NEW MICROBIOLOGICA, **31**, 409-416, 2008

Biotypes, genotypes and ketoconazole susceptibility of *Candida albicans* isolates from a group of Thai AIDS patients

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SUMMARY

A total of eighty-seven *Candida albicans* isolates from a group of Thai AIDS patients were characterized for phenotypic and genotypic profiles and antifungal susceptibility to ketoconazole. Phenotyping of the isolates was carried out by a biotyping method based on the enzyme profiles, carbohydrate assimilation patterns and boric acid resistance of the yeasts. Genotyping was performed through randomly amplified polymorphic DNA (RAPD) analysis. Antifungal susceptibility of ketoconazole was performed using the NCCLS broth microdilution method. Combination of the biotypic tests revealed a total of 49 different biotypes. The most predominant was A1S (31%), the remaining biotypes represented only few isolates in each. RAPD profiles identified 14 clusters of genotype among the 87 isolates. Almost every individual harboured his/her own specific isolate and in 25 of 26 (96.2%) harboured more than one clonal type. The heterogeneity of both phenotypic and genotypic profiles of *C. albicans* isolates in this study was similar to previous reports from other oral sources in different geographic areas. All isolates were susceptible to ketoconazole. The findings may be useful as baseline information of oral *C. albicans* colonization in Thai population living in the south of Thailand.

KEY WORDS: Candida albicans, Biotypes, RAPD, HIV infection, Thailand

Received March 03, 2007 *Accepted* May 05, 2008

INTRODUCTION

In healthy humans, *Candida albicans* presents as part of normal human microbial population of the oral cavity and gastrointestinal tract. However, *Candida* can cause severe mucosal or invasive diseases in immunosuppressed patients including AIDS patients (Odds, 1988). The high incidence of mucosal candidiasis in patients with AIDS may be due to infection with the same strains that are non-pathogenic in healthy subjects but that become pathogenic in AIDS patients due to impaired host defence mechanisms. Alternatively, it may be due to infection with unique or more virulent strains. In addition, the significance of oral candidiasis as a disease entity in HIV-related immunosuppression is its frequent recurrence. The mechanism behind the ability of this fungus to cause recurrent disease is unknown. A decrease in susceptibility to antifungal agents has been postulated (Samaranayake and Holmstrup, 1989; Larsen, 1990), and this may be related to certain types of *C. albicans* (Odds *et al.,* 1983).

Various phenotypic methods have been used to subtype *C. albican*s strains, such as serotyping (Hasenclever and Mitchell, 1971; Polonelli and Morace, 1986), morphotyping (Phongpaichit *et al.,* 1987), killer yeast typing (Polonelli *et al.,* 1983) and biotyping (Odds *et al.,* 1983, Williamson *et*

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al., 1987). Of these, the biotyping system developed by Williamson *et al*., (1987) has been widely used for typing *C. albicans* strains from various oral sites in different geographic areas (Tsang *et al.*, 1995; Xu and Samaranayake, 1995; Matee *et al.*, 1996; Hannula *et al.*, 1997; Teanpaisan *et al.*, 2000; Leung *et al.*, 2000; Waltimo *et al.*, 2001; Samaranayake *et al.*, 2003a; Pizzo *et al*., 2005). However, it has been suggested that phenotypic switching of *C. albicans* may have significant effects on the results of biotyping which is probably the cause of the poor reproducibility of this method (Syverson, 1981).

Numerous molecular methods have been used to subtype *Candida* species, including restriction endonuclease analysis of genomic DNA, Southern hybridization analysis, pulsed-field gel electrophoresis, and polymerase chain reaction-based approaches (Pfaller, 1995; Dassanayake and Samaranayake, 2003). Among these, the RAPD-based typing was commonly used to assess the diversity of *Candida* isolates, as it has been described as a simple, rapid and reliable discriminating method for clinical epidemiological studies of oral *Candida* colonization and infection (Barchiesi *et al.*, 1997; Jain *et al.*, 2001; Waltimo *et al.*, 2001; Dassanayake *et al.*, 2002; Hossain *et al.*, 2003; Samaranayake *et al.*, 2003b; Lattif *et al.*, 2004; Pizzo *et al*., 2005).

The aim of the present study was to characterize biotyping, genotyping and antifungal susceptibility of *Candida albicans* strains isolated from HIV-infected subjects.

