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Antifungal susceptibility of yeasts isolated from anthropogenic watershed

ANA C.M. MILANEZI, JOÃO P.D. WITUSK and SUELI T. VAN DER SAND

Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul/UFRGS, Rua Sarmento Leite, 500, 90050-170 Porto Alegre, RS, Brazil

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Abstract: Yeasts are unicellular fungi widespread in the environment, and studies suggest that there is a positive correlation between yeast and polluted aquatic environments. The aim of this study was to analyze the diversity and resistance of yeasts isolated from water samples collected along the Arroio Dilúvio in Porto Alegre to antifungals. Yeast isolates from the Arroio Dilúvio were subjected to susceptibility assays against antifungals using the minimum inhibitory concentration (MIC) test, and amplification of the ITS1-5.8S-IT2 region; in addition, the ITS-5.8S region was sequenced for some of the isolates. The amplification product was subjected to PCR-RFLP, and the restriction profile allowed the construction of a dendrogram. Susceptibility tests showed a high prevalence of resistance to azole antifungals, where 16.8% of the isolates had a resistance profile to amphotericin B. The sequence analysis allowed the identification of Candida species, including potentially pathogenic species, and species of the Debaryomyces spp. The resistance to antifungals in yeasts isolated from Arroio Dilúvio reinforces the importance of studies of environmental microbiota, and indicates that environmental degradation influences the phenotype displayed.

Key words: yeast, antifungal, watershed, resistance.

INTRODUCTION

Water availability and its quality have a significant influence on population growth, urbanization, industrialization and a broad range of social and economic consequences resulting from these processes. Water has a central role in sustainable development; however, its use is still neglected, compromising water supply and consumption. The intense urbanization observed in the last century has altered the natural structure of watersheds, increasing the deposition of pollutants, toxic substances, and other physical and chemical parameters (UNESCO 2015).

Yeasts are unicellular fungi that have a crucial role for humanity, with industrial, agricultural, environmental, and scientific applications (Johnson and Echavarri-Erasun 2011). These fungi can be found in diverse aquatic environments, free or in association with sediments, in other living organisms, extreme environments, oceans, soil and organic matter. Most studies evaluating the presence of yeasts on freshwater environments where conducted on the last three decades, especially in association with contaminated water, since unpolluted aquatic sources are scarce. Several studies have observed a significantly positive

Correspondence to**:** Sueli Teresinha Van Der Sand
E-mail: svands@ufrgs.br
ORCid: http://orcid.org/0000-0002-8591-6514

correlation between yeast counts and water quality indicators in aquatic environments, such as fecal coliforms, total coliforms and *Escherichia coli* (Hagler and Mendonça-Hagler 1981, de Almeida 2005, Medeiros et al. 2008, 2012, Coelho et al. 2010, Carneiro et al. 2015). Unpolluted water sources exhibit the prevalence of nonfermentative species from the genus *Cryptococcus* and *Rhodotorula* (Hagler et al. 1995). Species from the genus *Candida* are the main target on polluted freshwater environments and their recovery rate are prevalent. Other yeasts commonly recovered belongs to the genus *Cryptococcus*, *Rhodotorula*, *Saccharomyces*, and *Trichosporon*. Some species often are recovered in higher concentrations that fecal coliform isolates, such as *R. mucilaginosa*, *T. beigelii*, *Cryptococcus laurentii* and *Debaryomyces hansenii* (Brilhante et al. 2015).

According to Hoellein et al. (2014), few studies have analyzed the effects of pollution on yeast communities, particularly in freshwater sources in urban environments, and this highlights the importance of understanding the relationship between these two factors.

Despite growing interest relating to yeasts contamination in water, little of this knowledge is applied as practical measures (Hageskal et al. 2009). One reason for this is that the consumption of water contaminated with yeast rarely leads to clinical manifestations, unlike of what is observed for bacteria, viruses, and parasites. However, many yeast species found in water can behave as opportunistic pathogens, and thus pose a health risk to immunocompromised individuals (Al-Gabr et al. 2014).

