Restriction Fragment Length Polymorphism Analysis of *Cryptococcus neoformans* Isolates from Environmental (Pigeon Excreta) and Clinical Sources in New York City

BRIAN P. CURRIE,¹ LAWRENCE F. FREUNDLICH,² AND ARTURO CASADEVALL^{1,3*}

Departments of Medicine,¹ Laboratory Medicine,² and Microbiology and Immunology,³
Albert Einstein College of Medicine, Bronx, New York 10461

Received 6 October 1993/Returned for modification 7 December 1993/Accepted 29 January 1994

Restriction fragment length polymorphism analysis of environmental (pigeon excreta) and clinical *Cryptococcus neoformans* var. *neoformans* isolates in a limited geographic area distinguished 6 strains among 8 environmental isolates and 12 strains among 17 clinical isolates. Clusters of patients with three strain types accounted for 47% of clinical isolates. Despite this diversity, two strains were shared by environmental and clinical isolates.

The ecology of *Cryptococcus neoformans* and the epidemiology of cryptococcal infections are poorly defined (18). There are two varieties of *C. neoformans* which are pathogenic for humans, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. These can be distinguished genetically (14), serologically (12), biochemically (2, 30), and epidemiologically (8, 15).

The anamorph state (asexual haploid yeast) of *C. neoformans* var. *neoformans* has occasionally been isolated from a variety of natural sources including peach juice, milk, fruits, and vegetables but has been primarily associated with avian excreta and, to a lesser extent, with soil contaminated with avian excreta (1, 16, 18, 28). *C. neoformans* var. *neoformans* has been most frequently isolated from weathered pigeon excreta and occasionally from the guano of other avian species (chickens, parrots, sparrows, starlings, turtle doves, and canaries [16, 18, 28]). Since first described by Emmons in 1955 (9), the striking association between *C. neoformans* var. *neoformans* and pigeon excreta has been confirmed in many parts of the world (8, 11, 13, 16, 19, 20, 23, 27, 29). In contrast, the teleomorph state (filamentous diploid state resulting from the mating of a and α yeast forms and taxonomically designated *Filobasidiella neoformans* var. *neoformans*) has been observed only in the laboratory (15).

C. neoformans var. *neoformans* is believed to enter the human host via inhalation and subsequent alveolar deposition (18). Desiccated yeast cells have been proposed to be the infectious propagule, and infectious particles of a size consistent with alveolar deposition have been demonstrated in pigeon excreta (26). Serum anticryptococcal antibody is much more prevalent among pigeon breeders than in the general population, indicating that contact with pigeons and/or their excreta results in more frequent exposure to cryptococcal antigens (34). However, in spite of the strong association of *C. neoformans* var. *neoformans* and pigeon excreta, there are only rare anecdotal cases of human acquisition of cryptococcal infection that can be directly related to exposure to pigeon excreta (10, 20, 27). This discrepancy remains unexplained. Associations between exposure to pigeon excreta and clinical cryptococcal disease are complicated by the fact that it is not known whether clinical disease represents acute infection or

reactivation of a latent focus of infection (24, 25). If clinical infection is the result of reactivation of latent disease, then temporal associations between exposure to pigeon excreta and disease onset may be obscured. Other complex host-pathogen interactions, such as differing virulence for humans among environmental isolates, may also contribute to obscuring potential links between exposure to pigeon excreta and subsequent onset of disease.

In contrast to *C. neoformans* var. *neoformans*, the distribution of disease caused by *C. neoformans* var. *gattii* is restricted to semitropical and tropical regions and closely parallels the distribution of the red gum eucalyptus tree (*Eucalyptus camaldulensis*) and possibly other eucalyptus trees (7, 11). The anamorph state has been isolated exclusively from plant material collected under eucalyptus trees in bloom. Haploid basidiospores, which are the product of the teleomorph state (taxonomically designated *Filobasidiella neoformans* var. *bacillispora*), have been recovered by air sampling under flowering eucalyptus trees. Whether the association between *C. neoformans* var. *gattii* and eucalyptus trees is endophytic or epiphytic is uncertain, but the basidiospore has been proposed as the infectious propagule (6, 8). As a consequence, it has been suggested that basidiospores from *F. neoformans* var. *neoformans* associated with an as yet unidentified plant, as well as desiccated yeast cells from pigeon excreta, may serve as the infectious propagules of *C. neoformans* var. *neoformans* via alveolar deposition (6).

