CHAPTER IV

MATERIALS AND METHODS

Methods

1. Sample Size Estimation and Cluster Sampling

The pilot study was performed between March – May 2003 by surveying in some areas in Bangkok. After fifty samples of pigeon droppings from 5 regions were collected and cultured for *Cryptococcus neoformans*, the result showed that 4 samples out of the 50 samples were able to isolate this yeast. So the positive ratio of 8 (8%) was used to calculate the sample size in this study (6, 48, 50, 51).

$$N = Z^2_{\alpha/2} PQ / d^2$$

Where, Z= level of confidence = 1.96 (95% level of confidence)

N = sample size

P = proportion of positive or pilot study= 0.08

Q = 1 – P= 0.92

d = maximum permissible error= 0.03 (3%)

After calculation by this equation, the sample size for this study was 314 samples. Cluster sampling technique was performed. Twenty-one districts were randomed from 50 districts of Bangkok. Sixteen pigeon droppings were random collected in each district (totally 336 samples were studied). Pigeon droppings were random collected 2 locations at least in each district, and not more than 10 samples per location were taken to represent the sample in each district.

2. Sample Collection and Transportation

One gram, approximately, of pigeon droppings were picked up by sterile scalpel and kept in zip-lock plastic bags. The samples were stored in an ice box (0-4 ^{o}C) during the transportation, (4 - 5 hours).

3. Material Processing (9, 48)

One gram of pigeon droppings was suspended in a test tube containing 9 ml of sterile physiological saline (0.85% Normal saline). The suspension was vigorously shaken for 30 min and followed by stand at room temperature for 45 min. Each 0.1 ml aliquot of the 10 fold diluted (1:10) and undiluted supernatants were spreaded onto the Sabouraud dextrose agar with chloramphenicol plate (2 plates/sample) and differential medium, caffeic acid agar plate (2 plates/sample). The plates were incubated at 37 ^{o}C for 2-7 days.

4. Identification of *Cryptococcus neoformans* (*C. neoformans*) (27)

Randomized smooth mucoid colonies ($\leq$5 colonies/plate) from Sabouraud dextrose agar with chloramphenicol plates and dark-brown colonies from caffeic acid agar ($\leq$ 5 colonies/plate) were examined under microscopy using India-ink preparation for globose or oval encapsulated yeasts. The suspected encapsulated yeast isolates were tested for the urease production to confirm the genus level and were verified the species of *C. neoformans* by observing the function of phenol oxidase enzyme on caffeic acid agar, resulting as dark brown color of melanin pigment (9, 40, 48).

4.1 Urease Test

One loop of suspected cultures was streaked on the surface of urea agar and incubated at 37 ^{o}C for 24 hr. *C. neoformans* can produce urease enzyme and change the agar color from orange color to red-purple color (6, 48)

4.2 Phenol Oxidase Test

One loop of suspected cultures was streaked on the surface of caffeic agar plate. The plates were incubated at 37 ^{o}C for 7-14 days. *C. neoformans* produces

phenol oxidase, which oxidizes the caffeic acid into melanin. As a result, *C. neoformans* is selectively indicated by the dark-brown colonies (6, 48).

4.3 Canavanin glycine bromothymol blue reaction(1, 48, 49)

The variety of genus *Cryptococcus* was revealed by observing the reaction on Canavanin Glycine Bromothymol Blue (CGB) medium. To perform the test, one loop of the organisms was streaked on the CGB medium and incubated at 37 °C (or 30 °C) for 5 days. *C. neoformans* var *neoformans* provides a negative CGB reaction, resulting the changing of the color medium from yellow color to greenish yellow while *C. neoformans* var. *gattii* turns the medium into blue within 5 days.

5. The genetic diversity study

To delineate the genetic diversity of the *C. neoformans* in this study, rapid amplified polymorphic DNA using M13 primer to amplify genomic DNA was employed.

5.1 Genomic DNA Extraction by Wizard™ Genomic DNA Purification Kits (Promega, U.S.A)

The extraction method was followed the procedure of the Wizard genomic DNA purification kits (Promega, USA). In detail, the overnight culture of *C. neoformans* in 8 ml Sabouraud dextrose agar at 30°C on rotary shaker, was collected and washed 3 times in distilled water by centrifugation and then resuspended in 300 µl cell lysis buffer. More than 80 – 90% spheroplasts of these cells were prepared by vigorously shake in glass beads on vortex mixer (Scientific, USA). Then the mixture was centrifuged at 5,000 rpm for 0.5 min at 4 °C. The supernatant was transferred to a new 1.5 ml eppendorf tube and centrifuged at 13,000 rpm at 4 °C for 5 min. After the supernatant was removed, 300 µl of nuclei lysis buffer was added to the sample and mixed. Protein precipitate buffer was added to the suspension at 100 µl, mixed, and centrifuged and centrifuged at 13,000 rpm at 4 °C for 5 min. The supernatant was transferred to a new tube, 500 µl of isopropanol was added, and the suspension was incubated at -20 °C for 30 min. The suspension was then decanted; the extracted

DNA was rinsed with 70% ethanol, dried at room temperature, and dissolved in 50 µl of sterile distilled water.

5.2 RAPD Analysis (16)

The minisatellite-specific core sequence of the wild type phage M13 (5'GAGGGTGGCGGTTCT3') was used as a single primer in the PCR. The amplification reactions were performed in a volume of 50 µl containing 25 ng high-molecular-weight genomic DNA, 10 mM Tris-HCl, pH8.3, 50 mM KCl, 0.2 mM each of the dATP, dCTP, dGTP, and dTTP, 3 mM magnesium acetate, 30 ng primer, and 2.5 U of Taq polymerase (Promega, USA). PCR was performed for 35 cycles in Hybrid Express (Hybaid, USA) with 20 s of denaturation at 94 °C, 1 min of annealing at 50 °C, and 20 s of extension at 72 °C, followed by a final extension cycle for 6 min at 72 °C. Amplification products were separated by electrophoresis on 1.4% agarose gels (stained with ethidium bromide, 10 mg/ml stock) in 1X Tris-borate-EDTA (TBE) buffer at 60 V for 14 cm, and visualized under UV light. Molecular types (VNI-VNIV and VGI-VGIV) were assigned, according to the major bands in the patterns.

5.3. Data Analysis

Each individual RAPD patterns was compared to those of the standard molecular type strains (provided by Dr. W. Meyer, University of Sydney, Australia).

WM 148 *C.neoformans* var. *grubii* VNI, serotype A, Australia

WM 626 *C.neoformans* var. *grubii* VNII, serotype A, Australia

WM 628 *C.neoformans* var. *grubii*/var. *neoformans* hybrid VNIII, serotype AD, Australia

WM 629 *C.neoformans* var. *neoformans*VNIV, serotype D, Australia

The computer program, Quality one, version 4 (Biorad, USA) was used to determine the profiles.