

# 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<sup>®</sup> 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 10-10<sup>6</sup> 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

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 role in this setting (Jenkins et al. 2002, Razonable & Paya 2003, Kalpoe et al. 2006, Ogata 2009, Al Fawaz et al. 2014).

HHV-6 was first isolated by Salahuddin et al. (1986) from peripheral blood mononuclear cells of patients with lymphoproliferative disorders and has been identified as the causative agent of *exanthem subitum* (*roseola infantum*) (Yamanishi et al. 1988). HHV-6 is still considered to be emergent and is commonly associated with reactivation in patients undergoing HSCT (Ljungman 2002, Yoshikawa 2004, Sakai et al. 2011, Robles et al. 2014), but its clinical presentations in this setting remain obscure (Wang et al. 2006, Ogata 2012, Jeulin et al. 2013, Illiaquer 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<sup>®</sup> 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<sup>®</sup> 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.

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### 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 assistant 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  $\mu$ L 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  $\mu$ L nuclease free water and stored at -20°C 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 ( $1 \times 10^6$ ) were thawed, washed twice and suspended in phosphate-buffered saline (400  $\mu$ L) for DNA extraction, following the manufacturer's protocol (QIAamp DNA Blood Mini Kit) and eluted in 100  $\mu$ L of nuclease free water (Promega P1193).

**Primers and probe sequence** - The set of primers and TaqMan<sup>®</sup> probe for the hydrolysis approach has been described by Sugita et al. (2008). The primers (Fw: GA-CAATCACATGCCTGGATAATG, Rv: TGTAAGCGT-GTGGTAATGTACTAA) 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-AGCAGCTGGCGAAAAGTGCTGTGC-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 ( $10$ - $10^6$  copies) 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  $\mu$ L amplification reaction contained: HHV-6 Z29 DNA 5  $\mu$ L, 10X PCR Buffer without  $MgCl_2$  (total volume 5  $\mu$ L), 10 mM dNTP mixture 1  $\mu$ L, 50 mM  $MgCl_2$  2  $\mu$ L, 10  $\mu$ M Fw and Rv primers 1  $\mu$ L (Life Technologies), Platinum Taq DNA Polymerase 0.4  $\mu$ L and water nuclease free 34.6  $\mu$ L (Promega, P1193).

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

**PCR product purification** - The PCR product (40  $\mu$ L) was purified with MinElute Kit (Qiagen), which allows recuperation of 70 bp-4 kb fragments and was eluted in 10  $\mu$ L of nuclease free water (Promega, P1193). After purification step, the PCR product (1  $\mu$ L) 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<sup>®</sup> 2.0 Fluorometer) was made after purification, using Qubit<sup>®</sup> dsDNA HS (High Sensitivity)

TABLE I  
Standard curve dilution

HHV-6 copies/ $\mu$ L	HHV-6 PCR product amount ( $\mu$ L)	Dilution $H_2O$ amount ( $\mu$ L)	Final volume ( $\mu$ L)	Final concentration (copies/ $\mu$ L)
$42.5 \times 10^{10}$	2.4	47.6	50	$2 \times 10^{10}$
$2 \times 10^{10}$	100	900	1,000	$2 \times 10^9$
$2 \times 10^9$	100	900	1,000	$2 \times 10^8$
$2 \times 10^8$	100	900	1,000	$2 \times 10^7$
$2 \times 10^7$	100	900	1,000	$2 \times 10^6$
$2 \times 10^6$	100	900	1,000	$2 \times 10^5$
$2 \times 10^5$	100	900	1,000	$2 \times 10^4$
$2 \times 10^4$	100	900	1,000	$2 \times 10^3$
$2 \times 10^3$	100	900	1,000	$2 \times 10^2$
$2 \times 10^2$	100	900	1,000	$2 \times 10^1$
$2 \times 10^1$	100	100	200	$1 \times 10^1$
$2 \times 10^1$	100	900	1,000	$2 \times 10^0$
$2 \times 10^0$	100	100	200	$1 \times 10^0$
$2 \times 10^0$	100	900	1,000	$2 \times 10^{-1}$
$1 \times 10^0$	100	100	200	$5 \times 10^{-1}$

HHV: human herpesvirus; PCR: polymerase chain reaction.

