Full Length Research Paper

Heteroresistance to fluconazole among isolates of *Cryptococcus neoformans* in Northern Thailand

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We had determined that 190 *Cryptococcus neoformans* isolates (104 clinical and 86 environmental isolates from northern Thailand) were all susceptible to amphotericin B, fluconazole, itraconazole and ketoconazole. Of 14 selected isolates, one clinical isolate (CN4969) exhibited heterogeneity in fluconazole resistance, which produced two subpopulations of the fluconazole-resistant (CN4969HR) and -susceptible (CN4969S) subpopulations. To clarify the molecular mechanisms of fluconazole resistance, the expression of drug resistance genes was determined by using reverse transcription-polymerase chain reaction (RT-PCR). Both the *ERG11* and *MDR1* transcript levels showed at least a 2-fold increase in CN4969HR isolate compared to the fluconazole-susceptible isolates (H99, CN4901 and CN4969S), whereas the expression level of the *AFR1* gene in CN4969HR was equivalent to that of isolates H99, CN4901 or CN4969S. Collectively, this study demonstrates the existing of the fluconazole heteroresistant population among clinical isolates of *C. neoformans* in northern part of Thailand and molecular mechanism of fluconazole-susceptible and resistant isolates.

Key words: Heteroresistance, fluconazole, *C. neoformans*, Thailand.

INTRODUCTION

Cryptococcus neoformans is the etiologic agent of cryptococcosis in both immunocompetent and immunocompromised hosts, especially in patients infected with HIV. In Thailand, epidemiological data obtained from 1984 to 2008 by the bureau of epidemiology, Department Of Disease Control, Ministry of Public Health revealed 49,223 (12.72 %) cases of cryptococcosis, which was the second most common opportunistic fungal infection in Thai AIDS patients after *Pneumocystis carinii* pneumonia (PCP) (Bureau of

Epidemiology, 2008). In general, amphotericin B 0.7-1.0 mg/kg/day for two weeks and fluconazole 400 mg/day for 8 weeks are used for the primary treatment in Thai AIDS patient for cryptococcal meningitis. After completion of primary treatment, fluconazole 200 mg/day has been used until immune reconstitution occurs after antiretroviral therapy (Mootsikapun, 2004; Tansuphaswadikul et al., 2006).

Fluconazole-resistant strains of *C. neoformans* were reported by several investigators and strains of *C.*

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neoformans expressing heteroresistance to fluconazole were described (Armengou et al., 1996; Bii et al., 2007; Mondon et al., 1999; Paugam et al., 1994; Sionov et al., 2009; Yamazumi et al., 2003). Although cryptococcosis is still an important public health problem in northern Thailand, there is no information on the *in vitro* antifungal susceptibility of *C. neoformans* isolated in this area. The purpose of this study was i) to determine the *in vitro* susceptibilities of clinical and environmental isolates of *C. neoformans* to fluconazole, amphotericin B, itraconazole and ketoconazole ii) to investigate the presence of heteroresistant strain of *C. neoformans* among these isolates and iii) to compare the expression levels of the *ERG11*, *AFR1* and *MDR1* genes between fluconazole-resistant and fluconazole-susceptible subpopulations of the heteroresistant clone.

MATERIALS AND METHODS

Identification of *C. neoformans* from clinical and environmental samples

A total of 190 *C. neoformans* isolates which comprised of 104 clinical and 62 environmental isolates, was collected in Chiang Mai during 1999 - 2005 and 24 environmental isolates were collected in Nan province in 2005. All clinical isolates were obtained from Central Laboratory, Maharaj Nakorn Chiang Mai hospital and Nakornping hospital, Thailand. Identification was confirmed by microscopic morphology, India-ink preparation, melanin pigment production on L-dopa agar, urease production, carbon assimilation and fermentation tests.

