Phenotypic and Molecular Characterization of *Candida dubliniensis* Isolates from Clinical Specimens in Kuwait

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Key Words

Candida dubliniensis · *Candida albicans* · Isolation · Molecular characterization · rDNA sequencing

Abstract

Objective: This study was carried out to characterize *Candida dubliniensis* using phenotypic and molecular methods and to determine the occurrence of *C. dubliniensis* in clinical specimens in Kuwait. **Materials and Methods:** A total of 880 clinical specimens for isolation of fungi were processed according to standard procedures. Of these, 390 germ-tube-positive clinical isolates of *Candida* species were examined for rough colonies with hyphal fringes and chlamydospore production on simplified sunflower seed agar for their presumptive phenotypic identification as *C. dubliniensis*. The identification of *C. dubliniensis* isolates was further confirmed by the Vitek 2 yeast identification system, semi-nested (sn) PCR amplification of high-copy rDNA and direct DNA sequencing of the internally transcribed spacer 2 (ITS2) region. **Results:** Of the 390 isolates of *Candida* species investigated, 12 were identified as *C. dubliniensis*, giving an overall occurrence of 3%. All the *C. dubliniensis* isolates formed rough colonies with hyphal fringes and abundant chlamydospores on sunflower seed agar, did not assimilate trehalose, lactate and α -methyl-D-glucoside, and were isolated from human immunodeficiency virus (HIV)-negative patients. Four *C. dubliniensis* isolates utilized D-xylose. The species-specific primer derived from the ITS2 sequence of *C. dubliniensis* and used

together with the panfungal reverse primer in the reamplification step of the snPCR specifically amplified rDNA from reference and clinical *C. dubliniensis* isolates and not from *C. albicans* or other *Candida* species. The identity of two representative isolates was confirmed by DNA sequencing of the ITS2 region. **Conclusions:** The identity of 12 *C. dubliniensis* isolates was first established by phenotypic characteristics and then by snPCR using species-specific primers derived from ITS2 sequences. The recovery of *C. dubliniensis* from HIV-negative patients from Kuwait reinforces the existing view that this novel yeast species has a worldwide distribution and its occurrence is not restricted to any particular immunocompromised population.

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Introduction

Candida dubliniensis is a newly recognized species that is genotypically distinct from *C. albicans* but shares several phenotypic characteristics with the latter species [1]. It occurs as a minor constituent of the human oral microflora [2]. In the past, *C. dubliniensis* isolates have been misidentified as *C. albicans*, since both species are germ tube positive and produce chlamydospores on cornmeal Tween 80 agar [1–3]. Although initially *C. dubliniensis* was believed to be associated with oropharyngeal candidiasis in human immunodeficiency virus (HIV)-infected patients [4, 5], recent reports suggest that this species has a much wider epidemiologic significance [2]. The species

has been isolated from a variety of clinical specimens and anatomic sites of HIV-negative as well as HIV-positive individuals with and without symptoms of oral candidiasis [4–8].

Given the growing importance of *C. dubliniensis* as an opportunistic pathogen [7–9], development of rapid methods to discriminate *C. dubliniensis* from *C. albicans* is highly desirable. Recently, several seed-based and non-seed-based media have been described to discriminate *C. dubliniensis* from *C. albicans* on the basis of colony characteristics and chlamydospores production [10–15]. Rapid identification of clinical *C. dubliniensis* isolates has also been described by the use of specific primers in PCR [16, 17].

In this study, we report isolation and identification of 12 isolates of *C. dubliniensis* from non-HIV-infected patients from Kuwait during a 1-year period. The identification of the *C. dubliniensis* isolates was performed by phenotypic characteristics and by a semi-nested PCR (sn-PCR) using panfungal and *C. dubliniensis*-specific primers as described in the previous studies [18–20].

