



Mycological isolation from animal enclosures and environments in National Wildlife Rescue Centre and National Zoo, Malaysia

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ABSTRACT. It is important to provide a baseline of fungal composition in the captive wildlife environment to better understand their role in overall wildlife health. The objectives were to identify species of fungi existing within wildlife animal enclosures and their environment at the National Wildlife Rescue Centre (NWRC) and the National Zoo, Malaysia and to describe their medical and veterinary importance. Samples of air, wall or floor swab, enrichment swab and soil were taken from the animal enclosures, exercise yard and enrichments at NWRC and National Zoo respectively. All samples including those pre-treated samples were plated onto Sabouraud's Dextrose Agar (SDA). Numerous fungi were grown on all sampling SDA plates regardless by either single or multiple growth. Samples of air in both NWRC and National Zoo had the highest growth of *Penicillium* spp. with a prevalence of 31.2% and 83.7% respectively. Samples of swab from the wall, floor and enrichments were predominantly by *Candida* spp. (42.6%) in NWRC and *Penicillium* spp. (41.6%) in the National Zoo. Prevalence of multiple fungi isolated from the soil samples in NWRC were 57.9% and yeast species was the most common in National Zoo with a prevalence of 88.9%. Overall, 29 and 8 isolates were found in both samples from the NWRC and National Zoo with a predominant species of potential zoonotic fungi have been identified in both premises. The expected fungus *Aspergillus* spp. was not isolated in all samples in NWRC. Prevalent fungal species found in this study are known to cause disease in animals and humans as primary pathogen and also as opportunistic pathogens that may also cause infection. Thus, health safety precautions should be considered particularly in dealing with conservation of endangered wildlife species, along with personnel and public involvements.

KEY WORDS: captive, enclosure, fungal, wildlife, zoo

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Fungi are often overlooked as a potential cause of disease since more focus tends to fall on other pathogenic agents such as bacteria or viruses. However, fungal infections are on the rise and becoming an important cause of emerging diseases in wildlife worldwide [24]. In previous work by Seyedmousavi *et al.* [26], they reported that the zoonotic fungi are naturally transmitted between animals and humans, and that they can eventually cause significant public health concerns. Fungi are ubiquitous and reproduce by means of spores which can be inhaled or be transmitted via direct contact with body surfaces, especially the skin. Thus, fungal infections usually begin their pathogenic spread in the lungs or on the skin of animals and humans [23]. These pathogenic fungi impact human health just as greatly as they do with animals, leading to allergic responses, skin and mucosal infections, and even invasive diseases which can be extremely fatal.

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There are many factors that help in promoting the growth of fungus namely the level of humidity, temperature, pH, presence of oxygen and absence of light [14, 27]. Fungi need water to help them to obtain food, as they release enzymes to breakdown complex materials, water will dissolve these materials thus aiding with absorption [16]. They grow at temperatures within the range of 5°C to 35°C with optimum temperatures for growth between 25°C and 30°C [5]. Fungi in general prefer a neutral pH level as more fungal species grow around the neutral pH level compared to a higher or lower pH level [27]. Fungi can regulate the pH in their environment by secreting acid or alkali [33]. Fungi are best grown in dark places since the presence of light may induce stress for the fungal cells, thus inhibiting them from growing [9]. Oxygen is a critical requirement for all eukaryotic organisms as they help in maintaining the overall cellular metabolism of the fungal cells [10].

Captive wild animal settings or zoos are not just places for a wide range of biodiversity and entertainment, but they also harbor potential emerging infectious diseases [4]. It is estimated that 75% of these diseases are zoonotic, and of these, 70% originate in wildlife populations. Among these, fungal pathogens are emerging at an alarming rate worldwide and pose a significant threat to all wildlife species [6]. According to Sutherland *et al.* [29], there has been a marked increase in fatal fungal skin infections in wild snakes (Snake Fungal Diseases) since 2006 which caused a decline in the population of wild snakes in the eastern United States [2]. Cases of dermatophytosis in captive tigers have been reported in several countries including Thailand [17] and the United States of America [30]. Cryptococcosis is described in many wildlife species including wild birds [1, 19]. More recently, *Aspergillus flavus* has been isolated from the lung lesion of a Malayan tiger in the National Wildlife Rescue Centre in Malaysia (Kamarudin, Z., 2018, pers. comm.). According to Hedayati *et al.* [12], *A. flavus* is the second leading cause of invasive aspergillosis after *A. fumigatus* and the fungus is a saprotrophic and pathogenic residing in the soil.

