FREQUENCY OF Candida SPECIES IN A TERTIARY CARE HOSPITAL IN TRIANGULO MINEIRO, MINAS GERAIS STATE, BRAZIL

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SUMMARY

Infections by Candida species are a high-impact problem in public health due to their wide incidence in hospitalized patients. The goal of this study was to evaluate frequency, susceptibility to antifungals, and genetic polymorphism of Candida species isolated from clinical specimens of hospitalized patients. The Candida isolates included in this study were obtained from blood cultures, abdominal fluids, and central venous catheters (CVC) of hospitalized patients at the Clinical Hospital of the Federal University of Uberlândia during the period of July 2010 - June 2011. Susceptibility tests were conducted by the broth microdilution method. The RAPD-PCR tests used employed initiator oligonucleotides OPA09, OPB11, and OPE06. Of the 63 Candida isolates, 18 (28.5%) were C. albicans, 20 (31.7%) were C. parapsilosis complex species, 14 (22.2%) C. tropicalis, four (6.4%) C. glabrata, four (6.4%) C. krusei, two (3.3%) C. kefyr, and one (1.6%) C. lusitaniae. In vitro resistance to amphotericin B was observed in 12.7% of isolates. In vitro resistance to flucozanole, and itraconazole. High genetic polymorphisms were observed for isolates of C. albicans and C. parapsilosis complex species, mainly with the OPA09 marker.

KEYWORDS: Antifungal susceptibility; Candida species; Candidemia; Genotyping.

INTRODUCTION

In recent decades, candidiasis has increased significantly worldwide due to increased lifespans of immunosuppressed patients or transplant and HIV/AIDS patients. In many countries, the invasive infection of Candida yeast is a considerable public health problem, due to its severity, cause of increased hospital stays, cost, and contribution to high indexes of morbimortality. Some reports note that the mortality index caused by candidemia may reach 40-60% of hospital-admitted patients.

Invasive candidiasis is related to several factors that compromise patient conditions, such as neutropenia, organ transplantations, previous colonization by Candida species, prolonged use of antibiotics, presence of catheters for nasogastric feeding, use of urinary or parenteral probes for hemodialysis or mechanical ventilation, neoplasia, immunosuppressive diseases, drugs, and gastrointestinal surgeries.

For many years, C. albicans was regarded as the main cause of invasive fungal infections, but lately, non-C. albicans species have been reported to be predominant, especially in hospital environments.

According to some investigators, this is due to the selective pressure from the prophylactic use of fluconazole in patients at risk of developing invasive fungal infections. Variable frequencies of different species of Candida are identified depending on the hospital complexity and/or geographic region.

The choice of treatment for candidemia or invasive candidiasis is mainly based on two factors: Candida species and the condition of the host immune system. Depending on the protocol of the institution and the availability of antifungal agents, azoles (fluconazole, voriconazole, and posaconazole), polyene (amphotericin B), and/or echinocandins (caspofungin, anidulafungin, and micafungin) are used for the treatment. Echinocandins are recommended for prophylaxis and for the treatment of different groups of patients due to their efficacy and low toxicity in critical patients compared to other azoles and amphotericin B.

Candidiasis epidemiology has been studied by genotypic analysis, which employs molecular tools with high discriminating power to
distinguish different isolates, and thus allowing for improved accuracy in clinical and epidemiological studies\textsuperscript{29,34}. These studies attempt to relate the genotypes of isolates with pathogenicity and epidemiology. Genotypes with varying degrees of heterogeneity were found in different anatomical sites among various population groups, including patients and healthy individuals, and in different geographical areas\textsuperscript{4,10,33}.

The most commonly used molecular methods include polymorphism detection in the length of restriction fragments (RFLP) with hybridization (Southern blot) or amplification (AFLP), karyotyping in pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and other techniques based on polymerase chain reaction (PCR) of random amplified polymorphic DNA (RAPD-PCR)\textsuperscript{2,20,34}.

Regional peculiarities and hospital complexity services may influence the predominance of Candida species. These emphasize the need for studies on epidemiology, prevalence, and resistance to antifungals. This study aims to evaluate the frequency, in addition to testing the susceptibility to antifungals as well as genetic polymorphisms, of Candida species isolated from samples of blood, CVC, and abdominal fluids of hospitalized patients in a tertiary hospital in the Triangulo Mineiro region, Minas Gerais State, Brazil.