MATERIALS AND METHODS

Sources of *Candida albicans* strains

A total of 87 strains of *C. albicans* isolated from 26 HIV-infected patients with oral candidiasis. All 26 HIV-infected patients were at CDC stage IV and admitted to Songklanagarind Hospital, Songkhla, Thailand because of their diseases. All participants gave informed consent for the procedure used to obtain the oral samples and oral yeast isolates. The study was approved by the Medical Faculty Ethics Committee, Prince of Songkla University, Thailand.

The oral sample was obtained from individual subjects using 10 ml sterile phosphate buffered saline according to the method of Samaranayake *et al*. (1986). Colonies showing yeast-like morphology within 48 h incubation period were selected for study. All colonies showing variation in morphology, as well as identical colonies, were selected from the same isolation plate. The number of the chosen colonies depended on the density of growth recovered on the primary culture plate. In each patient, 2 to 5 isolates were collected, and all isolates were identified using production of chlamydospores, production of germ tubes and carbohydrate assimilation with API 20 C AUX (Bio Merieux, France).

Biotyping of *C. albicans* isolates

All *C. albicans* isolates were biotyped using the method of Williamson *et al*. (1987), which employed two commercially available kits, (API ZYM and API 20 C AUX (Bio Merieux)), and a boric acid resistance test. In brief, the API ZYM system evaluated the enzyme activity of the isolates by means of a set of 19 enzyme substrates contained in a tray of miniaturised plastic cupules. After inoculation of a standard suspension of the organism and incubation for 4 h at 37°C, the colour reactions in each cupule were read according to the manufacturer's instructions. The API 20 C AUX system utilised the ability of *C. albicans* isolates to assimilate 19 different carbohydrates as sole sources of carbon. The results were determined by comparison of the opacity in the test and control cupules. Finally, the boric acid resistance test assessed the sensitivity of the isolates to 1.8 mg/ml of boric acid incorporated into an agar medium.

Genotyping

Genotyping was performed by using randomly amplified polymorphic DNA (RAPD) analysis. DNA was extracted from each isolate by a modification method of Anthony *et al.* (1995).

In an initial study, several primers were used for RAPD analysis according to the instruction from previous studies. However, DNA fingerprints generated by primer RSD11 (5'GCATATCAATAAGCG-GAGGAAAAG-3') and RSD12 (5'-GGTCCGT-GTTTCAAGACG-3') (Samaranayake *et al.*, 2003a) showed adequately discriminatory and hence were used in this study.

Thermocycling was performed in a GeneAMP 2400 machine (Perkin Elmer, Foster City, USA). PCR master mix containing approximately 20 ng

of yeast DNA as template, 5 µl of 10x PCR buffer (200 mM Tris-HCl (pH 8.4) and 500 mM KCl), 200 mM of dNTPS, 2 mM $MgCl_2$, 1 µM of primer, 1.5 U Taq polymerase in 50 µl was used for PCR reaction. The first 5 cycles included 30 s of denaturation at 94°C, 2 min of annealing at 52°C, 2

TABLE 1 - *The distribution of 49 biotypes and 14 clusters of genotype of C. albicans isolated from 26 HIV-infected patients.*

Biotypes	*Number of*	*Biotypes isolates (%)*	*Number of isolates (%)*	*Clusters of Genotype*	*Number of isolates (%)*
A 1 S	27 (31.00)	B 6 S	1 (1.15)	1	2 (2.3)
A 6 S	1 (1.15)	B 15 S	1 (1.15)	2	6 (6.9)
A 6 R	1 (1.15)	B 16 S	1 (1.15)	3	2 (2.3)
A 7 S	1 (1.15)	B 19 S	1 (1.15)	4	8 (9.2)
A 8 S	3 (3.45)	B 21 R	1 (1.15)	5	9 (10.4)
A 13 S	3 (3.45)	C 1 S	2 (2.30)	6	11 (12.6)
A 14 S	3 (3.45)	C 1 R	1 (1.15)	7	7 (8.0)
A 15 S	1 (1.15)	C 2 S	2 (2.30)	8	4 (4.6)
A 16 S	1 (1.15)	C 13 S	1 (1.15)	9	4 (4.6)
A 17 S	1 (1.15)	C 22 R	1 (1.15)	10	2 (2.3)
A 17 R	1 (1.15)	C 26 S	1 (1.15)	11	8 (9.2)
A 18 S	1 (1.15)	C 29 S	1 (1.15)	12	7 (8.0)
A 19 S	1 (1.15)	D 1 S	1 (1.15)	13	9 (10.3)
A 21 S	1 (1.15)	D 8 S	1 (1.15)	14	3 (3.5)
A 22 S	1 (1.15)	D 14 S	1 (1.15)	uncluster	5 (5.8)
A 22 R	1 (1.15)	E 2 R	1 (1.15)		
A 23 S	1 (1.15)	E 14 R	1 (1.15)		
A 23 R	1 (1.15)	E 21 S	1 (1.15)		
A 24 S	1 (1.15)	E 29 R	1 (1.15)		
A 25 S	1 (1.15)	F 4 S	1 (1.15)		
A 26 S	1 (1.15)	G 1 S	1 (1.15)		
A 27 R	1 (1.15)	G 2 S	3 (3.45)		
A 30 S	1 (1.15)	G 5 R	1 (1.15)		
B 1 S	3 (3.45)	G 6 R	1 (1.15)		
B 4 S	1 (1.15)				