Broth microdilution susceptibility testing

The *in vitro* susceptibility to fluconazole, amphotericin B, itraconazole, ketoconazole of *C. neoformans* isolates was performed by the broth microdilution method according to NCCLS M27-A2 guidelines of the CLSI (formerly the National Committee for Clinical Laboratory Standards, 2002). Amphotericin B, itraconazole and ketoconazole were dissolved in dimethylsulfoxide (DMSO) (all from Sigma, St. Louis, USA), whereas fluconazole was dissolved with distilled water. Further dilutions of each antifungal agent were prepared with RPMI 1640 medium (Sigma) containing L-glutamine without sodium bicarbonate, and buffered to a pH 7.0 with 0.165 M morpholinopropanesulfonic acid, (MOPS), (Sigma). The suspension of 48-h-old cultures of yeast was prepared in sterile saline (0.85%) adjusted with a spectrophotometer to a cell density of 0.5 McFarland standard at a wavelength of 530 nm. This suspension was diluted at 1:50 followed by a 1:20 dilution in RPMI 1640 in order to obtain a final concentration of 1 to 5 x 10^3 CFU/ml. Microtiter plates were added with 100 µl of different concentrations of antifungal agents and 100 µl of the yeast suspension. The final concentration of inoculum was in the range of 0.5 to 2.5 x 10^3 CFU/ml. Plates were incubated at 37°C and read after 72 h. The interpretative criteria used for susceptibility to amphotericin B, fluconazole, itraconazole and ketoconazole were those of NCCLS (M27-A2). For fluconazole, isolates showing MICs ≤ 8 µg/ml were regarded as susceptible (S), 16-32 µg/ml as susceptible dose dependent (S-DD) and ≥ 64 µg/ml as resistant (R). Quality control organisms *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used to check the accuracy of the drug dilutions and the

reproducibility of the tests.

Screening of *C. neoformans* fluconazole heteroresistance

The screening method for identifying fluconazole-heteroresistant isolates was performed as described by Yamazumi et al. (2003). Briefly, cell suspensions (1 - 5 × 10^3 CFU/ml) of the isolates for which fluconazole MICs were 8-16 µg/ml were plated on PDA plates containing 64 µg of fluconazole/ml. Plates were incubated at both 30 and 37°C. The growth pattern was read after 72 h of incubation. Fluconazole heteroresistance was characterized by the ability to grow at 30°C but not at 37°C on PDA containing 64 µg of fluconazole/ml. The fluconazole-resistant and -susceptible subpopulations were isolated from the heteroresistant clone. Both the reference broth microdilution and E test methods were used to confirm the presence of *C. neoformans* fluconazole-resistant and -susceptible subpopulations. Finally, the *C. neoformans* fluconazole-resistant subpopulation was passaged in drug-free medium to determine the stability of resistance.

The E test was carried out according to the manufacturer's instructions (AB BIODISK) using RPMI 1640 (Gibco, Invitrogen Corporation, UK) supplemented with 1.5% agar and 2% glucose. Inocula were prepared from 24 h culture of *C. neoformans* and inoculated onto RPMI agar. Plates were dried at room temperature for 15 min before E test strips were applied. The plates were incubated at 35°C for 48 h. The minimum inhibitory concentrations (MICs) were read at the intersection (at the point of approximately 80% growth inhibition for fluconazole) of the zone edge and the E test strip. *C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028 were used as quality control strains.

Serotype and mating type identification

Genomic DNA was extracted by the method described by Del Poeta et al. (1999). To identify serotype and mating type of fluconazole-resistant and fluconazole-susceptible *C. neoformans* isolates, the polymerase chain reaction (PCR) method was performed using specific primers for *STE20* genes (STE20 Aα, STE20 Aa, STE 20 Dα and STE 20Da (Yan et al., 2002).