Materials and Methods

Isolation and Phenotypic Characterization

A total of 880 clinical specimens (vaginal swab: 410; urine: 123; bronchoalveolar lavage: 85; sputum: 81; cerebrospinal fluid: 46; blood culture: 40; tracheal secretion: 36; peritoneal and gastric fluid: 21; throat swab: 14; wound swab: 7; catheter tip: 7; tissue biopsies: 6, and bile fluid: 4) for the isolation of fungi received at the Mycology Laboratory during January to December 2003 were processed according to standard procedures as previously described [21]. All the yeast isolates ($n = 452$) were streaked on Sabouraud glucose agar (glucose 15 g, peptone 10 g and agar 15 g in 1 liter distilled water, pH 6.8) supplemented with chloramphenicol (50 mg/l) and checked for purity. The inoculum from the isolated yeast colonies was tested for germ tube formation in serum at 37°C. The germ tube-positive isolates (390) were examined for colonial and microscopic characteristics on simplified sunflower seed agar as previously described [10]. Isolates producing rough colonies with hyphal fringes and chlamydospores were provisionally identified as *C. dubliniensis*. They were further identified by their inability to assimilate trehalose and α -methyl-*D*-glucoside in the Vitek 2 yeast identification system (BioMerieux, Murey l'Etoile, France). Isolates were also tested for tolerance to 6.5% sodium chloride to distinguish *C. dubliniensis* from *C. albicans* [22].

Molecular Characterization

C. dubliniensis (CD 36), *C. albicans* (ATCC 76615), *C. parapsilosis* (ATCC 10233), *C. tropicalis* (ATCC 750), *C. glabrata* (ATCC 15545), *C. krusei* (clinical isolate) and *C. lusitanae* (clinical isolate) were used as reference *Candida* species for snPCR amplification of rDNA. In addition, DNA extracted from *Aspergillus fumigatus* (ATCC 13073), *A. terreus* (CBS 118.27), *Fusarium solani*

(ATCC 36031), *Trichosporon asahii* CBS 2530, *Cryptococcus neoformans* (ATCC 90112), *Toxoplasma gondii* (clinical isolate), human cells, *Mycobacterium tuberculosis* H₃₇Rv and *Escherichia coli* BL-21 was also included in the study.

snPCR Amplification Method

The genomic DNA was extracted from the grown cultures of the isolates as previously described [18, 19]. The sequences of the universal forward and reverse fungal primers, capable of amplifying the 3' end of 5.8S and 5' end of 28S rDNA including the internally transcribed spacer 2 (ITS2) region and the species-specific oligonucleotide primer, derived from the ITS2 region of *C. dubliniensis*, have already been described [18–20]. Amplification with universal fungal primers and species-specific primer together with universal fungal reverse primer in the first round and snPCR from *C. dubliniensis* CD 36 should yield DNA fragments of 350 bp and 105 bp, respectively [20]. Amplification of target DNA was carried out in thin-walled 0.2-ml PCR tubes in a total volume of 50 μ l containing 1 $\times$ AmpliTaq PCR buffer I, 1 U AmpliTaq DNA polymerase, 10 pmol each of CTSE and CTSR primers, 1 μ l of DNA and 0.1 mM of each dNTP. After amplification in the first step, 1 μ l of the product was further amplified using the initial reverse primer (CTSR) and *C. dubliniensis*-specific forward primer. For snPCR, the reaction mixture consisted of 1 $\times$ AmpliTaq PCR buffer I, 1 U AmpliTaq DNA polymerase, 5 pmol of CTSR together with 5 pmol of CDET; 1 μ l of the first PCR product and 0.1 mM of each dNTP. PCR cycling (total 30 cycles) was carried out in a Perkin-Elmer cyclor (GeneAmp PCR system 2400) under the following conditions: denaturation at 94°C for 1 min, annealing at 60°C for 30 s and extension at 72°C for 1 min. An initial denaturation step at 95°C for 3 min and a final extension step at 72°C for 10 min were also included. To detect amplified DNA fragments, agarose gel electrophoresis was performed as previously described [18]. The gels were exposed to UV light and photographed. Appropriate negative controls were included in each test run, including controls omitting the DNA template during PCR assays.

The DNA sequence of the ITS2 region of two representative clinical *C. dubliniensis* isolates from Kuwait (MF 1042 and MF 122) was determined by amplification of the ITS2 region with universal fungal primers. The sequencing reactions with amplified DNA as template and forward or reverse panfungal primer were performed as described elsewhere [23]. Briefly, the column-purified PCR-amplified fragment obtained with the panfungal primer pair was used as template in sequencing reactions using the cycle DNA sequencing kit (DTCS CEQ2000, Beckman Coulter). The reaction mixtures in a final volume of 20 μ l contained eluted DNA, 5 pmol of CTSE or CTSR primer and 8.0 μ l of the premix reaction components supplied in the kit. The cycling parameters for the sequencing reactions included an initial denaturation at 95°C for 5 min followed by 30 cycles of 1 min at 94°C, 30 s at 55°C and 2 min at 60°C. The reaction products were precipitated and loaded on the sequencer as directed by the manufacturer of the automated DNA sequencer (Beckman Coulter Model CEQ8000).