Assay Kit, following manufacturer protocol and the approximate copy numbers were calculated using the following formula:

$$\text{Copy n}^\circ = \frac{Q \times 6.022 \times 10^{23}}{T \times 1 \times 10^9 \times 650}$$

where Q = PCR product quantification and T= amplicon length.

The number of copies allowed the construction of the standard curve and was used to determine the linearity, limit of detection (LoD), inter-assay reproducibility, efficiency and specificity of the reaction.

Reaction linearity and LoD were determined analysing 12 replicates of 1-10<sup>6</sup> copies/reaction and the inter-assay reproducibility was assessed by calculating the coefficient of variation (CV) of cycle threshold (Ct) values from 10 replicates of 10-10<sup>6</sup> copies/reaction. The efficiency was determined by the slope of the reaction and the test specificity by testing the same sample for herpes simplex virus (HSV)1, HSV2, varicella zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), HHV-7 and HHV-8 qPCRs.

*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

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

CV: coefficient of variation; SD: standard deviation.

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 10<sup>10</sup> copies/µL.

*Real-time reaction* - The reaction linearity was 10-10<sup>6</sup> copies/reaction or 500-5 x 10<sup>7</sup> copies/plasma mL and the

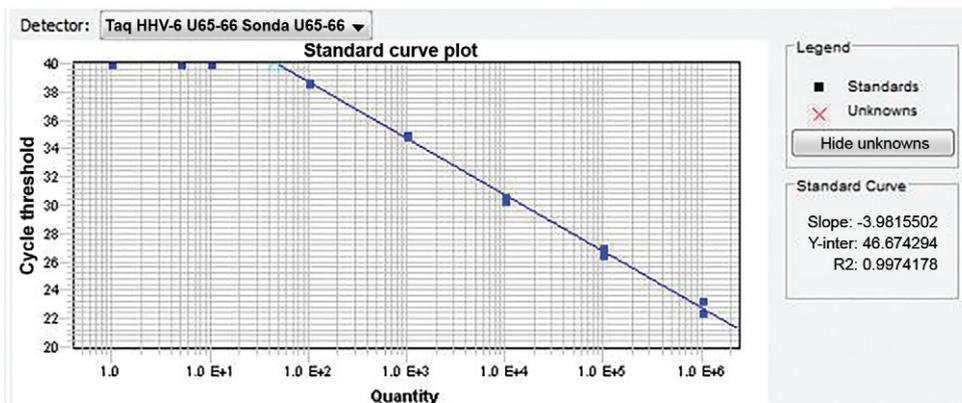


Fig. 1: human herpesvirus (HHV)-6 standard curve before optimisation.

