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ORIGINAL ARTICLE

Prevalence, antifungal susceptibility and virulence determinants of oral yeast species isolated from immunodeficient patients in Northeastern Brazil

Prevalência, suscetibilidade a antifúngicos e determinantes de virulência de leveduras orais isoladas de pacientes imunodeficientes do Nordeste do Brasil

Prevalencia, susceptibilidad antifúngica y determinantes de virulencia de levaduras orales aisladas de pacientes inmunodeficientes del Noreste de Brasil

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ABSTRACT

Background and Objectives: Oral candidiasis has a common occurrence in immunocompromised patients. However, other emergent infections have become increasingly common. The aim of this study was to investigate the prevalence, virulence determinants and the antifungal susceptibility of yeast colonizing the mucosa of immunocompromised patients in Northeastern Brazil. **Methods:** Samples from sixty HIV-positive patients seen at the Specialized Service / Hospital Dia - Hospital Universitário Prof. Alberto Antunes from the Federal University of Alagoas were collected from subgingival sites and seeded on CHROMagar for presumptive confirmation of *Candida* spp. followed by PCR and sequencing. In addition, we tested virulence determinants, phospholipase and protease and evaluated *in vitro* the Minimum Inhibitory Concentration of antifungals amphotericin B and fluconazole. This project was approved by the Research Ethics Committee of the Center for Higher Studies in Maceió. **Results:** Approximately 63% of the patients were colonized by yeasts, with *C. albicans* as the predominant species, while non-*Candida albicans* species accounted for 49% of the isolates, with *C. dubliniensis* and *C. parapsilosis* being the commonest, but *C. intermedia*, *Bullera penniseticola* and *Naganishia liquefaciens* were also found. The virulence determinants protease and/or phospholipase were also produced by Candida spp. and some uncommon opportunistic isolates such as *Kodamaea ohmeri, N. liquefaciens* and *Saitozyma podzolica*. Furthermore, most of *Candida* spp. strains and some uncommon opportunistic species showed high values of minimal inhibitory concentration. **Conclusion:** Results obtained indicate that *C. albicans* continues to be the predominant species in oral cavity of immunodeficient patients and along with other unusual species may present high resistance to the antifungals tested.

Keywords: *Virulence Factors. Oral Candidiasis. Acquired Immunodeficiency Syndrome. Yeasts. Candida*

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RESUMO

Justificativa e Objetivos: A candidíase oral tem uma ocorrência comum em pacientes imunocomprometidos. No entanto, outras infecções emergentes tornaram-se cada vez mais habituais. O objetivo deste estudo foi investigar a prevalência, os determinantes de virulência e a suscetibilidade a antifúngicos de leveduras que colonizam a mucosa de pacientes imunocomprometidos na região Nordeste do Brasil. **Métodos:** A amostra foi composta por 60 pacientes HIV positivos atendidos no Serviço de Atendimento Especializado/Hospital Dia do Hospital Universitário Prof. Alberto Antunes, vinculado à Universidade Federal de Alagoas. As amostras foram coletadas em regiões subgengivais e semeadas em CHROMagar para confirmação presuntiva de *Candida* spp., seguido por PCR e sequenciamento. Além disso, testamos os determinantes de virulência fosfolipase e protease e avaliamos *in vitro* a concentração inibitória mínima dos antifúngicos anfotericina B e fluconazol. Este projeto foi aprovado pelo Comitê de ética em pesquisa do Centro de Estudos Superiores de Maceió. **Resultados:** Aproximadamente 63% dos pacientes foram colonizados por leveduras. A espécie *C. albicans* foi predominante, enquanto as espécies de *Candida* não-*albicans* representaram 49% dos isolados, sendo *C. dubliniensis* e *C. parapsilosis* as mais comuns. Entretanto, *C. intermedia*, *Bullera penniseticola* e *Naganishia liquefaciens* também foram encontrados. Os determinantes da virulência protease e/ou fosfolipase também foram produzidos por *Candida* spp. e alguns isolados oportunistas incomuns como *Kodamaea ohmeri*, *N. liquefaciens* e *Saitozyma podzolica*. Além disso, a maioria dos isolados de *Candida* spp. e algumas espécies oportunistas incomuns apresentaram altos valores de concentração inibitória mínima. **Conclusão:** Os resultados obtidos indicam que *C. albicans* continua a ser a espécie predominante na cavidade oral de pacientes imunodeficientes e, juntamente com outras espécies incomuns, pode apresentar alta resistência aos antifúngicos testados.

