

A SURVEY OF ZOO AVIARIES FOR THE PRESENCE OF  
HISTOPLASMA CAPSULATUM AND CRYPTOCOCCUS NEOFORMANS

by

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## TABLE OF CONTENTS

	Page
INTRODUCTION . . . . .	1
MATERIALS AND METHODS . . . . .	1
Sample collection site . . . . .	1
Sample collections . . . . .	2
Mycological examination of dried feces samples . . . . .	3
Mycological examination of soil samples . . . . .	4
Mycological examination of fresh feces samples . . . . .	6
RESULTS . . . . .	6
Dried feces samples . . . . .	6
Soil samples . . . . .	9
Fresh feces samples . . . . .	9
DISCUSSION . . . . .	9
SUMMARY . . . . .	12
REFERENCES . . . . .	12
APPENDIX I . . . . .	15
Section 1. Review of the literature.	
<u>Cryptococcus neoformans</u> . . . . .	15
Section 2. Review of the literature.	
<u>Histoplasma capsulatum</u> . . . . .	23
Section 3. Literature cited.	
<u>Cryptococcus neoformans</u> . . . . .	32
<u>Histoplasma capsulatum</u> . . . . .	34
APPENDIX II. ACKNOWLEDGMENTS . . . . .	37

## INTRODUCTION

The pathogenic fungi, Cryptococcus neoformans and Histoplasma capsulatum, exist primarily as saprophytes in nature where they have been discovered most often around avian habitats. Man and animals become infected by inhaling spore contaminated air.<sup>1,14,19</sup>

C. neoformans displays a marked affinity for pigeon excreta, and has been isolated from in and around chicken houses.<sup>1</sup> H. capsulatum has been recovered from soil enriched with droppings from chickens, grackles, oil birds, pigeons, and starlings.<sup>2,20,21</sup> The association of these fungi with other avian species is open to further investigation.

A zoological park offers an unique opportunity to study the habitats of several avian species for the presence of C. neoformans and H. capsulatum. Also, due to the large number of people who visit a zoo, considerable public health significance might be involved if the organisms were recovered from zoo aviaries. Therefore, a survey was conducted to determine if C. neoformans or H. capsulatum were present in a zoo's aviaries, and to study the possible association of these organisms with some other avian species.

## MATERIALS AND METHODS

### Sample collection site

Samples of bird feces and soil were collected from indoor and outdoor aviaries at the Topeka Zoological Park, Topeka, Kansas. The zoo is located on the western border of an area considered highly endemic for histoplasmosis.<sup>10,15</sup> The zoo houses 87 species or subspecies of birds totaling 262 individual specimens (1968 Annual Report, Topeka Zoological Park). Most of the samples were collected from aviaries containing only one bird species.

Most indoor aviaries are constructed with a glass front, wooden walls, a cement floor covered with sand, and screen wire tops. Access to the inside of an aviary is through a door in the rear wall. Tree limbs and stumps are arranged in the aviaries to provide perches. All the outdoor aviaries are large screenwire enclosures with sod floors. Bird species which spend all or most of their life on the ground are provided a shelter house adjoining their outdoor enclosures.

#### Sample collections

Samples of dried bird feces were collected from six indoor and eight outdoor aviaries. Dried accumulations of feces on perches or in nesting boxes were scraped into plastic bags<sup>a)</sup> by using sterile tongue depressors, or a knife cleaned in 95% ethyl alcohol. Approximately four grams of dried feces were collected per sample.

Fourteen soil samples were collected including twelve from outdoor aviaries, one from under a tree where starlings roost, and one composited from several areas of the zoo located away from the aviaries. Soil samples were collected into sterile 1 quart Mason's jars using empty sterilized tin cans (cola, etc.) as scoops. A sample was the composite of soil collected to a 3 inch depth from several places in an aviary including areas under roosts.

Fresh fecal samples were collected from two indoor aviaries. Each fecal sample was collected on two sterile cotton swabs and placed in a screw-cap test tube (25X150 mm) containing 20 ml of sterile diluent (0.5% peptone in distilled water with chloramphenicol, 0.05 mg/ml).

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<sup>a)</sup> "Whirl-pak" bags, a product of Scientific Products, Evanston, Ill. 60201.



### Mycological examination of dried feces samples

Dried feces samples were examined for the presence of C. neoformans. A 4 gram portion of each sample was weighed on sterile paper and placed in a screw-cap test tube containing 36 ml of sterile distilled water. The tube was shaken for 5 minutes on a mechanical shaker that agitated at 260 rpm through a 1 1/2 inch stroke. A  $10^{-1}$  dilution was made by pipetting 1 ml of the suspension into a test tube containing 9 ml of sterile distilled water. The suspension and  $10^{-1}$  dilution were inoculated on media by streaking 0.1 ml aliquots of each on two plates of diphenyl agar<sup>16</sup> and two screw-cap tube slants (25X150 mm) of modified Littman oxgall agar<sup>4</sup> with 0.05 mg/ml of chloramphenicol. All plates and tubes were incubated at 37 C and examined daily for 21 days. Yeast-like colonies which turned brown were examined microscopically on slide mounts stained with lactophenol cotton blue and with India ink. These colonies were also subcultured on Sabouraud dextrose agar slants incubated at 25 C, and diphenyl agar plates incubated at 37 C. Subcultures that grew on Sabouraud dextrose agar as creamy-white yeast-like colonies without mycelial development were further subcultured on urea agar at 37 C. When a colony caused urease formation in urea agar, evidenced by the agar turning pink, a final test for identification of C. neoformans was performed. The colonies grown on Sabouraud dextrose agar was suspended in saline and 0.1 ml injected intracranially into each of two albino Swiss mice. Mice were sacrificed 14 days after injection. Brain tissue was stained on glass slides with India ink and viewed microscopically for the presence of C. neoformans.

A positive control sample was made by removing C. neoformans cells from a known culture and suspending them in a test tube containing 36 ml

of sterile distilled water. The control sample was tested in the same manner as the suspensions of dried feces samples.

