

Multicenter quality control study for human cytomegalovirus DNAemia quantification

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SUMMARY

Standardized protocols and methods for virological monitoring are mandatory for the correct surveillance of human cytomegalovirus (HCMV) infection in transplanted patients receiving pre-emptive therapy.

Fifteen Italian viral diagnostic laboratories belonging to different transplantation centers participated in the external Quality Control Programme for Molecular Diagnostics of HCMV-DNA by using two in-house and five commercial methods for HCMV-DNA quantification.

The different methods shared 100% specificity, and sensitivity reached 100% when samples containing >1,000 copies/ml were considered.

The variability range was wide (about $2 \log_{10}$) for samples containing a lower amount of HCMV-DNA (<1,000 copies/ml), but it decreased with increasing concentrations of HCMV-DNA. For HCMV-DNA levels $\geq 5,000$ copies/ml, the different methods provided results within a $\pm 0.5 \log_{10}$ variability range, while the 80% range (range in which 80% of results obtained will fall) was within $\pm 0.3 \log_{10}$ or less.

An acceptable level of variability was reached among different in-house and commercial methods for HCMV-DNA quantification in samples containing a clinically significant viral DNA amount. Based on these data, standardized cut-offs established for pre-emptive therapy in different transplantation centers should provide comparable clinical and virological results among centers.

KEY WORDS: Human cytomegalovirus, Transplantation, DNAemia, Standardization

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INTRODUCTION

Pre-emptive treatment of human cytomegalovirus (HCMV) infection is widely adopted to prevent HCMV disease in transplant recipients. Pre-emptive therapy consists of the administration of an-

tivirals before the onset of disease, at the time when HCMV load in blood reaches levels predictive of disease (Einsele *et al.*, 1995; Boeckh *et al.*, 1996; Egan *et al.*, 1998). Although universal prophylaxis is easier to implement, pre-emptive therapy offers advantages in avoiding late disease and reducing drug toxicity and patient treatment costs (Kusne *et al.*, 1999; Singh, 2006).

The antigenemia assay has been widely used to guide pre-emptive therapy (Boeckh *et al.*, 1992; Grossi *et al.*, 1995).

Although standardization of antigenemia is possible (Gerna *et al.*, 1998a), interpretation of test results remains subjective and the assay is not automatable. In addition, antigenemia quantification correlates only partially with actual viral replication, thus sometimes providing clinicians with misleading information (Gerna *et al.*, 1998b, 2000, 2003, 2005).

These limitations could be overcome by using molecular assays for HCMV-DNA quantification. However, since different systems (some developed in-house, others commercially available) are used, a standardized methodology is warranted, as well as periodical revision by external quality control programmes.

MATERIALS AND METHODS

The present report evaluates an external quality control programme (Quality Control for Molecular Diagnostics, QCMD) performed by 15 Italian centers, all of which are members of the SIV (Società Italiana di Virologia)-AMCLI (Associazione Microbiologi Clinici Italiani) Transplant Surveillance Group. Specific aims included the standardization of methods for HCMV-DNA quantification and the definition of variability ranges, with special reference to HCMV-DNA levels considered clinically significant for pre-emptive therapy.

Artificial standards and clinical samples

The control panel (QCMD 2007 Human Cytomegalovirus DNA Proficiency Programme, Glasgow, Scotland; www.qcmd.org) consisted of eight samples containing various amounts of lyophilized plasma or cell culture medium spiked with the HCMV AD169 strain along with two negative controls. Each sample was reconstituted by

adding 1 ml sterile water. The panel was independently tested by each of the participating laboratories with its own PCR method. In addition, 99 blood samples from 71 solid organ or haematopoietic stem cell transplant recipients were tested in parallel by one in-house and two commercial assays.

