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RESEARCH ARTICLE

Molecular epidemiology and antifungal susceptibilities of *Cryptococcus neoformans* in bird droppings from zoological gardens and public places in Peninsular Malaysia

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ABSTRACT

Cryptococcus neoformans, a ubiquitous fungus commonly found in bird droppings, poses a significant health risk by causing cryptococcal meningitis especially in immunocompromised individuals. In Malaysia, clinical reports from 1964–2010 documented an increasing incidence of *C. neoformans* cases, even in healthy individuals. Nevertheless, studies focusing on *C. neoformans* occurrence in birds are limited, with the last study conducted in 2005, focusing solely on the Klang Valley region. We aimed to update the molecular epidemiology and antifungal susceptibility of *C. neoformans* in bird droppings from public areas and zoological gardens across Peninsular Malaysia. Molecular identifications were performed using nested-PCR with CNLAC1 outer and inner primer pairs for the primary and secondary PCR. Antifungal susceptibility tests were conducted against Amphotericin B, Fluconazole, and Itraconazole. The hygiene and environmental conditions of the zoological gardens were recorded. A total of 509 bird droppings were collected: 257 (50.5%) from public places and 252 (49.5%) from zoological gardens. Most samples from public areas were from common pigeons (n = 144; 56.0%), while samples from Mandarin ducks predominated in zoological gardens (n = 40; 15.9%). The overall prevalence of *C. neoformans* was 42.6% (217/509), with a higher prevalence in zoological gardens (116/252; 46.0%) versus public places (101/257; 39.3%) (P = 0.125). Notably, common pigeons in zoological gardens showed a significantly higher carrier rate (80.8%) versus in public places (54.9%) (P = 0.013). Other species with high carrier rates in zoological garden included Indian peafowls (61.9%) and budgie birds (61.3%). In public areas, apart from pigeons; doves (43.5%) also exhibited a high prevalence. Enclosure density and Columbidae family were found to be associated with high positivity rate in zoological gardens. Two strains were identified: *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A). One isolate exhibited resistance to Itraconazole. This study highlights the need for ongoing public health surveillance and preventive measures particularly in settings where human-bird interactions are frequent.

Keywords: *Cryptococcus neoformans*; bird droppings; public places; zoological gardens; antifungal susceptibility.

INTRODUCTION

Cryptococcus neoformans is a ubiquitous fungi found particularly in tropical countries and the causative agent of cryptococcal meningitis primarily in immunocompromised individuals. Approximately 1.8 million individuals die annually to AIDS-related causes, including fatalities linked to this fungal infection (Nweze *et al*., 2015). It is estimated that around 220,000 cases of cryptococcal meningitis among HIV-infected patients are recorded each year (Rajasingham *et al*., 2017). Classified by the World Health Organization's (WHO) fungal priority list as a leading fungal pathogen (WHO, 2022), its impact on public health necessitates urgent research and development initiatives.

In Malaysia, historical clinical reports from 1964 to 1980 documented an increasing trend in *C. neoformans* cases, with 55 cases reported over a ten-year span by Richardson *et al*. (1976) and an additional 85 cases documented from 1974 to 1980 at the University Malaya Medical Centre (Pathmanathan & Soo-Hoo, 1982). Subsequent research by Tay *et al*. (2010) revealed a high incidence rate, reporting 96 cases over two years (2003-2004), indicating an alarming 100% increase. It is also estimated that Malaysia sees approximately 47 cases of cryptococcal meningitis annually

among healthy individuals and around 700 cases among those with HIV/AIDS (Velayuthan, 2022).

This encapsulated basidiomycetous yeast is closely associated with birds, especially pigeons and is commonly isolated from their droppings. Transmission to humans primarily occurs through exposure and inhalation of airborne propagules present in the environment. This mode of transmission emphasizes the potential risk posed by *C. neoformans* to individuals, particularly those with compromised immune systems. It highlights the need for increased awareness and preventive measures to mitigate its impact on public health, as evidenced by the recent rise in cases. However, studies on *C. neoformans* in birds in Malaysia are scarce and require further investigation. The last local research on its prevalence in birds dates back to 2005, reporting a 3.7% prevalence rate of *C. neoformans* isolated from bird droppings, but it was limited to the Klang Valley (Tay *et al*., 2005). This raises question about the situation in other regions of Malaysia.

Hence, our main objective was to assess the current epidemiology of *C. neoformans* in bird droppings from two environments: public places and zoological gardens in Peninsular Malaysia, updating the 2005 study. This will help assess the risk of transmission to zoo and farm handlers, as well as the public, especially as concerns grow over the spread of this fungus. The increasing number of zoological gardens and the ever-growing pigeon population, which has adapted to living closely with humans in urban environments, heighten this concern.

Cryptococcus neoformans is divided into three distinct strains and further subdivided into four serotypes distinguished by variation of the glucuronoxylomannan (GXM), the major capsular polysaccharides (Krangvichain *et al*., 2016). The serotypes are *C. neoformans* var. *grubii* (serotype A), which is the most common and highly pathogenic, especially in immunocompromised individuals, *C. neoformans* var. *gattii* (serotypes B and C), *C. neoformans* var. *neoformans* (serotype D) and a hybrid serotype AD, which results from the organism's ability to undergo recombination and form diploid or aneuploid intervarietal hybrids (Oh & Hwang, 2005; Machrumnizar *et al*., 2022). Therefore, our other objectives were to molecularly determine the strains of *C. neoformans* in the bird droppings along with determining their antifungal susceptibilities. We also aimed to investigate any association between *C. neoformans* occurrence in birds in zoological gardens with selected variables including hygiene and environmental factors.

MATERIALS AND METHODS

Ethical clearance

Ethical approval for the animal study was granted by Universiti Teknologi MARA (UiTM) Care Ethics Committee under verification number 600-UiTMCARE (PT.1/3/1). Permission was also obtained from The Department of Wildlife and National Parks of Peninsular Malaysia (PERHILITAN), a government body responsible for the conservation, regulation and preservation of wildlife and national parks under verification number JPHLTN.600-6/1/4 JLD2 (118).

