CHAPTER 5

RESULTS

5.1 To detect *Cryptococcus neoformans* in soil contaminated with avian and bat droppings by culture and nested-PCR

5.1.1 Detection of *C. neoformans* by culture and nested PCR

A total of 265 samples collected in Chiang Mai, Nakornsawan, Lampang and Maehongson during 2009-2010 were processed and cultured for *C. neoformans*. Suspected yeast colonies were isolated and identified by capsular production, phenol oxidase test on L-dopa agar (Figure 5), urease test, carbon assimilation and fermentation test (Figure 6).

Using culture isolation and identification, all soil samples were negative for *C. neoformans*. On the other hand, only one sample from soil contaminated with Myna dropping (MHC4/1)collected in Sripoom district, Chiang Mai was positive for *C. neoformans* by nested PCR (Figure 7) but *C. neoformans* could not be isolated from this soil sample by culture method (Table 7). Nucleotide sequencing analysis of PCR products showed 99 % identity to genes encoding 18S rRNA of *Cryptococcus neoformans*.

5.1.2 Sensitivity test of PCR condition to detect *C. neoformans*

The sensitivity of PCR system was determined by using different amounts of

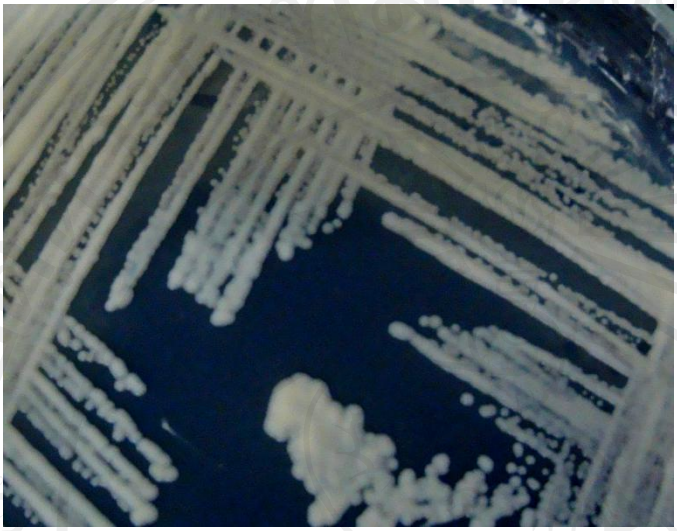
C. neoformans as a template. Figure 8A demonstrates the results utilizing primers Fungus I and Fungus II. These primers generate a PCR product of approximately 400 bp for most fungi (429 bp for *C. neoformans*). The detection limit was 10 ng per reaction (Figure 8A). Figure 8B shows the results of re-amplifying of the first round PCR reaction using *C. neoformans* specific primers, CrypI and CrypII. These primers produce a PCR product of approximately 278 bp. The sensitivity level for the nested PCR of *C. neoformans* was 10 fg of the purified DNA (Figure 8B).

The capacity to detect yeast cell in soil samples was simulated by adding a known number of *C. neoformans* cells to soil suspensions and purifying the suspensions for use in the PCR assay. The results of first round (Figure 9A) and second round (Figure 9B) PCR reactions, utilizing common fungal primers Fungus I and Fungus II for first round and Cryp I and Cryp II for second round, are demonstrated in Figure 9. The estimated limit of detection by this process is 2,000 cells.

5.1.3 Specificity test of PCR condition to detect *C. neoformans*

For specificity testing, DNAs of 1 bacteria and 7 fungal species including *M. avium, P. marneffei, A. fumigatus, C. albicans ATCC90028, C. tropicalis, C. dubliniensis, C. krusei ATCC6258* and *H. capsulatum*, microbial DNA were examined. No target DNA products were amplified when DNAs from other organisms described above were tested with primers, Fungus I-Fungus II, and Cryp I-Cryp II (Figure 10).

A



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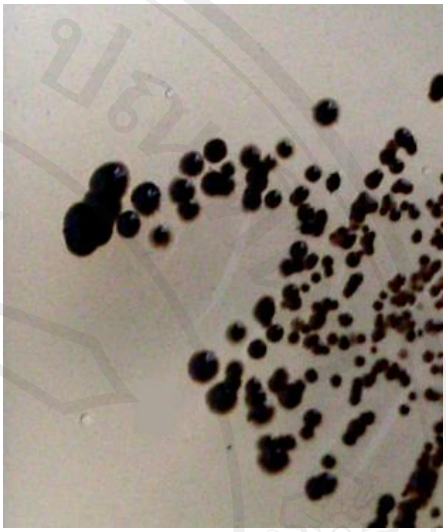

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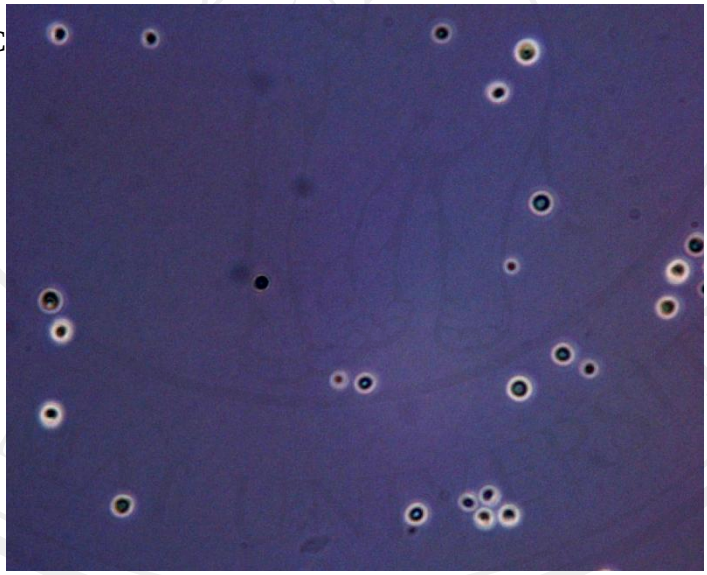

