

# Molecular Identification, Antifungal Susceptibility Profile, and Biofilm Formation of Clinical and Environmental *Rhodotorula* Species Isolates

Jorge Meneses Nunes, Fernando César Bizerra, Renata Carmona e Ferreira, Arnaldo Lopes Colombo

Laboratório Especial de Micologia, Disciplina de Infectologia, Universidade Federal de São Paulo, São Paulo, Brazil

*Rhodotorula* species are emergent fungal pathogens capable of causing invasive infections, primarily fungemia. They are particularly problematic in immunosuppressed patients when using a central venous catheter. In this study, we evaluated the species distribution of 51 clinical and 8 environmental *Rhodotorula* species isolates using the ID32C system and internal transcribed spacer (ITS) sequencing. Antifungal susceptibility testing and biofilm formation capability using a crystal violet staining assay were performed. Using ITS sequencing as the gold standard, the clinical isolates were identified as follows: 44 *R. mucilaginosa* isolates, 2 *R. glutinis* isolates, 2 *R. minuta* isolates, 2 *R. dairenensis* isolates, and 1 *Rhodospiridium fluviale* isolate. The environmental isolates included 7 *R. mucilaginosa* isolates and 1 *R. slooffiae* isolate. Using the ID32C system, along with a nitrate assimilation test, only 90.3% of the isolates tested were correctly identified. In the biofilm formation assay, *R. mucilaginosa* and *R. minuta* exhibited greater biofilm formation ability compared to the other *Rhodotorula* species; the clinical isolates of *R. mucilaginosa* showed greater biofilm formation compared to the environmental isolates ( $P = 0.04$ ). Amphotericin B showed good *in vitro* activity ( $MIC \leq 1 \mu\text{g/ml}$ ) against planktonic cells, whereas voriconazole and posaconazole showed poor activity ( $MIC_{50}/MIC_{90}$ , 2/4  $\mu\text{g/ml}$ ). Caspofungin and fluconazole MICs were consistently high for all isolates tested ( $\geq 64 \mu\text{g/ml}$  and  $\geq 4 \mu\text{g/ml}$ , respectively). In this study, we emphasized the importance of molecular methods to correctly identify *Rhodotorula* species isolates and non-*R. mucilaginosa* species in particular. The antifungal susceptibility profile reinforces amphotericin B as the antifungal drug of choice for the treatment of *Rhodotorula* infections. To our knowledge, this is the first study evaluating putative differences in the ability of biofilm formation among different *Rhodotorula* species.

*Rhodotorula* species are basidiomycetous yeasts that are widely distributed in nature. They have been isolated from a variety of environmental sources, including soil, air, aquatic ecosystems, plants, and fruits. In humans, these yeasts have been isolated from the nails, skin, sputum, urine, feces, and hands of health care workers (1–3). *Rhodotorula* species were traditionally considered to be nonvirulent saprophytes and common contaminant microorganisms. However, in the last 2 decades, these yeasts have emerged as opportunistic pathogens (4–7).

The increase in invasive fungal infections caused by emergent pathogens is related to several factors, including the increased occurrence of degenerative and malignant diseases in different populations, as well as the increased number of patients that undergo organ transplantation, immunosuppressive therapies, broad-spectrum antibiotic therapy, and invasive medical procedures (5, 6, 8). In addition, the availability of new tools for the identification of microorganisms has certainly played a role in the ever-increasing capability of labs to recognize emergent pathogens.

Invasive infections caused by *Rhodotorula* species are mostly associated with underlying immunosuppression or cancer and with the use of central venous catheters (CVCs) and other implantable medical devices. The most frequent infection caused by *Rhodotorula* species is fungemia, followed by eye infections, peritonitis, and meningitis (4, 7, 9–11).

Although *Rhodotorula* species are responsible for a small percentage of all nosocomial acquired fungemia, this pathogen has been reported as the third most common yeast isolated from blood cultures (9) and as the fourth most common infectious fungus according to the ARTEMIS Global Antifungal Surveillance Program (12). Overall, *R. mucilaginosa*, *R. glutinis*, and *R. minuta* have been recognized as the three most clinically relevant species of *Rhodotorula* isolated from blood cultures (4, 7, 12).

Conventional phenotypic methods are limited in accuracy and consistency for species identification of emergent pathogens, including *Rhodotorula* species (9, 13–17). Thus, limited published data are available on the species distribution of *Rhodotorula* species that cause invasive infections and the antifungal susceptibilities of *Rhodotorula* species isolates correctly identified by molecular methods.

In this study, we evaluated the species distribution of 51 clinical and 8 environmental *Rhodotorula* species isolates by using internal transcribed spacer (ITS) sequencing and by testing the antifungal susceptibilities of clinical *Rhodotorula* species isolates using the CLSI broth microdilution assay. In addition, we investigated the biofilm formation capabilities of different *Rhodotorula* species.

## MATERIALS AND METHODS

**Microorganisms.** We initially tested 59 yeast isolates previously identified as *Rhodotorula* species, including 51 clinical and 8 environmental isolates recovered from 14 different Brazilian hospitals during the period from 1995 to 2010. The 51 clinical strains included 39 samples obtained from blood cultures and 12 samples from different anatomical sites. Clinical strains were isolated from 50 different patients, and the yeasts were sent to

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Address correspondence to Arnaldo Lopes Colombo, arnaldolcolombo@gmail.com.

J.M.N. and F.C.B. contributed equally to this article.

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the Laboratório Especial de Micologia, Universidade Federal de São Paulo, São Paulo, Brazil, for identification and antifungal susceptibility testing. Reference strains of *R. mucilaginosa* (CBS 329), *R. pallida* (CBS 320), and *R. glutinis* (CBS 20) were also included as control organisms in all laboratory tests, totaling 62 *Rhodotorula* species isolates.

