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Normally Saprobic Cryptococci Isolated from *Cryptococcus neoformans* Infections

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We report two cases in which *Cryptococcus laurentii* was isolated from surgically resected pulmonary lesions but the cryptococcal cells in tissue reacted positively with a specific fluorescent antibody (FA) conjugate for *Cryptococcus neoformans*. Both patients had no apparent host defense defects. In both cases, multiple cryptococcal isolates were obtained from tissue, and yeastlike cells consistent with *C. neoformans* were seen in direct histology. The isolates were identified by assimilation patterns and standard procedures including phenoloxidase reactions. Since *C. laurentii* was consistently isolated by using stringent procedures, it was considered unlikely that the fungus represented surgical or laboratory contamination. Its presence may be the result of dual infection not detected by FA, but other possible explanations exist. The results show the value of the FA test in diagnostic mycology and call into question previous reports of cryptococci other than *C. neoformans* as agents of infection.

There have been numerous reports over the years of *Cryptococcus* species other than *C. neoformans* causing human infection (Table 1). Isolates in these cases have most often been obtained from body sites which characteristically have been associated with *C. neoformans* infection. For example, in 6 of 12 published cases, isolates were obtained from cerebrospinal fluid (Table 1) while in 3 other cases, non-*C. neoformans* isolates were recovered from pulmonary sources. In 11 of the 12 cases, the species isolated was either *C. albidus* or *C. laurentii*, both of which are commonly isolated from normal skin (14, 17) and indoor and outdoor air (20, 31).

Recently, we investigated two cases strongly suggestive of pulmonary infection by *C. laurentii*. In both instances, while multiple isolates of *C. laurentii* were obtained from surgically resected lung tissue, *C. neoformans* was not isolated. Fluorescent antibody staining (FA) techniques, however, indicated that the fungus present in the tissues was *C. neoformans*. These unusual findings suggest that a reassessment of the literature with respect to infections supposedly caused by *Cryptococcus* species other than *C. neoformans* is in order and that the clinical ecology of such infections requires clarification.

CASE REPORTS

Case 1. Over a 2-year period prior to admission, a 54-year-old woman resident of Ontario, Canada, experienced four episodes of hemoptysis, occasionally with purulent sputum. At presentation, the patient experienced dyspnea on exertion and transient right-sided chest discomfort. Chest X-rays did not reveal any anomalies. Bronchoscopy and cytological examination of bronchial washings were negative for fungi, bacteria, and mycobacteria. However, a computerized tomographic scan showed an ill-defined mass in the right upper lobe, as well as a small calcified granuloma elsewhere in the same lobe. Since percutaneous needle aspiration and repeat bronchoscopy failed to identify the nature of the lesion, an open lung biopsy was performed. Adhesions of the right upper lobe to the chest wall were found which necessitated resection of the apical segment of the lobe.

Microscopic studies of stained preparations of a portion of tissue removed on biopsy revealed small numbers of widely scattered budding yeastlike cells consistent with *Cryptococcus* spp. Culture media inoculated with a portion of the same tissue yielded numerous colonies of a yeastlike organism tentatively identified as *C. albidus*. However, upon extensive investigation the organism was found to be *C. laurentii* (Table 2). A representative isolate was deposited in the University of Alberta Microfungus Collection and Herbarium as UAMH 6255.

Sections stained with specific anti-*C. neoformans* fluorescein-tagged antibody showed that the tissue contained widely scattered, brightly fluorescing individual cells and clusters of yeastlike cells consistent with *C. neoformans* or a closely related, cross-reacting *Cryptococcus* species. After absorption of the conjugate with *C. laurentii* antigen, the cells continued to fluoresce, indicating that they were not *C. laurentii*. Cells also reacted positively to anti-*C. neoformans* conjugate absorbed with *C. albidus* antigens, indicating they were not *C. albidus*. On the basis of consistent reaction with these well-controlled, highly specific conjugates, the yeastlike cells were recognized as *C. neoformans*.

The patient was treated with amphotericin B (0.3 mg/kg/day for 4 weeks) and 5-fluorocytosine (75 mg/kg/day divided over four daily doses for 4 weeks). Four years after treatment was completed, the patient was in good health.

