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Antifungal Susceptibility and Molecular Typing of *Candida albicans* isolated from AIDS and non-AIDS Thai Patients

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Abstract : The antifungal susceptibility of *Candida albicans* isolated from 2 groups of Thai patients; AIDS patients and non-AIDS patients was investigated. Two hundred and seventeen *C. albicans* were isolated from the clinical specimens from 54 AIDS patients and 163 non AIDS patients. All isolates were included in the antifungal susceptibility test against amphotericin B, fluconazole, ketoconazole and nystatin. A hundred isolates were randomly selected from both groups for the electrophoretic karyotypes determination.

There was not much difference in the value of MIC, MIC₅₀ and MIC₉₀ of all antifungal agents for *C. albicans* isolates between AIDS and non-AIDS patients. The amphotericin B MIC for 61.0% of AIDS isolates and 71.0% of non-AIDS isolates were >0.5 mg/l, while ketoconazole MIC for 94.4% of AIDS and 74.9% of non-AIDS isolates were >0.125 mg/l and fluconazole MIC of 100% of AIDS and non-AIDS isolates were 2.0 mg/l. For nystatin, the MIC for more than 90% of both isolates was < 8 mg/l. The MIC₅₀ and MIC₉₀ of all antifungal agents for the two groups of isolates were almost at the same concentration except for fluconazole which showed a two-fold difference of MIC₅₀.

The chromosomal DNA karyotypic profiles of *C. albicans* indicated genetic diversity among all isolates. Twenty-three distinct pulsed-field gel electrophoresis karyotypes and molecular sizes ranging from 3.5 to 0.5 megabases were identified. Most isolates (61%) from both AIDS and non-AIDS isolates belong to types 1 and type 3. Among the AIDS isolates, 38.3% and 29.8% were type 1 and 3, respectively, and non-AIDS isolates, 20.8% and 33.9% were type 1 and 3, respectively. *C. albicans* isolates were shown to have 8 to 10 chromosomal DNA bands. Most of the isolates (78%) along with *C. albicans* FC18 control strain had 8 band patterns. The recovery of the common karyotypes in AIDS patients, as well as in non-AIDS patients, suggests that *C. albicans* infection may develop from a common source by the cross contamination between both groups of patients.

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เรื่องย่อ : การตรวจหาความไวต่อยาต้านเชื้อรา และการจำแนกสายพันธุ์โดยใช้วิธีระดับชีวโมเลกุลของเชื้อ *Candida albicans* ที่แยกได้จากผู้ป่วย
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การศึกษาความไวต่อยา amphotericin B fluconazole ketoconazole และ nystatin ต่อเชื้อ *Candida albicans* ที่แยกได้จากผู้ป่วยไทย 2 กลุ่ม คือผู้ป่วยโรคเอดส์ และผู้ป่วยอื่น โดยทดสอบกับเชื้อจำนวน 217 สายพันธุ์ ซึ่งแยกได้จากสิ่งส่งตรวจของผู้ป่วยโรคเอดส์ 54 รายและจากผู้ป่วยอื่น 163 ราย และเลือกโดยวิธีสุ่มมาจำนวน 100 สายพันธุ์เพื่อศึกษาสายพันธุ์ของเชื้อ *Candida albicans* 43 สายพันธุ์จากผู้ป่วยเอดส์ และ 57 สายพันธุ์จากผู้ป่วยอื่น โดยใช้วิธี electrophoretic karyotyping

ในการศึกษานี้ ไม่พบความแตกต่างของค่า MIC, ค่า MIC₅₀ และ MIC₉₀ ของยาต้านเชื้อราทั้ง 4 ชนิด ต่อเชื้อที่แยกได้จากผู้ป่วยทั้ง 2 กลุ่ม MIC ของยา amphotericin B ต่อเชื้อจำนวน 61% จากกลุ่มเอดส์ และ 71% จากกลุ่มอื่นมีค่ามากกว่า 0.5 mg/l และ MIC ของยา ketoconazole ต่อ 94.4% ของเชื้อกลุ่มเอดส์ และ 90.1% ของกลุ่มอื่นมีค่ามากกว่า 0.125 mg/l สำหรับยา fluconazole 100% ของเชื้อทั้งสองกลุ่มมีค่า MIC เท่ากับ 2.0 mg/l และค่า MIC ของยา nystatin มากกว่า 90% ของเชื้อทั้งสองกลุ่มมีค่าน้อยกว่า 8 mg/l ค่า MIC₅₀ และ MIC₉₀ ของยาทั้ง 4 ชนิด ก็มีค่าเท่ากัน ยกเว้นยา fluconazole ที่ค่า MIC₅₀ ต่อเชื้อ 2 กลุ่มต่างกันหนึ่งความเข้มข้น

