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Comparison of *Cryptococcus neoformans* Isolates from Clinical and Environmental Collections in South Central Kentucky and Surrounding Areas

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ABSTRACT

Cryptococcus neoformans is an opportunistic encapsulated yeast responsible for the invasive disease cryptococcosis in immunocompromised hosts. The importance of epidemiological studies on cryptococcosis has increased since the beginning of the AIDS epidemic. *Cryptococcus neoformans* exists in two varieties with four serotypes: *C. neoformans* var. *neoformans* (serotypes A and D) and *C. neoformans* var. *gattii* (serotypes B and C). In this research, *C. neoformans* var. *neoformans* has been associated with pigeon feces in those months having an average temperature of 17.8°C and above. Clinical and environmental isolates of *C. neoformans* were grouped into their variety status utilizing canavanine-glycine-bromthymol blue agar. Polyclonal antisera against *C. neoformans* serotypes A, B, C, and D were pooled from challenged rabbits. Serotyping *C. neoformans* isolates by using the polyclonal antisera resulted in 57% (20 of 35) of the serotypes confirmed with a direct immunofluorescent assay utilizing a single monoclonal antibody (E1). Assay data suggest that all *C. neoformans* obtained from regional hospitals (26 of 26) and those isolated from the environment (9 of 9) belong to the A serotype group. These data provide information on the origin of cryptococcosis in our region and may be beneficial to immunocompromised individuals.

INTRODUCTION

Cryptococcosis is a potentially harmful fungal disease in humans and animals. Usually it is listed as an opportunistic infection; however, the causative yeast, *Cryptococcus neoformans*, does occur in healthy persons (Sugar 1991). Cryptococcosis has recently been recognized as the most life-threatening mycosis in patients with Acquired Immunodeficiency Syndrome (AIDS) (Clancy et al. 1990). This mycosis generally manifests itself as meningitis, with the respiratory tract as the portal of entry for the yeast (Ellis and Pfeiffer 1990). Meningitis and to a lesser extent pneumonia have proven to be life-threatening manifestations of cryptococcosis, although morbidity may be due to involvement of virtually any organ system. Among the many species of *Cryptococcus*, the etiologic agent in virtually all cases of human cryptococcosis is *C. neoformans*. Previously, rare cases of infection were thought to be caused by *C. albidus* and *C. laurentii* (Krumholz 1972; Lynch et al. 1981); however, recent studies utilizing fluorescent antibody techniques suggest the need for a reassessment of past literature with respect to infections caused by *Cryptococcus* species other than *C. neoformans* (Sigmund et al. 1991).

On the basis of antigenic determinants of the polysaccharide capsule, four serotypes (A, B, C, and D) of *C. neoformans* have been recognized (Evans 1950; Wilson et al. 1968). Subsequent studies, using type specific antisera, have shown that serotypes A and D share similarities differentiating them from serotypes B and C, which are similar to one another.

Cryptococcus neoformans has a perfect or teleomorphic state, *Filobasidiella neoformans*, with two varieties. The teleomorph *F. neoformans* var. *neoformans* corresponds to the anamorph (asexual state) *C. neoformans* var. *neoformans* serotypes A and D. The teleomorph *F. neoformans* var. *bacillisporus* corresponds to the anamorph *C. neoformans* var. *gattii* serotypes B and C (Kwon-Chung 1975, 1976a, 1976b).

Extensive research conducted on epidemiological differences between these varieties (Bennett et al. 1977; Kwon-Chung and Bennett 1984) has shown that *C. neoformans* serotypes B and C are prevalent only in tropical and subtropical regions, whereas 85–100% of *C. neoformans* serotypes A and D are found in Europe and North America, excluding southern California, where serotypes B and C represented 41% of the isolates (Kwon-Chung et al. 1978).

