral strategy. A relevant issue in HCMV routine diagnostics is the interassay quantitation variability (Hirsch et al., 2013; Lilleri et al., 2009), possibly complicating the clinical management and therapeutic decision process in patients evaluated in different laboratories. Recently, the first World Health organization international standard for HCMV quantitative nucleic acid amplification-based assays has been made available (Freyer et al., 2010). Although the availability of the international standard should contribute to improve interassay agreement, specific issues of variability remain in terms of extraction methods and DNA elution, amplification efficiency, and operator-dependent variability (Hirsch et al., 2013; Kraft et al., 2012).

The aim of this study was to assess the performance characteristics of two nucleic acid extraction and testing systems for HCMV-DNA quantitation, the artus® CMV QS-RGQ kit by Qiagen, (Hilden, Germany) associated with a fully automated DNA extraction and assay set up by Qiagen and the Q-CMV Real Time Complete kit by Nanogen, Elitech Group (Milan, Italy) associated with a semiautomated nucleic acid extraction system by Biomérieux (Marcy l'Etoile, France). Using whole blood clinical specimens from transplant patients, the two systems were compared and the potential clinical implications were evaluated.

2. Materials and methods

2.1. Clinical specimens

One-hundred-fifty-six patients referred to the Virology Unit of the Azienda Ospedaliera Città della Salute e della Scienza di Torino, Italy, for routine HCMV load testing (90 solid organ transplant recipients and 66 hematopoietic stem cell transplant recipients) were included in the study. One-hundred-eighty-nine specimens of whole blood (EDTA tubes) were collected between January and June 2012. Ten HCMV quality control specimens (Quality Control for Molecular Diagnostics -QCMD- 2012 CMV panel) were also processed.

2.2 Study design

Two nucleic acid extraction and testing systems for HCMV-DNA were evaluated: the artus® CMV QS-RGQ kit (Qiagen), a commercial real-time PCR for HCMV-DNA associated with a fully automated DNA extraction from whole blood (Qiasymphony, Qiagen) and automated assay set up – system 1 - and the Q-CMV Real Time Complete kit, a commercial real-time PCR (Nanogen, Elitech Group) associated with a semiautomated nucleic acid extraction system from whole blood (Easymag, Biomérieux) and manual assay set up – system 2. Currently, the Q-CMV Real Time Complete kit, validated for EasyMAG extraction system by the manufacturer, is the routinely used method.

For system 1, nucleic acid extraction was performed according to the manufacturer's instructions; nucleic acid was purified with the QIAsymphony DNA Mini kit on the QIAsymphony instrument (Qiagen). Two-hundred microliters of whole blood were concentrated into a 60-µl eluate, and a 20 µl aliquot was used for the PCR on the real-time PCR cycler Rotor-Gene Q (Qiagen). The artus® CMV QS-RGQ kit targets the major immediate early (MIE) CMV gene. The reaction volume was 50-µl (20 µl of eluate plus 30 µl of master mix). Analytical sensitivity, as reported by the manufacturer, considering purification from whole blood (using the QIAsymphony DNA Mini kit) and the use of artus® CMV QS-RGQ kit on the Rotor-Gene Q, is 164.55 copies/ml.

For system 2, nucleic acid was purified with the NucliSENS® EasyMAG® instrument (Biomérieux), using the NucliSENS ® Nucleic Acid Extraction Reagents, according to the manufacturer's instructions.

One-hundred microliters of whole blood were concentrated into a 50-µl eluate, and a 5µl aliquot was used for PCR assay on the 7500 Real-Time PCR System (Applied Biosystems, Cheshire, United Kingdom). The Q-CMV Real Time Complete kit manufactured for Cepheid by Nanogen Advanced Diagnostics S.r.L. is specific for the exon 4 region of the CMV MIE gene (major immediate early HCMVUL123). The reaction volume was 25 µl (5 µl of eluate plus 20 µl of master mix). The limit of detection of the Q-CMV real time kit is 158 copies/ml, as reported by the manufacturer. The main technical features of the two systems are summarized in Table 1.

