Occurrence of Azole Resistant and Melanin Producing *Cryptococcus Neoformans* in Wild Birds Kept in a Zoological Garden

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ABSTRACT

Birds kept in zoological gardens and aviaries are aesthetically pleasing but may act as pollinators for pathogenic fungi such as *Cryptococcus neoformans*. Occurrence of cryptococcosis in human is associated with exposure to the avian droppings. The preference of avian species as hosts has not been fully explored for *C. neoformans*. The present study was undertaken to detect the occurrence and antifungal susceptibility of *C. neoformans* in the excreta of wild birds kept in a zoological garden in India. A total of 161 pooled weathered dry and moist droppings were collected from apparently healthy caged wild birds during summer. The study detected occurrence (5.59%) of *C. neoformans* in blue and yellow macaw, chattering lory, pigeons, Chinese silver pheasant, kalij pheasant, ring necked pheasant and golden pheasant. All the isolates produced mucoid creamy colonies in Sabouraud's dextrose agar (SDA) and Corn meal agar-Tween 80, and brown coloured colonies in bird seed agar. Negative staining with India ink showed characteristic encapsulated budding yeasts with blastoconidia. None of the isolates exhibited any growth in the Sabouraud's dextrose agar with cycloheximide or produced urease. All of the isolates produced *CNLAC1* outer gene in PCR. The MIC study revealed resistance to amphotericin B, ketoconazole, fluconazole, itraconazole, and flucytosine. Occurrence of *C. neoformans* in Chinese silver pheasant, kalij pheasant, ring necked pheasant, and golden pheasant has not been reported previously. The results are important from a public health point of view as the zoological garden is often situated in a crowded location and is frequently visited by children and the elderly.

Keywords: *Cryptococcus*; Macaw; Lory; Pheasant; Summer.

INTRODUCTION

Wild birds are established sources of zoonoses including the fungal hazards such as cryptococcosis, aspergillosis, and candidiasis (1). The basidiospores generated from the yeast can lodge into the lung alveoli of susceptible human population and in a situation of compromised health will flare up with multiplication and invasion into the central nervous system (2). Currently cryptococcal meningitis has become more prevalent throughout the world with an estimated 181,000 annual deaths especially in patients with compromised immunity (AIDS, lymphoma, hematologic malignancy and prolonged use of corticosteroids), and was found responsible for 15% of AIDS-associated deaths (3).

The genus *Cryptococcus* consists of 37 species including *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii* and *C. gattii* as major pathogens (4). *C. neoformans* remains viable in avian excreata specially of pigeons (*Columba livia*) for prolonged periods of time with occasional mating due to presence of creatinine, urea, uric acid, moisture, and protection from sunlight and ultraviolet ray (5, 6). *C. gattii* can also

grow in pigeon guano but is unable to mate and consequently it is not preferred as an ecological niche (7).

Whether the yeasts are pathogenic to pigeons is a debatable issue although the yeasts have been detected in the internal organs such as crop and external body parts that are exposed to the guano such as vent, feet and feathers of the pigeons (5, 8). *C. neoformans* var. *neoformans* and *C. gattii* have also been detected as a primary pathogen or commensal in other birds such as parakeets, thick billed parrots, ring necked parrot, African grey parrot, green-winged macaw, cockatoo, kiwi, Papua lories, blackcapped lories throughout the world especially in tropical and sub-tropical countries (9, 10, 11, 12, 13). The affinity of *Cryptococcus* to certain avian species has been correlated to the nutrient composition of their guano, and studies have revealed that the feces of seabirds and chicken were inhibitory to the growth of the yeasts (7).

The occurrence of cryptococcosis in human is associated with exposure to the avian droppings in immunocompromised patients and even in immunocompetent persons throughout the world including Kolkata (India) where the study was undertaken (14, 15, 16, 17, 18). The zoonotic transmission becomes more complicated if accompanied by antifungal resistant determinants. Resistance to azole fungicides has been detected in the environmental isolates of *C. neoformans* due to common use of fungicides in agriculture (19).

The birds kept in zoological gardens and aviaries are aesthetically pleasing but may act as sources for pathogenic fungi especially in crowded places. Moreover, the preference of avian species as hosts has not been fully explored in *C. neoformans*. The present study was undertaken to detect the occurrence and antifungal susceptibility of *C. neoformans* in the excreta of wild birds kept in a zoological garden situated in a congested location in Kolkata (India).

