CHAPTER VI
DISCUSSION

The preliminary study of *Cryptococcus* isolates from pigeon droppings from Bangkok area in molecular level has never been reported. In this study, the encapsulated yeast, *C.neoformans* was isolated with the rate of 8.9% (30 samples of 336 samples) from pigeon droppings. The positive percentage of cryptococcal isolates from pigeon droppings is different in individual countries and geographical region such as 175/983 (17.8%) (Northern Iran in 1997) (50), and 29/634 (4.6%) (Turkey in 1998) (51).This phenomenon was corresponded to this study (table 2). The predominant district was demonstrated in Sampantawong district. Wats in Sampantawong; Wat Bopitpimuk, Wat Trimitt, and Wat Kusonsamakorn have been showed highly positive percentage of Cryptococcal isolates,75% (6/8), 75% (3/4), and 100% (4/4), respectively. It was shown that this appearance may be due to the contamination of *C.neoformans* which attached the beak and foot of the pigeons to their droppings. Another possible factor was the pigeons who carrying *C.neoformans* in their intestinal tract migrated from one place to another place, introducing the distribution of *C.neoformans* to the various areas (52). Pigeon droppings allowed long-term survival of *C.neoformans*, and cultures have been demonstrated to remain viable for almost 2 years when maintained on desiccated pigeon droppings, and the fact that viable cryptococci 1 to 5.5 μm have been shown in the pigeon dropping, these cryptococci sizes can pass from bronchial trees to alveolar and deposit in alveolar (1, 52). In this study all isolated *C. neoformans* were recovered from dry pigeon droppings that was corresponded with the other studies. It implied that dry pigeon droppings were more likely to be suitable substrate for *C. neoformans* than fresh droppings (52).In this study, 20 out of 336 fresh pigeon droppings sample were collected and no encapsulated yeast was isolated. The reason might be the alkalization (ammonia production) was produced from bacteria in the droppings , resulting the growth inhibition of *C. neoformans* (13,18). In addition, it was observed that the color of the droppings did not show any correlation with the presence of the yeast. Another observation of the

sunlight exposure was not the significant factor for isolation of *C. neoformans*. This result was contrast to other studies which showed that sheltered environmental locations were more likely to be culture positive for *C. neoformans* than were locations exposed to sunlight (52). Interestingly, Wat Bopitpimuk has many trees, which related to high number of the isolated yeast. Only one *C. neoformans* var *gattii* was recovered from Pomprab Satrupai district probably due to contamination from the environment. It is noted that, most of the cryptococcal isolates in this study were recovered from the crowded areas and many building especially in Sampantawong, Yannawa, Ladpraw, and Pasri Jaroen districts. Probably the crowded building and the living style of the community, feeding the bird, might play the important role as their habitat.

It was found that VNI and VNII (*C.neoformans* var. *grubii*, serotype A) was the predominant serotype (98.2%). This result was corresponded many studies that showed the majority of serotype A from both clinical and pigeon dropping isolates (10, 15, 16, 53). From the result of many studies in clinical and environmental isolates, they concluded that *C.neoformans* var. *grubii* (serotype A) was the most common serotype distributed throughout the world (13,15,16,34,35,54,55). The distribution of any variety or serotype differs in their ecological and geographical regions. In India, patient showed serotype A and B to be present, respectively, in 92%, and 8% of 36 patients (54). Serotype D distributed around France but *C. neoformans* var *gattii* (serotype B and C) distributed limit in tropical and subtropical regions. In addition to differences in the distribution of the varieties, variation in pathogenicity has been reported (56).

From the results in this study, VNI (var. *grubii*, serotype A) was the predominant molecular type, accounting for 94.3% (n=50) of all isolates followed by VNII (var. *grubii*, serotype A) 3.8% (n=2) and *C. neoformans* var *gattii* 1.88% (n=1). Imwidthaya and Pongvarin in 2000(5) studied *C .neoformans* from AIDS patients in Siriraj hospital, Bangkok, by using M13-RAPD analysis. They found serotype A (99%) and 1% serotype B (23) that were in accordance with the results from this study and the other studies (16, 55). Meyer *et al* (2003) (16) showed that the majority of the cryptococcal isolates from patients and environment in IberoAmerican countries, 68.2% (n=232), were VNI (var. *grubii*, serotypeA). A smaller proportion, 5.6% (n=19), were VNII (var. *grubii*, serotype A); 4.1% (n=14), VNIII (AD hybrid) ; 1.8% (n=6), VNIV (var. *neoformans,* serotype D),

