Comparison of two commercial quantitative PCR assays for EBV DNA detection and their correlation with the first WHO International Standard for EBV

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Abstract

Purpose. There are few data on the performance of automated Epstein–Barr virus (EBV) PCR assays. This study compared EBV quantification for the kPCR PLX EBV DNA (kPCR; Siemens, France) and the EBV R-gene (R-gene; Argene, Biomerieux, France) assays and their correlation with the World Health Organization (WHO) standard.

Methodology. WHO International Standard for EBV (WHO standard) dilution panels in different matrices were submitted to nucleic acid extraction with Versant kPCR Molecular Systems SP followed by the kPCR assay, or to nucleic acid extraction with the MagNA Pure LC System or NucliSENS easyMag followed by the R-gene assay. Seventy-four clinical specimens were tested in both assays. Bland–Altman analysis and linear regression analysis were performed.

Results. The correlation between the WHO standard diluted in different matrices and the R-gene and kPCR assays was good (R^2 >0.96 and R^2 >0.92, respectively). A matrix effect was observed. The correlation of quantitative results between both assays yielded a coefficient of determination R^2 higher than 0.74. The quantification differences were within one log_{10} of the averaged results for 34 of the 38 specimens (89 %). Calibration to the WHO standard did not increase the comparability of quantitative results.

Conclusions. The quantitative results of both assays showed reasonable correlation with each other and a good correlation with the WHO standard.

INTRODUCTION

Primary Epstein–Barr virus (EBV) infection often causes the clinical picture of infectious mononucleosis. Like all members of the family Herpesviridae, EBV establishes lifelong persistence, and symptomatic or asymptomatic reactivations occur, especially in immunosuppressed patients [1, 2]. Laboratory diagnosis of primary infection in immunocompetent individuals is based on serological assays, but the detection/quantification of EBV DNA in blood can help to diagnose cases with clinically atypical presentations or unclear serological results. EBV DNA quantification is also useful in the diagnosis and treatment monitoring of EBV-associated haemophagocytic syndrome [2].

EBV can also lead to the development of lymphoid and epithelial cancers [3]. Posttransplant lymphoproliferative disorder (PTLD) is usually associated with a high EBV DNA load in the blood. Therefore, the quantification of EBV DNA in blood is used to monitor patients at risk for PTLD. The initiation and monitoring of treatment is typically decided while giving consideration to the EBV DNA load and the dynamics of its changes [2, 4–8].

The quantification of EBV DNA in specimens other than blood is also relevant. For example, EBV DNA quantification in bronchoalveolar lavage specimens is useful for PTLD diagnosis [9]. EBV DNA quantification in cerebrospinal fluid is relevant for the diagnosis of EBV infection of the central nervous system and central nervous system lymphomas [10–12]. Furthermore, EBV DNA quantification in nasopharyngeal specimens is helpful for the initial diagnosis and follow-up of patients with nasopharyngeal carcinoma [13, 14].

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Abbreviations: CV, coefficient of variation; EBV, Epstein–Barr virus; kPCR, kPCR PLX EBV DNA; PTLD, posttransplant lymphoproliferative disorder; R-gene, EBV R-gene; WHO standard, the first World Health Organization (WHO) International Standard for EBV.

Two supplementary figures and one supplementary table are available with the online version of this article.
EBV DNA quantification is widely performed by using quantitative PCR assays. However, there is substantial variation in the quantitative results obtained by different assays and different laboratories [15–17].

Recently, the first World Health Organization (WHO) International Standard for EBV [18] (WHO standard) was developed. Calibration to the WHO standard may help to improve the comparability of quantitative results between different laboratories and assays.

In this study we compared two assays for EBV DNA quantification, the EBV R-gene assay (Argene, Biomerieux, France) and the kPCR PLX EBV DNA assay (Siemens, France), and their correlation to the WHO standard.

METHODS

Definitions

Conversion factor: the factor calculated from the WHO standard dilutions to convert the EBV R-gene assay results from copies ml\(^{-1}\) to international units (IU) ml\(^{-1}\).

Correction factor: the factor calculated from the WHO standard dilutions to calibrate the kPCR PLX EBV DNA assay results from IU ml\(^{-1}\) (quantification by kPCR assay standards) to IU ml\(^{-1}\) (quantification by WHO standard).