2.3. Statistical analysis

The correlation between the two systems was determined by linear regression analysis and mean differences in quantitation for averaged logs by the Bland-Altman plot. Only viral loads positive by both assays were represented on the Bland-Altman graphs. Differences were considered significant for p value <0.05.

3. Results

The performance of the two systems was assessed and compared on 189 whole blood specimens from 156 transplant recipients undergoing routine testing in the first year post-transplantation. System 1 and system 2 detected HCMV-DNA in 118 and 99 samples, respectively, with a 80.4% concordance. In particular, 62/189 (32.8%) were concordantly negative by both systems; 90/189 (47.6%) concordantly positive; 28/189 (14.8%) positive by system 1 and negative by system 2; and 9/189 (4.8%) positive by system 2 and negative by system 1 (Table 2). For the 90 specimens that were positive by both tests, the population mean (SD) was 4.25 (4.58) $\log_{10}$ copies/ml with system 1 and 4.06 (4.29) $\log_{10}$ copies/ml with system 2. For all the specimens that were qualitatively discordant (by one of the two systems), mean viral load was lower or equal to 3 $\log_{10}$ copies/ml.

By referring to the 90 specimens that were concordantly positive, the correlation value between the two systems was r = 0.597 (Fig. 1). Bland-Altman analysis showed that differences between the two systems were within ± 1 $\log_{10}$ copies/ml of the averaged $\log_{10}$ results for 88.9% of the tested specimens (Fig. 2). Table 3 depicts the HCMV load results for the 10 specimens from the QCMD 2012 HCMV proficiency panel. The sample for which a negative result was expected was found negative by the two systems, whereas for the positive specimens the difference was below 0.7 log10 copies/ml for both the systems (mean $\Delta\log_{10}$ 0.1566 for system 1 and 0.2288 for system 2).

4. Discussion

The automation of nucleic acid extraction and availability of commercial real-time quantitative PCR assays have the potential to improve the agreement and clinical usefulness of HCMV-DNA measurement in routine transplant settings, thus promoting standardization across laboratories and enabling correlation with clinical study results.

In the present study, the HCMV –DNA load results obtained by two nucleic acid extraction and testing systems have been compared. Overall, both systems appear as reliable and user-friendly for monitoring HCMV-DNAemia in transplant recipients.

The two systems exhibited an overall concordance of 80.4% in qualitative terms. Considering specimens that were positive by both the systems, the mean viral load differs of 0.19 $\log_{10}$ copies/ml. On the other hand, when considering specimens that were qualitatively discordant, mean viral load was lower or equal to 3 $\log_{10}$ copies/ml.

Considering viral load measurement, system 1 gave earlier positives that system 2, as evidenced by the evaluation of frequency distribution for different log of viral load (not shown), with approximately 15% of specimens resulted positive at low viral loads with system 1 and negative with system 2. This is likely to be attributable to the fact that system 1 employs more blood derived material in comparison to system 2, thus improving the recovery ability in the nucleic acid purification phase. This should be taken into account in the monitoring of transplant recipients as it could be useful for prompt identification of patients at risk and could allow for rechecking on a subsequent specimen within a short period.

Monitoring of HCMV-DNAemia has become critical for early identification of viral reactivation with the aim of reducing the occurrence of systemic and/or organ disease in the post-transplant setting and of evaluating the response to antiviral therapy. Although both antiviral prophylaxis and pre-emptive therapy are useful strategies to prevent the occurrence of HCMV disease, the potential exposure to adverse events associated with prolonged antiviral drug administration has limited the utility of a universal prophylaxis strategy, thus suggesting its adoption only in high risk patients,

such as HCMV-seropositive donor/HCMV-seronegative recipients. On the other hand, viral load monitoring for guiding pre-emptive therapy is critical. The adoption of a pre-emptive strategy appears advantageous in terms of number of treated patients, appropriateness of antiviral administration and duration of therapy, costs, risks of onset of drug adverse events, as well as emergence of drug-resistant strains.

