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Use of Specialised Isolation Media for Recognition and Identification of *Candida dubliniensis* Isolates from HIV-Infected Patients

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During a study of oral rinses of 130 HIV-infected individuals, both typical and atypical *Candida albicans* colonies were isolated from ten patients on a yeast differential medium. Typical *Candida albicans* colonies were light green; atypical colonies were dark green. Both types of colonies were germ tube-positive and produced chlamydospores. However, DNA fingerprinting of the atypical isolates with the Ca3 *Candida albicans*-specific probe showed that they belonged to the recently described species *Candida dubliniensis*. *Candida dubliniensis* colonies could also be differentiated from *Candida albicans* colonies on isolation plates by the absence of fluorescence of colonies on methyl blue-Sabouraud agar under Wood's light. Among other phenotypic characteristics, only the absence of intracellular β-glucosidase activity reliably distinguished *Candida albicans* from *Candida dubliniensis*. *Candida dubliniensis* may be underreported in clinical samples because most currently used isolation and identification methods fail to recognize this yeast.

Oropharyngeal *Candida* infections are the most common opportunistic diseases in HIV-infected individuals, occurring in up to 90% during the course of their infection (1, 2). The majority of these infections are caused by *Candida albicans*, but other *Candida* spp., including *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, and *Candida glabrata*, are emerging as significant opportunistic pathogens (3, 4).

Recently, *Candida albicans* strains with abnormal characteristics have been isolated from HIV-infected individuals and AIDS patients (5–9). These isolates produced germ tubes and chlamydospores characteristic of *Candida albicans* but were considered atypical because of their unusual sugar assimilation patterns (5, 7–9) and their inability to grow at 42°C (9). Several molecular typing methods have been applied to these atypical yeasts. Southern blot hybridisation with the Ca3 (5, 7) and 27A oligonucleotide probes (6, 8, 9) specific for *Candida albicans* recognised the atypical isolates by their weak hybridisation reactions. Multilocus enzyme electrophoresis also revealed genetic differences between these yeasts and typical *Candida albicans* strains (5). Sullivan et al. (8, 9) described isolates resembling *Candida albicans* but with a distinctive atypical genomic organisation on the basis of oligonucleotide fingerprinting (8), random amplified polymorphic DNA analysis (8), karyotype analysis by pulsed-field gel electrophoresis (9), and rRNA gene nucleotide sequence analysis (9). They classified these strains as a new species, *Candida dubliniensis*, and deposited the type strain in the British National Collection of Pathogenic Fungi (NCPF) (Bristol, UK). This species differs phenotypically from *Candida albicans* by its production of abundant chlamydospores and its failure to grow at 42°C (9). However, phenotypic differentiation of *Candida dubliniensis* from *Candida albicans* appears to be more difficult and less dependable than differentiation on the basis of DNA fingerprinting with the Ca3 or 27A probe (5, 7, 8).

CHROMagar Candida medium is a novel differential culture medium that facilitates the isolation and presumptive identification of *Candida* spp. (10). Recently, we encountered several yeast colonies that produced unusually dark green-

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coloured colonies on CHROMagar Candida medium as compared with the expected light green colour of *Candida albicans* colonies. Further investigation showed the dark colonies to be *Candida dubliniensis*. The phenotypic and genotypic properties of these isolates are described.

Materials and Methods

Reference Strains. Candida albicans reference strain 3153A was kindly supplied by D.R. Soll, University of Iowa, Iowa City, Iowa, USA; *Candida dubliniensis*, type strain CD36, was obtained from the NCPF by courtesy of Dr. C.K. Campbell. Two oral isolates, 88/029 and 89/014, recovered in 1988 and 1989 from HIV-positive individuals in the UK and thus epidemiologically unrelated to the study isolates were also included. In a previous study these isolates were found to be "atypical *Candida albicans*" strains because of their weak hybridisation with the *Candida albicans*-specific Ca3 probe (7).

Specimen Collection, Culture, and Identification Tests. Between December 1994 and September 1995, 35 HIV-infected individuals and 95 AIDS patients attending the outpatient clinic of the Institute of Tropical Medicine, Antwerp, or hospitalised in the University Hospital of Antwerp were included in an epidemiologic survey after they gave verbal consent. Each patient rinsed his or her mouth with 10 ml of sterile saline and returned the mouthwash sample to a sterile container. CHROMagar Candida (CAC; CHROMagar, France) plates and Sabouraud agar (Difco, USA) with 0.01% methyl blue (11) were surface inoculated with 100 µl of the samples and incubated at 37°C in ambient air for 48 h.

