Invasive Mycosis Due to Species of *Blastobotrys* in Immunocompromised Patients with Reduced Susceptibility to Antifungals

Anil Kumar,[a] Rachana Babu,[a] Swapna Bijulal,[b] Mohan Abraham,[c] P. Sasidharan,[d] Shallu Kathuria,[e] Cheshta Sharma,[e]
Jacques F. Meis,[f,g] Anuradha Chowdhary[e]

Department of Microbiology, Amrita Institute of Medical Sciences & Research Centre, Kochi, Kerala, India[a]; Department of Microbiology, Regional Cancer Centre, Thiruvananthapuram, Kerala, India[b]; Department of Pediatric Surgery, Amrita Institute of Medical Sciences, Kochi, Kerala, India[c]; Department of Neonatology, Amrita Institute of Medical Sciences, Kochi, Kerala, India[d]; Department of Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India[e]; Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands[f]; Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands[g]

Cases of invasive mycosis due to *Blastobotrys serpentis* and *B. proliferans* identified by sequencing in a preterm patient and a rhabdomyosarcoma patient, respectively, are reported. Both species revealed elevated fluconazole and echinocandin MICs by the CLSI broth microdilution method. Additionally, *B. serpentis* exhibited high amphotericin B MICs, thus posing serious therapeutic challenges.

CASE REPORTS

Case 1. A 29-week gestation preterm male infant at birth was admitted on 24 April 2013 because of respiratory distress. The infant was born by normal vaginal delivery to a 24-year-old primigravida mother. On admission at day 1, the child was not active and had tachypnoea with a respiratory rate of 68/min and his chest X-ray showed mild haziness. Examination of the cardiovascular system revealed no abnormal heart sounds and equal bilateral peripheral pulses. The abdomen was soft, with no organomegaly noted. On day 5, a loud murmur was detected and an echocardiography revealed a large patent ductus arteriosus of 2.2 mm in size which was surgically closed. His total leukocyte count was elevated (24,000 cells/μl), and his C-reactive protein level was 0.1 mg/liter. Two days later, the infant developed abdominal distension and the X-ray revealed a pneumoperitoneum. This was managed with a peritoneal drainage followed by laparotomy. During surgery, a gangrenous gastric fundus with a sloughed greater curvature, leading to an approximately 40% to 45% loss of stomach volume, was seen. The necrotic tissues were removed; gastric anastomosis was done with placement of a gastrojejunal feeding tube via gastrotomy. The child underwent operation twice again, on days 14 and 29 of admission, because of anastomotic leakage and perforation due to a gangrenous bowel. Histopathological examination of tissue biopsy specimens of the distal ileum, terminal ileum, and stoma showed active inflammatory changes, but no granuloma and ganglion cells were present, ruling out Hirschsprung disease. The child was empirically treated with ampicillin and gentamicin starting from day 1 of admission along with prophylactic fluconazole (3 mg/kg of body weight twice weekly) due to a high risk for invasive candidiasis. Further during the course of treatment, meropenem therapy was included on day 3, cefoperazone-sulbactam therapy a week later, and ofloxacin therapy on day 17. The clinical course and the therapy instituted are depicted in [Fig. 1](#). Cultures of the discharge from the laprotomy incision site on day 15 of admission grew yeasts identified as *Stephanoascus ciferrii* (identification, 86%) by the use of a Vitek2 yeast ID system (bioMérieux, Marcy l'Etoile, France) which exhibited a high flucona-

zole MIC ($>$64 μg/ml) by AST-YS06 (bioMérieux). Blood cultures in Bactec Peds Plus/F vials taken on days 16, 18, 21, 23, and 26 of admission grew fluconazole-resistant *S. ciferrii* after 2 to 3 days of incubation at 37°C. The endotracheal aspirate collected on day 23 also grew *S. ciferrii*. The patient had already received fluconazole (6 mg/kg) for 17 days; the treatment was then changed to amphotericin B (1.5 mg/kg) due to clinical deterioration. Since blood culture results continued to be positive, voriconazole (2 mg/kg twice daily) was added on day 5 to the amphotericin B therapy. However, the neonate succumbed to the complications of fungemia on day 31 of admission.

