Journal section: Oral Medicine and Pathology
Publication Types: Research

Association between oral HSV-1 and survival in allogeneic hematopoietic stem cell transplanted patients

Andre Luiz Sena Guimaraes [1], Carolina Cavalieri Gomes [1], Luciano Marques da Silva [2], Jeane de Fatima Correia-Silva [2], Junia Maria Neto Victoria [2], Ricardo Santiago Gomez [2], Henrique Bittencourt [3]

[1] Department of Dentistry, Universidade Estadual de Montes Claros, Montes Claros, Brazil
[2] Department of Oral Surgery and Pathology, School of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
[3] Stem Cell Transplantation Unit, School of Medicine, Hospital das Clinicas, Brazil

Correspondence:
Prof. André Luiz Sena Guimarães
Universidade Estadual de Montes Claros
Hospital Universitário Clemente Faria
Av Cula Mangabeira, 562
Bairro Santo Expedito,
Montes Claros, MG
Brazil
cep 39401-001
andreluizguimaraes@gmail.com

Received: 31/03/2008
Accepted: 08/06/2008

Guimaraes ALS, Gomes CC, Marques-Silva L, Correia-Silva JF, Victoria JMN, Gomez RS, Bittencourt H. Association between oral HSV-1 and survival in allogeneic hematopoietic stem cell transplanted patients. Med Oral Patol Oral Cir Bucal. 2009 Feb 1;14 (2):E62-8.
http://www.medicinaoral.com/medoralfree01/v14i2/medoralv14i2p62.pdf

Article Number: 5123658800 http://www.medicinaoral.com/

eMail: medicina@medicinaoral.com
Indexed in:
-SCI EXPANDED
-JOURNAL CITATION REPORTS
-Index Medicus / MEDLINE / PubMed
-EMBASE, Excerpta Medica
-SCOPUS
-Indice Médico Español

Abstract

Introduction: This study was designed to investigate the effect of oral HSV-1 shedding on the survival of allogeneic hematopoietic stem cell transplanted (allo-HSCT) patients.

Methods: One hundred nineteen allo-HSCT patients were included in the study and divided in three groups: before transplant, 100 days after transplant and 1 year of allo-HSCT. Healthy volunteers matched by age and gender were also selected. Oral swabs were performed and the nested PCR was used to detect HSV-1 presence in the oral mucosa. In statistical analysis, chi-square test was used to test the distribution of HSV1 shedding among the three groups. Time to death after allo-HSCT was displayed by means of the Kaplan-Meier method and the results were compared by the log-rank test. Cox proportional hazards multivariate model was used to evaluate the survival.

Results: We observed that HSV-1 shedding was similar at different points after allo-HSCT. However, HSV-1 shedding before allo-HSCT was associated with worst survival rates after allo-HSCT in multivariate analysis.

Conclusion: Our data demonstrates that HSV-1 shedding in oral mucosa before transplant is associated with worst survival rate of allo-HSCT patients.

Key words: *Hematopoietic stem cell transplantation, oral infection, HSV-1, shedding and survival.*

Introduction

Allogeneic hematopoietic stem cell transplant (Allo-HSCT) is performed in patients for a range of underlying disorders, including haematological malignancies, severe aplastic anaemia and genetic diseases. Many evidences suggest that graft versus host disease (GVHD) and infectious diseases are the most important causes of mortality and morbidity after treatment with allo-HSCT (1-3).

