การเฝ้าระวังเชิงรุกของเชื้อ *Cryptococcus neoformans* ในสวนสัตว์นครราชสีมา ประเทศไทย

Active surveillance of *Cryptococcus neoformans* in Nakhon Ratchasima Zoo, Thailand

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บทคัดย่อ

Cryptococcus neoformans เป็นเชื้อราก่อโรค cryptococcosis ทั้งในคนและสัตว์ เชื้อชนิดนี้มักพบในมูลนก การติดเชื้อจากสัตว์สู่คนโดยการหายใจเอาเชื้อเข้าไปทำให้เกิดปอดอักเสบ เชื้อสามารถแพร่กระจายไปที่ระบบประสาทส่วนกลางทำให้เกิดโรคเยื่อหุ้มสมองอักเสบซึ่งอาจเป็นอันตรายถึงชีวิต เพื่อเป็นการเฝ้าระวังและป้องกันการแพร่กระจายของเชื้อชนิดนี้ในสวนสัตว์นครราชสีมา ประเทศไทย ทั้งจากนกสู่นกหรือนกสู่คน งานวิจัยนี้ได้สำรวจอุบัติการณ์ของเชื้อ *C. neoformans* ในมูลนกชนิดต่างๆ ของสวนสัตว์นครราชสีมาในช่วงเดือนเมษายน พ.ศ. 2564 โดยเก็บมูลนกสดในกรงเลี้ยงนก 29 ชนิด ของนกในกลุ่มต่างๆ ได้แก่ นกกระเรียน 5 ชนิด ไก่ฟ้า 8 ชนิด นกเงือก 4 ชนิด นกแก้ว 4 ชนิด นกกระสา 3 ชนิด นกกระทุง 1 ชนิด นกล่าเหยื่อ 1 ชนิด รวมทั้งนกบินไม่ได้ ได้แก่ นกอีมู นกคาสโซวารีและนกเพนกวิน อย่างละ 1 ชนิด ได้จำนวนมูลนก 67 ตัวอย่าง นำมาเพาะแยกเชื้อบนอาหารเลี้ยงเชื้อ Sabouraud dextrose agar (SDA) ที่เติมยาคลอแรมเฟนิคอลและสารสกัดจากเมล็ดทานตะวัน พบว่ามูลนกทั้งหมดตรวจไม่พบเชื้อ *C. neoformans* แต่อย่างไรก็ตาม การเฝ้าระวังเชื้อ *C. neoformans* ในสวนสัตว์ควรดำเนินการศึกษาอย่างต่อเนื่อง

คำสำคัญ: *Cryptococcus neoformans*, Cryptococcosis, Sabouraud dextrose agar (SDA), สารสกัดจากเมล็ดทานตะวัน

Abstract

Cryptococcus neoformans is an important pathogenic fungus causing cryptococcosis in humans and animals. This pathogen is usually found in avian droppings. Zoonotis infection occurs by inhalation of the fungus into the respiratory tract and causes pneumonia. The fungus can disseminate to the central nervous system and cause meningitis which can be life threatening. For active surveillance and prevention of *C. neoformans* in Nakhon Ratchasima Zoo, Thailand, either from bird to bird or bird to person, the present study investigated the incidence of *C. neoformans* in droppings of various captive birds in this zoo in April 2021. Dropping samples were collected from 29 avian species in various taxonomic groups which comprised cranes (5 species), pheasants (8 species), hornbills (4 species), parrots (4 species),

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storks (3 species), pelican (1 species), vulture (1 species), and flightless birds including emu, cassowary, and penguin (1 species each). A total of 67 fresh avian dropping samples were isolated on Sabouraud dextrose agar (SDA) with chloramphenicol and sunflower seed extract. No colony of *C. neoformans* was found in all samples. Nevertheless, active surveillance for *C. neoformans* should be performed continuously.

