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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× *g*. 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, were added 675 μL of phenol/chloroform/isoamyl alcohol (25:24:1) 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. These step, 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, were randomly selected five samples DNA of the isolates. 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.

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