To investigate whether pigeon excreta are a reservoir for human *C. neoformans* var. *neoformans* strains, we examined environmental and clinical isolates in a limited geographic area using restriction fragment length polymorphism (RFLP) analysis with two DNA probes. To our knowledge, there have been only limited attempts to characterize and compare cryptococcal isolates from pigeon excreta with those isolated from geographically related cases of human cryptococcal meningitis (11, 13, 19). These studies have been hampered by the lack of a sensitive technique for typing isolates which would allow meaningful comparison between environmental and clinical isolates. This study represents the first attempt to use molecular typing methods to examine the same issue.

(The findings reported here were presented at the 93rd General Meeting of the American Society for Microbiology, Atlanta, Ga., 1993 [3a].)

* Corresponding author. Mailing address: Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-4260. Fax: (718) 597-5814.

MATERIALS AND METHODS

Clinical and environmental isolates. Seventeen clinical *C. neoformans* isolates were obtained from the Bronx Municipal Hospital Center (Jacobi Hospital), Bronx, N.Y. All except one (J14) were obtained from patients with their initial diagnosis (not relapse) of cryptococcal infection. Fifteen of these patients had AIDS, one had chronic lymphocytic leukemia, and one had alcoholism as underlying causes of immunosuppression.

Eighteen outdoor locations in the Bronx and one in Manhattan which were heavily contaminated with desiccated pigeon excreta were sampled for *C. neoformans*. Only shaded locations where pigeon excreta were not in direct contact with soil were sampled by filling a sterile 50-cm³ conical tube with excreta collected with sterile tweezers and spatulas. An attempt was made to construct east-west and north-south sampling transect lines through the borough of the Bronx (an area approximately 12 by 15 km), as well as to locate samples as close as possible to each patient's home address at the time of diagnosis. All clinical and environmental samples were obtained in the course of 1 calendar year. *C. neoformans* was isolated from these samples by using tandem plating of distilled water suspensions of pigeon excreta on L-dopa agar and bird seed agar, both supplemented with biphenyl (Eastman Kodak Co., Rochester, N.Y.), vancomycin (Eli Lilly and Co., Indianapolis, Ind.), and imipenem/cilastin (Merck and Co. Inc., West Point, Pa.) (16, 22, 31). To prepare L-dopa agar plates, 4 g of KH₂PO₄, 2.5 g of MgSO₄ · 7H₂O, 10 mg of thiamine HCl, 1.0 g of glycine, 1.0 g of glucose, and 200 mg of 3,4-dihydroxyphenylalanine (L-dopa) (Sigma Chemical Co., St. Louis, Mo.) were dissolved in 1 liter of distilled water and sterilized by filtration through a 0.45-μm-pore-size filter (Millipore Corp., Bedford, Mass.). Equal volumes of this solution and 3% agar solution (sterilized by autoclaving and allowed to cool to 50°C) were mixed. Two grams of biphenyl dissolved in 30 cm³ of absolute ethanol, 500 mg of vancomycin suspended in 10 ml of distilled water, and 500 mg of imipenem/cilastin suspended in 10 ml of distilled water were sequentially added to the agar solution prior to pouring into plates. Bird seed agar was prepared by pulverizing approximately 50 g of *Guizotia abyssinica* seed (Amato Grain and Seed Co., Lodi, N.J.) and boiling this material for 30 min in 1 liter of distilled water. This mixture was filtered through filter paper. Fifteen grams of agar was then added to the filtrate, the volume was adjusted to 1 liter with distilled water, and the mixture was sterilized by autoclaving. When the molten agar had cooled to approximately 50°C, 1 g of biphenyl dissolved in 15 ml of absolute ethanol, 250 mg of vancomycin suspended in 5 ml of distilled water, and 250 mg of imipenem/cilastin suspended in 5 ml of distilled water were sequentially added prior to pouring into plates. Both media served as selective and indicator media for *C. neoformans*.