Case 2. An 8-year-girl was referred to a Regional Cancer Centre after being diagnosed with rhabdomyosarcoma. The biopsy specimen from the nasal cavity showed a malignant round-cell neoplasm consistent with rhabdomyosarcoma infiltrating the respiratory mucosa and glandular tissue. During the course of chemotherapy, a peripherally inserted central catheter (PICC) was placed for administering chemotherapeutic drugs. Two months after PICC line insertion, the patient developed fever without any localizing features. Her blood culture sent in a BacT/Alert bottle grew a *Candida* species. The isolate was misidentified as *Stephanoascus ciferrii* with a low discrimination profile by the use of a Vitek2 yeast ID system. The patient's PICC line was removed, and oral fluconazole (10 mg/kg/day) was given for 2 weeks. Her fever subsided, and she was progressing well after 6 months of follow-up.

Mycological investigations. The isolates originating from peritoneal and blood culture from case 1 (accession numbers VPCI 568/P/13 and VPCI 569/P/13) and blood culture from case
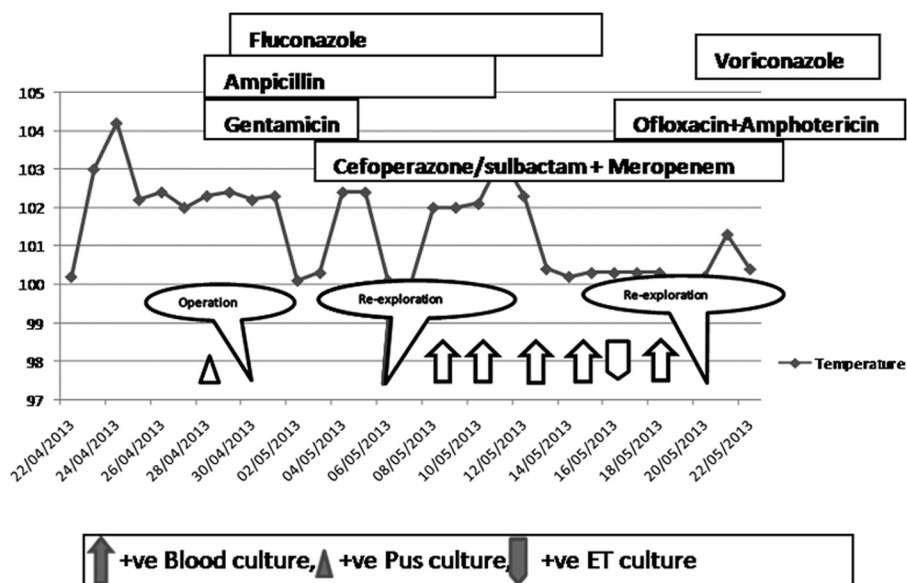
FIG 1 The drugs administered and the clinical course of a case 1 neonate with invasive mycosis due to *Blastobotrys serpentis*. +ve, positive; ET, endotracheal aspirate.

2 (accession number VPCI 573/P/13) were sent to the Mycology Reference Laboratory, V. P. Chest Institute, Delhi, India. They were identified as *Blastobotrys serpentis* and *Blastobotrys proliferans* from case 1 and case 2, respectively. A brief description of their morphological, biochemical, and molecular identification is given below.

Morphological and biochemical characteristics of *Blastobotrys* spp. (i) *Blastobotrys serpentis*. The clinical isolates (VPCI 568/P/13 and VPCI 569/P/13), along with the type strain of *B. serpentis* (CBS 10541[T]), revealed wrinkled white yeast-like colonies which showed multilateral budding and occasionally spherical yeast cells after 4 days of incubation at 28°C on potato dextrose agar (PDA). They revealed abundant, well-differentiated pseudohyphae with blastoconidia after 5 days of incubation at 28°C on rice Tween 80 agar (Fig. 2a). Septate hyphae were not observed. Also, the isolates grew in the presence of 0.01% cycloheximide and grew weakly on 50% glucose whereas the growth was inhibited on 6.5% NaCl. The carbon and nitrogen assimilation profiles of the isolates were determined by the microplate technique with yeast nitrogen base and yeast carbon base (Difco) (http://www.cbs.knaw.nl/yeast), respectively. The microtiter plates containing the medium were inoculated with 50 μl of yeast inoculum (1:10 MacFarland standard no. 2) into each well and incubated at 25°C for 3 to 5 days without agitation. The readings were taken by spectrophotometer (SpectraMax Plus; Molecular Devices) at 405 nm after shaking. Table 1 lists the assimilation profiles of *Blastobotrys serpentis* isolates. Notably, *Blastobotrys serpentis* clinical isolates, along with the type strain, grew at 37°C and 40°C.