Case 2. A 37-year-old male resident of Texas first presented in 1982 as an outpatient and was diagnosed as having an uncomplicated pneumonic process. He responded at that time to antibacterial agents.

One year later he received a routine physical examination.

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TABLE 1. Reported cryptococcal infections caused by species other than *C. neoformans*[a]

Reference	Age (yr)/sex	Species isolated	Infection	Predisposing condition	Therapy[b]	Outcome
3	4/F	*C. luteolus*	Pneumonia	Measles	None	Survived
4	45/M	*C. albidus*	Meningitis	None	Amph B	Survived
5	65/F	*C. albidus*	Fungemia	Leukemia, neutropenia	Amph B, 5-FC	Death
8	40/M	*C. laurentii*	Cutaneous granuloma	Atypical mycobacterial infection	Amph B, potassium iodide	Survived
11	68/M	*C. albidus*	Lung abscess	None	Amph B	Survived
12	55/F	*C. laurentii*	Lung abscess	Dermatomyositis, corticosteroids	Amph B	Survived
15	29/M	*C. albidus*	Meningitis	Corticosteroids, rheumatoid arthritis, alcoholic liver disease	Amph B	Death
25	13/F	*C. laurentii*	Peritonitis	Dialysis	Amph B	Survived
29	20/M	*C. albidus*	Meningitis	Chronic lung disease	NS	Survived
29	73/NS	*C. albidus*	Meningitis	Polycythemia rubra vera	NS	Death
29	48/NS	*C. albidus*	Meningitis	Glioblastoma	NS	NS
29	75/NS	*C. albidus*	Meningitis	Carcinoma (type not stated)	NS	Death

[a] NS, not stated.
[b] Amph B, amphotericin B; 5-FC, 5-fluorocytosine.

He was described as aymptomatic, but a chest X-ray revealed a persistent right-lobe pneumonic process. Sputum and skin tests for tuberculosis and coccidioidomycosis were negative. Routine laboratory tests were all within normal limits. A fiber-optic bronchoscopy was performed and did not reveal any abnormalities. Cultures and smears of bronchial brushings and washings were negative.

Because the lesion seen in the X-ray was suspected to be a malignancy, a thoracotomy was performed. A large mass in the middle lobe and a smaller mass in the right lower lobe were observed and biopsied. Histopathology with periodic acid-Schiff (24), Grocott methenamine silver (24), and hematoxylin and eosin (19) stains was initially viewed as being consistent with histoplasmosis, since small cells were seen and some were interpreted as being within macrophages. Surgery was performed to remove the masses, and histopathology of the resected material with mucicarmine stain (24) revealed the presence of a cluster of large budding yeasts suggestive of a cryptococcal fungus ball. Samples of lung material plated on Sabouraud peptone-dextrose agar yielded multiple cultures of *C. laurentii* var. *laurentii*. The identification was confirmed by two independent reference laboratories on the basis of characteristic morphological and physiological attributes (Table 2). Upon confirmation of this identification, a preliminary report on the case was presented indicating a diagnosis of *C. laurentii* pneumonia (30).

Subsequent FA studies done independently at two laboratories indicated that the organisms seen in tissue were *C. neoformans* var. *neoformans*. Cross-absorption studies with *C. laurentii* antigens confirmed this identification.

The patient was not treated with antifungal drugs and did well. At a 3-year follow-up he had no evidence of progressive or active disease.

TABLE 2. Identification characteristics of the cryptococcal isolates from cases 1 and 2

Test	Result for yeast isolates from:	
	Case 1	Case 2
Assimilation		
Glucose	+	+
Galactose	+	+
Sucrose	+	+
Maltose	+	+
Cellobiose	+	+
Trehalose	+	+
Lactose	+	+
Melibiose	+	+
Raffinose	+	+
Melezitose	+	+
Xylose	+	+
Rhamnose	+	+
Erythritol	−	+
Mannitol	+	+
Inositol	+	+
Potassium nitrate	−	−
Fermentation	−	−
Urease	+	+
37°C growth	−	V
Starch formation	ND[a]	+
Phenol oxidase	−	−
Colony coloration	Dull pinkish tan	Not recorded
Cellular encapsulation	Weak	Weak
Filamentation	Nil	Nil
Diagnosis	*C. laurentii*	*C. laurentii*

[a] ND, not done.