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

The yeast cells were grown to logarithmic phase in 250-mL Erlenmeyer flasks containing 50 ml of YEPD (1% yeast extract, 2% peptone and 2% dextrose) at 30°C in a shaking incubator at 150 rpm for 24 h. Fungal cells were harvested by centrifugation at 13,000 x *g* for 2 min. Total RNA was isolated from *C. neoformans* cells by mechanical disruption method using glass beads according to RNeasy mini kit (Qiagen GmbH, Germany). The expression levels of *ERG11, AFR1* and *MDR1* mRNAs from the fluconazole-resistant subpopulation were compared with those of the fluconazole-susceptible subpopulation and other representative fluconazole-susceptible isolates by RT-PCR. Using the Qiagen two-step RT-PCR Kit (Qiagen GmbH, Germany), total RNA (2 µg/reaction) in the presence of random hexamer (Gibco, BRL), oligo dT 18 primers and Omniscript Reverse Transcriptase was incubated at 37°C for 1 h. The resulting cDNAs of each gene were amplified using the specific primers as follows: *ERG11* (FERG2:5'-CCTATGATCACCAGCGAATG-3' and RERF2:5'-GGTCCTCGTATCTGTAGTCTCG-3'); *AFR1* (FAFR1:5'-CTCTTATCGTCGGCTCTGTT-3' and RAFR1:5'-GGTGTAGCTCGAGTCACAAG-3'); *MDR1* (FMDR1:5'-TCGCAGGTGAGGACAAGATAC-3' and RMDR1:5'-CTGTCTTAACGGCTCCAG-3'). The *C. neoformans* var. *grubii*

Table 1. *In vitro* antifungal susceptibilities of 190 isolates of *Cryptococcus neoformans* to amphotericin B, fluconazole, itraconazole and ketoconazole by broth microdilution method.

Antifungal drug concentration (μg/ml)	Number of susceptible isolates (%)			
	Amphotericin B	Fluconazole	Itraconazole	Ketoconazole
0.015	0	0	4(2.1)	0
0.03	0	0	74(38.9)	20(10.5)
0.06	0	0	85(44.7)	86(45.3)
0.125	0	0	21(11.1)	70(36.8)
0.25	0	3(1.6)	4 (2.1)	12(6.3)
0.5	21(11.1)	2(1.1)	2(1.1)	2(1.1)
1	169(88.9)	24(12.6)	0	0
2	0	82(43.2)	0	0
4	0	65(34.2)	0	0
8	0	13(6.8)	0	0
16	0	1(0.5)	0	0
32	0	0	0	0
64	0	0	0	0
MIC 50	0.5	2	0.03	0.06
MIC 90	1	4	0.06	0.125

(GenBank Accession No. AY2265353) *18S rRNA* genes were used as the internal controls for RT-PCR. They were F18s (5'-TCGATGGTAGGATAGAGG-3') and R18s (5'-AGCATACAGGACCACCAG-3'), which were forward and reverse primers, respectively. Each PCR reaction was carried out in a total volume of 25 μl containing 200 ng of cDNA of the RT product as a template, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM primers, and 0.1 U/μl *Taq* DNA polymerase (Invitrogen). Amplification was performed with an initial denaturing of 95°C for 5 min followed by 35 cycles for each individual transcript of 94°C for 45 s, 60°C for 30 s, 72°C for 1 min and a final extension step of 72°C for 10 min. PCR products were electrophoresed on 2% agarose gel and visualized by UV transillumination after staining with ethidium bromide. To quantify and compare mRNA levels, the gels were photographed utilizing a GelDoc 1000 (BIO-RAD, Hercules, CA, USA) and the intensity of bands was analyzed using densitometry. The relative band intensities in each drug resistance gene were normalized to the intensity of the internal control bands running in parallel. The ratio of the sample intensity to that of the corresponding *18S rRNA* internal controls was expressed as the relative gene expression level and plotted against the time points of the analysis.

RESULTS

Antifungal susceptibility testing

The *in vitro* susceptibilities of *C. neoformans* isolates to amphotericin B, fluconazole, itraconazole and ketoconazole by broth microdilution method were reported as the MIC values as shown in Table 1. Of the 190 isolates, only one was susceptible-dose dependent to fluconazole (S-DD for MICs of 16 to 32 μg/ml) while 189 isolates were susceptible to fluconazole (MICs ≤ 8 μg/ml). Six isolates were S-DD to itraconazole and 184 isolates were susceptible to itraconazole. All isolates were susceptible to amphotericin B (MICs ≤ 1 μg/ml) and

ketoconazole (MICs ≤ 1 μg/ml). The MIC range for all isolates was 0.5-1 μg/ml for amphotericin B, 0.25-16 μg/ml for fluconazole, ≤ 0.015-0.5 μg/ml for itraconazole and 0.03-0.5 μg/ml for ketoconazole. Most of *C. neoformans* isolates showed uniform patterns of susceptibility to the antifungal agents tested.