min of primer extension at 72°C, and then followed by 45 cycles of 30 s of denaturation at 94°C, 2 min at 57°C and 2 min at 72°C. The reaction was held at 72°C for 15 min. Control tube containing master mix but without template DNA was included in each run and reproducibility checked for each reaction. PCR products were electrophoresed in 7.5% polyacrylamide gel, stained with silver staining.

Analysis of fingerprints

RAPD gel profiles captured by a digital camera were then digitized using the Dendron software package version 3.0 (Solltech, Iowa City, Iowa) (Dassanayake *et al.*, 2006).

Antifungal susceptibility testing for ketoconazole

Minimal inhibitory concentration (MIC) for ketoconazole was performed using the NCCLS broth microdilution method according to CLSI guidelines (M27-A) (1997). A working suspension of an inoculum was prepared using a spectrophotometer at a concentration of each isolate 0.5×10^3 - 2.5×10^3 cells/ml in RPMI medium. Twofold dilutions of ketoconazole from 16 to 0.0075 µg/ml were prepared and inoculated with the working suspension. The trays were incubated at 37°C for 24 h and the MIC endpoint was determined by Microplate reader at the concentration that produced an 80% reduction of turbidity in comparison with a drug-free control.

RESULTS

The biotyping system employed in this study utilized three tests: the API ZYM test (the first letter of the code), the API 20 C system (the middle digit of the code), and resistance or sensitivity to boric acid, denoted as either R or S (the last letter of the code). A total of 49 biotypes were found among the 87 oral *C. albicans* isolates from HIV-infected patients. The major biotype, A1S, accounted for 31% of the isolates, and other 48 biotypes were generally distributed in quite less percentage (Table 1).

Overall, the results of RAPD showed that most patients exhibited varying and disparate genotypic patterns either within the same or different individuals (Figure 1). When genotypes of *C. albicans* from 26 patients were analyzed, it was shown that most patients 96.2% (25/26) harboured more than one clonal type, and a single patient could be colonized by up to 5 clonal types. The dendrogram based on S_{AB} values of 87 isolates from HIV-infected patients generated 14 clusters, contained 4 small clusters (clusters 1, 3, 10 and 14) and 10 larger clusters at the S_{AB} =0.02 (Table 1). Thus, results indicated that there was a loosely connection among this yeasts population, implying there was diversity of *C. albicans* isolated from Thai population.

All strains were examined for their MICs against ketoconazole using the NCCLS microdilution method, and the results are shown in Table 2. The mean MIC of ketoconazole was 0.45 ± 1.09 µg/ml, ranging from 0.0075 to 4.0 µg/ml. Only 17.2% (15 of 87 isolates) had MICs of ketoconazole more than the mean value (0.45±1.09 µg/ml). To assess the relationship of biotype, genotype and antifungal susceptibility, there was no association among biotypes, genotypes and antifungal susceptibility of ketoconazole (Table 2). It was noted that certain genotype or susceptibility of ketoconazole tended to relate to the host (Table 2).

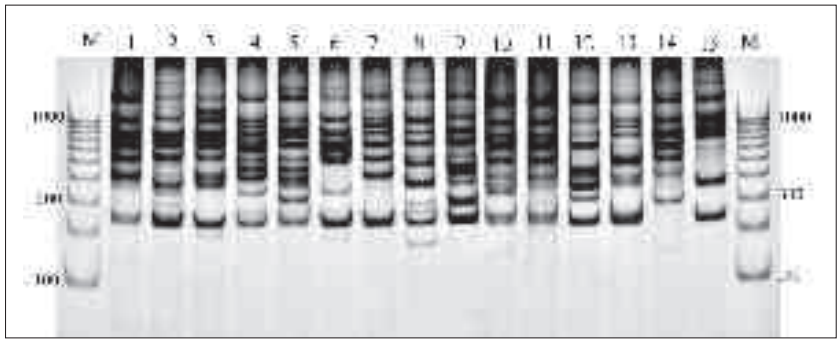

FIGURE 1 - *RAPD fingerprinting patterns (Lanes 1-15) generated with primer RSD 12 of Candida albicans isolates from 15 unrelated HIV-infected patients, each individual had a distinct pattern. M, Molecular size markers (Bio-Rad).*