The first WHO International Standard for EBV

The first WHO standard (NIBSC code 09/260) consists of \(5 \times 10^6\) IU of a whole-virus preparation of the EBV B95-8 strain (type 1) [18]. The lyophilized standard was reconstituted in 1 ml of sterile water, as recommended by the manufacturer. Three 10-fold serial dilutions were performed in different matrices (previously determined to be negative for EBV DNA) to obtain concentrations from 3.7 to 5.7 \(\log_{10}\) IU ml\(^{-1}\). In the case of the EBV R-gene assay, nucleic acids were extracted twice per dilution. The 6 nucleic acid extracts were tested in 3 separate PCR reactions, yielding a total of 18 data points. A conversion factor from copies ml\(^{-1}\) to IU ml\(^{-1}\) was calculated. First, each result was multiplied by the dilution factor, and second, the geometric mean of each dilution series was calculated. Finally, the overall geometric mean was calculated. The conversion factor was obtained by dividing the theoretical WHO standard value by this overall geometric mean.

In the case of the kPCR PLX EBV DNA assay, nucleic acid extraction and PCR reactions were performed in triplicate per dilution in 3 separate series, yielding a total of 27 data points. A correction factor was calculated by using the same approach as described above for the conversion factor.

Clinical specimens

Seventy-four specimens were referred to our laboratory for EBV DNA detection: 45 whole-blood specimens (EDTA tubes), 12 bronchoalveolar fluids, 1 expectoration, 15 biopsies and 1 specimen of undefined nature. These specimens were obtained from 70 patients (29 female, 41 male; median age 47 years, range 2 to 75 years). Fourteen patients (20 %) had undergone solid-organ transplantation and 20 (29 %) had undergone haematopoietic stem cell transplantation. Other causes of immunosuppression included HIV (n=2), chemotherapy (n=11) and malignant haematological diseases or cancer (n=7). Four patients suffered from haemophagocytic syndrome.

The specimens were tested with the EBV R-gene assay for routine diagnostics. The remaining volume was stored at \(-20\ ^\circ\mathrm{C}\) and retrospectively tested with the kPCR PLX EBV DNA assay.

The study was carried out in accordance with the Declaration of Helsinki and was approved by the institutional review board with a waiver of informed consent. It was a retrospective noninterventional study with no additional procedures.

Nucleic acid extraction

For the EBV R-gene assay nucleic acids from each specimen and WHO standard dilutions in whole blood were extracted by using the MagNA Pure LC System and the Magna Pure LC Total Nucleic Acid Kit – High Performance (Roche Diagnostics, France). Biopsies were pestled in Remel Microtest M4RT transport medium (Roche, France) and expectorations were diluted in DNase-free water before 200 µl was extracted as described above. In addition, nucleic acids from WHO standard dilutions in whole blood, cerebrospinal fluid and bronchoalveolar lavage were extracted with NucliSENS easyMag (Biomerieux, France) (Table S1, available in the online version of this article).

For the kPCR PLX EBV DNA assay nucleic acid extraction was performed using the Versant kPCR Molecular systems SP with the Versant sample preparation 1.2 Reagents (Siemens Healthcare Diagnostics, France) (Table S1). Biopsies were pestled in Remel Microtest M4RT transport medium (Roche, France) and expectorations were diluted in DNase-free water before being treated in the same way as described for specimens other than whole blood (Table S1).

EBV DNA detection

EBV R-gene assay

Quantitative EBV PCR was performed with the EBV R-gene assay (Argene, Biomerieux, France) according to the manufacturer’s instructions (Table S1).

kPCR PLX EBV DNA assay

Quantitative EBV PCR was performed with the kPCR PLX EBV DNA assay (Siemens Healthcare Diagnostics, France) according to the manufacturer’s instructions (Table S1).

