Biotypes of oral *Candida albicans* isolated from AIDS patients and HIV-free subjects in Thailand

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A short title:

Biotypes of *Candida albicans* in HIV infection in Thailand

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Abstract

This study was conducted to examine biotypes and antifungal susceptibility patterns of oral *Candida albicans* isolated from HIV-infected patients, HIV-free patients with candidiasis and healthy subjects. All isolates were biotyped using a typing system based on enzyme profiles, carbohydrate assimilation patterns and boric acid resistance. A total of 38 biotypes were found amongst 218 oral *C. albicans* isolates. The major biotype found was A1S, which accounted for 32.6% of all isolates and this biotypes was the most common in all groups. There were more different biotypes of *C.albicans* in HIV-infected groups than the in others; however, there was no statistically significant difference between the groups. The minimum inhibitory concentrations (MICs) of a total of 118 isolates were determined for amphotericin B, and ketoconazole using the National Committee for Clinical Laboratory Standards (NCCLS) broth macrodilution method and the E-test. When the antifungal susceptibility pattern among the groups were compared, a statistically significant difference was found only with amphotericin B. The median MIC of amphotericin B in the HIV-infected group was higher than in the healthy group ($p = 0.013$, NCCLS's method; $p = 0.002$, E-test). However, this difference in sensitivity was not restricted to any sub-type investigated.

Our results showed that the biotype patterns of *C.albicans* isolates that colonize HIV-infected patients are similar to HIV-free subjects, and there is no relation between antifungal susceptibility patterns and the biotypes.

Key words: Biotypes, *Candida albicans*, HIV infection, Thailand

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Introduction

A number of reports have revealed that the frequency of isolation of *Candida* and clinical signs of oral candidiasis increase with advancing HIV infection (1-4). Although among the *Candida* species isolated in these studies, *C.albicans* is the most common (1-4); relatively few details of the pathogenic features of this organism and AIDS associated oral candidiasis are known. In healthy humans, *C.albicans* is present as part of the normal floras of the oral cavity and gastrointestinal tract; however, in immunosuppressed patients, it can cause severe mucosal or invasive disease (5). 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 (6,7). The mechanism behind the ability of this fungus to cause recurrent disease is unknown. It is postulated that one factor that may influence this is a decrease in susceptibility to antifungal agents (7,8), and this may be related to certain types of *C.albicans* (9). Because of this, many attempts have been made to search for particularly virulent types of *C.albicans* using a number of techniques such as serotyping (10), biotyping (11), morphotyping (12, 13) and genotyping (14, 15). On account of the difficulty with some of these techniques, a standardised, simple and highly specific biotyping system developed by WIALLIAMSON *et al.* (11), which is technically undemanding and utilises relatively inexpensive commercially available quality controlled media, has been increasingly used in recent years (16-19).

In a previous paper (4) we showed that there is an association between candidal load and HIV infection. The aims of this study were to determine whether any sub-strains of *C.albicans*

isolated from HIV patients, HIV-free subjects with candidiasis and healthy subjects are particularly associated with health or disease using the biotyping method of WILLIAMSON *et al*. (11), and to compare isolates for their susceptibility to two commonly used antifungal agents (amphotericin B and ketoconazole).

Material and methods

Sources of *C.albicans*

A total of 218 isolates of *C.albicans* were included in this study which comprised 82 isolates from salivary samples of 15 HIV-infected patients, 76 isolates from 15 HIV-free patients with oral candidiasis and 60 isolates from 16 healthy subjects. The specimen collection and methods used for fungal cultivation and identification have been described previously (4). Briefly, an oral rinse specimen was obtained from each subject using 10 ml sterile phosphate buffered saline according to the method of SAMARANAYAKE *et al.* (20). Colonies showing yeast-like morphology within the 48-72 h incubation period were selected for study. All colonies showing variation in morphology, as well as identical colonies, were picked from the same isolation plate. The number colonies picked depended on the density of growth recovered on the primary culture plate. For examples, 10-20 colonies from 20-40 colonies on the same plate were picked for identification and biotyping but when recovery was lower (e.g. from healthy subjects), fewer colonies could be picked. All isolates were identified by using production of chlamydospores, production of germ tubes and carbohydrate assimilation with API 20 C AUX (Bio Merieux).

