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# Molecular subtyping of clinical isolates of *Candida albicans* and identification of *Candida dubliniensis* in Malaysia

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**Abstract**

The genotypes of 221 recent isolates of *Candida albicans* from various clinical specimens of 213 patients admitted to the University Malaya Medical Centre, Malaysia was determined based on the amplification of a transposable intron region in the 25 S rRNA gene. The analyses of 178 *C. albicans* isolated from nonsterile clinical specimens showed that they could be classified into three genotypes: genotype A (138 isolates), genotype B (38 isolates) and genotype C (2 isolates). The genotyping of 43 clinical isolates from sterile specimens showed that they belonged to genotype A (29 isolates), genotype B (10 isolates), genotype C (2 isolates) and genotype D (2 isolates). The overall distribution of *C. albicans* genotypes in sterile and nonsterile specimens appeared similar, with genotype A being the most predominant type. This study reported the identification of *C. dubliniensis* (genotype D) in 2 HIV-negative patients with systemic candidiasis, which were missed by the routine mycological procedure. The study demonstrated the genetic diversity of clinical isolates of *C. albicans* in Malaysia.

**Key words:** *Candida albicans, Candida dubliniensis,* genotyping, Malaysia, PCR

## Introduction

The increasing population of immunocompromised patients due to infection with human immunodeficiency virus, chemotherapy, organ transplantation and the common use of indwelling intravascular devices have significantly increased the incidence of candidiasis [1, 2]. Systemic candidiasis in hospitalized patients is a significant cause of morbidity and mortality among severely ill individuals and candidemia has been ranked the fourth most prevalent cause of bloodstream infections with its attributable mortality (~40%) exceeds that of bacteremia [3–6]. *Candida albicans* is generally accepted as being the most pathogenic member of the genus and the predominant causative agent of candidiasis and a major nosocomial pathogen [7–9]. However, atypical *C. albicans* strains have been described in an increasing number of reports [8, 10–12].

The strain delineation within *C. albicans* and its distribution among patients is important for identification of the predominant types of *C. albicans* responsible for candidiasis and to determine the relationships of their subtypes to human disease. Genotyping is highly sensitive and offers greater discrimination compared to biotyping, thus allowing more detail studies on the epidemiology and pathogenesis of microorganism [13–18]. McCullough et al. [17] reported the use of polymerase chain reaction (PCR) for differentiation of *C. albicans* using primers designed to span the 25 S rRNA gene (rDNA). The advantage of using the technique is that it can detect *C. dubliniensis* (genotype D) as well as determine the genotypes of *C.albicans* [17, 18]. McCullough et al. [17] confirmed that genotype B belongs to the same taxon as type I *C.stellatoidea* and genotype D belongs to the same taxon as *C. dubliniensis*. The use of this genotype analysis method is simple and

reproducible when reference *C. albicans* strains are used [18]. In addition, a new genotype of *C. albicans* with Group I intron, genotype E, was reported using the similar approach [18].

Analysis of *C. albicans* from various body sites of healthy women suggested that different body sites may select for certain genotypes [19]. There is also evidence suggesting that particular strains of *C. albicans* are associated with specific forms of oral candidiasis [16]. In addition, other types of associations between genotypes and special host or ecological conditions have been proposed [20]. The objectives of this study were to determine the genotypes of *C. albicans* isolated from various clinical specimens of patients admitted to a Malaysian hospital and to determine whether any specific genotypes were associated with the type of clinical specimens.

## Materials and methods

### *Clinical isolates*

A total of 221 *C. albicans* clinical isolates obtained from 213 patients admitted to the University Malaya Medical Centre, Kuala Lumpur, Malaysia, from August, 2002 to May, 2003 were selected for genotyping. These clinical isolates were identified as *C. albicans* on the basis of their cultural and morphological characteristics on Sabouraud's dextrose agar, germ tube production and production of chlamydopsore on cornmeal agar.

### *DNA extraction*

Yeast was grown on Sabouraud's dextrose agar medium for overnight at room temperature. The colony was resuspended in saline to obtain the turbidity of a 0.5 McFarland standard. Crude DNA extracts were prepared by a simple boiling method or using microLYSIS method (Microzone Limited, Australia). Briefly, to extract DNA by boiling method, a loopful of yeast colony was suspended in 0.5 ml of distilled water and boiled at 100 °C for 10 min before used for PCR. For the MicroLYSIS method, 2 $\mu$l of cell suspension was added to 18 $\mu$l of microLYSIS solution in a 0.2-ml Eppendorf tube and overlaid with 20 $\mu$l of sterilized mineral oil. The tube was heated in a thermal cycler (Perkin Elmer, USA) using the following temperature profile: 65 °C, 5 min; 96 °C, 2 min; 65 °C, 4 min; 96 °C, 1 min; 65 °C, 1 min; 96 °C, 30 s; and 30 °C, 5 min. After cycling, the lysis solution-DNA mixture was used directly for PCR amplification or stored at −20 °C for further use.

