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Rapid and Accurate Identification of *Candida albicans* and *Candida dubliniensis* by Real-Time PCR and Melting Curve Analysis

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Significance of the Study

- Accurate identification of *Candida albicans* and *Candida dubliniensis* is required as the two species vary in their pathogenicity and susceptibility to antifungal drugs. We have developed and evaluated a simple and rapid SYBR Green dye-based real-time PCR assay to differentiate these two species among clinical *Candida* spp. isolates. The method will help in the proper management of patients with invasive *Candida* infections.

Keywords

Candida albicans · *Candida dubliniensis* · Real-time polymerase chain reaction · Melting curve analysis

Abstract

Objective: *Candida albicans* and *Candida dubliniensis* are germ tube-positive pathogenic yeast species. Accurate identification of these two species is warranted since *C. albicans* is a highly pathogenic species while *C. dubliniensis* exhibits increased adherence to buccal epithelial cells, reduced susceptibility to azoles and resistance to flucytosine. We have developed a duplex real-time PCR assay for rapid detection and differentiation between clinical *C. albicans* and *C. dubliniensis* isolates. ***Materials and Methods:*** A duplex real-time PCR assay was developed by using two species-specific primer pairs and SYBR Green dye to differentiate *C. albicans* and *C. dubliniensis* isolates via melting curve analysis of real-time PCR amplicons. Amplification products were also analyzed by agarose gel electrophoresis to confirm real-time PCR results. ***Results:*** Melting temperatures (T_m) for reference strains of *C. albicans* and *C. dubliniensis* were 86.55 and 82.75 °C, respectively. No amplicon was obtained with DNA from reference strains of 8 other common *Candida* spp. When real-time PCR was applied on 226 clinical isolates previously identified by the Vitek 2 system and/or PCR sequencing of rDNA, T_m values for *C. albicans* (n = 113) and *C. dubliniensis* (n = 98) were 86.68 ± 0.529 and 82.616 ± 0.535 °C, respectively. The results were confirmed by agarose gel electrophoresis. No amplicon was obtained from 15 isolates belonging to 9 other *Candida* spp. ***Conclusions:*** The real-time PCR assay described here does not require prior identification of clinical yeast isolates as *C. albicans*/*C. dubliniensis* by germ tube formation and accurately reports results within 2 h. Detection of amplicons by agarose gel electrophoresis is also suitable for resource-poor settings devoid of real-time PCR facilities.

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Introduction

The predominant pathogenic *Candida* species in susceptible human hosts is *Candida albicans* [1–3]. However, recent years have also witnessed an increasing incidence of episodes of candidemia and invasive candidiasis by non-*albicans Candida* spp. including *Candida dubliniensis* [1–3]. *C. dubliniensis* shares many phenotypic characteristics such as the ability to form chlamydospores on cornmeal agar and germ tubes in serum with *C. albicans* [4, 5]. As the germ tube test is mainly used for the differentiation of *C. albicans* from other *Candida* species, routine identification of *C. albicans* based solely on this test leads to misidentification of some *C. albicans* isolates in routine mycology laboratories [6]. Accurate identification is desirable since *C. albicans* is the most prevalent and most pathogenic species while *C. dubliniensis* exhibits increased adherence to buccal epithelial cells, reduced susceptibility to azoles and genotype-specific resistance to flucytosine [4–7]. The role of *C. dubliniensis* as a bloodstream pathogen has been increasingly recognized in recent years as it was recorded as the third or fourth most common *Candida* spp. causing candidemia, usually surpassing *Candida tropicalis* and even *Candida parapsilosis* [8–13]. The isolation frequency of *C. dubliniensis* from the oral cavity is high, particularly from cancer patients, and it has been isolated in Kuwait from a variety of clinical specimens including blood, thus highlighting the increasing role of this non-*albicans Candida* species in human infections [9, 13–15].