ผลการศึกษา karyotype ของเชื้อด้วยวิธี pulsed-field gel electrophoresis พบว่าเชื้อมีความแตกต่างกันถึง 23 รูปแบบ และมีขนาดของ DNA อยู่ในช่วง 3.5 ถึง 0.5 megabases รูปแบบที่พบมากที่สุด (61%) ได้แก่ type 1 และ type 3 ไม่พบความแตกต่างชัดเจนระหว่างเชื้อ 2 กลุ่ม นอกจากนี้เชื้อส่วนใหญ่ (78%) มี chromosomal DNA 8 bands รวมทั้งเชื้อสายพันธุ์มาตรฐาน *C. albicans* FC18 การมี karyotype ที่พบได้บ่อยทั้งในผู้ป่วยโรคเอดส์และผู้ป่วยอื่นบ่งชี้ถึงการแพร่กระจายของเชื้อน่าจะมาจากแหล่งเดียวกัน โดยเป็น cross-contamination ทั้งในกลุ่มผู้ป่วยเอดส์และผู้ป่วยอื่น

INTRODUCTION

Candida albicans is one of the most common opportunistic pathogens causing nosocomial infection in chronic illness patients and immunocompromised, including HIV infected patients^{2,12,16,33}. Increasing incidence of candida infection common in AIDS patients, particularly *C. albicans*, has been reported in many countries including Thailand (Yamazaki et al. 1999)³³ Barnerjee et al.^{3,34,35}

Antifungal agents, including amphotericin B, fluconazole, ketoconazole and nystatin, are widely used in the treatment of fungal infections. At present, there are still therapeutic problems in the use of these antifungal agents such as amphotericin B, apart from its side effect.

The long-term treatment in fungal infection leads to the development of the resistant strains¹⁵. Thus, there are still insufficient data on the antifungal susceptibility. The reason is due to the difficulty in

performing the susceptibility testing including time consumption, reliable method and cost. However, there are many test methods which have been developed and provide the accurate results as compared to the gold standard method recommended by NCCLS.^{8,9,14,19} For epidemiologic study, there have been no data of isolates from Thai patients despite of the fact that the increase of incidence of *C. albicans* infection has grown steadily every year.^{34,35} At present, the epidemiologic study basically depends on the chromosomal DNA pattern of *C. albicans*. The pulsed-field gel electrophoresis (PFGE) technique has been widely used and is acceptable in the epidemiological study of various kinds of prokaryotes and eukaryotes including yeast.

The purpose of this study was to obtain data on the antifungal susceptibility to commonly used antifungal agents and on the karyotyping of *C. albicans* isolated from AIDS and non-AIDS groups of Thai patients. The results obtained from this study should provide helpful information for physicians in both treatment and control of *C. albicans* infections in Thai patients.

MATERIALS AND METHODS

Fungal strains

Of a total of two hundred and seventeen *C. albicans* isolates, 54 isolates from AIDS patients and 163 isolates from non AIDS patients obtained from Siriraj Hospital and King Chulalongkorn Memorial Hospital were included in this study. The isolates were identified by the standard microbiological methods according to Warren NG and Shadomy HJ 1991³² and confirmed for the species with the API 20C system (bioMérieux, Marcy, l'Etoile, France).

Susceptibility testing

All 217 *C. albicans* isolates were determined for the minimal inhibitory concentrations (MICs) against 4 antifungal agents including: amphotericin B, ketoconazole, fluconazole and nystatin by using the broth microdilution methods (NCCLS, M-27A)²⁰ and the colorimetric broth microdilution method.¹⁴

Karyotyping

One hundred *C. albicans* isolates were selected at random from the 217 isolates for the

karyotyping study. These included 47 isolates from AIDS patients and 53 isolates from non-AIDS patients. The karyotyping of *C. albicans* was performed according to modified methods described by Schwartz et al. (1984)³⁰, Doi et al.⁹ and Iwaguchi et al.¹⁵ The *C. albicans* FC18, *C. non albicans* and *Saccharomyces cerevisiae* ATCC 9763 strains were used as the size reference markers.