Descritores: *Fatores de Virulência. Candidíase Bucal. Síndrome de Inmunodeficiência Adquirida. Leveduras. Candida*

RESUMEN

Justificación y Objetivos: La candidiasis oral acomete con frecuencia a pacientes inmunocomprometidos. Sin embargo, otras infecciones emergentes se han vuelto cada vez más comunes. El objetivo de este estudio fue investigar la prevalencia, la producción de determinantes de virulencia y la susceptibilidad a antifúngicos de levaduras que colonizan la mucosa de pacientes inmunocomprometidos en la región Nordeste de Brasil. **Métodos:** Se colectaron muestras de sesenta pacientes VIH positivos atendidos en el Servicio de Atención Especializado/Hospital Día del Hospital Universitario Prof. Alberto Antunes, vinculado a la Universidad Federal de Alagoas. Se colectaron las muestras en las regiones subgingivales y las sembraron en CHROMagar para la presunta confirmación de *Candida* spp. seguido de PCR y secuenciación. Además, analizamos los determinantes de virulencia fosfolipasa y proteasa y evaluamos *in vitro* la concentración mínima inhibitoria de los antifúngicos anfotericina B y fluconazol. Este proyecto fue aprobado por el Comité de Ética en Investigación del Centro de Estudios Superiores de Maceió. **Resultados:** Aproximadamente el 63% de los pacientes fueron colonizados por levaduras, y la *C. albicans* fue la especie predominante, mientras que las especies de *Candida* no-*albicans* representaron el 49% de los aislamientos, de las cuales la *C. dubliniensis* y la *C. parapsilosis* fueron las más comunes. Sin embargo, también se encontraron *C. intermedia*, *Bullera penniseticola* y *Naganishia liquefaciens*. Los determinantes de virulencia de proteasa y/o fosfolipasa también fueron producidos por *Candida* spp. y algunos aislados oportunistas inusuales como *Kodamaea ohmeri*, *N. liquefaciens* y *Saitozyma podzolica*. Además, la mayoría de los asilados de *Candida* spp. y algunas especies oportunistas inusuales mostraron valores altos de concentración mínima inhibitoria. **Conclusión:** Los resultados obtenidos indican que *C. albicans* continúa siendo la especie predominante en la cavidad oral de pacientes inmunodeprimidos y, junto con otras especies poco comunes, puede presentar una alta resistencia a los antifúngicos evaluados.

Palabras clave: *Factores de Virulencia. Candidiasis Bucal. Síndrome de Inmunodeficiencia Adquirida. Levaduras. Candida*

INTRODUCTION

In advanced stages of HIV infection, patients become more vulnerable to opportunistic infections concurrent with progressive loss of CD4+ T-cells compared to those in early or intermediate stages.[1]with a focus on immune reconstitution inflammatory syndrome (IRIS Because of their weak immune systems, immunocompromised persons might be prone to opportunistic infections. Frequently, some microorganisms in endogenous or transitory microbiota may cause pathogenic clinical episodes such as candidiasis.[2]allergic mechanisms, or colonization of cavities, resulting in the diseases varying in severity and clinical course which may also depend upon the organs affected and the host. Apergilli are abundant in the environment. The genus Aspergillus now includes more than 130 recognized species, 1 all of which are world-wide in distribution. Their spores are produced in great abundance and are readily disseminated into the air by wind current. Although the spores are frequently inhaled by man, most of human aspergillosis are thought to be caused by members of the A. fumigatus group. Ho-

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wever, members of other groups, particularly, A. flavus, A. niger and A. terreus, have also been implicated.