To recover *C. neoformans* from pigeon excreta, approximately 0.1 to 1.0 g of desiccated excreta from each sample was placed in a 50-ml conical tube, and 5 ml of distilled water was added. Samples were gently shaken by hand and then allowed to stand for approximately 5 min. All samples were subsequently vortexed for 5 min and allowed to stand 3 min to permit heavier material to settle to the bottom of the tube. Aliquots of 20, 50, and 100 μl of the cloudy supernatant were then inoculated on both the antibiotic-biphenyl-supplemented L-dopa and bird seed agar plates and spread uniformly on each plate with a glass spreader. All plates were incubated at 37°C for 21 days, and colonies were harvested from positive plates. Each individual harvested colony was subsequently streaked on

FIG. 1. Partial map of New York City showing the boroughs of the Bronx and Manhattan. The map illustrates the geographic relationship between environmental isolates and patients' addresses at the time of diagnosis with *C. neoformans* infections. Environmental samples have the prefix M or B (Manhattan or Bronx, respectively) and are represented by closed circles. Clinical isolates have the prefix J (denoting Jacobi Hospital) and are represented by open circles.

a Sabouraud dextrose agar plate (Difco Laboratories, Detroit, Mich.), and a single colony was harvested for maintenance on a Sabouraud dextrose agar slant. Thus, each positive environmental site was represented by a single isolated colony with the exception of the M1 site, where 22 colonies were randomly harvested from the initial plates (Fig. 1).

Eight environmental *C. neoformans* isolates were obtained from the 18 locations sampled. Figure 1 illustrates the geographic relationship between positive environmental samples and each patient's address at the time of diagnosis. All clinical and environmental isolates were definitively identified as *C. neoformans* by a positive bird seed agar response, positive urease test, ability to grow at 37°C, and analysis with the API 20C system (Analytab Products, Plainview, N.Y.) (5), and all were determined to be *C. neoformans* var. *neoformans* by canavanine-glycine-bromothymol blue agar (17). All environmental isolates were determined to be pathogenic in BALB/c mice by intracerebral injection. All clinical isolates were able to grow after plating on L-dopa agar supplemented with biphenyl and antibiotics when incubated at 37°C for 21 days.

RFLP analysis. Genomic DNA was extracted from each isolate as previously described (3, 32) and examined by Southern blot analysis of *URA5* gene (3) and CNRE-1 (32) RFLPs. Genomic DNA was digested with *Hae*II, *Hind*III (Boehringer Mannheim, Indianapolis, Ind.), or *Sst*I (Sigma Chemical Co.), and restriction enzyme fragments were separated in a 1% agarose gel and transferred to a positively charged nylon membrane by using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After fixing by UV light cross-linking, the membranes were hybridized at 65°C for 16 h with either the *URA5* or the CNRE-1 probe labelled with [α -³²P]dCTP in a solution of denatured salmon sperm DNA (0.5 mg/ml) in 5× SSC, 7% sodium dodecyl sulfate, 10× Denhardt's reagent, and 10% dextran sulfate in 0.020 M sodium phosphate buffer (pH 7.2).

The *URA5* gene probe hybridizes to a single copy gene which is essential for uracil synthesis and which has been shown to be

FIG. 2. Representative Southern blot autoradiogram illustrating RFLPs resulting from *HaeII*-digested DNA probed with *UR45*. Four distinct RFLP patterns (types 1 through 4) that were present in environmental and clinical isolates are illustrated.

highly polymorphic in nucleic acid sequence (3). The CNRE-1 probe hybridizes to a repetitive element present in multiple copies in all chromosomes (32). RFLP patterns were used to define strains.

