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Isolation of *Cryptococcus neoformans* from Pigeon Excreta in Nakhon Ratchasima College, Nakhon Ratchasima Province

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Abstract

Cryptococcosis is an important opportunistic fungal disease in human. The disease is caused by *Cryptococcus neoformans* which is commonly found in pigeon droppings. The cryptococcal infection can become life-threatening diseases such as meningitis and pneumonia in human, especially in patient with immunodeficiency. This research aimed to determine the prevalence of *C. neoformans* from pigeon droppings in the area of Nakhon Ratchasima College, Nakhon Ratchasima province. In this study, 84 samples of pigeon droppings were collected from 11 different areas of the college from June to August 2020. The samples were cultured on the sunflower seed medium supplemented with chloramphenicol. The mucoid colonies with brown color formed on the agar media were examined with India ink preparation and Gram stain followed by the colony identification with urease test and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) determination, respectively. One of 84 pigeon droppings (1.2%) was identified as *C. neoformans*. This pigeon dropping isolate was collected from the area that had the accumulation of large amount of pigeon droppings. The results suggested that the accumulating area of pigeon droppings can become a potential reservoir of cryptococcal infection. Our data provide useful knowledge applied to propose the policy for prevention and control of cryptococcal infection in this college.

Keywords: *Cryptococcus neoformans*, pigeon droppings

1. Introduction

Cryptococcosis is a major opportunistic fungal infection in human that is commonly caused by *Cryptococcus neoformans*. (1, 2) The infection is a potentially life-threatening condition that is usually presented with meningoencephalitis in immunocompromised patients, especially human immunodeficiency virus (HIV) infected cases.(3) Recent estimates indicate approximately 1 million cases of cryptococcal meningitis occurring annually around the world. Among them estimated that 625,000 cases die after infection.(4) Although, the incidence of cryptococcal infections has decreased drastically over the last two decades due to advances in the anti-retroviral therapy. However, the incidence of the infection remains significant in population of immunosuppressed patients such as patients with transplantation and cancer.(1, 5-7) The infection of C. neoformans usually occurs through the inhalation of cryptococcal yeast cells from the environment. (1, 2) A potential environmental source of *C. neoformans* is pigeon droppings.(8-10) The accumulations of the dropping could be a reservoir in the spread of pathogenic yeasts into the environment and consequently infect humans. The isolation of *C. neoformans* from pigeon droppings can provide valuable information for ecological and epidemiological studies. Therefore, this study aims to examine the occurrence of *C. neoformans* in pigeon droppings collected from environmental samples gathered at Nakhon Ratchasima College in Nakhon Ratchasima province, Thailand.

2. Materials and Experiment

2.1 Study locations and Sampling

Eighty-four samples of pigeon droppings were collected from 11 different locations inhabited

by pigeons at Nakhon Ratchasima College (Figure 1) in Nakhon Ratchasima province, Thailand from June to August 2020. Each sample containing about 10 grams of pigeon droppings was collected with plastic spatulas, placed in a sterile container, and properly labeled according to site and date. The samples were processed within 24 hours after the collections.

Figure 1 The sample sites collected pigeon droppings from different areas in Nakhon Ratchasima College. (A) Education building (B) Cafeteria (C) Food façade

2.2 Isolation and identification of *Cryptococcus* strains

Approximately one gram of specimen from each site was suspended in 10 mL of sterile saline solution (0.9% NaCl). About 10 µL of the suspension of each sample was cultured on a selective medium containing sunflower seed medium (45 g/L) supplemented with chloramphenicol at a concentration of and 500 mg/L. (11) The culture plate was incubated at 37°C and observed for 2 days. The brown colonies with a mucous appetence were selected and performed subculture to isolate single colonies on Sabouraud's dextrose Agar (SDA) plate. The suspectedly isolated colony was revealed as budding yeast cells on India ink preparation and gram staining. To identify this isolated colony, the urease test was performed on urea agar medium. Finally, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics) was also performed in duplicate to confirm *C. neoformans* according to the manufacturer's recommendation. Briefly, a colony was picked and smeared as a thin film onto a MALDI-TOF MS target plate. Then, 1 µL of 70% formic acid was added to the smeared colony. After air drying, it was covered with 1 µL of Biotyper standard matrix solution, and then allowed to air dry prior to MALDI-TOF MS analysis. Following the manufacturer's recommendation, a score value of ≥ 2.0 is equivocal to a reliable species-level identification.