All yeast colonies with a different colour on CAC plates were identified to the species level. *Candida albicans* isolates were identified by germ tube formation in fetal calf serum after 3 h at 37°C and chlamydospore production on 1% rice-cream-1% Tween 80 agar after 48 h at 25°C. Germ tube- and chlamydospore-negative yeasts and those producing atypically dark green colonies on CAC medium were tested for assimilation patterns with the API ID32C yeast identification system (bioMérieux, France). One colony of each atypical isolate previously grown on CAC medium was subcultured on the same medium and incubated for 48 h at 37°C and 42°C. Light green colonies growing on these plates were also tested with API ID32C strips. Yeasts were identified by reference to the API APILAB database.

For β-glucosidase activity, yeast cells were inoculated into 5 ml of brain heart infusion broth (Difco) and incubated overnight at 37°C with gyratory shaking at 100 × *g*. One ml of these cultures was centrifuged for 2 min at 16,000 × *g* in a microcentrifuge; the cells were resuspended in 100 µl of 0.1 M sodium acetate, pH 5.5, containing 0.1% methylumbelliferyl-D-glucoside (Sigma, USA). Glass beads (0.4 g, 0.5 mm in diameter) were added, followed by mixing twice on a bench vortex mixer for 30 sec and centrifugation for 2 min at 16,000 × *g* in a microcentrifuge. The supernatants were transferred to the wells of a microdilution plate, allowed to stand for 15 min at room temperature, and examined on a UV transilluminator at 302 nm. Strains positive for β-glucosidase produced a bright fluorescence (5).

Serotyping. Candida isolates forming light or dark green colonies on CAC were mixed on glass slides with a commercially available serum raised against *Candida* antigenic factor no. 6 (Iatron Laboratories, Japan). Agglutinating isolates were classified as serotype A, nonagglutinating isolates as serotype B.

DNA Fingerprinting. DNA was prepared by the method of Scherer and Stevens (12) and treated with *Eco*RI as described by Schmid et al. (13). Fingerprinting with the moderately repetitive sequence Ca3 (a gift from D.R. Soll) was performed according to the method of Schmid et al. (13) and Soll et al. (14, 15). Restriction fragments were separated by electrophoresis in a 0.8% horizontal agarose gel overnight at 30 V and transferred to nitrocellulose membranes by vacuum blotting. The Southern blots were then hybridised with a digoxigenin-labelled Ca3 probe (16), and bands to which the probe bound were revealed by a coupled alkaline phosphatase reaction. Ca3 hybridisation patterns were analysed by means of Dendron software version 2.1 (13).

Blots were digitised into the Dendron data file with an Epson Scan/Mac GT-6000 flatbed scanner (Epson, Germany). Linear and nonlinear distortions in gel images were removed, when necessary, by the "unwarping" option of Dendron. After the gel image was processed, the lanes were automatically identified and scanned, and bands were automatically identified and assigned an intensity class from 0 (no bands) to 3 (highest intensity).

To compare lanes not in sequence in the same gel or on different gels, the neighbouring function of the Dendron software was used. Gels were normalised to a global standard in the Dendron database. Nonadjacent lanes of the same gel or lanes from different gels were then windowed and juxtaposed.

Results

Detection of Atypical Candida albicans Isolates. The oral rinses from ten of the 130 patients produced both highly distinctive dark green colonies on CAC medium and typical light green *Candida albicans* colonies. From five of these ten patients, mixtures of both light green and dark green colonies together with other *Candida* spp. were isolated, with the latter all appearing as pink-coloured colonies. The dark green colonies represented between 1.4 and 71.2% of all colonies isolated (median, 16%). They outnumbered other colonies on only one of the ten isolation plates. From each isolation plate, pairs of dark and light green-coloured colonies were subcultured separately for further study. After subculture at 37°C for 48 h on CAC medium, the atypical colonies lost their dark green colour and formed light green colonies typical of *Candida albicans*. On receipt, the *Candida dubliniensis* type strain initially produced dark green colonies on CAC medium. However, after storage at -70°C, the *Candida dubliniensis* type strain and the two atypical oral iso-

lates (88/029 and 89/014) produced only light green colonies.

On methyl blue-Sabouraud agar, *Candida albicans* can be distinguished from other yeasts by its yellow fluorescence under a Wood's lamp (11). On this agar the ten isolates producing dark green colonies, isolates 88/029 and 89/014, and the *Candida dubliniensis* type strain were macroscopically indistinguishable from *Candida albicans* colonies, but they did not fluoresce, whereas the paired light-green colonies fluoresced brightly.

