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Evaluation of four commercial extraction-quantification systems to monitor EBV or CMV viral load in whole blood

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ABSTRACT

Background: Measurement of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) viral loads is commonly used to monitor posttransplant patients. Two new systems (eMAG/eSTREAM and Versant/kPCR) have been recently commercialized.
Objectives: To evaluate the performance of four systems to quantify CMV and EBV in whole blood.
Study design: Three extraction and real-time PCR amplification systems: m2000SP/RT (M2000), eMAG/eSTREAM (EMAG), and Versant/kPCR (KPCR) were compared with our routine system Qiasymphony/RGQ (QS/RGQ). The 4 systems were tested using 3 dilutions in triplicate according to the WHO international standard (WHO-IS) for intra-assay reproducibility; 56 whole blood samples (24 patients, 4 follow-ups) for CMV and 45 samples (27 patients, 3 follow-ups) for EBV.
Results: For CMV, the mean of the WHO-IS (expected value: 4.7 Log IU/ml) was: QS/RGQ = 4.84, M2000 = 4.61, EMAG = 4.33, and KPCR = 4.79. One patient (10 samples) presented a major underquantification by QS/RGQ. Of the 46 remaining samples, 41 were quantified with QS/RGQ, 43 with M2000, 33 with EMAG and 24 with KPCR. For EBV, the mean of the WHO-IS was: QS/RGQ = 4.70, M2000 = 4.61, EMAG = 4.62, and KPCR = 4.57. Among the 45 samples, 43 were quantified with QS/RGQ, 39 with M2000, 40 with EMAG and 32 with KPCR.
Conclusion: The results obtained with the WHO-IS were very good. The results of patients' samples were well correlated with the announced sensitivity of each system. The elevated threshold of the KPCR CMV assay may be problematic for the follow-up of highly immunocompromised patients who require early introduction of treatment.

1. Background

Quantitative nucleic acid testing (QNAT) has become indispensable to measure viral load (VL) in the management of cytomegalovirus (CMV) infection. Indications include CMV infection during pregnancy and in newborns [1], viral surveillance of all immunocompromised patients [2] and follow-up of treatment efficacy. The use of Epstein-Barr Virus (EBV) VLs is more recent and allows the diagnosis, monitoring, and prevention of post-transplant lymphoproliferative disorders (PTLD) [3].

To respond to the need for QNAT, commercial assays have been developed on automated systems. The first reagents for CMV and EBV VLs in whole blood were Abbott RealTime kits on the m2000 SP/RT system (Abbott Molecular Inc, Des plaines, USA) (M2000) [4,5] and Artus QS-RGQ kits on the QIAsymphony RGQ system (Qiagen S.A.S., France) (QS/RGQ) [6]. Recently two new systems have been commercialized: i) the eMAG/eSTREAM system (Biomerieux, Marcy-l'Etoile, France) (EMAG) coupled with R-gene kits whose performance seems equivalent to the previous Nuclisens Easy Mag system [7] and ii) the Versant kPCR system (Siemens Healthcare Diagnostics, Saint Denis, France) with kPCR PLX kits (KPCR) [8]. A comparison of these systems is necessary to evaluate their performances.

2. Objectives

The objective of this single center study was to compare the results obtained with our routine system QS/RGQ and three other systems: M2000, EMAG and KPCR, to monitor CMV and EBV VL in whole blood. We assessed analytical performances using World Health Organization International Standards (WHO-IS) and clinical performances on samples with qualitative and quantitative analyses.

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3. Study design

3.1. Analytical assessment

3.1.1. Specificity and Inter-sample contamination

Inter-sample contamination was assessed by testing 10 negative clinical samples in runs with positive samples for each extraction system for both CMV and EBV.

3.1.2. Intra-assay reproducibility and accuracy

Intra-assay reproducibility was tested with an in-house Internal Quality Control (IQC) (corresponding to a sample from a positive patient). For CMV, IQC was tested 9 times in the same run and for EBV 8 times.