Phenotypic tests such as the formation of fringed/rough colonies on various seed agar-based media [16, 17] and tobacco agar [18], production of dark green colonies on CHROMagar Candida [19] and lack of growth in hypertonic Sabouraud dextrose broth [20] have been used to differentiate *C. dubliniensis* from *C. albicans*. However, these tests can only be applied on clinical isolates presumptively identified as *C. albicans/C. dubliniensis* by another test (such as germ tube formation), requiring a further 1–2 days and are not 100% specific [6]. Variations in growth conditions (incubation temperature, repeated subculturing and storage) may also impede accurate identification of these two species by phenotypic tests. Commercially available yeast identification systems (Vitek 2 ID-YST, API 20C and ID32C) based on assimilation of carbon/other compounds are useful for differentiating various *Candida* spp.; however, these methods are expensive and also take at least 1–2 days for results to be reported. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry has also been used for rapid discrimination between *C. albicans* and *C. dubliniensis* isolates. However, the method requires fresh cultures, and the Bruker Biotyper yields low scores and unacceptable identification for some *C. dubliniensis* isolates [21]. Molecular techniques offer unambiguous differentiation, and PCR-based methods are mostly employed. However, some PCR-based methods are complex and time-consuming while others are based on intronic sequences that may lead to erroneous results due to variations in intronic sequences among different strains [22–24]. In this study, we describe a duplex real-time PCR assay using primers derived from highly conserved rDNA sequences and used melting point analysis with SYBR Green dye for rapid detection and differentiation of strains of *C. albicans* and *C. dubliniensis*.

Material and Methods

Reference Strains and Clinical Isolates

Reference strains of *C. albicans* (ATCC 90029), *C. dubliniensis* (CD36), *C. parapsilosis* (ATCC 22019), *Candida orthopsilosis* (ATCC 96139), *Candida glabrata* (ATCC 15545), *Candida nivariensis* (CBS 9983), *C. tropicalis* (ATCC 750), *Candida lusitaniae* (CBS 1944), *Candida guilliermondii* (ATCC 6021) and *Candida krusei* (CBS 6258) were used. Clinical specimens including blood were collected from patients at various hospitals across Kuwait as part of routine patient care. Specimens were collected after obtaining consent from patients as part of the routine diagnostic workup. The isolates were sent to the Mycology Reference Laboratory, Department of Microbiology, Faculty of Medicine, Kuwait University, for identification and antifungal susceptibility testing. Clinical *Candida* isolates ($n = 226$) including *C. albicans* ($n = 113$), *C. dubliniensis* ($n = 98$) and other clinical *Candida* spp. ($n = 15$) were tested for evaluation of real-time PCR. All 226 clinical *Candida* spp. isolates were previously identified by the Vitek 2 yeast identification system and tested for germ tube formation in horse serum. All germ tube positive isolates were presumptively identified as *C. albicans/C. dubliniensis*.

Template DNA Preparation and Real-Time PCR Assay

A loopful of yeast colony was suspended in 1 mL of sterile water in a microcentrifuge tube containing 50 mg Chelex-100 (Sigma-Aldrich Co., St. Louis, MO, USA); the contents were heated at 95 °C for 20 min and then centrifuged. The supernatant was transferred to a new tube and then used as source of genomic DNA in real-time PCR. Two species-specific primer pairs derived from the internally transcribed spacer (ITS) region (comprising ITS-1, 5.8S rRNA and ITS-2) of ribosomal DNA (rDNA) were designed for differentiation of *C. albicans* and *C. dubliniensis* strains by real-time PCR. For this purpose, sequences of the ITS region of rDNA from different *Candida* species, *Aspergillus* species and other phylogenetically related fungi were downloaded from the GenBank database and aligned using clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Although we did not use any specific software for designing primers, species-specific sequences for primers SACALF and SACALR for *C. albicans* and SACDUF and SAC-

Table 1. Specific features and sequences of oligonucleotide primers used in this study and the melting temperature values of amplicons from *C. albicans* and *C. dubliniensis*

Primer	Target gene	Purpose	DNA sequence	Amplicon size, bp	Melting temperature T_m, ° C
SACALF	ITS-1	Forward primer for *C. albicans*	5′-TTTATCAACTTGTCACACCAGA-3′	~354	86.55
SACALR	ITS-2	Reverse primer for *C. albicans*	5′-GGTCAAAGTTTGAAGATATACGT-3′		
SACDUF	ITS-2	Forward primer for *C. dubliniensis*	5′-TTGGGTTTGCTTGAAAGATGAT-3′	~119	82.75
SACDUR	ITS-2	Reverse primer for *C. dubliniensis*	5′-AAAGTTTGAAGAATAAAATGGC-3′		
ITS-1	18S rRNA	Panfungal forward primer for ITS region	5′-TCCGTAGGTGAACCTGCGG-3′	~400–900	not done
ITS-4	28S rRNA	Panfungal reverse primer for ITS region	5′-TCTTTTCCTCCGCTTATTGATATG-3′		

The T_m values are shown for amplicons obtained from *C. albicans* ATCC 90029 and *C. dubliniensis* CD36 reference strains.

