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Azole resistance in *Candida* spp. isolated from Catú Lake, Ceará, Brazil: an efflux-pump-mediated mechanism

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ABSTRACT

Since, there is no study reporting the mechanism of azole resistance among yeasts isolated from aquatic environments; the present study aims to investigate the occurrence of antifungal resistance among yeasts isolated from an aquatic environment, and assess the efflux-pump activity of the azole-resistant strains to better understand the mechanism of resistance for this group of drugs. For this purpose, monthly water and sediment samples were collected from Catú Lake, Ceará, Brazil, from March 2011 to February 2012. The obtained yeasts were identified based on morphological and biochemical characteristics. Of the 46 isolates, 37 were *Candida* spp., 4 were *Trichosporon asahii*, 3 were *Cryptococcus laurentii*, 1 *Rhodotorula mucilaginosa*, and 1 was *Kodamaea ohmeri*. These isolates were subjected to broth microdilution assay with amphotericin B, itraconazole, and fluconazole, according to the methodology standardized by the Clinical and Laboratory Standards Institute (CLSI). The minimum inhibitory concentrations (MICs) of amphotericin B, itraconazole, and fluconazole were 0.03125–2 μg/mL, 0.0625 to ≥16 μg/mL, and 0.5 to ≥64 μg/mL, respectively, and 13 resistant azole-resistant *Candida* isolates were detected. A reduction in the azole MICs leading to the phenotypical reversal of the azole resistance was observed upon addition of efflux-pump inhibitors. These findings suggest that the azole resistance among environmental *Candida* spp. is most likely associated with the overexpression of efflux-pumps.

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Introduction

The quali-quantitative analysis of yeast microbiota is a promising tool to assess the eutrophication status of aquatic systems.[1–5] Medeiros et al.,[5] for example, studied the biodiversity of yeasts in the lakes and rivers of southeastern Brazil and found that the genus *Candida* accounted for the largest number of isolates, out of which 50% were resistant to itraconazole and 11% were resistant to fluconazole. Moreover, our group observed, in the freshwater prawn *Macrobrachium amazonicum* (Amazon River prawn) collected from its natural environment, that 33.3% of the *Candida* isolates from these prawns were resistant to fluconazole and itraconazole.[6]

However, none of the mentioned studies investigated the underlying mechanism of the azole resistance present in the *Candida* strains recovered from aquatic environments. It is well known that one of the main mechanisms of azole resistance among *Candida* spp. is the increased activity of efflux pumps. This increased activity of efflux pumps is conferred by genes, *CDR1* and *CDR2*, belonging to the superfamily of the ATP binding cassette, and *MDR1*, belonging to the major facilitator class. The overexpression of these genes and the subsequent increase in the activity of these pumps prevent the accumulation of the drug inside the cell at the site of action impairing its efficacy. The upregulation of *CDR1* and *CDR2* confers resistance to nearly all azoles, while that of *MDR1* provides specific resistance only to fluconazole.[7] Thus, the present study aims to investigate the occurrence of antifungal resistance among yeasts obtained from an aquatic environment, and assess the efflux-pump activity in the azole-resistant strains.

Methods

Study site and collections of the biological material

The samples were collected from Catú Lake, located in the municipality of Aquiraz, Ceará State, Brazil (UTM coordinates 0567000 E, 9561273 N and 0575000 E, 9569000 N). Catú Lake is a rich freshwater body with mangrove areas sheltering a large number of animal species. However, due to uncontrolled occupation of the surrounding area, water from different zones of this lake is mainly used for human and animal consumption, for industrial, commercial, farming activities, and for leisure activities, such as boat excursions.[8]

A total of 12 water samples were collected monthly from March 2011 to February 2012, according to the method described by Medeiros et al.,[5] with slight modification. The samples were obtained from four collection sites: recreational area point (point 1, 3°55′59.79″ S and 38°21′50.10″ W); agricultural wastewater point, with possible use of azoles (point 2, 3°55′47.25″ S and 38°22′14.16″ W); industrial wastewater point (point 3, 3°56′03.70″ S and 38°22′25.15″ W); Catú River confluence point, residential area with discharge of raw household sewage, (point 4, 3°56′56.72″ S and 38°22′31.57″ W) (Fig. 1). The water samples were collected in a 1-L Van Dorn bottle, which was rinsed three times with the water from each collection site before the collection. Two samples were collected from each point, one from the surface (SW sample) and the other from the bottom, including sediment (S sample). The study was approved by the Chico Mendes Institute for Conservation of Biodiversity/Biodiversity Authorization and Information System, SISBIO, under the process number 28175-1.

Mycological processing

The samples were processed in a biological safety level 2 laminar flow cabinet. Sabouraud agar with chloramphenicol (0.5 g/L) was used as the culture medium for primary isolation in Petri dishes. A 100-μL aliquot of the SW samples was spread on the medium after homogenization. The S samples were centrifuged for 20 min at 3000 rpm and the supernatant was removed and the sediment was resuspended in 2 mL of sterile 0.9% NaCl solution. Then, the suspension was agitated in a vortex mixer for 3 min and left to rest for 30 min at 25 °C. Afterwards, 100-μL aliquots of the supernatant of each sample were spread on the culture medium. The inoculated Petri dishes were incubated at 25 °C for 10 days, and were with daily observed daily to note any microbiological growth. The colony forming units (CFUs) were counted in all inoculated dishes.

Yeast identification

The colonies that appeared to be yeast were Gram stained and observed under a light microscope (400×) to check for the presence of blastoconidia, hyphae, or pseudohyphae, and to exclude bacterial contaminations. The yeast colonies were identified through specific macromorphological and micromorphological characteristics, including growth on chromogenic medium for the identification of mixed colonies, and biochemical tests, such as carbohydrate and nitrogen assimilation and urease production. VITEK 2™ microbial identification system (bioMérieux, USA) was used in case of dubious identification to aid the identification procedure.[6]

In vitro antifungal susceptibility tests

The antifungal minimum inhibitory concentrations (MICs) against these microorganisms were determined through broth microdilution method, as described by the Clinical and Laboratory Standards Institute (CLSI, 2008). Three drugs were tested against the isolates: amphotericin B (0.03125–16 μg/mL) (Sigma Chemical Corp.), itraconazole (0.03125–16 μg/mL) (Janssen Pharmaceutica, Belgium), and fluconazole (0.125–64 μg/mL) (Pfizer, Brazil). Inocula of all tested isolates were prepared from 1-day-old cultures grown on potato dextrose agar at 35 °C with RPMI 1640 medium supplemented with L-glutamine (HiMedia Laboratories) and buffered at pH 7 with 0.165 M morpholinepropanesulfonic acid. The inocula were adjusted to a final concentration of $0.5–2.5\times10^3$ cells/mL.[6,9] The microdilution plates were incubated at 35 °C for 48 h and were visually read.[9] For each isolate, drug-free and yeast-free controls were included and all the isolates were tested in duplicate. As quality control, for each test performed, *Candida parapsilosis* ATCC 22019 was included in each test as a quality control measure. The MIC of azole derivatives was defined as the lowest drug concentration capable of inhibiting 50% of growth, when compared with the growth control. For amphotericin B, the MIC was the lowest drug concentration at which no growth was observed. Isolates