Figure 5 *C. neoformans* cultured on Sabouraud dextrose agar displaying white colored, smooth, mucoid colonies at 37ºC for 3 days (A). Melanin pigment production of *C.neoformans* on L-dopa agar (B). India ink preparation of yeast cells of *C. neoformans* (C). The capsular material of cryptococci displaces the colloidal carbon particles of the ink so that the capsule appears as a clear halo around the microorganisms against a black background (C).

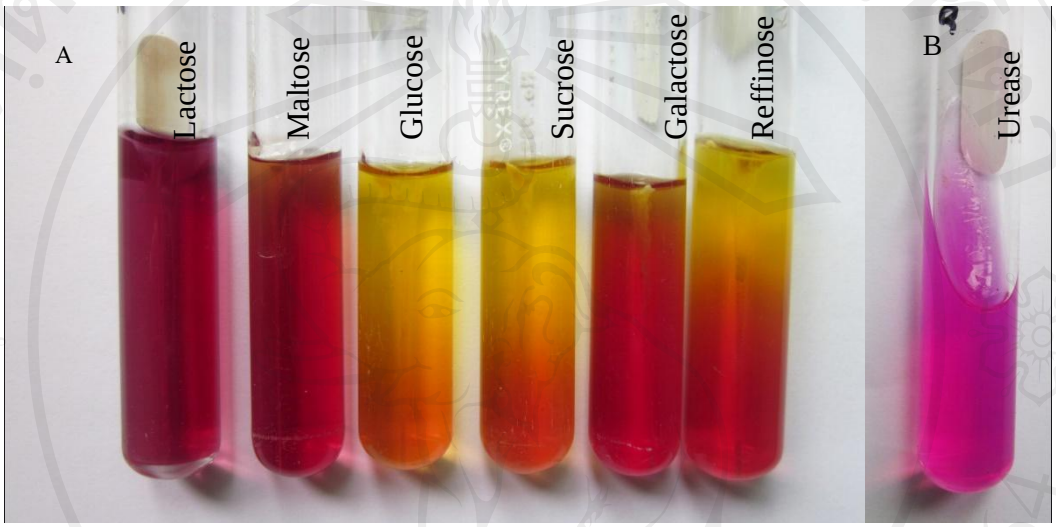

Figure 6 The carbon assimilation and fermentation tests for yeast identification. *C. neoformans* can assimilate glucose, galactose, sucrose, maltose and raffinose but not for lactose and unable to ferment all test sugars (A). Confirmatory test for the identification of *C. neoformans* is urease test (B). The urea agar turns to pink after 2 day inoculation at 37ºC.

