Comparison of Quantitative Competitive PCR with LightCycler-Based PCR for Measuring Epstein-Barr Virus DNA Load in Clinical Specimens

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Epstein-Barr virus (EBV), a lympho- and epitheliotropic gamma-1 herpesvirus, infects more than 90% of the population worldwide. In most individuals, EBV establishes a lifelong generally asymptomatic infection. However, in a minority of immunocompetent individuals, the virus is associated with several benign and malignant proliferative disorders including infectious mononucleosis, Hodgkin’s lymphoma, B- and T-cell non-Hodgkin’s lymphoma, and nasopharyngeal and gastric carcinoma. In immunosuppressed patients, active EBV infection is a strong risk factor for the development of posttransplantation lymphoproliferative disease (PTLD), AIDS-related lymphoma, and X-linked proliferative syndrome (reviewed in reference 7).

In transplant recipients, longitudinal monitoring of the EBV load in peripheral blood is increasingly recognized as a valuable diagnostic tool in prediction, diagnosis, and therapeutic management of PTLD (15, 21). Although semiquantitative assays are still frequently used (12, 16), recent data stress the importance of using calibrated quantitative PCR assays based on competitive coamplification of EBV with an internal standard added to the reaction in a known amount (1, 2, 14, 15, 18, 21). Although highly accurate and reproducible, such assays are rather laborious and require intensive post-PCR handling. Each sample has to be spiked with different amounts of internal standard to achieve precise quantification. To overcome the drawbacks of competitive PCR, a number of studies have used TaqMan-based real-time PCR assays for viral load determination, enabling fast, direct quantification of PCR products in a closed-tube assay (6, 8, 9, 13, 24). Such rapid assays are best suited for frequent and high-throughput monitoring of EBV load dynamics, which is essential in high-risk patients such as transplant recipients (15, 21, 23). However, real-time PCR assays involve a comparison of the fluorescence signal of a clinical specimen with that of external standard dilution series of plasmid or cell line DNA made in buffer instead of specimen. Consequently, reactions are not normalized as is the case with internal calibration standards and may be influenced by PCR inhibitors such as heparin, EDTA, and lipids or intertube differences in amplification efficiency (3, 10).

The aim of this study was to develop a real-time PCR assay for EBV load measurement. For this, we choose the LightCycler system (Roche Diagnostics, Mannheim, Germany), an ultrarapid PCR system based on real-time fluorimetric quantification of PCR products. The use of two labeled fluorescent hybridization probes for detection of PCR products in this assay ensures high specificity. The developed LightCycler assay is based on amplification of EBNA-1, a conserved single-copy gene of EBV. We compared the performance of the optimized EBV LightCycler assay with that of a well-validated quantitative competitive PCR (Q-PCR) which is based on the same EBNA-1 primer set. This Q-PCR was developed previously by our group (18) and has been shown to be of great clinical utility for transplant recipients (19) and human immunodeficiency virus (HIV)-infected patients (18, 22). In this study we show that real-time PCR is a suitable and time-saving technique as reported by others (6, 8, 9, 13, 24). A major drawback may be the lack of internally controlled amplifica-

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tion, leading to false-negative results in a small number of patients or to possible underestimation of EBV DNA load.

MATERIALS AND METHODS

Clinical specimens. Weekly follow-up whole-blood samples (n = 112) from five lung transplant recipients with PTLD were kindly provided by Erik Verschueren, Academic Hospital Groningen, Groningen, The Netherlands. Whole-blood samples (n = 7) from asymptomatic HIV-infected patients were kindly provided by Paul Smits, Slotervaart Hospital, Amsterdam, The Netherlands. Whole-blood samples (n = 21) from patients with infectious mononucleosis were kindly provided by Nadja Prang, Medizinische Immunologische Laboratorien, Muenchen, Germany. Whole-blood samples (n = 21) and parallel serum samples (n = 12) from juvenile African patients with Burkitt’s lymphoma were kindly provided by R. Broadhead, Blantyre, Malawi. Whole-blood samples from 15 healthy EBV-seropositive donors from our institute were included. Fresh whole-blood samples were diluted 1:9 in NASBA lysis buffer (Organon Teknika BV, Boxtel, The Netherlands) and stored at −80°C until used for DNA isolation. Blood samples from the Burkitt’s lymphoma patients were directly stored and transported in liquid nitrogen and lyzed in 9 volumes of lysis buffer prior to DNA isolation.

