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Candida bracarensis Detected among Isolates of *Candida glabrata* by Peptide Nucleic Acid Fluorescence In Situ Hybridization: Susceptibility Data and Documentation of Presumed Infection▽

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Molecular taxonomic studies have revealed new *Candida* species among phenotypically delineated species, the best example being *Candida dubliniensis*. This study was designed to determine the occurrence of two new molecularly defined species, *Candida bracarensis* and *Candida nivariensis*, which are closely related to and identified as *Candida glabrata* by phenotypic assays. A total of 137 recent clinical isolates of *C. glabrata* identified by phenotypic characteristics was tested with *C. bracarensis* and *C. nivariensis* species-specific peptide nucleic acid fluorescence in situ hybridization probes. Three of 137 (2.2%) isolates were positive with the *C. bracarensis* probe, whereas the control strain, but none of the clinical isolates, was positive with the *C. nivariensis* probe. D1/D2 sequencing confirmed the identification of the three isolates as representing *C. bracarensis*. Clinically, one *C. bracarensis* isolate was recovered from a presumed infection, a polymicrobial pelvic abscess in a patient with perforated diverticulitis. The other two isolates were recovered from two adult oncology patients who were only colonized. *C. bracarensis* was white on CHROMagar Candida, had variable API-20C patterns that overlapped with *C. nivariensis* and some *C. glabrata* isolates, and had variable results with a rapid trehalose assay. Interestingly, an isolate from one of the colonized oncology patients was resistant to fluconazole, itraconazole, voriconazole, and posaconazole in vitro. In summary, *C. bracarensis* was detected among clinical isolates of *C. glabrata*, while *C. nivariensis* was not. One *C. bracarensis* isolate causing a presumed deep infection was recovered, and another isolate was azole resistant. Whether clinical laboratories should identify *C. bracarensis* will require more data.

Although *Candida albicans* remains the *Candida* species most commonly recovered from clinical specimens including blood, non-*albicans Candida* species have become increasingly prevalent in multiple centers and geographic regions over the last two decades (11, 13). Indeed, at our institution (The Johns Hopkins Hospital), non-*albicans Candida* species accounted for 54% and 64% of yeast recovered from blood in 2005 and 2006, respectively (our unpublished data). Additionally, it is vital to identify these non-*albicans Candida* species correctly, as they are often less susceptible or resistant to antifungal agents used to treat *Candida* infections (2, 11).

Molecular diagnostic methods have the potential to provide rapid, accurate identification. In addition, molecular assays, particularly rRNA gene sequencing, have identified new *Candida* species that share significant phenotypic characteristics with frequently encountered *Candida* spp. (1, 3, 4, 7, 14). Perhaps the best example was the discovery of *Candida dubliniensis* in 1995, a yeast that shares many phenotypic characteristics with *C. albicans* (12). *Candida bracarensis* and *Candida nivariensis* are two recently described species that are phenotypically identified as *Candida glabrata* species (1, 3, 4). However, little is known about the prevalence, antifungal susceptibility patterns, and clinical significances of these two newly discovered species of the *C. glabrata* clade. Therefore, the goals of this study were to explore the prevalences, antifungal susceptibility patterns, and clinical significances of *C. bracarensis* and *C. nivariensis*. We utilized novel, species-specific, peptide nucleic acid fluorescence in situ hybridization (PNA FISH) probes to identify these new species among clinical isolates identified phenotypically as representing *C. glabrata*.