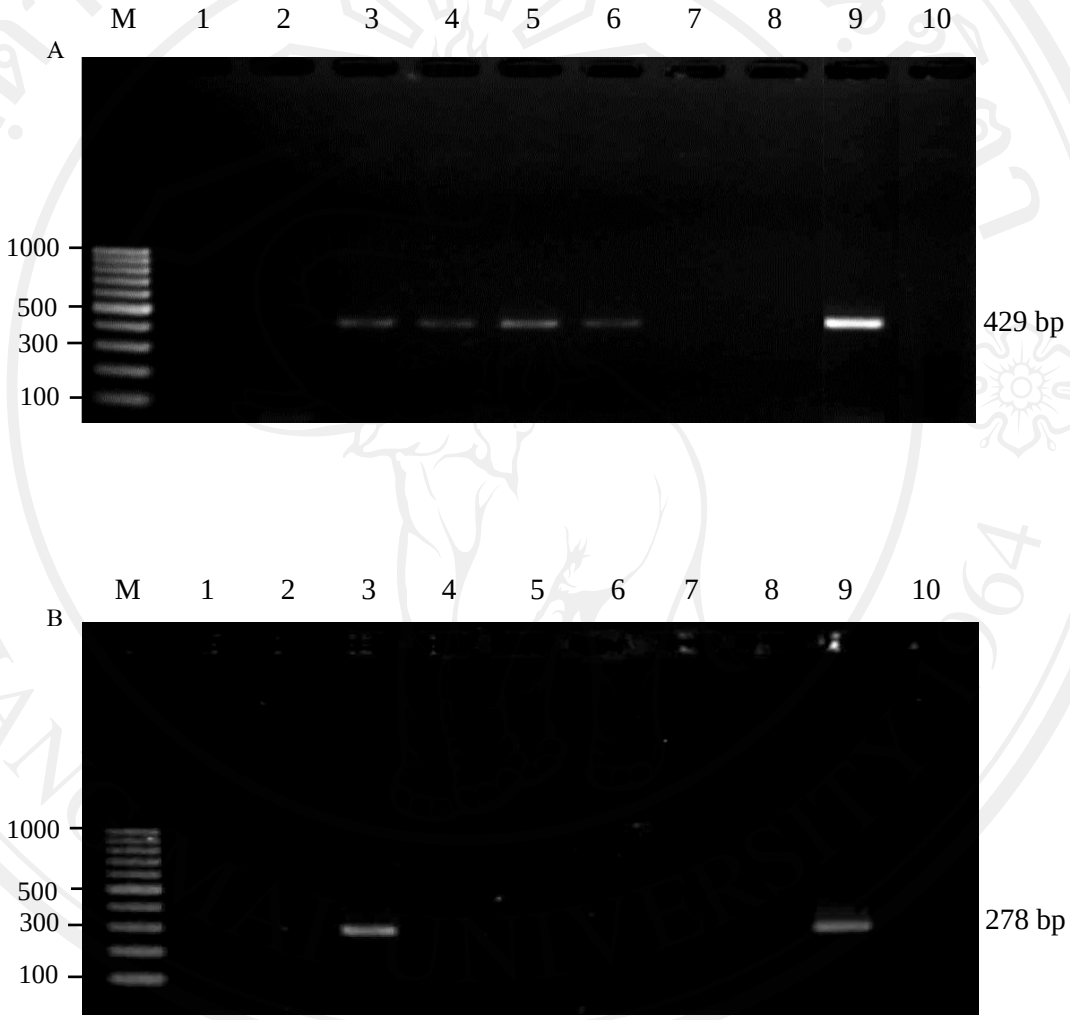

Figure 7 Representative gels of PCR analysed with Fungus I, Fungus II (A) and CrypI, CrypII (B) primers. Lane1-8, test samples (MHC3/5, MHC4/1, MHC4/2, MHC4/3, MHC4/4, MHC4/5, MHC5/1, MHC5/2) ; lane 9, positive control; lane 10, negative control; lane M, 100 bp molecular size ladder marker (Fermentas Applied Biosystems).

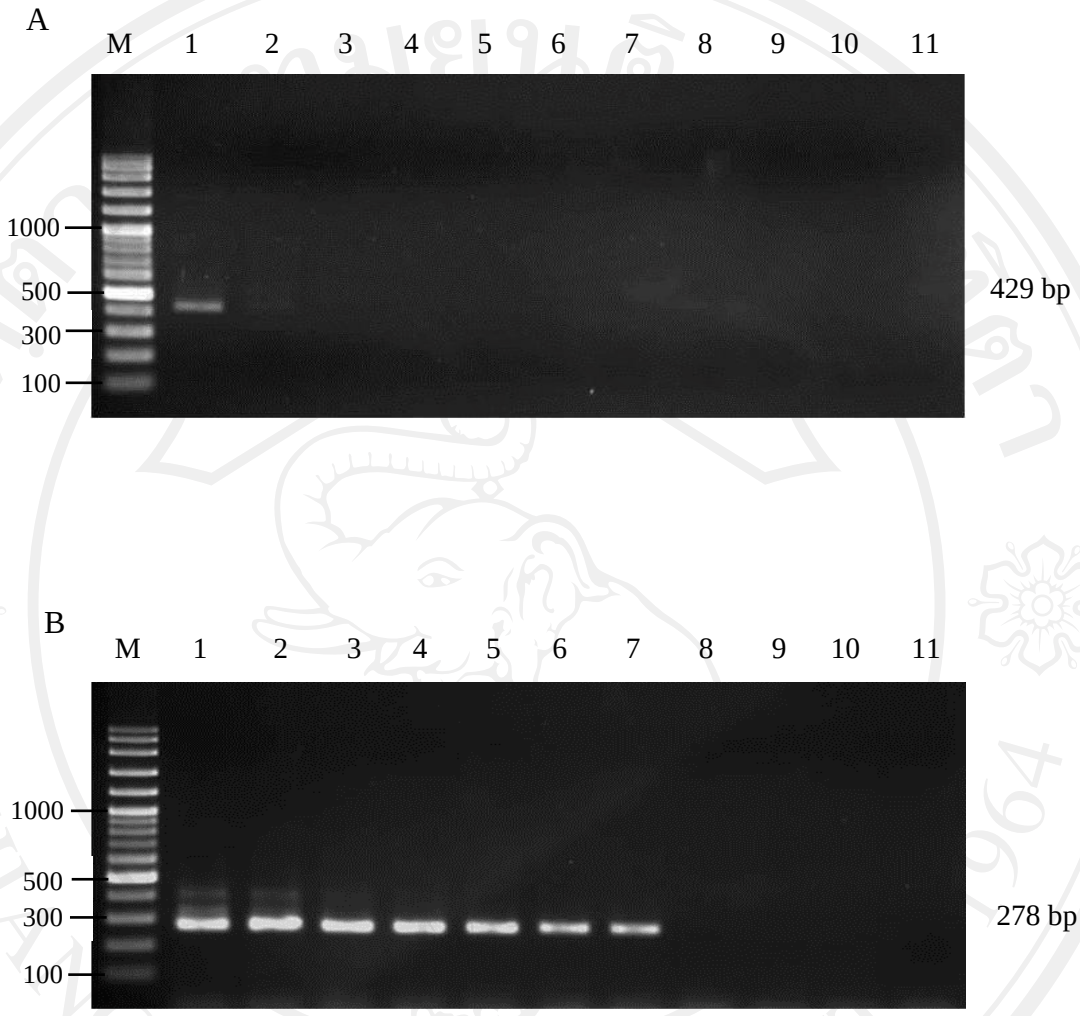

Figure 8 Sensitivity of polymerase chain reaction (PCR) using *C. neoformans* specific primers. First round PCR reactions utilizing the common fungal primers, Fungus I and Fungus II (A), with various amounts of template; lane 1-10: 10ng, 1ng, 100pg, 10pg, 1pg, 100fg, 10fg, 1fg,0.1fg, 0.01fg DNA respectively; lane 11, negative control; lane M, 100 bp molecular size ladder marker (Vivantis). Second round PCR using 1 µl of PCR reaction in A with *C. neoformans* specific primers, CrypI and CrypII (B).