DNA isolation. DNA was isolated from 1 ml of lysed whole blood by silica-based extraction as described by Boom et al. (5).

EBNA-1 based Q-PCR. Q-PCR for a highly conserved 213-bp region of EBNA-1, a single-copy gene of EBV, combined with enzyme immunoassay detection of PCR products was performed as described by our group previously, using the primer EBNA-1 QP1 and the biotinylated primer EBNA-1 QP2 (18). Clinical specimens were spiked with three different amounts of internal standard EBNA-1, a single-copy gene of EBV, combined with enzyme immunoassay detection of PCR products was performed as described by our group previously, using the primer EBNA-1 QP1 and the biotinylated primer EBNA-1 QP2 (18). Clinical specimens were spiked with three different amounts of internal standard EBNA-1, a single-copy gene of EBV, combined with enzyme immunoassay detection of PCR products was performed as described by our group previously, using the primer EBNA-1 QP1 and the biotinylated primer EBNA-1 QP2 (18).

EBNA-1-based LightCycler PCR. For LightCycler PCR, the same EBNA-1 QP1-QP2 primer set was used as for the Q-PCR, but instead a nonbiotinylated EBNA-1 QP2 primer was used. Two hybridization probes were designed. Probe EBNA-FLN is identical to the EBNA-1 wild-type probe used in Q-PCR (18), except for a 3’ fluorescein label. Probe EBNA-LCN (5’-ACCGGCGCCCAACCTGG-3’) flanks EBNA-FLN with a 1-base gap and is labeled at the 5’ end with LC Red640. Hybridization probes were purchased from TIB Molbiol (Berlin, Germany). Simultaneous hybridization of both probes on the PCR product leads to fluorescence resonance energy transfer (FRET) after excitation by a light-emitting diode (LED). The LC Red640 probe then emits red fluorescent light, whose amount is directly correlated with the amount of generated PCR product (26).

PCR runs were performed using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) in a volume of 20 μl consisting of 20 pmol of each primer, 4 pmol of each probe, 2 μl of LightCycler Fast Star DNA master hybridization probe reaction mixture (Roche Diagnostics), 4 mM MgCl2, and 5 μl of plasmid DNA or clinical specimen DNA. Reaction capillaries (Roche Diagnostics) were loaded and centrifuged before being placed in the LightCycler. The experimental PCR protocol was as follows: an initial 10-min denaturation at 95°C for Fast Start Taq DNA polymerase activation followed by 45 cycles of 10 s of denaturation at 95°C, 10 s of annealing at 55°C, and 10 s of extension at 72°C. Then the PCR samples were cooled to 40°C. Data were obtained during the annealing period in the “single” mode, with the channel setting F2/F1. The fluorescence gain settings were as follows: F1 gain, 1; F2 gain, 15; F3 gain, 30. For data analysis, the baseline adjustment was set at “arithmetic” and the fluorescence curve analyses were carried out in the “2 fit points” mode using LightCycler software version 3.5.

For quantification of EBV DNA, 10-fold dilutions of plasmid containing the EBNA-1 amplicon (18) were made both in water and in a DNA background equivalent to 5 μl of whole-blood DNA. At least 10 copies of plasmid DNA or 0.5 JY cell equivalent could be detected under both conditions, equivalent to <1 infected EBV cell (18) and indicating the LightCycler assay to be as sensitive as Q-PCR, which detects at least 10 copies of the plasmid as well (18).

Reproducibility of the LightCycler assay. The intra-assay variability of the LightCycler assay was determined by amplifying different numbers of copies of plasmid DNA in quadruplicate either in water or in a background of whole-blood DNA derived from a healthy donor. The results are summarized in Table 1.

The interassay variability of the LightCycler assay was determined by amplifying different amounts of EBNA-1 plasmid DNA in a background of whole-blood DNA derived from a healthy donor or in water. For this, 10, 100, 1,000, and 10,000 copies were quantified in quadruplicate in three independent experiments. The results are summarized in Table 2. The LightCycler variation observed at 1,000 copies was comparable to the Q-PCR variation, which is approximately 30%, as reported previously (18).