Despite being the most common fungal infection, candidiasis is not the only one; other species such as *Aspergillus flavus, Aspergillus nomius, Aspergillus tamarii, Cryptococcus neoformans* and *Cryptococcus gattii* have been identified as opportunistic fungal infections that can infect humans and may be responsible for some kind of pathogenesis.[3] There are also some yeasts, such as *Rhodotorula* spp, that are normally associated with environmental samples that have proven in recent years to be important emerging pathogens.[4]

Candidiasis is a type of infection caused by yeasts from the *Candida* genus, it has the ability to colonize the most diverse parts of the human body and has *C. albicans* species as its main etiologic agent. Current studies demonstrate the rise of other emerging species that can also cause these types of pathogenesis, such as *Candida tropicallis*, *Candida parapsilosis*, *Candida glabrata*, *Candida krusei*, *Meyerozyma guilliermondii* (= *Candida guilliermondii*), *Candida lusitaniae, Candida nivariensis, Candida haemulonii, Candida ciferrii* and *Candida auris*, which are able to cause skin, oral, vaginal, vulvovaginal or systemic candidiasis.[5]

Oral candidiasis has an opportunistic character and is closely related to HIV. This type of infection can be used to monitor the stages of immunosuppression, and although it is not a fatal disease, it can serve as a gateway to major complications. The pseudomembranous form, commonly known as thrush, is the most common type and can affect individuals of all ages.[6]

Candida genus yeasts can develop several virulence factors, such as adhesion, polymorphism and toxin production. The ability to produce hydrolytic enzymes, such as proteases and phospholipases, could be the most important features expressed in virulent *Candida* spp. phenotypes.[7]

Chromogenic selective media has been used to facilitate presumptive identification of *Candida* species.[8] However, there are many species with the same phenotypic characteristics within the genus as well as species from other genera of yeast that may cause similar injuries and can develop well in these culture media, forming similar staining colonies or often being equal to the standard for pathogenic yeasts. Therefore, the presumptive identification has often not been sufficient.[9]

Molecular methods could be used to obtain an efficient identification of clinical isolates, such as pathogenic *Candida* spp.. Multiplex PCR can be used to identify pathogenic fungi, such as *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. neoformans* and the nested multiplex PCR technique could be used as a tool for quick and accurate identification, providing a greater security to diagnosis and efficient treatment against candidiasis.[10] However, the identification of pathogenic yeast species by PCR of the RPS0 gene intron fragment may be a more viable alternative for providing higher speed and lower cost in identifications.[11]

The aim of this study was to investigate the prevalence, virulence determinants and the susceptibility of yeasts colonizing the mucosa of immunocompromised patients in northeastern Brazil.

METHODS

This was an observational cross-sectional study. From April to June 2014, oral mucosa from 60 patients older than 18 years who were HIV positive (diagnosed by rapid test, ELISA and/or Western blot) was sampled with a swab. Not all demonstrated signs of oral infections. These participants were from the program STD-AIDS (Sexually Transmitted Diseases - AIDS) from Prof. Alberto Antunes University Hospital - HUPAA / UFAL, an important reference center of the state. As inclusion criteria, participants must be enrolled in the Specialized Attendance Service / Hospital Dia do Hospital University / UFAL; 18 years of age or older and accept to participate in this study by signing the informed consent form (ICF). As exclusion criteria, the patients with conditions that may have association with vitamin D deficiency or insufficiency, such as: recent hospitalization; chronic renal failure; nephrotic syndrome; pancreatic failure; chronic liver disease, active hepatitis or cirrhosis; intestinal inflammatory disease, gastric or intestinal resection; recent or active use (within three months) of carbamazepine, systemic glucocorticoid, hormones, isoniazid, phenobarbital, phenytoin or rifampicin and patients receiving vitamin D or being treated for osteoporosis.

