# CHAPTER 4

# MATERIALS AND METHODS

## 4.1 Experimental design

An overview of the experimental plan was used to detect *Cryptococcus neoformans* and *Histoplasma capsulatum* in soil contaminated with avian and bat droppings by nested-PCR (Figure 4).

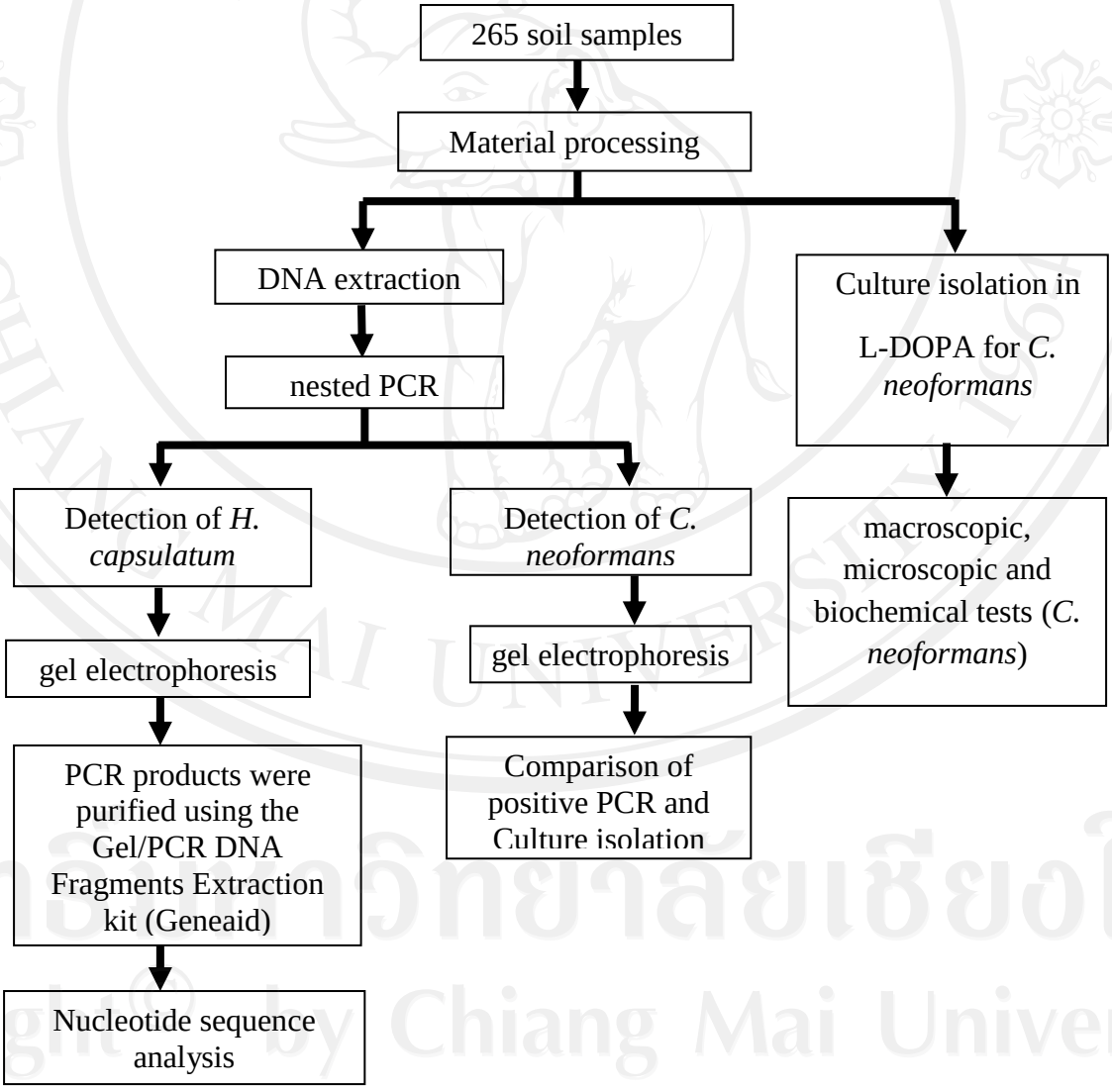


**Figure 4** Schematic representation of the detection of *C. neoformans* and *H.capsulatum* in soi1 contaminated with avian and bat droppings by nested PCR

