UNIVERSITI TEKNOLOGI MARA

MOLECULAR EPIDEMIOLOGY AND ANTIFUNGAL SUSCEPTIBILITY OF *CRYPTOCOCCUS NEOFORMANS* ISOLATED FROM BIRD DROPPINGS IN SELECTED PUBLIC PLACES AND ZOOLOGICAL GARDENS OF PENINSULAR MALAYSIA

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MSc

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HAZIQ BIN FAISAL

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CONFIRMATION BY PANEL OF EXAMINERS

I certify that a Panel of Examiners has met on 5 March 2026 to conduct the final examination of Haziq bin Faisal on his Masters of Science thesis entitled "Molecular Epidemiology and Antifungal Susceptibility of *Cryptococcus neoformans* isolated from bird droppings in selected Public Places and Zoological Gardens of Peninsular Malaysia" in accordance with Universiti Teknologi MARA Act 1976 (Akta 173). The Panel of Examiners recommends that the student be awarded the relevant degree. The Panel of Examiners was as follows:

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ABSTRACT

Cryptococcus neoformans is a ubiquitous fungus commonly associated with pigeon droppings. This organism poses a significant health risk, causing cryptococcosis in both immunocompromised and immunocompetent individuals. In Malaysia, clinical reports spanning 1953 to 2020 indicate a rising incidence of cryptococcal infections especially cerebral cryptococcosis. However, research on the occurrence of *C. neoformans* in bird hosts remains scarce, with the last study conducted in 2005 and restricted to the Klang Valley region. This study aimed to provide updated data on the molecular epidemiology of *C. neoformans* in bird droppings by encompassing its prevalence, associated risk factor in zoological garden, antifungal susceptibility and serotypes from samples collected in public places and zoological gardens across Peninsular Malaysia. Samples were first cultured on Bird Seed Agar (BSA) for presumptive identification based on melanin pigment production. Nested PCR employing *CNLAC1* gene-specific outer and inner primers was performed for molecular identification. Susceptibility of the positive isolates to antifungals Amphotericin B, Fluconazole and Itraconazole was assessed. Bird species, hygiene and environmental aspects of the zoological gardens were documented. Overall, 509 bird droppings were collected: public places [n=257 (50.5%)] and zoological gardens [n=252 (49.5%)]. Common pigeons were the predominant samples collected from public places (n = 144; 56.0%). Mandarin ducks represented the dominant source of samples from zoological gardens (n = 40; 15.9%). Common pigeons in zoological gardens exhibited a significantly higher positivity rate (80.8%) compared to the common pigeon in public places (54.9%) (P = 0.013). High positivity rates in zoological gardens were observed in Indian peafowls (61.9%) and budgies (61.3%). Doves in this study exhibited a high positivity rate (43.5%) in public places. The positivity rate of *C. neoformans* in zoological gardens were found to be associated by enclosure densities (P=0.02) and the Columbidae family (P= 0.002). Only one isolate showed resistance to Itraconazole, while another exhibited intermediate resistance to Fluconazole. Two serotypes in this study were identified, serotype A (*C. neoformans* var. *grubii*) and serotype D (*C. neoformans* var. *neoformans*). These findings reveal markedly high *C. neoformans* positivity rate, most notably in common pigeons but also in other bird species, with enclosure density in zoological gardens further contributing to colonization. The detection of clinically relevant serotypes and potential resistance to Itraconazole also highlighted the importance of continuous public health monitoring and the implementation of preventive strategies, especially in environments with frequent human-bird interactions.

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Final thanks to my family for always believing in me and being there whenever I am down.

Alhamdulillah

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LIST OF SYMBOLS

Symbols

%	Percentage
≥	More Than Or Equal To
≤	Less Than Or Equal To
+	Addition
=	Equals
&	And
℃	Degree Celsius
“	Inches
bp	Base Pair
cm	Centimetre
cm^3	Cubic Centimetre
g	G Force (Relative Centrifugal Force)
kb	Kilobase
mm	Micrometre
ml	Millilitre
Mg	Magnesium

pH	Potential Of Hydrogen
PSI	Pounds Per Square Inch
rpm	Revolutions Per Minute
s	Seconds
w/v	Weight Over Volume
x	Multiply
µg	Micrograms
µL	Microlitre
µM	Micrometre

LIST OF ABBREVIATIONS

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
BBB	Blood Brain Barrier
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
FPPL	Fungal Priority Pathogen List
FNAC	Fine Needle Aspiration Cytology
HIV	Human Immunodeficiency Virus
mtDNA	Mitochondrial Deoxyribonucleic Acid
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
PIC	Person In Charge
R&D	Research & Development
SDG	Sustainable Development Goals
SOP	Standard of Procedures
UiTM	Universiti Teknologi MARA
WHO	World Health Organisation

CHAPTER 1
INTRODUCTION

1.1 Research Background

Cryptococcus is a ubiquitous fungus belonging to the Basidiomycota division, characterized by its ability to produce basidiospores during sexual reproduction and club-shaped extensions known as basidia. There are two major Cryptococcal species complexes that are clinically significant in humans: *Cryptococcus neoformans* (*C. neoformans*) and *Cryptococcus gattii* (*C. gattii*), which were once classified as a single species (Hagen et al., 2015). These organisms are the causative agent of cryptococcal meningitis, a chronic fungal disease in humans. *C. neoformans* is more common and primarily affects immunocompromised individuals, particularly those with human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS) and the elderly. In contrast, *C. gattii* is less prevalent but can also cause disease in immunocompetent hosts (Ngamskulrungroj et al., 2012).

Humans may get this fungal infection by inhaling the airborne propagules or spores from environments contaminated with *Cryptococcus,* which are often associated with the accumulation of bird droppings especially from pigeons. The fungus is also found in decaying wood or plant materials which provide a nutrient-rich habitat for its growth (Cogliati et al., 2016). Once inhaled, the fungus primarily infects the lungs before disseminates to extrapulmonary sites such as the central nervous systems (CNS), via the hematogenous route. Although it can infect a wide range of human cells, this fungus has a strong affinity towards the CNS due to the ability to attach to endothelial cells of the blood brain barrier through several mechanisms including the trojan horse strategy, transcytosis or paracellular crossing (Chander, 2017; Zhao et al., 2019; Chen et al., 2022). Since *Cryptococcus* is widespread in the environment, humans are likely exposed to its propagules regularly. However, due to it being a naturally opportunistic pathogen, the fungus will be typically cleared from the body immune systems or become dormant, preventing disease manifestation in immunocompetent individuals (Zhao et al., 2019; Alanio, 2020; Pruitt et al., 2025).

Globally, an estimated 220,000 cases of cryptococcal meningitis occur every year, with a mortality rate ranging from 41.0% to 61.0%, primarily affecting patients with the human immunodeficiency virus (HIV) (Rajasingham et al., 2017). Additionally, approximately 1.8 million people die annually from acquired immunodeficiency syndrome (AIDS) -related diseases, with cryptococcal meningitis accounting for 15.0% of these deaths (Nweze et al., 2015; Rajasingham et al., 2017; Zhao et al., 2019). Given its significant global impact, *C. neoformans* has been classified in the critical group of the Fungal Priority Pathogen List (FPPL) by the World Health Organization (WHO) and ranked as the number one priority pathogen on this list (Figure 1.1) (WHO, 2022).

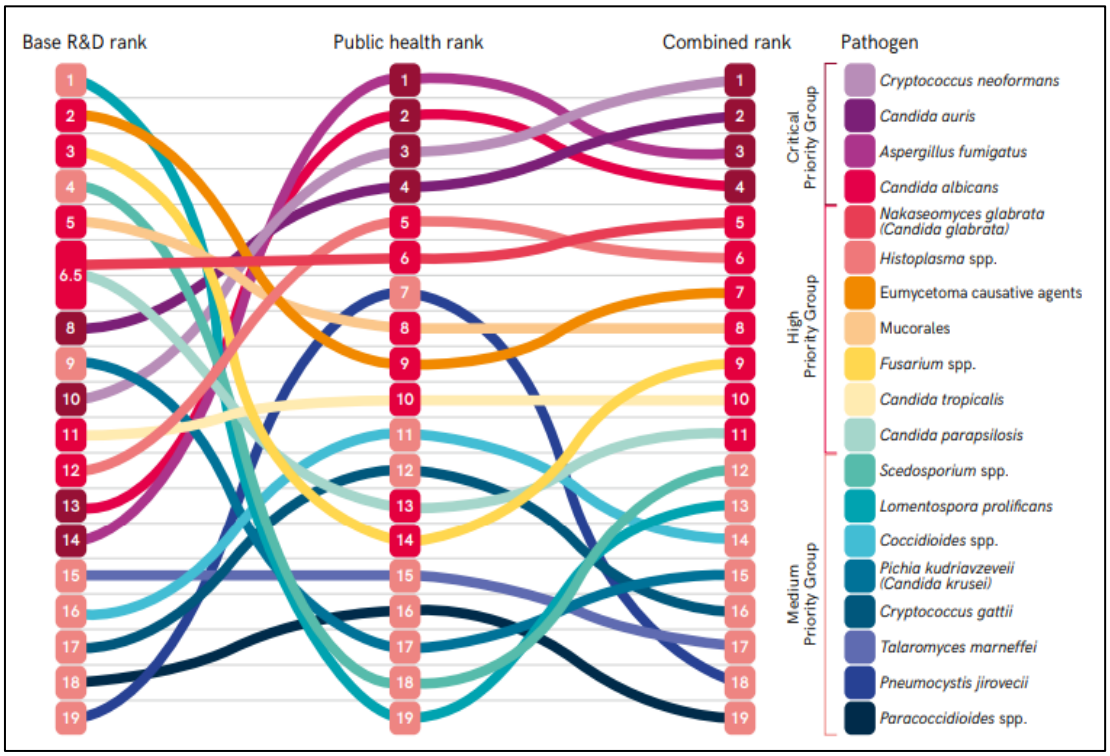

Figure 1.1 The Fungal Priority Pathogen List (FPPL) by the World Health Organization (WHO). Pathogens are Categorized into Medium, High, and Critical Priority Groups Based on Research and Development (R&D) Priorities and Public Health Significance. *Cryptococcus neoformans* is Ranked First on the List. Source: WHO, 2022

Due to the absence of vaccines for cryptococcosis, prevention relies on avoiding exposure to its propagules as the disease is not directly transmitted via human-to-human

transmission (WHO, 2022). One of the major risk factors of contracting this fungal infection is being exposed to birds frequently. These birds act as natural reservoirs and able to disseminate the fungus due to their mobility. Studies conducted in various countries such as Malaysia, Iran and Nigeria have reported the presence of *C. neoformans* in bird droppings with 3.0 % to 22.0% prevalent rates (Tay et al., 2005; Nweze et al., 2015; Mirpourian et al., 2022). Among bird species, pigeons pose the highest risk of harbouring *Cryptococcus*. This is mainly due to the high concentration of nitrogenous compounds particularly creatinine in their droppings which serve as a rich nutrient source that promotes the growth of the fungus (Chander, 2017).

Cryptococcus has several important variants or serotypes. Based on current classification, *C. neoformans* includes serotype A (*C. neoformans* var. *grubii)* and serotype D (var. *neoformans),* as well as the hybrid serotype AD. This species complex primarily causes infections in immunocompromised individuals (Mitchell & Perfect, 1995; Viviani et al., 2006). In contrast, *C. gattii* includes serotypes B and C and is more commonly associated with infections in immunocompetent individuals (Speed & Dunt, 1995). Genetic variation within these species arises from both unisexual and bisexual modes of reproduction (Zhao et al., 2019). These reproductive strategies lead to the redistribution of genetic material either through parent cells recombination or direct inheritance via basidiospores. Hence, mitochondrial DNA (mtDNA) which is responsible for virulence and pathogenicity also varies among strains, leading to different mechanisms of infection such as variation in spore germination rates and the formation of hyphae (Zhao et al., 2019). In general, the basidiospores produced by *C. neoformans* are resistant to many environmental stresses including oxidative stress, high temperatures and desiccations (Botts et al., 2009). Their small size helps them to reach in the lodging of spores deep into the lung alveoli, preventing airway clearance by the ciliated epithelium (Botts & Hull, 2010). It has been observed that serotype D (var. *neoformans*) spores are more pathogenic than their yeast counterparts, whereas the yeast cells of serotype A (var. *grubii*) exhibit greater virulence than their spores, the differences likely due to spore germination rate dynamics (Zhao et al., 2019).

Over the years, the main antifungal therapies for various forms of cryptococcosis including pulmonary and meningitis cases have been Amphotericin B, Fluconazole and Flucytosine (Chander, 2017). However, increasing reports of anti-fungal resistance have raised concerns, particularly with Fluconazole (Sionov et al.,

2009; Zafar et al., 2019). From 1997 to 2007, the prevalence of Fluconazole-resistant in clinical isolates from countries including Spain, Cambodia, and Africa increased from 7.3% to 11.7% (Bermas & McAlister, 2020). In 2022, WHO has highlighted Fluconazole resistance as a growing threat, further exacerbated by limited access to effective antifungal treatments in many countries. In addition to that, anti-fungal Amphotericin B is contraindicated in many patients due to its nephrotoxicity, making treatment even more challenging (WHO, 2022).

In Malaysia, this infection was first described clinically with 55 cases recorded between 1964-1975, followed by 85 cases (1974-1980) and 96 more cases in the year of 2003-2004 (Tay et al., 2005; Tay et al.,2010). Following these reports, Guna Segaran et al. (2021) documented an update on the cryptococcosis cases from a public hospital between 2013 and 2019, highlighting a growing number of cases in Malaysia with a higher prevalence among male patients. Although cryptococcosis is generally an opportunistic infection, not all reported cases involved immunocompromised individuals, suggesting that healthy individuals may also be at risk (IMR, 1953; Richardson et al., 1976; Pathmanatan & Soo-Hoo, 1982; Tay et al., 2010; Tan & Lim, 2019; Guna Segaran et al., 2021) (Figure 1.2).

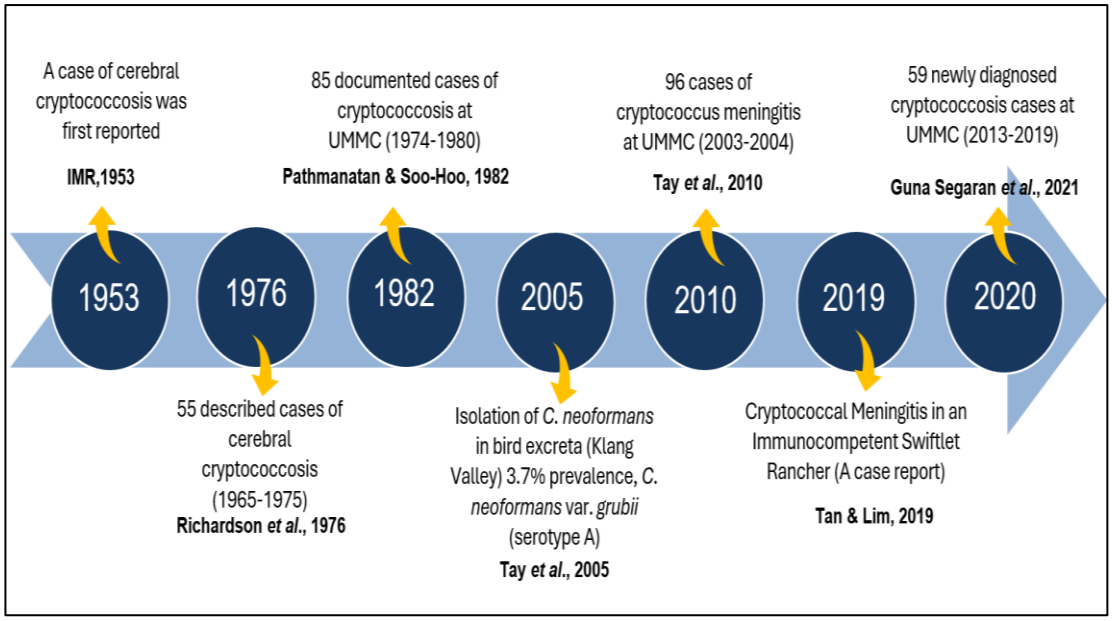

Figure 1.2 Chronology of Previously Reported *Cryptococcus* Cases in Malaysia

One of the possible contributing factors to the increasing incidence of cryptococcosis in Malaysia is widespread exposure to fungal propagules (IMR, 1953; Richardson et al., 1976; Pathmanatan & Soo-Hoo, 1982; Tay et al., 2010; Tan & Lim, 2019; Guna Segaran et al., 2021). Mainly found in bird droppings, *Cryptococcus* can easily contaminate the environments as birds mainly pigeons are highly seen in both rural and urban areas. In urban areas, dwelling pigeons pose a growing concern, as they tend to nest on rooftops and in the crevices of high-rise buildings and apartment complexes. The close proximity of these birds to densely populated residential areas may heighten the risk of human exposure. As a result, the rising bird population in urban settings may contribute to an increase in cryptococcal infections, affecting not only immunocompromised individuals but also those with healthy immune systems. Hence, the lack of current localized data on environmental exposure related to clinically significant *C. neoformans* in bird droppings warrants further investigation which forms the focus of this study.

1.2 Problem Statement

Cryptococcus infection has been clinically reported in Malaysia since 1964 with cases continuing to rise in recent years (IMR, 1953; Richardson et al., 1976; Pathmanatan & Soo-Hoo, 1982; Tay et al., 2010; Tan & Lim, 2019; Guna Segaran et al., 2021). Despite its recognized clinical relevance, environmental surveillance particularly its presence in bird droppings remains limited and warrants further investigation. To date, only one study (Tay et al., 2005) has reported about the occurrence of *C. neoformans* (serotype A) isolated from bird droppings in Peninsular Malaysia, however the study focused solely on the Klang Valley areas. This leaves a knowledge gap regarding the current status of *C. neoformans* in bird droppings after approximately 20 years since that study and its occurrence in other regions of Peninsular Malaysia. Moreover, comprehensive studies comparing its prevalence in bird droppings from zoological gardens and public areas in Peninsular Malaysia remain scarce.

The increasing number of zoological gardens, bird parks as well as coexistence of birds especially pigeons with humans in public places (e.g., on apartment rooftops, hospital balconies, and places of worship) raise concerns about high environmental exposure to cryptococcosis. Zoo or bird park workers in particularly, may face

heightened risk due to their routine contact with bird droppings. However, studies evaluating the hygienic or environmental conditions that may contribute to fungal presence in birds in such settings remain limited in Malaysia.

There is also limited understanding of the molecular characteristics of environmental *Cryptococcus* complexes isolates in Malaysia, including their serotypes and phylogenetic relationships. In 2005, Tay et al. (2005) reported all the *Cryptococcus* isolated from birds in Klang Valley area to be of serotype A (*C. neoformans* var. *grubii).* Following this discovery, another study by Tay et al. (2010) was conducted by isolating this fungus from human patients. The clinical isolates obtained in this study was also predominated by *C. neoformans* var. *grubii* serotype A accounting up to 88.5%, with the remaining being *C. gattii* species.

The antifungal susceptibility studies of *C. neoformans in* Malaysia are also rarely conducted. The last known report was by Tay et al. (2005) in which all isolates were found to be susceptible to Amphotericin B, Fluconazole and Itraconazole. Although no resistance was reported in Malaysia, given global trends in antifungal resistance (Sionov et al., 2009; Zafar et al., 2019), assessing current local antifungal susceptibility of *C. neoformans* isolates in bird dropping to anti-fungal agents like Amphotericin B, Fluconazole, and Itraconazole is also critical.

Hence, this study aimed to address gaps by investigating the current prevalence of *C. neoformans* in bird droppings from zoological gardens and public places in Peninsular Malaysia, the environmental and hygienic factors associated with its presence in bird droppings from zoological gardens, their susceptibility to common antifungal agents and the serotypes and phylogenetic relationships of the isolates. These findings are expected to provide valuable insights for public health awareness and occupational safety among individuals or zoo workers who are frequently exposed to bird droppings.

1.3 Research Questions

This study is motivated by the following research questions:

a) Given the limited data on *C. neoformans* in bird droppings in Malaysia, with the last study reported in 2005, what is the current prevalence of *C. neoformans* found in bird droppings from both zoological gardens and public

places in Peninsular Malaysia and how does its occurrence compare between these two areas?

b) Zoo workers are potentially at risk of exposure to *C. neoformans* due to their close interaction with bird dropping in zoological gardens, hence are there any potential hygienic or environmental factors that may associate with the presence of *C. neoformans* in this setting?

c) How susceptible are the *C. neoformans* isolates from bird droppings in Peninsular Malaysia to antifungal agents (Amphotericin B, Fluconazole and Itraconazole) and are there any potential resistance patterns?

d) What are the serotypes and phylogenetic relationships of *C. neoformans* isolated from bird droppings in Peninsular Malaysia?

1.4 Objectives

1.4.1 General Objective

The objective of this study was to investigate the molecular epidemiology of *C. neoformans* isolated from bird droppings in zoological gardens and public places in Peninsular Malaysia, the environmental and hygienic factors associated with its presence in bird droppings in zoological gardens, their antifungal susceptibility profiles, and the serotypes and phylogenetic relationships of the isolates.

1.4.2 Specific Objectives

a) To determine the current prevalence of *C. neoformans* in bird droppings collected from zoological gardens and public places in Peninsular Malaysia, and to compare its occurrence between these two environments.

b) To investigate potential hygienic and environmental risk factors associated with *C. neoformans* presence in bird droppings from zoological gardens.

c) To evaluate the antifungal susceptibility patterns of *C. neoformans* isolates from bird droppings in Peninsular Malaysia to assess potential resistance trends.

d) To identify the serotypes and phylogenetic relationships of *C. neoformans* isolated from bird droppings in Peninsular Malaysia.

1.5 Research Hypotheses

a) The prevalence of *C. neoformans* in bird droppings from Peninsular Malaysia is expected to be higher in zoological gardens compared to public places.
b) At least one environmental or hygienic factor is expected to influence the presence of *C. neoformans* in zoological gardens.
c) Some of the *C. neoformans* isolates from bird droppings will show resistance to certain antifungals.
d) Serotypes of clinically significant *C. neoformans* will be identified in bird droppings from Peninsular Malaysia.

1.6 Significance of Study

This study aimed to comprehensively update the status and knowledge of clinically significant *C. neoformans* species in bird droppings in Peninsular Malaysia, providing valuable insights into its current prevalence and potential public health risks. By evaluating the risks of transmission in zoological gardens, this study contributed to a better understanding of both occupational and environmental exposure to this fungal pathogen. In alignment with the United Nations Sustainable Development Goals (SDGs), this study supports SDG 3 (Good health and Well-Being) by raising awareness among the public and zoo workers about the risks of contracting *C. neoformans* by prolonged exposure to birds and the importance of preventive measures. By identifying which bird species harbour *C. neoformans* and their corresponding serotypes, the study can inform targeted public health strategies and risk mitigation efforts.

This study also addresses SDG 6 (Clean Water and Sanitation) by evaluating the cleanliness and environmental management practices (e.g., enclosure conditions and cleaning frequency) in zoological gardens. Based on these findings, recommendations and suggestions were shared with veterinarians and persons-in-charge (PICs) at the participating zoos, highlighting the need to improve hygiene measures to minimize potential transmission to the visitors and zoo workers. Beyond assessing the occurrence of this fungus in bird droppings, this study evaluated the antifungal susceptibility profiles of *C. neoformans* isolates. Determining whether these isolates remain susceptible to commonly used antifungals or exhibit resistance will provide important

baseline data and serve as a benchmark for monitoring antifungal resistance trends and providing essential data for future clinical management and epidemiological studies.

1.7 Scope of the Study

To answer the research questions, this study focused on the prevalence of *C. neoformans* in bird droppings collected from both zoological gardens and public places in Peninsular Malaysia (**objective 1**), hygiene and environmental factors associated with *C. neoformans* occurrence in birds of zoological garden (**objective 2**), antifungal susceptibility of *C. neoformans* isolates in bird droppings (**objective 3**) and identification of the serotypes and partial phylogenetic relationships (**objective 4**) across four regions (central, northern, western and eastern) of Peninsular Malaysia.

For **objective 1**, bird droppings were collected from selected zoological gardens based on their public popularity and the density of bird species they host. Sites for public places were chosen based on the higher abundance of birds observed in those locations. The samples then underwent both laboratory and molecular identification (nested PCR). For **objective 2**, a simple survey and observation were conducted on the environmental and hygiene factors that might be associated with the occurrence of *C. neoformans* in bird droppings in zoological gardens. For **objective 3**, fungal isolates from the bird dropping were tested against antifungals drugs; Amphotericin B, Fluconazole and Itraconazole to determine their susceptibility or resistance. For **objective 4**, PCR-positive samples were sent for sequencing to identify the serotypes of *C. neoformans.* The sequencing data were then used to construct a phylogenetic tree.

CHAPTER 2
LITERATURE REVIEW

2.1 Early Discoveries of *Cryptococcus neoformans*

Cryptococcus neoformans is an opportunistic yeast that has been infecting humans for over a century (Srikanta et al., 2014). It was first discovered in the late 1800s by Sanfelice, an Italian scientist who isolated the organism from fermenting peach juice and initially named the organism *Saccharomyces neoformans* (Sanfelice, 1894; Kwon-Chung et al., 2014; Srikanta et al., 2015). Around the same time, Busse described a similar *Saccharomyces*-like pathogen causing human infection, referring to it as *Saccharomyces hominis* (Busse, 1984). Barnett (2010) stated that in respect to both Busse's and Sanfelice's discovery; Vuillemin reclassified the organisms as *Cryptococcus hominis* and *C. neoformans* respectively in 1901, because the *Cryptococcus* did not produce ascospores like *Saccharomyces*. When scientists performed serological studies on these organisms, the infections belonged to one species but posed two varieties, conserving the name of the organism as *C. neoformans* (Barnett, 2010).