**Screening of *Rhodotorula* species by conventional methods.** Yeast isolates from stock cultures were initially plated on Sabouraud dextrose agar (SDA) (Difco; BD and Company) and incubated at 35°C for 48 h to ensure purity and viability. *Rhodotorula* species isolates were screened based on the texture and typical color exhibited by their colonies on SDA, as well as by their micromorphology after culturing each isolate on cornmeal-Tween 80 agar. All isolates that were able to produce carotenoid pigments conferring a salmon-pink to coral-red color to the colonies and presenting only spheroidal to oval budding cells without the rudimentary formation of hyphae were considered to belong to the genus *Rhodotorula* (18, 19).

**Microorganism identification using amplification and sequencing of the ITS region of ribosomal DNA (rDNA).** The genomic DNA was extracted using mechanical disruption with glass beads combined with the SDS-based enzymatic lysis method adapted from Wach et al. (20). PCR and sequencing were performed with the universal primers ITS1 and ITS4 as described previously, and amplicons were sequenced using the dideoxynucleotide method in an ABI Prism 3100 automated sequencer (Applied Biosystems, CA) (21, 22). The DNA sequences generated were edited using Sequencher 4.1.4 (Genes Code Co., MI) to obtain contigs for each sample. Species identification was performed using the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov>); an E value of  $<10^{-5}$  was used as the cutoff for species identification.

**Biochemical profile of yeasts evaluated by the ID32C galleries.** All of the yeasts were tested by the ID32C system (bioMérieux, Marcy l'Etoile, France), along with a nitrate assimilation test, for species-level identification of *Rhodotorula* isolates. The tests were performed rigorously according to the manufacturer's instructions. The galleries were incubated at 30°C for 72 h in an airtight box containing a small volume of water to create a humid atmosphere. The carbohydrate assimilation profile obtained for each tested isolate was compared to the database apiweb version 3.0 (bioMérieux, Marcy l'Etoile, France) to obtain the final yeast identification. The complementary nitrate assimilation tests were performed by culturing the isolates on yeast carbon agar, to which potassium nitrate and peptone were added as nitrogen test sources, at 30°C for up to 7 days (15, 23).

**In vitro susceptibility testing.** Antifungal susceptibility testing was performed using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) document M27-A3 (24). The antifungal agents and concentrations tested included the following: 0.125 to 64 µg/ml for fluconazole (FLC) (Pfizer, Inc., NY) and 0.03 to 16 µg/ml for amphotericin B (AMB) (Sigma Chemical Corporation, St. Louis, MO), caspofungin (CAS) (Merck & Co., Inc., Rahway, NJ), posaconazole (PSC) (Schering Plough Research Institute, Kenilworth, NJ), and voriconazole (VRC) (Pfizer Inc., NY). The antifungal compounds were provided as pure powders by the manufacturer. All of the plates were prepared with RPMI 1640 medium buffered with 0.165 M morpholinepropanesulfonic acid at pH 7.0 and frozen at -20°C until use.

The microplates were incubated at 35°C for 72 h. The MICs were determined visually after 48 h and 72 h of incubation. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains. The MIC endpoint for AMB was considered to be the lowest tested drug concentration able to prevent any visible growth. The MICs for CAS, FLC, PSC, and VRC were based on a prominent decrease ( $\geq 50\%$ ) in growth compared to that of the drug-free growth control. The MICs at which 50% (MIC<sub>50</sub>) and 90% (MIC<sub>90</sub>) of the tested isolates were inhibited for each drug after 72 h at 35°C were also determined.

**Biofilm production assays. (i) Growth conditions and biofilm formation.** The biofilm formation assay was adapted from previously described methods (25). The strains initially cultured in SDA at 35°C for 48 h were further subcultured into RPMI 1640 broth and grown for 24 h with shaking at 200 rpm at 37°C. The cell cultures were harvested, washed twice

with phosphate-buffered saline (PBS), and adjusted to a concentration of  $10^7$  cells/ml in RPMI 1640 medium. Biofilm formation was tested in sterile 96-well polystyrene flat-bottom plates (Techno Plastic Products, Switzerland).

For the attachment phase, 100 µl of the cell suspension was transferred to each well of the plates and then incubated at 37°C for 1 h 30 min at 75 rpm. Unattached cells were removed, the wells were washed twice with 150 µl of PBS, and 150 µl of fresh RPMI 1640 medium was added. The plate was incubated at 37°C for 72 h, with shaking at 75 rpm to allow biofilm growth. A test medium without cells was added to the final column of each plate and used as a negative control.

**(ii) Biofilm quantification by CV staining.** After biofilm formation, each well was washed twice with 150 µl PBS, and the plate was dried for 20 min at 35°C. The washed biofilms were stained with 110 µl of 0.4% aqueous crystal violet (CV) solution for 45 min. Afterwards, the wells were washed three times with 200 µl of Milli-Q sterile water and destained with 200 µl of 95% ethanol. After 45 min, 100 µl of destaining solution from each sample was transferred to a new plate and measured with a spectrophotometer plate reader (model 680; Bio-Rad) at 570 nm. The absorbance values ( $A_{570}$ ) of the negative controls (containing no cells) were subtracted from the values of the test wells to minimize background interference. Each strain was tested five times on three different days, and the biofilm production quantities were reported as the arithmetic means  $\pm$  standard deviations (SD) of the  $A_{570}$  values for 15 replicate tests.

**(iii) Scanning electron microscopy (SEM).** Biofilms were formed on sterile polyvinyl chloride (PVC) strips (surface area, 0.5 cm<sup>2</sup>) and placed in 24-well microtiter plates (Techno Plastic Products, Switzerland) as described previously. Biofilms formed on PVC strips were fixed overnight at 4°C with 4% formaldehyde plus 2% glutaraldehyde buffered at pH 7.2 with 0.1 M sodium cacodylate. After fixation, the samples were treated with 1% osmium tetroxide in cacodylate buffer for 1 h. Subsequently, the samples were treated with 1% tannic acid for 45 min, washed three times with distilled water for 15 min, and treated again with 1% osmium tetroxide in cacodylate buffer for 1 h. The samples were dehydrated with a graded series of ethanol washes, critical-point dried in CO<sub>2</sub>, coated with gold, and examined with a JEOL JSM-5300 scanning electron microscope (26).