Antifungal Susceptibility Testing

Antifungal susceptibility of the 12 *C. dubliniensis* isolates was determined against amphotericin B, fluconazole and voriconazole by E-test (AB BIODISK, Solna, Sweden). Each *C. dubliniensis* isolate was freshly subcultured and checked for purity. Five isolated colonies were uniformly suspended in sterile saline solution and

Fig. 1. a Simplified sunflower seed agar showing white rough colonies with hyphal fringes of *C. dubliniensis*, white smooth colonies of *C. albicans* and dark colonies of *C. neoformans* cultured from experimentally seeded bronchoalveolar lavage sample. **b** Slide culture on simplified sunflower seed agar showing chlamydospores of *C. dubliniensis* (magnification $\times 400$).

turbidity was adjusted to 0.5 on the McFarland standard scale. This inoculum was then streaked onto the Sabouraud dextrose agar Petri plates (150 mm diameter) and allowed to dry at room temperature for 10–15 min before the application of the E-test strips. The RPMI-1640 agar supplemented with 2% glucose and buffered with MOPS, 0.165 M, pH 7.0, was used for antifungal susceptibility testing (National Committee for Clinical Laboratory Standards, NCCLS, M-27A) [24]. The plates were incubated in an incubator at 35°C for 1–2 days and minimal inhibitory concentrations (MICs) were read after 24–48 h of incubation. The point where inhibition ellipses intercepted the scale on the antifungal strip was taken as MIC for each test isolate. Complete inhibition (100%) of growth for amphotericin B and marked decrease in growth intensity for fluconazole and voriconazole were observed [25, 26].

Results

Of the 390 germ tube-positive isolates of *Candida* species, 12 were identified as *C. dubliniensis* based on colonial and microscopic characteristics on simplified sunflower seed agar medium. All the 12 *C. dubliniensis* isolates formed rough colonies with hyphal fringes and abundant chlamydospores on this medium after 48 h of incubation at 30°C (fig. 1a, b), while none of the remaining 378 isolates exhibited these characteristics. The results of phenotypic and genotypic characterization and the antifungal susceptibility profile are presented in tables 1, 2 and figures 1–3. Among the 12 isolates of *C. dubliniensis*, 9 were recovered from the respiratory tract and 1

each from urine, vaginal swab and catheter tip. All the 12 *C. dubliniensis* isolates originated from HIV-negative patients and none of these patients had systemic *Candida* infection. None of the 12 *C. dubliniensis* isolates assimilated α -methyl-D-glucoside, lactate and trehalose after 24 h of incubation in the Vitek 2 yeast identification system. Four *C. dubliniensis* isolates assimilated D-xylose. The growth of *C. dubliniensis* isolates was inhibited in the presence of 6.5% sodium chloride (table 1).

The results of the E-test performed on the 12 *C. dubliniensis* isolates are presented in table 2. The E-test MIC ranges for the three antifungal agents were as follows: amphotericin B 0.002–0.038 $\mu\text{g/ml}$, fluconazole 0.094–0.75 $\mu\text{g/ml}$, and voriconazole 0.003–0.012 $\mu\text{g/ml}$. None of *C. dubliniensis* isolate was resistant to any of the three antifungal agents tested.

The PCR amplification of rDNA from the 7 *Candida* species, i.e., *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. lusitaniae*, using universal fungal primers (CTSF and CTSR) resulted in amplification of DNA fragments of expected sizes (fig. 2a). Similar results were obtained when the genomic DNA prepared from *A. fumigatus*, *F. solani*, *T. asahii* and *C. neoformans* was used as template (data not shown). Reamplification of the product of the first PCR with CTSR and CDEET resulted in specific amplification of a single DNA fragment of the expected size from the reference strain of *C. dubliniensis* only and not from the 6

Fig. 2. Agarose gel electrophoresis of the product of the first round of PCR amplification using universal fungal primers (CTSF and CTSR) (**a**) and snPCR amplification using CTSR and CDEET primers (**b**) of genomic DNA from *C. albicans* (lane 1), *C. dubliniensis* (lane 2), *C. tropicalis* (lane 3), *C. glabrata* (lane 4), *C. parapsilosis* (lane 5), *C. krusei* (lane 6) and *C. lusitaniae* (lane 7). Lane M is a 100-bp DNA ladder and the positions of migration of 100- and 600-bp fragments are marked.