2.2 Characteristics and Life Cycle

C. neoformans is found worldwide and is frequently isolated from pigeon droppings, while *C. gattii,* a closely related species is commonly associated with *Eucalyptus* trees (Lazera et al., 2000). It reproduces through either bisexual or unisexual mating which contributes to their genetic variation (Zhao et al., 2019). In the environment, *Cryptococcus* also interacts with potential predators such as the soil amoebas. *Cryptococcus* combat this predation through filamentation into pseudohyphae or hyphae (Steenbergen et al., 2001). In mammalian hosts, however, this fungus is dimorphic and grows almost exclusively in the yeast form (Lin et al., 2015; Steenbergen et al., 2001).

The pathogenicity of *Cryptococcus* relies on its life cycle and cellular differentiation in the host. Different forms trigger different host immune responses. For example, the hyphae form will trigger virulence attenuation from the host's protective

responses (Lin et al., 2015). Moreover, the sexual reproduction generates infectious spores, which responsible for the main pathogenesis of this organism in humans (Velagapudi et al., 2009). Therefore, the diversity of *Cryptococcus* genotype and phenotype arising from these sexual cycles and mating types contributes to the adaptability and virulence potential of this organism. Figure 2.1 shows the life cycle of *Cryptococcus* in the environment and in humans.

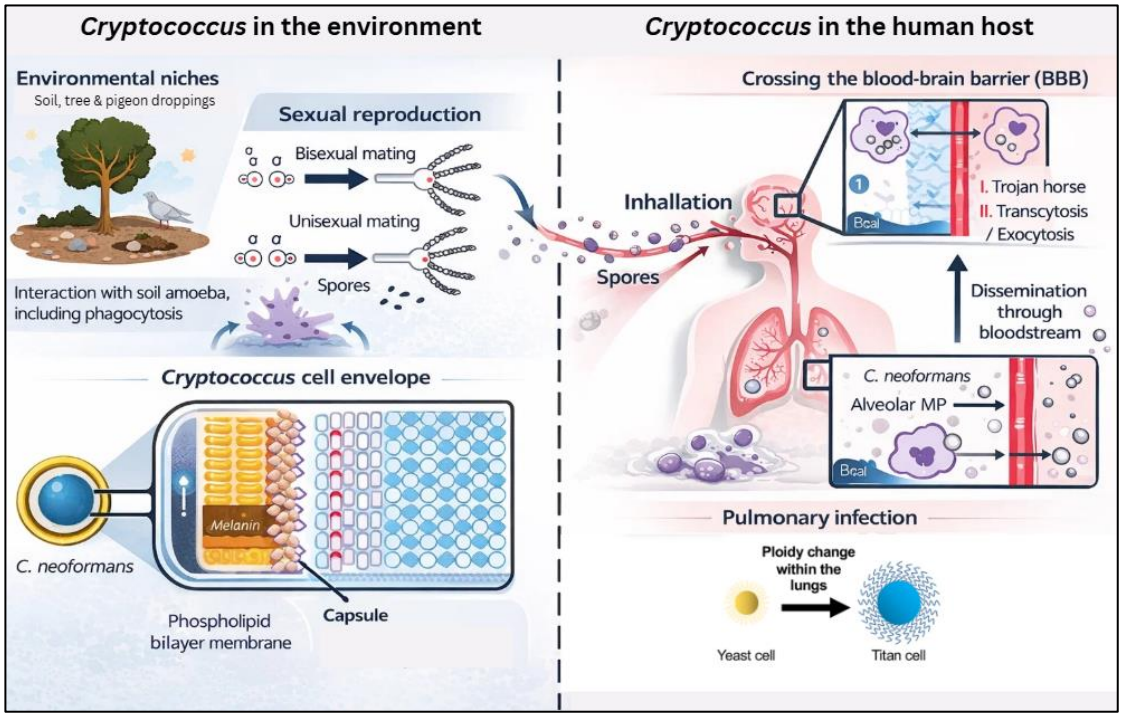

Figure 2.1 Life cycle of *C. neoformans*. Contraction of the Fungus from the Environment; Sexually Reproducing to Produce Spores and/or Yeast Cells. Both are Later Inhaled by Humans; the Fungus then Lodges itself in the Lungs and Disseminate into the Bloodstream which will later Crosses the Blood-Brain Barrier (BBB) Reaching the Brain. Source: (Bahn et al., 2020). Image created using Canva.

2.3 Serotypes of *C. neoformans*

The discovery of three serotypes (A, B and C) was discovered by Evans in 1950 (Evans, 1950), and the fourth serotype (serotype D) in 1968 by Wilson and team (Wilson et al., 1968). More efforts for looking at the diversity of *C. neoformans* isolates followed subsequently. There are several varieties of this organism, classified based on

their antigenic properties using specific antisera. These are divided into five serotypes: a classification that was used until 2015 (Chander, 2017; Hagen et al., 2015).

i. serotype A (*C. neoformans* var. *grubii)*

ii. serotypes B and C (*C. gattii)*

iii. serotype D (*C. neoformans* var. *neoformans)*

iv. serotype AD (*C. neoformans*)

Throughout the years, many studies of *C. neoformans* have been conducted inclusive of the taxonomic revisions of its varieties (May et al., 2016; Williamson et al., 2017; Friedman & Schwartz, 2019). The most notable taxonomic change was proposed by Hagen et al. (2015) based on the Multilocus Sequence Typing (MLST) and Amplified Fragment Length Polymorphism (AFLP) genotyping. As a result, *C. neoformans* var. *gattii* was reclassified as *Cryptococcus gatti* (serotypes B and C) (Hagen et al., 2015).

The varieties are mainly distinguished by differences in the major capsular polysaccharide; the glucuronoxylomannan (GXM) structure, which forms a key component of the fungal cell wall. More than 90% of the yeast cell is made of carbohydrates, predominantly GXM (Chander, 2017). The importance of these varieties proved to be detrimental as they have individual pathogenesis, in relation to their ecology and epidemiology (Zhao et al., 2019).

2.4 Clinical Epidemiology of *C. neoformans* Infection (Global Perspectives)

Globally, about 39 million are currently suffering from HIV /AIDS based on the latest report from the Joint United Nations Program and WHO (UNAIDS, 2025). These patients are highly susceptible to opportunistic infections, including those caused by *C. neoformans.* An approximately 600, 000 AIDS-related deaths occur every year, with a significant number associated with this fungal infection (UNAIDS, 2025).

The distribution of *Cryptococcus* species and serotypes varies across countries. In New Zealand, serotype A (*C. neoformans* var. *grubii*) predominates, accounting for 77.0% of human infections in both immunocompetent and immunosuppressed individuals (Chen et al., 2000). In contrast, reports from Australia show a nearly equal occurrence of *C. neoformans* var. *grubii* and *C. gattii* (Lee et al., 2019). In the Western

continents, Desnos-Ollivier et al. (2010) reported cases of mixed infections where different types of *C. neoformans* were found to hybridize, forming diverse mating types within the same patients. This indicates that a single serotype may not always be responsible for the cryptococcosis. In Italy, Cogliati et al. (2018) observed a notable increase of cryptococcosis from 7.0 to 38.0% between a year of 1997 to 2016 among immunocompetent individuals. Despite this trend, most affected patients (81.0%) had underlying conditions such as liver disease. The nearly five-fold increase of HIV-negative patients contracting cryptococcosis raises concerns over the potential for healthy individuals contracting cryptococcosis. Furthermore, the same study highlighted an increase of VNIII (Serotype AD) isolated from immunocompetent patients (11% to 41%).

In Europe, Tintelnot et al. (2004) reported that the clinical isolates from Austria and Germany were predominated by serotype A (74.5%), while Switzerland showed a lower proportion of the same serotype (52.0%). The remaining isolates belonging to serotypes AD and D at 26.0% and 22.0%, respectively. Similarly, Farrer et al. (2021) reported all clinical isolates in Scotland to be serotype A, and the recovered isolates have neither association to local environmental strains nor between patients, suggesting independent infection sources.

In North America, a *C. gattii* outbreak in British Columbia, Vancouver Island had infected 176 patients between 1999 to 2006. Approximately 27 cases were reported yearly infecting many immunocompetent patients (Galanis et al., 2007; MacDougall et al., 2007). A study by Ingroff-Espinel & Kidd (2015) reported that *C. gattii* infections and case reports in the USA and Canada showed a decreasing trend between 1999 and 2013. In their study, *C. gattii* was found more prevalent in immunocompromised patients instead.

In South America, serotype A remains the dominant type of *C. neoformans*. In a Brazilian university hospital, Aguiar et al. (2017) reported that 97.5% of isolates recovered from cryptococcosis patients between 2004 to 2013 were serotype A. Similarly, Bejar et al. (2014) isolated both clinical and environmental samples in Peru and found serotype A predominance (84.4%), while the remaining isolates belonged to serotype D. A prevalence study conducted in Argentina from 2009 to 2011 also showed a similar trend. Taverna et al. (2020) reported that serotype A was the predominant serotype (87.6%) out of 326 samples. Escadón et al. (2018) conducted a surveillance

study in Colombia and found that 87.5% of infections were from *C. neoformans* and 3.1% from *C. gattii*. For *C. neoformans* isolates, serotype A were the most isolated (96.1%). In terms of *C. gattii*, molecular type VGII predominates (54.3%). They also reported a male to female ratio of 3.9:1 indicating a higher prevalence among males.

In Asia, a similar dominance of serotype A has been observed. Chen & Lai (2011) reported serotype A to be the major isolate from clinical strains (93%) in China. In Japan, Umeyama et al. (2013) reported all clinical isolates to be serotype A as well. The majority of clinical isolates in Korea were also reported to be serotype A. Their genotyping report shows close genetic relationship between environmental and clinical isolates in the country (Park, et al., 2015).

In neighbouring countries like Singapore, Chan et al. (2014) reported predominance of serotype A, whereas only three of their cases were infected by *C. gatii*. About 81% of the patients were HIV positive. Similar to previous reports, the majority of patients in their study were male as well. This suggested that gender proportions of the local HIV infected individuals in Singapore, however, environmental exposure and other factors could play a role as well (Chan et al., 2014). In Thailand, the investigation was obtained from animals, humans and environment. Most of the *C. neoformans* var. *grubii* (serotype A) isolated in this study generally were from immunocompromised patients (88.5%), whereas only 37.5% of *C. gattii* isolates recovered in this study were from the immunocompetent. Therefore, this study shows that *C. neoformans* were isolated more in HIV positive patients compared to *C. gattii. C. gattii* is more likely found in the environment (Kaocharoen et al., 2013). In Laos and Vietnam, the predominant serotype found in these two countries were serotype A (Thanh et al., 2019). A summary of countries and their predominant isolates are shown in Table 2.1.

Table 2.1

Countries and Predominant *C. neoformans* Isolates.

Continent	**Country**	**Predominant Isolates**	**References**
Americas	Argentina	*C. neoformans* var. *grubii* (Serotype A)	Taverna et al., 2020
	Brazil	*C. neoformans* var. *grubii* (Serotype A)	Aguiar et al., 2017
	Canada	*C. gattii* (Serotype B & C)	Ingroff-Espinel & Kidd, 2015
	Colombia	*C. gattii* (Serotype B & C)	Escadón et al., 2018
	Peru	*C. neoformans* var. *grubii* (Serotype A)	Bejar et al., 2014
	United States of America	*C. gattii* (Serotype B & C)	Ingroff-Espinel & Kidd, 2015

Asia	China	*C. neoformans* var. *grubii* (Serotype A)	Chen & Lai, 2011
	Japan	*C. neoformans* var. *grubii* (Serotype A)	Umeyama et al., 2013
	South Korea	*C. neoformans* var. *grubii* (Serotype A)	Park, et al., 2015
	Laos	*C. neoformans* var. *grubii* (Serotype A)	Thanh et al., 2019
	Singapore	*C. neoformans* var. *grubii* (Serotype A)	Chan et al., 2014
	Thailand	*C. neoformans* var. *grubii* (Serotype A)	Kaocharoen et al., 2013
	Vietnam	*C. neoformans* var. *grubii* (Serotype A)	Thanh et al., 2019
Europe	Austria	*C. neoformans* var. *grubii* (Serotype A)	Tintelnot et al., 2004
	Germany	*C. neoformans* var. *grubii* (Serotype A)	Tintelnot et al., 2004
	Italy	*C. neoformans* (Serotype AD)	Cogliati et al., 2018
	Scotland	*C. neoformans* var. *grubii* (Serotype A)	Farrer et al., 2021
	Switzerland	*C. neoformans* var. *grubii* (Serotype A)	Tintelnot et al., 2004
Oceania	Australia	*C. neoformans* var. *grubii* & *C. gattii* (Serotype B & C)	Chen et al., 2000
	New Zealand	*C. neoformans* var. *grubii* (Serotype A)	Chen et al., 2000

2.5 *C. neoformans* Infection in Malaysia

In Malaysia, there are only several studies depicting the status and epidemiology of *Cryptococcus.* However, most of these studies focused on *C. neoformans*. Cryptococcosis was first reported in Malaysia by the Institute of Medical Research (IMR). This happened in 1953. Subsequently, in 1976, more cryptococcal infections were observed in the brain (Richardson et al., 1976). However, the authors did not report on the variations or type of *Cryptococcus* present. Only diagnostic tests were done in their study.

Tay et al. (2010) conducted molecular analysis on the serotypes of *Cryptococcus* in Malaysia. However, instead of bird droppings and environmental samples, the isolation of *Cryptococcus* in this study were recovered from patients with chronic meningitis who were admitted in hospitals across Malaysia between 2003 to 2004. A few demographic factors were taken into account from the patients in this study. Out of 96 patients, 77.1% of the patients were male and 22.9% were female (Tay et al., 2010). Pathmanathan et al. (1982) reported that 81% of cryptococcal patients in the year 1974-1980 were male, making males the majority of cryptococcal cases in Malaysia during those years. Next, in terms of age range, the majority were between 30 to 39 years (46.9%). For the serotypes found in this study, 88.5% were serotype A isolated from all regions in Malaysia. This discovery was in line with the previous study done in 2005

which reported the same serotype as well (Tay et al., 2005). The remaining isolates were *Cryptococcus gattii* (serotype B) isolated from all regions except Southern Malaysia (Tay et al., 2010).

From 2003 to 2004, two serotypes - A and B were recovered from cryptococcal patients in Malaysia. In 2005 however, only serotype A was isolated from bird droppings which is consistent with expectations. Since serotypes B and C are typically associated with infections in both immunocompetent and immunocompromised patients (Cogliati et al., 2012; Hoang et al., 2004; Springer et al., 2012).

In 2019, the first case report of cryptococcal meningitis in an immunocompetent patient was reported in Malaysia. The patient was a swiftlet rancher and often exposed to swiftlet droppings during work. However, the serotype of the isolated cryptococcal species were not identified by the authors (Tan & Lim, 2019). Guna Segaran et al. (2021) conducted a retrospective study from 2013 to September 2019 at Universiti Malaya Medical Centre and found that male patients were the majority of cases (83.1%) compared to females (16.9%). This finding was in line with the two previous studies by Pathmanathan et al. (1982) and Tay et al. (2010) in which the majority of cryptococcosis patients were male. With regards to the molecular typing, 81.4% of the cases were of *C. neoformans* var. *neoformans* otherwise known as serotype D, whereas serotype A was found in 3.4% and only 1 case from *C. gattii*.

2.6 Risk factors and *Cryptococcus* in Bird Droppings

Understanding the risk factors for cryptococcal disease is important in diagnosis, treatment and prevention. Clinically, apart from HIV/AIDS patients, several comorbidities have been identified as major predisposing factors. Lin et al. (2015) reported that liver cirrhosis, and cell-mediated immunity (CMI) suppressive regiments without calcineurin inhibitors were significantly associated with cryptococcosis Similarly, Spec et al. (2016) found that 10.8% of cryptococcosis patients had underlying liver disease. Nina et al. (2015) and Yoon et al (2020) found that 20.5% and 8.0% of cryptococcosis patients, respectively had liver cirrhosis. Yoon et al. (2020) also reported that 63% of cryptococcal cases over a 12-year period were among HIV infected patients consistent with Babamahmoodi et al. (2020), who reported 66.6% of cases in HIV-positive patients.

Hence, these comorbid individuals should be cautious of potential environmental sources of infection, particularly bird dropping. Birds, particularly pigeons are well documented reservoirs of *C. neoformans* (Chander, 2017). Other bird species, such as lovebirds and cockatiels, have also been reported as potential carriers. These are birds that carry the fungus but do not get infected (Elhariri et al., 2015). Therefore, continuous environmental surveillance especially in areas with dense bird populations is essential for risk assessment.

In Malaysia, research on environmental epidemiology of *C. neoformans* remains limited. To date, only one epidemiology study on bird droppings was conducted (Tay et al., 2005) despite many reports about clinical cryptococcosis have been addressed since 1953 (Richardson et al., 1976). Tay et al., (2005) reported *C. neoformans* serotype A using API 20C AUX and Canavanine Glycine Bromothymol (CGB) agar methods within Klang Valley areas.,. Since then, no updated epidemiological data have been reported in Malaysia, highlighting a significant research gap in the region.

Globally, several studies have addressed the findings in bird dropping. In Thailand, Kaocharoen et al. (2013) collected bird droppings from the year 2002-2005 in Bangkok and found the isolates to be serotype A (98.8%) and serotype D (1.2%). Similarly, Oh & Hwang (2005) in South Korea isolated *Cryptococcus* serotype A from pigeon droppings with no other serotypes detected. In India, Chowdhary et al. (2012) reported the predominance of serotype A in their study, with only a few serotypes B. These were isolated from pigeon droppings. In 2015, Mirpourian et al. (2021) reported that all of the isolates recovered from pigeon droppings to be serotype A. Their study involved bird droppings from pigeons, Passeriformes and Psittaciformes. Litvintseva et al. (2011) recovered serotype A isolates from pigeon droppings in South Africa and Botswana. Despite the many reports on serotype A being the predominant isolate worldwide, Nnadi et al. (2016) reported the predominance of *C. gattii* instead, isolated from pigeon droppings in Nigeria.

2.7 Pathogenesis and Clinical Features of Cryptococcosis

Cryptococcosis is commonly seen in patients among the age group of 30 to 60, probably due to host immune status instead of the progression of the microbe itself. However, the damage and virulence factors prove to play a part as well (Chander, 2017).

The severity of the disease is dependent on two types: cryptococcosis in immunocompetent or immunocompromised patients. The lungs and brain are the organs mostly affected by this fungus (Chander, 2017). Several factors depict the quick dissemination and pathogenesis of *Cryptococcus.* Being encapsulated, they evade phagocytic response, making opsonin rendered useless. However, phagocytosis still occurs, the presence of melanin serves as an antioxidant, protecting the fungus from intracellular killing. This prevents phagocytes from producing TNF-α as well, a required component for the protective *Cryptococcus* immune response, in cases where the immunocompetent patients are infected (Chander, 2017).

The portal of entry of *Cryptococcus* is through the respiratory route. Once inhaled, colonisation of the mucosa layer begins where it could remain localised (pulmonary cryptococcosis) or disseminate to extrapulmonary sites. This results in generalized cryptococcosis depending on the host's immune status. Clinical features vary by sites of involvement, most commonly presenting as pulmonary cryptococcosis or cryptococcal meningitis (infection in central nervous system). Disseminated infection such as visceral, osseous, and cutaneous cryptococcosis are called cryptococcaemia. This occurs when the yeast starts to spread throughout the whole body caused by three main predisposing conditions: AIDS, liver cirrhosis and immunosuppressive therapy. These patients will require an early diagnosis along with antifungal therapy. Patients suffering from liver cirrhosis usually have poor prognosis, despite their age, antifungal therapy timing, severity of sepsis and their APACHE II score (Mariarz & Perfect, 2016; Chander, 2017; Liao et al., 2023).

Pulmonary cryptococcosis often resembles other respiratory infections and may even asymptomatically render diagnosis dependent on laboratory investigation rather than clinical presentation alone. In patients who are immunocompromised or are having any underlying diseases, the assumption of cryptococcosis should not be ruled out (Chander, 2017; Howard-Jones et al., 2022). In symptomatic patients, clinical presentations include self-limiting pneumonitis with mild or no fever, chest pain or/and dry cough. Uncommon symptoms include hilar adenopathy, pleural effusions and calcification. Chest radiographs may indicate solitary nodules, diffuse infiltrates or interstitial patterns of the upper and lower lungs that can mimic pneumonia caused by *Pneumocystic jirovecii* infection (Mariarz & Perfect, 2018).

The most common and severe form of cryptococcosis is cryptococcal meningitis. The infection of the brain and meninges often leads to meningoencephalitis. The infection of CNS is believed to be caused by decreased or impaired phagocytic response of the patient's immune system. Nutritional factors that support yeast growth, such as creatinine and asparagine are present in CSF. In contrast, many inhibitory factors found in blood serum, are absent in the CSF, allowing the fungus to migrate and proliferate within the CSF rather than remaining the bloodstream (Chander, 2017).

Cryptococcal meningitis seems to have prolonged clinical course tubercular meningitis. Therefore, this characteristic helps in the differential diagnosis. An alteration of the mental state is a sign of poor prognosis, the diagnosis of cryptococcosis should be considered if there are patients showing signs of progressive cognitive decline or dementia-like symptoms. Despite antifungal therapy, disseminated cryptococcal meningoencephalitis is still fatal in about 30% of cases. While *C. neoformans* is more often associated with meningitis, *C. gattii* is more likely to infect he parenchyma of the CNS leading to diffuse brain lesions and meningoencephalitis rather than meningitis alone (Chander, 2017; Chastain et al., 2022).

Visual losses is a notable complication and typically presents in two distinct patterns: rapid and slow visual loss. The rapid visual loss is seen where there is profound visual loss within 12 hours, suggesting optic neuritis. The slow pattern visual loss is characterised by slow but progressive visual loss due to the increased intracranial pressure developed during cryptococcosis. The slow visual loss could progress to severe over a few weeks or months. The patterns of visual loss in these cases could be predicted if there is presence of elevated CSF pressure, encapsulated yeast cells in India ink preparation, and papilledema (Chander, 2017; Aldhaleei et al., 2019; Ristow & Davis, 2021).

In male patients suffering from chronic lymphocytic leukaemia, the prostate could also be affected and this is reported as cryptococcal prostatitis. Children have been reported to suffer from primary abdominal lymphonodular cryptococcosis. The dissemination of cryptococcosis also causes primary adrenal insufficiency showing bilateral adrenal masses and meningitis. (Chander, 2017; Liao et al., 2023).

Wood & Miedzinski (1996) reported that osseous cryptococcosis only occurs in 5% to 10% of patients with disseminated diseases. Hematogenous spread could be the main contributor of this dissemination to the bones from pulmonary infections. Other

potential sources could be from contiguous neural infection or skin infection, embolic phenomenon or bone related traumas. Osseous cryptococcosis can occur at any bony site, most commonly reported sites are the vertebrae, ribs and pelvis, occurring at either a single or multiple sites (Wood & Miedzinski, 1996).

Cutaneous or skin cryptococcosis are manifested in 10-15% of systemic cryptococcal infections. Manifestations of isolated cutaneous cryptococcal lesions are usually caused by serotype D (Neuville et al., 2003).

2.7.1 Clinical Features in Birds

Birds are usually seen as carriers of *cryptococcus* and cryptococcosis usually infect humans. However, there are a few cases where birds are infected. Symptoms include mycotic rhinitis or involvement of structures adjacent to the nasal cavity. This includes the break, choana, palate, and sinuses (Malik et al., 2003). In Australia, Malik et al. (2003) reported the localised disease in the upper respiratory tracts of captive parrots. These birds were presented with proliferative lesions near the beaks. Rhamphotheca, nasopharynx and paranasal sinuses were affected as well.

In 2004, it was reported that a cryptococcosis outbreak had occurred involving seven Psittacine birds. These were captive birds in Brazil made for breeding. All birds involved died with signs of progressive paralysis and flying difficulties. Histological examinations showed cryptococcal cells in various organs and tissues. This includes the brain, air sacs, intestines and the beaks. Biochemical analysis confirmed the presence of *C. gattii* which was the first report of *C. gattii* infection for Psittacine birds in Brazil (Raso et al. 2004).

In another report, an African grey parrot had succumbed to death by cryptococcosis. Laboratory examinations revealed presence of cryptococcal bodies in the air sac and coelum (Schunk et al. 2017). Velasco (2000) stated nonspecific signs include dyspnoea, weakness, listless-ness, diarrhoea and anaemia. When the eyes or rhinic are involved, clear nasal discharge are seen. If cryptococcosis involves the brain, paresis, paralysis and jaw limberness could occur.

Littman & Borok (1968) investigated the ability of *Cryptococcus* surviving passage through the gastrointestinal track of pigeons. The authors instilled *C. neoformans* into the crops of three Feral pigeons. The fungus appeared in the fresh

droppings within an hour and survived for another 24 hours. This study was conducted by the authors because *C. neoformans* was found on the beaks and feet of pigeons, but not on from their rectal swab.

2.8 Laboratory Diagnosis of *Cryptococcus*

Cryptococcus are identified in the laboratory by various methods. This includes clinical specimens under staining, culture, immunologic methods or animal pathogenicity testing (Chander, 2017).

2.8.1 Conventional

2.8.1.1 Direct Examination

In clinical specimens, the usual specimens will be CSF, however, *Cryptococcus* are also seen isolated from serum and other body fluids. A direct microscopy of these specimens using 10% nigrosin with formalin wet mount, or India ink staining will show globular or round budding yeast cells. These cells range in 5-20 µm with a distinct halo in size. Being an encapsulated yeast, the gelatinous capsule surrounding this organism can be seen being almost twice as thick in terms of diameter in comparison to the yeast itself (Dash et al., 2014).

India ink staining had been modified throughout the years. An addition of 2% chromium mercury may be done to allow clear distinction of internal and external structures of the organism (Zerpa et al., 1996). Due to India ink being a negative stain, it is important for lab personnel to be able to distinguish between the yeast cells and artifacts such as leukocytes and powder granules from latex gloves (Chander, 2017).

