DETECTION OF ZOONOTIC *CRYPTOCOCCUS NEOFORMANS* IN ZEBRA DOVE (*GEOPELIA STRIATA*) DROPPINGS BY NESTED-PCR

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Abstract. In southern Thailand, zebra doves (*Geopelia striata*) are raised as pet birds for singing (cooing) competitions. However, zebra dove droppings carry *Cryptococcus neoformans*, a causative agent of human cryptococcal meningitis. Here, pooled zebra dove droppings (*n* = 148) were collected from 14 farms in Songkhla Province, southern Thailand and cultured on Sabouraud dextrose agar. Putative *C. neoformans* colonies (mucoid appearance) positive to India ink staining were cultured on cornmeal agar and then subjected to carbohydrate assimilation and urease tests. Positive colonies (encapsulated, melanin formation, utilized a variety of carbohydrates except lactose, and demonstrated urease activity) were analyzed by nested-PCR using a universal primer pair ITS1-ITS4 and *C. neoformans*-specific primers. Among putative *C. neoformans* colonies (*n* = 46) positive by culture and biochemical tests, 7 (15%) samples were positive by *C. neoformans*-specific nested-PCR and were from only two farms. This is the first report of zoonotic *C. neoformans* in zebra dove droppings and due to its potential pathology in humans, zebra dove breeders and owners should take suitable measures to prevent possible cryptococcosis infection.

Keywords: *Cryptococcus neoformans*, nested PCR, zebra dove dropping, zoonosis

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INTRODUCTION

Zebra dove (*Geopelia striata*) or barred ground dove, brownish-grey in color with black-and-white barring and a long tail, are popular for their pleasant soft, staccato cooing calls. In southern Thailand, zebra doves are raised as pets for singing (cooing) competitions. In Songkhla Province, particularly in Chana District, there are more than 40 large zebra dove farms, each housing 200-400 breeding doves, while small farms (*n* = 107) have 20-40 breeding birds; altogether, about 3,000-4,000 zebra dove chicks are produced each month (information gathered from zebra dove farm owners). Zebra doves are raised in close contact with humans, with cages located inside or near homes.

Among zoonotic pathogens associated with pet birds, *Cryptococcus* spp complex is a primary pathogen of immunocompetent hosts (Boseret *et al*, 2013). *Cryptococcus* is a basidiomycete yeast, 4-6 µm diameter and forms a capsule of >25 µm in size. There are two species, namely, *C. gattii* and *C. neoformans* (Ngamskulrungroj *et al*, 2009), the latter being the main cause of cryptococcosis in humans and constitutes a global health problem. For example, in Nigeria, prevalence of *C. neoformans* from pigeon droppings is 22.0% (Nweze *et al*, 2015); in Iran, prevalence of *Cryptococcus* sp in bird guano is 20.4% (Bandalizadeh *et al*, 2020); and in India, prevalence of *C. neoformans* and *C. gattii* in the environment is 9 and 3% respectively (Prakash *et al*, 2018).

Cryptococcosis affects the respiratory tract, central nervous system, eyes and skin (Maziarz and Perfect, 2016). In animals, cryptococcosis is most commonly found in cats but can occur in dogs, cattle, horses, sheep, goats, birds and wild animals (Refai *et al*, 2017). *C. neoformans* is normally found in soil contaminated with avian droppings and decaying vegetation (Boseret *et al*, 2013). The infection can be easily transmitted when the host inhales yeast cells or basidiospores present in the environment, particularly dust containing avian excreta (Fessel, 1993; Lagrou *et al*, 2005). Cryptococcal cells from avian excreta are small and are deposited in the alveolae (Powell *et al*, 1972).

Cases of cryptococcosis infections have historically occurred mainly in tropical and subtropical regions, and at least 5-10% of patients with AIDS become infected with *Cryptococcus* sp (Levitz, 1991). Global prevalence of cryptococcosis and associated mortality is approximately 957,900 cases per year (ranges from 371,700 to 1,544,000 cases) and 181,000 deaths respectively (Park *et al*, 2009; CDC, 2020). In USA, infection has shifted to non-HIV-positive subjects (Barragan *et al*, 2014). Cases of cryptococcosis in Thailand is the second leading infection after tuberculosis in HIV-infected patients hospitalized with acute respiratory infections (Harris *et al*, 2012, Hatthakaroon *et al*, 2017).

