Presence of *Candida* spp. in the oral cavity of heart transplantation patients

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Received: May 28, 2009 - Modification: April 27, 2010 - Accepted: May 21, 2010

**ABSTRACT**

*Candida* spp. can lead to infections or even fungal sepsis particularly among immunocompromized individuals. Objective: The aim of the present study was to analyze the presence of *Candida* spp. among patients subjected to orthotopic heart transplantation. Material and Methods: Oral rinses from 50 patients subjected to orthotopic heart transplantation, aged 13 to 70 years, 40 males and 10 females, were examined. Sex-age-oral conditions matched-control included 50 individuals who were not subjected to any kind of transplantation and were not immunocompromized for any other reason. Counts of yeasts were expressed as median values of logarithm of cfu/mL and were statistically compared by Mann-Whitney’s test. The heart transplant and control groups were compared for the presence of *Candida* spp. by chi-square test (p<0.05). Results: The results showed statistically significant difference (p=0.001) in the prevalence of *Candida* spp. between the transplantation and control groups. Counts of yeasts (cfu/mL) in the transplanted group were significantly higher than in the control group (p=0.005). *Candida albicans* was the most prevalent species isolated from both groups. Conclusions: It was concluded that *Candida* yeast counts were higher in the heart transplant recipients than in the controls. There was higher variation of *Candida* species among the heart transplant patients and the most frequently isolated samples were: *Candida albicans, Candida glabrata* and *Candida tropicalis*. Isolates of *Candida dubliniensis* was not found in either of the groups.

Key words: *Candida*. Heart transplantation. Oral. Fungal infections.

**INTRODUCTION**

Candidiasis occurs in organ transplanted patients that are in immunosuppression treatment and carriers of acquired immunodeficiency virus¹,³,³,⁸,²⁶,³⁰. If disseminated, these infections are related to high morbidity and mortality levels¹,⁴. *Candida albicans* is the etiological agent of most of oropharyngeal candidiasis cases associated with immunosuppression, although infections caused by *C. tropicalis* and *C. dubliniensis* have also been reported¹,³,²¹.

A heart transplant patient undergoing immunosuppressive therapy receives cyclosporine, azathioprine and prednisone or tacrolimus (FK506), and rapamycin, among others¹⁰,²⁶. The main causes of death in the immediate period, up to the first year following heart transplantation, are infection and rejection³,²⁶. The incidence of fungal infection in patients receiving a heart transplant varies between 10% and 25%. Extreme cases have also been reported, where the cause of death was fungal sepsis⁵,⁶. The levels of *Candida* spp. in the oral cavity of heart transplant recipients can lead to a better understanding of the relationship among host, immunosuppression and the fungus development, improving the quality of life of these patients. The present study evaluated the prevalence of *Candida* spp. in the oral cavity of orthotopic heart transplant recipients.
MATERIAL AND METHODS

Ethic Aspects

This study was approved by the Research Ethics Committee of the São José dos Campos Dental School/UNESP (060/2001 PH/CEP) and the Medical School of the University of São Paulo/INCOR/HCFMUSP (1004/01).

Subjects

Inclusion criteria

One-hundred individuals were included in this study. Fifty patients were recipients of orthotopic heart transplantation, aged 13 to 70 years (mean age=48.6 years), 40 males (80%) and 10 females (20%). These patients received immunosuppressive therapy for a minimum period of 2 months and were under clinical follow-up at University of São Paulo Medical School’s Heart Institute (INCOR/HCFMUSP).

Sex-age-oral conditions matched control group included 50 individuals who were not subjected to any kind of organ transplantation. These individuals were aged 13 to 70 years (mean age=47.5 years), 40 males (80%) and 10 females (20%), with the closest oral conditions to those of the heart transplant patients (regarding the use of denture or orthodontic appliances). These individuals were under treatment at São José dos Campos Dental School, São Paulo State University, Brazil.

Exclusion criteria

Patients who presented clinical symptoms of oral candidiasis or were under antifungal or antibacterial therapy were excluded from the study. Patients under any kind of immunosuppressive therapy were excluded from the control group.

Oral Rinse Collection

Each individual received 10 mL of sterile phosphate-buffered saline (PBS; 0.1 M and pH 7.2) supplied in a disposable universal sterile container. They were required to mouthwash for 30 s and collect the oral rinse back into the previously identified container. This container was kept in ice in a thermal bag, and was forwarded to the microbiology laboratory within a maximum time span of 3 h between sampling and processing. Each sample was centrifuged at 2,300 xg for 10 min (Centrifuge MPW-350R; Biosystems, São José dos Pinhais, PR, Brazil) and the supernatant was kept on ice until the PCR analyses. Supernatant was transferred to 75 mL of zymoliase (0.5 mg/mL) (Sigma, St. Louis, MO, USA) solution (0.5 mg/mL in 1 M sorbitol buffer). Tubes were maintained at 95°C for 10 min. After this period, samples were centrifuged at 8,000 xg at 4°C for 15 min and the supernatant was kept on ice until the PCR analyses.