Briefly, each tested strain was grown at 30°C in a sabouraud dextrose broth. Early stationary phase cells were harvested, washed twice with 50 mM-sodium EDTA (pH 7.5), and resuspended in 1 ml 50 mM-Tris/HCl (pH 7.5). The cell suspension was lysed by enzyme solution in 50 mM Tris/HCl (pH 7.5). Then, the 1% (w/v) low-melting-point agarose (Amresco) in 0.125 M-sodium EDTA (pH 7.5) was added. After gently mixing, the mixture of the solution was poured into a mould chamber (Bio-Rad). The solidified agarose plugs were incubated in 10 mM-Tris/HCl (pH 7.5) containing 25 mM-sodium EDTA (pH 7.5) and 7.5% (v/v) 2-mercaptoethanol at 37°C for 18-24 h, then transferred to the agarose plug into 10 mM-Tris/HCl (pH 8.3) containing 25 mM-sodium EDTA, 0.06% sodium dodecyl sulphate and 0.17 mg ml⁻¹ proteinase K (USB, USA.). The plug was further incubated at 50°C for 18-24 hours and stored in 50 mM-sodium EDTA (pH 9.0) at 4°C until used. The chromosomal DNA was separated by contour-clamped homogeneous electric field (CHEF) electrophoresis in 0.5 X TBE, as a running buffer using the CHEF-DR III system (Bio-Rad) as described by Chu et al. 1986.⁶ The running condition was as follows : the buffer temperature was 14°C, switch time was 300 s at 140 V for 24 hours and then switched to 1,200 s at 80 V for 48 hours. The gels were stained with ethidium bromide and destained with distilled water; then the chromosomal DNA was observed and photograph under UV light (302 nm).

RESULT

The MIC values of antifungal agents

The results of antifungal susceptibility testing are shown in Tables 1 and 2. For amphotericin B, the MIC range was 0.125-1.0 mg/l, MIC for 71.0% of the non-AIDS isolates was > 0.5 mg/L, while the MIC for 61.0% of the AIDS isolates was > 0.5 mg/

L. All 217 isolates were susceptible to fluconazole with a MIC value range of 0.5 - 2.0 mg/L. For ketoconazole and nystatin, there was no difference of MIC values between AIDS and non-AIDS isolates and the MIC ranges were 0.5 - 2.0 mg/l and 2.337 - 9.250 mg/l, respectively. The MIC of ketoconazole for more than 90% of the isolates was > 0.125 mg/l and of nystatin, < 8.0 mg/l.

Karyotypic Patterns (Chromosomal patterns)

Karyotypic patterns determined by the use of pulsed-field gel electrophoresis (PFGE) based on the molecular size and the number of bands are shown in Table 3, Figures 1 and 2. The reference strains, *C. albicans* FC18, one clinical isolate of *C. non albicans* and *S. cerevisiae* ATCC 9763 demonstrated 8, 6 and 4 chromosomal DNA bands, respectively. Among the clinical isolates, there were 23 karyotypes which could be divided into 3 groups according to the number of DNA bands which showed 8, 9 and 10 bands, respectively. The molecular size ranged from 3.5 to 0.5 megabase. The first and largest group consisted of 78 out of 100 strains (78%) and demonstrated 8 DNA bands. In this group there were 7 karyotypes including type 1 (29 isolates) and type 3 (32 isolates) which were the most common types. Thirty six strains were isolated from AIDS patients

and 42 strains isolated from non-AIDS patients. The chromosomal pattern with 9 bands was classified as the second group. There were 20 strains (20%) in this group; 10 strains were isolated from AIDS patients and another 10 strains from non-AIDS patients. In the third group, there were only 2 strains which showed chromosomal pattern with 10 bands. One was an AIDS isolate and another was a non-AIDS isolate.

DISCUSSION

Since there are still a large number of HIV infections in Thailand, a high incidence of opportunistic infection by *C. albicans* is expected in our country. The commonly used antifungal agents are amphotericin B, azoles and nystatin.

