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Multicenter Evaluation of Whole-Blood Epstein-Barr Viral Load Standardization Using the WHO International Standard

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The first WHO international standard for Epstein-Barr virus (EBV) (WHO EBV standard) for nucleic acid amplification technology (NAT)-based assays was commercialized in January 2012 by the National Institute for Biological Standards and Control. In the study reported here, we compared whole-blood EBV DNA load (EDL) results from 12 French laboratories for seven samples (Quality Controls for Molecular Diagnostics 2013 proficiency panel) in order to determine whether expression in international units reduces interlaboratory variability in whole-blood EDLs. Each testing laboratory used a conversion factor to convert EDL results from copies per milliliter to international units per milliliter. This conversion factor was calculated from the WHO EBV standard according to the protocol described in this study (nine laboratories) or the recommendations of the PCR kit suppliers (three laboratories). The interlaboratory variability in whole-blood EDL results was reduced after standardization of the results using the WHO EBV standard. For the seven samples tested, standard deviations (SD) ranged from 0.41 to 0.55 when the results were expressed in log copies per milliliter, whereas the SD ranged from 0.17 to 0.32 when results were given in log international units per milliliter. Comparing the variance data (*F* test), we showed that the dispersion of whole-blood EDL results was significantly lower when they were expressed in log international units per milliliter ($P < 0.001$ for six of seven samples and $P < 0.05$ for one sample with a low mean EDL of 2.62 log IU/ml). This study showed that the use of the WHO EBV standard could improve the homogeneity of whole-blood EDL results between laboratories as well as the monitoring of patients at high risk of posttransplant lymphoproliferative disorders or other EBV-associated diseases.

Primary Epstein-Barr virus (EBV) infection is the cause of the vast majority of cases of infectious mononucleosis and the subsequent lifelong persistence of EBV in the host, which, although mostly asymptomatic, can lead to the development of several lymphoid and epithelial cancers in immunosuppressed and immunocompetent individuals (1, 2).

With the rapid development of real-time quantitative PCR, the measurement of EBV DNA load (EDL) during these EBV-associated diseases has been largely implemented in clinical practice (3–5). The monitoring of EDL in blood is required for transplant recipients at risk of posttransplantation lymphoproliferative disorders (PTLDs) and could also be a surrogate marker for the adjustment of the immunosuppressive regimen in these patients (6, 7). EDL measurement in plasma also appears to be a useful biomarker for the management of EBV-associated undifferentiated nasopharyngeal carcinoma (8). Although less clearly demonstrated, EDL measurements could also be helpful in other clinical situations such as severe or atypical infectious mononucleosis and other EBV-associated malignancies in immunosuppressed or immunocompetent patients (3, 9).

Besides the debates on the clinical utility and clinically relevant EBV DNA levels for various EBV-associated diseases, technical standardization of EDLs has not yet been entirely achieved (10). A wide range of methodologies to quantify EDLs are used in laboratories. Given this heterogeneity, comparison of EDL results between different laboratories is difficult (11–14). Interlaboratory variability in EDL testing should be reduced to establish consensus therapeutic or interventional thresholds between different medical centers for the management of PTLDs.

The first WHO international standard for Epstein-Barr virus (WHO EBV standard), intended to be used for the standardization of nucleic acid amplification technology (NAT)-based assays for EBV, was commercialized in January 2012 by the National Institute for Biological Standards and Control (NIBSC) (Hertfordshire, United Kingdom) (15).