Potassium Hydroxide (KOH) wet mount portrays budding yeast cells with numerous pus cells. However, a non-prominent capsule could be a false positive indicator for other yeasts. The use of calcoflour white stain prior to a KOH mount will help as the former acts as a fluorescent brightener, delineating the organism in the mount (Chander, 2017).

A routine Gram's staining is usually done in microbial laboratories; Gram-positive budding yeasts cells are seen. Giemsa stain for FNAC portrays yeast cells. The same can be seen for Papanicolaou preparations for the CSF. The capsular staining can

be done using Mayer's mucicarmine stain or Alcian-blue because *Cryptococcus'* capsule is carminophilic. To detect the production of melanin, Masson-Fontana stains can be performed, especially for *C. neoformans* that do not produce capsules in tissues (Chander, 2017).

2.8.1.2 Fungal Culture

C. neoformans is able to grow on various fungal agar, however, to provide a selective agar base, diphenyl is used in bird seed agars. This will inhibit other microbial growth whilst only supporting the growth of *C. neoformans*. Therefore, the bird seed agar is known to be the selective agar for *C. neoformans* (Denning et al., 1990). However, in some pathological cases, this fungus might be of an interest if grown together with other organisms. *C. neoformans* is able to grow well in Saboroud's dextrose agar (SDA), brain-heart infusion agar (BHI), cysteine-heart haemoglobin agar and sunflower seed agar. All these agars are also used for primary isolations as well, examined every day for three weeks. However, bird seed agar can be discarded if no growth is seen after two weeks (Nweze et al., 2015).

The colony morphology of *C. neoformans* on bird and sunflower seed agar is seen as light yellowish brown which can be seen growing as early as 48 hours. These can be incubated at various temperatures depending on the source of specimen; room temperature (environment) or 37℃ (humans) (Denning et al., 1990).

Suspecting yeast isolates can be identified by standard biochemical tests. Since all species of *Cryptococcus* assimilate inositol, produces urease and are non-fermentative, the differentiation between *C. neoformans* and other cryptococcus lies in the yellowing-browning of the colony on bird seed agar (Chander, 2017).

2.8.1.3 Immunodiagnosis

Cryptococcal polysaccharide capsular antigen (CrAg) and cryptococcal antibodies in CSF, serum and urine can be demonstrated via serological tests. Latex agglutination can detect the antigens in human samples called the Crypto-LA test. This latex agglutination could be done as a quantitative test by monitoring the levels of antigen. Adding serial dilutions of the serum during patient follow ups. Usually, the

titre is highest in serum, intermediate in CSF and the lowest titre being the urine. The antigen titre stays for longer duration in the CSF and it is proportional to the mortality of patients. However, there is no reported correlation for the mortality of patients and titres of urine and serum. False positive results could be misinterpreted in a few cases. Rheumatological disordered patients due to their rheumatoid factor, trichosporonosis caused by *Trichosporon* species, and bacterial infections from *Stomatococcus* and *Capnocytophaga* species. A CrAg titre greater than or equal to 1:8 is considered significant (Chander, 2017).

Eiken latex agglutination test to detect CrAg in serum, aimed at patients with pulmonary cryptococcosis. In this test, it is encouraged to pre-treat the serum with a pronase to eliminate false positive results that could arise in rheumatoid patients. (Tanaka et al., 1994).

2.8.1.4 Animal Pathogenicity Tests

To differentiate between pathogenic and non-pathogenic strains, the animal pathogenicity test is performed by injection of suspected clinical specimen or saline suspension of minimum two days into Swiss albino mice. This is aimed at providing "1 x 106" cells via intracerebral (0.02 ml), intravenous tail vein (0.1-0.2 ml), intraperitoneal (0.5 ml) or intrathecal. On average, the mice will die within seven to ten days. Mice who are still alive can be autopsied after two weeks of inoculation. The brain tissue of the autopsied mice should be grossly mucoid. Body fluids from the infected mice should exhibit yeast cells and capsules of *Cryptococcus*. Inoculation via intrathecal proved to be the best way as it stimulates the natural infection of the disease (Normile et al., 2020).

2.8.2 Molecular Techniques

There are several methods to diagnose *Cryptococcus* through molecular techniques. These techniques are more sensitive than conventional methods (Chae et al., 2012). Molecular techniques have been established by the Cryptococcal Working Group 1 of the International Society for Human and Animal Mycology (ISHAM). There

are a few genetic loci used for the genotyping of *Cryptococcus*. This includes *LAC1*, *PLB1*, *GPD1*, *CAP59*, *URA5*, *IGSI* and *SOD1* genes (Meyer et al., 2009).

The detection of *CNLAC1* gene by nested-PCR was introduced by Chae et al. (2012). Their study determined the rapid identification of *C. neoformans* in pigeon droppings. A molecular technique was done by Paschoal et al. (2004) by using the *ITS* region for PCR. It was possible to detect *Cryptococcus* from clinical samples such as CSF. The *CAP64* gene responsible for the formation of capsules in *Cryptococcus* can be used to detect the presence of the fungus, however, Chae et al. (2012) discovered that the fragment of the *ITS* and *CAP64* genes were present in other yeast-like fungi.

The *CNLAC1* gene was tested positive only on pathogenic *Cryptococcus* which are *C. neoformans* and *C. gattii*. Non-pathogenic *Cryptococcus* were negative, including *Cryptococcus laurentii* and *Cryptococcus uniguttulatus*. This makes the nested-PCR technique at least 10^7 times more sensitive than conventional PCR (Chae et al., 2012).

2.9 Treatment of Cryptococcosis

The treatment of cryptococcosis is usually dependent on the severity of disease and involvement sites. Prior to1990, the available antifungals were limited to Amphotericin B (AMB), Flucytosine and Miconazole (Chander, 2017). Now, whilst amphotericin B is still being used, newer antifungals agents namely Fluconazole and 5-Flucytosine have become the drugs of choice (Spadari et al., 2020). However, these drugs exhibit high toxicity to the patients especially AMB (Ellis, 2002).

Despite exhibiting toxicity, AMB remains the gold standard treatment for cryptococcosis. In cerebral cryptococcosis, it rapidly sterilises the CNS by binding onto the ergosterols in the fungal cell disrupting membrane function and integrity, and causing leakage of intracellular contents, and eventually leading to fungal cell death (Liu et al., 2017; Wilcock et al., 2013). To enhance efficiency, AMB is often combined with Flucytosine (Schwarz et al., 2006). There are two methods in the administration of antifungals: induction therapy and maintenance therapy. For induction, 0.7 mg/kg/day of AMB is paired with 100mg/kg/day of Flucytosine for two weeks in immunocompromised patients. Whilst immunocompetent patients are given Flucytosine at 150 mg/kg/day in maintenance therapy, Fluconazole is given 200 mg/day

to prevent relapses in both type of patients (Chander, 2017). Itraconazole is usually given to patients who had failed Fluconazole therapy, or unable to tolerate Fluconazole. 200 mg of Itraconazole are given twice daily (Saag et al., 2000).

In addition to antifungal agents, immunotherapy plays an important role particularly in treating immunocompromised and organ transplant patients (Antachopoulos & Walsh, 2012). This treatment emphasises on both the protective and non-protective aspects of cytokines (Chander, 2017). Other than cytokines, monoclonal antibodies were suggested by Mukherjee et al. (1995) by combining Mab 2H1 with Fluconazole. Based on their report, the combination therapy was able to reduce the numbers of CFU in the brain, however, it was conducted only *in vitro* and not in patients.

2.9.1 Rising Resistance to Antifungals

In Malaysia, the previous antifungal resistance study was conducted by Tay et al. (2005). In their report, all isolates were susceptible to the antifungals AMB, Fluconazole and Itraconazole. Other countries recorded antifungal resistance in *Cryptococcus*. In Germany, Selb et al. (2019) reported resistance to Fluconazole in clinical isolates of *C. neoformans*. Molecular investigations showed a mutation in the *ERG11*gene in one of the isolates. Allowing resistance to Fluconazole. Sionov et al (2010) suggested the resistance of Fluconazole without the mutations in the *ERG11* gene is probably due to multiple copies of chromosome 1 (Chr1). This genetic alteration reduces the intracellular Fluconazole concentration. In Netherlands, Hagen et al. (2012) conducted a study on antifungal resistance for *C. neoformans* isolated from two separate patients on the same day. One of the isolates had an MIC of >64 µg/ml and the other 8 µg/ml.

Whilst the common antifungal drugs such as AMB and Fluconazole prove to be effective in combating this opportunistic mycosis, recent reports highlighted the resistance exhibited by this organism. The resistance to Fluconazole has shown an increase from 7.3% to 11.7% between 1997 and 2007, as 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).

Moreover, some antifungals pose a threat to patients, especially the ones with impaired kidney functions (Saliba, 2006).

2.9.2 Alternative Treatments for Cryptococcosis

To address the increasing antifungal resistance potential toxicity to the patients globally, there are different approaches clinicians have taken to treat *Cryptococcus* infections. In Brazil, Pereira et al. (2023) reported that the combined use of Verapamil and AMB was able to reduce the size of *C. neoformans* capsule, resulting in enhanced antifungal activity against this fungus. However, the authors noted that this was an *in vitro* study and *In vivo* studies are still needed to confirm the clinical efficacy of this drug combination. Another study in Brazil reported that some strains of *C. neoformans* had shown a positive antifungal synergism when paired with various antifungals. These are; AMB + Azithromycin, AMB + Minocycline or AMB + Tigecycline (Rossato et al., 2015).

Sangalli-Leite et al. (2016) reported that the combination of Pedalitin (PED) and AMB has reduced the minimum inhibitory concentration (MIC) of both drugs by 4 times compared to what is required by both drugs individually. This will not only reduce the toxicity of the drugs on the patient, but will increase the efficacy in an allotted time. All three antifungal reports from Brazil were tested on patients.

2.10 Research Gap

Some limitations include scarce reports on the epidemiology of *Cryptococcus* from bird droppings compared to clinical isolates. The clinical isolates are already infecting the patients; clinicians will naturally diagnose and conduct the identification of this fungus. Next, the isolates obtained from bird droppings in all the previous studies ranges from dried droppings to fresh droppings, there are no consistency between the variations of bird droppings collected. The previous studies (Reference) do not indicate how old the dried droppings were which could pose as a confounding factor in the isolation of *Cryptococcus*. The molecular tests and identification methods varied cross reports, ranging from biochemical assays to PCR-based analyses. Limitations involving conventional methods are improper staining techniques (human error), limited

sensitivity, labour intensive and resource demanding. There needs to be more study indicating the risk factors of cryptococcosis.

2.11 Current Study

As discussed previously, there are worldwide evidence on the prevalence of *Cryptococcus* from bird droppings, its epidemiology, clinical impact and antifungal resistances. This phenomenon has garnered interest on the current status of *Cryptococcus* in Malaysia. whilst there are many aspects to determine the prevalence of this fungus, the differences of occurrence in bird droppings between public areas and zoological gardens needs to be assessed.

The central objectives of this study are to determine the prevalence of *Cryptococcus* isolated from bird droppings found in both public places and zoological gardens in Peninsular Malaysia. Public places selected in this study are crowded areas such as hospitals, places of worship and tourist hot spots. This will give a higher impact towards the positivity rate of *Cryptococcus* found in bird droppings from these places. The zoological gardens selected in this study were zoos that visitors are able to interact with the birds, regardless in an aviary or free flight.

The birds selected in this study were mainly common pigeons. Pigeons are found almost everywhere in Malaysia especially in the public places mentioned earlier. These pigeons have taken up residence in between building crevices, rooftops and balconies. The amount of pigeon flocks in public areas is of concern as well. There is a cultural tendency among Malaysians to feed birds in parks and other communal areas. This raise concerns as pigeons are usually associated with *Cryptococcus*. In zoological gardens, the birds primarily selected in this study are the ones that often interact with the visitors and zookeepers. A prime example will be the parrots. Visitors are able to feed and engage in close-proximity photographs. Incidence of the birds leaving droppings on the visitors increases the chances of contracting *Cryptococcus* from the birds.

For zoological gardens, hygiene and environmental factors were taken into consideration. This is to assess the relationship between any of these factors with the positivity rate of *Cryptococcus* isolated in the respective zoological gardens. There has not been any study related to the conditions of the zoological gardens with the positivity rate of *Cryptococcus.*

Amid the global increase in antifungal resistance, this study employed a disc diffusion method for the antifungal susceptibility test to update the study conducted by Tay et al. (2005). Although there are limitations towards this antifungal test method, this study acts as a screening result toward the antifungal resistance of *Cryptococcus*. Furthermore, the isolates tested against the antifungal agents in this study are recovered from bird droppings and not from clinical origin.

Finally, the molecular technique utilised in this study was the nested-PCR of the *CNLAC1* gene. This method was introduced by Chae et al. (2012) and proved the rapid testing and differentiations of pathogenic *Cryptococcus* such as *C. neoformans* and *C. gattii* against non-pathogenic ones such as *C. laurentii*. This allows the construction of a partial phylogenetic tree to further determine the serotypes of the isolates recovered in this study.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Overview of the Study Design

This research was designed as a cross-sectional study to address four specific objectives as illustrated in **Figure 3.1**. In **objective 1**, the epidemiology of the *C. neoformans* in bird droppings was investigated. In brief, bird droppings collected from both zoological gardens and public places across four regions (central, northern, southern, and eastern) of Peninsular Malaysia. The species of the birds was also recorded. Suspected colonies on bird seed agar were subjected to nested polymerase chain reaction (nested-PCR) for molecular identification. Only PCR-confirmed isolates were considered positive and included in the statistical analysis for prevalence and comparisons across environments, regions and bird species.

In **objective 2**, the possible risk factors associated with the occurrence of *C. neoformans* in birds from zoological gardens were investigated. Hygiene and environmental conditions were assessed based on five indicators: bird droppings cleaning frequency, air odour, drainage system, enclosure density, and frequency of encountering bird dropping stains. Logistic regression analyses were performed to determine potential associations.

In **objective 3**, all positive isolates confirmed by molecular identification were screened for antifungal susceptibility testing. The zone of inhibition was measured to assess susceptibility to Amphotericin B, Fluconazole, and Itraconazole. Finally, in **objective 4**, the serotypes of the selected positive isolates were determined using Sanger sequencing, followed by sequence comparison using BLAST against the NCBI nucleotide database. A partial phylogenetic tree was then constructed based on the aligned sequences.

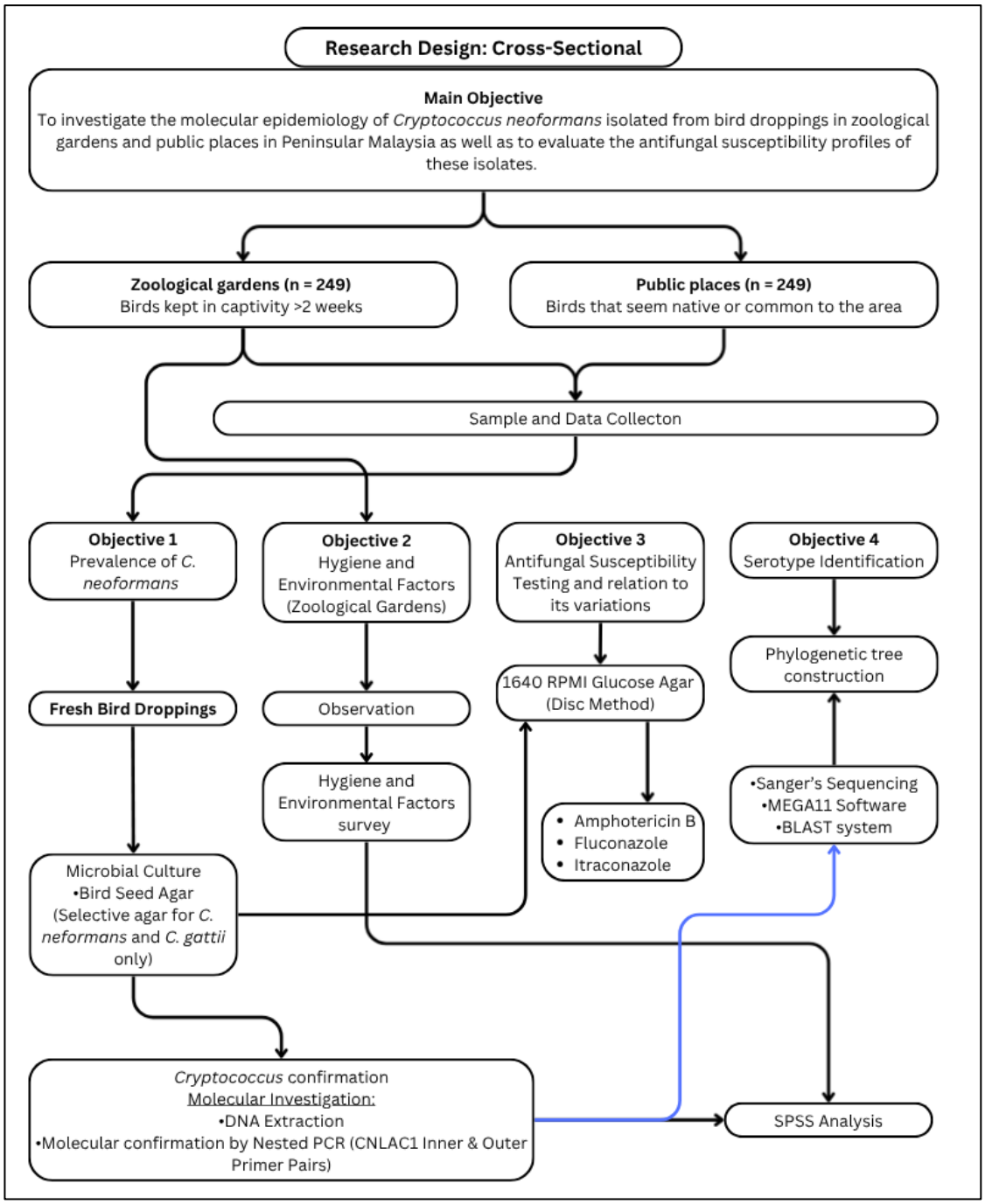

Figure 3.1 Overall Methodology Flowchart

3.2 Ethical Application and Approval

Ethical consideration was applied and approved from Research Ethics Committee (REC) of Universiti Teknologi MARA (UiTM) [reference number: 600-UiTMCARE (PT. 1/3/1)] prior to the research. Approval from Director of Department of Wildlife and National Parks (PERHILITAN) was also granted [reference number:

JPHLTN.600-6/1/4 JLD2 (118)]. Samples from zoological gardens were only collected with permission from the zoo authorities and in accordance with the provided guidelines.

3.3 List of Chemicals and Reagents, Consumables and Apparatus

The lists of chemicals and reagents, consumables and apparatus used in this study are shown in Table 3.1 and Table 3.2.

Table 3.12

List of Chemicals and Reagents

Chemicals & Reagents	**Brand**	**Country of Origin**
1% w/v aqueous diphenyl	HiMedia	India
50× Tris-Acetate-EDTA (TAE) Buffer, pH 8.0	1st Base	Singapore
India Ink	Beckton	United Kingdom
Sodium Hydroxide Solution	Sigma-Aldrich	United States of America

Table 3.23

List of Consumables and Apparatus

Consumables & Apparatus	**Brand**	**Country of Origin**
10 µl sterile disposable inoculation loops	Labchem	Malaysia
Antifungal Amphotericin B	Liofilchem	Italy
Antifungal Fluconazole	Liofilchem	Italy
Antifungal Itraconazole	Liofilchem	Italy
Autoclave SX-700	Tomy	United States of America
Biosafety cabinet Level 2	Nuaire	United Kingdom
Bird seed agar	HiMedia	India
Black permanent marker pens	Artline	Malaysia
Disposable Sample Containers	Zhejiang Runlab Technology	China
Disposable Spatula	Sigma-Aldrich	United States of America
DNA Ladder 100bp	SMOBIO	Taiwan
DNA Stain Florosafe	1st Base	Singapore

Glass slides	Hawach Scientific	China
Light Microscope	Olympus	Japan
RPMI 1640 Glucose Agar	HiMedia	India
Magnetic stirrer	FAVORIT	Malaysia
Masking Tape	NIKKO	Japan
Master Mix (2X)	Thermo Fisher Scientific	United States of America
Milli-Q integral water purification system	Milipore	United States of America
Petri dishes	FAVORIT	Malaysia
pH reader	Mettler Toledo	Malaysia
Polyethersulfone (PES) syringe filters 0.22 µm	Thermo Fisher Scientific	United States of America

3.4 Methodologies for objective 1: Current prevalence of *C. neoformans* in bird droppings from zoological gardens and public places

3.4.1 Study Design and Sample Size Calculation

This study employed a cross-sectional design. The sample size was calculated based on a previous study by Tay et al. (2005), which reported a 3.7% prevalence of *C. neoformans* in birds in the Klang Valley. The sample size calculation was done by using Select-Statistics website (https://select-statistics.co.uk/calculators/sample-size-calculator-two-proportions/) using the two-proportion sample size (equal) formula (Wang & Chow, 2007).

$$\mathbf{n = (Z_{\alpha/2}+Z_{\beta})^2 * (p_1(1-p_1) +p_2(1-p_2)) / (p_1-p_2)^2}$$

$Z_{\alpha/2}$ = critical value of the Normal distribution at $_{\alpha/2}$

Z_{β}= critical value of the Normal distribution at $_{\beta}$

p_1 and p_2 are the expected sample proportions of the two groups

p_1 = 3.7% (public places incidence based on previous study)

p_2 = 10% (expected incidence in zoological gardens)

Therefore,

$$\mathbf{n = (1.96+0.84)^2 \times (0.037(1-0.037) + 0.10(1-0.10)) / (0.037-0.10)^2}$$

= 249 sample size each for group 1 (public places) and group 2 (zoological gardens)

Hence, based on the final calculations, the minimum required sample size for this study is 498, with 249 samples in each group (n = 498).

3.4.2 Study Area

The study areas for sample collection for both zoological gardens and public places were divided into four regions of Peninsular Malaysia: Northern, Central, Southern, and Eastern Peninsula Malaysia.

3.4.2.1 Study Area (Public Places)

Public areas selected for this study were primarily urban locations known for having high densities of birds, particularly pigeons. These birds were observed to be long-term residents of the sites, often nesting in rooftops, balconies, and building crevices. High numbers of pigeons were also observed at hospitals and places of worship, largely due to consistent feeding by visitors and devotees.

For central region, a total of six locations were selected, comprising one commercial area, two public gardens, two places of worship, and one hospital. For commercial area, PJ New Town shop lots were chosen due to the dense bird population. Bird nests were seen at the rooftops and the birds are regularly fed by residents. Observations revealed that feedings typically occur three times daily: morning, afternoon and before sunset, resulting in large gatherings of birds and substantial accumulation of droppings on the public roads that are used as feeding grounds. Samples were also collected at two public gardens named as Kota Damansara Taman Rimba Riang and Tropicana Golf Club. These two public gardens are known for their

lush vegetation and high avian diversity, including doves, robins, Myna, crows and sparrows. While pigeons were less prevalent, bird droppings were frequently observed on verandas, benches, patios and ceilings. Bird nests were also frequently seen suggesting regular bird activity in these areas. Batu Caves (Gombak) and Sri Sithi Vinayagar Temple (Petaling Jaya Old Town) were also included in this study due to large number of birds, especially pigeons in these areas. Pigeon nests were commonly seen among the temple's statues, rooftops and surrounding buildings. As a major religious site and tourist attraction, Batu Caves experiences large volumes of visitors daily. Devotees regularly feed the birds, resulting in significant accumulation of droppings across the temple premises and surrounding walkways. Pigeon feeding occurs nearly every hour attracting large flocks and resulting extensive faecal contamination and increases the risk of environmental exposure to *Cryptococcus* in the area. In addition, Hospital Kuala Lumpur (HKL) was chosen due to the immense population of pigeons within the hospital vicinity. Numerous food stalls around the hospital contribute to unmanaged waste, attracting birds. Their proximity to patients and healthcare workers presents a notable public health concern.

In the northern area, this study focused on the George Town Shop Lots (Penang Island). This UNESCO World Heritage Site is characterized by low-rise, colonial-era buildings with minimal structural maintenance. Numerous holes and crevices provide ideal nesting sites for pigeons. The area is also known for its abundance of hawker stalls, leading to food waste accumulation, which attracts large pigeon flocks. These conditions create high human-pigeon contact potential.

In the Southern region, a place of worship known as Sri Maha Mariamman Temple Rasah (Negeri Sembilan) was selected for this study. Unlike other regions, Negeri Sembilan had noticeably fewer pigeons, likely due to the overall cleanliness of the area. Most observed pigeons perched on electric power lines without evidence of nesting or droppings, suggesting they were not resident birds. This temple was selected due to the presence of domesticated poultry, including chickens and geese. According to a local devotee, these birds are native to the surrounding area and regularly roam between the temple and nearby alleys. They are frequently fed by locals and devotees, resulting in regular human-bird contact.

In the Eastern region, two locations were selected: Kuantan MBK Park 2 (Pahang) and KTCC Mall area in Kuala Terengganu. The MBK Park features an open

field surrounded by numerous stalls and shop lots. Pigeons were observed flocking to the central area, where they are commonly fed by locals for leisure. Droppings were abundant throughout the park. Kuala Terengganu was found to have very few pigeons overall, likely due to effective waste management and urban cleanliness. However, a small group of pigeons was observed near KTCC Mall, close to the beach. Local individuals occasionally feed them, contributing to minor pigeon gatherings in the area. Table 3.3 presents a summary of all public places included in the study, according to region.

