Nation-wide measure of variability in HCMV, EBV and BKV DNA quantification among centers involved in monitoring transplanted patients

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ABSTRACT

Background: Inter-laboratory variability in quantifying pathogens involved in viral disease after transplantation may have a great impact on patient care, especially when pre-empitive strategies are used for prevention.

Objectives: The aim of this study was to analyze the variability in quantifying CMV, EBV and BKV DNA among 15 virology laboratories of the Italian Infections in Transplant Working Group (GLaIT) involved in monitoring transplanted patients.

Study Design: Panels from international Quality Control programs for Molecular Diagnostics (QCMD, year 2012), specific for the detection of CMV in plasma, CMV in whole blood (WB), EBV and BKV were used. Intra- and inter-laboratory variability, as well as, deviation from QCMD consensus values were measured.

Results: 100% specificity was obtained with all panels. A sensitivity of 100% was achieved for EBV and BKV evaluation. Three CMV samples, with concentrations below 3 $\log_{10}$ copies/ml, were not detected by a few centers. Mean intra-laboratory variability (% CV) was 1.6 for CMV plasma and 3.0 for CMV WB. Mean inter-laboratory variability (% CV) was below 15% for all the tested panels. An higher inter-laboratory variability was observed for CMV WB with respect to CMV plasma (3.0 vs 1.6% CV). The percentiles 87.7%, 58.6%, 89.6% and 74.7% fell within ± 0.5 $\log_{10}$ difference of the consensus values for CMV plasma, CMV WB, EBV and BKV panels, respectively.

*Conclusions***:** An acceptable intra- and inter-laboratory variability was observed in this study, in comparison with international standards. However, further harmonization in viral genome quantification is reasonable expectation for the future.

Keywords: multicenter evaluation; standardization; transplantation; CMV-DNA; EBV-DNA; BKV-DNA

1. Background

Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are major causes of post-transplant viral disease both in solid organ and hematopoietic stem cell transplantation, while Polioma BK virus reactivation, with virus-associated nephropathy, represents the most frequent cause of graft loss after renal transplantation [1,2]. The reliability and accuracy of viral load determination are therefore critical for the management of transplant patients. In fact, virological monitoring of transplanted patients is based on standardized protocols for genome quantification in order to apply clinical cut-offs in pre-emptive approaches for disease prevention [3-6]. However, measurements of viral load performed with commercially available assays might differ significantly, particularly according to the extraction method used, which is a source of variability with different clinical specimens. Finally, there is a need for an inter-laboratory comparison of results and evaluation of individual assays with standardized panels, particularly with collaborative multicentre networks.

2. Objectives

The aim of this study was to analyze the variability obtained among 15 Italian virology laboratories, belonging to the Working Group for Transplantation (Gruppo di Lavoro Infezioni nel Trapianto, GLaIT) of the Italian Association of Clinical Microbiologists (Associazione Microbiologi Clinici Italiani, AMCLI). Therefore, panels from international Quality Control programs for Molecular Diagnostics (QCMD, year 2012), specific for the detection and quantification of CMV in plasma, CMV in whole blood (WB), EBV and BKV were used.

3. Study design

3.1. QCMD samples

The QCMD panels used in the study were: QCMD 2012 CMV plasma, QCMD 2012 CMV WB, QCMD 2012 EBV and QCMD 2012 BKV-JCV. A number of samples with various amounts of the

different viruses suspended in an appropriate matrix and negative controls were tested in each panel. For a detailed description and composition of the panels see www.qcmd.org.

3.2. Extraction and quantitative real-time PCR assays

Each sample was tested by each of the laboratories using commercial and *in house* methods adopted for routinely virologic monitoring of transplanted patients (Table 1). Nucleic acid extraction was performed by the majority of the laboratories using automatic extraction with commercial with sometimes in-house modifications; Real-time PCR amplification was carried out by all the laboratories with commercially available kits, with only one exception. Quantitative results were expressed as $\log_{10}$ copies/ml for all three viruses tested. For positive samples detected below the lowest limit of quantification, when a detected number of copies was not available, an arbitrary value of half of the lowest limit of quantification was used.

