***Candida dubliniensis* Screening Using the Germ Tube Test in Clinical Yeast Isolates and Prevalence of *C. dubliniensis* in Korea**

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The aim of this study was to screen for *C. dubliniensis* using the germ tube test with human pooled serum (HPS) in clinical isolates and investigate the prevalence of *C. dubliniensis* in Korea. Among 1,854 yeast strains isolated, 1,404 strains of *C. albicans* (on the basis of positive results of the germ tube test) and 192 germ tube-negative yeast strains were examined. All 1,596 clinical isolates were examined using the germ tube test with HPS, the differential temperature, and NaCl tolerance test. Only 81 isolates that did not grow at 45°C nor on Sabouraud 6.5% NaCl broth were selected and tested using the VITEK 2 ID-YST

system and the multiplex-PCR assay for the study. The two strains, *C. dubliniensis* ATCC MYA-646 and KCTC 17427 failed to produce germ tubes in HPS but produced them in fresh rabbit serum (FRS) and fetal bovine serum (FBS). No *C. dubliniensis* was found in this study population. The results of this study suggest that the germ tube test with HPS in combination with FRS or FBS can be used for discriminating between *C. albicans* and *C. dubliniensis* strains and that the prevalence of *C. dubliniensis* appears to be extremely low in Korea. J. Clin. Lab. Anal. 24:145–148, 2010. © 2010 Wiley-Liss, Inc.

Key words: *Candida dubliniensis*; germ tube test; multiplex-PCR; Korea

INTRODUCTION

Although the genus *Candida* comprises more than 200 species, only dozen or so have been associated with human infections (1). Of these, *C. albicans* is the most commonly isolated clinical species, but in recent years there has been an increase in infections caused by other species including *C. glabrata* and *C. parapsilosis* (2). In 1995, *C. dubliniensis* was first described as a novel species in cases of oral candidosis in HIV-infected individuals (3) and has been reported in various geographic locations (4,5). However, despite the effort that has been made to identify *C. dubliniensis* in clinical samples, a definitive assessment of the prevalence of this species has yet to be conducted. Moreover, the prevalence of *C. dubliniensis* has not yet been reported in Korea.

In retrospective analyses of stock collections, *C. dubliniensis* was found to account for at least 1.2–2% of the yeast initially identified as *C. albicans* (6,7). Phenotypically, *C. dubliniensis* and *C. albicans* have many similarities, including their microscopic morphology and ability to form germ tubes in serum (4). As many laboratories use the germ tube test as the sole

method for the identification of *C. albicans*, isolates of *C. dubliniensis* have been overlooked and misidentified as *C. albicans* (3,5,6). However, recently described rapid and reliable identification tests, including the inability to grow at 45°C (8) and/or in Sabouraud dextrose broth with 6.5% sodium chloride (NaCl) (9), production of abundant pseudohyphae and chlamydospores on Staib agar (10), formation of dark green colonies on CHROMagar *Candida* medium (11), carbohydrate assimilation using commercial yeast identification systems (12), and DNA techniques (13), have significantly improved the recognition of *C. dubliniensis* in clinical specimens.

The aim of this study was to screen for *C. dubliniensis* using the germ tube test with human pooled serum (HPS) in clinical isolates and to investigate the

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prevalence of *C. dubliniensis* in various clinical specimens obtained from 17 university hospitals in Korea. In combination with simple screening tests, we attempted a species-specific multiplex-PCR to identify *C. dubliniensis*.

MATERIALS AND METHODS

Tested Organisms

A total of 1,854 yeast isolates were obtained from 17 university hospitals in South Korea during the 8-month period between July 2004 and February 2005. Among 1,854 isolated yeast strains, 1,404 strains of *C. albicans* (on the basis of positive results of the germ tube test) and 192 germ tube-negative yeast strains were examined for the subsistence of *C. dubliniensis*. As the 258 remaining isolated yeast strains were identified as *Candida* species other than *C. albicans* and *C. dubliniensis* using the VITEK 2 ID-YST system (bioMérieux Inc, Hazelwood, MO), they were excluded from the study. The 1,404 isolates of *C. albicans* were obtained from various clinical samples (534 isolates from sputum, 62 from the throat, 61 from the oral cavity, 20 from the suction tip, 12 from the bronchial washing, 292 from urine, 92 from the vagina, 53 from body fluid, 34 from blood, 28 from stool, 20 from the wound, and 196 from other sources), and the 192 germ tube-negative yeast strains were obtained from the upper respiratory tract and oral specimens. In addition, four reference strains were used in this study, including *C. albicans* ATCC 18804, *C. albicans* ATCC 14053, *C. dubliniensis* ATCC MYA-646, and *C. dubliniensis* Korean Collection for Type Cultures (KCTC) 17427. All isolates were saved on 50% glycerol at -70°C and sent to the Chung-Ang University Hospital for storage and further testing. Before testing, the isolates were subcultured twice on Sabouraud dextrose agar (SDA) plates at 35°C .