Table 3.34

List of Public Places Included in this Study According to Region

Region	**Public Places, State**	**Longitude**	**Latitude**
Central	Kota Damansara Taman Rimba Riang, Selangor	03°18'17.40" N	101°58'36.40" E
	PJ New Town Shop lots, Selangor	03°07'46.60" N	101°61'92.50" E
	Sri Sithi Vinayagar Temple, Selangor	03°09'11.50" N	101°64'56.20" E
	Tropicana Golf Club, Selangor	03°13'44.80" N	101°58'39.00" E
	Batu Caves, Kuala Lumpur	03°23'80.90" N	101°68'40.40" E
	Hospital Kuala Lumpur, Kuala Lumpur	03°17’15.70" N	101°70'25.40" E
Northern	George Town Shop Lots, Penang	05°42'00.10" N	100°33'27.20" E
Southern	Sri Maha Mariamman Temple Rasah, Negeri Sembilan	02°72'26.10" N	101°95'67.90" E
Eastern	Kuantan MBK Park 2, Pahang	03°80'64.70" N	103°32'63.70" E
	KTCC Mall, Terengganu	05°33'35.00" N	103°15'03.40" E

3.4.2.2 Study Area (Zoological Gardens)

A total of five zoological gardens were selected in this study: KL Bird Park, Farm in the City (FITC) and a privately owned zoo in Ijok (Central region); Kulim Bird Park (Northen region); and Zoo Melaka (Southern region). These sites were chosen based on their accessibility, the extent of human-bird interactions, and the presence of free-roaming birds, all of which are relevant for investigating the potential exposure to *Cryptococcus.* Selection also considered the logistical feasibility of sample collection and the availability of permission from the respective zoos. However, no samples were

collected from the eastern region due to the limited number of zoological gardens and in cases where such facilities existed, permission for sampling was not granted.

For the central region, KL Bird Park was chosen its due to its status as the largest walk-in aviary in Malaysia covering over 20 acres within the Kuala Lumpur Lake Gardens. It houses more than 3,000 local and foreign birds from approximately 200 species. (https://www.klbirdpark.com/about-us/). As a walk-in aviary, visitors can closely interact with numerous free-roaming birds, raising public health concerns regarding potential exposure to *C. neoformans.* This study specifically targeted bird species that were free-roaming and accessible to visitors. Birds kept in restricted enclosures, such as long-eared owls, bald eagles and cassowaries were excluded. Species of interest included the parrot family (e.g., lorikeets and eclectus), which are often fed and interact closely by visitors. Free-roaming migratory waterbirds within the aviary were also included, which adds an interesting aspect due to their possible role in spreading the fungus in the environment.

Located in Sri Kembangan, Selangor, FITC is a petting zoo that also features a walk-in aviary, albeit smaller in size than KL Bird Park. The aviary area can accommodate approximately 15 people at a time and contains multiple bird species including pigeons, ducks, parrots and some migratory birds. Visitors can interact closely with the birds and many of the parrots often land on visitors posing a higher likelihood of droppings coming into contact with humans. Similar to KL Bird Park, only free-roaming birds were included in this study, while those housed in isolated enclosures were excluded. Given the confined space and frequency of close human interaction, FITC presents a significant site for assessing the occurrence of *C. neoformans* in bird dropping.

A privately owned zoo established in 2023 in Ijok, Selangor was also included in this study. This zoo primarily houses poultry birds including chickens and turkeys, with a separate aviary for peacocks. According to the owner, the aviary was cleaned only once a month during the study period, leading to an accumulation of fresh and aged droppings. This study focused on collecting fresh droppings from the peacocks, given their consistent human contact and the infrequent cleaning schedule.

For the Northern region, only Kulim Bird Park was included in this study. This bird park houses other animals as well, such as capybaras and snakes. Located in Kulim, Kedah, this zoological garden is well-known for its mobile petting zoo service known

as “Zoo on Wheels”. This service brings animals including birds to schools, universities and public events, increasing public interaction beyond the park's premises. The mobile nature of this facility poses a potential risk for widespread exposure to avian-borne pathogens such as *Cryptococcus*. In terms of infrastructure, the park has both walk-in aviaries and bird enclosures. This study included free-roaming birds such as emus and budgies as well as birds involved in the mobile zoo service, such as the cockatoos and eclectus.

For the southern region, Zoo Melaka, a 53-acre zoo located in Bandar Melaka was selected. This Zoo offers a Night Safari as well to its visitors. Unlike the other zoological gardens mentioned, Zoo Melaka does not feature any walk-in aviaries. All birds are housed within enclosures, restricting direct contact with visitors. Zoo Melaka only hosts the parrot family such as cockatoos and budgie birds. Although visitors cannot handle the birds directly, closer proximity to enclosures still allows for potential indirect exposure to droppings. Details of all zoological gardens by region are summarized in Table 3.4.

Table 3.45

List of Zoological Gardens in this Study According to Region

Regions	**Zoological Garden, state**	**Latitude**	**Longitude**
Central	KL Bird Park, Kuala Lumpur	03°13'34.40" N	101°68'82.70" E
	Farm in the City, Selangor	02°98'03.50" N	101°66'17.10" E
	Ijok Private Zoo, Selangor	03°31'66.75" N	101°40'07.08" E
Northern	Kulim Bird Park, Kedah	05°35'94.40" N	100°55'26.30" E
Southern	Zoo Melaka, Melaka	02°27'67.30" N	102°29'86.70" E

3.4.3 Sample Collection

All sample were collected between August 2023 to June 2024. To collect the samples, both foraging or caged birds were monitored and observed closely. To encourage the birds in public places to stay and defecate, the birds were fed with Gardenia Original Classic White Bread 400g (Gardenia, Malaysia). Each feeding session and sample collection in public places spanned from 2 to 4 hours, whilst sample

collection for zoological gardens spanned for 4 to 6 hours. Their droppings were collected immediately after defecation to ensure the samples are fresh. It was then scooped using a spatula into sterile plastic containers. To minimize soil contamination risks, samples were only taken from the upper surface of the bird droppings carefully, ensuring no contact with the ground/soil. The identification of bird species was done by learning the species of the birds and their common names, along with cross checking with the zookeepers and veterinarians. Labelling of bird species was immediately done on the containers using 1.0 mm permanent black marker pens (Artline, Malaysia). Additional confirmation was based on the bird encyclopaedia by Alderton (2008).

Samples were transported and stored under cold chain conditions (2-8°C) until arrived at the laboratory IMMB and processed in the immediately. Overall, a total of 509 samples from both zoological gardens and public places were collected in this study as shown in Figure 3.2.

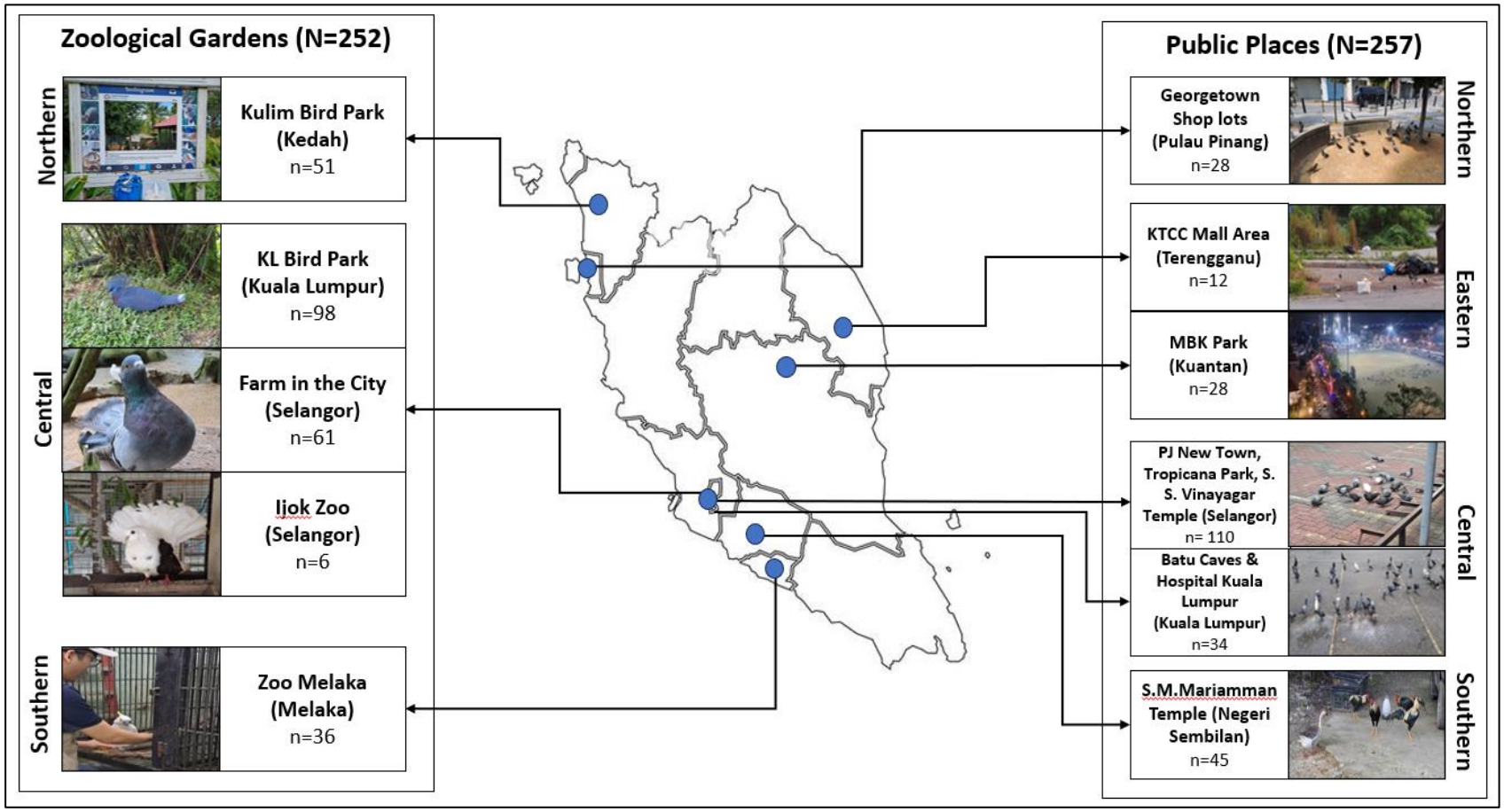

Figure 3.2 Map Illustrating the Sampling Locations and the number of bird droppings samples collected in this study

3.4.4 Culture and Microscopic Examination

In brief, about 0.5-1.0g bird droppings were mixed and vortexed (SASTEC, Malaysia) with 5 ml of sterile phosphate-buffered saline (PBS) for five minutes, as described by Tay et al. (2005). The resulting cloudy supernatant was then streaked onto Staib's Medium (bird seed agar) (HiMedia, India) using disposable inoculation loops. This medium was used for selective isolation and differentiation of *C. neoformans* (and *C. gattii*) from other *Cryptococcus* species and other yeasts. Labelling of bird species was immediately done on the agar plates using 1.0 mm permanent black marker pens and sealed with 1" masking tape (NIKKO, Japan). The culture plates were later incubated at room temperature for at least 7 and up to 10 days as highlighted in the manufacturer's guideline. Suspected colonies of *C. neoformans* were yellowish-brown to dark brown pigment due to the melanin production from the phenolic compounds in the bird seed agar (Figure 3.3). In terms of texture, they were mucoid, smooth and glossy due to the polysaccharide capsule. If no growth was seen, the cultures were autoclaved and discarded.

Figure 3.3 Suspected Colonies of *C. neoformans* on Bird Seed Agar

The colonies grown on the Bird Seed Agar were later used for India ink staining. One loop of colonies was smeared onto a clean glass slide and was spread evenly. Then a drop of Indian ink was added and was allowed to mix. The smear was covered with a coverslip and observed under light microscope at 1000× magnifications to detect the microscopic morphology of *Cryptococcus* (Figure 3.4). The yeast cells appeared spherical and surrounded by a clear capsule, which is a characteristic feature of *Cryptococcus* spp.

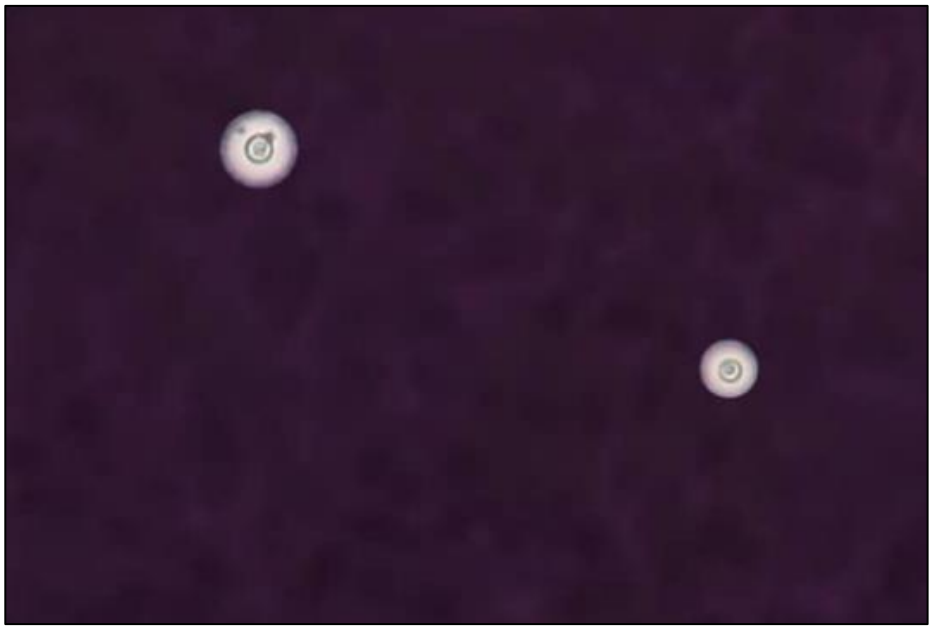

Figure 3.4 *C. neoformans* Stained with Indian Ink Under 1000x Magnification

3.5 Identification of *C. neoformans* by Molecular Technique

3.5.1 DNA Extraction

A total of 272 suspected colonies were subjected for molecular identification. Extraction of DNA from fungal culture was performed using NucleoSpin® Yeast (Macherey-Nagel GmbH & Co. KG, Germany) kit according to the manufacturer's instruction. The extraction method and buffer were optimized beforehand to choose the suitable buffer and to familiarise with the procedure. Prior to extraction, NucleoSpin® bead tubes, columns and collection tubes were labelled according to the samples to avoid any swapping error. About 100 mg of the cultured isolates was added into each the tube and centrifuged (Eppendorf, Germany) to obtain a yeast cell pellet. Then, 100

µl of Elution Buffer BE was added and cells were resuspended. To lyse the yeast samples, the cells were transferred into the provided MN Bead Tube Type C, and 40 µl of Buffer MG and 10 µl of Liquid Proteinase K were added. The suspension was allowed to agitate using Thermomixer Comfort (Eppendorf, Germany) for 20 minutes. The tube was centrifuged for 30s at 11,000 × *g* to clean the lid. 600 µl Buffer MG was added and the tube was vortexed and later centrifuged for 30s at 11,000 × *g*.

To bind the DNA, 650 µl of supernatant was withdrawn and transferred into NucleoSpin® DNA Yeast Column and placed into a 2 ml collection tube that was provided by the kit. Centrifugation was done for 30 s at 11,000 × *g* and the collection tube with flow through discarded after. To wash the silica membrane, the membrane was placed into a new collection tube. The silica membrane was washed twice using 500 µl Buffer BW and centrifuged for 30s at 11,000 × *g*. The flow through was discarded but the same collection tube was reused for a second wash by using 500 µl Buffer B5, followed by centrifugation for 30s at 11,000 × *g*. The flow-through was discarded and column was placed back into the same collection tube. The silica membrane was allowed to dry by centrifugation for 30 s at 11,000 × *g* to remove any residual wash buffer. In the last step, the DNA was eluted using 100 µl Buffer BE onto the column. The column was then incubated at room temperature for 1 min and centrifuged for 30s at 11,000 × *g*.

3.5.2 Optimisation of Nested-PCR and Specificity of Primers

DNA amplification was carried out using a nested PCR approach with primer sets described by Chae et al. (2012), targeting the *CNLAC1* gene using a thermocycler (AnalytikJena, Germany). This protocol utilized two sets of primers, yielding amplicons or PCR product of approximately 1386 and 690 base pairs (bp), respectively. The primers selected in this study was to amplify the *CNLAC1* gene encoding the laccase (Buchanan & Murphy, 1998). This gene is only detected in *C.neoformans* and not in other *Cryptococcus* species (Chae et al., 2012).

Prior to that, the nested PCR conditions were optimized to ensure reliable amplification and reproducible results. The optimization was performed following the nested PCR protocol also by Chae et al. (2012), as detailed in Table 3.5.

Table 3.5
Initial Protocol for Nested-PCR

Primers	Gene	Primer Sequence	Thermal Conditions	Expected bp
CNLAC1 (Outer)	Laccase	F-ACGGTGTCCCTGGTATAA R-GCGTTGGACGATTGAAAG	95°C -5 minutes, 30 cycles × (95°C -30 seconds, 58°C -30 seconds, 72°C - 90 seconds) 72°C-5 minutes (final extension)	1386
CNLAC1 (Inner)	Laccase	F-CACTCGCCCAATGAACC R-TATACCTCACCACCGCC	95°C -5 minutes, 30 cycles × (95°C -30 seconds, 58°C -30 seconds, 72°C - 45 seconds) 72°C-5 minutes (final extension)	690

The initial reaction mixture for the nested PCR in this study was also optimized based on the reference protocol by Chae et al. (2012). Table 3.6 shows the initial reaction mixtures for the nested-PCR (1st PCR and 2nd PCR respectively).

Table 3.6
Initial Reaction Mixture for Nested-PCR

1st PCR	2nd PCR	Reference
2 µl *CNLAC1* outer primer pair (1uM)	2 µl *CNLAC1* inner primer pair (1uM)	Chae et al., 2012
2.5 µl (20 ng) template DNA	0.1 µl of 1st PCR product	
8 µl distilled water	10.4 µl distilled water	
12.5 µl of 2X master mix	12.5 µl of 2X master mix	
Total Volume 25 µl	Total Volume 25 µl	

The optimisation began by adjusting the primer concentration from 1uM to 5uM. During this phase, there were still no bands seen. When the primer concentration was increased to 10uM, primer dimers were seen. Following the presence of primer dimers, the denaturation time was increased to 60 seconds from 30 seconds. This adjustment reduced the primer dimers to fainter bands. Later, the elongation time was increased to 90 seconds, utilising the gradient function from the thermocycler (AnalytikJena, Germany). To obtain a high chance of optimised annealing temperature, the gradient started from 48.1°C to 67.1°C. Given the gradient available in one run was only 8, therefore, the initial gradient temperatures are listed in **Appendix L**, indicating

the number of times the optimisation was conducted along with the temperatures in each run.

A few bands were finally obtained after the 13th run at the temperature of 58.4°C, 58.6°C and 58.8°C, with the brightest band being at 58.6°C. However, the band obtained was still faint. Therefore, the extension time was doubled from 45 seconds to 90 seconds, this in turn has made the brand brighter. The final extension time was also increased to 8 minutes and 10 minutes to see the difference in bands; the 8 minutes finals extension time had a brighter and more prominent band than 10 minutes.

For the second PCR, the optimisation started based on the optimised conditions of the first PCR. This time, the cycles were compared using x35, x36, x37 and x38. The faint bands were only seen on the x35 cycles with only primer dimers being present in the other cycles. To optimise further, the extension time was tested with 30, 45 and 60 seconds. A brighter band was seen with 60 seconds extension time. The final optimised conditions for the nested-PCR are shown in Table 3.7.

Nested PCR was performed using two sets of primers, with products of 1386 and 690 bp each, respectively. Primary amplification was done with a total volume of 25 µl reaction mixture. In this mixture, contains 2 µl *CNLAC1* inner primers (1 µl of forward and reverse each), 2.5 µl of template DNA, 8µl of purified distilled water and 12.5 µl of 2X master mix (Thermofischer DreamTaq Green PCR Master Mix with 20mM Mg^{2+} and dNTPs). The thermal conditions using thermocycler (AnalytikJena, Germany) for the reactions are described in Table 3.7. A positive nested PCR product was used as the positive control. Distilled water was used as a negative control. Secondary amplification was done with a total volume of 25 µl reaction mixture, consisting of 2 µl *CNLAC1* outer primer pairs (1 µl forward and reverse each), 0.1 µl of the first PCR set product, 10.4 µl of purified distilled water, and 12.5 µl of the same master mix. Both primary and secondary PCR was analysed by electrophoresis in 1.5% agarose gel conducted in tris acetate EDTA (TAE) 1× buffer. With an addition of FloroSafe DNA stain (1stBaseChemicals, Iran) and visualised by UV Transilluminator (Cleaver, United Kingdom).

Table 3.7

Final Optimised Thermal Conditions for Nested-PCR.

Primers	Gene	Primer Sequence	Thermal Conditions	PCR Product (bp)
CNLAC1 (Outer)	Laccase	F-ACGGTGTCCCTGGTATAA R-GCGTTGGACGATTGAAAG	95°C -5 minutes, 38 cycles × (95°C -60 seconds, 58.6°C -90 seconds, 72°C - 90 seconds) 72°C-8 minutes (final extension)	1386
CNLAC1 (Inner)	Laccase	F-CACTCGCCCAATGAACC R-TATACCTCACCACCGCC	95°C -5 minutes, 35 cycles × (95°C -60 seconds, 58.6°C -90 seconds, 72°C - 60 seconds) 72°C-8 minutes (final extension)	690

3.5.3 Agarose Gel Electrophoresis

The amplified DNA was detected using agarose gel electrophoresis. The preparation of 1.0% agarose gel was done by mixing 1.0 g of LE grade agarose powder (Vivantis Inc., USA) with 100 ml of fresh 1× TAE buffer (Vivantis Inc., USA). The mixture was boiled in a microwave until the agarose powder was completely dissolved. For every 100 ml of the agar slurry, 10 µl of the ViSafe red gel (Vivantis Inc, USA) stain was added. The agarose solution was poured onto a casting tray and gel comb for wells formation was inserted.

After the gel has already solidified, the comb was carefully removed. The gel was put into an electrophoresis chamber in the correct orientation (wells at negative terminal). The chamber was flooded with 1× TAE buffer until the gel was completely submerged. A volume of 5 µl of VC100 bp DNA ladder (Vivantis Inc., USA) and 10 µl of each DNA sample was loaded into each well.

Once loaded, the chamber was covered with its lid cover. The positive (red wire) and negative (black wire) electrodes were connected to a PowerPac™ basic power supply (Bio-Rad Inc., California, USA) and the gel was electrophoresed at 80 V. Upon completion, the gel was visualized by using UV Transilluminator (Cleaver, United Kingdom).

3.5.4 Positive Control Sequencing

After finalising the optimised thermal conditions for the Nested-PCR. The positive PCR product (Clivia PC44) was sent for sequencing to Apical Scientific Sdn. Bhd. to confirm the identity of the amplified PCR product. The nucleotide sequence was analysed using the online basic local alignment tool (BLAST) at the NCBI website. The sequence showed > 99% similarity with the *C. neoformans* reference gene (MG963886.1) in GenBank. The verified sequence was then used as the positive control for subsequent nested-PCR.

3.5.5 Statistical Analysis for Objective 1

The data obtained in this study inclusive of bird species, sampling locations, type of locations (zoological gardens vs public places), and *C. neoformans* infection status were systematically entered, recorded and analysed using IBM SPSS versions 27 (SPSS, Chicago II, USA). Descriptive analysis was utilised to summarise the data, in particularly, to determine the overall prevalence of *C. neoformans* across different variables in the samples. The prevalence in this study was expressed as percentages, stratified by bird species, location type and specific location sites to facilitate comparison.

To examine possible association between categorical variables such as infection status and bird species with location type, the Pearson chi-square test (χ^2) was applied. This test assessed the association and/or independence between groups within the dataset. For all statistical tests, a p-value of less than 0.05 ($P<0.05$) was considered statistically significant. Further cross-tabulations were generated to visualise distribution patterns and help interpret the chi-square results.

3.6 Methodologies for Objective 2: Hygiene and Environmental Factors That are Possibly Associated with the Occurrence of *C. Neoformans* in Zoological Gardens

In Objective 2, the hygiene and environment factors of the zoological gardens were observed (qualitative assessment) and recorded based on five criteria. For each criterion, the condition is divided into two categories. Assessment was then performed

based on the categories for each criterion. The following criteria were recorded for the hygiene and environmental factors of the zoological gardens.

i. Frequency of bird droppings cleaning: Low (0-2 Times daily) / High (> 2 times daily).
ii. Air odour: Bad odour (continuous unpleasant odour) / No odour (Fresh or neutral air).
iii. Drainage system: Fair (functioning, but with occasional blockages or areas of water stagnancy) / Excellent (well-maintained, not clogged and no visible stagnant water).
iv. Enclosure Density: Crowded (Less than or equal to 90 cm x 70 cm x 100 cm or 630,000 cm^3 in volume with more than one bird with sizes of 32-35 cm in length and 25-30 cm in height) / Spacious (More than 90 cm x 70 cm x 100 cm or 630,000 cm^3 in volume with only a single bird with sizes of 32-35 cm in length and 25-30 cm in height).
v. Frequency of bird droppings encountered: High (Visible in most areas with a minimum of two bird dropping stains in an approximately per square metre) / Low (fewer than two bird dropping stains in an approximately per square metre).

After each site visit, all observations were then reviewed and finalized through consensus among the observers and consultation with zoo representatives (veterinarians or personnel in charge).

3.6.1 Statistical Analysis for Objective 2

The evaluation for potential risk factors associated with *C. neoformans* positivity in bird droppings was conducted by binary logistic regression analysis using IBM SPSS Statistics version 27 due to the binary nature of the outcome variable (positive vs. negative for *C. neoformans*). The analysis was carried out in two stages: univariate and multivariate logistic regression. For univariate, each independent variable related to hygiene and environmental factors was tested for association with the infection outcome. Selected variables based on univariate analysis ($p < 0.25$) were

then included in multivariate logistic regression analysis. The backward likelihood ratio method was applied where non-significant variables were sequentially removed from the model until only variables with $p<0.05$ remained. Model fit was assessed using the Hosmer-Lemeshow goodness of fit test. This led to the control for confounding and to identify independent predictors of *C. neoformans* positivity. Odds ratios (ORs), 95% confidence intervals (CIs), and p-values were reported for each variable in the final model. Due to the insufficient sample size at Ijok Private owned Zoo (n=6), data from this location were excluded from this analysis. This is to avoid inconsistent estimates, ensuring statistical robustness.