MATERIALS AND METHODS

Animal Ethics Committee

The study was approved by the Institutional Animal Ethics Committee.

Sampling

A total of 161 pooled weathered dried and moist droppings were collected from apparently healthy caged wild birds such as bare eye cockatoo, gray parrot, chattering lory, blue and yellow mackaw, pigeons, pheasant, hill mynah, kept in a zoological garden in Kolkata (India) in sterile plastic zipper bags during March-April (Table 1).

The birds were housed in individual cages according to the family/species and the cages were situated close to each other. The cages were cleaned daily with detergent water (Trixie Cage cleaner, Texas) and weekly with a disinfectant such as copper sulphate at a concentration of 500mg/L. The birds were offered seeds, grains, fruits and small insects according to their preferences. The birds had no history of antifungal exposure for purposes of therapy.

Droppings were collected at the early morning before the daily cleaning of the cages. During the collection, the staff used masks and protective clothing. All the samples were brought into the laboratory within 15-20 minutes in ice packs and were processed within 6 to 8 hours after collection. Approximately 1 gm of each dropping was added to wide mouth sterile glass test tubes containing 20 ml of sterile distilled water with chloramphenicol (40 mg / 500 ml, chloramphenicol selective supplement, HiMedia, India) and was vortexed for 10 minutes to homogenize. The chloramphenicol was added to eliminate any bacterial contamination.

Isolation and identification of *Cryptococcus neoformans*

The supernatant (100 µl) of the homogenized mixtures was collected and was inoculated into Sabouraud's dextrose agar (SDA, HiMedia, India) after serial dilutions. The plates were incubated at 28°C for 2-7 days. Creamy white mucoid colonies appeared in SDA which were further streaked onto bird seed agar (HiMedia, India). The brown colonies appearing in birdseed agar was stained with India ink for the detection of encapsulated budding yeast cells. The sub-culturing was carried out on corn meal tween 80 agar (HiMedia, India). The isolates were phenotypically classified by a variety of biochemical tests such as urease test, sugar fermentation test, growth in presence of cycloheximide, assimilation test, and growth in L-canavanine-glycine–bromothymol blue (CGB) agar (HiMedia, India) (20).

PCR based characterization of *C. neoformans*

The chromosomal DNAs of all the biochemically confirmed *C. neoformans* isolates were extracted using a commercial kit (Genei, Merck). PCR for *CNLAC1* gene was carried out with 35 cycles consisting of 94°C for 1 minute, 57°C for 30 seconds and 72°C for 90 seconds with initial denaturation at 94°C for 5 minutes and final extension at 72°C for 7 minutes

Table 1: Distribution of *C. neoformans* in wild birds kept in a zoological garden in India

Birds	Numbers of pooled samples collected	*C. neoformans* isolates
Bare eyed cockatoo (*Cacatua sanguinea*)	4	0
Grey parrot (*Psittacus erithacus*)	10	0
Moluccan cockatoo (*Cacatua moluccensis*)	2	0
Blue and yellow macaw (*Ara ararauna*)	16	2
Red and blue macaw (*Ara chloroptera*)	2	0
Citron crested cockatoo (*Cacatua sulphurea citrinocristata*)	2	0
Goffin cockatoo (*Cacatua goffini*)	3	0
Yellow headed amazon parrot (*Amazona oratrix*)	1	0
Chattering lory (*Lorius garrulous*)	4	1
Eclectus parrot (*Eclectus roratus*)	2	0
Bhutan peacock Pheasant (*Polyplectron bicalcaratum)*	4	0
Reeve's pheasant (*syrmaticus reevesii*)	3	0
White parakeet (*Psittacula spp.*)	5	0
Pigeon (*Columba livia domestica*)	11	2
Blue parakeet (*Psittacula spp.*)	3	0
Black swan (*Cygnus atratus)*	2	0
Latino parakeet (*Psittacula spp.*)	7	0
Lady amherst's Pheasant (*Chrysolophus amherstiae*)	1	0
Western crowned Pigeon (*Goura cristata*)	2	0
Chinese silver Pheasant (*Lophura nycthemera*)	5	1
Indian pied hornbill (*Buceros bicornis*)	1	0
Kalij pheasant (*Lophura leucomelanos*)	4	1
Jungle fowl (*Gallus* spp.)	1	0
Ring necked pheasant (*Phasianus colchicus)*	2	1
Rosy pelican (*Pelecanus onocrotalus*)	1	0
Golden pheasant (*Chrysolophus pictus*)	5	1
Emu (*Dromaius novaehollandiae*)	4	0
Cockatiel (*Nymphicus hollandicus*)	10	0
Peacock (*Pavo cristatus)*	4	0
Australian budgerigars (*Melopsittacus undulatus*)	9	0
Asian openbill stork (*Anastomus oscitans*)	3	0
Greater adjutant (*Leptoptilus dubius*)	2	0
Alexandrine parakeet (*Psittacula eupatria*)	4	0
Dove (*Zenaida macroura*)	3	0
Green pigeon (*Treron* sp.)	5	0
Parrot (*Psittacula krameri*)	5	0
Hill mynah (*Gracula religiosa*)	5	0
Spoonbill (*Platalea* sp.)	4	0
Total	**161**	**9**