**(iv) Statistical analysis.** For a comparison of the biofilm formation capability between clinical and environmental strains of *R. mucilaginosa*, mean  $A_{570}$  values obtained for each group were compared using Student's *t* test with the GraphPad Prism version 5.00 for Windows (GraphPad Software, CA). A *P* value of  $<0.05$  was considered significant. Possible outliers were determined by Grubb's test using a *P* value of  $<0.05$  as the cutoff with GraphPad software. The test was performed until no outliers were detected in the replicates. Outliers were excluded from the analysis.

**Nucleotide sequence accession numbers.** The sequences generated in this study have been deposited in the GenBank (NCBI) database under the following accession numbers: JX499189, JX512667, JX512677, JX512680, JX512681, JX512682, JX512683, JX512684, JX512685, JX512686, JX512687, JX512688, JX512689, JX512690, JX512691, JX512692, JX512693, JX512694, JX512695, JX512696, JX512697, JX512698, JX512699, JX512700, JX512701, JX512702, JX512703, JX512704, JX512705, JX512706, JX512707, JX512708, JX512709, JX512710, JX512711, JX512712, JX512713, JX512714, JX512715, JX499188, JX512668, JX512669, JX512670, JX512671, JX512672, JX512673, JX512674, JX512675, JX512676, JX512678, JX512679, JX272795, JX272796, JX494370, JX494371, JX494373, JX494375, JX494374, and JX494372.

## RESULTS

**Screening of *Rhodotorula* species by conventional methods.** All 59 yeast isolates tested in this study plus the three reference strains were pure and viable. The individual colonies exhibited a salmon-pink to coral-red color on SDA and presented only spheroidal to oval blastoconidia with the absence of hyphae on corn meal-

**TABLE 1** Species distribution of clinical and environmental isolates of *Rhodotorula* species identified by ITS sequencing

Species	No. of isolates from:			Total
	Blood culture	Other source <sup>a</sup>	Environment	
<i>R. mucilaginosa</i>	34	10	7	51
<i>R. minuta</i>	2	—	—	2
<i>R. glutinis</i>	—	2	—	2
<i>R. dairenensis</i>	2	—	—	2
<i>R. slooffiae</i>	—	—	1	1
Total	38	12	8	58

<sup>a</sup> Other sources include pleural fluid, bronchoalveolar lavage, skin biopsy, secretion from a fistula hand, nasal mucus, scalp, sole of the foot, and catheter.

Tween 80 agar. Thus, all isolates were considered to belong to the genus *Rhodotorula*.

**Molecular identification of *Rhodotorula* species isolates by sequencing the ITS region of rDNA.** ITS sequencing was used as the gold standard for the species-level identification of all clinical and environmental *Rhodotorula* species isolates tested. DNA sequences of approximately 560 bp were obtained for all isolates tested. The sequences were subjected to BLAST searches to confirm the preliminary identification of the strains. BLAST alignments with the ITS sequences were successful in identifying all *Rhodotorula* species isolates, including the reference strains (100% query coverage,  $\geq 99\%$  identity, and an E value of 0.0 for all isolates). The 51 clinical isolates previously screened as *Rhodotorula* species were identified as the following: 44 *R. mucilaginosa* isolates, 2 *R. glutinis* isolates, 2 *R. minuta* isolates, 2 *Rhodotorula dairenensis* isolates, and 1 *Rhodospiridium fluviale* isolate. The environmental isolates were identified as *R. mucilaginosa* ( $n = 7$ ) and *Rhodotorula slooffiae* ( $n = 1$ ) (Table 1).

**Biochemical profile of *Rhodotorula* species isolates evaluated by ID32C and a nitrate assimilation test.** Using ITS identification as a reference, 56 out of the 62 (90.3%) isolates tested, including the reference strains, were correctly identified to the species level by the ID32C system combined with the nitrate assimilation test. However, even after the addition of the nitrate assimilation test, the ID32C resulted in discrepancies for 5 (8.1%) of the strains: 2 *R. dairenensis* strains were misidentified as *R. mucilaginosa*, 1 *Rhodospiridium fluviale* strain was misidentified as *R. glutinis*, 1 *R. slooffiae* strain was misidentified as *R. minuta*, and the reference *R. pallida* strain (CBS 320) did not assimilate nitrate and was identified with low discrimination between *R. glutinis* and the species *Candida sphaerica* and *Candida sake* (Table 2). This particular strain was phenotypically identified as a *Rhodotorula* species based on the salmon-pink color of the colonies and the compatible micromorphology. Moreover, 1 (1.6%) *R. minuta* isolate (2145) presented an unacceptable profile for final ID32C identification.

The ID32C system reported the final yeast species identification of 9 isolates without requesting the complementary nitrate assimilation test. These results were obtained with 1 *R. minuta* isolate and 2 *R. glutinis* isolates that were correctly identified and 6 other isolates that were misidentified, including 4 *R. mucilaginosa* isolates that were misidentified as *R. glutinis*, 1 *R. slooffiae* isolate that was misidentified as *R. minuta*, and 1 *Rhodospiridium fluviale* isolate that was misidentified as *R. glutinis*. For these isolates, after running the ID32C system combined with the nitrate assimilation

test, all 4 *R. mucilaginosa* isolates originally misidentified as *R. glutinis* had a final identification corrected to *R. mucilaginosa*.

