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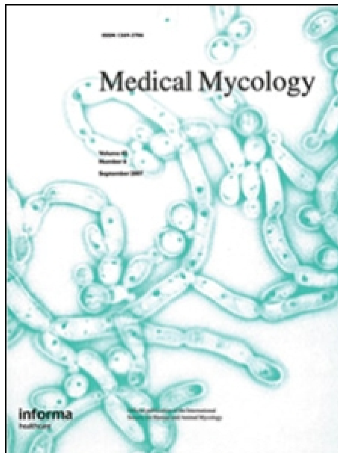

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Identification of a new species, Candida subhashii, as a cause of peritonitis

Heather Adam [a]; Marizeth Groenewald [b]; Subhash Mohan [c]; Susan Richardson [ad]; Ursula Bunn [e]; Connie Fe C. Gibas [f]; Susan Poutanen [ac]; Lynne Sigler [f]
[a] Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario [b] Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Netherlands [c] Department of Microbiology, Mount Sinai Hospital, [d] Department of Paediatric Laboratory Medicine, Hospital for Sick Children, Toronto, Ontario [e] Mycology Section, Laboratories Branch, Ministry of Health and Long Term Care, Toronto, Ontario [f] University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada

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Case Reports

Identification of a new species, *Candida subhashii*, as a cause of peritonitis

HEATHER ADAM*, MARIZETH GROENEWALD†, SUBHASH MOHAN‡, SUSAN RICHARDSON*§, URSULA BUNN+, CONNIE FE C. GIBAS$, SUSAN POUTANEN*‡ & LYNNE SIGLER$

**Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario, †Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Netherlands, ‡Department of Microbiology, Mount Sinai Hospital, §Department of Paediatric Laboratory Medicine, Hospital for Sick Children, Toronto, Ontario, +Mycology Section, Laboratories Branch, Ministry of Health and Long Term Care, Toronto, Ontario, and $University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada*

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We report a case of fungal peritonitis from which a novel *Candida* species was isolated. Phylogenetic analysis of DNA sequences from the internal transcribed spacer (ITS) region and the D1/D2 domains of the large subunit (LSU) rRNA gene show that the *Candida* species is distinct from, but related to, the human pathogenic species, *C. parapsilosis, C. orthopsilosis, C. metapsilosis, C. tropicalis, C. albicans,* and *C. dubliniensis*. *Candida subhashii* M. Groenewald, Sigler et Richardson sp. nov. is described.

Keywords *Candida subhashii*, fungal peritonitis, diabetes mellitus, peritoneal dialysis, molecular, ITS, D1/D2

Introduction

Candida species are the most common cause of fungal peritonitis, accounting for 43–89% of cases [1–6]. *Candida albicans* is frequently reported as the primary *Candida* species [2,7], but recent studies highlight that other *Candida* species are increasingly identified [1,8,9]. We report the identification of a novel *Candida* species isolated from a case of fungal peritonitis.

Case report

A 69-year-old man with end-stage renal failure developed fungal peritonitis in March 2006. He had a past history of diabetes mellitus since the late 1990s, complicated by retinopathy and coronary artery disease. In 2002, progressive renal insufficiency was noted with a rise in his creatinine from 591 to 741 μmol/l over two months. He was treated with peritoneal dialysis for progressive renal insufficiency in April of 2002. The patient continued on peritoneal dialysis for four years with few complications. During this time, he had only one episode of peritonitis from which a coagulase-negative *Staphylococcus* species was isolated in May of 2005.

On 22 March 2006, the patient was admitted with complaints of abdominal pain, nausea, vomiting, diarrhea and a history of cloudy dialysate. He was hemodynamically stable and afebrile, but tachycardic. Laboratory investigations of his peritoneal dialysis effluent revealed a white blood cell count of 3.5×10^9/l (92% neutrophils) and culture inoculated with the effluent yielded a *Candida* species other than *C. albicans*. The peritoneal dialysis catheter was removed on 25 March 2006 and the patient was begun on fluconazole and switched to hemodialysis. Culture of the peritoneal dialysis catheter grew only an *Enterococcus* species. Blood cultures were negative. The patient received a three week course of fluconazole

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Correspondence: Heather Adam, Department of Microbiology, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada. Fax: +1 416 586 8746; E-mail: hjadam@gmail.com

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and a two week course of intravenous ampicillin stepped down to oral amoxicillin, with resolution of his clinical symptoms. Upon completion of therapy, he remained on hemodialysis.

