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Molecular and phenotypic analysis of *Candida dubliniensis*: a recently identified species linked with oral candidosis in HIV-infected and AIDS patients

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The discovery and characterisation of a novel species of *Candida*, termed *Candida dubliniensis*, associated with oral candidosis in HIV-infected individuals is described. These organisms share several phenotypic characteristics in common with *Candida albicans* and *Candida stellatoidea*, including the ability to produce germ tubes and chlamydospores. However, in contrast to these latter two species, *C. dubliniensis* isolates produce abundant chlamydospores, which are often arranged in contiguous pairs, triplets and other multiples suspended from a single suspensor cell. They belong to *C. albicans* serotype A and exhibit atypical substrate assimilation profiles. Genomic DNA fingerprinting analysis with the *C. albicans*-specific probe 27A and five different oligonucleotide probes consisting of short repeat sequence-containing motifs, demonstrated that *C. dubliniensis* has a distinct genomic organisation relative to *C. albicans* and *C. stellatoidea*. This was confirmed by karyotype analysis and random amplified polymorphic DNA (RAPD) analysis. Comparison of 500 bp of the V3 variable region of the large ribosomal subunit genes from 14 separate *C. dubliniensis* isolates and the corresponding sequences from *C. albicans*, *C. stellatoidea*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. kefyr* and *C. krusei* demonstrated that the *C. dubliniensis* isolates formed a homogenous cluster (100% similarity), representing a discrete taxon within the genus *Candida* that was significantly different from the other species analysed.

Keywords: oral candidosis; *Candida dubliniensis*; molecular epidemiology; non-*albicans Candida* species; opportunistic pathogens; oral yeast

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Introduction

Oral candidosis is one of the most frequent manifestations of opportunistic fungal disease encountered in HIV-infected and AIDS patients (Holmberg and Meyer, 1986; Tavitian *et al*, 1986; Samaranayake, 1992; Coleman *et al*, 1993). *Candida albicans* is the most commonly recovered aetiological agent and the most pathogenic species of the genus *Candida* (Odds, 1988; Samaranayake, 1992; Coleman *et al*, 1993). However, over the past decade an ever increasing number of reports have documented the emergence of non-*albicans Candida* species as significant oral opportunistic pathogens in this patient group (Holmberg and Meyer, 1986; Alsina *et al*, 1988; Powderly, 1992; Warnock, 1992; Coleman *et al*, 1993; Law *et al*, 1994; Ruhnke *et al*, 1994; Hazen, 1995; Johnson *et al*, 1995; Rex *et al*, 1995; Sullivan *et al*, 1996). Studies from our collection of isolates recovered from Irish HIV-infected individuals indicate that, with protracted antifungal therapy, changes in the prevalence of *C. albicans* strains and other *Candida* species occur frequently (Coleman *et al*, 1995). In practice, identification and classification of *Candida* species has depended to a large extent on the analysis of a limited number of physiological traits and morphological features (Odds, 1988). It is therefore not surprising that a number of recent studies have described isolates of *Candida* whose characteristics do not correspond exactly with classical species definitions (Coleman *et al*, 1993; Sullivan *et al*, 1993; McCullough *et al*, 1994, 1995; Boerlin *et al*, 1995; Sullivan *et al*, 1995, 1996). Because many of these organisms were recovered from HIV-infected individuals, these findings have important implications, both for patient treatment and for epidemiological investigations.

This review summarises our experience with *Candida dubliniensis*, a new species of *Candida* associated with oral candidosis in HIV-infected and AIDS patients, which we recently identified and described, and which is now known to be prevalent in this patient group in many disparate geographic locations (Coleman and Sullivan, manuscript in preparation).