Recently, the commutability of the WHO EBV standard has been shown for two quantitative PCR methods and plasma specimens (16). One study including only four laboratories described a multicenter comparison of plasma EDL results using the WHO EBV standard as an external control but not as a calibrator (17). To our knowledge, a study evaluating the impact of the EBV WHO standard on the variability of EDL results has not yet been conducted with whole-blood specimens. In this study, (i) we propose a method to establish a conversion factor to convert whole-blood EDL results obtained with a

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TABLE 1 Methods used by 12 French laboratories for EBV DNA load measurement^a

Test	Manufacturer, extraction platform	Manufacturer, qPCR kit	Target	Manufacturer, qPCR platform	Conversion factor (protocol)
1	bioMérieux, EasyMag	bioMérieux, R-Gene	BXLF1	Applied Biosystems, ABI7500	1.57 (study design)
2	Roche, MagNaPure	bioMérieux, R-Gene	BXLF1	Roche, LC480	0.97 (study design)
3	bioMérieux, EasyMag	bioMérieux, R-Gene	BXLF1	Roche, LC480	2.09 (study design)
4	Qiagen, QIAAsymphony	bioMérieux, R-Gene	BXLF1	Applied Biosystems, ABI7500	0.46 (study design)
5	bioMérieux, EasyMag	bioMérieux, R-Gene	BXLF1	Qiagen, Rotorgene Q	0.31 (study design)
6	Abbott, m2000sp	Abbott, EBV PCR kit	EBNA	Abbott, m2000r	0.94 (study design)
7	Qiagen, QIAAsymphony	Qiagen, Artus	EBNA1	Qiagen, Rotorgene Q	0.14 (kit supplier)
8	Roche, MagNaPure Compact	Lab developed	BXLF1	Roche, LC480	2.05 (study design)
9	Qiagen, QIAxtractor	bioMérieux, R-Gene	BXLF1	Roche, LC480	0.952 (kit supplier)
10	Roche, MagNaPure	Roche, TibMolBiol	EBNA	Roche, LC480	0.71 (study design)
11	Qiagen, QIAAsymphony	Qiagen, Artus	EBNA1	Qiagen, Rotorgene Q	0.14 (kit supplier)
12	Qiagen, QIAAsymphony	Lab developed	BNRF1	Qiagen, Rotorgene Q	0.4 (study design)
13	Abbott, m2000sp	Abbott, EBV PCR kit	EBNA	Abbott, m2000r	0.91 (study design)
14	Abbott, m2000sp	Abbott, EBV PCR kit	EBNA	Abbott, m2000r	0.38 (study design)
15	bioMérieux, EasyMag	bioMérieux, R-Gene	BXLF1	Qiagen, Rotorgene Q	0.77 (study design)

^a Numbers in boldface type, italics, and underlining correspond to tests done in the same laboratory. Manufacturers included bioMérieux (Marcy l'Etoile, France), Roche (Meylan, France), Qiagen (Courtaboeuf, France), Abbott (Rungis, France), and Applied Biosystems (Cergy-Pontoise, France). R-Gene indicates the EBV R-Gene quantification kit, Artus indicates the EBV RG PCR kit, and TibMolBiol is a LightMix kit for the detection of Epstein-Barr virus. For the study design, the conversion factor was measured in each laboratory as recommended in Materials and Methods.

quantitative PCR (qPCR) platform (copies per milliliter) into international units per milliliter; (ii) we compared EDL results obtained from 12 French laboratories for the Quality Controls for Molecular Diagnostics whole-blood proficiency panel

(QCMD 2013), where each testing laboratory provided whole-blood EDL results in both log international units per milliliter and log copies per milliliter; and (iii) we showed that interlaboratory variability in whole-blood EDL testing could be reduced