In May of 2006, a follow-up CT scan demonstrated persistent fluid collections in the abdominal and suprapubic area. The patient was seen for pulmonary edema in June, but did not require hospital admission. He developed a urinary tract infection in July from which an unidentified yeast was isolated. Due to persistence of the abdominal and suprapubic collections they were drained in September 2006. Cultures of both collections were negative. A peritoneal dialysis catheter was re-inserted on 22 September 2006 and peritoneal dialysis was re-started. The patient has since remained well, without episodes of peritonitis.

From the March 2006 peritonitis episode described above, three specimens of peritoneal dialysis effluent and eight blood cultures were received in the microbiology laboratory over the course of a week. Following laboratory protocol, the peritoneal dialysis fluid was inoculated onto 5% sheep blood agar (Oxoid, Neapean, Ontario), chocolate agar (Oxoid), and MacConkey agar (Oxoid) and incubated in 5% CO_2 at 35°C for 4 days. The blood cultures were inoculated into BacT/Alert blood culture bottles and incubated for 5 days in the BacT/Alert system (bioMerieux, St. Laurent, Quebec). A *Candida* species was isolated from all peritoneal dialysis fluid samples, but the blood cultures were negative. The identification work-up involved the assessment of colonial features on Sabouraud modified agar (BD Biosciences, Mississauga, Ontario) and BBL CHROMagar Candida (Becton, Dickinson, and Company Canada, Oakville, Ontario), micro-morphological features on cornmeal tween 80 agar (Oxoid) after a 48 h incubation at 28°C, germ tube development in bovine serum incubated for less than 3 h at 35°C, urease detection on urea agar slants (Invitrogen, Burlington, Ontario), and the API 20C AUX profile (bioMerieux) provided after a 72 h incubation at 30°C. The colonial morphology on the Sabouraud modified agar was nonspecific appearing creamy-white as in other *Candida* species. On the BBL CHROMagar, the colonies were colourless (white). The organism was germ tube and urease negative. The morphology on cornmeal tween 80 agar displayed features most closely resembling *C. tropicalis* in producing long pseudohyphae with sparse blastoconidia (Fig. 1). The API 20C AUX was tested in triplicate, but the assimilation patterns (6756374, 2756375, 2766175) were not consistent and did not match to any profile within the database. Unsatisfactory profiles included 70.8% *C. guilliermondii* / 10.4% *C. tropicalis*, 79% *C. tropicalis* / 9.6% *C. lusitaniae*, and 68% *C. parapsilosis* / 26.5% *C. albicans*. As the isolate was unable to be identified, it was referred for reference identification, including physiological testing and molecular analysis.

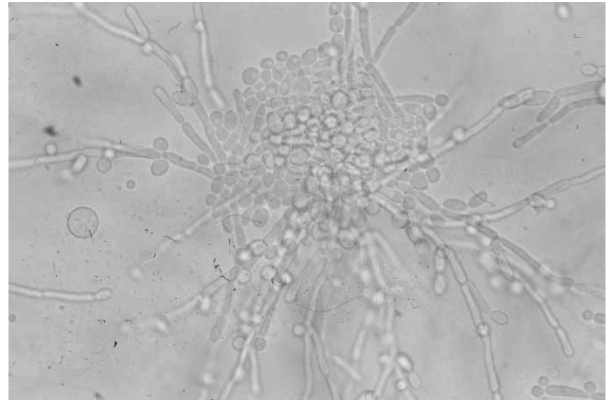

Fig. 1 Appearance of *Candida* species on cornmeal tween 80 agar (400× magnification).

Isolate characterization and sequencing

The isolate (FR 392) was deposited in the University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada as UAMH 10744 and in the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands as CBS 10753. For physiological testing, both conventional fermentation and assimilation tests in liquid media and elaborated assimilation tests were used. The conventional tests performed at the Laboratories Branch, Ministry of Health and Long Term Care, Toronto, Ontario included carbohydrates typically employed in Wickerham testing [10]. Elaborated tests evaluated utilization of 68 compounds in a microtiter plate incubated for 4 days at 25°C following standard methodologies (www.cbs.knaw.nl/yeast/). These tests included many compounds supplementary to those provided in reference books [11].