It is important to mention that some fungal diseases with zoonotic potential do not receive enough attention, which in turn would lead to inadequate preventive measures on a global scale. Fungi are extremely ubiquitous in the environment, therefore isolating and identifying those of veterinary and public health importance of protected wildlife could minimize the increase and spread of fungal diseases within wildlife species [18] by implementing specific preventive measures at the enclosures. Since there was lack of studies in Malaysia on this perspective, this study potentially served as an important baseline reference for fungal species distribution that is present in captive animal enclosures. In addition, any human and veterinary important fungi present in the captive wildlife setting can be identified earlier so that safety measure can be taken to prevent infection in animals and humans. Thus, this study was carried out to identify the type of fungi and to compare the presence of medical and veterinary important fungi in the wildlife enclosures and the environment of selected endangered wildlife species in Malaysia.

MATERIALS AND METHODS

Animal and sampling site

The sampling took place at the animal enclosures and exercise yards in National Wildlife Rescue Centre (NWRC), Sungkai, Perak (central south of Peninsular Malaysia), and the National Zoo, Ulu Kelang, Selangor (central of Peninsular Malaysia). The study was subjected to the approval from the Department of Wildlife and National Park Peninsular Malaysia (Ref. no.: JPHL&TN (IP): 100-34/1.24 Jld 12 (46 and 47) and National Zoo Student Research Program by the Education Department, National Zoo. Air samples, floor and enrichment swabs were taken from the captive enclosures or known as their night stall while samples taken from exercise yards or exhibits were the soil and air samples. The enrichment was referred to the animal enrichment provided by the two venues to the animals to enhance them to explore and interact with their environment, these include woods, balls, swing, etc. In NWRC, the captive enclosures and the exercise yards of the Malayan tiger (*Panthera tigris jacksoni*), Malayan sun bear (*Helarctos malayanus*) and clouded leopard (*Neofelis nebulosa*) were selected while Malayan tiger, Malayan sun bear and Orangutan (*Pongo pygmaeus*) enclosures and exhibits were selected in the National Zoo.

Collection and preparation of samples

A passive air sampling technique (gravitational sedimentation sampling) was employed by placing two Sabouraud's Dextrose Agar (SDA) plates at the animal's resting areas in the enclosures and exercise yards (Fig. 1). We used the short contact time 10–15 min based on Hashimoto and Kawakami [11] and our personal experience screening for the presence of fungal in our establishment (laboratory, offices, staff rooms, others). The amount of time is ample to isolate multiple fungi present in the air by carrying the plates around and also leaving the plates at the level of human heights and to limit the risk exposure to the personnel conducting the study. Sterile cotton swabs were used to take samples from the enrichments and floor/wall surface in the animal enclosures (see Fig. 1). The swabs were then placed inside a transport media before streaking onto SDA plate.

About 100 g soil sample from each exercise yard were collected by scraping the top soil using a clean disposable spoon and kept in a clean zip-locked plastic container separately before analysed. Each soil sample collected was mixed thoroughly using a Stuart scientific vortex machine and 10 g were taken and diluted with 10-fold dilution method using sterilized distilled water up until the third dilution. A 10 g of soil was mixed with 90 ml of sterilized distilled water for the dilution. One hundred microlitre (100 μ l) of the third dilution was streaked onto SDA plate, thus the detection of fungi was made from the lowest dilution (10^{-3}). The culture plate was then incubated at temperature between 25°C to 28°C in a dark cabinet and was checked for fungal growth on a weekly basis until week 4. The incubation temperature was established by the selected locations for sampling that had the temperature around 24°C to 32°C as the areas are surrounded by thick tall trees, hence the culture temperature was selected to mimic the condition present at the sampling areas.