Accuracy was tested with WHO-IS (code: 09/162 for CMV and 09/260 for EBV; NIBSC, Hertfordshire, Great Britain). Four dilutions (range 500 – 5×10^5 IU/ml, 2.7 – 5.7 Log IU/ml) of each WHO-IS were quantified in triplicate in the same run.

3.2. Clinical performances

Clinical performances were evaluated using blood samples collected on EDTA from hospitalized patients. Whole blood samples were tested with the QS/RGQ system (our routine system to monitor CMV or EBV VL) and frozen at −70 °C. For analysis on the M2000, EMAG and KPCR systems, 56 samples (conserved less than 6 months) were selected from 24 patients for CMV and 45 samples from 27 patients for EBV. Among these samples, we monitored the VL of 4 patients for CMV and of 3 patients for EBV.

3.3. Methods

All 4 assays were marked EC-IVD (European Conformity-In Vitro Diagnostic device) for whole blood except EBV with KPCR. In each sample, an internal control was included allowing the detection of potential inhibition. For QS/RGQ, M2000, and KPCR, the extraction automatons distributed the mix and the eluate in the Polymerase Chain Reaction (PCR) plate. For EMAG this step was performed by another automaton, the eSTREAM system. Results were calculated in IU/mL and Log10 IU/mL.

3.3.1. QIAsymphony RGQ system (Qiagen)

Whole blood samples (200 µl) were dispensed onto the QIAsymphony SP/AS automaton. This system performs all steps of the purification procedure and preparation of 60 µL of eluate with the extraction kit QIAsymphony DNA mini and the mix for DNA amplification (*artus* CMV QS-RGQ and *artus* EBV QS-RGQ). Then, the thermocycler Rotorgene enabled amplification of 72 samples. The linearity was between 2.9 and 7.6 Log IU/ml for CMV and between 2.2 and 6.8 Log IU/ml for EBV. The lower limit of detection (LOD) was 123 IU/mL (2.1 Log IU/ml) for CMV and 40 IU/mL (1.6 Log IU/ml) for EBV (Table 1).

3.3.2. m2000SP/RT system (Abbott)

Quantification was carried out on the m2000 system which includes the m2000SP instrument for automated extraction of DNA from 500 µl of sample using the *m*Sample preparation system DNA kit (eluate volume: 60 µl). Amplification was performed on the thermocycler m2000RT with Realtime CMV and EBV kits. The lower limit of quantification (LLQ) was 62.4 IU/mL (1.8 Log IU/ml) for CMV and 150 IU/mL (2.2 Log IU/ml) for EBV. The linearity extended from the LLQ to 8.2 Log IU/ml for CMV and 8.3 Log IU/ml for EBV.

3.3.3. eMAG/eSTREAM system (Biomerieux)

200 µL of sample were used. After extraction executed with a DNA extraction kit onto the eMAG system, 50 µl of eluate were obtained. Distribution was performed by the eSTREAM automaton using CMV R-gene and EBV R-gene quantification kits. Amplification was performed on an Abi Prism 7500 fast (Applied Biosystems, Foster City, USA). LOD was 535 IU/mL (2.7 Log IU/ml) for CMV and 328 IU/mL (2.5 Log IU/ml) for EBV. The linearity extended from the LLQ 2.8 to 9.4 Log IU/mL for CMV and to 3.0 to 6.7 Log IU/ml for EBV.

3.3.4. Versant kPCR system (Siemens)

Extraction was performed with Versant Sample Preparation 1.2 Reagents kit using 400 µL of sample. Quantification was carried out on the Versant kPCR Molecular System which includes the SP module for extraction and the AD module for real-time PCR. DNA amplification was performed with the CMV and EBV kPCR PLX DNA Assays (Altona Diagnostics, Hamburg, Germany). LOD was 835 IU/mL (2.9 Log IU/ml) for CMV in whole blood and 216.6 UI/mL (2.3 Log IU/ml) for EBV in plasma (not available because not validated in whole blood). The linearity extended from LOD to 6 Log IU/ml for CMV in whole blood and from 2.7 to 6 Log IU/ml for EBV in plasma.