By using the Bland-Altman analysis, differences between the two systems were within ± 1 $\log_{10}$ copies/ml of the averaged $\log_{10}$ results for almost 89% of the tested specimens. The occurrence of constant and variable quantitation differences among nucleic acid assays underlines the usefulness of a general quantitative standardization, that could also allow for a better evaluation of specific differences only related to different technical performances of the assays.

From an organizational point of view, system 1, being a full-automated system, provides benefits over a semi-automated system, in terms of less hands of time, fewer errors and reliability, that are relevant factors in a high-routine laboratory. It is to note that the present study compared two testing systems with two different extraction methods, therefore it is not known whether the differences came from the nucleic acid purification techniques or from the two HCMV tests or probably both; further studies including cross-test of both extraction methods with both PCR systems could help to clarify this. Further data on a larger number of specimens and evaluation of clinical management based on HCMV-DNAemia results will allow for better definition of the performance characteristics and clinical validation.

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Table 1

Performance characteristics of the two nucleic acid extraction and testing systems.

Features	System 1	System 2
Target	MIE	MIE
Extracted DNA volume in PCR reaction (µl)	20	5
Final volume (µl) in assay	50	25
N_o, type of quantitation standards	4, plasmid	4, plasmid
Analytical sensitivity*	164,55 copies/ml	158 copies/ml
Specificity*	100%	90%
Linear range*	$1x10^3$-$5x10^7$ copies/ml	20-$1x10^6$- copies/reaction

*Technical specifications as indicated in the manufacturer's report.

222**Table 2**

223Detection of HCMV-DNA obtained by systems 1 and 2 on 189 whole blood specimens.

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		System 1		
		POS	NEG	
System 2	POS	90 (47.6%)	9 (4.8%)	TOT 99
	NEG	28 (14.8%)	62 (32.8%)	
		TOT 118		

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243**Table 3**

244System 1 and 2 variability within the QCMD 2012 HCMV proficiency panel.

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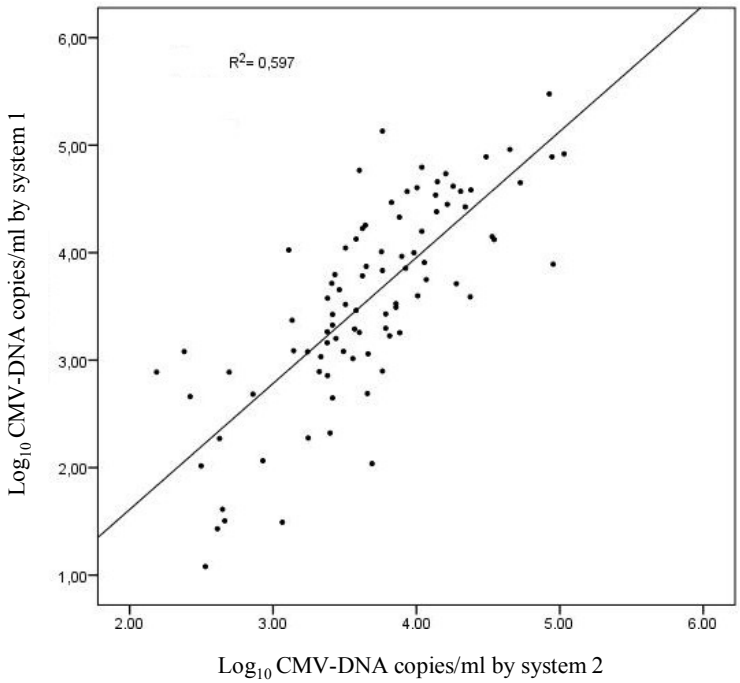
Samples	System 1 results log_{10} (copies/ml)	System 2 results log_{10} (copies/ml)	QCMD results log_{10} (copies/ml)	Difference system 1 / QCMD log_{10} (copies/ml)	Difference system 2 / QCMD log_{10} (copies/ml)
CMV12-01	4.36	4.46	4.30	**0.06**	**0.16**
CMV12-02	3.89	3.86	3.74	**0.15**	**0.12**
CMV12-03	2.30	2.14	2.24	**0.06**	**-0.10**
CMV12-04	1.43	1.47	2.07	**-0.64**	**-0.60**
CMV12-05	2.99	3.40	2.90	**0.09**	**0.50**
CMV12-06	3.33	3.51	3.30	**0.03**	**0.21**
CMV12-07	3.50	3.50	3.32	**0.18**	**0.18**
CMV12-08	negative	negative	negative	/	/
CMV12-09	3.62	3.82	3.67	**-0.05**	**0.15**
CMV12-10	2.88	2.69	2.73	**0.15**	**-0.04**