**4.2 Sample collection**

A total of 265 samples were collected in Chiang Mai, Nakornsawan, Lampang and Maehongson provinces during 2009-2010 as shown in Table 1

**Table 1** List of 265 soil contaminated with avian and bat dropping samples in this study

| Code | Source | location |
|---|---|---|
| CD1/1 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD1/2 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD1/3 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD1/4 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD2/1 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD2/2 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD2/3 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD2/4 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD3/1 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD3/2 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD3/3 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD3/4 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD3/5 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD4/1 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD4/2 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD4/3 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD4/4 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD4/5 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD4/6 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD5/1 | bat dropping | Chiang Dao Cave, Chiang Mai |
| CD5/2 | bat dropping | Chiang Dao Cave, Chiang Mai |
| PD1/1 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD1/2 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD1/3 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD1/4 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD1/5 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD1/6 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD1/7 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD1/8 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD1/9 | bat dropping | Pha Dang Cave, Chiang Mai |

**Table 1** (Continued)

| | | |
|---|---|---|
| PD2/1 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD 2/2 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD 2/3 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD 2/4 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD 3/1 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD 3/2 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD 3/3 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD 3/4 | bat dropping | Pha Dang Cave, Chiang Mai |
| PD 3/5 | bat dropping | Pha Dang Cave, Chiang Mai |
| CK1 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK2 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK3 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK4 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK5 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK6 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK7 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK8 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK9 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK10 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK11 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK12 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK13 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK14 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK15 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK16 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK17 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK18 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK19 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK20 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK21 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK22 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK23 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK24 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK25 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK26 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK27 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK28 | Chicken dropping | Sanpeeseu farm, Chiang Mai |

**Table 1** (Continued)

| | | |
|---|---|---|
| CK29 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK30 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK31 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK32 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK33 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK34 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK35 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK36 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK37 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK38 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK39 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK40 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK41 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK42 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK43 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK44 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK45 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK46 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK47 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK48 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK49 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK50 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK51 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK52 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK53 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK54 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK55 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK56 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK57 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK58 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK59 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK60 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK61 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK62 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK63 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK64 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK65 | Chicken dropping | Sanpeeseu farm, Chiang Mai |

**Table 1** (Continued)

| | | |
|---|---|---|
| CK66 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK67 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK68 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK69 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK70 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK71 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK72 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK73 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK74 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK75 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| CK76 | Chicken dropping | Sanpeeseu farm, Chiang Mai |
| NS1 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS2 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS3 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS4 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS5 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS6 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS7 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS8 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS9 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS10 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS11 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS12 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS13 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS14 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS15 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS16 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS17 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS18 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS19 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS20 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS21 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS22 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS23 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS24 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS25 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS26 | bat dropping | Nakornsawan Cave, Nakornsawan |

