Candida dubliniensis: An update

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The increased incidence of fungal infections during the last decade has been well-documented [1-4]. Given that one of the most important factors contributing to this phenomenon is the increased numbers of immunocompromised individuals, it is perhaps not surprising that species previously not associated with human disease and novel species previously unknown to science have been identified as potential pathogens (e.g., *Penicillium marneffei* [5], *Emmonsia pasteuriana* [6] and *Candida dubliniensis* [7]).

C. dubliniensis was first identified as a new species in 1995 [7]. As its name suggests this species was originally described in Dublin, Ireland. While performing an epidemiological investigation of oral candidosis in Irish HIV-infected individuals and AIDS patients in the early 1990s it was discovered that some germ tube- and chlamydospore-positive isolates, which were identified as *Candida albicans* on the basis of these characteristics, failed to hybridize efficiently with the *C. albicans*-specific DNA fingerprinting probe 27A [7,8]. Subsequent in-depth analysis of these organisms revealed that they constituted a distinct species clearly separate from, but closely related to, *C. albicans* [7]. In the intervening four years *C. dubliniensis* isolates have been identified in a range of clinical settings by many laboratories throughout the world [9-19].

The purpose of this short article is to review briefly the most recent data available on *C. dubliniensis*. In particular we wish to highlight the advances being made in the development of rapid and accurate tests to allow the discrimination of *C. dubliniensis* from other *Candida* species, especially *C. albicans*. With the introduction of these tests we hope that many other laboratories will be encouraged to search for this species in clinical specimens and culture collections and thus provide further information concerning the epidemiology and the true clinical significance of this newly identified opportunistic pathogen.

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Phenotypic characteristics

C. dubliniensis is closely related to and shares many phenotypic characteristics with *C. albicans* [7]. This close similarity has hindered differentiation between the two species in the clinical laboratory. Both species produce germ tubes and chlamydospores, features previously associated solely with, and used for the definitive identification of, *C. albicans*. It has been reported that *C. dubliniensis* strains can differ from *C. albicans* in that they often produce chlamydospores more readily and more abundantly on Rice agar Tween (RAT), Tween 80-oxgall-caffeic acid (TOC) or cornmeal agar [7,13,20]. However, this unusual chlamydospore presentation has not been shown to be reproducible in some laboratories [14,21]. In a recent study describing North American *C. dubliniensis* isolates it was shown that 16 of 23 (70%) *C. dubliniensis* isolates produced abundant chlamydospores, however, 1 of 28 (3.6%) *C. albicans* isolates examined also exhibited a similar phenotype [14]. Thus, while examination of chlamydospore production may be of some use as a confirmatory identification test for *C. dubliniensis* it should not be used as a primary means of identification. Comparative growth analysis at elevated temperatures such as 42°C and 45°C has also been suggested as a means of discriminating *C. dubliniensis* from *C. albicans* [7,22]. While all *C. dubliniensis* isolates tested so far do not grow at 45°C there is some confusion as to what proportion of *C. albicans* isolates can grow at this temperature. In our laboratory we have found that only 1 of 100 *C. albicans* isolates tested failed to grow at 45°C [22]. However, in another study it has been shown that 10 out of the 28 (36%) *C. albicans* isolates tested also failed to grow at this temperature [14]. The reason for this discrepancy is not clear, but may be a reflection of the inaccuracy of temperature readings and heat distribution in many laboratory incubators. Whatever the reasons, it would again appear that absence of growth at 45°C should only be used as a confirmatory test in conjunction with one or more other identification tests.

The recent introduction of the chromogenic medium CHROMagar Candida has proven to be particularly helpful in the identification of *C. dubliniensis* isolates, particularly following primary culture from clinical specimens. While *C. albicans* colonies are a light blue/green colour on this medium *C. dubliniensis* colonies are a much darker green colour [20,21,23]. This colour is particularly pronounced if plates are incubated for longer than 48 h (e.g., up to 72 h). Although CHROMagar Candida has been widely used in the identification of primary clinical isolates of *C. dubliniensis* it has been reported that the ability of *C. dubliniensis* to produce its distinctive dark green colour can be lost following subculture and storage [21]. One of the earliest observations

which suggested that *C. dubliniensis* was distinct from *C. albicans* was based on comparative analysis of substrate assimilation profiles using commercially available yeast identification kits such as the bioMérieux API ID 32C and API 20C AUX systems [7]. The data generated using these kits revealed that the range of carbohydrates assimilated by *C. albicans* and *C. dubliniensis* was significantly different. From these and other studies it is evident that *C. dubliniensis* isolates, unlike the great majority of *C. albicans* isolates, are unable to assimilate methyl-αD-glucoside, lactate or xylose [7,14,24]. In addition, *C. dubliniensis* grows much more slowly than *C. albicans* when trehalose is the only source of carbon. The recent inclusion of many specific *C. dubliniensis* carbohydrate assimilation profiles in the databases of the API ID 32C and API 20C AUX kits will certainly aid the identification of this species. *C. dubliniensis* and *C. albicans* can also be distinguished using a variety of other commercially available yeast identification techniques, including the RapID Yeast Plus, VITEK YBC and VITEK 2 ID-YST systems [25]. One interesting characteristic exhibited by *C. dubliniensis* is that cells grown at 37°C on Sabouraud's dextrose agar have the ability to coaggregate *in vitro* with cells of the oral bacterial species *Fusobacterium nucleatum* [26]. *C. albicans* cells grown under the same conditions fail to coaggregate with this species. The clinical significance of this finding is not clear, however, the authors who first described this phenomenon suggest that a test which they have developed to distinguish *C. dubliniensis* from *C. albicans* based on this phenomenon is rapid, specific and inexpensive [26].

