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Routine use of duplex real-time PCR assays including a commercial internal control for molecular diagnosis of opportunistic DNA virus infections

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A B S T R A C T

The aim of this work was to improve the validity of laboratory-developed real-time PCR protocols implemented in the laboratory for molecular diagnosis of opportunistic DNA virus infections using the Simplexa™ extraction and amplification control (SEAC) which allows the monitoring of the whole extraction and amplification process. Herpes simplex virus (HSV), varicella-zoster virus (VZV), human cytomegalovirus (CMV), Epstein–Barr virus (EBV), BK virus (BKV), and adenovirus (AdV) genomes were investigated in 152 different clinical specimens. The use of the SEAC did not influence the results of the different virus-specific PCRs. The SEAC results showed high reproducibility with a mean Cp value of 31.08 ± 1.44, and were not influenced by the virus-specific PCR performed or the type of clinical specimen tested. The SEAC in the DNA extracts showed high stability during storage at both +4 °C and −20 °C. These data allowed establishing a new procedure for the validation of viral PCR results. In conclusion, the SEAC provides a reliable option for improving the diagnosis of opportunistic viral infections by laboratory-developed real-time PCR assays in quality assurance programs.

1. Introduction

In the transplantation setting, opportunistic virus infections due to DNA viruses such as herpes simplex virus (HSV), varicella-zoster virus (VZV), human cytomegalovirus (CMV), Epstein–Barr virus (EBV), BK virus (BKV), and adenovirus (AdV) remain common complications with significant morbidity and occasional mortality (Feuchtinger et al., 2007; Hoffman, 2009; Miller and Dummer, 2007; Randhawa and Brennan, 2006; Razonable and Paya, 2003). Acute virological monitoring during the posttransplant period is essential in order to identify promptly incipient viral infections and to make the appropriate management decision, such as reducing the dose of maintenance immunosuppressive therapy or initiating an antiviral pre-emptive or curative chemotherapy.

The diagnosis of DNA viral infections based on the detection and/or quantitation of viral genome by real-time PCR has become the "gold-standard" in virology and is implemented widely in virological laboratories. The high sensitivity of real-time PCR enables early diagnosis of viral infections and therefore helps to prevent severe clinical complications. This technique allows virological diagnosis in a clinically relevant time frame. A wide variety of clinical samples can be tested, mainly depending on the type of viral infections: whole blood, plasma, cerebrospinal fluid, tissue biopsy, mucocutaneous lesion, bronchoalveolar lavage, stool, or urine. In the real-time PCR-based diagnostic setting, despite the availability of many commercial PCR kits, it remains necessary for end-user and reference laboratories to have access to "in-house" open-formula non-commercial PCRs for which information on target gene and reagents to use is available (Hoorfar et al., 2003). However, the choice of this home-made strategy makes it mandatory for monitoring the effectiveness of the whole technical process, including the DNA extraction step and the amplification step. The absence of PCR inhibitors can be checked by testing DNA extracts both undiluted and at a dilution of 1:10 (Deback et al., 2007; Najioullah et al., 2001). But this method is unsatisfactory since it is time-consuming and expensive because it requires two different amplifications for testing one single virus in a clinical sample. An alternative approach is to perform the detection of a housekeeping gene, such as albumin or β-actin genes, which is assumed to be present in all tested samples. However, since the initial copy number of housekeeping genes in the sample is unknown and can vary widely, this technique may not allow confirming the presence of low quantities of PCR inhibitors (Selvey et al., 2001; Stamey et al., 2001). The best strategy to monitor the whole process in order to detect systemic variation that may arise during both extraction and amplification steps of PCR includes the use of an exogenous internal extraction

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and amplification control (IEAC) (Hoorfar et al., 2004). The IEAC is added to the sample prior to DNA extraction. This type of approach has been reported previously for various DNA viruses such as HSV, VZV, CMV, human herpesvirus 8 (HHV-8), and hepatitis B virus (HBV) (Broccolo et al., 2002; Garson et al., 2005; Stranska et al., 2004; Sun et al., 2011; Tedder et al., 2002). However, the production of an IEAC in the laboratory requires good experience in the field. In consequence, the use of a commercial IEAC constitutes an attractive alternative.

The LightCycler® 480 (LC480) system (Roche Diagnostics, Meylan, France) constitutes a suitable and versatile real-time PCR instrument in a routine laboratory setting for the diagnosis and monitoring of opportunistic viral infections in transplant recipients using laboratory-developed PCR protocols (Deback et al., 2009). The aim of this study was to improve the validity of these home-made PCR protocols by the use of a universal and commercial IEAC in a routine laboratory setting for molecular diagnosis of opportunistic viral infections.

2. Materials and methods

2.1. Clinical samples

One hundred fifty-two clinical samples were tested: whole blood ($n = 77$), bronchoalveolar lavage ($n = 30$), viral transport medium containing mucocutaneous swabs ($n = 28$), urine ($n = 12$), and stool ($n = 5$). They were investigated by real-time PCR for HSV ($n = 40$), VZV ($n = 18$), CMV ($n = 54$), EBV ($n = 39$), BKV ($n = 36$), and AdV ($n = 5$). One hundred twelve clinical samples were tested for one single virus and 40 clinical samples were tested for two different viruses. The distribution of the clinical samples according to the type of sample, the number of viruses tested, and the PCR results is indicated in Table 1. All samples were stored at −80 °C and were selected retrospectively in order to obtain a representative panel of positive and negative samples considering the different viruses tested.