DNA extraction, PCR reaction parameters and sequencing protocols were completed as described previously with minor modification [12]. A heavy suspension of yeast cells in 2 ml of sterile distilled water was centrifuged and DNA extracted using the UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, California). The internal transcribed spacers and 5.8S rRNA gene (ITS) as well as the 28S rRNA gene (LSU D1/D2) were amplified using primers BMBC-R [13] and LR7 (http://www.biology.duke.edu/fungi/mycolab/primers.htm). PCR reactions were subjected to 30 cycles on a Perkin Elmer GeneAmp 9700 Thermal Cycler (Applied Biosystems, Foster, California). Sequencing of the ITS fragment

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was done with primers BMBC-R, ITS1, ITS2 and ITS4, and sequencing of the LSU region was done with primers LR5, LR16, LR0R, and LR3R using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and run on an ABI 377 Prism Automated Sequencer (Applied Biosystems). BLASTn searches [14] with sequences in the GenBank and in the CBS Yeast Database (online at http://www.cbs.knaw.nl/yeast/BioloMICS.aspx) were performed three times (November 2006; August 2007; May 2008). ITS and partial LSU sequences were assembled for comparison, aligned and analyzed as described by Groenewald *et al.* [15]. Sequences included in the analysis were from ex-type cultures of closest species according to BLAST results and for which both LSU and ITS sequences were available. Maximum parsimony analyses were done in PAUP (phylogenetic analysis using parsimony) 4.0b10 [16]. The robustness of the trees obtained was evaluated by 100 bootstrap replications [17]. Other measures calculated included tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC). The resulting trees were printed with TreeView v1.6.6 [18]. Sequences were deposited in GenBank as EU836707 (ITS) and EU836708 (LSU) and the alignment and trees in TreeBASE (http://www.treebase.org/treebase).

Susceptibility testing

Antifungal susceptibility testing was conducted by the broth dilution method in accordance with the National Committee on Clinical Laboratory Standards guidelines [19] and included amphotericin B, fluconazole, ketoconazole, itraconazole, and voriconazole.

Results

Physiological data in liquid media were recorded after 7, 14 and 21 days of incubation at 28°C. The isolate failed to ferment any carbohydrate. The assimilation results are listed in Table 1. The isolate grew on vitamin-free medium, peptone medium, in broth producing a sediment and on Sabouraud dextrose agar with chloramphenicol, cycloheximide and gentamicin. The isolate grew at 25°C, 37°C, and 40°C, but not at 42°C and was urease negative. The results of physiological tests performed in microtiter plates are listed also in Table 1 and are available online under the information of strain CBS 10753 (http://www.cbs.knaw.nl/yeast/BioloMICS.aspx). There are a few differences in substrate utilization that may be attributed to length of incubation time and temperature of incubation.

Table 1 Assimilation of carbon compounds by *Candida subhashii*

Assimilation of	25°C[a]	28°C[b]
D-Glucose	+	+
D-Galactose	+	+
L-Sorbose	−	+
D-Glucosamine	−	ND
D-Ribose	−	ND
D-Xylose	w	+
L-Arabinose	w	ND
D-Arabinose	−	ND
L-Rhamnose	−	−
Sucrose	+	+
Maltose	+	+
α,α-Trehalose	−	+
Me α-d-Glucoside	w	ND
Cellobiose	+	+
Salicin	w	ND
Arbutin	w	ND
Melibiose	−	−
Lactose	−	−
Raffinose	−	−
Melezitose	+	+
Inulin	−	ND
Starch	−	ND
Glycerol	−	ND
Erythritol	−	−
Ribitol	w	ND
Xylitol	−	ND
l-Arabinitol	−	ND
d-Glucitol	−	ND
d-Mannitol	−	+
Galactitol	−	ND
myo-Inositol	−	−
d-Glucono-1,5-lactone	−	ND
5-Keto-D-Gluconate	−	ND
d-Gluconate	−	ND
d-Glucuronate	−	ND
D-Galacturonate	−	ND
DL-Lactate	−	ND
Succinate	−	ND
Citrate	−	ND
Propane 1,2 diol	−	ND
Butane 2,3 diol	−	ND
Quinic acid	−	ND
D-glucarate	−	ND
D-Galactonate	−	ND
Palatinose	+	ND
Levulinate	−	ND
L-Malic acid	−	ND
L-Tartaric acid	−	ND
D-Tartaric acid	−	ND
meso-Tartaric acid	−	ND
Galactaric acid	−	ND
Uric acid	−	ND
Gentobiose	−	ND
Ethylene glycol	−	ND
Tween 40	w	ND
Tween 60	−	ND
Tween 80	−	ND
Nitrate	−	ND
Nitrite	−	ND
Ethylamine	w	ND