Biotyping of *C.albicans* isolates

All isolates of *C.albicans* were biotyped using the method of WILLIAMSON *et al.* (11) which employs 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 evaluates 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 instructions. The API 20 C AUX system utilises 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 the control cupules. Finally, the boric acid resistance test assesses the sensitivity of the isolates to 1.8 mg/ml of boric acid incorporated into agar medium.

Antifungal susceptibility testing

A total of 118 isolates of *C.albicans* were chosen to represent strains with either different or the same colony morphology and biotype. These included 52 isolates from the HIV-infected group, 33 isolates from the HIV-free candidiasis group and 33 isolates from the healthy group. For each isolate, minimal inhibitory concentrations (MIC) for amphotericin B and ketoconazole were determined using NCCLS macrodilution method (21) and the E-test strip (AB Biodisk, Sweden).

The NCCLS broth dilution method was performed according to NCCLS document M27 – P (21). A working suspension of the inoculum was made by a 1:100 dilution of the 0.5 McFarland standard yeast suspension in 0.85% saline followed by a 1:20 dilution in RPMI broth. Two fold dilutions of the antifungal agents from 64 to 0.015 ug/ml were prepared and inoculated with the working suspension. The tubes were incubated at 37°C for 48 h. The MIC was read as the concentration which inhibited growth (amphotericin B) or produced an 80% reduction of turbidity in comparison with drug-free control (ketoconazole). For the E-test, colonies of each yeast were suspended in saline to produce a turbidity equivalent to a 0.5 McFarland standard. The inoculum was swabbed on to Saboraud's Dextose Agar and allowed to dry for 10-15 min before each of the antifungal E-test strips were applied. Plates were read at 24 h and 48 h according to the E-test technical guide for antifungal susceptibility testing.

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, denoted as either R or S (the last letter of the code). The reproducibility of the results obtained by these methods has not been tested on a broad scale, but some isolates chosen at random were tested on multiple occasions and have shown clearly reproducible results. A total of 38 biotypes was found among the 218 oral *C.albicans* isolates (Table 1). The major biotype, A1S, accounted for 32.6% of the isolates and this biotype was commonly found in HIV-infected patients, HIV-free candidiasis and healthy subjects (31.7%, 32.8%, and 33.3% respectively). The second most common biotype was B4S, which represented 14.7% of total isolates. The other biotypes found were A4S, B1S and B1R (7.8%, 7.8% and 5.0% respectively). When the number of each biotype in each of the HIV-infected patients, HIV-free with candidiasis and healthy subjects was compared, the first group had a higher number of biotypes than the others (Fig.1); however, this difference was not statistically significant (Kruskal-Wallis test).

A total of 118 strains of *C.albicans* obtained from HIV-infection patients, HIV-free candidiasis and healthy subjects were compared for their MICs against amphotericin B and ketoconazole using the NCCLS macrodilution method and the E-test, and the results are shown in Table 2. Generally, results obtained by NCCLS broth method were largely in agreement with those obtained by the E-test. Pearson correlation coefficients of both methods for amphotericin B and ketoconazole were 0.89 and 0.57, respectively. The median MICs for amphotericin B in of isolates from the HIV-infected group was statistically significantly higher than in that of isolates from the healthy subjects ($p = 0.013$, NCCLS method; $p = 0.002$, E-test). Also, there were statistically significant differences in the mean MIC for

amphotericin B between the HIV-infected group and the HIV-free subjects with candidiasis ($p = 0.01$, NCCLS method) and between the HIV-free candidiasis group and the healthy group ($p < 0.03$, E-test). When the patients' history of antifungal therapy was taken into account, it showed that there was no significant difference between the MIC and whether a patient had taken amphotericin B previously. The mean MICs of ketoconazole among isolates from the three groups showed no significant differences using either the NCCLS method or the E-test.

