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Molecular Identification of *Cryptococcus neoformans* Isolates from House Environments of HIV-Infected Patients in an Urban Area, Indonesia: A First Report

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Abstract

Cryptococcus neoformans isolates were previously obtained from pigeon droppings in Jakarta. This study aimed to determine another source of infection and describe the supporting niche of the fungus. The occurrence of *C. neoformans* was evaluated in 110 samples of decaying wood and leaves, tree hollow debris, dust, and bird droppings. Yeasts isolates were collected from 22 houses of HIV-infected patients. The isolates were identified based on culture characteristics, an assimilation test, and URA5 restriction fragment length polymorphism polymerase chain reaction. The spatial analysis was conducted in geographical information system to determine dominant house and environmental factors. Seven of the 120 isolates (5.83%) were identified as *C. neoformans*, corresponding to four (18.2%) houses. All isolates were from house environments of HIV-infected patients with cryptococcal meningitis. Spearman's correlation analysis and McNemar's test revealed a significant association between cryptococcosis in HIV-infected patients and their environment. The clinical and environmental isolates were 100% identical based on molecular techniques, indicating that the patients acquired cryptococcosis from the environment. The spatial analysis revealed that house dust, soil, and leaves were the dominant distribution factors in terms of estimating disease prevalence. This study demonstrates that the house environment is a source of infection for cryptococcosis.

Keywords: cryptococcus neoformans, environment, GIS, HIV-infected patient, spatial analysis, URA5-RFLP PCR

Introduction

Cryptococcus neoformans and *C. gattii* are pathogenic yeasts that cause meningeal cryptococcosis, which is most common in immunocompromised (e.g., HIV-1/AIDS population) patients, with high mortality and morbidity [1]. The source of infection in humans is *Cryptococcus* spores, which are distributed in trees, soil, dust, and the droppings of pigeons and other animals [2, 3]. *C. neoformans* is widely distributed in many regions of the world. *C. neoformans* var. *grubii* is more frequently found in the environment than *C. neoformans* var. *neoformans*. The main habitat of this fungus is soil containing decomposing plant material, decayed wood in tree hollows, and bird droppings. This fungus has been identified in tree hollows of *Syzygium jambolana, Cassia grandis, Senna multijuga*, and *Ficus microcarpa* [4].

The distribution of *C. gattii* is more limited to tropical and subtropical regions, and there is a specific ecological relationship between *Eucalyptus camaldulensis* and *Eucalyptus tereticornis* trees [5]. The ecological niche of a species is the set of particular physical and biological conditions. The concept of ecological niche is defined based

on the differences between fundamental and realized niches. A fundamental niche consists of the total potential area that fulfills all physical and biological requirements of a species. The distribution of a species is determined by various factors, such as dispersal, history, and physical barriers, and this area is described as the realized niche [6]. The most common challenge in determining the geographical distribution and species variations of an organism is a lack of data. Data on *Cryptococcus* in the environment as a source of infection is very rarely reported in Indonesia, and there are only two reports in Jakarta [7, 8].

Indonesia is a tropical country with dry and wet seasons. The epidemiological description, genetic characterization, geographical distribution, and collection of *C. neoformans* samples in Indonesia have not yet been adequately explained, including its relationship with meningeal cryptococcosis. Knowledge about the natural habitat of *C. neoformans* in Indonesia and data sources concerning the house as a habitat or source of infection is also extremely limited. Two previous studies detected *Cryptococcus* in pigeon droppings but did not identify the organism to the species level [7, 8].

Cryptococcus is conventionally identified by studying morphology using Indian ink preparations and culture followed by physiological identification. The species can also be identified by molecular-based methods. The molecular identification technique that is widely used is restriction fragment length polymorphism (RFLP) analysis of the orotidine monophosphate pyrophosphorylase (URA5) and phospholipase (PLB1) genes [9–11].

The prevalence of cryptococcosis caused by the fungus *Cryptococcus spp*. is increasing due to the increasing number of immunocompromised patients with HIV infection and the use of immunosuppressant drugs. Cryptococcosis is not transmitted from person to person. Nature is the source of infection as a natural habitat of the fungus [4, 12, 13]. In this study, we examined the house environment of HIV-infected patients with cryptococcosis. In addition, a spatial analysis was carried out using the geographic information system (GIS) to determine the distribution of the fungus.