### *Amplification of transponsable intron region of the Candida 25S rRNA gene*

The primer pairs used were CA-INT-L (5′-ATAAGGGAAGTCGGCAAAATAGATCCGTAA-3′) and CA-INT-R (5′-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3′) as described previously [17]. The amplification mixture (total volume, 50 $\mu$l) contained 1.5 mM $MgCl_2$, 200 $\mu$M each dATP, dGTP, dCTP and dTTP, 0.1 $\mu$M primers, 1.25 U of *Taq* polymerase (Promega, USA) and 5 $\mu$l of template DNA. The conditions used were as follows: denaturation by incubation for 3 min at 94 °C prior to 30 cycles of 94 °C for 1 min, 65 °C for 1 min and 72 °C for 2.5 min and a final extension at 72 °C for 10 min in a thermocycler (Perkin–Elmer model 480). All reaction products were characterized by electrophoresis on 1.5% agarose gels in Tris–borate–EDTA buffer at 70 V for 100 min and were then stained in a solution of 0.5 $\mu$g of ethidium bromide per ml.

### *Identification of* C. dubliniensis *by phenotypic methods*

Suspected yeast organisms were examined for growth at 42 °C on Sabouraud's dextrose agar medium. Niger seed agar was inoculated with the organism and incubated at room temperature for observation of hyphal production as described by Lees and Barton [21]. Yeast was also grown on CHROMagar Candida agar medium (Paris, France) [22] at room temperature and colour reading was made after 48 h of incubation. API yeast identification test (Biomeriuex, France) was performed according to the recommendation of the manufacturer.

## Results

The PCR products of representative genotypes of *C. albicans* were demonstrated in Figure 1. The PCR resulted in the generation of a single product

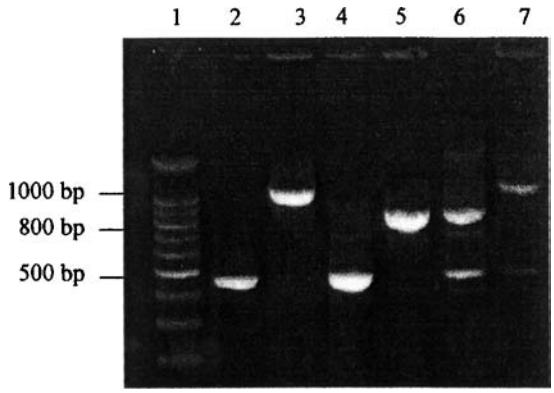


*Figure 1.* PCR genotyping of *C. albicans*. Lane 1, 100 bp DNA ladder; 2. genotype A, *Candida albicans* ATCC strain 90028; 3, genotype D (laboratory strain of *C. dubliniensis*), 4, genotype A; 5, genotype B; 6, genotype C; 7, genotype D.

for *C. albicans* genotypes A (~450 bp) and B (~840 bp). Genotype C isolates had 2 PCR products (~450 and ~840 bp) that were identical in size to the respective products from *C. albicans* genotypes A and B. Genotype D exhibited a band of approximately 1080 bp.

The genotype distribution of the clinical isolates according to various types of clinical specimens is demonstrated in Table 1. At least 2 different genotypes could be isolated from various clinical specimens obtained from the patients except for bronchoalveolar lavage (only genotype A). Three different genotypes could be isolated from the blood, sterile tissue and body fluids and vaginal specimens. The overall genotype distribution of *C. albicans* regardless of types of clinical specimens was: A (75.6%), B(21.7%), C(1.8%) and D(0.9%). Genotype A was the predominant *C. albicans* type that was isolated from all clinical specimens.

The 2 genotype D strains was characterized by the absence of growth at 42 °C on Sabouraud's dextrose agar medium and hyphal production on Niger seed agar. Both strains were identified as *C. dubliniensis* by API yeast identification system (profile 6172134, excellent identification, 99.9%). One strain formed bluish green colonies on CHROMagar Candida agar and the other produced darker green colonies. Repeat isolates of *C. albicans* were obtained from eight patients, of which different genotypes were isolated from the repeat specimens of three patients.