DUR for *C. dubliniensis* were designed based on multiple sequence alignment (Table 1). The sequences of the primers, particularly at the 3′ end, were also inspected carefully to ensure that they will not form primer dimers. Species specificity of SACALF + SACALR primers for *C. albicans* and SACDUF + SACDUR primers for *C. dubliniensis* was indicated by BLAST searches (http://www/ncbi.nlm.nih.gov/BLAST/blast.cgi?) as they showed complete identity with the corresponding sequences only from these two species and not with other *Candida* species, *Aspergillus* species or other fungi. The LightCycler FastStart DNA Master SYBR Green kit and instrument (Roche Diagnostics, Mannheim, Germany) were used for amplification and analysis as directed by the manufacturer. Reaction mixture (in 20 μL) contained LightCycler FastStart DNA Master SYBR Green, 5 pmol of each (SACALF, SACALR, SACDUF and SACDUR) primer, 3 mM $MgCl_2$ and 1 μL of template DNA. Cycling conditions included an initial denaturation at 95 °C for 2 min followed by 30 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 15 s. The amplified DNA was detected by fluorescence quantification of the double-stranded DNA binding dye SYBR Green by melting curve analysis. The melting curve analysis consisted of 1 cycle at 72 °C for 15 s and then an increase in temperature to 95 °C at 0.1 °C/s. Amplification products (10 μL) were also analyzed by agarose gel electrophoresis, performed as described previously [25], to confirm the results of real-time PCR.

PCR Sequencing of ITS Region of rDNA

The ITS region of rDNA from selected isolates was amplified by using panfungal ITS1 and ITS4 primers and the amplicons were sequenced by using internal (ITS1FS, ITS2, ITS3 and ITS4RS) primers, as described in detail previously [14].

Results

Melting temperatures (T_m) of amplicons were measured from melting curves derived by continuous monitoring of fluorescence during the temperature ramp. Recorded T_m values for *C. albicans* ATCC 90029 (86.55 °C) and *C. dubliniensis* CD36 (82.75 °C) showed a difference of nearly 4 °C which is sufficient for their accurate differentiation by real-time PCR (Fig. 1). Reference strains of other *Candida* species or human DNA extracted from the serum of healthy subjects or controls lacking genomic DNA yielded negative (no amplification) results. Although DNA samples from *Aspergillus* and other molds were not tested, based on the results of BLAST searches of primer sequences, they are not expected to yield amplicons either. Agarose gels of real-time PCR products confirmed the amplification of DNA fragments of expected size for both, *C. albicans* and *C. dubliniensis* (Table 1), and no primer dimer artifacts were visible indicating that T_m values were derived from expected amplicons only (data not shown). The analytical sensitivity was determined by extracting DNA from serial dilutions of pure cell suspension of *C. albicans* culture, and at least 5 colony-forming units (CFUs) were needed for a positive result in real-time PCR. Similar results were obtained when the assay was repeated demonstrating the reproducibility of the test. The amplicons from reference strains always yielded T_m values characteristic for *C. albicans* (approx. 86.5 °C) and *C. dubliniensis* (approx. 82.7 °C). When real-time PCR was applied on template DNA from 226 clinical isolates speciated by the Vitek 2 yeast identification system, the measured T_m values for *C. albicans* (n = 113) and *C. dubliniensis* (n = 98) were 86.68 ± 0.529 and 82.616 ± 0.535 °C, respectively (Fig. 1). Slight variations in T_m values are expected since the ITS region sequences vary slightly among different strains of both *C. albicans* and *C. dubliniensis* [5, 7]. Again, no amplification was obtained from other *Candida* species (Table 2). Identification of all 98 *C. dubliniensis*, 15 other *Candida* spp. and 73 selected *C. albicans* strains was confirmed by PCR sequencing of the ITS region of rDNA. With the rapid DNA extraction method from clinical isolates described here, the whole procedure could be completed within 2 h.