Herpes simplex virus type 1 (HSV-1) belongs to the Herpesviridae family, genus simplex virus and contains a 152,000 bp double-stranded DNA genome (4). HSV-1 is usually acquired during childhood and it is transmitted through direct mucocutaneous contact or droplet infection from infected secretions. As HSV is short-lived on external surfaces, the infection depends on intimate contact with an individual who is shedding live virus through secretions (5). Acute (primary) herpetic gingivostomatitis typically affects children and involves the gingiva, tongue, lips, buccal mucosa, as well as the hard and soft palate. Lymphadenopathy, fever and malaise are frequently reported (6). After primary infection, HSV-1 establishes a latent infection in neuronal cells of trigeminal ganglia. Then, it can reactivate causing the recurrent disease (7-10). Reactivation of HSV-1 in the oral cavity may be either symptomatically (recrudescence) or asymptomatically (shedding) (11). The HSV-1 shedding into the oral cavity occurs during the prodromal phase of recurrent herpes labialis before a lesion is clinically evident (12). Oral recrudescent HSV-1 infection is one of the major oral complications that follows allo-HSCT, although the incidence has decreased with the use of acyclovir prophylaxis. Unlike recrudescent infections in healthy patients, recrudescent lesions in immunocompromised patients are more extensive, aggressive, slow-healing, and painful (13,14). Recently, we have shown that recrudescent HSV-1 infection is an independent negative prognostic variable in allo-HSCT survival rate (15).

Although allo-HSCT patients are more susceptible to oral recrudescence of HSV-1, the impact of oral asymptomatic HSV-1 in allo-HSCT patients survival rate has not been studied yet. Therefore, the purpose of the present study was to investigate the frequency of asymptomatic HSV-1 oral shedding before and after allo-HSCT and its association with survival after allo-HSCT patients using nested polymerase chain reaction (PCR).

Material and Methods

The study protocol was approved by the appropriate local ethical committee and informed consent was obtained from all the patients and from parents if the patient was less than 18 years.

Patients

Three groups of patients were included in the study. For each case group there was a control group composed of healthy patients matched by age and gender. The first case group consisted of subjects that were being prepared to allo-HSCT (Group 1; n= 38), while the second group comprised individuals alive 100 days after allo-HSCT (Group 2; n= 45). The third group included subjects alive at 1 year after allo-HSCT (Group 3; n= 36). Considering that more than 90% of Brazilians older than 30 y.o. are HSV-1 seropositivity (16) no serologic test was not included in this study. All the patients were attended at the Dental Clinics in the Scholl of Dentistry of Universidade Federal de Minas Gerais. Patients were conditioned for transplantation with the following protocol: Cyclophosphamide 200mg/kg and Busulfan 4mg/kg (aplastic anemia) or Cyclophosphamide 120mg/kg and Busulfan 16mg/kg (malignant diseases). Methotrexate and cyclosporin were used for GVHD prophylaxis and steroids for GVHD treatment. All patients received acyclovir as HSV-1 prophylaxis from graft infusion until engraftment.

The medical records of the patients were reviewed and included the following information: gender and age of the patient, donor gender, primary disease, stem cell source (bone marrow or blood stem cells), and platelet counts. Biopsies of the lower lip were done in Group 2 patients for chronic GVHD staging in the oral mucosa and salivary glands as described elsewhere (15).

Samples:

Swabs were collected from all the subjects (buccal mucosa, lower lip and dorsum of the tongue). DNA extraction was carried out as described (17) and modified after (18). 450 µL lysis buffer (6.0M Gunidinium-SCN, 0.1 µL Tris-Cl,0.2M EDTA, 10% TritonX-100) and 20 µL silica (SiO2,Sigma S5631, washed with H2O, pH 2.5, corrected with HCl) in the pellet. The tubes were mixed for 10 s, incubated for 10 min at 56 C, centrifuged for 1 min and the supernatant was discarded. The pellet was washed twice with 450 ml washing buffer (6.0M Guanidinium-SCN, 0.1M Tris-Cl), twice with 70% ethanol and once with 450 µL acetone. After these steps the pellet was dried at 56 C for 10 min or until the silica became completely dry. Finally, 100 µL TE buffer (10mM Tris-Cl pH 8.0, 1.0mM EDTA) was added and incubated at 56 C for a minimum of 10 min and maximum of 24 h. The solution containing the silica and DNA was mixed for 5 s, centrifuged for 2 min and the supernatant was carefully transferred to a new tube.