**MATERIAL AND METHODS**

Isolates in the study: Candida samples included in the study were obtained from patients admitted to the Clinical Hospital of the Federal University of Uberlândia (UFU) in the city of Uberlândia located in the Triangulo Mineiro region, Minas Gerais State, Brazil, during the period of July 2010-June 2011. The isolates were from blood cultures, CVC, and abdominal fluids. Chromogenic agar (BD CHROMagar\textsuperscript{R} Candida, France) and Sabouraud dextrose agar were used to isolate the yeasts, which were identified by classical methods\textsuperscript{21} and confirmed by the Auxacolor\textsuperscript{R} system (Bio-Rad, France). Candida albicans and C. dubliniensis were differentiated by PCR utilizing specific primers, according to the technique described by Estrada-Barraza et al.\textsuperscript{14}. Samples were stored in BHI-glycerol broth at -20°C. Experiments were conducted after sample activation and incubation at 35°C for 24-48 h.

Antifungal susceptibility tests: The broth microdilution method described in document M27-A3, Clinical Laboratory Standard Institute (CLSI), was used for the tests. Antifungals amphotericin B (Fungizon, Bristol Myers Squibb, Brazil), fluconazole (Pfizer, Sandwich, UK), and itraconazole (Janssen, Beerse, Belgium) were tested in culture plates of RPMI-1640 medium containing glutamine, without sodium bicarbonate, and buffered using pH-7.0 MOPS with glucose (18g/L). The final concentrations of the antifungal agents were 0.03-16 µg/mL for amphotericin B and itraconazole and 0.25-64 µg/mL for fluconazole. Briefly, yeasts were inoculated in Sabouraud dextrose agar and incubated at 35°C for 24 h. Culture suspensions adjusted to 1-5x10^6 cells/mL were prepared in sterilized saline. Susceptibility tests were made in duplicates and the microdilution plates were incubated at 35°C for 48 h. Control strains were C. parapsilosis ATCC 22019 and C. krusei ATCC 6258. The minimum inhibitory concentration (MIC) was determined visually. For the azoles, MICs corresponded to the concentration inhibiting around 50% of growth for each microorganism compared to the control well (without antifungal); for amphotericin B, the MIC was the smaller drug concentration that inhibited 100% of yeast growth\textsuperscript{7}. For azoles, breakpoints were as indicated in CLSI\textsuperscript{43} and, for amphotericin B, due to lack of consensus, the values suggested by NGUYEN et al.\textsuperscript{29} were used.

Molecular typing: DNA extraction was performed according to the method of BOLANO et al.\textsuperscript{4}. The RAPD-PCR tests were performed with initiator oligonucleotides OPAP09 (5’TGGTTAAGGCCT3’), OPB11 (5’GTAGACCCGT3’), and OPB06 (5’AAAGCCCTCCT3’) (Invitrogen, São Paulo, Brazil). The reaction final volume was 25 µL and contained 2 µL DNA (60 ng/mL), 0.25 mmol of each deoxynucleotide (dATP, dCTP, dTTP, and dGTP) (Invitrogen), 1U Taq polymerase (Invitrogen), 2.5 mM MgCl\textsubscript{2}, and 2.5 mM initiator nucleotide. All amplifications were conducted in a thermalcycler (Eppendorf, Mastercycle Gradient, USA), consisting of an initial amplification cycle of four min at 92°C followed by 40 cycles of 40 s at 92°C, 40°C for 1.5 min, and 72°C for two min, and finally followed by five min at 72°C. Amplification fragments were separated by agarose gel (1.4%) electrophoresis for three h at 80V and 100mA. The gels were stained with ethidium bromide and visualized under UV light and the images were captured by a photo documentation system. Profiles for each sample were analyzed visually, and bands were classified as present (1) or absent (0). Genetic relationships (similarity coefficients) were calculated by the Jaccard coefficient equation (\(S_{J} = n_{ab}/(n_{a}+n_{b}+n_{ab})\)), where \(n_{ab}\) is the number of bands shared by two samples: \(a\), the number of exclusive bands for the first sample and \(b\), for the second sample\textsuperscript{40}. Values of \(S_{J}\) from 0.99-1.00 represent the same genotype, values from 0.800-0.99 represent clonally related samples (strongly similar but not identical), and values less than 0.800 indicate distinct samples. Dendrograms based on \(S_{J}\) values were generated for comparison by the unweighted pair group method with the arithmetical averages (UPGMA) method utilizing the multivariate statistical package program (MVSP).

