Isolation and molecular identification of *Candida dubliniensis* from non-human immunodeficiency virus-infected patients in Kuwait

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*Candida dubliniensis* is an emerging pathogen capable of causing oropharyngeal, vaginal and bloodstream infections. Although *C. dubliniensis* is similar to *Candida albicans* in several phenotypic characteristics, it differs from it with respect to epidemiology, certain virulence factors and the ability to develop resistance to fluconazole rapidly. In this study, the first seven isolations of *C. dubliniensis* from Kuwait are described, all originating from non-human immunodeficiency virus (HIV)-infected patients. The isolates were initially identified by the Vitek 2 yeast identification system, positive germ tube test, production of rough colonies and chlamydospores on Staib agar and by their inability to assimilate xylose, trehalose or methyl α-D-glucoside. The species identity of the isolates was subsequently confirmed by specific amplification of rDNA targeting the internally transcribed spacer 2 (ITS2), restriction endonuclease digestion of the amplified DNA and direct DNA sequencing of the ITS2. Using the E-test method, the MICs of *C. dubliniensis* test isolates were in the range 0.125–0.75 μg ml⁻¹ for fluconazole, 0.002–0.75 μg ml⁻¹ for itraconazole, 0.006–0.125 μg ml⁻¹ for ketoconazole, 0.002–0.5 μg ml⁻¹ for amphotericin B and 0.002–0.016 μg ml⁻¹ for voriconazole. Two of the isolates were resistant to 5-flucytosine (32 μg ml⁻¹), but none against fluconazole. The study reinforces the current view that *C. dubliniensis* has a much wider geographical and epidemiological distribution.

INTRODUCTION

Candidosis is a common opportunistic infection occurring in immunosuppressed individuals. Although *Candida albicans* is the most commonly isolated species, the emergence of non-*albicans* *Candida* species has contributed greatly to the dramatic increases in *Candida* infections in the last 2 decades (Coleman et al., 1998). *Candida dubliniensis* is phenotypically similar to but genotypically distinct from *C. albicans* (Coleman et al., 1998; Sullivan & Coleman, 1998; Sullivan et al., 1995). It is found as a minor constituent of the human oral microflora. Previously, *C. dubliniensis* was misidentified as *C. albicans*, since isolates of both species are germ-tube positive and produce chlamydospores (Sullivan & Coleman, 1998; Sullivan et al., 1995). *C. dubliniensis* is mainly associated with oropharyngeal candidosis in human immunodeficiency virus (HIV)-infected patients, particularly those with a history of recurrent oral candidosis (Jabra-Rizk et al., 2000; Moran et al., 1997). More recently, *C. dubliniensis* has also been isolated from faeces, urine, wounds, vaginal swabs and respiratory tract specimens of non-HIV-infected patients (Gee et al., 2002; Jabra-Rizk et al., 2000; Moran et al., 1997; Meis et al., 1999; Odds et al., 1998; Redding et al., 1999). The species exhibits enhanced adherence to buccal epithelial cells and rapidly develops resistance to fluconazole *in vitro* (Moran et al., 1997; Sullivan & Coleman, 1998). It is now regarded as an emerging pathogen of immunosuppressed individuals, capable of causing superficial and systemic disease among HIV-negative as well as HIV-positive patients (Brandt et al., 2000; Jabra-Rizk et al., 2000; Gutierrez et al., 2002; Polacheck et al., 2000; Sebti et al., 2001).

Here we report the first seven isolations of *C. dubliniensis* from non-HIV-infected patients from Kuwait. Besides positive phenotypic characteristics, the identity of the isolates was confirmed by a semi-nested PCR using universal and *C. dubliniensis*-specific primers (Ahmad et al., 2002), as well as by direct sequencing of the internally transcribed spacer 2 (ITS2) of rDNA in comparison with reference strains.