Influence of donor blood DNA background on LightCycler performance. In contrast to Q-PCR, LightCycler PCR is not internally corrected for PCR inhibitors that may be present in clinical specimens. Therefore we investigated the putative influence of different whole-blood DNA backgrounds on the performance of the LightCycler assay. DNA was extracted from whole blood of 10 healthy EBV-seropositive donors. All whole-blood samples were negative for EBV DNA in qualitative EBNA-1 PCR. The DNA equivalent of 5 μl of whole blood from each donor was spiked in triplicate with 1,000 copies of EBNA-1 plasmid DNA and quantified by LightCycler PCR; mean C values and quantified copy numbers are de-

### Table 1. Intra-assay reproducibility of the LightCycler assay in either a whole-blood DNA background or water

<table>
<thead>
<tr>
<th>No. of copies</th>
<th>Water background</th>
<th>Whole-blood DNA background</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean C (SD)</td>
<td>Mean copy no. (SD)</td>
</tr>
<tr>
<td>10,000</td>
<td>29.23 (0.31)</td>
<td>9,430 (1,767)</td>
</tr>
<tr>
<td>1,000</td>
<td>32.85 (0.27)</td>
<td>991 (169)</td>
</tr>
<tr>
<td>100</td>
<td>36.13 (0.85)</td>
<td>139 (62)</td>
</tr>
<tr>
<td>10</td>
<td>40.50 (1.99)</td>
<td>14 (15)</td>
</tr>
</tbody>
</table>

*Experiments were performed in quadruplicate.*
The mean Ct value found was 30.79, with a standard deviation of 0.81 (2.6%). For quantified copy numbers this was 1,290 ± 343 copies (26.5%). These results demonstrate the influence of different sample backgrounds on the accuracy of EBV DNA load quantification when using external calibration, leading to slightly higher variations in Ct values and quantified copy number than within one donor (Table 2). Thus, absolute values of EBV DNA load based on external calibration standard curves should be interpreted with caution.

Quantification of the EBV DNA load in clinical specimens.

(i) EBV DNA loads in healthy EBV-seropositive donors. None of the blood samples from healthy donors was above the cutoff value in LightCycler PCR, confirming the low EBV DNA loads and small number of EBV-positive B cells in peripheral blood of healthy carriers (21, 24) and the specificity of the LightCycler EBV load assay. When spiked with 10 copies of EBNA-1 plasmid DNA (18), all samples gave positive signals, indicating the absence of PCR inhibitors as a putative reason for EBV DNA negativity.

(ii) Influence of whole-blood DNA background in standard curves on quantification using LightCycler. Unfractionated whole blood is the preferred clinical specimen for EBV DNA load determination (20). However, standard curves in real-time PCR assays in most studies are generated from plasmid DNA diluted in water. Amplification efficiency may depend on clinical specimen DNA background, and therefore we compared LightCycler performance using standard curves generated in water or a DNA background equivalent to 5 μl of whole blood, the input amount used for clinical samples. Although the Ct value obtained in LightCycler PCR is not dependent on the type of standard curve that is used, the quantification may be influenced by the standard curve itself (Fig. 1). Therefore, follow-up samples from three PTLD patients (9, 26, and 27 samples) were analyzed by LightCycler PCR using standard curves generated either in water or in a DNA background equivalent to 5 μl of blood from EBV DNA-negative healthy donors. The results are shown in Fig. 2. Generating a standard curve by using either water or whole-blood DNA did not influence EBV DNA load dynamics, and correlation coefficients of 0.998, 0.988, and 0.734 (or 0.983 when corrected for one extreme outlier) were observed for quantifications based on either of the two different standard curves. Although the correlation between the two sets of conditions was very high, some differences were observed in the absolute amount of EBV quantified (data not shown). These results show the influence of sample background on the accuracy of DNA load quantification when using external calibrators. Therefore, absolute values determined in such systems should be interpreted with caution.

