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Candida albicans isolated from urine: Phenotypic and molecular identification, virulence factors and antifungal susceptibility

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ABSTRACT

Objective: To isolate *Candida albicans* (*C. albicans*) from the urine of hospitalized patients and assess the virulence factors and antifungal susceptibility profiles of the isolates.

Methods: Yeasts were identified using the chromogenic medium CHROMagar™, the VITEK® 2 system, hypertonic Sabouraud broth, tobacco agar, polymerase chain reaction, and DNA sequencing. The evaluated virulence factors were proteinase production, phospholipase production, and biofilm production on polystyrene. The broth microdilution technique was used to determine the minimum inhibitory concentration.

Results: All yeasts isolated from urine were identified as *C. albicans* using both classical and molecular methods. Although 91.3% of the isolates showed no phospholipase activity, 56.5% showed strong proteinase activity and 91.7% produced biofilm. All microorganisms were sensitive to fluconazole, voriconazole and amphotericin B, but 56.5% of the yeasts showed resistance to itraconazole.

Conclusions: *C. albicans* isolates from urine have a high capacity for virulence and can be associated with infectious processes. Furthermore, the high percentage of isolates resistant to itraconazole is important because this antifungal agent is commonly used to treat fungal infections in the hospital environment.

1. Introduction

Candida albicans (*C. albicans*) is the most frequent cause of fungal urinary tract infections in hospitals [1]. This yeast has phenotypic characteristics similar to those of *Candida dubliniensis* (*C. dubliniensis*), making it difficult to identify by morphological and biochemical methods [2]. Thus, specific methods such as growth on tobacco agar and hypertonic agar are required for differentiation. However, molecular methods based on amplification of highly conserved internal transcribed spacer (ITS) regions in yeast genomes allow quick and reliable identification and better discrimination between species [3].

Yeasts of the genus *Candida* can express virulence factors that assist in adhesion and infection in the host. Two such virulence factors, phospholipase and proteinase, are hydrolytic enzymes that promote tissue invasion and cell lysis [4]. The formation of biofilms on biomaterials is also an important virulence factor because it is associated with the persistence of *C. albicans* infection and increased resistance to antifungal drugs [5].

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Resistance of *Candida* spp. to antifungal agents is a frequent problem in the hospital environment [6]. *C. dubliniensis* develops resistance to antifungal agents more easily than *C. albicans* does. When subjected to the action of fluconazole, *C. dubliniensis* can secrete high levels of proteinase and increase its adherence to epithelial cells, leading to tissue invasion [7]. Identification of the virulence factors and defense mechanisms of both species makes it possible to determine their susceptibility to antifungal agents and develop effective antifungal therapies. Thus, the aim of this study was to isolate *C. albicans* from the urine of hospitalized patients and assess the virulence factors and antifungal susceptibility profiles of the isolates.

2. Materials and methods

2.1. Microorganisms

Twenty-three isolates from urine of hospitalized patients were presumptively identified as *C. albicans* by the automated system VITEK® 2 (bioMérieux) and used in this study. The following reference cultures, obtained from the American Type Culture Collection (ATCC), were also used in analyses: *C. albicans* (ATCC No. 90028), *C. dubliniensis* (ATCC No. MYA-646), and *Candida krusei* (ATCC No. 6258).

Yeasts were grown on CHROMagar™ *Candida* medium (Difco) and stored in cryogenic microtubes with Sabouraud dextrose broth (SDB) and 20% glycerol at −80 °C. For analyses, yeasts were reactivated in SDB for 48 h at 35 °C and subcultured on Sabouraud dextrose agar (SDA) for 48 h at 35 °C.

2.2. Phenotypic identification

2.2.1. Growth in hypertonic Sabouraud broth

The growth test in hypertonic Sabouraud broth was carried out as described by Alves *et al.* [8]. The microorganisms were inoculated in Sabouraud broth supplemented with sodium chloride and incubated at 28 °C. The cultures were visually examined for growth every 24 h. Growth indicates the isolate is *C. albicans*, while no growth after 96 h of incubation indicates the isolate is *C. dubliniensis*.