In addition to yeast recovery rate, another way of using them to infer environmental conditions is through the evaluation of phenotypic changes exhibited by yeast in anthropogenic environments. Environmental changes, such as the deposition of pollutants, could result in the induction of genetic mechanisms that might be responsible for the emergence of antifungal drug resistance in microorganisms. These phenotypic changes could also be used as a tool to evaluate environmental conditions (Brilhante et al. 2015). Recent studies evaluated the susceptibility of yeasts isolated from freshwater samples, and they observed antifungal drug resistance isolates to at least one of the drugs tested in the assays (Medeiros et al. 2008, Brandão et al. 2011, Feltrin 2014).

Most yeasts prefer growth temperatures of 20-30°C. Therefore, it is important that isolates that do not exhibit the expected pathogenic potential risk to human health be also studied concerning its resistance to antifungal drugs, allowing the investigation of the influence of environment and pollution on the phenotype of this community.

Since most studies involving yeasts in freshwater and their resistance to antifungal agents focus on clinical species, the aim of this study was to investigate the manifestation of antifungal resistance in yeasts that do not express pathogenic characteristics. Furthermore, it discusses how pollution may affect these microorganisms, which could affect selection of resistance to antifungal agents in the environment and contribute to the study of taxonomy and diversity of yeasts.

MATERIALS AND METHODS

YEAST ISOLATES

The Dilúvio Stream is part of an important watershed in Porto Alegre, Rio Grande do Sul, Brazil, which has suffered from intense urbanization since its canalization in 1930. The stream receives a significant amount of solid deposition every year, including irregular domestic and hospital sewage. It flows into the Guaíba Lake, the main water supply for drinking water treatment plants of the city. Samples were collected along its course, and their isolation is described in previous work (Feltrin 2014). The 104 yeast isolates used in this work belong to the Environmental Microbiology

Laboratory of Universidade Federal do Rio Grande do Sul. The isolates were recovered in plates containing Sabouraud agar incubated at 28°C for 48 h.

ANTIFUNGAL DRUG SUSCEPTIBILITY

Isolate susceptibility to antifungal agents were analyzed using a minimum inhibitory concentration (MIC) assay following the M27-A3 document from the Clinical and Laboratory Standards Institute CSLI (2008). Fluconazole (FCZ), amphotericin B (AMP-B), voriconazole (VCZ), ketoconazole (KCZ), and itraconazole (ICZ) were the antifungal drugs tested. Yeast suspensions of 2.5 x 10^3 cells/mL were inoculated into sterile 96-well U-shaped microplates containing one of the five antifungal agents. *Candida krusei* ATCC 6258 was used as a control. The final concentrations of fluconazole ranged from 0.125 to 64 $\mu g.mL^{-1}$, while itraconazole, amphotericin B, voriconazole and ketoconazole concentrations ranged from 0.031 to 16 $\mu g.mL^{-1}$. Interpretations of the results were based on cutoff values provided by MS27-S4 of CSLI (2012).

DNA EXTRACTION

Genomic DNA extraction of the isolates followed the protocol of Osorio-Cadavid et al. (2009). The cells were grown on Sabouraud broth for 16 h at 28°C precipitated by centrifugation and resuspended in 400 µL of lysis buffer (0.15 M NaCl, 50 mM Tris-HCl, 10 mM EDTA, 2% SDS, pH 8.0) followed by incubation at 65°C for 1 h. After that, 200 µL of 5 M potassium acetate (pH 4.8) was added, and the samples were homogenized for at least 30s before being placed in an ice bath for 30 min. Following this, the samples were centrifuged for five minutes at 14.000 rpm, and the supernatants were transferred to new tubes. Sample cleaning steps were as follows: one time extraction with chloroform; 1X phenol; 1X phenol/chloroform (1:1); and finally, 1X chloroform/isoamyl alcohol (24:1). The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of isopropyl alcohol and incubation at -20°C for 30 min. Samples were then centrifuged at 14.000 rpm for 10 min, the supernatant was discarded, and the pellet was washed with 500 µL chilled 70% ethanol. The samples were again centrifuged and DNA was resuspended in 50 µL TE (10 mM Tris, 1 mM EDTA, pH 7.4). DNA was visualized on a 1% agarose gel, and the concentration was determined using a NanoDrop spectrophotometer Lite (Thermo Fisher Scientific).