Keywords: *Cryptococcus neoformans*, Cryptococcosis, Sabouraud dextrose agar (SDA), Sunflower seed extract

Introduction

Cryptococcus neoformans is an important pathogenic encapsulated yeast which causes cryptococcosis in humans and animals (Maziarz & Perfect, 2016). This fungus is found worldwide in environments such as soil, decaying wood, tree hollows, and avian droppings which results in potential zoonotic transmission (Rosario *et al.*, 2008). Infection commences after inhaling its basidiospores into the lungs of the host. The spores are deposited in alveoli and terminal bronchioles and cause pneumonia. The fungus can disseminate to the central nervous system and cause meningitis which can be life threatening especially in HIV/AIDS patients (Maepa, 2019). The incidence of cryptococcosis in AIDS is high, around 18.5%, which is the third most common opportunistic infection in AIDS in Thailand following tuberculosis and pneumocystis pneumonia (Chariyalertsak *et al.*, 2001). The annual number with cryptococcal infection globally is high (almost 300,000) (Rajasingham *et al.*, 2017). In addition, *C. neoformans* resistant to antifungal drugs has been increasingly reported worldwide (Tangwattanachuleeporn *et al.*, 2013; Kobayashi *et al.*, 2005).

C. neoformans can be classified into 3 varieties with 5 serotypes based on polysaccharide capsular structure: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *gattii* (serotype B and C), *C. neoformans* var. *neoformans* (serotype D), and hybrid of serotypes A and D (serotype AD) (Levitz, 1998; Franzot *et al.*, 1999; Nelson & Lodes, 2006; Ito-Kuwa *et al.*, 2007). In 2002, *C. neoformans* var. *gattii* was reclassified as another species, *C. gattii*, which is associated with eucalyptus trees (Kwon-Chung *et al.*, 2002). According to previous studies, these two species have distinct differences in epidemiology, ecology, molecular patterns, geography, and clinical characteristics (Kwon-Chung *et al.*, 2014; Faganello *et al.*, 2009).

A zoo is a potential source of various infectious diseases such as bacterial, mycobacterial and fungal infections (Ahasan *et al.*, 2008). There are many reports showing that animals in zoos suffered from cryptococcosis. A report of Bauwens *et al.* (2004) revealed cryptococcosis in rodents and lizards of Antwerp Zoo, Belgium. All the isolated strains were identified as *C. neoformans* var. *neoformans* serotype A (Bauwens *et al.*, 2004). In Asia, a report of Ahasan *et al.* (2014) showed animals in Dhaka Zoo, Bangladesh, such as monkeys and antelopes suffered from cryptococcosis. Clinically, affected animals manifested frequent cough and anorexia, weakness followed by death. Necropsy findings showed granulomatous lesions of the affected organs (Ahasan *et al.*, 2014). There is a report of Caicedo *et al.* (1999) indicating that birds are reservoirs of *C. neoformans*. This investigation used a sunflower seed agar culture medium for fungus isolation which showed the presence of *C. neoformans* in bird excreta (prevalence = 0.9%) and in the air circulating inside cages (prevalence = 0.7%) in the City Zoo of Cali, Colombia. All isolates were *C. neoformans* var. *neoformans* (Caicedo *et al.*, 1999). In Thailand, there is a report of Keerativasee *et al.* (2008) about *C. neoformans* in avian droppings from Zoo. The periods of sample collection were in winter and summer (December 2005 to May 2006, respectively). A total of 97 avian dropping samples from 27 avian species in Chiang Mai Zoo showed a 1.03% prevalence of *C. neoformans*. One strain was isolated from red-billed hornbill droppings (Order Bucerotiformes) (Keerativasee *et al.*, 2008). Although there are many reports of associations of *C. neoformans* with avian species, cryptococcosis in birds is rare. A report of Griner and Walch (1978) demonstrated *C. neoformans* from digestive tract of healthy birds in San Diego Zoo, United States (Griner & Walch, 1978). According to these reports, *C. neoformans* rarely affects captive birds in zoos but can cause epizootic infections in other animals and zoonotic infections in humans.