#### Mycological examination of soil samples

Soil samples were examined for the presence of C. neoformans and H. capsulatum by using direct and indirect methods of isolation. A soil sample was mixed by manually shaking the sample container. A 10 gram portion of soil was weighed on sterile paper and placed in a screw-top jar containing 160 ml of sterile distilled water. After mechanically shaking the jar for 5 minutes the soil suspension was poured through sterilized brass strainer cloth (60 mesh)<sup>b)</sup> shaped into a cone and mounted in a Seitz filter apparatus attached to a vacuum pump. The filtrate, free of large soil particles and other debris, was poured into a sterilized glass waring blender container and blended for one minute to reduce the soil particle size. The blended suspension was distributed in 4 sterile screw-cap tubes and centrifuged at 400 g for 15 minutes. The supernatant fluid was poured off and the soil resuspended in 20 ml of sterile physiological saline solution (PSS) containing 10,000 units of penicillin per ml, 2 mg of streptomycin per ml, and 10 micrograms of polymyxin B per ml. Resuspension was accomplished by suspending the soil in one centrifuged tube, pouring the suspension into another centrifuged tube, and repeating until all the soil was suspended in the fourth centrifuged tube. This tube was held at 37 C for 2 hours then used for direct and indirect isolation techniques.

Direct isolation attempts entailed making  $10^{-1}$  and  $10^{-2}$  dilutions of the soil suspension in tubes containing 9 ml of sterile PSS. The soil

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<sup>b)</sup> C. O. Jelliff Mfg. Corp., Southport, Conn.

suspension and two dilutions were inoculated on culture media, subcultured, and examined microscopically by the techniques described under "Mycological examination of dried feces samples."

Indirect isolation procedures were begun by injection 1.0 ml of the soil sample suspension intraperitoneally into each of 4 albino Swiss mice. Mice that died during the first week after injection were discarded, but mice which died after the first week were held in a freezer at -17 C for later examination. If fewer than two mice survived the first 7 days, the sample group was reestablished by injecting 3 more mice IP with 0.8 ml of a 1:4 dilution of the original inoculum in PSS (with antibiotics).

Mice in a sample group were euthanized 4 weeks after injection and necropsied. Portions of their livers and spleens were liquified in sterile tissue grinders containing 1.0 ml of sterile PSS. One-tenth ml aliquots of liquified tissue were inoculated on 3 slants of each of the following media; Sabouraud dextrose agar with chloramphenicol (0.05 mg/ml) and cycloheximide (0.5 mg/ml), and brain-heart infusion agar containing the same antibiotics. The media was incubated at 25 C for 4 weeks and examined weekly for growth of H. capsulatum by studying teased slide mounts of mycelial growth stained with lactophenol cotton blue stain.

Pieces of liver and spleen from each mouse were smeared on glass slides and then placed in a 10% aqueous formalin solution. Slide smears were stained with Giemsa stain and examined microscopically. The formalin-fixed tissues were stained by the McManus periodic-acid-Schiff method and prepared for histopathological examination.

A brain smear was made from each mouse and stained with India ink. The smear was immediately viewed microscopically for the presence of

C. neoformans.

A soil sample containing H. capsulatum was obtained from the Soil Ecology Unit, Center for Disease Control, Kansas City, Kansas, and used as a positive control for the soil testing system.

Mycological examination of fresh feces samples

Examination of samples for C. neoformans began 3 hours after collection. A  $10^{-1}$  dilution of the swab sample suspension was made in a test tube containing 9.0 ml of sterile diluent (0.5% peptone in distilled water). Inoculation of media, subculturing, and microscopic examination followed the procedures detailed under "Mycological examination of dried feces samples."

A positive control sample was made by suspending a loop of C. neoformans cells in 9.0 ml of sterile diluent. The control was tested in the same manner as the other samples.

RESULTS

Dried feces samples

Results in Table I show that C. neoformans was isolated from samples number 3 and 4 which were collected from aviaries housing one White-breasted Toucan and two Concave-casqued Hornbills. Positive identification of C. neoformans was based on: 1) yeast-like growth and brown coloring of the colony on diphenyl agar and modified Littman oxgall agar incubated at 37 C, 2) appearance of creamy-white yeast-like colonies without mycelial development when cultured on Sabouraud dextrose agar at 25 C, 3) urease production in urea agar, 4) and detection of thick-capsuled yeast cells in mouse brain smears stained with India ink.

TABLE I

Results of tests used to isolate and identify Cryptococcus neoformans in the dried feces of several avian species.

Smp1 <sup>*</sup> No.	Species	No. in aviary	Yrs. in aviary	Media (agar)				Mouse- brain smear (India ink stain)
				Primary <sup>**</sup>		Subculture <sup>***</sup>		
				MLO	DP	SD	U	
1.	Finch	20						
	Quail	2	2.3	(-)	(+)	(-)	(-)	
2.	Dove	3	1.3	(-)	(+)	(-)	(-)	
3.	White-breasted Toucan	1	1.5	(+)	(+)	(+)	(+)	(+)
4.	Concave-casqued Hornbill	2	5.0	(+)	(+)	(+)	(+)	(+)
5.	Lorikeet	2	1.7	(-)	(-)			
6.	Greater Sulfur- crested Cockatoo	2	3.3	(-)	(+)	(-)		
7.	Barred Owl	1						
	Barn Owl	2	1.8	(-)	(+)	(-)		
8.	Duck & Goose brooder			(-)	(+)	(-)	(-)	
9.	Parasitic Jaeger	1	1.6	(-)	(+)	(-)		
10.	Peafowl	5	5.2	(-)	(+)	(-)		
11.	Horned Owl	2	4.0					
	Swainson's Hawk	1	2.0					
	Red-tailed Hawk	3	2.0	(-)	(-)			
	Turkey Vulture	1	3.0					
	Black Vulture	2	3.0					
12.	Andean Condor	2	2.0	(-)	(-)			
13.	Emu	2	3.0	(-)	(+)	(-)		
14.	North American Turkey	4	3.6	(-)	(+)	(-)		
CONTROL - <u>C. neoformans</u> streaked on media				(+)	(+)	(+)	(+)	

\* Samples 1-6 from inside aviaries. Samples 7-14 from outside aviaries.

