ORAL DISEASES

Oral Diseases (2010) 16, 210–216. doi:10.1111/j.1601-0825.2009.01637.x

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ORIGINAL ARTICLE

# Saliva as a source of HCMV DNA in allogeneic stem cell transplantation patients

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**OBJECTIVE: The purpose of this study was to investigate the use of saliva for the identification of human cytomegalovirus (HCMV) in allogeneic hematopoietic stem cell transplant patients by real time PCR compared with blood.**

**MATERIALS AND METHODS: Saliva and blood samples were sampled weekly in 30 allogeneic hematopoietic stem cell transplant patients until 100 days after transplant. Total genomic DNA, extracted from saliva and whole-blood samples, was used for HCMV real time PCR. Nonparametric tests were performed, and *P* value ≤0.05 was considered statistically significant.**

**RESULTS: Human cytomegalovirus DNA load in saliva showed a high correlation with viral DNA in the blood (*R* = 0.858; *P* < 0.0001). Blood DNA levels also correlated with HCMV antigenemia (*R* = 0.773; *P* < 0.0001). The HCMV levels in saliva (*P* = 0.015) and blood (*P* = 0.008) showed higher levels at the beginning of antiviral treatment, with clear reduction after this period. Saliva showed earlier HCMV reactivation than blood detected by real time PCR and antigenemia assay in 11 out of 22 subjects.**

**CONCLUSIONS: This study shows that the real time PCR test could be useful to identify HCMV DNA in saliva and to monitor patients at risk of cytomegalovirus disease after allogeneic hematopoietic stem cell transplant. However, further studies are necessary to confirm this data.**

*Oral Diseases* (2010) **16**, 210–216

**Keywords:** human cytomegalovirus; allogeneic hematopoietic stem-cell transplantation; saliva

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Received 10 June 2009; revised 29 July 2009; accepted 9 September 2009

## Introduction

Human cytomegalovirus (HCMV), a member of the Herpesviridae family, has the ability to establish lifelong persistence and latent infection following primary exposure (Nichols and Boeckh, 2000). HCMV may remain latent in polymorphonuclear cells, dendritic cells, endothelial cells and salivary glands (Grefte *et al*, 1993; Wagner *et al*, 1996; Nichols and Boeckh, 2000). Salivary glands ductal epithelium and blood vessels endothelium are infected by HCMV (Kanas *et al*, 1987), but the virus can be shed in saliva (Canto *et al*, 2000) blood and other secretions (Kano and Shiohara, 2000). Direct exposure to infected saliva is the main mechanism responsible for transmitting HCMV (Canto *et al*, 2000).

Primary and recurrent HCMV disease is considered the single most important infectious complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT) (Allice *et al*, 2008). The majority of active HCMV infection cases are usually due to reactivation of latent infection. HCMV infections are ubiquitous in Brazilian population and about 95% of the adult individuals are HCMV seropositive (Canto *et al*, 2000). We have previously demonstrated that immunosuppression related to allo-HSCT affects HCMV reactivation in the oral mucosa (Correia-Silva *et al*, 2007).

Preemptive therapy in HSCT patients, a procedure mainly based on HCMV pp65-antigen detection on peripheral blood leukocytes, is associated with a significant reduction in the incidence of HCMV disease (Boeckh and Boivin, 1998). However, pp65-antigenemia assay is labor intensive and is not usually performed when leukopenia is present (Kalpoe *et al*, 2004). Quantification of HCMV DNA in blood using real time PCR technique has demonstrated a relationship between viral load and the risk of HCMV disease (Emery, 2001). Considering that salivary glands harbor latent HCMV infection and that evidences point that immunosuppression is related to its reactivation, the purpose of this study was to investigate the viability of applying saliva test by real time PCR for identification of HCMV DNA in allo-HSCT patients and to compare it to blood.

## Materials and methods

*Patients and samples*

The study was approved by the local Ethics Committee. Thirty consecutive allo-HSCT recipients at Hospital das Clinicas (HC-UFMG), between October 2006 and November 2007, were included in this prospective and longitudinal study. Recipients were conditioned for allo-HSCT according to the protocols of the allo-HSCT Unit of HC-UFMG. Cyclosporin (CSA), in combination with Methotrexate (MTX) or Mycophenolate mofetil, was used for graft *vs* host-disease (GVHD) prophylaxis and methylprednisolone 2 mg $kg^{-1}$ for GVHD treatment. Hemocomponents were leukodepleted only for patients with a negative HCMV serology to prevent HCMV infection and reactivation after HSCT.