TABLE 2 - *Details of biotypes, clusters of genotype and ketoconazole susceptibility of 26 patients.*

Patient	*Number of isolates*	*Biotypes*	*Clusters of genotype*	*MIC (μg/ml)*
1	3	A1S (1), A6S (1), A13S (1)	3 (2), 13 (1)	0.031 (3)
2	4	A1S (3), B19S (1)	8 (4)	0.25 (4)
3	3	A1S (1), A17R (1), C13S (1)	11 (3)	0.015 (3)
4	4	A1S (2), A14S (1), B4S (1)	1 (1), 6 (3)	0.015 (2), 1.0 (1), 0.031 (1)
5	4	A1S (2), A7S (1), A15S (1)	1 (1), 6 (1), 7(2)	0.015 (4)
6	2	A16S (1), A24S (1)	10 (2)	0.015 (2)
7	2	A1S (1), C1S (1)	2 (2)	0.031 (1), 0.062 (1)
8	2	B1S (2)	14 (2)	0.25 (1), 0.031 (1)
9	5	A1S (1), A8S (2), A21S (1), A25S (1)	2 (1), 11 (1), uncluster (3)	0.031 (1), 0.0075 (4)
10	3	A13S (2), A23S (1)	2 (3)	0.125 (2), 0.062 (1)
11	4	C1S (1), C2S (2), A22S (1)	6 (2), 14 (1), uncluster (1)	0.125 (1), 0.25 (1), 0.5 (1), 4.0 (1)
12	2	A1S (1), A6R (1)	4 (2)	0.062 (2)
13	3	A1S (3)	4 (3)	0.031 (2), 0.015 (1)
14	5	A1S (5)	6 (5)	0.015 (3), 0.031 (2)
15	2	A17S (1), A18S (1)	11 (2)	0.031 (2)
16	3	A1S (2), B1S (1)	5 (3)	0.015 (2), 0.031 (1)
17	3	A8S (1), D1S (1), D8S (1)	5 (2), 9 (1)	0.015 (2), 0.062 (1)
18	2	B21R (1), E2R (1)	5 (2)	0.031 (1), 0.25 (1)
19	3	A22R (1), A23R (1), G6R (1)	5 (2), 11 (1)	0.015 (2), 0.031 (1)
20	2	A27R (1), G5R (1)	4 (2)	0.25 (1), 4.0 (1)
21	5	C1R (1), C22R (1), E14R (1), E21S (1), E29R (1)	11 (1), 12 (4)	4.0 (5)
22	5	A14S (1), A19S (1), A26S (1), A30S (1), C26S (1)	7 (5)	1.0 (3), 2.0 (2)
23	5	C29S (1), G1S(1), G2S (3)	4 (1), 12 (3), uncluster (1)	0.015 (1), 0.031 (3), 0.062 (1)
24	5	A1S (3), A14S (1), B6S (1)	13 (5)	0.031 (1), 0.062 (3), 0.5 (1)
25	2	A1S (2)	13 (2)	0.031 (1), 0.125 (1)
26	4	B15S (1), B16S (1), D14S (1), F4S (1)	9 (3), 13 (1)	0.015 (1), 0.031 (3)

MIC = minimal inhibitory concentration of ketoconazole

DISCUSSION

In the present study, we used a standardized biotyping system (Williamson *et al.*, 1987), RAPD genotyping method and antifungal susceptibility to characterize strains *C. albicans* isolated from HIV-infected patients living in the Southern Thailand. The biotyping system we utilized is simple to perform, technically undemanding, relatively inexpensive and has been shown to have a good discriminatory power (Williamson *et al.*, 1987; Tsang *et al.*, 1995; Pizzo *et al.* 2005). For all of these reasons, this biotyping method has been widely employed to characterize oral *C. albicans* isolates from different geographic regions (Williamson *et al.*, 1987; Tsang *et al.*, 1995; Xu and Samaranayake, 1995; Matee *et al.*, 1996; Hannula *et al.*, 1997; Teanpaisan *et al.*, 2000; Waltimo *et al.*, 2001; Samaranayake *et al.*, 2003a; Pizzo *et al.* 2005). The percentage distribution of biotyping detected in our study was consistent with those reported in other studies in which A1S was the most predominant biotype (Williamson *et al.*, 1987; Xu and Samaranayake, 1995; Teanpaisan *et al.*, 2000). Data from the current and other studies imply that the phenotype A1S may be more widely distributed in China and Thailand (Xu and Samaranayake, 1995; Teanpaisan *et al.*, 2000).