(iii) Qualitative comparison of Q-PCR and LightCycler PCR assays. A total of 253 clinical samples from different patient populations were tested in the LightCycler PCR assay using standard curves of plasmid DNA diluted in water. Of these, 194 were above the cutoff value of 2,000 EBV DNA

![FIG. 1. Performance of LightCycler EBNA-1 PCR when amplifying 1,000 copies of EBNA-1 plasmid DNA in a background of whole-blood DNA from 10 different donors (all experiments were done in triplicate). (A) Mean Ct value and standard deviation. (B) Mean quantified EBV-DNA copy number and standard deviation.](http://jcm.asm.org/ on October 14, 2017 by guest)
copies/ml in Q-PCR and the viral load ranged from 2,000 to 3,192,168 EBV DNA copies/ml. Using the 2,000-copies/ml cutoff value, the overall sensitivity of LightCycler compared to Q-PCR was 91%. For HIV patients, the sensitivity of LightCycler was 91%; for transplant recipients, it was 88%; and for both infectious mononucleosis and Burkitt’s lymphoma patients (whole blood and serum tested separately), it was 100%. In LightCycler-negative samples, the EBV DNA load determined by Q-PCR ranged from 2,000 to 28,000 EBV DNA copies/ml.

(iv) Comparison of LightCycler and Q-PCR quantifications using follow-up samples from transplant recipients. Absolute values of EBV DNA load may not be informative (22), and therefore we performed longitudinal analysis of EBV DNA load dynamics by LightCycler compared to Q-PCR. For this, five lung transplant recipients were selected from whom frequent follow-up samples were available, which exhibited fluctuating EBV DNA loads in unfraccionated whole blood as determined by Q-PCR. All follow-up samples were monitored for viral load by the LightCycler assay using a standard calibration curve generated with plasmid DNA diluted in water. Patient A experienced three episodes of PTLD after primary EBV infection and showed increasing EBV DNA loads before diagnosis (as determined by Q-PCR), with peak levels occurring at diagnosis (19). In the LightCycler assay, nearly identical dynamics of EBV DNA load in time were observed (Fig. 3A). The diagnostic value of EBV DNA load monitoring in this patient is obvious with either Q-PCR or LightCycler, and the viral load dynamics correspond very well with the clinical course of EBV infection and the development of PTLD.

All follow-up samples from patient B were above the cutoff value of 2,000 EBV DNA copies/ml of blood in Q-PCR. All samples were also strongly positive in the LightCycler assay, and similar viral load kinetics were observed, with the exception of sample 3, which gave a higher reading in LightCycler. Other samples were within 30% of the Q-PCR value, which is the mean standard deviation observed in both Q-PCR (18) and LightCycler (see above).

In the samples from patient C, no correlation was observed in the two assays. The overall levels remained positive at rather low and fluctuating levels throughout the follow-up period.

Patient D developed PTLD after a switch of immunosuppressive treatment from cyclosporin A to FK506 (19). This switch led to a peak in EBV load measured in both Q-PCR and LightCycler (sample 16). The EBV DNA load dynamics were similar in the two assays, taking into account the variability of 30% in the assays.

Patient E showed the same trend of increases and decreases in EBV DNA load in both assays, with the exception of sample 1, which was repeatedly negative in LightCycler (in two independent experiments performed in triplicate) but strongly positive in Q-PCR. When spiked with 10 copies of EBNA-1 plasmid DNA, this sample remained negative in LightCycler, indicating the presence of inhibitors that interfere with real-time PCR. The EBV load fluctuations were larger in Q-PCR, but the trend of decreases and increases was the same in the two assays.

Although the longitudinal EBV DNA load dynamics in four of the five transplant recipients were similar in Q-PCR and LightCycler PCR, the correlation with absolute values of EBV DNA load was low (patient A, \( r^2 = 0.551 \); patient B, \( r^2 = 0.316 \); patient C, \( r^2 = 0.02 \); patient D, \( r^2 = 0.42 \); patient E, \( r^2 = 0.31 \) or 0.895 with sample 1 excluded).

