Antifungal Drug Susceptibilities of Oral *Candida dubliniensis* Isolates from Human Immunodeficiency Virus (HIV)-Infected and Non-HIV-Infected Subjects and Generation of Stable Fluconazole-Resistant Derivatives In Vitro

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Candida dubliniensis is a recently described species of *Candida* associated with oral candidiasis in human immunodeficiency virus (HIV)-infected individuals. Nineteen oral isolates of *C. dubliniensis* recovered from 10 HIV-positive and 4 HIV-negative individuals and one vaginal isolate from an additional HIV-negative subject were assessed for fluconazole susceptibility by broth microdilution (BMD), hyphal elongation assessment, and Etest. The susceptibilities of these 20 isolates to itraconazole and amphotericin B and of 10 isolates to ketoconazole were also determined by BMD only. Sixteen of the *C. dubliniensis* isolates were susceptible to fluconazole (MIC range, 0.125 to 1.0 μg ml^{-1}), and four (recovered from two AIDS patients) were fluconazole resistant (MIC range, 8 to 32 μg ml^{-1}). Fluconazole susceptibility data obtained by hyphal elongation assessment correlated well with results obtained by BMD, but the corresponding Etest MIC results were one to four times higher. All of the isolates tested were found to be sensitive to itraconazole, ketoconazole, and amphotericin B. Sequential exposure of two fluconazole-sensitive (MIC, 0.5 μg ml^{-1}) *C. dubliniensis* isolates to increasing concentrations of fluconazole in agar medium resulted in the recovery of derivatives which expressed a stable fluconazole-resistant phenotype (BMD-determined MIC range, 16 to 64 μg ml^{-1}), even after a minimum of 10 consecutive subcultures on drug-free medium and following prolonged storage at -70°C. The clonal relationship between the parental isolates and their respective fluconazole-resistant derivatives was confirmed by genomic DNA fingerprinting and karyotype analysis. The results of this study demonstrate that *C. dubliniensis* is inherently susceptible to commonly used antifungal drugs, that fluconazole resistance does occur in clinical isolates, and that stable fluconazole resistance can be readily induced in vitro following exposure to the drug.

Recent studies have shown that during the 1980s there was a significant increase in the diagnosis of opportunistic and systemic *Candida* infections (5, 14). This was particularly evident in human immunodeficiency virus (HIV)-infected and AIDS patients (3, 8). Although *Candida albicans* is by far the most common cause of candidal infection, the incidence of candidiasis caused by other species, such as *C. glabrata*, *C. tropicalis*, and *C. krusei*, has also increased (15, 16). These latter species tend to be less susceptible to commonly used antifungal agents, such as fluconazole, and it has been suggested that this might account for their emergence as significant pathogens (1, 9, 29).

Recently, Sullivan et al. (21, 24) described a group of atypical *Candida* isolates from the oral cavities of HIV-infected and AIDS patients, many of whom had a history of recurrent oral candidiasis and had received fluconazole therapy. These organisms were shown to constitute a new species of *Candida* for which the name *Candida dubliniensis* was proposed. *C. dubliniensis* isolates are phenotypically similar to *C. albicans* as they are germ tube positive, produce chlamydospores, and can be easily misidentified at the time of primary isolation. How-

ever, in contrast to *C. albicans*, *C. dubliniensis* isolates hyperproduce chlamydospores, which are often arranged in contiguous pairs, triplets, and other multiples attached to a single suspensor cell. They belong exclusively to *C. albicans* serotype A and give substrate assimilation profiles with the API ID 32C yeast identification system which do not correspond to any known species. The genomic organization of these isolates has also been shown to be unique when compared with those of *C. albicans* and *C. stellatoidea* by DNA fingerprint analysis with the *C. albicans* mid-repeat sequence probe 27A and a number of oligonucleotide probes homologous to eukaryotic microsatellite repeat sequences. Randomly amplified polymorphic DNA (RAPD) analysis and karyotype analysis further confirmed the distinct genetic nature of these organisms. Furthermore, phylogenetic studies involving nucleotide sequence analysis of 500 bp of the V3 variable region of the large ribosomal subunit genes of nine epidemiologically unrelated *C. dubliniensis* isolates and the corresponding sequences from *C. albicans*, type I *C. stellatoidea*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. kefyr*, *C. krusei*, and *Aspergillus fumigatus* revealed that *C. dubliniensis* isolates form a homogeneous cluster representing a distinct taxon within the genus *Candida*, clearly separated from the other species tested (24).

C. dubliniensis has been recovered from a significant proportion of the HIV-infected Irish population (23). Recently, several other papers have described the isolation of atypical oral

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TABLE 1. Fluconazole susceptibility of oral *C. dubliniensis* isolates and reference *Candida* strains as determined by BMD, HEA, and Etest[a]