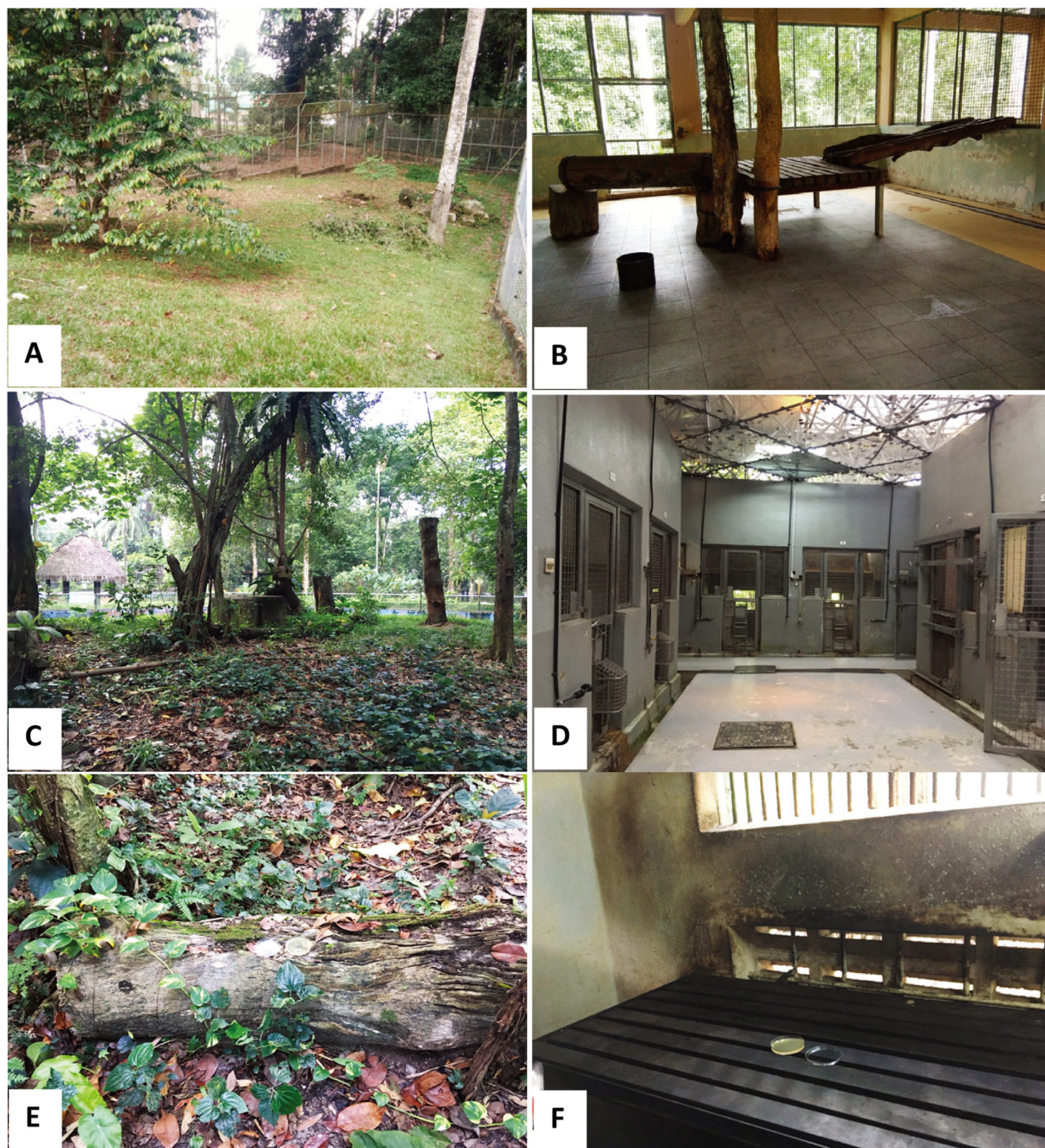


Fig. 1. Sample collection from the animal enclosures and their environment in National Wildlife Rescue Centre (NWRC) and National Zoo. (A) Exercise yard for animal roaming during the daytime in NWRC. (B) Animal enclosure or night quarters for animal resting and keeping animal at night in NWRC. (C) Animal display enclosure in National Zoo. (D) Animal holding area or night quarters in National Zoo. (E–F) Examples of enrichment (tree trunk) in the display enclosure and furniture (wooden platform) in the night quarters.