METHODS

Reference and clinical *Candida* isolates. *C. dubliniensis* (type strain CD361\(^{7}\)), *C. dubliniensis* (CBS 7987), *C. albicans* (ATCC 76615), *Candida parapsilosis* (ATCC 10233), *Candida tropicalis* (ATCC 750\(^{1}\)), *Candida glabrata* (ATCC 15545), *Candida krusei* (clinical isolate) and *Candida lusitaniae* (clinical isolate) were used as reference *Candida* species. Seven local clinical isolates of *C. dubliniensis* were included in the study. They originated from sputum (five isolates), vagina (one isolate) and urine (one isolate) (Table 1).

Abbreviation: ITS2, internally transcribed spacer 2.
Source of isolation and phenotypic characteristics of clinical C. dubliniensis isolates from Kuwait

SLE, Systemic lupus erythematosus; Treh, trehalose; Xyl, xylose; MDG, methyl ß-D-glucoside; +, positive; /C6, weakly positive; /C0, negative.

<table>
<thead>
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Growth and biochemical characterization. Clinical specimens were processed according to standard procedures (McGinnis, 1994). All cultures were made on Sabouraud glucose agar (SGA; glucose 15 g, peptone 10 g and agar 15 g in 1 l distilled water, pH 6-8) and grown at 30°C. Yeast isolates were examined by wet mount and tested for germ tube formation and chlamydospore production on Staib agar. Germ-tube-positive isolates were provisionally identified as C. albicans and their identity was further determined by the Vitek 2 yeast identification system (bioMérieux) (Table 1). The sodium chloride tolerance test to differentiate between C. albicans and C. dubliniensis was performed as described by Alves et al. (2002).

Isolation of genomic DNA and PCR amplification. Genomic DNA was extracted from cultures of the isolates according to a method described previously (Ahmad et al., 2002). 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 ITS2, and the species-specific oligonucleotide primers, derived from the ITS2 region of C. albicans, C. parapsilosis, C. tropicalis and C. glabrata have already been described (Ahmad et al., 2002; Elie et al., 1998; Fujita et al., 1995). The sequence of the species-specific primer, CDDET (5'-GCTAAGGCCTCTCTGCGGTGCG-3'), was derived from the conserved sequence of the ITS2 from several strains of C. dubliniensis (Williams et al., 2001; Gee et al., 2002). Amplification with universal fungal primers and species-specific primers together with universal fungal reverse primer in the first round, followed by semi-nested PCR of C. dubliniensis CD36^2 should yield DNA fragments of 350 and 105 bp, respectively. Amplification of target DNA was carried out in thin-walled 0.2 ml PCR tubes in a total volume of 30 l containing 1X AmpliTaq PCR buffer I, 1 U AmpliTaq DNA polymerase, 10 pmol each of CTSR and CTSR primers, 1 µl of DNA extracted from culture and 0.1 mM each dNTP. After amplification in the first step, 1 µl of the product was further amplified using the initial reverse primer (CTSR) and the C. dubliniensis-specific forward primer (CDDET). For semi-nested PCR, the reaction mixture consisted of 1X AmpliTaq PCR buffer I, 1 U AmpliTaq DNA polymerase, 5 pmol CTSR together with 5 pmol CDDET and 0.1 mM each dNTP. PCR cycling was carried out in a Perkin-Elmer cycler (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 94°C for 3 min and a final extension step at 72°C for 10 min were also included. Amplification was determined to be optimum with 30 cycles of the first PCR followed by 20 cycles of the semi-nested PCR.

The amplified products (20 µl) were resolved by electrophoresis in agarose gels carried out in TBE buffer (89 mM Tris/HCl, 89 mM boric acid, 20 mM EDTA, pH 8:0) at 110 V for 60–90 min on 1% (w/v) agarose (Gibco-BRL) gels. After electrophoresis, the gels were exposed to UV light and photographed. The sizes of amplified DNA fragments were determined by comparison with molecular mass marker DNA (100 bp DNA ladder). To avoid the risk of contamination of PCR samples, precautions and guidelines advocated by Kwok & Higuchi (1989) were followed. The area where the PCR mixtures were prepared was physically separated from the laboratory where DNA extraction was performed. Amplicon carryover was prevented by using aerosol-guarded pipette tips. Appropriate negative controls were included in each test run, including controls omitting the DNA template during PCR assays.