DNA AMPLIFICATION

Amplification of the ITS1-5.8S-ITS2 region was performed in a final volume of 25 µL. Reaction mixtures contained 10 pmol of each primer, ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (White et al. 1990), 2.5 µL of reaction buffer (10X), $MgCl_2$ 2 mM, 1 µL of each dNTP 2.5 mM, 1.0 U of Taq polymerase, 50 ng of genomic DNA and sterile deionized water to the final 25 µL volume. Amplification reactions were performed in a thermocycler ProFlex™ PCR System (Thermo Fisher Scientific) with initial denaturing at 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 53°C for 45 s and 72°C for 1 min, followed by a final extension of 72°C for 5 min. PCR products were observed in a 1.2% agarose gel electrophoresis and visualized under UV light using an L-Pix Touch (Loccus Biotecnologia) system. All amplifications were repeated at least twice for each isolate in separate experiments.

PCR-RFLP

The amplified products were subjected to digestion with the restriction endonucleases *Hinf*I, *Hae*III, and *Cfo*I (Promega) according to the manufacturer's recommendations for each enzyme. The digestion products were separated on a 3% agarose gel

electrophoresis and visualized under UV light using an L-Pix Touch (Loccus Biotecnologia) system.

ITS-5.8S DNA SEQUENCING

All isolates with a resistance profile to amphotericin B, and one isolate from each cluster formed with the PCR-RFLP assay, were chosen for sequencing of the ITS-5.8 region. Amplification was performed following the same conditions described above. Amplification products were purified with a PureLink® Quick Gel Extraction PCR Purification Kit Combo (Invitrogen) and sequenced on an ABI 3500 Genetic Analyzer with 50 cm capillaries and POP7 polymer (Applied Biosystems) at Ludwig Biotec Company. The sequences obtained were analyzed and aligned with sequences available in the database MycoBank.org (http://www.mycobank.org/) and GenBank database using BLAST (http://www.ncbi.nlm.nih.gov).

DATA ANALYSIS

The fragment patterns generated from each PCR-RFLP technique were clustered using the CLIQS 1D Pro program (Total lab). A binary data matrix was built according to the presence or absence of each fragment generated by the restriction enzymes. The comparison of the isolates was performed using the Dice similarity coefficient. Dendrogram construction was carried out using the unweighted pair group method using arithmetic averages (UPGMA).

RESULTS

MINIMUM INHIBITORY CONCENTRATION

One hundred and four isolates were recovered from glycerol at -20°C in agar Sabouraud. All isolates were cultivated in RPMI 1640 medium at 28°C for 48 h. Of these, nine isolates did not grow after incubation. From the resulting 95 isolates that grew in the medium, 44.2% were resistant to fluconazole (42/95), 18.9% had a dose-dependent sensitivity (18/95), and 36.8% were susceptible to the drug (35/95). For ketoconazole, only 5.3% (5/95) showed resistance. For the antifungal voriconazole, 24.2% of the strains were resistant (23/95), 14.7% had a dose-dependent sensitivity (14/95), and 61.1% were susceptible (58/95). For the itraconazole assay, 7.4% of the isolates were sensitive to the antifungal (7/95), 34.7% had a dose-dependent sensitivity (33/95), and 57.9% were resistant (55/95). Finally, 16.8% isolates were resistant to amphotericin B (16/95) and 83.2% exhibited drug sensitivity (79/95) (Table I).

Regarding the coexistence of resistance to the antifungal drugs, 31.6% (30/95) showed no resistance to any of the drugs, 3.2% (3/95) were resistant to all drugs tested, 5.3% (5/95) showed resistance to four drugs, 14.7% (14/95) showed resistance to three, 22.1% (21/95) were resistant to two drugs, and 23.2% (22/95) were resistant to only one drug. Of the 22 isolates that showed resistance to only one drug, two isolates, I2C12 and

TABLE I
Number of isolates showing each susceptibility profile for each of the five antifungals tested in the minimum inhibitory concentration assay.

