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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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1COMPARISON OF TWO NUCLEIC ACID EXTRACTION AND TESTING SYSTEMS
2FOR HCMV-DNA DETECTION AND QUANTITATION ON WHOLE BLOOD
3SPECIMENS FROM TRANSPLANT PATIENTS

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21Abstract

22Quantitative detection of human cytomegalovirus (HCMV) DNA on whole blood is currently the
23primary choice for virological monitoring in transplant patients and for determining the appropriate
24antiviral strategy, however specific issues of variability remain in terms of extraction methods,
25amplification efficiency, and variability. This study compared the performance characteristics of
26two nucleic acid extraction and testing systems for HCMV-DNA quantitation, the artus® CMV QS-
27RGQ kit, associated with a fully automated DNA extraction and assay set up by Qiagen (system 1)
28and the Q-CMV Real Time Complete kit by Nanogen, associated with a semiautomated nucleic
29acid extraction system by Biomérieux (system 2) in 189 specimens from transplant patients and 10
30from 2012 HCMV Quality Control for Molecular Diagnostics (QCMD). The two systems exhibited
31a 80.4% concordance. Differences between the two systems were within $\pm 1 \log_{10}$ copies/ml of the
32averaged \log_{10} results for 88.9% of the tested specimens. For all qualitatively discordant specimens,
33mean viral load was $\leq 3 \log_{10}$ copies/ml. Considering viral load measurement, system 1 gave earlier
34positives than system 2, with a 14.8% of specimens resulting positive at low viral loads with system
351 and negative with system 2. In QCMD specimens, difference was below $0.7 \log_{10}$ copies/ml for
36both the systems.

37In conclusion, the two systems provided reliable and comparable results. Some specific
38performance characteristic and automation could be taken into account in terms of less hands of
39time, fewer errors and reliability.

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42**Keywords:** human cytomegalovirus; nucleic acid; extraction; quantitative PCR; efficiency;
43variability

441. Introduction

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

69The aim of this study was to assess the performance characteristics of two nucleic acid extraction
70and testing systems for HCMV-DNA quantitation, the artus® CMV QS-RGQ kit by Qiagen,
71(Hilden, Germany) associated with a fully automated DNA extraction and assay set up by Qiagen
72and the Q-CMV Real Time Complete kit by Nanogen, Elitech Group (Milan, Italy) associated with
73a semiautomated nucleic acid extraction system by Biomérieux (Marcy l'Etoile, France). Using
74whole blood clinical specimens from transplant patients, the two systems were compared and the
75potential clinical implications were evaluated.

762. **Materials and methods**

772.1. *Clinical specimens*

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

842.2 *Study design*

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

93 For system 1, nucleic acid extraction was performed according to the manufacturer's instructions;
94 nucleic acid was purified with the QIASymphony DNA Mini kit on the QIASymphony instrument
95 (Qiagen). Two-hundred microliters of whole blood were concentrated into a 60- μ l eluate, and a 20
96 μ l aliquot was used for the PCR on the real-time PCR cycler Rotor-Gene Q (Qiagen). The artus®
97 CMV QS-RGQ kit targets the major immediate early (MIE) CMV gene. The reaction volume was
98 50- μ l (20 μ l of eluate plus 30 μ l of master mix). Analytical sensitivity, as reported by the
99 manufacturer, considering purification from whole blood (using the QIASymphony DNA Mini kit)
100 and the use of artus® CMV QS-RGQ kit on the Rotor-Gene Q, is 164.55 copies/ml.

101For system 2, nucleic acid was purified with the NucliSENS® EasyMAG® instrument
102(Biomérieux), using the NucliSENS ® Nucleic Acid Extraction Reagents, according to the
103manufacturer's instructions.

104One-hundred microliters of whole blood were concentrated into a 50- μ l eluate, and a 5 μ l aliquot
105was used for PCR assay on the 7500 Real-Time PCR System (Applied Biosystems, Cheshire,
106United Kingdom). The Q-CMV Real Time Complete kit manufactured for Cepheid by Nanogen
107Advanced Diagnostics S.r.L. is specific for the exon 4 region of the CMV MIE gene (major
108immediate early HCMVUL123). The reaction volume was 25 μ l (5 μ l of eluate plus 20 μ l of master
109mix). The limit of detection of the Q-CMV real time kit is 158 copies/ml, as reported by the
110manufacturer. The main technical features of the two systems are summarized in Table 1.

1112.3. *Statistical analysis*

112The correlation between the two systems was determined by linear regression analysis and mean
113differences in quantitation for averaged logs by the Bland-Altman plot. Only viral loads positive by
114both assays were represented on the Bland-Altman graphs. Differences were considered significant
115for p value <0.05.

