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-
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 NCDF 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 agar after 48 h at 25°C. Germ tube- and chlamydospore-negative yeasts and those producing atypically dark green colonies on CAC medium as compared with the expected light green colour of Candida albicans colonies were tested for assimilation patterns with the API ID32C yeast identification system (bioMerieux, 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 362 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 (Jatrom 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 EcoRI 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-