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Evaluation of a commercial real-time PCR assay for quantitation of Epstein-Barr virus DNA in different groups of patients

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Abstract

The aim of this study was to evaluate the performance of a molecular assay for quantitation of Epstein-Barr virus (EBV) DNA based on real-time PCR, and to determine EBV DNA levels in EDTA whole blood samples derived from different groups of patients. Following a manual DNA extraction protocol, real-time PCR was performed using the LightCycler EBV Quantification Kit, which demonstrated sufficient accuracy and linearity. Coefficients of variations were found to be between 6 and 42% and 5 and 34%, respectively, for interassay and intra-assay variations.

In clinical specimens, EBV DNA was detected in all patients with acute EBV infection ($n=34$), in one of 25 adults with past EBV infection, in 16 out of 25 (64%) anti-HIV antibody positive persons, in 10 out of 25 (40%) solid organ transplant recipients, and in none of the 23 infants without history of EBV infection. When EBV DNA levels in positive specimens were compared between different groups, statistically significant differences were not found. The LightCycler EBV Quantification Kit was found to be useful for determination of EBV DNA levels in EDTA whole blood.

Key words: *real-time PCR; Epstein-Barr virus; quantitation; viral load; immunosuppression*

1. Introduction

Epstein-Barr virus (EBV) is a human herpesvirus classified in *Lymphocryptovirus* genus of the *Gammaherpesviridae* subfamily. EBV infects B-lymphocytes and oropharyngeal epithelial cells. After primary infection, EBV establishes life-long latency. The expression of specific latency-associated genes determines whether the virus will be maintained in a latent state or turn into active viral replication (Cohen et al., 2000; Johannsen et al., 2005).

Epstein-Barr virus is the etiologic agent of infectious mononucleosis but may also act as an oncogene associated with nasopharyngeal carcinoma and Burkitt's lymphoma. In the immunocompromised host, EBV is associated with posttransplant lymphoproliferative disorders or AIDS-related lymphomas. Posttransplant lymphoproliferative disorders may occur in 1-20% of transplant recipients depending on several issues including type of transplantation, intensity and duration of immunosuppressive therapy and CMV infection (Andreone et al., 2003).

Diagnosis of EBV infection is usually based on serological testing and clinical presentation. However, serology may be unreliable in immunocompromised patients (Verschuuren et al., 2003). In these patients, the diagnostic method should be able to differentiate latency from disease. Recently, distinct patterns of viral gene expression at the RNA level were described in EBV-associated diseases (Stevens et al., 2005). Although profiling EBV mRNA expression might be of interest, these methods could be demanding for routine clinical use.

Molecular detection of EBV DNA has been shown to be useful for monitoring of dynamic EBV-load fluctuations in immunocompromised patients

(Bai et al., 1997; Meerbach et al., 2001, Stevens et al., 2001a). The EBV DNA level has been found to correlate with the severity of illness (Rowe et al., 1997; van Esser et al., 2001). Molecular assays for detection of EBV DNA are usually based on home-designed protocols (Baldanti et al., 2000; Limaye et al., 1999; Riddler et al., 1994). These assays involve commonly laborious extraction protocols and do not include appropriate postamplification detection methods. Some of these methods lack inclusion of an internal control and they usually are not designed for accurate quantitation of EBV DNA. Recently, molecular assays for detection of EBV DNA based on real-time PCR have been reported (Brenzel-Pesce et al., 2002; Jebbink et al., 2003; Kimura et al., 1999; Niesters., 2000; Stevens et al., 2002a). Compared to conventional PCR-based assays, real-time PCR offers several important advantages. It combines amplification of target DNA with detection of amplification products in the same closed reaction vessel. Therefore, the potential for contamination is reduced significantly. With real-time PCR assays, the analytical turnaround time is significantly shorter than that required for conventional PCR assays. In contrast to conventional PCR, real-time PCR allows for log-phase analysis. Therefore, the quantitation range for real-time PCR assays is significantly broader than that for conventional PCR assays.

Recently, the LightCycler EBV Quantification Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) has been introduced. This assay is based on real-time PCR employing the hybridization probes technology, and it contains an internal control in order to detect possible inhibition. Although this kit is intended for quantitative detection of EBV DNA in various human samples (whole blood, plasma, cerebrospinal fluid, cell culture), EBV DNA isolated from EDTA whole blood showed better test

sensitivity when compared to plasma. EDTA whole blood has been found to reflect the viral load in the defined unit of circulation better than any other matrices (Stevens et al 2001a).