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1183. Results

119The performance of the two systems was assessed and compared on 189 whole blood specimens
120from 156 transplant recipients undergoing routine testing in the first year post-transplantation.
121System 1 and system 2 detected HCMV-DNA in 118 and 99 samples, respectively, with a 80.4%
122concordance. In particular, 62/189 (32.8%) were concordantly negative by both systems; 90/189
123(47.6%) concordantly positive; 28/189 (14.8%) positive by system 1 and negative by system 2; and
1249/189 (4.8%) positive by system 2 and negative by system 1 (Table 2). For the 90 specimens that
125were positive by both tests, the population mean (SD) was 4.25 (4.58) \log_{10} copies/ml with system 1
126and 4.06 (4.29) \log_{10} copies/ml with system 2. For all the specimens that were qualitatively
127discordant (by one of the two systems), mean viral load was lower or equal to 3 \log_{10} copies/ml.
128By referring to the 90 specimens that were concordantly positive, the correlation value between the
129two systems was $r = 0.597$ (Fig. 1). Bland-Altman analysis showed that differences between the two
130systems were within $\pm 1 \log_{10}$ copies/ml of the averaged \log_{10} results for 88.9% of the tested
131specimens (Fig. 2). Table 3 depicts the HCMV load results for the 10 specimens from the QCMD
1322012 HCMV proficiency panel. The sample for which a negative result was expected was found
133negative by the two systems, whereas for the positive specimens the difference was below 0.7 \log_{10}
134copies/ml for both the systems (mean $\Delta\log_{10}$ 0.1566 for system 1 and 0.2288 for system 2).

1354. Discussion

136The automation of nucleic acid extraction and availability of commercial real-time quantitative PCR
137assays have the potential to improve the agreement and clinical usefulness of HCMV-DNA
138measurement in routine transplant settings, thus promoting standardization across laboratories and
139enabling correlation with clinical study results.

140In the present study, the HCMV –DNA load results obtained by two nucleic acid extraction and
141testing systems have been compared. Overall, both systems appear as reliable and user-friendly for
142monitoring HCMV-DNAemia in transplant recipients.

143The two systems exhibited an overall concordance of 80.4% in qualitative terms. Considering
144specimens that were positive by both the systems, the mean viral load differs of 0.19 log₁₀
145copies/ml. On the other hand, when considering specimens that were qualitatively discordant, mean
146viral load was lower or equal to 3 log₁₀ copies/ml.

147Considering viral load measurement, system 1 gave earlier positives than system 2, as evidenced by
148the evaluation of frequency distribution for different log of viral load (not shown), with
149approximately 15% of specimens resulting positive at low viral loads with system 1 and negative
150with system 2. This is likely to be attributable to the fact that system 1 employs more blood derived
151material in comparison to system 2, thus improving the recovery ability in the nucleic acid
152purification phase. This should be taken into account in the monitoring of transplant recipients as it
153could be useful for prompt identification of patients at risk and could allow for rechecking on a
154subsequent specimen within a short period.

155Monitoring of HCMV-DNAemia has become critical for early identification of viral reactivation
156with the aim of reducing the occurrence of systemic and/or organ disease in the post-transplant
157setting and of evaluating the response to antiviral therapy. Although both antiviral prophylaxis and
158pre-emptive therapy are useful strategies to prevent the occurrence of HCMV disease, the potential
159exposure to adverse events associated with prolonged antiviral drug administration has limited the
160utility of a universal prophylaxis strategy, thus suggesting its adoption only in high risk patients,

161such as HCMV-seropositive donor/HCMV-seronegative recipients. On the other hand, viral load
162monitoring for guiding pre-emptive therapy is critical. The adoption of a pre-emptive strategy
163appears advantageous in terms of number of treated patients, appropriateness of antiviral
164administration and duration of therapy, costs, risks of onset of drug adverse events, as well as
165emergence of drug-resistant strains.

166By using the Bland-Altman analysis, differences between the two systems were within $\pm 1 \log_{10}$
167copies/ml of the averaged \log_{10} results for almost 89% of the tested specimens. The occurrence of
168constant and variable quantitation differences among nucleic acid assays underlines the usefulness
169of a general quantitative standardization, that could also allow for a better evaluation of specific
170differences only related to different technical performances of the assays.

171From an organizational point of view, system 1, being a full-automated system, provides benefits
172over a semi-automated system, in terms of less hands of time, fewer errors and reliability, that are
173relevant factors in a high-routine laboratory. It is to note that the present study compared two
174testing systems with two different extraction methods, therefore it is not known whether the
175differences came from the nucleic acid purification techniques or from the two HCMV tests or
176probably both; further studies including cross-test of both extraction methods with both PCR
177systems could help to clarify this. Further data on a larger number of specimens and evaluation of
178clinical management based on HCMV-DNAemia results will allow for better definition of the
179performance characteristics and clinical validation.

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210 **Table 1**

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

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	Features	System 1	System 2
	Target	MIE	MIE
214	Extracted DNA volume in PCR reaction (µl)	20	5
	Final volume (µl) in assay	50	25
215	No. type of quantitation standards	4, plasmid	4, plasmid
	Analytical sensitivity*	164,55 copies/ml	158 copies/ml
216	Specificity*	100%	90%
	Linear range*	1x10 ³ -5x10 ⁷	20-1x10 ⁶ -
217		copies/ml	copies/reaction

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221 *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.