RAPD analysis has proved to be a rapid, simple, cost-effective and discriminatory technique for the molecular typing of *C. albicans*, and has also been demonstrated to be highly reproducible. Thus, it was accepted that RAPD analysis of genomic DNA is a versatile tool for epidemiological studies of *C. albican*s (Pfaller, 1995; Dassanayake and Samaranayake, 2003; Pizzo *et al.,* 2005). In the present study, fingerprinting generated by RSD 11 and RSD 12 was further evaluated using a well-proven computer-assisted data analysis system, showing diversity of genotypes. This confirmed previous reports on the discriminatory power of DNA–based methods for strain delineation of *Candida*. RAPD profiles of isolates collected from different participants were generally distinct, and almost every individual harboured his/her own specific isolate. Such broad spectrum of genetic heterogeneity within isolates is quite similar to previous reports from other oral and non-oral sources (Waltimo *et al.*, 2001; Pizzo *et al.*, 2002; Dassanayake *et al.*, 2003; Samaranayake *et al.*, 2003b; Pizzo *et al*., 2005). It has been postulated that variations in the genotype could strategically enhance the survival of an organism in the oral cavity (Dassanayake *et al.,* 2006). Although, it is not our purpose to compare the study of HIV-infected patients to non-HIV infected subjects, the genetic diversity of *C. albicans* isolated from 6 healthy individuals showed similar profile, heterogeneity of phenotypic and genotypic profiles, as HIV-infected patients (data not shown).

Susceptibilities to ketoconazole varied among the isolates tested. However, all strains in this study were (in vitro) susceptible to ketoconazole (MIC ≤8.0 µg/ml) according to Pfaller *et al*. (1997) criteria. It is known that the emergence of resistant strains of *C. albicans* frequently occurs in patients previously exposed to azoles (Rex *et al*., 1995; Masiá Canuto *et al*., 2000). This study showed that ketoconazole could be the drug of choice for treatment of oral candidiasis in the south of Thailand.

The phenotypic and genotypic profiles and antifungal susceptibility were also correlated to evaluate the congruence of the typing systems used and antifungal susceptibility. As already reported (Waltimo *et al.*, 2001, Pizzo *et al*. 2005), such typing systems were not in agreement, since different genotypes could be detected in the same phenotype while the isolates in the same genotype could belong to different biotypes. In this study, the most prevalent biotype A1S could be distributed in several different genotypes, and the isolates in the same genotypic cluster, e.g. five isolates of cluster 7 of patient no. 22, could be detected as 5 different biotypes. Moreover, the susceptibility of ketoconazole was not restricted to either biotype or genotype. This confirms previous studies which showed that there was no association between drug susceptibility and biotypes or genotypes (Teanpaisan *et al*.; 2000; Pizzo *et al.,* 2002).

Despite the wide variety of sub-typing methods developed for *C. albicans*, no single procedure has become the accepted standard. Typing methods based on phenotypes could be discounted for their occasional poor discrimination and uncertain reproducibility (Hunter, 1991). It has been argued that phenotypic methods are now no longer needed when DNA-typing methods are available. However, DNA-typing methods are rel-

atively expensive and require special equipment. The results of this study showed that both biotyping and RAPD are applicable for sub-typing of *C. albicans* strains. We suggest that biotyping remains in use, as it is relatively inexpensive and requires no special equipment. Biotyping is probably a method of choice for laboratories that cannot afford equipment for DNA-typing. However, genotyping with computer analysis should become the preferred typing for *C. albicans* where it is available.

In conclusion, the current study provided data on the biotypes, RAPD profiles and antifungal susceptibility of 87 *C. albicans* isolates recovered from 26 HIV-infected patients living in Southern Thailand. A total of 49 biotypes and 14 clusters of genotype were found. The results of phenotyping and genotyping suggested high heterogeneity among *C. albicans* isolates, and all isolates were found susceptible to ketoconazole. Such findings may be useful as baseline information on oral *C. albicans* colonization in the Thai population living in the south of Thailand.

ACKNOWLEDGEMENT

We would like to give the special thanks to Professor LP Samaranayake for his kind advice for the work and for providing the laboratories facility and Dendron software for analysis of DNA fingerprints.

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