Materials and Methods

Ethical clearance. This study was approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia under No. 101/UN2.F1/ ETIKA/II/ 2015. All patients and families were provided explanations and information concerning this study and agreed to participate by signing informed consent.

Study area. The study areas were DKI Jakarta (*Daerah Khusus Ibukota Jakarta*, Jakarta Special District), Depok, Bekasi, and Tangerang (areas surrounding Jakarta). DKI Jakarta comprises a land area of 662 km^2 (256 sq. mi) and a sea area of 6,977 km^2 (2,694 sq. mi), located at the coordinates 6°12′S 106°49′E. The metropolitan area of Jakarta Raya occupies an area of 6,392 km^2 encompassing two provinces neighboring West Java and Banten. The Jabodetabek region comprises three neighboring districts *(Kabupaten)* (the districts of Bekasi, Tangerang, and Bogor) and five adjacent cities (Bogor, Depok, Bekasi, Tangerang, and South Tangerang). The map of the study area showing the spatial distribution of the sampling points is shown in Figure 1.

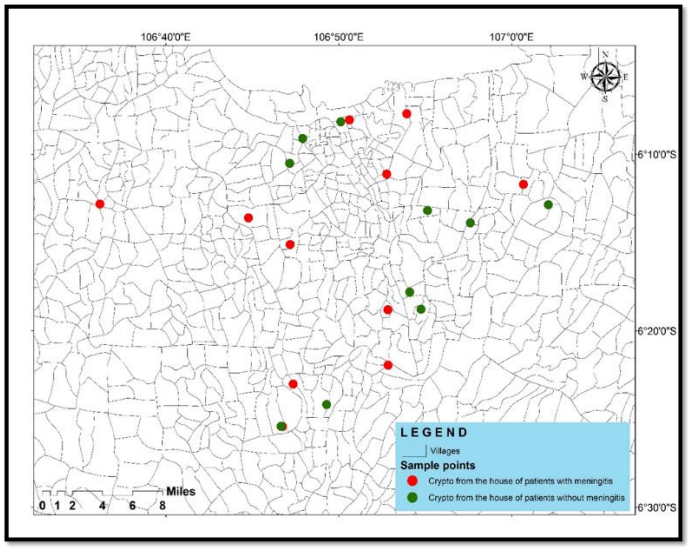

Figure 1. Map Showing the Distribution of the *Cryptococcus* Sampling Points Among HIV Patients with and without Meningitis

Materials. The study sample consisted of debris from tree hollows, the soil under trees consisting of decaying leaves and wood, household dust, and bird droppings collected from the house of HIV-infected patients with meningeal cryptococcosis. Samples were collected from a house of patients with HIV but without cryptococcosis as controls.

Patients' houses. This study was conducted from August 2014 to May 2015 in the environment around the houses of 22 HIV-infected patients, consisting of 11 HIV-infected patients with meningeal cryptococcosis as the group of cases (HIV-CMpos) and 11 HIV-infected patients without meningeal cryptococcosis as the control group (HIV-CMneg). These houses were located in the cities of Jakarta, Tangerang, Depok, and Bekasi, which are urban areas.

Environmental sample collection. Samples of bird droppings, dust, water, soil contaminated with decayed wood and decomposing leaves, and debris in the hollows of various tree species, were collected from the 22 patients' houses and their environments. The house and the environmental conditions have not changed until now.

Five types of samples were collected from each home, resulting in a total of 110 samples from each house (30 samples were from inside the house and 80 samples were from outside). The samples collected from outside the house consisted of a mango tree (26), soil (28), and bird droppings (26). The samples were collected from locations that were protected from direct sunlight. Dust was collected from ventilation openings in the house and places where dust collects, such as the lower parts of cupboards and beds. Environmental samples were obtained from the droppings of pigeons and other birds. Soil samples were collected with sterile tweezers and placed in labeled sterile plastic bags (SPBs). The samples from the tree hollows were collected using a sterile spatula. The samples from clefts in trees were taken by scraping with a sterile scalpel or spatula or were picked with sterile tweezers if the sample was large. Tree cuttings (bark, leaves, and seeds) were collected from the lower parts of trees with sterile tweezers. Environmental samples were examined in the laboratory using the method modified from Kidd *et al.* (2007) [14].

