

CHAPTER 3

OBJECTIVES

1. To detect *Cryptococcus neoformans* in soil contaminated with avian and bat droppings by culture and nested-PCR
2. To detect *Histoplasma capsulatum* in soil 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 chloramphenicol 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 55°C for 60 min and 95°C for 10 min and then cooled at RT, a small amount (20U) of westase (Glucanase,

Takara) was added, and suspensions were incubated at 37°C 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 °C for at least 2 h. After centrifugation at 13,000 xg for 30 min at 4°C, the DNA pellet was washed with 70% ethanol, air dried, dissolved in 50 µl of TE buffer and kept at -20°C. 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 37°C 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*

	<i>C. neoformans</i>
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 94°C for 5 min, the 35 cycle amplification of the first-round PCR consisted of 94°C for 1 min, 50°C for 30 sec and 72°C for 1 min. A final elongation step was applied at 72°C for 5 min. One µl of the first PCR reaction was used as the template for second round PCR. Following initial denaturation at 94°C for 5 min, the 35 cycle amplification of the second-round PCR consisted of 94°C for 1 min, 65°C for 30 sec and 72°C for 1 min. A final elongation step was applied at 72°C 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)
1 st PCR	Fungus I	GTAAAAAGCTCGTAGTTG	617 - 635	429
	Fungus II	TCCCTAGTCGGCATAGTTTA	1045 - 1026	
2 nd 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(μ l)	Final conc.
10x PCR buffer	5	1x
50mM MgCl ₂	2.5	2.5mM
10mM dNTP mix	0.5	100 μ M
50 μ M primer Fungus I	1	1 μ M
50 μ M primer Fungus II	1	1 μ M
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 μ g/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 (μ l) of QG

solubilization and binding buffer were added to 1 volume (mg) of the gel slice and incubated at 50°C 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 8.05) 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 μ l of each suspension were used to extract DNA as described above and PCR was performed using 10 μ l 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 marneffeii*, *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 μ l reaction volume

consisting of PCR mixture according to Table 7. Following an initial denaturation at 94°C for 5 min, the 35 cycles amplification of the first-round PCR consisted of 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min was performed. A final elongation step was applied at 72°C 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 94°C for 5 min, the 35 cycles amplification of the second-round PCR consisted of 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec was consequently performed. A final elongation step was applied at 72°C 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, *Candida 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 MgCl ₂	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	