Stability of RFLP patterns obtained with *UR45* and CNRE-1 probes has been previously documented in initial and relapse isolates from human patients (33). In addition, two CNRE-1 RFLP patterns (A and C) obtained from environmental samples in this study were found to be stable after 4 months of passage in mice and after 7 months of desiccation or serial passage on Sabouraud dextrose agar (data not shown).

RESULTS

HaeII-digested DNA probed with *UR45* revealed four types (1 through 4) of RFLPs, which are illustrated in Fig. 2. *HindIII*-digested DNA probed with *UR45* revealed five types of RFLPs (I through V) (not shown). *SstI*-digested DNA probed with CNRE-1 revealed 16 RFLP patterns (A through P). Representative autoradiograms illustrating 11 of these RFLP patterns are shown in Fig. 3.

At the initiation of this study it was thought that using two parallel strain typing techniques (*UR45* and CNRE-1 RFLPs) would increase the ability to discriminate between strains. *UR45* and CNRE-1 RFLPs had previously been used concurrently for only a limited number of isolates, and comparative data regarding the efficiency of strain typing by each of these probes were not available. The results indicate that the CNRE-1 probe was significantly more discriminatory in typing isolates than the *UR45* probe. This is not surprising given that the CNRE-1 probe examines a repetitive element present in multiple copies in all chromosomes whereas the *UR45* probe hybridizes to a single-copy gene. These results indicate that the CNRE-1 probe is a powerful epidemiological tool.

Table 1 summarizes the RFLP patterns for all isolates. Sixteen strains (i through xvi) were identified by RFLPs from the 25 environmental and clinical isolates.

RFLP patterns revealed six strains of *C. neoformans* var. *neoformans* from eight environmental sites. There were different strains at sites less than 0.5 km apart (B4 and B5; B6 and B10). However, some strain types, such as i and v were found at multiple environmental sites separated by up to 9 km, indicating wide distribution of those strains within the sample

FIG. 3. Representative Southern blot autoradiograms illustrating RFLPs resulting from *SstI*-digested DNA probed with CNRE-1 from 14 *C. neoformans* isolates. (A) Composite photograph from a single blot illustrating the wide diversity in RFLP patterns obtained with the CNRE-1 DNA probe. Ten different RFLP patterns are illustrated. Isolates J15, J17, and J24 have identical RFLPs. (B) Composite photograph from a single blot illustrating identical RFLP patterns obtained from an environmental sample (B3) and a clinical isolate (J12).

area. At one site (M1), two strains were isolated from a 1-g sample of pigeon excreta. Analysis of 22 colonies from this site revealed 21 colonies (M1A) corresponding to strain type v and 1 colony (M1B) corresponding to strain type xvi.

RFLP analysis revealed 12 strains among the 17 clinical isolates. Three of those strains (vi, vii, and viii) were isolated from more than one patient and accounted for 47% of the clinical isolates.

Two strains (i and iv) were found at environmental sites and were isolated from two patients as well (Table 1). Strains shared by patients and environmental samples were separated by approximately 6 km in the study area when the patient's address was used as a potential marker for geographic location of exposure. Lack of geographic association in microanalysis situations (distances of less than 170 m between environmental samples and patients' homes) may simply reflect the extensive

TABLE 1. RFLP patterns

Strain	Isolate(s)	RFLP		
		<i>UR45</i>		CNRE-1
		<i>HaeII</i>	<i>HindIII</i>	
i	B3, B7, B10, J12	1	III	A
ii	B4	2	I	B
iii	B5	4	II	C
iv	B17, J16	4	II	D
v	B6, M1A	4	II	E
vi	J21, J22	2	I	F
vii	J19, J26	4	V	G
viii	J15, J17, J24, J25	4	II	H
ix	J18	4	V	I
x	J20	4	II	J
xi	J23	4	II	K
xii	J14	3	IV	L
xiii	J11	1	III	M
xiv	J10	4	II	N
xv	J13	4	II	O
xvi	M1B	4	II	P

genetic diversity between and within environmental sites, as well as the highly variable nature of potential human exposure to pigeon excreta (i.e., place of employment, travel routes, travel modes, etc.).