**Table 1** (Continued)

| | | |
|---|---|---|
| NS27 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS28 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS29 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS30 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS31 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS32 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS33 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS34 | bat dropping | Nakornsawan Cave, Nakornsawan |
| NS35 | bat dropping | Nakornsawan Cave, Nakornsawan |
| PP1 | White wagtail dropping | Lampang city, Lampang |
| PP2 | White wagtail dropping | Lampang city, Lampang |
| PP3 | White wagtail dropping | Lampang city, Lampang |
| PP4 | White wagtail dropping | Lampang city, Lampang |
| PP5 | White wagtail dropping | Lampang city, Lampang |
| PP6 | White wagtail dropping | Lampang city, Lampang |
| PP7 | White wagtail dropping | Lampang city, Lampang |
| PP8 | White wagtail dropping | Lampang city, Lampang |
| PP9 | White wagtail dropping | Lampang city, Lampang |
| PP10 | White wagtail dropping | Lampang city, Lampang |
| PP11 | White wagtail dropping | Lampang city, Lampang |
| PP12 | White wagtail dropping | Lampang city, Lampang |
| PP13 | White wagtail dropping | Lampang city, Lampang |
| PP14 | White wagtail dropping | Lampang city, Lampang |
| PP15 | White wagtail dropping | Lampang city, Lampang |
| CPA1 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CPA2 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CPA3 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CPA4 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CPA5 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CUA1 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUA2 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUA3 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUA4 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUA5 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUB1 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUB2 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUB3 | wagtail dropping | Chiang Mai city, Chiang Mai |

**Table 1** (Continued)

| | | |
|---|---|---|
| CUB4 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUB5 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUB6 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CUB7 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CUB8 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CUB9 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CUB10 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CUB11 | pigeon dropping | Chiang Mai city, Chiang Mai |
| CUB12 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUB13 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CUB14 | wagtail dropping | Chiang Mai city, Chiang Mai |
| CKA1 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA2 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA3 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA4 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA5 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA6 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA7 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA8 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA9 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA10 | Chicken dropping | Chiang Mai city, Chiang Mai |
| CKA11 | Chicken dropping | Hangdong, Chiang Mai |
| CKA12 | Chicken dropping | Hangdong, Chiang Mai |
| CKA13 | Chicken dropping | Hangdong, Chiang Mai |
| CKA14 | Chicken dropping | Hangdong, Chiang Mai |
| CKA15 | Chicken dropping | Hangdong, Chiang Mai |
| CJA1 | Red whiskered Bulbul dropping | Hangdong, Chiang Mai |
| CJA2 | Red whiskered Bulbul dropping | Hangdong, Chiang Mai |
| CJA3 | Red whiskered Bulbul dropping | Hangdong, Chiang Mai |
| CPA6 | Pigeon dropping | Hangdong, Chiang Mai |
| CPA7 | Pigeon dropping | Hangdong, Chiang Mai |
| CPA8 | Pigeon dropping | Hangdong, Chiang Mai |
| CPA9 | Pigeon dropping | Hangdong, Chiang Mai |
| CPA10 | Pigeon dropping | Hangdong, Chiang Mai |
| CPA11 | Pigeon dropping | Hangdong, Chiang Mai |
| CPA12 | Pigeon dropping | Hangdong, Chiang Mai |
| CPA13 | Pigeon dropping | Hangdong, Chiang Mai |