MATERIALS AND METHODS

Lung tissues were ground in sterile glass mortars in physiological saline, and 2 drops (approx. 60 μl) of a heavy suspension was spread by means of a sterile loop on each of several routine fungal isolation media: bovine blood agar; Sabouraud glucose agar with 100 μg of cycloheximide per ml, 100 μg of chloramphenicol per ml, and 50 μg of gentamicin per ml; and Littman's oxgall agar (Difco Laboratories, Detroit, Mich.) with 100 μg of streptomycin per ml. Plates were incubated at 25°C for up to 3 weeks. Yeast colonies which became apparent during that time were subcultured from isolation media to Sabouraud glucose agar with 100 μg of chloramphenicol per ml and 50 μg of gentamicin per ml only and then examined microscopically for morphological features. In addition, isolates were subjected to assimilation auxanography and fermentation tests by using the techniques outlined by Kreger-van Rij (10). Isolates obtained were also subcultured to Staib agar (*Guizotia abyssinica*-based phenoloxidase test agar) for detection of the brown reaction typical of *C. neoformans* (13, 26).

Sections of lung tissue stained with mucicarmine and Grocott methenamine silver (24) were examined for the presence of fungal cells. For FA (7) studies, paraffin-embedded tissue was sectioned and the sections were floated on

cold water and attached to glass slides with dilute commercial Elmer's Glue-All (7). Mounted sections were then directly stained with specific rabbit anti-*C. neoformans* fluorescein isothiocyanate (7). All FA reagents were prepared in the Laboratories for Mycology, Wadsworth Center for Laboratories and Research, New York State Department of Health. Sections of tissue from animals experimentally infected with *C. neoformans* served as positive controls in all FA studies. FA investigations were conducted with antigens prepared from identified isolates of *C. neoformans* and *C. laurentii* and also from unabsorbed and *C. laurentii*-cross-absorbed (7) *C. neoformans* FA reagents. The preparations were overlaid with coverslips and examined with a Nikon microscope equipped with an epifluorescence attachment. Results of control studies clearly indicated that the cross-absorbed FA specifically reacted only with *C. neoformans* antigen preparations.

RESULTS AND DISCUSSION

Both of the cases discussed here demonstrate an unusual situation in that numerous colonies of *C. laurentii* were isolated from pulmonary lesions which were demonstrated by FA to be most likely caused by *C. neoformans*. However, *C. neoformans* was never isolated from clinical material. These results cast doubt on the accuracy of previous reports of lung abscesses caused by *Cryptococcus* species other than *C. neoformans*. The *C. laurentii* lung infection reported by Lynch et al. (12) was similar to the present cases in that exclusive growth of *C. laurentii* from a pulmonary abscess strongly suggested a diagnosis of *C. laurentii* cryptococcosis. Unfortunately, neither conventional histopathology nor FA were done in the study (12) to further evaluate the diagnosis. In addition, the *C. albidus* pulmonary infection reported by Krumholz et al. (11), despite ambiguity about the species identification (6), is strikingly similar to the present cases. In both cases no. 2 and the case reported by Krumholz et al. (11), histopathological examination of resected material was initially interpreted as consistent with histoplasmosis because of the presence of clusters of relatively small yeast cells.

A broader examination of the 12 reported infections caused by *Cryptococcus* species other than *C. neoformans* reveals numerous problems in affirming the pathogenic status of the fungi isolated in culture. In addition, in all but two cases (5, 12), the identification of the isolates recovered is either questionable or at least not verifiable from published data. In Table 3, the cryptococcal isolates noted in Table 1 are again listed along with information pertinent to the confirmation of their pathogenic status and identification.

In only 5 of the 12 cases listed in Table 3 (3, 4, 8, 29 [2 cases]) were *Cryptococcus*-like cells seen upon direct microscopic examination of patient specimens. In the remaining seven cases, only the isolation of *Cryptococcus* colonies in pure culture suggested the identification of the etiologic agent. Although the fungus was isolated in multiple cultures in 8 of 12 cases (3, 4, 8, 11, 12, 25, 29 [2 cases]), in three of these (3, 11, 12) it was recovered from normally contaminated sources such as sputum (3, 11, 12), bronchial wash (11), and gastric lavage (11). The diagnosis of one fungemia case with nonspecific symptoms was based solely on a single positive blood culture and the positive response of the patient to amphotericin B therapy (5).