Nested PCR

The sequence of DNA was obtained from National Centre for Biotechnology Information and the Mac-Vector program was used to construct and analyze the primers as previously described (18). In summary, two sets of primers were used for HSV-1 on the nested PCR (outer primers F: 5' TGC TGG AGG ATC ACG AGT TTG 3'R: 5' CAT CGT CTT TGT TGG GAA CTT 3' and inner primers: F:5' TGC AGA GCA ACC CCA TGA AG 3' and R: 5' ATG ACCA TGT CGG TGA CCT TGG 3'). Two microliters (100 ng) of DNA purified as described

above were subjected to PCR using outer primers. PCR was carried out in a 50 µL mixture containing Taq DNA polymerase (1 unit/reaction), PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.4, 1.5 mM MgCl2), deoxynucleoside triphosphates (0.1 mM/reaction of each dNTP) and primers (10 pmol/reaction). All samples were amplified using a DNA thermal cycler (Eppendorf Mastercycler). After PCR, 2 µL of the final product was transferred to the reaction mixture of the second round PCR (nested) and reamplified with the inner pair of primers. Recurrent oral herpes lesion in a bone marrow transplantation patient was used as positive control for HSV-1. Negative controls included PCR without DNA.

Agarose gel eletrophoresis

10 µL of each reaction product was added to 2 µL of gel loading dye (0.25% bromophenol blue, 30% glycerol, 10 mM EDTA), loaded into the 1.5% agarose gel and electrophoresis was carried out using 1X TAE buffer. DNA fragments were visualized after staining with ethidium bromide (0.5µg/ml) and using the photo documentation system BIO-RAD GEL-DOC 1000. The molecular weight of the DNA was estimated using k HindIII DNA and 100 bp ladder markers.The human β-globin gene was amplified in order to assess the adequacy of each specimen (19).

Statistical analyses:

Chi-square test was used to test the distribution of HSV1 shedding into the three groups and controls. Time to death after allo-HSCT was displayed by means of the Kaplan-Meier method for the following variables: gender and age of the patient (≤ 30 and >30 years), donor gender, primary disease (malignant versus non-malignant), stem cell source (bone marrow versus blood stem cell), platelet count (≤ 100,000/mm3 vs > 100,000/mm3), and presence of HSV-1 in the saliva. Chronic Graft Versus Host Disease (cGVHD) at the salivary glands and oral mucosa (no vs mild vs moderate/severe) were included in the group 2 analysis. The results of Kaplan-Meier were compared by the log-rank test. Variables with $p \leq 0.25$ alongside with HSV-1 DNA positivity (irrespective of p value) were included in the Cox proportional hazards multivariate model. Statistical significance was set at $p<0.05$. The records of each patient were reviewed 36 months after the oral swab collection of all patients in the three groups of the study. The survival analyses were assessed using SPSS (SPSS Inc., Chicago), version 13.0.

Results

The Group 1 was formed by 18 females and 20 males with median age of 37 years (range 1 to 58, standard deviation 14.7). The Group 2 (+100 days after allo-HSCT) presented 20 females and 25 males subjects with median age of 27 years (range 9 to 55, standard deviation 13.2). The group 3 consisted of 15 females and 21 males with median age of 27 years (range 4 to 49, standard deviation 12.5). Since the control of each group was matched by age and gender, they had the same results related to age and gender distribution. None of the patients presented clinical HSV oral lesions (neither suspect) at the moment of sample collection. No difference in the presence of HSV-1 was observed between controls and the case groups. The frequencies of HSV positive swabs in each group of study are shown in Figure 1. The frequency of