Strain or isolate[b]	Source and/or comments[c]	Previous fluconazole therapy[d]	Fluconazole MIC			Reference
			BMD	HEA	Etest	
C. albicans						
132A	Oral reference strain	NA[e]	0.25	0.5	1	7
Y0.109	Fluconazole susceptibility testing reference strain	NA	0.25	0.5	2	7
C. dubliniensis						
CD36[f]	Type strain	NA	0.5	0.5	1.5	24
CM1	Patient 1; homosexual with AIDS [+]	Yes	0.5	0.5	3	24
CM2 (18)	Patient 1; homosexual with AIDS [−]	Yes	32	32	32	24
CD48-I	Patient 2; homosexual with AIDS [+]	Yes	0.5	0.5	3	This study
CD48-II (6)	Patient 2; homosexual with AIDS [+]	Yes	0.5	0.5	4	This study
CD42	Patient 3; homosexual with AIDS [+]	Yes	1	0.5	4	This study
CD47-I	Patient 4; IVDU with AIDS [+]	Yes	16	8	256[h] (24)	This study
CD47-IIa[g] (6)	Patient 4; IVDU with AIDS [+]	Yes	8	8	128[h] (12)	This study
CD47-IIb[g] (6)	Patient 4; IVDU with AIDS [+]	Yes	16	32	24	This study
CD46	Patient 5; HIV[+] IVDU [+]	No	0.5	0.5	4	This study
CD65	Patient 6; HIV[+] IVDU [−]	No	≤0.125	0.5	0.75	This study
CD54	Patient 7; hemophiliac with AIDS [+]	No	0.25	0.25	1	This study
CD51-I	Patient 8; HIV[+] hemophiliac [+]	Yes	0.5	0.5	6	This study
CD51-II (3)	Patient 8; HIV[+] hemophiliac [−]	Yes	0.25	0.5	2	This study
CD53	Patient 9; HIV[+] hemophiliac [−]	No	≤0.125	≤0.125	0.75	This study
CD55	Patient 10; HIV[+] hemophiliac [+]	No	0.5	0.5	1.5	This study
CD66	Patient 11; HIV[−] IVDU [−]	No	≤0.125	0.5	1.5	This study
CD67	Patient 12; HIV[−] IVDU [−]	No	≤0.125	0.5	1.5	This study
CD68	Patient 13; HIV[−] IVDU [−]	No	0.25	0.5	3	This study
CD41	Patient 14; HIV[−] healthy carrier [−]	No	0.25	0.5	4	24
CD57	Patient 15; HIV[−] vaginitis patient {+}	No	0.5	0.5	3	This study

[a] The *C. dubliniensis* isolates were recovered from the oral cavity, except for CD57, which was recovered from a high vaginal swab of a patient who presented with vaginal candidiasis.

[b] The notations I and II refer to isolates recovered from the same patient during each of two consecutive episodes of oral candidiasis. The time period elapsed (in months) on recovery of recurrent isolates is shown in parentheses.

[c] [+], patient presented with clinical symptoms of oral candidiasis; [−], patient presented without clinical symptoms of oral candidiasis; {+}, patient presented with clinical symptoms of vaginal candidiasis; IVDU, intravenous drug user; HIV[+], HIV positive; HIV[−], HIV negative.

[d] Indicates patients who received fluconazole therapy within a 3- to 18-month period prior to the recovery of the *C. dubliniensis* isolates listed in column 1 (see Materials and Methods).

[e] NA, not applicable.

[f] The *C. dubliniensis* type strain CD36 has been lodged with the British National Collection of Pathogenic Fungi under accession number NCPF 3949 and with the Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands, under accession number CBS 7987.

[g] These isolates were recovered from the same clinical specimen on primary isolation media but could be differentiated on the basis of colony morphology. CD47-IIa colonies were smooth and white on PDA, whereas colonies of CD47-IIb had a wrinkled appearance on PDA.

[h] End points were determined following 48 h of incubation because of poor growth of isolates CD47-I and CD47-IIa on Casitone agar. Both isolates grew well on RPMI agar, and end points determined after 24 h of growth on this medium are shown in parenthesis.

Candida isolates from HIV-infected and AIDS patients in disparate geographic locations (2, 11, 12), representative isolates of which have been identified as *C. dubliniensis* by this laboratory (2a, 24). Many of the HIV-infected individuals colonized by this organism have a history of recurrent oral candidiasis and have received protracted therapy with the antifungal drug fluconazole (21). The purpose of the present study was to determine the susceptibilities to fluconazole and other antifungal drugs of a selection of clinical isolates of *C. dubliniensis* from the oral cavities of HIV-infected and non-HIV-infected individuals. A single vaginal isolate from a non-HIV-infected individual was included for comparison. In the case of fluconazole, results obtained with three separate susceptibility testing techniques, including broth microdilution (BMD), hyphal elongation assessment (HEA), and fluconazole Etest, were compared. Experiments were also performed to determine whether *C. dubliniensis* could develop stable resistance to fluconazole in vitro following prolonged exposure to this antifungal drug.

MATERIALS AND METHODS

Candida strains and clinical isolates. Twenty clinical isolates of *C. dubliniensis* were included in the study. Thirteen were oral isolates recovered from nine Irish HIV-infected and AIDS patients (patients 2 to 10 [Table 1]) of the Dublin Dental Hospital or the Hemophilia Centre at St. James's Hospital, Dublin, between January 1994 and January 1996. Three were oral isolates recovered from separate HIV-negative intravenous drug users (patients 11 to 13 [Table 1]) who were patients at the Dublin Dental Hospital in January 1996. One isolate was recovered from a high vaginal swab from a patient (patient 15 [Table 1]) with clinical symptoms of *Candida* vaginitis at the Department of Genitourinary Medicine, St. James's Hospital, Dublin, in 1992. One isolate was recovered from the oral cavity of a healthy HIV-negative Irish subject (patient 14 [Table 1]) in March 1994 and has been described previously (24). The two remaining oral isolates were recovered 18 months apart from a single AIDS patient (patient 1 [Table 1]) at the Fairfield Hospital, Melbourne, Australia, between August 1990 and December 1991 (11, 12). Reference strains of *C. albicans* and *C. dubliniensis* used in the study are listed in Table 1. None of the HIV-negative subjects included in the study presented with clinical symptoms of oral candidiasis at the time of sampling. In contrast, seven of the nine Irish HIV-infected and AIDS patients included in the study had clinical symptoms of oral candidiasis at the time of initial sampling (Table 1). Of the three of these who were sampled on a second occasion, two (patients 2 and 4 [Table 1]) had clinical symptoms of oral candidiasis. Five of the HIV-infected patients received fluconazole therapy within a 3- to 18-month period prior to the recovery of oral *C. dubliniensis* (Table 1). Of these, patients 2, 3, 4, and 8 received between one and three courses of fluconazole (50 to 100 mg orally per day for a week). Patient 1 had been enrolled as part of a concurrent clinical trial and had received at least one course of fluconazole therapy (100 mg per day for a week, followed by maintenance therapy of 50 mg per day for approximately 2 months) (12).

Except for patients 1 and 15, samples were recovered from the mid-dorsum of the tongue with sterile swabs which were processed as described previously (21).