Of note, 37 out of the 52 (71.2%) *R. mucilaginosa* isolates identified by the ID32C system along with the nitrate assimilation test generated the codes 5461750113 and 5461750111. Apparently, both carbohydrate assimilation profiles are reliable in the identification of *R. mucilaginosa* isolates.

A single yeast clinical isolate molecularly identified as *Rhodospiridium fluviale* was excluded from further experiments.

**Biofilm formation by *Rhodotorula* species isolates.** The CV staining assay for the quantification of biofilm formation of the remaining 61 *Rhodotorula* species strains tested, including the reference strains, had absorbance values at 570 nm ( $A_{570}$ ), ranging from 0.007 to 1.739. To better illustrate the differences in biofilm formation capability among isolates of *Rhodotorula* species, we arbitrarily established three categories for the quantification of biofilm production: low ( $A_{570} < 0.160$ ), medium ( $A_{570}$ , 0.160 to 0.632), and high ( $A_{570} > 0.632$ ). These categories were defined based on the quartile values of the full range of *Rhodotorula* species  $A_{570}$  values.

The rank scale for *Rhodotorula* species biofilm formation determined by CV staining was as follows: *R. minuta* > *R. mucilaginosa* > *R. glutinis* > *R. slooffiae* > *R. pallida* > *R. dairenensis*. According to the interpretive criteria adopted in this study, *R. minuta* and *R. mucilaginosa* were classified as medium biofilm producers, while the isolates from the remaining species were considered low biofilm producers (Fig. 1).

In addition, clinical isolates of *R. mucilaginosa* showed higher biofilm formation capability than that of environmental isolates (Fig. 2). The clinical isolates exhibited an  $A_{570}$  value of  $0.497 \pm 0.353$  (mean  $\pm$  SD), and the environmental isolates had an  $A_{570}$  value of  $0.210 \pm 0.109$  ( $P = 0.04$ ).

Of note, during the period from 1997 to 2010, the storage time at  $-70^\circ\text{C}$  for all *R. mucilaginosa* isolates did not affect their biofilm formation capability (data not shown).

**Biofilm SEM.** SEM images of the biofilms were used to validate the CV staining interpretive criteria used in our study to quantify the biofilms of *Rhodotorula* species isolates. For SEM, we used three isolates representing high, medium, and low biofilm producers: isolate 2991B ( $A_{570} = 1.739$ ), isolate 3166 ( $A_{570} = 0.394$ ), and isolate 320 ( $A_{570} = 0.072$ ). It was possible to verify in the SEM images (Fig. 3) that isolate 2991B (Fig. 3E and F) had a higher level of biofilm formation than isolate 3166 (Fig. 3C and D). The isolate 320 presented only microcolonies that did not have the multilayered architecture of a mature biofilm (Fig. 3A and B). An examination of the SEM images revealed that the degree of biofilm formation by high, medium, and low producers was highly correlated to the results of the CV assay.

**Susceptibilities of clinical isolates of *Rhodotorula* species to 5 antifungal agents.** Table 3 summarizes the MIC<sub>50</sub>, MIC<sub>90</sub>, and MIC ranges ( $\mu\text{g/ml}$ ) obtained for the *Rhodotorula* species isolates for the five antifungal agents after 48 and 72 h of incubation.

The MICs of the 50 clinical isolates of *Rhodotorula* species after 72 h of incubation ranged from 0.5 to 1  $\mu\text{g/ml}$  for AMB, 4 to  $>16$   $\mu\text{g/ml}$  for CAS, 0.25 to 4  $\mu\text{g/ml}$  for VRC, 1 to 8  $\mu\text{g/ml}$  for PSC, and  $\geq 64$   $\mu\text{g/ml}$  for FLC.

Of note, all *Rhodotorula* species isolates tested exhibited AMB MICs of  $\leq 1$   $\mu\text{g/ml}$ . The CAS MICs for all isolates were consistently high (MIC  $\geq 4$   $\mu\text{g/ml}$ ). The VRC and PSC MICs were similar (MIC<sub>50</sub>/MIC<sub>90</sub>, 2/4  $\mu\text{g/ml}$ ). The FLC MICs were very high for all isolates tested ( $\geq 64$   $\mu\text{g/ml}$ ).



TABLE 2 Concordance between the genotypic and phenotypic identification of 62 yeast isolates previously identified as *Rhodotorula* species<sup>a</sup>