DNA Fingerprinting. DNA fingerprints were obtained from the ten dark green oral isolates, the two atypical *Candida albicans* 88/029 and 89/014 isolates, the ten paired light green isolates, the *Candida dubliniensis* type strain CD36, and the *Candida albicans* reference strain 3153A.

The Ca3 probe hybridised efficiently with the *Eco*RI-digested DNA extracted from the *Candida albicans* reference strain and the ten light green isolates, yielding 15 to 25 bands ranging in size from approximately 2.25 kb to 7.9 kb (Figure 1). The same probe bound poorly to DNA purified from the ten dark green isolates, the two atypical oral isolates 88/029 and 89/014, and the *Candida dubliniensis* type strain, yielding hybridisation profiles containing maximally four weak bands ranging in size from 6.5 to 7.9 kb and one or two bands heavier than 7.9 kb (Figure 1).

The DNA fingerprinting patterns suggested to a high degree of probability that the isolates forming dark green colonies and the two older isolates matched the pattern of the *Candida dubliniensis* type strain. We now refer to these isolates as *Candida dubliniensis*.

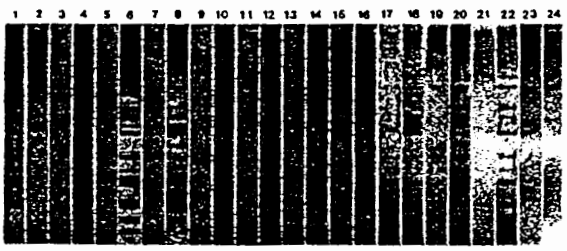

Figure 1: DNA fingerprints of yeasts from dark green and light green colonies on CHROMagar Candida. DNA digested with *Eco*RI was electrophoresed and Southern blots probed with the oligonucleotide Ca3. The blots were scanned, introduced into a computer, and analysed with Dendron software. The gel illustrated was reconstructed by the software from the details of band patterns obtained in the analysis. Lane 1, *Candida albicans* 3153A reference strain; lane 2, *Candida dubliniensis* type strain CD36. Lanes 3–22 show, side by side, the patterns from dark green (odd-numbered lanes) and light green (even-numbered) colonies from each of ten HIV-positive patients. Lane 23, strain 88/029; lane 24, strain 89/014.

Phenotypic Characterisation of the Isolates. The ten light green isolates produced germ tubes in serum and chlamydospores on 1% rice-cream-1% Tween 80 agar. Their assimilation profiles obtained with the API ID32C confirmed their identification as *Candida albicans*. All ten *Candida albicans* strains grew at 42°C; six agglutinated with Iatron factor 6 antiserum and were classified as serotype A, and four were classified as serotype B.

The 12 *Candida dubliniensis* isolates and the *Candida dubliniensis* type strain produced germ tubes and chlamydospores, although the latter did not produce them in unusual abundance. The *Candida dubliniensis* type strain CD36 and eight of the 12 *Candida dubliniensis* isolates grew at 42°C, but the remaining four isolates did not. All of the *Candida dubliniensis* isolates and the type strain agglutinated with Iatron factor 6 antiserum and were classified as serotype A.

The assimilation profiles of the 12 *Candida dubliniensis* isolates and the *Candida dubliniensis* isolate differed in duplicate tests with two batches of API ID32C test kits. In the first tests, five of the 12 isolates and the *Candida dubliniensis* type strain produced assimilation profiles typical for *Candida albicans*, three gave a possible identification of *Candida sake*, and four gave an inconclusive result for identification with the API APILAB database. The *Candida dubliniensis* type strain and three of the new isolates assimilated α-methyl-D-glucoside, and five of the new isolates (but not the type strain) assimilated DL-lactate. These positive reactions are in contrast to the uniformly negative assimilation of these substrates reported previously (9). These assimilation patterns appeared to be unstable, however, since the 12 *Candida dubliniensis* isolates and the *Candida dubliniensis* type strain all produced an identical carbohydrate assimilation profile upon retesting with a new lot of API ID32C strips. This profile did not correspond with any known *Candida* spp. in the API APILAB database.

The ten *Candida albicans* isolates producing light green colonies on the primary isolation plates as well as the *Candida albicans* reference strain 3153A were β-glucosidase positive. The ten originally dark green isolates, strains 88/029 and 89/014, and the *Candida dubliniensis* type strain were β-glucosidase negative.