The recovery of oral samples from patient 1 by an oral rinse technique has been described in a recent Australian paper (12). A high vaginal swab was taken from patient 15 and processed in the same manner as the oral swab specimens. *Candida* isolates were routinely cultured on potato dextrose agar (PDA) medium (Oxoid) at 37°C for 48 h as described previously (3, 21). Colonies of different *Candida* species were distinguished on the basis of colony morphology on PDA primary isolation plates, the relative abundance of each colony morphology type was recorded, and representatives were purified for formal identification (3). All of the Irish HIV-infected and AIDS patients who presented with clinical signs of oral candidiasis yielded greater than 300 CFU of semiconfluent growth of yeasts upon primary isolation on PDA medium. The HIV-negative subjects included in the study and the Irish HIV-infected and AIDS patients who presented without clinical signs of oral candidiasis yielded between 1 and 48 CFU upon primary isolation on PDA medium, except for patient 8, who yielded 100 CFU at the second sampling (Table 1, column 3). Data on the relative abundance of yeasts recovered from patient 1 (an Australian patient [12]) and 15 (an Irish vaginitis patient [this study]) were not available.

Candida isolates were identified on the basis of their ability or inability to produce germ tubes and chlamydospores and on their substrate assimilation profiles obtained with the API ID 32C yeast identification system (bioMérieux, Marcy l'Etoile, France) as described previously (21, 24). The identities of *C. dubliniensis* isolates were confirmed by their ability to produce abundant chlamydospores on rice-agar-Tween medium (RAT medium; bioMérieux), by their poor growth rate at 42°C, and, in the case of 14 of the 20 clinical *C. dubliniensis* isolates which were tested in the study, by their characteristic DNA fingerprints following hybridization with the *C. albicans* mid-repeat sequence probe 27A, all as described previously by Sullivan et al. (24).

C. dubliniensis either was the only *Candida* species or was the predominant *Candida* species recovered from the Irish HIV-infected and AIDS patients as shown by the following data (the percent relative abundance of each species recovered in each case is shown in parentheses): patient 2, *C. dubliniensis* (100%, primary and secondary samples); patient 3, *C. dubliniensis* and *C. albicans* (70 and 30%, respectively); patient 4, *C. dubliniensis* and *C. albicans* (95 and 5%, respectively, at primary sampling) and *C. dubliniensis* only (100%, at secondary sampling); patient 5, *C. dubliniensis* (100%); patient 6, *C. dubliniensis*, *C. albicans*, and *C. tropicalis* (66, 24, and 6%, respectively); patient 7, *C. dubliniensis* (100%); patient 8, *C. dubliniensis* (100%, primary and secondary samples); patient 9, *C. dubliniensis* (100%); and patient 10, *C. dubliniensis* (100%). Data on the relative abundance of *C. dubliniensis* and other *Candida* species (if any) from patient 1 (an Australian AIDS patient) at the time of sampling were not available. *C. dubliniensis* was the only *Candida* species recovered from three of the five HIV-negative subjects included in the study (patients 12, 13, and 15 [Table 1, column 2]), and it was recovered in a mixed culture with other *Candida* species from the remaining two subjects as follows (the percent relative abundance of each species recovered in each case is shown in parentheses): patient 11, *C. dubliniensis*, *C. tropicalis*, and *C. albicans* (16, 82, and 2%, respectively); and patient 14, *C. dubliniensis* and *C. albicans* (5 and 95%, respectively).

Antifungal drugs. Fluconazole powder (a gift from Pfizer Central Research, Sandwich, Kent, United Kingdom) was dissolved in 10% (vol/vol) dimethyl sulfoxide at a concentration of 5 mg ml^{-1}. Ketoconazole, itraconazole (both gifts from Janssen Pharmaceutical, Cork, Republic of Ireland), and amphotericin B (E. R. Squibb, Swords, Republic of Ireland) were dissolved in 100% (vol/vol) dimethyl sulfoxide, with the aid of heating to 70°C, at 5 mg ml^{-1}. All drugs were divided into aliquots and stored at −70°C.

Susceptibility testing procedures. (i) BMD. Microdilution susceptibility testing with fluconazole was carried out by the method of Rodriguez-Tudela and Martinez-Suárez (18), which is a modification of the broth macrodilution method outlined in the National Committee for Clinical Laboratory Standards document M-27P (13). The medium (RPMI–2% glucose) consisted of RPMI 1640 medium supplemented with 18 g of glucose liter^{-1}, buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma) and adjusted to pH 7. Doubling dilutions of the stock antifungal drug were prepared in RPMI–2% glucose medium, dispensed into 96-well microtiter plates (Corning), and inoculated with the test organism as described previously (18). End points were determined spectrophotometrically by measuring the A_{405} with an automated microplate reader (Spectra I; SLT-Labinstruments, Salzburg, Austria). An end point (IC$_{50}$), termed the MIC, was determined by the method of Galgiani and Stevens (6) as the lowest fluconazole concentration which fulfilled the criterion percent $T \geq$ percent T_k + 0.5(100 − percent T_k), where T is the transmission from a microtiter plate well with medium containing fluconazole and the organism being tested and T_k is the transmission from a microtiter plate well containing drug-free medium and the organism being tested. The IC$_{50}$ represents the drug concentration that inhibited growth by 50%, as determined by transmission, compared with growth of controls, as described by Rodriguez-Tudela and Martinez-Suárez (18). All tests were performed in duplicate on two separate occasions. All isolates were tested additionally for susceptibility to itraconazole and amphotericin B by BMD, and 10 isolates were tested for susceptibility to ketoconazole by BMD. Ketoconazole and itraconazole susceptibility tests were carried out in essentially the same manner as those described for fluconazole. Amphotericin B susceptibility testing was carried out in a similar fashion with Antibiotic Medium 3 (Difco Laboratories, Detroit, Mich.), at pH 7, as described by Rex et al. (17).

