Issues in Identifying Germ Tube Positive Yeasts by Conventional Methods

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Candida speciation is vital for epidemiology and management of candidiasis. Non-molecular conventional methods often fail to identify closely related germ tube positive yeasts from clinical specimens. The present study was conducted to identify these yeasts and to highlight issues in conventional versus molecular methods of identification. A total of 98 germ tube positive yeasts from high vaginal swabs were studied over a 12-month period. Isolates were examined with various methods including growth at 42°C and 45°C on Sabouraud dextrose agar (SDA), color development on CHROMagar *Candida* medium, chlamydospore production on corn meal agar at

25°C, carbohydrate assimilation using ID 32C system, and polymerase chain reaction using a single pair of primers targeting the hyphal wall protein 1 (Hwp1) gene. Of all the isolates studied, 97 were molecularly confirmed as *C. albicans* and one isolate was identified as *C. dubliniensis*. No *C. africana* was detected in this study. The molecular method used in our study was an accurate and useful tool for discriminating *C. albicans*, *C. dubliniensis*, and *C. africana*. The conventional methods, however, were less accurate and riddled with many issues that will be discussed in further details. J. Clin. Lab. Anal. 28:1–9, 2014. © 2013 Wiley Periodicals, Inc.

Key words: *C. africana*; *C. albicans*; *C. dubliniensis*; Hwp1; Malaysia

INTRODUCTION

The genus *Candida* comprises many different species of which *C. albicans* is considered the most common and important. However, in recent years, other species, such as *C. krusei*, *C. glabrata*, and *C. tropicalis*, have also been introduced as important pathogens (1,2). Additionally, in the past two decades, many reports have defined atypical isolates of *C. albicans* (3–5), which show similar phenotypic and biochemical characteristics making them difficult to identify by conventional methods. A famous example is *Candida dubliniensis* that was first introduced by Sullivan et al. (3) from the oral cavity of HIV-infected patients. However, this organism has also been observed in other immunosuppressed (6) and healthy individuals (7). Isolation of this yeast has not been limited to the oral cavity; it has also been recovered from specimens from different body sites, including blood, feces, lungs, and vagina (3,6,8). *Candida dubliniensis* shares many phenotypic similarities with *C. albicans*, both species have the ability to produce germ tube in serum and produce chlamydospores on corn meal agar. Frequently these similarities have led to the misidentification of *C. dubliniensis* as *C. albicans*

in routine laboratories (6,9). *Candida africana* is another newly introduced species or variant of *C. albicans* that is known to be a germ tube positive and chlamydospore negative yeast (10). This yeast was first isolated from patients with positive vaginal yeast cultures in Angola, Madagascar, and Germany (4). *Candida africana* has also been reported in vaginal samples from Saudi Arabia (5), Spain (11), Italy (12), United Kingdom (13), Nigeria (14), and Senegal (15) and is likely to have a worldwide distribution. Moreover, Odds et al. (16) identified *C. africana* in one blood sample from a patient in Chile, suggesting

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that this yeast may be associated with a wider clinical distribution.

To achieve a better understanding of the clinical importance and epidemiological role of *C. dubliniensis* and the newly introduced *C. africana* in human infections, it is important for medical mycology laboratories to rapidly and accurately identify these closely related germ tube positive yeasts. However, over the past years, many variable results have been reported from phenotypic methods when used to identify these germ tube positive yeasts (17–21). In contrast, many molecular methods based on polymerase chain reaction (PCR) assay have been reported to be reliable for identifying *C. albicans* and *C. dubliniensis* (22–24). However, no *C. africana* strain was included in the previous studies. Nevertheless, in 2008 Romeo and Criseo (25) described a new molecular technique by using a single pair of primers that were extracted from the hyphal wall protein 1 (Hwp1) genes. Hwp1 gene is highly promoted during germ tube production and appears to be characteristic of *C. dubliniensis*, *C. africana*, and *C. albicans* (25). The method described by Romeo and Criseo is simple, cost-effective, and offers a high throughput in the correct identification of these closely related germ tube positive yeasts (25).

