Recovery of *Candida dubliniensis* from Non-Human Immunodeficiency Virus-Infected Patients in Israel

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Candida dubliniensis is a recently discovered yeast species principally associated with carriage and disease in the oral cavities of human immunodeficiency virus (HIV)-infected individuals. To date the majority of isolates of this species have been identified in Europe and North America. In this study, five *Candida* isolates recovered from separate HIV-negative hospitalized patients in Jerusalem, Israel, were presumptively identified as *C. dubliniensis* on the basis of their dark green coloration when grown on CHROMagar Candida medium. Their identification was confirmed by a variety of techniques, including carbohydrate assimilation profiles, absence of growth at 45°C, positive reaction with *C. dubliniensis*-specific antibodies as determined by indirect immunofluorescence analysis, and positive amplification with *C. dubliniensis*-specific PCR primers. All five strains were shown to be susceptible to a range of antifungal agents, including fluconazole. One of the five isolates was recovered from urine specimens, while the remaining four were recovered from upper respiratory tract and oral samples. While none of the patients was HIV positive, all were receiving broad-spectrum antibacterials at the time isolates of *C. dubliniensis* were obtained from clinical specimens. This study describes the first isolates of *C. dubliniensis* from the Middle East and confirms that this yeast can be associated with carriage and infection in the absence of HIV infection.

Candida dubliniensis, which was first established as a novel yeast species in 1995, is phenotypically and genotypically closely related to the most frequently identified human fungal pathogen, *Candida albicans* (25). This close similarity between the two species has proved problematic in the identification of *C. dubliniensis* in clinical samples and in retrospective analyses of laboratory stock collections, with many isolates being misidentified as *C. albicans* (5, 18). However, the recent description of reliable and rapid identification tests, including the observation of differentially colored primary colonies on CHROMagar Candida medium and the use of *C. dubliniensis*-specific PCR primers, will greatly facilitate the identification of this species in clinical samples and establish its epidemiologic significance (6, 24). In addition, accurate species identification has been aided by the inclusion of *C. dubliniensis*-specific carbohydrate assimilation profiles in the databases of commercially available yeast identification kits, such as the API ID 32C and the API 20C AUX systems (20, 24). To date the majority of *C. dubliniensis* isolates have been identified in Western Europe and North America (1–3, 7, 10–14, 17, 18, 23, 25, 26). Most of these isolates were associated with oral carriage and oropharyngeal infection in human immunodeficiency virus (HIV)-infected individuals. In a recent study of an Irish subject group, 26% (48 of 185) of HIV-positive individuals and 32% (26 of 82) of AIDS patients with oral candidiasis yielded *C. dubliniensis*. In approximately 25% of these cases *C. dubliniensis* was the only species detected (5, 24). However, *C. dubliniensis* is not exclusively associated with HIV-infected indi-

viduals. In the same study *C. dubliniensis* was also identified in clinical specimens recovered from HIV-negative individuals, both with and without symptoms of oral candidiasis. In an analysis of oral samples taken from healthy individuals without any signs of oral disease, 3.5% (7 of 202) of subjects yielded *C. dubliniensis*, suggesting that this species is a minor constituent of the normal human oral flora. *C. dubliniensis* was also identified in 12.5% (16 of 128) of cases of oral candidiasis in HIV-negative individuals (5, 24). Although the oral cavity is the human niche from which *C. dubliniensis* has been recovered most frequently, there have been reports of isolates being recovered from other anatomical sites and specimens, including the vagina, the lung, feces, and sputum (16, 18, 21). Furthermore, *C. dubliniensis* was recently identified as the cause of three cases of systemic disease in HIV-negative Dutch patients receiving post-bone marrow transplantation immunosuppressive treatment or cytotoxic chemotherapy for the treatment of rhabdomyosarcoma (14). In this study we describe the application of routine phenotypic and rapid molecular methods to the identification of *C. dubliniensis* isolates from five separate HIV-negative hospitalized patients in Israel. All five isolates were shown to be susceptible to a range of antifungal agents, including fluconazole. This is the first report of the identification of this novel species in the Middle East.