Samples collection and sample size calculation

In this cross-sectional study, bird droppings were collected from public places and zoological garden across four regions: Central, Northern, Eastern, and Southern of Peninsular Malaysia, from August 2023 to June 2024 (Figure 1). The species of birds was recorded. The selection of public locations was based on the observed presence of large flocks of birds, such as pigeons foraging or nesting in these areas. These locations are also popular tourist destinations and frequented by locals. Examples of public places included in the study are places of worship like temples, shoplots, surrounding markets, and hospitals in town areas. For zoological gardens, three parks/zoos in the central region, one in the northern, and one in the southern region were included in the study. The hygiene level

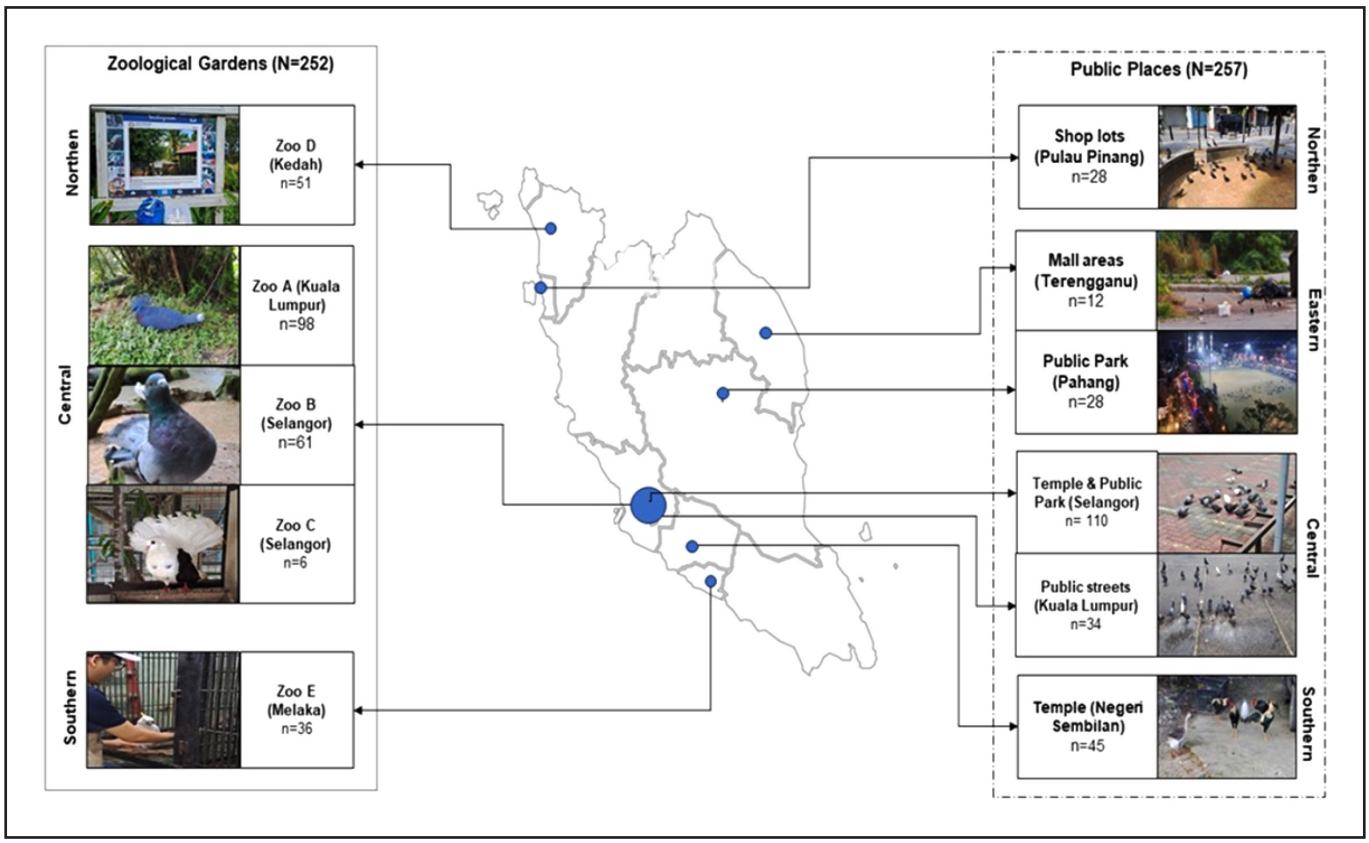

Figure 1. Sampling locations in both zoological gardens and public places across four regions in Peninsular Malaysia.

for all five zoological gardens in this study were also observed and recorded based on the following criteria:

a. Frequency of bird droppings cleaning- Low frequency (0-2 times daily) / High frequency (more than 2 times daily).
b. Air odour- Bad odour (indicated by a persistent foul smell) / No odour (fresh or neutral air).
c. Drainage system- Fair (functioning, but with occasional blockages or areas of stagnant water) / Excellent (well-maintained, not clogged and no visible stagnant water).
d. Enclosure density- Crowded (high number of birds per unit area with limited roaming space)/ Spacious (adequate space allowing birds to roam freely with clear separation between birds).
e. Frequency of encountering bird dropping stains- High (visible in most areas with approximately a minimum of two bird dropping stains per square metre)/ Low (fewer than two bird dropping stains per square metre or confined to specific spot).

Observations were finalized after each site visit, following agreement with all observers and discussions with representatives (veterinarians or personnel in charge) from the respective zoos.