## Discussion

This study showed that the clinical strains of *C. albicans* in Malaysia could be classified into 4 genotypes: A, B, C, and D. The genotype distribution of *C. albicans* clinical isolates seemed to be in agreement with the Japanese isolates [18], in which genotype A was the most predominant genotype isolated from clinical specimens, followed by genotype B, C and D. However, no

*Table 1.* Genotype distribution of clinical isolates of *C. albicans* in Malaysia according to type of clinical specimens

| Clinical specimens | No. tested | Genotype | | | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| Sterile | 43 | 29 (67.4%) | 10 (23.2%) | 2 (4.7%) | 2 (4.7%) |
| Blood | 20 | 13 | 5 | 0 | 2 |
| Tissue and body fluids | 15 | 10 | 3 | 2 | 0 |
| Bronchoalveolar lavage | 4 | 4 | 0 | 0 | 0 |
| Others[a] | 4 | 2 | 2 | 0 | 0 |
| Non-sterile | 178 | 138 (77.5%) | 38 (21.3%) | 2 (1.1%) | 0 (0%) |
| Vagina | 120 | 93 | 25 | 2 | 0 |
| Sputum | 24 | 20 | 4 | 0 | 0 |
| Urine | 18 | 16 | 2 | 0 | 0 |
| Pus | 7 | 3 | 4 | 0 | 0 |
| Others[b] | 9 | 6 | 3 | 0 | 0 |
| Total | 221 | 167 (75.6%) | 48 (21.7%) | 4 (1.8%) | (0.9%) |

[a] Includes swabs taken from placental.
[b] Includes nasal swabs, throat swabs and wound swabs.

genotype E was detected. No specific association of genotype with types of clinical specimens was noted in this study.

The genotyping method used in this study allowed the identification of 48 strains of genotype B (type I *C. stellatoidea,* according to McCullough et al. [17]). The yeast was the second most predominant *C. albicans* isolated from the patients in this study (Table 1). *C. stellatoidea* is very closely related to *C. albicans* and is indistinguishable from *C. albicans* by the routine mycological procedures. The organism is not conferred with a species status as it is not possible to differentiate the organism from *C. albicans* using molecular and nonmolecular methods [11, 17, 23]. Genotype C was isolated from four clinical specimens; i.e., peritoneal fluid, placental tissue and two vaginal swabs from the patients in this study. The role and clinical significance of each genotype in candidiasis has not been elucidated.

This study reported the identification of two strains of *C. dubliniensis* which were initially identified as *C. albicans* by routine laboratory procedures. The organisms were isolated from 2 HIV negative patients with systemic candidiasis. As *C. dubliniensis* is phylogenetically closely related to *C. albicans,* both species share many morphological and physiological characteristics such as germ tube formation which makes discrimination between these two species difficult in a clinical laboratory [10]. This could have contributed to the absence of *C. dubliniensis* reported in the general hospital populations in previous studies [24, 25]. The overall prevalence of *C. dubliniensis* between germ tube positive yeasts from clinical samples in this study was 0.9%, which seems to be in agreement with that of a previous study [12]. *C. dubliniensis* occurs in the oral cavity of mostly HIV positive individuals [10, 26]. However, there were also reports on the isolation of the organism from the blood of transplant patients with neutropenia, patients undergoing broad-spectrum antibiotic therapy and patients suffering from gastric carcinoma [27], indicating that this fungal infection may not necessarily be associated with AIDS. The organism has most recently been identified in cases of systemic disease in Europe, United States and Australia [10], and also in the Southeast Asia [28].

Although CHROMagar Candida agar has been widely used for differentiation of *C.dubliniensis* from *C. albicans* [22], the reliability of the use of the CHROMagar Candida medium was questioned as not all produced the typical dark green colonies on the agar [29]. In this study, only one of the *C. dubliniensis* strain produced typical dark-green colonies.

As the yeast microflora can be very dynamic, a single host could harbor multiple yeast species or multiple genotypes of the same species at the same or different body sites. It will be interesting to determine whether the different genotypes of *C. albicans* isolated from the repeat specimens of three patients in this study were acquired recently or they were the ones persisting in the hosts.

The genotyping of *C. albicans* in this study showed that the majority of the clinical isolates have identical genotypes, with a single genotype (A) predominating in majority of isolates. A correlation between the *Candida* genotype and antifungal susceptibility has been indicated, with strains of genotype A were reported as less susceptible to flucytosine than either genotype B or genotype C strains [17, 18]. Due to the large number of this genotype A present in the clinical specimens, the heterogeneity of this genotype should be further determined to allow more detail epidemiological analysis.

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