The Germ Tube, Differential Temperature and NaCl Tolerance Test

The germ tube test was performed by incubating the specimen in HPS for 3 hr at 37°C and then confirming by microscopy at $40\times$ magnification. For the differential temperature test, all isolates were cultured on SDA and incubated at 45°C for 96 hr (8). For the NaCl tolerance test, 20 μl of yeast suspension (0.5 McFarland) was added to each of the 1 ml of sterile Sabouraud broth supplemented with 6.5% NaCl and then statically incubated for 96 hr (9). The cultures for the differential temperature and NaCl tolerance test were visually examined to detect fungal growth at 24 hr intervals.

VITEK 2 ID-YST System and Multiplex-PCR Amplification

Among 1,596 clinical isolates, we selected 81 strains that formed cream-colored, pasty, smooth colonies on SDA at 37°C but did not grow at 45°C nor on Sabouraud 6.5% NaCl broth, regardless of germ tube formation. These 81 strains were tested using the VITEK 2 ID-YST system and the multiplex-PCR assay.

The VITEK 2 ID-YST system was used according to the manufacturer's directions, and the organisms were identified by using instrument software.

The Multiplex-PCR assay was developed using internally transcribed spacers and topoisomerase II genes for accurate identification of eight *Candida* spp. (*C. albicans*, *C. dubliniensis*, *C. krusei*, *C. glabrata*, *C. guilliermondii*, *C. tropicalis*, *C. parapsilosis*, and *C. lusitanae*). We designed species-specific Dual Specificity Oligo primers for multiplex-PCR (14). Genomic DNA from yeast isolates grown overnight in SDA plates was extracted using a High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's directions. Multiplex-PCR followed by 2% agarose gel electrophoresis and specific PCR products were detected by visual comparison.

RESULTS

C. dubliniensis strains, ATCC MYA-646 and KCTC 17427, failed to produce germ tubes in HPS and did not grow at 45°C nor on Sabouraud 6.5% NaCl broth, whereas *C. albicans* ATCC 18804 and ATCC 14053 produced germ tubes and grew both at 45°C and on Sabouraud 6.5% NaCl broth. To check for germ tube formation of *C. dubliniensis*, we performed the germ tube test with fresh rabbit serum (FRS) in combination with fetal bovine serum (FBS). All type strains of *C. albicans* and *C. dubliniensis* were clearly positive for the germ tube test with FRS in combination with FBS within 3 hr. In addition, HPS consistently differentiated all *C. albicans* strains from *C. dubliniensis* type strains.

The results of the germ tube, differential temperature, and NaCl tolerance test for 1,596 clinical yeast isolates are presented in Table 1. To identify *C. dubliniensis*, 81 isolates that formed cream-colored, pasty, smooth colonies on SDA at 37°C but did not grow at 45°C nor on Sabouraud 6.5% NaCl broth were selected and tested using the VITEK 2 ID-YST system and the multiplex-PCR assay. All germ tube-positive isolates in HPS (16 isolates) were confirmed as *C. albicans*, regardless of the results of the differential temperature and NaCl tolerance test. Germ tube-negative isolates were identified as *Candida* spp. or were left unidentified. However, no *C. dubliniensis* was found in this study population (Table 2).

DISCUSSION

The *C. dubliniensis* species is phylogenetically closely related to but clearly distinct from *C. albicans*. These two *Candida* species share several phenotypic characteristics, including microscopic morphology and the ability to form germ tubes in serum (4,6,7). Therefore, most previous studies have been conducted to identify *C. dubliniensis* among *C. albicans* or germ tube-positive *Candida* species (6,7,15–18). This study was attempted to screen the isolates of *C. dubliniensis* using the differential temperature and NaCl tolerance test. The isolates were identified as *C. albicans* on the basis of positive results of the germ tube test. However, in this

study, both *C. dubliniensis* ATCC MYA-646 and KCTC 17427 failed to produce germ tubes, whereas *C. albicans* ATCC 18804 and ATCC 14053 produced. The germ tube test was performed with fresh HPS independently by two well-trained technicians. In order to detect *C. dubliniensis*, the germ tube, differential temperature, and NaCl tolerance test were additionally conducted with 192 strains of yeast isolated from the upper respiratory tract and oral specimens that were reported to be non-*albicans* *Candida* based on the negative result of the germ tube test. However, no *C. dubliniensis* was found in this study population.