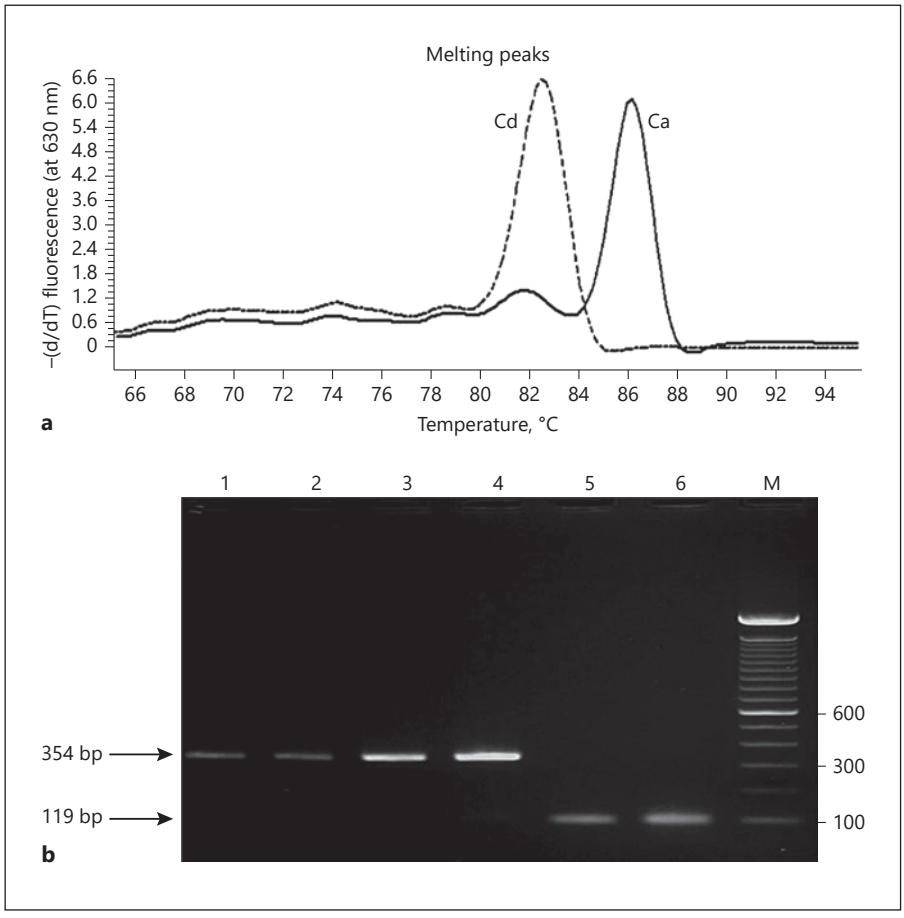

Fig. 1. a Melting curve analysis of amplicons of real-time PCR assay with SYBR Green I dye. The solid line (Ca) is *C. albicans* ATCC 90029; the dashed line (Cd) is *C. dubliniensis* CD36. **b** An agarose gel of real-time PCR amplified product with DNA from 4 different clinical *C. albicans* isolates (lanes 1–4) and 2 different clinical *C. dubliniensis* isolates (lanes 5 and 6). Amplicons from *C. albicans* (354 bp) and *C. dubliniensis* (119 bp) are marked by arrows. DNA from reference/clinical isolates of other *Candida* species was not amplified in real-time PCR (data not shown). Lane M is a 100-bp DNA ladder, and positions of migration of 100-, 300- and 600-bp fragments are marked.

Table 2. Evaluation of real-time PCR for detection and differentiation of *C. albicans* and *C. dubliniensis* among 226 clinical *Candida* isolates speciated by Vitek 2 yeast identification system

Candida species by Vitek 2	Clinical isolates tested, *n*	T_m by melting curve analysis (mean + SD), ° C	*C. albicans* or *C. dubliniensis* isolates correctly detected by real-time PCR, *n*	Isolates sequenced, *n*
C. albicans	113	86.680±0.529	113	73
C. dubliniensis	98	82.616±0.535	98	98
C. glabrata	2	no amplification detected	not applicable	2
C. parapsilosis	2	no amplification detected	not applicable	2
C. orthopsilosis	2	no amplification detected	not applicable	2
C. tropicalis	2	no amplification detected	not applicable	2
C. lusitaniae	2	no amplification detected	not applicable	2
C. krusei	2	no amplification detected	not applicable	2
C. haemulonii	1	no amplification detected	not applicable	1
C. famata	1	no amplification detected	not applicable	1
C. kefyr	1	no amplification detected	not applicable	1