Discussion

Among the numerous AIDS-associated oral diseases, oral candidiasis is the most frequent, with up to 90% of HIV-infected patients being affected (22, 23). The clinical syndrome is not life-threatening; but it is painful and its recurrent nature makes it of importance. Oral candidiasis is caused mainly by *C.albicans*, although its pathogenesis is still unclear. The condition of the patient is probably the major factor governing the development of clinical candidiasis, and this is often associated with immunodeficiencies (24). However, as recently shown for many other microbial pathogens, the possibility that certain strains or groups of strains are more likely to be involved in clinical disorders cannot be excluded (25). Whether or not HIV-patients are colonized with selected strains of *C.albicans* has been a matter of debate. Using DNA fingerprinting, the results of two studies showed that no particular strain was associated with HIV-infected patients and that *C.albicans* populations from the oral cavities of HIV-infected and HIV-negative persons have a similarly disparate clonal origin (26, 27). Some researchers used a DNA probe to track the *C.albicans* isolates from oral lesions in HIV-seropositive individuals and their data suggested that each patient carriers a unique strain of *C.albicans*. Furthermore, it was shown that the strains present during both symptomatic and asymptomatic states of candidiasis were the same (28, 29). However, others have produced evidence that there is increased genetic variation of *C.albicans* isolates in HIV-infection compared to controls (30, 31). And the differences were noted when the appropriate molecular technique coupled with appropriate analyses were used (31).Also, SWEET *et al.* (32, 33) showed that more biotypes of *C.albicans* were present in HIV/AIDS groups than in control subjects, and that almost all *Candida* species isolated from HIV subjects adhered to buccal epithelial cells in higher numbers than did those strains isolated from controls.

Here, we used the biotyping method of WILLIAMSON *et al.* (11) to evaluate the biotypes of isolates from a range of patient groups. It is known that genetic typing methods provide more sensitive and specific means to discriminate among isolates. However, biotyping has the advantages of being simple to perform, technically undemanding and inexpensive compared to molecular techniques. In addition, it also has the advantage of allowing more meaningful comparison of the present studies with those of previous studies of a similar nature. The first study of *C.albicans* biotypes in HIV-infected patients was conducted by KORTING *et al.* (34) and employed the API 20 C (carbohydrate assimilation) system. Their results showed that from a total of 61 oral *C.albicans* strains isolated from HIV-infected individuals, with or without signs of candidiasis, the majority (64%) of isolates belonged to group 1. However, there was no detail given of the incidence and proportions of each of the biotypes between the groups with and without signs of clinical candidiasis and no HIV-negative subjects were included in the study. Since the API 20 C carbohydrate assimilation system has a relatively poor discriminatory power, API ZYM and boric sensitivity tests were added to complement the API 20 C profiles (11). Results from previous studies of others (16-19) and ours demonstrated that this system serves to further differentiate the biotypes into smaller sub-groups. Using this biotyping system, TSANG *et al.* (17) showed that there are many different sub-strains of oral *C.albicans* in HIV-infected patients. However, no data on healthy subjects were provided. Our results showed that there were 38 biotypes among 218 strains. When the results were compared among the groups, HIV-infected patients had many sub-types (1 - 8) more than HIV-free candidiasis (1 - 4) or healthy subjects (1 - 4), and there were no significant differences in the biotypes between the three groups. The results of statistical analysis suggested that this is may be affected by the small sample size in this study. Thus, larger numbers of patients will be need to be studied for further confirmation.