	Susceptibility profile			Total
	Resistant	Sensitive-dose-dependent	Susceptible	
Fluconazole	42 (44.2%)	18 (18.9%)	35 (36.8%)	95
Cetoconazole	5 (5.3%)	0	90 (94.7%)	95
Voriconazole	23 (24.2%)	14 (14.7%)	58 (61.1%)	95
Amphotericin B	16 (16.8%)	0	79 (83.2%)	95
Itraconazole	55 (57.9%)	33 (34.7%)	7 (7.4%)	95

V3B8, were resistant to amphotericin B. Therefore, 63 isolates were resistant to at least one antifungal of the azole class. There was a predominance of resistance to the antifungal itraconazole (55/95), followed by fluconazole (42/95), voriconazole (23/95) and ketoconazole (5/95).

ITS-PCR-RFLP AND DNA SEQUENCING

DNA extraction was performed for all 104 isolates. Six isolates grew very slowly, and it was not possible to extract good quality DNA with the protocol used. Thus, DNA of 98 isolates was amplified. Amplification products using ITS1 and ITS4 primers generated fragments ranging in size: 400–950 bp. Eleven isolates showed two fragments for the ITS-5.8S region after two separate repetitions of the assay, and these isolates were excluded from this assay. Thus, 87 isolates were submitted to digestion with the restriction endonucleases *Hinf*I, *Hae*III, and *Cfo*I. With the resulting restriction pattern, a dendrogram was constructed (Figure 1). Sixteen clusters, considering a cutoff of 80% similarity, were observed. Forty-one isolates showed 100% similarity to at least one other isolate, and 45 did not cluster with any isolate considering the 80% similarity cutoff (Figure 1). Isolates IB217 and I2B9 cluster together in group 4 and showed different profile for antifungal resistance (Table II). Thus, I2B16 and V3A2 showed the same antifungal resistance profile and cluster in the same group (Group 10). Also this isolates belong to the same genera in the sequencing results (Table II). Isolates V3B12, V3B9, V3B8, V2A10, V2C12 did not cluster with any other isolate and share the resistance profile to amphotericin B (Figure 1, Table II).

Isolates that exhibited resistance to amphotericin B (polyene), and at least one isolate resistant to an azole class from each cluster formed in the dendrogram (Figure 1), were selected for sequencing of the ITS1-5.8 region. Isolate V2A10 (*Zygoascus bituminiphila)* showed the lowest identity (80%) with the sequences available in GenBank (Table II). The other isolates showed at least 94% identity with sequences available in the database (Table II).

Seven isolates showed identity with different species of the genus *Candida*. Six isolates exhibited a high degree of similarity to different species within the genus *Debaryomyces*.

DISCUSSION

Water quality is a topic that requires the attention of government agencies, water providers, health authorities, and the general population. Microbiological indicators are used to ensure the safety of water for consumption since the presence of microorganisms suggests contamination from feces and other pathogens. However, the main concern regarding the quality of water using microbiological indicators refers mainly to water intended for consumption and recreational activities, among others, but often disregards aspects of the environment. Several studies indicate that an increased concentration of yeasts accompanies increased pollution of aquatic environments. However, little of this knowledge is used to examine and evaluate the quality of aquatic ecosystems related to yeast presence (Hageskal et al. 2009).

A study carried out in Dilúvio stream analyzed the parameters of water quality from the period 1995 to 2011 and it observed a constant and elevated presence of fecal coliforms related to high incidence of phosphorus; and high levels of biochemical oxygen demand associated with low dissolved oxygen coefficients (Dal Forno and De Matos 2016). The authors state that the water quality parameters and its temporal evolution in the Dilúvio stream are the results of urban pollution, especially from the sewage, depriving it of its natural properties. To date, the study conducted by