2.2.2. Growth on tobacco agar

Species differentiation on tobacco agar was performed as described by Khan *et al.* [9]. After inoculation of isolates on tobacco agar, plates were incubated at 28 °C and colony characteristics were observed daily for 96 h. Species were differentiated by the morphology and coloration of the colonies: *C. dubliniensis* colonies are rough and yellowish brown, whereas *C. albicans* colonies are smooth and white to cream.

2.3. Molecular identification

2.3.1. DNA extraction

The DNA of isolates was extracted from three colony-forming units (2.40×10^7 cell/cm^3) reactivated and grown in Sabouraud dextrose broth and subsequently incubated in a shaker at a speed of 50 rpm at 25 °C for 24 h. We divide the extraction protocol in four large steps. In step 1 all centrifugations were carried out in room temperature at 5 000 rpm. This step, the tubes containing cells were centrifuged for 5 min and supernatant was discarded. We added 5 mL of MilliQ water in the tubes containing cells. The tubes were centrifuged for 5 min and supernatant was discarded. This step was repeated twice. After, the tubes were again centrifuged for 1 min for sedimentation of cells.

In step 2, the cells were resuspended in 350 μL of phosphate buffered saline (PBS) and transferred into 2 mL microtubes. These microtubes were added 60 μL of β-mercaptoethanol and 800 μL of extraction buffer (3% CTAB, 5% PVP, 2 N NaCl, 100 mM Tris HCl, 25 mM EDTA pH 8). Subsequently, the microtubes were incubated in a water bath at 60 °C for 1 h.

In step 3, 675 μL of phenol/chloroform/isoamyl alcohol (25:24:1) were added in microtubes. The samples were mixed for 10 min by inversion and subsequently centrifuged for 10 min at 12 000 rpm at 4 °C. The aqueous phase was transferred to other microtubes and was added 600 μL of sodium acetate/absolute ethanol (0.625:0.25), and stored in the freezer (−20 °C) for 10 min.

In step 4 all centrifugations were carried at 12 000 rpm. In these steps, the microtubes were removed from the freezer and centrifuged at for 5 min at 4 °C. The supernatant was discarded, and was added 500 μL of 70% cold ethanol in the microtubes. The samples were homogenized by inversion and centrifuged again for 5 min at 4 °C. The supernatant was discarded and the microtubes were inverted on paper towels for 10 min to dry the precipitated DNA. After drying, the DNA was resuspended in TE buffer (10 mM Tris HCl pH 7.61 and 1 mM EDTAm pH 8.7) plus RNase (Promega). To finish, the DNA ware incubated in a water bath (37 °C) for 60 min and after stored at −20 °C. The purity (260 nm/280 nm) and concentration (ng/μL) of the extracted DNA were determined using a nanophotometer (NanoPhotometer™ P-300 UV–Vis, Implen GmbH, Schatzbogen, Germany).

2.3.2. Polymerase chain reaction (duplex PCR) and DNA sequencing

Molecular identification was performed by amplifying the ITS1 and ITS2 regions of rDNA. To this end, duplex PCR was performed with species-specific primers for *C. albicans* and *C. dubliniensis* described by Ahmad *et al.* [10]. The primer sequences are shown in Table 1.

The amplification reactions were performed using MyCycler™ thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification solutions were prepared in a final volume of 25 μL containing 12.5 μL of PCR Master Mix (Kapa Biosystems, Capetown, South Africa), 1 μL of each primer (10 pmol) and 2 μL of genomic DNA (10–20 ng).

The amplification program was as follows: initial denaturation at 95 °C for 5 min; 30 denaturation cycles at 95 °C for 1 min, annealing 55 °C at 30 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The resulting amplification products were subjected to 2% agarose gel electrophoresis [10].

Sequencing was performed to validate duplex PCR. For these analyses, five samples DNA of the isolates were randomly selected. The PCR was performed in the same conditions described above, but use the universal ITS primers (Table 1). The amplicons were purified with isoamyl alcohol and sequenced by the Sanger method on an ABI 3500 automated DNA sequencer (Applied Biosystems) with the same primers used for PCR and a BigDye Terminator Cycle Sequencing Kit.