Screening for fluconazole heteroresistant isolate

To investigate the presence of fluconazole heteroresistance among clinical and environmental isolates, a total of 14 isolates of *C. neoformans* for which the MICs of fluconazole ranged from 8 to 16 μg/ml were selected for further investigation. Fluconazole heteroresistance was characterized by the ability to grow at 30°C for 72 h on PDA containing 64 μg of fluconazole per ml. Of the 14 *C. neoformans* tested isolates, only one clinical isolate (CN4969) exhibited a fluconazole-heteroresistant phenotype, defined as the co-existence of fluconazole-susceptible (CN4969S) and -resistant (CN4969HR) subpopulations in the same isolate (Figure 1). The fluconazole-resistant subpopulaton (CN4969HR) was able to grow on PDA containing 64 μg of fluconazole per ml at 30°C for 72 h (data not shown). No growth was found at 37°C on this fluconazole containing PDA. The fluconazole MIC of CN4969HR was ≥ 64 by broth microdilution and ≥ 256 μg/ml by E test methods, respectively (Figure 1). The fluconazole MIC for CN4969HR reverted to the baseline after subculture for seven passages (data not shown). In contrast, the fluconazole-susceptible (CN4969S) subpopulation was not able to grow on PDA containing 64 μg of fluconazole per ml. The fluconazole MIC of CN4969S was 8 μg/ml by
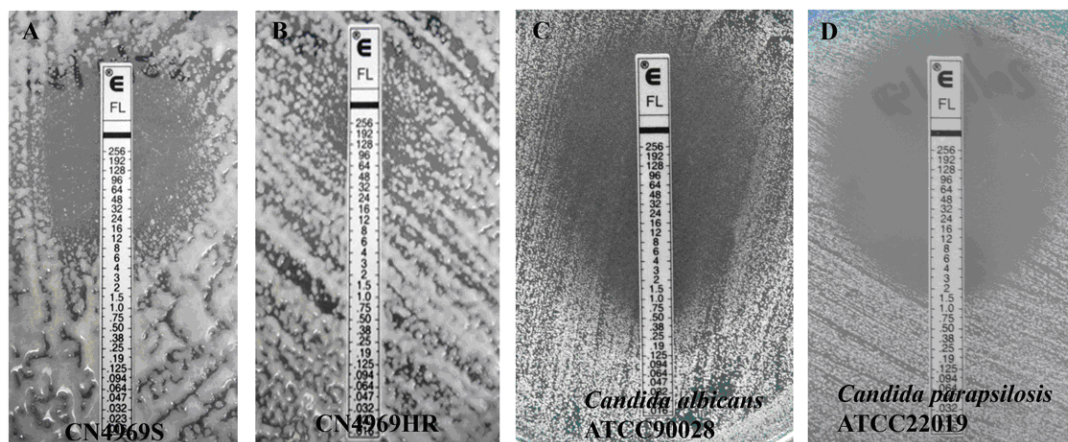
Figure 1. Characteristics of CN4969S (susceptible) and CN4969HR (heteroresistant) isolates in the E test for fluconazole. The fluconazole MICs of CN4969S (A) and CN4969HR (B) were 8 and ≥ 256 µg/ml, respectively. The fluconazole MICs of control organisms, *Candida albicans* ATCC 90028 (C) and *Candida parapsilosis* ATCC 22019 (D) were 0.5 and 1.5 µg/ml, respectively.

both broth microdilution and Etest methods (Figure 1).

To clarify the mechanisms of fluconazole resistance, the fluconazole-resistant subpopulation (CN4969HR), the fluconazole-susceptible subpopulation (CN4969S), one representative fluconazole-susceptible clinical isolate (CN4901) and standard *C. neoformans* serotype A (H99) were subjected to compare the expression of drug resistant genes by RT-PCR. The MICs of antifungal susceptibility tests of these isolates are shown in Table 2. Serotype and mating type identification indicated that CN4901, CN4969S and CN4969HR strains belonged to serotype A and mating type α.