Household dust was collected with a broom or taken with a sterile spatula/anatomic tweezers, or cotton swabs for fine dust. Bird droppings were collected with a sterile spatula. Larger or dry droppings were taken with sterile anatomical tweezers. Water samples were collected in bottles. After the bottle was filled, it was recapped while still under water. Soil samples were taken with a sterile spatula from the tree trunk and roots outward to a distance of 30 cm and a depth of 1–1.5 cm, including decayed leaves and aboveground twigs. All samples were placed in SPBs, and the bags were tightly closed to prevent contamination. Each plastic bag was labeled with the subject's name and address, the season (rainy or dry), and the location of the sample (from inside the house, outside the house, and the name of the tree). The samples were placed in cold boxes containing ice packs and were transported immediately to the laboratory for examination.

Isolation, identification, and genotyping of the *C. neoformans* isolates. One g of sample was placed in a 50-mL conical tube containing 45 mL of sterile distilled water. Large-sized samples were comminuted with sterile scissors before being placed in the tube. Water samples of 50 mL were first homogenized (shaken) before being placed in the conical tubes.

Subsequently, all samples, i.e., bird droppings, dust, soil contaminated with decayed wood or leaves, and debris from tree hollows (except water) were placed in conical tubes and homogenized by vortexing for 1 min, so that the fungus was freed from the wood/leaves and left to stand for 5–10 min. A volume of 1.5 mL was taken from the upper layer of the suspension and plated on Sabouraud dextrose agar medium with antibiotics (SDA+) and without antibiotics (SDA-), as well as bird seed agar (BSA) to a volume of 0.5 mL respectively. Plating was performed in duplicate; one set [SDA (−), SDA (+), BSA] was incubated at a temperature of 30 °C, and another set was incubated at a temperature of 37 °C for 7 days to observe fungal growth, and the cultures were observed daily. Yeast colonies growing in media and suspected of being *C. neoformans* based on macroscopic and microscopic identification using India ink were subcultured in fresh BSA medium and detected as dark brown yeast colonies suspected of being *C. neoformans*. Subsequently, all yeasts were identified based on the macroscopic and microscopic morphology of the colonies and an assimilation test using a commercial kit (Integral Yeasts System Plus, Liofilchem, cat. no. 71822-79822, Roseto degli Abruzzi, Italy). Isolates of *C. neoformans* were further identified based on melanin synthesis in BSA, the presence of a thick capsule on the India ink preparations, and genotyping by URA5 restriction fragment length polymorphism polymerase chain reaction (URA5-RFLP PCR) according to Mora *et al.* (2010) [9]

Spatial analysis. Inverse distance weighted (IDW) interpolation was used for the spatial analysis to determine values using a linearly weighted combination of a set of sample points. The weight is a function of the inverse distance from the sampled point [15]. Surface interpolation assumes that the variable being mapped has less influence with distance from its sampled location. So, a technique was used to create a continuous (or prediction) surface from the sampled point values for the

house and environment to understand the spatial distribution and predict the prevalence of infection.

Results and Discussion

Houses. Twenty-two houses were occupied by 11 HIV-CMpos and 11 HIV-CMneg individuals. *C. neoformans* was isolated from the environments of four houses with HIV-CMpos patients, whereas the remaining seven houses were negative. No *C. neoformans* were found in the 11 houses of the HIV-CMneg patients. The *Cryptococcus*-positive houses were occupied by the patients, their parents, and other family members. All HIV-CMpos subjects were males, 30–40-years-of-age, and most were employed and single. Among them, seven patients came from Jakarta, two patients resided in Depok, one patient resided in Tangerang, and one lived in Bekasi. The characteristics of the HIV-CMneg group were similar to those of the HIV-CMpos group, except that there were also females in the negative group (Table 1). *Cryptococcus* in the house environment is the most feasible source of infection. *Cryptococcus* spores in nature reside in a dehydrated state within a thin capsule, and the smaller size allows the spores to be dispersed by the wind and inhaled into the respiratory tract. The results of the GIS analysis were used to determine the possibility of an HIV-CMneg patient getting an infection from the nearest neighbor if no *Cryptococcus* was found in the house (Figure 1).