Several antifungal susceptibility studies of *Candida* species have been reported from many countries, except for Thailand.^{4,11,17,19,22-24,31} One problem in performing the test is the result interpretation, that is, whether the test strain is sensitive or resistant. Up until now, there is no definite MIC breakpoint for those commonly used antifungal agents, including amphotericin B, ketoconazole and nystatin.

Table 1. Antifungal susceptibility of *Candida albicans* isolated from AIDS and non-AIDS patients.

Antifungal agent/number of AIDS/ Non-AIDS isolate (%)	MIC (mg/L)			
	0.125	0.25	0.5	1.0
Amphotericin B				
Number of AIDS isolates	6 (11.1)	15 (27.7)	24 (44.4)	9 (16.6)
Number of non-AIDS isolates	15 (9.2)	32 (19.6)	51 (31.2)	65 (39.8)
Fluconazole				
Number of AIDS isolates	3 (5.6)	30 (55.5)	21 (38.9)	-
Number of non-AIDS isolates	8 (4.9)	69 (42.3)	86 (52.7)	-
Ketoconazole				
Number of AIDS isolates	3 (5.6)	35 (64.8)	16 (29.6)	0
Number of non-AIDS isolates	41 (25.1)	98 (60.1)	23 (14.1)	1 (0.6)
Nystatin				
Number of AIDS isolates	0	50 (92.5)	4 (7.4)	-
Number of non-AIDS isolates	8 (4.9)	147 (90.1)	8 (4.9)	-

* Interpretation breakpoint criteria of the NCCLS (M27-A, 1997) ; fluconazole resistant = > 64mg/l

MIC = minimal inhibitory concentration

Table 2. MIC values of 4 antifungal agents for *Candida albicans* isolated from AIDS and non-AIDS patients.

Antifungal agents	MIC (mg/L)					
	AIDS patients (n = 54)			Non-AIDS patients (n = 163)		
	Range	MIC ₅₀	MIC ₉₀	Range	MIC ₅₀	MIC ₉₀
Amphotericin B	0.125-1.0	0.5	1.0	0.125-1.0	0.5	1.0
Fluconazole	0.5-2.0	1	2	0.5-2.0	2	2
Ketoconazole	0.125-1.0	0.25	0.5	0.125-1.0	0.25	0.5
Nystatin	2.337-9.250	4.675	4.675	2.337-9.250	4.675	4.675

Table 3. Karyotypic patterns of clinical *Candida albicans* isolates from AIDS and non-AIDS patients.

Pattern	Band		No. of isolates			
	Size (megabase)		Number AIDS	Non-AIDS	Total	
<i>C. albicans</i> FC18	3.5, 3.14, 2.2, 1.8, 1.63, 1.4, 1.13, 1.02		8			
<i>C. non albicans</i>	3.2, 2.8, 2.5, 1.4, 1.2, 1.0		6			
<i>S. cerevisiae</i> ATCC 9763	2.2, 1.6, 1.2, 1.0, ...		4			
1	3.5, 3.1, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		8	18	11	29
2	3.5, 3.4, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1, 1.0		9	0	2	2
3	3.5, 3.2, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		8	14	18	32
4	3.5, 3.4, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		8	1	5	6
5	3.5, 3.0, 2.2, 2.0, 1.8, 1.6, 1.4, 1.2, 1.1		9	0	1	1
6	3.5, 2.6, 2.3, 1.6, 1.4, 1.3, 1.1, 1.0		8	0	1	1
7	3.5, 2.6, 2.3, 1.8, 1.6, 1.4, 1.2, 1.1		8	1	1	2
8	3.8, 3.5, 3.1, 2.2, 1.8, 1.6, 1.4, 1.1, 1.0, 0.5		10	1	0	1
9	3.5, 3.3, 3.0, 2.5, 1.8, 1.6, 1.4, 1.2, 1.1		9	1	2	3
10	3.5, 2.9, 2.2, 1.8, 1.6, 1.4, 1.2, 1.1		8	1	6	7
11	3.5, 3.1, 2.6, 1.8, 1.6, 1.4, 1.2, 1.1, 0.8		9	1	0	1
12	3.5, 3.2, 3.0, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		9	1	2	3
13	3.5, 3.1, 2.9, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		9	1	1	2
14	3.5, 3.3, 3.1, 2.9, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		10	0	1	1
15	3.5, 3.4, 3.0, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		9	0	1	1
16	3.5, 3.0, 2.2, 2.0, 1.8, 1.6, 1.4, 1.1, 1.0		9	0	1	1
17	3.5, 3.1, 2.8, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		9	1	0	1
18	3.5, 3.2, 2.5, 1.8, 1.6, 1.5, 1.4, 1.2, 1.1		9	1	0	1
19	3.8, 3.5, 2.3, 2.0, 1.8, 1.6, 1.2, 1.1, 1.0		9	1	0	1
20	3.5, 3.1, 2.5, 2.3, 1.8, 1.6, 1.4, 1.2, 1.1		9	1	0	1
21	3.7, 3.5, 2.9, 2.3, 1.8, 1.6, 1.4, 1.2, 1.1		9	1	0	1
22	3.7, 3.5, 2.4, 1.8, 1.6, 1.4, 1.2, 1.1		8	1	0	1
23	3.5, 3.1, 2.4, 1.8, 1.6, 1.5, 1.4, 1.2, 1.1		9	1	0	1
Total				47	53	100