Patient demographic information, as well as clinical and laboratory data included source of stem cells (bone marrow or peripheral blood stem cell), gender and age of the patient, donor gender, and HCMV-serostatus of donor and recipient before transplant, conditioning regimens, HLA matching (matched related, mismatch related or unrelated matched), pp65 antigenemia and use of ganciclovir (Table 1). Patients were followed up before from 7 days until 100 days after allo-HSCT (day +100) or until death of recipient. Saliva and whole-blood samples were attempted to be simultaneously obtained from each recipient once a week. To collect saliva sample, swabs were placed on normal oral mucosa (labial, buccal, and tongue) of the HSCT subjects using sterile citobrush (Kolplast Ltda, Brazil), placed immediately in 500 $\mu$l of Krebs buffer (NaCl 20%, KCl 2%, CaCl22H2O 2%, MgSO4, KH2-PO4, C6H12O6), and then stored at −20°C until processing. Peripheral blood (4 ml) was collected in an EDTA tube and stored at −20°C until processing. The collection of saliva samples was not possible in some situations due to hyposalivation or xerostomia.

**Table 1** Clinical characteristics of alo-HSCT patients ($n$ = 30)

| | |
|---|---|
| Median age in years (range) | 30.5 (11–54) |
| Recipient gender | |
| Male | 18 (60%) |
| Primary disease | |
| Malignant | 21 (70%) |
| Chronic myeloid leukemia | 9 (45%) |
| Acute myeloid leukemia | 6 (30%) |
| Hodgkin's lymphoma | 2 (10%) |
| Other[a] | 4 (20%) |
| Severe aplastic anemia | 9 (30%) |
| Stem cell source | |
| Bone marrow | 19 (64%) |
| Peripheral blood stem cell | 11 (36%) |
| HLA match | |
| HLA matched related | 25 (83%) |
| HLA matched unrelated | 3 (10%) |
| HLA mismatched related | 2 (7%) |
| HCMV serology status recipient/donor | |
| Positive/Positive | 26 (87%) |
| Positive/Negative | 2 (7%) |
| Negative/Positive | 1 (3%) |
| Negative/Negative | 1 (3%) |

[a]Acute lymphoid leukemia ($n$ = 1), multiple myeloma ($n$ = 1), myelodysplastic syndrome ($n$ = 1) and myelofibrosis ($n$ = 1).

*DNA extraction*

Total genomic DNA was extracted from saliva and whole-blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Final elution of saliva DNA and blood DNA was performed in 50 $\mu$l of specific buffer AE of the Kit and stored at −20°C until further use. Two hundred nanograms of extracted DNA were used for HCMV real time PCR. The concentration of the extracted DNA was quantified by spectrophotometric measurement at a wavelength of 260 nm using a BioSpec-mini (Shimadzu Corporation, Kyoto, Japan).

*Real time PCR*

Human cytomegalovirus specific PCR primers used in the assay were selected from the US17 region of HCMV AD169 (Machida *et al*, 2000). Quantification of HCMV DNA copies was performed using SYBR Green PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA). Each reaction mixture contained 5 $\mu$M dATP, 5 $\mu$M dCTP, 5 $\mu$M dGTP, and 10 $\mu$M dUTP, 2.5 $\mu$l of 10× SYBR Green PCR Buffer, 6.25 $\mu$M $MgCl_2$ and 0.5 U AmpliTaq Gold DNA Polymerase, 5 $\mu$M of each HCMV primer, and 200 ng of the extracted DNA and water was added to a total volume of 25 $\mu$l. PCR was performed in ABI Prism 7900 instrument (PE Applied Biosystems) using 96-well microtiter plates at the following conditions: 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Negative controls contained reagents only. The mean value of the duplicates was used in HCMV DNA copies calculations.