HCMV-DNA quantification

The 15 laboratories performed 17 test runs (Table 1) using two in-house real-time PCR methods [in-house PV (Gerna *et al.*, 2006), targeting the HCMV US8 region, performed by 3 centers, and in-house PD (Mengoli *et al.*, 2004), targeting UL122, performed by one center] and four commercial real-time PCR kits: Nanogen Q-CMV Real time Complete kit (Nanogen Advanced Diagnostics, Torino, Italia; n=8; target region: UL123), Cepheid Affigene CMV treader (Cepheid AB, Bromma, Sweden; n=2; target region: UL123), QIAGEN Artus CMV PCR kit (QIAGEN GmbH, Hilden, Germany; n=1), and Abbott CMV PCR kit (Abbott Molecular, Abbott Park, Illinois, USA; n=1).

These two latter methods were grouped together (and named "Artus") since they represent two versions of the same test (target region: UL123) distributed by the two companies. A single center used also the end-point PCR COBAS Amplicor CMV Monitor test (Roche Diagnostic Systems, Pleasanton, CA, USA; target region: UL54). Different methods were adopted for nucleic acid extraction (QIAamp DNA Mini Kit, QIAamp DNA Blood BioRobot MDx Kit and BioSprint 15 DNA Blood Kit, QIAGEN; NucliSENS EasyMAG, bioMerieux, Durham, NC, USA; EXTRAgen, Nanogen Advanced Diagnostics; X-tractor Gene, Corbett Life Science, Sidney, Australia; MagNA Pure Systems, F. Hoffmann-La Roche Ltd, Basel, Switzerland; Abbott m2000sp Abbott Molecular; ABI PRISM 6700 Nucleic Acid Automatic Workstation, Applied Biosystems, Foster City, CA, USA).

Sequence analysis of the HCMV US8 region

PCR amplification products were obtained from a subset of 15 patients with primers external to the target region (US8, nt 226-290) of the in-house PV.

The sequence reaction was performed with ABI PRISM™ Big Dye Terminator kit (Applied

TABLE 1 - Participating centers and methods for HCMV DNA quantification.

<i>Participating center</i>	<i>DNA extraction method</i>	<i>Commercial or in-house method</i>	<i>HCMV-DNA quantification method</i>
Roma-Spallanzani I*	QIAGEN	Affigene	Real-time PCR
Roma-Spallanzani II*	QIAGEN	Artus	Real-time PCR
Pisa I	QIAGEN	Affigene	Real-time PCR
Pisa II	QIAGEN	COBAS	End-point PCR
Siena	QIAGEN	Artus	Real-time PCR
Torino	QIAGEN	Nanogen	Real-time PCR
Modena	Abbott m2000sp	Nanogen	Real-time PCR
Udine	QIAGEN	Nanogen	Real-time PCR
Bologna	EasyMag	Nanogen	Real-time PCR
Milano	EasyMag	Nanogen	Real-time PCR
Cosenza	X-tractor Gene	Nanogen	Real-time PCR
Novara	EXTRAgen	Nanogen	Real-time PCR
Roma-La Sapienza	EasyMag	Nanogen	Real-time PCR
Padova	ABI-Prism 6700	In-house PD	Real-time PCR
Bergamo	MagNa Pure	In-house PV	Real-time PCR
Pavia	EasyMag	In-house PV	Real-time PCR
Siena	QIAGEN	In-house PV	Real-time PCR

*I and II indicate that the same lab performed two different assays.

TABLE 2 - Qualitative analysis of results within the European QCMD.

<i>Sample</i>	<i>Estimated HCMV DNA content</i>	<i>"EQA" log₁₀ consensus</i>	<i>No. positive samples/tested (%)</i>
Q-03	negative	negative	0/17 (0)
Q-05	negative	negative	0/17 (0)
Q-09	weak positive	2.03	6/17 (35)
Q-10	weak positive	2.41	13/17 (76)
Q-02	weak positive	2.41	14/17 (82)
Q-06	positive	3.00	16/16* (100)
Q-08	positive	3.54	17/17 (100)
Q-01	positive	4.34	17/17 (100)
Q-04	strong positive	5.35	17/17 (100)
Q-07	strong positive	6.35	17/17** (100)

EQA: European Quality Assessment. *One invalid result. **One result exceeding the quantification range was excluded from the analysis.