Previous studies have reported that the prevalence of *C. dubliniensis* among the strains, which were isolated and identified as *C. albicans* using the germ tube test with HPS, was 0–3.3% (15–18). However, in this study, the two type strains of *C. dubliniensis* failed to produce germ tubes in HPS and did not grow at 45°C nor on Sabouraud 6.5% NaCl broth. To check for germ tube formation of *C. dubliniensis*, we performed the germ tube test with FRS or FBS. All strains of *C. albicans* and *C. dubliniensis* were clearly positive for the germ tube test with FRS or FBS within 3 hr. In addition, among 81 isolates, the strains that produced germ tubes in HPS were confirmed as *C. albicans*. Thus, although germ tube formation was not confirmed in clinical isolates of *C. dubliniensis*, it is thought that *C. dubliniensis* may not produce germ tubes in HPS. It is suggested that HPS in combination with FRS or FBS can be used for

TABLE 1. Phenotypic Characteristics of 1,596 Yeast Isolates Obtained from Clinical Specimens

No.(%) of yeasts	GT	Growth at	
		45°C	6.5% NaCl
1261 (79.01)	+	+	+
36 (2.26)	+	+	–
9 (0.36)	+	–	+
9 (0.36)	+	–	–
184 (11.53)	–	+	+
11 (0.69)	–	+	–
72 (4.51)	–	–	+
14 (0.88)	–	–	–

GT, germ tube with human pooled serum.

TABLE 2. Identification Results by the VITEK 2 ID-YST System and Multiplex-PCR for 81 Isolates and 4 Type Strains

No. of yeasts	GT	45°C	6.5% NaCl	Identification results (No. of isolates)	
				VITEK 2 ID-YST	Multiplex-PCR
9	+	–	–	<i>C. albicans</i> (6) UnID (3)	<i>C. albicans</i> (9)
3	+	+	–	<i>C. albicans</i> (3)	<i>C. albicans</i> (3)
9	+	–	+	<i>C. albicans</i> (9)	<i>C. albicans</i> (9)
10	–	–	–	<i>C. parapsilosis</i> (1) <i>C. tropicalis</i> (1) <i>S. cerevisiae</i> (3) <i>C. colliculosa</i> (1) UnID (4)	<i>C. parapsilosis</i> (1) <i>C. tropicalis</i> (1) UnID (8)
5	–	+	–	<i>C. albicans</i> (2) <i>C. lusitaniae</i> (2) UnID (1)	<i>C. albicans</i> (2) <i>C. lusitaniae</i> (2) UnID (1)
15	–	–	+	<i>C. parapsilosis</i> (6) <i>C. glabrata</i> (3) <i>C. tropicalis</i> (3) <i>C. guilliermondii</i> (2) <i>S. cerevisiae</i> (1)	<i>C. parapsilosis</i> (6) <i>C. glabrata</i> (3) <i>C. tropicalis</i> (3) <i>C. guilliermondii</i> (2) UnID (1)
2 (TS)	+	+	+	<i>C. albicans</i> (2)	<i>C. albicans</i> (2)
2 (TS)	–	–	–	<i>C. dubliniensis</i> (2)	<i>C. dubliniensis</i> (2)

GT, germ tube with human pooled serum; 45°C, growth at 45°C (differential temperature test); 6.5% NaCl, tolerance test to 6.5% NaCl, UnID, Unidentification; TS, Type strain.

discriminating between *C. albicans* and *C. dubliniensis* strains. Moreover, the differential temperature and NaCl tolerance test is convenient but it requires a long cultivation time of 3 days and they may be negative for *Candida* species other than *C. dubliniensis* including *C. albicans*. For these reasons, it is conceivable that the two aforementioned tests are not suitable to differentiate *C. dubliniensis* from other *Candida* species.

The results of this study suggested that there is a low possibility that *C. dubliniensis* can be erroneously identified as *C. albicans* because the germ test with HPS has been conducted for presumptive identification in most microbiology laboratories in Korea. In addition, the prevalence of *C. dubliniensis* is estimated to be extremely low ($\leq 0.1\%$), which may be attributed to the geographic difference in the distribution of *Candida* species, low infection rate of HIV, and the difference in the patterns of usage of antibiotics or antifungal agents.

In conclusion, the results of this study suggest that the germ tube test with HPS in combination with FRS or FBS can be used for discriminating between *C. albicans* and *C. dubliniensis* strains and that the prevalence of *C. dubliniensis* is extremely low in Korea. In addition, to confirm the usefulness of the germ tube test with HPS and FRS or FBS for discriminating between *C. albicans* and *C. dubliniensis* and the exact prevalence of *C. dubliniensis* in Korea, further prospective studies with a larger sample size of clinical isolates of *Candida* species are needed.

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