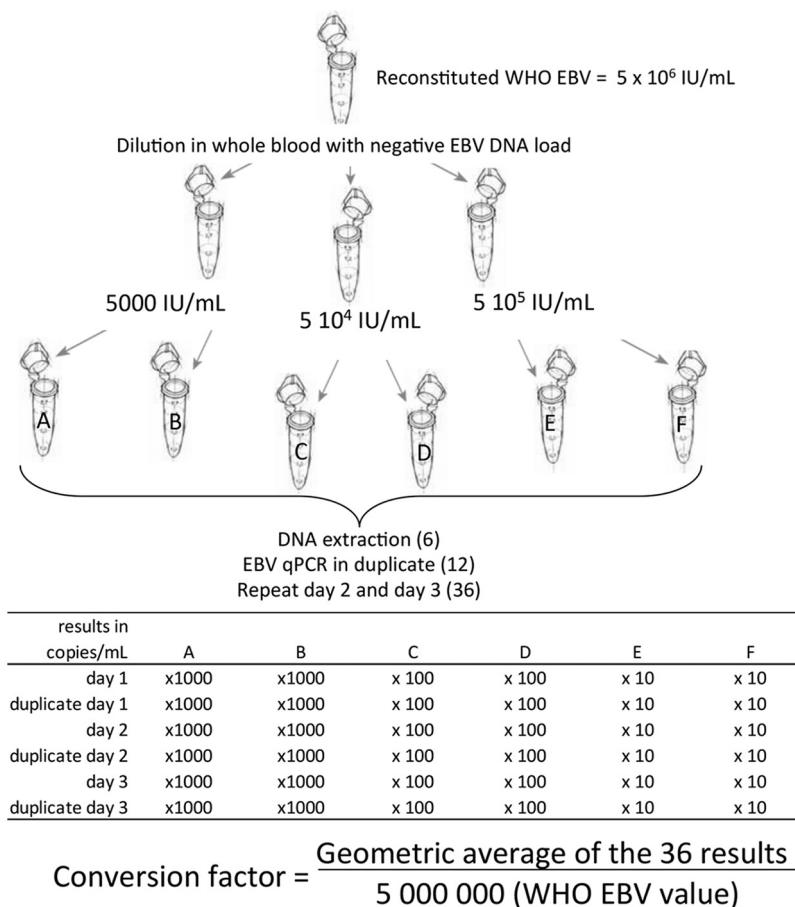


FIG 1 Protocol for determination of the conversion factor for conversion of expression of results in copies per milliliter to expression in international units per milliliter.

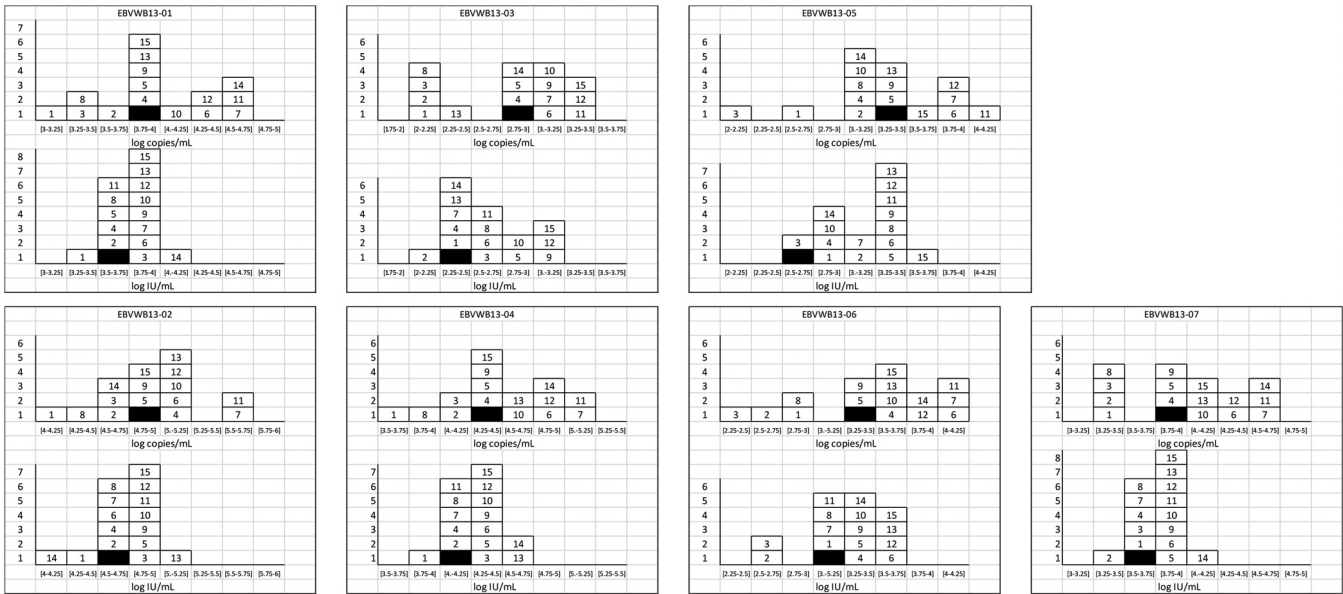


FIG 2 EBV DNA loads in log copies per milliliter or in log international units per milliliter of seven whole-blood samples from the QCMD 2013 Epstein-Barr virus whole-blood EQA pilot study (EBVWB13) measured in 12 French laboratories. Each box represents the results from one laboratory with its code number. The black box represents the expected values from QCMD.