There are several reports showing that pigeons (Order Columbiformes) are the major reservoir for *C. neoformans.* Their droppings and dusts are potential sources of *C. neoformans* transmission to other hosts. However, *C. neoformans* can be found in other birds such as passerine birds (Order Passeriformes), psittacine birds (Order Psittaciformes), Hornbills (Order Bucerotiformes) and others (Rosario *et al.*, 2008; Tangwattanachuleeporn *et al.*, 2013; González-Hein *et al.*, 2010; Caicedo *et al.*, 1999; Keerativasee *et al.*, 2008; Griner & Walch, 1978).

In laboratory, culture is the gold standard for cryptoccocosis diagnosis (Prariyachatigul *et al.*, 1996). *C. neoformans* can grow on most standard media used for fungal isolation except media with cycloheximide (Kwon-Chung & Bennett 1992). For fungal isolation according to Pal *et al.* (1990) SDA is a suitable medium for fungal culture (Pal *et al.*, 1990). Sunflower seed extract comprises phenolic and polyphenolic compounds as precursors of melanin pigment which can be utilized in phenoloxidase activity by cryptococcal yeasts which show as brown-pigmented colonies. (Krema *et al.*, 2018). Chloramphenicol can inhibit other organisms especially bacteria. SDA with chloramphenicol and sunflower seed extract is useful to identify and differentiate cryptococcal colonies from other yeasts (Pal *et al.*, 1990; Krema *et al.*, 2018). For confirmatory testing, matrix assisted laser desorption/ ionization-time of flight mass spectrometry (MALDI-TOF MS) was high accuracy and enables quick and easy identification of (McTaggart *et al.*, 2011).

Northeast region of Thailand was reported to have the highest percentage of cryptococcosis in AIDS patients in Thailand (Chariyalertsak *et al.*, 2001). Thus, this region is an interesting area in which to study *C. neoformans*. In 2021, the prevalence of *C. neoformans* was 1.2% in pigeon droppings at Nakhonratchasima College, Nakhon Ratchasima province, northeast region of Thailand (Thunyaharn *et al.*, 2021). This incidence emphasizes the occurrence of this fungus in Nakhon Ratchasima province. To extend the study of the incidence of *C. neoformans*, the present study was designed to perform active surveillance for this fungus in the Nakhon Ratchasima Zoo, located near the college, where might contain public environmental reservoirs of *C. neoformans* in Nakhon Ratchasima province.

Materials and methods

Sample collection

The fresh avian droppings were collected in cages of 29 captive bird species (Figure 1) from Nakhon Ratchasima Zoo, Thailand, during April 2021.

Each sample of avian droppings was at least 5 g collected using a sterile plastic spatula (Figure 2) and placed in a sterile plastic container labeled with site and date of collection (Figure 2).

Sample processing and isolation for *C. neoformans*

All samples were processed within 4 hours of collection. Approximately 1 gram of samples was suspended vigorously in 5 mL of saline solution (0.85% NaCl) with penicillin G (4 mg/mL) (Figure 2) and allowed to settle for 30 minutes at room temperature. Each sample was inoculated using a sterilized cotton swab and cultured on a selective medium which was Sabouraud dextrose agar (SDA) containing chloramphenicol (0.05 mg/mL) and sunflower seed extract (45 mg/mL) (Figure 2), incubated at 37 °C according to the method of Pal *et al.* 1990 (Pal *et al.*, 1990). Selective media were used for quality control by standard strains of *Candida albicans* ATCC10231 and *C. neoformans* ATCC90112 as the positive control, and *Escherichia coli* ATCC25922 as the negative control. The media were daily observed for brown mucoid colonies of *C. neoformans* for 3 days.