Expression analysis of drug resistant genes (ERG11, AFR1 and MDR1) in fluconazole-resistant and fluconazole-susceptible *C.neofromans* isolates

Expression of *ERG11, AFR1* and *MDR1* genes was compared between fluconazole-susceptible (H99, CN4901 and CN4969S) and -resistant (CN4969HR) strains by a quantification of mRNA levels based on RT-PCR method. The expression pattern of the *ERG11, AFR1* and *MDR1* genes was analyzed from cells taken during exponential growth of both fluconazole-susceptible and -resistant strains. To normalize the amount of RNA of each strain, the expression level *18S rRNA* was used for comparison (Figure 2). The relative *ERG11* gene expression level in CN4969HR was found to be higher than in H99, CN4901 and CN4969S. The *ERG11* gene in CN4901 was minimally expressed. The *ERG11* gene expression level of CN4969HR was 2, 10 and 2 -fold higher than those in H99, CN4901 and CN4969S, respectively. This result shows overexpression of *ERG11* gene in CN4969HR that was at least 2-fold higher than in the H99, CN4901 and CN4969S isolates.

The *AFR1* gene expression was undetectable in CN4901 and CN4969S. The relative *AFR1* gene expression level in CN4969HR was similar to that in H99. Additionally, the result shows an increased *MDR1* gene expression in CN4969HR compared with expression in H99 and CN4969S after normalization. Quantification of mRNA for the *MDR1* efflux pump showed that the CN4969HR isolate contained approximately 4- and 2- fold more *MDR1* mRNA than the susceptible H99 and CN4969S strains, respectively. There was at least a 2-fold increase of both *ERG11* and *MDR1* expression levels in fluconazole-resistant strain when compared with the fluconazole-susceptible strains. In contrast, the *AFR1* gene expression level in the fluconazole-resistant strain was similar to that of the fluconazole-susceptible strains.

DISCUSSION

At present, although the decreasing incidence of cryptococcosis among HIV-infected patients has been documented after the introduction of highly active antiretroviral therapy (HAART), cryptococcosis is still an important opportunistic infection in Thailand. Little is known about the antifungal susceptibility of *C. neoformans* from patient and environmental samples in different regions of Thailand. A previous study in Thailand showed that 50 clinical isolates of *C. neoformans* were susceptible to 5 antifungal agents including amphotericin B, fluconazole, flucytosine, itraconazole and miconazole except for one isolate that was resistant to flucytosine (Poonwan et al., 1997). Sukroongreung et al. (2001) reported fluconazole MICs of 7/22 isolates from 9 AIDS patients in Bangkok, Thailand and untreated primary cryptococcosis were ≥ 256 µg/ml by using E test method. Approximately, 30% of clinical

Table 2. MICs of *Cryptococcus neoformans* isolates determined by broth microdilution. All these strains were used in RT-PCR experiment.