Phenotypic characterization

Fungal isolation

The patients' oral swabs were inoculated in chro--mogenic yeast-specific plates (BBL CHROMagar Candida; Becton Dickinson) and incubated for five days at 25ºC. The growth test at 42ºC was conducted in order to differentiate presumptive isolates of C. albicans from C. dubliniensis. We observed positive growth of C. albicans species and no C. dubliniensis growth.

Hydrolytic enzymes production assays

Isolates were submitted to protease and phospholipase enzyme tests. In order to test phospholipase production, the isolates were inoculated in petri dishes containing culture mediums with egg yolk as a substrate and incubated for seven days at 37°C.[12] The production of protease enzymes was checked utilizing the same test method for phospholipase, with bovine serum albumin as a substrate.[12] The activity zone (Pz) was assessed by dividing the colony diameter by colony diameter plus the precipitation zone. The Pz index values are as follows: high (Pz < 0.40), medium (Pz = 0.41–0.60), low (Pz = 0.61–0.80) and none (Pz = 1.00).

Molecular characterization

DNA extraction

For DNA extraction, the isolates were inoculated in liquid medium GYP (2% glucose (*w*/v), 0.5% yeasts extract (*w*/v), 0.5% peptone (*w*/v) and incubated at 25°C for 48

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hours. After precipitation and washing, the cells were suspended in 0.5 mL of extraction buffer (0.15 M NaCl, 50 mM Tris-HCl, 10 mM EDTA and 2% SDS (*w*/v) [pH 8.0]). The tube was incubated for 1 hour at 65°C. Total DNA was extracted with phenol-chloroform (v/v) and precipitated with 1 volume of isopropanol. After standing for 5 min at room temperature, precipitated DNA was collected by centrifugation (10,000 X g, 15 min), washed in ice-cold 70% ethanol for 5 min and air-dried. The final DNA pellet was suspended in 100 µL of TE (10 mM Tris-HCl and 1 mM EDTA adjusted to pH 8.0) and stored at -20°C. An agarose gel at concentration of 1% was used to analyze the integrity of the DNA extracted.

PCR assay

The presumptive identifications were confirmed by molecular methods based on the methodology previously reported, utilizing the polymerase chain reaction (PCR) with specific primers to amplify the RPS0 gene of pathogenic yeasts.[11]

Sequencing of the ITS region and the D1/D2 domain of the LSU rDNA gene

The ITS region was amplified with ITS1 and ITS4 primer pairs and the D1/D2 domain of the LSU rDNA gene was amplified with primer pair NL1 and NL4. PCR amplification process occurred in a T100 Thermal Cycler (Bio-Rad) under the following conditions: initial denaturation (95ºC, 5 min); 35 cycles of denaturation (95ºC, 45s), annealing (56ºC, 30s), extension (72ºC, 1 min) and final extension (72ºC, 7 min).[10]

Sequencing was performed on an ABI 3730 automated DNA gene analyzer (Applied Biosystems) according to the manufacturer's instructions. The nucleotide sequences were processed on Staden Package (version 2.0.0b10) and edited with the software. All sequences were checked manually. Consensus sequences obtained were compared against the GenBank database utilizing the nucleotide Blast program.

Antifungal Susceptibility Testing

Antifungal susceptibility testing of the strains was performed using Clinical Laboratory Standards Institute method (CLSI) protocol M27-A3. The isolates were cultured on YEPD medium at 35°C for 48h. The inoculum was prepared by the suspension of colonies in saline solution (0.85%) and the number of cells was measured with a hemocytometer and they were diluted to obtain 5 x 10^2 to 2.5 x 10^3 CFU/mL in each well. Fluconazole was tested in the range of 0.125–64 µg/mL and amphotericin B was utilized in the final concentration range of 0.03–16 µg/mL. The incubation was at 35°C for 48 hours and the minimal inhibitory concentrations were determined as the minimal antifungal concentration that no visible growth (100% of inhibition) was observed when compared with the control growth (wells without antifungal agents). For *C. albicans* isolates, we defined as resistant those with MIC > 2 for amphotericin B and > 1 for fluconazole, while non-*Candida albicans* species were set according to the reference strains of each species present in document M27-A3. Non-conventional clinical isolates with MICs of > 1 and > 16 µg/mL were considered resistant to amphotericin B and fluconazole, respectively. As a control to the experiment, we used *Candida albicans* ATCC 90028 strain according to MIC ranges of 0.5–2.0 µg/mL to amphotericin B and 0.25–1.0 µg/mL to fluconazole.