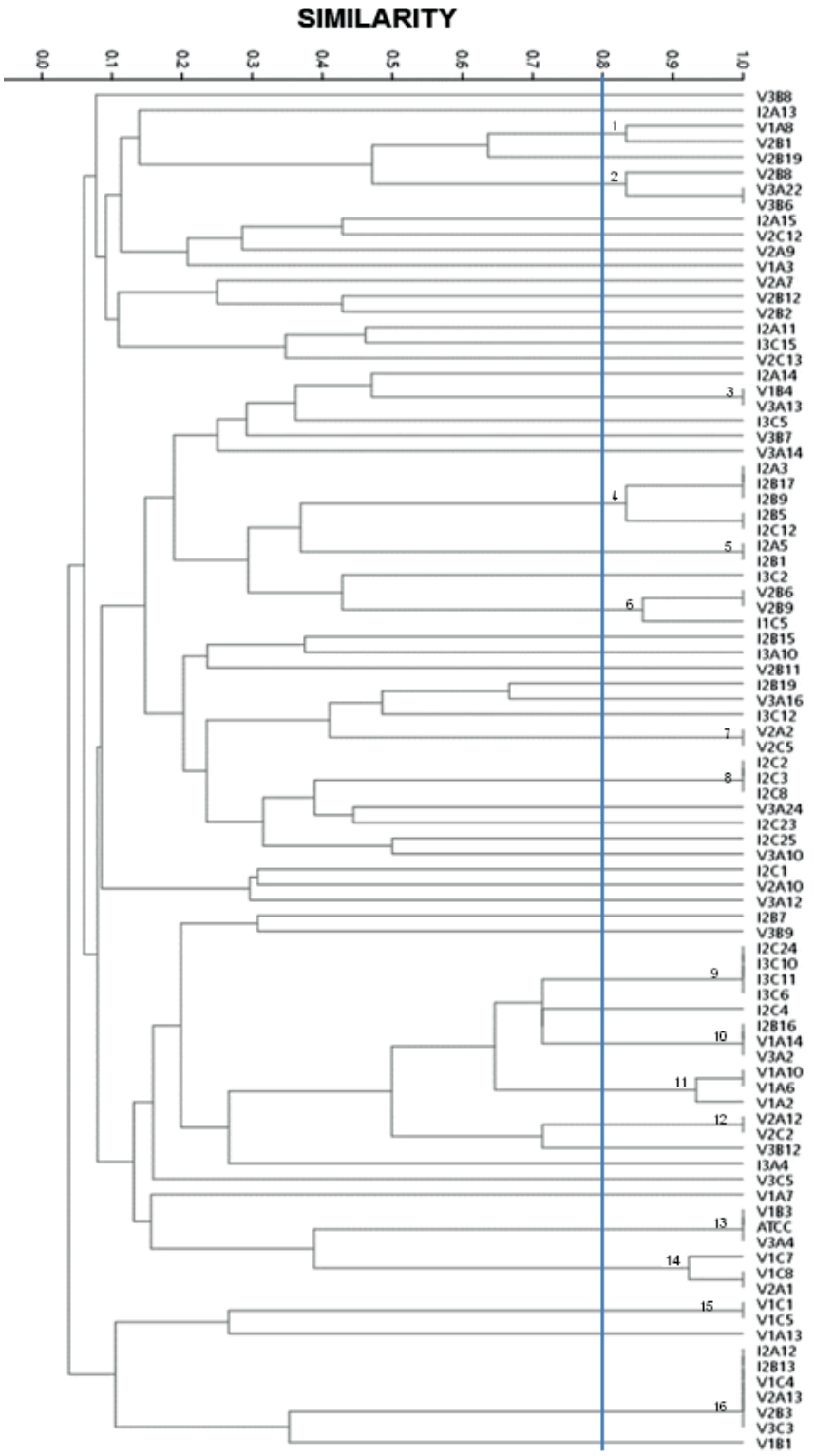

Figure 1 - Dendrogram of samples submitted to PCR-RFLP using the enzymes *Hinf*I, *Hae*III and *Cfo*I. The similarity matrix was calculated using the Paleontological Data Analysis (PAST) software using the Dice coefficient by the unweighted pair group method using arithmetic averages (UPGMA).

TABLE II
Isolates submitted to sequencing and their species or genera, sequence identity values (Id), overlay and accession number in database and the antifungals in which they presented resistance. FCZ = Fluconazole, KCZ = Ketoconazole, VCZ= Voriconazole, AMP-B = Amphotericin B, ICZ= itraconazole.