Samples. The samples were collected from soil, bird droppings, decaying trees, household dust, and water. The tree species examined were trees commonly found around the houses of Indonesian inhabitants. Bird droppings came from birds kept in cages in the houses and were taken from the accumulated droppings in the cages. Positive samples came from *rambutan* (*Nephelium lappaceum*) and mango *(Mangifera indica)* trees. In addition, positive samples also came from household dust and canary *(Serinus canaria)* droppings (Table 2). The results of this study add a new niche, indicating that *Cryptococcus* can grow on *Syzygium jambolana*, *Cassia grandis*, *Senna multijuga*, *Ficus microcarpa*, *Eucalyptus camaldulensis*, *Eucalyptus tereticornis*, *Syzygium cumini* (water guava trees), almonds (*Prunus dulcis*), golden shower (*Cassia fistula*), spruce, cedar, and maple [4, 16].

Isolation, identification, and genotyping of the *C. neoformans* isolates. All samples were directly streaked on SDA and BSA. Of the 110 environmental samples, 297 isolates were found, consisting of 62.29% yeasts (185 isolates) and 37.71% filamentous fungi (112 isolates). Of the 185 yeast isolates, 16.2% (48 isolates) were obtained from soil containing decaying tree material. Of the 185 yeast isolates, only 120 were recovered and seven colonies were growing as dark brown colonies on BSA medium. A microscopic examination using India ink staining revealed round cells with capsules indicating *C. neoformans*-specific characters (Figure 2). These results agree with previous findings describing that the colonies produce characteristic pigments on Niger seed (*Guizotia abyssinica*) medium, which is brown or dark brown to black. *Cryptococcus* appears as a typical yeast cell on Indian ink staining, which may be round, oval, or elongated, and surrounded by a capsule in the form of a clear zone [17, 18].

Table 1. Socio-demographic Distribution of the HIV-Infected Patients

Characteristic	Group of Cases		Control Group	
	Total (n = 11)	Percent (%)	Total (n = 11)	Percent (%)
Gender				
Male	11	100	4	36.36
Female	0	0	7	63.64
Age				
< 30 years	0	0	1	9.09
30–40 years	11	100	9	81.82
> 40 years	0	0	1	9.09
Employment				
Employed	6	54.6	5	45.45
Unemployed	5	45.4	6	54.55
Marital status				
Married	4	36.3	6	54.55
Single	7	63.64	5	45.45
Area				
Jakarta (five areas)	7	63.7	6	54.55
Outside Jakarta	4	36.3	5	45.45

Table 2. Distribution of the Samples from Houses of HIV-Infected Patients with Cryptococcosis (HIV-CMpos)

Type of sample	Number of samples	Cryptococcus (+)
Household dust	11	2
Tree hollows	12	
*Rambutan**	3	1
Banana	1	0
Jackfruit	1	0
Mango*	3	1
Oil palm	1	0
Matoa (oceanic lychee)	1	0
Star fruit	1	0
Cananga	1	0
Soil with material	9	
*Rambutan**	2	1
Banana	1	0
Jackfruit	1	0
Mango	3	1
Oil palm	1	0
Matoa (oceanic lychee)	1	0
Star fruit	1	0
Cananga	1	0
Bird droppings	7	
Canary*	4	1
Pleci (Zosterops spp.)	1	0
Ciblek (bar-winged Prinia)	1	0
Woodpecker	1	0
Water	11	0