Molecular characterization of the ITS2 of C. dubliniensis. The amplified DNA fragments of 345 and 350 bp obtained from C. albicans and C. dubliniensis using universal fungal primers contain two and one restriction sites for restriction enzyme MspA11, respectively (Williams et al., 2001; Gee et al., 2002). Thus, the amplified fragment from C. albicans should be digested into three fragments of 35, 143 and 167 bp, while that from C. dubliniensis should be digested into two fragments of 35 and 315 bp by MspA11. Restriction digestion with MspA11 to generate RFLPs

<table>
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was performed as described by Williams et al. (2001). The DNA sequence of the ITS2 region from two clinical C. dubliniensis isolates from Kuwait (Kw74 and Kw848) was determined by amplification of the ITS2 region with universal fungal primers. The PCR products were run on agarose gels in TBE buffer, the DNA band corresponding to the amplified DNA was cut out and the DNA was eluted. The eluted DNA was used as a template in a sequencing reaction using the cycle DNA sequencing kit (Applied Bio-Systems). The reaction mixtures in a final volume of 20 μl contained: eluted DNA, 5 pmol of primer CTSF and 9-5 μl of the pre-mix reaction components supplied in the kit. The cycling parameters for the sequencing reaction included an initial denaturation at 94 °C for 2 min followed by 45 cycles of 1 min at 94 °C, 30 s at 55 °C and 1 min at 72 °C. The reaction products were extracted with phenol/chloroform, precipitated with ethanol and the pellet DNA was loaded on the sequencing gel as described in the instructions supplied with the sequencing kit.

Antifungal susceptibility. E-test was carried out on freshly prepared RPMI agar plates. The plates were prepared by adding sterile liquid RPMI 1640 (4·6 %) (Life Technologies), buffered with 0·164 M MOPS (Sigma), to Bacto agar (1·5 %; Difco). The growth suspension of the test isolate at 0·5 McFarland density was swabbed in three directions across the entire RPMI-agar plate with E-test strips (AB Biodisk, Solna, Sweden) of fluconazole, itraconazole, voriconazole, ketoconazole, amphotericin B and flucytosine. The plates were incubated at 35 °C, and MICs were read after 24 h. The MICs were read at the intersection of the ellipse on the E-test strip and for azoles at the point of 80 % growth inhibition. Interpretation of susceptibility was based on NCCLS breakpoints (NCCLS, 1997) as susceptible, susceptible-dose dependent/intermediate and resistant: fluconazole ≤8, 16–32, >32 μg ml⁻¹; itraconazole ≤0·125, 0·25–0·5, >1 μg ml⁻¹ and flucytosine ≤4, 8–16, >32 μg ml⁻¹ (NCCLS, 1997). No NCCLS break-points are available for voriconazole, ketoconazole and amphotericin B.

RESULTS AND DISCUSSION

Of the seven isolates of C. dubliniensis reported in this study, five originated from sputum and one each from vaginal swab and urine (Table 1). Five of the patients had cancer, one systemic lupus erythematosus and one diabetes mellitus. None of our isolates originated from HIV-positive patients. As our understanding of the epidemiology of C. dubliniensis is increasing, it is becoming apparent that this pathogen has the potential to colonize, as well as cause superficial or invasive disease in HIV-negative, as well as HIV-positive patients; such as those receiving cytotoxic chemotherapy for cancer, organ transplant recipients or patients with other underlying conditions (Brandt et al., 2000; Gee et al., 2002; Jabra-Rizk et al., 2000; Polacheck et al., 2000; Redding et al., 1999; Sebti et al., 2001).