Discussion

Although several end point PCR-based methods targeting rDNA have been described previously for the detection and differentiation of *C. albicans* and *C. dubliniensis*, they usually involve two separate PCR reactions for each strain [22, 26, 27] or further manipulations (such as restriction digestion of amplicons to generate restriction fragment length polymorphism or DNA sequencing) are needed for species-specific identification [22, 23, 26–29]. These additional steps add to the cost of the test and/or consume additional time, thus delaying the results. In a previous study, we developed a thermal duplex PCR assay with two pairs of species-specific primers for detection and differentiation of strains of *C. albicans* and *C. dubliniensis*, and the amplicons were detected by agarose gel electrophoresis [30]. However, when the same primers were used in real-time PCR, the difference in T_m values between *C. albicans* and *C. dubliniensis* strains was small which was not suitable for routine use as the ITS region sequences vary among clinical *C. albicans* and *C. dubliniensis* strains [5, 7, 31]. Thus, other sets of primers were tested, and the primers used in this study yielded a T_m difference of nearly 4 °C between reference strains of *C. albicans* and *C. dubliniensis* which was most suitable for accurate screening of a large number of samples. A novel real-time PCR assay was described previously for the detection of 6 *Candida* species including *C. albicans* and *C. dubliniensis* [32]. The assay involved amplification of the ITS region of rDNA with pan-*Candida* primers and detection of amplicons by the LightCycler with 6 specific probe primers corresponding to *C. albicans, C. dubliniensis, C. tropicalis, C. parapsilosis, C. glabrata* and *C. krusei* [32]. The DNA was extracted by the High Pure PCR template preparation kit, the probe primers were labeled with Cy5 fluorescent dye and the analytical sensitivity of the assay was reported as 1 CFU per PCR [32]. Although the analytical sensitivity of our assay was slightly lower (5 CFU per PCR in our assay), the DNA was extracted by a more rapid and inexpensive but less efficient boiling method, and the amplicons were detected directly with SYBR Green dye without reliance on expensive probe primers. Another real-time PCR assay using melting point analysis has also been described for detection and differentiation of *C. albicans*, *C. dubliniensis, C. tropicalis* and *C. glabrata* strains [33]. The target chosen in this study was 18S rRNA gene, the DNA was obtained from *Candida* isolates by using a commercial DNA extraction kit and PCR amplification was performed in LightCycler with pan-*Candida* primers followed by detection of amplicons by hybridization with 4 separate species-specific probe primers labeled with a fluorescent dye [33]. Three real-time PCR assays using SYBR Green dye have also been described [34–36]. Two of these real-time PCR assays used panfungal primers targeting the ITS region of rDNA [34, 35]. The panfungal nature of the primers makes prior identification of strains of *C. albicans*/*C. dubliniensis* by another rapid test (such as the germ tube test) absolutely essential to avoid misidentification of other *Candida*/yeast species (false-positive results) and the additional step is also time-consuming [34, 35]. Furthermore, the difference in T_m values for *C. albicans* and *C. dubliniensis* strains in these reports was either small (approx. 0.5 °C) or negligible which may lead to misidentification of some isolates since ITS region sequences vary among clinical *C. albicans* and *C. dubliniensis* isolates [5, 7, 31]. The other method is a nested PCR involving a first round of amplification with pan-*Candida* primers followed by real-time PCR amplification with 8 different species-specific primer pairs for the identification of 8 different *Candida* species including *C. albicans* and *C. dubliniensis* [36]. The additional requirement of the nested step in this procedure [36] is time-consuming, increases cost and the possibility of false-positive results due to amplicon carry-over. Our real-time PCR assay involves only one round of amplification and the method can also be used as a thermal PCR assay with detection of amplicons by agarose gels (Fig. 1b) for detection and differentiation of *C. albicans* and *C. dubliniensis* for resource-limited mycology laboratories that do not have a real-time PCR machine.

A limitation of our study is that the real-time PCR assay described here was only tested with culture isolates but was not tested for the detection of *C. albicans* and *C. dubliniensis* directly in clinical specimens obtained from patients in Kuwait.

