Optimisation of a quantitative polymerase chain reaction-based strategy for the detection and quantification of human herpesvirus 6 DNA in patients undergoing allogeneic haematopoietic stem cell transplantation

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Human herpesvirus 6 (HHV-6) may cause severe complications after haematopoietic stem cell transplantation (HSCT). Monitoring this virus and providing precise, rapid and early diagnosis of related clinical diseases, constitute essential measures to improve outcomes. A prospective survey on the incidence and clinical features of HHV-6 infections after HSCT has not yet been conducted in Brazilian patients and the impact of this infection on HSCT outcome remains unclear. A rapid test based on real-time quantitative polymerase chain reaction (qPCR) has been optimised to screen and quantify clinical samples for HHV-6. The detection step was based on reaction with TaqMan® hydrolysis probes. A set of previously described primers and probes have been tested to evaluate efficiency, sensitivity and reproducibility. The target efficiency range was 91.4% with linearity ranging from 10-106 copies/reaction and a limit of detection of five copies/reaction or 250 copies/mL of plasma. The qPCR assay developed in the present study was simple, rapid and sensitive, allowing the detection of a wide range of HHV-6 loads. In conclusion, this test may be useful as a practical tool to help elucidate the clinical relevance of HHV-6 infection and reactivation in different scenarios and to determine the need for surveillance.

Key words: human herpesvirus 6 - real-time PCR - viral load

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Haematopoietic stem cell transplantation (HSCT) has become an important treatment modality of malignant and nonmalignant haematological diseases (Kernan et al. 1993, Chapenko et al. 2012), but immunosuppression may allow the development of infections that remain a concerning cause of post-transplant morbidity and mortality (Cordonnier 2008). Viral infections are frequent after HSCT and may be life threatening, especially when affecting the lung, liver or central nervous system in allogeneic HSCT and solid organ recipients (Boutolleau et al. 2003, de Pagter et al. 2008a, Schonberger et al. 2010, Pollack et al. 2011, Gotoh et al. 2014). Human herpesvirus (HHV) appears to play an important a role in this setting (Jenkins et al. 2002, Razonable & Paya 2003, Kalpoe et al. 2006, Ogata 2009, Iliaquer et al. 2014) due to a lack of standardised diagnostic methods and appropriate follow-up, which make the definition and interpretation of HHV-6 infection symptoms difficult (Zerr et al. 2005, Leibovitch et al. 2014).

In Brazil, the first population-based study reporting the prevalence of HHV-6 antibodies was performed by Linhares et al. (1991), who found rates of 76.5% for Brazilians and 77.2% for Japanese immigrants. Since then, studies on HHV-6 seroprevalence and DNA detection (SybrGreen® based technology) have been reported in solid organ transplant recipients (de Freitas et al. 1994, de Freitas & Linhares 1997, Oliveira et al. 2001, Canto et al. 2008, Cavalcanti 2011, Magalhães et al. 2011, Guardia et al. 2012, 2014), but the detection by real-time quantitative polymerase chain reaction (qPCR) (TaqMan® technology based) in HSCT recipients has not been systematically performed in Brazilian patients. The development of diagnostics and immunology methods for HHV-6 detection can provide better surveillance of HHV-6 reactivation (de Pagter et al. 2008b, 2010, Betts et al. 2011, Gerdemann et al. 2013, Leibovitch et al. 2014). The aim of this study is to optimise a qPCR assay for HHV-6 detection and quantification in plasma samples.


SUBJECTS, MATERIALS AND METHODS

Samples - Peripheral blood samples from 98 patients undergoing HSCT were collected in sterile EDTA k3 treated tubes. In total, 1,082 plasma samples were included in the study. Samples were collected starting on D0 (transplantation day), weekly until D+100, unless otherwise requested by the attending physician.

HSCT patients’ samples were used to validate the technique, as they were part of a clinical study to evaluate the impact of HHV-6 infection in these patients.

DNA - DNA extraction was performed from 400 µL of plasma using the QIAamp DNA Blood Mini QIAcube Kit and the QIAcube robot (Qiagen) following the manufacturer’s protocol. Purified DNA was eluted in 100 µL nuclease free water and stored at -20ºC until use.

Positive control - Frozen MOLT-3 cells infected with HHV-6 (Z29 strain) were kindly provided by the HHV-6 Foundation. The infected cells (1 x 10⁶) were thawed, washed twice and suspended in phosphate-buffered saline (400 µL) for DNA extraction, following the manufacturer’s protocol (QIAamp DNA Blood Mini Kit) and eluted in 100 µL of nuclease free water (Promega P1193).