Table 1
Oligonucleotide primers used for duplex PCR and sequencing of the ITS regions of *C. albicans* and *C. dubliniensis*.

Species	Primer	Sequence	Fragment (bp)	Ref
C. albicans	CalF	5′-TGGTAAGGCGGGATCGCTT-3′	~100	Ahmad *et al.* [10]
	CalR	5′-GGTCAAAGTTTGAAGATATAC-3′		
C. dubliniensis	CduF	5′-AAACTTGTCACGAGATTATTTTT-3′	~325	
	CduR	5′-AAAGTTTGAAGAATAAAATGGC-3′		
Universal[a]	ITS1 F	5′-TCCGTAGGTGAACCTGCGG-3′	Variable	White *et al.* [11]

[a] Primers used for sequencing.

2.4. Virulence factors

2.4.1. Phospholipase production

Phospholipase production was assessed as described by Samaranayake *et al.* [12]. Yeasts were seeded in egg yolk and incubated at 37 °C for 48 h. The results were expressed as the ratio of the diameter of the colony to the diameter of the colony plus the precipitation zone. A ratio (PZ value) of 1.0 indicates no enzyme activity, $0.63 < PZ < 1.0$ indicates moderate enzyme activity, and $PZ \leq 0.63$ indicates strong enzymatic activity [13].

2.4.2. Proteinase production

For determination of proteinase production, yeast cells were seeded in petri plates containing Bacto Agar plus bovine serum albumin, yeast extract, and yeast carbon base and incubated at 37 °C for 48 h [14]. The results were calculated according to Price *et al.* [13].

2.4.3. Biofilm production

Biofilm formation was assessed using the polystyrene adhesion method. Yeasts were cultured in 96-well microplates in SDB at 35 °C for 24 h, and then absorbance was read in a microplate reader (TP Reader; ThermoPlate) at a wavelength of 450 nm. The results were converted to transmittance [15].

2.5. Susceptibility to antifungal agents

Susceptibility testing by the broth microdilution technique was carried out as recommended by the Clinical and Laboratory Standards Institute [16,17]. The tested antifungal agents were amphotericin B, fluconazole, itraconazole, and voriconazole. The results are reported as the minimum inhibitory concentration, which was defined as the lowest concentration of antifungal agent capable of inhibiting 90% of visible yeast growth (for amphotericin B) or 50% of visible yeast growth (for the other antifungal agents).

3. Results

All 23 yeasts grew in hypertonic Sabouraud broth and formed white-cream colonies with a smooth appearance on tobacco agar; thus, they were phenotypically identified as *C. albicans*. All isolates were molecularly identified as *C. albicans* by duplex PCR. These results were confirmed by sequencing, which showed 100% similarity between the ITS sequences of the isolates and the sequences of *C. albicans* in GenBank.

Assessment of virulence factor production showed that 8.7% of the *C. albicans* isolates had moderate phospholipase activity and 91.3% had no phospholipase activity. In contrast, 56.5% of the isolates showed strong proteinase activity, 39.1% had moderate proteinase activity, and 4.4% had no proteinase activity. Finally, 91.7% of the *C. albicans* isolates formed biofilms.

Table 2
Antifungal susceptibility profiles of *C. albicans* isolated from urine and minimum inhibitory concentrations of the antifungal agents.

Antifungal agent	No. of isolates			MIC (μg/mL)			
	S	DD	R	Range	MIC_{50}	MIC_{90}	GM
Amphotericin B	23	–	–	0.25–2	1	1	0.595
Fluconazole	23	–	–	0.25–2	0.25	1	0.515
Itraconazole	3	7	13	0.03–4	0.5	1	0.528
Voriconazole	23	–	–	0.03–8	0.03	0.06	0.053

S: sensitive; DD: dose-dependent; R: resistant; MIC: minimum inhibitory concentration; MIC_{50} and MIC_{90}: minimum concentration capable of inhibiting 50% and 90% of the isolates, respectively; GM: geometric mean.