*Clinical definitions*

Antigenemia was measured by quantification of neutrophils positive nuclei for the HCMV lower matrix phosphoprotein pp65 in a cytospin preparation of $10^5$ blood leukocytes after neutrophil recovery (≥500 neutrophils $mm^{-3}$). Assay antigenemia was used in the routine of allo-HSCT Unit of HC-UFMG for guided preemptive ganciclovir treatment when 2 or more HCMV positive blood cells were detected according to the protocols of the HC-UFMG. HCMV disease was defined by clinical symptoms or radiological evidence consistent with HCMV and organ infection together with histologic features of HCMV infection on tissue biopsy specimens. In the presence of new or changing pulmonary infiltrates, detection of HCMV in bronchoalveolar lavage by direct fluorescence antibody was considered supportive evidence of HCMV pneumonitis (Boeckh *et al*, 2003).

*HCMV quantification assay setup*

Real time PCR conditions were adjusted so that single and specific amplification products could be obtained from both saliva and blood samples. Sequencing of these reaction products confirmed the HCMV specificity. A HCMV standard curve was then generated by using 10-fold serial dilutions of prequantified US17 target sequences (AD169) ranging between $10^0$ and $10^6$

copies per well. When these dilutions were subjected to real time PCR analyses, the corresponding threshold cycle ($C_T$) values defined a linear XY dispersion plot with a slope of −3.19, which indicated a PCR efficiency of 100%.

*Statistical methods*

Reproducibility between samples duplicates was measured with the Intraclass Correlation Coefficient (ICC). Nonparametric Spearman correlation coefficients were used to assess the association between continuous variables (HCMV DNA load saliva/blood, and HCMV DNA load blood/pp65-antigenemia values). Nonparametric tests Mann–Whitney and Kruskal–Wallis were used to compare two groups of cases and more than two groups of cases on one variable, respectively. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA) and $P$ value ≤0.05 was considered statistically significant.

## Results

*HCMV DNA levels detected with the assay*

The 30 enrolled patients were followed up for a median of 100 (ranging from 28 to 100) days. A median of 12 (ranging from 3 to 15) saliva samples and 12 (ranging from 3 to 16) blood specimens per patients were analyzed. In all patients, HCMV copies in saliva and/or blood were detected anytime during the study course. A total of 331 saliva and 353 blood samples were tested for HCMV using real time PCR. The virus was detected in 140 saliva and 192 blood samples, and the HCMV copies number ranged from 1 to 3400 copies per 200 ng DNA and from 1 to 151 874 copies per 200 ng DNA, respectively. The ICC for the HCMV result was 0.996 between saliva samples and 0.995 between blood samples.

*Correlation between HCMV realtime PCR results from saliva and blood*

A total of 304 sample pairs (saliva–blood) simultaneously obtained were tested by real time PCR. HCMV DNA load in saliva was plotted against the HCMV DNA load in blood (Figure 1a). The correlation coefficient ($r$) of 0.858 and R Square ($r^2$) of 0.736 indicated a high correlation (Spearman rank test; $P < 0.0001$). However, HCMV DNA load in saliva tended to be lower than that in blood. An overall difference of approximately 41 times was found.

*Correlation between HCMV DNA loads in blood and pp65 antigenemia assay*

Correlation between HCMV DNA loads of 151 blood samples and pp65 antigenemia values, in samples collected simultaneously, was also evaluated. HCMV DNA copy numbers in blood were plotted against pp65 positive cells number (Figure 1b). A correlation coefficient ($r$) of 0.773 and $R$ Square ($r^2$) of 0.598 showed a good correlation between HCMV DNA loads and pp65 antigenemia in blood (Spearman rank test; $P < 0.0001$). One positive pp65 cell was equivalent to approximately 413 HCMV DNA copies per 200 ng DNA in blood.