In this research, we studied 98 germ tube positive yeasts that were isolated from 155 patients with positive high vaginal swabs (HVSs) at the Universiti Kebangsaan Malaysia Medical Centre (UKMMC) in Kuala Lumpur, Malaysia. The isolates were compared on phenotypic and biochemical bases against molecular confirmation by the method described by Romeo and Criseo (25). Therefore, the main objectives of this study were to evaluate the performance of various conventional methods and to highlight the issues in identifying germ tube positive yeasts in comparison to a newly described molecular method.

MATERIAL AND METHODS

Isolates

From October 2010 until September 2011 a total of 155 HVS cultures were performed at the Medical Mycology Unit at UKMMC by standard methods. All of the samples were tested for a positive reaction to the germ tube test for preliminary identification (presumptive of *C. albicans*, *C. dubliniensis*, and *C. africana*). A total of 98 germ tube positive isolates were studied.

Reference Strain

Three reference strains, *C. albicans* ATCC 10231, *C. dubliniensis* NCPF 8809, and *C. africana* ATCC MYA 2669, were included in this study.

Phenotypic Identification

All of the 98 germ tube positive isolates were tested for their growth ability on Sabouraud dextrose agar (SDA) (Oxoid, Cambridge, United Kingdom) at 42°C and 45°C, colony color appearance on CHROMagar Candida medium (CHROMagar, Paris, France), chlamydospore production on corn meal agar with added 1% Tween 80 at 25°C (Becton, Dickinson and Company, Sparks, Maryland, USA) and carbohydrate assimilation tests (ID 32C yeast identification system; bioMerieux, Marcy-l'Étoile, France).

Germ Tube

Small inoculum of yeast isolates were added to 0.5 ml horse serum (Oxoid, Cambridge, United Kingdom) and incubated at 37°C for 2 hr to determine their germ tube production. Subsequently each sample was examined for germ tube production for up to 5 min by using a light microscope with a $\times 10$ objective.

Growth on SDA at 42°C and 45°C

All of the germ tube positive isolates were subcultured on SDA (Oxoid, Cambridge, United Kingdom) at 42°C and 45°C for 48 hr to determine their growth ability at these temperatures. If the growth of the colonies were enhanced to the last two streaking areas of the plate, the growth was considered as good (++), if the growth was limited to the primary streaking area of the plate, it was considered as poor (+), and if no growth was observed on the plate, no growth (-) was considered.

CHROMagar Candida Medium

All of the germ tube positive isolates were subcultured on CHROMagar Candida medium (CHROMagar, Paris, France) at 37°C for 48 hr. Isolates were reported as *C. albicans*, *C. dubliniensis*, or *C. africana* if the colony color was light blue-green (9), dark green (9), or slight green (10), respectively.

Chlamydospore Production

All of the germ tube positive isolates were subcultured on corn meal agar with added 1% Tween 80 (Becton, Dickinson and Company, Sparks, Maryland, USA) using the Dalmau technique (26). Isolates were incubated at 25°C and were microscopically examined on days 2, 3, and 7, suggesting *C. albicans*, that is, mainly single chlamydospores on elongated hyphae, or *C. dubliniensis*, that is, abundant chlamydospores, in contiguous pairs or clusters on short branching pseudohyphal elements (3), and

C. africana producing no chlamydospores (10). Inspection was done with a light microscope at $\times 40$ objectives.

Carbohydrate Assimilation

All of the germ tube positive isolates underwent a carbohydrate assimilation test using the ID 32C yeast identification system (bioMérieux, Marcy-l'Étoile, France). The test was performed according to the manufacturer's instructions. The test strips were incubated at 30°C and were observed after 48 hr of incubation. Final identification was obtained from analysis of numerical results by the identification software provided by the manufacturer.