(21). The amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 1.2% (W/V) agarose (SRL, India) gel containing ethidium bromide (0.5µg/ ml) (SRL, India).

Detection of minimum inhibitory concentration (MIC) for antifungals

For detection of MIC, the confirmed samples, 48 hours after growth of *C. neoformans* colonies in SDA plates were homogenized in 0.85% NaCl to achieve 1 McFarland turbidity (22). The homogenized mixture for each colony was spread evenly over the media (RPMI 1640, glucose and bacteriological agar) with sterile cotton swabs (HiMedia, India). The MIC strips of fluconazole, itraconazole, ketoconazole, voriconazole, amphotericin B, posaconazole, flucytosine and caspofungin (EZY ™ MIC strips, HiMedia, India) were used for detection of antifungal sensitivity of the isolates. The agar plates with MIC strips were incubated at 35°C for 2 to 3 days (22). The interpretation of MIC was performed according to the manufacturer's instructions.

RESULTS

The present study detected the occurrence (9/161, 5.59%) of *C. neoformans* in the captive wild birds (blue and yellow macaw, chattering lory, pigeons, Chinese silver pheasant, kalij pheasant, ring necked pheasant, golden pheasant) kept in a zoological garden in Kolkata, India. All the nine isolates produced mucoid creamy colonies in SDA and corn meal agar-Tween 80, and brown coloured colonies in bird seed agar (Figure 1). Negative staining with India ink revealed characteristic encapsulated budding yeasts with blastoconidia (Figure 2).

All the isolates did not exhibit any growth in the Sabouraud's dextrose agar with cycloheximide or produced urease. All of them produced fermentation of glucose, galactose, sucrose, cellobiose, trehalose, raffinose, L-arabinose, and inositol. None of the sample produced a blue colour on the CGB agar, which would have been indicative of

Figure 1: Brown coloured colonies of *Cryptococcus neoformans* in bird seed agar isolated from macaw in India

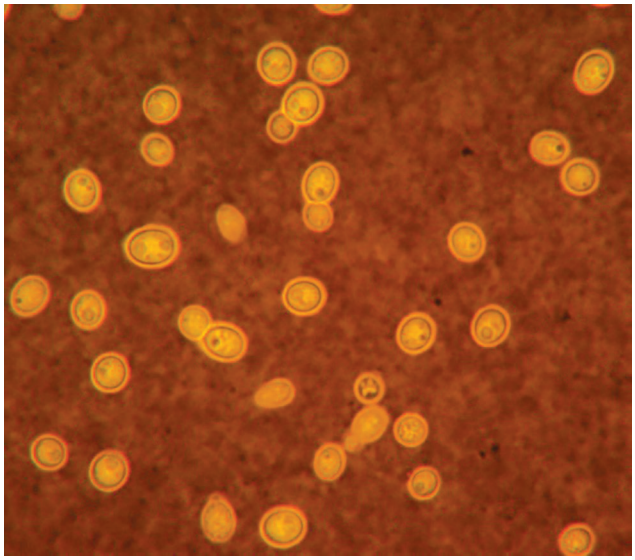

Figure 2: India ink staining showing encapsulated budding *Cryptococcus* with blastoconidia

C. neoformans var. *gattii.* Moreover, all of the nine isolates produced *CNLAC1* outer gene (1200 bp) by PCR.

The MIC study revealed resistance to amphotericin B (0.03-32 µg/ml), ketoconazole (0.064-1.5 µg/ml), fluconazole (1.5->256 µg/ml), itraconazole (0.03-1.5 µg/ml), and flucytosine (0.094->32 µg/ml) (Figure 3). All the isolates were found susceptible to voriconazole and most of the isolates were susceptible to posaconazole. Few isolates showed intermediate resistance to posaconazole.