*Types of samples containing *Cryptococcus neoformans*

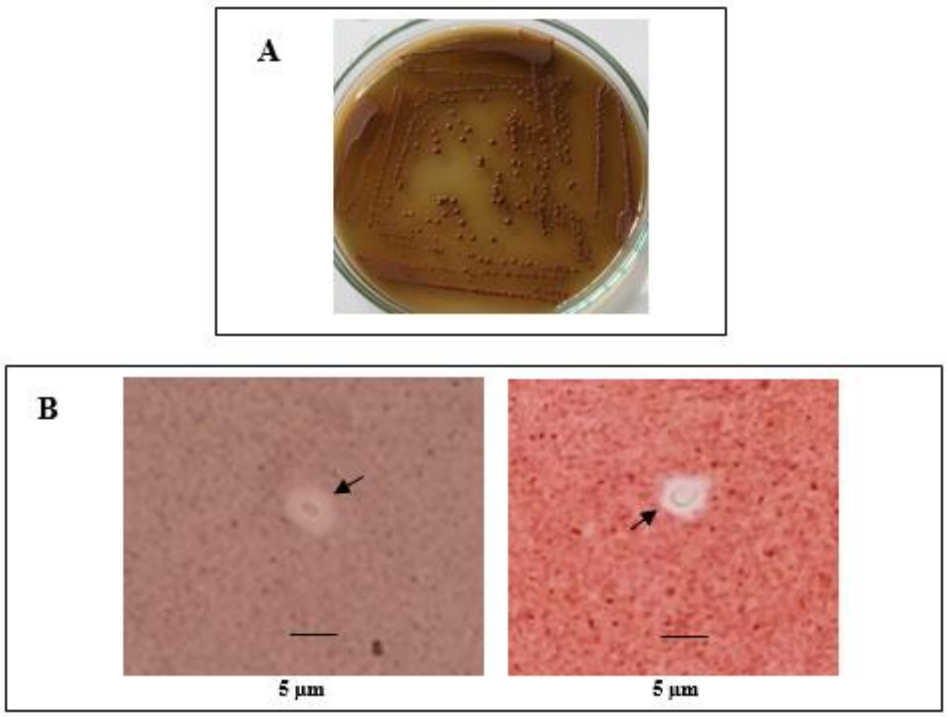

Figure 2. Colonies of *Cryptococcus* on (A) Bird Seed Agar: Colonies were Dark Brown and were Readily Differentiated from other Yeast Colonies, (B) Microscopic Examination with an India Ink Preparation Yielded Positive Results, *Cryptococcus* Spores have Capsules (Arrow)

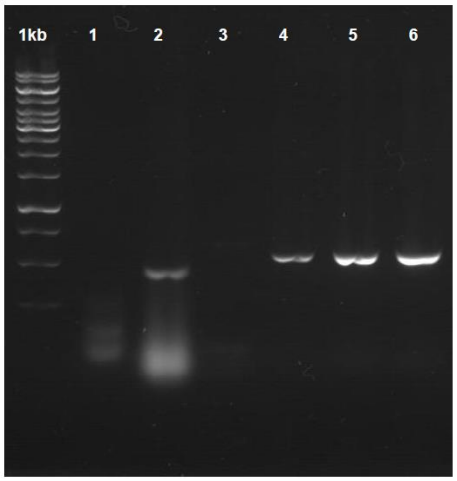

Figure 3. Electrophoresis of URA5 gene Restriction Fragment Length Polymorphism (RFLP) Profiles was Identified by Double Digestion of the Gene using Sau96I and HhaI. [1. LTT; 2. H1; 3. H006; 4. AJ; 5. AY; 6. RB]