3.7 Methodologies for Objective 3: Evaluation of Antifungal Susceptibility Patterns of *C. neoformans* Isolates from Bird Droppings

For objective 3, all positive isolates confirmed by nested-PCR were screened for their antifungal susceptibility tests using the disc diffusion method. The susceptibilities were measured in micrometre (mm) for their diameter of inhibition. In brief, 1640-RPMI glucose agar (HiMedia, India) plates were spread with 48-hour positive isolates standardized to 0.5 McFarland turbidity (Tay et al., 2005). The three antifungal discs; Amphotericin B (AMB) (20 µg/disk), Itraconazole (ITC) (50 µg/disk), and Fluconazole (FLU) (25 µg/disk) (Liofilchem, Italy) were applied using flame sterilized medical forceps onto the agar, with enough spacing between each antifungal disc (24 mm from centre disc to another centre disc) and not less than 15 mm of each disc from the edge of the plate. The plates were then sealed and incubated in room temperature for two days. Room temperature used due to environmental isolates (Chander, 2017). Susceptibility was assessed based on the manufacturer's guidelines in which the resistances are defined as resistant≤13mm, intermediate resistance at 14-16 mm and susceptible as ≥17 mm in diameter based on the zone of inhibition formed measured from edge to edge with the antifungal in the centre (Liofilchelm, 2021).

3.8 Methodologies for Objective 4: Identification of Serotypes and Phylogenetic Relationships of *C. neoformans* Isolated from Bird Droppings

A total of 16 positive PCR products were selected for sequencing (including the PCR product used for positive control establishment in this study). The selection of the

PCR products for sequencing will be mainly based on the bird species the fungus was isolated from, along with any antifungal resistance the isolates might show.

PCR products were sequenced with forward and reverse primer (Apical Sdn Bhd service (Malaysia). After conversion to FASTA format, alignment using the online basic local alignment tool (BLAST) was done at the NCBI website. A phylogenetic tree was constructed and the corresponding regions of the other *C. neoformans* using the maximum likelihood method with MEGA10 software. The partial phylogenetic trees were built according to the protocol on the building of phylogenetic trees from molecular data with MEGA (Hall, 2013) by using MEGA7 program (Kumar et al., 2016). The nucleotide sequences were aligned with selected reference sequences (**Appendix M**) obtained from GenBank by using ClustalW method (Thompson et al., 1994). Then, the tree was built by Neighbour Joining (NJ) analysis and the tree reliability was supported by bootstrap method and shown by bootstrap percentage on the tree branches.

CHAPTER 4
RESULTS

This chapter describes the findings of the study, organized into four main sections based on the specific objectives. Section 4.1 reports the current prevalence of *C. neoformans* based on molecular identification which includes a comparison of findings between zoological gardens and public areas. Section 4.2 highlights the potential hygiene and environmental factors associated with the occurrence of *C. neoformans* in bird droppings in zoological gardens. In Section 4.3, the results of the antifungal susceptibility profiles of the positive isolates against Amphotericin B, Fluconazole, and Itraconazole are presented. Finally, section 4.4 details the serotyping and partial phylogenetic analysis findings.

4.1 Current Prevalence of *C. neoformans* in Bird Droppings in Peninsular Malaysia

4.1.1 Sample Characteristics

In this study, a total of 509 bird droppings were collected from four regions (central, northern, southern and eastern) of Peninsular Malaysia. Of these, 252 (49.5%) were collected from zoological gardens, while 257 (50.5%) were obtained from public places. Majority of the sample were collected from the central region (60.7%), which is the most densely populated region in Peninsular Malaysia and hosts the highest number of zoological gardens. In the eastern region, only samples from public places were included, as no samples from zoological gardens were collected due to the limited number of zoos and challenges in obtaining access permissions.

Overall, the sample collection involved droppings from 24 bird species belonging to 12 taxonomic families, with the Columbidae (comprising pigeon and dove species) being the most prominent family. Nine bird species dominated by common pigeons (*Columba livia*) (56.0%), followed by chickens (*Gallus gallus domesticus*) (12.1%) and house sparrows (*Passer domesticus*) (8.2%) were sampled from public areas. In contrast, the zoological garden samples represented a more diverse range of

bird species, with 17 species recorded. The most frequently sampled species in zoos were Mandarin ducks (*Aix galericulata*) (15.9%), budgie birds (*Melopsittacus undulatus*) (12.3%), common pigeons (10.3%) and rainbow lorikeets (*Trichoglossus moluccanus*) (9.9%).

Out of the 24 bird species, only droppings from only two species, common pigeons and chickens were collected from both public areas and zoological gardens. Notably, this study also included samples from a migratory bird species, the Great White Pelican (*Pelecanus onocrotalus*), which accounted for 4.8% of the zoo samples. A detailed breakdown of the sample collection by region, location type, and bird species is presented in Table 4.1.

Table 4.1
Number of Samples Based on Bird Species and Regions from Public Places and Zoological Gardens (N=252)

Bird Species, Total Sample (N=509)	**Public Places (N=257)**					**Zoological Garden (N=252)**			
Family	Total	C	Nr	S	E	Total	C	Nr	S
Species, TOTAL (N)	n (%)	(n=144)	(n=28)	(n=45)	(n=40)	n (%)	(n=165)	(n=51)	(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

Note: C= Central; Nr= Northern; S = Southern; E = Eastern

Table 4.1
Continued

Bird Species, Total Sample (N=509)	**Public Places (N=257)**					**Zoological Garden (N=252)**			
Family Species, TOTAL (N)	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)
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

4.1.2 Prevalence of *C. neoformans* in Bird Droppings

The prevalence of *C. neoformans* found in bird droppings in this study was determined by molecular identification (Figure 4.1).

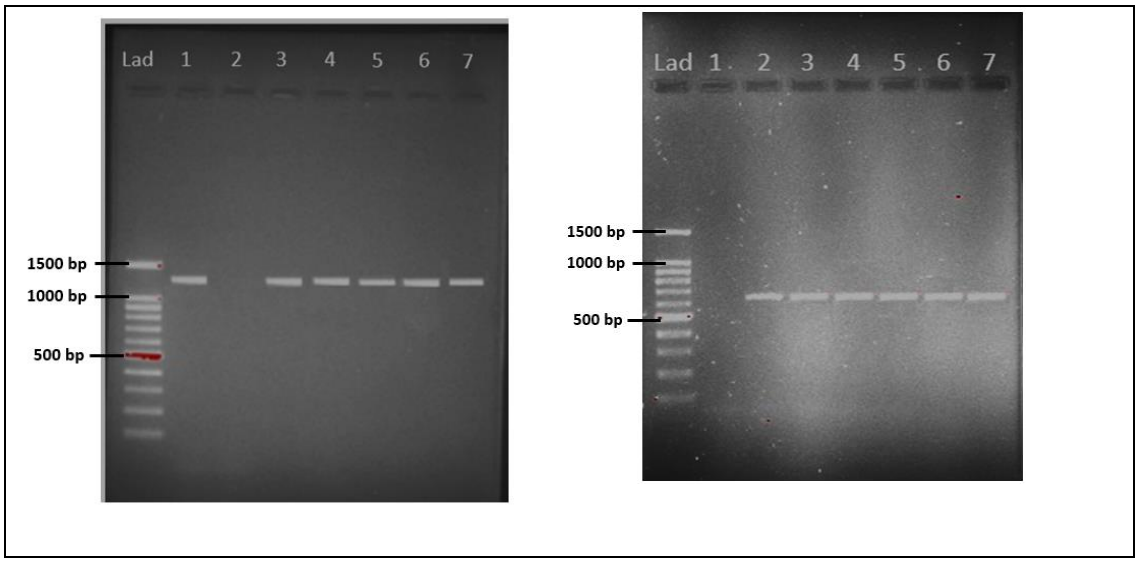

Figure 4.1 Agarose Gel Electrophoresis of Nested PCR. (Left) Primary Amplification using the *CNLAC1* Outer Primer (1386 bp). Lanes: 1 - Positive Control (Sequenced PCR product), 2 - Negative Control (Nuclease-free water), 3-7 - Positive Samples. (Right) Secondary Amplification using the *CNLAC1* Inner Primer Set (690 bp) to Identify *C. neoformans* in the Samples. Lanes: 1 - Negative Control (Nuclease-free water) 2 - Positive Control (Sequenced PCR product), 3-7 - Positive Samples. Ladder: 100-bp Molecular Marker (SMOBIO, Taiwan)

The overall prevalence of *C. neoformans was* 42.6% (217/509). In terms of regions, the Northern region recorded the highest prevalence (44/79; 55.7%), followed by the Central region (138/309; 44.7%), Southern (25/81; 30.9%) and lastly, Eastern (10/40; 25.0%).

Among the bird families examined, the Columbidae family (pigeons and doves) showed the highest prevalence, (115/205; 56.1%) followed by the parrot family (Psittaculidae) (32/65; 49.2%) and Cacatuidae (cockatoos) (9/20; 45.0%). For bird species, the Indian peafowl showed the highest positivity rate of *C. neoformans* (13/21; 61.9%). This is followed by budgies (19/31; 61.3%), common pigeons (100/170; 58.8%). Nicobar pigeons (5/12; 41.7%), rainbow lorikeets (10/25; 40.0%) and

Mandarin ducks (16/40; 40.0%). Notably, three bird species that were negative from *Cryptococcus* in this study were swan geese, Oriental-magpie robins and the emu.

Comparatively, *C. neoformans* prevalence was higher in zoological gardens (116.252; 46.0%) compared to public places (101/257; 39.3%), however this difference was not statistically significant (P=0.125). For public places, the highest positivity rate was observed in the Northern region (24/28; 85.7%), followed by Central (63/144; 43.8%), Eastern (10/40; 40.0%) and Southern (4/45; 8.9%). In zoological gardens, the highest positivity rate was at the Southern region (21/36; 58.3%), followed by Central (76/165; 46.1) and lastly, Northern (19/51; 37.3%). Further downstream analysis showed that the positivity rate of common pigeon in zoological garden (21/26; 80.8%) was significantly higher compared to public places (79/144; 54.9%) (P=0.013). A similar trend was observed in chickens, with higher positivity in zoological gardens (3/6; 50.0%) than in public places (4/31; 12.9%). A summary for the positivity rates of *C. neoformans* found in bird droppings in this study is detailed in Table 4.2.

Table 4.2
The Occurrence of *Cryptococcus* in Bird Droppings According to Bird Species, Places and 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 (42.6%)	101 (43.8)	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 Family & Species									
Columbidae									
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)	0
Zebra & Spotted Dove (N=23)	10/23 (43.5)	10/23 (43.5)	0	0	0	0	0	0	0
Nicobar Pigeon (N=12)	0	0	0	0	0	5/12 (41.7)	5/12 (41.7)	0	0
Anatidae									
Mandarin duck (N=40)	0	0	0	0	0	16/40 (40.0)	16/40 (40.0)	0	0
Mallard (N=18)	0	0	0	0	0	5/18 (27.8)	5/18 (27.8)	0	0
Phasianidae									
Chicken (N=37)	4/31 (12.9)	3/5 (60.0)	0	1/26 (3.8)	0	3/6 (50.0)	2/2 (100)	1/4 (25.0)	0
Indian peafowl (N=21)	0	0	0	0	0	13/21 (61.9)	13/21 (61.9)	0	0
Turkey (N=6)	0	0	0	0	0	2/6 (33.3)	0	2/6 (33.3)	0
Psittaculidae									
Budgie (N=31)	0	0	0	0	0	19/31 (61.3)	0	9/15 (60.0)	10/16 (62.5)

Table 4.2
Continued

Samples (N=509)	Number of birds positive Public Places					Number of birds positive Zoological Gardens			
	Total Positive n (%)	C (N=144)	Nr (N=28)	S (N=45)	E (N=40)	Total Positive n (%)	C (N=165)	Nr (N=51)	S (N=36)
By Bird Family & Species									
Rainbow Lorikeet (N=25)	0	0	0	0	0	10/25 (40.0)	10/25 (40.0)	0	0
Moluccan eclectus (N=9)	0	0	0	0	0	3/9 (33.3)	0	2/5 (40.0)	1/4 (25.0)
Passeridae									
House sparrow (N=21)	4/21 (19.0)	4/17 (23.5)	0	0	0/4 (0.0)	0	0	0	0
Sturnidae									
Common Myna (N=16)	3/16 (18.8)	1/8 (12.5)	0	2/8 (25.0)	0	0	0	0	0
Psittacidae									
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)
Cacatuidae									
Sulphur-crested & Solomons cockatoo (N=20)	0	0	0	0	0	9/20 (45.0)	0	1/7 (14.3)	8/13 (61.5)
Pelecanidae									
Great white pelican (N=12)	0	0	0	0	0	2/12 (16.7)	2/12 (16.7)	0	0
Corvidae									
House crow (N=7)	1/5 (20.0)	1/5 (20.0)	0	0/2 (0.0)	0	0	0	0	0

4.2 Association Between Hygiene and Environmental Factors with the Occurrence of *C. neoformans* in Bird Droppings in Zoological Gardens

In this study, several factors on environmental and hygiene conditions were explored to identify the possible risk factors associated with the positivity rates of *C. neoformans* in zoological gardens. Due to the low sample size in the Ijok Private Zoo, it was excluded from the analysis. Details of the hygiene and environmental conditions of each zoo are summarized in Table 4.3. Generally, Zoo Melaka had the highest positivity rate (58.3%). Even though the zoo is frequently cleaned, other factors such as limited space (crowded conditions) and frequent bird stains were seen. Conversely, in KL Bird Park, despite its less frequent cleaning schedule, recorded a lower positivity rate, likely due toits spacious enclosures. Kulim Bird Park had the lowest positivity rate (39.2%), as expected because the birds were living in better overall hygiene conditions. The zoo gets cleaned frequently, no odour unobstructed drainage system, along with the spacious enclosures for the welfare of the birds.

Table 4.3

Hygiene and Environmental Observations with Number of Positive Samples from Zoological Gardens

Zoological gardens	Cleaning Frequency	Air Odour	Drainage System	Enclosure density	Frequency of bird stain	Positivity status (%)
KL Bird Park (N=98)	Low	No odour	Fair	Spacious	High	41.8
Farm in The City (N=61)	High	No odour	Excellent	Moderate	Low	50.8
Kulim Bird Park (N=51)	High	No odour	Excellent	Spacious	Low	39.2
Zoo Melaka (N=36)	High	Mild odour	Fair	Crowded	High	58.3

A risk factor associated with *C. neoformans* positivity in zoological garden was performed using logistic regression analysis. In the univariate analysis, zoological gardens that houses a large number of Columbidae birds (pigeons and doves) had 2.9 times the odds (P=0.004) to exhibit *C. neoformans* positivity.

Further multivariate analysis included three factors with P values < 0.25 from the univariate analysis; the presence of Columbidae birds, air odour and enclosure density. Two factors have been identified as significant predictors for the high positivity rate of *C. neoformans* in birds from zoological gardens. These predictors included the presence of many Columbidae birds (AOR = 3.3) and crowded enclosures (AOR = 2.6). These findings suggest that both Columbidae birds and environmental closures play important roles in facilitating *C. neoformans* presence in zoological gardens (Table 4.4).

Table 4.4

Potential Risk Factors Associated with the Positivity Rate in Birds from Zoological Gardens

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	0.002*
No	209	88 (42.1)	(1)		(1.6,7.0)	
Cleaning frequency						
High	148	72 (48.6)	(1)	0.294	1.4	0.338
low	98	41 (41.8)	0.8 (0.5,1.3)		(0.7, 2.6)	
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	0.020*
Spacious	210	92 (43.8)	(1)		(1.2, 5.9)	
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)			

AOR = Adjusted Odd Ratio; CI = Confidence Interval; COI = Crude Odd Ratio; NC = Not Calculated

4.3 Antifungal Susceptibility Tests

In this study, a total of 217 positive isolates were subjected to antifungal susceptibility screening against Amphotericin B, Itraconazole and Fluconazole using disc diffusion method (Figure 4.2).

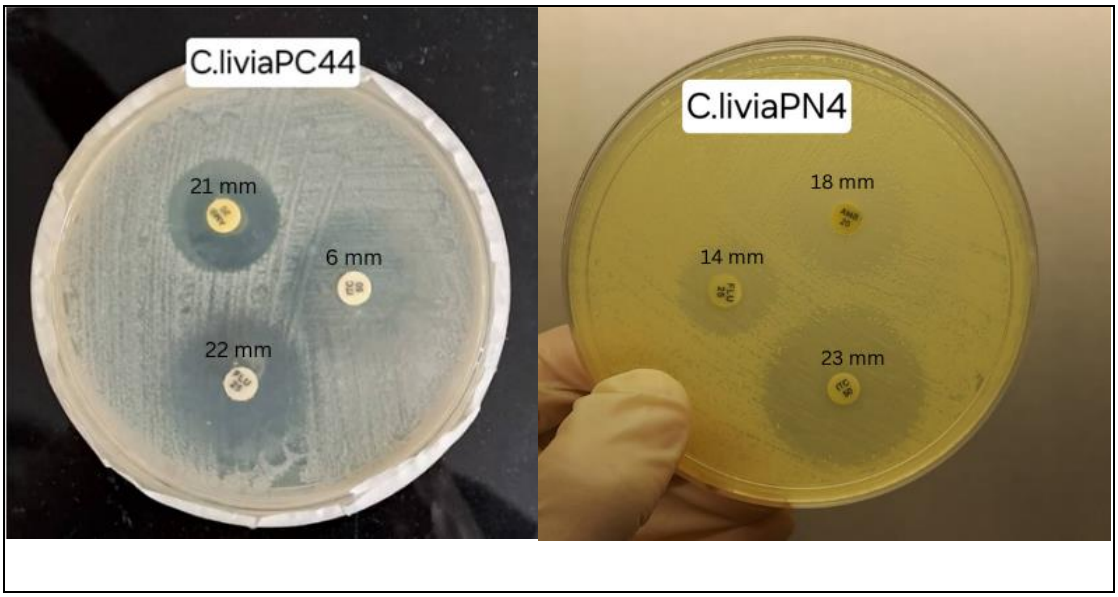

Figure 4.2 Antifungal Resistances of C.liviaPC44 (left) and C.liviaPN4 (right)

Of these, two isolates exhibited resistance to Itraconazole (50 µg/disk) (zone of inhibition, 6 mm) and intermediate resistance to Fluconazole (25 µg/disk) (zone of inhibition, 14 mm), respectively. These samples were C.liviaPC44 and C.liviaPN4 respectively. Both samples were obtained from common pigeons (Columbidae family) in public places (PC44 from the Central region and PN4 from the Northern region). The remaining 215 samples were susceptible to all antifungals without any resistance observed (Appendix N). Detailed antifungal susceptibility results are shown in Table 4.5.

Table 4.510
Antifungal Susceptibility Tests of C.liviaPC44 & C.liviaPN4

Sample	Amphotericin B (mm)	Itraconazole (mm)	Fluconazole (mm)
C.liviaPC44	21	**6**	22
C.liviaPN4	18	23	**14**

mm = millimetre

Due to the extremely low number of resistant isolates, statistical comparison between groups were not performed since tests such as chi-square will lack distinct values and violate assumptions. Instead, the results are presented descriptively to demonstrate the continuous effectiveness or resistance of the isolates to antifungal agents in Peninsular Malaysia.

Unlike other antifungal tests which relies on concentration and minimum inhibitory values (MIC), the disc diffusion test does not provide any MIC values. Therefore, it should not act as a final confirmatory test on the antifungal resistance for *C. neoformans*. Instead, the disc diffusion test conducted in this study serves as a screening test on the antifungal resistance of *C. neoformans* in this study. Antifungal Tests Results in Millimetre (mm). Those resistance to Itraconazole of C.liviaPC44 and Intermediate resistance to Fluconazole for C.liviaPN4 have been bolded and underlined.

4.4 A Partial Phylogenetic Tree Construction

In this study, 16 positive isolates were selected for purification and sequencing (Apical Sdn. Bhd). Amongst the 16 included was the sample resistant to Itraconazole (sample ID: C.liviaPC44) and another sample with intermediate susceptibility against Fluconazole (sample ID: C.liviaPN4). The other 14 samples were chosen based on high interaction with public, such as those present in big flocks or birds from zoological gardens that the public are able to interact with (either touching or taking close up pictures with the birds). These are detailed in Table 4.6.

Table 4.6
Characteristics of Sequenced Isolates and Their Homology

Location	Region	ID	Justification for Sequencing	Bird species	Sample Accession number	Similarity (%)	Ref. Accession number	Strain
Public places	Central	C.liviaPC44	Isolate showed resistance to Itraconazole	Common pigeon	SRR30002292	99.57	MG963886.1 Pigeon nest, Denmark	*C. neoformans* var. *neoformans* Serotype D
		S. chinensisPC 3	Third highest prevalence for public places	Spotted dove	SRR30002297	97.03	AB273979.1 Human Japan	*C. neoformans* var. *grubii* Serotype A
		G. striataPC3	Second highest prevalence in public places	Zebra dove	SRR30002301	99.52	AB273959.1 AIDS Patient Thailand	*C. neoformans* var. *grubii* Serotype A
	Northern	C.liviaPN4	Isolate showed intermediate resistance to Fluconazole	Common pigeon	SRR30002291	99.71	MG963878.1 Lab Strain	*C. neoformans* var. *neoformans* Serotype D
Zoological gardens	Central	C.liviaZC18	Highest prevalence in zoological gardens	Common pigeon	SRR30002290	99.19	MG963884.1 Pigeon nest Denmark	*C. neoformans* var. *neoformans* Serotype D
		C.nicobarica ZC1	High public interaction (Walk in aviary) and closest living relative to the dodo bird (Heupink et al., 2014)	Nicobar pigeon	SRR30002289	99.46	AB273959.1 AIDS Patient Japan	*C. neoformans* var. *grubii* Serotype A
		A. solstitalisZC 1	High public interaction (walk in aviary)	Sun Conure	SRR30002294	98.35	MG963842.1 Plane Tree Greece	*C. neoformans* var. *neoformans* Serotype D
		T. moluccanus ZC1	High prevalence in zoo and public interaction	Rainbow lorikeet	SRR30002296	89.96	AB273992.1 AIDS Patient Brazil	*C. neoformans* var. *neoformans* Serotype D
		P. cristatusZC7	Third highest prevalence in zoo	Indian peafowl	SRR30002298	98.49	AB273959.1 AIDS Patient Thailand	*C. neoformans* var. *grubii* Serotype A

Table 4.6
Continued

Zoological Gardens	Central	A. galericulata ZC1	High prevalence in zoo and public interaction	Mandarin Duck	SRR30002302	98.36	AB273989.1 AIDS Patient Italy	*C. neoformans* var. *neoformans* Serotype D
	Northern	E. roratusZN1	High prevalence in zoo and for petting	Moluccan Eclectus	SRR30002288	99.15	AB273970.1 AIDS Patient Thailand	*C. neoformans* var. *grubii* Serotype A
		A. oratrixZN1	High interaction with public for petting	Yellow Headed Amazon	SRR30002295	98.60	MG963879.1 Laboratory Strain	*C. neoformans* var. *neoformans* Serotype D
		M. undulatusZN1	High prevalence in zoo and public interaction	Budgie	SRR30002300	98.15	AB273959.1 AIDS Patient Thailand	*C. neoformans* var. *grubii* Serotype A
	Sr	M. undulatusZS1	High prevalence in zoo and public interaction	Budgie	SRR30002299	97.63	AB273979.1 Human Japan	*C. neoformans* var. *grubii* Serotype A
		C. galeritaZS4	High prevalence in zoo and petting	Sulphur-Crested Cockatoo	SRR30002293	99.56	AB273975.1 AIDS Patient Italy	*C. neoformans* var. *grubii* Serotype A
		A. ararаunaZS1	High prevalence in zoo	Blue-and-yellow Macaw	SRR30002303	95.08	MT892612.1 Common pigeon, Iran	*C. neoformans* var. *grubii* Serotype A

All 16 sequences obtained in this study were submitted to GenBank, under the accession numbers SRR30002288-SRR30002303. Majority of the sequenced isolates showed >97% similarity, belonging to either *C. neoformans* var. *grubii* (Serotype A) or *C. neoformans* var. *neoformans* (Serotype D). The two samples that were resistant to Itraconazole and the other with intermediate susceptibility to Fluconazole exhibited >99% similarity to *C. neoformans* var. *neoformans* (Serotype D).

A partial phylogenetic tree was then constructed (Figure 4.3) based on the 16 sequenced samples along with the reference sequences in **Appendix M**. The isolates clustered closely with *C. neoformans* var. *grubii* (9 isolates) and *C. neoformans* var. *neoformans* (7 isolates), portraying their genetic similarities to these lineages. Interestingly, based on the reference strains, the majority isolated from environment are var. *neoformans,* whereas human clinical isolates are predominantly var. *grubii*. The majority of var. *grubii* references trains included in the analysis were derived from AIDS/HIV infected patients, denoting the similar genetic strains between clinical and environmental isolates in Peninsular Malaysia. Two out of 9 var. *grubii* isolates in this study were isolated from public parks with both being dove birds, or Columbidae family.