Table 1 Isolation of oral *Candida dubliniensis* from different cohorts of Irish subjects

Cohort	*HIV status*	*Total No. of subjects*	*No. subjects yielding* C. dubliniensis
Intravenous drug users	HIV-positive	351	85 (24.2%)
Intravenous drug users	HIV-negative	32	6 (18.8%)
Haemophiliacs	HIV-positive	20	6 (30%)
Haemophiliacs	HIV-negative	14	0 (0%)
Homosexuals	HIV-positive	11	3 (27.3%)
Normal healthy subjects	HIV-negative	150	5 (3.3%)
Prisoners	Unknown	98	23 (23.5%)
Totals		676	128

Characterisation of C. dubliniensis oral isolates
Atypical oral *Candida* organisms (now termed *C. dubliniensis*) were first detected among isolates recovered from separate HIV-infected individuals with a history of recurrent oral candidosis attending the Dublin Dental Hospital between March 1991 and September 1992 (Sullivan *et al*, 1993). Since this preliminary study, we have recovered a total of 128 oral isolates of *C. dubliniensis* from separate individuals, the majority (73%) of whom were HIV-infected individuals (Table 1), who were predominantly intravenous drug users. Unexpectedly, the frequency of isolation of *C. dubliniensis* from prisoners of unknown HIV serostatus was similar to that of the HIV-infected intravenous drug using cohort tested. The significance of this finding is unclear and is currently being investigated.

Phenotypic properties *C. dubliniensis* isolates were investigated in comparison with reference strains of *C. albicans* and its synonym *C. stellatoidea* (Sullivan *et al*, 1995; Table 2). *C. stellatoidea* can be divided into two distinct types (termed type I and type II), both of which are sucrose-negative, based on karyotype pattern, DNA fingerprint profiles and pathogenicity for mice (Kwon-Chung *et al*, 1989, 1990; Sullivan *et al*, 1995). However, whereas type I *C. stellatoidea* are naturally sucrose-negative and genetically distinguishable from *C. albicans*, type II *C. stellatoidea* are considered to be merely sucrose-negative variants of *C. albicans* with similar karyotype patterns. The ability to produce chlamydospores and germ tubes are features previously associated with only two *Candida* species, *C. albicans* and *C. stellatoidea* (Odds, 1988). However, *C. dubliniensis* is unusual because of the production of large numbers of chlamydospores on Rice Agar Tween agar (RAT agar, bioMérieux, Marcy L'Etoile, France) and by their frequent arrangement in contiguous pairs, triplets or larger multiples attached to a single suspensor cell (Figure 1). Typically these are attached to short pseudohyphae with abundant lateral branching, and the chlamydospores can be stained with 1% (w/v) lactophenol cotton blue, which differentiates them from blastospores, suspensor cells and

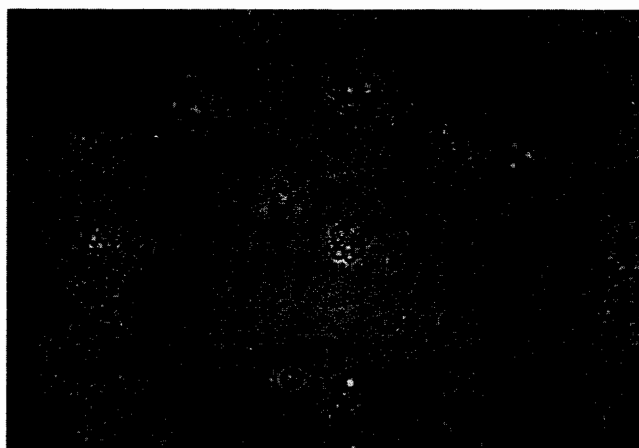

Figure 1 Light micrograph showing production of pseudohyphae and abundant chlamydospores by *C. dubliniensis* following growth on RAT agar. The letters a, b, c and d indicate examples of terminal chlamydospore arrangements comprising 1, 2, 4 and multiple chlamydospores, respectively. Bar: 5 μm

Table 2 Comparative phenotypic properties of *C. dubliniensis, C. albicans* and *C. stellatoidea*

