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BACTERIAL FUNGAL AND VIRUS MOLECULAR BIOLOGY - SHORT COMMUNICATION

Molecular identification and antifungal susceptibility testing of *Pucciniomycotina* red yeast clinical isolates from Rio de Janeiro, Brazil

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Abstract

Infections caused by *Rhodotorula* spp. are increasing worldwide. This study identified, through the light of the new taxonomic advances on the subphylum *Pucciniomycotina*, 16 isolates from blood cultures and compared their antifungal susceptibility on microdilution and gradient diffusion methods. Internal transcriber spacer sequencing identified *Rhodotorula mucilaginosa* (n = 12), *Rhodotorula toruloides* (n = 2), *Rhodotorula dairenensis* (n = 1), and *Cystobasidium minutum* (n = 1). Amphotericin B was the most effective drug. A good essential agreement was observed on MIC values of amphotericin B and voriconazole determined by the two methods. Therefore, the gradient method is useful for susceptibility tests of *R. mucilaginosa* against these drugs.

Keywords Opportunistic fungi · *Rhodotorula* · Bloodstream infections · ITS sequencing · Antifungal susceptibility

Fungal infections by yeasts belonging to the genus *Rhodotorula* are increasingly reported in the scientific literature. Fungemia accounts for more than half of *Rhodotorula* infections, especially in patients with malignancy, and the mortality rate among *Rhodotorula*-infected patients can reach 12% [1]. This genus has been reported as the third most frequent yeast isolated from blood cultures in some centers [2].

For several years, the genus *Rhodotorula* comprised ubiquitous basidiomycetous yeasts producing large amounts of carotenoid pigments, which results in the production of red to pink yeast colonies during in vitro growth [3]. However, molecular studies have shown that this genus was polyphyletic and, to fulfill the "one fungus = one name" concept, a multilocus sequence typing was used to recognize monophyletic clades within the subphylum *Pucciniomycotina*, which resulted in the description of new genera, for example, *Rhodosporidiobolus*, and in the emended of others, for example, *Rhodotorula* and *Cystobasidium* [4, 5].

Amphotericin B is the main drug used to treat *Rhodotorula* infections, with good rates of cure. Other antifungal drugs used include fluconazole, 5-flucytosine, itraconazole, voriconazole, and ketoconazole [1]. However, there are no clinical guidelines for the management of the infections caused by *Rhodotorula* or by its sibling genera. The paucity of literature data is indeed a challenge to the development of clinical guidelines to treat Pucciniomycotina red yeast infections. The information about the response of these fungi to the antifungal drugs is based on reports of in vitro antifungal susceptibility tests performed with clinical strains. However, strains included in some studies are not properly identified [6, 7] or the susceptibility is determined with methods other than the gold standard microdilution technique [8, 9].

The aim of this study was to identify, through the light of the new taxonomic advances on the subphylum *Pucciniomycotina*, red yeasts isolated from blood cultures of

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Table 1 Minimal inhibitory concentration values, geometric mean, and essential agreement between Etest and CLSI broth microdilution of 16 clinical *Pucciniomycotina* red-yeasts isolates from patients with fungemia to amphotericin B

Species (number of isolates)	Method	Amphotericin B (μg/ml)			
		MIC range	MIC_{50}/MIC_{90}	GM	%EA
Rhodotorula mucilaginosa (12)	CLSI	0.5-1	0.5/1	0.53	
	Etest	0.12-0.75	0.5/0.75	0.34	100
Rhodotorula dairenensis (1)	CLSI	2	-	-	
	Etest	0.75	-	-	100
Rhodotorula toruloides (2)	CLSI	0.5	-	0.5	
	Etest	0.25-0.5	-	0.35	100
Cystobasidium minutum (1)	CLSI	0.5	-	-	
	Etest	0.25	-	-	100

MIC_{50}/MIC_{90} minimal inhibitory concentration of the antifungal able to inhibit the growth of 50 and 90% of fungal isolates, respectively, GM geometric mean of the minimal inhibitory concentrations values, EA essential agreement

patients from a tertiary hospital in Rio de Janeiro, Brazil. Also, their in vitro antifungal susceptibility was evaluated using the gold standard microdilution and the gradient diffusion methods.

Sixteen strains deposited at the fungal culture collection at the Instituto Nacional de Infectologia Evandro Chagas, Fiocruz, Brazil, were studied. They were isolated from the bloodstream of 14 patients admitted to the Instituto Nacional de Cancer in Rio de Janeiro, Brazil, between 2000 and 2012. *Rhodotorula* sp. identification was performed using standard procedures, such as the production of red to pink yeast colonies on Sabouraud Dextrose Agar (Difco, Becton, Dickinson and Company, USA) and biochemical analysis using the API 20C AUX or the Vitek 2 system (bioMérrieux, France).

Genomic DNA was extracted from strains grown on Sabouraud Dextrose agar for 5 days at 30 °C and used as a template for the amplification of the internal transcriber spacer (ITS) region using primers ITS1 and ITS4 and PCR conditions described for the fungal barcoding protocol [10]. For antifungal susceptibility, amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin (Sigma Chemical Corporation, St. Louis, MO, USA) were prepared and tested according to the CLSI M27-A3 document [11]. Etest® strips (bioMérieux, France) containing the abovementioned antifungal drugs were used to test the in vitro susceptibility with the gradient diffusion method, according to the manufacturer recommendations. Minimal inhibitory concentrations (MICs) of both methods were determined after 72 h of incubation at 35 °C [12].