**Table 1** (Continued)

| | | |
|---|---|---|
| CPA14 | Pigeon dropping | Hangdong, Chiang Mai |
| CPA15 | Pigeon dropping | Hangdong, Chiang Mai |
| CUB1 | wagtail dropping | Sansai, Chiang Mai |
| CUB2 | wagtail dropping | Sansai, Chiang Mai |
| CUB3 | wagtail dropping | Sansai, Chiang Mai |
| CUB4 | wagtail dropping | Sansai, Chiang Mai |
| MHC1/1 | common myna dropping | Sripoom, Chiang Mai |
| MHC1/2 | common myna dropping | Sripoom, Chiang Mai |
| MHC1/3 | common myna dropping | Sripoom, Chiang Mai |
| MHC1/4 | common myna dropping | Sripoom, Chiang Mai |
| MHC1/5 | common myna dropping | Sripoom, Chiang Mai |
| MHC2/1 | common myna dropping | Sripoom, Chiang Mai |
| MHC2/2 | common myna dropping | Sripoom, Chiang Mai |
| MHC2/3 | common myna dropping | Sripoom, Chiang Mai |
| MHC2/4 | common myna dropping | Sripoom, Chiang Mai |
| MHC2/5 | common myna dropping | Sripoom, Chiang Mai |
| MHC3/1 | common myna dropping | Sripoom, Chiang Mai |
| MHC3/2 | common myna dropping | Sripoom, Chiang Mai |
| MHC3/3 | common myna dropping | Sripoom, Chiang Mai |
| MHC3/4 | common myna dropping | Sripoom, Chiang Mai |
| MHC3/5 | common myna dropping | Sripoom, Chiang Mai |
| MHC4/1 | common myna dropping | Sripoom, Chiang Mai |
| MHC4/2 | common myna dropping | Sripoom, Chiang Mai |
| MHC4/3 | common myna dropping | Sripoom, Chiang Mai |
| MHC4/4 | common myna dropping | Sripoom, Chiang Mai |
| MHC4/5 | common myna dropping | Sripoom, Chiang Mai |
| MHC5/1 | common myna dropping | Sripoom, Chiang Mai |
| MHC5/2 | common myna dropping | Sripoom, Chiang Mai |
| MHC5/3 | common myna dropping | Sripoom, Chiang Mai |
| MHC5/4 | common myna dropping | Sripoom, Chiang Mai |
| MHC5/5 | common myna dropping | Sripoom, Chiang Mai |
| MHC6/1 | common myna dropping | Sripoom, Chiang Mai |
| MHC6/2 | common myna dropping | Sripoom, Chiang Mai |
| MHC7/1 | common myna dropping | Sripoom, Chiang Mai |
| MHC7/2 | common myna dropping | Sripoom, Chiang Mai |
| MHC8 | common myna dropping | Sripoom, Chiang Mai |
| RO1/1 | bat dropping | Lod Cave, Mae Hong Son |

**Table 1** (Continued)

| | | |
|---|---|---|
| RO1/2 | bat dropping | Lod Cave, Mae Hong Son |
| RO1/3 | bat dropping | Lod Cave, Mae Hong Son |
| RO1/4 | bat dropping | Lod Cave, Mae Hong Son |
| RO1/5 | bat dropping | Lod Cave, Mae Hong Son |
| RO2/1 | bat dropping | Lod Cave, Mae Hong Son |
| RO2/2 | bat dropping | Lod Cave, Mae Hong Son |
| RO2/3 | bat dropping | Lod Cave, Mae Hong Son |
| RO2/4 | bat dropping | Lod Cave, Mae Hong Son |
| RO3/1 | bat dropping | Lod Cave, Mae Hong Son |
| RO3/2 | bat dropping | Lod Cave, Mae Hong Son |
| RO3/3 | bat dropping | Lod Cave, Mae Hong Son |
| RO3/4 | bat dropping | Lod Cave, Mae Hong Son |
| RO3/5 | bat dropping | Lod Cave, Mae Hong Son |

**4.3 Material processing**

Five-gram portion of each sample was placed in a screw-capped test tube containing 25 ml of sterile phosphate buffer saline (PBS) with 50 µg/ml of chloramphenical and 0.01% tween 80. The soil suspensions were vigorously shaken for 5 min and allowed to settle for approximately 1 h. Suspensions were collected for DNA extraction and cultured on L-DOPA agar (for the isolation of *C. neoformans*).

**4.4 DNA extraction**

Genomic DNA was extracted by Phenol-chloroform method (Haynes et al., 1995; Kersulyte et al., 1992) with some modifications. Equal volume of lysis buffer was added to the soil suspension from 4.3 and proteinase K was added to the final concentration of 60µg/ml. The mixture were incubated at 55ºC for 60 min and 95ºC for 10 min and then cooled at RT, a small amount (20U) of westase (Glucanase,

Takara) was added, and suspensions were incubated at 37ºC for 60 min. After centrifugation for 10 min at 12,000 xg, the aqueous phase was transferred to a new microcentrifuge tube, 1 volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and then the mixture was vortexed for 2 min. After centrifugation for 10 min at 12,000 xg, the supernatant was carefully transferred to a new microcentrifuge tube, 2 volume of 100% ethanol was added, and stored at -20 ºC for at least 2 h. After centrifugation at 13,000 xg for 30 min at 4ºC, the DNA pellet was washed with 70% ethanol, air dried, dissolved in 50 µl of TE buffer and kept at -20ºC. The DNA concentration was determined with a UV spectrophotometer.