(ii) HEA. HEA for fluconazole susceptibility testing of clinical isolates of *C. dubliniensis* was carried out according to the method of Gallagher et al. (7). Briefly, to 9-cm-diameter petri dishes containing 19.8 ml of the hypha-promoting *N*-acetylglucosamine–yeast nitrogen base–proline (NYP) medium were added doubling dilutions of fluconazole ranging in concentration from 0.125 to 32 µg ml^{-1}. Drug-free medium was used as a growth control in all experiments. A 0.2-ml aliquot of a suspension containing 10^6 CFU of each isolate ml^{-1} was used to inoculate each plate to a final cell density of 10^4 CFU ml^{-1}. Plates were incubated at 37°C with 5% (vol/vol) CO$_2$ in a humidified atmosphere for 24 h to promote hyphal growth. Each plate was examined visually to assess hyphal growth. The MIC of fluconazole was then determined spectrophotometrically by measuring the A_{600} after thoroughly aspirating each plate culture to dislodge and disperse any yeast cells adhering to the bases of the petri dishes. An MIC (IC$_{50}$) was then calculated as described above for each organism being tested. All tests were carried out in duplicate on at least two separate occasions.

Etest fluconazole susceptibility testing. The Etest (AB Biodisk; Dalvägen, Solna, Sweden) is based on the use of a plastic strip impregnated with a concentration gradient of the appropriate drug (4). Strips containing fluconazole ranging in concentration from 0.016 to 256 µg ml^{-1} were placed on the surfaces of agar plates on which lawns of the test organism were grown. Casitone medium was used for Etest fluconazole susceptibility testing as recommended by the manufacturer. Plates were incubated at 35°C for 24 h or until sufficient growth was obtained to determine an end point. Following the manufacturer's guidelines, end points were taken as the point of 80% inhibition (MIC). A number of isolates referred to in the text were additionally tested on RPMI agar (10.4 g of RPMI 1640 liter^{-1} plus 18 g of glucose liter^{-1}, buffered with 0.2 M phosphate; pH 7) due to poor growth on Casitone agar.

Exposure of oral isolates of *C. dubliniensis* and *C. albicans* 132A to fluconazole in vitro. One hundred colonies of selected fluconazole-sensitive clinical isolates of *C. dubliniensis* and the oral *C. albicans* reference strain 132A were inoculated onto yeast peptone dextrose (YPD) agar medium (per liter, 10 g of yeast extract [Oxoid], 20 g of peptone (Difco), 20 g of glucose, and 15 g of agar [Difco]; pH 5.5) containing fluconazole at a concentration of 0.5 µg ml^{-1} with sterile wooden toothpicks. Following 48 h of incubation at 37°C, each colony was then aseptically transferred, with sterile toothpicks, to fresh medium, also containing 0.5 µg of fluconazole ml^{-1}, and incubated for a further 48 h at 37°C. Each colony was further subcultured twice on YPD agar containing fluconazole at 1, 5, 10, 25, and 50 µg ml^{-1}. Following each subculture, the plates were incubated for 48 h at 37°C. Colonies which grew on medium containing the highest concentration of fluconazole (50 µg ml^{-1}) were deemed to be putative fluconazole-resistant derivatives, and these were subcultured twice on fluconazole-free PDA medium and incubated on each occasion for 48 h at 37°C. These single-colony derivatives were then assessed for their susceptibility to fluconazole by determining their fluconazole MICs by the microbroth assay in RPMI–2% (wt/vol) glucose. Any derivative which exhibited an elevated MIC was subcultured repeatedly (at least 10 times) on PDA medium without fluconazole, after which MIC determinations were repeated by BMD to evaluate the stability of the resistance phenotype.

Chemicals, enzymes, radioisotopes, and oligonucleotides. Analytical-grade or molecular biology-grade chemicals were purchased from Sigma, BDH (Poole, Dorset, United Kingdom), or Boehringer Mannheim (Lewes, East Sussex, United Kingdom). Enzymes were purchased from the Promega Corporation (Madison, Wis.) or Boehringer Mannheim and used according to the manufacturer's instructions. [α-^{32}P]dATP (3,000 Ci mmol^{-1}; 110 TBq mmol^{-1}) was purchased from Amersham International Plc. (Little Chalfont, Buckinghamshire, United Kingdom).

Isolation of genomic DNA and DNA fingerprinting. Total genomic DNA of *Candida* isolates was prepared from cells grown overnight in YPD broth as described by Gallagher et al. (7). Restriction endonuclease *Eco*RI-digested total genomic DNA was separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with the *C. albicans* dispersed mid-repeat sequence probe 27A (20) by the method of Sullivan et al. (24).

RAPD analysis. RAPD analysis was carried out on *C. dubliniensis* genomic DNA samples with the oligonucleotide primer 5′-GCGATCCCCA-3′ as described by Sullivan et al. (24). Amplifications were carried out in a Perkin-Elmer Cetus DNA thermal cycler as described previously (24).

Pulsed-field gel electrophoresis. Yeast chromosomes were prepared for electrophoresis in agarose plugs by the method of Vazquez et al. (28) and separated in 1.4% (wt/vol) agarose gels by using the contour-clamped homogeneous electric field CHEF-Mapper pulsed-field gel electrophoresis system (Bio-Rad) as described by Sullivan et al. (24).

RESULTS

Identification of *C. dubliniensis* isolates. All 20 of the *C. dubliniensis* isolates included in this study (Table 1) were identified as described previously by Sullivan et al. (24) on the basis of their ability to produce germ tubes in normal human serum and to produce abundant chlamydospores, often arranged in multiples attached to single suspensor cells, on RAT medium, by their poor growth at 42°C, and by their unusual substrate

assimilation profiles obtained with the API ID 32C yeast identification system. Four isolates yielded the ID 32C assimilation profile code 7143140015, which is identical to the code obtained with the *C. dubliniensis* type strain CD36 (24). Twelve isolates yielded the ID 32C assimilation profile code 7142140015, and four yielded the code 7142100015. None of these three codes corresponded to definite identification of any known *Candida* species in the API APILAB database, and all three codes have been previously reported for oral isolates of *C. dubliniensis* (23, 24). Furthermore, of the 14 of the 20 isolates tested, all 14 yielded DNA fingerprint profiles characteristic of *C. dubliniensis* following hybridization analysis of *Eco*RI-digested genomic DNA with the *C. albicans* fingerprinting probe 27A as described by Sullivan et al. (24) (data not shown).