The aim of this study was to evaluate the performance of a commercially available real-time PCR assay for quantitation of EBV DNA. Accuracy was tested with the Quality Control for Molecular Diagnostics 2002 Epstein-Barr Virus Proficiency Panel and linearity with a dilution series of a routine EBV DNA positive sample. Both interassay and intra-assay variations were determined. The utility of the molecular assay for quantitation of EBV DNA in EDTA whole blood was evaluated on clinical samples derived from five groups of patients with different clinical and serological presentation of Epstein-Barr virus infection. Although there are similar reports concerning EBV DNA quantitative detection in EBV-related diseases with different real-time PCR tests, the data on LightCycler EBV Quantification Kit are uncommon.

2. Materials and Methods

2.1. Molecular Assay

Blood was collected using 3.5 ml-EDTA tubes (BD, Franklin, Lakes, NJ, USA). Within 24 hours after blood collection, EBV DNA was isolated with the QIAamp blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's package insert. The input volume was 200 µl. For each sample, 10 µl of an EBV DNA quantitative internal standard was added to 200 µl of lysis reagent. Extracted DNA was eluted in 100 µl of elution buffer.

Real-time PCR was carried out using the LightCycler EBV Quantification Kit on the LightCycler Instrument, version 1, Software version 3.3, according to the manufacturer's package insert. The test employs hybridization probes to detect a gene fragment encoding a single copy of EBV gene EBNA 1. The primers and probes in the kit are specific for amplification and detection of the EBV DNA, so unspecific amplification of other agents is avoided (Auer et al., 2002). The LightCycler capillaries were filled with 15 µl of master mix and 5 µl of extracted DNA. Three external controls (negative, low positive, and high positive) were included in each run.

2.2. Study Design

In the first step, the accuracy of the molecular assay was determined with original samples ($n=8$) of the Quality Control for Molecular Diagnostics (QCMD) 2002 Epstein Barr virus proficiency panel. This panel contained

samples with different concentrations of EBV DNA copies per ml (range, 1.0×10^2 to 5.0×10^4) as well as a negative control.

In the second step, the linearity of the molecular assay was determined. A routine clinical EDTA whole blood sample with high viral load was used for the determination of linearity. A dilution series (0.5 log steps, i.e., 1:3.16 dilutions) was prepared by using EBV DNA negative-EDTA whole blood. Each dilution was analyzed three times and the mean EBV DNA copy number of each sample was determined.

In the third step, the interassay variation of the molecular assay was determined. In eight clinical routine samples that contained different concentrations of EBV DNA (ranging from 9.6×10^3 to 2.3×10^4 copies per ml), DNA was re-isolated five times and tested once on each of five different days.

In the fourth step, the intra-assay variation of the molecular assay was determined. Four clinical routine samples with different EBV DNA concentrations ranging from 3.4×10^3 to 2.0×10^4 copies per ml were aliquoted, and each aliquot was tested five times in one run.

2.3. Clinical Samples

A total of 132 clinical samples were studied. All samples were obtained from patients for routine clinical diagnosis. The patients were diagnosed and treated at the Dr Fran Mihaljević University Hospital for Infectious Diseases, Zagreb, Croatia, between January 2003 and October 2004. The local ethics committee approved the study.

Samples were obtained from five different groups of patients. Group 1 ($n=34$) consisted of children and adults with acute EBV infection based on clinical presentation and serological testing. All patients had anti-viral capsid antigen (VCA)-specific immunoglobulin M (IgM), anti-VCA-specific IgG, and anti-EBV early antigen (EA)-specific IgG. Anti-Epstein Barr nuclear antigen (EBNA)-specific antibodies were absent. Group 2 ($n= 25$) consisted of adults without clinical signs of EBV infection. All patients in Group 2 had both anti-EBNA-specific and anti-VCA-specific IgG antibodies, indicating past EBV infection. Group 3 consisted of anti-HIV positive patients ($n=25$) and Group 4 consisted of solid organ transplant recipients ($n=25$). All patients of group 3 and 4 had both anti-EBNA-specific and anti-VCA-specific IgG antibodies. The Group 5 ($n=23$) consisted of infants without clinical and serological signs of past or present EBV infection.

2.4. Statistical Considerations

The median values of positive results of EBV DNA levels in each of the patient groups were calculated. For comparative analysis, the Mann-Whitney U test was used and a finding of $P<0.05$ was considered statistically significant.