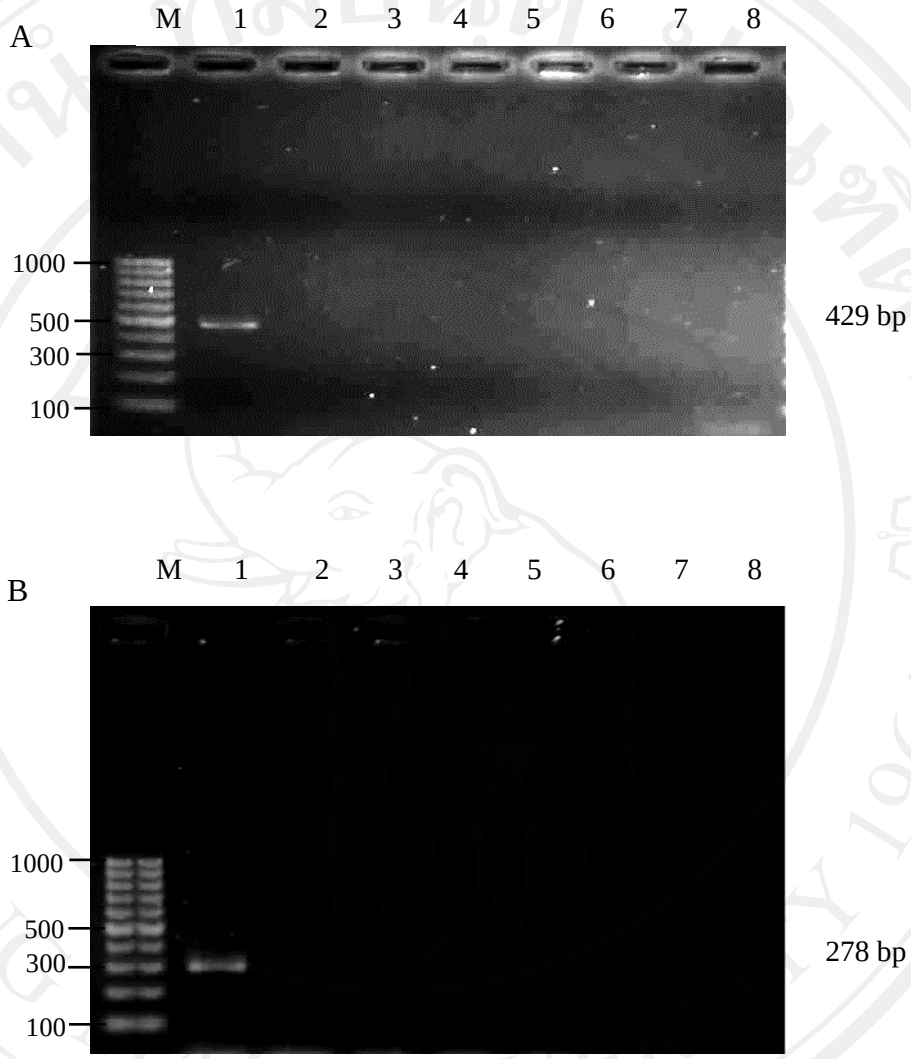

Figure 9 Ability to detect *Cryptococcus neoformans* cells in soil samples by nested PCR. First round PCR reaction utilizing common fungal primers, Fungus I and Fungus II (A) with various amounts of template; lane 1-7 : 2,000 cells, 200 cells, 20 cells, 2 cells, 0.2 cell, 0.02 cell, 0.002 cell respectively; lane 8, negative control; lane M, 100 bp molecular size ladder marker (Fermentas Applied Biosystems) (A). Second round PCR reactions using 1 µl of PCR reaction in A with *C. neoformans* specific primers, CrypI and CrypII (B).