Descriptive statistical analyses were performed with the GraphPad Prism 7.0 software, to obtain MIC ranges, MIC50 and MIC90 values, and geometric means. The MIC50 and MIC90 values correspond to the lowest concentration of the antifungal drug able to inhibit the growth of 50% and 90% of all fungal strains tested, respectively. MICs obtained for each strain under different methodologies were compared using the essential agreement criterion [13]; that is, agreements were recorded when discrepancies between the MIC values determined by the different methods were within two drug dilutions (e.g., 0.5 μg/mL and 2.0 μg/mL).

ITS sequencing identified all strains: 12 as *Rhodotorula mucilaginosa*, two as *Rhodotorula toruloides*, one as *Cystobasidium minutum*, and one as *Rhodotorula dairenensis*. The sequences presented 99% to 100% homology with sequences from the CBS database (Table S1).

Table 2 Minimal inhibitory concentration values, geometric mean, and essential agreement between Etest and CLSI broth microdilution of 16 clinical *Pucciniomycotina* red-yeasts isolates from patients with fungemia to azoles (Voriconazole, Itraconazole and Posaconazole)

Species (number of isolates)	Method	Voriconazole (μg/ml)				Itraconazole (μg/ml)				Posaconazole (μg/ml)			
		MIC range	MIC_{50}/MIC_{90}	GM	%EA	MIC range	MIC_{50}/MIC_{90}	GM	%EA	MIC range	MIC_{50}/MIC_{90}	GM	%EA
Rhodotorula mucilaginosa (12)	CLSI	2-8	4/8	3.77		2-8	4/8	3.36		2-8	4/8	5.04	
	Etest	0.5-32	2/16	1.97	100	0.75->32	8/32	7.65	61.5	0.5->32	16/32	8.25	76.9
Rhodotorula dairenensis (1)	CLSI	1	-	-		0.12	-	-		2	-	-	
	Etest	2	-	-	100	0.75	-	-	0	0.75	-	-	100
Rhodotorula toruloides (2)	CLSI	0.25-2	-	0.70		0.12-2	-	0.49		0.25-0.5	-	0.35	
	Etest	1.5	-	1.5	100	0.125-3	-	0.61	100	0.125->32	-	2	50
Cystobasidium minutum (1)	CLSI	2	-	-		0.25	-	-		0.5	-	-	
	Etest	1.5	-	-	100	1.5	-	-	100	>32	-	-	0

MIC_{50}/MIC_{90} minimal inhibitory concentration of the antifungal able to inhibit the growth of 50 and 90% of fungal isolates, respectively, GM geometric mean of the minimal inhibitory concentrations values, EA essential agreement

The in vitro antifungal susceptibilities of the strains as determined by the two studied methods are summarized in Table 1, for amphotericin B, and Table 2, for azoles. In general, fluconazole (MIC ≥ 64 μg/mL) and caspofungin (MIC ≥ 4 μg/mL) were unable to inhibit the growth of all species and amphotericin B was the most effective antifungal drug. Essential agreement between MIC values obtained by broth microdilution and gradient diffusion was excellent (100%) for amphotericin B and voriconazole, but essential agreement percentages obtained by Etest® were lower for itraconazole and posaconazole.

The main limitation for comparative studies on infections caused by *Pucciniomycotina* yeasts is their low incidence. As a result, there is a paucity of knowledge on species distribution, epidemiology, antifungal susceptibility, and clinical aspects of these infections. To the best of our knowledge, this is the second study describing antifungal susceptibility data obtained with the gold standard method in a collection of clinical *Pucciniomycotina* red yeasts identified by means of ITS sequencing [12]. However, the lack of access to clinical data limited a clinical-microbiological correlation.

As described before, the ITS sequencing was a reliable methodology to identify *R. mucilaginosa*, *R. dairenensis*, and *C. minutum*. It appears that *R. mucilaginosa* is the most prevalent species among the *Pucciniomycotina* red yeasts that causes human infections [1, 12, 14]. Infections caused by *R. dairenensis* and *C. minutum*, formerly *Rhodotorula minuta*, were already described by some authors [1, 12], always with a low frequency, as observed in this study. *R. toruloides* is a yeast largely used by industries due to its potential to produce significant amounts of several compounds of interest, such as carotenoids and lipids [15]. In the present study, this species is presented, for the first time, as the agent of bloodstream infections. This species is probably misdiagnosed by clinical laboratories due to the limitations of the routine identification systems [16] and its real frequency in human infections remains to be elucidated.

The results of this study corroborate the inefficacy of fluconazole and caspofungin against clinical *Pucciniomycotina* red yeasts [12], including *R. toruloides* to the list of resistant species to these drugs. Amphotericin B is the most used antifungal drug used to treat infected patients [1, 17], and the in vitro results support its clinical use.

The gold standard microdilution method is laborious and its use by routine clinical laboratories is limited. Gradient diffusion methods are usually used as its substitute due to the easiness in performance and in the determination of MIC values. The usefulness of gradient diffusion methods is already described for *Candida* and *Cryptococcus* species [18, 19], but for other fungi, the gradient diffusion MICs do not correlate with the gold standard method [20]. This study has shown that Etest® can be a fast and reliable methodology for the MIC values' determination of amphotericin B and voriconazole against *R. mucilaginosa* and thus, it could be applied to a diagnosis laboratory routine as well as to the epidemiological resistance surveillance. Due to the low strain number of other species, further studies are needed to confirm if good essential agreement between these two methods also occurs with *Pucciniomycotina* yeasts rather than *R. mucilaginosa*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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