\*\* Primary culture media: MLO = Modified Littman oxgall agar (37 C). DP = Diphenyl agar (37 C). Growth of brown yeast-like colonies recorded as (+).

\*\*\* Subculture media: SD = Sabouraud dextrose agar (25 C). Creamy-white yeast-like growth with no mycelial development is recorded as (+). U = Urea agar (37 C). Pink color change indicating urease formation is recorded as (+).

TABLE II

Results of tests used to isolate and identify Cryptococcus neoformans and Histoplasma capsulatum in soil associated with several avian species.

Soil smpl No.	Avian species	No. in aviary	Yrs. in aviary	Media (agar)				Mouse tissues		
				Direct *		Indirect **		Liver, spleen		Brain
				MLO	DP	SD	BHI	Smears (Giemsa stain)	Tissue (PAS stain)***	Smear (India ink)
1.	Parasitic									
	Jaeger	1	1.6	(-)	(-)	(-)	(-)	(-)	(-)	(-)
2.	Silver Pheasant	2	2.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
3.	Night Heron	2	2.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
4.	Peafowl	5	5.2	(-)	(-)	(-)	(-)	(-)	(-)	(-)
5.	Horned Owl	2	4.0							
	Swainson's Hawk	1	2.0							
	Red-tailed Hawk	3	2.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	Turkey Vulture	1	3.0							
	Black Vulture	2	3.0							
6.	Golden Eagle	2	3.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
7.	Andean Condor	2	2.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
8.	Ducks, Geese	130	3.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
9.	N. Am. Turkey	4	3.6	(-)	(-)	(-)	(-)	(-)	(-)	(-)
10.	Masai Ostrich	2	2.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
11.	Emu	2	3.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
12.	Common Rhea	7	5.0	(-)	(-)	(-)	(-)	(-)	(-)	(-)
13.	Soil under starling roost			(-)	(-)	(-)	(-)	(-)	(-)	(-)
14.	Composite sample from zoo premises			(-)	(-)	(-)	(-)	(-)	(-)	(-)
CONTROL 1. Positive <u>H.</u> <u>capsulatum</u> control soil				(-)	(-)	(+)	(+)	(+)	(+)	(-)
CONTROL 2. <u>H. capsulatum</u> streaked on media				(+)	(+)	(+)	(+)			

\* Media used in direct isolation procedures: MLO = Modified Littman oxgall agar (37 C). DP = Diphenyl agar (37 C). Each medium was streaked with soil suspension.

\*\* Media used in indirect isolation procedures: SD = Sabouraud dextrose agar with cycloheximide and chloramphenicol (25 C). BHI = Brain-heart infusion agar with cycloheximide and chloramphenicol (25 C). Each medium was inoculated with a saline suspension of liquified mouse liver and spleen tissues.

\*\*\* PAS = Periodic-acid-Schiff stain.

Some other microorganisms developed brownish colonies on diphenyl agar, and two of the genera identified were Rhodotorula and Geotrichum. However, C. neoformans was the only organism which turned brown on modified Littman oxgall agar.

#### Soil samples

Table II shows that C. neoformans and H. capsulatum were not isolated from any of the soil samples. However H. capsulatum was successfully isolated from a positive control sample, Control 1, indicating that the soil suspension technique and indirect isolation procedures made a reliable isolation system. The direct isolation procedure was not successful even though Control 2 results indicated that H. capsulatum would grow on the media used.

#### Fresh feces samples

Fresh feces samples were collected from the two aviaries where C. neoformans had been isolated from dried feces (see Table I). C. neoformans was not isolated from fresh feces. The organism was reisolated on culture media from a positive control sample.

### DISCUSSION

The techniques used in this survey to isolate C. neoformans and H. capsulatum from samples of soil and dried feces were modifications of isolation techniques described by other authors. Emmons<sup>5,7,8,9</sup> isolated these fungi by making sample suspensions in PSS and injecting albino Swiss mice intraperitoneally, later culturing their livers and spleens. An indirect isolation technique such as this is necessary to isolate the fastidious

H. capsulatum. However, C. neoformans grows on most laboratory media and can be isolated by direct techniques, especially if a carbohydrate is included in the substrate.<sup>3,14</sup> Dried feces samples collected in this survey were suspended in sterile distilled water, but only the direct isolation technique (for C. neoformans) was performed since injection of fecal suspensions into mice usually kills them. It was noted during incubation of cultured samples that modified Littman oxgall agar retarded the growth of fungal contaminants much better than diphenyl agar. Both media performed equally well in producing recognizable C. neoformans colonies.

Several indirect isolation techniques have been described for the isolation of H. capsulatum from soil. The main variations from Emmons' method involve the way in which soil is suspended to liberate the fungal spores prior to mouse injection. Emmons allowed the soil suspended in PSS to settle out, believing that most H. capsulatum spores would rise toward the top. He injected the supernatant fluid into mice. Larsh, et al<sup>14</sup> found that the spores adhere to soil particles, so they modified Emmons' method by injecting mice with the unsettled soil suspension. A method described by Klite, et al<sup>11</sup> involved filtering the soil suspension to remove large soil particles so that the small-gage needles used for mouse injection would not become plugged. Their final filtration step was through a millipore filter to discard the PSS used for soil suspension along with any toxins washed from the soil. However, they discovered by control sample studies that about 70% of the fungal spores were lost during filtration. Smith and Furcolow<sup>17</sup> reported a method which included in part the blending of soil suspensions in a blender to reduce soil particle size.

The soil suspension technique used in this survey was a modification



of the methods just mentioned. Soil was suspended in sterile distilled water; filtered once to remove large particles and debris; blended to reduce the particle size; and centrifuged so that the supernatant fluid could be discarded and replaced with PSS containing antibiotics. Polymyxin B was included with the penicillin and streptomycin because Smith and Furcolow<sup>17</sup> reported that it reduced the number of mice dying due to bacterial infection.