3.4. Statistical analysis

Bland and Altman plots were used to represent the degree of agreement between the Log10-transformed VLs obtained with the reference system and the three other systems using MedCalc Statistical Software version 17.6 (MedCalc Software, Ostend, Belgium).

4. Results

4.1. Analytical performances

4.1.1. Specificity and inter-sample contamination

The 10 negative samples tested on each system for CMV and EBV were all undetected and no inter-sample contamination was observed with the 4 systems.

4.1.2. Intra-assay reproducibility and accuracy

Standard deviations (SD) were: i) for CMV, QS/RGQ = 0.04; M2000 = 0.07; EMAG = 0.12 and KPCR = 0.06 with a coefficient of variation from 1.09 to 2.65% and ii) for EBV, QS/RGQ = 0.14; M2000 = 0.15; EMAG = 0.29 and KPCR = 0.20 with a coefficient of

Table 1
Limit of detection (LOD), lower limit of quantification (LLQ) and target genes for each assay.

	CMV				EBV			
	QS/RGQ	M2000	EMAG	KPCR	QS/RGQ	M2000	EMAG	KPCR
LOD Log IU/ml	2.1	1.8	2.7	2.9	1.6	2.2	2.5	NA
LLQ Log IU/ml	2.9	1.8	2.8	2.9	2.2	2.2	3.0	NA
Target genes	ND	UL34 and UL80.5	ppUL83	ND	ND	BLLF1	BXLF1	ND

NA: Not available ND: information kept confidential by the manufacturer.

Table 2
Results of intra-assay reproducibility for CMV (N = 9) and EBV (N = 8).

	System	Mean concentration (IU/ml)	Mean concentration (Log IU/ml)	Standard deviation (Log IU/ml)	Coefficient of variation (%)
CMV	QS/RGQ	91203	4.96	0.04	1.09
	M2000	115267	5.06	0.07	1.38
	EMAG	35392	4.53	0.12	2.65
	KPCR	61593	4.79	0.06	1.18
EBV	QS/RGQ	2569	3.39	0.14	4.13
	M2000	1041	3.00	0.15	5.00
	EMAG	3002	3.41	0.29	8.50
	KPCR	1921	3.25	0.20	6.15

variation from 4.13 to 8.50% (Table 2). SD were below 0.25 Log IU/ml for all the assays except for EBV EMAG (SD = 0.29).

4.1.3. Performances on WHO international standard

In contrast with the other assays, the KPCR system detected only 2 of the 3 replicates for CMV and 1 of the 3 for EBV for the lowest dilution (Table 3). Two points were answered "inhibited" by EMAG. Due to a higher dispersion of the values mainly for KPCR and EMAG, this dilution was excluded from the calculation of the mean. For an expected value of 4.70 Log IU/ml the mean values of WHO-IS CMV were QS/RGQ = 4.84, M2000 = 4.61, EMAG = 4.33, KPCR = 4.79 and of WHO-IS EBV were QS/RGQ = 4.70, M2000 = 4.61, EMAG = 4.62, KPCR = 4.57.

4.2. Performances on clinical samples

4.2.1. A patient with discrepant results

Ten of the 56 samples of whole blood tested with CMV assays were from Patient 1. This patient's samples were repetitively underquantified with QS/RGQ with a mean of 1.62 Log IU/ml for these 10 measures, whereas M2000 = 2.77, EMAG = 2.59 and KPCR = 3.33. These samples were excluded from further analyses.