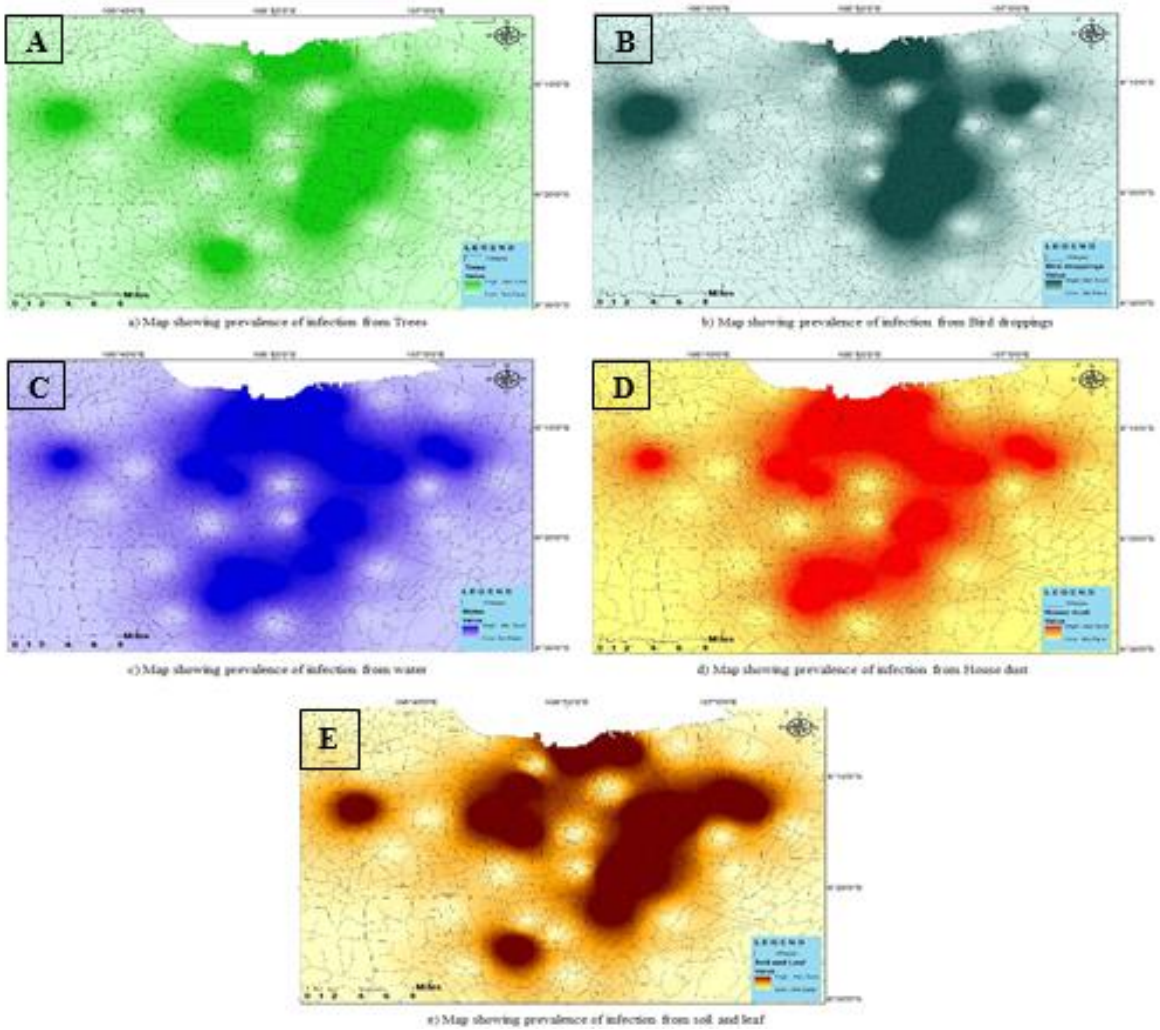

Figure 4. Interpolated Maps Showing the Continuous (or Prediction) Surface from the House and Environmental Sample Points, A. Map Showing the Prevalence of Infection in Trees, B. Map Showing the Prevalence of Infection in Bird Droppings, C. Map Showing the Prevalence of Infection in Water, D. Map Showing the Prevalence of Infection in House Dust, E. Map Showing the Prevalence of Infection from Soil and Leaves

Table 3. Correlation between *C. neoformans* in the Environment with Cryptococcosis

		C. neoformans
HIV-infected patient, Cryptococcosis (+)	r	0.47
	p	p = 0.013 *
	n	22

* Spearman correlation test, $p < 0.05$

Table 4. Association between Cryptococcosis and the Presence of *C. neoformans*

		Environmental *C. neoformans*		**Total**	**p-Value**
		Positive	**Negative**		
HIV Patient	Crypto (+)	4	7	11	0.016*
	Crypto (-)	0	11	11	
Total		4	18	22	