The two aviaries with dried feces positive for C. neoformans were located inside the same building, but not in close proximity. Fecal droppings had accumulated on perches in the Toucan aviary for 1 1/2 years, and in the Hornbill aviary for 5 years. Most of the feces drops onto the sand covered floors and is removed daily. Periodic disinfection of the inside aviaries is attempted by brushing on a disinfectant solution. Zoo personnel were directed to an article by Walter and Coffee<sup>18</sup> which relates a method for control of C. neoformans in pigeon coops by alkalization.

C. neoformans is commonly isolated from dried pigeon feces and considered cosmopolitan in occurrence, thus the public health significance in finding this fungus in a zoo's aviaries is difficult to determine. The exact public health impact of exposure to pigeon feces remains unclear. The greatest hazard must be to the zoo attendants who have close daily contact with these birds and their habitats. This survey does reveal other possible sources of exposure to C. neoformans and indicates two more bird species with which it is associated.

Topeka, Kansas, is situated within a histoplasmosis endemic area of the United States. Just prior to this survey H. capsulatum was isolated from soil collected in a wooded section of the city where starlings have

roosted for several years.<sup>22</sup> Thus, with H. capsulatum known to be present in the area, failure to isolate the fungus from the zoo aviaries surveyed lends support to an assumption that H. capsulatum is not associated with the avian species confined in those aviaries.

#### SUMMARY

A zoo's aviaries were surveyed for the presence of H. capsulatum and C. neoformans. Dried feces samples were collected in 14 aviaries and tested for C. neoformans by using direct cultural methods. This pathogen was isolated from an indoor aviary housing one White-breasted Toucan, and from another indoor aviary housing two Concave-casqued Hornbills. Soil samples were collected in 12 outdoor aviaries and two other areas of the zoo premises and tested for H. capsulatum and C. neoformans by direct and indirect cultural methods. Neither fungal pathogen was isolated from the soil samples. Fresh feces samples were collected from the two aviaries where C. neoformans had been isolated in dried feces. Tests for the presence of C. neoformans were made by direct culture methods, however the organism was not isolated.

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Appendix I, Section 1: Review of the literature.

Cryptococcus neoformans

History

Busse and Buschke reported from Germany in 1894-1896 isolating a yeast-like organism from a woman with a "sarcoma-like" lesion of the tibia, lymphadenopathy and secondary skin lesions. Busse referred to the disease as saccharomycosis hominis (Littman and Zimmerman, 1956). In Italy, at this time (1894), San Felice isolated a yeast from fermenting peach juice, demonstrated its pathogenicity for laboratory animals, and named the organism Saccharomyces neoformans because of its so called tumor-forming characteristics in experimental infections (Dubos and Hirsch, 1965). The following year, Curtis reported from France the recovery of a pathogenic yeast from a myxomatous tumor in a patient's hip and called the organism Saccharomyces subcutaneus tumefaciens (Littman and Zimmerman, 1956). In 1901, after failing to find ascospores typical of Saccharomyces, Vuillemin transferred the fungus to the genus Cryptococcus and used the name Cryptococcus hominis (Emmons, et al, 1963). In 1902 Weis compared 4 pathogenic fungi: S. plimmeri, isolated by Plimmer from cancer of the breast; Torula sanfelice from an adenocarcinoma of a human ovary; S. neoformans of Sanfelice; and a yeast isolated from milk by Kline. He found the 4 organisms to be microscopically and culturally identical, and placed them in the genus Torula (Dubos and Hirsch, 1965). The first human case of nervous system cryptococcosis said to have been correctly diagnosed antemortem was reported in 1914 by Versé (Littman and Zimmerman, 1956). The synonym, European blastomycosis, may have come into use at that time, a point not clear in the literature.

In 1916 Stoddard and Cutler defined the pathology and clinical picture of cryptococcosis and differentiated the infection from other deep mycoses. However, they misinterpreted the mucoid capsule of the fungus as evidence of histolysis of host tissue and misnamed it Torula histolytica (Emmons, et al, 1963). Benham, in 1935, compared most of the above isolates and found them to be biologically similar. He also compared them with other budding fungi which cause deep mycoses and clearly differentiated them. The organism was reclassified and Benham suggested retaining the name Cryptococcus hominis. In the 1952 taxonomic study of the yeast by Lodder and Kreger-Van Rij, the organism was classified as Cryptococcus neoformans (Littman and Zimmerman, 1956). The consensus among medical mycologist at present is that the most valid name, on the basis of usage and priority, is Cryptococcus neoformans.

#### Epidemiology

Cryptococcosis is not confined to a geographical area but occurs throughout the world. The largest number of cases have been reported from the United States, but no highly endemic areas have been found as compared with coccidioidomycosis, histoplasmosis and North American blastomycosis (Littman and Zimmerman, 1956).

The fungus, C. neoformans, has been isolated from many sources including peach juice, milk (Kline, 1901; Carter and Young, 1950), air (Lodder and Kreger-Van Rij, 1952), soil (Emmons, 1951; Ajello, 1958), pigeon excreta (Emmons, 1951, 1954, 1955, 1960; Littman and Schneierson, 1959), and other avian habitats, particularly in and around chicken houses (Ajello, 1958). The organism has been the cause of infection in man and a wide variety of animals (Ajello, 1958).

Although the fungus was readily isolated from pigeon's nests by Emmons he was unable to culture the organism from pigeon organs and tissues (Emmons, 1955). It was believed that the body temperature of pigeons at 41 - 43 C inhibited growth of the organism. However, two German investigators have reported success in isolating the organism from naturally infected pigeons. The infected tissues included heart, lungs, liver, and intestinal contents (Weiland and Bohm, 1968). At about this time it was reported that both human and pigeon strains of C. neoformans had been grown in vitro on Sabouraud dextrose yeast extract agar slants at temperatures up to 44 C (Littman and Borok, 1968); and pigeons fed a virulent strain of the organism contained viable cells of the fungus in fresh droppings after a period of 3 weeks (Sethi and Randhawa, 1968).