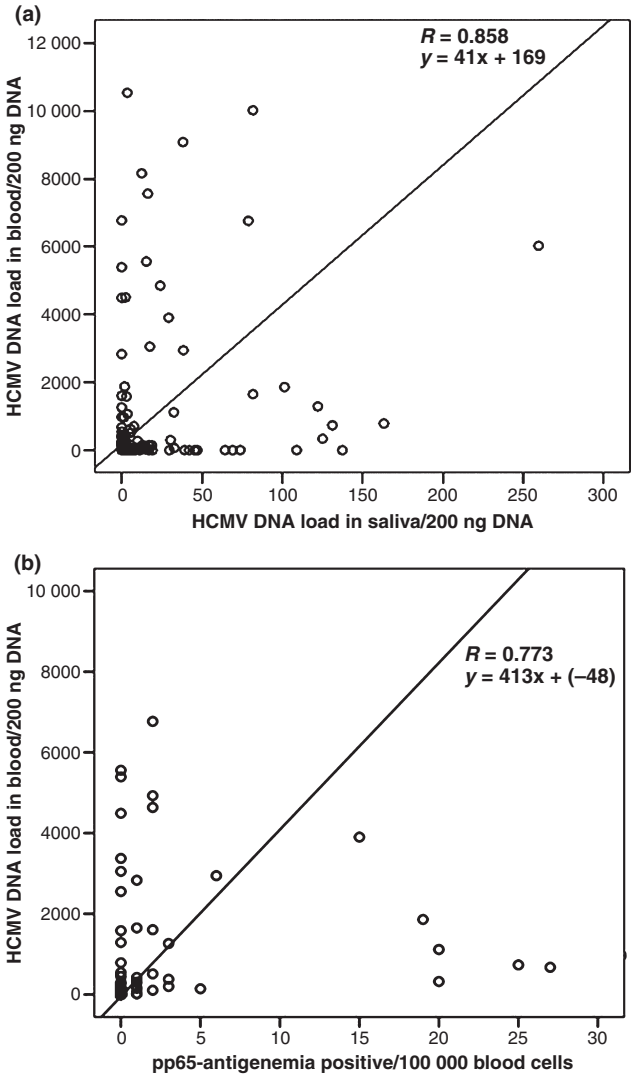


**Figure 1** (**a**) Correlation between HCMV DNA load in saliva and blood determined by real time PCR. (**b**) Correlation of results of real time PCR and pp-65 antigenemia

All pp65-positive samples showed a detectable HCMV DNA load in blood, but 119 samples showed negative blood specimens by pp65 antigenemia. Only 40 (34%) samples with a negative pp65 antigenemia presented HCMV DNA load with zero copy per 200 ng DNA. In 79 (66%) specimens tested, a detectable HCMV DNA load with a median of 19 (range: 1–5558) viral copies per 200 ng DNA was demonstrated.

*Determination of HCMV DNA salivary load threshold values*

The pp65 assay was used for guiding HCMV therapy with ganciclovir in the current study when two or more positive cells in $10^5$ leukocytes were detected. However, studies have demonstrated that pp65 antigenemia test presents a low sensitivity before engraftment due to the

lack of leukocytes readily examinable at this period (Allice *et al*, 2008). Thus, antigenemia was not used to establish the corresponding threshold values for the HCMV load in saliva. The amount of viral load in blood corresponding to one pp65 positive cell in the antigenemia was 413 copies HCMV DNA per 200 ng DNA). As viral blood load measurement corresponds to 41 times the viral load in the saliva (Figure 1a), it was established in the corresponding threshold of 10 copies per 200 ng DNA in saliva for predicting HCMV reactivation.

*Impact of the preemptive therapy for prevention of HCMV disease based in pp65 antigenemia*

Twenty two out of 30 patients presented a positive pp65 antigenemia positive (at least 1 positive cell per $10^5$ leukocytes). Among these 22 patients, 17 (77%) had a positive antigenemia test showing 2 or more positive cells (median of 22 pp65 positive cells per $10^5$ leukocytes; range: 2–208) and received preemptive therapy with gancyclovir. Saliva and blood samples were available to be analyzed in 13 and 17 out of the 17 patients submitted to the preemptive therapy, respectively. The samples were divided in the following groups: – Group 1 corresponded to samples collected in the first and second weeks before antiviral treatment; – Group 2 corresponded to samples collected at the very beginning of antiviral treatment; – Group 3 included samples collected in the first or second week after antiviral treatment. The median HCMV DNA load in saliva was 6 (range, 0–640) copies per 200 ng DNA (group 1), 18 (range, 0–3400) copies per 200 ng DNA (group 2), and 1 (range, 0–81) copies per 200 ng DNA (group 3). The median HCMV DNA load in blood was 147 (range, 8–7560) copies per 200 ng in the first and second weeks before antiviral treatment (group 1), 1877 (range, 142–151 874) copies per 200 ng at the very beginning of antiviral treatment (group 2), and 1248 (range, 0–10 015) copies per 200 ng in the first or second week after antiviral treatment (group 3). The HCMV levels in saliva and blood showed higher levels at the beginning of antiviral treatment, with clear reduction after this period (Kruskal–Wallis test, $P = 0.015$ in saliva and $P = 0.008$ in blood) (Figure 2).