(ii) *Blastobotrys proliferans*. The VPCI 573/P/13 isolate showed dry, hairy, white colonies on potato dextrose agar (PDA) after 4 days of incubation at 28°C. Slide cultures of 7 days growth on malt extract agar at 28°C showed conidiophores bearing pear-shaped mother cells (Fig. 2b). The mother cells were single, and each was crowned with secondary conidia (Fig. 2b). In addition, globose, lateral conidia and hyaline, thick-walled terminal and intercalary chlamydospores were observed (Fig. 2c). Like *B. ser-*

pentis, *B. proliferans* assimilated sugars such as D-glucose, D-galactose, D-xylose, maltose, etc., but failed to assimilate nitrate (Table 1). However, unlike *B. serpentis*, this species grew in the presence of melibiose. Also, the isolate revealed growth at 37°C.

***In vitro* antifungal susceptibility testing.** *In vitro* antifungal susceptibility testing of all isolates was done using the Clinical and Laboratory Standards Institute broth microdilution method, following the CLSI M27-A3 guidelines (1). The antifungals tested were amphotericin B (Sigma, St. Louis, MO), fluconazole (Pfizer, Groton, CT), itraconazole (Lee Pharma, Hyderabad, India), voriconazole (Pfizer), posaconazole (Merck, Whitehouse Station, NJ), isavuconazole (Basilea Pharmaceutica, Basel, Switzerland), 5-flucytosine (Sigma), caspofungin (Merck), micafungin (Astellas, Toyama, Japan), and anidulafungin (Pfizer). *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) were used as quality control strains. The MIC endpoints were defined as the lowest drug concentrations that caused a prominent (50%) decrease in growth vis-à-vis the controls and were read visually after 24 h for azoles and echinocandins as recently validated by Pfaller et al. (2, 3, 4). For amphotericin B, the MIC was defined as the lowest concentration at which there was 100% inhibition of growth compared with the drug-free control well results. The *in vitro* antifungal susceptibility data for both the species are presented in Table 2. *Blastobotrys serpentis* (VPCI 568/P/13 and VPCI 569/P/13) isolates exhibited high fluconazole, amphotericin B, caspofungin, micafungin, and anidulafungin MICs. They had low isavuconazole, itraconazole, voriconazole, 5-flucytosine, and posaconazole MICs. However, *B. proliferans* VPCI 573/P/13 revealed reduced susceptibility to all the antifungals tested except amphotericin B (MIC, 0.125 μg/ml).

Sequencing of ITS and D1/D2 regions. Genomic DNA was extracted from all of the test yeast isolates as described by Xu et al. (5). Extracted DNA was amplified using the ITS-1 (5′-TCCGTA GGTGAACCTTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTG ATATGC-3′) primers, which amplify the internal transcribed spacer (ITS) region of the ribosomal subunit, and the NL-1 (5′-G
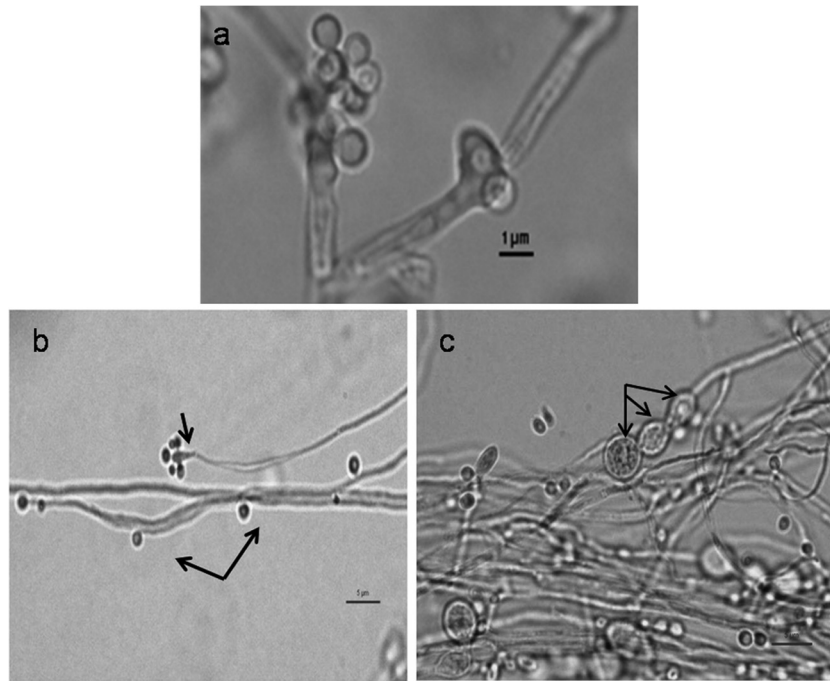
FIG 2 (a) Slide cultures on malt extract agar of 5 days growth at 28°C of the *Blastobotrys serpentis* (VPCI 568/P/13) isolate collected from blood culture of case 1, showing well-differentiated pseudohyphae with abundant blastoconidia. Septate hyphae were not observed. Magnification, ×1,000. (b) Slide cultures on malt extract agar of 7 days growth at 28°C of the *Blastobotrys proliferans* (VPCI 573/P/13) isolate collected from blood culture of case 2, showing conidiophores bearing pear-shaped mother cells and crowned with secondary conidia (downward arrow) and lateral conidia (upward arrows). Magnification, ×400. (c) Thick-walled, terminal, and intercalary chlamydospores (arrows). Magnification, ×1,000.

CATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCC GTGTTTCAAGACGG-3′) primers, which amplify the ~600-bp D1/D2 region of the large ribosomal subunit (6, 7). Amplified DNA was sequenced in both strands on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA) using a BigDye Terminator kit, v3.1 (Applied Biosystems). Sequences were aligned using Sequencing Analysis 5.3.1 software (Applied Biosystems). GenBank basic local alignment search tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) were performed for species identification. ITS and D1/D2 region sequences of the two isolates VPCI 568/P/13 and VPCI 569/P/13 from case 1 showed 99% homology (query coverage, 99%) with *Candida* sp. strain YS W113A (GenBank accession no. AM410670 [ITS] and AM410667 [D1/D2]), which was named species nov. *Blastobotrys serpentis* in 2008 (8). However, the ITS and D1/D2 region sequences of isolate VPCI 573/P/13 showed 99% homology (query coverage, 99%) with those of *Blastobotrys proliferans* CBS 522.75[T] (accession no. EU343812 [ITS] and DQ442684 [D1/D2]).

For phylogenetic analyses, the D1/D2 region sequences of the type and reference strains of *B. serpentis* CBS 10541[T] and W113B and *B. proliferans* CBS 522.75[T], along with those of *B. chiroptero-rum* CBS 6064[T] and *Trichomonascus ciferrii* CBS 5295 (synonym *Stephanoascus ciferrii*), were retrieved from the NCBI database. The sequences of the test and reference isolates were aligned using the ClustalW program (version 1.82) (9), and the final alignments were edited manually. A maximum-likelihood tree based on D1/D2 gene sequences using 2,000 bootstrap replications was constructed using MEGA version 5 (10). The phylogenetic tree enabled the differentiation of *B. serpentis* and *B. proliferans* from each other and from the closely related species *B. chiroperorum*

with a good bootstrap support value (99%). *Trichomonascus ciferrii* was also classified apart from clusters of both of the species (Fig. 3).

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Fungal infections due to species of *Blastobotrys* are extremely rare, and a solitary case of peritonitis due to *B. proliferans* in a patient undergoing continuous ambulatory peritoneal dialysis is on record (11). Recently, the role of *B. rhaffinosifermentans* causing a significant airway inflammation in a cystic fibrosis patient before lung transplant and pleural space infection post-lung transplant was highlighted (12). The genus *Blastobotrys* von Klo-potek (1967) was originally treated as a hyphomycete, and it was believed to be closely related to the genus *Sporothrix*, from which it was distinguished on the basis of morphological criteria (13). On the basis of the D1/D2 large-subunit (LSU) rRNA gene sequences, Kurtzman and Robnett placed the genus *Blastobotrys* in the saccharomycetales and suggested that *Blastobotrys*, *Arxula*, and *Sympodiomyces* may represent the same anamorphic genus (14). Later, this suggestion was confirmed using multilocus analysis and the species *Arxula* and *Sympodiomyces* were therefore transferred to the *Blastobotrys* genus (teleomorph *Trichomonas-cus*) (14). The two species of *Blastobotrys* reported here, *viz.*, *B. serpentis* and *B. proliferans*, were initially misidentified as *Stephanoascus ciferrii* by the use of a Vitek2 system. Kurtzman and Robnett, by using multigene analysis in 2007, showed that *Stephanoascus ciferrii* (synonym *Candida ciferrii*) is in the *Trichomonascus* clade, and the genus *Stephanoascus* became a synonym of *Trichomonascus* (14). However, this taxonomic yeast nomencla-