Soil contaminated with avian excreta, especially from pigeons, is thought to be the primary source of infection (Littman and Borok, 1968; Littman and Walter, 1968). The mode of infection is by inhalation of spore contaminated air (Littman and Zimmerman, 1956). Clinical evidence supports this opinion as the majority of cryptococcosis cases have a primary, pulmonary focus indicating inhalation of the organism (Ajello, 1958). The organism is reported to exist in its saprophytic sites in an unencapsulated form (Choundhry, et al, 1967; Wolstenholme and Porter, 1968), a form which could easily become airborne and be inhaled.

In view of the ubiquity of C. neoformans, it is surprising that clinical cases are not seen more frequently. However, like other systemic fungus infections, cryptococcosis is commonly associated with diseases of the reticuloendothelial system, such as leukemia or Hodgkin's disease, and the systemic administration of corticosteroids (Hildick-Smith, et al, 1964;

Littman and Schneierson, 1959).

### Mycology

Cryptococcus neoformans is a spherical to oval yeast-like cell of 4 to 7 microns in diameter, but which in tissue may occasionally reach 15 microns. It is surrounded by a refractile mucinous capsule which is readily demonstrable in India ink mounts. The capsule thickness varies considerable so that it may be very thin or may reach a thickness of 7 microns (Littman and Zimmerman, 1956). The fungus reproduces by budding at any point on the surface, sometimes producing several buds simultaneously at various points. Under conditions of rapid growth, the buds break free early so that cells found in tissue and in culture vary widely from 4 to 20 microns in diameter (Emmons, et al, 1963). No true mycelium is produced, but several cells in chains, or elongated yeast-like cells representing rudimentary pseudo-mycelium may be formed in culture (Littman and Zimmerman, 1956).

C. neoformans may be cultured at room temperature or at 37 C on most laboratory media. Colonies may appear within 48 hours, but may take 10 to 14 days to develop. Colonies will develop much more abundantly when a carbohydrate such as dextrose (1-2 percent) is included in the substrate. They are soft and creamy in texture, or mucilaginous if considerable capsular material is present. Colonies are white or shades of cream to tan (Ajello, et al, 1963; Littman and Zimmerman, 1956). An important cultural difference from other systemic fungi is that C. neoformans forms smooth yeast colonies not only at 37 C but also at 20 C (Littman and Zimmerman, 1956). Its ability to grow at 37 C also distinguishes this species from nonpathogenic Cryptococci (Dubos and Hirsch, 1965).

Media commonly employed to cultivate the fungus includes Sabouraud



dextrose agar and Littman oxgall agar for incubation at 20 C, and brain-heart infusion blood agar or Littman liver-spleen glucose blood agar for incubation at 37 C (Littman and Zimmerman, 1956). Cryptococcus neoformans is sensitive to cycloheximide, thus isolation should not be attempted on media containing that antibiotic (Ajello, et al, 1963). A media for selective isolation of C. neoformans, called diphenyl agar, was described by Shields and Ajello (1966), which included an extract of Guizotia abyssinica seed in the substrate. This extract was absorbed by the fungus and caused it to become brown. Other Cryptococcus species did not develop a brown color when grown on the media. Similarly, Botard and Kelley (1968) modified Littman oxgall agar by adding this same extract. They reported that C. neoformans became brown as with diphenyl agar, and that the modified Littman oxgall agar was superior to diphenyl agar in suppressing growth of fungal contaminants.

C. neoformans is an aerobic yeast which does not produce gas with any known carbohydrate. It is urease positive on urea medium as are all species of Cryptococci, but unlike other Cryptococci, it cannot assimilate nitrate. Sugar assimilation tests also help in differentiating C. neoformans from other Cryptococci. C. neoformans does not assimilate lactose but does digest glucose, maltose, sucrose and galactose (Littman and Zimmerman, 1956; Dubos and Hirsch, 1965).

The only pathogenic species of the genus Cryptococcus is C. neoformans. Its virulence is unaltered by growth on culture media but seems to vary with different strains. Serial passage through white Swiss mice increases its virulence. Although white Swiss mice and rats exhibit definite individual variations in susceptibility to infection, they are the most

susceptible of all animal species. Guinea pigs are less prone to infection, while rabbits are relatively resistant (Littman and Zimmerman, 1956).

The affinity of C. neoformans for bird feces, especially pigeon feces, is due to the chemical constituents of bird manure serving as natural selective media. Creatinine, in particular, is a source of nitrogen utilized by C. neoformans but not other Cryptococci or most other yeast (Wolstenholme and Porter, 1967).

#### Pathology

Cryptococcal infection is associated with minimal inflammatory response. The characteristic lesion consists of an aggregate of encapsulated budding cells intermixed with a reticulum of lightly formed connective tissue which enlarges to compress surrounding tissues. In some instances the aggregates or cells provoke a granulomatous tissue reaction containing lymphocytes, plasma cells and giant cells, with budding cryptococcal cells seen within the large phagocytic cells (Hildick-Smith, et al, 1964; Ajello, et al, 1963; Littman and Zimmerman, 1956).

Any tissue of the body may be involved in disseminated cryptococcosis, but it commonly presents with infection of the central nervous system, and less commonly with involvement of the lungs, skin, and osseous tissue (Hildick-Smith, et al, 1964). Cryptococcus neoformans first forms tubercles in the lungs about one centimeter in diameter. As the tubercle ages, the organisms are digested within giant cells and fibrosis occurs, without calcification. Occasionally the pulmonary lesions are extensive and require surgical removal. Sometimes the Cryptococcus passes from the lung to the brain via the bloodstream, and causes cryptococcal meningitis (Wolstenholme and Porter, 1967). This is when cryptococcosis is most likely to be

diagnosed, through examination of the cerebrospinal fluid.

There is a much higher incidence of cryptococcosis in patients with malignant diseases of the reticuloendothelial system such as Hodgkin's disease, lymphosarcoma, and leukemia. However, patients with malignant lymphoma or leukemia are known to be more susceptible to infectious diseases. In these patients the cryptococci are widely disseminated in the body. Such dissemination is unusual in the ordinary case of cryptococcal meningoencephalitis (Littman and Zimmerman, 1956).