Statistical analysis

The correlation of both assays with the WHO standard was determined by linear regression analysis. The qualitative results for the two tests were compared by determining Cohen’s kappa coefficient. Quantitative analysis was restricted to specimens that are routinely analysed quantitatively (i.e. blood and bronchoalveolar fluids) and were
positive in both assays. The quantitative results for the two assays were compared by using linear regression analysis and plotting the differences in quantification against mean values by using Bland–Altman plots. To determine the intra-assay variability three replicates of the three WHO standard dilutions were analysed simultaneously. To determine the inter-assay variability three replicates of the three WHO standard dilutions were analysed in three independent series on different days. The coefficients of variation (CV) were calculated by dividing the standard deviation by the mean of the results in log_{10} IU ml^{-1}.

RESULTS

Correlation of EBV PCR assays with the WHO standard

EBV R-gene assay

The dilution panels of the WHO standard in whole blood extracted with MagNA Pure or NucliSSENS easyMag, as well as in bronchoalveolar lavage and cerebrospinal fluid extracted with NucliSSENS easyMag, were submitted to the EBV R-gene assay. There was a significant correlation between the observed and expected results (Spearman r=0.944, P<0.0001; Spearman r=0.945, P<0.0001; Spearman r=0.944, P<0.0001; and Spearman r=0.944, P<0.0001, for whole blood extracted with MagNA Pure, whole blood extracted with NucliSSENS easyMag, bronchoalveolar lavage extracted with NucliSSENS easyMag and 0.91 for cerebrospinal fluid extracted with NucliSSENS easyMag. Therefore, a matrix effect was detected for whole blood versus other specimen types.

kPCR PLX EBV DNA assay

The WHO standard dilution panel in whole blood and bronchoalveolar lavage was subjected to nucleic acid extraction with Versant kPCR Molecular Systems SP followed by quantitative PCR with the kPCR PLX EBV DNA assay. There was a significant correlation between the observed and expected results (Spearman r=0.944 and P<0.0001, and Spearman r=0.944 and P<0.0001 for the whole blood and bronchoalveolar lavage specimens, respectively). Linear regression analysis yielded coefficients of determination R^2 higher than 0.92 (Fig. 2). A correction factor between the values obtained with the kPCR PLX EBV DNA assay and the theoretical values of the WHO standard was calculated; this was 4.31 for whole blood and 0.26 for bronchoalveolar lavage. This shows that the matrix effect was stronger when the kPCR PLX EBV DNA assay was used. The intra- and inter-assay variability of the kPCR PLX EBV assay was determined with the WHO standard dilution panel in whole blood. The CV was between 1.87 and 4.15% and 2.26 and 14.40 % for the intra-assay and inter-assay variability, respectively (Table 1).

Comparison of the kPCR PLX EBV DNA assay and the EBV R-gene EBV assay

Seventy-four clinical specimens were tested in both assays. The internal controls of both assays were valid in all cases with the exception of one specimen. This specimen was a biopsy with a high virus load (Ct 22.9). Its internal control was invalid in the kPCR PLX EBV assay, but according to the package insert this was due to competition and the assay results were considered valid. The correlation coefficient kappa of the qualitative results between both assays was 0.82 (Table 2). Three of the five discordant results were weakly positive, with a viral load below the limit of quantification.

The quantitative results for concordantly positive specimens were compared between the two assays (whole blood, n=28; bronchoalveolar lavage, n=10) (Fig. 3). There was a significant correlation between the results for both assays (Spearman r=0.905, P<0.0001 for whole blood; Spearman r=0.770, P=0.013 for bronchoalveolar lavage). Bland–Altman analysis revealed that the differences in quantification were within +/− 1 log_{10} of the averaged log_{10} results for 24 of the 28 whole-blood specimens (86 %) and for 10/10 (100 %) of the bronchoalveolar lavage specimens (Fig. 3b, d).

Calibration of EBV DNA assays to the WHO standard

Calibration to the WHO standard by using the calculated conversion/correction factors

Because it could be more meaningful to compare quantitative values with the same unit, we converted the EBV R-gene assay results from copies ml^{-1} to IU ml^{-1} by using the conversion factors calculated above (1.93 for blood and 0.90 for bronchoalveolar lavage) and repeated linear regression analysis. This did not affect the coefficients of determination or the slopes, but increased the intercept for whole-blood specimens (Fig. S1a). Bland–Altman analysis revealed a slightly increased percentage of whole-blood specimens with differences in quantification within +/− 1 log_{10} of the averaged log_{10} results (25/28, 89 %) and no change for bronchoalveolar lavage specimens (Fig. S1b, f). We further reasoned that calibration of the kPCR PLX EBV DNA assay results to the WHO standard by using the calculated correction factor might improve the comparability of quantitative results. This was not the case, because only 17 of the 28 whole-blood specimens (61 %) and 9/10 (90 %) of the bronchoalveolar lavage specimens were within +/− 1 log_{10} of the averaged log_{10} results (Fig. S1d, h).