TABLE 3 - Variability ranges within the QCMD.

Sample	Italian \log_{10} consensus \pm SD	\log_{10} range			95% range			80% range			"EQA" \log_{10} consensus \pm SD
		Min	Max	Δ	Min	Max	Δ	Min	Max	Δ	
Q-09	1.87 \pm 0.47	neg	2.44	2.44	0.96	2.79	1.83	1.28	2.47	1.20	2.03 \pm 0.74
Q-10	2.13 \pm 0.49	neg	2.78	2.78	1.17	3.09	1.93	1.50	2.76	1.26	2.41 \pm 0.46
Q-02	2.18 \pm 0.54	neg	3.10	3.10	1.12	3.23	2.11	1.49	2.86	1.38	2.41 \pm 0.53
Q-06	3.02 \pm 0.37	2.07	3.42	1.36	2.30	3.74	1.44	2.55	3.49	0.94	3.00 \pm 0.34
Q-08	3.69 \pm 0.29	3.20	4.08	0.89	3.11	4.26	1.16	3.31	4.06	0.75	3.54 \pm 0.34
Q-01	4.48 \pm 0.28	4.12	4.89	0.77	3.94	5.03	1.09	4.13	4.84	0.71	4.34 \pm 0.34
Q-04	5.55 \pm 0.22	5.21	5.93	0.72	5.11	5.98	0.87	5.26	5.83	0.57	5.35 \pm 0.34
Q-07	6.51 \pm 0.17	6.24	6.88	0.64	6.18	6.85	0.66	6.30	6.73	0.43	6.35 \pm 0.43

EQA: European Quality Assessment.

Biosystems, Foster City, CA, USA). Sequences were analyzed using the Genetic Analyzer 3100 System (Applied Biosystems). The US8 sequences from reference strains were obtained from GenBank (see figure 3), except for VR1814 which was provided by Andrew J. Davison (Institute of Virology, University of Glasgow, UK).

Statistical analysis

A consensus DNA content was established as the \log_{10} mean of each participant's results. Boundaries for two-sided 95% and 80% ranges were determined from the normal distribution as the mean \pm 1.96 or \pm 1.28 standard deviation, respectively.

The unpaired *t*-test was adopted for comparison of means for unpaired data.

RESULTS

Qualitative analysis

The two negative QCMD control samples were negative in all test runs (specificity 100%; Table 2). Three "weakly positive" samples containing a low HCMV-DNA amount (consensus concentration <1,000 copies/ml) were detected as positive in 35%,

76% and 82% of test runs, respectively. Conversely, all samples containing \geq 1,000 HCMV-DNA copies/ml were detected as positive (except for one sample which was scored as invalid). Thus, the sensitivity of HCMV-DNA measurement reached 100% in the presence of \geq 1,000 copies/ml. No significant difference was observed between in-house and commercial systems (data not shown).

Quantitative analysis

The mean ratio between each test result (expressed as copies/ml) and the consensus DNA amount ranged from 0.41 to 2.68 (data not shown).

This indicates that the DNA amount tested in different runs ranged from about half to less than threefold with respect to the consensus values. One single sample was quantified in a test run as 3.7 fold higher than the consensus. Thus, it was considered an outlier and excluded from the calculation for variability ranges.