In birds, *C. neoformans* has been detected in 9.09% of pigeon excreta (Soogarun *et al*, 2006). In Thailand, 6.71% of pigeon droppings from Chiang Mai Province show the presence of *C. neoformans*/*C. gattii* species complex (Khayhan, 2017). However, there has been no report on *Cryptococcus* sp in zebra dove droppings. Here, the occurrence of *C. neoformans* in zebra dove droppings in Songkhla Province, Thailand was investigated using culture, biochemical tests and molecular assay to determine the risk of cryptococcosis associated with raising zebra doves.

MATERIALS AND METHODS

Sample collection

Sample size (n = 139) was calculated as previously described (Soogarun *et al*, 2006) using a prevalence of 9.09%. A total of 148 zebra dove dropping samples from 14 farms in Chana District, Songkhla Province, southern Thailand during June 2019 to April 2020 were collected. The sample was taken from each cage containing a flock of birds by using a sterile spoon, placed in a sterile plastic bag and kept on ice during transfer to the laboratory.

Detection of *Cryptococcus* sp

Five grams of sample were mixed with 30 ml of phosphate-buffered saline (PBS) containing 4 mg/ml penicillin G and 2 mg/ml streptomycin and incubated at ambient temperature for 20-30 minutes. A series of solutions by10-fold dilutions in PBS (from 10^{-1} to 10^{-5}) was prepared and 0.1 ml aliquot of 10^{-3}, 10^{-4} and 10^{-5} dilutions were spread on Difco™ Sabouraud dextrose agar (BD, Sparks, NJ) containing 0.5 mg/ml chloramphenicol and incubated under aerobic conditions at 37°C for 7 days. Five presumptive *Cryptococcus* sp colonies, creamy white with a mucoid appearance, were streaked on cornmeal agar (Himedia, Mumbai, India) and incubated at 30-32°C for 7 days to observe melanin formation. These colonies were then stained with India ink to visualize budding.

Biochemical tests

Presumptive colonies were tested for urease activity and carbohydrate assimilation. The urease test was carried out using a Christensen agar medium (Himedia, Mumbai, India), a positive result indicated by a color change from light orange to magenta (pink). Carbohydrate assimilation assay was performed using glucose, lactose, maltose, raffinose, and sucrose, presence *C. neoformans* indicated by growth on all carbohydrate sources except lactose (Alcamo, 1997).

Nested-PCR assay

DNA was extracted in 40 µl distilled water from 2-3 fresh colonies by a boiling method (da Silva *et al*, 2012). For nested-PCR, the outer primers were ITS1 (5′-TCCG-TAGGTGAACCT GCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)

targeting ITS15.8SITS2 rDNA of fungi and inner *C. neoformans*-specific primers were CN4 (5′-ATCACCTTC CCACTATTCACACATT-3′) and CN5 (5′-GAAGGGCATGCCTGTTT-GAGAG-3′) (Mitchell *et al*, 1994). The first-round PCR mixture contained 10 µl of KAPA PCR Ready Mix (Kapa Biosystem, Tokyo, Japan), 1 µl of 10 µM each primer, and 4 µl of DNA template. Thermocycling was conducted in Bio-Rad Thermal Cyclers (Bio-Rad Laboratories Inc, Hercules, CA) as follows: 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 60 seconds; and a final heating at 72°C for 7 minutes. Samples with required amplicon (500-600 bp) were subjected to second-round PCR in a mixture containing 5.0 µl of KAPA PCR Ready Mix (Kapa Biosystem, Tokyo, Japan), 0.25 µl of 10 µM each primer, 4.0 µl of ultra-pure water, and 2.0 µl of the first-round reaction solution. Thermocycling was performed as described above with the following conditions: 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 55°C for 60 seconds and 72°C for 2 minutes; and a final heating at 72°C for 5 minutes. Amplicons were analyzed by 1.5% (first-round PCR) and 2.5% (second-round PCR) agarose gel-electrophoresis and staining with ethidium bromide (Thermo Fisher Scientific, Waltham, MA). *C. neoformans* samples generated a 136-bp amplicon.

RESULTS

There were 46 samples of zebra dove droppings from 11 farms producing presumptive *Cryptococcus* sp colonies, which were encapsulated, produced melanin and capable of budding (Fig 1). Based on urease activity and carbohydrate assimilation, all 46 samples were characterized as *Cryptococcus* sp (Table 1). In the first round nested-PCR, 32 samples from 10 farms were indicative of *Cryptococcus* sp (data not shown), but results from second round nested-PCR indicated only seven samples from two farms (six from one of the farms) carried *C. neoformans* (Table 1). A representative agarose gel-electrophoresis of *C. neoformans*-specific amplicon (136 bp) is shown in Fig 2.