For PCR, two pairs of primers were used: two universal primers, Uni-f: 5’-GCATATCAATAAGCGAGAAAAG-3’ and Uni-r: 5’GGTCCGTGTGTTTCAAGACG-3’; and two C. dubliniensis-specific ones, DUBF Act-f: 5’GTATTTGTCGTTCCCCTTTC-3’ and DUBR Act-r: 5’-GGTGGTGTGTCATCAAGCTC-3’. The amplification was carried out in a 10 μL final volume containing five picomoles of each, 5.0 μL PCR Master Mix (Promega, Madison, WI, USA), 3.2 μL of ultra-pure water and 1 μL of DNA template. Cycling conditions consisted of 3 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 58°C, 60 s at 72°C, followed by 72°C for 10 min (Mastercycler, Eppendorf, Hamburg, Germany). In all reactions, C. albicans (ATCC 18804) and C. dubliniensis (NCPF 3108) were included as control. Amplification products were separated by electrophoresis through 2% (w/v) agar (Difco Laboratories, Detroit, MI, USA) with chloramphenicol (0.1 mg/mL of culture medium) and also in duplicate on CHROMagar (CHROMagar; Microbiology, Paris, France). Plates were incubated at 37°C for 48 h and kept at room temperature for the following 5 days. After, the number of colony-forming unities per milliliter (cfu/mL) of yeasts was calculated. Stained smears were obtained by Gram method for microscopic confirmation and yeast cfu were transferred to Sabouraud dextrose agar, so as to obtain pure cultures that were later identified. All cfu that showed different color or morphology in CHROMagar were isolated.

Candida Species Identification

Initial presumptive identification was based on colonies morphology and colour observed in CHROMagar. Isolates were phenotypically identified according to Samaranayake and Macfarlane (1990) and Sandvén (1990). The following tests were performed: germ-tube production, chlamidiosconidia, hyphae/pseudohyphae production on Corn Meal agar (Difco), carbohydrate fermentation and assimilation. Orange-red colonies with Rhodotorula spp. features were confirmed by urease testing.

Genotypic Identification of C. dubliniensis (Polymerase Chain Reaction - PCR)

Isolates identified phenotypically as C. albicans or C. dubliniensis were subjected to molecular detection of C. dubliniensis. These isolates were analyzed by PCR, according to the methodology proposed by Donnelly, et al. (1999) and Mähnb, et al. (2005), with modifications. Briefly, the isolates were plated on Sabouraud dextrose agar and incubated for 24 h at 37°C. Then, a single colony was transferred to 75 mL of zymoliase (0.5 mg/mL) (Sigma, St. Louis, MO, USA) solution (0.5 mg/mL in 1 M sorbitol buffer). Tubes were maintained at 95°C for 10 min. After this period, samples were centrifuged at 8,000 xg at 4°C for 15 min and the supernatant was kept on ice until the PCR analyses.

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agarose gels containing 25 \( \mu M \) ethidium bromide (Sigma) and visualized on a UV transilluminator (Foto/UV 26; Bioresearch, Upland, CA, USA). A DNA "ladder" 100 pb (BRL; Gibco, Gaithersbur, MD, USA) was used as molecular size standard. The gels were photographed for documentation (Kodak Photo Documentation System, DC290; Sigma).

Statistical Analysis

The heart transplant and control groups were compared for the presence (or absence) of Candida spp. by chi-square test in order to compare proportions.

Counts of yeasts were expressed as median values of logarithm of cfu/mL and were statistically compared by Mann-Whitney’s test, using the Minitab Statistical Software version 13.1 (Minitab Inc., State College, PA, USA). A significance level of 5% was set for all analyses.

RESULTS

The heart transplant group differed significantly \((p=0.001)\) from the control group for the presence of Candida spp. (Table 1). There was no statistically significant difference \((p=0.104)\) between the heart transplant and the control group for the proportion of the presence of C. albicans and other species.

Counts of yeasts (cfu/mL) in the transplanted group were significantly higher than in the control group \((p=0.005)\) (Table 2).

C. albicans was the prevalent species in both groups of subjects. Among the transplanted patients, other isolated Candida species and Rhodotorula rubra was found. C. dubliniensis was not found in either of the groups. Larger counts of Candida species were found in the transplanted individuals in relation to the controls (Table 3). Four isolates of R. rubra were observed in the heart transplant group and 7 in the control group.

Associations of Candida species observed in the transplant recipients and controls are shown in Figure 1. More associations were observed in the transplantation group.