With regard to the geographic distribution of the different biotypes, it has been shown that some biotypes are globally prevalent. However, almost one third of the biotypes reported here have not been previously described and may reflect geographical exclusivity. TSANG *et al.* (17) has reported that A1R (18%) and A1S (11%) are the most common biotypes among the oral *C.albicans* isolates deriving from HIV-infected patients in Hong-Kong, Australia, England and Germany. Another previous study in healthy individuals in Britain, found that A1R and A1S were also the commonest biotypes, accounting for 23% and 26% of the total isolates, respectively (11). The biotypes A1S and J1S have been found to predominate in China (18) and in Tanzania (19). Our results concur with the findings in China and Tanzania that the most common biotypes is A1S, accounting for 32.6% in all groups investigated. It is noted that in previous studies (17-19), only a single representative isolate was selected from each culture plate; whereas, multiple colonies from each plate were collected in this study. It is assumed that the colonies examined represent the predominant strains present on the plate. No clear explanation for the widespread overrepresentation of such biotypes has been given. It may be hypothesized that they are better adapted than other sub-types to life on or in the human body. They may also be more easily transmitted between humans than are other sub-types. When compared with previous data of new biotypes from the foregoing countries, our results showed that almost one third (65 of 218) of isolates were previously undescribed new biotypes. As the previous reports were from Scotland (11), Germany, Australia, England and Hong-Kong (17), China (18) and Tanzania (19), it is likely that there are geographical variations in *C.albicans* biotypes.

Up to now, there is no general agreement about a standardized method of *in vitro* antifungal susceptibility testing, since results show great inter- and intra- laboratory variations. The NCCLS has established a broth macrodilution method as a reference method for antifungal

susceptibility testing; however, it is labor-intensive and time consuming. We are in agreement with the previous studies that E-test appears to be equivalent to the NCCLS reference macrobroth method for testing of *Candida* species susceptibility to azole antifungal agents (35, 36). WAGNER *et al.* (37) has concluded that the E-test is comparable to the NCCLS method for testing of susceptibility to amphotericin B and fluconazole; in addition, E-test appears to be superior for the detection of resistance to amphotericin B. Our results have shown that the NCCLS reference macrodilution method and the E-test gave very similar results for amphotericin B and showed moderate agreement for ketoconazole. Generally, the endpoints obtained were identical or different by no mean than 2 twofold dilutions. However, when the MIC level is high, the results of both methods show greater difference: if the MICs given by NCCLS method is more than 1-4 μg/ml, it would be >32 μg/ml by the E-test. This may be due to the problem of diffusion of the agent through the agar medium necessary for the E-test. In the present study, we have shown that susceptibility of our isolates to amphotericin B was significantly different between the patient groups but there was no difference in sensitivity to ketoconazole. It was found that isolates from the AIDS group were more resistant to amphotericin B than were isolates from the HIV-free candidiasis group and the healthy group. This resistance is not associated with a history of amphotericin B therapy or restricted to any sub-type investigated (data not shown). GALLAGHER *et al.* (38) have shown that phenotypically switched variants of *C.albicans* can develop decreased azole susceptibility even though these strains remained genetically identical. The results of MCCULLOUGH (39) showed that the same *C.albicans* genotypes tended to persist during the course of disease progression, but that the colonial morphologies of the isolates changed. Also, SOLL *et al.* (40) found that despite a high frequency of phenotypic switching by *C.albicans*, nucleic acid hybridization of DNA from multiple phenotypes from a single

culture site consistently yielded identical genotypes. These observations have shown that it is not necessary to have alterations in the type of strain present for there to be changes in the drug susceptibility of *C.albicans*. This may explain our finding of increased resistance to amphotericin B among *C.albicans* strains isolated from HIV-infected patients but no difference in biotypes. However, further work on genetic analysis is required to clarify this.

To conclude, it is worth emphasizing here that the present data are the first base line information for the studies of oral candidal infection in different cohorts (AIDS patients, HIV-free healthy subjects and HIV-free with candidiasis) of the Thai population. Our results show that the biotype patterns of *C.albicans* that colonize AIDS patients are similar to those in normal Thai subjects. This suggests that there may be no particular biotypes of *C.albicans* linked with specific clinical characteristics. Similarly, the oral *C.albicans* isolates showing *in vitro* susceptibility to amphotericin B and ketoconazole were not restricted to any sub-group investigated. However, the concept of phenotypic switching of *C.albicans* among HIV-infected patients could not be excluded and the resistance of these isolates to amphotericin B needs further explanation.