Table 1 (*Continued*)

Assimilation of	25°C[a]	28°C[b]
L-Lysine	+	ND
Cadaverine	w	ND
Creatine	–	ND
Creatinine	–	ND
Glucosamine	–	ND
Imidazole	–	ND
D-Tryptophan	–	ND
D-Proline	–	ND

[a]Elaborated physiological tests done on a microtiter plate recorded after 4 days at 25°C.
[b]Classical physiological tests recorded after 21 days at 28°C.
–, Negative; +, positive; w, weak.
ND, not determined.

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The minimum inhibitory concentrations determined by antifungal susceptibility testing were: amphotericin B, 0.25 mg/l; fluconazole, 1 mg/l; ketoconazole, 0.03 mg/l; itraconazole, 0.25 mg/l; voriconazole, 0.03 mg/l.

BLASTn searches using ITS and partial LSU sequences yielded no match to sequences of any known yeast species in either the GenBank or CBS databases. Sequence similarity with nearest *Candida* species was not very high. The LSU sequence was 91% similar to that of *C. orthopsilosis* (EU564208) and the ITS sequence was 82% similar to that of *C. tropicalis* (U45749). The ITS and LSU alignments contained sequences of 13 strains, including the outgroup. The ITS had a total length of 558 characters, of which 243 were constant, 112 were parsimony uninformative and 203 were parsimony informative. Parsimony analysis of the ITS data resulted in a single parsimonious tree (TL 727 steps; CI 0.729; RI 0.631; RC 0.460) (Fig. 2). The LSU had a total length of 570 characters, of which 378 were constant, 60 were parsimony uninformative and 132 were parsimony informative. Parsimony analysis of the LSU data resulted in a single parsimonious tree (TL 398 steps; CI 0.656; RI 0.609; RC 0.399) (Fig. 2). The phylogenetic analyses grouped the peritoneal isolate together with *C. parapsilosis, C. orthopsilosis, C. metapsilosis, C. tropicalis, C. albicans*, and *C. dubliniensis* with bootstrap support values of 89% (ITS) and 73% (LSU) (Fig. 2). Similar topologies were found for the ITS and LSU trees using neighbor-joining method (data not shown). On the basis of these results, a new species of *Candida* is proposed.

Description of Candida subhashii M. Groenewald, Sigler et Richardson sp. nov.

Latin diagnosis: *Coloniae in agaro maltoso post* 7 *dies* 25°C 10–25 *mm diametro, cultura cremea pallida, laevis, convexa, teres margine. Cellulae* 3.0–6.0 × 3.1–6.1 μm, *globosae, ovoideae, ellipsoideae, citriformes, leviter, allantoideae vel irregulares, singulae vel binae. Conidiophora brevia, sympodialiter proliferentia, numerosas, gemmarum cicatrices ferentia, rhachidis similia, conidia singula vel plura ferentia. Pseudohyphae parcae, hyphae verae non observatae. Ascosporae, ballistosporae, arthroconidia et teliosporae absentes. Charactera physiologica in Table 1. Ad crescentiae vitaminae non necessariae sunt. Urea non finditur. Augmentum ad* 25°C et 40°C. *Typus: stirps* CBS 10753^T^ (=UAMH 10744^T^) *isolatus ex humanus masculin patiens. Holotypus lyophilus conservatur in collectione culturarum* Centraalbureau voor Schimmelcultures, *Trajectum ad Rhenum*. Etymology: *subhashii* in honor of Subhash Mohan for contributions to clinical laboratory mycology in Canada.