* McNemar's comparative test, $p < 0.05$

All of the isolates were collected from four houses of HIV-CMpos patients. The positive samples came from tree hollows (two isolates), household dust (two isolates), bird droppings (one isolate), and two isolates of *Cryptococcus* were from soil containing decaying wood and leaves (Table 2). Three of these seven isolates were recognized as *C. neoformans* var. *grubii* (serotype A), and the remaining isolates were *Candida* sp., *Cryptococcus uniguttulatus*, *C. albidus*, *C. gastricus*, *C. terreus*, *C. laurentii*, *Rhodotorula rubra*, and *Saccharomyces cerevisiae*. *Cryptococcus neoformans* was not found in the samples from the houses of the HIV-CMneg patients. Three *C. neoformans* environmental isolates were further analyzed by URA5-RFLP PCR and confirmed to be *C. neoformans*. A URA5-RFLP profile comparison among the clinical isolates showed similarity to one environmental isolate. The *C. neoformans* clinical isolate was *C. neoformans* var. *grubii* (serotype A). This result indicated that the environmental isolate was *C. neoformans* var. *grubii* or serotype A (Figure 3).

Spearman's correlation analysis indicated a positive correlation ($r = 0.47$ and $p = 0.013$) between cryptococcosis in HIV-infected patients and their environment (Table 3). The McNemar test was carried out to assess the presence of *C. neoformans* in the house environment, which played a role in causing cryptococcosis. The test revealed a significant relationship between the presence of this fungus in the environment and cryptococcosis ($p = 0.016$) (Table 4). Based on the finding of *C. neoformans* in the environment, a significant difference was found in the proportions between the two groups ($p < 0.05$). Three of the 11 HIV-CMpos house environments (18.2%) were positive for *C. neoformans*. The spatial analysis using IDW of the 110 samples collected from 22 HIV infected patients with and without meningitis was done to evaluate the role of house environmental factors in the geographic distribution and prevalence of the infection. The interpolated maps showing the spatial distribution at the village level of the house environmental factors are shown in Figure 4 (a–e). The prevalence of cryptococcus infection as evident from household sampling from the spatially interpolated maps showed variation in the prevalence of infection at village levels as evident from that household samples from soil, water, house dust, trees, and bird droppings respectively. This spatial information can be effectively used for prioritization and containment of the infection. The prevalence evident from the spatial maps clearly shows the potential to spread the infection to surroundings as shown in Figure 4 (a–e). The bird droppings from the environment indicated less spatial distribution and prevalence, but posed a high risk of spreading the infection due to the dispersion of birds to distant areas (Figure 4 (b)). The results of the spatial interpolation predicted the areas where the infection prevailed. The co-location of neighborhoods identified in the spatial analysis delineated areas with high localized risk. These results could be an effective tool for monitoring and intervention to prevent infection.

In this study, all patients who had meningeal cryptococcosis were males (100%) and were in the reproductive age range of 30–40 years. The report of the Directorate General for Disease Management and Environmental Sanitation (Ditjen PP & PL), Ministry of Health (2013) showed that males comprise 58% of HIV-positive patients with an age range of 25–49 years (72%) [19]. Kaocharoen *et al.* (2013) reported that 68.9% of

HIV-infected patients with cryptococcosis in Thailand are males with a mean age of 37.97 years [5]. This agreed with the epidemiology of HIV-infected patients, most of whom were of reproductive age. Several studies have demonstrated that *Cryptococcus* can readily pass the blood-brain barrier of male than female test animals. This is associated with the presence of high estrogen concentrations and the observation that phagocytosis of *Cryptococcus* by macrophages in females inhibits the growth of *Cryptococcus* more efficiently than in males [20,21].