Calibration to the WHO standard by using the regression equations

Another way of calibrating to the WHO standard is to use the regression equations obtained by linear regression analysis of the WHO standard dilutions in the two assays. We applied this approach by using the regression equations in Fig. I(a, c) for the EBV R-gene assay and those in Fig. 2 for
20 of the 28 (71%) whole-blood specimens and 9 of the 10 (90%) bronchoalveolar lavage specimens were within $+/−1\log_{10}$ of the averaged $\log_{10}$ results (Fig. S2). These percentages were lower than when using the unconverted results.

**DISCUSSION**

Few data have been published on the performance of automated extraction and EBV DNA quantification and, to the best of our knowledge, this is the first study on the performance of the kPCR PLX EBV DNA assay. In this assay both nucleic acid extraction and the preparation of the PCR reaction were automated. In contrast, the EBV R-gene assay was used in combination with automatic nucleic acid extraction on a MagNA Pure LC System and the preparation of the PCR reaction was done manually. The quantitative results varied substantially between the two assays because in 14% (4/28) of the whole-blood specimens tested, the results differed by more than one $\log_{10}$ from the averaged $\log_{10}$ results (Fig. 3). This percentage is in the same range as reported for a comparison...
between the EBV PCR Kit V1 on the Abbott m2000 system and an in-house assay [19]. The coefficient of determination between the results of the EBV R-gene assay and the kPCR PLX EBV DNA assay was also in the same range as reported recently by two studies comparing EBV quantification between different assays [19, 20]. The differences in quantification were higher for low virus loads (Fig. 3), as was the intra- and inter-assay variability of the kPCR PLX EBV DNA assay (Table 1) and the EBV R-gene assay [21]. The higher variability in the quantification of EBV DNA in specimens with low virus load has previously been reported [21, 22]. In general, the kPCR PLX EBV DNA assay yielded higher values than the EBV R-gene assay (Fig. 3).

Recently, the WHO standard [18] was developed in order to improve the comparability of quantitative results from different assays. To the best of our knowledge, this is the first study to investigate WHO standard dilutions in different clinically relevant matrices. Interestingly, the WHO standard performed differently when diluted in whole blood as compared to other body fluids, resulting in a conversion/correction factor that was higher than one for blood and lower than one for other specimen types. To the best of our knowledge, this study is the first to report a matrix effect for the WHO standard for EBV. A matrix effect has been reported previously for the first WHO International Standard for human CMV [23, 24]. Taken together, our results show that both assays underquantify the WHO standard in

### Table 1. Intra- and inter-assay variability of the kPCR PLX EBV DNA assay

<table>
<thead>
<tr>
<th>WHO standard</th>
<th>Theoretical values (log(_{10}) IU ml(^{-1}))</th>
<th>kPCR PLX EBV DNA assay</th>
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<tr>
<td></td>
<td>Mean (log(_{10}) IU ml(^{-1}))</td>
<td>Standard deviation (log(_{10}) IU ml(^{-1}))</td>
<td>CV (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intra-assay variability</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.699</td>
<td>5.146</td>
<td>0.135</td>
<td>2.63</td>
</tr>
<tr>
<td>4.699</td>
<td>4.142</td>
<td>0.077</td>
<td>1.87</td>
</tr>
<tr>
<td>3.699</td>
<td>2.976</td>
<td>0.124</td>
<td>4.15</td>
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<tr>
<td><strong>Inter-assay variability</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.699</td>
<td>5.134</td>
<td>0.121</td>
<td>2.36</td>
</tr>
<tr>
<td>4.699</td>
<td>4.183</td>
<td>0.095</td>
<td>2.26</td>
</tr>
<tr>
<td>3.699</td>
<td>2.876</td>
<td>0.414</td>
<td>14.40</td>
</tr>
</tbody>
</table>

CV, coefficient of variation.
whole blood, while the kPCR PLX EBV DNA assay over-quantifies the WHO standard in bronchoalveolar lavage specimens. Although there is currently no consensus on whether plasma or whole blood should be used for EBV DNA quantification [4], the observed matrix effect might favour the use of plasma.