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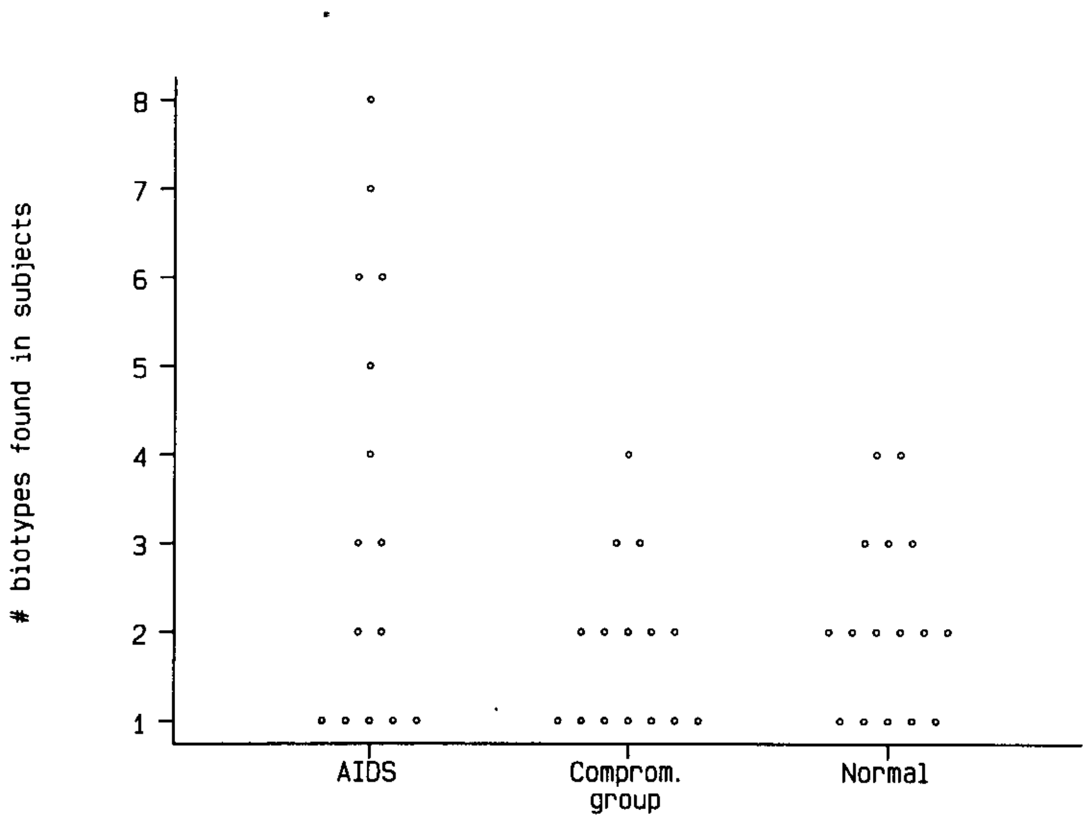

Fig.1 Number of biotypes identified in AIDS groups, HIV-free with candidiasis (comprom.) group and normal subjects.

Table 1. Biotype profiles of oral *Candida albicans* isolated from HIV-infected patients, HIV-free with candidiasis and healthy subjects