Molecular Identification

Genomic DNA was extracted from all of the germ tube positive isolates as described by Michelle and Wei (27). The primers (CR-f 5'-GCTACCACTTCAGAA TCATCATC-3', CR-r 5'-GCACCTTCAGTCGTAGA GACG-3') were selected based on a previously conducted study (25). The PCR mixture (total volume of 20 μ l) included 5 μ l of genomic DNA template, 4 μ l PCR mixture (5 $\times$ HOT FIREPol® Blend Master Mix Solis BioDyne, Tartu, Estonia), 0.5 μ l forward primer, 0.5 μ l reverse primer, and 10 μ l molecular grade water. PCR reaction conditions were as follows: denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for

45 sec, primer annealing at 58°C for 40 sec, and extension at 72°C for 55 sec, followed by a final extension at 72°C for 10 min in a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany; 25). PCR products were visualized on a 1.3% agarose gel in TAE buffer at 80 V for 55 min. The PCR products were stained with GelStar™ Nucleic Acid Gel Stain (Lonza Rockland, Inc. Rockland, Maine, USA) and photographed with a UV transilluminator.

RESULTS

Of 155 vaginal isolates, 98 isolates were germ tube positive (prevalence of 63.2%). Ninety-seven (99%) isolates were molecularly identified as *C. albicans*, one (1%) isolate was identified as *C. dubliniensis*, and none of the isolates were identified as *C. africana*. Cultivation characteristics of the 98 molecularly confirmed germ tube positive clinical isolates and the reference strains are summarized in Table 1.

From the 97 confirmed *C. albicans* isolates tested, 92 (94.9%) isolates showed good growth and five (5.1%) isolates showed poor growth at 42°C. The only isolate that showed no growth at this temperature was confirmed as *C. dubliniensis*. Of the 97 confirmed *C. albicans* isolates, 35 (36.1%) isolates showed good growth, 19 (19.6%) isolates showed poor growth, and 43 (44.3%) isolates failed to grow at 45°C. The only confirmed *C. dubliniensis*

TABLE 1. Cultivation Characteristics of 98 Molecularly Confirmed Germ Tube Positive Clinical Isolates and the *Candida* Reference Strains

Characteristics	Clinical isolates		Reference strain		
	<i>Candida albicans</i> (n = 97)	<i>Candida dubliniensis</i> (n = 1)	<i>C. albicans</i> ATCC 10231 (n = 1)	<i>C. dubliniensis</i> NCPF 8809 (n = 1)	<i>C. africana</i> ATCC MYA 2669 (n = 1)
Growth at 42°C					
Good	92	—	—	—	—
Poor	5	—	1	—	—
None	—	1	—	1	1
Growth at 45°C					
Good	35	—	—	—	—
Poor	19	—	1	—	—
None	43	1	—	1	1
Colony color on CHROMagar Candida medium					
Light-blue green	93	1	1	1	—
Dark green	4	—	—	—	—
Slight green	—	—	—	—	1
Chlamydospore production					
Single on elongated pseudohyphae	34	—	1	—	—
In contiguous pairs, clusters on elongated pseudohyphae	60	—	—	—	—
Abundant chlamydospore in pairs on short branching pseudohyphae	3	1	—	1	—
No chlamydospore production	—	—	—	—	1

isolate also failed to grow at this temperature. The *C. albicans* reference strain ATCC 10231, showed poor growth at 42°C and 45°C, whereas the *C. dubliniensis* reference strain NCPF 8809 and the *C. africana* reference strain ATCC MYA 2669 were unable to grow at 42°C and 45°C.

Of the 97 confirmed *C. albicans* isolates tested, 93 (95.9%) and the only confirmed *C. dubliniensis* isolate showed light blue-green colonies on CHROMagar Candida medium (CHROMagar, Paris, France) that is suggestive of *C. albicans* (9). Four (4.1%) confirmed *C. albicans* isolates showed dark green colonies that are suggestive of *C. dubliniensis* (9). The ATCC 10231 *C. albicans* reference strain showed light blue-green colonies at 37°C after 48 hr of incubation. Interestingly, the *C. dubliniensis* reference strain NCPF 8809 also showed light blue-green colonies. The *C. africana* ATCC MYA 2669 reference strain showed a slight green colony color when cultured on CHROMagar Candida medium (CHROMagar, Paris, France).