Samples	System 1 results	System 2 results	QCMD results	Difference system 1 /	Difference system 2 /
	log₁₀ (copies/ml)	log₁₀ (copies/ml)	log₁₀ (copies/ml)	QCMD log₁₀ (copies/ml)	QCMD log₁₀ (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

246**Fig. 1.** Linear regression plot for log values of system 1 and system 2, by referring to the 90 double
247positive samples.

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249**Fig. 2.** Mean differences in HCMV-DNA quantitation of 90 positive specimens with system 1 and 2
250by Bland-Altman analysis.

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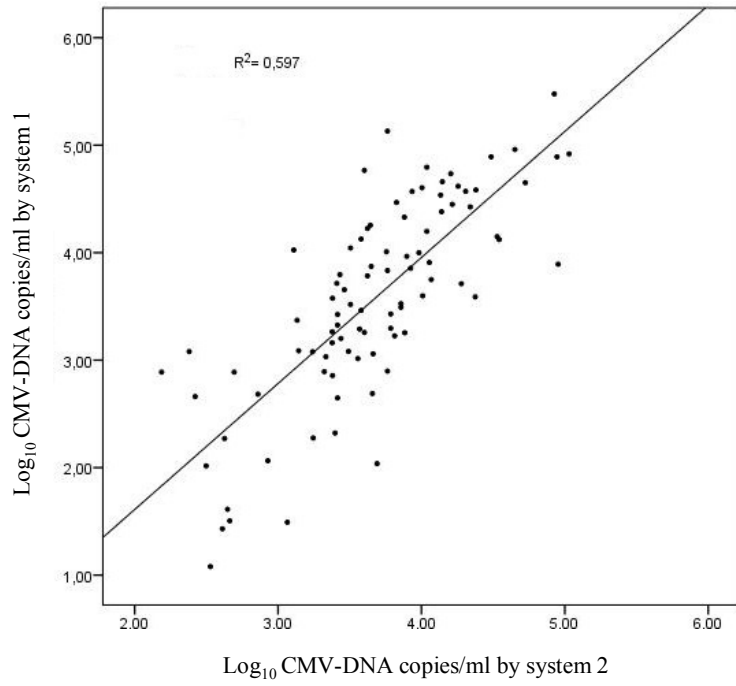
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272 **Figure 1**



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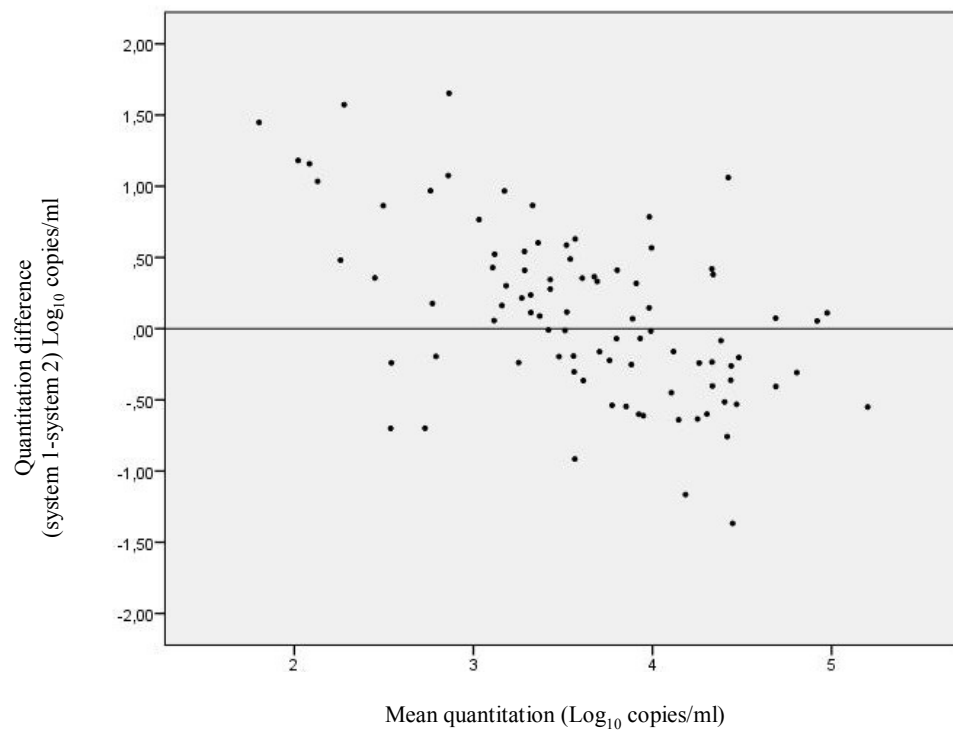
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283 **Figure 2**



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