Candida species	*Chlamydospore production*	*Germ tube production*	*Serotype*	*Growth at*			*Assimilation of sucrose*	*Colony colour*[d] *on CHROMagar®*
				30°C	*37°C*	*42°C*		
C. dubliniensis	++[a]	+	A	+	+	+/–[b]	+	Dark green
C. albicans	+	+	A or B	+	+	+	+	Light green
C. stellatoidea type I	+	+	B	+	+	–	–	Blue-green
C. stellatoidea type II[c]	+	+	A or B	+	+	+	–	Light green

[a]*C. dubliniensis* isolates characteristically hyperproduce chlamydospores, often in multiples of 2, 3 or more attached to single suspensor cells
[b]*C. dubliniensis* isolates grow poorly at 42°C
[c]*C. stellatoidea* type II are considered to be sucrose-negative mutants of *C. albicans* (Kwon-Chung *et al*, 1988)
[d]Following 48 h growth at 37°C (see Figure 2)

pseudomycelium which stain poorly or not at all. Although we have observed the formation of contiguous pairs of chlamydospores by *C. albicans* isolates cultured on RAT medium, this occurrs infrequently compared to *C. dubliniensis* (Sullivan *et al*, 1995).

C. albicans, *C. stellatoidea* and *C. dubliniensis* all grow well at both 30°C and 37°C. In contrast, *C. dubliniensis* grows poorly at 42°C, a feature shared with type I *C. stellatoidea* (Kamiyama *et al*, 1989) but not with *C. albicans* or type II *C. stellatoidea* (sucrose-negative mutants of *C. albicans*; Kwon-Chung *et al*, 1990), both of which grow well at 42°C. However, *C. dubliniensis*, which belongs to *C. albicans* serotype A (ie, can be agglutinated with antisera raised against *Candida* antigenic factor number 6; Sullivan *et al*, 1993, 1995), can be readily distinguished from type I *C. stellatoidea*, which belongs to serotype B (ie, cannot be agglutinated with antisera raised against *Candida* antigenic factor number 6; Kwon-Chung *et al*, 1989; Sullivan *et al*, 1993, 1995). *C. dubliniensis* also differs from type I *C. stellatoidea* by its ability to assimilate sucrose, although it should be noted that the latter can shed sucrose-positive variants upon prolonged exposure to sucrose (Wickes *et al*, 1991).

All of the 128 *C. dubliniensis* isolates indicated in Table 1 yielded one of four very similar substrate assimilation profile codes with the API ID 32C yeast identification system, including 7143140015, 7142140015, 7142100015 and 7143100015, none of which corresponded precisely with any known *Candida* species in the API APILAB database (Sullivan *et al*, 1993, 1995; Coleman, unpublished data). All of the isolates assimilated glucose, galactose, sucrose, maltose, sorbitol, mannitol, 2-keto-gluconate, *N*-acetylglucosamine and glucosamine. None of the isolates assimilated L-arabinose, D-xylose, ribose, α-methyl-D-glucoside, sorbose, rhamnose, cellobiose, lactose, melibiose, melezitose, raffinose, glycerol, erythritol, inositol, glucuronate, DL-lactate, levulinate or gluconate. Approximately half of the isolates did not assimilate trehalose and a minority did not assimilate palatinose. None of the isolates could hydrolyse aesculin or assimilate nitrate and all of them could grow in the presence of cycloheximide.

C. dubliniensis can be readily differentiated from *C. albicans* and both type I and type II *C. stellatoidea* on the basis of colony colour following growth on CHROMagar® Candida (CHROMagar® Candida, Paris, France) medium (Table 2, Figure 2). *C. dubliniensis* colonies appear dark green in colour following 48 h growth on the medium at 37°C in contrast to *C. albicans* and type II *C. stellatoidea* colonies which appear as a lighter green colour and to type I *C. stellatoidea* colonies which appear blue-green in colour (Table 2). CHROMagar Candida medium has previously been shown to be valuable for the presumptive identification of several clinically important *Candida* species (Odds and Bernaerts, 1994).

The results of all of these studies indicated that although *C. dubliniensis* shares many phenotypic characteristics in common with *C. albicans* and both types of *C. stellatoidea*, *C. dubliniensis* also exhibits significant differences.