Antifungal susceptibility testing showed that all isolates were sensitive to fluconazole, voriconazole, and amphotericin B. For itraconazole, 13.1% of the isolates were sensitive, 30.4% were dose-dependently sensitive, and 56.5% were resistant (Table 2).

4. Discussion

Hypertonic Sabouraud broth can be used to differentiate *C. albicans* and *C. dubliniensis*, since only *C. albicans* can grow in this medium [8]. *C. dubliniensis* is emerging as a pathogen that can cause invasive disease in hospitalized patients. However, accurate data on the incidence of infection with this yeast species are still scarce because *C. dubliniensis* is often confused with *C. albicans* in clinical laboratories [18]. Our results agree with those of Alves *et al.*, who found that 248 *C. albicans* isolates grew in hypertonic Sabouraud broth and 18 *C. dubliniensis* isolates exhibited no visually detectable growth [8], thereby demonstrating the effectiveness of the methodology described by those authors.

Tobacco agar was first developed for the presumptive identification of *Cryptococcus neoformans* and was subsequently used to differentiate isolates of *C. albicans* and *C. dubliniensis* with 100% accuracy [9,19]. Species are differentiated on this medium by the color and appearance of colonies. Khan *et al.* analyzed 50 isolates of *C. albicans* and 28 isolates of *C. dubliniensis*, and all had the features expected for the studied species [9]. Ribeiro *et al.* examined 200 isolates and considered tobacco agar satisfactory for differentiating *C. albicans* and *C. dubliniensis*, as identifications on this medium showed 92% agreement with the results of PCR [20].

A fast, relatively inexpensive, and precise molecular method for differentiating *C. albicans* and *C. dubliniensis* involves the amplification of two specific and independent target DNA sequences in a single reaction (duplex PCR). In a study by Ahmad *et al.*, this method was used to discriminate 55 *C. albicans* and 67 *C. dubliniensis* isolates among a total of 134 isolates of *Candida* species, and no amplification was observed with other *Candida* species [10]. Thus, the study showed that duplex PCR has high sensitivity and specificity for the identification of *C. albicans* and *C. dubliniensis* isolates.

Phospholipase and proteinase are enzymes produced by *Candida* species. They are considered important virulence factors because they can degrade host tissue and allow tissue invasion. The level of expression of these enzymes can indicate the virulence of *Candida* species [21]. Previous studies have demonstrated that *C. albicans* can produce proteinase and phospholipase [22,23].

Biofilm formation by *Candida* species and biofilm structure are influenced by the contact surface, environmental factors and the *Candida* species involved [24]. Other studies have reported biofilm production by *C. albicans* on surfaces of different materials [25,26].

The results of this study confirm that *C. albicans* has the capacity for virulence and may be associated with urinary tract infections in hospitalized patients, which could lead to the formation of biofilms in the urethra.

Itraconazole, an azole antifungal agent, is one of the first therapeutic choices for the treatment of inpatients [27]. Almost 90% of the *C. albicans* isolates in our study showed resistance or dose-dependent sensitivity to itraconazole. Resistance or dose-dependent sensitivity of yeast to itraconazole indicates that care must be taken regarding the use of this drug in hospitalized patients. Although such findings are not frequently reported, surveillance of isolates resistant to antifungal agents is important because drug resistance has a great influence on morbidity and the management of infected patients [28]. Moreover, determining the susceptibility profile is important for obtaining a quick and accurate diagnosis and initiating safe treatment using an appropriate antifungal agent for each case.

In conclusion, *C. albicans* isolates from urine have a high capacity for virulence and can be associated with infectious processes. The high number of isolates resistant to itraconazole is worrisome, since this antifungal agent is a drug of first choice in the treatment of infections in the hospital setting. Hypertonic Sabouraud broth and tobacco agar are effective media for phenotypic characterization of *C. albicans* and *C. dubliniensis*. The use of these media for yeast identification requires little cost and minimal infrastructure; thus, it is an attractive strategy for routine laboratory analysis.

Conflict of interest statement

We declare that we have no conflict of interest.

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