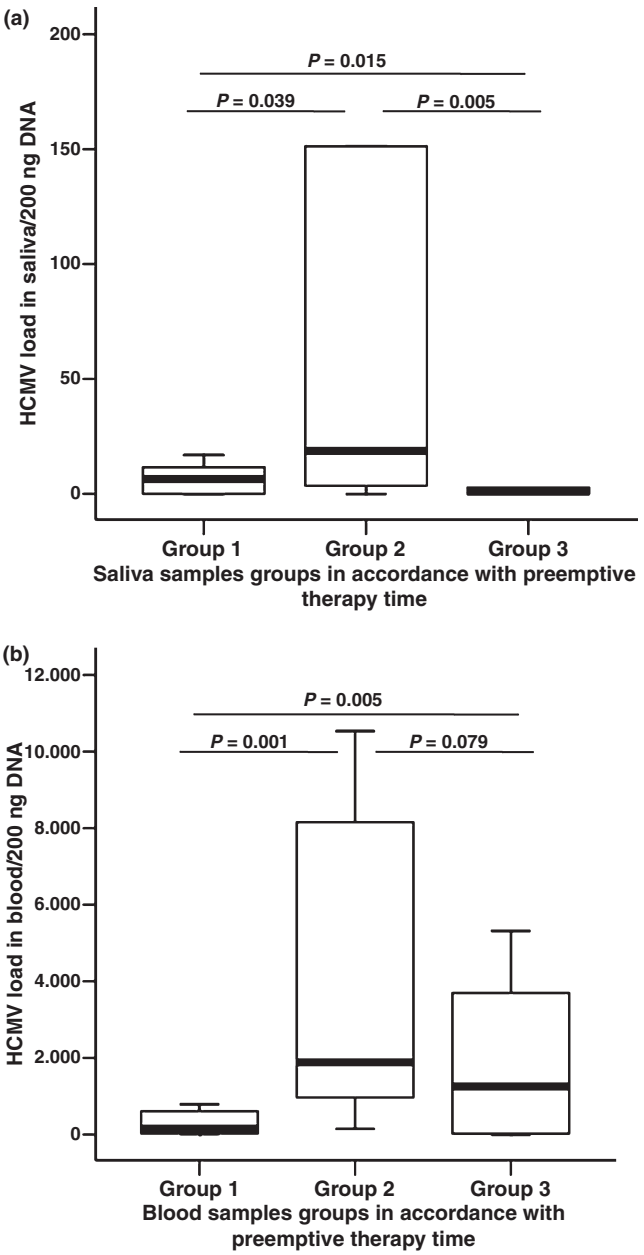


**Figure 2** (**a**) Comparison of the HCMV DNA loads detected by real time PCR in saliva samples in accordance with ganciclovir preemptive therapy. (**b**) Comparison of the HCMV DNA blood load detected by real time PCR in blood samples in accordance with gancyclovir preemptive therapy. Group 1 corresponded to samples collected in the first and second weeks before antiviral treatment. Group 2 corresponded to samples collected at the very beginning of antiviral treatment. Group 3 included samples collected in the first or second week after antiviral treatment. The HCMV levels in saliva and blood showed higher levels at the beginning of antiviral treatment (Group 2), with clear reduction after this period (Group 3)