Among dissimilarities between the varie-

ties—including epidemiologic, biochemical, and genetic aspects—the most striking is in their ecology. Cultures of *C. neoformans* var. *neoformans* are regularly isolated throughout the world from pigeon excreta and waste from other birds including chickens, parrots, sparrows, starlings, turtledoves, canaries, and skylarks (Leitz 1991). Until recently, isolates of *C. neoformans* var. *gattii* serotypes B and C have been cultured only from clinical specimens. Environmental isolations established that *C. neoformans* var. *gattii* may have a specific ecological association with the tree *Eucalyptus camaldulensis* (Ellis and Pfeiffer 1990), which could explain its tropical and subtropical distribution. Investigations have shown that creatinine assimilation in *C. neoformans* plays a role in these ecological differences (Kwon-Chung 1991). The biochemical basis for the differences was found in the deiminase enzyme system common to both varieties. Upon deimination of creatinine, two compounds are produced: methylhydantoin and ammonia, the latter of which may raise the pH of the surrounding environment. A regulatory system that has evolved in *C. neoformans* var. *neoformans* but not in *C. neoformans* var. *gattii* is repression of creatinine deiminase once small amounts of ammonia are detected. This mechanism of creatinine metabolism seems to suit the conditions of avian guanos in which *C. neoformans* var. *neoformans* occurs (Kwon-Chung 1991).

In our study, we prepared antiserum against each of the four *C. neoformans* serotypes A, B, C, and D. Environmental samplings of plant materials and pigeon roosts were analyzed to determine the serotypes most prevalent in the sampling area and to identify natural habitats for serotypes A and D. Serological comparison of clinical specimens, obtained from regional hospitals, with the environmental isolates was conducted utilizing polyclonal antisera and a highly specific monoclonal antibody against the cryptococcal capsular polysaccharide (Dromer et al. 1993; Dromer et al. 1987)).

MATERIALS AND METHODS

Environmental Samples

Random environmental samples were collected monthly from October 1991 through December 1992; the average temperature of each month was recorded. The environmental test area was a 30-km sampling site along Highway 31-W including Bowling Green, Woodburn, and Franklin, Kentucky.

Plant collections, as well as pigeon roost samples, were collected throughout the 15 months. Plant collections from selected sites included tree bark, tree leaves, and tassels and stalks from corn and sorghum; they were kept in plastic bags.

Plants to be sampled were swabbed with the culturette collection system (American Scientific Products), which contained modified Stuarts Bacterial Transport Medium. Samples taken from pigeon roosts were nest material, feces, soil, and swabs of surrounding surfaces; they were stored as described for the plant collections.

Sample preparation. An 11 g portion of each plant collection was suspended in 99 ml of 0.1M phosphate buffer (pH 6.8), shaken on an Eberbach shaker at 270 cycles/min for 5 min, and allowed to stand for 10 min. A 0.1 ml aliquot of the dilution was spread-plate inoculated onto *Guizotia abyssinica* medium (niger seed agar) (Staib and Seeliger 1966) containing 0.5% diphenyl as a mold inhibitor. All plates were incubated at 26°C and observed daily for phenoloxidase-producing colonies, which synthesize melanin. To prepare the sample collections from pigeon roosts a 1.0 g portion of the sample was suspended into 99 ml of phosphate buffer and treated as described for plant materials. Sample collections acquired with the culturette collection system were either directly plated onto agar or suspended in 9 ml 0.1 M phosphate buffer (pH 6.8) prior to plating. After plating, the medium was incubated at 26°C and observed daily for phenoloxidase-producing colonies.

All isolates were stored on 2% (w/v) dextrose, 1% neopeptone agar slants and maintained at 26°C. For long-term storage, 3-day slant cultures were kept at −70°C.

Clinical Isolates

Clinical isolates were obtained from University of Louisville School of Medicine, Department of Pathology, Louisville, Kentucky; University of Kentucky School of Medicine, Department of Pathology, Lexington, Kentucky; T.J. Samson Community Hospital, Glas-

gow, Kentucky; Greenview Hospital, Bowling Green, Kentucky; and Vanderbilt University Medical Center, Department of Pathology, Nashville, Tennessee. These hospitals are within a 240-km radius of Bowling Green.

