

Research Article

A New screening medium for environmental recovering of *Cryptococcus neoformans*/*C. gattii* species complex

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ABSTRACT

Cryptococcus neoformans is the most common causative agent of fungal meningitis in human especially AIDS patients. Avian droppings in communitarian areas are considered as an environment reservoir for this organism. In this study, we developed a new screening medium, the red holy basil extract agar, for environmental survey of the yeast. The new medium successfully induced melanin production of *C. neoformans* controls but not in *Candida* spp. For a field test, fifty-three avian droppings were collected from 37 districts around Bangkok, Thailand. Twenty yeast isolates were recovered on the red holy basil extract agar and, subsequently, identified by using conventional biochemical methods and *URA5RFLP*. Nineteen isolates from six districts were identified as *C. neoformans*, VNI molecular type and one isolate from one district were identified as *Cryptococcus* spp. The new screening medium, red holy basil extract agar, is affordable in Thailand as the raw material can be readily found in local market and inexpensive. Moreover, the high prevalence of the VNI molecular type are in line with all previous cryptococcal epidemiological studies in Asia. However, further molecular genotyping antifungal drug susceptibility and strains samplings are warranted to monitor an emergence of any high virulent genotype.

1. INTRODUCTION

Cryptococcus neoformans/*C. gattii* species complex is an encapsulated yeast causing mainly opportunistic fungal infections especially pneumonia and meningitis. This yeast produces infections both in immunocompetent patients without any known underlying disease¹ and in severely immunocompromised hosts such as HIV infection, organ transplantation, malignancy and treatment with corticosteroids or sarcoidosis². Approximately, 1 million AIDS-related cases of cryptococcosis, occur throughout the year globally with more than 600,000 associated deaths, especially within the region and countries where highly active antiretroviral therapy (HAART) is less available³. Patients who recover from disease may have long – term effect related to their infections such as focal neurologic deficits, blindness, deafness, cranial nerve palsies and memory deficits. They may need prolonged therapy for preventions of disease relapses⁴.

C. neoformans is worldwide distribution agent and has been associated with a variety of environmental sources in particular, avian droppings and decaying wood⁵. Several studies have confirmed association between *C. neoformans* and avian droppings, especially pigeon droppings. The yeast can be isolated readily from environments contaminated with bird guano. In Thailand, *C. neoformans* strains were isolated from pigeon guano in Bangkok, Chiang Mai, Ubon Ratchatani and Chonburi provinces⁶⁻⁹. Molecular typing approaches have been used in epidemiology studies. Molecular characterizations were determined by using M13-PCR fingerprinting, multilocus sequence typing (MLST), restriction fragment length polymorphism (RFLP) analysis of the *URA5* gene or *PLB1* gene and multiplex PCRs¹⁰⁻¹³. At present, each specie is divided into four major molecular types. VNI, VNII, VNIII and VNIV molecular types were designated to *C. neoformans* while VGI, VGII, VGIII and VGIV were designated to *C. gattii*. Three new species names were recently proposed for *C. gattii*, namely *C. deuterogattii* (VGII), *C. bacillisporus* (VGIII), *C. tetragattii* (VGIV) and the original *C. gattii* sensu stricto (VGI), but is still debatable^{11-13, 14}. VNI is generally the most common molecular type worldwide among strains from clinical samples and environment. Conventionally, the organism is identified as an encapsulated yeast which is able to produce urease, synthesize melanin, and grow at 37°C.

Melanin production by phenoloxidase activity is a distinctive property of the *Cryptococcus* species. This production is one of the most used criteria for the identification of *C. neoformans*. Previously, environmental surveys used birdseed agar, niger seed agar or modified of Dichloran Rose-Bengal Chloramphenicol (DRBCm) agar with antibiotics as a primary screening plate¹⁵⁻¹⁷. These media allow this organism to grow and produce brown pigments on phenolic substrates. However, they are costly and need complex preparation. Thus, there is a need for an affordable media with simple preparation for such screening task. For example, in 2014, Ajah showed apple leaves and eggplant leaves agar could be used as differential isolation media for the rapid identification of *C. neoformans*¹⁸. Red holy basil (*Ocimum tenuiflorum* L.) is an ingredient and medical herbs belonging to the family Labiatae which contains phenolic compounds^{19,20}. Red holy

basil is a local plant, easy to grow and low-cost. Therefore, we showed here that the red holy basil extract agar can be used as a screening medium for environmental survey for *Cryptococcus* species from wide area in Bangkok, Thailand.