Sample	Species or Genus	Overlay (%)	Id (%)	Access	Antifungal resistance
V1B3	*Candida tropicalis*	93	96	KP675019.1	FCZ, VCZ, AMP-B, ICZ
V2A1	*Candida parapsilosis*	98	99	KY102320.1	FCZ, AMP-B, ICZ
V2A10	*Zygoascus bituminiphila*	78	80	NR137545.1	VCZ, AMP-B, ICZ
V2A13	*Candida glabrata*	96	99	KY102104.1	FCZ, AMP-B
V2C12	*Candida* sp.	92	95	EF621564	FCZ, KCZ, VCZ, AMP-B, ICZ
V3A2	*Debaryomyces hansenii*	96	99	KY103264.1	
	Debaryomyces fabryi	98	99	NR138186.1	AMP-B, ICZ
	Debaryomyces prosopidis	98	99	NR077067.1	
V3B8	*Candida pseudolambica*	96	94	KY102347.1	AMP-B
V3B9	*Candida tropicalis*	95	94	KY102470.1	FCZ, KCZ, VCZ, AMP-B, ICZ
V3B12	*Debaryomyces hansenii*	99	99	NR120016.1	FCZ, VCZ, AMP-B, ICZ
	Debaryomyces fabry	99	98	KY103196.1	
I2B9	*Debaryomyces hansenii*	98	98	FR686595.1	
	Debaryomyces nepalensis	96	98	KY103283.1	FCZ, VCZ, AMP-B, ICZ
	Debaryomyces subglobosus	98	98	FN675240.1	
I2B16	*Debaryomyces hansenii*	96	99	KY103229.1	
	Debaryomyces subglobosus	98	99	FN675240.1	AMP-B, ICZ
	Debaryomyces fabryi	98	99	KY103196.1	
I2B17	*Debaryomyces subglobosus*	98	99	FN675240.1	
	Debaryomyces fabryi	98	99	KY103196.1	AMP-B, ICZ
	Debaryomyces vindobonensis	96	99	KY103298.1	
I2C12	*Debaryomyces fabryi*	99	99	KY103196.1	
	Debaryomyces hansenii	98	99	KY103264.1	AMP-B
	Debaryomyces subglobosus	99	98	FN675240.1	
I2C24	*Candida railenensis*	95	99	NR077080.1	FCZ, AMP-B
I2B1	*Rhodotorula mucilaginosa*	97	99	KY104848.1	FCZ, VCZ, AMP-B, ICZ
V3A22	*Candida intermedia*	98	98	KY102152.1	ICZ

Feltrin (2014) has been the only study of the Dilúvio stream that has considered the evaluation of yeast present in this highly degraded environment. In this work, the author determined the susceptibility of 50 yeasts isolates that grew at 37°C, and presented virulence factors, therefore potentially pathogenic to human health. In the present work, yeasts capable of growing at different temperatures were tested. The results obtained with the MIC assay were consistent with results obtained in previous studies that evaluated the susceptibility profile of yeasts isolated from the environment. Feltrin (2014) observed that 30% of the isolates showed resistance to fluconazole and itraconazole. Similarly, Medeiros et al. (2008) observed that 50% of the yeasts isolated from water samples of the lakes and rivers of the Rio Doce Basin (Minas Gerais/BR) were able to grow at 37°C and showed resistance to itraconazole. Although to a lesser degree, Medeiros et al. (2008) also observed species resistance to fluconazole, including potentially pathogenic species that were previously considered susceptible

to the drug in clinical trials. Interestingly, there was a prevalence of resistant yeasts isolated from water sources with higher levels of pollution (Medeiros et al. 2008). Brandão et al. (2010) also reported of the presence of yeasts isolated from freshwater lakes in southeastern Brazil that grew at 37ºC, with resistance to itraconazole and amphotericin B. These studies reinforce the relationship between water quality and yeast counts, since they also registered a positive correlation with the concentrations of coliforms present at the collection sites (Brandão et al. 2010).