Identification

The suspected brown mucoid colonies, which grew on the selective medium, were picked up and sub-cultured on SDA. The pure cultures were identified by India ink preparation and urease test as well as the method of Canteros *et al.* (1996), and then *C. neoformans* and C. *gattii* were differentiated by culture on canavanine glycine bromothymol blue (CGB) agar following the method of Kwon-Chung *et al.* 1982 (Kwon-Chung *et al.*, 1982). According to the method of McTaggart *et al.* (2011) MALDI-TOF MS was used to identify species of organism. The colonies were smeared on the target and irradiated with the laser. The matrix absorbed power from the laser resulting in protein ionization. The detection ionized proteins spectra in the region 2,000 to 20,000 m/z were compared with protein spectra profile analysis to confirm identity as *C. neoformans* (McTaggart *et al.*, 2011).

Results and discussion

A total of 67 avian droppings from 29 captive bird species were collected from Nakhon Ratchasima Zoo as shown in Table 1. All samples showed colonies of various microorganisms but colonies of *C. neoformans* was absent (Figure 2). *C. neoformans* was not found in this study and might be considered for many reasons. First, samples were collected using plastic spatulas scooping on the fresh parts of avian droppings. The fresh droppings were collected as same as the study of Keerativasee *et al.* (2008) (Keerativasee *et al.*, 2008). However, the samples might contain various organisms which can grow alongside the fungus. To solve this problem, the samples were kept in sterile containers. Specimens were processed within 2 hours using saline solution with penicillin G to reduce bacterial contamination. This method is similar to that described in the study of Keerativasee *et al.* (2008) (Keerativasee *et al.*, 2008) which discovered *C. neoformans* in avian droppings. In the laboratory, isolation of *C. neoformans* by the culture method is the gold standard (Prariyachatigul *et al.*, 1996). According to the method of Pal *et al.* (1990) SDA with sunflower seed extract as a plant supplement supports growth of *C. neoformans,* preventing / limiting growth of other organisms from unsterilized samples. Therefore, this medium with the supplement is very sensitive and highly specific for *C. neoformans* (Pal *et al.*, 1990; Krema *et al.*, 2018). However, the present study revealed various organisms such as bacteria and other yeasts that grew on the media (Figure 2) and might inhibit *C. neoformans* according to the suggestion of Pal *et al.* (1990).

Besides, lack of a more extensive sampling might be another reason. The present survey was conducted in April representing the summer season. Interestingly, the presence of *C. neoformans* in avian droppings is related to seasonal variations. A study of Chae *et al.* (2012) reported significantly different prevalence of *C. neoformans* in avian droppings between fall and spring seasons (Chae *et al.*, 2012). However, Kuroki *et al.* (2004) reported *C. neoformans* from chicken faces in dry season higher than rainy season. (Kuroki *et al.*, 2004).

In the present study, *C. neoformans* might be absent in the zoo because of high standards of sanitization. The studies of Caicedo *et al.* (1999) and Keerativasee *et al.* (2008) reported that the prevalence of *C. neoformans* in avian droppings was 0.9% from the City Zoo of Cali, Colombia (Caicedo *et al.*, 1999), and 1.03% from Chiang Mai Zoo, Thailand (Keerativasee *et al.*, 2008). These previous studies suggested adequate cleaning and disinfection were the key factors of *C. neoformans* absence in zoos possibly.

Table 1 Isolation of *C. neoformans* from avian droppings collected in Nakhon Ratchasima Zoo, in 2021

Order (bird groups) and species (common name)	No. of collected sample	No. of positive sample	% positive
Gruiformes (Cranes)			
Antigone antigone (Eastern sarus crane)	24	0	0
Anthropoides virgo (Demoiselle crane)	1	0	0
Balearica regulorum (Grey crowned crane)	1	0	0
Balearica pavonina (Black crowned crane)	1	0	0
Grus paradise (Blue crane)	1	0	0
Galliformes (Pheasants)			
Lophura nycthemera (Silver pheasant)	4	0	0
Lophura ignita (Crested fireback)	1	0	0
Numida meleagris (Guinea fowl)	1	0	0
Rollulus rouloul (Crested partridge)	1	0	0
Syrmaticus reversii (Reeves's pheasant)	1	0	0

Table 1 Isolation of *C. neoformans* from avian droppings collected in Nakhon Ratchasima Zoo, in 2021 (cont.)