Molecular properties Molecular studies strengthened the conclusion, based on phenotypic analysis, of the unique nature of *C. dubliniensis* relative to other species of the genus *Candida*, particularly in regard to *C. albicans* and *C. stellatoidea*. *C. dubliniensis* isolates and reference strains of *C. albicans* and *C. stellatoidea* were subjected to karyotype analysis (analysis of chromosome banding patterns following separation by pulsed-field gel electrophoresis). The *C. albicans* and the *C. stellatoidea* type II strains tested gave rise to seven distinct chromosome-sized bands. Both the

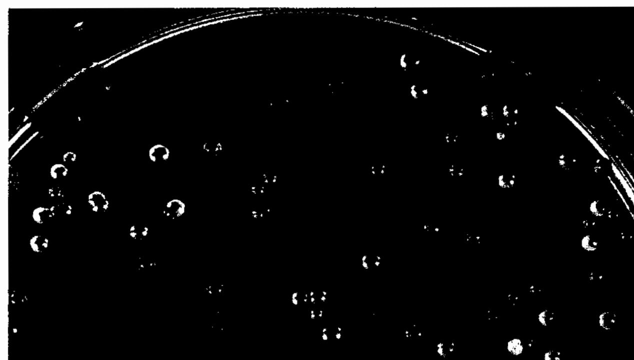

Figure 2 Colony colours exhibited by *C. dubliniensis* and *C. albicans* following 48 h growth at 37°C on CHROMagar® Candida medium. *C. dubliniensis* colonies are dark green (an example is indicated by an arrowhead) and *C. albicans* colonies are a lighter shade of green (an example is indicated by an arrow)

Table 3 Comparative molecular properties of *C. dubliniensis*, *C. albicans* and *C. stellatoidea*

Candida *species*	*Hybridization with probe 27A*[a]	*No. of chromosome-sized bands in karyotype*	*Percentage*[b] *sequence divergence within V3 region of large ribosomal subunit genes relative to* C. albicans
C. albicans	Strong	7	—
C. stellatoidea type I	Strong	9	0
C. stellatoidea type II	Strong	7	0
C. dubliniensis	Weak	9–11	2.25

Data taken from Coleman *et al*, 1993; Sullivan *et al*, 1995, 1996

[a]Probe 27A is a *C. albicans*- and *C. stellatoidea*-specific mid-repeat sequence probe used for DNA fingerprinting. *C. albicans* clinical isolates (Scherer and Stevens, 1988). This probe hybridizes strongly to 10 or more bands of *Eco*R1-digested genomic DNA from *C. albicans* and *C. stellatoidea* type I and type II strains, but only to between 4–7 bands of *Eco*R1-digested genomic DNA from *C. dubliniensis*, most of which hybridize weakly (see Figure 4).

[b]Values correspond to percentage of difference corrected for multiple base changes following multiple sequence alignments as described by Sullivan *et al*, 1995

type I *C. stellatoidea* reference strain used and the *C. dubliniensis* clinical isolates tested yielded 9–10 bands and one or more bands of <1 Mb (Figure 3; Sullivan *et al*, 1995). Chromosome-sized bands <1 Mb are not usually present in the karyotype profiles of *C. albicans* isolates, although similar small sized chromosomes are a feature of type I *C. stellatoidea* (Kwon-Chung *et al*, 1988).

Restriction endonuclease-digested chromosomal DNA from *C. dubliniensis* hybridized poorly with the *C. albicans*- and *C. stellatoidea*-specific mid-repeat sequence probe 27A developed by Scherer and Stevens (1988), yielding weak hybridization profiles composed of a small number of bands compared to the large number of strongly hybridizing bands obtained with genomic DNA from *C. albicans* isolates (Sullivan *et al*, 1995; Figure 4). These findings indicated that the genomic organisation of *C. dubliniensis* is significantly different to that of *C. albicans* and *C. stellatoidea* because probe 27A and related probes hybridize well with repetitive DNA sequences dispersed throughout the genomes of both of these species (Scherer and Stevens, 1988; Kwon-Chung *et al*, 1989; Sullivan *et al*, 1995). Furthermore, *C. dubliniensis* can be readily distinguished from *C. albicans* and both type I and type II *C. stellatoidea* on the basis of significant differences in *Hin*fI-generated restriction fragment length polymorphism (RFLP) patterns in agarose gels, without the requirement for hybridization with probe 27A (Sullivan *et al*, 1995). These results strengthened the conclusions drawn from 27A fingerprinting data.