Identification of fungus

The incubated plates were examined for fungal growth starting on Day 3 post inoculation (PI) and on a weekly basis. The examination involved two stages: macroscopic and microscopic examination [7, 20]. Macroscopic examination consists of description of consistency, ridges and grooves, as well as the color or pigmentation of the colony morphology observed on top and reverse side of SDA plate. Microscopic examination involved the observation of fungal structures such as conidia, conidiophore, hyphae and the presence of other unique characteristics of the species such as chlamydoconidia or macroconidia, wet-mount preparations was used to visualize the fungal structures. Briefly, a clear or colorless clean cellophane tape was touched onto the surface of mycelia and placed onto a clean glass slide and stained with lactophenol cotton blue (LCB). *Candida* spp. was identified using the available commercial identification kit API 20 C AUX (bioMérieux, Durham, NC, USA) only for isolation from the NWRC. Descriptive statistics on the percentages of fungal isolation were performed for each type of sample in each premise.

RESULTS

A total of air samples (n=57; n=25), swab samples (n=38, n=25) and soil (n=10, n=6) were collected from the NWRC and National Zoo, respectively (details in Table 1). Fungi were grown on all sampling SDA plates regardless by either single or multiple growth in both NWRC and National Zoo. In NWRC, 58.6% of fungi were isolated from the animal enclosure and 86.2% of the exercise yard with 86.2%, 36% and 39.9% being isolated from the air samples, swabs and soil respectively. Multiple fungi were isolated from the soil samples in NWRC with a prevalence of 57.9%. *Penicillium* spp. and *Candida guilliermondii* were the most prevalent in NWRC with a prevalence of 76.2 and 70.5%, respectively. *Penicillium* spp. were mostly isolated from the air samples and enrichment swabs in the enclosure with the average prevalence of 31.2% (the average % prevalence of both air samples from the enclosures and exercise yards) and 30%, respectively, while they represented only 3.7% of each isolate from the swabs of the enclosure and soil samples. Samples of swab from the wall, floor and enrichments were predominantly represented by *Candida* spp. (42.6%) in NWRC, especially *C. guilliermondii* (45.3%) and *C. tropicalis* (40%). Another 27 isolates were identified in all samples in NWRC with a prevalence range from 0.9 to 49.5% (see detail in Table 2).

In National Zoo, fungi were isolated from all night quarters and exhibit samples with the prevalence from the air, swabs

Table 1. Distribution of samples taken from the animal environments, enclosures, enrichments and soil in National Wildlife Rescue Centre (NWRC) and National Zoo (NZ)

	Animal enclosure/night quarters (n)			Exercise yard/Display enclosure (n)		Total of samples (n)
	Air	Wall/ floor swab	Enrichment swab	Air	Soil	
NWRC	37	19	19	20	10	105
NZ	15		30	10	5	60

Table 2. Fungal isolates from the animal enclosure (floor, wall and enrichment swab) and environments (air and soil) in the National Wildlife Rescue Centre