DISCUSSION

This study represents the first attempt to compare *C. neoformans* var. *neoformans* isolates from pigeon excreta with those from geographically related human isolates by using molecular typing methods. A striking finding was the extensive genetic diversity among environmental and clinical *C. neoformans* var. *neoformans* isolates from a limited geographic area (16 strains identified from 25 environmental and clinical isolates). The finding of 12 strains among 17 clinical isolates at a single hospital confirms a previous observation which suggested extensive genetic diversity among clinical isolates in New York City (3). The recovery of six strains from eight environmental sites in a relatively small geographic area indicates extensive genetic diversity among *C. neoformans* var. *neoformans* environmental isolates that has not been previously documented. In addition, the results suggest a complex distribution of strain types within the geographic area investigated, as evidenced by the recovery of strains i and v from sites separated by up to 9 km, unique strains being found less than 0.5 km apart, and two strains isolated from a 1-g sample of pigeon excreta from one site. The factors responsible for this genetic diversity are not understood, but the findings are intriguing and warrant further research concerning possible contributions of asexual and sexual reproduction in *C. neoformans* var. *neoformans*.

Despite the genetic diversity in clinical isolates, three strains were isolated from more than one patient and accounted for 47% of the clinical isolates. Human-to-human transmission is an unlikely explanation for these clusters since it has never been documented except in rare cases of organ transplant (24). Given that 88% of the clinical isolates came from human immunodeficiency virus-infected patients and that such patients were infected with unique strain types, as well as the strain types found in multiples, it would appear that underlying host factors did not account for selection of prevalent strains. Alternatively, the preponderance of cases associated with strains vi, vii, and viii suggests that these may be genetic markers for differences in virulence, and this may have implications for ongoing research to develop vaccines (4) and antibody-based strategies (21).

The fact that isolates from environmental sites heavily contaminated with pigeon excreta and from patients had identical RFLPs implies that some patients are infected by strains of *C. neoformans* var. *neoformans* which are genetically related to those found in pigeon excreta. This observation suggests that pigeon excreta could potentially be a reservoir for pathogenic *C. neoformans* var. *neoformans* for immunocompromised individuals but does not conclusively prove that acquisition of human cryptococcal infection in this study occurred by exposure to pigeon excreta. Consider, for example, an alternative explanation which proposes that human cryptococcal infection is the result of exposure to a yet undefined environmental reservoir of *C. neoformans* var. *neoformans* (6, 8, 16). In this scenario, clinical isolates and isolates from pigeon excreta with identical RFLPs could only reflect the ability of excreta to support the growth of *C. neoformans* var. *neoformans* after inoculation from cryptic environmental reservoirs (6). The fact that 10 RFLP strain types found in patients were not recovered from environmental sites could reflect insufficient environmental sampling, acquisition of in-

fection elsewhere, reactivation of disease, or exposure to an alternative and as yet undefined environmental reservoir of *C. neoformans* var. *neoformans*.

Our results are preliminary, and they must be considered within the limitations imposed by small sample sizes, the extensive genetic variation among isolates, and uncertainties in the knowledge of the pathogenesis of cryptococcal infection and the life cycle of *C. neoformans* var. *neoformans* in the environment. Continued research will result in larger sample sizes and better define the distribution of genetic variability within the environment, outcomes both of which will permit the development of meaningful statistical models and statistical analysis of results. The answer to the question of whether pigeon excreta are a reservoir for human *C. neoformans* var. *neoformans* infection continues to be obscure, and the significance of the fact that some strains in pigeon excreta are related to some clinical strains by RFLPs remains difficult to interpret. Nonetheless, it would seem prudent to reinforce existing recommendations (18, 19) that immunosuppressed patients avoid exposure to sites heavily contaminated with pigeon excreta.