3. Results

3.1. Performance of the molecular assay

Detection of EBV DNA in clinical samples and QCMD 2002 EBV proficiency panel was performed with LightCycler EBV Quantification Kit with primers and probes specific for amplification and detection of the EBV DNA.

When the original samples of QCMD 2002 EBV proficiency panel were tested with the LightCycler EBV Quantification Kit, the results obtained from 4 samples were found to be within ± 0.50 log unit of those obtained by reference laboratories (Table 1). One sample that contained 1.0×10^3 copies per ml showed a 0.58 log unit difference from the expected result. Two samples that contained 1.0×10^2 and 5.0×10^2 copies per ml as well as the negative control were found to be below the detection limit.

Linearity was tested with a dilution series of a high-titer EBV DNA routine clinical EDTA whole blood sample. A quasilinear curve was observed between 1.1×10^3 and 9.5×10^6 copies per ml (Figure 1).

For determination of interassay variation, the mean EDTA whole blood EBV DNA titers ranged from 9.6×10^3 to 2.3×10^4 copies of EBV DNA per ml when samples were tested once on each of five different days. Coefficients of variation were found to be between 6 and 42%. Intra-assay variation was determined by testing four routine clinical samples with mean titers ranging from 3.4×10^3 to 2.0×10^4 copies of EBV DNA per ml, five times in one run. Coefficients of variation were found to be between 5 and 34% (Table 2).

3.2. Quantitation of EBV DNA in Different Groups of Patients

EDTA whole blood EBV DNA levels of patient groups 1 to 5 are shown in Figure 2. In patients with acute EBV infection (group 1), the median EDTA whole blood EBV DNA load was 1.7×10^4 copies/ml (range, 2.2×10^3 to 1.9×10^6 copies/ml). Samples of adults with past EBV infection (group 2) tested below the lower limit of detection, except of one sample with an EBV DNA level of 1.2×10^4 copies/ml. When EDTA whole blood EBV DNA levels of anti-HIV antibody positives (group 3) were determined, 9 of 25 (36%) patients were found to be below the lower limit of detection. The remaining 16 patients were positive for EBV DNA and the median EBV DNA load in these samples was found to be 1.6×10^4 copies/ml (range, 8.4×10^3 to 5.0×10^4 copies/ml). When EDTA whole blood EBV DNA levels of solid organ transplant recipients (group 4) were determined, 15 of 25 (60%) patients were found to be below the lower limit of detection. The remaining 10 patients were positive for EBV DNA; the median EBV DNA load was found to be 1.0×10^4 copies/ml (range, 4.5×10^3 to 4.8×10^4 copies/ml). All samples obtained from infants without clinical and serological signs of past or present EBV infection (group 5) were found to be below the lower limit of detection of the employed molecular assay.

When median EDTA whole blood EBV DNA levels of patients with acute EBV infection (group 1) were compared to those of anti-HIV antibody positives (group 3) or solid transplant recipients (group 4), no statistically significant differences were found ($p=0.7399$).

4. Discussion

The LightCycler EBV Quantification Kit found to be accurate. For samples of an EBV proficiency panel, correct results (i.e., within ± 0.5 log units) were obtained except for one result with a slightly increased deviation (+ 0.58 log units). According to the manufacturer's package insert, the lower limit of detection is ≤ 10 copies per reaction (i.e., ≤ 1000 copies per ml when using 200 μ l of input sample volume and 100 μ l of elution volume). Panel samples that contained 100 and 500 copies/ml were below the lower limit of detection. According to the QCMD 2002 EBV Proficiency Panel Program report, only 3 of 12 and 4 of 12 laboratories reported correct results for both samples, respectively, using this assay (Niesters H., 2003).

It should be emphasized that this assay allows more reliable and accurate EBV DNA quantification when compared to methods targeting the BAMHI-W repeats on the EBV genome.

The linearity was found to be sufficient between 1.1×10^3 and 9.5×10^6 copies per ml. The interassay variation ranged from 6 to 42% and intra-assay variation ranged from 5 to 34%. These results correspond to those reported for other molecular assays based on PCR amplification (Kessler et al., 2000; Stelzl et al., 2004).

When the utility of this assay was evaluated on clinical samples, EDTA whole blood was taken. Similar to other viruses of the herpesvirus family, EBV is associated with cell compartments. EDTA whole blood has been found to combine the viral load in both cell and plasma sources and ml of blood is a well-standardized unit of sample (Stevens et al., 2001b). Depending on type

and stage of disease EBV DNA may also be found in plasma, usually representing fragmented cell-derived material. However, during active infection EBV DNA can be detected for a longer period of time in whole blood than in plasma (Fafi-Kremer et al., 2004).