C. dubliniensis isolates have also been discriminated from *C. albicans* using a number of more sophisticated techniques. Firstly, Bikandi *et al.* have developed a *C. dubliniensis*-specific antiserum [9]. In this study, antiserum raised against *C. dubliniensis* was adsorbed with *C. albicans* blastospores and subsequently used in an indirect immunofluorescence assay. In this test the antiserum reacted with blastospores and germ tubes of *C. dubliniensis*, but not with *C. albicans* blastospores, suggesting that there are differences in the cell wall architecture of the two species. Interestingly, the antiserum also reacted, albeit weakly, with *C. albicans* germ tubes and hyphae. However, this did not interfere with the results obtained in a blind trial when the antiserum correctly discriminated between 83 *C. dubliniensis* and 43 *C. albicans* isolates. This test is very rapid and specific, however, its potential for widespread use is limited by the availability of the antiserum and the necessity to use immunofluorescence microscopy. Other tests which allow the discrimination of *C. dubliniensis* and *C. albicans* include pyrolysis mass spectrometry (PyMS) and Fourier transform infrared (FT-IR) spectroscopy [27]. However, the technology required to perform these techniques is not widely available thus precluding their usefulness in routine clinical diagnostic laboratories.

Genotypic characteristics

The first isolates now known to be *C. dubliniensis* were first noticed and distinguished from *C. albicans* isolates because of their unusual DNA fingerprint patterns generated using the *C. albicans*-specific DNA fingerprinting probe 27A [7,8]. That there are significant differences in the chromosomal arrangement of sequences in each species was confirmed using a wide range of DNA profiling techniques, including fingerprinting with oligonucleotides homologous to microsatellite sequences, pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) PCR analysis [7]. These data indicated that the genomic organisation of *C. dubliniensis* is readily distinguishable from that of *C. albicans*. Recently, a species-specific repetitive DNA element has been identified in *C. dubliniensis* which shows promise for use as a specific fingerprinting probe for this species and will greatly aid in the epidemiological analysis of *C. dubliniensis* infections [28]. Interestingly, preliminary data using this probe suggest that *C. dubliniensis* isolates can be subdivided into two distinct groups, one of which forms a cluster of closely related strains [28]. However, DNA fingerprinting techniques, such as restriction endonuclease (REA) analysis, PFGE analysis and DNA fingerprinting using specific probes are expensive, time consuming and not readily applicable to routine use for identification purposes in most clinical microbiology diagnostic laboratories.

Demonstrating that *C. dubliniensis* has a distinct genomic organisation was insufficient for the delineation of *C. dubliniensis* as a species separate from *C. albicans*. To determine the phylogenetic relationship of these organisms it was necessary to demonstrate that, in addition to differences in genomic organisation, there is a significant nucleotide sequence divergence between the two species. The final and most conclusive evidence that *C. dubliniensis* is a *bona fide* species came from the comparative analysis of ribosomal RNA (rRNA) gene sequences from a variety of *Candida* species. In the original paper describing *C. dubliniensis* it was found that a 600 bp region encompassing the V3 variable region of the large rRNA (lrRNA) genes of *C. dubliniensis* and *C. albicans* differed by 2.3% [7]. Similar analysis of the D1/D2 region of the lrRNA genes of both species also revealed a significant degree of nucleotide divergence [29]. In addition, comparison of the sequence of the self-splicing group I introns present in the lrRNA genes of both species revealed that the *C. dubliniensis* intron is almost identical to that of *C. albicans* except for two widely divergent stem-loop regions [11]. The unique phylogenetic position of *C. dubliniensis* was further established by comparison of the sequences of the entire small rRNA genes (approximately 1.8 kb) of *C. dubliniensis* and *C. albicans* which revealed a difference of 1.4% [30]. In addition to ribosomal RNA sequences, the *ACT1* gene, which encodes the structural protein actin, has been used extensively in phylogenetic studies. Comparison of the *ACT1* genes from *C. albicans* and *C. dubliniensis* showed that the coding sequences differ by 2.1% while the less highly conserved *ACT1*-associated introns differ by 16.6% [31]. These findings strongly suggest that *C. albicans* and *C. dubliniensis* diverged from each other in the distant past.