Fungal species	Isolated species (n=105)	Prevalence %	Prevalence of each isolated fungi from each samples				
			Enclosure (air) %, n=37	Enclosure (wall & floor) %, n=19	Enclosure (enrichment swab) %, n=19	Exercise yard (air) %, n=20	Exercise yard (soil) %, n=10
<i>Penicillium</i> spp.	80	76.2	38.7	3.7	30.0	23.7	3.7
<i>Candida guilliermondii</i>	74	70.5	5.4	52.7	37.8	4.1	0
<i>Fusarium</i> spp.	52	49.5	26.9	30.7	7.7	17.3	17.3
<i>Madurella grisea</i>	33	31.4	36.4	0	36.4	27.3	0
<i>Paecilomyces</i> spp.	25	23.8	16.0	32.0	16.0	24.0	12.0
<i>Cladosporium</i> spp.	19	18.1	42.1	0	21.1	36.8	0
<i>Fonsecaea</i> spp.	18	17.1	66.7	22.2	0	11.1	0
<i>Acremonium</i> spp.	16	15.2	6.3	0	0	62.5	31.3
<i>Exophiala jeanselmi</i>	15	14.3	26.7	0	0	20.0	53.3
<i>Kloeckera</i> spp.	14	13.3	14.3	57.1	28.6	0	0
<i>Microsporium ferrugineum</i>	10	9.5	80.0	0	0	20.0	0
<i>Rhizopus</i> spp.	9	8.6	0	0	0	0	100.0
<i>Gliocladium</i> spp.	6	5.7	0	0	0	100.0	0
<i>Candida ciferrii</i>	5	4.7	0	0	0	40.0	60.0
<i>Candida tropicalis</i>	5	4.7	0	0	80.0	20.0	0
<i>Geotrichum klebahnii</i>	5	4.7	40.0	0	0	0	60.0
<i>Aspergillus fumigatus</i>	3	2.8	33.3	0	0	66.7	0
<i>Conidiobolus</i> spp.	3	2.8	0	0	0	100.0	0
<i>Exophiala werneckii</i>	3	2.8	0	0	0	0	100.0
<i>Microsporium gypseum</i>	3	2.8	0	0	0	0	100.0
<i>Trichophyton</i> spp.	3	2.8	0	0	0	0	100.0
<i>Aspergillus</i> spp. ^{a)}	2	1.9	0	0	0	100.0	0
<i>Microsporium mycetomi</i>	2	1.9	100.0	0	0	0	0
<i>Onychocola canadensis</i>	2	1.9	0	0	0	100.0	0
<i>Streptomyces</i> spp.	2	1.9	0	0	0	100.0	0
<i>Trichosporon asahii</i>	2	1.9	100.0	0	0	0	0
<i>Trichoderma</i> spp.	2	1.9	0	0	0	100.0	0
<i>Verticillium</i> spp.	2	1.9	0	0	0	100.0	0
<i>Basidiobolus</i> sp.	1	0.9	100.0	0	0	0	0

a) Excluding *Aspergillus fumigatus*

Table 3. Fungal isolates from the animal enclosure (enrichment swab) and environments (air and soil) in the National Zoo

Animal species and samples (n=60)	<i>Penicillium</i> spp. (n) %	<i>Trichophyton</i> spp. (n) %	<i>Conidiobolus coronatus</i> (n) %	<i>Paecilomyces</i> spp. (n) %	<i>Fonsecaea</i> sp. (n) %	<i>Aspergillus</i> spp. (n) %	Yeast (n) %
Orang utan							
Night quarters (air), n=7	(6) 85.7	(2) 28.6	(0) 0	(0) 0	(1) 14.3	(0) 0	(1) 14.3
Exhibit (air), n=6	(4) 66.7	(5) 83.3	(2) 33.3	(0) 0	(0) 0	(0) 0	(2) 33.3
Enrichment (swabs), n=12	(7) 58.3	(3) 25.0	(0) 0	(1) 8.3	(0) 0	(1) 8.3	(5) 41.7
Exhibit (soil), n=3	(0) 0	(1) 33.3	(0) 0	(1) 33.3	(0) 0	(0) 0	(2) 66.7
Malayan tiger							
Night quarters (air), n=4	(4) 100	(1) 25.0	(1) 25.0	(1) 25.0	(0) 0	(2) 50.0	(0) 0
Exhibit (air), n=2	(2) 100	(1) 50.0	(0) 0	(1) 50.0	(0) 0	(0) 0	(0) 0
Enrichment (swabs), n=9	(4) 44.4	(1) 11.1	(0) 0	(2) 22.2	(0) 0	(3) 33.3	(4) 44.4
Exhibit (soil), n=1	(1) 100	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0	(1) 100
Malayan sun bear							
Night quarters (air), n=4	(2) 50.0	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0
Exhibit (air), n=2	(2) 100	(1) 50.0	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0
Enrichment (swabs), n=9	(2) 22.2	(2) 22.2	(0) 0	(0) 0	(0) 0	(4) 44.4	(2) 22.2
Exhibit (soil), n=1	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0	(0) 0	(1) 100
Overall % prevalence of fungi	60.6	27.3	4.8	11.6	1.2	5.7	35.2