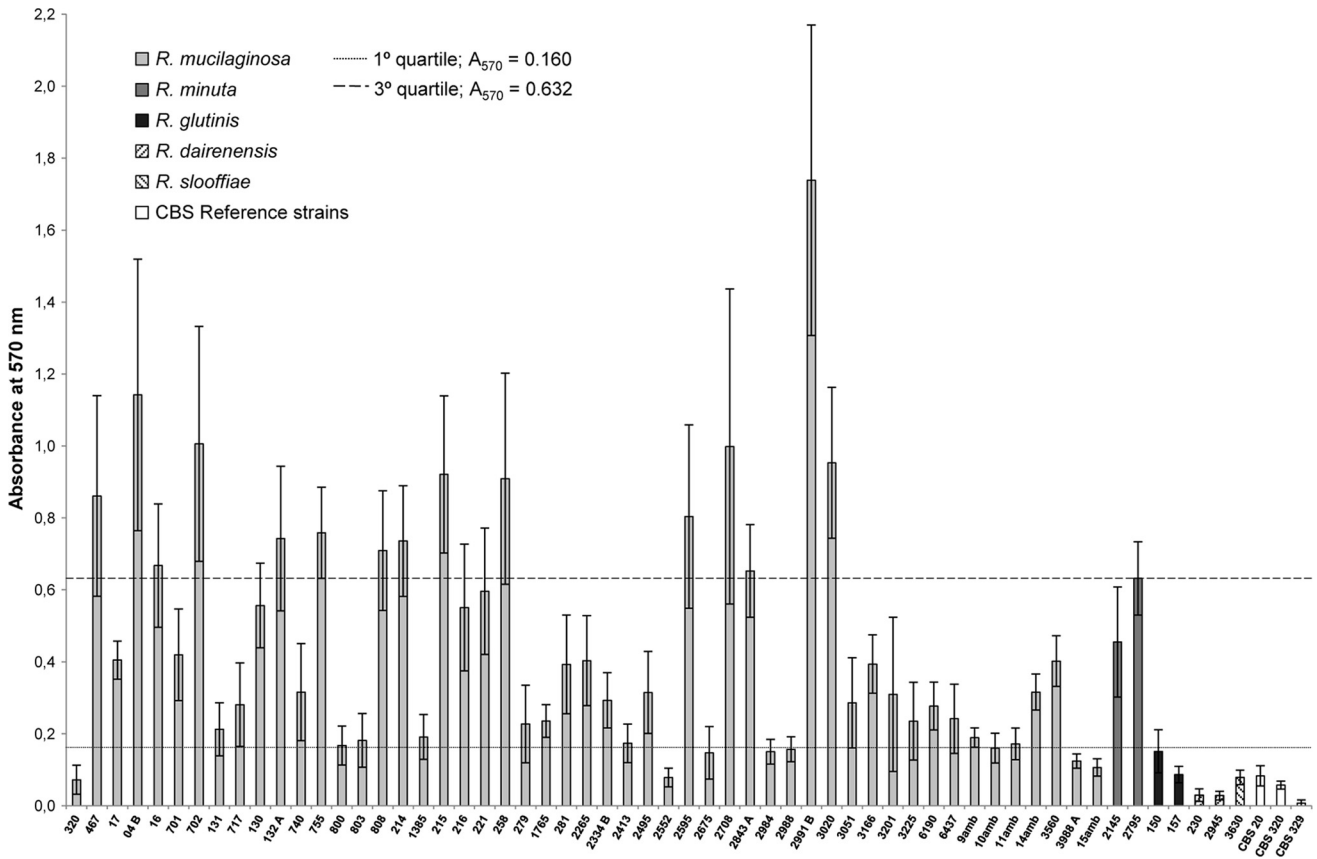
Isolate	Source	ITS sequencing		ID32C system + nitrate assimilation	
		Yeast species	Accession no.	Yeast species	Identification code
16	Blood culture	<i>R. mucilaginosa</i>	JX499189	<i>R. mucilaginosa</i>	<b>5461750113</b>
17	Blood culture	<i>R. mucilaginosa</i>	JX512667	<i>R. mucilaginosa</i>	5461750311
230*	Blood culture	<i>R. dairenensis</i>	JX512677	<i>R. mucilaginosa</i>	5061710113
281	Blood culture	<i>R. mucilaginosa</i>	JX512680	<i>R. mucilaginosa</i>	<b>5461750113</b>
320	Blood culture	<i>R. mucilaginosa</i>	JX512681	<i>R. mucilaginosa</i>	<b>5461750111</b>
369*	Blood culture	<i>Rhodospiridium fluviale</i>	JX512682	<i>R. glutinis</i>	5465750313
467	Blood culture	<i>R. mucilaginosa</i>	JX512683	<i>R. mucilaginosa</i>	7461750111
701	Blood culture	<i>R. mucilaginosa</i>	JX512684	<i>R. mucilaginosa</i>	<b>5461750111</b>
702	Blood culture	<i>R. mucilaginosa</i>	JX512685	<i>R. mucilaginosa</i>	<b>5461750113</b>
717	Blood culture	<i>R. mucilaginosa</i>	JX512686	<i>R. mucilaginosa</i>	<b>5461750113</b>
740	Blood culture	<i>R. mucilaginosa</i>	JX512687	<i>R. mucilaginosa</i>	<b>5461750111</b>
755	Blood culture	<i>R. mucilaginosa</i>	JX512688	<i>R. mucilaginosa</i>	<b>5461750111</b>
800	Blood culture	<i>R. mucilaginosa</i>	JX512689	<i>R. mucilaginosa</i>	<b>5461750111</b>
803	Blood culture	<i>R. mucilaginosa</i>	JX512690	<i>R. mucilaginosa</i>	<b>5461750111</b>
808	Blood culture	<i>R. mucilaginosa</i>	JX512691	<i>R. mucilaginosa</i>	5461710113
1385	Blood culture	<i>R. mucilaginosa</i>	JX512692	<i>R. mucilaginosa</i>	<b>5461750111</b>
1765	Blood culture	<i>R. mucilaginosa</i>	JX512693	<i>R. mucilaginosa</i>	<b>5461750111</b>
2145*	Blood culture	<i>R. minuta</i>	JX512694	Unacceptable profile	5313514331
2265	Blood culture	<i>R. mucilaginosa</i>	JX512695	<i>R. mucilaginosa</i>	<b>5461750113</b>
2334B	Blood culture	<i>R. mucilaginosa</i>	JX512696	<i>R. mucilaginosa</i>	5061550313
2413	Blood culture	<i>R. mucilaginosa</i>	JX512697	<i>R. mucilaginosa</i>	<b>5461750111</b>
2495	Blood culture	<i>R. mucilaginosa</i>	JX512698	<i>R. mucilaginosa</i>	5461650111
2552	Blood culture	<i>R. mucilaginosa</i>	JX512699	<i>R. mucilaginosa</i>	5061350113
2595	Blood culture	<i>R. mucilaginosa</i>	JX512700	<i>R. mucilaginosa</i>	<b>5461750111</b>
2675	Blood culture	<i>R. mucilaginosa</i>	JX512701	<i>R. mucilaginosa</i>	<b>5461750113</b>
2708	Blood culture	<i>R. mucilaginosa</i>	JX512702	<i>R. mucilaginosa</i>	<b>5461750113</b>
2795	Blood culture	<i>R. minuta</i>	JX512703	<i>R. minuta</i>	4513314331
2843A	Blood culture	<i>R. mucilaginosa</i>	JX512704	<i>R. mucilaginosa</i>	7461360113
2945*	Blood culture	<i>R. dairenensis</i>	JX512705	<i>R. mucilaginosa</i>	5421710013