3.3. Statistical analysis

Intra- and inter-laboratory variability was calculated, as well as, variation with respect to the QCMD consensus as the coefficient of variation (%CV). The Pearson correlation analysis and the Bland-Altman analysis were performed to examine the level of agreement between the 15 laboratories' results and the QCMD samples. Results were considered to be quantitatively discordant when the results of the Bland-Altman analysis were discordant by more than ±0.5 Log_{10} of the QCMD consensus values. Statistical analysis was performed using Graph Pad Prism software, version 5.00.288.

4. Results

The results obtained by the different GLaIT laboratories were analyzed to obtain a description of intra- and inter-laboratory variability and a quantitative comparison with respect to the consensus values reported by the different QCMD panels.

For each of the four panels tested (QCMD CMV plasma, CMV WB, EBV and BKV) no false positive results were obtained by any of the GLaIT laboratories (specificity 100%). A sensitivity of 100% was achieved with the EBV and BKV evaluations. Concerning the CMV plasma panel, sample #3 (2.24 $\log_{10}$ copies/ml) was not detected by 1/15 (6.6%) centres and sample #4 (2.08 $\log_{10}$ copies/ml) was not detected by 4/15 (26.6%) centres. For the CMV WB panel only sample #8 (2.58 $\log_{10}$ copies/ml) was not detected by 4/13 (30.8%) centres. For the BKV/JCV panel, no cross reactivity with the JCV virus was observed (6 samples) and all of the centres detected all the five samples containing BKV.

The QCMD CMV plasma and WB panels contained duplicate samples to allow intra-laboratory variability evaluation. The results indicated that the mean intra-laboratory % of the coefficient of variation (CV) was 1.6 for CMV plasma and 3.0 for CMV WB.

In Table 2, for each sample the mean, standard deviation (SD), CV (%), median and range of $\log_{10}$ copies/ml are reported. The mean SD for CMV plasma, CMV WB, EBV and BKV were respectively 0.27, 0.49, 0.25 and 0.37. The mean % CV for CMV plasma, CMV WB, EBV and BKV were respectively: 9.4%, 13.7%, 6.71% and 13.3%. The mean Delta $\log_{10}$ for CMV plasma, CMV WB, EBV and BKV were respectively: 0.93, 1.40, 0.97 and 1.29. It should be emphasized that the variability was usually larger when considering samples with a virus concentration lower than 3 $\log_{10}$ copies/ml.

In order to compare the results obtained by the different GLaIT laboratories with those of the international quality control study, consensus values for each QCMD panel were extrapolated and used for comparison. Significant correlations were observed in CMV WB, CMV plasma, EBV and BKV panel results with the Spearman coefficient which ranged from 0.82 to 0.96 (data not showed). Bland-Altman plots were used to describe the $\log_{10}$ difference between the GLaIT laboratory results and the consensus values (Figure 1). According to previous reports [7-9], ±0.5 $\log_{10}$ was considered an acceptable variability. In CMV the plasma panel, 114/130 (87.7%) of the determinations were within ±0.5 $\log_{10}$ difference, while in the CMV WB panel only 51/87 (58.6%)

were within ±0.5 $\log_{10}$ difference. In the CMV plasma panel, the majority of the discordant results (14/16, 87.5%) were observed in samples with a <3.0 $\log_{10}$ DNA copies number (Figure 1A), while in the CMV WB panel discordant results were observed for all sample concentrations (Figure 1B). In the EBV panel (Figure 1C), 120/134 (89.6%) of the measurements were within a ±0.5 $\log_{10}$ difference, with no evident differences among different sample concentrations. In the BKV panel (Figure 1D), a total of 56/75 (74.7%) determinations fell within ±0.5 $\log_{10}$ difference; and for the EBV panel, no differences among the different sample concentrations were detected.

5. Discussion

Since significant inter-laboratory variability in quantifying CMV, EBV and BKV genomes may impact the quality of transplanted patient care, especially when pre-empitive strategies are used for prevention, initiatives aimed at harmonizing viral genome quantification among different laboratories should always be encouraged. In fact, transplant centres collect patients from all Italians regions while post-transplant monitoring may be carried out by local laboratories. To the best of our knowledge, this is the first report which simultaneously measures variability in quantifying CMV, EBV and BKV DNA which represent the three major viral pathogens responsible for disease in solid organ transplantation.