MATERIALS AND METHODS

Control strains and clinical isolates tested. One hundred fifty recently collected clinical isolates that were previously identified as being *C. glabrata* isolates were selected for this study (Table 1). Criteria for inclusion were as follows: there was only one isolate per patient, and there was an attempt to represent consecutive isolates. These yeasts were identified by the staff of the Microbiology Laboratory at The Johns Hopkins Hospital using standard laboratory methods. Characteristics used for the identification of isolates as being *C. glabrata* isolates included negative germ tube test, small yeasts with no hypha production and no melanin production on cornmeal agar with caffeic acid, and positive results for glucose and trehalose fermentation. When identification remained uncertain, carbohydrate assimilation patterns were determined using the API-20C yeast

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TABLE 1. Isolates of yeasts phenotypically identified as being *C. glabrata* and their clinical sources

Specimen source	No. (%) of isolates
Urine	53 (39)
Stool	38 (28)
Throat	16 (12)
Wounds	7 (5)
Blood	6 (4)
Miscellaneous fluid	5 (4)
Bile	4 (3)
Abscesses	3 (2)
Gynecological[a]	3 (2)
Respiratory[b]	2 (1)
Total	137 (100)

[a] Gynecological indicates samples from endometrium, cervix, and urethra.
[b] Respiratory indicates samples from trachea, induced sputum, and pleural cavity.

identification system (BioMerieux, Hazelwood, MO). Purity and viability were ascertained before testing by subculturing on CHROMagar Candida (BD Diagnostics, Sparks, MD). The results for the 137 isolates on which all tests were performed are reported: 13 of the original 150 isolates failed to grow when subcultured.

Control strains included *C. bracarensis* type strain (TS) NRRL Y-48270 (CBS 10154), reported previously by Correia et al. (3); *C. bracarensis* HOP-15 (NRRL Y-27794), described previously by Page et al. (9); *Candida nivariensis* type strain NRRL Y-48269 (CBS 9983); described previously by Alcoba-Florez et al. (1); *Nakaseomyces* (*Kluyveromyces*) *delphensis* type strain NRRL Y-2379 (CBS 2170 or ATCC 24205); and *C. glabrata* CLC (a well-characterized control strain used for quality control of internal tests at The Johns Hopkins Clinical Mycology Laboratory).

PNA FISH. Species-specific probes for *C. bracarensis* and *C. nivariensis* (kindly provided by AdvanDx, Inc., Woburn, MA) were used according to the manufacturer's protocol for positive blood cultures (8). Briefly, an inoculum of a yeast culture phenotypically identified as being *C. glabrata* was suspended in saline at a concentration of at least a 0.5 McFarland standard; a drop of the suspension was placed onto Teflon-coated microscope slides, air dried, and fixed with methanol. One drop of hybridization solution was added, a coverslip was applied, and the slides were incubated on a slide warmer at 55°C for 90 min. The slides were then submerged for 30 min in a stringent wash solution (55°C) in a water bath, during which time the coverslips were removed. Slides were mounted with mounting fluid, and coverslips were again applied before examination with a fluorescence microscope (oil objective, 100×) equipped with a fluorescein isothiocyanate-Texas Red dual-band filter (filter no. AC003; AdvanDx). Two readers independently examined each slide. Distinct green fluorescence in multiple fields was considered to be a positive result (8).

D1/D2 LSU rRNA gene sequencing. Isolates that were reactive with the *C. bracarensis* or *C. nivariensis* probes and all control strains were sequenced using methods previously described by Kurtzman and Robnett (5, 6) for domains 1 and 2 (D1/D2) of the large-subunit (LSU) rRNA gene.

Patient information. Following institutional review board approval, the records for individuals with a positive culture for *C. bracarensis* were reviewed. Data collected included patient demographics, underlying illness, length of hospitalization, antifungal usage, signs and symptoms of infection, and other positive cultures for *C. glabrata*.

Mycologic studies. For control strains and *C. bracarensis* and *C. nivariensis* probe-positive isolates, colony color was assessed on CHROMagar Candida (Becton Dickinson Diagnostics, Sparks, MD) after incubation at 37°C for 24 and 48 h. Carbohydrate assimilation patterns were compared using API-20C (BioMerieux, Hazelwood, MO) according to the manufacturer's directions. Rapid trehalose assimilation was performed using rapid trehalose assimilation broth (Remel, Lenexa, KS) according to the manufacturer's directions.