All isolates were initially tested for production of phenoloxidase and urease (Urease Test Media, Difco Laboratories). Those environmental isolates presumptively identified as *C. neoformans* were further characterized by the API 20C, as directed by the manufacturer. Excluding Greenview Hospital and T.J. Samson Community Hospital, which utilized the Vitek system and Microscan, respectively, all remaining participating hospitals confirmed *C. neoformans* with API 20C. All clinical and environmental isolates of *C. neoformans* were identified to variety with canavanine-glycine-bromthymol blue (CGB) agar prepared according to the methods of Kwon-Chung et al. (1982).

Preparation of Vaccines

Cultures of *C. neoformans* var. *neoformans* strains A68 and D52 and of *C. neoformans* var. *gattii* strains B112 and C18 were donated by K.J. Kwon-Chung (National Institute of Allergy and Health, Bethesda, Maryland). Strain B112 was a mutant of the original Evans Strain 1523 (Ellis and Pfeiffer 1990).

Each of the four *C. neoformans* serotypes was grown in 300 ml of 2% (w/v) dextrose/1% neopeptone broth on a rotary shaker at 140 RPM for 72 hours at 26°C. Cells were pelleted by centrifugation at 1200 g and washed twice in 0.85% saline. Cell suspensions were heat-killed by submersion in a water bath at 100°C for 1 hour, pelleted, and resuspended in 20 ml of sterile 0.85% saline. Vaccines were prepared from heat-killed cells by adjusting the number of cells/ml to 2×10^8 as determined by hemocytometer counts.

Immunization of Rabbits

Rabbits weighing 2 kg or more were given intravenous injections of 2×10^8 cells in sterile 0.85% saline. All rabbits received a course of six injections consisting of two 3-day series, 4 days apart, then no injections for 2 weeks. The course of injections was repeated four times, and the rabbits were given one injection a day for 14 consecutive days. Seven days after the last injection, the rabbits were bled and the antisera pooled. Those weighing less than 2 kg (1800–1999 g) were given 10^8 cells per injection.

Absorption and Agglutination

Heterologus titers were determined by slide agglutination with suspensions of cells corresponding to McFarland standards 1 and 3. Slide agglutinations were carried out by mixing 0.05 ml of cells (2×10^8 cells/ml) and 0.05 ml of antisera, diluted with 0.85% NaCl, on a glass slide. Agglutination titers were observed after 5, 10, and 15 min on a Fisher Clinical Rotator, model 341, rotating 120 times/min. Antibody titers were expressed as final dilutions of antiserum with agglutinating cells.

Serotyping of isolates was performed with antiserum absorbed with a mixture of cells from the other serotypes. The volume of packed cells was equal to the volume of antiserum to be absorbed. Three absorptions were incorporated for each of the four antisera. A fourth absorption was required if the antisera cross reacted with more than one of the four serotypes. Following serotyping of isolates, utilizing polyclonal antiserum, all *C. neoformans* serotypes were confirmed with a monoclonal antibody against type A cryptococcal cells conjugated with fluorescein isothiocyanate. The monoclonal antibody technique was developed and generously carried out by Dr. Françoise Dromer (Institute Pasteur, Paris, France) (Dromer et al. 1993, 1987).

RESULTS

Environmental Isolates

Isolation of *C. neoformans* from environmental sources occurred only on months with an average temperature of 17.8°C and above. Nine environmental isolates were collected from May 1992 through September 1992 from the sampling area (Table 1). Attempts to isolate *C. neoformans* from barks and leaves of various trees and other plant materials were unsuccessful. *Cryptococcus laurentii* was isolated in April 1992 from a Kentucky coffeetree (*Gymnocladus dioicus*) on the campus of Western Kentucky University in Bowling Green.

Clinical Isolates

Twenty-six clinical isolates were obtained during our study (Table 2). All were acquired

Table 1. *Cryptococcus neoformans* isolates from environmental sample collections conducted October 1991 through December 1992.