Additional DNA fingerprint evidence supporting the unique genomic organisation of *C. dubliniensis* was obtained following hybridization analysis of *Eco*RI-cleaved genomic DNA from a representative sample of *C. dubliniensis* isolates and reference strains of both types of *C. stellatoidea* with each of the five synthetic oligonucleotide probes $(GT)_8$, $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$ and $(GTG)_5$. One advantage of this method is that the same DNA sample used to obtain a profile with probe 27A can then be successively hybridized with each of the oligonucleotide probes to produce five additional fingerprint profiles. In each case the overall fingerprint profiles of the *C. dubliniensis* isolates were very similar to each other but quite distinct from the profiles obtained with the *C. albicans* and the *C. stellatoidea* strains (Sullivan *et al*, 1995). These findings were further confirmed by random amplified polymorphic DNA (RAPD) analysis employing a range of oligonucleotide primers (Sullivan *et al*, 1995).

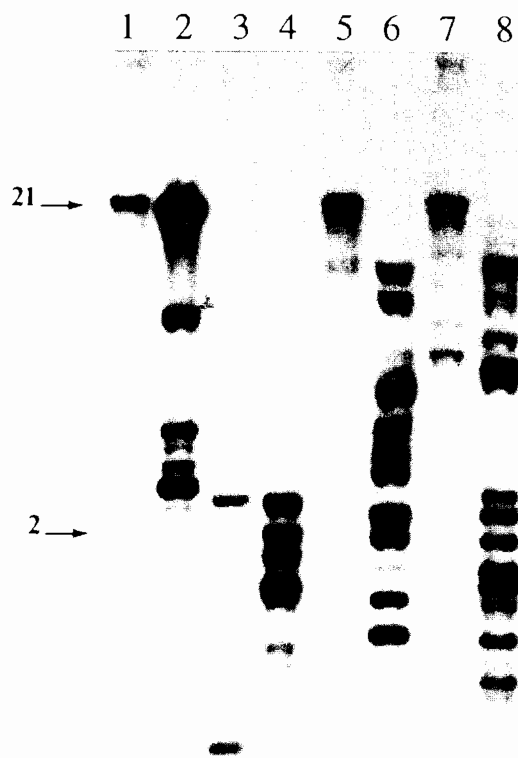

Figure 4 Southern blot analysis of restriction endonuclease-digested total genomic DNA from a *C. albicans* isolate and a *C. dubliniensis* isolate probed with the ^{32}P-labelled *C. albicans*-specific probe 27A. The fingerprints shown in lanes 1, 3, 5 and 7 were generated from *C. dubliniensis* DNA, whereas those shown in lanes 2, 4, 6 and 8 were generated from *C. albicans* DNA. DNA corresponding to the lanes was digested with restriction endonucleases as follows: lanes 1 and 2, *Eco*RI; lanes 3 and 4, *Dde*I; lanes 5 and 6 *Msp*I; lanes 7 and 8, *Cla*I. Size reference markers are indicated in kb on the left of the figure