Statistical analysis

Risk factors were compared for the presence (or absence) of yeasts by chi-square test in order to compare the proportions. The level of significance was established as 5% ($p \leq 0.05$).

Ethical aspects

This study is part of a project and was approved by the Research Ethics Committee of the *Centro de Estudos Superiores de Maceió – CESMAC*, No. 623.311, CAAE 29924714.7.0000.0039. Patient's age in the study ranged from 22 to 62 years old and they signed a free and informed consent form (ICF).

RESULTS

From sixty immunocompromised patients in this study, 38 showed colonization of the oral mucosa by one or more yeast species. Sex-related identification was not possible as men and women represented the same proportion of 50% (19 men and 19 women). Almost 23% of the participants (n = 14) were smokers and from those individuals, 93% (n = 13) showed clinical symptoms of fungal infection, which indicates that smoking is directly related to the appearance and colonization of the oral mucosa by yeasts.

From the presumptive identification obtained with chromogenic cultures (CHROMagar BBL *Candida*; Becton Dickinson), we assumed that approximately 65% (n = 32) of the isolates with a greenish pattern belonged to the *C. albicans* (49%) or *C. dubliniensis* (16%) species and around 35% of the isolates were *Candida krusei* and *Candida tropicalis* species (Table 1).

The growth test was conducted at 42ºC to presumptively distinguish the species *C. albicans* and *C. dubliniensis*. We observed that among the 32 isolates, 73% (n = 24) grew at a temperature close to 42°C. We tentatively identified them as *C. albicans* and noted that 16% (n = 8) did not grow at this temperature. There are other fungi species of the *Candida* genus, such as *C. tropicalis*, with the ability to grow at this temperature.

Phospholipase and protease activities were tested and both enzymes are claimed as virulence factors produced by species of *Candida* spp.[12] When subjected to phospholipase test, approximately 33% (n = 16) of the isolates showed positive results. For the protease assay, 35% (n = 17) were positive. Notwithstanding, 10% (n = 5) of isolates were positive for both enzymatic tests and all were presumptively and molecularly identified as *C. albicans* (Table 1).

The method based on the amplification of RPS0

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Table 1. Enzymatic activities, presumptive, molecular identification and in vitro antifungal susceptibility of oral yeasts.