Mission of the GLaIT group is to improve standardization of diagnostic procedures for microbiological monitoring of solid organs and stem cell transplant recipients. Two different studies aimed to measure variability in CMV and EBV DNA quantification have already been performed [9,10]. Concerning CMV, the present study, in contrast with the 2009 report [9], takes into account both CMV DNA quantification in plasma and WB. As for the former CMV study, no false positive samples were obtained and a sensitivity of 100% was obtained in samples with a DNA load greater than 3 $\log_{10}$ copies/ml. Although in the past a variability of less than 1 $\log_{10}$ was obtained only in samples with a viral load greater than 3.7 $\log_{10}$ copies/ml, the results reported here ranged from 0.93 to 1.40 $\log_{10}$ variation for plasma and WB even when considering samples with concentrations

below 3.0 log_{10} copies/ml. A greater variability was observed for CMV in WB with respect to plasma; this is in line with the more complex matrix represented by blood, where nucleic acid extraction is more laborious. At the same time, the CMV blood panel results, although more variable, display a greater linearity. Overall, the % accuracy measured fell within ±0.5 log_{10} and ranged from 58.6% to 89.6%. This accuracy measured in a multicentre study is considered acceptable and is higher than those observed in a similar study including fewer centres (n=4) [11].

It should be underlined that in the present CMV quantification analysis, in contrast with previous studies, all of the laboratories, with only one exception, used a commercial real-time PCR method and only three different real-time methods were used. These real-time methods were however associated with a variety of different manual or automated commercial and in house modified protocols for nucleic acid extraction. It is reasonable to suppose that much of the variability observed among the different quantifications was associated with the extraction procedures, rather than the PCR amplification. This was also the case for EBV and BKV DNA determinations, where as for CMV DNA, no false positive results were obtained for any samples. In the present study, the best results (lower variability) were obtained with the EBV DNA panel. However, no direct comparison can be made with the previous EBV study [10] due to the different composition of the panels used for the evaluation. For BKV DNA, no cross reactivity was observed for samples positive for the other Poliomavirus (JCV), included in the same QCMD panel and, although this represents the first study by our group on quantification of this virus, an acceptable level of variability was achieved. International standards are available since 2011 for CMV DNA and 2012 for EBV DNA [12, 13]; this represents an opportunity to improve harmonization in CMV and EBV genome quantification.

In conclusion, the results of this multicentre study indicate that CMV, EBV and BKV DNAemia are quantified with acceptable variability using a variety of extraction volumes and protocols with different commercial and in-house molecular protocols.

Competing interests

None declared.

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Ethical approval

None. All experiments were performed with samples made available by QCMD 2012.

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Appendix A. AMCLI-Infections in the Transplant Working Group (GLaIT) - list of other participants.

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Figure Legend

Figure 1. Bland-Altman plots are used to describe the Log difference between the GLaIT laboratory results and QCMD consensus values. A (CMV plasma panel), B (CMV WB panel), C (EBV panel) and D (BKV panel).

Table 1. Methods for viral DNA extraction and quantification.