As shown in Table 3, the observed result ranges were extremely wide ($>2 \log_{10}$) for the three "weakly positive" samples (<1,000 copies/ml). Result homogeneity improved when the sample DNA amount increased, as shown by the finding that results relevant to samples containing \geq 5,000

copies/ml ($3.7 \log_{10}$) ranged within less than 1 \log_{10} variation (i.e. $\pm 0.5 \log_{10}$ from the consensus). The result range decreased from 0.89 to 0.64 \log_{10} with the increase in viral DNA from the $3.7 \log_{10}$ DNA copies/ml to the $6.5 \log_{10}$ DNA copies/ml. The estimated 95% and 80% ranges

for these samples (ranges within which 95% and 80% of results were estimated to fall) were within 1.16 to 0.66 and 0.75 to 0.43 \log_{10} , respectively. When compared with the global QCMD 2007 programme (www.qcmd.org), results provided by the Italian Transplant Surveillance Group were

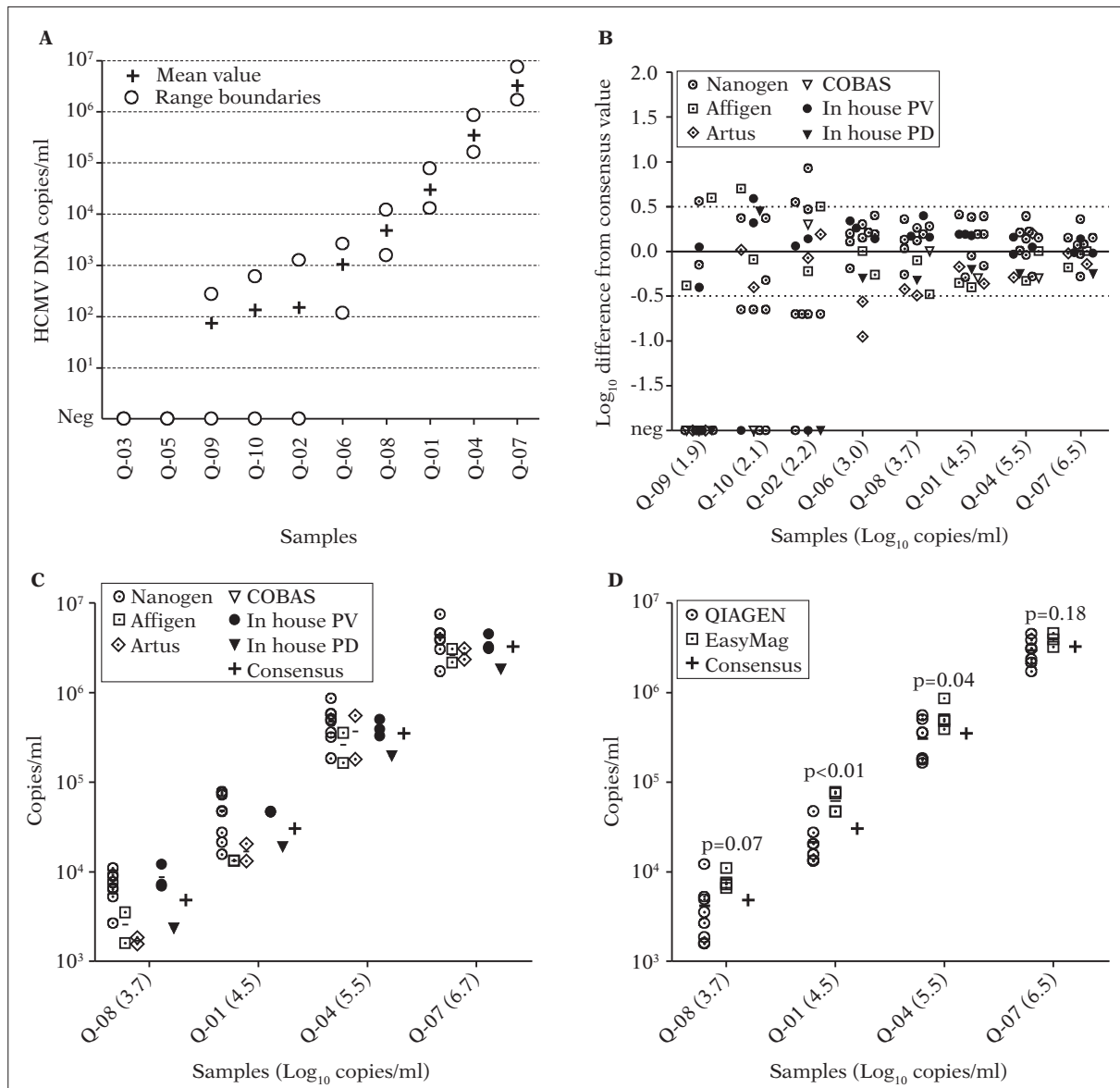


FIGURE 1 - HCMV-DNA quantification variability in QCMD samples using different extraction and PCR amplification methods. A: consensus values and range boundaries (minimum and maximum) for each sample in the panel. B: the positive or negative \log_{10} distance of each sample from the consensus is reported for the different QCMD samples. The solid line represents the concordance between test results and consensus values, while dotted lines indicate $\pm 0.5 \log_{10}$ variations from the consensus. Comparative results obtained on the 4 QCMD samples containing $\geq 3.7 \log_{10}$ HCMV DNA copies/ml with the different PCR (C) or extraction (D) methods are reported. Manual and automated QIAGEN extraction methods are grouped together.

similar in the consensus DNA content, with a somewhat smaller standard deviation (Table 3). The ranges of the DNA quantification results obtained for the panel samples are also illustrated in Figure 1A, whereas the \log_{10} differences from the consensus values for the different results are reported in Figure 1B. For samples containing low DNA concentrations ($<3.7 \log_{10}$, Q-09, Q-10, Q-02, Q-06) a number of results exceeded a $0.5 \log_{10}$ difference from the consensus, while all values for samples with higher DNA content (Q-08, Q-01, Q-04, Q-07) were within $\pm 0.5 \log_{10}$ (or even less) from the consensus. Figure 1C compares the results provided by different PCR methods for the four panel samples containing $\geq 3.7 \log_{10}$ DNA copies/ml.