TABLE 1 Assimilation profile and growth characteristics of *Blastobotrys serpentis* and *B. proliferans*

Characteristic or condition	Result[a]	
	B. serpentis (n = 2 sources [peritoneal wound swab, blood])	*B. proliferans* (n = 1 source [blood])
Carbon assimilation		
D-Glucose	+	+
D-Galactose	+	+
L-Sorbose	+	+
N-Acetyl-glucosamine	+	+
D-Ribose	+	−
D-Xylose	+	+
L-Arabinose	+	+
L-Rhamnose	+	+
Sucrose	+	+
D-Maltose	+	+
D-Trehalose	+	+
D-Cellobiose	+	+
D-Melibiose	−	+
D-Mannose	+	+
D-Melezitose	−	−
Lactose	−	±
D-Sorbitol	+	+
Raffinose	+	+
Gentiobiose	+	+
Palatinose	+	+
D-Melezitose	−	−
Glycerol	−	−
Erythritol	+	+
Xylitol	+	+
Inositol	+	+
D-Gluconate	+	+
L-Lactate	+	+
Citrate	−	−
L-Malate	+	+
D-Galactouronate	+	+
Acetate	+	+
Urease	−	+
Glucuronate	+	+
2-Keto-D-gluconate	+	
Nitrogen assimilation		−
Nitrate	−	−
L-Lysine	−	+
Leucine	+	+
Arginine	+	±
Tyrosine	−	+
L-Proline	+	
Growth under different conditions		+
37°C	+	+
40°C	+	+
0.01% cycloheximide	+	−
0.1% cycloheximide	−	W^c
50% glucose	W	−
6.5% NaCl	−	

[a] +, growth; −, no growth; W, weak growth.

TABLE 2 *In vitro* antifungal susceptibility profiles of the *Blastobotrys* species isolates

Species identified	MIC (µg/ml)[a]									
	AMB	FLU	ITC	VRC	ISA	POS	CAS	MFG	AFG	5-FC
B. serpentis VPCI 568/P/13	2	32	0.25	0.5	0.25	0.5	1	0.25	0.5	0.25
B. serpentis VPCI 569/P/13	1	32	0.25	0.25	0.125	0.25	1	0.25	0.5	0.5
B. proliferans VPCI 573/P/13	0.125	64	1	4	4	2	4	1	4	32

[a] Abbreviations used: AMB, amphotericin B; FLU, fluconazole; ITC, itraconazole; VRC, voriconazole; ISA, isavuconazole; POS, posaconazole; CAS, caspofungin; MFG, micafungin; AFG, anidulafungin; 5-FC, flucytosine.
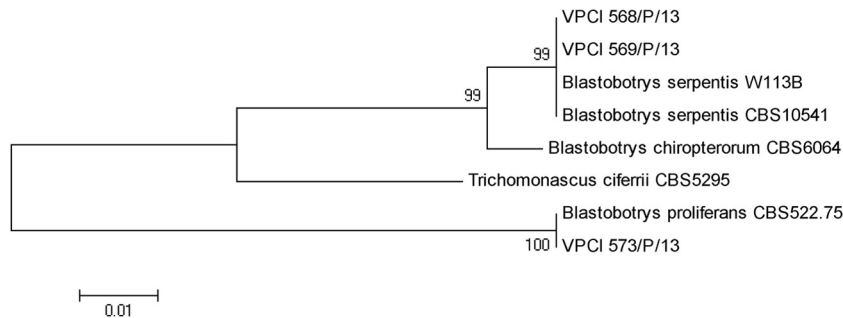
ture change has clearly not been updated in the Vitek2 system database.

Case 1 reported here involved a severely immunocompromised neonate who had undergone multiple surgeries due to in-

FIG 3 Phylogenetic tree based on D1/D2 sequences of the type/reference strains of *B. serpentis* CBS 10541^T and W113B, *B. proliferans* CBS 522.75^T, *B. chiropterorum* CBS 6064^T, and *Trichomonascus ciferrii* CBS 5295, obtained by using maximum-likelihood phylogenetic analyses with 2,000 bootstrap replications.