3. Results

A total of 84 samples of the pigeon droppings collected from 11 areas of Nakhon Ratchasima College were included in this study. Regarding the culture on the selective medium, any colonies but not the suspected one appeared on the medium of 73 samples after the incubation at 37°c. In contrast, no colony appeared on the medium of 11 samples. Based on the observation of colony morphology, from 84 samples, only one sample collected from the 9th floor of education building, that had the accumulation of large amount of pigeon droppings, was suspected as cryptococcal colonies due to the occurring dark brown pigmented colonies (Figure 2A). Under microscopic morphology, the colonies revealed encapsulated- and gram positive budding yeast cells by India ink preparation and gram staining (Figure 2B and 2C). Moreover, the isolate also produced urease (Figure 2D) and was correctly identified as *C. neoformans* by MALDI-TOF MS, with identification scores higher than 2.0 (Table 1). Therefore, our results indicated that the presence of *C. neoformans* was rare in the environment contaminated by pigeon droppings of Nakhon Ratchasima College with a prevalence of 1.2% (Table 2).

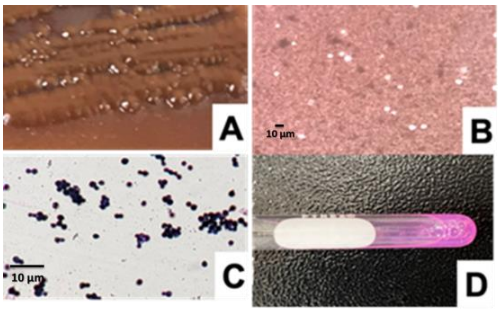

Figure 2 Characteristics of suspected cryptococcal colonies. (A) Colony growth on sunflower seed medium supplemented with chloramphenicol. (B) Encapsulated budding yeast cells seen on India ink preparation. (C) Gram's stain revealed the presence of encapsulated budding yeast cells seen. (D) Urease test showed the positive result.

Table 1 Scores obtained for identification of *Cryptococcus neoformans* using MALDI-TOF MS.

Sample name	Sample ID	Organism (best match)	Score value
A1	Unknown 1	*Cryptococcus neoformans_var_grubii*	2.02
A2	Unknown 2	*Cryptococcus neoformans_var_grubii*	2.08
A3	Control	*Escherichia coli*	2.07

Table 2 Sample sources and prevalence of *Cryptococcus neoformans*

Sample sites	No. of samples	Number of samples detected with *Cryptococcus neoformans* (%)
1st floor of education building	13	0
2nd floor of education building	6	0
3rd floor of education building	6	0
4th floor of education building	5	0
5th floor of education building	4	0
6th floor of education building	5	0
7th floor of education building	3	0
8th floor of education building	2	0
9th floor of education building	5	1 (1.2)
Cafeteria	32	0
Food facade	3	0
Total	84	1 (1.2)

4. Discussion

Since the first report about the ecological relationship of *C. neoformans* in pigeon droppings was revealed by Emmons in 1955 (12), many researchers have continued to confirm this finding in different regions of the world. (10, 13-16) In Thailand, the first recovery of *C. neoformans* from the pigeon droppings was reported in 1968 by Taylor. (17) Subsequently the occurrence of *C. neoformans* in the pigeon droppings was studied in several areas of Thailand. (18-21) These studies implied that pigeon habitats serve as a major saprobic reservoir for cryptococcal infection. Therefore, the survey of *C. neoformans* from pigeon droppings in various regions can provide useful information for ecological and epidemiological studies. Moreover, these data are also valuable for developing effective approaches to prevent and control the infection of *C. neoformans*, especially in immunocompromised patients. Currently, the information of cryptococcal occurrence is unavailable in Nakhon Ratchasima province. To reveal the ecological niche of the pathogen in this region, this study investigated the prevalence of *C. neoformans* in Nakhon Ratchasima College to be representative prevalence data of the province. Based on our results, the prevalence of *C. neoformans* (1.2%) was at a lower percentage than reports for different regions of Thailand with the prevalence range from 9.1 to 16.4%. (19-21) However, the reason for our low prevalence of *C. neoformans* could be affected by various conditions which depend on abiotic components as in previous reports. (22-25) In addition, the immigration of the pigeon might be also associated with the difference in prevalence.(25) According to our study, the results indicated a possible link between pigeon droppings and cryptococcosis with lower risk at Nakhon Ratchasima College and might represent a lower risk for transmission of the infection in Nakhon Ratchasima province. Nevertheless, more concern and strict control measures are required to prevent the spread of the pathogen.

5. Conclusion

Although the occurrence of *C. neoformans* in pigeon droppings is rare in Nakhon Ratchasima College, this study is the first report of *C. neoformans* contaminating in pigeon droppings in this province. The presence of this organism should be carefully concerned, especially about the improvement of college policy for prevention and control strategies to eradicate their reservoir.

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Declaration of conflicting interests

The authors declared that they have no conflicts of interest in the research, authorship, and this article's publication.

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