by the use of the WHO EBV standard and the expression of results in international units.

MATERIALS AND METHODS

Study design and samples. This multicenter study included 12 French university hospital laboratories. Nine laboratories returned one panel of results. Three laboratories using two different methodologies for EDL quantification returned two panels of results. The 15 panels of results and the characteristics of the methods used by each testing laboratory are shown in Table 1 (see Fig. 2).

The QCMD 2013 Epstein-Barr virus whole-blood external quality assessment pilot study (Glasgow, United Kingdom) is a proficiency panel from QCMD including seven whole-blood samples (samples EBVWB13-1 to EBVWB13-7) that were positive for EBV DNA. Samples EBVWB13-1 and -7 were duplicate samples and were a 10-fold dilution of sample EBVWB13-2. The expected results were given by QCMD and were calculated from the data returned by all participants in either interna-

tional units per milliliter (22 data sets) or copies per milliliter (77 data sets), once outliers had been removed.

Determination of a site-specific conversion factor using the WHO EBV standard. The first WHO international standard for Epstein-Barr virus (product code 09/260; NIBSC, Hertfordshire, United Kingdom) contains the lyophilized equivalent of 1 ml of EBV strain B95-8 (type 1) in 10 mM Tris buffer (pH 7.4), 0.5% human serum albumin, and 0.1% trehalose. After being reconstituted with 1 ml of deionized, nuclease-free, molecular-grade water, the reconstituted material was assigned a final concentration of 5,000,000 IU/ml.

The protocol for the determination of a conversion factor was previously proposed by Sophie Alain for the cytomegalovirus DNA load (available at the National Cytomegalovirus Reference Center website [see <http://cnr-cytomegalovirus.fr/>]). Each laboratory was requested to determine its own conversion factor, which would be specific for the method of EBV quantification used by the laboratory. The conversion factor is the ratio between the geometric mean from 36 separated EDL measurements of a range of dilutions in whole blood from the international standard and the given value of the WHO standard (Fig. 1). Briefly, the WHO EBV standard was diluted to 1/10 (500,000 IU/ml), 1/100 (50,000 IU/ml), and 1/1,000 (5,000 IU/ml) in whole-blood samples that previously tested negative for EBV by qPCR. These dilutions were aliquoted and tested in duplicate in three separate experiments. After checking that the relative standard deviation (SD) for each range of dilutions was congruent with the manufacturer's recommendations or with in-house assay validation criteria and after elimination of inappropriate values, the qPCR results were multiplied by their initial dilution to obtain 36 experimental WHO EBV values (Fig. 1). To obtain the results in international units per milliliter, the results obtained in copies per milliliter have to be multiplied by the conversion factor. Nine laboratories (12 assays) used the protocol recommended in this study to determine the conversion factor. For testing laboratories 7 and 11, the conversion factor provided by the kit supplier was established by a regression analysis of multiple dilution series of the WHO EBV standard compared against a reference method reporting results in international units per milliliter. Testing laboratory 9 calculated the conversion factor by using a method that was very similar to ours and recommended by the supplier. In this protocol, the WHO EBV standard was diluted 10-fold in whole blood, aliquoted, and tested in triplicate in four