**4.5 Detection of *Cryptococcus neoformans* in soil contaminated with avian and bat droppings by culture and nested-PCR**

**4.5.1 Isolation of *C. neoformans* by culture method**

**4.5.1.1 Culture isolation**

The undiluted suspensions from 4.3 were plated directly by spreading 0.1 ml over the surface of Sabouraud's Dextrose Agar (SDA) and L-Dopa agar. All plates were incubated at 37ºC and observed daily for one week.

**4.5.1.2 Identification**

All suspected smooth yeast colonies on SDA and smooth black pigmented colonies on L-Dopa agar were examined by India ink preparations for encapsulated globose or oval yeasts. The positive isolates were then identified by

carbohydrate assimilation and fermentation tests. The biochemical characteristics of *C. neoformans* were positive for urease, negative fermentation tests for all carbohydrates, sugar assimilations were positive for glucose, galactose, sucrose, maltose and raffinose but not for lactose (Table 2).

**Table 2** Biochemical characteristics of *C. neoformans*

| | *C. neoformans* |
|---|---|
| Culture | |
| - Sabouraud's Dextrose Agar (SDA) | Cream colored, smooth, mucoid colonies |
| - L-Dopa agar | Smooth black colonies |
| - Urea agar | + |
| Fermentation | |
| - Glucose, galactose, sucrose, maltose, raffinose, lactose | - |
| Assimilation | |
| - Glucose, galactose, sucrose, maltose, raffinose | + |
| - Lactose | - |

+, Positive; -, Negative

### 4.5.2 Detection of *C. neoformans* by nested PCR

The two-stage PCR according to Bialek's method (2002) was performed to detect *C. neoformans* using specific primers for 18S rDNA gene, as shown in Table 3.

Amplification reactions were performed in a 50 µl reaction volume consisting of PCR mixture as shown in Table 4. Amplification was carried out in an automatic thermocycler (Eppendorf, Germany). Following an initial denaturation at 94ºC for 5 min, the 35 cycle amplification of the first-round PCR consisted of 94ºC for 1 min, 50ºC for 30 sec and 72ºC for 1 min. A final elongation step was applied at 72ºC for 5 min. One µl of the first PCR reaction was used as the template for second round PCR. Following initial denaturation at 94ºC for 5 min, the 35 cycle amplification of the second-round PCR consisted of 94ºC for 1 min, 65ºC for 30 sec and 72ºC for 1 min. A final elongation step was applied at 72ºC for 5 min. The PCR products were electrophoresed in 1.5% agarose gel and visualized with UV transilluminator after staining with ethidium bromide. Band sizes were estimated from comigration of 100 bp DNA ladder (Fermentas Applied Biosystems, USA) molecular size standard.

**Table 3** Oligonucleotide primers for 18S rDNA gene of *C. neoformans* (Bialek et al., 2002c).

| PCR | Primer name | Nucleotide sequence (5'→3') | Position | Length (bp) |
|---|---|---|---|---|
| 1st PCR | Fungus I | GTTAAAAAGCTCGTAGTTG | 617 - 635 | 429 |
| | Fungus II | TCCCTAGTCGGCATAGTTTA | 1045 - 1026 | |
| 2nd PCR | Cryp I | TCCTCACGGAGTGCACTGTCTTG | 661 - 683 | 278 |
| | Cryp II | CAGTTGTTGGTCTTCCGTCAATCTA | 938 - 914 | |

