

***Helicobacter pylori* in the oral mucosa of patients submitted to allogeneic haematopoietic stem cell transplantation**

Helicobacter pylori na mucosa oral de pacientes submetidos ao transplante de células-tronco hematopoiéticas

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ABSTRACT: This study was designed to investigate the impact of haematopoietic stem cell transplantation (HSCT) on *Helicobacter pylori* colonization of the oral mucosa by nested polymerase chain reaction (nested-PCR). Forty six consecutive patients submitted to HSCT and 46 healthy volunteers were included in the study. Oral swabs were taken from the oral mucosa of the patients and control group. The medical records of the patients were reviewed and the following information was retrieved: gender and age of the patient, donor gender, primary disease, stem cell source (bone marrow or blood stem cells), leukocyte, neutrophil and platelet counts, and chronic graft *versus* host disease (cGVHD) of salivary glands. The results demonstrated an increased frequency of *H. pylori* in the oral mucosa of HSCT patients compared to controls ($p = 0.002$). The presence of *H. pylori* in the oral mucosa was not related to the severity of cGVHD. The median counts of platelet/mm³, leukocytes/mm³ and neutrophils/mm³ in the group of HSCT patients positive for *H. pylori* were not statistically different from those of the patients negative for it. In conclusion, the present study shows increased frequency of *H. pylori* in the oral mucosa of HSCT patients compared to non-transplanted healthy volunteers.

DESCRIPTORS: *Helicobacter pylori*; Bone marrow transplantation; Mouth mucosa.

RESUMO: O objetivo do estudo é investigar o impacto do transplante de células-tronco hematopoiéticas (TCTH) na colonização da mucosa bucal pela *Helicobacter pylori* através do "nested-PCR". Quarenta e seis pacientes submetidos ao TCTH e 46 indivíduos saudáveis foram incluídos no estudo. Raspados de mucosa bucal foram realizados nos pacientes do grupo de estudo e grupo controle. Os dados médicos dos pacientes foram revisados e as seguintes informações foram coletadas: gênero e idade do paciente, gênero do doador, doença primária, fonte de células-tronco (medula óssea ou células-tronco sanguíneas), número de leucócitos, neutrófilos e plaquetas, doença do enxerto contra o hospedeiro crônica (DECHc) de glândulas salivares. Os resultados demonstram aumento na frequência de *H. pylori* na mucosa bucal de pacientes submetidos ao TCTH comparado com grupo controle ($p = 0.002$). A presença da *H. pylori* na mucosa bucal não teve relação com a severidade da DECHc. As medianas de número de plaquetas/mm³, leucócitos/mm³ e neutrófilos/mm³ no grupo de pacientes TCTH positivos para *H. pylori* não foram estatisticamente diferentes das medianas dos pacientes negativos. Concluindo, o presente estudo mostra um aumento da frequência da *H. pylori* na mucosa bucal de pacientes submetidos ao TCTH quando comparada com a de um grupo de voluntários não transplantados saudáveis.

DESCRIÇÕES: *Helicobacter pylori*; Transplante de medula óssea; Mucosa bucal.

INTRODUCTION

Helicobacter pylori is now accepted as the major cause of chronic active gastritis, duodenal and gastric ulceration⁴, and is associated with the development of gastric carcinoma¹⁸. *H. pylori* has been detected in dental plaque, saliva and the subgingival

region^{10,15}, and there have been reports that *H. pylori* strains in the mouth and stomach are identical¹⁷. The mouth has been considered as an alternative reservoir for *H. pylori*¹⁶. Although people carrying *H. pylori* have an increased risk of developing peptic

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ulcers and stomach cancer, it has been recently demonstrated that this bacterium may actually protect humans against diseases of the esophagus².

Haematopoietic stem cell transplantation (HSCT) is being used to treat a variety of malignant and non-malignant diseases. Various complications may interfere with treatment during the neutropenic phase, as well as with engraftment of the new marrow. The most common problems are infections and graft-versus-host disease (GVHD)⁹. The oral cavity is a frequent site of infections, immunologic reactions, and other side effects of HSCT. The oral manifestations subsequent to HSCT include oral lichenoid lesions, mucosal atrophy, erythema, ulcers, and xerostomia^{13,14}.

Although it is known that the oral cavity may function as a reservoir for *H. pylori* and that this organism has an important role in the protection or development of diseases of the gastrointestinal mucosa, the impact of immunosuppression on *H. pylori* colonization of the oral mucosa has not yet been addressed. Therefore, the purpose of the present study was to investigate the presence of this bacterium in the oral mucosa of HSCT patients. In addition, as a recent study demonstrated a reduced incidence of acute GVHD of the gut in Chinese carriers of *H. pylori* during allogeneic bone transplantation¹, we studied the effect of the presence of *H. pylori* on the severity of oral GVHD. Finally, as *H. pylori* has an influence on the platelet count in HSCT patients²⁰, the association between platelets number and the presence of *H. pylori* was also studied.