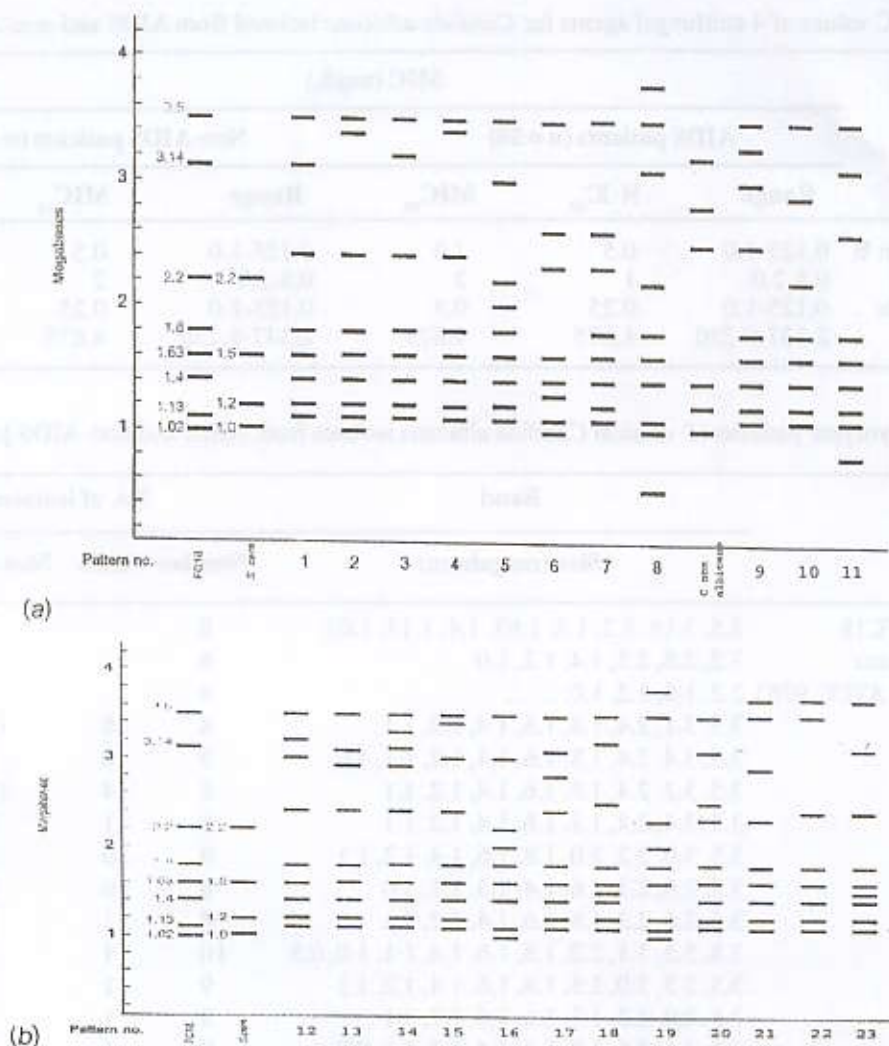


Figure 1. Schematic diagram of pulsed-field gel electrophoresis patterns (karyotypes : size and number of bands) of chromosomal DNA of *Candida albicans* FC18, *Saccharomyces cerevisiae* and 23 karyotypes of clinical *C. albicans* isolates : (a) *C. albicans* FC18, *S. cerevisiae*, *C. non albicans* and karyotype 1-11 of clinical *C. albicans* isolates, (b) *C. albicans* FC18, *S. cerevisiae* and karyotype 12-23 of clinical *C. albicans* isolates.

For amphotericin B, many studies on MIC have been reported.^{21,25} As mentioned above, no definite breakpoint had been established until the year 2001. Rex et al. (2001)²⁹ had reviewed many reports and commented that besides the MIC value there were several factors involved in the outcome of the treatment of fungal infections such as patient's conditions and therapeutic doses. However, a

proposed breakpoint has been introduced for guidance in the determination of amphotericin B and ketoconazole²⁹ which was > 0.5 mg/l. According to the breakpoint, more