4.2.2. Qualitative analysis

Comparisons were performed using 46 CMV samples and 45 EBV samples (Table 4). For CMV, quantifiable results, above the threshold announced by the manufacturers, were found for QS/RGQ n = 41, M2000 n = 43, EMAG n = 33 and KPCR n = 24. Samples were undetectable for QS/RGQ n = 1 (238 IU/ml with M2000, 404 IU/ml with EMAG), M2000 n = 3 (24, 27 and 2412 IU/ml with QS/RGQ, negative or invalid with the others), EMAG n = 5 (VL maxi = 505 IU/ml with QS/RGQ), and KPCR n = 14 (VL maxi = 2130 IU/ml with QS/RGQ). For EBV, quantifiable results were found for QS/RGQ n = 43, M2000 n = 39, EMAG n = 40 and KPCR n = 32. Samples were undetectable for M2000 n = 2 (77 and 759 IU/ml with QS/RGQ), EMAG n = 4 (VL maxi = 759 IU/ml), and KPCR n = 13 (VL maxi = 9438 IU/ml). Two samples of 77 and 759 IU/ml were undetectable in all assays except QS/RGQ.

4.2.3. Quantitative analysis

The samples quantifiable by 2 assays were plotted for Bland and Altman representation (Fig. 1). One sample with very discordant results was excluded: CMV VL of 5.15 Log IU/ml with KPCR and 2.97, 3.19 and 3.89 Log IU/ml with QS/RGQ, M2000 and EMAG respectively. Between QS/RGQ and M2000 the differences in mean were -0.06 Log IU/ml with SD = 0.58 for CMV and 0.16 Log IU/ml with SD = 0.57 for EBV (Fig. 1A, 1D). Three deviations greater than 0.5 Log IU/ml were observed for CMV and 5 for EBV. Between QS/RGQ and EMAG the differences in mean were -0.27 Log IU/ml with SD = 0.57 for CMV and -0.27 Log IU/ml with SD = 0.67 for EBV (Fig. 1B, 1E). Seven deviations greater than 0.5 Log IU/ml were observed for CMV and 10 for EBV. Between QS/RGQ and KPCR the differences in mean were 0.01 Log IU/ml with SD = 0.83 for CMV and 0.52 Log IU/ml with SD = 1.45 for EBV (Fig. 1C, 1 F). Six deviations greater than 0.5 Log IU/ml were observed for CMV and 22 for EBV.

4.2.4. Comparison of the four assays for patient monitoring

The clinical performances of the 4 assays were evaluated following the kinetics of VL in 4 patients for CMV and 3 for EBV (Fig. 2). The CMV VLs of patient 1 were always underquantified with QS/RGQ. Patient 1 also replicated EBV but with QS/RGQ VLs similar to the other systems. In patients 2 and 4, the first CMV VL was not detected with KPCR. Samples of patient 5 became undetectable for EBV after 2 VLs with KPCR, after 3 VLs with EMAG whereas they stayed detected with M2000 and QS/RGQ. Patient 6 was underquantified by KPCR with 3 positive EBV VLs with a mean of 2.4, whereas the mean of the same VLs was 3.7 Log IU/ml with QS/RGQ.

5. Discussion

5.1. Technical features that matter

To evaluate automated systems it is important to take into account their simplicity, flexibility and ergonomics. In the QS/RGQ system, Qiasymphony SP/AS automaton works in series from 1 to 24 samples but uses two different kits to extract DNA from whole blood and acellular samples. The Rotorgene thermocycler uses small strips or tubes

Table 3
Values in triplicate obtained with the four systems on dilutions of WHO-IS CMV and EBV.

Viral Load Log IU/ml	Expected Value WHO IS	CMV				EBV			
		QS/RGQ	M2000	EMAG	KPCR	QS/RGQ	M2000	EMAG	KPCR
STD 1	2.70	2.73	2.76	1.91	ND	3.02	3.02	3.03	2.46
		2.99	2.70	2.63	2.72	2.94	2.90	2.88	ND
		2.56	2.80	1.64	2.91	3.02	3.09	IN	ND
STD 2	3.70	3.82	3.65	3.55	3.71	3.73	3.88	3.67	3.26
		3.77	3.64	3.33	3.77	3.76	3.88	3.89	3.41
		3.79	3.62	3.37	3.82	3.73	3.86	3.64	3.46
STD 3	4.70	4.82	4.63	4.31	4.74	4.70	4.87	4.42	4.76
		4.85	4.59	4.32	4.77	4.68	4.89	4.44	4.74
		4.82	4.59	IN	4.83	4.73	4.86	4.80	4.72
STD 4	5.70	5.90	5.55	5.26	5.80	5.61	5.92	IN	5.68
		5.89	5.58	5.25	5.84	5.64	5.91	5.23	5.50
		5.90	5.60	5.24	5.84	5.67	5.94	5.70	5.61
Mean	4.70	4.84	4.61	4.33	4.79	4.70	4.61	4.62	4.57

ND = not detected IN = inhibited STD = Standard
Values in the gray zone have been excluded from the mean calculation. Values in bold deviate by more than 0.25 Log from the reference value.