Fluconazole susceptibility testing. The *C. albicans* reference strains Y0.109 and 132A, the *C. dubliniensis* type strain CD36, and 20 isolates of *C. dubliniensis* recovered from 10 HIV-infected and 5 HIV-negative subjects were assessed for their susceptibilities to fluconazole by BMD, HEA, and Etest. Y0.109, 132A, and CD36 and the vast majority (16 of 20) of the isolates examined exhibited fluconazole-sensitive phenotypes (MIC range, 0.125 to 1.0 μg ml^{-1}) when tested by BMD (Table 1). However, four isolates from two different AIDS patients (CM2 from patient 1; CD47-I, CD47-IIa, and CD47-IIb from patient 4) exhibited significantly higher MIC values (8 to 32 μg ml^{-1}), indicating that these isolates were significantly less susceptible to fluconazole (Table 1). Interestingly, isolates CD47-IIa and CD47-IIb, which were recovered from the same clinical specimen on primary isolation, yielded similar fluconazole MIC values (Table 1). Both isolates were identified as *C. dubliniensis*, but they could be distinguished on the basis of colony morphology; CD47-IIa grew as smooth white colonies on PDA agar, whereas CD47-IIb yielded wrinkled colonies on this medium. Furthermore, the colony phenotypes of both isolates were stable following 10 consecutive subcultures. The fluconazole susceptibility data obtained by BMD was found to be highly reproducible, as all of the isolates yielded fluconazole MIC values within ±1 doubling dilution of the original result upon repeat testing.

All the *C. dubliniensis* isolates studied consistently grew in the pseudohyphal form under the conditions of the HEA procedure. When the isolates tested by BMD were tested independently by HEA, fluconazole MIC values within ±2 doubling dilutions were obtained for each isolate by both methods, despite the differences in the media and growth conditions used (Table 1). The reproducibility of MIC data obtained by HEA was also found to be good, with 16 of 20 isolates tested yielding MIC values ±1 doubling dilution and the remaining 4 isolates yielding MIC values ±2 doubling dilutions in repeat experiments.

All of the isolates tested by BMD and HEA were also tested for fluconazole susceptibility on Casitone agar by the commercially available Etest. Fluconazole MIC results were determined following 24 h of growth, with the exception of isolates CD47-I and CD47-IIa, which grew poorly on this medium and hence were incubated for 48 h to obtain sufficient growth for end point determination. The data obtained allowed the fluconazole-sensitive and fluconazole-resistant *C. dubliniensis* isolates determined by both BMD and HEA to be readily distinguished, although the Etest results were, in general, 1 to 4 doubling dilutions of fluconazole higher. *C. dubliniensis* isolates identified as being fluconazole resistant by BMD and HEA (MIC, ≥8 μg ml^{-1}) all yielded MIC values of ≥24 μg ml^{-1} when assessed by Etest (Table 1). However, the fluconazole MIC results obtained with isolates CD47-I and CD47-

TABLE 2. Antifungal drug susceptibilities of parental *C. dubliniensis* isolates and in vitro-generated fluconazole-resistant derivatives after 10 consecutive subcultures on drug-free medium[a]

Isolate or derivative	Comment	BMD MIC (μg ml^{-1})		
		Fluconazole	Itraconazole	Amphotericin B
CD51-II	Parental isolate	0.25	0.015	0.015
CD51-IIA	Flu^r derivative of CD51-II	16	0.015	0.06
CD51-IIB	Flu^r derivative of CD51-II	64	0.03	0.06
CD51-IIC	Flu^r derivative of CD51-II	64	0.03	0.03
CD57	Parental isolate	0.5	0.12	0.015
CD57A	Flu^r derivative of CD57	16	0.12	0.03
CD57B	Flu^r derivative of CD57	32	0.25	0.03

[a] The data shown are from one set of experiments. Identical results were obtained in duplicate experiments performed on two separate occasions. Results similar to those shown here were obtained following storage of the isolates and derivatives at −70°C for a 10-week period. Flu^r, fluconazole resistant.

IIa, (128 and 256 μg ml^{-1}, respectively) differed significantly from the MIC values of 16 and 8 μg ml^{-1}, respectively, obtained by BMD and from the MIC values of 8 μg ml^{-1} obtained by HEA for each isolate (Table 1). However, when these two isolates were retested by the Etest with RPMI agar instead of Casitone agar, good growth was obtained following 24 h of incubation and MIC values to within ±1 doubling dilution of those obtained by BMD were achieved (Table 1).

Itraconazole, ketoconazole, and amphotericin B susceptibility testing. All of the isolates and reference strains listed in Table 1 were tested for susceptibility to itraconazole and amphotericin B by BMD. Ten of the 20 *C. dubliniensis* isolates (including all of the fluconazole-resistant isolates) and the reference strains were also assessed for susceptibility to ketoconazole by BMD. All of the reference strains and clinical isolates tested were found to be susceptible to itraconazole (MIC range, <0.03 to 0.5 μg ml^{-1}), ketoconazole (MIC range, ≤0.03 to 0.25 μg ml^{-1}), and amphotericin B (MIC range, ≤0.03 to 0.125 μg ml^{-1}). Interestingly, none of the fluconazole-resistant isolates exhibited cross-resistance to itraconazole, ketoconazole, or amphotericin B.