(v) Comparison of LightCycler and Q-PCR quantifications using cross-sectional samples. The sensitivity of LightCycler is highly comparable to that of Q-PCR, with the majority of Q-PCR-positive samples also being above the cutoff value of LightCycler (see “Qualitative comparison of Q-PCR and LightCycler PCR assays” above). Overall, the correlation between absolute values of EBV DNA load determined in the two assays was rather low (\( r^2 = 0.37 \)) but significant (\( P < 0.001 \)), as can be seen in Fig. 4. The correlation between LightCycler- and Q-PCR-determined viral loads was calculated for cross-sectional samples from individual patient groups: for
HIV patients, $r^2 = 0.61$; for infectious mononucleosis patients, $r^2 = 0.36$; for Burkitt’s lymphoma patient whole-blood samples, $r^2 = 0.16$; for Burkitt’s lymphoma patient serum samples, $r^2 = 0.20$. Low correlation coefficients were also observed in the follow-up samples from most transplant recipients (see “Comparison of LightCycler and Q-PCR quantification using follow-up samples from transplant recipients” above) despite a good correlation between EBV DNA load dynamics over time. This indicates that comparison of absolute EBV DNA load values is not very informative when comparing two assays that use different calibration methods. Longitudinal analysis by a single method, preferably using an internal coamplified calibrator, is recommended as the method of choice.

**DISCUSSION**

In this study we developed a real-time LightCycler PCR assay for rapid determination of EBV DNA loads in clinical specimens. This LightCycler assay is less laborious than Q-PCR, omits the need for post-PCR detection and handling steps, and is extremely rapid, which is important when frequent monitoring is required, such as in patient management post-transplantation (19). The LightCycler PCR is a very fast closed-tube system, eliminating the risk of PCR contamination by product carryover and thus making this assay very suitable for high-throughput screening and frequent monitoring of patients at risk for developing EBV-associated diseases. The assay is based on amplification of a highly conserved region of EBNA-1, a single-copy gene of EBV that is essential for maintenance of the virus in infected cells. Mutation hot spots in EBNA-1 have been mapped in clinical isolates of EBV (4, 17, 27), enabling primer selection in a highly conserved region of this gene (18). In previous studies, this region of the viral genome was shown to allow reliable EBV DNA quantification in samples from patients with various EBV-associated disease worldwide (19, 22).

The LightCycler assay is analytically as sensitive as the Q-PCR assay (18) and shows high intra- and interassay reproducibility when amplifying plasmid DNA in either the presence or absence of a whole-blood DNA background (Tables 1 and 2; Fig. 1). This is in agreement with other studies that have
reported real-time PCR to be a reproducible technique and found similar variations in $C_t$ values (11). However, the variation based on quantified copy number is considerably higher because for calculations the $C_t$ values of standard dilutions are plotted against $\log_{10}$ input used in PCR, yielding a linear relationship between the two variables. Thus, small variations in $C_t$ have large effects on copy number calculation. Nevertheless, the observed variations in quantified copy number compare favorably with those of other quantitative PCR assays (28).

The correlation between Q-PCR- and LightCycler-determined EBV DNA load dynamics in follow-up samples is better than the correlation between absolute values of EBV DNA load. This confirms the limited relevance of absolute EBV DNA load values when comparing the results of different viral load assays. Previously we showed that it is more important to monitor dynamic decreases and increases in viral load and correlate these with clinical events rather than to depend on absolute EBV levels (19, 22).

As input for EBV DNA load assays, we prefer to use unfractionated whole-blood since it combines all components that may harbor EBV, e.g., serum, and circulating EBV-harbor (tumor) cells. Whole blood gives an absolute measure of the circulating viral load, irrespective of artifacts due to cell isolation or serum or plasma preparation and variations in cell counts (20). However, some studies using real-time PCR have reported that whole-blood DNA preparations may influence the PCR results. Wagner et al. (24) showed, using TaqMan PCR, that the amplification efficiency is lower for whole-blood DNA than for peripheral blood mononuclear cell or B-cell DNA, indicating inhibitory factors influencing the reaction. Kimura et al. (9) showed that heparin in plasma DNA preparations had inhibitory effects on the real-time TaqMan PCR performance that could not be removed by a commercial kit for DNA extraction from clinical specimens. Therefore, the purity of the isolated DNA from clinical specimens is a pivotal factor in quantitative PCR systems based on external calibration curves. Because of this, we prefer silica-based DNA extraction from whole-blood (5), which very efficiently removes exogenous and endogenous interfering substances such as heparin, EDTA, zidovudine, and hemoglobin (25).