Primers and probe sequence - The set of primers and TaqMan® probe for the hydrolysis approach has been described by Sugita et al. (2008). The primers (Fw: GA-CAATCACATGCTTGATATGT, Rv: TGTAAGCGTAGTGTGAAT) amplify a 173-176 bp sequence within the U65-U66 genes of HHV-6A and HHV-6B and the probe was labelled with 5’carboxyfluorescein (FAM) and carboxytetramethylrhodamine (TAMRA) at the 3’ (P: FAM-AGCAGTGCCGAAAAAGTGCTGTC-TAM-RA) (Gautheret-Dejean et al. 2002, Sugita et al. 2008).

Standard curve - PCR products were purified with MinElute PCR Purification kit (Qiagen) and 10-fold serial dilutions (10⁻¹⁰copies) were used as standard curve (Table I). To avoid and reduce the loss of amplicons, nuclease-free water with carrier (100 ng Yeast tRNA, Invitrogen, 15401-011) was used as the diluent.

In house PCR - Template DNA was obtained by conventional PCR reaction using Platinum Taq DNA Polymerase Kit (Invitrogen, 10966-026). Each 50 µL amplification reaction contained: HHV-6 Z29 DNA 5 µL, 10X PCR Buffer without MgCl₂, (total volume 5 µL), 10 mM dNTP mixture 1 µL, 50 mM MgCl₂, 2 µL, 10 µM Fw and Rv primers 1 µL (Life Technologies), Platinum Taq DNA Polymerase 0.4 µL and water nuclease free 34.6 µL (Promega, P1193).

A Veriti® Thermal Cycler (Applied Biosystems) was used to perform the reactions as follows: 95ºC for the 5 min initial denaturation step, 40 cycles (95ºC for 45 s, 55ºC for 45 s and 72ºC for 1 min) and 72ºC for the 7 min final extension.

PCR product purification - The PCR product (40 µL) was purified with MinElute Kit (Qiagen), which allows recovery of 70 bp-4 kb fragments and was eluted in 10 µL of nuclease free water (Promega, P1193). After purification step, the PCR product (1 µL) was submitted to electrophoresis for integrity evaluation (Bioanalyzer-Agilent) using Agilent DNA 1000 kit. The purification and electrophoresis were made according to the manufacturer protocol.

PCR product quantification - The PCR product quantification (Qubit® 2.0 Fluorometer) was made after purification, using Qubit® dsDNA HS (High Sensitivity)

<table>
<thead>
<tr>
<th>HHV-6 copies/µL</th>
<th>HHV-6 PCR product amount (µL)</th>
<th>Dilution H₂O amount (µL)</th>
<th>Final volume (µL)</th>
<th>Final concentration (copies/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42.5 x 10¹⁰</td>
<td>2.4</td>
<td>47.6</td>
<td>50</td>
<td>2 x 10¹⁰</td>
</tr>
<tr>
<td>2 x 10⁹</td>
<td>100</td>
<td>900</td>
<td>1,000</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>2 x 10⁸</td>
<td>100</td>
<td>900</td>
<td>1,000</td>
<td>2 x 10⁵</td>
</tr>
<tr>
<td>2 x 10⁷</td>
<td>100</td>
<td>900</td>
<td>1,000</td>
<td>2 x 10⁴</td>
</tr>
<tr>
<td>2 x 10⁶</td>
<td>100</td>
<td>900</td>
<td>1,000</td>
<td>2 x 10³</td>
</tr>
<tr>
<td>2 x 10⁵</td>
<td>100</td>
<td>900</td>
<td>1,000</td>
<td>2 x 10²</td>
</tr>
<tr>
<td>2 x 10⁴</td>
<td>100</td>
<td>900</td>
<td>1,000</td>
<td>2 x 10¹</td>
</tr>
<tr>
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<td>2 x 10⁰</td>
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<td>200</td>
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<tr>
<td>2 x 10⁻¹</td>
<td>100</td>
<td>900</td>
<td>200</td>
<td>5 x 10⁻¹</td>
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<td>5 x 10⁻¹</td>
<td>100</td>
<td>900</td>
<td>200</td>
<td>5 x 10⁻¹</td>
</tr>
</tbody>
</table>

HHV: human herpesvirus; PCR: polymerase chain reaction.
HHV-6 positive reactions were confirmed by a second independent analysis in duplicates using the same detection method.