C. neoformans was detected in 5% (7/148) samples of zebra dove droppings obtained from 14 large breeding farms in Songkhla Province, southern Thailand. However, only 2/14 (14%) farms contained *C. neoformans*-positive bird droppings.

DISCUSSION

The study shows, using nested-PCR, that only 5% of samples from zebra dove droppings were *C. neoformans*-positive. This is to be compared with 31% when based on conventional culture or biochemistry tests. It is worth noting only 14% of breeding farms contained *C. neoformans*-positive bird droppings.

Hayeeyusoa *et al* (2015) reported a *C. neoformans* prevalence of 20% in zebra dove droppings in Yala province, southern Thailand using conventional culture method and biochemical tests. This high prevalence is not surprising given the poor specificity of the two techniques as shown in our study. Prevalence of *C. neoformans* var. neoformans in bird excreta ranges 6.71-9.09% (Soogarun *et al*, 2006; Khayhan, 2017).

That only 2/14 breeding farms harbored *C. neoformans*-infected zebra doves might be ascribed to their being large commercial breeding farms, where owners are aware of the need for good farming practice. The two farms with infected bird droppings allowed droppings to accumulate, which were removed only once every three months and the owners did not appear to clean cages using disinfectants.

C. neoformans has been identified in many species of pet birds, such as pigeons, parrots and cockatoos (Kielstein *et al*, 2000; Nosanchuk *et al*, 2000; Soogarun *et al*, 2006). Zebra dove must be added to this list. Although zebra doves showed no clinical signs of infection, the presence of *C. neoformans* in bird droppings constitutes an environmental reservoir of this pathogen. The frequency of infected bird droppings was low, but the sampling size was relatively limited, and as owners of zebra doves keep cages close to or inside homes, there is

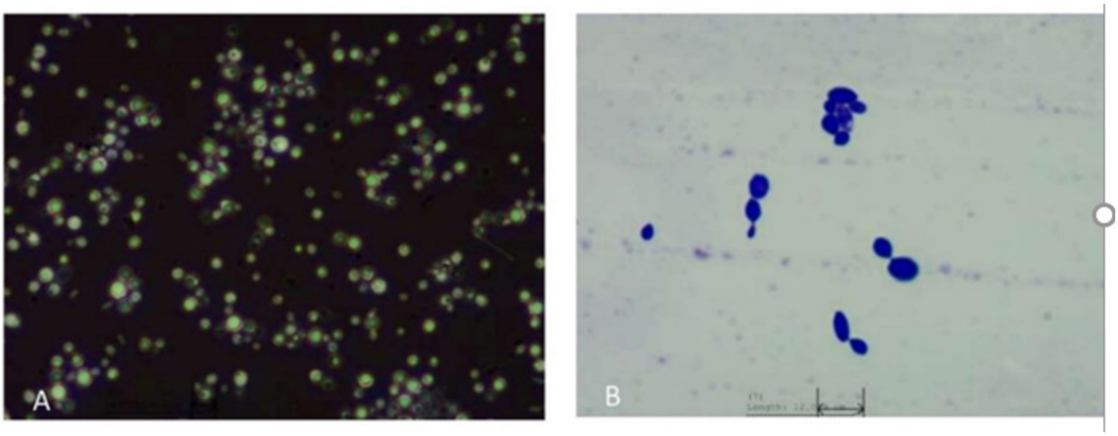

Fig 1 - Light micrographs of *Cryptococcus* sp colonies grown on cornmeal agar

(A) spherical, encapsulated cells with a zone of clearance or 'halo' by India ink staining (40x magnification) and (B) stained with methylene blue showing budding forms (100x magnification)

Table 1

Identification by conventional culture, biochemical tests and nested-PCR of *Cryptococcus neoformans* in zebra dove (*Geopelia striata*) droppings from breeding farms in Chana District, Songkhla Province, southern Thailand (during June 2019 to April 2020)

Farm Number	Number of zebra doves	Number of samples of bird dropping	Number of positive colonies			
			Conventional culture	Biochemical tests	1st round nested PCR	2nd round nested PCR
1	200	20	5	5	3	0
2	1,000 (30 cages)	30	1	1	1	0
3	200	14	6	6	1	0
4	30	10	4	4	4	0
5	60	4	2	2	1	0
6	200 (3 cages)	3	0	0	0	0
7	300 (4 cages)	4	0	0	0	0
8	20	2	2	2	2	0
9	20	2	1	1	1	0
10	30	3	2	2	2	1
11	10	1	0	0	0	0
12	200	20	15	15	14	6
13	300 (5 cages)	5	3	3	3	0
14	100	30	5	5	0	0
Total	2,670	148	46	46	32	7