2984	Blood culture	<i>R. mucilaginosa</i>	JX512706	<i>R. mucilaginosa</i>	<b>5461750111</b>
2988	Blood culture	<i>R. mucilaginosa</i>	JX512707	<i>R. mucilaginosa</i>	<b>5461750113</b>
2991B	Blood culture	<i>R. mucilaginosa</i>	JX512708	<i>R. mucilaginosa</i>	<b>5461750113</b>
3020	Blood culture	<i>R. mucilaginosa</i>	JX512709	<i>R. mucilaginosa</i>	<b>5461750113</b>
3051	Blood culture	<i>R. mucilaginosa</i>	JX512710	<i>R. mucilaginosa</i>	5021610003
3166	Blood culture	<i>R. mucilaginosa</i>	JX512711	<i>R. mucilaginosa</i>	<b>5461750113</b>
3201	Blood culture	<i>R. mucilaginosa</i>	JX512712	<i>R. mucilaginosa</i>	<b>5461750113</b>
3225	Blood culture	<i>R. mucilaginosa</i>	JX512713	<i>R. mucilaginosa</i>	<b>5461750113</b>
6190	Blood culture	<i>R. mucilaginosa</i>	JX512714	<i>R. mucilaginosa</i>	<b>5461750113</b>
6437	Blood culture	<i>R. mucilaginosa</i>	JX512715	<i>R. mucilaginosa</i>	<b>5461750113</b>
04B	Secretion from a fistula hand	<i>R. mucilaginosa</i>	JX499188	<i>R. mucilaginosa</i>	5061750113
130	Bronchoalveolar lavage	<i>R. mucilaginosa</i>	JX512668	<i>R. mucilaginosa</i>	<b>5461750113</b>
131	Bronchoalveolar lavage	<i>R. mucilaginosa</i>	JX512669	<i>R. mucilaginosa</i>	<b>5461750111</b>
132A	Bronchoalveolar lavage	<i>R. mucilaginosa</i>	JX512670	<i>R. mucilaginosa</i>	<b>5461750113</b>
150	Scalp	<i>R. glutinis</i>	JX512671	<i>R. glutinis</i>	7067750113
157	Sole of the foot	<i>R. glutinis</i>	JX512672	<i>R. glutinis</i>	7067750113
214	Nasal mucus	<i>R. mucilaginosa</i>	JX512673	<i>R. mucilaginosa</i>	5441750113
215	Pleural fluid	<i>R. mucilaginosa</i>	JX512674	<i>R. mucilaginosa</i>	<b>5461750113</b>
216	Pleural fluid	<i>R. mucilaginosa</i>	JX512675	<i>R. mucilaginosa</i>	<b>5461750113</b>
221	Pleural fluid	<i>R. mucilaginosa</i>	JX512676	<i>R. mucilaginosa</i>	<b>5461750113</b>
258	Skin biopsy	<i>R. mucilaginosa</i>	JX512678	<i>R. mucilaginosa</i>	5061750113
279	Catheter	<i>R. mucilaginosa</i>	JX512679	<i>R. mucilaginosa</i>	5461640101
9amb	Env.	<i>R. mucilaginosa</i>	JX272795	<i>R. mucilaginosa</i>	<b>5461750113</b>
10amb	Env.	<i>R. mucilaginosa</i>	JX272796	<i>R. mucilaginosa</i>	<b>5461750111</b>
11amb	Env.	<i>R. mucilaginosa</i>	JX494370	<i>R. mucilaginosa</i>	<b>5461750113</b>
14amb	Env.	<i>R. mucilaginosa</i>	JX494371	<i>R. mucilaginosa</i>	5061750113
3560	Env.	<i>R. mucilaginosa</i>	JX494373	<i>R. mucilaginosa</i>	<b>5461750113</b>
3630*	Env.	<i>R. slooffiae</i>	JX494375	<i>R. minuta</i>	4513214311
3988A	Env.	<i>R. mucilaginosa</i>	JX494374	<i>R. mucilaginosa</i>	7061750113
15amb	Env.	<i>R. mucilaginosa</i>	JX494372	<i>R. mucilaginosa</i>	<b>5461750113</b>
CBS 20	CBS	<i>R. glutinis</i>		<i>R. glutinis</i>	5061310013
CBS 320*	CBS	<i>R. pallida</i>		<i>Rhodotorula</i> sp.	7067350113
CBS 329	CBS	<i>R. mucilaginosa</i>		<i>R. mucilaginosa</i>	5661750311