The most satisfactory stains for demonstration of C. neoformans in tissue are the periodic-acid-Schiff stains (Hotchkiss-McManus, Bauer, or Gridley modifications), and Mayer's mucicarmine stain (Ajello, et al, 1963).

#### Serology

Serologic test for cryptococcosis met with little or no success for many years for reasons attributed to (1) poor antigenicity of C. neoformans, (2) immunologic "paralysis," (3) neutralization of antibodies by excessive fungal antigens and (4) insensitivity of available serologic test. With the development of newer serologic techniques, investigators have been successful in demonstrating the presence of free cryptococcal antigens and antibodies in the body fluids of patients with cryptococcosis. Antibodies have been detected by hemagglutination test, indirect immunofluorescent antibody test, tube agglutination test, complement fixation fluorescent antibody test, and slide agglutination test. Cryptococcal antigens were detected by a latex fixation test, and a complement fixation test (Littman and Walter, 1968).

Circulating cryptococcal antigens have been demonstrated in the serum

and spinal fluid of patients with meningeal and disseminated cryptococcosis at the time their cultures are positive. Antigen levels declined during the recovery phase of the disease when circulating antibodies appeared. Appearance of antibodies is thus considered to be a favorable prognostic sign. Antibodies were also encountered in patients with early or low grade infections, or with solitary pulmonary lesions, or shortly after therapy with amphotericin B. These findings tend to disprove the theories of the nonantigenicity of Cryptococcus and immunologic "paralysis." They support the concept of antibody neutralization or a masking effect caused by an abundance of circulating antigens (Littman and Walter, 1968).

Skin hypersensitivity to C. neoformans has been reported in man, but an adequate skin testing antigen is still unavailable (Littman and Walter, 1968). In a recent study, using an improved cryptococcin antigen prepared by Bennett and Atchison of the National Institute of Health, it was reported that 31% of a group of pigeon breeders showed dermal sensitivity reactions to this antigen as compared to 4% in a control group with little or no contact with pigeons (Newberry, et al, 1967).

#### Techniques for isolation of Cryptococcus neoformans from nature

Emmons reported isolating the fungi from soil samples and pigeon excreta samples by making a sample suspension in physiological saline and injecting white Swiss mice intraperitoneally with 1 ml of the suspension. Penicillin and streptomycin were incorporated in the pigeon excreta suspension. Mice were killed 4 to 6 weeks after inoculation and their spleens and livers cultured on modified Sabouraud's agar slants. Pigeon excreta was also cultured directly onto agar slants and incubated at 37 C (Emmons, 1951, 1955, and 1960).

Appendix I, Section 2. Review of the literature.

Histoplasma capsulatum

History

A study of the monograph on histoplasmosis by Negróni (1965) and a special article on the history of histoplasmosis by Schwarz and Baum (1957) provided an excellent historical résumé.

In 1905 Dr. Samuel T. Darling, a pathologist working in Panama, observed protozoan-like bodies resembling leishmania in autopsy material from a man who, at autopsy, showed generalized involvement by the disease with hepatosplenomegaly and anemia. The organism had multiplied inside of large mononuclear cells in the spleen, liver, lung, bone marrow, and lymph nodes. He made two similar observations the following year (1906) and realized it was a new disease. He named the organism Histoplasma capsulatum and the disease histoplasmosis.

Richard P. Strong, in the Philippine Islands, observed and described organisms consistent with H. capsulatum before the report of Darling's observation. Strong thought the organism was H. farciminosum. Because Darling's descriptions were so complete, Strong's observation has been completely overshadowed (Schwarz and Baum, 1957).

In 1912-1913, da Rocha-Lima studied Darling's histologic preparations and concluded that H. capsulatum was a fungus rather than a protozoa. He emphasized the staining properties and the presence of budding forms (Schwarz and Baum, 1957).

In 1932 Dr. Katherine Dodd asked Dr. Edna H. Tompkins to study a blood smear of a child suffering from an unusual anemia. Dr. Tompkins had previously studied a tissue section from Darling's original material and

recognized the organism in the circulating monocytes on the blood smear. Her finding was immediately corroborated by Drs. Meleney and Goodpasture. This was the first ante-mortem diagnosis of human histoplasmosis (Schwarz and Baum, 1957).

Dr. William A. DeMonbreun, alerted to the case by Dr. Tompkins, cultured the organism from the spleen of the sick child. His description of the growth characteristics, morphology, and nutritional requirements of H. capsulatum was a masterpiece (DeMonbreun, 1934). Dr. DeMonbreun fulfilled Koch's postulates using experimental animals, and also showed that H. capsulatum is a dimorphic fungus with a pathogenic yeast phase and a saprophytic mycelial phase. He concluded that the saprophytic form probably existed free in nature (Negroni, 1965; Schwarz and Baum, 1957).

Histoplasmosis was considered to be a rare disease for many years. Dr. Amos Christie in 1934 noted the occurrence of pulmonary calcifications in the absence of positive tuberculin test, and Dr. Charles E. Smith suggested that histoplasmosis might be the cause. Christie skin tested 180 children during 1944 and many reacted positively to histoplasmin though signs of infection were not apparent (Christie and Peterson, 1945).

Dr. Carroll E. Palmer, an officer in the U. S. Public Health Service, after a visit with Christie in 1945, immediately obtained histoplasmin and conducted a nationwide project of skin testing of student nurses. He discovered a great variation in histoplasmin skin sensitivity between persons from different regions in the United States. An overwhelming majority of persons tested by Palmer (and by Christie) were asymptomatic, strengthening an earlier belief of DeMonbreun that histoplasmosis was fairly common and occurred in a mild, nonfatal form (Schwarz and Baum, 1957).

Many other investigators have contributed to the volume of knowledge about histoplasmosis, including Dr. M. L. Furcolow who studied many epidemics and found that exposure to spore-laden dust was a major factor (Furcolow and Grayston, 1952; Grayston and Furcolow, 1953); and Dr. C. W. Emmons who was the first to culture H. capsulatum from soil (Emmons, et al, 1949).