CASE REPORTS

Case 1. A 39-year-old female with a past history of thalassemia major, splenectomy, and transfusion associated hemochromatosis initially presented with acute respiratory tract infection. The physical examination on admission was notable only for the presence of a purulent postnasal drip. Given the patient's asplenic condition, she was placed on intravenous cefuroxime, 750 mg every 8 h. Oral and upper respiratory

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specimens were inoculated onto blood agar (5% [vol/vol] defibrinated sheep blood), chocolate agar, and MacConkey agar media and incubated at 35°C for 48 h in an atmosphere of 5% (vol/vol) CO₂. With the recovery of beta-hemolytic streptococcus (Lancefield group G), *Staphylococcus aureus*, *C. albicans*, and *Candida krusei*, treatment was altered to cephalexin, 500 mg every 6 h, for a total duration of 14 days.

While the patient's respiratory symptoms slowly resolved, initial symptoms of oropharyngeal candidiasis were observed 4 days after the initiation of antibiotic therapy. As a result, the patient was placed on clotrimazole troches (10 mg), five times per day. Five days after the initiation of antifungal therapy and while the patient was still being treated for her respiratory infection, additional oral and upper respiratory specimens were obtained and cultured at 30°C for 48 h on Emmon's modified Sabouraud glucose agar (SGA) supplemented with 50 µg of chloramphenicol and 5 µg of gentamicin/ml. Pure cultures of *C. dubliniensis* were recovered from all specimens, resulting in the alteration of the antifungal therapy to oral fluconazole, 200 mg daily for 5 days. Cultures inoculated with respiratory specimens collected 10 days after completion of antifungal therapy were negative for yeasts, and a physical examination at this same time disclosed only mild glossitis and perlèche.

Case 2. A 19-year-old man was admitted to the hospital due to a urinary tract infection complicating type I neurofibromatosis. Massive retroperitoneal tumors were known to obstruct the ureters and deform the bladder, with secondary renal failure and recurrent episodes of urinary tract infection. In view of previous infection with resistant bacteria (prior to hospitalization the patient was repeatedly treated for urinary tract infection, with eventual culture of antibiotic-resistant bacteria), the patient was treated with broad-spectrum antibiotics, including vancomycin, meropenem, and co-trimoxazole, and was subsequently referred for surgical revision of his urinary tract. Peri- and postoperatively, during removal of drains, the patient was frequently given broad-spectrum antibiotics as a prophylactic measure, including ampicillin, ofloxacin, and metronidazole. Urine cultures during that period grew numerous bacteria, including *Stenotrophomonas maltophilia*, *Citrobacter koseri*, *S. aureus*, and *Enterococcus faecalis*. Forty days after admission, when a suprapubic catheter was removed without adequate alternative urinary drainage, the patient became febrile. A urine culture was positive for *Enterococcus faecium* and *Trichosporon beigelii*. Administration of antibacterial agents was continued, drainage of the urinary tract was restored by intermittent catheterizations, and the patient defervesced. During this time the patient did not receive any antifungal agent. Repeated urine cultures in the following 10 days were positive for *T. beigelii*, and antibiotic treatment was eventually stopped. Follow-up urine culture 5 days later yielded *T. beigelii* and *C. dubliniensis*. Because the patient was asymptomatic, he was not treated. A urine culture 2 weeks later was negative for fungi.

Case 3. A 21-year-old woman with cystic fibrosis and thalassemia minor was admitted to the hospital due to worsening dyspnea, productive sputum, and fever. The patient had recurrent episodes of *Pseudomonas aeruginosa* respiratory tract infection, the last being 1 month prior to this admission, for which she was treated with ceftazidime and amikacin and later switched to ciprofloxacin. Bronchoscopy revealed purulent secretions, with a Gram stain of lavage disclosing gram-positive cocci and gram-negative rods. Cultures of this lavage fluid were positive for *S. aureus*, *P. aeruginosa*, and *C. dubliniensis*. The patient was treated with ceftazidime and amikacin and improved from the bacterial infection within 5 days. Previous

bronchoalveolar lavage and follow-up sputum cultures were persistently positive only for *S. aureus* and *P. aeruginosa*.