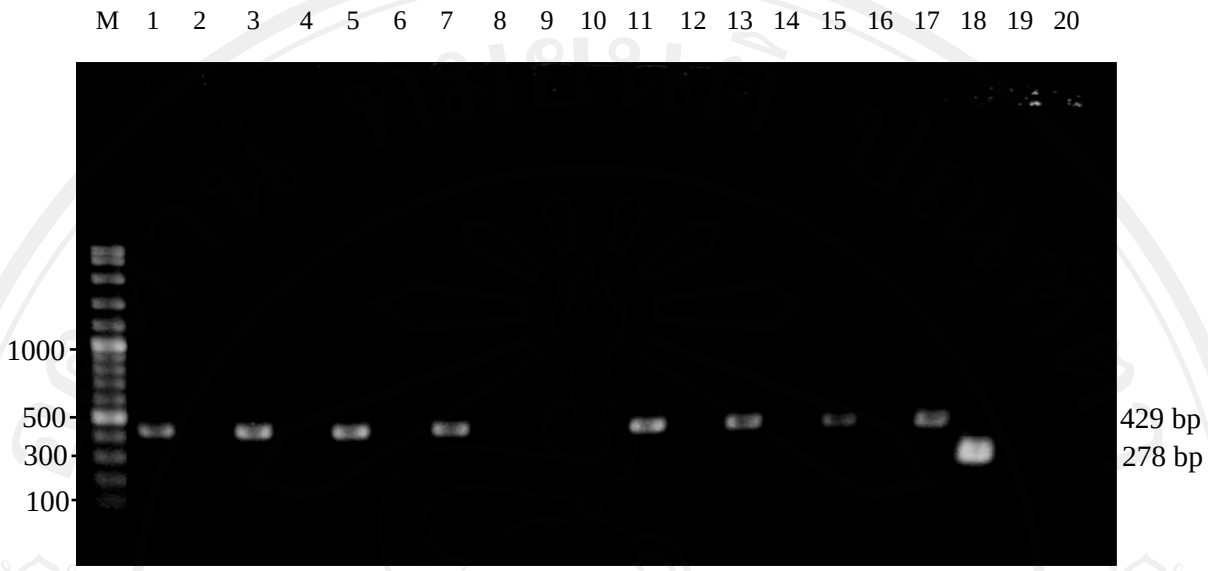

Figure 10 Specificity of PCR assay with DNA from various pathogenic organisms by using Fungus I-Fungus II and Cryp I-Cryp II primers. ; lane 1, *C. albicans* 1st round; lane 2, *C.albicans* 2nd round; lane 3, *C. tropicalis* 1st round; lane 4, *C. tropicalis* 2nd round; lane 5,*C. krusei* 1st round; lane 6, *C. krusei* 2nd round; lane 7, *C. dubliniensis* 1st round; lane 8, *C. dubliniensis* 2nd round; lane 9, *M. avium* 1st round; lane 10, = *M. avium* 2nd round; lane 11, *A. fumigatus* 1st round; lane 12, *A. fumigatus* 2nd round; lane 13, *P. marneffei* 1st round; lane 14, *P. marneffei* 2nd round; lane 15, *H. capsulatum* 1st round; lane 16, *H. capsulatum* 2nd round; lane 17, *C. neoformans* 1st round; lane18, *C. neoformans* 2nd round; lane 19, negative control 1st round; lane 20, negative control 2nd round; lane M, 100 bp molecular size ladder marker (Vivantis).

Table 7 Detection of *C. neoformans* from soil contaminated with avian and bat droppings by culture and nested PCR**.**

Source	No. of collected samples	No. of positive samples		% positive
		Culture	PCR	
Bat	88	0	0	0
Bird				
- White wagtail (*Motacilla alba*)	15	0	0	0
- Wagtail (genus *Motacilla*)	17	0	0	0
- Pigeon (*Columba livia*)	21	0	0	0
- Myna (*Acridotheres tristis*)	30	0	1	3.33
- Red whiskered Bulbul (*Pycnonotus jocosus*)	3	0	0	0
Chicken (*Gallus gallus*)	91	0	0	0
total	265	0	1	0.37

5.2 To detect *Histoplasma capsulatum* in soil contaminated with avian and bat droppings by nested-PCR

5.2.1 Detection of *H. capsulatum* by nested PCR

A total of 265 samples collected in Chiang Mai, Nakornsawan, Lampang and Maehongson during 2009-2010 were processed and determined for the presence of *H. capsulatum* by nested PCR. Seven of 88 (7.95%) soil contaminated with bat guano samples, 1/21 (4.76%) soil contaminated pigeon dropping samples, 10/ 91 (10.99%) soil contaminated with chicken dropping samples (Table 8) were positive for *H.capsulatum* (Figure 11, 12). Nucleotide sequencing analyses of PCR products showed 94 to 99 % identity to gene encoding 100 kDa-like protein of *Ajellomyces capsulatus* (Table 9).

5.2.2 Sensitivity test of PCR condition to detect *H. capsulatum*

The sensitivity of PCR system was determined by using different amounts of *H. capsulatum* as a template. Figure 11A demonstrates the results utilizing *H. capsulatum* specific primers, Hc I and Hc II. These primers generate a PCR product size of approximately 391 bp for *H. capsulatum* based on 100 kDa- like protein. The detection limit was 100 pg per reaction (Figure 13A). Figure 10B shows the results of re-amplifying of the first round PCR reaction using Hc III and Hc IV primers. These primers produce a PCR product of approximately 210 bp. The sensitivity level for the nested PCR of *H. capsulatum* was 1 pg of the purified DNA (Figure 13B).

The capacity to detect yeast cells in soil samples was simulated by adding a known number of *H. capsulatum* cell to soil suspensions and purifying the suspensions to be used in the PCR assay. The results of first round (Figure 14A) and second round (Figure 14B) PCR reaction, utilizing fungal primers, Hc I and Hc II for first round, and Hc III and Hc IV for second round, are demonstrated in Figure 14. The estimated limit of detection by this process is 2,000 cells.

5.2.3 Specificity test of PCR condition to detect *H. capsulatum*

For specificity testing, DNAs of 1 bacterial and 7 fungal species including *M. avium*, *P. marneffei*, *A. fumigatus*, *C. albicans* ATCC90028, *C. tropicalis*, *C. dubliniensis*, *C. krusei* ATCC6258 and *C. neoformans*, were determined. No target DNA products were amplified when DNA from each organism described above was tested with primers, Hc I - Hc II, and Hc III - Hc IV (Figure 15).