### Epidemiology

Histoplasmosis occurs around the world in the temperate and tropical zones, and has been reported from more than 30 countries (Emmons, et al, 1963). In the United States the Central and Eastern States appear to be the most densely infected, and includes the states of Tennessee, Kentucky, Arkansas, Missouri, Indiana, and certain regions of Ohio, Illinois, Kansas, and Louisiana. In these areas up to 90 per cent of adults react positively to histoplasmin, an index which decreases rapidly in adjacent areas (Negroni, 1965).

Ecological and epidemiological studies have established that classical histoplasmosis is an airborne disease and that H. capsulatum lives as a saprophyte in soil. Emmons was the first investigator to isolate the organism from soil (Emmons, et al, 1949). He also found the presence of tuberculate spores in the soil showing that H. capsulatum grows actively as a saprophyte in soil.

Histoplasma capsulatum has specific ecological requirements for growth in soil it was discovered by Zeidberg and associates (Zeidberg, et al, 1952; Zeidberg and Ajello, 1954). They found the fungus most often in and around chicken habitats. H. capsulatum has also been found in soil enriched with droppings of other birds: grackles (Quiscalus quiscula), oil

birds (Steatornis caripensis), pigeons (Columbia livia), and starlings (Sturnus vulgaris). The factors which influence this association with avian habitats have yet to be discovered (Ajello, 1967).

Natural infections among birds have not been demonstrated, so the relationship to H. capsulatum is indirect. It is believed that soil enriched with avian feces provides conditions in which the fungus thrives and produces its infectious spores in abundance. When the soil dries, the spores are disseminated in the air, seeding other suitable habitats or infecting animals and people who inhale the spores (Wolstenholme and Porter, 1967). The occurrence of H. capsulatum spores in air was demonstrated when the fungus was isolated from the air in chicken coops in Kansas and Missouri (Iback, Larsh and Furcolow, 1954).

Bat habitats also favor development of H. capsulatum. The fungus has been isolated from bat guano many times (Ajello, et al, 1962; Ajello, et al, 1967; Emmons, 1958). It has also been successfully isolated from infected bats, and in the Americas, 14 species of bats have yielded this fungus (Ajello, et al, 1967). However bats, like birds, are thought to serve only an indirect role in the transmission of histoplasmosis.

#### Mycology

Histoplasma capsulatum is a dimorphic fungus whose natural habitat is soil or composted plant material enriched by fecal or other nitrogenous substances (Emmons, et al, 1963). It grows as a mold in the soil and on culture media held at room temperature (25 C), but transforms into a yeast-form in living tissues or on culture media at 37 C (Negroni, 1965).

The mold form on culture media appears white and cottony, but when much sporulation occurs the cultures become velvety textured and cinnamon



colored. The reverse side of the culture remains uncolored (Negroni, 1965). Most isolates grow slowly, requiring at least 10 to 14 days incubation before the development of a distinct colony (Ajello, et al, 1963).

The saprophytic mold form exhibits branching, septate hyphae which develop two types of spores; microconidia and macroconidia. The microconidia usually appear first, and are sessile or stalked, round to pyriform structures measuring 2 to 6 microns in diameter. Most of these small spores are smooth, but a few may be echinulated. Some of them may bud forming one or more secondary microconidia. The macroconidia are large (7 - 25 microns), thick-walled, round or pyriform spores borne on short, lateral conidiophores, or sessile on the side of the hyphae. Immature macroconidia are smooth or echinulated, but after further development they become tuberculate with numerous blunt projections, 1 to 8 microns in length, radiating from the surface. The presence of these tuberculate macroconidia are considered diagnostic for H. capsulatum. Sepedonium spp. may also form pyriform tuberculate spores, but these saprophytic fungi are monomorphic molds, and they do not form microconidia (Negroni, 1965; Ajello, et al, 1963).

The yeast phase of H. capsulatum, grown on suitable media at 37 C, will appear white to cream-colored, pasty, and cerebriform. Microscopically one sees small (2 - 4 microns), oval, single-budding blastospores (Negroni, 1965; Army TM 8-277-8). If viewed during the conversion stage the fungus will appear as bead-like chains of elongate cells which fragment to form individual yeast cells. Some budding will be seen (Ajello, et al, 1963).

The tissue phase of H. capsulatum appears as small, round or oval yeast cells, one to four microns in diameter, usually located inside of

mononuclear cells. The appearance of the fungus varies with the staining method used (Ajello, et al, 1963):

a. Giemsa or Wright stain. A pale, light blue ring indicates the cell wall which surrounds the darker blue cell protoplasm. A clear space is seen between the protoplasm and cell wall, the result of shrinkage of the protoplasm. This halo effect is the so-called "capsule" of H. capsulatum.

b. Periodic-Acid-Schiff stains. The cell walls are clearly stained pink to purplish red. The poorly stained protoplasm fills the entire cell so that no "capsule" effect is noted. (In the Gomori methenamine-silver stain the cell walls stain black.)

c. Hematoxylin-Eosin stain. H. capsulatum stains very poorly and is difficult to differentiate from surrounding tissues according to Ajello, et al (1963). However, Emmons, et al (1963), report that the fungus cells can readily be seen with hematoxylin-eosin stain.

The mycelial phase of H. capsulatum develops at 25 C on a variety of simple media, and Sabouraud dextrose agar is an excellent sporulation media for most strains. This characteristic development will be inhibited though, if serum or blood is added to the media, or if incubated at temperatures higher than 32 C (Ajello, et al, 1963).

The yeast-phase is highly fastidious requiring enriched media, incubation at 37 C, and the presence of adequate moisture. A variety of enriched media have been recommended, including brain-heart infusion agar (BHI), BHI with blood added, and liver-spleen glucose blood agar (Ajello, et al, 1963).

H. capsulatum isolations should not be attempted on media containing

cycloheximide if incubated at 37 C, as no growth or very poor growth is obtained. If cycloheximide is necessary for suppression of saprophytic fungi, the cultures should be incubated at 25 C (McDonough, et al, 1960).