**Table 4** PCR master mix preparation for the detection of *C. neoformans*

| PCR reagent | 1x(µl) | Final conc. |
|---|---|---|
| 10x PCR buffer | 5 | 1x |
| 50mM $MgCl_2$ | 2.5 | 2.5mM |
| 10mM dNTP mix | 0.5 | 100µM |
| 50 µM primer Fungus I | 1 | 1µM |
| 50 µM primer Fungus II | 1 | 1µM |
| 5 U Taq DNA polymerase (invitrogen) | 0.4 | 2.0U |
| DNA template | 10 | |
| DNase free water | 29.6 | |
| Total volume | 50 | |

### 4.5.3 Nucleotide sequence analysis of *C. neoformans*

The PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN, Germany) according to the manufacturer's protocol. Initially, the PCR products were electrophoresed in 1.5% agarose gel containing 0.5 µg/ml ethidium bromide, followed by excision of the gel containing the DNA fragment of expected sizes under UV transilluminator. After weighing the gel slice, 3 volumes (µl) of QG

solubilization and binding buffer were added to 1 volume (mg) of the gel slice and incubated at 50ºC for 10 min until the gel slice had completely dissolved. Then, the mixture was applied onto a QIAquick spin column in a 2 ml collecting tube and centrifuged at 12,000 rpm for 1 min. The flow-through filtrate was discarded and the column was placed back in the same collecting tube. To wash DNA that binding to the column, 750 µl of PE buffer was added and centrifuged for 1 min. The flow-through filtrate was discarded again, and the column was centrifuged for an additional 1 min to remove residual ethanol. The QIAquick spin column was placed into a clean 1.5 ml microcentrifuge tube and then, 30 µl of EB elution buffer (10 mM Tris-HCL, pH 805) was added to the center of QIAquick membrane to elute DNA from the column. The column was standed for 1 min and then centrifuged for 1 min. Finally, the quantity of the purified DNA was assessed by spectrophotometric analysis at 260 and 280 nm as well as by electrophoresis in 1.5% agarose gel. Then, the purified PCR products and sequencing primers (listed in Table 3) were sent to 1st BASE (Selangor Darul Ehsan, Malaysia), the DNA sequencing service provider for sequencing.

**4.5.4 Sensitivity of PCR for the detection of *C. neoformans***

Sensitivity of PCR was tested for cellular DNA and DNA isolated from *C. neoformans* cell. For sensitivity testing of the PCR for DNA isolated from *C. neoformans* cell, the DNA concentration was quantified using a viable cell count. Ten-fold serial dilutions of DNA were subjected for nested PCR to determine sensitivity of the method. PCR was performed by using 1 µl of each sample as

described above. In order to simulate detection of cells in contaminated soil, the cells of *C. neoformans* were counted with a hemocytometer and resuspended at a concentration of $10^6$ cells/ ml. Ten-fold serial dilutions ($10^5$-$10^0$) in the soil suspension were used, 200 µl of each suspension were used to extract DNA as described above and PCR was performed using 10 µl DNA suspension as template DNA. PCR products were analysed by electrophoresis in 1.5% agarose gel.

### 4.5.5 Specificity test of PCR for detection of *C. neoformans*

For specificity testing, DNAs of 1 bacteria and 7 fungal species including *Mycobacterium avium, Penicillium marneffei, Aspergillus fumigatus, Candida albicans ATCC90028, Candida. tropicalis, Candida dubliniensis, Candida krusei ATCC6258* and *Histoplasma capsulatum*, were separately extracted by using DNA extraction protocol as described above and subjected for nested PCR to determine specificity of the method.