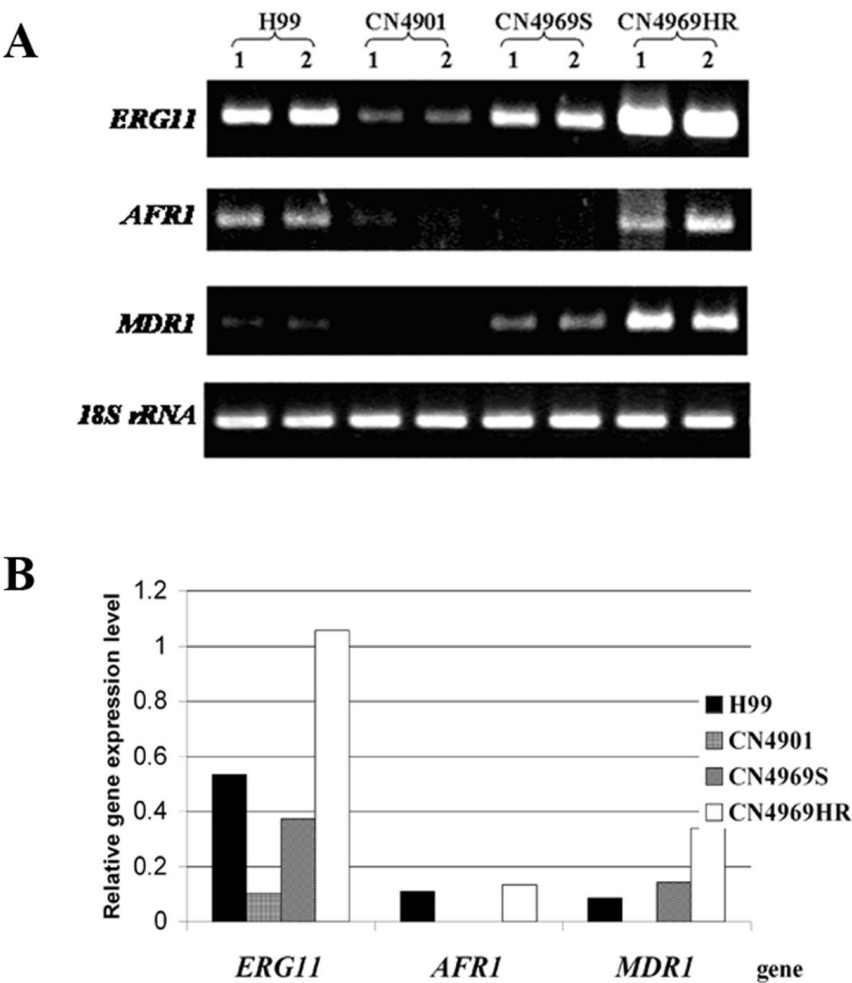| Isolate	Source and/or comment	MIC (µg/ml) according to broth microdilution			
		AmphotericinB	Fluconazole	Itraconazole	Ketoconazole
H99	Standard strain or Fluconazole-susceptible	1	2	0.06	0.06
CN4901	Fluconazole-susceptible	1	2	0.03	0.06
CN4969S	Fluconazole-susceptible	1	8	0.06	0.125
CN4969HR	Fluconazole- resistant	1	≥64	0.06	0.125

Figure 2. Evaluation of differential expression by RT-PCR of *ERG11*, *AFR1* and *MDR1* genes of H99, CN4901, CN4969S and CN4969HR in two independent experiments (A); RT-PCR of *C. neoformans 18S rRNA* was performed as expression control. The relative levels of *ERG11, AFR1* and *MDR1* gene expression normalizing with *18S rRNA* expression in fluconazole-susceptible (H99, CN4901 and CN4969S) and -resistant (CN4969HR) isolates calculated from two independent experiments (B).

isolates of *C. neoformans* in Bangkok had MICs of fluconazole in the range of 16-32 µg/ml as determined by broth microdilution method (Manosuthi et al., 2006).   In the present study, the *in vitro* susceptibilities of the majority of the 190 *C. neoformans* isolates from northern Thailand were susceptible (MIC < 1 µg/ml) to ampho-tericin B, itraconazole and ketoconazole. Only one clinical isolate was susceptible dose dependent (MIC 16 µg/ml) to fluconazole.

The phenomenon of heteroresistance has been observed in many microorganisms including many bacteria and yeasts such as *C. albicans* (Marr et al., 2001). Heteroresistance in *C. neoformans* was defined as a subpopulation of cells that showed variable growth at a certain concentration of an antimicrobial agent which inhibited a majority of the cells; when exposed to the drug. The individual cells may either grow well, slowly, or not at all. The resistant subpopulation can adapt in a stepwise manner to higher concentrations of the drug. Fluconazole heteroresistance in *C. neoformans* is universal and intrinsic phenomenon that may contribute to relapse of cryptococcosis during azole maintenance therapy (Sionov et al., 2009).