Case 4. A 52-year-old woman was admitted for right-side pneumonia leading to acute respiratory failure. Her past medical history was significant for indicating type II polyglandular autoimmune syndrome (Schmidt's syndrome) treated with prednisone (7.5 mg daily), insulin, and thyroxine; ischemic heart disease; and perforated appendicitis 5 months prior to admission. The patient was treated with cefuroxime and ciprofloxacin, and prednisone was replaced by hydrocortisone (300 mg daily). Bronchial washings performed on admission through the endotracheal tube disclosed few granulocytes without bacteria and no significant growth. Bronchoscopy done 4 days later disclosed a minute amount of purulent secretions. A Gram stain was negative, while a culture was positive for *C. dubliniensis*. Thereafter, acute renal failure developed, and peritoneal dialysis was initiated. The patient died 19 days later with evidence of *C. albicans* peritonitis and bloodstream infection (specimens from both sources yielded *C. albicans*). An autopsy was not performed. HIV serology was negative.

Case 5. A 31-year-old otherwise healthy woman was treated for postpartum endometritis with ampicillin, gentamicin, and metronidazole for 10 days. One week after completion of therapy she noticed a burning sensation on the tongue accompanied by a whitish discoloration that persisted. Three weeks later a diagnosis of oral candidiasis was made. A superficial tongue specimen disclosed mixed bacterial morphotypes with few leukocytes. A culture was positive for mixed bacteria and *C. dubliniensis*. She was not treated, and at a follow-up examination 4 weeks later all symptoms and signs had resolved. Microscopic analysis and culture of specimens taken from the endometrium were both negative for fungi.

MATERIALS AND METHODS

Yeast isolates. Yeast isolates from clinical specimens were recovered following primary culture for 48 h at 30°C on Emmon's modified SGA supplemented with 50 µg of chloramphenicol and 5 µg of gentamicin/ml. Following incubation, confluent or semiconfluent areas of yeast growth were sampled with a sterile wire loop and streaked on CHROMagar Candida medium (CHROMagar, Paris, France) to yield single colonies. Selected colonies exhibiting different colony colors were transferred to and maintained on SGA at 30°C. A single isolated colony of each species grown on SGA was transferred after 48 h of incubation at 30°C to CHROMagar medium, incubated at 30°C, and examined for colony color after 24 and 48 h. Colonies from 48-h SGA cultures were similarly used as inocula in the following standard morphological and physiological tests: (i) chlamydoconidia formation on corn meal agar supplemented with 1% (wt/vol) Tween 80, (ii) germ tube development in human serum incubated for 3 h at 37°C, (iii) sensitivity to cycloheximide as determined by growth on Mycosel agar (BBL, Cockeysville, Md.), and (iv) growth at 37, 42, and 45°C on SGA. Carbohydrate source and nitrogen source assimilation patterns were evaluated by using the API ID 32C and the API 20C AUX yeast assimilation systems (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions, with an inoculum derived from 48-h SGA cultures.

Serotyping. *C. dubliniensis* isolates were serotyped on the basis of agglutination reactions with antiserum raised against *Candida* antigenic factor no. 6 (Iatron Laboratories, Inc., Tokyo, Japan) as described previously (25).

Chemicals, enzymes, and oligonucleotides. Analytical-grade or molecular biology grade chemicals were purchased from Sigma-Aldrich, BDH (Poole, Dorset, United Kingdom) or Boehringer Mannheim (Lewes, East Sussex, United Kingdom). Enzymes were purchased from Boehringer Mannheim or the Promega Corporation (Madison, Wis.) and used according to the manufacturer's instructions. Custom-synthesized oligonucleotides were purchased from Genosys Biotechnologies (Pampisford, Cambridgeshire, United Kingdom).