For sample collection, foraging birds and caged birds were observed and monitored closely and their droppings were collected immediately (fresh samples) using spatula into sterile plastic containers. To minimize the risk of soil contamination, samples were carefully taken from the upper surface of the dropping ensuring no contact with the ground during collection. The bird species were identified by learning about the bird names and species and cross checked by the help of the zookeepers and the bird encyclopaedia by Alderton (2008). Samples were processed within 1-3 hours, as described by Tay *et al*. (2005). Approximately 0.5-1g of the isolates were directly cultured onto Staib's Medium (bird seed agar) (HiMedia, India) using sterile disposable inoculation loops. The agar plates were labelled and sealed with masking tape to prevent contamination, then incubated at room temperature and monitored daily for the growth of *C. neoformans* for up to five days. Suspected colonies (light yellow-brown colonies) were examined microscopically using Indian ink staining to observe the morphology of the encapsulated yeast. DNA extraction and antifungal susceptibility testing were performed immediately on suspected colonies.

Molecular characterization

All suspected colonies according to manufacturer for Staib's agar (light yellow-brown colonies) were subjected to DNA extraction using the NucleoSpin® Yeast kit (Macherey-Nagel GmbH & Co. KG, Germany), according to the manufacturer's instructions with slight modifications at incubation elution time (90 seconds) and an extra elution step. The extracted DNA was stored in a -20°C freezer prior to DNA amplification.

DNA amplification was performed using nested PCR with primers as described by Chae *et al*. (2012) The specific gene targeted in this PCR protocol was the CNLAC1 gene. This nested-PCR protocol utilized two sets of primers, producing PCR products of 1386 and 690 base pairs, respectively. The primary amplification was carried out in a 25 µl reaction mixture containing 2 µl of CNLAC1 outer primer pair (10 µM), 2.5 µl of template DNA, 12.5 µl of 2x Master Mix (Thermo Fisher, USA: DreamTaq Green PCR Master Mix with 20 mM Mg^{2+} and dNTPs), and 8 µl of PCR-grade water. PCR-grade distilled water served as a negative control. The secondary amplification was performed in a 25 µl reaction mixture, consisting of 2 µl of CNLAC1 inner primer pair (10 µM), 0.2 µl of the primary amplification PCR product, 12.5 µl of 2x Master Mix (Thermo Fisher, USA: DreamTaq Green PCR Master Mix with 20 mM Mg^{2+} and dNTPs), and 10.3 µl of PCR-grade water. The optimized thermal cycling conditions for the thermocycler (Analytik Jena, Germany) for both amplifications are described in Table 1.

Both the primary and secondary PCR products were analysed using 1.5% agarose gel in 0.5x Tris-Acetate-EDTA (TAE) buffer (1st Base, Singapore) with safe stain (1st Base Biochemicals, Singapore), and visualized by transillumination under ultraviolet (UV) light (Cleaver Scientific, UK). The amplified products were compared against a 100 bp DNA ladder (SMOBIO, Taiwan) for size estimation. To establish the positive control in this methodology, one of the positive PCR-amplified products, initially selected based on the microscopic identification was sent for Sanger's sequencing (Apical Scientific Sdn Bhd). The resulting sequenced product was then analysed using BLAST (https://blast.ncbi.nlm.nih.gov/) to confirm its identity and subsequently used as a positive control for the other nested PCR in this study. Only samples confirmed by molecular approaches are considered positive.

Antifungal susceptibility test

All positive samples underwent antifungal susceptibility testing using the disc diffusion method as an initial screening to detect potential resistance. Positive colonies were suspended into 0.5 McFarland turbidity and lawned onto 1640 RPMI glucose agar (HiMedia, India). Three antifungal discs; Amphotericin B (AMB) (20 µg/disk), Itraconazole (ITC) (50 µg/disk), and Fluconazole (FLU) (25 µg/disk) (Liofilchem, Italy) were added using flame sterilised forceps as described by Tay *et al*. (2005). The plates were incubated at room temperature for 48 hours. Susceptibility was assessed based on the manufacturer's guidelines which were based on the Clinical & Laboratory Standards Institute: CLSI Guidelines with resistance defined as a zone of inhibition <13 mm, intermediate as 13-17 mm, and susceptibility as >17 mm.

Data and phylogenetic analysis

Data on bird family and species, sampling locations, type of location (zoological garden vs. public places), hygiene and environmental status (zoological garden only) and positivity status were recorded,

Table 1. Primer sequences for the molecular identification of *C. neoformans* and the optimized thermal cycling conditions (nested PCR) used in this study

Primer	Gene	Primer Sequence (5'-3')	PCR Product (bp)	Optimised Thermal Conditions
CNLAC1 (Outer)	laccase	F-ACGGTGTCCCTGGTATAA R-GCGTTGGACGATTGAAAG Chae *et al*. (2012)	1386	95°C -5 minutes, 95°C -60 seconds (x38 cycles), 58.6°C -90 seconds, 72°C - 90 seconds, 72°C -8 minutes (final extension)
CNLAC1 (Inner)	laccase	F-CACTCGCCCAATGAACC R-TATACCTCACCACCGCC Chae *et al*. (2012)	690	95°C - 5 minutes, 95°C - 60 seconds (x35 cycles) 58.6°C- 90 seconds, 72°C - 60s- seconds, 72°C-8 minutes (final extension)

entered, and analysed using IBM SPSS version 27 (SPSS, Chicago, IL, USA). Descriptive analysis (percentages) was used to describe the prevalence of *C. neoformans* in the samples. The Pearson chi-square test (χ^2) was applied to assess associations between selected variables, with statistical significance set at $P < 0.05$. Logistic regression analysis (univariate and multivariate) was also performed to investigate the risk factors associated with the positivity rate in bird droppings. For this downstream analysis, Zoo C was removed from the calculation due to the low sample size (n=6).

Some amplified DNA were then selected for purification and sequencing. The resulting sequences were converted into FASTA format, aligned, and identified using the online Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). A partial phylogenetic tree was then constructed for the corresponding *C. neoformans* strains using the neighbour-joining method and the maximum-composite likelihood in the MEGA11 (64-bit). Software bootstrap support was assisted by 1000 repetitive analyses.

