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Fungemia in a cancer patient caused by fluconazole-resistant *Cryptococcus laurentii*

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We report the recent isolation of *Cryptococcus laurentii* from the blood of a patient given the diagnosis of ganglioneuroblastoma. The organism was identified using physiological and molecular characteristics, including morphology, carbohydrate and nitrate assimilation, urease activity, inability to form melanin on appropriate media, positive staining with diazonium blue B and sequence analysis of the D1/D2 domain of 26S ribosomal DNA. The isolate was resistant to fluconazole and 5-fluorocytosine using both the Etest and a broth microdilution assay. Repeated recovery of the organism from different blood cultures, and the patient's good response to treatment with amphotericin B support its etiological role. *C. laurentii* has rarely been implicated as a cause of clinically significant infections. The identity of reported isolates has not always been adequately documented, and some appear to have been isolated from lesions caused by *Cryptococcus neoformans*, emphasizing the true rarity of disease due to this fungus.

Keywords antifungal(s), *Cryptococcus laurentii*, cryptococcosis, fungemia

Introduction

Cryptococcosis was rare until the last decade. However, its medical importance increased dramatically as a consequence of the AIDS epidemic [1–3]. The major risk factors in non-AIDS patients are lymphoproliferative disorders, corticosteroid therapy, sarcoidosis, and organ transplantation [2–4]. Infections caused by non-*Cryptococcus neoformans* cryptococci have been reported rarely in humans in recent years, although reports of cases of infections due to *Cryptococcus laurentii* have been increasing [5–9].

We report the isolation of *C. laurentii* from the blood of a patient with a solid tumor. In addition, we review the literature of infections caused by this rare pathogen and discuss the problem of the emergence of fungi resistant to antimycotic agents. Finally, we discuss the salient characteristics of this yeast and the factors contributing to its appropriate classification.

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Material and methods

Case report

A 16-year-old male given the diagnosis in 1998 of ganglioneuroblastoma presented with a large abdominal tumor and metastases to the skull, right humerus, ribs, pelvis and both femurs. He received ten courses of chemotherapy through a Hickman catheter, with partial response. One month after the last course of chemotherapy he was admitted to hospital for elective resection of the abdominal mass. On admission, the physical examination was unremarkable except for the presence of a large abdominal mass and hepatomegaly. Laboratory tests revealed a peripheral leukocyte (WBC) count of 6000 mm^{-3} with 70% granulocytes, hemoglobin 13.7 g%, and platelet count 378 000 mm^{-3}. One day after admission he developed fever (38.2°C), which lasted for several hours. Antibiotic treatment with cefuroxime and gentamicin was initiated. Blood cultures from two different blood samples, obtained at the onset of fever, yielded a pure culture of yeast that was later identified as *C. laurentii.* No other pathogens were isolated. The fever subsided and the antibiotic treatment was discontinued. One week later, pyrexia (39°C) recurred, with chills and hypotensive episodes that lasted for several days, in spite

of reinstitution of the antibiotic therapy. In addition, leukopenia of 2500 mm^{-3} WBC, with 47% granulocytes, was observed. Therefore, the Hickman catheter was removed and treatment with amphotericin B (AmB) (0.5 mg kg^{-1} day^{-1}) was administered for three weeks. The fever subsided and WBC count gradually increased to normal levels. After recovering from this infectious episode, the patient underwent the planned laparotomy for resection of his tumor.

Although *C. laurentii* was not isolated again from repeated blood cultures, it was suspected to be the most likely cause of the infectious episode as it was isolated from different blood samples at the onset, and because there was no response to antibacterial antibiotics but prompt response to amphotericin B.

Isolation and characterization of the organism

Blood was incubated at 37°C in aerobic and anaerobic BacTAlert® blood culture bottles (Organon Teknika Corp., Durham, NC, USA). After two days, broth from the positive BacTAlert® culture was streaked for isolation over the surface of Emmons' modified Sabouraud's glucose agar (SGA) supplemented with chloramphenicol (50 μg ml^{-1}) and gentamicin (5 μg ml^{-1}). Melanin formation was investigated on L-DOPA agar [1], incubated at 30°C and norepinephrine media incubated at 25°C; colony color was examined after 2, 3 and 5 days. Selected colonies were transferred to and maintained on SGA at 30°C. Colonies from 48-h SGA cultures were evaluated for urease activity on Christensen's urea medium, and for carbohydrate and nitrogen assimilation patterns using the API ID 32C and the API 20C AUX yeast assimilation systems (bioMérieux, Marcy-l'Etoile, France). Fermentative capabilities and growth responses were further investigated using standardized methods and results were compared with those in recently published yeast monographs [10,11]. Conidial morphology and ontogeny were examined microscopically after 7–10 days' incubation at 30°C on cornmeal–Tween agar (Difco Laboratories, Detroit, MI, USA) slide cultures. Sequence analysis was performed of the D1/D2 domains of the 26S ribosomal DNA according to Fell *et al.* [12]. The *C. laurentii* isolate (P-6723) is maintained in the CBS culture collection (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) as CBS 8833.