TABLE 2 EBV DNA load values obtained from the 12 participating laboratories

Sample	EBV DNA load					
	Log copies/ml (<i>n</i> = 15) ^a			Log IU/ml (<i>n</i> = 15) ^a		
	Mean	SD	Range	Mean	SD	Range
EBVWB13-01	3.98	0.44	3.24–4.62	3.78	0.17	3.43–4.19
EBVWB13-02	4.91	0.43	4.09–5.64	4.72	0.24	4.10–5.02
EBVWB13-03	2.81	0.47	2.14–3.43	2.62	0.32	2.16–3.15
EBVWB13-04	4.47	0.41	3.70–5.06	4.28	0.17	3.90–4.55
EBVWB13-05	3.34	0.51	2.24–4.18	3.15	0.3	2.56–3.61
EBVWB13-06	3.46	0.55	2.35–4.10	3.28	0.32	2.59–3.70
EBVWB13-07	3.98	0.47	3.27–4.68	3.79	0.2	3.35–4.11

^a *n* = 15 EDL results for each of the seven samples tested (three laboratories tested the samples with two different methodologies). Comparison of variances (*F* test) between results in log copies per milliliter and log international units per milliliter showed significant differences for all samples (*P* < 0.001 except for sample EBVWB13-03 [*P* < 0.05]).

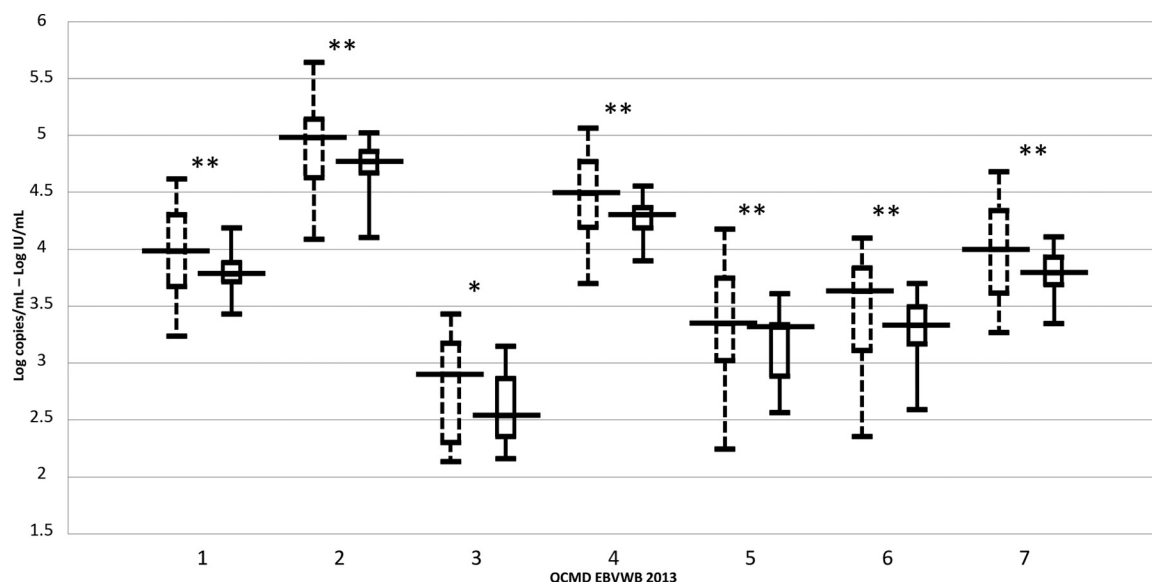


FIG 3 Distribution of the EBV DNA load in log copies per milliliter or in log international units per milliliter for seven whole-blood samples from the QCMD 2013 Epstein-Barr virus whole-blood EQA pilot study (EBVWB13) measured in 12 French laboratories. Large bars are medians, boxes are 25th and 75th percentiles, small bars are maxima and minima, dashed boxes indicate copies per milliliter, and solid boxes indicate international units per milliliter. *, $P < 0.05$; **, $P < 0.001$ (determined by difference of variances [F test]).

separate experiments (the conversion factor was the ratio between the arithmetic mean value from 12 EDL results and the value of the WHO EBV standard). In routine analysis, the conversion factor should be recalculated at least whenever the kit lot number changes and/or a new standard curve is made with the calibrators. Each qPCR run should be performed and validated according to the manufacturer's instructions (or in-house validation criteria) and according to the kit-specific (or home-specific) calibrators and controls.