RESULTS

Samples characteristics

In this study, a total of 509 bird droppings were collected from 24 bird species across 12 taxonomic families in four regions of Peninsular Malaysia. Of these, 257 (50.5%) were collected from public places and 252 (49.5%) samples from zoological gardens. In public places, droppings were collected from nine bird species, with the Columbidae family particularly common pigeons (56.0%) accounting for majority of the samples (Table 2). In contrast, samples from zoological garden were collected from a more diverse range of 17 bird species dominated mainly by Mandarin ducks (15.9%) followed by budgie birds (12.3%), common pigeons (10.3%) and rainbow lorikeets (9.9%). Only two bird species, the common pigeon and chicken, were collected from both public places and zoological gardens. The majority of the samples (60.7%) were collected from the Central region which is the most densely populated region of Peninsular Malaysia. This higher collection rate was primarily due to the greater number of zoological gardens and crowded public areas in this area. No samples from zoological gardens were available from the Eastern region.

Prevalence of *C. neoformans* in bird droppings

The overall prevalence of *C. neoformans* in bird droppings was 42.2% (217/509). Comparatively, the prevalence rate was higher in zoological gardens (116/252; 46.0%) compared to public places (101/257; 39.3%), although this difference was not statistically significant (P= 0.125). The positive samples involved the majority of bird species, involving 21 out of 24 species. No *C. neoformans* was detected in the swan goose (*Anser cygnoides*) species (9 samples) and Oriental magpie robin (*Copsychus saularis*) species (6 samples). One sample from the emu (*Dromaius novaehollandiae*) bird, was also negative.

As expected, more than half of the common pigeons in this study were carriers of *Cryptococcus* (58.8%). A notably high positivity rate was observed among common pigeons in zoological gardens (21/26; 80.8%) compared to public places (79/144; 54.9%) (P = 0.013). A similar pattern was observed in chickens, with 3/6 samples (50.0%) from the zoo found to be positive, contrary to only 4/31 samples (12.9%) from public places (P=0.034). Interestingly, a high positivity rate was also found in budgie birds (61.3%), Indian peafowls (61.9%), Mandarin ducks (40.0%), rainbow lorikeets (40.0%), cockatoos (45.0%), and Nicobar pigeons (41.7%) in zoological gardens. In public places, apart from pigeons (54.9%), a high prevalence was also noted in doves (43.5%), which are from the same Columbidae family as pigeons.

In public places, the highest positivity rate was observed in the northern region (24/28; 85.7%), followed by the central region (63/144; 43.8%). The high infection rate in the northern region can be attributed to the fact that only common pigeon samples were collected there. In zoological gardens, the positivity rate ranged from 37.3% in the northern region to 58.3% in the southern region, depending on the type of birds collected at each zoo. A summary of the positivity rates of *C. neoformans* in this study is detailed in Table 3.

Association between hygiene and environmental factors in zoological gardens with the occurrence of *C. neoformans* in bird droppings.

The association between hygiene and environmental factors in zoological gardens and the positivity rate of *C. neoformans* was further explored. Due to a low sample size, Zoo C was excluded from the analysis. Details regarding the hygiene and environmental conditions for each zoo are presented in Table 4. In general, Zoo Melaka had the highest positive rate (58.3%). Despite its high cleaning frequency, limited space (crowded conditions) and frequent bird staining may have contributed to the higher positivity rate among its birds. In contrast, Zoo A, although with low cleaning frequency, had a lower positivity rate, possibly due to its spacious area. We then conducted a risk analysis to confirm this using logistic regression that included the presence of birds from the Columbidae family, the primary group affected by *C. neoformans*. In univariate analysis, zoos with a high number of Columbidae birds had 2.9 times the odds (P = 0.004) of exhibiting a high positivity rate. Notably, in multivariate analysis, two factors were identified as significant predictors for high positivity rate of birds in zoological gardens: the presence of many Columbidae birds (AOR = 3.3) and crowded conditions (AOR = 2.6) (Table 5).

Antifungal susceptibility tests

All 217 positive samples were subjected to antifungal susceptibility testing against Amphotericin B, Itraconazole and Fluconazole. Of these, one sample (C.liviaPC44) exhibited resistance to Itraconazole (50 µg/disk) (zone of inhibition, 6mm), and another one sample (C.liviaPN4) showed intermediate resistance to Fluconazole (25 µg/disk) (zone of inhibition, 14mm). Both samples were from common pigeon species collected from public places in the central and northern regions. The remaining 215 samples were susceptible (zone of inhibition, > 17mm) to all three types of antifungal agent, with no resistance observed.

A partial phylogenetic tree construction

A total of 16 amplified DNA samples were selected for purification and sequencing (Apical). This set included the isolate resistant to Itraconazole (sample ID: C.liviaPC44), the isolate with intermediate susceptibility to Fluconazole (sample ID: C.liviaPN4), and 14 additional isolates chosen due to the high public interaction of the bird species from which the fungus was isolated, as detailed in S1 Table. These 16 sequences were submitted to GenBank with accession numbers SRR30002288-SRR3000. The majority of the sequenced isolates, showing >97% similarity, belonged to *C. neoformans* var. *grubii* or *C. neoformans* var. *neoformans*. Both samples, one resistant to Itraconazole and the other with

Table 2. Detailed number of samples according to the bird species and regions in both public places (N=257) and zoological gardens (N=252)