Susceptibility to antifungal drugs

The *in vitro* antifungal susceptibility of the yeast isolate was determined using the Etest system (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions and as described previously [13]. Agar formulations used for the Etest were: RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 1.5% agar and 2% glucose (RPG) and buffered to pH 7.0 with 0.165 M morpholinepropane sulfonic acid buffer (MOPS) for the azoles; and modified Casitone agar for the amphotericin B and 5-fluorocytosine [9 g l^{-1} Bacto Casitone (Difco), 5 g l^{-1} yeast extract (Difco), 10 g l^{-1} sodium citrate (Sigma) and 20 g l^{-1} glucose]. For the Etest, 90-mm-diameter plates containing agar at a depth of 4.0 mm were used. The inoculum was prepared from 48-h culture. Cell suspension was prepared in sterile 0.85% NaCl adjusted to the turbidity of a 1.0 McFarland standard. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antifungal agent at which the border of the elliptical inhibition zone intercepted the readable scale on the strip. *Candida krusei* ATCC 6258 (American Type Culture Collection, Manassas, VA, USA) and *Candida parapsilosis* ATCC 22019 served as quality controls for all tests.

The *in vitro* susceptibility of the isolates was also determined by the broth microdilution method according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS M27-A) [14] as described by Espinel-Ingroff *et al.* [15] for *Cryptococcus* spp. These tests involved the use of 0.2 ml of RPMI-1640 broth medium (Sigma) buffered to a final pH of 7.0 with 0.165 M MOPS and 1 M NaOH, filter sterilized solutions, inoculum size of 10^4 cells per ml. The microtitration plate was incubated at 35°C with agitation for 48 h. The MIC was defined as the lowest drug concentration that resulted in complete inhibition of visible growth.

Results

Identification of the clinical isolates

White to cream-colored mucoid colonies with a smooth and glossy surface were recovered on SGA plates. Microscopic examination of cells from these colonies, as well as from slide cultures, showed round to oval, budding, encapsulated yeasts, without pseudo- or true hyphae. The overall micro- and macroscopic appearance was consistent with the genus *Cryptococcus*. The isolates grew on SGA at 30 and 37°C but not at 45°C, were sensitive to cycloheximide, hydrolyzed urea and lacked fermentative ability. However, the colonies did not produce any brown to black color on L-DOPA and norepinephrine agars and remained hyaline after 5 days. Identification profiles for *C. laurentii* were excellent with the ID 32C yeast assimilation test system (biocode 5577766375), and good with the API 20C AUX system (biocode 27455773). The growth pattern on a wide variety of carbon and nitrogen sources, growth at 37°C,

growth in 0.1% cycloheximide, the production of starch-like compounds, urease activity and a positive staining with diazonium blue B were all in agreement with known characters of *C. laurentii* [10,11]. The D1/D2 sequence of the 26*S* rDNA of the isolate was found to be identical with two other isolates of *C. laurentii*, namely CBS 2174 (AB035040) originally isolated from a tumor, and CBS 7140 isolated from soil. The sequence differed in one nucleotide from that of CBS 139 (AF075469), the type strain of *C. laurentii*, and in two nucleotides from CBS 7235 [16].

Antifungal susceptibility

The MIC values (mg l^{-1}) of antifungal drugs were detected according to the Etest method and the NCCLS microbroth dilution method (Table 1), and compared with these of *C. laurentii* type strain (CBS 139). It is clear that our isolate (CBS 8833), contrary to the type strain, was *in vitro* resistant to fluconazole according to both methods (MIC value > 256 mg l^{-1} – Etest, or 50 mg l^{-1} – NCCLS).

Discussion

Molecular characterization, phylogenetic status and ecology of C. laurentii

Morphological, physiological and molecular data point to the identity of the isolate as *C. laurentii.* This species differs phylogenetically from *C. neoformans*, as its type strain (CBS 139) belongs to a different cluster in the order Tremellales (cluster Indecorata) [12]. *C. laurentii,* as currently defined, is genetically heterogeneous [10,11,16,17]. Consequently it is difficult to identify it phenotypically, e.g. with the ID 32C or API 20C AUX systems. Sequence analysis of the D1/D2 domain of the 26*S* rDNA is required for the accurate identification of clinical isolates belonging to this complex. According to a recent molecular phylogenetic analysis of *C. laurentii* based on the D1/D2 sequence, our isolate belongs to phylogenetic group I [17].