2. MATERIALS and METHODS

2.1. Preparation of red holy basil extract agar

The red holy basil extract agar (5% red holy basil with 2% agar, pH 6.0) were prepared based on the apple leaf agar recipe¹⁸. Freshly red holy basil leaves (*Ocimum tenuiflorum* L.) were washed and cut into little pieces. These pieces were dried in hot air oven at 70°C for 3 hours and then grinded in a domestic blender to fine powder. Fifty grams of the red holy basil powder were mixed with one liter of distilled water and boiled for 30 minutes. After cooled to room temperature, the solution was filtered through gauze and the volume was readjusted to 1 liter with distilled water. The pH of the solution was adjusted to 6.0 and 20 grams of agar-granulated (Difco) was added before autoclaved at 121°C for 15 min. The medium was allowed to cool to 50–55 °C and dispensed into sterile Petri dishes. The medium was kept in dark at 4°C until used.

2.2. Environmental sampling

Avian droppings were collected from 37 districts in Bangkok between March – April 2017. The samples were collected from temples (such as : Tonchai temple in Saun Luang district) and public parks (such as : Suanluang Rama IX in Saun Luang district) with high human and pigeon populations. Approximately, 2-5 g of droppings were put into sterile bags and processed according to a previous study²¹. Briefly, 1 g of dropping were dissolved in 0.85% sterile normal saline, vigorously vortex and stranded for 10 minute. The supernatant was diluted, then spread onto red holy basil extract agar and incubated for 7 days at room temperature and observed for brown yeast colonies as evidences of melanin production every day. The grown brown yeast colonies were then transferred to Sabouraud dextrose agar with 0.5% chloramphenicol (SDA+C agar) and underwent species identification. *Cryptococcus neoformans*/*C. gattii* species complex was identified with positivity of both capsule and urease production based on India ink preparation and urease test, respectively. Melanin production tests were also repeated on caffeic acid ferric

citrate agar (CAFC agar; Himedia laboratories, India)

2.3. Reference strains

Three isolates of *C. neoformans* and two isolates of *Candida albicans* which were used as positive and negative controls, respectively, for melanin production tests on the red holy basil extract agar were provided by microbiology laboratory, faculty of medical technology, Huachiew Chalermprakiet University. The eight major molecular types standard strains were used as references for molecular typing, WM148 (VNI), WM626 (VNII), WM628 (VNIII), WM629 (VNIV), WM179 (VGI), WM178 (VGII), WM175 (VGIII) and WM779 (VGIV)²². The reference strains were recovered from the culture collections of the Molecular Mycology Research Laboratory, Westmead Hospital, Westmead, NSW, Australia.

2.4. DNA extraction

The isolates were grown on Sabouraud dextrose broth (SDB) at room temperature for 24 hours and then centrifuged for 5 min at 2,500 rpm. The packed cell was collected, then washed in 0.9% sterile normal saline twice. Cells were re-suspended with 400 μ l of TE buffer and the cell was mechanically disrupted. The sample was vortexed vigorously for 50 seconds and purified with the phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v) method as described previously¹¹. DNA is kept in minus 20°C until used.

2.5. Species identification and molecular typing

The species identification and major molecular type were determined by *URA5*RFLP analysis as previously described¹³. Briefly, *URA5* gene was amplified with the following primers *URA5* (5' ATGCCTCCCAAGCCCTCGACTCCG3') and *SJ01* (5'TTAAGACCTCTGAACACCGTACTC3'). The amplified products were digested with the restriction enzyme *HhaI* and *Sau96I*. The patterns of the digested PCR products were compared to the reference strains to designate the cryptococcal species and molecular types.

2.6. Statistical and data analysis

Descriptive statistical analysis was calculated by the Microsoft Excel 2010 program (Microsoft company (Thailand), Bangkok, Thailand) and used to present all findings. Percentage was calculated for the environmental sampling and molecular typing results.

3. RESULTS

3.1. The red holy basil extract agar could induce melanin production in the *C. neoformans* controls

All three isolates of *C. neoformans* produced dark colonies on the red holy basil extract agar and CAFC agar at 48-72 hours post-inoculation. On the other hand, the creamy color colonies of the two isolates of *Candida albicans* were not changed at the maximum period of 7-day incubation on the melanin inducing media.