Order (bird groups) and species (common name)	No. of collected sample	No. of positive sample	% positive
Polypectron bicalcaratum (Grey peacock-pheasant)	2	0	0
Argusianus argus (Great argus)	1	0	0
Pavo muticus (Green peafowl)	1	0	0
Bucerotiformes (Hornbills)			
Buceros rhinoceros (Rhinoceros hornbill)	4	0	0
Anthracoceros albirostris (Pied hornbill)	3	0	0
Buceros bicornis (Great hornbill)	2	0	0
Rhyticeros undulates (Wreathed hornbill)	2	0	0
Psittaciformes (Psittacines)			
Eclectus roratus (Eclectus parrot)	3	0	0
Melopsittacus undulates (Zebra parakeet)	1	0	0
Psittacus erithacus (African grey parrot)	1	0	0
Psittacula eupatria (Rose-ringed parakeet)	1	0	0
Ciconiiformes (Storks)			
Ciconia episcopus (White-necked stork)	1	0	0
Eudocimus ruber (Scarlet ibis)	1	0	0
Mycteria cinerea (Milky stork)	1	0	0
Pelecaniformes (Pelicans)			
Pelecanus philippinensis (Spot-billed pelican)	1	0	0
Accipitriformes (Vultures)			
Sarcogyps calvus (Red-headed vulture)	1	0	0
Casuariiformes (Ratites)			
Casuarius casuarius (Southern cassowary)	2	0	0
Dromaius novaehollandiae (Emu)	1	0	0
Sphenisciformes (Penguins)			
Sphenicus humboldti (Humboldt penguin)	2	0	0
Total	**67**	**0**	**0.00**

Figure 1 Example of captive birds, in Nakhon Ratchasima Zoo, which were included in this study; A: Eastern sarus crane (*Antigone antigone*), B: Eclectus parrot (*Eclectus roratus*), C: Guinea fowl (*Numida meleagris*), D: Great hornbill (*Buceros bicornis*), E: Cassowary (*Casuarius casuarius*) and F: Humboldt penguin (*Sphenicus humboldti*)