Yeasts codes	Phospholipase	Protease	Presumptive identification	Molecular identification	Antifungal susceptibility	
					Amphotericin B (MIC µg/mL)	Fluconazole (MIC µg/mL)
LHU 01	++	++	*C. albicans*	*C. albicans*[a]	16	64
LHU 02	-	-	*C. albicans*	*C. albicans*[a]	0.03	0.125
LHU 03	-	-	*C. tropicalis, C. krusei*	*C. dubliniensis*[a]	16	8
LHU 04	-	++	*C. dubliniensis*	*K. ohmeri*[b]	NT	NT
LHU 05	-	-	*C. albicans*	*C. albicans*[a]	8	2
LHU 06	-	-	*C. dubliniensis*	*C. tropicalis*[a]	16	64
LHU 07	-	-	*C. albicans*	*C. albicans*[a]	0.5	1
LHU 09	-	++	*C. albicans*	*C. albicans*[b]	8	8
LHU 10	-	-	*C. tropicalis, C. krusei*	*D. hansenii*[a]	0.5	4
LHU 11	-	++	*C. albicans*	*C. albicans*[b]	0.25	1
LHU 12	++	-	*C. albicans*	*C. albicans*[a]	1	2
LHU 13	-	-	*C. tropicalis, C. krusei*	*C. glabrata*[b]	4	32
LHU 14	-	-	*C. albicans*	*C. glabrata*[b]	4	4
LHU 15	-	-	*C. tropicalis, C. krusei*	*K. ohmeri*[b]	16	4
LHU 16	-	-	*C. dubliniensis*	*K. ohmeri*[b]	NT	NT
LHU 17	-	-	*C. tropicalis, C. krusei*	*P. kudriavzevii*[b]	16	8
LHU 18	-	-	*C. krusei*	*C. orthopsilosis*[b]	4	2
LHU 19	-	-	*C. tropicalis*	*B. penniseticola*[b]	1	2
LHU 20	-	-	*C. albicans*	*C. albicans*[a]	NT	NT
LHU 21	-	++	*C. krusei*	*C. parapsilosis*[b]	4	4
LHU 22	++	-	*C. albicans*	*C. albicans*[a]	NT	NT
LHU 23	-	++	*C. dubliniensis*	*C. parapsilosis*[c]	16	16
LHU 24	-	++	*C. tropicalis, C. krusei*	*C. albicans*[a]	NT	NT
LHU 25	-	-	*C. dubliniensis*	*C. intermedia*[b]	0.03	0.125
LHU 26	++	-	*C. albicans*	*C. albicans*[a]	4	4
LHU 27	++	++	*C. albicans*	*C. albicans*[a]	1	2
LHU 28	++	-	*C. albicans*	*C. albicans*[b]	4	16
LHU 29	++	-	*C. albicans*	*C. albicans*[b]	2	0.5
LHU 30	++	-	*C. albicans*	*C. albicans*[b]	8	0.5
LHU 31	-	++	*C. tropicalis, C. krusei*	*C. dubliniensis*[b]	NT	NT
LHU 32	++	-	*C. albicans*	*C.albicans*[a]	16	2
LHU 33	-	-	*C. krusei*	*C. tropicalis*[a]	0.25	4
LHU 34	++	-	*C. tropicalis, C. krusei*	*C. albicans*[a]	2	0.125
LHU 35	++	-	*C. dubliniensis*	*N. liquefaciens*[b]	2	32
LHU 36	-	++	*C. albicans*	*S. podzolica*[b]	8	2
LHU 37	++	++	*C. albicans*	*C. albicans*[a]	16	1
LHU 38	++	-	*C. albicans*	*C. albicans*[a]	NT	NT
LHU 39	-	++	*C. tropicalis, C. krusei*	*C. parapsilosis*[b]	2	2
LHU 40	-	-	*C. tropicalis, C. krusei*	*C. intermedia*[b]	4	4
LHU 41	-	-	*C. tropicalis, C. krusei*	*C. albicans*[a]	16	64
LHU 42	-	++	*C. albicans*	*C. albicans*[a]	NT	NT
LHU 43	-	-	*C. dubliniensis*	*C. dubliniensis*[a]	8	1
LHU 44	-	++	*C. dubliniensis*	*C. dubliniensis*[a]	8	4
LHU 45	++	-	*C. albicans*	*C. albicans*[a]	0.03	0.5
LHU 46	++	++	*C. albicans*	*C. albicans*[a]	0.03	0.125
LHU 47	-	-	*C. albicans*	*C. albicans*[a]	2	8
LHU 48	++	++	*C. albicans*	*C. albicans*[a]	0.03	0.125
LHU 49	-	-	*C. krusei*	*C. orthopsilosis*[b]	16	8
LHU 50	-	++	*C. krusei*	*C. parapsilosis*[b]	16	16

LHU = "Leveduras Hospital Universitário"
(++) Strong production of the enzyme (-) negative (NT) not tested.
[a]RPS0 gene amplification
[b]D1/D2 region 26S rDNA sequencing
[c]ITS region sequencing

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with a specific primer was used to confirm presumptive identifications. All isolates were subjected to this method, but only 53% (n = 26) of the isolates were identified. The D1/D2 domain of the LSU rDNA gene sequencing was also performed with the isolates not identified by the previous methodology.

The remaining 47% (n = 23) of the samples analyzed were not successfully identified using the D1/D2 domain and were submitted to the ITS sequencing, where the strain LHU 23 was identified as *C. parapsilosis* (Table 1). These results confirm that PCR with specific primers is only useful for conventional oral yeasts.