Using the E-test method, the MICs of C. dubliniensis test isolates were in the range 0·125–0·75 μg ml⁻¹ for fluconazole, 0·002–0·75 μg ml⁻¹ for itraconazole, 0·006–0·125 μg ml⁻¹ for ketoconazole, 0·002–0·5 μg ml⁻¹ for amphotericin B and 0·002–0·016 μg ml⁻¹ for voriconazole. Two of the isolates (Kw41 and Kw848) were found to be resistant to 5-flucytosine (>32 μg ml⁻¹). It has been suggested that the recent emergence of C. dubliniensis as a human pathogen may have resulted from widespread use of antifungal drug therapy (Coleman et al., 1998). However, none of our isolates was resistant to fluconazole or amphotericin B (taken as susceptible at ≤1 μg ml⁻¹). These results were in agreement with those reported by several other investigators who have also found that the great majority of C. dubliniensis isolates are susceptible to commonly used antifungal agents, including voriconazole (Odd et al., 1998; Pfaller et al., 2002; Quindos et al., 2000). Resistance to 5-flucytosine in two of our isolates by E-test (also subsequently confirmed by broth microdilution test) is an unusual observation, since C. dubliniensis isolates have been found to be highly susceptible to this agent (Quindos et al., 2000) with most of the strains showing MICs ≤0·12 μg ml⁻¹ (Pfaller et al., 2002). Five of our seven isolates originated from cancer patients. The precise reason for the increased occurrence of C. dubliniensis among cancer patients is not known, although it has been suggested that this species has a greater propensity to adhere to mucosal epithelium (Gillilan et al., 1998).

Persistent exposure to fluconazole probably leads to enhanced expression of proteinase antigen on the surface of C. dubliniensis cells (Borg-von Zepelin et al., 2002). Thus, prolonged chemoprophylaxis with fluconazole to prevent oral Candida colonization in HIV and cancer patients could contribute to this phenomenon.

Consistent with the known phenotypic characteristics of C. dubliniensis (Gales et al., 1999; Sullivan & Coleman, 1998), our isolates were positive for germ tube formation, produced chlamydoospores as well as rough colonies on Staib agar (Al Mosaid et al., 2001), did not assimilate xylose, trehalose or methyl α-D-glucoside and showed no growth at 45 °C (Table 1). Likewise, the growth of our isolates was inhibited in the presence of 6·5 % sodium chloride in Sabouraud broth, an observation that has been used to discriminate C. dubliniensis from C. albicans (Alves et al., 2002).

A recently developed semi-nested PCR, for the detection of Candida species DNA isolated from culture or clinical specimens, which targets the multicopy rDNA (Ahmad et al., 2002), was utilized for the identification of the local C. dubliniensis isolates. This was further confirmed by molecular characterization and direct DNA sequencing of the ITS2 of the rDNA. The PCR, using CTSF and CTSR primers (Ahmad et al., 2002), performed with reference C. albicans strain ATCC 76615 (Fig. 1a, lane 1), C. dubliniensis reference strains CD36 (Fig. 1a, lane 2), and CBS7987 (data not shown) and the seven clinical isolates of C. dubliniensis recovered from Kuwait (data from three isolates are shown in Fig. 1a, lanes 3–5), resulted in specific amplification of a single DNA fragment of the expected size in each case.

Since the amplified DNA fragments of 345 and 350 bp obtained with CTSF and CTSR primers from reference C. albicans and C. dubliniensis strains contain two and one restriction sites for restriction enzyme MspAI1, respectively (Gee et al., 2002; Williams et al., 2001), restriction digestion with MspAI1 was also performed to distinguish the clinical C. dubliniensis isolates from C. albicans. When subjected to restriction endonuclease digestion with the enzyme MspAI1, amplified DNA fragments obtained from C. albicans reference strain ATCC 76615 (Fig. 1b, lane 1) and C. dubliniensis

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**Fig. 1.** (a) Agarose gel electrophoresis of the products of the first round of PCR amplification using universal fungal primers (CTSF and CTSR); (b) restriction digestion of amplified products with Msp A1I and (c) semi-nested PCR amplification using CTSR and CDDET primers of genomic DNA from: C. albicans ATCC 76615 (lanes 1), C. dubliniensis CD36T (2), C. dubliniensis Kw74 (3), C. dubliniensis Kw239 (4) and C. dubliniensis Kw848 (5). Undigested amplified DNA from C. dubliniensis CD36T (b, lane 2’) and C. dubliniensis Kw74 (b, lane 3’) is also shown. Lane M shows a 100 bp DNA ladder; the positions of migration of the 100 and 600 bp fragments are marked.