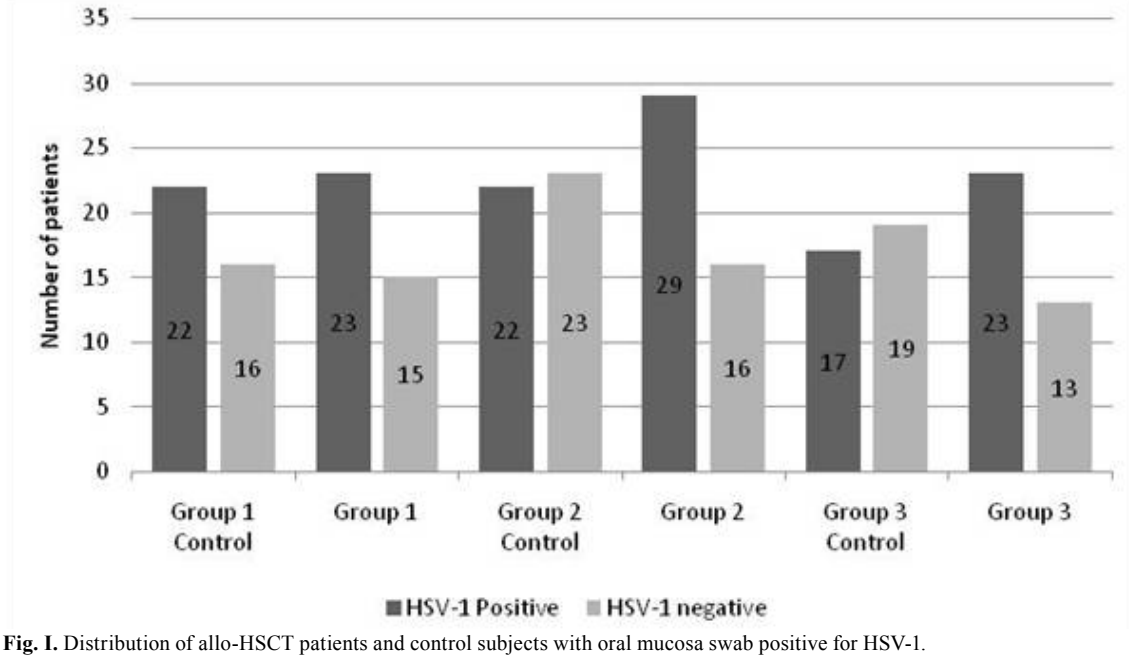

Fig. I. Distribution of allo-HSCT patients and control subjects with oral mucosa swab positive for HSV-1.

Group 1 – before allo-HSCT; Group 2- at the day +100 after allo-HSCT; Group 3- 1 year after allo-HSCT. No significant difference in distribution was observed between the groups.