Table 4
Qualitative analysis of results obtained on 46 CMV samples and 45 EBV samples.

	CMV				EBV			
	QS/RGQ	M2000	EMAG	KPCR	QS/RGQ	M2000	EMAG	KPCR
LLQ	123 IU/ml	62.4 IU/ml	455 IU/ml	835 IU/ml	40 IU/ml	150 IU/ml	349 IU/ml	NA
Undetectable	1	3	5	14	0	2	4	13
Quantifiable	41	43	33	24	43	39	40	32
Detected	4	0	5	5	2	4	0	0
Invalid or inhibited	0	0	3	3	0	0	1	0

NA = not available.

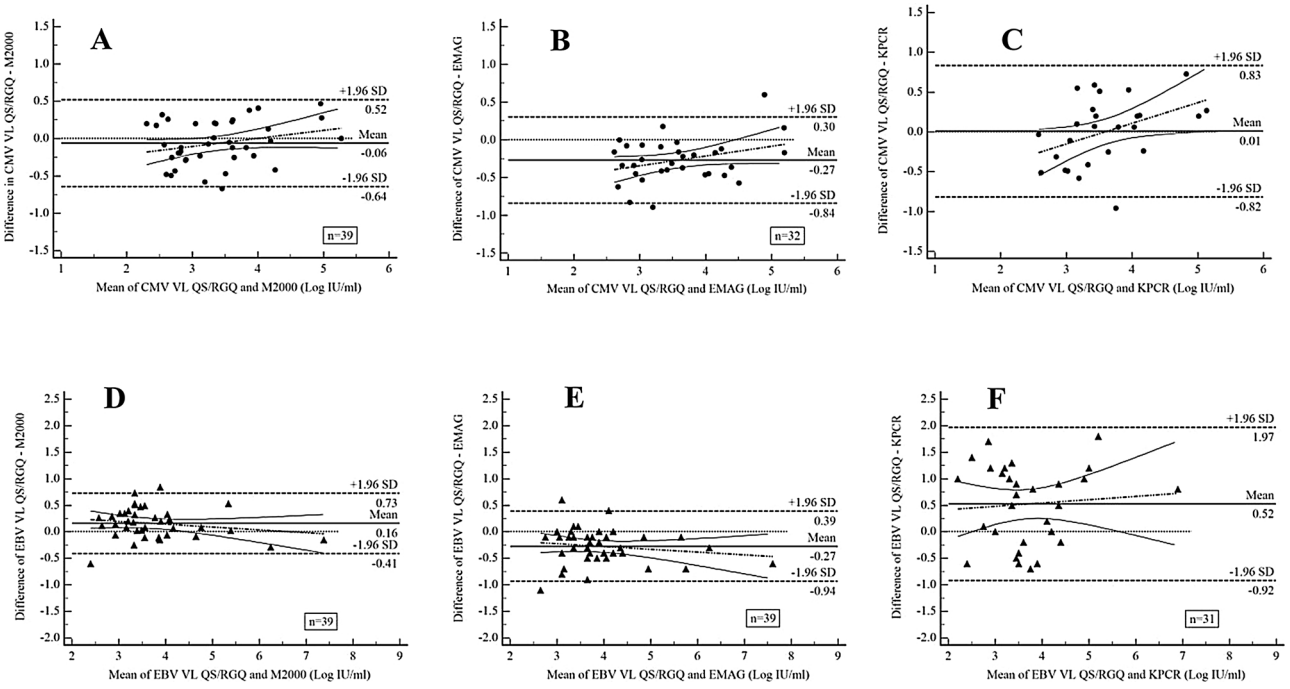

Fig. 1. Degree of agreement in Log IU/ml between the different CMV VL (●) and EBV VL (▲) assays. (A) and (D) between QS/RGQ and M2000, (B) and (E) between QS/RGQ and EMAG, (C) and (F) between QS/RGQ and KPCR. For Bland and Altman curves, the mean values for each sample quantifiable by the 2 techniques are plotted on the x axis. The differences between the values obtained by the 2 techniques are plotted on the y axis. The solid lines show the differences in mean between the values, and the dotted lines show the differences in mean plus or minus 1.96 SD (95% limits of agreement).

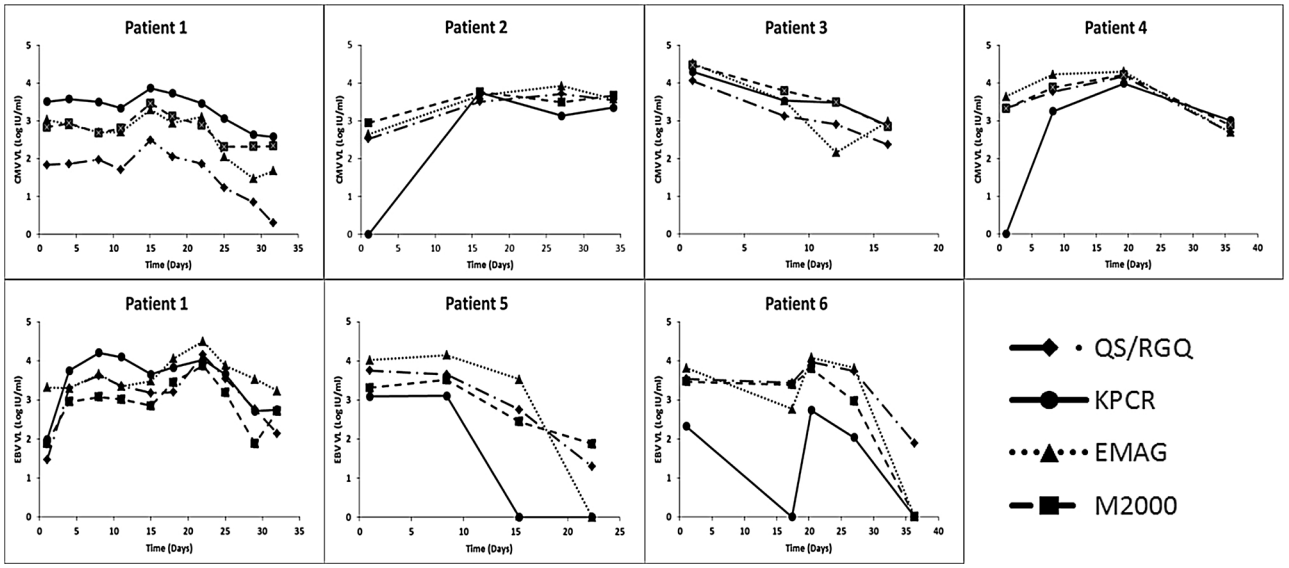

Fig. 2. Follow-up of VL in patients with at least 4 measurements in the 4 assays (◆ QS/RGQ,● KPCR,▲ EMAG, ■ M2000). Patients 1 to 4 were followed for CMV and Patients 1, 5 and 6 for EBV.

which require some dexterity to close and handle. The Abbott system m2000 SP/RT is a robust and well-known system with a capacity of 96 tests/runs also used in many laboratories to quantify HIV, HBV and HCV. M2000 can be used to extract DNA from a variety of samples therefore without validation of Abbott. The main complaint of M2000 is the duration of the extraction step which is the longest of the four systems (more than 3 hours). The Biomerieux eMAG extractor has been validated for most types of samples using only one extraction kit (serum, CSFs, BALs, urine, biopsies and amniotic fluids); it allows extraction of 1 to 48 samples in consumables of eight wells but does not distribute, mix and extract in the PCR tubes (step ensured by eSTREAM). The Siemens system, Versant kPCR, is probably the easiest and fastest system to use despite a dilution step for all the blood samples before extraction. Particular attention should be paid to the preparation of reagents to avoid mistakes with this system.