C. laurentii is found in the environment in diverse habitats, e.g. in soil, certain vegetable skins, and as a contaminant during the fermentation process of wine and beer [11]. It was recently isolated at a high frequency from wild pigeons, suggesting that these birds are a possible reservoir in nature [18]. The species is a very rare pathogen in humans. Only 16 cases of infections from which *C. laurentii* was isolated have been reported. Most were in cancer patients or immunocompromised hosts, including seven cases of fungemia (Table 2).

As Krajden *et al.* have indicated [19], interpretation of published reports of these rare clinical isolations is problematic. Two main categories of problems are encountered: the absence of adequate demonstration of the organism in tissues, and the uncertainty of its identification as *C. laurentii*. The two cases reported by Krajden *et al.* were of particular interest, as *C. laurentii* was undoubtedly grown from tissue in which *C. neoformans* was shown to be present by fluorescent antibody techniques but from which tissue it did not grow in culture. A further example is the repeated isolation of *C. laurentii* from peritoneal fluid in a patient undergoing peritoneal dialysis via a Tenckhoff catheter [20]. Whereas the peritoneum is usually regarded as a sterile site, the presence of a long-term catheter raises questions as to the etiological significance of the isolates in this case. In addition, without proof of tissue involvement, colonization of skin or catheter could not be excluded.

Despite these caveats, and in view of the paucity of information regarding the species in general, we have included all published reports of clinical isolations. We believe that all the information should be available (Table 2), especially for clinicians who are required to make real-time judgments based on laboratory results. However, we would emphasize the need to evaluate each report critically.

Clinical manifestations of C. laurentii

The clinical manifestations of *C. laurentii* infection vary from local skin lesions or asymptomatic lung infection [5,21] to systemic symptoms with fever or hypothermia and hypotension, as probably occurred in our case [8], or meningitis [7]. Fever was noted in all patients with fungemia, except in one who had hypothermia [9]. Other

Table 1 Susceptibility of *Cryptococcus laurentii* isolates to antifungal drugs

	Antifungal MIC (mg l^{-1})				
Isolate	AmB	Flu	Itra	Keto	5FC
CBS 8833 (current case)	0.032 (0.03)*	> 256 (50)	0.5 (ND)	0.016 (ND)	>32 (>500)
CBS 139 (type strain)	0.006 (0.03)	8 (6)	0.006 (ND)	0.008 (ND)	> 32 (4)

AmB, amphotericin B; Flu, fluconazole; Itra, itraconazole; Keto, ketoconazole; 5-FC, flucytosine. The MIC values are according to the Etest method.
*Numbers in parentheses are the MIC value that were determined according to the NCCLS microbroth dilution method. ND, not detected.

Table 2 Reported proven and strongly suggestive cases of *Cryptococcus laurentii* infection, including cases later concluded to be caused by *Cryptococcus neoformans*

Age/sex	Underlying disease	Risk factor	Presentation	Infection site	Specimen*	Treatment	Outcome	Ref.
16/M	Solid tumor	CVC	Fever, hypotension	CVC	Blood (1)	AmB, CR	Cure	This study
17/M	Post BMT	CVC	Fever	Fungemia	Blood (1)	Flu, CR	Cure	[6]
Neonate/M	Hypoplastic lungs, hydronephrosis	CVC, urinary catheter	Fever, hypotension	Fungemia	Blood (1)	AmB, 5FC CR	Cure	[8]
27/F	Drug use, bipolar disorder	PIC, IV drug use	Fever, chills, skin nodules	Fungemia, skin nodules	Blood (1)	Flu CR	Cure	[8]
50/M	Non-Hodgkin lymphoma	CVC, steroids, antibiotics, neutropenia	Fever	Fungemia	Blood (2)	AmB, CR	Death	[24]
Premature/F	Prematurity	PIC, antibiotics, total parenteral alimentation	Hypothermia, circulatory and respiratory insufficiency	Fungemia	Blood (2)	AmB CR	Cure	[9]
57/M	Acute myelogenous leukemia	CVC, steroids antibiotics, neutropenia, mucositis	Fever	Fungemia	Blood (2)	AmB, CR	Cure	[25]
26/M	Solid tumor	CVC, antibiotics, neutropenia	Fever	Fungemia	Blood (2)	Flu, CR	Cure	[24]
34/M	HIV	Exposure to mould	Acute febrile illness, meningeal signs	Meningitis	CSF (1)	AmB, 5FC, Flu	Cure	[7]
55/F	Dermatomyositis	Steroids	Nonspecific	Lung	Bronchial biopsy (1) sputum (1)	AmB	Cure	[21]
54/F	None	None	Hemoptysis dyspnea, chest discomfort	Lung	Lung biopsy (1)	AmB, 5FC	Cure	[19]†
37/M	None	None	Persistent asymptomatic pulmonic process	Lung	Lung biopsy (1)	None	Cure	[19]†
14/F	Chronic renal disease	Peritoneal dialysis	Fever, abdominal pain, vomiting, diarrhea	Peritoneum	Peritoneal fluid (2)	CR	Cure	[23]
13/F	Chronic renal disease	Peritoneal dialysis	Fever, abdominal pain	Peritoneum	Peritoneal fluid (2)	5FC, Mic, AmB, CR	Cure	[20]
61/F	Chronic uveitis	Topical steroids	Chronic uveitis	Endophthalmitis	Vitreous (1)	Flu	Cure	[22]
40/M	Mycobacterial skin lesions	None	Granulomatous lesions	Lower limb	Lesion biopsy (2)	AmB	Improve-ment	[5]