#### Pathology

H. capsulatum has a predilection for reticuloendothelial tissue, and is seen microscopically as an endoparasite within cells of this system. Primary lesions are in the lungs where one or more granulomatous nodules, closely resembling tuberculous lesions, form in the lungs and peribronchial lymph nodes (Hildick-Smith, et al, 1964). These lesions usually become calcified in 3 to 5 years (Ajello, et al, 1963) and are visible on thoracic radiographs (Wolstenholme and Porter, 1967). In the vast majority of cases, the infection is benign, self-limiting and inapparent, and revealed only by a positive skin test to histoplasmin. A few pulmonary infections become chronic and result in fibrocaseous lesions with cavity formation in the upper lobes (Dubos and Hirsch, 1965).

The fungus may disseminate throughout the body from the primary pulmonary infection via the circulatory system. Lesions occur chiefly in those organs or tissues which are rich in reticuloendothelial cells, especially liver, spleen, adrenals, lymph nodes, mucous membranes of the mouth or gastrointestinal tract, and bone marrow. There is a greater than random association between disseminated histoplasmosis and lymphoma, leukemia and Hodgkin's disease (Emmons, et al, 1963).

#### Allergy and Immunity

Two or three weeks after primary infection, a person becomes hypersensitive to the antigens of H. capsulatum. This allergy can be revealed

by intradermal injection of histoplasmin which causes a delayed tuberculin-type positive reaction. Histoplasmin is the sterile, standardized extract of liquid cultures of the fungus (Negroni, 1965). Some sensitized people will also cross react with blastomycin and coccidioidin, therefore skin test procedures should include simultaneous use of these antigens to determine which antigen provokes the most significant reaction (Hildick-Smith, et al, 1964).

Circulating antibodies are also produced, and precipitins, agglutinins, and complement fixing antibodies may be demonstrated, depending on the severity of the infection. Complement fixing antibodies usually appear last but persist for longer periods. The presence of complement fixing antibodies denotes a good prognosis while their persistence is not a good sign (Hildick-Smith, et al., 1964).

#### Isolation of H. capsulatum from soil

Emmons, when he first isolated H. capsulatum from soil, modified the technique used to isolate C. immitis (Stewart and Meyer, 1932). He prepared a 1:10 mixture of dirt in sterile saline, agitated the mixture, then let the suspension settle for 1/2 to 2 hours. He injected 1 ml aliquots of the supernatant liquid into the peritoneal cavity of white Swiss mice. The mice were sacrificed in 3 to 5 weeks and culture media seeded with pieces of liver and spleen (Emmons, 1949).

Larsh, et al (1953), studied the efficiency of this method and concluded it was not satisfactory because the spores adhere to the soil particles. It had previously been thought that spores of H. capsulatum would rise toward the top of the saline solution. The authors modified Emmon's technique by injecting mice with 1 ml aliquots of the 1:10 suspension,

not allowing the soil to settle. Also, the physiological saline used for soil suspension contained penicillin and streptomycin at 8,000 units of each per milliliter.

Larsh, et al (1956), found that the use of mucin as a vehicle for the inoculum suspension was not advisable since it added greatly to mouse mortality following injection.

Klite, et al (1965), reported a new soil sampling technique utilizing three filtration steps to reduce the soil particle size remaining in the physiological saline suspension. In the final filtration step a millipore filter ( $1.5 \pm 0.5$  micron porosity) was employed which allowed the original suspension liquid to be discarded along with any toxic substance which might have dissolved in it. The soil was resuspended from the filter pad into fresh saline which contained 10,000 units of penicillin and 0.1 mg streptomycin per milliliter. As in other techniques, mice were injected with 1 ml portions of the final suspension, and finally sacrificed so that livers and spleens could be cultured.

Smith and Furcolow (1964) also developed an isolation method in which saline suspensions of soil were mixed with sterile liquid petrolatum and blended. The mixture was allowed to separate in a separatory funnel where most of the soil remained in the water phase, while organic material separated into the oil phase. The oil phase was collected, remixed with saline, and centrifuged to force the organic material into the saline. The oil was discarded and the saline treated with penicillin (10,000 units/ml) and streptomycin (4,000 micrograms/ml). Mice were injected I.P. and necropsied after 4 weeks. Media was inoculated with liver and spleen.

## Appendix I, Section 3: Literature cited.

Cryptococcus neoformans

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A SURVEY OF ZOO AVIARIES FOR THE PRESENCE OF  
HISTOPLASMA CAPSULATUM AND CRYPTOCOCCUS NEOFORMANS

by

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Cryptococcus neoformans and Histoplasma capsulatum are fungal pathogens that exist primarily as saprophytes in nature and have been discovered mostly around avian habitats. Man and animals become infected primarily by inhaling the fungal spores. C. neoformans shows a marked affinity for pigeon excreta. H. capsulatum has been recovered from soils enriched with feces from chickens, grackles, oil birds, pigeons, and starlings. Other avian species with which these pathogens may be associated have remained undiscovered, therefore, a zoo's aviaries containing many avian species were surveyed for the presence of C. neoformans and H. capsulatum.

Samples of dried feces were collected from 6 indoor and 8 outdoor aviaries. Attempts to isolate C. neoformans were made by direct culture at 37 C on diphenyl agar and modified Littman oxgall agar with chloramphenicol added. C. neoformans was isolated from 2 of the samples. One sample was from an indoor aviary housing a White-breasted Toucan. The other sample was from an indoor aviary housing two Concave-casqued Hornbills.

Soil samples were collected in 12 outdoor aviaries and tested for H. capsulatum and C. neoformans by direct and indirect cultural methods. Soil was suspended by a new method in physiological saline containing antibiotics. White Swiss mice were injected IP, and after 4 weeks the mice were sacrificed. Portions of liver and spleen were triturated and cultured on Sabouraud dextrose agar with chloramphenicol and cycloheximide, and brain-heart infusion agar containing the same antibiotics. Media was incubated at 25 C. Sections of liver and spleen were also subjected to histopathological examination. H. capsulatum and C. neoformans were not isolated from the soil samples.

Fresh feces samples were collected from the two aviaries where

C. neoformans had been previously isolated in dried feces. Tests for C. neoformans were made by direct cultural methods, but the organism was not isolated from fresh feces.