Figure 3: Minimum inhibitory concentration (MIC) study of azole antifungals against *Cryptococcus neoformans*

DISCUSSIONS

The present study detected the occurrence of (5.59%) *Cryptococcus neoformans* in pooled excreta samples collected from the wild birds kept in a zoological garden in Kolkata, India. Earlier studies suggested collection of pooled faecal samples from migratory birds for better isolation of *C. neoformans* (23). The occurrence rate of *C. neoformans* varied between 0-2% in the excreta of wild birds collected from the aviaries, rescue centres and clinics in different countries such as in Italy, Malaysia, Austria, and the Czech Republic (24, 25, 26). The lower occurrence (0-1.8%) of *C. neoformans* was detected in different internal organs such as crop, lower part of the intestine, cloacal swabs in wild migratory birds and pigeons due to high body temperature and high concentration of ammonia in the fresh avian faeces both of which are inhibitory to the yeast growth (5, 27, 28, 29). Collection of pooled samples and their processing immediately after collection was carried out due to the distance between the zoological garden and the departmental laboratory and were the probable reasons of higher isolation rate in the present study where the laboratory is situated close-by.

The occurrence of yeasts in cloaca of migratory and wild birds depends on several factors such as habitat in swampy areas and preference of vegetables and seeds as observed in coots, teals and garganeys (28). Whereas food habits with the preference for insects as detected in woodcocks was inversely correlated with the occurrence of yeasts in cloaca (28).

The present study detected the occurrence of *C. neoformans* in blue and yellow macaw, chattering lory, pigeons, Chinese silver pheasant, kalij pheasant, ring necked pheasant, and golden pheasant. Occurrence of *C. neoformans* in pigeons, macaw and lory was detected previously in several studies (13) but no study in Chinese silver pheasant, kalij pheasant, ring necked pheasant, and golden pheasant was to the best knowledge of the authors available for comparison. The food habits of golden pheasants and ring-necked pheasants was mostly seed and cereal based especially during the summer season when the samples were collected that might have been the reason for the occurrence of *C. neoformans* in the cloaca (30).

The *CNLAC1* gene harboured by *C. neoformans* isolates is considered as a laccase associated gene required for melanin production and it is possessed by all pathogenic varieties of *C. neoformans* (21). All the isolates in the present study produced brown coloured colonies in birdseed agar and possessed *CNLAC1* gene in PCR, which suggested their capacity of melanin production. Production of melanin by *C. neoformans* isolates acts as a marker for pathogenic strains as is correlated with central nervous system invasion where the dopamine is used as a substrate (31).

The MIC study of *C. neoformans* isolates revealed resistance to amphotericin B (0.03-32 µg/ml), ketoconazole (0.064-1.5 µg/ml), fluconazole (1.5->256 µg/ml), itraconazole (0.03-1.5 µg/ml), and flucytosine (0.094->32 µg/ml). The MIC cut-off value of *C. neoformans* was described earlier for itraconazole (0.12 µg/ml), posaconazole (0.25 µg/ml) and fluconazole (8 µg/ml) (32). Resistance to azoles as detected in the present study was probably associated with contaminated seeds and cereals used as feed, as the birds did not receive any antifungal therapy. Similarly, the environmental isolates of *C. neoformans* showed resistance against azole compounds due to its common use in agriculture in Brazil (19).

The present study identified the occurrence of pathogenic and azole resistant *C. neoformans* in wild birds such as blue and yellow macaw, chattering lory, pigeons, Chinese silver pheasant, kalij pheasant, ring necked pheasant, and golden pheasant kept in a zoological garden. Occurrence of *C. neoformans* in Chinese silver pheasant, kalij pheasant, ring necked pheasant, and golden pheasant has not been reported prevously. The results are interesting from the sociological and public health point of view as the zoological garden is situated in a crowded place and is frequently visited by children and the elderly who are considered as at high risk for transmission of mycotic zoonoses.

ACKNOWLEDGEMENTS

The authors express sincere gratitude to the Honorable Vice Chancellor for infrastructure and the Indian Council of Agricultural Research (ICAR) for the necessary funds under the project entitled 'Outreach Programme on Zoonotic Diseases'.

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