Virus target	Center #	Nucleic acid extraction		Input volume (µl)	Output volume (µl)	Amplification method	Real-time PCR instrument
		Instrument	Protocol				
CMV	1	QIA Symphony	DSP virus/pathogen (modified)	400 (200 for WB)	90 (90 for WB)	CMV Trender Affigene	Stratagene xp3000
	2	NucliSENS EasyMag	generic 2.0.1	250 (100 for WB)	25 (25 for WB)	CMV Alert Real-Time, ELITechGroup	ABI Prism 7300
	3	QIA Symphony	DSP virus/pathogen (modified)	1000	110	CMV ELITe MGB Kit, ELITechGroup	ABI Prism 7300
	4	NucliSENS EasyMag	generic 2.0.1 and specific 2.0 for WB	500 (200 for WB)	55 (55 for WB)	in house PCR (target US8)[14]	ABI Prism 7300
	5	NucliSENS EasyMag	generic 2.0.1	100	50	CMV ELITe MGB Kit, ELITechGroup	ABI Prism 7300
	6	x-tractor gene UV light	Helix DNA Corbet	400	60	CMV Alert Real-Time, ELITechGroup	ABI Prism 7300
				200 for WB	150 for WB	CMV ELITe MGB Kit, ELITechGroup	
	7	NucliSENS EasyMag	generic 2.0.1	500	50	CMV ELITe MGB Kit, ELITechGroup	ABI Prism 7500
		QIA Symphony	blood 200V6 for WB	200 for WB	200 for WB		
	8	NucliSENS EasyMag	generic 2.0.1	400 (200 for WB)	60 (85 for WB)	CMV Alert Real-Time, ELITechGroup	ABI Prism 7300
	9	NucliSENS EasyMag	generic 2.0.1	1000 (200 for WB)	25 (55 for WB	CMV Alert Real-Time, ELITechGroup	ABI Prism 7300
	10	QIA Symphony	DSP virus/pathogen and DSP DNA for WB	500 (200 for WB)	90 (90 for WB)	CMV ELITe MGB Kit, ELITechGroup	ABI Prism 7300
	11	QIA Symphony	DSP virus/pathogen and DSP DNA for WB	500 (200 for WB)	140 (90 for WB)	CMV ELITe MGB Kit, ELITechGroup	ABI Prism 7300
	12	NucliSENS EasyMag	specific 2.0 (modified for WB)	500 (100 for WB)	100 (50 for WB)	CMV ELITe MGB Kit, ELITechGroup	ABI Prism 7300
	13	NucliSENS EasyMag	generic 2.0.1	500 (100 for WB)	55 (55 for WB)	CMV Alert Real-Time, ELITechGroup	ABI Prism 7300
	14	NucliSENS EasyMag	generic 2.0.1	500 (200 for WB)	55 (55 for WB)	CMV Alert Real-Time, ELITechGroup	ABI Prism 7300
	15	Manual extraction	QIAamp blood mini kit	200	100	CMV r-gene Argene-Biomerieux	ABI Prism 7500
EBV	1	QIA Symphony	DSP virus/pathogen	400	90	EBV Trender Affigene	Stratagene xp3000
	2	NucliSENS EasyMag	generic 2.0.1	250	25	EBV Alert Real-Time, ELITechgroup	ABI Prism 7300
	3	NucliSENS EasyMag	generic 2.0.1	500	55	EBV ELITe MGB Kit, ELITechgroup	ABI Prism 7300
	4	NucliSENS EasyMag	generic 2.0.1	500	55	in house PCR (target EBNA-1)[15]	ABI Prism 7300
	5	NucliSENS EasyMag	generic 2.0.1	100	100	EBV ELITe MGB Kit, ELITechgroup	ABI Prism 7300
	6	x-tractor gene UV light	Helix DNA Corbet	400	60	EBV Alert Real-Time, ELITechgroup	ABI Prism 7300
	7	NucliSENS EasyMag	generic 2.0.1	500	50	EBV ELITe MGB Kit, ELITechgroup	ABI Prism 7500
	8	NucliSENS EasyMag	generic 2.0.1	400	60	EBV Alert Real-Time, ELITechgroup	ABI Prism 7300
	9	NucliSENS EasyMag	generic 2.0.1	1000	25	EBV Alert Real-Time, ELITechgroup	ABI Prism 7300
	10	NucliSENS EasyMag	generic 2.0.1	500	55	EBV ELITe MGB Kit, ELITechgroup	ABI Prism 7300
	11	NucliSENS EasyMag	generic 2.0.1	1000	60	EBV Alert Real-Time, ELITechgroup	ABI Prism 7300
	12	NucliSENS EasyMag	specific 2.0	500	100	EBV ELITe MGB Kit, ELITechgroup	ABI Prism 7300
	13	NucliSENS EasyMag	generic 2.0.1	500	55	EBV Alert Real-Time, ELITechgroup	ABI Prism 7300
	14	NucliSENS EasyMag	generic 2.0.1	500	55	EBV Alert Real-Time, ELITechgroup	ABI Prism 7300
	15	Manual extraction	QIAamp blood mini kit	200	100	EBV R-gene Argene-Biomerieux	ABI Prism 7500
BKV	1	QIA Symphony	DSP virus/pathogen (modified)	400	90	BKV Trender Affigene	Stratagene xp3000
	2	NucliSENS EasyMag	generic 2.0.1	250	25	BKV Alert Real-Time, ELITechgroup	ABI Prism 7300
	3	QIA Symphony	DSP virus/pathogen (modified)	1000	110	BKV ELITe MGB KIT, ELITechGroup	ABI Prism 7300
	4	NucliSENS EasyMag	generic 2.0.1	500	55	in house PCR (target large T region)[16]	ABI Prism 7300
	5	NucliSENS EasyMag	generic 2.0.1	1000	100	BKV ELITe MGB KIT, ELITechGroup	ABI Prism 7300
	6	x-tractor gene UV light	Helix DNA Corbet	400	60	BKV ELITe MGB KIT, ELITechGroup	ABI Prism 7300
	7	NucliSENS EasyMag	generic 2.0.1	500	50	BKV ELITe MGB KIT, ELITechGroup	ABI Prism 7500
	8	NucliSENS EasyMag	generic 2.0.1	400	60	Light mix Polyomaviruses JC and BK (TibMolBiol)	Lightcycler 2.0
	9	NucliSENS EasyMag	generic 2.0.1	1000	25	BKV Q-PCR Alert Kit, ELITechGroup	ABI Prism 7300
	10	NucliSENS EasyMag	generic 2.0.1	500	55	BKV ELITe MGB KIT, ELITechGroup	ABI Prism 7300
	11	NucliSENS EasyMag	generic 2.0.1	1000	60	BKV Alert Real-Time, ELITechgroup	ABI Prism 7300
	12	NucliSENS EasyMag	specific 2.0	500	100	BKV ELITe MGB KIT, ELITechGroup	ABI Prism 7300
	13	NucliSENS EasyMag	generic 2.0.1	500	55	BKV ELITe MGB KIT, ELITechGroup	ABI Prism 7300
	14	NucliSENS EasyMag	generic 2.0.1	500	55	BKV ELITe MGB KIT, ELITechGroup	ABI Prism 7300
	15	Manual extraction	QIAamp blood mini kit	200	100	JC primers - probe + BKV R-gene, Argene-Biomerieux	ABI Prism 7500