TYPES OF BIRD, OVERALL SAMPLE, N=509	PUBLIC PLACES (N=257)					ZOOLOGICAL GARDEN (N=252)			
Family- Species	**Total N (%)**	**C (n=144)**	**N (n=28)**	**S (n=45)**	**E (n=40)**	**Total N (%)**	**C (n=165)**	**N (n=51)**	**S (n=36)**
Columbidae									
Common pigeon (*Columbia livia*), N=170	144 (56.0)	78 (54.2)	28 (100)	2 (4.4)	36 (90.0)	26 (10.3)	24 (14.5)	2 (3.9)	0
Nicobar pigeon (*Caloenas nicobarica*), N=12	0	0	0	0	0	12 (4.8)	12 (7.3)	0	0
Zebra dove (*Geopelia strata*), N=14	14 (5.4)	14 (9.7)	0	0	0	0	0	0	0
Spotted dove (*Spilopelia chinensis*), N=9	9 (3.5)	9 (6.3)	0	0	0	0	0	0	0
Anatidae									
Mandarin duck *(Aix Galericulata)*, N=40	0	0	0	0	0	40 (15.9)	40 (24.2)	0	0
Mallard (*Anas platyrhynchos)*, N=18	0	0	0	0	0	18 (7.1)	18 (10.9)	0	0
Swan goose (*Anser cygnoides*), N=9	9 (3.5)	2 (1.4)	0	7 (15.6)	0	0	0	0	0
Phasianidae									
Chickens (*Gallus gallus*), N=37	31 (12.1)	5 (3.5)	0	26 (57.8)	0	6 (2.4)	2 (1.2)	4 (7.8)	0
Indian peafowl (*Pavo cristatus*), N=21	0	0	0	0	0	21 (8.3)	21 (12.7)	0	0
Turkey (*Meleagris gallopavo*), N=6	0	0	0	0	0	6 (2.4)	0	6 (11.8)	0
Psittaculidae									
Budgie (*Melopsittacus undulatus*), N=31	0	0	0	0	0	31 (12.3)	0	15 (29.4)	16 (44.4)
Rainbow lorikeet (*Trichoglossus moluccanus*), N=25	0	0	0	0	0	25 (9.9)	25 (15.2)	0	0
Moluccan eclectus (Eclectus roratus), N=9	0	0	0	0	0	9 (3.6)	0	5 (9.8)	4 (11.1)
Passeridae - House sparrow (*Passer domesticus*), N=21	21 (8.2)	17 (11.8)	0	0	4 (10.0)	0	0	0	0
Sturnidae - Common myna (*Acidotheres tristis*), N=16	16 (6.2)	8 (5.6)	0	8 (17.8)	0	0	0	0	0
Psittacidae									
Sun conure *(Aratinga solstitialis)*, N=15	0	0	0	0	0	15 (6.0)	15 (9.10)	0	0
Yellow-headed Amazon *(Amazona oratrix)*, N=7	0	0	0	0	0	7 (2.8)	0	7 (13.7)	0
Blue-yellow macaw *(Ara ararauna)*, N=3	0	0	0	0	0	3 (1.2)	0	0	3 (8.3)
Cacatuidae									
Sulphur-crested cockatoo *(Cacatua galerita)*, N=14	0	0	0	0	0	14 (5.6)	0	7 (13.7)	7 (19.4)
Solomons cockatoo *(Cacatua ducorpsii)*, N=6	0	0	0	0	0	6 (2.4)	0	0	6 (16.7)
Pelecanidae - Great white pelican *(Pelecanus onocrotalus)*, N=12	0	0	0	0	0	12 (4.8)	12 (7.3)	0	0
Corvidae - House crow *(Corvus splenden)*, N=7	7 (2.7)	5 (3.5)	0	2 (4.4)	0	0	0	0	0
Muscicapidae - Oriental-magpie robin *(Copsychus saularis)*, N=6	6 (2.3)	6 (4.2)	0	0	0	0	0	0	0
Casuariidae - Emu *(Dromaius novaehollandiae)*, N=1	0	0	0	0	0	1 (0.4)	0	1 (2.0)	0

C=Central; N= Northern; S= Southern; E= Eastern.

Table 3. The occurrence of *C. neoformans* in bird droppings (positivity rate) according to bird species, places, regions

Samples (N= 509)	Number of birds positive - Public Places					Number of birds positive - Zoological gardens			
	Total positive	C (N=144)	Nr (N=28)	S (n=45)	E (N=40)	Total positive	C (N=165)	Nr (N=51)	S (N=36)
	n (%)					n (%)			
Total number of birds positive: 217/509 (42.2%)	101 (39.3)	63 (43.8)	24 (85.7)	4 (8.9)	10 (25.0)	116 (46.0)	76 (46.1)	19 (37.3)	21 (58.3)
By bird species									
Common pigeon (N=170)	79/144 (54.9)	44/78(56.4)	24/28 (85.7)	1/2 (50.0)	10/36 (27.8)	21/26 (80.8)	19/24 (79.2)	2/2 (100)	0
Mandarin duck (N=40)	0	0	0	0	0	16/40 (40.0)	16/40 (40.0)	0	0
Chickens (N=37)	4/31 (12.9)	3/5 (60.0)	0	1/26 (3.8)	0	3/6 (50.0)	2/2 (100)	¼ (25.0)	0
Budgie (N=31)	0	0	0	0	0	19/31 (61.3)	0	9/15(60.0)	10/16(62.5)
Indian peafowl (N=21)	0	0	0	0	0	13/21 (61.9)	13 (61.9)	0	0
Rainbow lorikeet (N=25)	0	0	0	0	0	10/25 (40.0)	10/25 (40.0)	0	0
House sparrow (N=21)	4/21 (19.0)	4/17 (23.5)	0	0	0/4 (0.0)	0	0	0	0
Mallard (N=18)	0	0	0	0	0	5/18 (27.8)	5/18 (27.8)	0	0
Zebra & Spotted dove (N=23)	10/23 (43.5)	10/23 (43.5)	0	0	0	0	0	0	0
Sulphur-crested & Solomons cockatoo (N=20)	0	0	0	0	0	9/20 (45.0)	0	1/7 (14.3)	8/13 (61.5)
Nicobar Pigeon (N=12)	0	0	0	0	0	5/12 (41.7)	5/12 (41.7)	0	0
Sun Conure, Yellow-headed Amazon & Blue-yellow macaw (n=25)	0	0	0	0	0	8/25 (32.0)	4/15 (26.7)	2/7 (28.6)	2/3 (66.7)
Moluccan eclectus (N=9)	0	0	0	0	0	3/9 (33.3)	0	2/5 (40.0)	1/4 (25.0)
Common myna (N=16)	3/16 (18.8)	1/8 (12.5)	0	2/8 (25.0)	0	0	0	0	0
Great white pelican (n=12)	0	0	0	0	0	2/12 (16.7)	2/12 (16.7)	0	0
House crow (N=7)	1/5 (20.0)	1/5 (20.0)	0	0/2 (0.0)	0	0	0	0	0
Turkey (N=6)	0	0	0	0	0	2/6 (33.3)	0	2/6 (33.3)	0

C=Central; Nr= Northern; S= Southern; E= Eastern.