In vitro antifungal susceptibility tests. The in vitro antifungal susceptibilities of *C. dubliniensis* isolates were determined by using the Etest system (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. The MIC was defined as the lowest concentration of antifungal agent at which the border of the elliptical inhibition zone intercepted the readable scale on the strip (4). All tests were quality controlled by using *C. krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019.

Immunofluorescence. An indirect immunofluorescence assay (IFA) was performed as described previously (1). Briefly, the blastospores from *C. dubliniensis* isolates, including the *C. dubliniensis* type strain, CD36 (CBS 7989) (25), and

TABLE 1. Substrate assimilation profiles^b of Israeli *C. dubliniensis* isolates

Strain or isolate	Strain type (reference) or date of isolation	API ID 32C results			API 20C AUX results		
		Profile	Species	Predictive value (%) ^a	Profile	Species	Predictive value (%) ^a
Reference strains							
<i>C. dubliniensis</i> CD36	Type strain (25)	7142140015	<i>C. dubliniensis</i>	99.3	6172134	<i>C. dubliniensis</i>	99.9
<i>C. albicans</i> 179A	Serotype A strain (9)	7347340015	<i>C. albicans</i>	99.9	2776174	<i>C. albicans</i>	98.3
<i>C. albicans</i> 132A	Serotype B strain (9)	7347140015	<i>C. albicans</i>	99.9	2376174	<i>C. albicans</i>	99.2
Clinical isolates							
P-6265	December 1998	7042140011	<i>C. dubliniensis</i>	99.9	6152034	<i>C. dubliniensis</i>	99.9
P-6785	March 1999	7042140011	<i>C. dubliniensis</i>	99.9	6142034	<i>C. dubliniensis</i>	99.5
P-7073	May 1999	7142140015	<i>C. dubliniensis</i>	99.3	2172174 ^c	<i>C. albicans</i>	97.6
P-7266	June 1999	7142100015	<i>C. dubliniensis</i>	99.9	6172134	<i>C. dubliniensis</i>	99.9
P-7276	June 1999	7042140015	<i>C. dubliniensis</i>	99.8	6152034	<i>C. dubliniensis</i>	99.9

^a bioMérieux APILAB Plus database, version 3.3.3.^b All profiles were read following 48 h incubation at 30°C.^c Isolate P7073 is *C. dubliniensis* as determined by IFA and by PCR amplification with the primer set of DUBF and DUBR (this study). This isolate assimilated trehalose. However, in the current database the expected percentage for trehalose assimilation by *C. dubliniensis* is 0%. A recent in-depth study has demonstrated that many proven *C. dubliniensis* isolates were misidentified or unidentified by the API 20C AUX system due to positive trehalose reactions and that incorporation of this variability in a future database would permit correct identification of such isolates as *C. dubliniensis* (20).

reference oral *C. albicans* isolate 132A (9) were grown on Sabouraud agar (Oxoid, Poole, Dorset, United Kingdom) plates for 48 h at 37°C, resuspended in phosphate-buffered saline (PBS) at a cell density of 10⁶ cells/ml, and placed on Teflon-coated immunofluorescence slides. The slides were incubated with anti-*C. dubliniensis* rabbit serum (1) diluted 1:5 in PBS supplemented with Evans blue (0.05% [wt/vol]) and Tween 20 (0.05% [vol/vol]) and washed, and the reacting antibodies were revealed by incubation with fluorescein-conjugated goat anti-rabbit immunoglobulin G (Sigma).