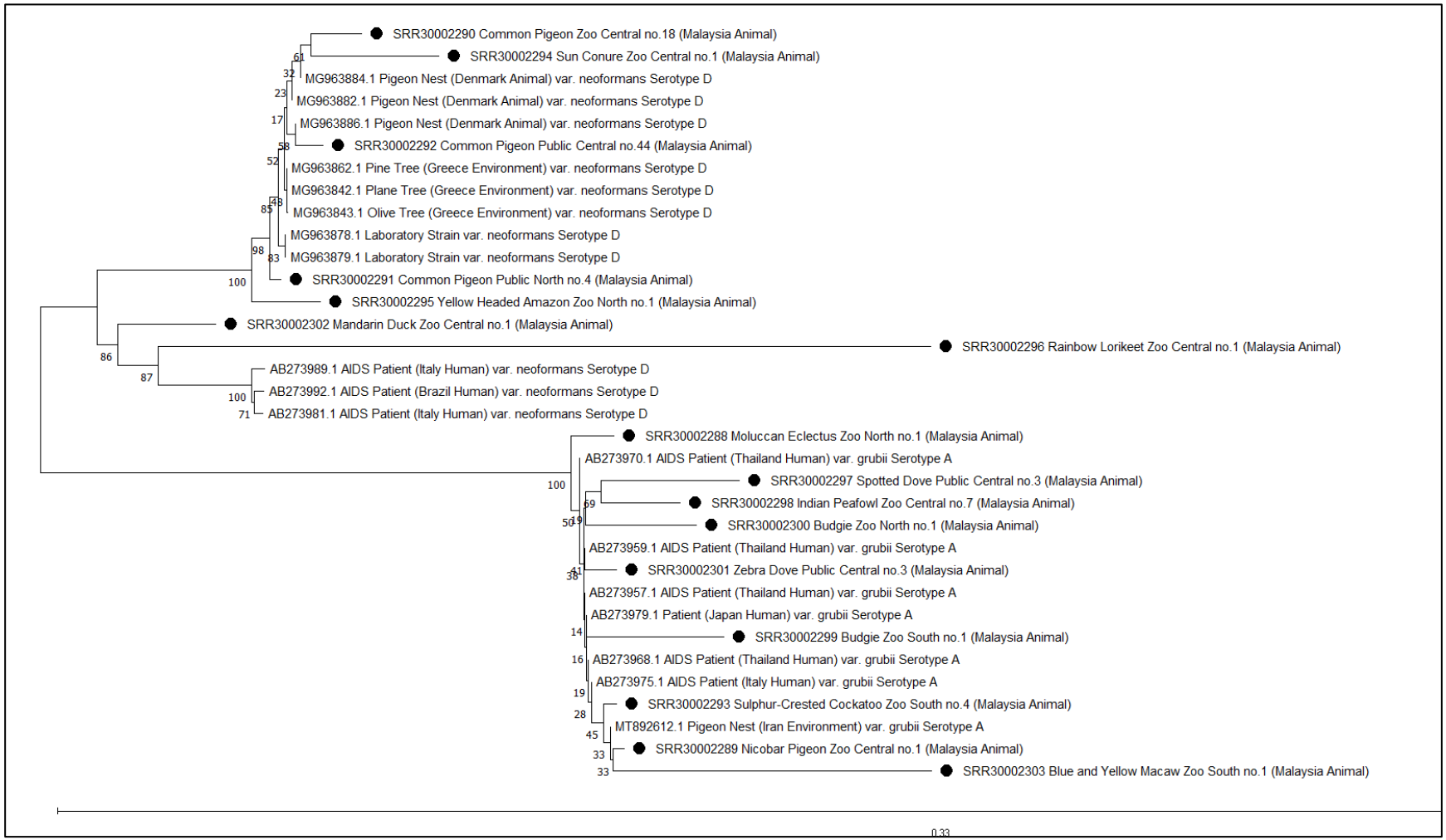

Figure 4.3 Partial Phylogenetic Tree of all 16 *Cryptococcus* Strains sequenced in this Study. Showing relations with Serotype A and D

CHAPTER 5
DISCUSSION

This study investigated the current prevalence of *C. neoformans* in bird droppings collected from both public areas and zoological gardens across four regions in Peninsular Malaysia (objective 1). In addition to that, this study also examined hygienic and environmental factors that are potentially associated with the occurrence of *C. neoformans* in zoological gardens (objective 2). The study further determined the antifungal susceptibility testing against Amphotericin B, Fluconazole, and Itraconazole (objective 3), along with serotypes and evaluated partial phylogenetic analysis of the isolates (objective 4). This chapter discusses the key findings in relation to existing literature, highlighting significant findings and observations as well as discuss the potential implications for public health and environmental management related to *C. neoformans*.

5.1 Current Prevalence of *C. neoformans* in Bird Droppings in Peninsular Malaysia

Despite the known role of birds, particularly pigeons as reservoirs for *C. neoformans*, there are limited current data on its prevalence across different ecological and geographical settings in Malaysia. The only prior surveillance study in birds was conducted over two decades ago (Tay et al., 2005) and restricted to Klang Valley with no documentation on the specific bird species of the collected droppings or excreta. This significant gap in national surveillance especially given higher interaction between human population and birds warranted a comprehensive update.

To achieve this, a total of 509 fresh bird droppings involving 24 bird species representing 12 taxonomic families were collected from both public places and zoological gardens across four regions (Central, Northern, Eastern, and Southern) of Peninsular Malaysia. Unlike earlier research (Tay et al., 2005), this study specifically documented the bird species associated with each sample by collecting fresh droppings immediately after defecation, ensuring reliable species attribution. This strategy

allowed us not only to determine updated prevalence data but also to associate fungal occurrence with specific bird species.

Overall, the prevalence of *C. neoformans* in bird droppings in this study was 42.2% (217/509) which is markedly higher than the 3.7% as reported by Tay et al. (2005). This high prevalence is concerning and highlights a potential public health risk, particularly in areas where close contact between humans and birds is common. However, several key factors may account the discrepancy of the prevalence. Firstly, the present study had broader geographic coverage as compared to previous study which was limited to Klang Valley only. This present study also included samples from both public places and zoological gardens in Peninsular Malaysia, providing more diverse representation of birds' population. Next, the type of samples and diagnostic approaches differed as well. The samples collected in this study were fresh droppings to minimize the risk of environmental or soil contamination in contrast to the previous study (Tay et al., 2005) which utilized dried bird droppings. This present study also used nested-PCR, a higher sensitivity diagnostic tests to detect *C. neoformans* DNA compared to the culture and biochemical identification techniques employed by Tay et al. (2005), which in general are less sensitive.

It is important to note that the species of birds included in this study differed between public places and zoological garden. Out of the 24 bird species, nine species were from public places. This study emphasised on native and backyard bird species which were identified by observing their nesting grounds The common pigeons (*Columba livia*) was the predominant species sampled in public places. This species is well-documented globally for harbouring *C. neoformans* a including China (Li et al., 1993), Iran (Ghaderi et al., 2019) and Brazil (Ribeiro et al., 2019). Other important bird species found in public places were spotted doves (*Spilopelia chinensis*) and zebra doves (*Geopelia strata*), both belonging to the Columbidae family (Soares et al., 2016). Following the pigeons, the poultry birds were of high importance in this study, such as the chickens (*Gallus gallus*). Chickens were raised by the locals as farm animals for both food and pest control. Therefore, these chickens would be in small or big flocks, increasing the chances of *C. neoformans* transmission to their owners and the neighbourhood. Lastly for public places, house sparrows (*Passer domesticus*) were also commonly seen and closely associated with urban landscapes and are a widespread species (Summers-Smith, 1988).

The species of bird in zoological garden are more diverse and rarer. For the remaining 15 bird species in zoological gardens, the highest number collected was from Mandarin ducks (*Aix galericulata*). These ducks are highly distinctive with their ornate plumage, making it an attraction for visitors at KL Bird Park. Visitors were able to get close to these ducks and feed them. Following the ducks, budgies (*Melopsittacus undulatus*) were the second highest sample collected from zoological gardens. These parrots are popular in Kulim Bird Park and Zoo Melaka. For Kulim Bird Park, budgies were located in a walk-in aviary, visitors were able to hand feed these birds and notorious for perching on top of the visitors. According to the Kulim Bird Park's zookeeper, many of these birds would just excrete and leave their droppings on the visitors. Thus, raising concern of the exposure of *C. neoformans* from these small parrots. Not to be missed, common pigeons were also available both for show and for hand feeding in the zoological gardens. These pigeons however, occurred in smaller flocks compared to the ones found in public. These common pigeons are considered fancy pigeons, also known as common fantail pigeons. Thus, becoming popular among visitors and being social in nature, these pigeons tend to get very close to the visitors in hopes of getting fed. Another bird species that tends to get close with the visitors was the rainbow lorikeets (*Trichoglossus moluccanus*). These loris are able to be hand fed a special type of milk by the visitors in KL Bird Park. The bird will perch on the visitors and sometimes excrete on them. Furthermore, these birds like to fly and circle around the visitors, since *C. neoformans* is primarily spread via airborne propagules, the repeated flapping from the lorikeets along with their droppings on visitors could potentially increase the exposure of *C. neoformans* to the visitors.

In this study, despite not having any significance difference in the overall prevalence between public areas and zoological gardens, the prevalence in zoological gardens is still higher than public places. A study done in Antwerp's Zoo in 1986 provided evidences for the presence of *C. neoformans* isolated from bird droppings in the zoo. These birds were mainly of the parrot family (Bauwens et al., 1986). In this study, the parrot's family mainly; the Psittacidae and Cacatuidae had a prevalence of 32.0% and 45.0%, respectively. Aligning with the previous study in Belgium where the parrot family had a higher chance of harbouring *C. neoformans* in their zoo. The common pigeons had a higher infection rate of *C. neoformans* in zoological gardens. These findings align with previous research that identifies pigeons as common

reservoirs for this fungus. Interestingly, this study documents the higher infection rate of *C. neoformans* in chickens from zoological gardens as compared to public places (50.0% versus 12.9%, respectively). This aligns with the previous study from Thailand on bird droppings from chickens whereby Kuroki et al. (2004) reported a prevalence of 24% of *C. neoformans* from chicken droppings.

In this study, other bird species contributes to the environmental contamination as well. Fascinatingly, a high prevalence of *C. neoformans* were found in Indian peafowl droppings (61.9%). For this species of bird, no prior data on *C. neoformans* prevalence existed. The same can be said for Mandarin ducks as this study documented a prevalence of 40.0% with no prior data. The findings from these two bird species suggests that a wider range of avians may serve as reservoirs for *C. neoformans*.

This study was conducted across four regions of Peninsular Malaysia, namely Central, Northern, Eastern and Southern. In terms of sample collection, a large proportion of samples was collected from the central region primarily in the Klang Valley. As of 2023, the Klang Valley houses about 8 million people who are registered. This does not include the unregistered numbers (Department of Statistics Malaysia, 2023). On top of that, the sample collected from the Klang Valley updates the current prevalence for this area that was done in 2005 by Tay et al. (2005). Being an urban area and densely populated, the number of pigeons and other birds tend to increase as well. The availability of food scraps and litter are a reliable food source for these urban birds, on top of the shelter that the birds are able to nest within the structures of Klang Valley buildings. This includes hospitals, places of worships and train stations. Bird droppings can be seen almost everywhere in the Klang Valley.

Hospital Kuala Lumpur (HKL) is one of the main government hospitals in the Klang Valley. This hospital not only serves as a treatment building, it gave rise to many business platforms, particularly, stalls and markets. These stalls are scattered all around HKL. Their litters and food scraps serve as an abundance of food source for pigeons. During the daytime, these pigeons will scavenge the area, leaving droppings all around. They will then nest in nearby buildings especially in HKL. Pigeons are seen in balconies and even patient wards. They will nest on top of every crevasse they can find. Based on direct observations and informal interviews with nursing staff, HKL has open wards, these pigeons tend to enter the wards and wonder in with the patients, flapping around and potentially exposing the already sick patients to *C. neoformans*.

In the Klang Valley, Batu Caves is one of the most iconic buildings located in Kuala Lumpur. This place serves primarily as a place of worship, however, the iconic 140ft statue has turned Batu Caves into a popular tourist spot. Here, the locals and devotees feed the pigeons deliberately in keeping with their faith of being kind to animals will result in a better life after death. Great flocks of pigeons (estimated more than 30 birds) will gather in a great feast offered by the devotees. Like any other buildings, the pigeons will then nest in between the smaller statues, crevasses of buildings or any open areas they can find.

In Petaling Jaya, PJ New Town is one of the oldest cities. Here lies old buildings and structures dating back to the 1960s. Despite being named "new town" the buildings are still kept the way they are to maintain the heritage ambience. The lowly maintained buildings with multiple fissures housed many pigeons. A few initiatives of the local municipal; Majlis Bandaraya Petaling Jaya (MBPJ) includes campaigns that impedes feeding the birds, especially pigeons. Along with the addition of anti-nesting spikes seen outside the buildings. However, much to the municipal's surprise, these pigeons saw these spikes as fences for their nesting materials, hence, turning it into a foundation for their nests. Pigeon droppings are everywhere in PJ New Town shop lots. Many of the building paint and wall murals were "decorated" with bird droppings. Furthermore, being one of the oldest cities in PJ, this area houses many senior citizens, some of the locals enjoy feeding the pigeons as well, either on their morning routine or on their evening stroll. The pigeons here are fed a lot of times daily. Growing and populating rapidly in the heart of PJ. Not far from PJ New Town, Sri Sithi Vinayagar temple is a Hindu temple where the devotees and locals regularly feed pigeons. The pigeons are notoriously known for nesting in between the statues of the buildings. These pigeons also stay and linger in nearby buildings of the temple.

Kota Damansara is known as one the few thriving urban cities in the Klang Valley. In section 9, a local recreational park leads up to a hiking trail. This park is famous among the locals for outdoor activities. Encompassing a few districts of Kota Damansara, this park houses many trees and gazebo. These are the main nesting areas of the local backyard birds in KD especially for the doves. Visitors and locals of the park are potentially exposed to *C. neoformans* if they sit and rest underneath the gazebo as there are bird dropping stains and nests underneath the roofs of the structures.

Following the local park in KD, Tropicana Golf Club is another recreational park but serves primarily as a golf course. The same settings can be observed, countless trees housing many bird nests and gazebo scattered throughout the park. Much like the gazebo in KD park, there were many bird droppings stains and signs of nesting materials underneath the rooftops. Not to mention there are many more bird dropping stains in between the buildings of the Golf Club. Having a high-rise ceiling, the bird nests are more difficult to be cleaned and removed. Hence, bird dropping stains are seen periodically on the floor underneath the nests. However, to preserve the status of the golf club, cleaners mop and clean the floor every hour.

In the Eastern region, samples were collected from two areas involving two states, namely MBK field in Kuantan, Pahang and KTCC mall in Kuala Terengganu, Terengganu. MBK field was selected for being a place of gathering and feeding for the pigeons. At night, this area becomes a night market. The scraps and litters from the night carries forward into the day serve as a feeding ground for the pigeons. Locals on the other hand, would sometimes feed these pigeons during the evening around 4pm whilst waiting for the night market to be set up. In Terengganu, KTCC mall's outskirts was the only place in Terengganu that pigeons are often seen. The pigeons tend to feed at the malls' disposable or rubbish collection sites. The reason behind this is that Kuala Terengganu's streets are very clean with proper litter management. Pigeons are only able to eat from the scraps of the rubbish area in the outskirts of KTCC Mall.

The Northern region's sampling area is in Georgetown, Penang. The area's town centre or shop lot area spans miles in Penang Island. Here, numerous hawker stalls, restaurants and cafes spread across Georgetown. Thus, food scraps and unmanaged wastes spread across the area as well. Great flocks of pigeon scatter about flying everywhere in the city and are able to nest in between buildings of the shop lot area. These pigeons will scavenge and eat at multiple areas in the city. Sometimes, locals will feed the pigeons as well.

Lastly, the Southern region's sampling site is at Sri Maha Mariamman Temple located in Seremban, Negeri Sembilan. There are not many hawker stalls in Seremban, therefore, barely any food scraps or unmanaged litter were seen. Since restaurants are required to be kept clean, the waste is managed properly by the municipal council, discouraging pigeons from eating on the streets. Instead, many birds flock to the temples. The locals of Seremban tend to raise poultry birds that roam the

neighbourhood. Chickens and geese travel in flocks from one neighbourhood to the other and often stay near the temples. Nevertheless, devotees of the temple feed the birds every time the birds visit the temple. In this study, no sample was available in the Eastern region's Zoological Gardens.

The occurrence of *C. neoformans* is documented in many other countries. Neighbouring countries such as in Bangkok, Thailand reported a prevalence of 11% from pigeon droppings (Krangvichain et al., 2016). In Jakarta, Indonesia, Machrumnizar et al. (2022) isolated *C. neoformans* from various bird droppings, including canaries, white eyes, prinia and woodpeckers with a prevalence of 14.3% (1/7). Other Asian countries such as in Korea, Oh & Hwang (2005) reported a prevalence of *C. neoformans* at 41.7% isolated from pigeon shelters in Busan. Abou-Elmagd et al. (2011) isolated *C. neoformans* from various parts of chickens, including their droppings. They recorded a prevalence of 2.54%, as compared to this study however, the isolation of *C. neoformans* from chickens were at 18.9%, showing a higher rate. Recently in Iran, Mirpourian et al. (2021) reported the prevalence to be 13.0% in various bird species.

In other parts of the globe such as in Spain reported that the prevalence of *C. neoformans* in pigeon samples to be t 7.8% (Rosario et al., 2005). Nweze et al. (2015) later isolated this fungus from bird droppings based on locations or environments in Nigeria, with a prevalence of 22%. Other than pigeons and chickens, the presence of *C. neoformans* were studied in other type of birds. In Iran, Amirrajab et al. (2016) had reported one positive case of *C. neoformans* in mallards. However, the prevalence was not obtained as the authors did not mention how many mallards were sampled. In this study, mallards were recorded with a prevalence of 27.7% from KL Bird Park. Although these findings indicate regional differences, the role of mallards as potential reservoirs of *C. neoformans* appears to be significant.

However, there are differences in the sampling techniques and molecular approaches, suggesting that the limitations of different type of approaches could have different outcomes in terms of prevalence. Despite the differences, public awareness and surveillance for this fungus should be practiced and emphasised. The need to stay vigilant especially for bird handlers, zookeepers and pet owners such as proper PPE when handling birds or the use of air purifiers in an enclosed space.

In this study, the pigeon family Columbidae and enclosure densities play distinct roles in the positivity of *Cryptococcus* in zoological gardens. The chances of having *Cryptococcus* are 3.3 times higher if there are pigeons in the zoological gardens. This does not constitute the species of pigeons, but the pigeon family as a whole. At the same time, a crowded enclosure increases the chances of *Cryptococcus* presence in the zoological gardens by 2.9 times. This enclosure density is categorised as dense if the birds are not able to roam or fly freely in their enclosures. Despite the other environment or hygiene factors are not statistically significant in this study, regular cleaning of the zoological gardens still proves to be the key to managing the occurrence of *Cryptococcus*. Furthermore, hygiene and sanitisation help mitigate more than just the spread of this fungus, other pathogenic microbes would pose less risks to the public and zookeepers.

5.2 Association Between Hygiene and Environmental Factors with the Occurrence of *C. neoformans* in Bird Droppings from Zoological Gardens

Other than prevalence, this study highlights the association between hygiene and environmental conditions of the zoological gardens with the positivity rate of *C. neoformans* in bird droppings. The pigeon family also known as the Columbidae and enclosure densities were found to be significant predictors of *C. neoformans* positivity. In terms of the Columbidae family, as anticipated pigeons are commonly associated with the fungus. Many studies have discussed about the relation between pigeons and the presence of *C. neoformans* worldwide (Chander, 2017; Ghaderi et al., 2019; Ribeiro et al., 2019).

Enclosure densities significantly proved to be a factor for the positivity of *C. neoformans* in bird droppings as well. A crowded enclosure has a higher positivity rate for this fungus compared to a spacious one. This was seen in Zoo Melaka having the highest prevalence amongst all zoological gardens at 58.3%. Too many birds were kept in a certain space, and it was crowded. Furthermore, birds were only allowed to leave their crowded enclosures to roam in a bigger one three times weekly. Concerns were escalated as the amount of bird droppings increases a lot in a short time, piling and concentrated in the enclosures. If bird droppings are not cleaned frequently, their accumulation can lead to a higher concentration of bird dropping stains, thereby

increasing the likelihood of fungal growth in the enclosures. This not only poses a health hazard to visitors and zoo staff, but also make the area look unpleasant to visitors. This study highlights the importance of enclosure density for the positivity of *C. neoformans* as compared to other environmental factors. In another study by Raso et al. (2004), a cryptococcosis outbreak occurred in a breeding enclosure for various Psittacine birds. These birds were kept in a small breeding cage, further enforcing the importance of enclosure density in relation to *C. neoformans* positivity rate.

Despite enclosure density and the Columbidae family being a significant factor for the presence of *C. neoformans* in this study, other factors in the survey that were not significant could potentially be predictors of *C. neoformans* positivity. The cleaning frequency of the enclosures although not significant (P=0.338) could potentially be a factor for *C. neoformans* positivity. Uncleaned bird droppings that have become dried are shown to be reservoirs of this fungus. Such were reported by a few studies globally, notably collecting dried bird droppings for the prevalence of *C. neoformans* (Mirpourian et al., 2021; Nweze et al., 2015; Tay et al., 2005). These studies reported a prevalence of 3.7%, 10.7%, and 22% from Malaysia, Iran and Nigeria, respectively. This further escalates the potential reservoir of *C. neoformans* in dried bird droppings regardless of country.

This finding could be related to another hygiene factor which is the frequency of bird droppings stains. The factor was estimated based on the approximate number of bird droppings observed per square metre. In this study, both low and high bird dropping stains frequencies were not statistically significant. This is likely due to the sample collection method whereby neither dried droppings nor stains of the bird droppings were collected. Although not significant, dried bird droppings can still serve as potential reservoirs for *C. neoformans* as discussed earlier.

Air odour in this study does not contribute to the positivity of *C. neoformans*. Despite Zoo Melaka appear to have mild odour with the highest positivity rate among zoological gardens (58.3%), the minimal sample size of mild odour against no odour does not allow this study to produce any significance based on this factor alone. Furthermore, this odour does not pinpoint specifically to the source of the said odour. An unpleasant odour smelled in the zoological gardens might not be from a bird dropping, many potential factors could be the reason for such vexatious smell. Such as unmanaged litter or stagnant drain water. Furthermore, it was observed that most

zoological gardens had acceptable odour to no odour in this study. This highlights that despite having acceptable odour, *C. neoformans* positivity was still high in the respective zoological gardens as seen in Farm in The City (50.8%).

This proved to be a limitation for this part of the study as confounding factors play a major role in the air odour of the zoological gardens. This factor could be correlated with the drainage system instead, which is another hygienic factor in this study. Both fair and excellent drainage systems were seen in both high and low positivity status for *C. neoformans*. Therefore, this factor is not a significant factor in relating to the positivity of *C. neoformans*. The samples obtained in this study were fresh bird droppings and not from drains, rendering this factor highly unrelated to the positivity of *C. neoformans*. However, if samples were to be obtained from excellent or fair drainage systems, the significance could be proved otherwise.

Studies assessing the hygiene and environmental factors of *C. neoformans* are lacking as well. Previous studies which mentioned about the prevalence of *C. neoformans* only state about the condition of the sampling area, not comparing these conditions as seen in this study (Mirpourian et al., 2021; Nweze et al., 2015; Tay et al., 2005).

In terms of bird health, all birds and their droppings collected in this study appeared to be healthy, there were no significant signs or symptoms of infection or injuries. This suggest that, whilst the birds are reservoirs of *C. neoformans*, they may not always exhibit symptoms of cryptococcosis. A case of cryptococcosis however, was documented from captive parrots in Sydney, Australia (Malik et al., 2003), highlighting the zoonotic potential of this fungus.

5.3 Antifungal Susceptibility Test

The previous study conducted by Tay et al. (2005) reported that all isolates were susceptible to antifungals such as Amphotericin B, Fluconazole and Itraconazole. However, in this study, two isolates from the public places were showing resistance or intermediate susceptibility to Fluconazole and Itraconazole. These isolates were SRR30002291 and SRR30002292, respectively. Both these isolates were from common pigeon droppings, highlighting the potential evolution of antifungal resistances in *C. neoformans* over the last 20 years (Chander, 2017). These two isolates are both

serotypes D (*C. neoformans* var. *neoformans*) and commonly found in the environment of Northern European countries (Casadevall & Perfect, 1998). However, three isolates of serotype D were recovered from patients with AIDS, two from Italy and another in Brazil. These isolates share a common ancestor with the two isolates from this study, despite their geographical separation. However, the partial phylogenetic tree in this study only acts as a preliminary insight.

Both the isolates were recovered from different locations, the isolate resistant to Itraconazole was recovered from a bird dropping nearby Hospital Kuala Lumpur (Central) and the other; intermediate susceptibility to Fluconazole was from a bird dropping nearby George Town, Penang (North). However, the limitations in the phylogenetic tree in this study does not confirm the evolutionary change, such as any mutations in the ERG11 gene (Odiba et al., 2022).

An *in vitro* study on the antifungal resistance of *C. neoformans* was conducted in Cambodia. This study reported in the neighbouring country portrayed resistance to fluconazole (MIC>=256 mg/L). The isolates were recovered from HIV-infected individuals. However, 98.5% of the isolates were serotype A (var. *grubii*) (Sar et al., 2004). Another study done in Southeast Asia was in Thailand where Tangwattanachuleeporn et al. (2013) reported decreased susceptibility to fluconazole. All *C. neoformans* isolates recovered from pigeon droppings were of serotype A.

In a period of over 10 years, an increased resistance to fluconazole was seen from 7.3% to 11.7% between 1997 to 2007 as recorded by the ARTMIS DISK Global Antifungal Surveillance Study. This was reported by Cambodia, Africa and Spain for *C. neoformans* (Bermas & Geddes-McAlister, 2020). Concerns are on the rise on the current status of fluconazole resistance globally, as this data was only reported in 2007. In Malaysia, the state of antifungal resistance of *C. neoformans* remains a concern as well. Furthermore, Cambodia and Malaysia are both in the same region and have almost similar climate. Climate is an important factor in the growth of *C. neoformans* (Rajasingham et al., 2017). Based on the previous reports from other Southeast Asian countries, the trend of increasing resistance to Fluconazole is without a doubt the next globally emerging threat towards the clinical impact of cryptococcosis.