Biotypes	HIV-infected group	HIV-free with candidiasis	Healthy subjects	Total
	No. (%)	No. (%)	No. (%)	No. (%)
A1R	3 (3.6)	0	1 (1.7)	4 (1.8)
A1S	26 (31.7)	25 (32.8)	20 (33.3)	71 (32.6)
A4R	1 (1.2)	0	1 (1.7)	2 (0.9)
A4S	6 (7.3)	6 (7.8)	5 (8.3)	17 (7.8)
A6S*	0	0	1 (1.7)	1 (0.5)
A7S*	1 (1.2)	0	0	1 (0.5)
A8S	4 (4.9)	2 (2.6)	0	6 (2.8)
A14R	1 (1.2)	0	0	1 (0.5)
A17S	0	1 (1.3)	1 (1.7)	2 (0.9)
A18S	0	2 (2.6)	3 (5.0)	5 (2.3)
A19S*	1 (1.2)	0	0	1 (0.5)
A20S*	1 (1.2)	0	0	1 (0.5)
A23S*	0	1 (1.3)	1 (1.7)	2 (0.9)
A24S*	0	0	1 (1.7)	1 (0.5)
B1R	0	6 (7.9)	5 (8.3)	11 (5.0)
B1S	4 (4.9)	6 (7.9)	7 (11.7)	17 (7.8)
B2S*	0	0	1 (1.7)	1 (0.5)
B4R*	1 (1.2)	2 (2.6)	0	3 (1.4)
B4S*	13 (15.9)	12 (15.8)	7 (11.7)	32 (14.7)
B6S*	2 (2.4)	1 (1.3)	0	3 (1.4)
B15S*	0	0	1 (1.7)	1 (0.5)
B16S*	0	0	1 (1.7)	1 (0.5)
B18R*	0	2 (2.6)	0	2 (0.9)
B18S*	1 (1.2)	0	0	1 (0.5)
B19S*	1 (1.2)	0	0	1 (0.5)
B20S*	2 (2.4)	0	0	2 (0.9)
B21S*	1 (1.2)	1 (1.3)	0	2 (0.9)
B22S*	1 (1.2)	0	0	1 (0.5)
B24S*	1 (1.2)	0	0	1 (0.5)
C1S	1 (1.2)	2 (2.6)	0	3 (1.4)
D1R	1 (1.2)	1 (1.3)	0	2 (0.9)
D1S	6 (7.3)	2 (2.6)	0	8 (3.7)
D6S*	0	2 (2.6)	1 (1.7)	3 (1.4)
D8S	3 (3.6)	0	0	3 (1.6)
D14S*	0	0	1 (1.7)	1 (0.5)
E1R	0	0	1 (1.7)	1 (0.5)
F4S*	0	0	1 (1.7)	1 (0.5)
I4S*	0	2 (2.6)	0	2 (0.9)
Total	**82 (100)**	**76 (100)**	**60 (100)**	**218 (100)**
***New biotypes**	**26 (31.7)**	**23 (30.3)**	**16 (26.7)**	**65 (29.8)**

Table 2. Median of MICs (µg/ml) of amphotericin B and ketoconazole

Group	Amphotericin B				Ketoconazole	
	NCLLS	p-value	E-test	p-value	NCLLS	E-test
HIV-infection:	0.500 (0.062 – 4.0)	0.013[a]	0.315 (0.032 - >32)	0.002[a]	0.062 (0.015 – 16.0)	0.094 (0.012 - >32)
Taken antifungals	0.500 (0.250 – 1.0)		0.380 (0.032 - >32)		0.062 (0.031 – 1.0)	0.094 (0.012 - >32)
No antifungals	0.500 (0.125 – 0.500)		0.250 (0.190 – 0.500)		0.062 (0.015 – 4.0)	0.125 (0.032 – 0.500)
HIV-free candidiasis:	0.250 (0.062 – 1.0)	0.01[b]	0.250 (0.038 – 0.500)	0.49[b]	0.062 (0.015 – 8.0)	0.094 (0.032 - >32)
Taken antifungals	0.310 (0.125 – 0.500)		0.380 (0.125 – 0.500)		0.062 (0.062 – 0.125)	0.079 (0.032 – 0.094)
No antifungals	0.250 (0.062 – 1.0)		0.250 (0.038 – 0.500)		0.062 (0.015 – 8.0)	0.125 (0.032 - >32)
Healthy subjects	0.250 (0.031 – 1.0)	0.96[c]	0.190 (0.032 – 0.500)	0.03[c]	0.062 (0.015 – 8.0)	0.094 (0.047 - >32)

[a] Median MICs of HIV group vs healthy subjects

[b] Median MICs of HIV group vs HIV-free candidiasis

[c] Median MICs of HIV-free candidiasis vs healthy subjects