Generation of fluconazole-resistant derivatives in vitro. In order to determine whether stable fluconazole resistance could be induced in *C. dubliniensis* by exposure to the drug, 100 colonies of two separate fluconazole-sensitive isolates (MIC, 0.5 μg ml^{-1}), CD51-II and CD57, were exposed to increasing doses of fluconazole by subculture on fluconazole-containing YPD agar. On initial subculture, at 0.5 μg of fluconazole ml^{-1}, all 100 colonies of each isolate tested grew satisfactorily. Following an additional subculture on YPD agar containing 0.5 μg of fluconazole ml^{-1} and two successive subcultures on YPD agar containing progressively increasing concentrations of fluconazole (1, 5, 10, 25, and 50 μg ml^{-1}), three clones derived from CD51-II and two clones derived from CD57 were found to be able to grow satisfactorily on YPD agar containing fluconazole at a concentration of 50 μg ml^{-1}. Each of these was subcultured on drug-free PDA and assessed for its susceptibility to fluconazole by BMD. The two clones derived from CD57 (CD57A and CD57B) and the three clones derived from CD51-II (CD51-IIA, CD51-IIB, and CD51-IIC) exhibited fluconazole MIC values significantly higher than those of their respective parental isolates, although they remained susceptible to both itraconazole and amphotericin B (ketoconazole susceptibility was not tested) (Table 2). The stability of the
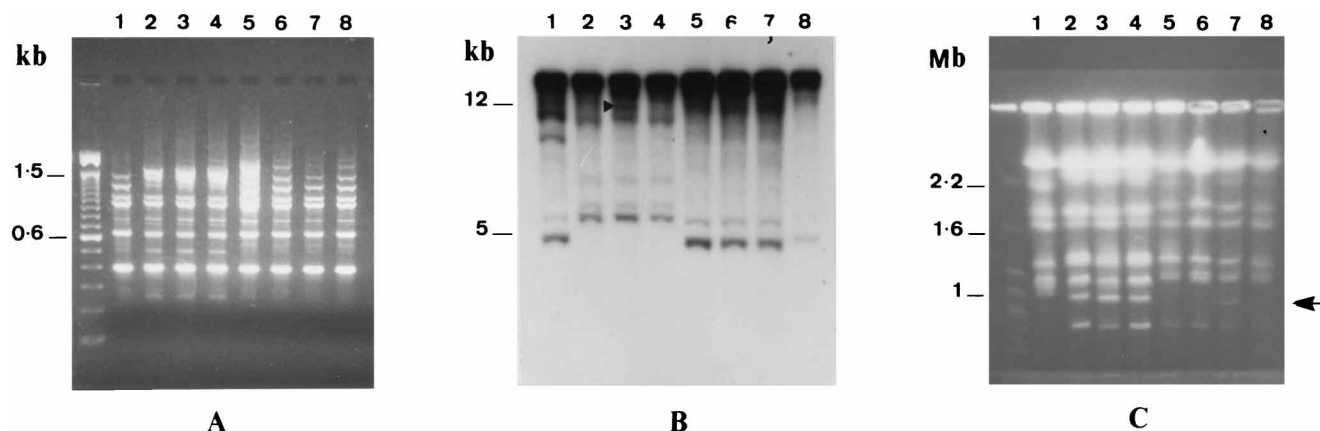
FIG. 1. DNA fingerprint analysis of fluconazole-sensitive clinical isolates of *C. dubliniensis* and in vitro-generated fluconazole-resistant derivatives. (A) Agarose gel containing RAPD products from fluconazole-susceptible *C. dubliniensis* clinical isolates and in vitro-generated fluconazole-resistant derivatives amplified with oligonucleotide primer 5′-GCGATCCCCA-3′. One hundred-base-pair DNA ladder size standards (Promega) are shown on the left of the panel. (B) Autoradiogram of *Eco*RI-digested total cellular DNA from fluconazole-susceptible *C. dubliniensis* clinical isolates and in vitro-generated fluconazole-resistant derivatives following hybridization analysis with the ^{32}P-labelled 27A probe. The positions of representative molecular weight reference markers are shown on the left of the panel. A polymorphic hybridization band of approximately 12 kb (indicated by an arrowhead) was detected in genomic DNA of derivative CD57A (lane 3). (C) Electrophoretic karyotypes of fluconazole-susceptible *C. dubliniensis* clinical isolates and in vitro-generated fluconazole-resistant derivatives. The polymorphic chromosome-sized DNA band of approximately 1 Mb present in the karyotype of *C. dubliniensis* derivative CD51-IIB (lane 7) is indicated by an arrow. The molecular sizes of chromosomes from *Saccharomyces cerevisiae* used as molecular mass standards (Bio-Rad) are shown on the left of the panel. Lanes: 1, *C. dubliniensis* type strain CD36 (A and B) or *C. albicans* oral reference strain 132A (C); 2, *C. dubliniensis* parental isolate CD57; 3, *C. dubliniensis* derivative CD57A; 4, *C. dubliniensis* derivative CD57B; 5, *C. dubliniensis* parental isolate CD51-II; 6, *C. dubliniensis* derivative CD51-IIA; 7, *C. dubliniensis* derivative CD51-IIB; 8, *C. dubliniensis* derivative CD51-IIC.

fluconazole-resistant phenotype of these five derivatives was assessed by monitoring their fluconazole MIC values following each of 10 successive subcultures on fluconazole-free PDA medium. For each of the five derivatives examined, no change in fluconazole MIC was detected by BMD following each of the 10 subcultures or following storage at −70°C for 10 weeks (data not shown).

Additional experiments were carried out with the fluconazole-susceptible oral *C. albicans* reference strain 132A. Following a similar regime of subculturing on fluconazole-containing YPD agar, four derivatives which could grow on medium containing 10 μg of fluconazole ml^{-1} were recovered but none were obtained which could grow on YPD agar containing higher concentrations of fluconazole. However, after a single subculture on drug-free PDA or YPD agar followed by BMD testing for susceptibility to fluconazole, all four of the derivatives were found to be susceptible to fluconazole and yielded fluconazole MIC values similar to those of their parental strain, 132A (MIC, 0.25 μg ml^{-1}).

