VI. DISCUSSION

Genetic differences among *C. neoformans* strains have been detected by several typing methods, including restriction fragment length polymorphism (RFLP) analysis (Currie et al., 1994 ; Dromer et al., 1994 ; Varma et al., 1995), electrophoretic karyotyping (Dromer et al., 1994 ; Perfect et al., 1993), allele sequencing (Casadevall et al., 1992), multilocus enzyme electrophoresis (Brandt et al., 1995). These techniques are time-consuming and not readily adaptable in the clinical laboratory for epidemiologic purposes. Recently, a method was developed to discriminate between strains of this pathogenic yeast by amplifying polymorphic DNA with the polymerase chain reaction (PCR) using arbitrarily chosen oligonucleotide primers or random amplified polymorphic DNA analysis (RAPD) (Mitchell et al., 1994a ; Yamamoto et al., 1995). Optimization of RAPD for typing of *C. neoformans* strains would have several advantages over other molecular methods, including speed, cost, ease of performance, sample volume throughput, and a requirement for little specialized equipment. Khayhan (2001) studied 132 *C. neoformans* isolated from clinical and environmental sources in Chiang Mai for their serotype and PCR-fingerprints by using $(GACA)_4$ as a primer. All clinical and environmental isolates were identified as *C. neoformans* var. *grubii* serotype A, except one isolate belonged to serotype AD. Most clinical and environmental isolates (130 of 132), including one isolate of *C. neoformans* serotype AD, showed the identical PCR-fingerprinting pattern (designated as profile AI). Profile AII was observed in two environmental isolates. Fewer variations of PCR-fingerprinting profiles in his study might be due to the fact that isolates from these restriction areas in Chiang Mai were less heterogeneous. On the other hand, using only one primer may not be enough to discriminate among these isolates. Using the random amplified polymorphic DNA (RAPD) with the four primers, Passo et al. (1997) reported the genetic relatedness of clinical and environmental *C. neoformans* strains in the Maltese Islands of Italy. The clinical strains isolated over the period of 1 year from AIDS patients showed identical fingerprints. The electrophoretic patterns of the two clinical

strains were also the most common patterns among the environmental strains, but the patterns among the environmental strains showed a wide variability and no correlation with the site of isolation. Yamamoto et al. (1995) used RAPD profile analysis with three primers that revealed six patterns among 21 clinical isolates and three patterns among 8 environmental (pigeon excreta) isolates in the southern Japanese prefecture of Nagasaki. Two environmental isolates from two locations associated strongly with two patients revealed identical RAPD patterns for isolates from each patient. These results suggested that clinical and environmental isolates belonged to the same pool of *C. neoformans* isolates and that these isolates had certain geographic locations, although the number of isolated strains was limited. Thus, analysis by using three arbitrary primers may reveal variable profiles for better discrimination on strain typing of *C. neoformans*.

In this study, the RAPD analysis used the method of Yamamoto et al. (1995) with some modifications of the amplification program, and it was able to discriminate isolates of *C. neoformans* serotype A in Chiang Mai. Because of the genetic difference of isolates from various geographical areas, the amplification products of each primer yielded different electrophoretic profiles from the results of Yamamoto et al. The RAPD profiles obtained with the three primers revealed five patterns (pattern I, II, III, IV and V) among 112 isolates. For isolates belonged to *C. neoformans* serotype A, three patterns (pattern I, II and III) were found among 50 clinical Chiang Mai isolates, two patterns (pattern I and II) among 50 environmental Chiang Mai isolates. One eucalyptus (*Eucalyptus deglubta*) flower sample in Chiang Mai was positive for *C. neoformans* var. *grubii*, no *C. neoformans* var. *gattii* was found in the previous study (Khayhan, 2001). The presence of *C. neoformans* serotype A in eucalyptus sample suggests the possibility of contamination of bird droppings, since only *C. neoformans* var. *gattii* has been reported as associated with eucalyptus material (Ellis and Pfeiffer, 1990 ; Pfeiffer and Ellis, 1992). This result is similar to the previous study in Maltese, Italy by Passo et al., 1997 that *C. neoformans* var. *neoformans* was isolated from flowers of *E. camaldulensis*. They concluded that the presence of *C. neoformans* serotype A in the eucalyptus samples might be due to contamination from the avian dropping. This isolate also produced an identical RAPD pattern (pattern I) to isolates from bird excreta. In Chiang Mai, pigeons