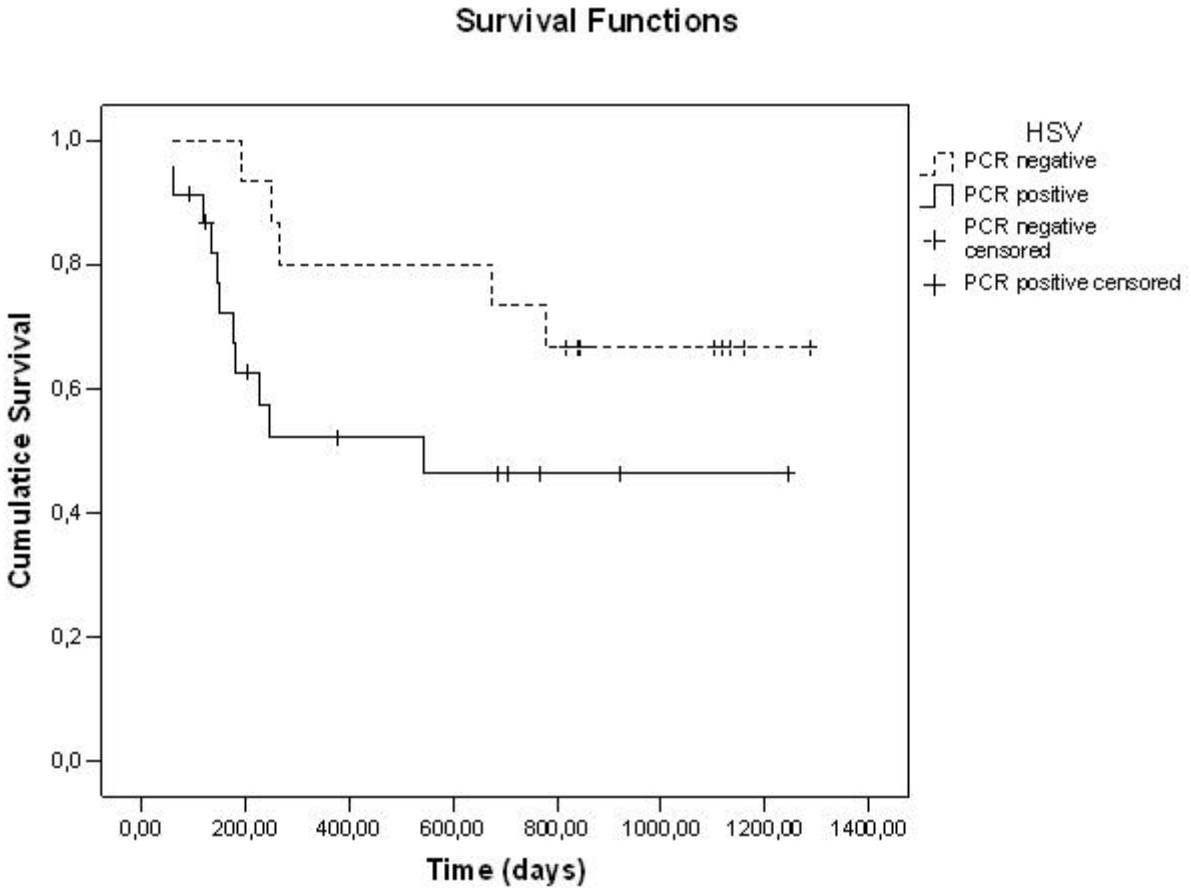

Fig. 2. Survival according to HSV-1 PCR before transplant (Group 1) (p = 0.07).

HSV-1 DNA detection in the groups before allo-HSCT, 100 days and 1 year after allo-HSCT were not statistically different from the control group. The frequencies of HSV positive swabs between the three groups were not statistically different either (p = 0.92 - figure 1). The number of platelets in allo-HSCT patients positive for HSV-1 was not statistically different from the patients negative for HSV-1 (data not shown).

Univariate analysis of the different variables is shown in table 1. Only female donor gender was associated with a better survival rate in patients one year after allo-HSCT in univariate analysis. While in the univariate analysis HSV-1 shedding before transplant revealed only a slightly trend to a worst survival rate (p=0.07) (figure 2), in multivariate analysis it was associated with a significant worst survival rate (table 2). On the other hand, a positive HSV1 shedding one hundred days and one year after transplant was not associated with survival rate even in uni- nor in multivariate analysis (table 2).

Discussion

Detection of viral DNA using polymerase chain reaction has demonstrated greater sensitivity for identifying HSV-1 than the traditional viral culture (12, 20). In some circumstances HSV-1 is only detected by nested PCR (21). Therefore, the nested PCR was used to identify HSV-1 DNA in the present study and others (11, 22, 18).

The pathogenesis of oral HSV infection in immunosuppressed patients is unclear. Different prevalence of HSV-1 has been identified in different immunosupression states (23, 24). HSV-1 was present in saliva of 24% to 70% of HIV-infected patients (25). HSV-1 was also detected in the oral mucosa of 3.6% of patients undergoing head and neck radiation therapy (24) and in 28% of patients with aplastic anemia using cell cultures (26). In another study, 60% of cell cultures from oral mucosa samples of bone marrow transplantation patients were positive for HSV-1 (27). Moreover, recipients of matched unrelated/mismatched allo-SCT may be more at risk of acquiring breakthrough infections with HSV than patients who have undergone matched related allo-SCT (28). Imunos-

Table 1. Baseline characteristics of patients and p value of log-rank test (univariate analysis).