*Number of positive cultures is given in parentheses. CVC, central venous catheter; PIC, peripheral intravenous catheter; CR, catheter removal; BMT, bone marrow transplantation; IV, intravenous; 5-FC, flucytosine; AmB, amphotericin B; Flu, fluconazole; Mic, miconazole.
†Case later concluded via immunohistochemistry to be caused by *Cryptococcus neoformans*.

clinical manifestations in the fungemic patients were infrequent (Table 2).

The fungus can be isolated from the site of infection, such as the skin, respiratory tract, peritoneal fluid, or CSF [5,7,20–23], or, as in our case, from blood cultures [6,8,24,25]. It may be argued that in our case the isolation of *C. laurentii* represents colonization by a saprobe rather than true infection. However, no other infectious agent was associated with the recrudescence of fever and recurrent episodes of hypotension during leukopenia. Moreover, there was no response to antibacterial antibiotics or to removal of the Hickman catheter, whereas there was a prompt response to amphotericin B. Thus it is likely that our case was indeed a true infection with *C. laurentii*.

Therapeutic approaches

Six of the seven reported patients with fungemia and the case described here had a central venous catheter in place [6,8,24,25] that was removed as part of the treatment. However, there is no definite evidence that the central venous catheter was the source of the fungemia. Culturing of the tip of the Hickman catheter in our case, as well as in three other patients [6,8], failed to reveal the pathogen. Removal of a central line upon microbiologically documented fungemia is common practice.

There are various therapeutic approaches to treating patients with infection caused by *C. laurentii.* Our patient, three others with fungemia and patients with respiratory tract disease were treated successfully with AmB [21,24,25]. Others with similar clinical syndromes were successfully treated with fluconazole [6,8,24], or a combination of AmB and flucytosine [8]. The duration of treatment for fungemia varied from 10 days to 4 weeks [6,8,24,25].

Antifungal resistance

There are few reports on the MICs of antifungal drugs for *C. laurentii*. Ryder *et al.* [26] reported that the MIC of seven isolates for fluconazole ranged between 1 and 4 mg l^{-1}, compared with 50 mg l^{-1} (NCCLS method) in our case. The MIC of AmB in three clinical isolates was 0.037–0.25 mg l^{-1} (0.03 mg l^{-1} in our case) [7,8]. Johnson *et al.* [8] reported two blood-culture isolates that were resistant to flucytosine, as in our case. They also had relatively high MICs to fluconazole (8–16 mg l^{-1}), which, however, are significantly lower than our isolate, and below the breakpoint for other known yeasts such as *Candida albicans* [27]. Breakpoints for *Cryptococcus* spp. are not available. To our knowledge, our strain is the only clinical isolate of *C. laurentii* that has proven resistant to both fluconazole (50 mg l^{-1}, NCCLS or >256 mg l^{-1}, Etest) and flucytosine ($>$ 32 mg l^{-1}). We assume that this type of resistance is innate and is unrelated to drug exposure, as our patient had never been treated with azoles or flucytosine. This case resembled another, recently reported from Israel, in which *C. neoformans* that was resistant to antimycotic agents was obtained from a patient who had not received these drugs previously [28,29].

It is becoming clear that clinically relevant isolates of *C. laurentii* show different degrees of susceptibility to antifungal agents. Opportunistic fungal infections due to species tending to be less susceptible to antifungal agents in clinical use have been increasing in recent years. Resistance to azole antifungals among *Candida* and *Cryptococcus* spp. constitutes by far the most significant problem, and we might expect to see resistant *C. laurentii* added to the emerging list of species causing opportunistic disease in vulnerable patients exposed to antifungal agents [17,29–31].

In conclusion, *C. laurentii*, which is usually considered to be a saprobe, may cause clinically significant fungemia and other localized infections, especially in immunocompromised hosts. Patients with malignant diseases with a central venous catheter seem to be at particular risk for fungemia with this organism. The clinical manifestations of *C. laurentii* fungemia are usually not severe, and there is usually a favorable response to appropriate antifungal therapy and catheter removal. Finally, special consideration should be paid to the emergence of *C. laurentii* strains resistant to antifungals.

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