Phenotypic and molecular analysis of fluconazole-resistant derivatives. When cultured on fluconazole-free PDA, the in vitro-generated fluconazole-resistant derivatives of *C. dubliniensis* isolates CD51-II and CD57 exhibited the same smooth, white colony morphology and ID 32C substrate assimilation profiles (7142100015 for CD51-II and its derivatives CD51-IIA, CD51-IIB, and CD51-IIC; 7142140015 for CD57 and its derivatives CD57A and CD57B) as their respective parental isolates.

Several molecular genetic studies were performed in order to determine whether the fluconazole-resistant derivatives were closely related to their parental isolates or whether they could have been exogenous fluconazole-resistant contaminants. The parental isolates CD57 and CD51-II were shown to possess different RAPD DNA fingerprint profiles (Fig. 1A, lanes 2 and 5). However, each derivative exhibited an RAPD fingerprint profile which was identical to the profile of its parental isolate (Fig. 1A). Similar results were obtained following hybridization analysis of *Eco*RI-digested total cellular

DNA from CD51-II and CD57 and their derivatives with the *C. albicans* fingerprinting probe 27A. This probe, which was used by Sullivan et al. (21, 24) to discriminate between *C. dubliniensis* isolates, hybridizes less frequently to *C. dubliniensis* DNA but has been shown to produce discriminatory profiles. The fingerprint profiles obtained with the *C. dubliniensis* parental isolates CD57 and CD51-II were significantly different (Fig. 1B, lanes 2 and 5). However, the profiles obtained with CD51-IIA, CD51-IIB, and CD51-IIC were identical to each other and to the profile of their parent, CD51-II (Fig. 1B, lanes 5 to 8). Similarly, the profile of CD57B was identical to the profile of its parental isolate, CD57 (Fig. 1B, lanes 2 and 4). The fingerprint profile obtained with the derivative CD57A exhibited a single polymorphism at approximately 12 kb when compared to the profiles of CD57 and CD57B (Fig. 1B, lanes 2 to 4). These findings supported the phenotypic data described above which indicated that the fluconazole-resistant derivatives were derived from their respective fluconazole-susceptible isolates and were not exogenous contaminants.

Karyotype analysis was also used to compare the genomic organization of the fluconazole-sensitive clinical isolates CD57 and CD51-II and their respective resistant derivatives. The karyotype profile of *C. albicans* 132A contained seven distinct chromosome-sized DNA bands (Fig. 1C, lane 1). In contrast, the fluconazole-sensitive *C. dubliniensis* isolates CD51-II and CD57 and their fluconazole-resistant derivatives, CD51-IIA, CD51-IIB, CD51-IIC (all CD51-II derivatives), CD57A, and CD57B (both CD57 derivatives), all contained nine chromosome-sized DNA bands, including one or more bands of <1 Mb (Fig. 1C), a characteristic feature of *C. dubliniensis* isolates (24). Each fluconazole-resistant derivative had a karyotype similar to that of its parental isolate, indicating the close relatedness of these organisms. Derivative CD51-IIB (Fig. 1C, lane 7) however, exhibited a single polymorphism at approximately 1 Mb, which allowed it to be differentiated from its parental isolate, CD51-II (Fig. 1C, lane 5). The high degree of similarity between the karyotype profiles of the fluconazole-resistant derivatives and their respective fluconazole-suscepti-

ble parental isolates also makes it more likely that the former were not contaminants.

DISCUSSION

In order to determine the susceptibility of *C. dubliniensis* to fluconazole and other antifungal drugs, several methods, i.e., BMD, HEA, and Etest, which have been previously shown to be reliable for testing the susceptibility of *C. albicans* isolates, were applied to the analysis of *C. dubliniensis* isolates. BMD and HEA yielded equivalent fluconazole susceptibility data with the same panel of reference strains and clinical isolates, although BMD was found to be more suitable for routine testing of clinical isolates due to its less-cumbersome format. When the results obtained are considered, it is apparent that *C. dubliniensis* is inherently susceptible to azole and polyene antifungal drugs. However, four isolates from two different AIDS patients (patients 1 and 4 [Table 1]) were found to have significantly reduced susceptibilities to fluconazole (BMD-determined MIC range, 8 to 32 μg ml^{-1}) and were deemed to be fluconazole resistant (Table 1).

The fluconazole susceptibilities of the reference strains and clinical isolates were also assessed by the commercially available Etest. Tests were performed on Casitone agar medium, as recommended by the manufacturer, as it gives clear end points with azole antifungal agents. All of the reference strains and clinical isolates which were found to be fluconazole susceptible by BMD and HEA (MIC range, $\leq$0.125 to 1.0 μg ml^{-1}) were also found to be fluconazole susceptible by the Etest (MIC range, 0.75 to 6.0 μg ml^{-1}) (Table 1). Similarly, the *C. dubliniensis* clinical isolates which were found to be fluconazole resistant by BMD and HEA (MIC range, 8 to 32 μg ml^{-1}) were also found to be resistant by the Etest (MIC range, 24 to 256 μg ml^{-1}) (Table 1). However, the Etest MIC results were generally 1 to 4 doubling dilutions of fluconazole higher than the equivalent data obtained by BMD or HEA. This is perhaps not surprising because of differences in media and the methods of end point determination used with the three procedures. However, two of the *C. dubliniensis* isolates, CD47-I and CD47-IIa, which were found to be fluconazole resistant by BMD and HEA (MIC, 8 to 16 μg ml^{-1}) grew poorly on Casitone agar and had to be incubated for 48 h instead of 24 h in Etest experiments before sufficient growth was obtained for end point determination, after which they yielded MIC results which were unsatisfactorily high (MIC, 128 to 256 μg ml^{-1}) in comparison to those obtained by BMD and HEA (Table 1). However, when these isolates were retested by the Etest with the richer RPMI agar being used as the growth medium, as described previously for the Etest with *C. albicans* isolates (4), MIC end points within 1 doubling dilution of the corresponding MIC data obtained by BMD were obtained (Table 1).