Based on the D1/D2 domain of the LSU rDNA gene sequencing, we observed that 18% (n = 4) of the isolates were identified as *C. albicans* and 82% (n = 18) as non-*Candida albicans* and non-conventional yeasts species (Table 1). These results indicate that the chromogenic culture (CHROMagar Candida) had 96% of *C. albicans* identification efficiency and 25% of *C. dubliniensis* identification efficiency. The identification based on PCR with specific primers demonstrated 80% of *C. albicans* identification efficiency. However, 10% (n = 5) of the isolates from immunodeficient patients did not belong to the genus *Candida*.

The minimum inhibitory concentration test revealed a high number of isolates resistant to amphotericin B, including clinically uncommon species and three isolates resistant to fluconazole, from which two strains of *C. albicans* and one of *C. tropicalis*, according to the break point adopted (Table 1). Among *C. albicans*, 48% of isolates showed resistance to amphotericin B, while this value was 75% among *C.* non-*albicans* species, revealing an uncommon increase in the rate of resistance to traditional antifungals.

DISCUSSION

There is a growing interest in correctly identifying yeasts of hospital interest, as more and more species in this group are being considered emerging. The development of chromogenic methods brought a certain speed to the diagnosis, but as we show here, they lack precision when compared to molecular methods. The results of the comparison between chromogenic and molecular methods showed that some species can grow in CHROMagar and not belong to the genus *Candida*, as previously reported.[13]

At the time of collection, not all patients had symptoms related to the yeasts isolated from their samples. According to previous studies, asymptomatic carriage of *Candida* species is a common finding in HIV positive patients and is a possible risk factor for subsequent oral infection. For instance, Mun et al. observed colonization or infection by yeasts in 98 (48.3%) of HIV/AIDS patients and from these, 83 (84.7%) patients carried *C. albicans*.[14]

In our results, smoking was the only habit that showed a possible relationship with colonization by yeast in the type of patients evaluated ($p \leq 0.05$), and this relationship has already been observed in the literature.[14] Cigarette smoking, in varying concentrations, can modulate the expression of genes, such as HWP1, EAP1 and SAP2, which can enhance the growth of biofilm formation and adhesiveness, a characteristic which is connected to the virulence of *Candida* spp. as an important virulence factor.[15]

In addition, we found some species of uncommon genera to this type of infection, which shows two things: the medium used is not as selective as imagined, since it allows the growth of these yeasts, and the diagnosis obtained through it can lead to mistakes in adopting necessary measures, such as in choosing the antifungal to be used. The yeast *Kodameae ohmeri* is an emerging pathogen reported in immunocompromised patients, and it was associated with patients' oral cavity and medical devices, such as an intravenous catheter. *K. ohmeri* has also shown resistance to azole type antifungals that can cause nosocomial clusters and it is associated with virulence factors, such as production of extracellular enzymes as phospholipase and protease.[16] The presence of this yeast species in this study, mainly a strain considered resistant to amphotericin B (MIC = 16µg/mL), highlights its importance as an emerging pathogen of immunocompromised individuals, although it does not produce phospholipase and protease *in vitro*.

One strain (LHU 36) of the species *Saitozyma podzolica* (=*Cryptococcus podzolicus*), considered resistant to amphotericin B (MIC = 8µg/mL), was found in the oral cavity of immunocompromised patients. Usually, this species is found in soil samples, sediment and lake waters.[17] *S. podzolica* was able to grow at 42°C and has showed the ability to produce extracellular protease enzyme. Together, these characteristics can help this species to be responsible for pathogenic episodes in humans.

The species *Debaryomyces hansenii* is a hemiascomycetous yeast commonly found in natural substrate, but has already been described in human infections, and it is considered part of the growing list of opportunistic fungi frequently found in severely immunocompromised patients.[18] Because of the great similarity in their biochemical and morphological characteristics, the species is extremely difficult to be phenotypically differentiated from *P. guilliermondii*, a constituent species of the normal human microbiota, and molecular identification was already proposed to prevent mistakes.[18] One of our strains, LHU 10, was susceptible to amphotericin B (MIC = 0.5µg/mL) and fluconazole (MIC = 4µg/mL), but was not able to produce phospholipase or protease.