<sup>a</sup> Isolates that showed differences between the phenotypic and genotypic identifications are marked with an asterisk. The identification codes in bold represent the two most common biochemical profiles among all *R. mucilaginosa* isolates tested. Env., air from hospital environment; CBS, reference strains.

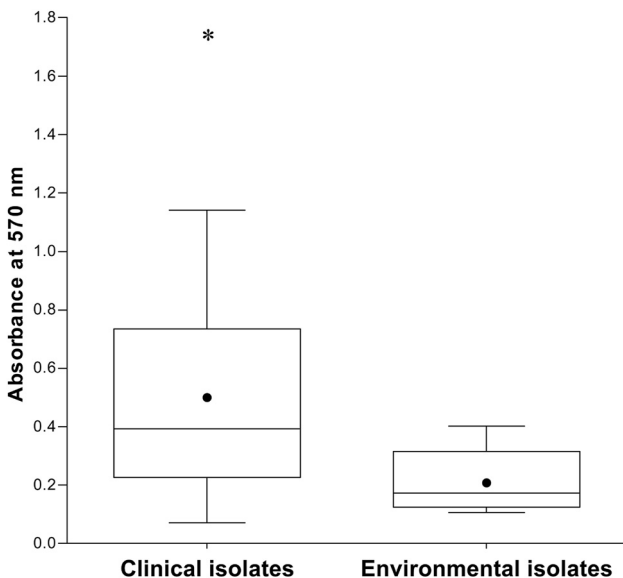
## DISCUSSION

*Rhodotorula* species is an emergent pathogen capable of causing invasive infections in humans, particularly in immunocompromised patients (7, 9, 27–29). In spite of the increased number of invasive infections by *Rhodotorula* species described in the last decades, only a

small number of epidemiological studies have used molecular methods for the identification of *Rhodotorula* species. To our knowledge, this is the first study in which ITS sequencing was used as the gold standard for the species-level identification of a large collection of clinical and environmental *Rhodotorula* species isolates.



**FIG 1** Biofilm production of 61 *Rhodotorula* species isolates, including the reference strains of *R. mucilaginosa* (CBS 329), *R. pallida* (CBS 320), and *R. glutinis* (CBS 20). The histograms represent the means  $\pm$  SD of the absorbance values at 570 nm ( $A_{570}$ ) obtained from 15 replicate tests for each sample. According to the interpretive criteria adopted in this study, *R. mucilaginosa* (mean  $A_{570}$ , 0.449) and *R. minuta* (mean  $A_{570}$ , 0.543) were classified as medium biofilm producers, whereas the other species of *Rhodotorula* were considered low biofilm producers ( $A_{570}$ ,  $<0.160$ ).

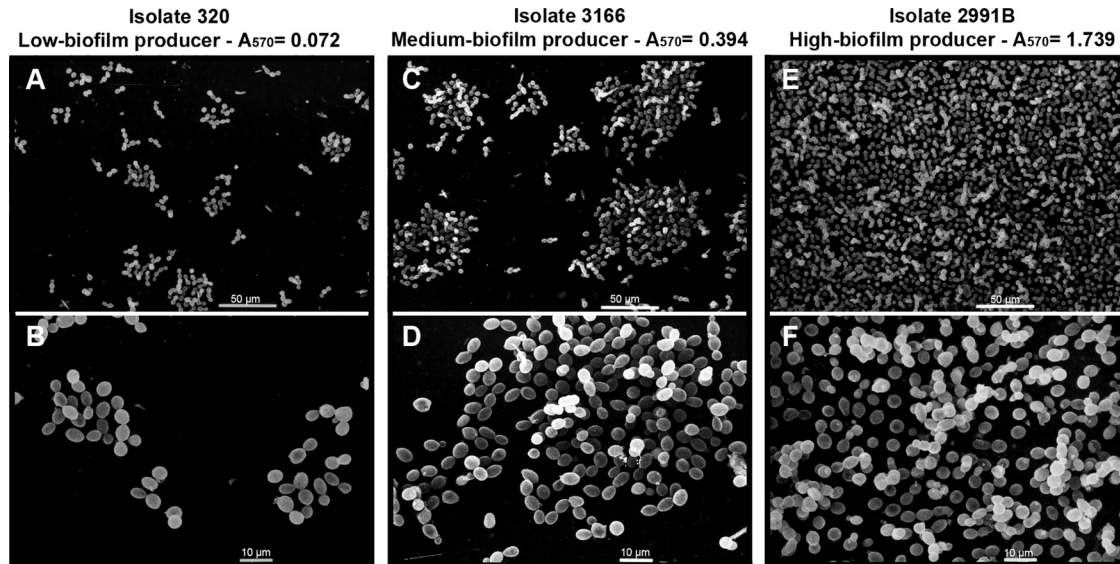


**FIG 2** Boxplots of the absorbance values obtained for the clinical and environmental isolates of *R. mucilaginosa* in the biofilm formation assays. The clinical isolates presented a greater ability to form biofilms than did the environmental isolates ( $P = 0.04$ ). The solid circle indicates the mean absorbance obtained for the group of clinical ( $A_{570}$ , 0.497) and environmental ( $A_{570}$ , 0.21) isolates. The asterisk indicates that isolate 2991B showed significantly greater biofilm formation capability ( $P < 0.05$ ).

ITS sequencing was a reliable method for the identification of *Rhodotorula* species, and it was more accurate than conventional phenotypic tests. In our study, the *Rhodotorula* species distribution among clinical isolates was similar to that reported previously: *R. mucilaginosa* ( $n = 44$ ; 88%) was the most prevalent species, followed by *R. glutinis* ( $n = 2$ ; 4%), *R. minuta* ( $n = 2$ ; 4%), and *R. dairenensis* ( $n = 2$ ; 4%) (7, 9). It is worth mentioning that there are no reports in the literature regarding infections caused by the species *R. dairenensis* and *Rhodospiridium fluviale*. In addition, *R. mucilaginosa* was also the most prevalent species found among environmental samples, suggesting that this species is well adapted to both free-living growth and human hosts.

According to the CBS site (<http://www.cbs.knaw.nl>), *Rhodospiridium diobovatum*, *Rhodospiridium sphaerocarum*, and *Rhodospiridium toruloides* represent sexual forms of *R. glutinis*. Indeed, the ITS sequences of our clinical isolates, 150 and 157, matched the sequences of *R. glutinis* and *Rhodospiridium diobovatum* when BLAST searches were performed. These BLAST matches showed the same scores for all variables, including the E value, max score, and max identity. Because both taxa appeared to be the same species, we considered the final identification of the isolates 150 and 157 to be *R. glutinis*.