of the enrichment and soil of 83.7, 66.7 and 25% respectively. *Penicillium* spp., yeast species and *Trichophyton* spp. were predominant with a prevalence of 60.6, 35.2 and 27.3% respectively. *Penicillium* spp. were mostly isolated from the air samples with a prevalence of 83.7%, while they were also isolated from 41.6 and 33.3% of the swabs of the enrichment and soil samples, respectively. Yeast species was the most commonly isolated in soil samples with a prevalence of 88.9%, while *Trichophyton* spp. were predominant in air samples with 39.5%. Another 5 isolates were identified in samples in National Zoo with prevalence ranging from 1.2 to 11.6%, including isolation of *Aspergillus* spp. (see detail in Table 3).

DISCUSSION

This study was conducted to isolate fungi species that are present in the NWRC and National Zoo wildlife enclosure and environment and to identify other medical and veterinary importance fungus. Because of the recent finding of *A. flavus* in the NWRC, this species was expected to be found, surprisingly, *A. flavus* was not isolated in the present study. In contrast, other *Aspergillus* species, namely *A. fumigatus* and *A. niger*, were detected at a prevalence of 1.9% and 0.9%, respectively. In the National Zoo, the detection of *Aspergillus* spp. occurred in 5.7% of the collected samples. Fungi can easily infect animals and humans as they are ubiquitous, often by inhalation and penetration through un-intact skin. It is reported that previously, *A. flavus* had been isolated from the pulmonary lesions of an incidental finding in the necropsy of a tiger in NWRC, indicating that this fungus might be present in the wildlife enclosures and the environment. *Aspergillus flavus* produces aflatoxin, a very powerful hepatocarcinogenic mycotoxin and are known to cause human and animal infection [12], being more commonly found in the air compared to *A. fumigatus*. *Aspergillus* is a saprophytic mold that is closely associated with agriculture and other human activities that make nutrients available to fungi. Aspergillosis is not contagious, nevertheless when the human is immunocompromised, *Aspergillus* can cause rapid developing acute infection following environmental exposure. Chronic forms of aspergillosis causing respiratory tract infections in wild birds have been reported since at least back in the year 1813 [8]. In general, molds reported in this study are easily transmitted through inhalation of spore-containing air.

According to the result of this study (Tables 2 and 3), the most prevalent fungus isolated from both NWRC and National Zoo wildlife enclosure (floor, wall and enrichment swab) and environment (air and soil) is *Penicillium* spp. In this study, *Penicillium* spp. is the most abundant fungus present in the environment, it is mostly present on the air sample cultured plates from both inside animal enclosure and the exercise yard of the animal. This is most probably due to the spore dispersal carried by the wind into the animal enclosure from the exercise yard (outdoor). The air sample was taken by placing the plate on the ground and *Penicillium* spp. are known to be very abundant in soil. Thus, this can explain the higher number of spores present from the air sample of an exercise yard as the plates are closer to the soil. The factors that might affect the prevalence of fungus species include the ease of spore dispersal as spore can be widely dispersed especially by the wind intensity. In addition, animals can also be the mechanical vectors that help spreading the spores. *Penicillium* spp. are ubiquitous soil fungi and usually regarded as unimportant in terms of pathogenicity in human and animals, for most of its species. One of the *Penicillium* spp., *P. marneffeii*, however, is known to commonly infect immunocompromised individuals [25]. It was only recognized as important when the human immunodeficiency virus (HIV) pandemic occurred in Asia [32] and untreated cases are usually fatal. Infection by *Penicillium* spp. are rare in domestic animals, however animals such as cat have been reported to get infected with *Penicillium* spp. [28]. In addition, the organism's natural habitat is in soil endemic to Southern China and South-East Asia [3, 32]. *Penicillium* is globally recognized as the organism responsible for the production of the Penicillin antibiotic, but little is known that one of its species, *P. marneffeii* is an emerging

pathogenic fungus particularly in immunocompromised patients with HIV infection [32].