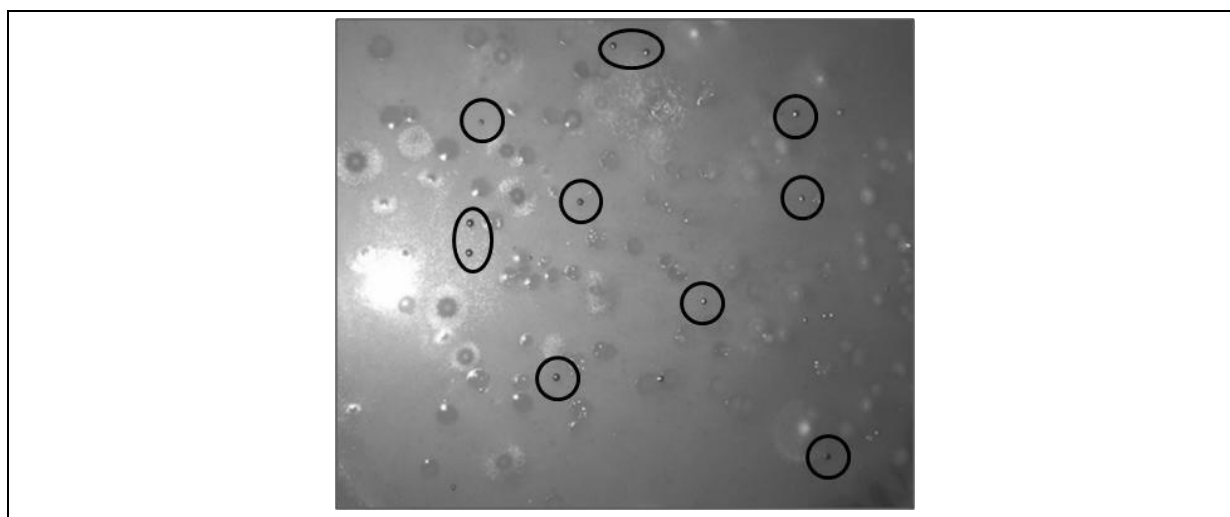


Figure 1. Dark brown colonies (illustration in black circle) growth from avian droppings on red holy basil extract agar.

Table 1. Details on environmental samplings

Districts	Sampling results	Amount (CFU/g)	Species identification	Molecular type
Phra Nakhon	Brown colonies	1.8x10 ²	N/A	N/A
Bang Ruk	NF	0	N/A	N/A
Bang Na	Brown colonies	1x10 ³	<i>C. neoformans</i>	VNI
Bang Kapi	Brown colonies	2x10 ¹	N/A	N/A
Bang Kho Laem	NF	0	N/A	N/A
Bangkok Noi	Brown colonies	3x10 ²	N/A	N/A
Bangkok Yai	NF	0	N/A	N/A
Bueng Kum	Brown colonies	2.2x10 ²	N/A	N/A
Bang Khen	Brown colonies	2.4x10 ³	N/A	N/A
Chom Thong	NF	0	N/A	N/A
Chatuchak	Brown colonies	1.4x10 ³	N/A	N/A
Din Daeng	Brown colonies	2.5x10 ³	<i>C. neoformans</i> / <i>Cryptococcus</i> spp.	VNI ND
Don Mueang	NF	0	N/A	N/A
Dusit	NF	0	N/A	N/A
Huai Khwang	NF	0	N/A	N/A
Khlong Toei	Brown colonies	1x10 ³	<i>C. neoformans</i>	VNI
Khan Na Yao	NF	0	N/A	N/A
Khlong Sam Wa	Brown colonies	2x10 ³	<i>C. neoformans</i>	VNI
Lat Krabang	Brown colonies	1x10 ¹	N/A	N/A
Lat Phrao	NF	0	N/A	N/A
Lak Si	NF	0	N/A	N/A
Min Buri	NF	0	N/A	N/A
Phra Khanong	Brown colonies	3.2x10 ¹	N/A	N/A
Pathum Wan	NF	0	N/A	N/A
Phasi Charoen	Brown colonies	2.8x10 ²	N/A	N/A
Phaya Thai	Brown colonies	1.5x10 ²	N/A	N/A
Prawet	Brown colonies	1x10 ²	<i>C. neoformans</i>	VNI
Pom Prap Sattru	NF	0	N/A	N/A
Ratchathewi	Brown colonies	1.5x10 ²	N/A	N/A
Rat Burana	NF	0	N/A	N/A
Sathon	NF	0	N/A	N/A
Suan Luang	Brown colonies	7x10 ⁴	<i>C. neoformans</i>	VNI
Sai Mai	Brown colonies	6x10 ²	N/A	N/A
Thung Khru	Brown colonies	2.2x10 ²	N/A	N/A
Vadhana	NF	0	N/A	N/A
Wang Thonglang	NF	0	N/A	N/A
Yan Nawa	Brown colonies	1.8x10 ³	N/A	N/A

NF = not found, N/A= no analysis due to no isolates were recovered on SDA+C

3.2. The red holy basil extract agar could be used for environmental samplings

Fifty-three samples from thirty-seven districts of Bangkok were collected. Samples from 20 districts (54.05%) were positive for the dark-brown colonies on the red holy basil extract agar within 3-5 days (Figure 1). The number of colonies were ranged between 1x10¹ to 7x10⁴ CFU/g of samples (Table 1). After culturing on SDA+C agar, twenty isolates from 6 districts grew. Microbial identification by India ink preparations and urease tests revealed that 20 isolates out of the 20 isolates (100%) were identified as *C. neoformans*/*C. gattii* species complex (Table 1). Finally, the *URA5RFLP*

revealed 19 strains (95%) were *C. neoformans* molecular type VNI (Figure 2, Table 1) whereas 1 isolate was *Cryptococcus* species.