Currently, yeast identification in clinical laboratories is performed using commercial and automated systems (ID32C and Vitek 2, respectively). These systems show high accuracy for the



**FIG 3** Scanning electron microscopy (SEM) images of 3 *R. mucilaginosa* isolates after the induction of biofilm formation on a PVC strip. Isolate 320, low biofilm producer (A and B); isolate 3166, medium biofilm producer (C and D); and isolate 2991B, high biofilm producer (E and F). Absorbance values at 570 nm obtained for each isolate subjected to the crystal violet staining assay are provided at the top of micrographics. Micrographics B, D, and F are higher magnifications of A, C, and E, respectively. SEM images confirmed the CV staining interpretive criteria to indirectly quantify bulk biofilm production by *Rhodotorula* species isolates.

identification of the most common yeast species causing human infections, such as *Candida* species. However, they are unable to distinguish genetically similar species such as the *Candida parapsilosis* species complex and show limited accuracy for identification of emergent pathogens, including *Trichosporon* species and *Rhodotorula* species (9, 13–17).

Despite a number of limitations, the ID32C system along with a nitrate assimilation test was able to correctly identify 90.3% of *Rhodotorula* species isolates. The complementary nitrate assimilation test was crucial to the final species identification, suggesting that this test should be performed even when not requested by the ID32C system report. Inconsistent results in phenotypic identification were mostly related to non-*R. mucilaginosa* isolates. Indeed, the database of the commercial system ID32C does not include all species in the genera *Rhodotorula* and *Rhodospiridium*.

*Rhodotorula* species infections are frequently associated with the presence of CVCs and other implantable medical devices (9–11, 30). These devices provide the necessary surfaces for biofilm

formation and are currently responsible for a significant percentage of human infections. In contrast to the extensive literature dealing with *Candida* species biofilms (25, 26, 31–33), little attention has been paid to emergent fungal pathogens such as *Rhodotorula* species.

This is the first study to evaluate the biofilm formation ability of different *Rhodotorula* species and the putative differences between clinical and environmental isolates. Using CV staining, we were able to demonstrate that clinical isolates of *R. mucilaginosa* were better at forming biofilms than the environmental isolates. Moreover, we verified that *R. mucilaginosa* and *R. minuta* were the best biofilm producers.

The main limitation of our study was the small number of environmental strains and non-*R. mucilaginosa* species tested. Further studies are needed to confirm if these microorganisms really have a lower capability to produce biofilm than clinical strains of *R. mucilaginosa*.

Overall, the CV staining was a useful method to indirectly mea-

**TABLE 3** *In vitro* activity of five antifungal agents against 50 clinical isolates of *Rhodotorula* species using the CLSI broth microdilution assay

<i>Rhodotorula</i> species (no. of isolates)	Cell incubation period (h)	MIC ( $\mu\text{g/ml}$ ) for indicated antifungal agent <sup>a</sup>														
		AMB			CAS			VRC			PSC			FLC		
		MIC <sub>50</sub>	MIC <sub>90</sub>	Range	MIC <sub>50</sub>	MIC <sub>90</sub>	Range	MIC <sub>50</sub>	MIC <sub>90</sub>	Range	MIC <sub>50</sub>	MIC <sub>90</sub>	Range	MIC <sub>50</sub>	MIC <sub>90</sub>	Range
<i>R. mucilaginosa</i> (44)	48	1	1	0.25–1	8	8	1–16	1	2	0.25–2	2	2	0.5–4	64	>64	$\geq 64$
	72	1	1	0.5–1	8	16	4–16	2	4	0.5–4	2	4	1–8	64	>64	$\geq 64$
<i>R. glutinis</i> (2)	48	ND	ND	0.5	ND	ND	4	ND	ND	0.25	ND	ND	1–2	ND	ND	64
	72	ND	ND	0.5–1	ND	ND	8	ND	ND	0.25	ND	ND	2	ND	ND	64
<i>R. minuta</i> (2)	48	ND	ND	0.5	ND	ND	2	ND	ND	4	ND	ND	2	ND	ND	64
	72	ND	ND	1	ND	ND	4	ND	ND	4	ND	ND	2	ND	ND	64
<i>R. dairenensis</i> (2)	48	ND	ND	0.5–1	ND	ND	16	ND	ND	0.25–0.5	ND	ND	2	ND	ND	64
	72	ND	ND	0.5–1	ND	ND	>16	ND	ND	0.5–1	ND	ND	1–2	ND	ND	64

<sup>a</sup> AMB, amphotericin B; CAS, caspofungin; VRC, voriconazole; PSC, posaconazole; FLC, fluconazole; ND, not determined.

sure bulk biofilm, and the results of biofilm production were confirmed by SEM. It was also observed that the storage time of the samples did not affect the biofilm formation capability of *R. mucilaginosa* clinical isolates. This finding conflicts with the hypothesis that the expression of virulence factors by fungi may be substantially impaired by long-term storage of strains (34, 35).

The antifungal susceptibility profiles obtained strongly suggest that the genus *Rhodotorula* is not a target for FLC or CAS. Other azole agents also showed poor activity ( $\geq 2$   $\mu\text{g/ml}$ ) against the majority of the isolates tested. In contrast, AMB showed the best activity *in vitro*. Overall, the results obtained here are in agreement with previous studies and show that it is more appropriate to read the susceptibility test of *Rhodotorula* species after 72 h of incubation (4, 7, 9, 12, 29, 36, 37).

In conclusion, we emphasized the importance of molecular methods to correctly identify *Rhodotorula* species isolates and non-*R. mucilaginosa* species in particular. *R. mucilaginosa* was the most prevalent species among the clinical and environmental samples. All *Rhodotorula* species isolates tested were susceptible to AMB, suggesting that it should be considered the antifungal drug of choice for the treatment of *Rhodotorula* species invasive infections. Finally, we demonstrated that *Rhodotorula* species are able to form biofilms, which may play a role in the pathogenesis of infections by this species.

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