Fungemias due to *Pichia kudriavzevii* are rare, but the species has already been reported from a HIV-infected patient with cirrhosis in Spain.[19] The strain LHU 17 of *P. kudriavzevii* has not shown the ability to produce phospholipase or protease and was susceptible to fluconazole (MIC = 8µg/mL), but it was considered resistant to amphotericin B (MIC = 16µg/mL).

The most unusual isolate found was of the species *B. penniseticola*, originally isolated and described from the phylloplane of Thailand plants, and until then no research demonstrated the association of this species with human substrates.[20] The strain LHU 19 has not shown the ability to produce phospholipase or protease and was susceptible

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tible to amphotericin B (MIC = 1µg/mL) and fluconazole (MIC = 2µg/mL).

We have also observed the isolation of environmental fungal species *N. liquefaciens*. Although it is associated with extremophile environments, *N. liquefaciens* has been responsible, in recent years, for clinical episodes in humans. In our study, the strain LHU 35 was considered resistant to amphotericin B (MIC = 2µg/mL). It has been reported to be colonizing the skin in patients with atopic dermatitis, and may be responsible for fungemia in blood streams related to central venous catheter and it could also be responsible for fatal cases of meningitis in immunodeficient patients.[21]

C. tropicalis alone or in association with *C. parapsilosis* is the second most prevalent *Candida* species after *C. albicans*. Resistance of clinical isolates to the azoles has been reported, while fewer studies related the resistance of this species to other antifungal drugs, such as amphotericin B. Fluconazole resistance has been reported among isolates obtained from different Spanish hospitals and among isolates obtained from one of the largest public hospitals in Malaysia, for example.[18,22] In Brazil, among 51 isolates of *C. tropicalis* collected from the General Hospital of Fortaleza, three strains were resistant to fluconazole, and none of the strains showed resistance to amphotericin B.[23]

The mechanisms responsible for drug resistance in *Candida* species have been elucidated and acquired resistance to azole in *C. tropicalis* could be due to overexpression of CtERG11 associated with a missense mutation in the gene, also to the presence of CtMDR1 gene, associated to efflux pumps that confer cross-resistance to the entire class of azoles.[24] It is noteworthy the presence of isolates resistant to amphotericin B among isolates considered clinically rare, such as *K. ohmeri, N. liquefaciens* and *S. podzolica*. The resistance of these species to amphotericin B had not been previously reported by any research, although resistance to fluconazole was not observed.

Corroborating results seen in the literature, *C. albicans* remains the species with the highest prevalence among fungal infections in oral mucosa of immunodeficient patients and has a great virulence potential. This study also highlights the need for molecular identification by sequencing of pathogenic species, considering that the presumptive identification is still not enough for accurate diagnosis of opportunistic species that are prone to being pathogenic.[13]

Some species isolated in the present study, normally associated with environmental samples, have already been found in emerging infections in humans.[19] The identification of these non-conventional yeasts is very important, but it is difficult to perform with standard laboratory methods, mainly in laboratories without the specific technology and because of that, they can be misidentified. In addition, many strains were resistant to the antifungal agents tested, reinforcing the importance of correctly characterizing these isolates in clinical practice, including molecular identification and *in vitro* testing for antifungals agents.

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AUTHORS' CONTRIBUTION

João Pedro Monteiro, Gustavo Vasconcelos Bastos Paulino, Patrícia Valente and Thayná Melo de Lima Morais contributed for the conception, design, analysis and writing of the article;

Sonia Maria Soares Ferreira, Leonardo Broetto, Melissa Fontes Landell contributed for the planning and design, review and final approval of the article;

All authors approved the final version of the article to be published and are responsible for all aspects of the study, including the assurance of precision and integrity of the results.

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