Ethical committee: This study was approved by the Ethical Committee for Human Research of the Federal University of Uberlândia (UFU) under the number 317/10.

Statistical analysis: Qualitative variables were compared using the chi-square test, and the \(G\) test was used for quantitative results. In both tests, statistical significance was considered when \(p < 0.05\).

RESULTS

During the study period, 63 cultures of body fluids from individuals with suspected systemic candidiasis were positive for Candida spp., of which 47 were in blood, nine were in CVC, and seven in abdominal fluids, all obtained from 58 hospitalized patients at the Clinical Hospital of Federal University of Uberlândia. Thirty-four were from males and 24 from females. Ages of the patients ranged from one day to 94 years, with a mean age of 42 years. Most patients who developed systemic candidiasis and who had a positive culture were older than or equal to 21 years (Fig. 1).

Of the 63 Candida isolates, 18 (28.5%) were identified as C. albicans and 45 (72.5%) as non-C. albicans, distributed as follows: 20 (31.7%) C. parapsilosis complex species; 14 (22.2%) C. tropicalis; four (6.4%) C. glabrata; four (6.4%) C. krusei; two (3.3%) C. kefyr; and one (1.6%) C. lusitaniae. Candida dubliniensis was not identified by PCR. Except for C. albicans (\(p = 0.050\)), the distribution of species between males and
Molecular analyses by RAPD-PCR with primers OPA09 and OPB11 produced different molecular profiles. Primer OPE06 did not amplify any genome fragments of the isolates included in the study. Analysis of the dendrogram generated from band profiles among isolates of the same species showed isolate groups with $S_j = 1.00$ (identical isolates with the same profile) and $S_j < 0.80$ (distinct samples). Table 3 shows frequencies of profiles generated with each primer for the most frequent species. Each profile relates isolates showing the same genotypes ($S_j = 1.00$). Candida parapsilosis complex strains showed five (A-E) and four (A-D) profiles with OPA09 and OPB11, respectively.

Profile A of each primer was composed of the higher number of isolates. Candida albicans isolates presented six and two profiles, respectively, with primers OPA09 and OPB11 (Table 3). Candida tropicalis isolates produced only one profile with OPA09 and two unrelated ones ($S_j < 0.8$) with OPB11 (A-B) (Table 3). Two C. kefyr strains were demonstrated to be distinct strains with both primers ($S_j < 0.80$). Candida krusei showed two profiles with OPA09, each one with two isolates with similarity indexes that the indicated strains were clonally related ($0.99 > S_j > 0.80$); OPB11 produced only one profile, with 100% similarity among isolates. Candida glabrata produced two profiles with each one of the primers; OPA09 and OPB11 grouped three isolates in profile A and another isolate in profile B, with A and B being unrelated ($S_j < 0.80$) for both primers.

DISCUSSION

The predominance of Candida species non-C. albicans observed in this study confirms results reported in other studies from different Brazilian regions. The C. parapsilosis complex occurred at the highest frequency compared to other species, including C. albicans. Observations from other Latin American countries and Tunisia show that C. parapsilosis-induced infections increased significantly in the past two decades. Candida albicans, C. tropicalis, and C. parapsilosis complex species are the most frequent species isolated in candidemia cases and constitute 82.5% as a whole of the isolates in this study and, in some other instances, represent more than 90% of etiologies.