Initially, we also used TaqMan PCR (Applied Biosystems) for EBV DNA load quantification in unfractionated whole-blood, using the method exactly as described recently (13). This assay gave good results when plasmid DNA or cell-derived DNA was spiked in PCR. However, poor results were observed when unfractionated whole-blood samples were used as input. The EBV DNA load quantified in TaqMan was always lower than in Q-PCR, and a large number of samples were negative in TaqMan, despite highly elevated EBV DNA loads as found in Q-PCR and LightCycler in the same DNA isolates (unpublished data). This indicates that silica-based DNA extraction may not completely remove all substances interfering with TaqMan PCR and that TaqMan PCR may be inhibited by whole-blood DNA preparations. Inhibition of TaqMan PCR by whole-blood DNA was also reported by several other groups (8, 9, 24). Since this effect is much less frequently seen in the LightCycler assay, we prefer LightCycler PCR as an alternative real-time PCR assay. Possibly, the TaqMan technology, which uses hydrolysis probes and is based on both the 5’ exonuclease and polymerase activity of the Taq polymerase enzyme, is more sensitive to inhibition than is the LightCycler technology, which is based on simultaneous hybridization of two labeled probes without enzymatic degradation of the probes by exonuclease activity.

For four of five lung transplant recipients, LightCycler PCR gave similar EBV DNA load dynamics to Q-PCR when unfractionated whole-blood was used as input. However, some discrepant results were observed, e.g., in patient E (sample 1), where the LightCycler results were repeatedly negative despite a high viral load in Q-PCR. These discrepancies could be caused by PCR-inhibitory factors. Other studies using real-time PCR have reported a negative influence of endogenous and exogenous inhibitory factors on EBV PCR (8, 9, 24). Therefore, it may be advisable to include an internal control in LightCycler, e.g., a fixed amount of the internal standard that is used in Q-PCR (18). This standard has identical length and base composition, ensuring equal amplification efficiency to the wild-type target and an internally randomized base stretch for identification. Detection of such internal standards is possible by using a second probe and measurement via a second available fluorescence channel on the LightCycler apparatus. Inclusion of such a standard would normalize each reaction and correct for any variations or inhibitors in PCR and could indicate whether a sample is true EBV negative or negative as a consequence of substances interfering with amplification.

When DNA derived from healthy donor blood was spiked to 1,000 copies of EBNA-1 plasmid in LightCycler, no clear influence of whole-blood DNA on PCR performance was found (Tables 1 and 2; Fig. 1). In addition, generating a standard quantification curve of plasmid DNA in either the presence or absence of a whole-blood DNA background did not have profound effects on the quantified viral load in follow-up samples from PTLD patients (Fig. 2). In the two experiments described, however, only single blood samples from 1 or 10 healthy donors were included. Discrepancies between Q-PCR and LightCycler PCR when testing patient samples are rather rare, taking into account the variability of both assays and the totally different mechanisms of quantification on which the
assays are based. We conclude that a negative influence of the clinical specimen (whole-blood DNA) on LightCycler PCR performance is rare. However, PCR inhibition should not be neglected since it may lead to an underestimation of the EBV DNA load or even to false-negative results. Especially when large amounts of samples are screened and the risk of false-positive results increases, this aspect of real-time PCR should receive further attention. Absolute values of the DNA load determined in real-time PCR should be interpreted with some caution, because external calibration used in this system may influence quantification.

At present a wide variety of PCR assays are available for quantification of EBV DNA loads, including semiquantitative PCR (12, 16), quantitative-competitive PCR (1, 2, 14, 18), TaqMan PCR (6, 8, 9, 13), and LightCycler PCR (this study). Although all these assays have proven to be of excellent diagnostic value within a given clinical setting, basic interlaboratory standardization of EBV DNA load monitoring has yet to be achieved. Reported viral loads, defined clinical cutoff values, type and amount of input material, and standard curves used differ widely among institutes. Future standardization of EBV DNA load assays may be aided by using internally controlled real-time PCR assays and the development of an international standard.

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