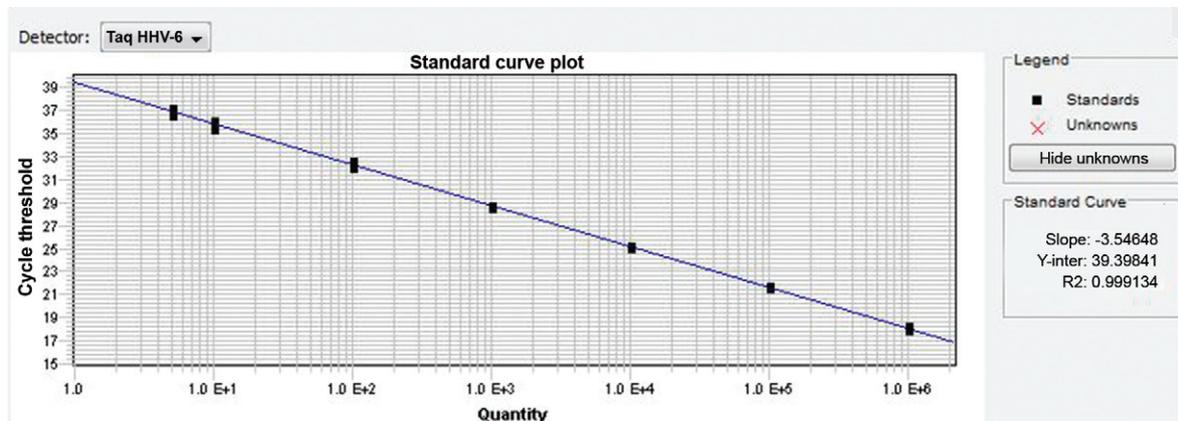


Fig. 2: human herpesvirus (HHV)-6 standard curve after optimisation.

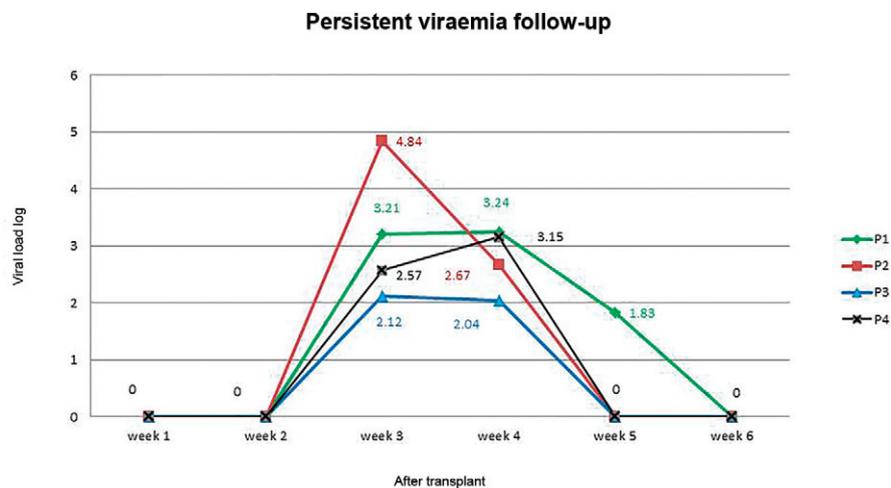


Fig. 3: persistent viraemia follow-up. P: patients.

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

Monitoring HHV-6 and providing precise, rapid and early diagnosis of related clinical diseases constitutes an essential measure to improve disease outcome on several backgrounds, such as HHV-6 reactivation after umbilical cord blood transplantation (Chevallier et al. 2010, Mori et al. 2010, Ogata et al. 2013, Scheurer et al. 2013, Illiaquer et al. 2014, Le Bourgeois et al. 2014), and the unexplained presence of HHV-6 DNAemia associated to specific clinical conditions (Zerr et al. 2005, Ogata 2009, Meyding-Lamadé & Strank 2012, Al Fawaz et al. 2014, Shoji et al. 2014, Ahluwalia et al. 2015). Additionally, it has potential to elucidate the clinical significance of recent reported presence of genomic integration (Pellett et al. 2012, Panty et al. 2013, Bell et al. 2014, Hill et al. 2015).

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

TABLE III  
Reproducibility analysis

Copies/reaction	Cts mean	SD	CV (%)
10 <sup>6</sup>	18.30	0.17	0.94
10 <sup>5</sup>	21.89	0.17	0.78
10 <sup>4</sup>	25.46	0.24	0.94
10 <sup>3</sup>	29.07	0.36	1.25
10 <sup>2</sup>	32.77	0.52	1.58
0 <sup>1</sup>	36.67	1.03	2.82

Ct: cycle threshold; CV: coefficient of variation; SD: standard deviation.

to the results of a qPCR standardisation reaction using a standard curve constructed with clones inserted into the plasmid, which is a well-described technique in the literature (Locatelli et al. 2000, Collot et al. 2002, Gautheret-Dejean et al. 2002, Ogata et al. 2006, Isegawa et al. 2007).