*Incidence of HCMV reactivation and disease*

According to HCMV reactivation threshold, 22 (73%) patients (threshold of 10 and 413 HCMV DNA copies per 200 ng DNA for saliva and blood, respectively) showed HCMV reactivation in one or both specimens (patients #1, #2, #3, #4, #5, #6, #7, #8, #9, #10, #11, #12, #13, #14, #15, #16, #17, #18, #19, #20, #21, #22). However, one patient presented 3 pp65-positive cells, 198 HCMV DNA copies in blood, but no DNA copy in the saliva (patient #23). Saliva samples showed earlier HCMV reactivation than blood samples detected by real time PCR and antigenemia assay in 11 cases (patients #1, #2, #6, #7, #10, #14, #15, #16, #17, #18, #19) (Figure 3, Table 2). Three recipients developed HCMV disease: two developed pneumonia (patients #11 and #21) and one developed colitis (patient #22) (Figure 3, Table 2), confirmed by biopsy and radiological exam. Thirteen (patients #1, #2, #3, #4, #9, #11, #12, #13, #14, #17, #19, #21, #22) out of 22 patients positive in HCMV real time PCR died, including three patients with HCMV

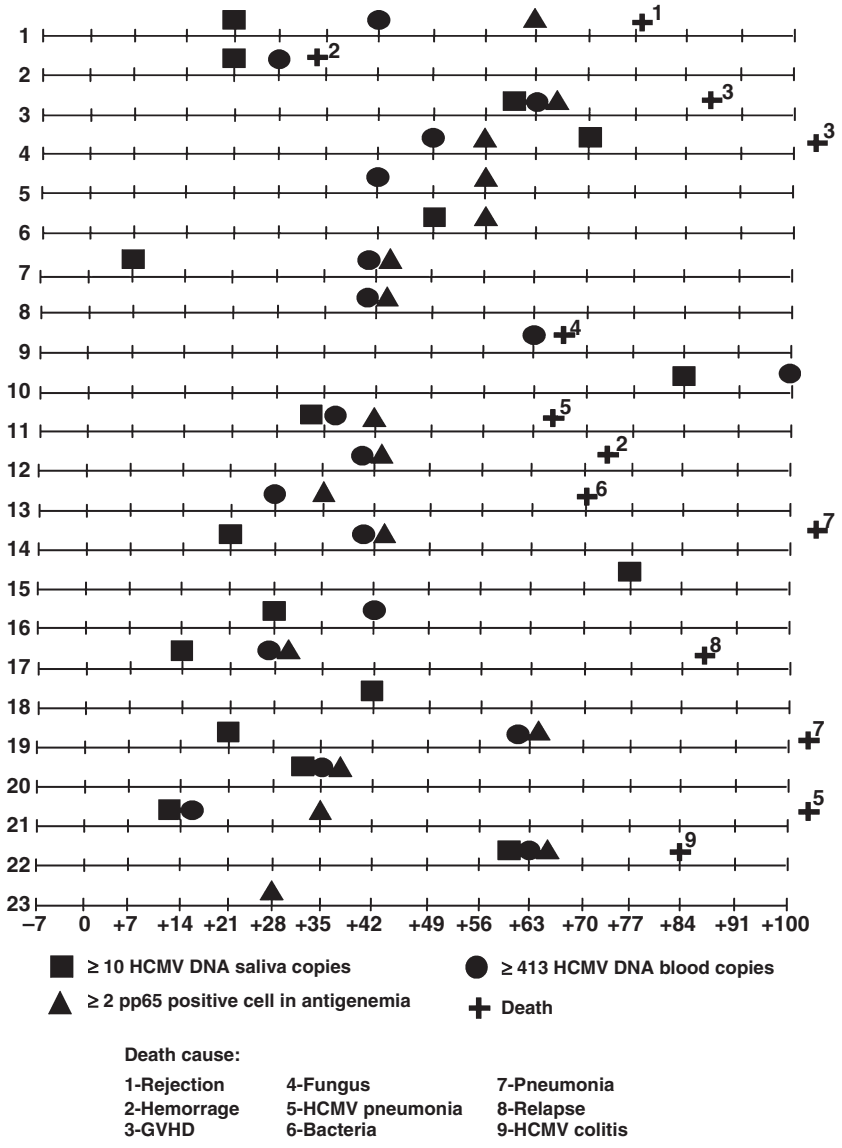


**Figure 3** Evolution of the 22 patients who developed a positive real time PCR results in accordance with HCMV reactivation threshold in saliva (10 HCMV copies per 200 ng DNA) and blood (413 HCMV copies per 200 ng DNA). One patient was positive only for antigenemia (patient #23)

disease. None of the patients presented oral lesions suggestive of HCMV infection, despite the HCMV detection in saliva.