WB, whole blood

Table 2. Summary of quantitative performance for CMV plasma, CMV WB, EBV and BKV panels.

QCMV 2012 panel	Sample	No. of values	Mean ± SD (Log copies/ml)	Inter-lab. CV (%)	Median (Log copies/ml)	Range (Log copies/ml)
CMV plasma	CMV12-01	15	4.43±0.17	3.93	4.43	4.12-4.65
	CMV12-02	15	3.82±0.29	7.56	3.89	3.29-4.36
	CMV12-03	14	2.19±0.33	12.65	2.17	1.56-2.53
	CMV12-04	11	2.05±0.46	19.56	2.00	1.28-2.35
	CMV12-05	15	3.24±0.48	14.84	3.40	2.04-3.66
	CMV12-06	15	3.43±0.21	6.19	3.47	2.98-3.80
	CMV12-07	15	3.46±0.18	5.15	3.50	3.16-3.70
	CMV12-09	15	3.80±0.21	5.60	3.83	3.29-4.14
	CMV12-10	15	2.74±0.26	9.30	2.72	2.18-3.14
CMV WB	CMV12-01	13	3.82±0.59	15.33	3.78	2.78-4.56
	CMV12-02	13	4.83±0.57	11.90	4.96	3.89-5.50
	CMV12-03	13	3.01±0.43	13.95	3.09	2.32-3.65
	CMV12-04	13	3.85±0.54	14.06	3.82	3.15-4.51
	CMV12-05	13	3.03±0.41	13.03	3.08	2.38-3.54
	CMV12-07	13	4.55±0.50	10.91	4.44	3.84-5.17
	CMV12-08	9	2.63±0.44	16.57	2.73	1.81-3.05
EBV	EBV12-01	15	2.52±0.30	11.92	2.56	1.97-3.16
	EBV12-02	15	3.57±0.24	6.89	3.59	3.22-4.20
	EBV12-03	15	4.93±0.25	5.12	4.87	4.59-5.53
	EBV12-04	15	4.55±0.23	5.17	4.53	4.26-5.12
	EBV12-05	15	4.12±0.23	5.61	4.11	3.80-4.68
	EBV12-06	15	4.26±0.21	5.10	4.20	3.98-4.77
	EBV12-07	15	4.58±0.23	5.19	4.49	4.32-5.21
	EBV12-08	15	3.26±0.23	7.02	3.23	2.81-3.81
	EBV12-09	15	3.09±0.26	8.33	3.10	2.52-3.72
BKV	BK12-02	15	3.58±0.38	10.59	3.64	2.92-4.19
	BK12-03	15	2.43±0.34	14.54	2.43	1.81-2.90
	BK12-07	15	1.72±0.48	26.25	1.76	0.90-2.35
	BK12-08	15	4.64±0.39	8.51	4.66	3.81-5.26
	BK12-12	15	5.14±0.34	6.58	5.15	4.35-5.56