In one case (25) where the fungus is listed in Table 3 as being from a normally sterile site, this site is in fact an unusually vulnerable one: the peritoneal fluid of a patient with a Tenckhoff catheter. In another case involving a normally sterile site, *Cryptococcus* colonies were obtained from subcutaneous tissue of a patient with an infection resembling Madura foot (8). In this case, an unclassified isolate resembling an atypical mycobacterium was also recovered and the patient responded to antibacterial and antimycobacterial agents (8). In the remaining three cases in which the fungus was repeatedly isolated (4, 29 [2 cases]), the normally sterile site was cerebrospinal fluid.

TABLE 3. Evaluation criteria for assessment of etiologic status and identification of putative non-*C. neoformans* agents of cryptococcosis

Reference	Result for criterion					
	Isolation			Positive direct microscopy of specimen	Published data conclusively rule out *C. neoformans*	Published data suggest identification is questionable
	From normally sterile site	From normally sterile site, repeatedly	From normally unsterile site, repeatedly			
3	−	−	+	+	−	+
4	+	+	NA[a]	+	−	−
5	+	−	−	−	+	−
8	+	+	NA	+	−	−
11	+	−	+	−	−	+
12	+	−	+	−	+	−
15	+	−	−	−	−	−
25	+	+	NA	−	−	−
29	+	+	NA	+	−	−
29	+	−	NA	−	−	−
29	+	+	NA	+	−	−
29	+	−	NA	−	−	−

[a] NA, criterion not applicable to case in question.

In three cases (5, 12, 15), negative results with slide latex agglutination tests for *C. neoformans* diffusible antigen were reported for cases diagnosed as non-*C. neoformans* cryptococcosis. In none of these cases, however, were *Cryptococcus*-like cells found in the cerebrospinal fluid (12, 15) or serum (5) assayed. Hence the substantiation of any cryptococcal colonization whatsoever in these cases is weak. Wieser (29), using the antibody detection techniques of Vogel et al. (28), demonstrated the presence of antibodies in cerebrospinal fluid which reacted with *C. albidus* antigen even after prior absorption with a *C. neoformans* homogenate. The titer of the reaction was much lower after absorption with *C. neoformans* than before. Wieser did not state, however, whether the homogenate used for absorption included all serotypes of *C. neoformans*. Thus, it remains uncertain that the diminished quantity of reactive antibodies remaining after absorption were truly specific to *C. albidus*.

The identification of many of the cryptococcal isolates listed in Tables 1 and 3 is also problematic. The putative *C. luteolus* reported by Binder et al. (3) grew abundantly at 37°C. Yet, recent taxonomic texts (1, 23), indicate this species cannot grow at this elevated temperature. Using the characters described by Binder et al. (3) and the taxonomic keys of Rodrigues de Miranda (23), we tentatively identified the isolates as *C. neoformans*. However, the isolates recovered by Binder et al. (3) were atypical of *C. neoformans* in that colonies were yellowish-brown, cells were ellipsoidal in shape, and the fungus was not pathogenic in an experimental mouse model.

Doubts as to the identity of the *C. albidus* isolated from lung by Krumholz (11) have already been summarized by Gordon (6).

The remaining identifications listed in Table 1 are likely to have been accurate, but in most cases their accuracy is not verifiable because of a lack of published data. This was the case both for identifications simply credited to reference laboratories and for those credited, without specific result information, to commercial yeast test strips. Only in two cases (5, 12) was the relatively specific phenoloxidase test conducted to rule out atypical *C. neoformans*. In some other cases, the isolates in question were shown to have characters atypical of *C. neoformans*, such as avirulence in the mouse model (11, 29 [1 of 4 cases]), weak encapsulation (11, 25, 29 [1 of 4 cases]), and poor growth at 37°C in vitro (5, 29 [1 of 4 cases]). As Gordon (6) has pointed out, however, "a single negative virulence test does not exclude *C. neoformans*"; moreover, a few *C. neoformans* isolates are known to have weak encapsulation (21) and weak growth at 37°C in vitro (1). Unfortunately, there is no record of any isolate from a putative case of non-*C. neoformans* cryptococcosis being deposited in a recognized fungal culture collection. Hence the taxonomic reliability of such reports cannot be independently confirmed by the reexamination of isolates.