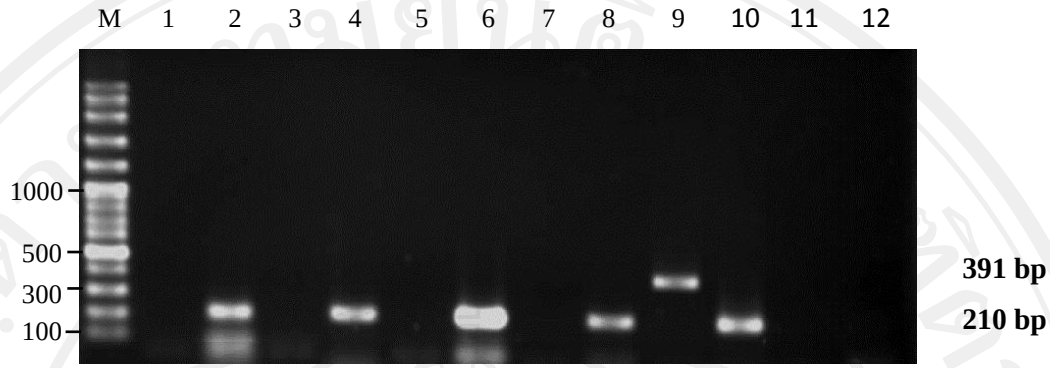

Figure 11 Representative gel of PCR analyses with Hc I, Hc II (first round) and Hc III, Hc IV (second round) primers. Lane 1, 3, 5, 7, 1st round PCR from soil contaminated with bat guano samples (CD1/1, CD2/2, CD2/4, CD3/1 Respectively); Lane 2, 4, 6, 8, 2nd round PCR from soil contaminated with bat guano samples (CD1/1, CD2/2, CD2/4, CD3/1 Respectively); lane 9, 1st round PCR positive control (391bp); lane 10, 2nd round PCR positive control (210bp); lane 11, 1st round PCR negative control ; lane 12, 2nd round PCR negative control; lane M, 100 bp molecular size ladder marker (Vivantis).

Note: CD; soil contaminated with bat guano collected from Chiang Dao cave, Chiang Mai

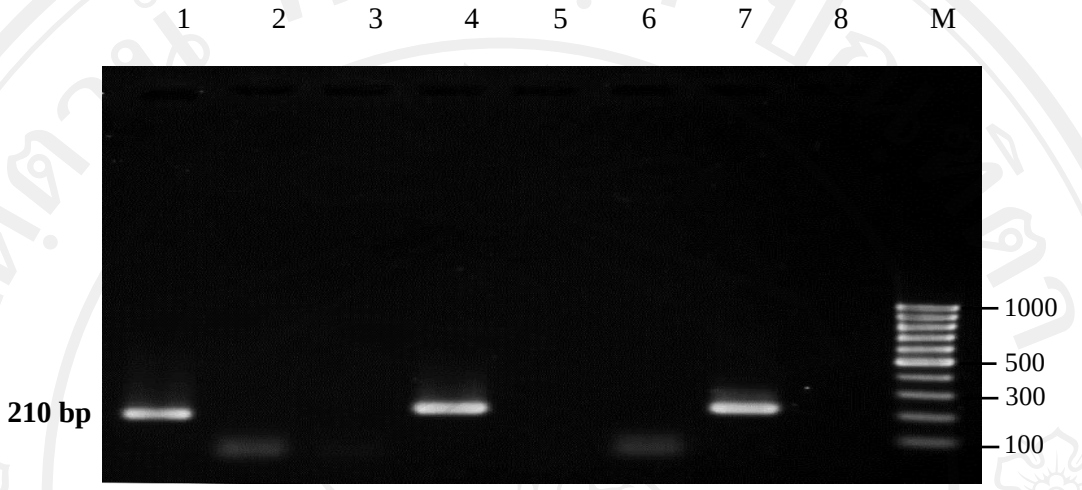

Figure 12 Representative gel of PCR analyse with Hc III and Hc IV (second round) primers. ; Lane 1-6, 2nd round PCR from soil contaminated with chicken dropping samples (CK17, CK18, CK19, CK20, CK21, CK22 Respectively); lane 7, 2nd round PCR positive control (210bp); lane 8, 2nd round PCR negative control; lane M, 100 bp molecular size ladder marker (Fermentas Applied Biosystems).

Note: CK; soil contaminated with chicken dropping collected from Sanpeesua farm, Chiang Mai