Statistical analysis. Mean viral loads, variances, SD, minimum and maximum viral load values, and range (maximum minus minimum) were calculated for each sample. The differences of variances were tested by using the F test (Fisher-Snedecor test). The mean EDL values were compared by using the Wilcoxon signed-rank test. Statistical analyses were performed by using Statview 5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

The 12 laboratories participating in the study gave EDL results in both log copies per milliliter and log international units per milliliter for the seven whole-blood QCMD 2013 samples. Whatever method that was used for the calculation, all laboratories were able to obtain their own conversion factor to convert quantification results from copies per milliliter to international units per milliliter (Table 1). For the seven samples, the variability of EDL results was reduced when the laboratories expressed the results in log international units per milliliter compared to when the results were expressed in log copies per milliliter (Fig. 2). The SD ranged from 0.41 to 0.55 when the results were expressed in log copies per milliliter, whereas it ranged from 0.17 to 0.32 when the results were expressed in log international units per milliliter (Table 2). The means of EDL ranges were 1.53 and 0.89 when the results were expressed in log copies per milliliter and log international units per milliliter, respectively (Table 2). By analyzing the variances (F test of the equality of variances), we showed that the dispersions of EDL results were significantly different depending on the mode of expression in log copies per milliliter or log international units per milliliter (Fig. 3). The reduction of the variability was better for

samples with an EDL of >3.5 log IU/ml (samples EBVWB13-1, -2, -4, and -7) than for those with an EDL of <3.5 log IU/ml (EBVWB13-3, -5, and -6). Note that for sample EBVWB13-3, which had the lowest EDL (mean EDL = 2.62 log IU/ml), the difference in variances was at the limit of statistical significance ($P < 0.05$). The lower impact on variability for the samples with lower EDLs should not be considered a limitation to the use of expression in international units. Indeed, low EDLs are less often associated with EBV lymphoproliferative diseases (7). Figure 3 also shows that the median EDLs expressed in log international units per milliliter were lower than the medians expressed in log copies per milliliter (with a smaller difference for sample EBVWB13-5). Comparison of the two groups confirmed that the mean EDL values were statistically different for each of the seven samples tested ($P < 0.05$ by a Wilcoxon signed-rank test). The EDL values expressed in log international units per milliliter were lower than the EDL values expressed in log copies per milliliter (mean difference, 0.2 logs). Clinicians should be informed of these differences since they may have an impact on the interpretation of results of viral load monitoring in patients at high risk of PTLDs.

An EDL variation of 0.5 logs is commonly considered technically significant for qPCR, and in whole blood, clinicians consider a variation of 0.5 or 1 log clinically significant (7). We analyzed the frequency of EDL results differing by more than 0.5 logs or 1 log between laboratories according to the mode of expression. Overall, 50% and 17% of EDLs expressed in log copies per milliliter and log international units per milliliter, respectively, differed by >0.5 logs. The ranges over 1 log concerned 15% of EDL results expressed in copies per milliliter and 0.5% of EDL results expressed in international units per milliliter (Fig. 2 and data not shown).

Although this comparison is based on a low number of testing laboratories (statistical analysis was not feasible), we also noted that the reductions of variability in EDL values seemed similar between the centers having used conversion factors provided by

kit suppliers and the centers having followed the protocol described in this study (Table 1 and Fig. 2).

The major limitations of this study were (i) the use of a commercial panel of external quality controls to evaluate interlaboratory variability and (ii) that the seven samples were probably analyzed in a single run. The use of clinical samples analyzed at different times by different operators would have been more appropriate to approach the conditions of routine analysis.

The standardization of whole-blood EDL results in log international units per milliliter should improve management of patients at high risk of PTLDs and may facilitate comparison of EDLs obtained from other centers. The use of the WHO EBV standard in different medical centers may be useful to establish interventional and/or therapeutic viral load thresholds, which could guide the timing of the initiation of preemptive therapy.

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