	Group 1		Group 2		Group 3	
Parameters	Cumulative Proportion Surviving in Three years	*P*-value log-rank test	Cumulative Proportion Surviving in Three years	P-value log-rank test	Cumulative Proportion Surviving in Three years	P-value log-rank test
Recipient gender						
Male	61.1%	0.33	69.6%	0.77	88.6%	0.77
Female	42.8%		75.0%		87.5%	
Donor gender						
Male	66.0%	0.30	75.6%	0.30	76.9%	0.05*
Female	41.9%		66.7%		100%	
Age of patient (years)						
≤ 30	64.3%	0.13*	75.0%	0.26	93.3%	0.09*
> 30	43.6%		64.7%		76.9%	
Primary disease						
Non-malignant disease	44.5%	0.10*	68.4%	0.26	87.3%	0.67
Malignant (neoplastic) disease	83.3%		79.1%		89.0%	
Stem cell						
Peripheral stem cell	47.0%	0.48	66.3%	0.13	95.0%	0.33
Bone Marrow	63.6%		81.6%		81.2%	
Platelet number						
≤100,000/mm3	59.3%	0.87	54.5%	0.07*	75.0%	0.39
>100,000/mm3	51.8%		78.0%		88.6%	
Mucosal GVHD						
No			62.8%	0.31		
Mild	X	X	77.2%		X	X
Moderate /Severe			50.0%			
Oral Gland GVHD						
No			54.8%	0.77		
Mild	X	X	75.0%		X	X
Moderate /Severe			76.92%			
HSV-1 DNA						
Negative	66.6%	0.07*	100%	0.76*	84.6%	0.65*
Positive	46.4%		90.9%		88.3%	

* - included in multivariate analyses

Group 1 – before allo-HSCT; Group 2- at the day +100 after allo-HSCT; Group 3- 1year after allo-HSCT.
GVHD: Graft versus host disease.

Table 2. Survival according in an adjusted model of Cox proportional hazards multivariate model.

Group (n)	Analised variables	OR§	95%CI§		§*p*-value
			Min	Max	
Group 1 (38)	Age	2.18	0.810	5.86	0.123
	Primary Disease (ref. no neoplasic)	0.60	0.191	1.900	0.387
	PCR HSV-1 (ref PCR negative)	6.16	19.68	1.93	0.002*
Group 2 (45)	Platelets number (ref > $1x10^6$ mm^3)	2.82	1.03	7.74	0.043*
	PCR HSV-1 (ref PCR negative)	1.85	0.81	4.23	0.140
Group 3 (36)	Donor (ref female)	0.00	0.00	NA	0.957
	Age (ref <30 years)	4.28	0.43	42.477	0.213
	PCR HSV-1 (ref PCR negative)	0.814	0.11	5.94	0.839

*significant values, NA= Not applicable

supression after allo-HSCT, present in group 2 and 3, did not modify HSV-1 shedding. It is important to note that in all periods no HSV-1 lesions were observed.