All the 98 germ tube positive isolates tested produced chlamydospore on corn meal agar at 25°C after 48–72 hr of incubation. Thirty-four (35%) confirmed *C. albicans* isolates produced single chlamydospores on elongated hyphae typical for *C. albicans* (3), whereas 60 (61.9%) confirmed *C. albicans*, produced chlamydospores in contiguous pairs or clusters on elongated pseudohyphae. Three (3.1%) confirmed *C. albicans* isolates produced abundant chlamydospores on short branching pseudohyphae typical for *C. dubliniensis* (3), the only confirmed *C. dubliniensis* isolate also showed similar results. The ATCC 10231 *C. albicans* reference strain produced single and contiguous pairs of chlamydospores on elongated hyphae, the *C. dubliniensis* reference strain NCPF 8809 produced abundant chlamydospores on short branching pseudohyphae, and *C. africana* reference strain ATCC MYA 2669 was unable to produce any chlamydospores.

A total of 92 of 97 (94.8%) confirmed *C. albicans* isolates showed a database code of 7347340015 that is described as *C. albicans* 1 in the ID 32C system database (percentage of identification [%id], 99.9). Two (2%) of the isolates failed to assimilate lactate (LAT) and showed a database code of 7147340015 (%id 99.9). One (1%) isolate failed to assimilate xylose (XYL) and showed a database code of 7347140015 (%id 99.9). One (1%) isolate failed

to assimilate α -methyl-D-glucoside (MDG) and showed a database code of 7343340015 (%id 99.9). Interestingly, one (1%) isolate in addition to not being able to assimilate LAT showed a positive reaction toward glycerol (GLY) and potassium gluconate (GNT). This isolate showed a database code of 7147350215 (%id 45.9). The only confirmed *C. dubliniensis* isolate in our study was tested on four different occasions with the ID 32C system. The following ID 32C database codes were detected: 7142140015, 7143140015, 7342140015, and 7347140015. In all of the occasions tested, variable assimilation results toward five carbohydrate substrates including LAT, trehalose (TRE), MDG, XYL, and palatinose (PLE) were observed. Our confirmed *C. dubliniensis* isolate showed reliable identification value only in the first, second, and third occasions when tested with the ID 32C system and interestingly, in the fourth occasion the isolate was misidentified as *C. albicans*. The ID 32C results for the only confirmed *C. dubliniensis* isolate in this study are summarized in Table 2. The *C. albicans* ATCC 10231 reference strain was unable to assimilate LAT but was able to assimilate GLY and showed a database code of 7147350015 (%id 95.1). The *C. dubliniensis* reference strain NCPF 8809 was able to assimilate TRE, MDG, XYL, and PLE and showed a database code of 7147340015 (the isolate was identified as *C. albicans* with a %id of 99.6). The *C. africana* reference strain ATCC MYA 2669 was unable to assimilate N-acetylglucosamine (NAG), LAT, TRE and glucosamine (GLN) and showed a database code of 7046340011 (a code for *C. africana* was not available in the ID 32C system database). It was instead reported as *Candida sake* with no percentage of identification.

From the total of 98 germ tube positive yeast isolates molecularly tested, 96 isolates showed a DNA fragment of ~1,000 bp identical to the *C. albicans* reference strain, ATCC 10231. One isolate showed a DNA fragment of ~800 bp that was not identical to any of the reference strains used in this study. Only one isolate showed a DNA fragment of 569 bp, identical to the *C. dubliniensis* NCPF 8809 reference strain. None of the isolates tested showed a DNA fragment of ~700 bp identical to the *C. africana* reference strain ATCC MYA 2669 (25; Fig. 1).

The only isolate that showed a DNA fragment of ~800 bp was sequenced (AITbiotech Pte Ltd., Singapore) to

TABLE 2. ID 32C System Results for the Only Confirmed *Candida dubliniensis* Isolate.