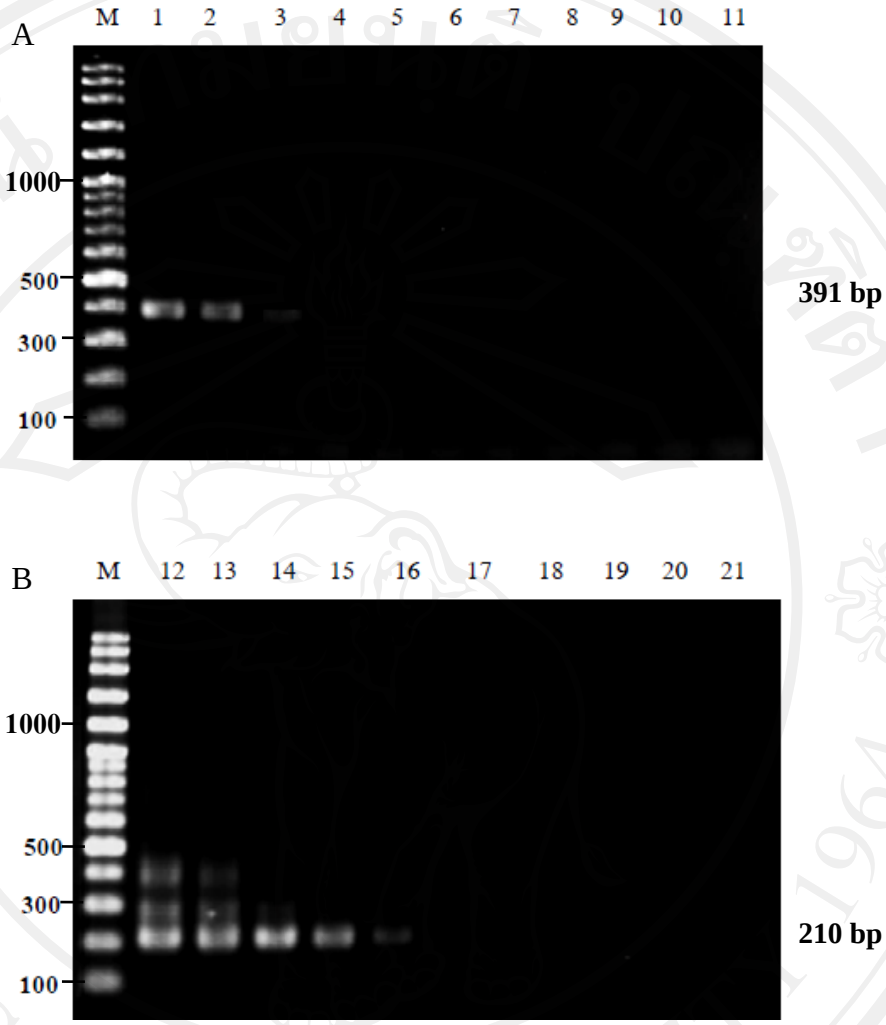

Figure 13 Sensitivity of polymerase chain reaction (PCR) using *H. capsulatum* specific primers. First round PCR reactions utilizing primers Hc I and Hc II (A)with varied amounts of template; lane 1-10: 10ng, 1ng, 100pg, 10pg, 1pg, 100fg, 10fg, 1fg,0.1fg, 0.01fg DNA; lane 11, negative control; lane M, 100 bp molecular size ladder marker (Vivantis). Second round PCR reactions using 1 µl of PCR reaction in A with *H. capsulatum* primers, Hc III and Hc IV (B).

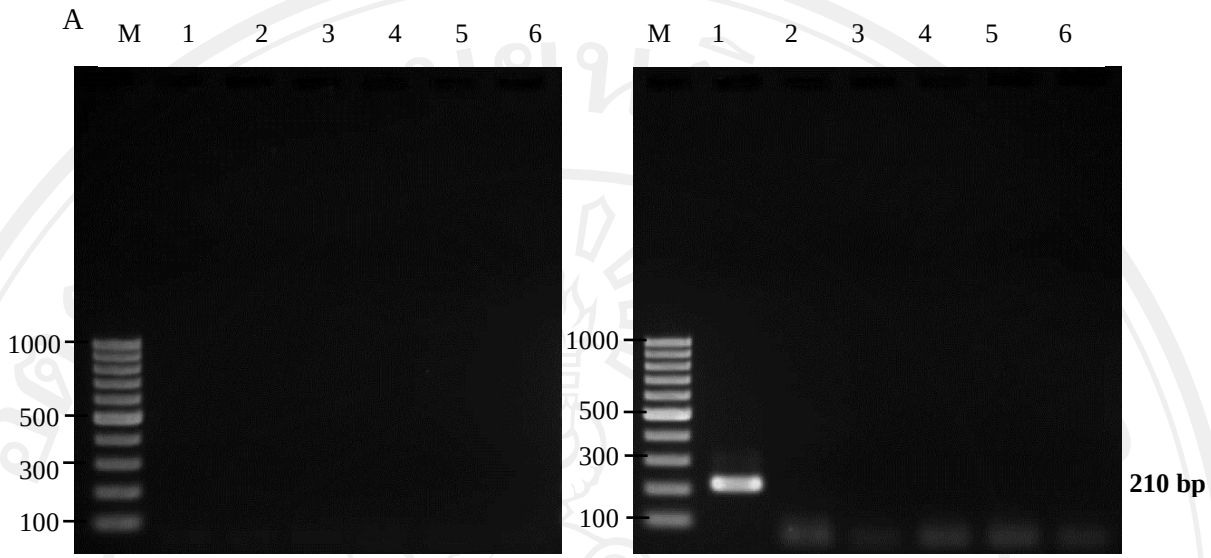

Figure 14 Ability to detect *Histoplasma capsulatum* cells in soil suspensions by nested PCR. First round PCR reactions utilizing *H. capsulatum* specific primers, Hc I and Hc II (A) with various amounts of template; lane 1- 6 : 2,000 cells, 200 cells, 20 cells, 2 cells, 0.2 cell; lane 6, negative control; lane M, 100 bp molecular size ladder marker (Fermentas Applied Biosystems) (A). Second round PCR reactions using 1 µl of PCR reaction in A with *H. capsulatum* primer Hc III and Hc IV (B).