MATERIALS AND METHODS

Subjects and sample collection

Forty six consecutive patients who underwent HSCT at the Clinical Hospital, Federal University of Minas Gerais, were included in the study. Oral mucosa swabs were taken from three oral sites (labial mucosa, buccal mucosa and tongue) at 100 days after transplantation. The study group was formed by 19 females and 27 males with median age of 27.4 years (range 11 to 56). The main clinical data of the patients are in Table 1. The swabs were taken with sterile plastic tips, placed immediately in Eppendorf microtubes containing 500 µl of Krebs buffer (20% NaCl, 2% KCl, 2% CaCl₂·2H₂O, MgSO₄, KH₂PO₄, C₆H₁₂O₆), and the pellet obtained after 10 min of centrifugation at 10,000 g was stored at -20°C until processing. The same procedure was performed with the oral mucosa swabs of the control group. The control group was composed of 46 non-transplanted healthy

TABLE 1 - Summary of the clinical data of haematopoietic stem cell transplantation patients included in the study.

Median age, years (range)	27.4 years (11 - 56)
Recipient gender	
Male, n° (%)	27 (58.7)
Female, n° (%)	19 (41.3)
Donor gender	
Male, n° (%)	24 (52.2)
Female, n° (%)	22 (47.8)
Primary disease	
Malignant, n° (%)	32 (69.6)
Non-malignant, n° (%)	14 (30.4)
Stem cell Source	
Blood stem cells, n° (%)	20 (43.4)
Bone Marrow, n° (%)	26 (56.6)

volunteers attending the Restorative Dentistry Clinic. The sex and age of both groups were matched.

The patient group was conditioned for transplantation according to the following protocol. Patients received cyclophosphamide (50 mg/kg/day for 4 days for patients with aplastic anemia or 60 mg/kg/day for 2 days for patients with leukemia or lymphoma) and busulfan (4 mg/kg/day for 1 day for patients with aplastic anemia or 4 mg/kg/day for 4 days for patients with leukemia or lymphoma). Methotrexate and cyclosporin were used for GVHD prophylaxis after allo-HSCT.

The medical records of the patients were reviewed and the following information was retrieved: gender and age of the patient, donor gender, primary disease, stem cell source (bone marrow or blood stem cells), leukocyte, neutrophil and platelet counts. Biopsies of the lower lip were done in patients at +100 day for chronic GVHD staging in the salivary glands as previously described⁸.

The study protocol was approved by the local ethics in research committee, and informed consent was obtained from all the patients, or from the patients' parents if they were under 18 years of age.

DNA isolation

The DNA extraction was carried out as described by Boom *et al.*⁵ (1990) and modified as described below. We added 450 µl of lyses buffer (6.0 M GuSCN, 65 mM Tris HCl pH 6.4, 25 mM EDTA, 1.5% TritonX-100) and 20 µl of silica (SiO₂, Sigma S-5631) to the microcentrifuge tube containing the oral mucosa

swab pellet. The tube was vortexed and incubated for 30 min at 56°C, centrifuged at 10,000 g for 1 min, and the supernatant was discharged. The pellet obtained (DNA adsorbed to the silica) was washed twice with 450 µl of washing buffer (6.0 M GuSCN, 65 mM Tris HCl), twice with 70% ethanol, once with 450 µl of acetone and dried at 56°C for 10 min. Finally, 100 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) were added and the solution was incubated at 56°C overnight to elute the DNA. After incubation the solution was homogenized and centrifuged at 10,000 g for 2 minutes and the supernatant containing DNA was transferred to a new tube.

Nested Polymerase Chain Reaction (PCR)

The PCR reactions were performed as described elsewhere¹⁹. Six µl of DNA solution were subjected to PCR with 2 sets of primer pairs from a genome of *Helicobacter pylori* strain 26695. The outer primer pair was 5'CAGTTATTTGGTGGCTACAACCG 3' and 5' CCCATCAATAGAGACGCTTAATCC 3'. The 50 µl of reaction mixture containing buffer, *Taq* DNA polymerase, primers, and deoxyribonucleoside triphosphates were subjected to 40 cycles at 95°C for 30 seconds, at 57°C for 45 seconds, and at 72°C for 30 seconds performed in an Eppendorf-Master Cycler (Eppendorf, Westbury, NY, USA). After the first round of PCR, 2 µl of the final product were used as a template for the second PCR with the inner primer pair 5' GCTGTAATTTAAGGGTGGGGGTTG 3' and 5' TGCCGTAATTCAAAGCTCAAGCG 3'. The same procedure as described earlier was used except for the annealing temperature, which was 56°C, and for the use of 30 cycles instead of 40.

Agarose gel electrophoresis

Ten microliters of each reaction product were added to 2 µl of gel loading dye (0.25% bromophenol blue, 30% glycerol, 10 mM EDTA) to visualize the specific product (345 base pairs) in a 1.5% agarose gel, and electrophoresis was carried out using 1x TAE buffer. DNA fragments were visualized after staining with ethidium bromide (0.5 µg/ml) and using the photo documentation system Vilber Lourmat (Torcy, France). The molecular weight of the DNA was estimated using 100 bp ladder markers.