Candida parapsilosis has been reported as the second or third most frequent Candida species in candidemias. In fact, in 2005, the C. parapsilosis complex was reclassified into three species: C. parapsilosis sensu stricto, C. orthopsilosis, and C. metapsilosis. These three species may exhibit, according to some researchers, differences in

Table 1

| Clinical specimens | C. parapsilosis | C. albicans | C. tropicalis | Others* | Total |
|--------------------|----------------|-------------|---------------|---------|-------|
| Blood              | 11 (17.4%)     | 9 (14.3%)   | 10 (15.9%)    | 5 (7.9%)| 35 (55.6%)|
| CVC                | 6 (9.5%)       | 1 (1.6%)    | 1 (1.6%)      | 1 (1.6%)| 9 (14.3%)|
| Blood + CVC        | 3 (4.8%)       | 5 (7.9%)    | 2 (3.2%)      | 2 (3.2%)| 12 (19.0%)|
| Abdominal fluids   | 0 (0.0%)       | 3 (4.8%)    | 1 (1.6%)      | 3 (4.8%)| 7 (11.1%)|
| Total isolates     | 20 (31.7%)     | 18 (28.6%)  | 14 (22.2%)    | 11 (17.4%)| 63 (100%)|

*Other species: C. krusei (4); C. glabrata (4); C. lusitaniae (1); and C. kefyr (2). CVC = central venous catheter.
patterns of susceptibility to antifungal and biofilm production\(^1\). Of all the \textit{Candida} isolates, they were detected in 55.6\% of samples from blood cultures, 14.3\% from CVC, 11.1\% from abdominal fluids, and 19\% from blood and CVC simultaneously. Positive results in blood cultures are considered the main indicators of invasive infections. Although cultures of samples obtained from other organic sites may be secondary in the diagnostics of hospital infection, these \textit{Candida} isolates may have a predictive value for the occurrence of candidemias\(^1,50\). Similar to what happened with bacteria, the indiscriminate use of antifungal drugs has stimulated the occurrence of fungi with decreased susceptibility or even \textit{in vitro} resistance, especially among \textit{Candida} species\(^6\). In this study, the susceptibility of isolates in relation to fluconazole, itraconazole, and amphotericin B, which were the antifungals used for treatment of invasive candidiasis in the service during the period studied, was analyzed. However, recent studies have pointed primarily to the use of echinocandins\(^26,31,49\). Most isolates were susceptible to the three antifungals evaluated.

\textit{Candida krusei} and \textit{C. glabrata} are known to be resistant and less susceptible to fluconazole, respectively\(^31,32,34,45,49\). \textit{In vitro} resistance of \textit{Candida} species, notably non-\textit{C. albicans}, to fluconazole has been reported in different hospital studies\(^13,16,31,32,36,39\). Itraconazole has been recently utilized in the treatment of candidemia in neutropenic patients because it is less toxic than amphotericin B, as well as having shown a similar

| Table 2 |
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| \textit{In-vitro} susceptibility of \textit{Candida} species to three antifungal agents |
| Species (n) | Antifungal agents | MIC (µg/mL) | Resistant n (%) |
| | | Range | MIC\(_{50}\) | MIC\(_{90}\) | |
| \textit{C. parapsilosis} (20) | Amphotericin B | 0.5-2.0 | 1.0 | 1.0 | 1 (5\%) |
| | Fluconazole | 0.125-1.0 | 0.5 | 0.5 | 0 |
| | Itraconazole | 0.03-0.125 | 0.03 | 0.03 | 0 |
| \textit{C. albicans} (18) | Amphotericin B | 0.5-2.0 | 0.5 | 1.0 | 1 (5.6\%) |
| | Fluconazole | 0.125-0.5 | 0.25 | 0.5 | 0 |
| | Itraconazole | 0.03-0.06 | 0.03 | 0.06 | 0 |
| \textit{C. tropicalis} (14) | Amphotericin B | 0.5-1.0 | 1.0 | 1.0 | 0 |
| | Fluconazole | 0.125-0.5 | 0.25 | 0.5 | 0 |
| | Itraconazole | 0.03-0.06 | 0.03 | 0.06 | 0 |
| \textit{C. krusei} (4) | Amphotericin B | 1.0-2.0 | - | - | 3 (75\%) |
| | Fluconazole* | - | - | - | 4 (100\%) |
| | Itraconazole | 0.03-0.12 | - | - | 0 |
| \textit{C. glabrata} (4) | Amphotericin B | 1.0-2.0 | - | - | 2 (50\%) |
| | Fluconazole | 0.5-4.0 | - | - | 0 |
| | Itraconazole | 0.25-0.3 | - | - | 0 |
| \textit{C. kefyr} (2) | Amphotericin B | 0.5-2.0 | - | - | 1 (50\%) |
| | Fluconazole | 0.125-0.5 | - | - | 0 |
| | Itraconazole | 0.06-0.125 | - | - | 0 |
| \textit{C. lusitaniae} (1) | Amphotericin B | 1 | - | - | 0 |
| | Fluconazole | 0.25 | - | - | 0 |
| | Itraconazole | 0.03 | - | - | 0 |