Characteristics of *Candida subhashii* sp. nov. After 7 days on malt extract agar (Difco-BD, France) at 25°C, colonies are 10–25 mm in diameter, pale cream coloured, smooth, convex, with a glistening surface, a smooth margin and soft texture. Budding is mainly unipolar or bipolar. Budding cells 3.0–6.0 × 3.1–6.1 μm are globose to ovoid (Fig. 3a). Conidiophores are 1.8–3.5 μm in diameter and the distance between septa ranges from 22.5 and 29.7 μm. Blastoconidia are produced on a sympodially proliferating apex that, at maturity, shows a rachis-like structure with numerous slightly denticulate scars left after the dehiscence of conidia (Fig. 3b). Conidia are more irregular in shape than budding cells and can be globose, ovoid to elongated. Pseudomycelium is present but not abundant. Ascospores and arthroconidia were not observed. The type strain, CBS 10753^T^ (=UAMH 10744^T^) has been isolated from peritoneal dialysis fluid from a 69-year-old man with end-stage renal failure. Mycobank number MB 512099.

Discussion

Fungal peritonitis is a rare but serious complication of peritoneal dialysis and is associated with significant morbidity and mortality. Of all peritonitis episodes, fungal causes are reported in 3–6% of cases [1,3–7]. Forty to 100% of cases require catheter removal and conversion to hemodialysis [1,7,20]. The associated mortality rate is 5–25% [1,7,21]. The International Society for Peritoneal Dialysis (ISPD) recommends prompt catheter removal following laboratory confirmation of fungal peritonitis as it may reduce mortality [21]. The ISPD recommends 4 weeks of antifungal therapy, or treatment for at least 10 days after catheter removal, for peritonitis. In our case, peritonitis resolved after prompt catheter removal and therapy with fluconazole for three weeks. The organism was subsequently

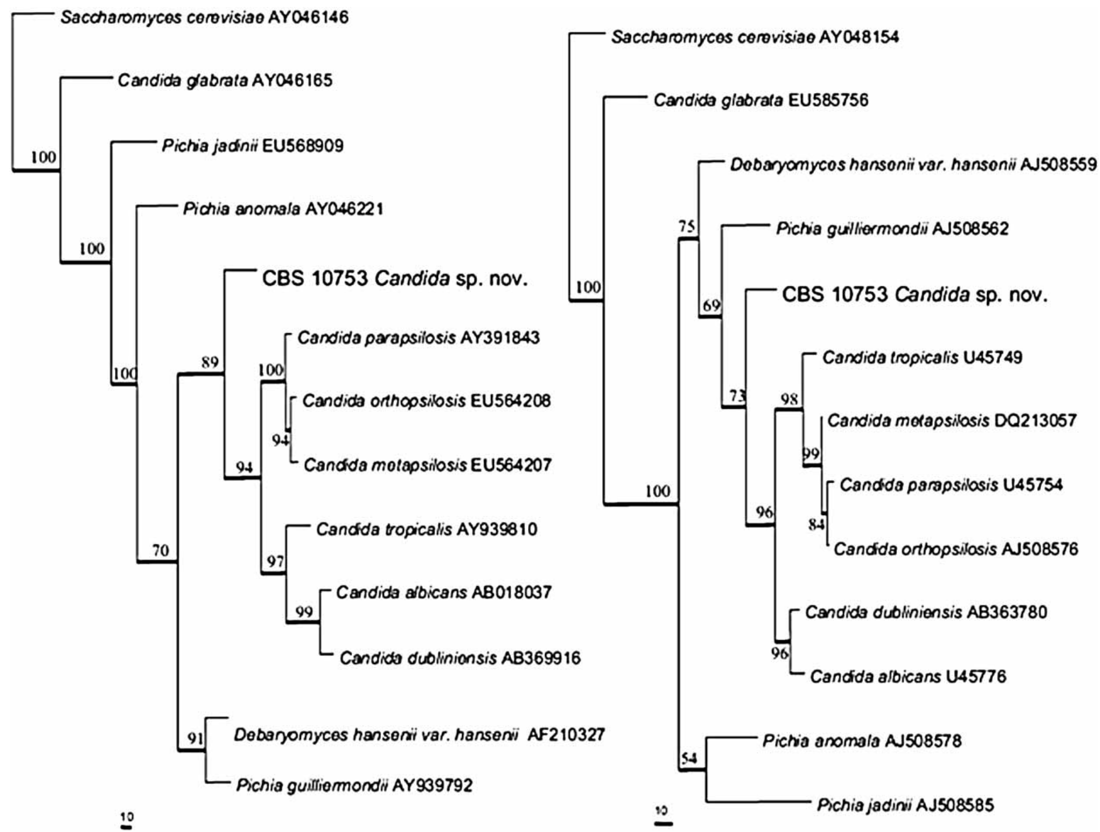

Fig. 2 A single most parsimonious tree obtained from a heuristic search with 100 random taxon additions of the ITS (left) and LSU (right). The scale bar shows ten changes and bootstrap support values from 100 replicates are shown at the nodes. The trees were rooted to *Saccharomyces cerevisiae*. Sequences for strain CBS 10753 are deposited as EU836707 (ITS) and EU836708 (LSU).

determined to be susceptible to fluconazole and all other agents tested.