Occasions	Lactate (LAT)	Trehalose (TRE)	α -methyl-D-glucoside (MDG)	Xylose (XYL)	Palatinose (PLE)	ID 32C code	%id	Species identified
First	–	–	–	–	+	7142140015	99.6	<i>C. dubliniensis</i>
Second	–	+	–	–	+	7143140015	87.7	<i>C. dubliniensis</i>
Third	+	–	–	–	+	7342140015	94.6	<i>C. dubliniensis</i>
Fourth	+	+	+	–	+	7347140015	99.9	<i>C. albicans</i>

Fig. 1. Molecular characterization of samples isolated from hospital patients and reference strains. Amplification of Hwp1 gene. Lanes 1 to 4, *C. albicans* samples isolated from hospital patients; lane 5, the only *C. dubliniensis* sample isolated from a hospital patient; lane 6, *C. albicans* sample (~800 bp) isolated from a hospital patient; lane 7, *C. albicans* ATCC 10231; lane 8, *C. dubliniensis* NCPF 8809; lane 9, *C. africana* ATCC MYA 2669; lane M, molecular size marker.

obtain its identity. The sequence analysis obtained from this isolate showed a 97% similarity with the *C. albicans* Hwp1 gene isolate (accession no. EU044787/GenBank database) when used as the query in the BLASTN program (version 2.2.27). The sequence of this isolate was deposited in the GenBank database (accession no. KC736934).

The confirmed *C. dubliniensis* PCR product was sequenced (AITbiotech Pte Ltd., Singapore) to assure its certainty. The sequence analysis of this isolate showed a 99% similarity with the *C. dubliniensis* Hwp1 gene isolate (accession no. AJ632273/GenBank database) when used as the query in the BLASTN program (version 2.2.27). The sequence of only *C. dubliniensis* isolated in our study was deposited in the GenBank database (accession no. KC736935).

DISCUSSION

Despite the fact that *C. albicans* remains the main species of yeast isolated from vaginal specimens (28) over the past few years, other non-*albicans* species have also been isolated (29, 30). Vaginal colonization of *Candida* species can be influenced by many factors, one of which is pregnancy. During this period, high levels of estrogen cause glycogen to increase in the epithelium, which can be a nutritional source for *Candida* growth.

As reported by Vidotto et al. (31), although *C. albicans* isolates were more adherent to vaginal epithelial cells, *C. dubliniensis* isolates also showed high levels of adherence to this epithelium. The adherence of these two

species could be attributed to the interaction with receptors of the vaginal cells, such as glycolipids, glycoproteins, and carbohydrates (32). Little information is still known about the adherence abilities of *C. africana* in human infections. However, Romeo et al. (33) reported that *C. africana* showed poor adhesion ability to human Hela cells when compared to *C. albicans* and *C. dubliniensis*. Few epidemiological studies in the past have provided information about *C. dubliniensis* and especially *C. africana* being recovered from vaginal samples (8,10,11,34). Kurzai et al. (35) screened 30 vaginal isolates and could not find any *C. dubliniensis*. Acikgoz et al. (36) reported only one *C. dubliniensis* isolate among 600 stored vaginal *Candida* isolates tested, while Al-Sweih et al. (37) tested an even more significant amount of germ tube positive *Candida* isolates ($n = 1,644$) and could only find one *C. dubliniensis* isolate from a HVS sample. Moreover, Romeo and Criseo (38) reported the first detailed epidemiological data concerning the occurrence of *C. africana* from clinical samples. From all of the vaginal samples tested, 139 isolates were identified as *C. albicans*, two isolates were identified as *C. dubliniensis* and 27 were identified as *C. africana*.

Until today no accurate phenotypic method has been introduced for the discrimination of these closely related germ tube positive yeasts. Phenotypic methods can be time-consuming and require three or more days for conformation to the species level. Therefore, as until now some *C. dubliniensis* and *C. africana* strains are still misidentified as typical *C. albicans* strains in clinical medical mycology laboratories.