Table 1- Number and percentage of individuals positive to Candida spp. in the oral cavity

| Groups   | n   | Positive to Candida spp. | Negative to Candida spp. |
|----------|-----|--------------------------|--------------------------|
| Transplanted | 50  | 44 (88%)*                | 6 (12%)                  |
| Control  | 50  | 28 (56%)                 | 22 (44%)                 |

*Statistically different proportion in relation to the control (chi-square test, \(p=0.001\))

Table 2- Values of logarithm of colonies forming units per millilitre (log cfu/mL) in the studied groups

| Groups   | n   | Median 25% | 75% |
|----------|-----|------------|-----|
| Transplanted | 50  | 5.66*      | 6.27|
| Control  | 50  | 5          | 5.55|

*Statistically significant difference in relation control group (Mann-Whitney, \(p=0.005\))

Table 3- Candida species isolated from the oral cavity of orthotopic cardiac transplantation patients and species isolated from the oral cavity of control individuals

| Species              | Transplanted (n=67) | Control |
|----------------------|---------------------|---------|
|                      | n       | %       | n | % |
| C. albicans          | 31      | 49.21   | 25 | 75.75 |
| C. glabrata          | 9       | 14.28   | 3  | 9.1  |
| C. tropicalis        | 7       | 11.11   | 2  | 6.06 |
| C. krusei            | 4       | 6.35    | 2  | 6.06 |
| C. parapsilosis      | 4       | 6.35    | -  | -    |
| C. kefyr             | 3       | 4.76    | -  | -    |
| C. lipolitica        | 3       | 4.76    | -  | -    |
| C. guilliermondii    | 1       | 1.59    | 1  | 3.03 |
| C. lusitaniae        | 1       | 1.59    | -  | -    |
| Total                | 63      | 100     | 33 | 100  |
C. tropicalis most frequently related to immunosuppression were reported that, in addition to individuals, point to infections. C. albicans was responsible for 29% of such cases occurred among heart transplant recipients. The overall mortality rate of transplant recipients show an increased prevalence of yeasts, and this was confirmed by our results. According to Ruskin, et al. (1992), the reduction of C. albicans in the oropharyngeal area could potentially decrease the incidence of disseminated candidiasis that is often associated to death cases. Dictar, et al. (1992) agree that orthotopic heart transplant patients show an increased prevalence of Candida species isolated from the oral cavity of orthotopic heart transplant patients (Tx) and control individuals (C).

**DISCUSSION**

Transplantation is a treatment option for patients who have lost the normal function of their vital organs. Infectious complications are still among the major causes of morbidity and mortality during the period immediately following surgery. Many authors agree that orthotopic heart transplant recipients show an increased prevalence of C. albicans yeasts, and this was confirmed by our results. According to Ruskin, et al. (1992), the reduction of C. albicans in the oropharyngeal area could potentially decrease the incidence of disseminated candidiasis that is often associated to death cases. Dictar, et al. (1992) have reported that the mortality rate due to fungal infections in recipients of transplanted organs increased to 23.7%, and that 75% of these cases occurred among heart transplant recipients. C. albicans was responsible for 29% of such infections.

Many studies on immunosuppressed individuals point to C. albicans as the most prevalent species, which agrees with the results of the present study. A greater variety of species was found in the heart transplant group, and this results also agree with to previous findings in literature. Furthermore, some authors have reported that, in addition to C. albicans, the species most frequently related to immunosuppression were C. tropicalis, C. glabrata and C. dubliniensis. In the present study, C. albicans was the most prevalent species, followed by C. glabrata and C. tropicalis. C. dubliniensis was not found in either of the groups. In the control group, C. albicans, R. rubra and C. glabrata were the most prevalent species.

C. dubliniensis has many phenotypic features with C. albicans and the incidence of candidemia due C. dubliniensis is unknown mainly because of difficulty in distinguishing these two strains. C. dubliniensis has been frequently associated to oral and systemic candidiasis in HIV-positive patients under chemotherapy and bone marrow transplant patients, sometimes liable for death cases. Thus, it is very important to identify among the population of immunoosuppressed patients those who have C. dubliniensis in their oral microbiota, and how big is this ratio (cfu/mL).

Sabouraud dextrose agar with chloramphenicol and CHROMagar Candida were effective for Candida growth and, in both media, there was a higher prevalence of yeasts in the heart transplant group. This group presents species variation one third higher in relation to control group and these results were similar to those obtained for others authors. Being chromogenic, CHROMagar allowed the identification of distinct colonies. This fact offered a better visualization of the various shapes of the yeasts.

There was higher isolation (cfu/mL) on Sabouraud dextrose agar with chloramphenicol and CHROMagar Candida for both groups. It is likely that this culture medium causes partial inhibition of some Candida isolates, which might be attributed to a higher selectivity of chromogenic components. We used a reliable, simple, fast, and noninvasive method for studying the prevalence of Candida yeasts in oral cavity of patients subjected orthotopic heart transplantation. This method was also able to detect, among the studied population, those individuals with higher Candida cfu/mL counts in saliva, and who might be more susceptible to candidiasis development. In such cases, the
use of a proper antifungal prophylaxis may be indicated, aiming at avoiding the clinical oral manifestation of the disease, as well as its systemic dissemination\textsuperscript{14,20}.

CONCLUSION
The number of \textit{Candida} yeasts was significantly larger in the oral cavity of orthotopic heart transplant patients than in non-immunosuppressed subjects. There was higher prevalence of \textit{C. albicans} in saliva isolates from both groups, but the proportion between this species and others was not statistically different. The heart transplantation group showed higher variation of \textit{Candida} species in relation to the control group.

ACKNOWLEDGEMENTS
The authors would like to thank the State of São Paulo Research Foundation/FAPESP (04/10654-9 and 05/55135-1) for financial support.

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