PCR: polymerase chain reaction

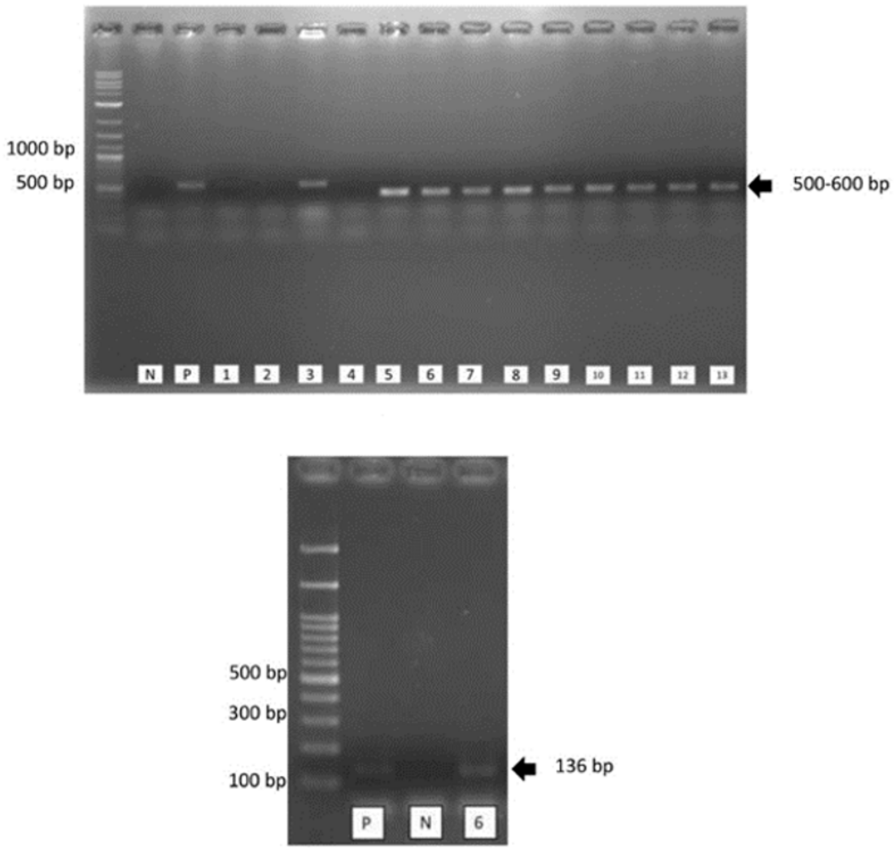

Fig 2 - Nested-PCR detection of *Cryptococcus* neoformans in zebra dove (*Geopelia striata*) droppings from a breeding farm in Chana District, Songkhla Province, southern Thailand

DNA extracted from putative *C. neoformans* colonies were subjected to nested-PCR employing outer primers targeting fungal rDNA and inner *C. neoformans*-specific primers (Mitchell et al, 1994), and amplicons were analyzed by 2.5% agarose gel-electrophoresis and ethidium bromide dye staining. The first round PCR showed an amplicon of approximately 500-600 bp and nested PCR showed 136 bp of *C. neoformans*-specific amplicon.

Left lane: 100 bp DNA size markers; Lane P: positive control (DNA of *C. neoformans*); Lane N: negative control (Sterile water); Lane 6: colony from zebra dove droppings from Farm No. 10 (Table 1)

bp: base pair; PCR: polymeras chain reaction always a finite risk of cryptococcosis from inhalation of dust from *C. neoformans*-infected bird droppings. A study in Colombia of re-isolation of *C. neoformans/C. gattii* from environmental samples stored for 10 years revealed the pathogens still remain viable (Escandón and Castañeda, 2015).

Given the popularity of zebra dove rearing for pleasure or for export, it is important from the public health aspect that these birds are free of any zoonotic pathogens, albeit yeast, bacteria or virus. Our study highlights the usefulness of nested-PCR in definitive identification of *C. neoformans*. Although standard gel-electrophoresis is sufficient for identifying *C. neoformans*-specific amplicon, for screening purposes, quantitative PCR provides a high throughput assay platform with rapid turn-around time and requiring less labor input.

In conclusion, the authority should encourage prevention of *C. neoformans* transmission by promoting biosecurity and good practices for setting up standard zebra dove farms in southern of Thailand. This endeavor may lead to the production of birds that meet appropriate standards so that they can be exported to foreign countries after issues of avian influenza are settled.

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CONFLICTS OF INTEREST DISCLOSURE

The authors declare no conflicts of interest.

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