For the first time in Malaysia in this study, we tested a significant number of positive vaginal yeast cultures for

the possibility of being *C. dubliniensis* or *C. africana*. The performance of five phenotypic methods including growth at 42°C and 45°C on SDA, chlamyospore production on corn meal agar at 25°C, colony color appearance on CHROMagar Candida medium, and carbohydrate assimilation profiles were evaluated in comparison to a newly described molecular method. With the help of the new molecular method previously described, we were able to identify one *C. dubliniensis* isolate from a HVS sample in a 32-year-old pregnant woman. Our results emphasize the importance of applying molecular methods when identifying these closely related germ tube positive yeasts (38). Conventional methods could not accurately identify the *C. dubliniensis* isolate in our study.

Good and limited growth (17, 39, 40) at 42°C has been reported in several studies for *C. albicans* isolates. Poor or no growth at all has been reported for *C. dubliniensis* isolates (3). In our study, 92 of 97 (94.9%) confirmed *C. albicans* isolates showed good growth and only five of 97 (5.1%) showed poor growth at 42°C. The only isolate that showed no growth at 42°C was confirmed as *C. dubliniensis*.

Candida albicans and *C. dubliniensis* isolates have shown variable growth abilities, such as good, poor, and no growth at all, at 45°C (17, 18, 41). In our study, 35 of 97 (36.1%) confirmed *C. albicans* isolates showed good growth, 19 of 97 (19.6%) showed poor growth, and 43 of 97 (44.3%) showed no growth at 45°C. The only confirmed *C. dubliniensis* isolate did not grow at this temperature. Growth at 42°C seemed to be more selective in our study, due to fact that the only isolate that did not grow at 42°C was confirmed as *C. dubliniensis*. However, the overall results obtained from our study indicate that failure to grow at 42°C or 45°C cannot be reliable confirmation methods for identifying germ tube positive yeasts from one another and can lead to misidentification (17, 21, 41–43).

CHROMagar Candida medium has been used to discriminate *C. albicans* from *C. dubliniensis* and the newly introduced *C. africana* on the basis of colony color following growth at 37°C after 48 hr of incubation (10). On this medium *C. albicans* colonies appear as light blue-green, whereas *C. dubliniensis* colonies appear as dark green (9) and *C. africana* colonies show a slight green color with a retarded growth (10). However, CHROMagar Candida medium cannot always be relied upon as a distinct phenotypic test for the differentiation of these closely related germ tube positive yeasts. Previous studies have shown that *C. albicans* has the potential to produce dark green colonies on this medium (21, 35, 42) and that *C. dubliniensis* can lose its ability to produce dark green colonies on this medium after subculture and storage (20). It has also been reported that this medium can only be useful for indentifying colonies of *C. dubliniensis* following

primary plating from clinical specimens after 48 hr of incubation (20, 44). In our study, four isolates produced dark green colonies typical for *C. dubliniensis*, but none of these isolates were confirmed as *C. dubliniensis*. Ninety-three confirmed *C. albicans* isolates showed light blue-green colonies typical for *C. albicans*, and the only confirmed *C. dubliniensis* isolate also showed light blue-green colonies on CHROMagar Candida medium. None of the isolates tested showed slight green colonies typical for *C. africana*. Nevertheless, discriminating *C. dubliniensis* and *C. africana* from *C. albicans* on CHROMagar Candida medium in our study seemed to be unreliable due to the different results that were obtained. Our results are in accordance with other studies (17, 20, 42). Due to the variable colony colors that are produced on this medium (20), identifying germ tube positive yeasts on CHROMagar Candida medium can be a challenging task. Therefore, it has been suggested that this medium can be more useful when used as a distinguishing test for primary and mixed isolations, especially when involving non-*albicans* *Candida* species (45).