Table 4. Hygiene and environmental observations and number of positive samples for each zoo in zoological gardens

Zoological garden	Cleaning Frequency	Air Odour	Drainage System	Enclosure density	Frequency of bird stain	Positivity status (%)
ZooA_Kuala Lumpur (N=98)	Low	No odour	Fair	Spacious	High	41.8
ZooB_Selangor (N=61)	High	No odour	Excellent	Moderate	Low	50.8
ZooD_Kedah (N=51)	High	No odour	Excellent	Spacious	Low	39.2
ZooE_Melaka (N=36)	High	Mild odour	Fair	Crowded	High	58.3

Table 5. Potential risk factors associated with the positivity rate in birds in zoological garden

Selected variables	N	Bird Positivity status	Univariate logistic regression		Multivariate logistic regression	
			COI (CI)	P value	AOR (CI)	P value
Columbidae family						
Yes	37	25 (67.6)	2.9 (1.4,6.0)	0.004*	3.3 (1.6,7.0)	0.002*
No	209	88 (42.1)	(1)			
Cleaning frequency						
High	148	72 (48.6)	(1)	0.294	1.4 (0.7, 2.6)	0.338
low	98	41 (41.8)	0.8 (0.5,1.3)			
Air odour						
No odour	210	92 (43.8)	(1)	0.106	-	-
Mild odour	36	21 (58.3)	1.8 (0.9, 3.7)			
Enclosure density						
Crowded	36	21 (58.3)	1.8 (0.9, 3.7)	0.106	2.6 (1.2, 5.9)	0.020*
Spacious	210	92 (43.8)	(1)			
Drainage system						
Excellent	112	51 (45.5)	(1)	0.909	NC	NC
fair	134	62 (46.3)	1.0 (0.6,1.7)			
Dropping stain frequency						
High	134	62 (46.3)	1.0 (0.6, 1.7)	0.909	NC	NC
low	112	51 (45.5)	(1)			

COI= Crude odd ratios; AOR=Adjusted odd ratios; CI= Confidence Interval; *P value <0.05 is considered significant; (1) = reference value.

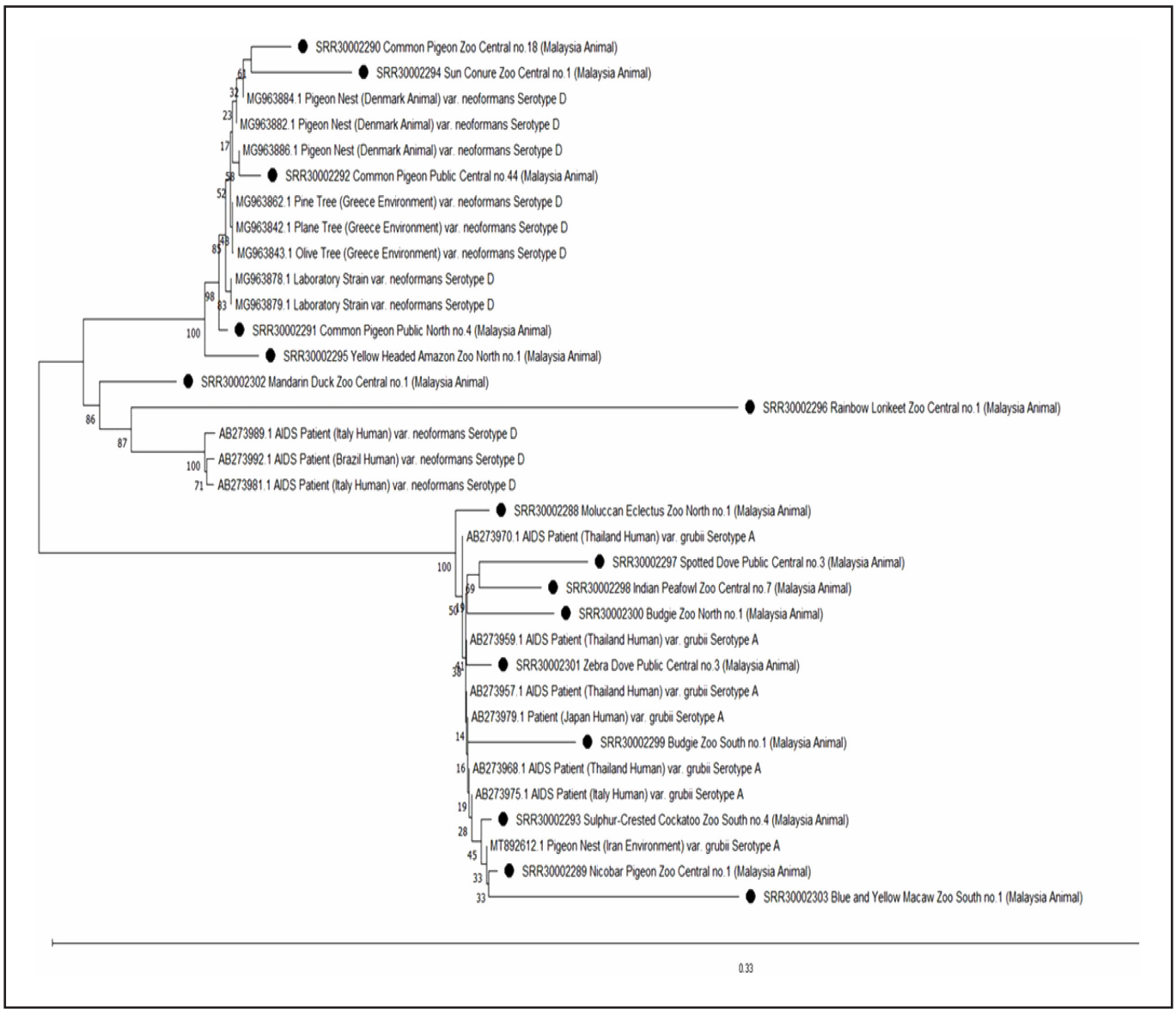

Figure 2. Phylogenetic analysis of the nucleotide sequences (samples from this study are indicated with bullets).