C. neoformans was isolated from the hollows of mango trees (*M, indica*) that grew in the garden of one of the houses of the HIV-CMpos patients. Tree hollows are ideal habitats because they are protected from sunlight and contain decayed wood, which is important to the life cycle of *C. neoformans* [22]. Swinne *et al.* (1991) hypothesized that wood may be a natural habitat of *C. neoformans,* which was successfully demonstrated in *C. gattii* by Lazera *et al.* (2000), who investigated wood hollows of several tree species, such as *Moquilea tomentosa, Cassia grandis, Ficus* sp., and *Mangifera indica*. However, in that study, *C. neoformans* was successfully isolated from decayed wood in clefts and hollows of *S. jambolana* [4,23]. In the present study, *C. neoformans* was also isolated from soil containing *rambutan* tree (*Nephelium lappaceum*) material in the form of decayed twigs and leaves. In line with the study by Kidd *et al.* (2007), who isolated *C. gattii* from trees, soil, air, and water, Randhawa *et al.* (2008) isolated *C. neoformans* and *C. gattii* from the soil surrounding the roots of *Polyalthia longifolia, Mimusops elengi*, and *Manilkara hexandra* trees [14,24]. This study showed that there was a wide spectrum of host tree species for *C. neoformans* and that decaying wood from various tree species is a potential fungal habitat. *Cryptococcus* was also successfully isolated from canary (*Serinus canaria*) droppings in the house of one HIV-CMpos patient. Pal (1995) isolated *C. neoformans* from a wood canary cage, while Criseo *et al.* (1995) stated that canary droppings are an important source of *C. neoformans* and a potential natural habitat for the growth of *C. neoformans* [25,26]. Leite Jr *et al.* (2012) successfully identified seven species of *Cryptococcus* from dust in three public libraries, whereas Swinne *et al.* (1991) isolated *C. neoformans* in dust from a house of an AIDS patient with cryptococcosis [3,23]. In line with these studies, the present study found *C. neoformans* in household dust samples taken from patients' rooms. This household dust was presumably contaminated with the droppings of birds, which frequented the trees near the patients' houses, but the bird species were unknown. This result indicates that dust is a potential biotope for isolating *C. neoformans*.

These are novel findings because there are no previous reports about environmental isolates associated with HIV-infected patients suffering from cryptococcal meningitis in Indonesia, except for the studies conducted by Susilo (1968) and Wahyuningsih *et al.* (2006) on pigeon droppings [7,8]. However, there are several differences with respect to the bird species investigated, the lack of *Cryptococcus* identification at the species level, and the use of different methods. The results of spatial interpolation of the samples from the house environments of the patients with and without meningitis corroborated our findings for the dominant transfer method for infections. The spatial analysis successfully demonstrated the importance of house environmental factors, which need to be supported and validated by more samples. The key assumption of the interpolation analysis is that the distribution of the value was spatially correlative [27,28]. Most infectious diseases are spatially correlated depending on the environment, meteorological factors, economic level, local customs, and other factors. The similarity between the contaminated factors from the house environment and the exposure to HIV makes the case spatially correlative and the interpolation analysis feasible. Additional samples are needed to evaluate the assumption of the model. The studies we have described did not collect samples from trees, soil, or dust from the surrounding environment. We collected samples from positive trees commonly found in the houses of HIV-infected patients with and without cryptococcal meningitis. More attention must be paid, particularly to HIV/AIDS-infected patients with lowered immunity in areas with a high prevalence of cryptococcal infection. Further studies are necessary to confirm our results. Additional studies will produce more complete data on the environmental sources of cryptococcal infection and their relationship with cryptococcosis.

Conclusion

The results of this study show that the clinical and environmental isolates were 100% identical according to a molecular technique. The results indicate that the patients acquired cryptococcosis from the environment by inhaling the fungal spores. Thus, the house environment of the HIV patients with cryptococcosis was the source of the cryptococcal infection. We concluded that there was a relationship between the clinical isolates and the environment. The spatial analysis using GIS demonstrated cross-scale prediction of various house environmental variables to address the context of spatial distribution and prevalence at a fine scale. Further studies are needed to confirm the results and to provide more complete data on the environmental sources of cryptococcal infection and their relationship with cryptococcosis. HIV-infected patients should be aware of the *C. neoformans* environmental sources of infection found in this study.

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Conflicts of Interest Disclosure

None declared.

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