CHAPTER VII

SUMMARY

1. A total of 190 *C. neoformans* isolates, including 166 clinical and environmental isolates from Chiang Mai province and 24 environmental isolates from Nan province, were tested for their susceptibilities to amphotericin B, fluconazole, itraconazole and ketoconazole. The MICs were determined by using the standard NCCLS broth microdilution methods (M27-A2). All 190 isolates were susceptible to four antifungal drugs tested. The MICs50 and MICs90 of amphotericin B, fluconazole, itraconazole and ketoconazole were 0.5 and 1 µg/ml, 2 and 4 µg/ml, 0.03 and 0.06 µg/ml and 0.06 and 0.125 µg/ml, respectively. The results indicated that most of *C. neoformans* clinical and environmental isolates in northern Thailand were susceptible to amphotericin B, fluconazole, itraconazole and ketoconazole. No resistant strains of fluconazole were found.

2. To investigate the presence of fluconazole heteroresistance among these isolates, a total of 14 isolates of *C. neoformans* for which the MICs of fluconazole ranged from 8 to 16 µg/ml were selected to screen. Fluconazole-heteroresistance was characterized by the ability to grow at 30°C on agar containing 64 µg of fluconazole/ ml. Of the 14 *C. neoformans* isolates tested, one clinical isolate (CN4969) exhibits heterogeneity in fluconazole resistance. One subpopulation of this isolate (CN4969HR) which grew at 30°C on agar containing 64 µg of fluconazole/ ml exhibits fluconazole resistant phenotype (MIC ≥ 64 µg/ml by broth microdilution method and ≥ 256 µg/ml by E-test). This CN4969HR could not grow at 37°C on agar containing 64 µg of fluconazole/ml. Another subpopulation (CN4969S) exhibits fluconazole susceptible phenotype (MIC = 8 µg/ml by both broth microdilution method and E-test). The prevalence of heteroresistant phenotype among northern *C. neoformans* isolates was 7.1% (1 of 14 isolates). This study demonstrated the existing of the fluconazole heteroresistant population among clinical isolates of *C. neoformans* in northern part of Thailand and the expression of heteroresistant phenotype was influenced by temperature.

3 The representative fluconazole susceptible isolates (H99; standard strain of *C. neoformans* serotype A, CN4901; one of clinical isolates, CN4969S; subpopulation of CN4969 and fluconazole resistant isolate (CN4969HR) were determined their serotype and mating type by PCR using STE20Aα primer. This primer amplified a 588 bp DNA fragment from H99, CN4901, CN4969S and CN4969HR strains. Thus, these isolates belonged to serotype A and *MAT*α or *C. neoformans* var. *grubii*.

4. Sequences of the *ERG11* gene of H99, CN4901, CN4969S and CN4969HR were analyzed to determine point mutations. The DNA fragment of 2,268 bp containing the full length *ERG11* genomic sequence from each isolate was obtained by PCR amplification. The coding sequences of the entire *ERG11* genes encoding 547 deduced amino acids consisted of 1,644 bp in length. Alignment of deduced amino acid sequences showed the presence of the G484S amino acid substitution in lanosterol 14α-demetylase in CN4969S and CN4969HR comparing with H99, CN4901 and other fungi. This result indicated that the amino acid substitution may play a role in fluconazole resistance. It also supports the possibility that all cells in the heteroresistant population carry the genetic marker(s) for resistance, but phenotypic expression of such resistance occurs in a very small fraction of the population.

5. Analysis by the RT-PCR showed an increase at least 2-fold in both *ERG11* and *MDR1* expression levels in fluconazole-resistant (CN4969HR) comparing with susceptible isolates (H99, CN4901 and CN4969S) but *AFR1* gene expression level in CN4969HR was equivalent to the expression in H99 or CN4901 or CN4969S. This result indicated the overexpression of *ERG11* and *MDR1* genes in the resistant isolate. By using northern blot analysis, gene expression level in *ERG11*, *AFR1* and *MDR1* of H99, CN4901, CN4969S and CN4969HR could not be detected. Due to the fact that these drug resistant genes may produce low-abundance mRNAs and the sensitivity of northern blot analysis was lower and lesser than RT-PCR.

6. Analysis of point mutation and RT-PCR suggest that molecular mechanisms involving drug efflux and alterations in the structure or cellular amount of lanosterol 14α- demethylase may play a role in the resistance to fluconazole.