Of the 15 individual subjects included in the study from whom *C. dubliniensis* was recovered, fluconazole-resistant isolates were recovered only from two of the five HIV-infected patients who had received prior therapy with fluconazole (Table 1). Two *C. dubliniensis* isolates were recovered from one of these patients (patient 1 [Table 1]) at separate clinical evaluations approximately 18 months apart. The initial isolate (CM1) was found to be susceptible to fluconazole (MIC, 0.5 μg ml^{-1}), whereas the secondary isolate (CM2) was fluconazole resistant (MIC, 32 μg ml^{-1}) (Table 1). There are three possible explanations for these results. First, resistance to fluconazole could have developed in the *C. dubliniensis* harbored by patient 1 between clinical evaluations. Second, this patient could have been harboring both fluconazole-susceptible and fluconazole-resistant organisms at both clinical evaluations,

with a representative of only one of these types being sampled on each occasion. Third, patient 1 may have become colonized or infected with a new strain of *C. dubliniensis* between clinical evaluations. DNA fingerprinting analysis of genomic DNA from isolates CM1 and CM2 with the *C. albicans* fingerprinting probe 27A revealed that the fingerprint profiles of each isolate were significantly different, suggesting that the isolates were different strains of *C. dubliniensis* (data not shown). These results discount the first scenario described above, but it is not possible to distinguish between the other two possibilities from the available epidemiological data. The remaining three fluconazole-resistant *C. dubliniensis* isolates were all recovered from patient 4 (Table 1). The first of these isolates, CD47-I, was recovered 6 months prior to the recovery of the second two isolates, CD47-IIa and CD47-IIb. DNA fingerprinting analysis with the 27A probe indicated that all three isolates belonged to the same strain (data not shown), suggesting that the second episode of oral candidiasis in this patient was due to persistence of, or reinfection with, this strain. Although isolates CD47-IIa and CD47-IIb were recovered from the same clinical specimen, shared identical fingerprint profiles, and were both resistant to fluconazole, they could be distinguished on the basis of colony morphology on PDA medium; CD47-IIa possessed a normal smooth, white colony morphology, whereas CD47-IIb colonies were wrinkled in appearance. Furthermore, CD47-IIa grew poorly on Casitone agar, in contrast to CD47-IIb, which grew well. It is possible that CD47-IIb is a phenotypic variant of CD47-IIa as many *C. dubliniensis* isolates have been observed to exhibit the phenomenon of phenotypic switching in vitro (2a).

None of the four fluconazole-resistant *C. dubliniensis* clinical isolates exhibited cross-resistance either to the azole drugs tested, including itraconazole and ketoconazole, or to the polyene antifungal drug amphotericin B. Although several reports describing fluconazole resistance in *C. albicans* isolates have also reported cross-resistance to other azole compounds (9, 27), a number of recent studies have reported the recovery of clinical isolates of *C. albicans* which exhibited resistance to fluconazole without showing cross-resistance to other azole drugs (25).

In order to determine whether *C. dubliniensis* could develop resistance to fluconazole following exposure to the drug, 100 colonies each of two fluconazole-susceptible (MIC, 0.5 μg ml^{-1}) clinical isolates, CD51-II and CD57 (Table 1), were sequentially exposed to progressively increasing concentrations of the drug in agar medium, up to a maximum concentration of 50 μg ml^{-1}. This resulted in the generation of stable resistant derivatives of both isolates which exhibited significantly reduced susceptibilities to fluconazole as determined by BMD (MIC range, 16 to 64 μg ml^{-1}) (Table 2). This phenomenon was shown to be reproducible by repeating the experiment with *C. dubliniensis* CD57 and with an epidemiologically unrelated isolate, CD41 (data not shown). Genetic fingerprinting analysis demonstrated that these derivatives were clonally closely related to their respective parental isolates as they exhibited similar RAPD profiles, similar karyotype profiles, and similar DNA fingerprints generated with the 27A probe (Fig. 1).

Attempts to generate fluconazole-resistant derivatives from the fluconazole-sensitive *C. albicans* oral reference strain 132A were unsuccessful. Similar experiments have been performed with *C. albicans* by Vanden Bossche et al. (26), who utilized a ketoconazole-containing medium in an attempt to induce ketoconazole resistance. However, only a slight change in azole susceptibility was detected following 148 sequential subcultures on this medium. These findings suggest that *C. dubliniensis*, unlike *C. albicans*, possesses the ability to rapidly develop

stable resistance to fluconazole following direct exposure to the drug in vitro. Although the mechanism of resistance in these fluconazole-resistant derivatives has not yet been elucidated, its development over a relatively short period of time in vitro suggests that this organism may possess a readily inducible fluconazole resistance mechanism. This mechanism could be similar to those recently described for *C. albicans* which involve efflux of fluconazole from the cell by specific multidrug transporters (19), although some degree of cross-resistance with other azole or triazole antifungals would be likely in this case. It is possible that fluconazole resistance may be induced in patients as a result of treatment with the drug for protracted periods. Interestingly, in two of the in vitro-generated fluconazole-resistant derivatives (CD57A and CD51-IIb) (Fig. 1B and C, lanes 3 and 7, respectively), induction of drug resistance was accompanied by DNA polymorphisms, indicating that genetic rearrangements may occur under strong selective pressures, such as exposure to antifungal drugs. Particular genetic changes may be beneficial to the organisms under harsh environmental conditions. Similar phenomena have been described for *C. albicans* and *Cryptococcus neoformans* in vivo (10, 22).

C. dubliniensis, a recently identified species closely related to *C. albicans*, has been shown to be susceptible to the same range of antifungal drugs as *C. albicans*, but significantly, results presented here show that the former is capable of developing stable fluconazole resistance in vitro at a high frequency. Oral candidiasis in HIV-infected and AIDS patients is associated with a range of *Candida* species, particularly *C. albicans*, but more frequently involves other species, including those inherently less susceptible to azole antifungal drugs, such as *C. krusei*, and *C. dubliniensis*, which may conceivably develop resistance during courses of fluconazole treatment. We are currently examining the occurrence and susceptibility of *C. dubliniensis* isolates in other patient groups.

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