## Discussion

The quantification of HCMV by real time PCR technology is useful for predicting disease and monitoring response to antiviral therapy (Gault *et al*, 2001; Machida *et al*, 2000). However, HCMV may be also detectable in patients with asymptomatic infection who never progress to disease because of its high sensitivity (Tanaka *et al*, 2000). In this study, a positive correlation was found between HCMV DNA loads in saliva and blood. HCMV is commonly detected in salivary glands and in endothelium of the blood vessels, while the epithelium-lined surface of oral cavity is usually spared (Kanas *et al*, 1987). Therefore, oral swabs probably represent the presence of the virus in saliva (Correia-Silva *et al*, 2007). Among the reasons for use of saliva as a diagnostic fluid is the demand for noninvasive and easy-to-use diagnostic tests. An increase of HCMV DNA load in saliva may be valuable for predicting risk of patients developing HCMV disease as previously described in blood (Cortez *et al*, 2003). We have reported that immunosuppression related to allo-HSCT affects HCMV reactivation detection in the oral cavity after transplantation compared with non-transplanted healthy individuals (Correia-Silva *et al*, 2007). In this study the median HCMV DNA load detected in blood of the 30 HSCT recipients was significantly higher than in saliva. Considering that HCMV may be latent in the blood of healthy subjects (Kano and Shiohara, 2000), we did not use the same threshold for both fluids. The biological significance of each viral source should be individually evaluated.

Real time PCR allows a more reliable quantification of HCMV DNA level with more precise information on

**Table 2** Number of copies of HCMV in the saliva, in the blood, and number of cells pp65 positive in 22 patients who developed a positive real time PCR results in accordance with HCMV reactivation threshold in saliva (10 HCMV copies per 200 ng DNA) and blood (413 HCMV copies per 200 ng DNA). One patient was positive only for antigenemia (patient #23)

| *Patients* | *Saliva # copies* | *Days* | *Blood # copies* | *Days* | *pp65 #cells* | *Days* |
|---|---|---|---|---|---|---|
| 1 | 19 | +21 | 6756 | +42 | 19 | +63 |
| 2 | 902 | +21 | 1651 | +28 | Negative | |
| 3 | 427 | +63 | 49 227 | +63 | 79 | +63 |
| 4 | 81 | +70 | 3372 | +49 | 53 | +56 |
| 5 | Negative | | 983 | +42 | 32 | +56 |
| 6 | 15 | +49 | Negative | | 5 | +56 |
| 7 | 69 | +7 | 1116 | +42 | 20 | +42 |
| 8 | Negative | | 511 | +42 | 2 | +42 |
| 9 | Negative | | 546 | +63 | Negative | |
| 10 | 27 | +84 | 420 | +100 | Negative | |
| 11 | 16 | +35 | 7560 | +35 | 96 | +42 |
| 12 | Negative | | 1877 | +42 | 34 | +42 |
| 13 | Negative | | 1066 | +28 | 2 | +35 |
| 14 | 32 | +21 | 151 874 | +42 | 208 | +42 |
| 15 | 16 | +77 | Negative | | Negative | |
| 16 | 13 | +28 | 3052 | +42 | Negative | |
| 17 | 13 | +14 | 1264 | +28 | 3 | +28 |
| 18 | 11 | +42 | Negative | | Negative | |
| 19 | 29 | +21 | 4928 | +63 | 2 | +63 |
| 20 | 12 | +35 | 8156 | +35 | 43 | +35 |
| 21 | 163 | +14 | 787 | +14 | 25 | +35 |
| 22 | 38 | +63 | 2945 | +63 | 6 | +63 |
| 23 | Negative | | Negative | | 3 | +28 |

viral load kinetics that is useful for predicting HCMV disease and monitoring response to antiviral therapy (Allice *et al*, 2008; Machida *et al*, 2000). We found that HCMV load in saliva detected by real time PCR had a similar kinetics compared with blood, and that HCMV loads decreased significantly after antiviral treatment. Therefore, quantification of HCMV DNA by real time PCR of saliva seems to be a useful alternative tool to the diagnosis of HCMV infection and to monitor antiviral response.