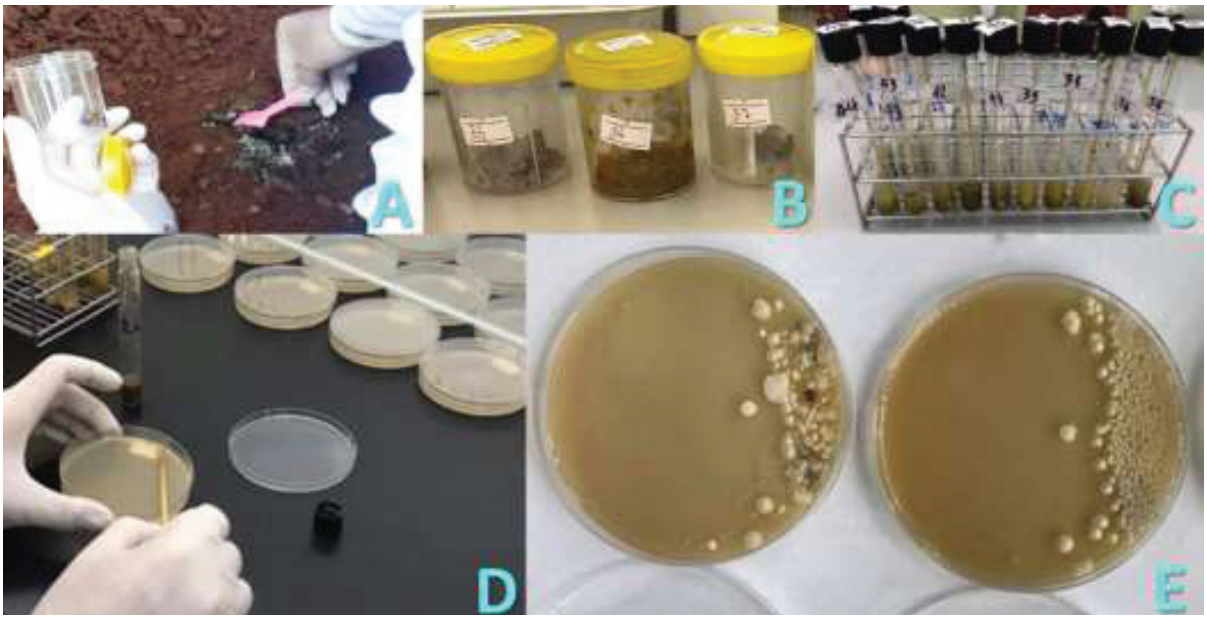

Figure 2 Sample collection, processing and isolation for *C. neoformans*; A: A fresh portion of avian dropping was collected using a plastic spatula, B: Avian dropping storage in sterile containers, C: Samples in 0.85% NaCl with penicillin G, D: Isolation on Sabouraud dextrose agar (SDA) with sunflower seed extract and chloramphenicol, E: Colonies of various organisms grow on the selective SDA.

It has been acknowledged that pigeons (Order Columbiformes) are the major reservoirs of *C. neoformans* (Lugarini *et al.*, 2008; Mirpourian *et al.*, 2021; Bertout *et al.*, 2022). However, the present study investigated 29 captive bird species in 9 taxonomic groups of birds; cranes, pheasants, hornbills, psittacines, storks, pelicans, vultures, ratites, and penguins (Table 1). The negative results for *C. neoformans* in 5 bird groups (cranes, pheasants, storks, pelicans, and vultures) are similar to the report of Keerativasee *et al.* (2008) (Keerativasee *et al.*, 2008). However, the present study had negative results for *C. neoformans* in 2 bird groups (psittacines and hornbills), whereas previous reports showed the prevalence of *C. neoformans* in psittacines and hornbills was 6.67% (Lugarini *et al.*, 2008) and 11.11% (Keerativasee *et al.*, 2008) respectively. However, there have been several reports in Thailand indicating that pigeon droppings were the common habitat of *C. neoformans* (Khayhan *et al.*, 2018; Sriburee *et al.*, 2004; Krangvichain *et al.*, 2016) similar to the reports in many countries (Weber & Schäfer 1991; Nielsen *et al.*, 2007; Oh & Hwang 2005). These reports suggested that taxonomic group, especially pigeons, were important factors relating to the presence of *C. neoformans*.

However, the quality of captive animal management should not be overlooked. A report of Gilad *et al.* (2015) did not find *Cryptococcus* spp. in environments and animals from a zoo in Israel, although this zoo imported animals that were introduced from Australia. A previous study discussed that captive animal management is important to prevent *Cryptococcus* spp. (Gilad *et al.*, 2015).

In the present study, fungal identification was confirmed using MALDI-TOF MS. However, molecular techniques such as multiplex PCR (Ito-Kuwa *et al.*, 2007) and DNA sequencing (Kaocharoen *et al.*, 2013) should be considered for further epidemiology study. These techniques not only distinguish between *C. neoformans* and *C. gattii* but also identify *Cryptococcus* spp. and their serotypes.

In summary, *C. neoformans* was negative in the present study because of possible factors such as avian dropping collection, sanitization, bird species, and quality of animal captivity. However, the active surveillance of *C. neoformans* in zoos should be concerned about these factors, and should be employ an adequate number of samples relating to each captive bird species.

Declaration of interests

The authors declare no conflicts of interest for this article, and they alone are responsible for the content.

Ethical considerations

This study was reviewed and approved by the Ethics Committee of the Nakhonratchasima College (approval no. S029b/57).

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