PCR identification of *C. dubliniensis*. PCR identification of *C. dubliniensis* with the *C. dubliniensis*-specific primer pair DUBF and DUBR (6) was carried out in a 50-μl final volume containing 10 pmol each of the forward and reverse primers, 2.5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0 at 25°C), 10 mM KCl, 0.1% (vol/vol) Triton X-100, 2.5 U of *Taq* DNA polymerase (Promega), and 25 μl of template DNA-containing cell supernatant (prepared as described below). The primer pair of DUBF and DUBR is complementary to sequences within the *ACT1*-associated intron sequence of *C. dubliniensis* and yields an amplicon of 288 bp. Each reaction mixture also contained 10 pmol each of the universal fungal primers RNAF and RNAR (8), which amplify a fragment of approximately 610 bp from all fungal large-subunit rRNA genes, as an internal positive control. Cycling conditions consisted of 6 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, followed by 72°C for 10 min. Amplification products were separated by electrophoresis through 2.0% (wt/vol) agarose gels containing 0.5 μg of ethidium bromide/ml and were visualized on a UV transilluminator.

Preparation of template DNA. *Candida* template DNA for use in PCR experiments with the *C. dubliniensis*-specific primer pair DUBF and DUBR was prepared as described by Donnelly et al. (6). Briefly, a single colony from a culture grown for 48 h at 37°C on potato dextrose agar or CHROMagar *Candida* medium was suspended in 50 μl of sterile distilled water. Cell suspensions were boiled for 10 min, and the lysed cells were subjected to a clearing spin for 5 min at 20,000 × g. Template DNA contained in 25 μl of supernatant was used for PCR amplification.

RESULTS AND DISCUSSION

Phenotypic characterization of putative *C. dubliniensis* isolates. Clinical specimens from five separate hospitalized Israeli patients yielded yeast colonies which were dark blue-green in color on CHROMagar medium. These were all found to produce germ tubes in normal human serum, to form abundant chlamydoconidia following growth on corn meal agar, and to grow in the presence of cycloheximide on Mycosel agar. Based on these findings and in accordance with previous studies (5, 22, 25) these isolates were presumptively identified as *C. dubliniensis*. In order to confirm this identification, all five isolates were then subjected to substrate assimilation profile analysis with the API ID 32C and 20C AUX yeast identification systems. The profiles of four of the five corresponded to excellent identification of *C. dubliniensis* (Table 1). The profile of one

isolate corresponded to good identification of *C. albicans*. Since *C. dubliniensis* was only first described as a new species in 1995, it has only recently been added to the API 20C AUX and the API ID 32C databases. The results of a recent study show that these systems have excellent potential as a means of identifying this yeast but that database modifications are required to avoid its misidentification as *C. albicans* or unidentified results (20). The largest discrepancy observed was the positive trehalose assimilation results found with 15 and 30% of the 80 *C. dubliniensis* isolates tested with the API 20C AUX and the API ID 32C systems, respectively. In that study the authors concluded that it is reasonable to assume that incorporation of this variability in a future database would correct this problem (20). Indeed, if isolate P-7073 had not assimilated trehalose, it would have been identified as *C. dubliniensis* with the API 20C AUX system with the current database.

All five presumptive Israeli *C. dubliniensis* isolates and the type strain grew on SGA at 37 and 42°C, but none grew at 45°C. In contrast, isolates and reference strains of *C. albicans* grew well at all temperatures. These findings provided supporting evidence that the five Israeli isolates were *C. dubliniensis*. Previous studies have shown that *C. dubliniensis* isolates do not grow at 45°C and that many grow poorly or not at all at 42°C (21, 25). Furthermore, all five presumptive *C. dubliniensis* isolates and the type strain belonged to *C. albicans* serotype A, as determined by latex agglutination with rabbit antiserum raised against *Candida* antigenic factor no. 6. To date all *C. dubliniensis* isolates tested belong exclusively to *C. albicans* serotype A (22, 23, 25). All of these findings strongly suggested that the five Israeli isolates were *C. dubliniensis*.

To further investigate the identity of the five putative *C. dubliniensis* isolates, blastospores of each were tested by IFA with anti-*C. dubliniensis* serum adsorbed with *C. albicans* blastospores. The adsorbed serum had been shown in previous studies to differentially label *C. dubliniensis* isolates (1). The antiserum reacted with blastospores of all five putative *C. dubliniensis* isolates and the type strain, CD36, but did not label blastospores of reference *C. albicans* isolate 132A. These results confirmed that the five Israeli isolates were *C. dubliniensis*.