intermediate susceptibility to Fluconazole, showed over 99% similarity to *C. neoformans* var. *neoformans.* We then constructed a partial phylogenetic tree (Figure 2) based on the 16 sequenced samples along with the reference sequences (S2 Table). Most of the isolates clustered closely with *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*, confirming their genetic similarities with these strains.

DISCUSSION

In this study, we aimed to determine the current epidemiology, molecular characterization, associated risk in zoological gardens and antifungal susceptibility of *C. neoformans* in bird droppings, expanding upon previous surveillance that was limited to the Klang Valley region of Malaysia. To date, no studies have reported *C. neoformans* prevalence across the entirety of Peninsular Malaysia. Our study addresses this gap by providing a surveillance in four regions, comparing samples collected from both public areas and zoological gardens. We observed a significantly high prevalence of *C. neoformans* (42.2%) in bird droppings, updating earlier data from nearly two decades ago, where only 3.7% prevalence was reported in 2005 (Tay *et al*., 2005). Several factors may account for the difference in prevalence findings including sampling location since the earlier study was limited to sites such as pet shops, zoos and public areas within the Klang Valley region, whereas our study covered a broader range of locations across Peninsular Malaysia. The sample types and diagnostic methods also differed as we specifically collected fresh bird droppings and used Nested-PCR which is known for its higher sensitivity and ability to detect low fungal loads. In contrast, the previous study included weathered or dried samples and employed culture and biochemical identification techniques which are generally less sensitive.

Comparatively, the occurrence of *C. neoformans* in bird droppings from neighbouring countries such as Thailand were done in Bangkok with a prevalence of 11% from pigeon droppings (Krangvichain *et al*., 2016). In Jakarta, Indonesia, Machrumnizar *et al*. (2022) had previously isolated *C. neoformans* from various sources including bird droppings from Canaries, White Eyes, Prinia, and woodpeckers, with a prevalence of 14.3%. Elsewhere in other countries such as in Busan, Korea, a 41.7% prevalence of *C. neoformans* in pigeon shelters was reported (Oh & Hwang, 2005). While in Ahvaz, Iran, the rate was 13.0% out of 161 samples in various bird species (Mirpourian *et al*., 2021). In Spain, a study found *C. neoformans* in 7.8% of pigeon samples (Rosario *et al*.,

2005). In Nigeria, Nweze *et al*. (2015) collected bird excreta samples from various locations and environments with a 22.0% prevalence of *C. neoformans* among 144 samples. In Africa, *C. neoformans* was isolated from 44 out of 420 samples collected from pigeon droppings, *Eucalyptus* and olive trees (Ellabib *et al*., 2016). Another study in Egypt discovered a 6.3% prevalence of *C. neoformans* in chicken samples (Abou-Elmagd *et al*., 2011). This study was not consistent with our findings as we have isolated 7 positive isolates out of 37 (18.9%) in chicken droppings. The prevalence of *C. neoformans* were also studied in migratory birds with one positive case was reported in Mallards (Amirrajab *et al*., 2016). In our study, Mallards had a prevalence of 27.7% recovered from Central Zoo A. These figures demonstrate regional differences, and our findings contribute important data from Malaysia.

We also highlight that, although there was no significant difference in overall prevalence between public areas and zoological gardens, common pigeons in zoological gardens had a significantly higher infection rate. This finding aligns with previous research identifying pigeons as common reservoirs for *C. neoformans* (Chander, 2008). Of concern, other bird species also contribute to environmental contamination. In public areas, pigeons nest near humans, frequently occupying rooftops, hospital balconies, and places of worship, where they rely on human structures for shelter and human activity for food. Interestingly, we found a high prevalence of *C. neoformans* in Indian peafowl droppings (61.9%), a species for which no prior data on *C. neoformans* prevalence exists. Similarly, Mandarin ducks had a prevalence of 40.0%. These findings suggest that a wider range of avian species may serve as reservoirs for the fungus.

We also explored the association between hygiene practice and environmental condition with the positive rate of *C. neoformans* in bird dropping. Columbidae family and enclosure density were found as significant predictors of *C. neoformans* positivity. For Columbidae family, the finding was not surprising as pigeons are commonly associated with this fungus. However, in our study we were also able to determine that the enclosure densities show significance as well. Crowded enclosures show a higher positivity rate for *C. neoformans* as compared to spacious ones. For example, the Southern Zoo E in this study had the highest prevalence amongst all zoological gardens at 58.3%. This zoo had the highest bird density in an enclosure, too many birds are kept together in certain space. Furthermore, each bird was only allowed to leave their cages to roam free in a bigger enclosure three times weekly. This raise concerns as the amount of bird droppings increases a lot in a short time, being concentrated in the particular enclosures. Not being cleaned in time leads to a high frequency of bird dropping stains within the enclosures, increasing the probability of C. neoformans growth. Whilst it poses health hazards, it also makes the zoological garden appear unsightly to visitors. This shows that enclosure density plays a major role for the positivity of this fungus compared to other factors. Whilst there are not many reports on enclosure densities and infection positivity rate, Raso *et al*. (2004) reported a cryptococcosis outbreak in a breeding enclosure for various Psittacine birds that are kept in a small breeding cage.

In this study, all birds from which samples were collected appeared healthy, suggesting that while birds act as reservoirs, they may not always exhibit symptoms of cryptococcosis. However, one case of avian cryptococcosis with mycotic rhinitis was reported in captive parrots in Sydney, Australia, reinforcing the zoonotic potential of this fungus (Malik *et al*., 2003).