ACKNOWLEDGMENTS

We are grateful to Eric Spitzer and Silvia Spitzer for supplying the CNRE-1 probe and for many helpful discussions and suggestions.

B.P.C. was supported by National Institutes of Health training grant AI07183-14 ST32 and A.C. was supported in part by a Pfizer postdoctoral fellowship, a James S. McDonnell Scholar award, and NIH grants RO1-AI33774 and RO1-AI13342. This support is gratefully acknowledged.

REFERENCES

1. Ajello, L. 1958. Occurrence of *Cryptococcus neoformans* in soils. *Am. J. Hyg.* **67**:72-77.
2. Bennett, J. E., K. J. Kwon-Chung, and T. S. Theodore. 1978. Biochemical differences between serotypes of *Cryptococcus neoformans*. *Sabouraudia* **16**:167-174.
3. Casadevall, A., L. Freundlich, L. Marsh, and M. D. Scharff. 1992. Extensive allelic variation in *Cryptococcus neoformans*. *J. Clin. Microbiol.* **30**:1080-1084.
- 3a. Currie, B. P., L. F. Freundlich, and A. Casadevall. 1993. Genetic analysis of environmental and clinical *Cryptococcus neoformans* isolates from New York City. abstr. F-43, p. 534. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993. American Society for Microbiology, Washington, D.C.
4. Devi, S. J. N., R. Schneerson, W. Egan, T. J. Ulrich, D. Bryla, J. B. Robbins, and J. E. Bennett. 1991. *Cryptococcus neoformans* serotype A glucuronoxylomannan-protein conjugate vaccines: synthesis, characterization, and immunogenicity. *Infect. Immun.* **59**:3700-3707.
5. Dimech, W. J. 1991. Diagnosis, identification, and epidemiology of *Cryptococcus neoformans* infection. *Aust. J. Med. Lab. Sci.* **12**:13-21.
6. Ellis, D. H., and T. J. Pfeiffer. 1990. Ecology, life cycle, and infectious propagule of *Cryptococcus neoformans*. *Lancet* **336**:923-925.
7. Ellis, D. H., and T. J. Pfeiffer. 1990. Natural habitat of *Cryptococcus neoformans* var. *gattii*. *J. Clin. Microbiol.* **28**:1642-1644.
8. Ellis, D., and T. J. Pfeiffer. 1992. The ecology of *Cryptococcus neoformans*. *Eur. J. Epidemiol.* **8**:321-325.
9. Emmons, C. W. 1955. Saprophytic sources of *Cryptococcus neoformans* associated with the pigeon (*Columba livia*). *Am. J. Hyg.* **62**:227-252.
10. Fessel, W. J. 1993. Cryptococcal meningitis after unusual exposures to birds. *N. Engl. J. Med.* **328**:1354-1355.
11. Fromtling, R. A., G. Abruzzo, and A. Ruiz. 1989. Virulence and antifungal susceptibility of environmental and clinical isolates of *Cryptococcus neoformans* from Puerto Rico. *Mycopathologia* **106**:163-166.
12. Ikeda, R., T. Shinoda, Y. Fukuzawa, and L. Kaufman. 1982.

- Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. *J. Clin. Microbiol.* **36**:22–29.
13. Imwidthaya, P., P. Dithaprasop, and C. Egtasaeng. 1989. Clinical and environmental isolates of *Cryptococcus neoformans* in Bangkok (Thailand). *Mycopathologia* **108**:65–67.
 14. Kwon-Chung, K. J. 1976. A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. *Mycologia* **68**:942–946.
 15. Kwon-Chung, K. J., and J. E. Bennett. 1984. Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. *Am. J. Epidemiol.* **120**:123–130.
 16. Kwon-Chung, K. J., and J. E. Bennett. 1992. Cryptococcosis, p. 397–446. In K. J. Kwon-Chung and J. E. Bennett (ed.), *Medical mycology*. Lea & Febiger, Philadelphia.
 17. Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett. 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). *J. Clin. Microbiol.* **15**:535–537.
 18. Levitz, S. M. 1991. The ecology of *Cryptococcus neoformans* and the epidemiology of cryptococcosis. *Rev. Infect. Dis.* **13**:1163–1169.
 19. Littman, M. L., and S. S. Schneierson. 1959. *Cryptococcus neoformans* in pigeon excreta in New York City. *Am. J. Hyg.* **69**:49–59.
 20. Muchmore, H. G., E. R. Rhoades, G. E. Nix, F. G. Felton, and R. E. Carpenter. 1963. Occurrence of *Cryptococcus neoformans* in the environment of three geographically associated cases of cryptococcal meningitis. *N. Engl. J. Med.* **268**:1112–1114.
 21. Mukherjee, J., L. S. Zuckier, M. D. Scharff, and A. Casadevall. 1994. Therapeutic efficacy of monoclonal antibodies to *Cryptococcus neoformans* glucuronoxylomannan alone and in combination with amphotericin B. *Antimicrob. Agents Chemother.* **38**:580–587.
 22. Paliwal, D. K., and H. S. Randhawa. 1978. Evaluation of a simplified *Guizotia abyssinica* seed medium for differentiation of *Cryptococcus neoformans*. *J. Clin. Microbiol.* **7**:346–348.
 23. Partridge, B. M., and H. I. Winner. 1965. *Cryptococcus neoformans* in bird droppings in London. *Lancet* **i**:1060–1061.
 24. Perfect, J. R. 1989. Cryptococcosis. *Infect. Dis. Clin. N. Am.* **3**:77–102.
 25. Perfect, J. R., D. T. Durack, and H. A. Gallis. 1983. Cryptococcosis. *Medicine (Baltimore)* **62**:98–109.
 26. Powell, K. E., B. A. Dahl, R. J. Weeks, and F. E. Tosh. 1972. Airborne *Cryptococcus neoformans*: particles from pigeon excreta compatible with alveolar deposition. *J. Infect. Dis.* **125**:412–415.
 27. Procknow, J. J., J. R. Benfield, J. W. Rippon, C. F. Diener, and F. L. Archer. 1965. Cryptococcal hepatitis presenting as a surgical emergency. First isolation of *Cryptococcus neoformans* from point source in Chicago. *JAMA* **191**:93–98.
 28. Rippon, J. W. 1988. Medical mycology: the pathogenic fungi and pathogenic actinomycetes, 3rd ed., p. 582–609. W. B. Saunders Co., Philadelphia.
 29. Ruiz, A., R. A. Fromtling, and G. S. Bulmer. 1981. Distribution of *Cryptococcus neoformans* in a natural site. *Infect. Immun.* **31**:560–563.
 30. Salkin, I. F., and N. J. Hurd. 1982. New medium for differentiation of *Cryptococcus neoformans* serotype pairs. *J. Clin. Microbiol.* **15**:169–171.
 31. Shields, A., and L. Ajello. 1966. Medium for selective isolation of *Cryptococcus neoformans*. *Science* **151**:208–209.
 32. Spitzer, E. D., and S. G. Spitzer. 1992. Use of a dispersed repetitive DNA element to distinguish clinical isolates of *Cryptococcus neoformans*. *J. Clin. Microbiol.* **30**:1094–1097.
 33. Spitzer, E. D., S. G. Spitzer, L. F. Freundlich, and A. Casadevall. 1993. Persistence of the initial infection in recurrent cryptococcal meningitis. *Lancet* **341**:595–596.
 34. Walter, J. E., and R. W. Atchinson. 1966. Epidemiological and immunological studies of *Cryptococcus neoformans*. *J. Bacteriol.* **92**:82–87.