No statistical analysis on the performance of the different PCR methods was performed due to the small number of replicates available for each method. Instead, it was possible to compare results provided by two different DNA isolation systems (QIAGEN, grouping together both manual and automated extraction, and EasyMag), regardless of the PCR method used (Figure 1D). Except for the sample with the highest DNA concentration (Q-07), centers adopting EasyMag for DNA isolation had significantly higher results (Q-08, $p=0.07$; Q-01, $p<0.01$; Q-04, $p=0.04$).

Comparison of one in-house and two commercial methods

Results obtained using one in-house PV and two commercial methods (Nanogen and Artus) on a series of clinical samples from transplant recipients were also compared (Figure 2). According to these results, patients could be clustered into two groups: those whose blood samples (open circles) showed a difference among assays roughly comprised within $\pm 0.5 \log_{10}$ (the great majority), and patients in whom the difference among assays exceeded $0.5 \log_{10}$ (closed circles).

Nanogen and in-house PV were compared on 60 blood samples obtained from 32 patients (Figure 2A). In 26 patients (41 samples), the difference between the HCMV-DNA quantification provided by the two tests was between -0.57 and $0.54 \log_{10}$ (mean difference -0.05 ; standard deviation $\pm 0.25 \log_{10}$). On the other hand, in 19 samples from the remaining 6 (19%) patients, Nanogen gave consistently higher results than in-house PV, with a mean difference between the two assays of $0.75 \pm 0.20 \log_{10}$, ranging from 0.62 to 1.14.

Nanogen and Artus were performed in parallel on 39 samples from 39 other patients (Figure 2B). Similar to what was observed in the comparison of Nanogen and in house-PV, in 31 blood samples, the mean difference between the two assays