Conidiobolus coronatus has been isolated markedly in National Zoo while a small proportion detected in NWRC. This fungus has a worldwide distribution but more often seen in tropical rainforests. It is commonly found in soil and decaying leaves and possess a distinctive morphological feature for identification—a short conidiophore that bears conidia that produces hair-like appendages known as villae. Since the mold is powdery, the infection may result from inhalation of the spores hence lesions found in human and animals usually concentrate at the rhino-facial area. *Conidiobolus coronatus* is known to cause entomophthoromycosis in human, that is usually characterized by nasal cavity tumor due to the aggressive invasiveness of the fungus [31]. *Paecilomyces* spp. are also found worldwide and has always been regarded as contaminants of the air, but for an immunocompromised host, it may lead to fatal events [34].

Fusarium spp. are widely distributed in soil where they are commonly considered as a contaminant. In humans, this fungus is commonly reported to cause infection in both immunocompetent and immune compromised hosts [15]. It can cause superficial infection as well as invasive and disseminated infection. According to Jain *et al.* [15], about 15 *Fusarium* spp. have been identified to cause infections in animals and humans. Example of superficial infection by *Fusarium oxysporum* is onychomycosis in humans while in animals, *Fusarium* spp. infection is uncommon. *Candida guilliermondii* was the second most prevalent fungus identified from NWRC and markedly isolated from the swab samples from the wall, floor and enrichment within the enclosure. This fungus is part of the human skin and mucosal normal flora, and this fungus appeared to be the least virulent amongst *Candida* species [22] where the author classified *Candida* species into 3 virulence group of decreasing pathogenicity and *C. guilliermondii* placed on the third group with the least virulence species. The assumption of high prevalence of this fungus would be the circulation and contact with personnel with the animal and the environment is higher in the NWRC premises. As it is a normal inhabitant of human, the yeast becomes opportunistic pathogen when immunological mechanisms are disturbed and the proliferation of the fungus is higher than normal thus leading to disease formation [21]. In animal, infection with the yeast species has been reported in dogs, in which the normal protective barrier was disturbed [26].

Wildlife populations worldwide are under increasing threat from a variety of processes, ranging from climate change to habitat loss that can lead to a physiological stress response [13]. This has become a concern because captive wildlife is more prone to stress due to their unnatural environments. When stressors act for a prolonged time, or when effects accumulate, it is harmful to the animal especially because these environmental fungi can cause systemic mycoses and can be fatal. Zoos are places where all walks of life visit all year round. Hence, it is important to ensure that proper awareness is displayed at the ticket counter and provide the vulnerable visitors such as unhealthy individuals, children or elderly with basic PPE (e.g. facial mask) prior to entry in addition to hand sanitizer at the exits. Zoo personnel (e.g. keepers, veterinarians, curators and biologists) must also be continually reminded that all husbandry practices should be based on principles which minimize stress to the wildlife.

Even though it is known that fungi are presented ubiquitously in the environment, most of the fungus may or may not be causing disease in humans and animals. Animals may be carriers of certain agents including fungi, therefore if they are translocated from one place to another, it may seed their new environment with agents. Other than being presence in the environment, they need other factors such as presence in extremely abundant amount to eventually cause disease. As for the fungi species found in both NWRC and National Zoo animal enclosure and environment in this study, this preliminary result should consider the animal and public health disease prevention especially to immunocompromised animals and humans. There may not be many fungus species under a genus that may cause disease, therefore it is important to identify fungus up to the species level in order to know appropriate and practical action to be taken when a pathogenic fungal species is present in the environment. Molecular technique is seen as the best technique to be used for fungal identification up to the species level. Besides that, active air sampling technique can also be implanted for air sampling rather than passive air sampling. This technique can help to capture a fungus species that is presence at a very low amount in the environment. In conclusion, prevalent fungal species found in this study are known to cause disease in animals and humans as primary pathogens and also as opportunistic pathogens that may also cause infection, therefore health safety precautions should be emphasized by the management.

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