Countries which are in the lower income group tend to suffer the casualties of cryptococcosis for not being able to afford or obtain access to first-life antifungal drugs. Nevertheless, the growing antifungal resistance portrayed by *C. neoformans* only makes

matter worst. Most countries affected by this would be some of the Asian and sub-Saharan African countries (Bermas & Geddes-McAlister, 2020). In Malaysia however, the availability and accessibility of these antifungals are not an issue, instead, since there are potential antifungal resistance patterns in Malaysia, the awareness of this fungus to the community should be taken into consideration. As cryptococcal meningitis proves to be lethal if untreated or undertreated.

In this study, the antifungal susceptibility test only portrays initial screening data and not the final antifungal resistances. This test does not qualify for minimum inhibitory concentrations (MICs) based on a standardized reference method such as the CLSI M27 broth microdilution protocol. Furthermore, active and constant surveillance on the antifungal resistance both locally and globally should be emphasised. Not to mention, the stewardship of antifungals for the public and agricultural use.

5.4 Molecular Typing and Phylogeny

Cryptococcus has a few serotypes which include serotypes A, B, C, D and AD (Ito-Kuwa et al., 2007). There have been a few trends in the distribution and predominance of serotypes globally, for example, *C. gattii* (serotype B and C) being predominantly present in tropical and subtropical climates (Kwon-Chung & Bennett, 1984). Meanwhile, serotypes A are present globally whereas Serotype D constitutes about 50% of the isolates in Northern European countries (Casadevall & Perfect, 1998). Serotype AD has been reported in Europe and North America (Lengeler et al., 2001).

In Malaysia, Tay et al. (2005) reported the presence of serotype A (*C. grubii*) in their study. Notwithstanding as this serotype have been reported from other Southeast Asian countries (Machrumnizar et al., 2022; Poonwan et al., 1997; Sar et al., 2004). Following the study done in 2005, Tay et al. (2010) reported the presence of serotype B (C. *gattii*) in Malaysia. This discovery after a 5-year gap showed the movement of *Cryptococcus* in this country. However, serotype A is still predominant in their report (88.5%).

In this present study, after 15 years, the serotypes of *Cryptococcus* have been updated in Malaysia. The molecular typing in this study identified all *Cryptococcus* strains as either *C. neoformans* var. *grubii* or var. *neoformans* (serotype A and D, respectively). As expected, *C. neoformans* var. *grubii* predominates in this study (nine

isolates) due to being the most common *Cryptococcus* strain isolated globally, primarily in infected humans (Franzot et al., 1999; Ito-Kuwa et al., 2008). The majority of this strain emphasizes the need for protective measures and raised awareness for both zookeepers and visitors. The exposure of bird droppings with this strain especially, poses a public health risk. Surprisingly, only three out of nine isolates are recovered from the pigeon family (Nicobar pigeon, spotted and zebra dove). The remaining six isolates were recovered from the parrot order Psittaciformes and an isolate from the Indian peafowl. This suggests the potential dangers of keeping parrots as pets as compared to pigeons or other birds. Notwithstanding the suggested divergence portrayed by the isolate recovered from the blue and yellow macaw in Zoo Melaka.

In terms of *C. neoformans* var. *neoformans*, the isolation of this strain in Malaysia is an eye-opener as this strain is usually associated with decaying woods in the Northern Europe (Franzot et al., 1999; Lazéra et al., 1996). The presence of var. *neoformans* in this study serves as an unexpected clade in the phylogeny. Seven of the isolates were identified as var. *neoformans*, two were from a Mandarin duck and rainbow lorikeet droppings in KL Bird Park. These two strains however, are closely related to previous strains recovered from patients with AIDS with the rainbow lorikeet isolate showing a big distance in the clade, suggesting a significant divergence. This raise concerns on the potential human infections these isolates could cause given the accumulated genetic variations (Ito-Kuwa et al., 2008). Three of the isolates were closely related to isolates collected from pigeon nests in Denmark. Out of these three, two of them in this study were from pigeons and another from a sun conure. However, the isolate recovered from the sun conure proved to be quite a distance, suggesting a greater divergence from the other pigeon isolates.

Next, another greater divergence is seen from an isolate recovered from a yellow headed amazon in Kulim Bird Park. This isolate appears to share a common ancestor with the laboratory strains and isolates recovered from two types of trees in Greece.

Previously, Tay et al. (2010) reported that *C. gattii* were isolated from patients in 2003-2004 in Malaysia, however, var. *neoformans* showed predominance over the former. In this study however, no *C. gattii* were isolated from bird droppings. Nevertheless, *C. gattii* are usually isolated from the red gum trees, including the River Red Gum Tree (Jenney et al., 2004). Following a 15-year gap, the absence of *C. gattii* in this study suggests that an epidemiological fade-out has occurred. However,

comparing different sampling approaches between the former and this study, in theory, *C. gattii* could be found more in dried bird droppings or environment as opposed to fresh ones because *C. gattii* is closely associated more with trees instead of bird droppings (Torre et al., 2022). The discovery of serotype D in the present study gives a new insight on the genetic movement of *Cryptococcus* as serotype D isolates are usually present in Europe (Casadevall & Perfect, 1998). Next, no *C. gattii* was isolated in this study, despite being present in Malaysia as reported by Tay et al. (2010) years ago. However, the phylogenetic tree in this study is a partial tree with only 16 isolates submitted for sequencing. A full phylogenetic tree with more sample isolates would give a better understanding on the molecular epidemiology of *Cryptococcus* in Malaysia. The chances of all serotypes to be present in Malaysia still warrants further investigation and future works.

5.5 Discussion Summary

In summary, this study provides an update on the status of *Cryptococcus* in Malaysia in terms of prevalence in bird droppings, their molecular epidemiology and their antifungal susceptibility test. Not to mention the difference in prevalence between public places and zoological gardens. Furthermore, this study emphasised the relationship and potential factors for the prevalence of *Cryptococcus* in zoological gardens.

In terms of prevalence, 217 out of 509 bird dropping samples were positive for *Cryptococcus* (42.6%) in the whole of Peninsular Malaysia. The prevalence in public places is at 39.3% (101/257) and in zoological gardens at 46.0% (116/252). Although not statistically significant in difference, both areas were higher than the previous study in Malaysia conducted by Tay et al. (2005) which was only 3.7%.

For the molecular epidemiology of *Cryptococcus*, this study has mapped the partial phylogenetic tree for 16 isolates recovered in Peninsular Malaysia. Nine of these isolates are serotype A (*Cryptococcus neoformans* var. *grubii*) and the remaining seven are serotype D (*Cryptococcus neoformans* var. *neoformans*). Comparing with the previous reports in 2005 and 2010, the isolates found in Malaysia were of the serotype A and serotype B (*Cryptococcus gattii)* (Tay et al., 2005; Tay et al., 2010).

In terms of antifungal susceptibilities, one of the isolates recovered in this study showed intermediate susceptibility to Fluconazole and another, resistant to Itraconazole. In relating to their molecular epidemiology, both of these isolates are serotype D. Whilst mostly associated with environment, there are still chances of serotype D to infect humans as three isolates in the tree were recovered from AIDS patients in Italy and Brazil (Ito-Kuwa et al., 2008). There have been reports of fluconazole resistance globally including in Africa, Cambodia, Spain and Thailand (Bermas & Geddes-McAlister, 2020; Tangwattanachuleeporn et al., 2013). However, none of these studies reported any resistance to Amphotericin B, in line with this current study where all isolates were susceptible to this antifungal. Despite the availability of Amphotericin B in Malaysia, Lee at al. (2024) stated that the regular Amphotericin B is accessible, however, the alternative Amphotericin B called Liposomal Amphotericin B (LAmB) which is less toxic and has a greater efficacy could be used if it was affordable. The limitation of this study however, is that the antifungal susceptibility test used in this study is only a screening test and not a final confirmation because of mainly two factors. One, the disc diffusion test does not provide the MIC values, hence, the right concentration of susceptibility or resistance are not recorded. Second, the isolates obtained in this study are from bird droppings and not actual isolates recovered from patients suffering from cryptococcosis.

Future studies relating to the occurrence of *Cryptococcus* from East Malaysia will update the molecular epidemiology and trend of this fungus in Malaysia as a whole. A complete phylogenetic tree will provide a clearer view of the genetic trend of this fungus as well. To further enhance the epidemiology, isolates should also be obtained from infected individuals be it immunocompromised or healthy. A more specific and impactful antifungal susceptibility tests should be done for their MIC values. Antifungal stewardship programs in veterinary and clinical settings should be considered for future antifungal awareness.

CHAPTER 6
CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

6.1 Conclusion

The objective of this study was to investigate the molecular epidemiology of *C. neoformans* isolated from bird droppings in zoological gardens and public places in Peninsular Malaysia, the environmental and hygienic factors associated with its presence in bird droppings in zoological gardens, their antifungal susceptibility profiles, and the serotypes and phylogenetic relationships of the isolates. This study has accomplished all objectives and hypothesis, as well as addressed the gaps.

This study highlighted a high overall prevalence (42.6%) of *C. neoformans* from both public places and zoological gardens in peninsular Malaysia, indicating a potential public health concern due to the close interaction of human and birds in both environmental settings. While pigeons (Columbidae family) were expected as main carriers of *C. neoformans.* This study also showed high positivity rates in Indian peafowls, budgies and doves. In addition to that, 21 out of 24 bird species were found as carriers, an observation that expands current understanding of the avian hosts associated with this fungus.

Advancing the knowledge of *C. neoformans* occurrence in zoological gardens, this study has established the association between hygiene and environmental factors that significantly contributes to the positivity rate of the fungus. Specifically, the abundance of Columbidae birds and high enclosure densities were identified to be significant predictors of *C. neoformans* occurrence in zoological gardens. This finding emphasizing the importance of proper enclosure factors and type of bird species in minimizing infection risks of *C. neoformans* in this setting.

Antifungal susceptibility testing revealed that most of the isolates were susceptible to Amphotericin B, Itraconazole and Fluconazole. However, two isolates exhibited resistance and intermediate resistance to Itraconazole and Fluconazole, respectively. Both isolates belonging to serotype D. This finding is concerning as it suggests the potential of antifungal resistance for *C. neoformans* in the environment.

Continuous surveillance is therefore essential to detect resistant strains for the public health strategies.

An insight on the serotypes and variations of *Cryptococcus* was obtained through the partial phylogenetic tree. Serotype A (*C. neoformans* var. *grubii*) and serotype D (*C. neoformans* var. *neoformans*) were identified from both public places and zoological gardens. The detection of these serotypes confirms their wide environmental distribution and zoonotic potential. In addition, the phylogenetic analysis demonstrated close similarities between environmental isolates and clinically derived reference strains, suggesting the possibility of bird droppings as source of human infection. In short, the findings of this study highlight the need for integrated control measures to mitigate the risk of cryptococcosis in humans and avian populations.

6.2 Limitations

This study had several limitations that may influence the interpretation of the findings:

a) As this study employed a cross-sectional design, the results reflect only a snapshot of *C. neoformans* prevalence during the sampling timeframe and do not capture trends over time as provided by a longitudinal study design.
b) Second, no samples were collected from zoological gardens in the eastern region of Peninsular Malaysia due to restricted access permissions from the selected zoos and the limited number of zoological gardens in that region. As a result, the samples in this study were primarily collected in the central region, potentially introducing a regional imbalance that may limit the generalizability of the findings.
c) Another sampling limitation is that this study was based on the presence of specific bird species encountered during the sampling period, which may not represent the overall bird diversity across all regions and this could introduce sampling bias. Geographical and environmental differences (e.g., elevation, climate, vegetation types) between states or regions may influence the diversity of the bird species encountered.
d) For objective 2, the evaluation of environmental and hygiene factors that might be associated with *C. neoformans* in zoological gardens was conducted based

on observation and questionnaire-based interview with respective veterinarians and person-in-charge (PICs) (qualitative assessments using categorical variables) only. Quantitative data from standard of procedures (SOPs) such as cleaning frequency logs, exact enclosure measurements and precise bird counts per enclosure were not collected.

e) For objective three, antifungal susceptibility testing was performed using the disc diffusion method which is non-specific and serves only as a preliminary screening tool for antifungal resistance pattern. This method does not provide minimum inhibitory concentration (MIC) values which could be acquired using methods such as CLSI M27 broth microdilution assay which was not employed in this study.
f) Furthermore, Flucytosine was not obtainable in this study due to licensing issues from the vendors.
g) Lastly, only a small number of isolates were selected for sequencing due to financial limitations resulting in partial phylogenetic tree construction and limited insights into full genetic diversity.

Despite these limitations, this study serves as a valuable baseline and offers important insights for future investigations into *C. neoformans* studies in Malaysia.

6.3 Recommendations and Future Studies

In future studies, besides the epidemiology of *Cryptococcus* from bird droppings, samples should be collected from clinical isolates as well. This is to address the gap of *Cryptococcus* status in Malaysia as a whole and not only from a single type of source. Furthermore, clinical isolates recovered from patients have greater significance. as they represent strains actively infecting humans.

This study did not obtain any samples from the zoological gardens from the Eastern region of Peninsular Malaysia. Therefore, the final occurrence of *Cryptococcus* in zoological gardens could be different. Furthermore, the inclusivity of East Malaysia should be considered to fully address the status of *Cryptococcus* in Malaysia as a whole. To add to the significance of sampling locations, droppings from captive birds as pets should be considered as the owner of the pet birds are constantly exposed to the droppings at home.

More isolates should be sequenced to provide a complete phylogenetic tree. This is to give a wider view of the possible genetic variances and mutations of *Cryptococcus* in Malaysia.

A more specific antifungal resistance approach such as the CLSI M27 should be employed in the future to denote the final MIC values of antifungal resistance of *cryptococcus*. This study only employed the disc method for antifungal susceptibility which only acts as a screening test and devoid of the final value.

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APPENDICES

APPENDIX A

UiTM CARE APPROVAL LETTER

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UNIVERSITI TEKNOLOGI MARA

Pejabat
Timbalan Naib Canselor
(Penyelidikan dan Inovasi)

Ref No : 600-UiTMCARE (PT.1/3/1)
Date : 24/2/2023

Dr Azdayanti Muslim (P.I)
Haziq Faisal
Assoc. Prof Dr Vellayan Subramaniam
Prof Dr Yvonne Lim Ai Lian
Faculty of Medicine
Universiti Teknologi MARA
Selangor Branch,
47000 Jalan Hospital, Sungai Buloh,
SELANGOR

Dear Prof/ Assoc. Prof/ Dr/ Mr/ Mdm

The Committee on Animal Research and Ethics (UiTM CARE) reviewed your research proposal entitled below on the 3rd February 2023. The committee has taken note of the application, and advises applicants to obtain consent from the relevant authorities and facilities, and to also ensure their safety during data collection.

Project Title: Molecular epidemiology and antifungal susceptibility test of Cryptococcus neoformans from zoological gardens and public places in Peninsular Malaysia.

Thank you.

Yours truly

PROF DR. HARBINDAR JEET SINGH
Chairperson
Committee on Animal Research and Ethics
Universiti Teknologi MARA (UiTM CARE)

cc: Asst Vice Chancellor (Research & Inovation) TNCPI Office

Please be informed that failure to comply with the conditions may result in withdrawal of the approval for the project

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UiTM di hatiku

APPENDIX B

PERHILITAN PERMIT

IBU PEJABAT
JABATAN PERLINDUNGAN HIDUPAN LIAR DAN
TAMAN NEGARA (PERHILITAN) SEMENANJUNG MALAYSIA
HEADQUARTERS
DEPARTMENT OF WILDLIFE AND NATIONAL PARKS (DWNP)
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Tarikh: 08 Februari 2023

Encik Haziq bin Faisal
33 Jalan Cecawi 6/4
Kota Damansara
47810 Petaling Jaya
SELANGOR

Tuan,

KEPUTUSAN PERMOHONAN MENJALANKAN PENYELIDIKAN

Dengan hormatnya saya diarah merujuk kepada perkara di atas dan keputusan Mesyuarat Jawatankuasa Penyelidikan Jabatan PERHILITAN Bil.01/2023 bertarikh 25/01/2023 adalah berkaitan.

2. Sukacita dimaklumkan bahawa Jabatan **meluluskan** permohonan tuan untuk menjalankan penyelidikan seperti butiran di bawah:

Nama Pemohon	:	**Encik Haziq bin Faisal**
Institusi Pemohon	:	**Insititute of Medical Molecular Biotechnology Universiti Teknologi MARA Sg. Buloh, Selangor**
Tajuk	:	**Molecular epidemiology and Antifungal susceptibility test of *Cryptococcus neoformans* from Zoological Gardens and public places in Peninsular Malaysia**
Lokasi	:	***Seperti di lampiran B***
Tempoh kajian	:	**November 2022 – November 2024**

3. Sehubungan itu, tuan dipohon untuk melakukan beberapa perkara seperti berikut :

a. Rakan Saing & *co-author* Jabatan yang dilantik ialah Dr. Hamidah binti Helman, Pegawai Veterinar (Bahagian Konservasi Ex-Situ);

b. Berkongsi hasil penyelidikan seperti laporan/penerbitan kertas saintifik/tesis dengan Jabatan melalui rakan saing;

APPENDIX C

RESEARCH PAPER

Tropical Biomedicine 42(3): 1-11 (2025)
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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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ARTICLE HISTORY

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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

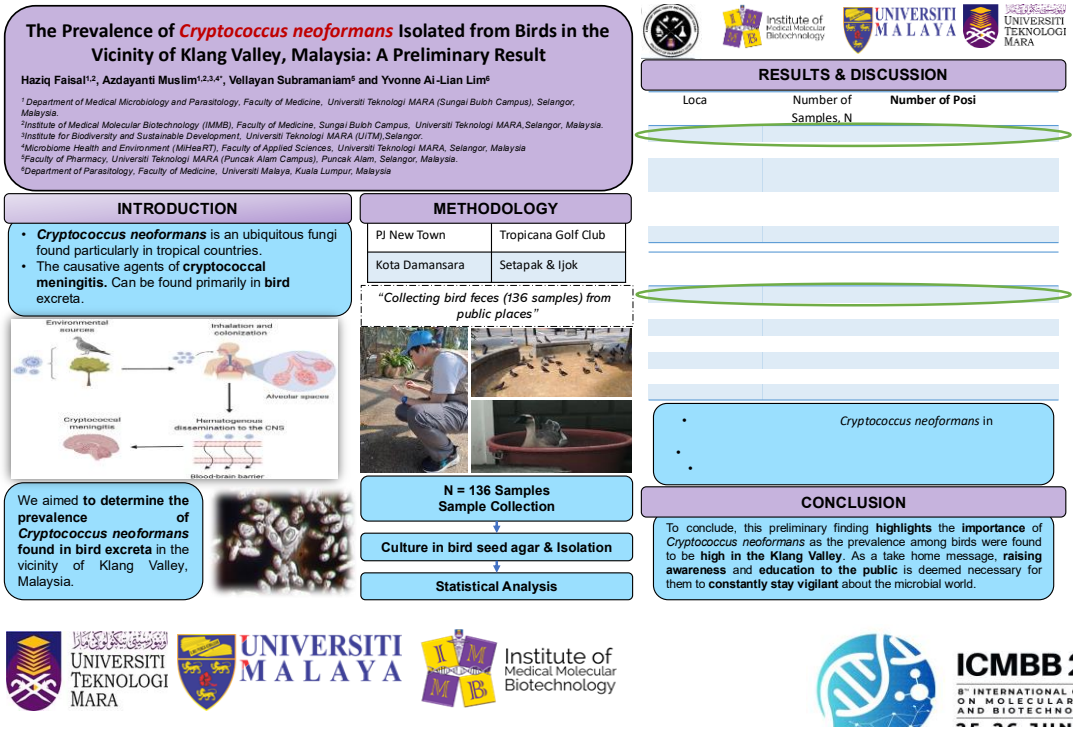
Cryptococcus neoformans is a ubiquitous fungi found particularly in

its impact on public health necessitates urgent research and development initiatives.