In vitro susceptibility testing. In vitro susceptibility testing was performed on all control strains and *C. bracarensis* or *C. nivariensis* probe-positive isolates. The Sensititre YeastOne system (Trek Diagnostic Systems, Inc., Cleveland, OH) was used according to the manufacturer's directions. Antifungal drugs tested on the Sensititre panel were fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), posaconazole, caspofungin (CASP), and flucytosine (5FC). Amphotericin B (AMB) was tested using E-strips (AB BIODISK, Solna, Sweden); the MIC was determined in accordance with the manufacturer's instructions.

RESULTS

PNA FISH results for detection of *C. bracarensis* and *C. nivariensis*. Of the 137 *C. glabrata* isolates screened with the species-specific *C. bracarensis* PNA FISH probe, three isolates (Cagl-78, Cagl-112, and Cagl-121) were reactive, with fluorescent yeast cells detected. Two *C. bracarensis* control strains (*C. bracarensis* TS and *C. bracarensis* HOP-15) were also PNA FISH probe positive. On the other hand, none the 137 isolates showed any fluorescence with the *C. nivariensis* probe, whereas the control strain was positive. There was no noticeable cross-reactivity with the *C. bracarensis* or *C. nivariensis* probes.

D1/D2 sequence results. Sequencing of the LSU D1/D2 regions confirmed the species identity of control strains and the three clinical isolates as being *C. bracarensis* isolates. The three clinical isolates differed from the type strain by 2 nucleotides, with a CT inversion near the middle of the sequence. The earlier clinical *C. bracarensis* control strain from our institution (9), Cagl-HOP-15, did not have this inversion.

Patient information. Pertinent clinical and demographic information for the three patients from whom the isolates were recovered is summarized in Table 2. One of the *C. bracarensis* strains (Cagl-121) was recovered from throat and another (Cagl-78) was recovered from stool from surveillance cultures of two oncology patients undergoing treatment for hematological malignancies. Both patients had received antifungal agents (both with AMB and VRC and one also with CASP) prior to the isolation of *C. bracarensis*. One of these patients had multiple previous surveillance cultures that were positive for *C. glabrata*, and both patients had subsequent positive *C. glabrata*

TABLE 2. Demographic and clinical characteristics of patients from whom *C. bracarensis* was isolated

Organism	Body site of isolation	Age (yr)/sex/race[a]	Underlying illness[b]	Evidence of infection	Length of hospital stay (days)	Antifungal(s) used within previous 30 days	Source or reference
Cagl-78	Stool	62/F/W	AML	No	36	VRC, AMB	This study
Cagl-112	Abscess	67/F/W	Perforated diverticulitis	Yes	13	None	This study
Cagl-121	Throat	31/M/B	ALTCL	No	95	VRC, AMB, CASP	This study
C. bracarensis HOP-15	Stool	17/F/B	AIDS	No	22	FLC	11
C. bracarensis TS	Vagina	Unknown		Yes	Unknown	Unknown	3

[a] F, female; M, male; W, white (Caucasian); B, black (African American).
[b] AML, acute myelogenous leukemia; ALTCL, anaplastic large-T-cell lymphoma.

TABLE 3. Comparison of characteristics of *C. bracarensis* with *C. glabrata*, *C. nivariensis*, and *N. delphensis*[a]

Yeast species	Assimilation at 24 h/48 h			Rapid trehalose	Color on CHROMagar Candida (at 37°C)	Growth at:	
	Gluc	Glyc	Treh			37°C	42°C
C. glabrata (control)	+/+	−/−	−/−	+	Pink	+	+
C. bracarensis HOP	+/+	−/+	−/−	−	White	+	+
C. bracarensis Y48270	+/+	−/+	−/+	−	White	+	+
C. nivariensis (control)	+/+	−/+	−/−	−	White	+	+
N. delphensis	−/+	−/−	−/−	−	White	+	+
Cagl-78	+/+	−/−	−/+	+	White	+	−
Cagl-112	+/+	−/−	−/−	+	White	+	+
Cagl-121	+/+	−/−	−/−	+	White	+	−

[a] Glu, glucose; Glyc, glycerol; Treh, trehalose.

cultures from their gastrointestinal tracts. These isolates were not available for testing with the *C. bracarensis* probe.