It is also important to consider the existence of at least three hospitals in the vicinity of the Arroio stream environment, which would possibly indicate the occurrence of hospital sewage disposal directly into the stream. This sewage could contain resistant yeast from clinical sources, or antifungal drugs that would act as a selection factor, representing a possible hypothesis for the observation of a consequent increase of antifungal resistance strains. However, it is expected that hospital sewage receives the appropriate treatment before disposal. Despite the undeniable importance of assessing these microorganisms and their resistance in a clinical setting, the variability in the resistance profiles from water samples, and other sources registered in the literature, suggests a great influence of pollution and environmental factors in the different phenotypes observed. However, the factors that increase selection for resistant microorganisms in an environment where the presence of drugs is not expected to be a direct selection factor remain unclear.

Some studies suggest that the continued and indiscriminate use of antifungals and fungicides of the azole class in agriculture, and other products routinely used by the population, results in the contamination of water sources and soil, acting as selecting agents in the environment (Hof 2008, Azevedo et al. 2015, Chen and Ying 2015). Since azoles interact and target the same active site of the lanosterol 14-α-demethylase enzyme, both azoles used in clinical and agriculture settings share the same mode of action (Azevedo et al. 2015). Thus, one could hypothesize that there is cross-resistance to one or more antifungal agents belonging to the azole class, and the appearance of mechanisms capable of circumventing the action of a drug is applied to other drugs from the same class. This scenario seems likely as fungicides have been indiscriminately used for many years, they have a long half-life, remaining and accumulating in the environment, and allowing a gradual selection of resistance mechanisms by the microorganisms (Azevedo et al. 2015).

Another hypothesis of how yeasts can acquire resistance in the environment is raised by Brilhante et al. (2015). The authors argue that the results observed by Medeiros et al. (2008) and Brandão et al. (2010) could be related to anthropogenic activity and the highly degraded environment from which the yeasts were isolated. The presence of industrial waste and other pollutants could promote altered expression of genes and other nucleotide sequences, which may occasionally occur in pathways related to resistance, such as increased expression of efflux pumps encoded by the CDR1 and CDR2 genes in the cell membrane. Since azoles can passively enter a fungal cell, increasing the expression of these proteins would indirectly cause a decrease in it's concentration and consequent increase of resistance (Brilhante et al. 2015).

The hypothesis that antifungal resistance of yeasts could be achieved through exposure to nonspecific compounds derived from various sources of pollution has been reinforced in a recent study published by Brilhante et al. (2016). In their work, the authors observed that yeast derived from freshwater lakes with resistance to fluconazole and itraconazole had the activity of its efflux pumps inhibited by the addition of promethazine and haloperidol. MIC tests performed after this test resulted in a reversal of the azole-resistant

phenotype, indicating a direct involvement of the efflux pumps in the drug sensitivity of yeast (Brilhante et al. 2016).

The sequencing of isolates was performed in this study to identify samples with resistance to amphotericin B, resulting in seven isolates belonging to the genus *Candida*. The resistance profiles against itraconazole and fluconazole of *C. tropicalis*, *C. parapsilosis,* and *C. glabrata* in water samples was also observed in previous studies (Medeiros et al. 2008, Brandão et al. 2010, Brilhante et al. 2016).

In recent years, there has been an increased incidence of yeasts resistant to antifungal agents in non-albicans candidiasis, such as *C. parapsilosis*, *C. glabrata* and *C. tropicalis* (Silva et al. 2012), and their occurrence at the Dilúvio stream is worth attention. The Dilúvio stream water flows into Guaíba Lake, which is the largest source of water supply for the city of Porto Alegre (Brandão and Kindel 2010). The parameters used to ensure microbiological quality of drinking water does not consider the presence of yeasts and other fungi, and thus, the presence of these species can be a hazard to the population.