*\textit{C. krusei} is intrinsically resistant to fluconazole.

| Table 3 |
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| Frequency of cluster profiles and isolates per cluster with primers OPA09 and OPB11 |
| Species (n) | OPA09 | OPB11 |
| Molecular profile* | Frequency of isolates | Molecular profile* | Frequency of isolates |
| \textit{Candida parapsilosis} (20) | A | 10 | A | 13 |
| B | 3 | B | 4 |
| C | 3 | C | 1 |
| D | 3 | D | 1 |
| E | 1 |
| \textit{Candida albicans} (18) | A | 7 | A | 14 |
| B | 4 | B | 4 |
| C | 4 |
| D | 1 |
| E | 1 |
| F | 1 |
| \textit{Candida tropicalis} (14) | A | 14 | A | 13 |
| B | 1 |

*A cluster was considered when it grouped isolates with 100% similarity.
effectiveness to that presented by other azoles. One isolate (25%) of *C. glabrata* showed a dose-dependent susceptibility to itraconazole, while NEUFELD et al. reported a dose-dependent susceptibility in only 3.4% of the isolates in their studies.

Resistance to amphotericin B has not been reported among isolates of different regions. In this study, a MIC of 2 µg/mL was determined for some isolates, especially non-*C. albicans* ones, characterizing *in vitro* resistance. Data on the clinical outcomes of patients were not generated in this isolate, as *in vitro* results do not mean resistance, due to the fact that the cut-off point for amphotericin B is not established by the standardization committee due to technical difficulties related to the antifungal and culture media, as reported in the literature. The results should be considered an alert and they should emphasize the importance of continuous surveillance to detect occasional isolates that are resistant to one or more antifungals. Future vigilance studies, including monitoring of patients, on antimicrobial resistance will show if these results were occasional or common occurrences.

The genetic variability of clinical isolates has been used to demonstrate cases of cross infection that occur in health care, but also to determine if the isolates of one anatomical site are identical to isolates from other sites of the same patient. In this study, the RAPD methodology was utilized in an attempt to reveal molecular variants of *Candida* spp. Based on the gel patterns and on the dendrograms obtained (data not shown), six profiles (A-F) were determined with primer OPA09 while OPB11 allowed only two (A-B) for isolates of *C. albicans*. Primer OPA09 had a higher discriminatory power especially for *C. albicans* and *C. parapsilosis* complex (Table 3). Neither of the two primers was able to discriminate isolates of *C. tropicalis*. Isolates of other species occurred in small numbers, so it is not possible to discuss this. Several studies have shown the discriminatory power of different primers and have suggested the use of multiple primers to improve the sensitivity of the results.

This study identified a variety of strains in the patients involved, especially for isolates *C. albicans* and *C. parapsilosis* complex. However, it was not possible to show a cross infection at all. However, in 12 patients who had blood and CVC, positive cultures were isolated to the same species, and these exhibited the same genotype when blood and CVC isolates were compared. This might be evidence of hematological dissemination of this particular microorganism from the CVC, but also blood-to-CVC. Identifying the source of infection is an important way to prevent infection. However, it suggests that prospective studies, including clinical data of patients and correlating these data with the microbiological characteristics of isolated samples may provide important insights for *Candida* spp. epidemiology in patients.

In conclusion, of the *Candida* species isolated during the study period, the most frequent were *C. parapsilosis* complex species followed by *C. albicans* and *C. tropicalis*. Most samples were susceptible to antifungals fluconazole, itraconazole, and amphotericin B. The genotypic markers seemed efficient at discriminating the isolates of *C. albicans* and *C. parapsilosis*; high genetic polymorphism was observed for isolates of *C. albicans* and *C. parapsilosis* complex species, mainly with the OPA09 marker.

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