than 60% of our isolates were resistant to amphotericin B. Marchetti et al. (2004)¹⁸ recently reported that most of the isolates from a tertiary care hospital in Switzerland were amphotericin B sensitive. Because amphotericin B

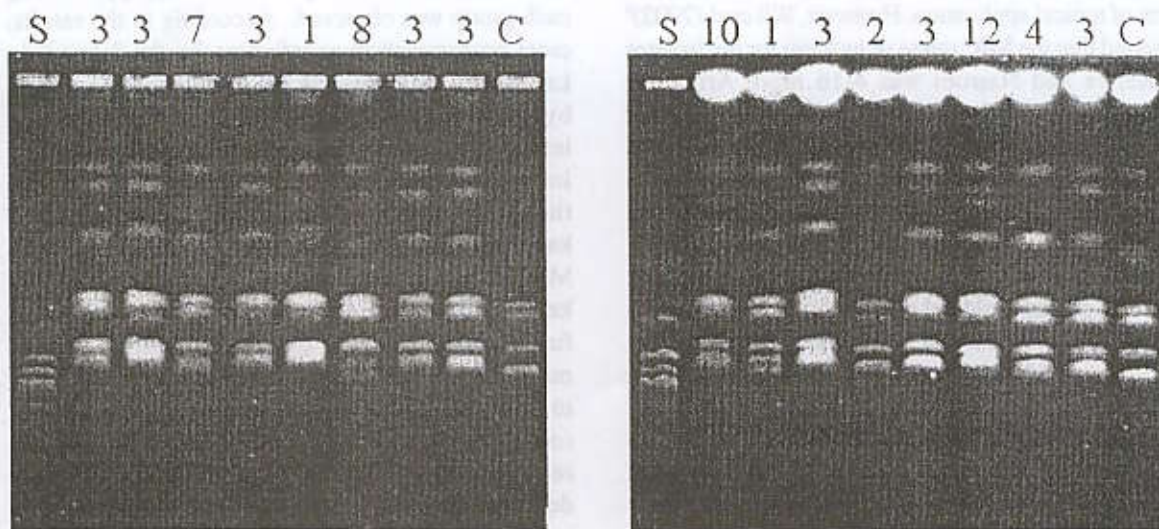


Figure 2. Pulsed-field gel electrophoresis patterns of chromosomal DNA of *Candida albicans* FC18 (C), *Saccharomyces cerevisiae* (S) and clinical isolates of *C. albicans* (number represents karyotype).

is still commonly used in many hospitals in Thailand, further study, including drug resistant mechanism and resistant gene transfer should be made in order to find out why there were so many amphotericin B resistant strains in Thailand.

Rex et al. (2001)²⁹ also proposed that the ketoconazole MIC breakpoint that isolates with the MIC > 0.125 mg/l were less likely to respond to treatment. According to our study, 90% of our isolates should be interpreted as resistant to ketoconazole. The use of these agents has already decreased. Instead, fluconazole is now commonly used.