The V3B8 and I2C24 isolates were identified as *C. pseudolambica* and *C. railenensis* respectively, and no pathogenicity or resistance records of these species were found in the literature. In this work, isolate V3B8 showed resistance to amphotericin B and I2C24 to fluconazole and amphotericin B. Both species are unable to grow at 37°C, have a general ecology allowing the use of various habitats, such as in association with insects, rotting trees, soil and bivalves (Lachance et al. 2011). In addition to these environments, *C. pseudolambica* species was also isolated from artificial lakes (Silva-Bedoya et al. 2014), hyper acidic freshwater (Libkind et al. 2014), and along with other yeast strains, it is used for bioaugmentation in effluent treatment (Hoellein et al. 2014). Bedoya-Silva et al. (2014) argue that in addition to water quality indicators, the presence of yeast in degraded environments offers the possibility to explore physiological aspects of biotechnology and industrial applications, due to the great enzymatic adaptation of these microorganisms. In this sense, studies that aim to analyze the resistance of yeasts to antifungal agents are relevant. The antifungal resistance observed in this study suggests that the acquisition of this phenotype is related to environmental pressures.

The susceptibility against amphotericin B observed in our work, although at lower prevalence, should be given attention. The acquisition of resistance to this drug is shown to be rare, even after 50 years of its introduction into fungal therapy (Vincent et al. 2013). Sokol-Anderson et al. (1988) observed that the resistance against amphotericin B, besides changes in the sterol content in the cell membrane, can also be the result of better adaptation to oxidative effects caused by the drug, with a higher expression of the catalase enzyme in resistant *Candida* strains. Linares et al. (2013) observed increased expression of superoxide dismutase and catalase in *C. albicans* and *C. dubliniensis* strains resistant to amphotericin B and fluconazole. Since highly contaminated environments can induce an oxidative response in organisms (Mahboob et al. 2014), it can be hypothesized that the simultaneous resistance observed in the isolates in this study against two antifungal classes is also a result of adaptation to stress caused by the oxidative environment.

Regarding the analysis of fragments generated by the PCR-RFLP technique, the dendrogram indicates the presence of 16 clusters with 80% similarity. Esteve-Zarzoso et al. (1999) suggested the use and standardization of the assay to identify yeast species. However, the emergence of more accurate molecular techniques, coupled with the difficulty of reproducing the technique conditions in different laboratories make PCR-RFLP more appropriate for the study of diversity (Kurtzman et al. 2015). Furthermore, the number of yeast

species described in recent years has increased considerably, and the ITS-PCR-RFLP database (https://www.yeast-id.org) does not include a significant proportion of currently known yeasts.

Despite its limitation for direct identification, PCR-RFLP may be coupled with other molecular identification techniques to allow differentiation between strains. In this study, six of the 14 isolates submitted to sequencing showed between 95% and 99% similarity with more than one species of the *Debaryomyces* genus available at GeneBank and MycoBank.org. The different restriction patterns produced by PCR-RFLP might suggest that they represent other species of the genus that also had a high percentage of similarity in sequence.Wrent et al. (2015) observed that the species *D. hansenii*, *D. fabryi*and *D. subglobosus* produced the same restriction patterns after digestion with *Hinf*I, *Hae*III, and *Cfo*I endonucleases. Thus, it is not possible to determine whether the different restriction patterns obtained in this study represent polymorphisms between species of *Debaryomyces* genus.

The ITS region is highly conserved in *Debaryomyces*. Martorell et al. (2005) observed that the *D. castellii, D. coudertii, D. hansenii, D. nepalensis, D. polymorphus, D. pseudopolymorphus, D. robertsiae, D. udenii* and *D. vanrijia* species presented sequences of the ITS region with similarity above 99% with one or more species of the genus. Therefore, several alternative molecular markers have been proposed for identification of species of this genus, such as the use of the intron sequences of ACT1 genes (Martorell et al. 2005), RPL33 (Jacques et al. 2009) and the putative *PAD1* homologous gene (Wrent et al. 2015).

Besides the potentially pathogenic species exhibiting resistance to antifungal drugs, yeasts with biotechnological potential were also found in this work. Together, these results confirm the importance of assessing biological diversity in degraded environments and the relationship between contaminants and yeast adaptation. The study of resistance to antifungal drugs in environmental yeast is extremely relevant, since the acquisition of these mechanisms by fungi occurs at much lower rates than is observed in bacteria, for example. Nevertheless, studies analyzing environmental yeasts indicate that this phenotype is not as rare as expected.

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