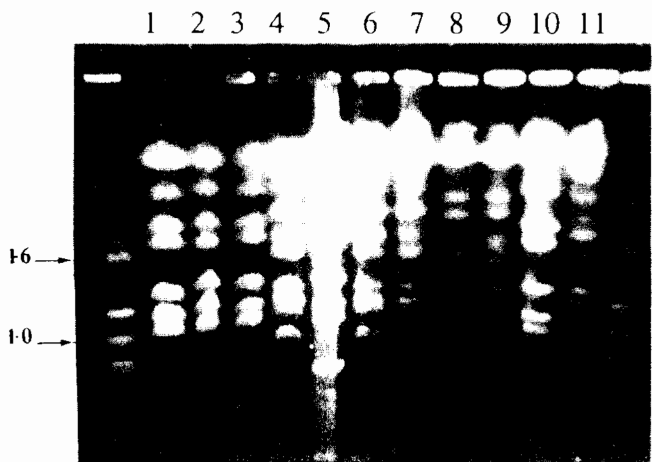

Figure 3 Electrophoretic karyotypes of *Candida* isolates. Lanes: 1, 2 and 10, *C. albicans*; 3, type II *C. stellatoidea*; 4, type I *C. stellatoidea*; 5–11, *C. dubliniensis*. *Saccharomyces cerevisiae* chromosomes, used as molecular mass standards (Bio-Rad), are on the left of the figure and the molecular masses are in Mb

Phylogenetic analysis of C. dubliniensis

Much of the nucleotide sequence of ribosomal DNA is highly conserved but the V3 region of the large subunit genes has been shown to be variable. The phylogenetic relationships between a number of marine yeast species have been determined previously by comparing corresponding V3 sequences (Fell *et al*, 1992; Fell, 1993). The nucleotide sequence of 500 bp of DNA from this region was determined, following amplification using the polymerase chain reaction (PCR), from a selection of 14 separate *C. dubliniensis* isolates recovered in Ireland, the UK, Switzerland, Australia, and South America and reference strains of *C. albicans*, type I and type II *C. stellatoidea*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. kefyr*, *C. krusei* and *Aspergillus fumigatus*. These sequences were aligned and com-

pared using computer-assisted techniques and an evolutionary tree constructed from an evolutionary distance matrix based on these sequences, indicating the phylogenetic relationships between the species (Sullivan *et al*, 1995). These studies revealed that the *C. dubliniensis* isolates formed a homogenous cluster (100% similarity) that was unambiguously separated from the other species tested (Sullivan *et al*, 1995; Coleman, Sullivan and Haynes, unpublished data). The closest species linked to the cluster were *C. albicans* and *C. stellatoidea*, both of which exhibited 2.25% sequence divergence from the *C. dubliniensis* sequence. In contrast, the *C. albicans* and *C. stellatoidea* sequences were virtually identical. Bootstrap analysis of the aligned sequences was performed in order to establish levels of confidence for the separation of the species as indicated by the evolutionary tree. In 987 of the 1000 trees generated (98.7%) *C. dubliniensis* was grouped separately from *C. albicans*, *C. stellatoidea* and the other species tested (Sullivan *et al*, 1995). These results provided convincing evidence that *C. dubliniensis* constitutes a discrete taxon within the genus *Candida* and further demonstrated that *C. dubliniensis* has diverged significantly from *C. albicans* and *C. stellatoidea*, and that this divergence is evident in isolates recovered in diverse geographic locations.

Concluding remarks

C. dubliniensis oral isolates (McCullogh *et al*, 1995; Sullivan *et al*, 1995, 1996; Coleman, Sullivan manuscript in preparation) and similar organisms (Anthony *et al*, 1995; Boerlin *et al*, 1995; Le Guennec *et al*, 1995) have been recovered from HIV-infected individuals in many different centres around the world. These organisms are phenotypically very similar to *C. albicans* and very likely have been misidentified as unusual forms of *C. albicans* in many instances. In addition, *C. dubliniensis* shares some genotypic properties with type I *C. stellatoidea* (ie, similar karyotype profile). However, genomic fingerprinting analysis and phylogenetic analysis based on V3 rRNA sequences demonstrated that both organisms are taxonomically distinct. Because of the significant genetic divergence found between *C. albicans* and *C. dubliniensis*, these organisms require intensive study to determine their precise role in disease, their response to antifungal therapy and to further determine their phenotypic and molecular properties.

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