A previous study conducted by our group showed that in healthy individuals, HSV-1 shedding in the oral cavity occurs independently of herpes labialis recrudescence (11). In the past, we observed that the presence of oral recrudescent lesions of HSV-1 alongside with a platelet count below 100,000/mm3 at the day 100 were independent negative prognostic variables in allo-HSCT patients' survival rates (15). So the main objective of the present study was to determine the impact of HSV-1 shedding on the oral mucosa and on survival of allo-HSCT patients. The present study demonstrated that the presence of HSV-1 shedding before transplant was associated with a higher risk of death after allo-HSCT. The absence of viral prophylaxis during this period might predispose the patient to a higher risk of gastrointestinal lesions and this could explain the association between HSV-1 shedding and survival rate. On the other hand, we did not observe a worst survival in groups 2 and 3, both in uni- and multivariate analysis. This could be explained by a selection bias, since most patients will die in the first one hundred days after allo-HSCT. Moreover a great number of patients die after engraftment, mainly due to GVHD or infections secondary to the intense imunossupression necessary to its treatment. Late mortality of patients is associated with relapse of the primary disease and late effects of allo-HSCT, but not with infections. We were unable to show any correlation between platelets and leukocytes counts and HSV-1 shedding. GVHD severity was also not associated with the presence of HSV-1 in the oral mucosa. A major drawback of this study was the fact that the samples were collected at outpatient department and the samples were collected during a specific time before or after HSCT. Thus, we were unable to verify the presence of HSV-1 in the same patient during the interval before and 100 days after HSCT.

In conclusion, our data demonstrates that HSV-1 shedding in oral mucosa represents a very common event that seems to be associated with a short survival when detected before allo-HSCT. As our study is cross-sectional and the groups are not the same, we have to analyze this data with cautions. Prospective studies are necessary to confirm the effects of HSV-1 shedding in the oral mucosa on allo-HSCT survival rate.

References

1. Sviland L, Pearson AD, Green MA, Baker BD, Eastham EJ, Reid MM, et al. Immunopathology of early graft-versus-host disease--a prospective study of skin, rectum, and peripheral blood in allogeneic and autologous bone marrow transplant recipients. Transplantation. 1991;52:1029-36.

2. Baldanti F, Lilleri D, Gerna G. Monitoring human cytomegalovirus infection in transplant recipients. J Clin Virol. 2008;41:237-41.

3. Tian Y, Deng YB, Huang YJ, Wang Y. Bone marrow-derived mesenchymal stem cells decrease acute graft-versus-host disease after allogeneic hematopoietic stem cells transplantation. Immunol Invest. 2008;37:29-42.

4. Vestey JP, Norval M. Mucocutaneous infections with herpes simplex virus and their management. Clin Exp Dermatol. 1992;17:221-37.

5. Chauvin PJ, Ajar AH. Acute herpetic gingivostomatitis in adults: a review of 13 cases, including diagnosis and management. J Can Dent Assoc. 2002;68:247-51.

6. Whitley RJ. Herpes simplex virus infection. Semin Pediatr Infect Dis. 2002;13:6-11.

7. Barkvoll P, Attramadal A. Recurrent herpes labialis in a military brass band. Scand J Dent Res. 1987;95:256-8.

8. Scully C. Orofacial herpes simplex virus infections: current concepts in the epidemiology, pathogenesis, and treatment, and disorders in which the virus may be implicated. Oral Surg Oral Med Oral Pathol. 1989;68:701-10.

9. Yeung-Yue KA, Brentjens MH, Lee PC, Tyring SK. Herpes simplex viruses 1 and 2. Dermatol Clin. 2002;20:249-66.

10. Arduino PG, Porter SR. Herpes Simplex Virus Type 1 infection: overview on relevant clinico-pathological features. J Oral Pathol Med. 2008;37:107-21.

11. Da Silva LM, Guimarães AL, Victória JM, Gomes CC, Gomez RS. Herpes simplex virus type 1 shedding in the oral cavity of seropositive patients. Oral Dis. 2005;11:13-6.

12. Scott DA, Coulter WA, Biagioni PA, O'Neill HO, Lamey PJ. Detection of herpes simplex virus type 1 shedding in the oral cavity by polymerase chain reaction and enzyme-linked immunosorbent assay at the prodromal stage of recrudescent herpes labialis. J Oral Pathol Med. 1997;26:305-9.