In this study, only one clinical isolate (CN4969) exhibited heterogeneous patterns of resistance. The fluconazole-resistant subpopulation (CN4969HR) grew on PDA containing 64 µg of fluconazole per ml at 30°C. Similar to the study of Yamazumi *et al.*(2003), heterore-sistance was demonstrable only at 30°C but not at 37°C by agar-based tests, and was reversible through serial transfers (7 passages) on drug-free medium. We used only one concentration of fluconazole in the medium (64 µg/ml) to select the highly fluconazole-heteroresistant population.   We successfully isolated fluconazole-susceptible (CN4969S) and -resistant subpopulations (CN4969HR) from a heteroresistant clone (CN4969). Their fluconazole MICs were confirmed by broth microdilution and E test methods. These fluconazole-susceptible and -resistant subpopulations were used to compare expression levels of *ERG11*, *AFR1* and *MDR1* by RT-PCR. The molecular mechanism of fluconazole resistance in *C. neoformans* has not been elucidated except a report of the amino acid substitution G484S of the 14-α lanosterol demethylase gene (*ERG11*) (Rodero et al., 2003). It has been reported that fluconazole-resistant *C. albican*s isolates express higher *ERG11* mRNA than susceptible strains in the presence of the drug (Franz et al., 1998). Until now, it is not known whether overexpression of *ERG11* causes resistance to fluconazole in *C. neoformans*. Overexpression of the *ERG11* gene results in the production of high concentrations of target enzyme, thus creating the need for higher intracellular fluconazole concentrations to inhibit the enzyme in the cell (Balkis et al., 2002). Another important mechanism of fluconazole resistance is a reduced intracellular accumulation of the drug. Fluconazole is actively transported out of the cells in an energy-dependent manner and genes encoding membrane transport proteins such as *CnAFR1* (Posteraro et al., 2003) and *CneMDR1* (multidrug resistance1) (Thornewell et al., 1997) may relate in fluconazole resistance of *C. neoformans*. Both genes are classified as ATP-binding cassette (ABC) transporters, which use adenosine triphosphate (ATP) as energy source. In this study, at least 2-fold increase in both *ERG11* and *MDR1* expression levels occurred in CN4969HR (fluconazole-resistant subpopulation) com-pared to CN4969S (fluconazole-susceptible subpopula-tion), another representative fluconazole-susceptible clini-cal isolate (CN4901) and the standard *C. neoformans* serotype A (H99). Interestingly, this is the first report of overexpression of the *ERG11* gene in a fluconazole-resistant *C. neoformans*. Similarly, a 1.2- to 2.3-fold change in expression of *ERG11* occurred in fluconazole-resistant isolates of *C. albicans* (Lyons and White, 2000). Recently, up-regulation of *ERG11* gene among flucona-zole-resistant *C. albicans* generated *in vitro* has ever been reported (Ribeiro and Paula, 2007).

In *C. albicans,* overexpression of multidrug resistant gene (*MDR1*) was detected in the fluconazole-resistant isolate during logarithmic growth (Lyons and White, 2000). *CneMDR1*, a gene encoding a protein related to several eukaryotic multidrug resistance proteins, was identified in a clinical isolate of *C. neoformans* (Thornewell et al., 1997). The possibility of a mechanism similar to multidrug resistance involved in the resistance phenotype of *C. neoformans* has ever been proposed (Joseph-Horne et al., 1995).

Other than *MDR1* gene, an ATP Binding Cassette (ABC) transporter-encoding gene called *C. neoformans* antifungal resistance 1 (*CnAFR1*) was identified and characterized. Disruption of *CnAFR1* gene in the resistant isolate resulted in an enhanced susceptibility of the knock-out mutant *cnafr1* against fluconazole (Posteraro et al., 2003). In our experiment, the *AFR1* gene expression level in a fluconazole-resistant strain was similar to the expression in fluconazole-susceptible one. Our data are different to those reported by Sanguinetti et al.(2006) who reported that the *AFR1* gene is involved in the *in vitro* fluconazole resistance of *C. neoformans*. Expression levels in fluconazole-resistant strains were over 10 times higher than those observed in fluconazole-susceptible strains (Sanguinetti et al., 2006). However, the fluconazole-resistant subpopulation used in our study was isolated from heteroresistant clone and the ABC transporter *AFR1*, known to efflux fluconazole, might not be involved in the heteroresistance mechanism (Sionov et al., 2009).

In conclusion, antifungal susceptibility tests using four antifungal agents including amphotericin B, fluconazole, itraconazole and ketoconazole against 190 isolates of *C. neoformans* in northern Thailand showed that those isolates were sensitive to all of the antifungal agents tested. One was susceptible dose dependent (S-DD)

against fluconazole. The strain that exhibited heteroresistant phenotype to fluconazole could be selected by the ability to grow on agar containing 64 µg/ml of fluconazole at 30°C. Both over expression of *ERG11* and *MDR1* gene were observed in the fluconazole-resistant subpopulation.

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