Fig. 3. Agarose gel electrophoresis of the product of the first round of PCR amplification using universal fungal primers (CTSF and CTSR) (**a**) and snPCR amplification using CTSR and CDEET primers (**b**) of genomic DNA from the 4 clinical *C. dubliniensis* isolates MF 1042, MF 41, MF 48 and MF 122 (lanes 1–4, respectively). Other details are the same as in the legend of figure 2.

other *Candida* species, i.e., *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. lusitaniae* (fig. 2b), or other fungal pathogens (e.g. *A. fumigatus*, *F. solani*, *T. asahii* and *C. neoformans*; data not shown). No amplification was detected with genomic DNA from nonfungal organisms in the first as well as the second step of snPCR (data not shown).

The PCR amplification of rDNA from all the 12 clinical isolates of *C. dubliniensis* recovered from Kuwait, using CTSF and CTSR primers, resulted in specific amplification of a single DNA fragment of the expected size (the data from four isolates are shown in figure 2a, lanes 1–4) from each isolate. Further, reamplification of the products of the first PCR with CTSR and CDEET resulted again in specific amplification of a single DNA

Table 1. Phenotypic characteristics of *C. dubliniensis* isolates recovered from clinical specimens in Kuwait

Isolate No.	Source	Characteristics on SSA		Growth at or on					
		colony	chlamydo-spores	xylose	MDG	lactate	trehalose	6.5% NaCl	
MF 1042	urine	rough	+	–	–	–	–	–	
MF 41	sputum	rough	+	–	–	–	–	–	
MF 48	sputum	rough	+	–	–	–	–	–	
MF 122	sputum	rough	+	–	–	–	–	–	
S 4	sputum	rough	+	–	–	–	–	–	
S 12	vaginal swab	rough	+	+	–	–	–	–	
S 59	sputum	rough	+	+	–	–	–	–	
MF 272	sputum	rough	+	–	–	–	–	–	
MF 311	catheter tip	rough	+	–	–	–	–	–	
MF 426	BAL	rough	+	+	–	–	–	–	
MF 460	tracheal secretion	rough	+	+	–	–	–	–	
MF 523	throat swab	rough	+	–	–	–	–	–	

SSA = Sunflower seed agar; BAL = bronchoalveolar lavage; MDG = α -methyl-*D*-glucoside.

Table 2. Antifungal susceptibility data of 12 isolates of *C. dubliniensis* by E-test

Antifungal agents	Antifungal susceptibility, μ g/ml			
	range	MIC ₅₀	MIC ₉₀	% resistance
Amphotericin B	0.002–0.38	0.023	0.047	0
Fluconazole	0.094–0.75	0.19	0.25	0
Voriconazole	0.003–0.012	0.006	0.012	0

fragment (the data from the same four isolates are shown in figure 2b, lanes 1–4) of the same size as that obtained from the reference strain of *C. dubliniensis*. Finally, the identity of two of the representative clinical isolates (MF 1042 and MF 122) as *C. dubliniensis* was confirmed by DNA sequencing of the ITS2 region of rDNA. The DNA sequence of the ITS2 region of these isolates completely matched only with the *C. dubliniensis* strain CD 36 but not with *C. albicans* or any other *Candida* species. These data confirmed the isolates recovered from HIV-negative patients in Kuwait as *C. dubliniensis*.