## 4.6 Detection of *H. capsulatum* in soil contaminated with avian and bat droppings by nested PCR

### 4.6.1 Detection of *H. capsulatum* by nested PCR

The two-stage PCR according to Bialek's method (2002) was performed to detect *H. capsulatum* using specific primers for 100 kDa like protein gene, as shown in Table 6. Amplification reactions were performed in a 50 µl reaction volume

consisting of PCR mixture according to Table 7. Following an initial denaturation at 94ºC for 5 min, the 35 cycles amplification of the first-round PCR consisted of 94ºC for 30 sec, 65ºC for 30 sec and 72ºC for 1 min was performed. A final elongation step was applied at 72ºC for 5 min. One µl of the first PCR reaction was used as the template for a second. Following with the initial denaturation step at 94ºC for 5 min, the 35 cycles amplification of the second-round PCR consisted of 94ºC for 30 sec, 65ºC for 30 sec and 72ºC for 30 sec was consequently performed. A final elongation step was applied at 72ºC for 5 min. Amplification was carried out in automatic thermocycler (Eppendorf, Germany). The PCR products were electrophoresed in 1.5% agarose gel and visualized with UV transilluminator after staining with ethidium bromide. Band sizes were estimated from comigration of 100 bp DNA ladder (Fermentas Applied Biosystems, USA) molecular size standard.

**4.6.2 Nucleotide sequence analysis of *H. capsulatum***

The PCR product of 100 kDa like protein gene were purified using QIAquick Gel Extraction Kit (QIAGEN, Germany) with the same protocol as previously described in nucleotide sequence analysis of *C. neoformans*. The purified PCR products and sequencing primers listed in Table 5 were sent to 1st BASE (Selangor Darul Ehsan, Malaysia), the DNA sequencing service provider, for sequencing.



**4.6.3 Sensitivity test of PCR for the the detection of *H. capsulatum***

Sensitivity of PCR was tested for cellular DNA and for DNA isolated from

*H. capsulatum* cells. For sensitivity testing of the PCR on cellular DNA, the DNA concentration was quantified using a spectrophotometer. Ten-fold serial dilutions were subjected to analysis. PCR was performed by using 1 µl of each sample as described above. In order to simulate detection of cells in contaminated soil, the *H. capsulatum* concentration were measured with spectrophotometer and then the cell suspension was adjusted to McFarland 1.0 ($10^5$ cells/ml). Ten-fold serial dilutions ($10^5$-$10^0$) in the soil suspension were used, 200 µl of each suspension were used to extract DNA as described above and PCR was performed using 10 µl DNA suspension as template DNA. PCR products were analysed by electrophoresis in 1.5% agarose gel.

**4.6.4 Specificity test of PCR for the detection of *H. capsulatum***

For specificity testing, DNAs of 1 bacterial and 7 fungal species including *Mycobacterium avium, Penicillium marneffei, Aspergillus fumigatus, Candida albicans ATCC90028,* C*andida tropicalis, Candida dubliniensis, Candida krusei ATCC6258* and *Cryptococcus neoformans* were extracted by using DNA extraction protocol as described above and 10 µl DNA suspension was subjected for PCR.

**Table 5** Oligonucleotide primers for 100 kDa like protein gene of *H. capsulatum* (Bialek et al., 2002b).

| PCR | Primer name | Nucleotide sequence (5'→ 3') | Length (bp) |
|---|---|---|---|
| 1st PCR | Hc I | GCGTTCCGAGCCTTCCACCTCAAC | 391 |
| | Hc II | ATGTCCCATCGGGCGCCGTGTAGT | |
| 2nd PCR | Hc III | GAGATCTTAGTCGCGGCCAGGTTCA | 210 |
| | Hc IV | AGGAGAGAACTGTATCGGTGGCTTG | |

**Table 6** PCR master mix preparation for the detection of *H. capsulatum*

| PCR reagent | 1x(µl) | Final conc. |
|---|---|---|
| 10x PCR buffer | 5 | 1x |
| 50mM MgCl2 | 2.5 | 2.5mM |
| 10mM dNTP mix | 0.5 | 100µM |
| 50 µM primer Hc I | 1 | 1µM |
| 50 µM primer Hc II | 1 | 1µM |
| 5 U Taq DNA polymerase (invitrogen) | 0.4 | 2.0U |
| DNA template | 10 | |
| DNase free water | 29.6 | |
| Total volume | 50 | |