Date	Avg. mon. temp. °C	Sample collection	Location	Isolate
12 Oct 91	Oct 15.2	Sorghum tassel, stalk and leaf	Woodburn	None
18 Oct 91		Pigeon feces	Bowling Green	None
3 Nov 91	Nov 11.9	Tree bark	Franklin	None
3 Nov 91		Tree bark	Bowling Green	None
10 Nov 91		Pigeon feces	Bowling Green	None
11 Dec 91	Dec 5.4	Pigeon feces	Bowling Green	None
2 Feb 92	Feb 6.6	Pigeon feces	Bowling Green	None
2 Feb 92		Pigeon feces	Bowling Green	None
9 Mar 92	Mar 8.6	Pigeon feces	Bowling Green	None
6 Apr 92	Apr 14.3	Tree swabs	Bowling Green	None
11 May 92	May 17.8	Thistle swabs	Bowling Green	None
20 May 92	May 17.8	Bark, tree swabs, pine cones	Bowling Green	None
29 May 92		Pigeon feces	Bowling Green	SSE3, SSE4
20 June 92	Jun 22.2	Pigeon roost swabs	Bowling Green	SSE5, SSE6
21 Jul 92	Jul 26.4	Pigeon feces	Bowling Green	PE1, PE2
12 Sep 92	Sep 20.4	Pigeon feces	Franklin	FE1
14 Sep 92		Pigeon roost swabs	Bowling Green	None
14 Sep 92		Pigeon feces	Bowling Green	None
24 Oct 92	Oct 14.5	Pigeon feces	Franklin	None
24 Oct 92		Thistle swabs	Woodburn	None
24 Oct 92		Pigeon feces	Bowling Green	None
28 Nov 92	Nov 8.9	Pigeon feces	Franklin	None
9 Dec 92	Dec 3.7	Pigeon feces	Bowling Green	None

during the 15-month course, except those obtained from Vanderbilt Medical School, which had been stored frozen at −70°C for periods not exceeding 2 years.

Identification of Isolates

All nine isolates producing phenoloxidase on niger seed agar, modified with 0.5% diphenyl and giving a positive urease test, were confirmed by the API 20C system (Kwon-Chung and Rhodes 1986; Polacheck et al. 1990). Seven gave an API 20C profile index numbr of 2557373, which confirmed the identification as *C. neoformans*. The two remaining isolates (SSE5 and SSE2) gave an index number of 2757373, which also indicated *C. neoformans*.

All clinical and environmental isolates were identified to their variety by utilizing CGB agar. A positive test, indicated by pH change from 5.8 (greenish yellow) to at least 7.0 (cobalt blue), was exhibited by all isolates tested. Thus, all clinical and environmental isolates were identified as *C. neoformans* var. *neoformans*.

Absorption and Agglutination

The heterologous titers of the non-absorbed antisera failed to reach levels described in past literature (Ikeda et al. 1982; Wilson et al.

Table 2. *Cryptococcus neoformans* obtained from regional hospitals.

Hospital	Isolates	Isolate identification
University of Louisville School of Medicine, Louisville, KY	4	UL-1, UL-2, UL-3, UL-4
University of Kentucky School of Medicine, Lexington, KY	5	UK-1, S120, X77, 136R, S325
T.J. Samson Community Hospital, Glasgow, KY	1	TJ-1
Greenview Hospital, Bowling Green, KY	1	GH-1
Vanderbilt Medical School, Nashville, TN	15	V1334, V1347, V6415, V1459, V1733, V2153, V3493, V3933, V2667, V3176, V2409, V5928, V6824, V6626, V6627

1968). Rabbits injected with the A68 cells and those injected with the D52 cells gave the highest heterologous titers, 256 and 128, respectively. A dramatic decrease in titers of the antisera was experienced after absorptions were performed. Antisera for A and D serotyping expressed homologous titers of 16, whereas B and C antisera gave homologous titers of 4 and 8, respectively, which correlates with their low heterologous titer. The polyclonal antisera developed in rabbits were utilized and separated all isolates into either A, D, or AD serotypes. From the 35 total isolates obtained, 57% (20 of 35) were typed as being serotype A, 17% (6 of 35) were D serotype, 20% (7 of 35) typed AD, and 5% (2 of 35) were untypeable (Table 3). After data were obtained from monoclonal antibody typing, variability in the polyclonal antisera was discovered. All 35 isolates were found to be serotype A by utilizing a direct immunofluorescence assay with a single monoclonal antibody (E1) specific for cryptococcal polysaccharide (data obtained from Dr. Françoise Dromer, Institute Pasteur, Paris, France) (Table 3).