Discussion

According to the original description (9), the characteristics of the new *Candida dubliniensis* species closely resemble those of *Candida albicans*. The main differences are the weak reaction of its DNA with the oligonucleotide probe Ca3 specific for *Candida albicans*, its failure to grow at 42°C, and the production of unusually abundant, multiple chlamydospores in triplets or contiguous pairs (9). None of these characteristics allows a rapid and easy specific recognition of *Candida dubliniensis*; it is therefore quite possible that many isolates of this species have been misidentified as *Candida albicans*. Today, most diagnostic clinical laboratories use the following approach for yeast identification: first, recognition of *Candida albicans* on the basis of a single characteristic (rapid production of germ tubes in serum), followed by other morphologic and physiologic tests only with germ tube-negative isolates. This strategy ignores *Candida dubliniensis*, since this species produces germ tubes that cannot be differentiated from those of *Candida albicans*.

The DNA from the unusually dark green colonies on CAC described in the present study reacted faintly or not at all with the oligonucleotide Ca3 probe, a property characterising *Candida dubliniensis* with a very high degree of probability (9). Normally, green colonies on CAC medium are *Candida albicans* (10), but the colonies studied here produced a green colour noticeably darker than the usual one. On methyl blue-Sabouraud agar, *Candida dubliniensis*, in contrast to *Candida albicans*, formed colonies that did not fluoresce under Wood's light (11). Either or both of these properties in a yeast isolate should therefore alert investigators to the possible presence of *Candida dubliniensis*.

It must be emphasised that the differential character of the dark green colonies formed on CHROMagar Candida can be reliably seen only after two days of incubation at 37°C: the colour of *Candida albicans* colonies also tends to darken upon longer incubation. Neither the dark green nor the nonfluorescent colony properties were retained in subcultures of *Candida dubliniensis*: these properties allow recognition of the species only at the point of primary isolation. We have no explanation for the loss of these distinctive properties. The ability to recognise *Candida dubliniensis* in primary culture and to confirm its identity by DNA fingerprinting and a negative β-glucosidase test was particularly convincing, since the fingerprints of light green colonies developing on the same plates as *Candida dubliniensis* showed the multiple band patterns typical of *Candida albicans* (Figure 1) and were positive for β-glucosidase.

It is remarkable that *Candida dubliniensis* did not appear even once in pure culture. The earliest descriptions of yeasts with a *Candida albicans* morphologic phenotype but virtually no reaction with the oligonucleotide probe Ca3 in Southern blots were for yeasts isolated from AIDS patients in Leicester, UK (7). These isolates were randomly chosen colonies from Sabouraud agar isolation plates. They showed the unusual genotype and were repeatedly isolated from the same two patients (7). No *Candida albicans* isolate was ever recovered from either patient. It seems unlikely that such repeated isolation of *Candida dubliniensis* from the same patient could have occurred if the species always grew in association with *Candida albicans*, as in the present study.

The phenotypic characterisation of *Candida dubliniensis* is controversial. Our investigations failed to confirm some of the distinguishing properties of the species set forth in the original description (9). We saw no unusually abundant or triplet chlamydospore formation, and several *Candida dubliniensis* isolates, including the type strain, grew at 42°C. We confirmed the accuracy of our incubator set to this temperature by placing a mercury thermometer inside as well as by relying on the reading of the electronic thermometer built into the apparatus. The positive reaction of the *Candida dubliniensis* isolates with antiserum recognising *Candida albicans* type A confirms the findings in the original description (9).

Like other investigators (5, 9), we found that our *Candida dubliniensis* isolates produced inconsistent and often uninterpretable assimilation patterns with the API ID32C yeast identification gallery. Moreover, the results of duplicate tests with these isolates suggest that the physiological phenotype of the species is unstable. In contrast, the *Candida dubliniensis* isolates, including the type strain, were always negative for β-glucosidase, whereas *Candida albicans* was positive. These findings agree with those of Boerlin et al. (5) with their atypical *Candida albicans* isolates. We consider the absence of β-glucosidase activity a more consistent phenotypic characteristic of *Candida dubliniensis* than the other properties formally attributed to the species (9).

All the yeast isolates reported thus far as "atypical *Candida albicans*" or identified as *Candida dubliniensis* have been cultured from the mouths of HIV-positive patients in Europe (5–9). We are aware of many other anecdotal reports of such yeasts from other European, but not North American, HIV-infected individuals. No clinical factor appeared to distinguish the patients from whom *Candida dubliniensis* was cultured in the present study: the presence of *Candida dubliniensis* was not correlated with prior antifungal treatment, clinical symptoms, CD4+ cell count, sex, sexual orientation, or intravenous drug abuse. Others have noted an association between *Candida dubliniensis* and intravenous drug use (5). It is not yet known whether *Candida dubliniensis* can colonise the oral cavity in healthy individuals or whether it is associated with anatomical sites other than the mouth. The approaches we have described should favour the recognition of this previously unknown yeast and improve the study of its clinical importance.

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