5.2. Analytical performances

No problem of specificity or contamination was observed with the four systems. The use of a dilution of 500 IU/ml of WHO-IS allows evaluation of the announced sensitivity of the assays. KPCR assays are less sensitive than the others particularly for EBV with two replicates out of three not detected. The concordance of the results with the expected values of WHO-IS was very good for the four EBV assays (maximum discrepancy = 0.13 Log IU/ml) and for three of the four CMV assays with a slightly low mean for EMAG (discrepancy of 0.37 Log IU/ml). These results confirm those obtained in other studies [9,10] and are very good news because in some pathologies there is a major difficulty in determining a specific threshold value. For example, the ECIL-6 (Sixth European Conference on Infections in Leukemia) cannot recommend a threshold of EBV DNAemia to give preemptive therapy in patients with a high risk for EBV-PTLD because of the large variety of threshold values used by the authors [11]. However, in order to establish these thresholds, it will be necessary to use data obtained on the same matrix (plasma or whole blood) because differences between these two matrices have been observed [12].

These results comparing four systems pave the way for future studies comparing results between different laboratories and allowing the determination of universal thresholds for each type of pathology to better manage CMV or EBV infections.

5.3. Clinical performance

CMV and EBV are known to have lower genetic variability than others viruses like HIV. The possibility of underquantification because of mismatch of the primers is rare but has already been described for CMV [13]. We found one patient repetitively underestimated by QS/RGQ CMV system. The major clinical risk is not detecting a low CMV VL under antiviral treatment and stopping the treatment too early. Indeed, many patients have recurrent CMV viremia and/or CMV disease following initial therapy [2] and the consensus guidelines on the management of CMV in solid-organ transplantation recommend two consecutive undetectable VLs to ensure viral clearance before stopping treatment [14]. One option for dealing with this problem is the use of dual target PCRs but only one assay (the M2000) amplifies two targets.

On clinical samples, the results were well correlated with the sensitivity announced by the manufacturers of the assays with the greatest number of CMV samples quantifiable with M2000 (Threshold: 62.4 UI/ml) and the lowest obtained with KPCR (Threshold: 835 UI/ml). It should be noted that this elevated threshold for KPCR can be problematic for the follow up of high risk patients receiving T-cell depleted or umbilical cord blood grafts, indeed in these cases it is common to treat when CMV viremia reaches 100 copies/mL to 1000 copies/mL [15]. For EBV the best sensitivity was with the QS/RGQ system.

A good correlation between the QS/RGQ and M2000 systems was observed with a small number of samples with more than 0.5 Log IU/ml of difference (7.3% of CMV and 12.8% of EBV). This percentage was higher between QS/RGQ and EMAG and reached 71% between QS/RGQ and KPCR for EBV.

Results of sequential samples confirm our previous results of sensitivity with no detection of the majority of samples with KPCR. The sensitivity issues of the KPCR system are probably explained by less efficient extraction in whole blood than on the other matrices. Indeed, during an evaluation integrating different types of matrices Engelmann et al [8] found lower performance in whole blood for CMV VL confirmed by our findings.

To conclude, each laboratory should select a system based on the importance given to the sensitivity and the types of usable matrices. The four systems assessed here provide concordant results compatible with routine monitoring of CMV DNA in whole blood. For EBV if we exclude KPCR which was not validated in this matrix, the three other techniques were also concordant.

CRediT authorship contribution statement

Adeline Baron: Formal analysis, Writing - original draft. **Albane Gicquel:** Investigation, Validation. **Jean-Christophe Plantier:** Supervision. **Marie Gueudin:** Conceptualization, Methodology, Software, Writing - review & editing.

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