Despite these problems, at least two of the reported cases of non-*C. neoformans* cryptococcosis, i.e., the case described by Kamalam et al. (8) and the primary case reported by Wieser (29), amass sufficient anomalous information that it would be difficult to reinterpret them as misconstrued cases of infection caused by *C. neoformans* or another yeast. These cases include a tissue demonstration of cryptococcal cells, a repeat culture from a normally sterile site, and an extensive effort to achieve a correct identification in consultation with independent reference laboratories. On the other hand, our own cases would be equally difficult to interpret as *C. neoformans* infection if not for the direct evidence of FA. Therefore, all existing reports of non-*C. neoformans* cryptococcosis must be regarded as questionable until a direct and unequivocal demonstration of non-*C. neoformans* cryptococcal cells in tissue is made.

The circumstantial evidence for non-*C. neoformans* cryptococcal infection in our cases and similar instances requires some explanation. Certainly the isolation of non-*C. neoformans* cryptococci from percutaneous samples such as blood and lumbar punctures may be at least partly explained by the very common presence of these organisms as contaminants of normal skin (14, 17). The occasional fortuitous isolation of these fungi in blood cultures is well documented (2, 22). Limited colonization around catheterization sites may also be expected. In such cases, the isolation of cryptococcal contaminants may coincide with infection by other, unrecovered fungi. However, none of these factors related to skin colonization affect the interpretation of pulmonary infections supposedly caused by cryptococci other than *C. neoformans*.

The isolation of significant numbers of non-*C. neoformans* cryptococci from *C. neoformans* infections may be tentatively explained in a number of ways, none of which can be definitively substantiated. Simple laboratory contamination must be ruled out in our cases, as growth of the non-*C. neoformans* yeasts was consistent in repeat samples, occurred only from sample materials (not, for example, on areas not touched by the loop in streaked agar plates), and was of an intensity uncharacteristic of aerial contamination. Also, although non-*C. neoformans* cryptococci are generally among the most common airborne contaminants in normal indoor and outdoor air (20, 31), they are uncommon in the clinical laboratories involved in this study, which possess modern filtered air systems. A year-round survey of fungi isolated from air in the hospital where our case no. 1 was observed showed that *C. laurentii* accounted for only 3 of the approximately 7,000 fungal isolates identified.

On the other hand, it is certainly known that naturally occurring contaminant fungi may be recovered from deep within the lungs (16, 18) in recently deceased individuals. In regions of the lung affected by the immunosuppressive (9) *C. neoformans*, non-*C. neoformans* cells may not be rapidly cleared. Viable yeast cells in excised tissue specimens are well known to proliferate rapidly, and psychrotolerant species like *C. laurentii* (27) may even proliferate in material refrigerated at the permissive temperature of 5°C. A yeast proliferating in such a fashion regularly increases in CFU, whereas a mold contaminant, growing as an intact nonsporulating mycelium, does not. Hence a pulmonary specimen with a few cells of an airborne psychrotolerant *Cryptococcus* species, upon transport or overnight refrigeration, might well generate enough CFU to mimic an infection and to overgrow a nonpsychrotolerant etiologic agent. The exact storage and processing conditions of the specimens in our cases are now untraceable, but in case no. 1 specimens were processed in a public health laboratory at some distance from the collecting hospital. Similar specimens sent between the two facilities are not infrequently refrigerated overnight before transport.

While we cannot provide a definite explanation of our findings, we hope that future studies will shed additional light on possible infections by non-*C. neoformans* cryptococci and on the potential diagnostic problems caused by naturally occurring yeast contaminants in pulmonary specimens. In the interim, our results suggest that FA studies should be employed in assessing the etiology of pulmonary and other infections which would appear to be caused by cryptococci other than *C. neoformans*.

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