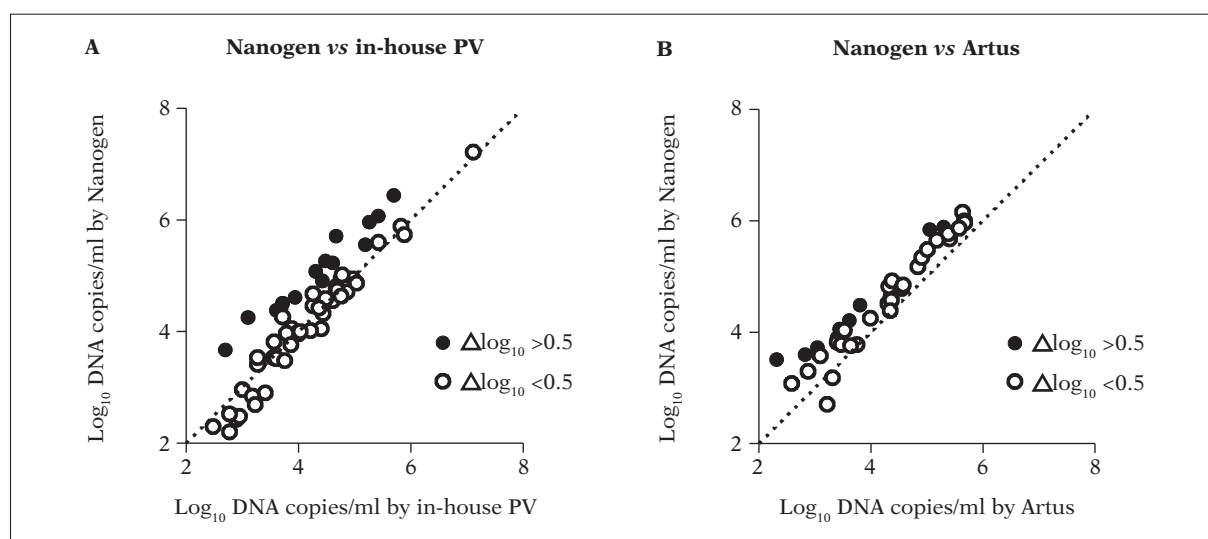


FIGURE 2 - HCMV-DNA quantification variability in clinical samples using different PCR methods. The correlations between results obtained in clinical samples by Nanogen and in-house PV (A), and by Nanogen and Artus (B) are reported. Open circles represent samples with variation in quantification ranging within $\pm 0.5 \log_{10}$, while closed circles represent sample variations exceeding this range. Dashed lines indicate the correspondence between the two assays.

	Forward primer										Probe										Reverse primer									
Reference strains																														
#AD169_BR000394	GCG	TGT	GGG	ACG	CTG	TGT	GGC	GG	TAC	AGG	GTA	CGT	TCT	ATC	CCG	AGA	AGG	GGA	TAG	TGG	CTC	GGG	TCG	GAT	GGT	CGG	GCC	GTC		
#AD169_X17403	
#TB40EBAC_EF999921	
#TOLEDO_AY486480	
#VR1814_(AJ Davison)	
#FIXBAC_AC146907	
#MERLIN_AY446894	
Clinical samples																														
#pt. 1 (0.11)*	
#pt. 2 (0.17)	
#pt. 3 (-0.11)	
#pt. 4 (0.18)	
#pt. 5 (0.09)	
#pt. 6 (-0.20)	
#pt. 7 (-0.05)	
#pt. 8 (0.74)	
#pt. 9 (0.67)	
#pt. 10 (0.73)	
#pt. 11 (1.04)	
#pt. 12 (0.62)	
#pt. 13 (0.78)	
#pt. 14 (-0.15)	
#pt. 15 (-0.12)	

*In brackets the \log_{10} difference between HCMV DNA quantification by Nanogen and in-house PV methods is reported

FIGURE 3 - Sequence analysis of the in-house PV target region (US8, nt 226-290) from seven reference and 15 clinical HCMV strains. Nucleotide changes in HCMV reference or clinical strains with respect to AD169 are reported. Substitution of GG with AA at the end of the probe sequence (nt 271-272) characterizes the clinical strains (pt #8-13) with a $>0.5 \log_{10}$ (0.62-1.04 \log_{10}) difference in the quantification provided by the two methods. GenBank accession nos. for the reference strains are reported.

was $0.30 \pm 0.22 \log_{10}$, ranging from -0.52 to 0.54 . In the remaining eight samples (21%) the mean difference between Nanogen and Artus was $0.74 \pm 0.19 \log_{10}$, ranging from 0.59 to 1.18 .