Candida dubliniensis is known to produce abundant chlamydo spores in pairs, triplets, or clusters on short branching pseudohyphae (3), whereas *C. albicans* produces single chlamydo spores on elongated pseudohyphae (3), and *C. africana* does not produce any chlamydo spores on corn meal agar (10). However, in our study three (3.1%) of 97 confirmed *C. albicans* isolates showed abundant chlamydo spore productions typical for *C. dubliniensis*. The only confirmed *C. dubliniensis* isolate also showed similar results. From the total of 97 confirmed *C. albicans* isolates, 34 (35%) isolates showed chlamydo spore productions typical for *C. albicans*. A large number of the confirmed *C. albicans* isolates ($n = 60$, 61.9%) produced chlamydo spores in contiguous pairs or clusters on elongated pseudohyphae. Our results are in accordance with other studies (17–19, 21, 35). Other laboratory culture media have been introduced for discriminating these two species on the basis of chlamydo spore production, but none of them seemed to be accurate (46–48). Interestingly, in our study chlamydo spore diameter measurement on corn meal agar seemed to be an efficient marker to discriminate *C. dubliniensis* from *C. albicans*. The average chlamydo spore diameter for the only confirmed *C. dubliniensis* was 4.69 μm (4.52–5.03 μm), whereas for the confirmed *C. albicans* isolates it was 3.72 μm (3.50–3.92 μm ; P -value = 0.006). After only 24 hr of incubation on corn meal agar with added 1% Tween 80, the only confirmed *C. dubliniensis* isolate produced abundant chlamydo spores in doublets and triplets on short branching pseudohyphal elements, whereas the confirmed *C. albicans* isolates produced different chlamydo spore productions (abundant on short branching pseudohyphae, single and in pairs or

clusters on elongated pseudohyphae) after 72 hr of incubation. This could indicate that the speed of chlamydospore production on this medium could be considered as a discriminatory marker.

As reported by Sullivan et al. (3), *C. dubliniensis* is unable to assimilate LAT, MDG, and XYL and has a variable reaction toward TRE in contrast to a high number of *C. albicans* isolates when tested with the ID 32C system. However, later studies have reported positive reactions to these carbon sources (20,21,42,49). In our study, some of the confirmed *C. albicans* isolates showed variable results to some carbohydrate substrates, such as LAT, XYL, MDG, GLY, and GNT. From the 97 confirmed *C. albicans* isolates, two (2%) isolates were unable to assimilate LAT, one (1%) isolate was unable to assimilate XYL, and one (1%) isolate was unable to assimilate MDG. Interestingly, one (1%) isolate was unable to assimilate LAT, however, it was able to assimilate GLY and GNT. The ID 32C system database percentages of assimilation for LAT, MDG, XYL, GLY and GNT for *C. albicans* are 96%, 98%, 98%, 10% and 2%, respectively. The only confirmed *C. dubliniensis* isolate was tested on four separate occasions with four different batches of the ID 32C system after 48 hr of incubation. We observed that our *C. dubliniensis* isolate could show variable results toward five substrates, which included LAT, TRE, MDG, XYL, and PLE. The ID 32C system database percentages for *C. dubliniensis* are 10%, 11%, 0%, 3%, and 1%, respectively, for these carbon sources. Interestingly, in all of the four occasions our *C. dubliniensis* was able to assimilate palatinose (PLE). As described in the ID 32C system database, the assimilation percentage for this carbon source is only 1% (50). Tintelnot et al. (21) reported that due to high number of assimilation of LAT (87%), MDG (55%), and XYL (58%) these carbon sources could not be used as an assimilation marker for discriminating *C. dubliniensis* from *C. albicans*. While some other reports have described the ID 32C system as a poor discriminatory test for *C. dubliniensis* and *C. albicans* (19,42), similar results have also been observed in other commercially available yeast identification systems (42,50). The numerical profiles for the *C. dubliniensis* isolate in our study were similar to a previous study (3).