and 20.3% (n=69) (*C. neoformans* var *gattii*). Casali *et al* (2003) (15) found predominant VNI from clinical and environmental isolates in Brazil (89.5% and 52.6%) that accorded studied of *C. neoformans* from Tamil Nadu in India by Kid *et al*(2002) (55). They found that VNI was the predominant molecular type, comprising 14 of the 17 isolates (82.3%). The fact that VNI was the predominant molecular type in this study was corresponded to the other studies that studied molecular type in cryptococcosis patient, and the results revealed high VNI molecular type. It may be concluded that the pigeon were thought to provide a major reservoir of *C. neoformans* (13, 15, 57).The recovery of *C. neoformans* from pigeon droppings in this study accorded with the other reports (9,15,50,51) that showed that *C. neoformans* from pigeon droppings revealed the molecular pattern similar to *C. neoformans* from cryptococcosis patient. The size of *C. neoformans* from desiccated pigeon droppings, Highly number of cryptococcal isolates from pigeon droppings, and the similarity of molecular patterns between patient and pigeon droppings can be implied that pigeon droppings are the potential sources of cryptococcosis in human. In addition, *C. neoformans* in pigeon droppings can transmitted to the patient by inhalation (1, 37, 40, 52). The recommendation from several authorities suggested that immunosuppressed patients should avoid pigeons and pigeon dropping contaminated areas because Cryptococcus can transmitted by inhalation of aerosolized organisms (52, 58).

The RAPD analysis by M13 clearly distinguished variation among strains of *C. neoformans* at species level, variety level and individual level (13, 56). There were difference among the major bands of VNI, VNII, VNIII, and VNIV. VNI differed from VNII due to VNII does not bear the size of 1,787 and 1,569 bp, and differed from VNIV due to VNIV revealed the bands higher than 1,787 bp. The genetic diversity of pigeon droppings isolate showed greater variation within VNI type. This diversity was revealed by using M13-RAPD, resulting the strain variation. Each *C. neoformans* strain could have different in virulence or different response to therapy or high affinity for particular population at risk for infection (48, 52). In addition to RAPD, RFLPs of *C. neoformans* by Currie *et al* (59) showed extensive genetic diversity among clinical and environmental isolates, and showed similarity between two strains from clinical and environmental isolates. The identification of the same strain in clinical and environmental isolates

showed in many reports such as Horta *et al* (2002), Yamamoto *et al* (1995), Sriburee *et al* (2004), Casali *et al* (2003), Meyer *et al* (2003), Sorrell *et al* (1996) etc. (6, 9, 13, 15, 16, 60) supported that the Cryptococcus in human derived from environmental reservoir. In addition to M13, $(GTG)_5$ and $(GACA)_4$ are microsatellite single primers that can amplify hypervariable repetitive DNA sequences in *C. neoformans* and can generate polymorphic bands (52). Although RAPD by M13 is reliable method, RAPD is very sensitive PCR technology for strain identification in *C. neoformans.* It is essential to control every step. Meyer *et al* (2003) (16) and Casali *et al* (2003) (15) used M13 as single primer, can separated *C. neoformans* into 8 molecular types but the pattern of the band are not the same (the reference standard strain in Meyer and Casali reports are the same strain). This evidence revealed that RAPD using M13 as a primer may be showed the variation of the minor bands, based on the reaction condition and model of thermal cycle in individual laboratories. This is disadvantage of RAPD. It is possible that similar sized fragment may not be homologous because RAPD need to use highly standardized experimental procedures because of sensitivity to reaction condition (61).

It is surprising that *C. neoformans* var. *gattii* was found in pigeon dropping because it usually was isolated from animal, Eucalyptus species, or variety of tree species in tropical and subtropical areas. In our knowledge, it is the first time that *C. neoformans* var. *gattii* was found in pigeon dropping in Thailand. Studies by Kidd *et al* (2004) (62), they found a rare genotype of *C. neoformans* var. *gattii* caused the Cryptococcus outbreak on Vancouver Island in Canada. They found predominant VGII from all environmental isolates and almost clinical and veterinary isolates. Their results are very interesting because temperature in Vancouver Island is different from tropical and subtropical areas, including Thailand.

From our results demonstrated local geographic differences in the molecular epidemiology of *C. neoformans* that could result from natural selection by specific environmental at individual geographic sites. It is interesting that what is the reason that Wats in Samphantawong have been showed *C. neoformans* in the pigeon droppings. In addition, pigeons live in Wats in Samphantawong are the same flock or not.

In the future study, *C. neoformans* isolates from pigeon droppings will be examine the correlation with *C. neoformans* from patients in Bangkok. The extensive

study, *C. neoformans* will be defined the mating types of them because mating type is important for determination the relationship between *C. neoformans* from pigeon droppings and *C. neoformans* from patients because from many reports (3, 21, 52, 53), the most specimen from AIDS patients were α-mating type which is the major type in the environment that can found in pigeon droppings.