EBV DNA was extracted by a standardized, commercially available manual protocol. The assay used allows extraction within 45 minutes and is relatively easily to handle. With this method, both intra- and extracellular EBV DNA can be isolated in a rather simple procedure.

As expected, all patients with acute infection (group 1) tested positive with a relatively wide distribution of EBV DNA values. In group of adults with past EBV infection (group 2), no EBV DNA could be detected in EDTA whole blood, except of one patient. This patient had CMV infection detected by serology just one week earlier, so that CMV infection could have caused T-cell suppression and EBV reactivation.

In the group of anti-HIV antibody positive patients without apparent EBV-related disease (group 3), 16 out of 25 (64%) patients were found to be positive for EBV DNA. The corresponding number for solid organ recipients (group 4) was 10 out of 25 (40%). In the group of infants without serological and clinical signs of acute or earlier EBV infection (group 5), EBV DNA was not detected in any of the patients.

The results for anti-HIV antibody positive patients are in agreement with those reported recently (Fafi-Kremer et al., 2004). Similar results were obtained when peripheral blood mononuclear cells were tested for EBV DNA (Brenzel-Pesce et al., 2002). Positive results for EBV DNA may be caused indirectly by immunosuppression through either retroviral infection or immunosuppressive therapy. In addition, EBV DNA levels in peripheral blood

appear to be independent from both CD 4+ T-cell counts and HIV-1 RNA levels in plasma. It is documented that elevated EBV DNA is a common phenomenon for HIV patients, reflecting rather insufficient immune-mediated control of EBV replication than underlying lymphoproliferative disease (Stevens et al., 2002a). In organ transplant recipients, EBV DNA monitoring allows early diagnosis of threatening posttransplant lymphoproliferative disease, which is preceded by high viral load several months before outbreak of disease (Stevens et al., 2001a; Stevens et al., 2002b). Therefore, clinical relevance of increased EBV DNA levels in different groups of immunocompromised patients may be different.

On the other hand, single determination of the EBV DNA level has limited clinical relevance. Therefore, follow-up of EBV DNA dynamic fluctuations in patients with EBV-related diseases would be helpful for the monitoring of disease development and therapy response.

The LightCycler EBV Quantification Kit was found to be useful for determination of EBV DNA levels in EDTA whole blood.

Acknowledgments

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transplant recipients with primary EBV infection and/or post-transplant lymphoproliferative disease. *J. Med. Virol.* 69, 258-266.

Tables

Table 1. Results obtained by the LightCycler EBV Quantification Kit in comparison with those obtained by reference laboratories with members from the Quality Control for Molecular Diagnostics Epstein-Barr Virus Proficiency Program Panel 2002.

| Member no. | Results obtained by reference laboratories (copies/ml) | Results obtained by the LightCycler EBV Quantification Kit (copies/ml) | Log unit difference |
|------------|--|--|---------------------|
| 1 | 1.0×10^2 | UDL ^a | - |
| 2 | 0 | UDL ^a | - |
| 3 | 5.0×10^3 | 1.4×10^4 | +0.45 |
| 4 | 5.0×10^3 | 5.7×10^3 | +0.06 |
| 5 | 1.0×10^3 | 3.8×10^3 | +0.58 |
| 6 | 5.0×10^4 | 4.7×10^4 | -0.03 |
| 7 | 5.0×10^2 | UDL ^a | - |
| 8 | 1.0×10^4 | 1.8×10^4 | +0.26 |

^aUDL, under detection limit

Table 2. Results of interassay testing (one replicate of each sample was tested once on five different days) and intra-assay testing (five replicates were tested in one run).

| Interassay variation | | | |
|----------------------|---------------------------------|--------------------|------------------------------|
| Sample no. | Mean no. of EBV DNA (copies/ml) | Standard deviation | Coefficient of variation (%) |
| 1 | 9.6×10^3 | 4.0×10^3 | 42 |
| 2 | 1.7×10^4 | 5.6×10^3 | 34 |
| 3 | 1.2×10^4 | 3.5×10^3 | 28 |
| 4 | 1.4×10^4 | 2.9×10^3 | 20 |
| 5 | 1.4×10^4 | 2.9×10^3 | 20 |
| 6 | 1.8×10^4 | 3.8×10^3 | 21 |
| 7 | 2.1×10^4 | 1.4×10^3 | 6 |
| 8 | 2.3×10^4 | 8.9×10^3 | 38 |

| Intra-assay variation | | | |
|-----------------------|---------------------------------|--------------------|------------------------------|
| Sample no. | Mean no. of EBV DNA (copies/ml) | Standard deviation | Coefficient of variation (%) |
| 1 | 3.4×10^3 | 1.2×10^3 | 34 |
| 2 | 9.5×10^3 | 4.6×10^2 | 5 |
| 3 | 1.2×10^4 | 1.1×10^3 | 9 |
| 4 | 2.0×10^4 | 3.9×10^3 | 20 |