In conclusion, the real-time PCR method based on melting point analysis with SYBR Green dye described here is a simple and rapid molecular tool for accurate detection and differentiation of strains of *C. albicans* and *C. dubliniensis* without relying on germ tube or other such tests. The method described here also does not require the use of expensive hybridization probes specific for *C. albicans* and *C. dubliniensis*.

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Statement of Ethics

Specimens were collected after obtaining consent from patients as part of the routine diagnostic workup.

Disclosure Statement

No conflict of interest to declare.

References

1 Leroy O, Gangneux JP, Montravers P, et al: Epidemiology, management, and risk factors for death of invasive *Candida* infections in critical care: a multicenter, prospective, observational study in France (2005–2006). Crit Care Med 2009;37:1612–1618.
2 Neofytos D, Fishman JA, Horn D, et al: Epidemiology and outcome of invasive fungal infections in solid organ transplant recipients. Transpl Infect Dis 2010;12:220–229.
3 Pfaller MA, Moet GJ, Messer SA, et al: Geographic variations in species distribution and echinocandin and azole antifungal resistance rates among *Candida* bloodstream infection isolates: report from the SENTRY Antimicrobial Surveillance Program (2008 to 2009). J Clin Microbiol 2011;49:396–399.
4 Moran GP, Coleman DC, Sullivan DJ: *Candida albicans* versus *Candida dubliniensis*: why is *C. albicans* more pathogenic? Int J Microbiol 2012;2012:205921.
5 McManus BA, Coleman DC: Molecular epidemiology, phylogeny and evolution of *Candida albicans*. Infect Genet Evol 2014;21:166–178.
6 Ells R, Kock JLF, Pohl CH: *Candida albicans* or *Candida dubliniensis*. Mycoses 2011;54:1–16.
7 Asadzadeh M, Ahmad S, Al-Sweih N, et al: Population structure and molecular genetic characterization of 5-flucytosine-susceptible and -resistant clinical *Candida dubliniensis* isolates from Kuwait. PLoS One 2017; 12:e0175269.
8 Odds FC, Hanson MF, Davidson AD, et al: One year prospective survey of *Candida* bloodstream infections in Scotland. J Med Microbiol 2007;56:1066–1075.
9 Khan Z, Ahmad S, Joseph L, et al: *Candida dubliniensis*: an appraisal of its clinical significance as a bloodstream pathogen. PLoS One 2012;7:e32952.
10 Idelevich EA, Grunewald CM, Wüllenweber J, et al: Rapid identification and susceptibility testing of *Candida* spp. from positive blood cultures by combination of direct MALDI-TOF mass spectrometry and direct inoculation of Vitek 2. PLoS One 2014;9:e114834.
11 Pannanusorn S, Fernandez V, Römling U: Prevalence of biofilm formation in clinical isolates of *Candida* species causing bloodstream infection. Mycoses 2013;56:264–272.
12 Orasch C, Marchetti O, Garbino J, et al: *Candida* species distribution and antifungal susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing and new vs old Clinical and Laboratory Standards Institute clinical breakpoints: a 6-year prospective candidaemia survey from the fungal infection network of Switzerland. Clin Microbiol Infect 2014;20:698–705.
13 Aslani N, Janbabaei G, Abastabar M, et al: Identification of uncommon oral yeasts from cancer patients by MALDI-TOF mass spectrometry. BMC Infect Dis 2018;18:24.
14 Mokaddas E, Khan ZU, Ahmad S: Prevalence of *Candida dubliniensis* among cancer patients in Kuwait: a 5-year retrospective study. Mycoses 2011;54:e29–e34.
15 Ellepola AN, Joseph BK, Altarakemah Y, et al: In vitro adhesion of oral *Candida dubliniensis* isolates to acrylic denture surfaces following brief exposure to sub-cidal concentrations of polyenes, azoles and chlorhexidine. Med Princ Pract 2015;24:58–64.
16 Lees S, Barton RC: The use of Niger seed agar to screen for *Candida dubliniensis* in the clinical microbiology laboratory. Diagn Microbiol Infect Dis 2003;46:13–17.