Fig. 1. Linear regression plot for log values of system 1 and system 2, by referring to the 90 double positive samples.

Fig. 2. Mean differences in HCMV-DNA quantitation of 90 positive specimens with system 1 and 2 by Bland-Altman analysis.

272**Figure 1**



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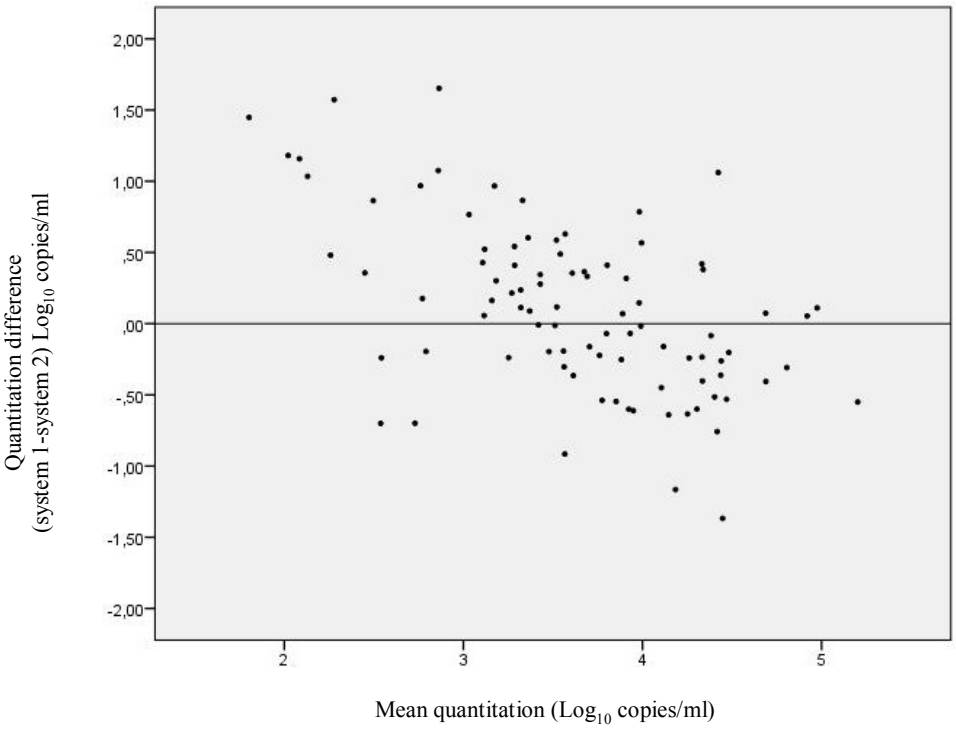
283**Figure 2**


Quantitation difference
(system 1-system 2) Log10 copies/ml
2,00
1,50
1,00
,50
,00
-,50
-1,00
-1,50
-2,00
2
3
4
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Mean quantitation (Log10 copies/ml)

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