Figures

Figure 1. Linearity of the results for a 0.5-log-unit dilution series of a routine clinical EDTA whole blood sample with high titer of EBV DNA obtained by the LightCycler EBV Quantification Kit.

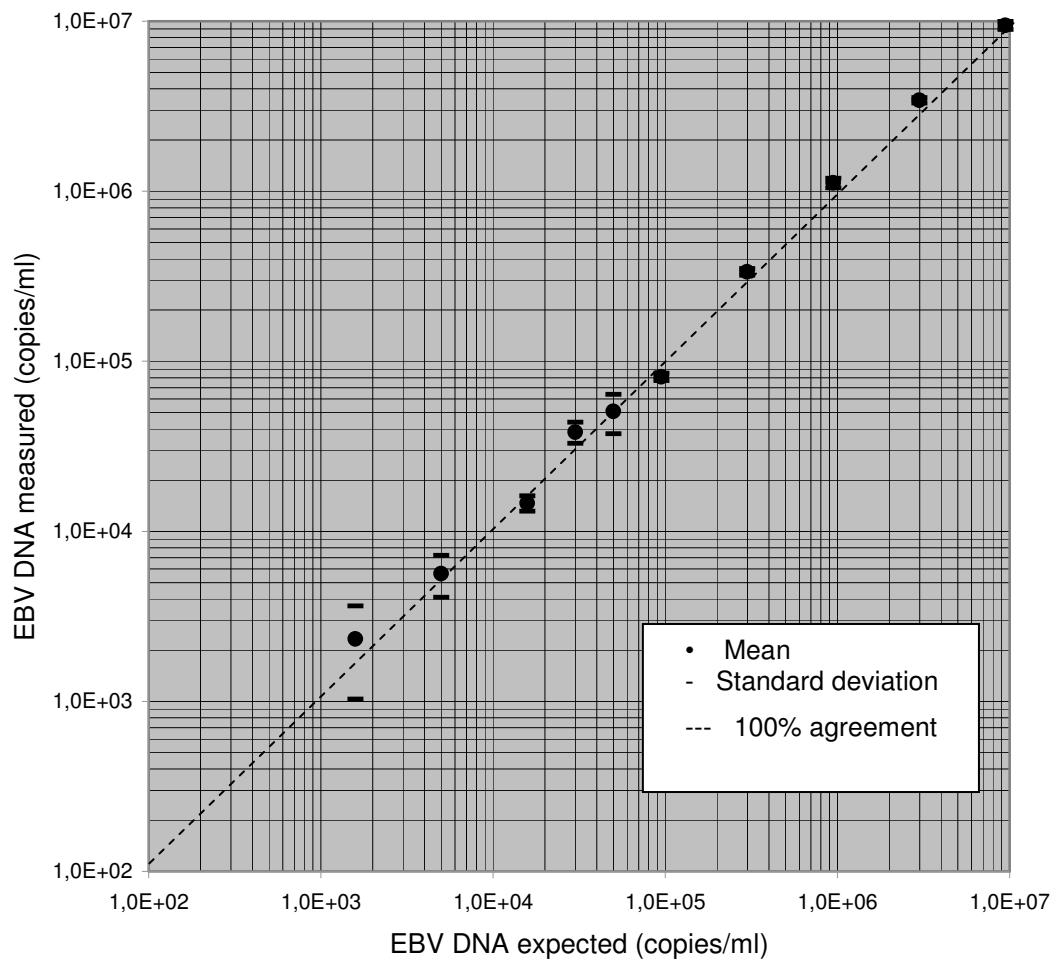


Figure 2. EBV DNA levels (copies/ml) in patients with acute EBV infection (group 1), in EBV-seropositive adults without clinical signs of EBV infection (group 2), in immunocompromised patients including anti-HIV antibody positives (group 3), in solid organ transplant recipients (group 4), and in infants without clinical and serological signs of past or present EBV infection (group 5). The dashed line indicates the lower detection limit.