Many studies have attempted to identify risk factors associated with fungal peritonitis; however, most potential risk factors remain highly controversial. The majority of studies have identified previous bacterial peritonitis episodes and recent antibiotic treatment as important risk factors [1,4,5,7,9,20]. Additionally, increased length of time on peritoneal dialysis has been associated with drop-out due to fungal peritonitis [3]. A

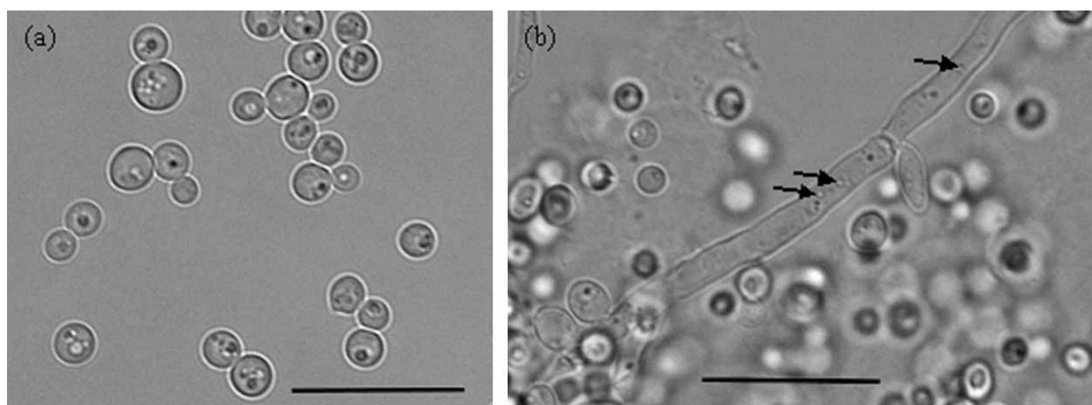

Fig. 3 (a) Vegetative cells of *Candida subhashii* CBS 10753, (b) conidia and conidiophores with scars, see arrows. Growth on malt extract agar at 25°C for 1 week. Bars, 10 μm.

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history of diabetes mellitus, as in our patient, has been suggested as a risk factor [20], but a significant association has not been confirmed [2,4,7].

Isolation of a novel *Candida* species from this case of peritonitis is a highly unusual finding. The morphological features in Dalmau conditions were most suggestive of *C. tropicalis*, which has been identified as a common species causing non-*C. albicans* peritonitis in some reports [6–8]. The organism could not be matched to any known *Candida* species by physiological or molecular characteristics. BLASTn results (date May 2008) together with the phylogenetic position of the newly identified *Candida* species show that the closest related sister taxa are the human pathogenic species, *C. parapsilosis, C. orthopsilosis, C. metapsilosis, C. tropicalis, C. albicans*, and *C. dubliniensis* [Fig. 2]. This correlates with the API 20C AUX profiles that identified *C. guillermondii*, *C. albicans, C. tropicalis*, and *C. parapsilosis* as the closest matches among species that can be tested with this system.*Candida subhashii* differs from all these species in being non-fermentative, but additional isolates need to be tested to determine the reliability of physiological data in distinguishing the species. The topologies of the trees obtained from both the ITS and LSU data compare well with the ITS tree obtained by Tavanti *et al*. [22] who suggested that *C. parapsilosis* group II and III be described as *C. orthopsilosis* and *C. metapsilosis,* respectively. It is clear that molecular data are invaluable in distinguishing novel yeast pathogens that may not be well recognized by conventional tests alone. Phylogenetic analyses as well as the data obtained with the API 20C AUX system confirm that *C. subhashii* has not been described before and that it is indeed a novel species isolated from human specimens. Although *C. subhashii* is thus far represented by only a single strain, it is desirable to provide a name to facilitate the discovery of additional strains and to further elucidate the phenotypic properties, potential virulence and drug susceptibilities of this new species.

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