Reference strain CD36T (Fig. 1b, lane 2) yielded three and two DNA fragments of the expected sizes, respectively, based on the presence of two and one restriction sites for this restriction enzyme in the amplified DNA fragments. C. dubliniensis reference strain CBS7987 also yielded the same results as CD36T (data not shown). Msp A1I restriction endonuclease digestion of the amplified fragments from the seven clinical isolates of C. dubliniensis from Kuwait exhibited the same RFLP pattern as that exhibited by C. dubliniensis CD36T and CBS7987, but not C. albicans (Fig. 1b, lanes 3–5). The data showed that all seven clinical C. dubliniensis isolates from Kuwait exhibited the same RFLP pattern as that exhibited by the C. dubliniensis reference strains CD36T and CBS7987. Although not specific for C. dubliniensis, the PCR-RFLP assay of the ITS2 with MspA1I discriminates C. dubliniensis from C. albicans (Gee et al., 2002; Williams et al., 2001). This is because the amplified DNA fragments from other clinically important Candida species e.g. C. parapsilosis, C. tropicalis, C. glabrata, C. krusei and C. lusitaniae also contain a single restriction site for the restriction endonuclease MspA1I. However, as the sizes of the amplified DNA fragments obtained with CTSF and CTSR primers from the latter Candida species vary considerably from those obtained from C. dubliniensis or C. albicans, their RFLP patterns are different and can be easily distinguished from those obtained from C. dubliniensis (Gee et al., 2002; Williams et al., 2001).

Reamplification of the products of the first PCR from C. albicans reference strain ATCC 76615 and C. dubliniensis reference strains CD36T and CBS7987, resulted in the specific amplification of a single DNA fragment of the expected size from CD36T (Fig. 1c, lane 2) or CBS7987 (data not shown) but not from reference C. albicans strain ATCC 76615 (Fig. 1c, lane 1). Reamplification of the products of the first PCR from the seven clinical C. dubliniensis isolates from Kuwait also resulted in the specific amplification of a single DNA fragment of same size as that obtained from the C. dubliniensis reference strains CD36T or CBS7987 (Fig. 1c, lanes 3–5). The results of these studies showed that a specific product of the same size was obtained from all seven clinical C. dubliniensis isolates from Kuwait as that obtained from the C. dubliniensis reference strains CD36T and CBS7987.

The identity of two of the clinical isolates (Kw74 and Kw848) as C. dubliniensis was further confirmed by direct DNA sequencing of the ITS2 of the rDNA. The DNA sequence of the ITS2 from these isolates matched completely with C. dubliniensis strain CD36T, but not with other subtypes of C. dubliniensis or with C. albicans or any other Candida species (Gee et al., 2002; Williams et al., 2001). These data unequivocally confirmed the isolates recovered from Kuwait as C. dubliniensis since the DNA sequence of the ITS2 of the rDNA is regarded as species-specific for various Candida species (Elie et al., 1998; Gee et al., 2002; Fujita et al., 1995).

In conclusion, we describe the isolation and identification of the first seven C. dubliniensis isolates from Kuwait recovered from HIV-negative, immunosuppressed patients. To the best of our knowledge, this is also the first report of the isolation of C. dubliniensis from the Persian Gulf region.

**ACKNOWLEDGEMENTS**

We thank H. C. Gugnani for providing C. dubliniensis CD36T (type strain) and R. Chandy and D. Farhat for excellent technical assistance. This work was supported by Kuwait University Research Administration grant MI 118.
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