Few studies have investigated whether calibration to the WHO standard improves the comparability of quantitative results. Two recent studies found that the quantitative agreement between assays was unchanged by calibration to the WHO standard [25, 26]. On the other hand, the use of the WHO standard was reported to have reduced the inter-laboratory variability of EBV DNA quantification in a multicentre study [27]. Of note, there are different methods to calibrate results to the WHO standard, e.g. the mean or the median can be used to calculate conversion factors [24, 26–28], or regression equations can be used [25, 29–31]. In the current study, we first used the mean [27] to calculate the conversion/correction factors, and found that this did not improve the comparability of the quantitative results (Fig. 3 compared to Fig. S1). For this reason, we applied a second approach, in which we used the regression equations

Table 2. Comparison of the kPCR PLX EBV DNA assay to the EBV R-gene assay using clinical samples (n=74)

<table>
<thead>
<tr>
<th>Assay type and results</th>
<th>EBV R-gene assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>kPCR PLX EBV DNA assay</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>53</td>
</tr>
<tr>
<td>Negative</td>
<td>4†</td>
</tr>
</tbody>
</table>

*One specimen with a virus load below the limit of quantification.
†Two of these specimens had a virus load below the limit of quantification.

**Table 2.** Comparison of the kPCR PLX EBV DNA assay to the EBV R-gene assay using clinical samples (n=74)

**DNA quantification**

**Fig. 3.** Comparison of quantitative results obtained with the EBV R-gene and the kPCR PLX EBV DNA assays. Linear regression plot comparing the EBV DNA quantification obtained with EBV R-gene assay in log_{10} copies ml$^{-1}$ and kPCR PLX EBV DNA assay in log_{10} IU ml$^{-1}$ for whole-blood specimens (n=38) (a) and bronchoalveolar lavage specimens (n=10) (c). Mean differences in EBV DNA quantification plotted against mean values using Bland–Altman analysis (b, d). The perfect fit line is shown as a dotted line.
obtained from the testing of WHO standard dilutions in both assays. However, this did not improve comparability of quantitative results either (Fig. S2). The same has also been reported for CMV DNA assays and other standard preparations [23, 28, 31]. A potential cause of this could be the standards’ lack of commutability [31, 32], which may be due to the presence of both cell-free and cell-associated EBV DNA in clinical specimens as opposed to cell-free EBV DNA in the WHO standard. The finding that calibration to the WHO standard did not improve the comparability of the quantitative results demonstrates once again that universally valid EBV DNA load thresholds for the initiation of preemptive therapy cannot yet be defined. However, EBV DNA load dynamics may be more relevant than single EBV load measurements for the diagnosis of PTLD [2]. Slight quantification differences between different assays therefore do not represent a major problem as long as EBV DNA load monitoring is performed by the same assay over time.

A point that has to be taken into account is the fact that the clinical specimens were stored at −20°C before testing in the kPCR PLX EBV DNA assay in our study. DNA is stable for several years at −20°C [33]. Quantitative PCRs usually have short amplicon lengths and the kPCR PLX EBV DNA assay has an amplicon length of less than 100 base pairs (Philippe Boulenger, personal communication). Therefore, the possibility of DNA fragmentation induced by freezing and thawing is not expected to have a substantial impact on EBV DNA quantification. As a result, we do not think that the storage of clinical specimens had a relevant impact on the quantification results reported in this study.

In conclusion, our study demonstrated that both assays showed reasonable correlation with each other and with the WHO standard. A matrix effect was observed. Calibration to the WHO standard did not improve the comparability of the quantitative results. Further efforts are needed to develop commutable standards without a matrix effect that closely resemble clinical specimens in order to achieve the standardization and improved comparability of quantitative results between different assays and laboratories, and thus to establish universally valid EBV DNA load thresholds that can guide the initiation of preemptive therapy.

**References**