2.2. Simplexa™ extraction and amplification control set

The Simplexa™ extraction and amplification control (SEAC) set (Eurobio, Courtaboeuf, France) was evaluated in this study. This internal control corresponds to a 577-base pair DNA fragment derived from the gene encoding ribulose-1,5-bisphosphate carboxylase oxygenase large unit *N*-methyltransferase of the plant *Arabidopsis thaliana*. This is a noncompetitive internal control with its own mix (called primer mix) containing primers and a Quasar® 670 labeled-probe specifically designed for its amplification.

2.3. Nucleic acid extraction methods

DNA extraction was performed using two different automated instruments according to the routine process implemented in the laboratory for virological diagnosis: MagNA Pure Compact instrument (Roche Diagnostics, Meylan, France) for DNA extraction from whole blood, virus transport medium, and urine samples, and NucliSENS EasyMAG instrument (BioMérieux, Lyon, France) for DNA extraction from bronchoalveolar lavage and stool samples. For the SEAC evaluation, DNA extraction was performed from 400 μL of clinical sample in duplicate: one extraction without SEAC DNA and one extraction with SEAC DNA. For the SEAC DNA spiking, 5 μL of SEAC DNA were added directly to the sample, according to the manufacturer's instructions, and sample vortexing was performed during 5 s just before the start of DNA extraction process. DNA was eluted in a final volume of 100 μL and stored at −20 °C until use.

2.4. Real-time PCR protocols

Viral DNA amplifications were performed on the LC480 system using laboratory-developed real-time PCR assays based on hydrolysis probe technology implemented in the laboratory for virological diagnosis activity. PCR protocols for CMV, EBV, and BKV were carried out as described previously (Deback et al., 2009). For HSV, VZV, and AdV, LC480 protocols were developed after slight adaptations from the initial protocols previously reported (Cohrs et al., 2000; Heim et al., 2003; Kessler et al., 2000). The sequences of all primers and probes used in LC480 protocols are indicated in Table 2. Ten μL of extracted DNA were used for each amplification. For testing DNA extracts without SEAC, real-time PCRs were performed in 25 μL of mixture containing 1 × LightCycler® 480 Probes Master, 200 nM forward primer, 200 nM reverse primer, 100 nM hydrolysis probe, 0.40 units uracil-N-glycosylase (Invitrogen, Cergy-Pontoise, France). For BKV, primer concentration was 400 nM and probe concentration was 200 nM. For AdV, primer and probe concentrations were 500 nM. Primers and probes were purchased from Eurofins MWG Operon (Ebersberg, Germany). All probes for viral detection were FAM-labeled. For testing DNA extracts with SEAC, the composition of the PCR mix was identical to the one described above, except the addition of 0.5 μL of SEAC primer mix (replacing 0.5 μL of NAT water), according to the manufacturer's recommendations. For all viruses but AdV, cycling conditions on LC480 were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles, each consisting of 15 s at 95 °C and 40 s at 60 °C, and a final cooling step of 30 s at 40 °C (ramp rate of 4.4 °C/s). AdV assay was performed with the following cycling parameters: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles, each consisting of 15 s at 95 °C, 10 s at 55 °C, 60 s at 65 °C, and a final cooling step of 30 s at 40 °C (ramp rate of 4.4 °C/s). For SEAC unspiked samples, the viral DNA target amplification was monitored using the mono color hydrolysis probe FAM detection format on LC480 (monoplex PCR), and for SEAC spiked samples, both viral and SEAC DNA target amplifications were monitored using the dual color hydrolysis probe FAM/Cy5 detection format (duplex PCR). Viral quantitative results (i.e., CMV, EBV, and BKV loads in whole blood) were obtained using standard curves and results were expressed in log (viral copy number/mL). Given the extraction dilution, the sensitivity threshold was 1.40 log. Qualitative results for virus (in all samples different from whole blood) and for SEAC (in all samples) were expressed using the crossing point (Cp) value. For SEAC unspiked samples, DNA extracts were tested undiluted and 1:10 diluted in order to detect potential inhibitors, whereas DNA extracts from SEAC spiked samples were tested undiluted only.

2.5. Evaluation of SEAC stability in DNA extracts during storage

The stability of SEAC in DNA extracts was evaluated for different storage conditions: one week at +4 °C for 30 clinical samples (whole blood, $n = 10$; bronchoalveolar lavage, $n = 10$; urine, $n = 10$) and one month at −20 °C for 20 whole blood samples.

2.6. Statistical analyses

Viral loads in whole blood samples were analyzed for the correlation between assays without and with SEAC. A nonparametric Spearman correlation test was conducted to assess the strength of linear association between viral loads obtained with both assays. A Bland–Altman plot was used to assess graphically the magnitude of disagreement between both assays. The nonparametric Mann–Whitney *U* test was used to compare continuous variable from different groups (i.e., SEAC Cp values from positive and negative samples). The nonparametric Wilcoxon rank test was used to compare continuous variable from related samples (i.e., SEAC Cp