In Malaysia, historical clinical reports from 1964 to 1980

APPENDIX D

PRESENTATIONS AT CONFERENCES



APPENDIX E

PICTURES OF BIRDS & ENVIRONMENT IN KL BIRD PARK












APPENDIX F

PICTURES OF BIRDS & ENVIRONMENT IN FARM IN THE CITY

APPENIX G

PICTURES OF BIRDS & ENVIRONMENT IN KULIM BIRD PARK





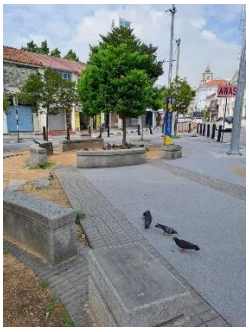



APPENDIX H

PICTURES OF BIRDS & ENVIRONMENT IN ZOO MELAKA

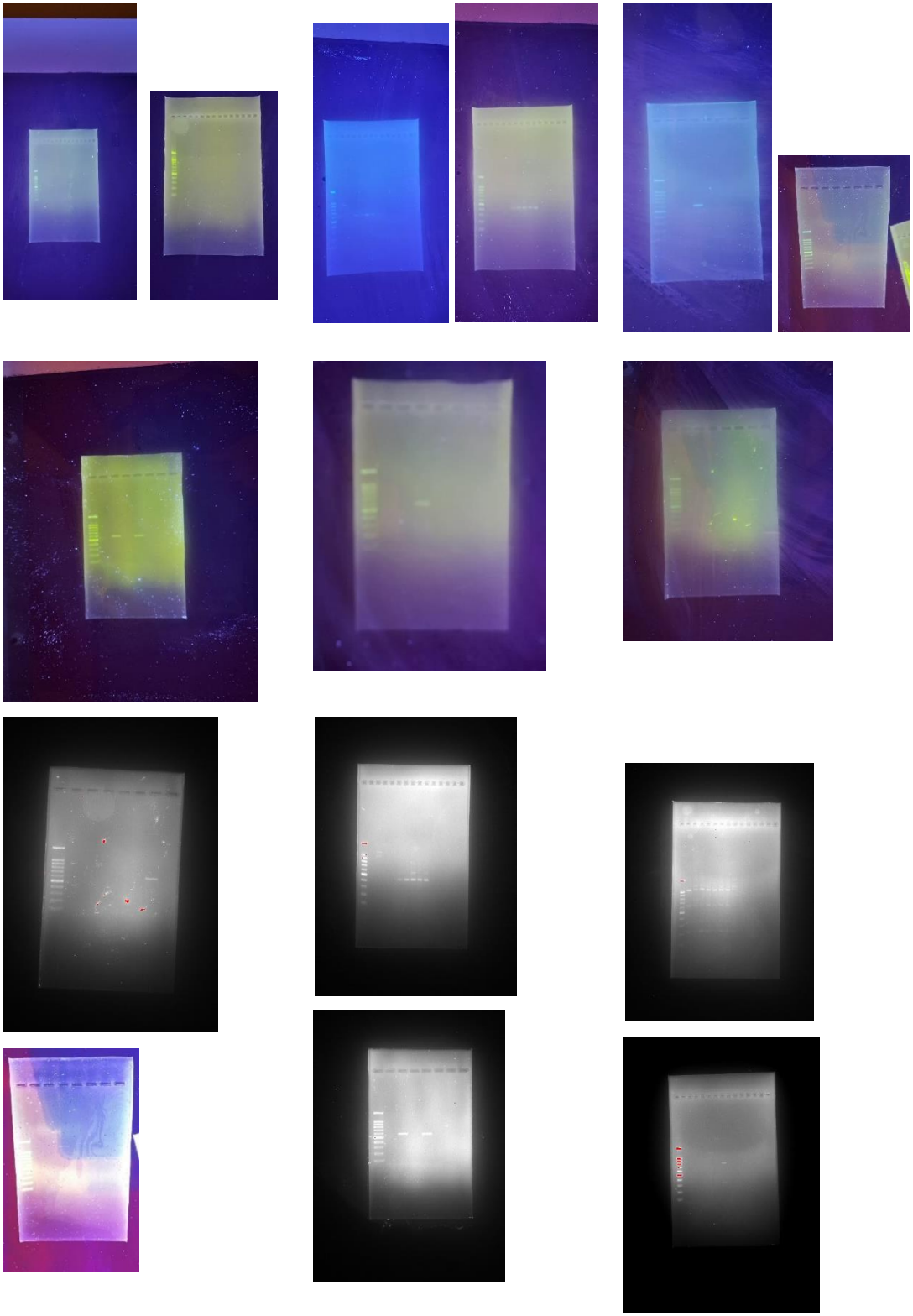
APPENDIX I

PICTURES OF BIRDS & ENVIRONMENT IN PUBLIC PLACES



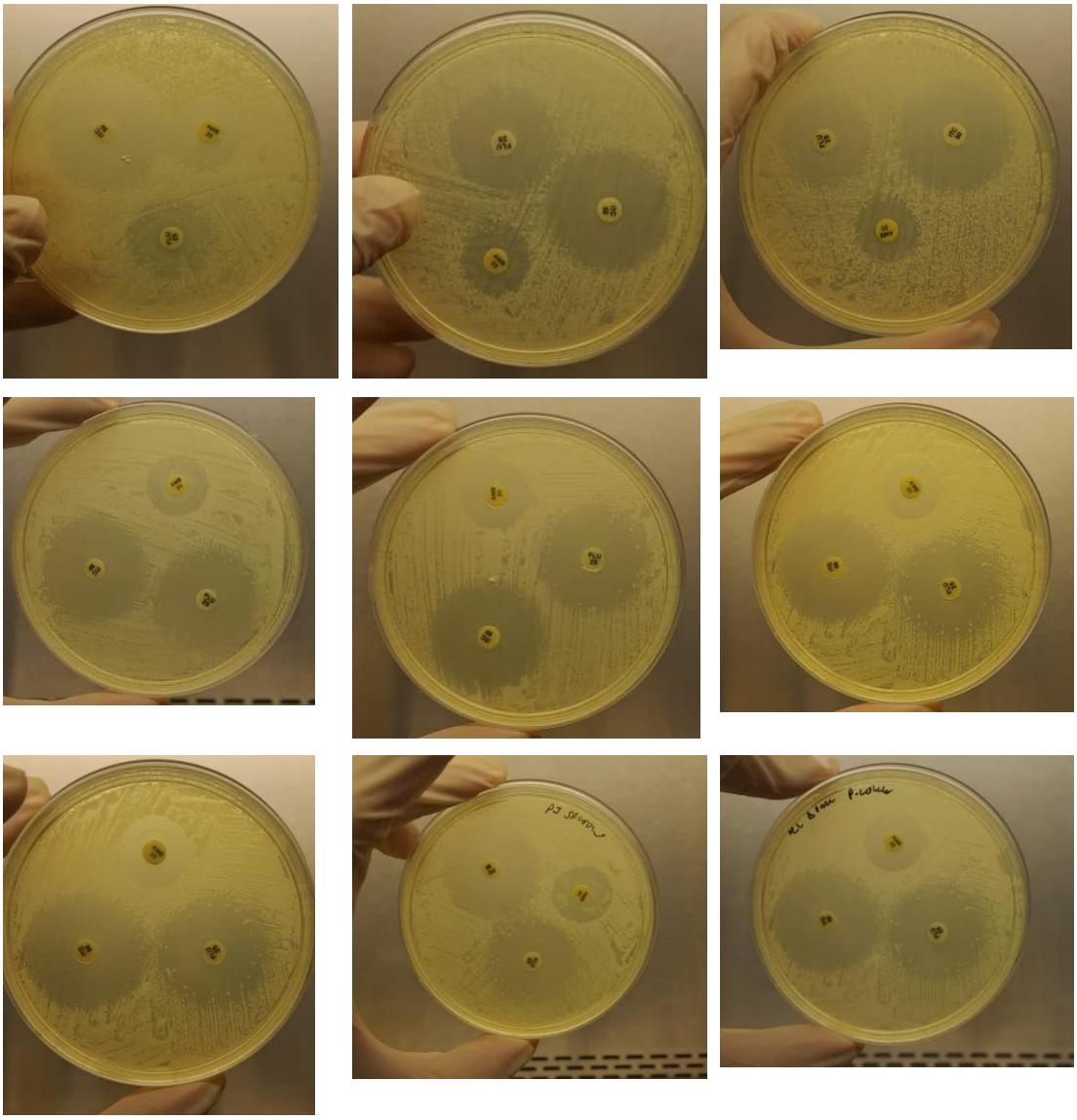
APPENDIX J

VARIOUS PICTURES OF NESTED-PCR ELECRTOPHORESIS



APPENDIX K

VARIOUS PICTURES OF ANTIFUNGAL SUSCEPTIBILITY TESTS



APPENDIX L

GRADIENT TEMPERATURES DURING OPTIMISATION

Runs	Gradient Temperatures							
1	54.5°C	54.7°C	55.1°C	55.8°C	56.7°C	57.4°C	57.8°C	58.0°C
2	54.2°C	54.3°C	54.6°C	54.9°C	55.4°C	55.8°C	56.1°C	56.2°C
3	48.1°C	48.3°C	48.7°C	49.3°C	50.0°C	50.6°C	51.0°C	51.2°C
4	51.0°C	51.1°C	51.4°C	51.8°C	52.3°C	52.6°C	52.9°C	53.0°C
5	54.1°C	54.5°C	55.3°C	56.7°C	58.4°C	59.8°C	60.6°C	61.0°C
6	48.0°C	48.5°C	49.6°C	51.4°C	53.6°C	55.4°C	56.5°C	57.0°C
7	66.2°C	66.3°C	66.4°C	66.5°C	66.8°C	66.9°C	67.0°C	67.1°C
8	65.2°C	65.3°C	65.4°C	65.5°C	65.8°C	65.9°C	66.0°C	66.1°C
9	64.2°C	64.3°C	64.4°C	64.5°C	64.8°C	64.9°C	65.0°C	65.1°C
10	54.0°C	54.5°C	55.4°C	57.0°C	59.0°C	60.5°C	61.5°C	62.0°C
11	65.2°C	65.3°C	65.4°C	65.5°C	65.8°C	65.9°C	66.0°C	66.1°C
12	58.1°C	58.2°C	58.5°C	58.8°C	59.1°C	59.7°C	60.0°C	60.1°C
13	58.3°C	58.4°C	**58.6°C**	58.8°C	59.1°C	59.3°C	59.5°C	60.0°C

APPENDIX M

ASCENSION NUMBERS REFERENCES IN THIS STUDY

Ascension numbers	Strain	Country	Source	References
MG963842.1	GRAKI13HO1 C. neoformans var. neoformans Serotype D	Greece	Plane Tree	Samarasinghe et al., 2017
MG963843.1	GRAKI28HO1 C. neoformans var. neoformans Serotype D	Greece	Olive Tree	Samarasinghe et al., 2017
MG963862.1	GRACP15SO1 C. neoformans var. neoformans Serotype D	Greece	Pine Tree	Samarasinghe et al., 2017
MG963878.1	JEC20 *C. neoformans* var. *neoformans* Serotype D	-	Laboratory Strain	Samarasinghe et al., 2017
MG963879.1	JEC21 *C. neoformans* var. *neoformans* Serotype D	-	Laboratory Strain	Samarasinghe et al., 2017
MG963882.1	NIH-424 *C. neoformans* var. *neoformans* Serotype D	Denmark	Pigeon Nest	Samarasinghe et al., 2017
AB273975.1	CN1525 *C. neoformans* var. *grubii* Serotype A	Italy	AIDS Patient	Ito-Kuwa et al., 2008
AB273979.1	TIMM1313 *C. neoformans* var. *grubii* Serotype A	Japan	Human Patient	Ito-Kuwa et al., 2008
AB273981.1	AS121GL C. neoformans var. grubii Serotype A	Italy	AIDS Patient	Ito-Kuwa et al., 2008
AB273989.1	GHIR1 C. neoformans var. neoformans Serotype D	Italy	AIDS Patient	Ito-Kuwa et al., 2008
AB273992.1	1FM5845 (B-3502) C. neoformans var. neoformans Serotype D	Brazil	AIDS Patient	Ito-Kuwa et al., 2008
MT892612.1	CN1525 *C. neoformans* var. *grubii* Serotype A	Iran	Common Pigeon	Ito-Kuwa et al., 2008
MG963884.1	NIH-430 *C. neoformans*	Denmark	Pigeon Nest	Samarasinghe et al., 2017

	var. *neoformans* Serotype D			
MG963886.1	NIH-432 *C. neoformans* var. *neoformans* Serotype D	Denmark	Pigeon Nest	Samarasinghe et al., 2017
AB273957.1	T1 *C. neoformans* var. *grubii* Serotype A	Thailand	AIDS Patient	Ito-Kuwa et al., 2008
AB273959.1	T4 *C. neoformans* var. *grubii* Serotype A	Thailand	AIDS Patient	Ito-Kuwa et al., 2008
AB273968.1	T49 *C. neoformans* var. *grubii* Serotype A	Thailand	AIDS Patient	Ito-Kuwa et al., 2008
AB273970.1	T73 *C. neoformans* var. *grubii* Serotype A	Thailand	AIDS Patient	Ito-Kuwa et al., 2008

APPENDIX N

ANTIFUNGAL SUSCEPTIBILITIES OF OTHER SAMPLES

Antifungal Susceptibilities of Other Samples

C.liviaPC1	18	21	21
C.liviaPC2	18	20	20
C.liviaPC3	18	25	19
C.liviaPC4	18	23	18
C.liviaPC5	20	21	19
C.liviaPC6	21	24	18
C.liviaPC7	20	25	18
C.liviaPC8	19	25	19
C.liviaPC9	18	20	19
C.liviaPC10	19	20	21
C.liviaPC11	18	21	20
C.liviaPC12	18	20	20
C.liviaPC13	19	21	21
C.liviaPC14	19	20	20
C.liviaPC15	21	25	18
C.liviaPC16	20	23	19
C.liviaPC17	20	21	20
C.liviaPC18	21	24	21
C.liviaPC19	20	25	20
C.liviaPC20	19	25	19
C.liviaPC21	18	20	18
C.liviaPC22	19	20	19
C.liviaPC23	20	21	20
C.liviaPC24	21	20	21
C.liviaPC25	20	21	20
C.liviaPC26	19	20	19
C.liviaPC27	18	25	18
C.liviaPC28	19	23	19
C.liviaPC29	20	21	20
C.liviaPC30	21	24	21
C.liviaPC31	20	25	20
C.liviaPC32	19	25	20
C.liviaPC33	18	20	21
C.liviaPC34	19	20	20
C.liviaPC35	20	21	19
C.liviaPC36	21	20	18
C.liviaPC37	20	20	19
C.liviaPC38	19	19	20
C.liviaPC39	18	18	21
C.liviaPC40	19	19	20
C.liviaPC41	20	20	21
C.liviaPC42	21	21	20
C.liviaPC43	20	20	19
C.liviaPN1	18	21	19
C.liviaPN2	19	20	21

C.liviaPN3	20	25	20
C.liviaPN5	21	21	20
C.liviaPN6	20	24	19
C.liviaPN7	19	25	18
C.liviaPN8	18	25	19
C.liviaPN9	19	20	20
C.liviaPN10	21	20	20
C.liviaPN11	20	21	21
C.liviaPN12	21	20	19
C.liviaPN13	20	19	20
C.liviaPN14	19	20	20
C.liviaPN15	18	20	21
C.liviaPN16	19	21	20
C.liviaPN17	20	20	19
C.liviaPN18	20	19	18
C.liviaPN19	21	18	19
C.liviaPN20	20	19	20
C.liviaPN21	19	20	21
C.liviaPN22	18	21	20
C.liviaPN23	19	19	21
C.liviaPN24	20	20	20
C.liviaPE1	21	21	20
C.liviaPE2	20	20	21
C.liviaPE3	19	20	20
C.liviaPE4	20	21	19
C.liviaPE5	21	20	20
C.liviaPE6	20	19	21
C.liviaPE7	20	20	20
C.liviaPE8	21	21	19
C.liviaPE9	20	20	21
C.liviaPE10	19	21	19
C.liviaPS1	20	20	20
G.strataPC1	21	25	21
G.strataPC2	20	23	20
G.strataPC3	19	21	20
G.strataPC4	21	24	21
G.strataPC5	20	25	20
G.strataPC6	21	25	19
G.strataPC7	20	20	20
S.chinensisPC1	19	20	21
S.chinensisPC2	18	21	20
S.chinensisPC3	19	20	19
P.domesticusPC1	20	20	21
P.domesticusPC2	20	21	20
P.domesticusPC3	21	20	21
P.domesticusPC4	20	19	21
A.tristisPC1	19	18	20
A.tristisPS1	18	19	19
A.tristisPS2	21	20	20
G.gallusPC1	20	20	21
G.gallusPC2	21	21	20
G.gallusPC3	20	20	19
G.gallusPS1	19	25	20

C.splendenPC1	18	18	21
C.liviaZC1	19	21	20
C.liviaZC2	20	20	19
C.liviaZC3	20	21	18
C.liviaZC4	21	20	21
C.liviaZC5	20	20	20
C.liviaZC6	20	21	21
C.liviaZC7	21	20	20
C.liviaZC8	20	19	19
C.liviaZC9	19	20	18
C.liviaZC10	20	21	19
C.liviaZC11	21	20	20
C.liviaZC12	20	19	20
C.liviaZC13	19	21	21
C.liviaZC14	21	20	20
C.liviaZC15	20	21	20
C.liviaZC16	21	20	21
C.liviaZC17	20	21	20
C.liviaZC18	19	20	19
C.liviaZC19	18	25	20
C.liviaZN1	19	23	21
C.liviaZN2	20	21	20
C.nicobaricaZC1	20	24	19
C.nicobaricaZC2	21	25	21
C.nicobaricaZC3	20	25	21
C.nicobaricaZC4	19	20	20
C.nicobaricaZC5	18	20	19
G.gallusZC1	21	21	20
G.gallusZC2	20	20	21
G.gallusZN1	20	20	20
P.cristatusZC1	21	21	19
P.cristatusZC2	20	20	21
P.cristatusZC3	19	19	20
P.cristatusZC4	20	20	21
P.cristatusZC5	21	21	20
P.cristatusZC6	20	20	19
P.cristatusZC7	19	19	18
P.cristatusZC8	21	21	19
P.cristatusZC9	20	20	20
P.cristatusZC10	21	21	20
P.cristatusZC11	20	20	21
P.cristatusZC12	19	21	20
P.cristatusZC13	18	20	19
M.gallopavoZN1	19	25	18
M.gallopavoZN2	20	23	21
A.galericulataZC1	20	21	20
A.galericulataZC2	21	24	20
A.galericulataZC3	20	25	21
A.galericulataZC4	19	25	20
A.galericulataZC5	18	20	19
A.galericulataZC6	21	20	20
A.galericulataZC7	20	21	21
A.galericulataZC8	20	20	20

A.galericulataZC9	21	19	19
A.galericulataZC10	20	20	21
A.galericulataZC11	19	20	20
A.galericulataZC12	20	21	21
A.galericulataZC13	21	20	20
A.galericulataZC14	20	21	19
A.galericulataZC15	19	20	18
A.galericulataZC16	21	25	19
A.platyrhynchosZC1	20	23	20
A.platyrhynchosZC2	21	21	20
A.platyrhynchosZC3	20	24	20
A.platyrhynchosZC4	19	25	20
A.platyrhynchosZC5	18	25	21
P.anacratalusZC1	19	20	20
P.anacratalusZC2	20	20	19
A.solstitalisZC1	20	21	18
A.solstitalisZC2	21	20	21
A.solstitalisZC3	20	21	20
A.solstitalisZC4	19	20	20
A.oratrixZN1	18	19	21
A.oratrixZN2	21	18	20
A.araraunaZS1	20	21	19
A.araraunaZS2	20	20	20
T.moluccanusZC1	21	20	20
T.moluccanusZC2	20	21	21
T.moluccanusZC3	19	20	20
T.moluccanusZC4	20	19	19
T.moluccanusZC5	21	20	18
T.moluccanusZC6	20	20	19
T.moluccanusZC7	19	21	20
T.moluccanusZC8	21	20	20
T.moluccanusZC9	20	19	21
T.moluccanusZC10	21	18	20
M.undulatusZN1	20	19	19
M.undulatusZN2	19	20	21
M.undulatusZN3	18	20	20
M.undulatusZN4	19	21	19
M.undulatusZN5	20	20	18
M.undulatusZN6	20	19	21
M.undulatusZN7	21	21	20
M.undulatusZN8	20	21	20
M.undulatusZN9	19	20	21
M.undulatusZS1	18	25	20
M.undulatusZS2	21	23	19
M.undulatusZS3	20	21	20
M.undulatusZS4	20	24	21
M.undulatusZS5	21	25	20
M.undulatusZS6	20	25	19
M.undulatusZS7	19	20	21
M.undulatusZS8	20	20	20
M.undulatusZS9	21	21	21
M.undulatusZS10	20	20	20
E.roratusZN1	19	21	19

E.roratusZN2	21	20	18
E.roratusZS1	20	25	19
C.galeritaZN1	21	23	20
C.galeritaZS1	20	21	20
C.galeritaZS2	19	24	21
C.galeritaZS3	18	25	22
C.galeritaZS4	19	25	18
C.galeritaZS5	20	20	19
C.galeritaZS6	20	20	18
C.ducorpsiiZS1	21	21	19
C.ducorpsiiZS2	22	20	18

APPENDIX O

ANTIFUNGAL SUSCEPTIBILITY REFERENCES

TABLE OF CONTENTS

Antifungal Disc

ENGLISH

Discs for antifungal susceptibility test of yeasts

DESCRIPTION

Antifungal Disc are paper discs with special features that are impregnate with antimicotics, used for the susceptibility test of yeasts according to the CLSI method. Each antimicotic is available in packages of 50 and 250 discs.

CONTENTS OF THE PACKAGES

Discs in cartridge

The 50-test package contains 1 cartridge with 50 discs packaged in blister with silica gel.
The 250-test package contains 5 cartridges with 50 discs, packaged in blister with silica gel.
Each package also contains a transparent resealable bag.

Discs in canister

The canister contains 250 discs and a desiccant tablet.

METHOD PRINCIPLE

The discs are applied to the surface of a culture medium inoculated with the suspension containing pure colonies of the microorganism under examination. After incubation, the plates are examined, the inhibition halos around each disc are examined and compared with the standard inhibition haloes: in this way the microorganisms are defined as being susceptible, susceptible-dose dependent, resistant or non susceptible to the tested antimicrobial agents.

COMPOSITION

The discs are made of high-quality paper in compliance with WHO and FDA specifications. Each disc is impregnated with a standardised amount of antimicotic.

GATHERING AND KEEPING SAMPLES

The colonies that are to be subjected to the susceptibility test are taken up by culture media that have been previously swabbed with the sample under examination. In the case of mixed colonies the strains must be purified in blood medium or Sabouraud Dextrose Agar before they are swabbed on the plates for the susceptibility test.

TEST PROCEDURE

For procedural details of the test, see the attached bibliography or the microbiology literature. A brief description of the CLSI method follows.

1. Allow discs to equilibrate to room temperature before opening the container (cartridge or canister) in order to minimize the condensation on the discs, which could affect long-term stability.
2. Make a suspension of the test organism to the density of a 0.5 McFarland turbidity standard for *Candida* spp. and 1 Mcfarland for *C. neoformans*.
3. Immerse a sterile buffer in the suspension and squeeze it on the wall of the test tube to eliminate excess liquid. Drag it along the surface of the susceptibility test medium of yeasts (Mueller Hinton agar + glucose + methylene blue).
4. Position the antifungal discs within 15 minutes from inoculation of the plates, pressing them with a sterile pliers on the surface of the agar and within 15 minutes of depositing of the discs leave the plate to incubate at 35 ± 2°C for 24 hours; read at 48 hours only when insufficient growth is observed after 24 hours of incubation.

EVALUATING THE RESULTS

At the end of the incubation period, measure the inhibition halos and compare them with the diameters of the standard CLSI inhibition halos. Pinpoint microcolonies at the zone edge or large colonies within a zone are encountered frequently and should be ignored.

CLINICAL INTERPRETATION

The susceptibility test carried out *in vitro* cannot exactly reproduce *in vivo* conditions. Nevertheless, it shows the effect of the concentration of the antimicotic, which varies in the culture medium in relation to the growth of the microbial population. The final choice of antimicotic to administer to the patient is the responsibility of the clinician who possesses all the information on the patient.

QUALITY CONTROL

Each batch of Antifungal Disc is subjected to precise and thorough checks in compliance with CLSI standards using the following strains: *Candida albicans* ATCC 90028; *Candida parapsilosis* ATCC 22019; *Candida tropicalis* ATCC 750; *Cryptococcus neoformans* ATCC H99.

LIMITS

Diffusion susceptibility tests use an *in vitro* technique and cannot therefore reproduce the extremely complex *in vivo* conditions. Nevertheless, it is a useful and important tool that helps the clinician choose the correct therapy. Many variable factors influence the final result of the diffusion susceptibility test. The main ones are: the culture medium used, impregnation of the discs, inoculation of the medium, temperature, time and incubation atmosphere of the plates, pre-incubation and pre-diffusion conditions, depth of the medium, etc.

PRECAUTIONS

The Antifungal Disc cannot be classified as being hazardous according to current legislation. Antifungal Disc are disposable products. Antifungal Disc are for *in vitro* diagnostic use only and are intended for professional use. They must be used in the laboratory by properly trained operators using approved aseptic and safety methods for pathogenic agents.

STORAGE

The unopened package of Antifungal Disc can be stored in most cases at −20°C to +8°C till the expiry date. Some products have to be stored at −20°C as maximum storage temperature. The recommended temperature limits can be found both on the product envelop and on the box label. Leftover discs from an opened CARTRIDGE need to be stored at 2-8°C for no more than 7 days. The cartridge containing unused discs should be returned into its desiccant envelope and then inserted into the resealable bag. Discs in a CANISTER can be used for up to 2 months from first opening and must be stored at the label storage temperature. Dispose of expired discs.

ELIMINATING USED MATERIAL

After use, Antifungal Disc and the material that comes into contact with the sample must be decontaminated and disposed of in accordance with current laboratory techniques for the decontamination and disposal of potentially infected material.

NOTE: See the **Antifungal Disc Interpretative Criteria and Quality Control** table at the end of this document.

Antifungal Disc Interpretative Criteria and Quality Control.

ANTIFUNGAL AGENT		µg	Species	S ≥	I	SDD	R ≤	CLSI QUALITY CONTROL		Zone Diameter Ranges (mm)
				Zone Diameters (mm)						
Amphotericin B	AMB	20	*C. albicans*	17	15-16		14	*C. albicans*	ATCC 90028	18-27
			C. guilliermondii	13	11-12		10	*C. krusei*	ATCC 6258	19-26
			C. krusei	17	15-16		14	*C. parapsilosis*	ATCC 22019	14-23
			C. parapsilosis	13	11-12		10	*C. tropicalis*	ATCC 750	20-27
			C. neoformans	17	14-16		13	*C. neoformans*	ATCC H99	14-23
Fluconazole	FLU	25	*C. albicans*	17	15-16		13	*C. albicans*	ATCC 90028	28-39
			C. neoformans	17	14-16		13	*C. neoformans*	ATCC H99	22-33
			C. parapsilosis	17	15-16		13	*C. parapsilosis*	ATCC 22019	26-37
			C. tropicalis	17	11-12		13	*C. tropicalis*	ATCC 750	14-24
Posaconazole	POS	5						*C. albicans*	ATCC 90028	24-34
								C. krusei	ATCC 6258	23-31
								C. parapsilosis	ATCC 22019	25-36
								C. tropicalis	ATCC 750	23-33
Itraconazole	ITC	50	*C. albicans*	17	15-16		14	*C. albicans*	ATCC 90028	31-42
			C. neoformans	17	14-16		13	*C. neoformans*	ATCC H99	16-25
			C. parapsilosis	17	15-16		14	*C. krusei*	ATCC 6258	28-37
			C. tropicalis	17	11-12		14	*C. parapsilosis*	ATCC 22019	14-33

S= Susceptible I= Intermediate S-DD = Susceptible-Dose Dependent R = Resistant

Description			CLSI	Ref.*
Amphotericin B	AMB	20 µg		9071
Amphotericin B	AMB	10 µg		9137
Caspofungin	CAS	5 µg	√	9165 •
Clotrimazole	CLO	50 µg		9097
Econazole	ECN	10 µg		9072
Fluconazole	FLU	100 µg		9069
Fluconazole	FLU	25 µg	√	9166
Flucytosine	AFY	1 µg		9073
Flucytosine	AFY	10 µg		9148
Griseofulvin	AGF	10 µg		9074
Itraconazole	ITC	50 µg		9107
Itraconazole	ITC	8 µg		9139
Ketoconazole	KCA	10 µg		9075
Ketoconazole	KCA	15 µg		9140
Miconazole	MCL	10 µg		9077
Nystatin	NY	100 units		9078
Posoconazole	POS	5 µg	√	9167
Voriconazole	VO	1 µg	√	9168

•, to be stored at -20°C

* Packaging

5 cartridges of 50 discs (5x50 discs = 250 discs).
Single cartridges of 50 discs are available: add /1 to the catalogue ref. no. to indicate the relevant item.
Example: ref. 9168/1 indicates Voriconazole 1 µg in one single cartridge of 50 discs.

250 discs in a CANISTER: add /2 to the catalogue ref. no. to indicate the relevant item.
Example: ref. 9168/2 indicates Voriconazole 1 µg in a canister of 250 discs.

The above list of products might be out-of-date, **view the complete range of Antimicrobial Discs on Liofilchem's website**

REFERENCES / BIBLIOGRAFIA / BIBLIOGRAPHIE / REFERENCIAS

1. CLSI. Performance Standards for Antifungal Susceptibility Testing of Yeasts. 2nd ed. CLSI supplement M60. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.
2. CLSI. Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts. 3rd ed. CLSI Guideline M44. Wayne, PA: Clinical and Laboratory Standards Institute; 2018.

TABLE OF SYMBOLS / TABELLA DEI SIMBOLI / SYMBOLE / TABLA DE SÍMBOLOS

LOT	Batch code / Numero di Lotto / Charge / Código de lote
REF	Catalogue number / Numero di catalogo / Artikelnummer / Número de catálogo
IVD	*In vitro* Diagnostic Medical Device / Dispositivo Medico Diagnostico *in vitro* / Für die *in vitro* Diagnostik / Dispositivo médico diagnóstico *in vitro*
	Temperature limitation / Limiti di Temperatura / Temperaturbereich für die Lagerung / Limites de temperatura
	Fragile, handle with care / Fragile, maneggiare con cura / Zerbrechlich, vorsichtig behandeln / Frágil, manipular con cuidado
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LIST OF PUBLICATION

Faisal, H., Muslim, A. Subramaniam, V. & Lim, Y. A. L. (2025). Molecular epidemiology and antifungal susceptibilities of *Cryptococcus neoformans* in bird droppings from zoological gatrdens and public places in Peninsular Malaysia. *Tropical Biomedicine, 42*(3), 1-11.

LIST OF SCIENTIFIC PRESENTATIONS

Faisal, H., Muslim, A. Subramaniam, V. & Lim, Y. A. L. (2025, June 25 -26). *Molecular Identification, Risk Factors, and Antifungal Susceptibility of Cryptococcus neoformans Isolated from Bird Droppings in Peninsular Malaysia: Current Epidemiological Insights* [Conference Session]. 8th International Conference on Molecular Biology and Biotechnology 2025.

Faisal, H., Muslim, A. Subramaniam, V. & Lim, Y. A. L. (2023, November 22 -23). *The Prevalence of Cryptococcus neoformans Isolated from Birds in the Vicinity of Klang Valley, Malaysia: A Preliminary Result* [Conference Session]. The 36th Symposium of the Malaysian Society for Microbiology 2023. Bangi, Selangor, Malaysia.