Table 3. Serotypic identification of *Cryptococcus neoformans* isolates.

Isolate	*Variety* CGB agar[1]	Polyclonal antisera	*Serotype* Monoclonal antibody
SSE6	—	A	A
PE1	—	A	A
FE1	—	A	A
UL-2	—	A	A
UL-3	—	A	A
GH1	—	A	A
V5928	—	A	A
V1733	—	A	A
V6627	—	A	A
V2409	—	A	A
V1459	—	A	A
V1347	—	A	A
V3176	—	A	A
V2153	—	A	A
V6824	—	A	A
V3933	—	A	A
V2667	—	A	A
V6626	—	A	A
V3493	—	A	A
V1334	—	A	A
SSE2	—	D	A
SSE5	—	D	A
UK-1	—	D	A
S325	—	D	A
TJ-1	—	D	A
UL-4	—	D	A
SSE1	—	AD	A
SSE3	—	AD	A
SSE4	—	AD	A
PE2	—	AD	A
S120	—	AD	A
UL-1	—	AD	A
136R	—	AD	A
X77	—	Untypeable	A
V6415	—	Untypeable	A

[1] Negative test denotes *C. Neoformans* var. *neoformans*.

DISCUSSION

In the collection of *C. neoformans* from the environment, an average monthly temperature 17.8°C and above appeared to be required for isolation of the yeast. Further observation of samples collected that did possess *C. neoformans* seemed to suggest the yeast would best be found in areas where a high available water was present. In terms of preventing exposure for HIV infected persons or those who have severe immunosuppresion, these individuals should avoid environments with abundant pigeon feces. Samples collected from arid locations, and expected to contain *Cryptococcus*, did not contain the yeast in numbers high enough to isolate. Those samples possessed high mold counts, which may have inhibited the recovery of any *C. neoformans*. Attempts to isolate *C. neoformans* from plant collections were unsuccessful; however, *C. laurentii* was found to be associated with the Kentucky coffeetree, isolated in April 1992, when the average monthly temperature was 14.3°C.

By use of the CGB medium described by Kwon-Chung et al. (1982), the presumptive serotype group was determined for each of the 35 isolates obtained our study. The results of this testing show that 100% of *C. neoformans* isolated in the environmental sampling area and all regional hospitals were *C. neoformans* var. *neofomans* serotype A or D. These data are more than needed in hospitals since treatment appears to be the same for both varieties; however, in epidemiological studies more precise data require serological testing in order to differentiate serotypic groups within the species.

In typing the 35 strains of *C. neoformans* with the polyclonal antisera, only 57% were typed accurately in comparison to data obtained from the immunofluorescent monoclonal antibody (Table 3). Similar inaccuracies were noted by Bennett et al. (1977) when typing 106 strains with their polyclonal antisera.

The above authors found five isolates (4.7%) untypeable and four isolates (3.7%) reacting with A and D typing sera.

To omit these discrepancies when when serotyping *C. neoformans*, a monoclonal antibody should be used; this has proven to be a reproducible and reliable way of screening multiple isolates (Spiropulu et al. 1989). The difference between the conventional serotyping methods and the direct immunofluorescence assay using a monoclonal antibody was mainly the disappearance of the ambiguous AD serotype along with any strains that cannot be typed. All the environmental isolates corresponded serologically to the clinical isolates obtained from regional hospitals. Some degree of serotypic variability was experienced in that 43% of the isolates did not type specifically by conventional polyclonal antisera methods.

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