Sequence analysis of HCMV US8 (the PCR target region for the in-house PV) was conducted on blood samples from 15 of the patients tested in parallel with Nanogen corresponding to the 6 patients with $>0.5 \log_{10}$ difference between the two assays and 9 of the 26 patients with concordant results (Figure 2A).

Sequence alignment was compared also with sequences from seven reference strains available in GenBank (Figure 3).

Interestingly, the six patients with discordant DNAemia results harboured HCMV strains showing a substitution (from GG to AA) of the 3' nucleotides (nt positions 271-272) of the genome region recognized by the TaqMan probe for the in-house PV, while in the remaining patients (as well as in all the AD169 reference samples) this region matched the probe sequence.

DISCUSSION

Results of the present study indicate that the PCR methods for HCMV-DNA quantification examined share 100% specificity and sensitivity for samples

from the QCMD containing $\geq 1,000$ HCMV-DNA copies/ml. The variability range was higher for samples containing a low HCMV-DNA concentration, but variability decreased with an increase in HCMV-DNA content. For HCMV-DNA levels $\geq 5,000$ copies/ml, the methods analyzed provided results within $\leq 1 \log_{10}$ ($\pm 0.5 \log_{10}$) difference from the consensus. The estimation of the 80% range suggests that 80% of results would fall within a $0.5 \log_{10}$ range or even less than $0.5 \log_{10}$ for higher HCMV-DNA amounts. These results support the conclusion that HCMV-DNA levels predictive for risk of HCMV disease (Cope *et al.*, 1997) (and corresponding to the cut-offs for pre-emptive therapy previously validated in prospective clinical trials) (Gerna *et al.*, 2007, 2008; Lilleri *et al.*, 2007) are homogeneously and reliably quantified by different methods (both commercial and in-house) and by different laboratories.

A similar degree of variability was observed in clinical samples. However, in less than 20% of patients, figures provided by different assays may exceed the $\pm 0.5 \log_{10}$ range. To explain this finding, it can be hypothesized that in some HCMV field-strains genetic polymorphisms may involve genomic regions that are preferentially selected as a target for PCR, thus affecting DNA quantification. Sequence analysis of the HCMV-DNA region targeted by the in-house PV assay, seems to sup-

port this hypothesis. Investigations regarding this issue are ongoing.

Thus, the standardization of HCMV-DNA PCR methods implies that cut-offs for the pre-emptive treatment of HCMV infection could be exported to different transplantation centers where patients would receive the same level of surveillance for HCMV infection (provided that it is mandatory for laboratories to participate in an external quality assessment programme).

For this reason, the SIV-AMCLI Transplant Surveillance Group has recently elaborated a "Consensus" on the management of HCMV infection in solid organ transplant recipients (www.amcli.it; www.siv-virologia.it). These guidelines recommend starting preemptive therapy in the presence of >100,000 HCMV DNA copies/ml blood, according to a recently published study (Gerna *et al.*, 2007).

Although different nucleic acid extraction methods were adopted and better performance was observed for EasyMag with respect to QIAGEN (both manual and automated), the results of this study cannot conclusively evaluate this issue. The sample matrix adopted in this study was lyophilized culture medium or plasma (reproducing the characteristics of fresh plasma), thus the present analysis cannot evaluate the performance of DNA extraction systems on whole blood, which is the specimen of choice in the transplant setting, since it permits determination of both cell-free and cell-associated HCMV (Razonable *et al.*, 2002; Weinberg *et al.*, 2002; Cortez *et al.*, 2003; Mengelle *et al.*, 2003) The adoption of a blood-like matrix for the external quality assessment panels is the next step required to better standardize and optimize the surveillance of HCMV infection among centers.

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