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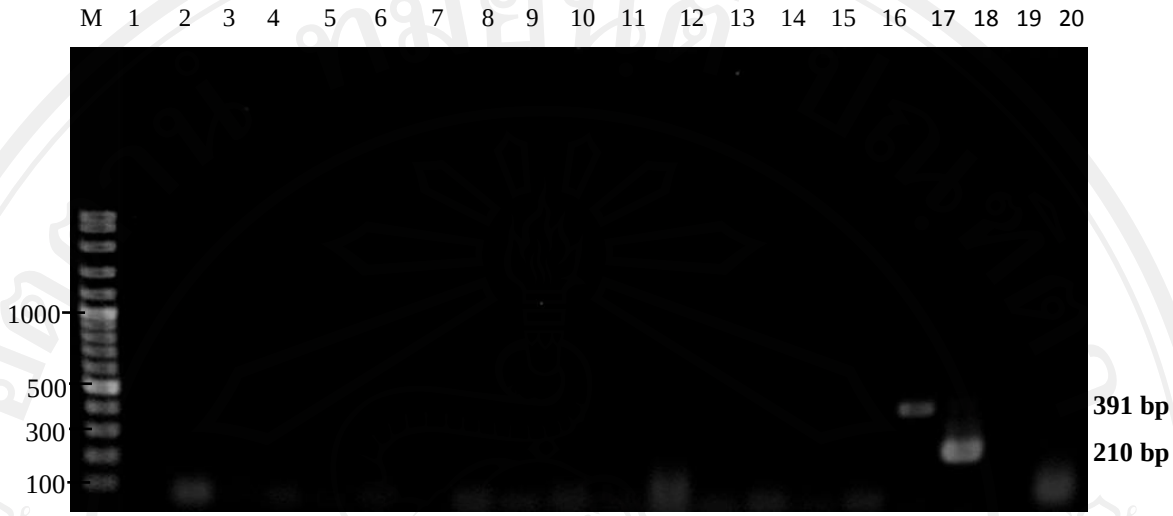

Figure 15 Specificity of PCR assays with DNAs from various pathogenic organisms by using Hc I - Hc II and Hc III - Hc IV primers. Lane 1, *C .albicans* 1st round; lane 2, *C. albicans* 2nd round; lane 3, *C. tropicalis* 1st round; lane 4, *C. tropicalis* 2nd round; lane 5,*C. krusei* 1st round; lane 6, *C. krusei* 2nd round; lane 7, *C. dubliniensis* 1st round; lane 8, *C. dubliniensis* 2nd round; lane 9, *M. avium* 1st round; lane 10, *M. avium* 2nd round; lane 11, *A. fumigatus* 1st round; lane 12, *A. fumigatus* 2nd round; lane 13, *P. marneffei* 1st round; lane 14, *P. marneffei* 2nd round; lane 15, *C. neoformans* 1st round; lane 16, *C. neoformans* 2nd round; lane 17, *H. capsulatum* 1st round; lane18, *H. capsulatum* 2nd round; lane 19, negative control 1st round; lane 20, negative control 2nd round; lane M, 100 bp molecular size ladder marker (Vivantis).

Table 8 Detection of *H. capsulatum* in soil contaminated with bat and avian droppings by nested PCR

Soil contaminated with	No. of collected samples	No. of positive samples	% positive
Bats	88	7	7.95
Birds			
- White wagtail (*Motacilla alba*)	15	0	0
- Wagtail (genus *Motacilla*)	17	0	0
- Pigeon (*Columba livia*)	21	1	4.76
- Myna (*Acridotheres tristis*)	30	0	0
- Red whiskered Bulbul (*Pycnonotus jocosus*)	3	0	0
Chicken (*Gallus gallus*)	91	10	10.99
total	265	18	6.79

Table 9 Nucleotide sequencing analyses of PCR products.

Code	Source	Location	Percent of sequence identity to *H. capsulatum* 100kDa protein
CD1/1	Bat dropping	Chiang Dao Cave, Chiang Mai	97
CD2/2	Bat dropping	Chiang Dao Cave, Chiang Mai	97
CD2/4	Bat dropping	Chiang Dao Cave, Chiang Mai	96
CD3/1	Bat dropping	Chiang Dao Cave, Chiang Mai	95
PD3/1	Bat dropping	Phadang Cave, Chiang Mai	94
PD3/2	Bat dropping	Phadang Cave, Chiang Mai	98
PD3/5	Bat dropping	Phadang Cave, Chiang Mai	97
CPA7	Pigeon	Hangdong, Chiang Mai	99
CK3	Chicken	Sanpeesua farm, Chiang Mai	98
CK5	Chicken	Sanpeesua farm, Chiang Mai	97
CK7	Chicken	Sanpeesua farm, Chiang Mai	98
CK8	Chicken	Sanpeesua farm, Chiang Mai	98
CK10	Chicken	Sanpeesua farm, Chiang Mai	98
CK17	Chicken	Sanpeesua farm, Chiang Mai	98
Ck20	Chicken	Sanpeesua farm, Chiang Mai	98
CK22	Chicken	Sanpeesua farm, Chiang Mai	98
CK30	Chicken	Sanpeesua farm, Chiang Mai	98
CK34	Chicken	Sanpeesua farm, Chiang Mai	98