The third clinical *C. bracarensis* isolate (Cagl-112) was recovered from a specimen collected surgically in a case of pelvic abscess in a woman with a history of perforated diverticulitis but no underlying immunosuppressive conditions. In contrast to the other two cases, the patient had clinical signs and symptoms in addition to radiographic evidence of infection. This was a polymicrobial infection; *Enterococcus faecium* and coagulase-negative *Staphylococcus* were also recovered from the wound. Following percutaneous abscess drainage, the patient underwent a hemicolectomy and diverting ileostomy. She never received antifungal therapy, and no prior or subsequent cultures grew yeast.

Mycologic studies. The phenotypic characteristics of the three newly identified *C. bracarensis* isolates as well as those of all of the control isolates are summarized in Table 3. All of the organisms in question shared phenotypic morphological properties with no germ tube formation, no hypha production on cornmeal agar with caffeic acid, no phenoloxidase activity, and no urease activity. The recently collected clinical isolates of *C. bracarensis* as well as the *C. bracarensis* controls showed similar but not identical carbohydrate assimilation profiles; the isolates differed in their abilities to assimilate trehalose. Our control culture of *C. nivariensis* had phenotypic properties similar to those of *C. glabrata* and *C. bracarensis* and was trehalose assimilation negative.

In the original and subsequent descriptions of *C. nivariensis*, it was noted that this species produced white colonies, not the pink-purple colonies typically produced by *C. glabrata*, on CHROMagar Candida (1, 4). All of the three newly discovered clinical isolates and all control strains of *C. bracarensis*, *C. nivariensis*, and *N. delphensis* produced white colonies. The *C. glabrata* control strain produced pink colonies.

In vitro susceptibility studies. In vitro susceptibility testing of the clinical isolates and controls is presented in Table 4. The *C. bracarensis* isolate recovered from throat (Cagl-121) was resistant to all triazole agents tested, and the control strain of *N. delphensis* was resistant to FLC (128 μg/ml), ITC (8 μg/ml), and VRC (4 μg/ml). In contrast, the other two *C. bracarensis* isolates as well as the control strain of *C. nivariensis* displayed susceptibilities similar to those of our *C. glabrata* control strain. All isolates tested had low MICs of CASP, 5FC, and AMB.

DISCUSSION

We identified three isolates of *C. bracarensis* among 137 yeast isolates identified phenotypically as representing *C. glabrata*, for a frequency of 2.2%. This equates to approximately 1 patient with a *C. bracarensis* isolate for every 50 patients with positive cultures for yeasts phenotypically identified as *C. glabrata*. D1/D2 sequencing confirmed the identification of the three recently discovered isolates. These findings confirm the original description by Correia and colleagues that *C. bracarensis* is a mimicker of *C. glabrata* and also suggest that it may have a wide geographic distribution, since it has been recovered from clinical specimens from Portugal, the United Kingdom, and the United States (3, 9; this study). Although *C. bracarensis* is a newly described species, we hypothesize that this species would be found among *C. glabrata* strains from well-maintained stock culture isolates collected decades ago. It should be emphasized that our study examined a limited number of isolates from one tertiary care medical center. Further studies from multiple geographical locations are needed to

TABLE 4. Comparison of in vitro antifungal susceptibility of *C. bracarensis* with those of *C. glabrata*, *C. nivariensis*, and *N. delphensis*