4. DISCUSSION

Melanin-inducing media for *Cryptococcus* has already been well established. However, preparations of these media are complicated, need special ingredients and can be costly, especially for developing countries. Our red holy basil extract agar is simple to be prepared. The key ingredient, red holy basil, is readily available in any local market in Thailand. Our quality test verified that the red holy basil extract agar is comparable to the standard CAFC

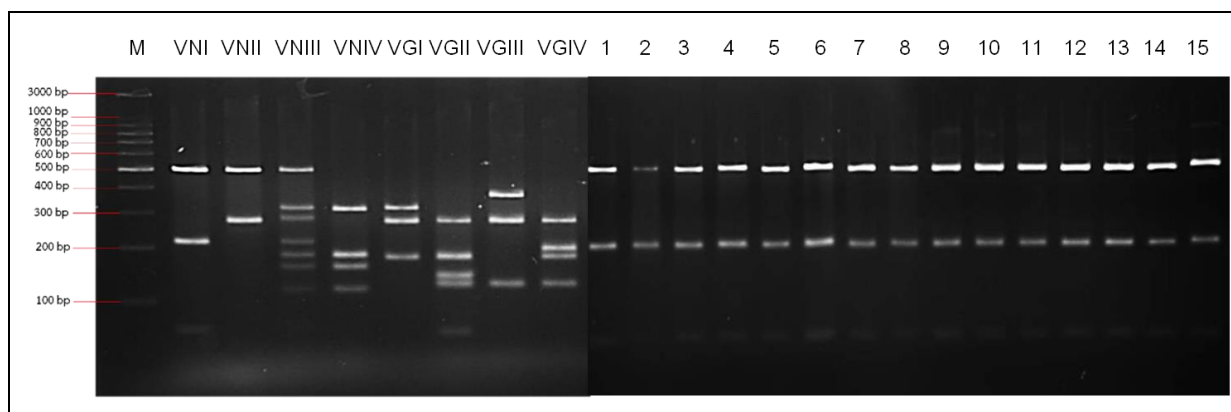


Figure 2. *URA5* RFLP patterns of standard strains VNI – VNIV and VGI – VGIV. No. 1-15 were samples of *URA5*RFLP patterns of isolates in this study. M; 100 bp. Ladder (Biotechrabbit, Germany).

agar in term of melanin induction. Moreover, environmental samples typically contaminated with other environmental bacteria and fungi, adding of anti-microbial drugs is needed which increase cost of the media further^{15, 23-25}. This is a problem of the apple leaves agar and eggplant leaves agar^{18, 26} which have never been tested for cryptococcal environmental samplings. However, our red holy basil extract agar could be efficiently used for such tasks without any need for the drug adding.

Many studies showed isolations of *C. neoformans* from environment in many parts of Thailand^{7, 27-29}. The highly populated area, such as temples and public parks, with high amount of avian droppings pose risk for people with immune-compromising conditions. These people can inhale cryptococcal spores entering the lung and, eventually, causes fungal meningitis. Therefore, area of high concentration *Cryptococcus* has to be precisely identified so that a warning for people with immune-compromising conditions can be set up. However, a previous environmental study⁷ surveyed only in Khlong Toei district causing a limit in its application. Thus, our wide-province survey was conducted and revealed specifically six districts that consider high risk area for people with immune-compromising conditions namely, Suan Luang, Din Daeng, Khlong Sam Wa, Bang Na, Khlong Toei and Prawet, respectively. Notably, Khlong Toei district was also contaminated with the organism similar to the previous survey⁷. This suggests that the yeast can stay in its ecological niche for a long time.

Finally, the molecular typing of *C. neoformans* using *URA5*RFLP analysis showed all strains were VNI molecular type. This molecular type was considered the most common

genotype in Asia and worldwide including in Thailand^{6, 9, 13, 27, 29-34}. However, more detailed molecular typing methods, especially multilocus sequence typing (MLST), showed large genetic variations among VNI molecular type especially the ST5 genotype which causes diseases mainly in apparent normal people.

In conclusion, *C. neoformans* produces brown colonies whereas *C. albicans* maintained normal morphology and did not produce pigment on red holy basil extract agar. Therefore, the red holy basil extract agar was developed as an valuable tool for screening *C. neoformans* from avian droppings based on melanin production. Further molecular genotypings, antifungal drug susceptibility and strains samplings are warranted to monitor an emergence of any high virulent genotype and ongoing with an attempt to find other plants native to our country that could contribute to such survey.

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Conflict of interest

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