Human cytomegalovirus DNA copy number in the blood showed a good correlation with pp65 antigenemia. Correlation between DNAemia and antigenemia has been shown in some studies (Ikewaki *et al*, 2003), but it has not been confirmed by other authors (Machida *et al*, 2000). Studies using real time PCR showed that DNAemia presents a higher sensitivity than antigenemia, and detects HCMV reactivation earlier in the clinical course (Ikewaki *et al*, 2003; Kearns *et al*, 2001), but this data has not been confirmed by others (Gerna *et al*, 2008). These controversial results were probably due to the higher threshold value used for real time PCR. An important issue is, thus, to establish the best cut-off value for determining HCMV reactivation and initiate antiviral therapy. Success of preemptive therapy depends on the availability of appropriate diagnostic tests to detect early stages of HCMV infection (Kalpoe *et al*, 2004).

According to the threshold established, an increased or diminished incidence of HCMV infection may be found. In this study, a threshold of 10 HCMV DNA copies in 200 ng DNA for HCMV reactivation in saliva was established. If this threshold was reduced to 2 HCMV copies in 200 ng DNA, an increased number of HCMV positive patients would be noticed, which could permit early diagnosis of HCMV reactivation. Gerna *et al* (2008) using a higher threshold for DNAemia showed a lower HCMV reactivation with PCR than with antigenemia. However, one patient of the DNAemia-guided group developed HCMV gastritis. Saliva real time PCR sensitivity cannot be based in the blood real time PCR test because of the limitations to define HCMV disease. The sensitivity of the test must be calculated from the HCMV disease diagnosis. The tests sensitivity was not calculated because only three patients presented HCMV disease. In addition, despite the difference between threshold values, our findings indicate a high correlation between the tests. It is also important to state that negative effects may occur due to under- or over-treatment of HCMV reactivation (development of late HCMV disease or inhibition of the immune system reconstitution, respectively). Furthermore, besides threshold, kinetics of HCMV during patient monitoring should also be considered before starting antiviral therapy.

We observed in some patients early HCMV reactivation in saliva compared with blood, but others presented early reactivation in the blood. While leukopenia associated with HSCT before engraftment may affect the detection of the virus in the blood, xerostomia could also compromise the identification of HCMV in saliva. Leukopenia associated with allo-HSCT before engraftment (Kano & Shiohara, 2000) may affect the use of blood as a diagnosis source of HCMV infection at least in some individuals. On the other hand, reduction of saliva quantity associated with allo-HSCT therapy (Alborghetti *et al*, 2005) could also compromise the identification of HCMV in saliva of some patients during opioid use or with xerostomy associated with GVHD. In some patients, the early HCMV reactivation in saliva may be partly explained by the fact that salivary glands are important reservoir of the virus, which may be a source for reinfection of internal tissues and organs, via small lacerations of the oral/pharyngeal mucosa, as it was previously demonstrated in animal model (Kloover *et al*, 2002). In conclusion, this study shows that saliva can be a useful source for real time PCR in the monitoring and diagnosis of HCMV disease in patients submitted to HSCT. As a result of the preliminary nature of this datum, these results must be confirmed in a larger series of patients, in the context of a randomized clinical trial.

## Acknowledgements

This study was supported in part by grants from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. Dr. R. S. Gomez is research fellow of CNPq. Potential conflicts of interest: The authors do not have any association that might pose a conflict of interest.

## Author contributions

JF Correia-Silva, O Bruna-Romero, H Bittencourt, RS Gomez: study concepts and design; JF Correia-Silva, H Bittencourt, RS Gomez: definition of intellectual content; JF Correia-Silva, H Bittencourt, RS Gomez: literature research; JF Correia-Silva, O Bruna-Romero, RG Resende, LPM Miranda, FE Oliveira, SG Xavier, SP Figueiredo-Neves, HC Almeida: data acquisition; JF Correia-Silva, O Bruna-Romero, FO Costa, H Bittencourt, RS Gomez: data analysis; JF Correia-Silva, FO Costa, H Bittencourt: statistical analysis; JF Correia-Silva: manuscript preparation and editing; JF Correia-Silva, H Bittencourt, RS Gomez: manuscript review.

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