The first tests with the primers and probe showed that the linearity between 10-100 copies/reaction (500-5,000 copies/plasma mL) were negatively affected when using 500 nM primers and 300 nM probe concentrations. The results of primers and probe test optimisation using 100 HHV-6 copies/reaction (target) with 500 nM of each primer and 300 nM of probe concentrations, initially showed a 37.9 Ct reaction. When these concentrations were modified to 250 nM of each primer and 200 nM of probe, the Ct reaction decreased to 32.4, showing an efficiently improvement of primers and probe. Other target concentrations (50 and 10 HHV-6 copies/reaction) were tested with 250 nM and 200 nM of primers and probe concentrations, respectively and similar Ct reaction reductions were observed. Gautheret-Dejean et al. (2002) have used 200 nM of each primer and 100 nM probe.

As previously mentioned, a purified PCR product was used to build a standard curve. The purified PCR product was submitted to electrophoresis for integrity evaluation (Bioanalyzer-Agilent) and a 180 bp amplicon was found, close to 176 bp amplicon founded by Gautheret-Dejean et al. (2002). The fluorometric PCR product quantitation (80.8 ng/ $\mu$ L) was used to estimate the copy number/ $\mu$ L ( $42.5 \times 10^{10}$  copies/ $\mu$ L) that allowed the generation of a standard curve.

The 10-fold dilution curve presented linearity ranging from 10-10<sup>6</sup> copies/reaction (500 to  $5 \times 10^7$  copies/plasma mL). Collot et al. (2002) and Isegawa et al. (2007) have founded linearity ranging 10<sup>1</sup>-10<sup>8</sup> copies/reaction.

The LoD determined was five copies/reaction or 250 copies/plasma mL, which means the lowest analyte amount present in a sample that could be detected with 95% probability, although not quantified as an exact value. All results between two-five copies/reaction comprised a "gray zone" (linearity range out) and have been tested for a second time. These results have all been confirmed. Our results were similar to the LoD reported by de Pagter et al. (2008b) of 250 copies/mL approximately. Tavakoli et al. (2007) detected five gene copies/reaction

or 200 gene copies/sample mL, and the LoD related by Yao et al. (2009) was 10 copies/reaction in a nested PCR reaction. Sugita et al. (2008) defined a cut-off of 50 copies per tube and the sensitivity described by Gautheret-Dejean et al. (2002) was 10 genomic equivalent copies/reaction. The lack of HHV-6 quantification standardisation represents a substantial issue on this scenario, which makes comparisons difficult.

The reaction efficiency before primers optimisation was 78.3% (slope -3.98) and after optimisation a good improvement could be observed; the target efficiency obtained was 91.4% (slope -3.546).

The slope values presented by Watzinger et al. (2004) and Wada et al. (2009) were slope -3.374 and slope -3.135, respectively.

Dilutions from 10<sup>1</sup>-10<sup>6</sup> copies/reaction have been evaluated to provide the reproducibility or inter-assay variation and the CV mean found was 1.39 (range 0.78-2.82). Collot et al. (2002) found similar results, range of 0.80-0.96 and Watzinger et al. (2004) presented a CV = 1.60% in inter-assay variation whereas Isegawa et al. (2007) related CV = 1.36% of intra-assay variation. No cross-reaction or false positive results were detected.

All samples with results lower than 250 copies/mL were tested a second time. The samples with more than five copies/reaction (or 250 copies/mL) on the second test were considered "positive" and the samples with less than five copies/reaction (or 250 copies/mL) were considered "negative". The significance of the HHV-6 positive result remains unclear, however, we believe that all patients whose VL is around 200 copies/mL should provide another blood sample after at least three-four days after the first sample was collected and get tested again in order to observe the VL trend (increasing or decreasing).

In conclusion, the test developed in the present study was simple, rapid and sensitive, allowing the detection of a wide range of HHV-6 loads. It 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.

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