(*Columba livia*) are found around temples, schools and parks, while doves (Spotted dove, *Streptopelia chinensis* ; Zebra dove, *Geopelia striata* and Collared dove, *Streptopelia decaocta*) are usually domestic birds. Identification of genetically related clinical and environmental isolates indicates a link between environmental (dove and pigeon excreta) sources and cryptococcosis in this area of Thailand. In addition, the most common pattern (pattern I) was also found among 8 clinical Khon Kaen isolates while the pattern II was found among 2 clinical Japanese isolates. One isolate from Khon Kaen (K38) produced pattern IV, since it belonged to serotype B. Two untypeable isolates of the same province (K25 and K97) produced patterns V and I, respectively. The other patterns such as patterns III, IV and V were not evaluated because of the small number of isolates, but it would be meaningful if more isolates could be collected in the future. It is interesting that RAPD patterns of 8 of 10 clinical Khon Kaen isolates and 2 clinical Japanese isolates were identical to Chiang Mai isolates, pattern I and II, respectively. These results demonstrate local geographic differences in the molecular epidemiology of *C. neoformans* in Chiang Mai. The results also suggest that there may be widespread global distribution of certain pathogenic *C. neoformans* strains of serotype A.

The reproducibility of the RAPD profile is important. Thus, reproducibility of this method was confirmed by subculturing the original isolate three times and repeating the DNA isolation and PCR amplification. However, reproducibility was assessed by PCR amplification on two different occasions. When the fragments that were produced were less intense, they were not assumed to be pattern-discriminant elements.

Pigeon excreta are the saprophytic source most commonly associated with *C. neoformans*, but the fungus has also been isolated from a variety of other avian excreta, including that of dove (Tharavichitkul et al., 1973), canaries (Griseo et al., 1995), parrots (Lopez-Martinez and Castanon-Olivares, 1995), chickens (McDonough et al., 1961 ; Swinne et al., 1986), and other species. Although cryptococcosis is one of the most common opportunistic infection among AIDS patients living in Chiang Mai, there was no information on the environmental sources of the disease. Thus, a study on the domestic environment of cryptococcosis patient is also set up to determine sources of *C. neoformans* around the patients' homes area. In this study, *C.*

neoformans were isolated from only 2 (0.99 %) of 202 of avian dropping, including pigeon, dove, chicken, duck, goose, Hill Myna and Budgerigars. Of these two isolates, one was recovered from samples collected from dove dropping, the other was recovered from samples collected from pigeon dropping, whereas none of the other species samples yielded isolates of the fungus, particularly, chicken droppings which were collected from all of the patients' homes in Chiang Mai. Failure to isolate *C. neoformans* from chicken droppings may be due to the inhibitory nature of chicken droppings. This result is similar to the previous study, in 1962 a pilot comparative study was undertaken for the isolation of *C. neoformans* in pigeon and chicken habitats. The fungus was recovered from 14 of 75 pigeon fecal samples while no isolations were made from 277 samples of chicken droppings. Also in 1962, Fragner reported that *C. neoformans* was recovered only from pigeon habitats in a comprehensive study on the habitats of 15 different avian species. Staib (1963) reported of evidence showing that pigeon manure may be an enrichment medium for *C. neoformans*. All *Cryptococcus* species and other yeasts tested were able to assimilate urea, uric acid, xanthine and guanine which were present in the dropping. However only *C. neoformans* could assimilate creatinine, also a constituent of pigeon dung. These findings do not explain the rare occurrence of *C. neoformans* in chicken droppings. In 1968, Walter and Yee reported that infusions of field-collected pigeon droppings were shown to be excellent media for the growth of *C. neoformans*. In contrast, infusions of field-collected chicken droppings inhibited the growth of the fungus. Failure in attempts to isolate *C. neoformans* from chicken habitats may be due the result of high alkalinity and the presence of a low molecular weight, thermostable growth-suppressing substance (s). Under natural conditions, alkalinization of pigeon droppings did not occur. This latter factor together with the growth supporting substances in pigeon excreta may explain the high prevalence of *C. neoformans* in pigeon habitats.

The present study obtained two environmental isolates (Do 3/2 and Pge 12/2) from two locations strongly associated with isolates from two patients (Cne 3 and Cne 12). These isolates were analyzed by RAPD with three primers. The RAPD patterns of Cne 3 and Do 3/2 were identical (pattern II). This may suggest a relationship between clinical and environmental isolates. In contrast, the other isolates (Cne 12

and Pge 12/2) revealed different in RAPD patterns. This may suggest a different and as yet unknown source of infection for this patient. In fact, fungus may be spread by air currents, and, perhaps, by rodents, cockroaches, acarids and insects (Swinne et al., 1986), it is possible to suppose that heavily contaminated places in the vicinity of a house favour domestic contamination by *C. neoformans*. Further epidemiologic studies should be carried out to investigate new domestic environment sources of cryptococcosis patients living in Chiang Mai.

In conclusion, the results of the RAPD analysis suggest that most clinical and environmental isolates belong to the same pool of *C. neoformans* isolates. The relationship between environmental sources and cryptococcosis is shown in this area of Thailand. Immunocompromised patients especially HIV-infected patients should be avoid keeping pet birds or exposing to contaminated sites.