Discussion

This study reports recovery of 12 isolates of *C. dubliniensis* from a range of clinical samples and originating from patients with different underlying conditions within

a span of 1 year. The identity of the isolates has been unequivocally established as *C. dubliniensis* by known phenotypic and molecular characteristics. In most of the published studies, differences in the assimilation of xylose, trehalose, and α -methyl-*D*-glucoside have been used to separate *C. dubliniensis* from *C. albicans* [1, 27, 28]. However, some recent reports suggest that assimilation characteristics of carbohydrates between the 2 species are not as discriminatory as initially reported and may vary depending upon the assimilation method employed [29, 30]. The ability of 4 of our *C. dubliniensis* isolates to assimilate *D*-xylose within 24 h with the Vitek 2 yeast identification system also suggests the inconsistency of the assimilation profiles in distinguishing these two closely related *Candida* species. Using a novel automated assimilation system, Kurzai et al. [30] reported that *C. dubliniensis* isolates were able to utilize *D*-xylose and trehalose. In another study [29], while the 24-hour profiles obtained with the Vitek 2 yeast identification system showed that α -methyl-*D*-glucoside, *D*-xylose and glycerol could be used to separate *C. dubliniensis* from *C. albicans*, at 48 h only *D*-xylose (6 vs. 95%) was useful for separating the two species. Following comparative evaluation of different commercial systems, these investigators [29] concluded that the assimilation of α -methyl-*D*-glucoside, *D*-xylose and trehalose was most useful for the differentiation of the two species by the majority of commercial systems. Moreover, it was the rapidity of the assimilation achieved with the commercial systems that allowed the differentiation of *C. dubliniensis* from *C. albicans*. It is apparent

that while most of the *C. dubliniensis* isolates can be distinguished by carbohydrate assimilation results, some strains may give inconsistent results justifying application of PCR-based methods for unequivocal identification [29–32].

As our understanding on the occurrence and pathogenesis of this novel species is increasing, it is becoming apparent that this pathogen has the potential to cause a wide spectrum of clinical conditions both in HIV-infected and non-HIV-infected patients such as those receiving chemotherapy for cancer, organ transplant recipients or patients with other underlying conditions [2, 4, 6, 8, 33, 34]. A review of published reports suggests that *C. dubliniensis* is widely distributed in the population but forms only a minor constituent of the microbial flora in HIV-negative individuals [1, 2, 32]. Although the species may be found at any anatomic site, it probably exhibits greater propensity to colonize oral mucosa particularly of HIV-infected patients [2, 4, 5, 34, 35]. In our study, none of the *C. dubliniensis* isolates came from HIV-infected patients, but as many as 9 of the 12 (75%) were isolated from respiratory tract specimens (table 1). The other three isolates were recovered from catheter tip, urine and vaginal specimens. Other studies have also reported the isolation of *C. dubliniensis* from a wide range of clinical specimens including bronchoalveolar lavage, urine and catheter tip [37, 38]. In a study of the Irish population of normal healthy individuals, only 3.5% were found to carry *C. dubliniensis* in the oral cavity, while the prevalence of this species in the vagina was found to be even lower (2.7%) [34]. In contrast, 26% of HIV-infected and 32% of AIDS patients with symptoms of oropharyngeal candidiasis yielded *C. dubliniensis*, whereas in patients without symptoms of oropharyngeal candidiasis the colonization levels were 18 and 25%, respectively [35]. Like in other regions of the world [2, 32], occurrence of *C. dubliniensis* has also been recognized in the Middle East [20, 33, 34, 36–38].

Fotadar and Al-Hedaithy [38] studied 823 germ tube-positive and chlamydospore-positive *C. albicans* isolates at the University Hospital in Riyadh, Saudi Arabia. On reexamination, 27 (3.3%) of them were identified as *C. dubliniensis*. The prevalence of *C. dubliniensis* amongst germ tube-positive isolates was almost similar (3%) in the present study.

It has been suggested that the recent emergence of *C. dubliniensis* as a human pathogen may have resulted due to the widespread use of azole antifungal therapy in HIV-infected patients, in a manner similar to that suggested for *C. glabrata* [1, 2]. However, this assumption may not be correct since it has been found that the great majority of *C. dubliniensis* isolates are inherently susceptible to commonly used antifungal agents [5, 39]. Similarly, all the 12 *C. dubliniensis* isolates of this study were susceptible to fluconazole and voriconazole and also to amphotericin B.

Conclusions

We have described the isolation and identification of 12 isolates of *C. dubliniensis* recovered mostly from the respiratory tract of HIV-negative patients in Kuwait during a 1-year period. As our understanding of the epidemiology of *C. dubliniensis* is increasing, it is becoming apparent that this pathogen can colonize different anatomic sites with a potential to cause superficial or invasive disease.

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