13. Cohen SG, Greenberg MS. Chronic oral herpes simplex virus infection in immunocompromised patients. Oral Surg Oral Med Oral Pathol. 1985;59:465-71.

14. Epstein JB, Sherlock C, Page JL, Spinelli J, Phillips G. Clinical study of herpes simplex virus infection in leukemia. Oral Surg Oral Med Oral Pathol. 1990;70:38-43.

15. Gomez RS, Carneiro MA, Souza LN, Victória JM, De Azevedo WM, De Marco L, et al. Oral recurrent human herpes virus infection and bone marrow transplantation survival. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001;91:552-6.

16. Cowan FM, French RS, Mayaud P, Gopal R, Robinson NJ, De Oliveira SA, et al. Seroepidemiological study of herpes simplex virus types 1 and 2 in Brazil, Estonia, India, Morocco, and Sri Lanka. Sex Transm Infect. 2003;79:286-90.

17. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-Van Dillen PM, Van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990;28:495-503.

18. Victória JM, Guimarães AL, Da Silva LM, Kalapothakis E, Gomez RS. Polymerase chain reaction for identification of herpes simplex virus (HSV-1), cytomegalovirus (CMV) and human herpes virus-type 6 (HHV-6) in oral swabs. Microbiol Res. 2005;160:61-5.

19. Gall-Troselj K, Mravak-Stipetić M, Jurak I, Ragland WL, Pavelić J. Helicobacter pylori colonization of tongue mucosa--increased incidence in atrophic glossitis and burning mouth syndrome (BMS). J Oral Pathol Med. 2001;30:560-3.

20. Tateishi K, Toh Y, Minagawa H, Tashiro H. Detection of herpes simplex virus (HSV) in the saliva from 1,000 oral surgery outpatients by the polymerase chain reaction (PCR) and virus isolation. J Oral Pathol Med. 1994;23:80-4.

21. Knaup B, Schünemann S, Wolff MH. Subclinical reactivation of herpes simplex virus type 1 in the oral cavity. Oral Microbiol Immunol. 2000;15:281-3.

22. Marques-Silva L, Castro WH, Gomez EL, Guimarães AL, Silva MS, Gomez RS. The impact of dental surgery on HSV-1 reactivation in the oral mucosa of seropositive patients. J Oral Maxillofac Surg. 2007;65:2269-72.

23. Kameyama T, Sujaku C, Yamamoto S, Hwang CB, Shillitoe EJ. Shedding of herpes simplex virus type 1 into saliva. J Oral Pathol. 1988;17:478-81.

24. Epstein JB, Gorsky M, Hancock P, Peters N, Sherlock CH. The prevalence of herpes simplex virus shedding and infection in the

oral cavity of seropositive patients undergoing head and neck radiation therapy. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2002;94:712-6.
25. Fons MP, Flaitz CM, Moore B, Prabhakar BS, Nichols CM, Albrecht T. Multiple herpesviruses in saliva of HIV-infected individuals. J Am Dent Assoc. 1994;125:713-9.
26. Brennan MT, Sankar V, Baccaglini L, Pillemer SR, Kingman A, Nunez O, et al. Oral manifestations in patients with aplastic anemia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001;92:503-8.
27. Montgomery MT, Redding SW, LeMaistre CF. The incidence of oral herpes simplex virus infection in patients undergoing cancer chemotherapy. Oral Surg Oral Med Oral Pathol. 1986;61:238-42.
28. Van Kraaij MG, Verdonck LF, Rozenberg-Arska M, Dekker AW. Early infections in adults undergoing matched related and matched unrelated/mismatched donor stem cell transplantation: a comparison of incidence. Bone Marrow Transplant. 2002;30:303-9.

Acknowledgments
This study was supported in part by grants from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Programa de Excelência (PRONEX) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. Dr. R.S. Gomez is research fellow of CNPq. Dr. A.L.S. Guimaraes is research fellow of FAPEMIG.