Molecular typing in this study identified all isolates as either *C. neoformans* var. *grubii* or var. *neoformans*. The predominance of *C. neoformans* var. *grubii* (9 isolates) was expected, as it is the most common variety globally especially in infected humans (Franzot *et al*., 1999; Ito-Kuwa *et al*., 2008). This emphasizes the need for heightened awareness and protective measures for both zookeepers and visitors, as exposure to bird droppings poses a public health risk. However, the presence of *C. neoformans* var. *neoformans* in Malaysia is surprising, as this variety is typically associated with decaying wood in Northern Europe (Lazera *et al*., 1996; Franzot *et al*., 1999). Seven of our isolates were identified as var. *neoformans*, including two from Mandarin duck and rainbow lorikeet droppings in a central Peninsular Malaysia zoo. These isolates were closely related to strains previously recovered from AIDS patients raising concerns about potential human infections (Ito-Kuwa *et al*., 2008). Zoo visitors and zookeepers may be at risk due to prolonged exposure to bird droppings.

Previous study by Tay *et al*. (2010) showed that *C. neoformans* var. *gattii* were isolated from patients in 2003-2004 in Malaysia but was predominated by infections by *C. neoformans* var. *neoformans*. In our study however, no *C. neoformans* var. *gattii* were isolated from bird droppings, this is expected as *C. neoformans* var. *gattii* are usually isolated from the red gum trees such as River Red Gum tree or Forest Red Gum Tree (Krangvichain *et al*., 2016).

Earlier studies reported all *C. neoformans* isolates as susceptible to antifungals such as Amphotericin B, Fluconazole and Itraconazole (Tay *et al*., 2005). In contrast, our study identified two isolates from public areas (SRR30002291 and SRR30002292) showing resistance or intermediate susceptibility to Fluconazole (25 µg/disk) and Itraconazole (50 µg/disk). These isolates were derived from common pigeon droppings, highlighting the possibility of evolution of antifungal resistance in *C. neoformans* over the past two decades (Chander, 2008). However, it is important to note that our finding only represent initial screening data and confirmation of antifungal resistance or intermediate susceptibility would require determination of minimum inhibitory concentrations (MICs) using a standardized reference method, such as the CLSI M27 broth microdilution protocol which were not included in this study.

Resistance to fluconazole has shown an increase from 7.3% to 11.7% between 1997 and 2007, this was recorded by the ARTEMIS DISK Global Antifungal Surveillance Study and were reported to be *C. neoformans* isolates from Cambodia, Africa and Spain (Bermas & Geddes-McAlister, 2020). These shows rising concerns on the current status of fluconazole resistance globally, as this information was last reported in 2007. Same goes for the state of antifungal resistance in Malaysia, since Cambodia and Malaysia both share the same climate, as climate proves to be significant in being a factor for *C. neoformans* growth (Rajasingham *et al*., 2017). People living in countries who are on the lower income group tend to suffer the fatality of cryptococcosis due to not being able to get access to first-line antifungal drugs, notwithstanding the growing antifungal resistance portrayed by *C. neoformans*. The countries that are mainly affected by this phenomenon are the sub-Saharan African countries along with some Asian countries (Bermas & Geddes-McAlister, 2020). Since cryptococcal meningitis proves to be fatal if left untreated immediately, a resistant *C. neoformans* will make the treatment ineffective. The prognosis will already be detrimental by the time clinicians discover antifungal resistance. Our study highlights the chances of antifungal resistance portrayed by *C. neoformans* in Malaysia to bring awareness to the health community.

In conclusion, this study demonstrates that *C. neoformans* is prevalent in both public areas and zoological gardens in Peninsular Malaysia. These findings emphasize the importance of periodic surveillance and preventive measures, particularly for immunocompromised individuals, zookeepers, and zoo visitors (Rao, 2023). Regular sanitization, the use of protective gear, and the control of bird populations in public areas such as places of worship and hospitals are crucial to reducing exposure and the risk of cryptococcosis.

This study has several limitations that should be considered when interpreting the findings. First, the sampling was based on the presence of specific bird species encountered during the sampling period, which may not represent the overall diversity of bird across all regions and may introduce sampling bias. Additionally, geographical and environmental factors vary not only between states but also between districts within Peninsular Malaysia, potentially influencing the distribution and prevalence of *C. neoformans* in bird droppings. Our sampling was also highly concentrated in the central region with no samples from the eastern region due to lack of access permissions. This regional imbalance limits the generalizability of our findings. In addition, the cross-sectional design of the study provides only a snapshot of *C. neoformans* prevalence during the sampling period and does not capture temporal changes. Longitudinal studies, particularly in zoological gardens and other high-risk environments, would be valuable for tracking trends over time and improving our understanding of the epidemiology of this infection. Another limitation of this study is the absence of quantitative data related to hygiene in zoological gardens such us as cleaning frequency logs, exact enclosure measurements and precise bird counts per enclosure. Instead, our assessment was based on observational categorical variables only. While these provided baseline insights for risk analysis, the lack of quantitative data may limit the precision of our findings. Incorporating such data in future studies would strengthen the reliability of risk factor analysis and allow better statistical comparisons. Furthermore, only a few isolates were selected for sequencing due to financial constraints, resulting in partial phylogenetic trees and limiting insights into the full genetic diversity of the isolates. Regarding antifungal susceptibility testing, we employed the disc diffusion method, which is non-specific and serves only as a preliminary screening tool to identify potential resistance. This approach does not provide precise inhibitory values such as MIC which would require a standardized method like the CLSI M27 broth microdilution assay. Future studies should incorporate MIC determination for a more accurate assessment of antifungal resistance patterns. Despite these limitations, this study serves as a valuable baseline, offering insights and a benchmark for future investigations into *C. neoformans* studies in Malaysia.

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Conflict of Interest Statement

The author declares that they have no conflict of interests.

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