testinal perforation and developed persistent fungemia due to *B. serpentis*, which was isolated from multiple blood cultures, peritoneal drainage, and endotracheal aspirate. *Blastobotrys serpentis* has never previously been reported as an etiologic agent of invasive mycoses in humans. However, the species was first described in 2008 as a novel member of genus *Blastobotrys* isolated from the intestine of a dead trinket snake in India (8). Notably, both species of *Blastobotrys* reported here, i.e., *B. proliferans* and *B. serpentis*, had reduced susceptibility to commonly used antifungals, i.e., fluconazole and caspofungin. Additionally, *B. serpentis* isolates had relatively high anidulafungin (0.5 μg/ml) and micafungin (0.25 μg/ml) MICs, considering that the recent epidemiological cutoff values for 5 *Candida* species, barring *C. parapsilosis*, for both the echinocandins range from 0.03 to 0.25 μg/ml (4). Furthermore, *B. serpentis* also exhibited high amphotericin B MICs, thus raising serious concerns about the therapeutic options for patients with *Blastobotrys* infections. Due to the rarity of clinical cases of infection by *Blastobotrys*, there is paucity of information on the susceptibility data of this genus. In the present report of case 1, the newborn developed breakthrough fungemia due to *B. serpentis* while he was on fluconazole prophylaxis and could not be successfully treated with amphotericin B, for which the isolate had high MICs. The neonate had all the risk factors reported for development of candidemia, including prematurity, prolonged antibiotic treatment, total parenteral nutrition, mechanical ventilation, and an extended stay in the neonatal intensive care unit (15). Case 2 with fungemia due to *B. proliferans* probably responded to removal of the PICC line. Although the patient was also treated with fluconazole, the isolate showed a high (64 μg/ml) fluconazole MIC. Previously, Quirin et al. reported that *B. proliferans* isolated from a case of peritonitis showed decreased susceptibility to all antifungals tested, except amphotericin B (11). Similarly to our experience, the removal of the Tenckhoff catheter and administration of oral fluconazole resulted in a successful outcome in the case reported by Quirin et al. (11). Notably, *B. rhaffinosifermentans* also had reduced susceptibility to fluconazole in a recently reported case (12). The patient was treated successfully with aggressive antifungal therapy, including multiple courses of intravenous caspofungin, oral voriconazole, and nebulized L-amphotericin B extending for 22 months (12). The sources of both *Blastobotrys* species in the present cases remain enigmatic, especially in the newborn, who possibly acquired the infection while in the hospital, whereas in case 2, the child may have obtained the fungus from her surroundings, as *B. proliferans* has been previously isolated from garden soil and house dust (13). However, *B. serpentis* has so

far been reported to have been isolated only from the gut of a dead snake in a zoo in India, suggesting that the yeasts of the genus *Blastobotrys* may be acquired by humans and animals from the environment (8).

Of the 21 species of *Blastobotrys* previously described in a taxonomic study of yeasts (13), 12 were reported to grow at 37°C, and these three species, namely, *B. proliferans*, *B. chiropterorum*, and *B. rhaffinosifermentans*, have been isolated from humans (11–13, 16). Notably, a report in the latest edition of a monograph describing yeasts (13) asserting that growth of the *B. serpentis* type strain (CBS 10541) is negative at 37°C is erroneous, as it, along with the two clinical strains (VPCI 568/P/13 and VPCI 569/P/13), showed luxuriant growth at 37°C in the present study. Furthermore, species of the genus *Blastobotrys* reveal diverse morphological and biochemical characteristics and are therefore difficult to identify by standard mycological procedures in routine laboratories. Additionally, they exhibit reduced susceptibility to antifungals. Therefore, any significant clinical yeast isolate originating from sterile sites which is identified as *Stephanoascus ciferrii* by the Vitek2 system should be evaluated further by molecular techniques and *in vitro* antifungal susceptibility testing should be performed. Finally, the emergence of *Blastobotrys* spp. as opportunistic fungal pathogens capable of causing invasive disease, with reduced susceptibility to antifungals, is noteworthy, as it poses serious diagnostic and therapeutic challenges.

Nucleotide sequence accession numbers. VPCI 568/P/13, VPCI 569/P/13, and VPCI 573/P/13 ITS sequences were submitted to GenBank with accession numbers KM056339, KM056340, and KM056341, respectively, and LSU sequences with accession numbers KM056336, KM056337, and KM056338, respectively. The 2 strains *B. serpentis* VPCI 568/P/13 and *B. proliferans* VPCI 573/P/13 were deposited in the CBS-KNAW Fungal Biodiversity Centre, The Netherlands, with accession numbers CBS 13871 and CBS 13872, respectively.

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