**Ethics** - The study protocol was approved by the Research Ethical Committee of Federal University of São Paulo (protocol CEP 1297/08) and of all participating centres’ local committees and written informed consent was obtained from all subjects.

### RESULTS

#### Primers and probe

An optimisation approach was used to achieve the optimal concentration of primers and probe, starting with 500 nM from each primer (Fw/Rv) and 300 nM probe. The combination of primers (Fw/Rv) at 250 nM and probe at 200 nM was the optimal concentration, resulting in a decrease of Ct from 37.9-32.4.

**Positive control** - The PCR product electrophoresis (Bioanalyzer-Agilent) showed a unique and well defined band around 180 bp and the fluorometric quantification (Qubit® 2.0 Fluorometer) was 80.8 ng/µL or 42.5 x 10¹⁰ copies/µL.

**Internal control** - A commercial internal positive control (IPC) (Life Technologies) was used as an internal control. The virus probe was labelled with FAM™, while the IPC probe was labelled with VIC® dye. 2 μL IPC DNA 50X (1:20 water diluted) was spiked in 400 μL of plasma sample before the extraction and its detection was performed in a duplex reaction with HSV1 (data not shown).

**Real-time reaction** - The qPCR reaction (25 μL reaction) contained: extracted DNA solution or water in the no template control (NTC) reactions 5 μL, 2X TaqMan Universal Master Mix 12.5 μL (Life Technologies), 10 nM primer (Fw and Rv) 0.625 μL (Life Technologies), 10 nM probe 0.5 μL (Life Technologies) and water 5.75 μL. All qPCR reactions were set up in a semi-automated workflow Qiagility (Qiagen) and performed in a 7900HT (Life Technologies). Thermal cycling consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

#### Table II: Detection rate and cycle threshold (Ct) values of standard curve

<table>
<thead>
<tr>
<th>Copies/reaction</th>
<th>Detection rate (%)</th>
<th>Cts mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000,000</td>
<td>100</td>
<td>18.10</td>
<td>0.14</td>
<td>0.80</td>
</tr>
<tr>
<td>100,000</td>
<td>100</td>
<td>21.71</td>
<td>0.06</td>
<td>0.27</td>
</tr>
<tr>
<td>10,000</td>
<td>100</td>
<td>25.19</td>
<td>0.12</td>
<td>0.47</td>
</tr>
<tr>
<td>1,000</td>
<td>100</td>
<td>28.74</td>
<td>0.06</td>
<td>0.21</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>32.35</td>
<td>0.21</td>
<td>0.64</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>35.86</td>
<td>0.45</td>
<td>1.24</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>37.63</td>
<td>0.82</td>
<td>2.19</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>38.11</td>
<td>0.69</td>
<td>1.82</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>38.67</td>
<td>0.69</td>
<td>1.77</td>
</tr>
</tbody>
</table>

CV: coefficient of variation; SD: standard deviation.

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**Real-time reaction** - The reaction linearity was 10-10⁶ copies/reaction or 500-5 x 10⁷ copies/plasma mL and the

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**Fig. 1**: human herpesvirus (HHV)-6 standard curve before optimisation.
LoD found was approximately five copies of target DNA/reaction, representing 250 copies/plasma mL (Table II). The reaction efficiency was 78.3% (slope -3.98) when the primers and probe concentration was 500 nM and 200 nM, respectively (Fig. 1). After primers optimisation (250 nM of primers and 200 nM of probe concentrations), the efficiency result was 91.4% and slope -3.546 (Fig. 2).

The inter-assay variation (reproducibility) range was 0.78-2.82% (Table III) and the test resulted to be specific as no false positive were detected even in the presence of other herpesviruses fragments (HSV1, HSV2, VZV, EBV, CMV, HHV-7 and HHV-8).

Quantitation of HHV-6 DNA in plasma samples - HHV-6 genome sequences were detected in 2% (18/1,082) of plasma samples. Among these samples, 39% (7/18) yielded more than 250 HHV-6 copies/plasma mL (LoD value) and the highest HHV-6 mean viral load (VL) detected was 69,163 copies/mL.

Despite the low LoD, samples with results lower than 250 copies/mL were retested a second time and all results were confirmed.

Four patients (P1, P2, P3 and P4) presented persistent viraemia after transplant and all cases are illustrated in Fig. 3.

DISCUSSION


In this study a strategy to optimise a qPCR technique using a standard curve constructed with PCR product was applied, which seemed to be quite effective when compared...
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