17 Khan ZU, Ahmad S, Mokaddas E, et al: Simplified sunflower (*Helianthus annuus*) seed agar for differentiation of *Candida dubliniensis* from *Candida albicans*. Clin Microbiol Infect 2004;10:574–592.
18 Khan ZU, Ahmad S, Mokaddas E, et al: Tobacco agar, a new medium for differentiating *Candida dubliniensis* from *Candida albicans*. J Clin Microbiol 2004;42:4796–4798.
19 Odds FC, Davidson A: "Room temperature" use of CHROMagar Candida. Diagn Microbiol Infect Dis 2000;38:147–150.
20 Alves SH, Milan EP, de Laet Sant'Ana P, et al: Hypertonic Sabouraud broth as a simple and powerful test for *Candida dubliniensis* screening. Diagn Microbiol Infect Dis 2002;43:85–86.
21 Jamal WY, Ahmad S, Khan ZU, et al: Comparative evaluation of two matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) systems for the identification of clinically significant yeasts. Int J Infect Dis 2014;26:167–170.
22 Ahmad S, Khan Z, Mokaddas E, et al: Isolation and molecular identification of *Candida dubliniensis* from non-human immunodeficiency virus-infected patients in Kuwait. J Med Microbiol 2004;56:633–637.
23 Graf B, Trost A, Eucker J, et al: Rapid and simple differentiation of *Candida dubliniensis* from *Candida albicans*. Diagn Microbiol Infect Dis 2004;48:149–151.
24 Romeo O, Criseo G. First molecular method for discriminating between *Candida africana, Candida albicans* and *Candida dubliniensis* by using *hwp1* gene. Diagn Microbiol Infect Dis 2008;62:230–233.
25 Ahmad S, Mustafa AS, Khan Z, et al: PCR-enzyme immunoassay of rDNA in the diagnosis of candidemia and comparison with amplicon detection by agarose gel electrophoresis. Int J Med Microbiol 2004;294:45–51.
26 Ahmad S, Mokaddas E, Al-Sweih N, et al: Phenotypic and molecular characterization of *Candida dubliniensis* isolates from clinical specimens in Kuwait. Med Princ Pract 2005; 14(suppl 1):77–83.
27 Lau A, Halliday C, Chen SC, et al: Comparison of whole blood, serum and plasma for early detection of candidemia by multiplex-tandem PCR. J Clin Microbiol 2010;48:811–816.
28 Than LT, Chong PP, Ng KP, et al: Detection of 10 medically important *Candida* species by seminested polymerase chain reaction. Diagn Microbiol Infect Dis 2012;72:196–198.
29 Rezazadeh E, Moazeni M, Sabokbar A: Use of cost effective and rapid molecular tools for identification of *Candida* species, opportunistic pathogens. Curr Med Mycol 2016;2:1–4.
30 Ahmad S, Khan Z, Asadzadeh M, et al: Performance comparison of phenotypic and molecular methods for detection and differentiation of *Candida albicans* and *Candida dubliniensis*. BMC Infect Dis 2012;12:230.
31 Ahmad S, Khan ZU, Joseph L, et al: Genotypic heterogeneity and molecular basis of 5-flucytosine resistance among *Candida dubliniensis* isolates recovered from clinical specimens in Kuwait. Med Mycol 2012;50:244–251.
32 Schabereiter-Gurtner C, Selitsch B, Rotter ML, et al: Development of novel real-time PCR assays for detection and differentiation of eleven medically important *Aspergillus* and *Candida* species in clinical specimens. J Clin Microbiol 2007;45:906–914.
33 Fricke S, Fricke C, Schimmelpfennig C, et al: A real-time PCR assay for the differentiation of *Candida* species. J Appl Microbiol 2010; 109:1150–1158.
34 Somogyvari F, Doczi I, Serly J, et al: Rapid discrimination between *Candida albicans* and *Candida dubliniensis* by using real-time polymerase chain reaction. Diagn Microbiol Infect Dis 2007;58:367–369.
35 Khan ZU, Mustafa AS, Alam FF: Real-time LightCycler polymerase chain reaction and melting temperature analysis for differentiation of clinically important *Candida* species. J Microbiol Immunol Infect 2009;42:290–295.
36 Zhang J, Hung GC, Nagamine K, et al: Development of *Candida*-specific real-time PCR assays for the detection and identification of eight medically important *Candida* species. Microbiol Insights 2016;9:21–28.