Statistical analysis

Statistical analysis was performed by the *chi*² and Mann-Whitney tests. A significance level of $P \leq 0.05$ was used.

RESULTS

The distribution of positive HSCT patients and controls according to the presence of *H. pylori* is presented in Table 2. The results demonstrate a positive association between HSCT patients and the presence of *H. pylori* in the oral mucosa ($p = 0.002$).

The patients were scored for chronic GVHD of salivary glands and categorized according to the presence of *H. pylori* in the oral mucosa (Table 3). There was no association between cGVHD of salivary glands and the presence of *H. pylori*. The median counts of leukocytes/mm³ and neutrophils/mm³ in the group of HSCT patients positive for *H. pylori* were not statistically different from those of the patients negative for it (Table 4). Although a decreased count of platelets/mm³ in the

TABLE 2 - Distribution of HSCT (haematopoietic stem cell transplantation) patients and control subjects according to the presence of *Helicobacter pylori* in the oral mucosa.

	<i>H. pylori</i> + n (%)	<i>H. pylori</i> - n (%)
HSCT (n = 46)	23 (50.0)	23 (50.0)
Control group (n = 46)	8 (17.4)	38 (82.6)

$p = 0.002$.

TABLE 3 - Distribution of haematopoietic stem cell transplantation patients according to cGVHD (chronic graft-versus-host disease) severity of salivary glands and the presence of oral swabs positive for *Helicobacter pylori*.

GVHD	<i>H. pylori</i> + n (%)	<i>H. pylori</i> - n (%)
Absence	5 (22.7)	5 (22)
Mild	14 (63.6)	9 (39)
Moderate/Severe	3 (13.7)	9 (39)

$p = 0.13$. In one HSCT patient, cGVHD staging was not possible due to insufficient sample collection.

TABLE 4 - Median number of leukocytes, neutrophils and platelets in HSCT subjects, positive and negative for *H. pylori*.

	<i>H. pylori</i> +	<i>H. pylori</i> -	P-value
Leukocytes (cels/mm ³)	4,680	4,500	0.95
Neutrophils (cels/mm ³)	2,420	2,340	0.11
Platelets (cels/mm ³)	147,000	181,000	0.10

group positive for that organism was observed, the difference was not statistically significant.

DISCUSSION

Helicobacter pylori is a microaerophilic, spiral, gram-negative bacterium that colonizes the human stomach. Although not invasive, it causes inflammation of the gastric mucosa and it is linked to gastric ulcers and carcinomas³. In most cases, the acquisition of the infection would be during childhood⁷. Previous studies have identified this microorganism in dental plaque and saliva^{6,11}, which would implicate the oral cavity as a potential reservoir for *H. pylori* or as a possible route of transmission to other sites. The possible role of *H. pylori* in the oral cavity is a highly controversial issue.

Although *H. pylori* infection has been reported worldwide, improved sanitation and antibiotics have dropped this bacterium prevalence in the developed world over the past century³. Recent evidence has demonstrated that the decline of *H. pylori* has reduced the incidence of stomach cancer but may be triggering an upsurge in diseases of the esophagus, such as acid reflux, Barrett's esophagus (a premalignant lesion) and adenocarcinoma². In addition, *H. pylori* infection can be involved in the pathogenesis of the idiopathic interstitial pneumonia after stem cell transplantation²¹. In the present study a higher presence of *H. pylori* in the oral mucosa of HSCT patients was detected. This fact may be due to poor oral hygiene conditions of the patients during transplantation and/or immunosuppression related to HSCT therapy. It is well known that the oral cavity is a frequent site of local infections and also an important port of entry for systemic infections

in HSCT recipients. The presence of *H. pylori* in the oral cavity may be a risk factor for infection or reinfection of the stomach of these patients¹². Although the importance of *H. pylori* in the oral cavity has not been established, this finding may be relevant to the gastrointestinal pathology of HSCT patients.

In the present investigation GVHD severity was not associated with the presence of *H. pylori* in the oral mucosa. However, in a study of Chinese carriers of *H. pylori* during allogeneic bone marrow transplantation, a positive association between *H. pylori* carriage and reduced risk of gut acute GVHD was demonstrated¹. Although a previous study has shown that *H. pylori* infection accelerates recovery of the platelet count after HSCT, possibly by stimulating IL-6 production²⁰, we have not identified any association between platelet, leukocyte and neutrophil counts and the presence of *H. pylori* in the oral mucosa.

CONCLUSION

The present study showed an increased frequency of *H. pylori* in the oral mucosa of HSCT patients when compared to that of non-transplanted healthy volunteers. The systemic implications related to the presence of this bacterium in the oral mucosa of HSCT patients needs further investigation.

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