Organism	MIC (μg/ml)						
	FLC	ITC	PSC[a]	VRC	CASP	5FC	AMB
C. glabrata (control)	16	0.5	1	0.25	0.12	0.03	0.064
C. bracarensis (HOP-15)	16	0.5	0.5	0.25	0.12	0.03	0.032
C. bracarensis (TS)	8	0.5	0.5	0.12	0.25	0.03	0.023
C. nivariensis (TS)	16	2	2	0.25	0.25	0.5	0.5
N. delphensis (TS)	128	>16	8	4	0.25	0.25	0.032
Cagl-78	8	0.25	0.5	0.06	0.25	0.12	1
Cagl-112	4	0.25	0.25	0.06	0.25	0.12	0.094
Cagl-121	256	>16	>8	8	0.12	0.25	0.19

[a] PSC, posaconazole.

elucidate the epidemiology of infection, colonization, and antifungal resistance of *C. bracarensis*.

In contrast, *C. nivariensis* was not found among the 137 isolates of *C. glabrata* in the present study. Although the incidence of *C. nivariensis* remains unknown, our data suggest that this species is less common than *C. bracarensis*. Currently, to the best of our knowledge, this species has been recovered in Spain, Japan, and Indonesia (1, 4). Studies in multiple laboratories and geographic locations will be needed to address this question.

Evaluation of the clinical characteristics at the time of specimen collection for the patients from whom *C. bracarensis* was recovered yielded interesting findings. Specifically, there were additional "*C. glabrata*"-positive cultures recovered from the two oncology patients; it is likely that they were *C. bracarensis* cultures. If confirmed, these findings plus one reported previously in the literature (9) suggest that persistence of gastrointestinal tract colonization may occur with this species.

The other significant finding was that one of the three patients from whom we recovered *C. bracarensis* had evidence of a presumed deep infection; the type strain was the causal agent of vaginitis. This is the first report of recovery of *C. bracarensis* from a deep anatomic site and supports a presumption of infection since the organism was collected directly from the abscess and not from drainage tubes. The presence of this organism in a pelvic abscess also implies that it was probably a component of this individual's gastrointestinal tract. Preabscess cultures would have provided important data on whether this patient was colonized.

Our evaluation of common mycological characteristics of the three new *C. bracarensis* strains, as well as the control strains for the members of the *C. glabrata* clade, yielded no phenotypic test(s) that could distinguish these closely related species. It is interesting that all the members of the *C. glabrata* clade produced white colonies on CHROMagar Candida; these data suggest the potential utility for this medium as a screening tool for members of the *C. glabrata* clade.

The antifungal susceptibility data were particularly noteworthy. The *C. bracarensis* isolate recovered from the throat of one of the oncology patients (Cagl-121) was resistant to all triazoles tested. This patient had the longest hospital stay (95 days) of the three patients studied and had received prior AMB, VRC, and CASP treatment. Additionally, the control strain of *N. delphensis* was found to be resistant to FLC, ITC, and VRC. Finally, although the type strain that we used as our *C. nivariensis* control was susceptible to all agents tested, Fujita and colleagues previously reported pan-azole resistance in an isolate of *C. nivariensis* recovered from blood (4). These findings are clinically relevant because selection of antifungal therapy for candidiasis is often species based, and azoles are considered by many to be first-line antifungal drugs for many types of infections (2, 10). More studies will be needed to determine the extent of azole resistance in the newly described members of this clade.

In conclusion, three isolates of *C. bracarensis*, a member of the *C. glabrata* clade, were identified among isolates of *C. glabrata*. One patient in the present study had evidence of an infection, whereas the other two patients were colonized. One of the colonized patients' isolates was pan-azole resistant. Whether it is important to identify the individual species within the *C. glabrata* clade is unclear. Further data are needed to determine whether these species have clinically significant differences in antifungal susceptibilities and responses to antifungal therapy compared to *C. glabrata*.

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