CHAPTER 6
DISCUSSION

Although cryptococcosis is the third most common opportunistic infection among HIV positive patients in northern Thailand, little is known about the epidemiology of this diseases. In Chiang Mai, Tharavichitkul et al (1973) isolated *C. neoformans* from dove (*Streptopelia* spp. and *Geopelia* sp) and pigeon (*Columba livia*) excreta which were collected in the area of Amphoe Muang Chiang Mai. Both dove and pigeon excreta were found to harbor this yeast in high percentages (69.40% of dove and 57.14% of pigeon excreta). During 1999 to 2000, *C. neoformans* were isolated from environmental sources collected in various areas of Chiang Mai by isolation on Sabouraud dextrose agar and Littman's oxgall agar. *C. neoformans* was isolated from 45% (45 /100) of dove excreta, 16% (9 /55) of pigeon excreta and 0.9 % (2/ 230) of eucalyptus flower samples (Sriburee et.al, 2004). Recently, during 2005 to 2006, *C. neoformans* was isolated from avian droppings collected in Chiang Mai area and from Chiang Mai Zoo. Using the isolation on L-dopa agar plates, *C. neoformans* was isolated from 26.2% (16/61) of pigeon droppings, 20.0%(2/10) of dove droppings, 0.5% (1/189) of chicken droppings and 1/9 (11.1%) of the red-billed hornbill droppings (Keerativasee et al, 2008).

In this present study, *C. neoformans* could not be isolated from all soil contaminated bat and avian dropping or even though in pigeon dropping samples by using culture method on L-Dopa agar. The reason why this organism could not be isolated from soil samples contaminated with pigeon droppings may be not so many

samples (only 21) were collected. In addition, most samples were also collected from outdoor sites , it is the fact that direct sunlight can significantly reduce the survival of cryptococci in soils (Isaq, et al, 1968) and may account for the fact that pigeon excreta from outdoor sites are less heavily contaminated than pigeon excreta from indoor sites (Hubalek, 1975; Littman and Borok, 1968). Denton and Di Salvo (Denton and Di Salvo, 1968) demonstrated that sheltered environmental locations are more likely to be culture positive for *C. neoformans* than are locations exposed to sunlight. Although soil contaminated chicken droppings were previously reported as the saprophytic source of *C. neoformans* (Kuroki et al., 2004), the absence of this fungus was shown in this study. This may be because the growth of *C. neoformans* was inhibited in chicken dropping by a combination of high pH and the presence of thermostable low- molecular- weight substances (Walter and Yee, 1968).

Since the nested PCR may be more useful and sensitive for *C. neoformans* detection in environmental sources than the culture isolation, all soil contaminated with bat and avian droppings were used to detect this organism by using primers specific for a gene encoding 18s rRNA of the fungus. Only one sample (soil contaminated with myna droppings) was positive for *C. neoformans* by nested PCR, this sample was negative by culture method. Although nested PCR is more sensitive method than culture isolation, breakdown products of heme, such a bilirubin, as well as bile salt can inhibit PCR in sample containing feces (Widjojoatmodjo et al., 1992). In addition, humic substances are also interfering with the PCR. Humic substances, mainly humic acid and fulvic acid, are commonly found in aquatic, soil, and sediment environments. As such, humic substances are ubiquitous in soil and water and can contaminate any materials exposed to the environment (Tsai and Olson, 1992a). It

has been reported previously that trace amounts of humic substances can inhibit the PCR and cause false-negative results (Tsai and Olson, 1992b).

The conventional method for isolation and identification of *H. capsulatum* in environmental samples are mice inoculation (Emmons, 1949). Unfortunately, the method is slow (approximately 2 months) and expensive. In 1999, two-stage PCR methods have been developed to detect *H. capsulatum* in soil suspension (Reid and Schafer, 1999). The two-stage PCR protocol was followed employing both fungal-specific primers and nested primers specific for the internal transcribed spacer (ITS) region of the 5·8S rRNA gene of *H. capsulatum*. The estimated limit of detection of this method is 10 spores. In this study, a nested PCR assay using primers specific for a 100 kDa-like protein gene of *H. capsulatum* as described by Bialek et al (2002) was used to detect this fungus in soil contaminated with bat guano and avian droppings. The result revealed that only DNA of *H. capsulatum* was positive with these primers, whereas negative results were shown in other fungi and bacteria. This indicated that the primers used in this study are specific to *H. capsulatum*. In addition, the sensitivity of *H. capsulatum* detection using this nested PCR assay was limited to 2,000 cells per sample which was less sensitive comparing to previous study (Reid and Schafer, 1999). This result may be explained by the fact that humic substances, which can inhibit PCR as described above. Bovine serum albumin (BSA) is also known to bind lipids via hydrophobic forces and anions by virtue of its high lysine content (Loomis, 1974). Therefore, BSA may be able to scavenge a variety of substances and thereby prevent their binding and inactivation of *Taq* DNA polymerase (Kreader, 1996). BSA was also added to PCR to relieve inhibition from samples containing endogenous protease activity in previous study. Furthermore,

they used primer targeting specific for the internal transcribed spacer (ITS) region of the 5·8S rRNA gene of *H. capsulatum*, which have multiple copies within a single genome. However, ribosomal genes are conserved regions bearing the risk of nonspecific amplifications (Bialek et al., 2000).

In Thailand, there has never been reported of environmental sources of *H. capsulatum* and this is the first study to detect environmental sources of this organism in Chiang Mai or in Thailand by using molecular technique. Surprisingly, 18 of 265 (6.79%) soil samples were positive for *H. capsulatum* and nucleotide sequencing analyses of PCR products showed 94 to 99 % identity of genes encoding 100 kDa-like protein of *Ajellomyces capsulatus*. Bat species inhabited in Chiang Dao cave were *Hipposideros armiger* and *Aselliscus stoliczkanus* and species inhabited in Pha Dang cave were *Hipposideros lylei*, *Myotis chinensis*, *Taphozous melanopogon*, *Hipposideros armiger* and *Aselliscus stoliczkanus.* The results indicate the association of soil contaminated bat and avian droppings especially and *H. capsulatum* in this area of Thailand. Moreover, soil contaminated with chicken droppings may be more likely to be the natural reservoir of this fungus in Chiang Mai since bat inhabiting caves are far apart from Chiang Mai city. The nested PCR is rapid and useful method for the detection of *H. capsulatum* in natural reservoirs.