All five of the Israeli *C. dubliniensis* isolates were susceptible to antifungal drugs as determined with Etest strips and yielded

FIG. 1. Agarose gel showing ethidium bromide-stained amplicons from PCRs with the *C. dubliniensis*-specific primers DUBF and DUBR (288-bp product) and the fungal universal primers RNAF and RNAR (610-bp product). The amplicons shown in the lanes were obtained from template DNA from yeast strains and isolates as follows: lane 2, *C. dubliniensis* type strain CD36; lane 3, *C. albicans* reference strain 132A, lanes 4 to 8, Israeli *C. dubliniensis* isolates P-6265, P-6785, P-7073, P-7266, and P-7276, respectively. Lane 1, molecular weight size markers (100-bp ladder); lane 9, negative control lacking template DNA.

MICs in the range of 0.004 to 0.047 (amphotericin B), 0.008 to 0.032 (5-fluorocytosine), 0.008 to 1.0 (itraconazole), 0.008 to 0.032 (ketoconazole), and 0.38 to 1.0 $\mu\text{g/ml}$ (fluconazole). These results are in agreement with previous studies, which demonstrated that the majority of *C. dubliniensis* clinical isolates are susceptible to commonly used antifungal drugs (16, 19).

PCR-based identification of *C. dubliniensis* isolates. In order to confirm the definitive identification of the five Israeli *C. dubliniensis* isolates, template DNA from each was subjected to PCR analysis with a set of primers (DUBF and DUBR) complementary to *C. dubliniensis* *ACT1*-associated intron sequences (6). These primers amplify a DNA fragment of 288 bp from *C. dubliniensis*, but do not yield an amplicon from *C. albicans*, *Candida stellatoidea*, or any other *Candida* species. Each PCR mixture also contained the fungal universal primer pair RNAF and RNAR (8), which amplify a product of approximately 610 bp from the fungal large-subunit ribosomal RNA gene and which served as an internal positive amplification control. All five Israeli *C. dubliniensis* isolates and the *C. dubliniensis* type strain, CD36, yielded amplicons of 288 bp and approximately 610 bp (Fig. 1). In contrast, *C. albicans* reference strain 132A yielded an amplicon of approximately 610 bp only (Fig. 1). These findings unequivocally confirmed the results of the phenotypic tests that the five isolates were *C. dubliniensis*.

This study constitutes the first report of the isolation of *C. dubliniensis* in the Middle East and broadens our knowledge of the widespread geographic distribution of this organism (23). Additionally, the results confirm previous findings that *C. dubliniensis* is usually an opportunistic pathogen which is mainly associated with colonization and infection of the oral cavity and upper respiratory tract (5, 22). In three of these cases *C. dubliniensis* appears to have been responsible for infection. Most of the patients had underlying disease, but all were treated with broad-spectrum antibiotics, which may have been a contributory factor in the outgrowth of *C. dubliniensis*. All five isolates of *C. dubliniensis* were susceptible to commonly used antifungal drugs, including fluconazole. This was not surprising since fluconazole resistance has only been reported previously for clinical isolates of *C. dubliniensis* from HIV-infected individuals previously treated with the drug (15, 16, 19). Furthermore, all of these data add to the present limited evidence (14, 16, 18, 25) that infection and colonization with *C. dubliniensis* are not confined to HIV-infected individuals. The use of CHROMagar *Candida* medium (12) as a means of

preemptively identifying *C. dubliniensis* in clinical specimens on primary culture should help, when combined with other phenotypic techniques described in this study, to facilitate the detection and identification of additional cases of *C. dubliniensis* colonization and infection.

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ADDENDUM IN PROOF

Since this article was submitted for publication, an additional six isolates recovered from six separate non-HIV-infected patients have been definitively identified as *C. dubliniensis* in the same hospital in Jerusalem, Israel. Two isolates were recovered from vaginal tract specimens, two were recovered from respiratory tract specimens, and one each was recovered from a wound specimen and a sputum specimen.

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