In this study, all isolates were determined as fluconazole sensitive strains since the MIC was not higher than 64 mg/l. For a clinical response, there were several reports on clinical correlation between fluconazole MIC and therapeutic dosage. Although the recommended M27-A MIC breakpoint was > 64 mg/l, Danaoui et al. (2001)⁷ reported the failure of using fluconazole 100 to 200 mg per day in patients infected by isolates with MIC > 48 mg/l. Cartledge et al. (2001)⁵ also reported that infections due to the isolates with MIC > 8 mg/l were unlikely to respond

to 100 mg of fluconazole per day. However, Revankar et al. (2001)²⁸ reported that with increasing doses of fluconazole from 100 to as high as 800 mg per day as needed, clinical failure occurred only when the MIC of the isolate was > 64 mg/l. Differences in fluconazole MIC values among the isolates were continuously reported. For instance, Pfaller et al.²⁵ reported that the MIC₅₀ and MIC₉₀ of fluconazole were 0.25 and 16 mg/l, respectively, while this study and also the study by Bill et al. (2002)⁴ showed that the fluconazole MIC₉₀ of the isolates was only 2 mg/l. Variation in the MIC values to some extent depends on the time of specimen collection since resistant strains could commonly be isolated from the patients with long term treatment of the antifungal agents. This reason was supported by the work of Emmanuelle et al. (1995)¹¹ and Michael et al. (1999)¹⁹ who could isolate fluconazole resistant strains from the patients who received long-term prophylactics and prolonged or repeated treatment with fluconazole before the microbiologic studies were performed.

There were not many susceptibility data of nystatin. This may be due to the fact that nystatin is

not absorbed and with this property it is used in the form of topical application. However, Bill et al.(2002)⁴ reported that the MIC range of nystatin for the isolates in Kenya and Nairobi was 4-16 mg/l. Arikan et al.(2002)¹ reported that the nystatin MIC range for clinical isolates was 0.5-2.0 mg/l. In contrast to our study, the nystatin MIC range of isolates was 2.337-9.250 mg/l. It seems like nystatin should be an effective agent

The molecular study of chromosomal DNA by using pulsed-field gel electrophoresis showed the number of chromosomal DNA bands detected from *C. albicans*. Most of the strains (78%) had 8 DNA bands including a control strain of *C. albicans* FC18. Although the variation in the number of DNA bands was observed, which were 9 and 10 bands, a small number of strains were demonstrated. Actually *Candida* is a diploid, mostly like an aneuploid cell, and a variation of the number of bands is unavoidable. The results were supported by the report of Lupetti et al. (1995)¹⁷ who also found that the 8-band pattern was the predominate pattern of *C. albicans*.

Epidemiologic study of *C. albicans* has been reported by several investigators using PFGE. At least 3 to 7 karyotypes had been reported.^{10,13,17} In addition, Lupetti et al. (1995)¹⁷ showed that there were 2 common karyotypes among the isolates from AIDS and non-AIDS patients. One patient could harbor more than one karyotype^{2,26,31} and cross infection by one type was also reported.¹⁰ Among the 100 isolates, 23 karyotypes were shown. There were also 2 common karyotypes (designated as types 1 and 3) of the isolates obtained from AIDS and non-AIDS groups. The finding was similar to the work of Lupetti et al.¹⁷ In a comparison between AIDS and

non-AIDS isolates, no particular karyotype among each group was observed. According to the results, cross contamination or infection by the 2 common karyotypes was possible, as mentioned previously by Dupont et al.¹⁰ In addition, colonization or infection by an endogenous strain was also possible. In conclusion, there was no significant difference in the MIC values of the antifungal agents and karyotype between AIDS and non-AIDS isolates. Most of the isolates resisted amphotericin B and ketoconazole but all isolates were sensitive to fluconazole. Although the susceptibility testing is not so easy to perform and is not an infallible answer to questions about treatment of fungal infection, routine candida susceptibility testing is recommended. The term susceptibility dose dependent has also been introduced to the antifungal susceptibility system in order to assist clinicians in considering the adjustment of an appropriate dose for a good clinical outcome. Eight bands of chromosomal DNA is a characteristic of *C. albicans*. Two predominant karyotypes of the Thai isolates were observed. Karyotyping using PFGE is still recommended for epidemiologic study. Further study on factors involved in the increase of drug resistance particularly amphotericin B and fluconazole, should be considered.

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