We were surprised to see that the *C. albicans* ATCC 10231 reference strain was unable to assimilate LAT but was able to assimilate GLY. In addition, the *C. dubliniensis* reference strain NCPF 8809 was able to assimilate TRE, MDG, XYL, and PLE and was identified as *C. albicans* with the ID 32C system. Tietz et al. (10) reported that all of their *C. africana* isolates that were tested were unable to assimilate N-acetylglucosamine (NAG), glucosamine (GLN), lactate (LAT) and trehalose (TRE); the main ID 32C profile number for the *C. africana* isolates in their study was 7046340011; the ID 32C profile

number for their study was 7046340011. We also observed similar results with the *C. africana* reference strain ATCC MYA 2669. In two separate cases of *C. africana* infections from Spain (11) and Italy (12) the ID 32C assimilation profile showed identical codes (7042340011). These profile codes were very similar to the one reported by Tietz et al. (10). The difference between these two codes and the first code introduced by Tietz et al. (10) was the variable assimilation of MDG. Moreover, it is important to note that these assimilation profile codes do not exactly exist in the ID 32C system database and due to this fact *C. africana* can be misidentified as *C. dubliniensis*, *C. sake*, or *C. colliculsa* (10,11). However, newly introduced biochemical systems, such as Micronaut-Candida (Merlin Diagnostika GmbH, Bornheim, Germany), CANDIDAtest 21 system (PLIVA-Lachema Diagnostika, Brno, Czech Republic) have included *C. africana* in their database. But, more time and research is needed for the widespread use of these biomedical systems in routine laboratories. Nevertheless, in our study the ID 32C system showed a good validity for *C. dubliniensis* in the first, second, and third occasion of identification. However, in the forth occasion our *C. dubliniensis* isolate was misidentified as *C. albicans*. This could be due to some specific gene mutation that is present in the genome that can cause differences in metabolic pathways (4). Some authors have stated that the density of the inoculums can influence the final results and that evaluation can depend on technical skills (41,42). Although, the ID 32C system was carefully performed in our study but variable results were still observed. In our study, identifying *C. dubliniensis* with the ID 32C system showed some limitations. Therefore, this phenotypic method was not relied on as an accurate technique for the identification of the germ tube positive yeasts that were tested.

Interestingly, from all of the confirmed *C. albicans* isolates tested, one isolate showed a DNA fragment of ~800 bp that was not identical to any of the reference strains used in our study. This isolate was sequenced and confirmed as *C. albicans*. Our molecular data showed that a 800 bp DNA fragment is characteristic of some *C. albicans* isolates including *C. stellatoidea* type I, a pathogenic yeast previously known as a variant of *C. albicans* but currently considered as a synonym of this latter (25, 50; Table 3).

CONCLUSION

With the results obtained from our study, it can be stated that due to budget or physical limitation of applying molecular methods in identifying germ tube positive yeasts in routine laboratories, fairly reliable but less accurate phenotypic methods, such as chlamydospore production (on the basis of diameter measurement and growth

TABLE 3. Phenotypic Characteristics of the Only Confirmed *Candida albicans* That Showed a DNA Fragment of ~800 bp

Characteristics	Confirmed <i>C. albicans</i> with a DNA fragment of ~800 bp
Growth at 42°C	No growth
Growth at 45°C	No growth
Colony color on CHROMagar <i>Candida</i> medium	Light blue-green
Chlamydospore production	Abundant chlamydospores in pairs on short branching pseudohyphae
ID 32C system database code	7347340015

speed) on corn meal agar and the ID 32C system, can still be used as presumptive phenotypic techniques for discriminating these closely related germ tube positive yeasts.

Few studies have reported *C. dubliniensis* in Southeast Asia (51, 52) and to our knowledge, this is the first well-documented report of *C. dubliniensis* identified with molecular methods in Malaysia from HVS samples. Although we did not find any *C. africana* in our investigation, our study is the first to deal with the epidemiology of *C. africana* in vaginal samples in Southeast Asia and especially Malaysia. In conclusion, infections especially vaginal disorders due to *C. dubliniensis* and *C. africana* are still not widely known. Therefore, discrimination of these closely related germ tube positive yeasts with newly introduced molecular methods is essential for further epidemiological studies.

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