As well as direct evidence of significant sequence divergence in specific genes there is also evidence of genome-wide sequence divergence based on data obtained using multilocus enzyme electrophoresis (MLEE) analysis. This technique, which measures the relative electrophoretic mobility of specific proteins, was used to differentiate a subgroup of Swiss atypical *Candida* isolates, which were later identified as *C. dubliniensis*, from *C. albicans* [10]. In the original study by Boerlin *et al.* it was observed that, in contrast with *C. albicans*, *C. dubliniensis* isolates did not appear to produce β-glucosidase activity. This led to the design of a simple method to differentiate between the two species based on the ability of *C. albicans* to generate fluorescence in the presence of methyl-umbelliferyl-labelled β-glucoside [10]. This technique has been used quite successfully in a number of studies, although in a recent analysis of an archival stock collection 67 of 537 (12.5%) *C. albicans* isolates were

found to be βglucosidase negative [17]. Another technique based on genetic sequence divergence that shows great potential for use in the rapid identification of *C. dubliniensis* is the polymerase chain reaction (PCR). To date *C. dubliniensis*-specific primers have been designed on the basis of the sequence of the D1/D2 region of the lrRNA gene [29] and the *ACT1*-intron [31]. In the latter study, the *ACT1 C. dubliniensis*-specific primers have been tested successfully in an extensive blind trial including greater than 120 *C. dubliniensis* and 50 *C. albicans* isolates from a range of clinical specimens recovered from patients around the world (Figure 1). Using this test *C. dubliniensis* isolates can be identified accurately in less than 4 h. *C. albicans*-specific primers have also been designed based on *PHR1* sequences which do not yield amplimers when used with *C. dubliniensis* template DNA [32]. Restriction fragment length polymorphism analysis of amplimers obtained using PCR primers flanking various regions of the rRNA locus have also been demonstrated to allow the discrimination of *C. dubliniensis* from *C. albicans* [33]. In addition, a PCR enzyme immunoassay (PCR-EIA) using a *C. dubliniensis*-specific DNA probe derived from the ITS2 region of the rRNA locus has also been developed [12]. These techniques are specific, rapid, easy to perform and applicable to large numbers of isolates and should enhance the rapid and accurate identification of *C. dubliniensis* in the future.

Epidemiology

Originally identified in specimens recovered from the oral cavities of HIV-infected individuals with recurrent oral candidosis in Ireland, *C. dubliniensis* has since been identified in a wide variety of clinical settings throughout the world. Details of the isolation of this species from different subject cohorts in our own study population are presented in Table 1. In addition to the recovery of *C. dubliniensis* in Ireland, there have been many recent reports of the identification of this species in laboratories around the world [9-19,24,28,33,34]. Most of these isolates have been recovered from cases of oral candidosis in HIV-infected individuals. From our own experience

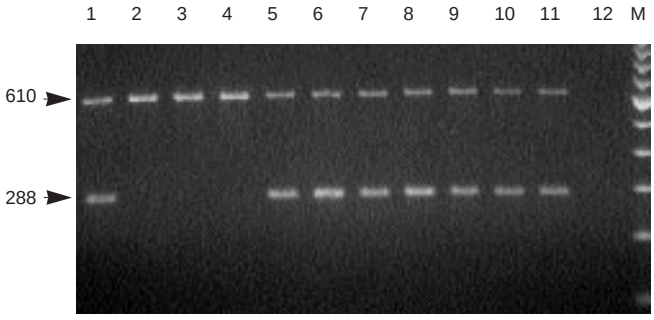

Figure 1. Agarose gel with ethidium bromide-stained amplimers from PCR reactions using fungal-specific primers (610 bp product) and *C. dubliniensis*-specific primers derived from the *ACT1* intron sequence (288 bp product) [31]. Lane 1; *C. dubliniensis* type strain, CD36, Lane 2; *C. albicans* 132A, Lane 3; *C. albicans* SC5314, Lane 4; Type 1 *C. stellatoidea* ATCC11006, Lanes 5-11; clinical isolates of *C. dubliniensis*. Lane 12; negative control lacking template DNA. Lane M; 100 bp molecular weight ladder.

C. dubliniensis appears to be most often associated with recurrent episodes of the erythematous form of oral candidosis. Interestingly, in a recent study, *C. dubliniensis* was implicated in an unusual form of linear gingival erythematous candidosis [35]. We have also identified this species as a cause of oral disease in non-HIV-infected individuals and have detected it at low incidence levels in normal healthy individuals (Table 1). In addition, there have also been reports of the recovery of *C. dubliniensis* isolates from vaginal and faecal samples [7,17]. Isolates have also been recovered from cases of systemic disease in non-HIV-infected patients [16,22]. In a recent report one patient receiving cytotoxic chemotherapy for relapsed rhabdomyosarcoma and two patients following allogeneic haematopoietic stem cell transplants yielded *C. dubliniensis*-positive blood cultures [16].

The earliest known isolates of *C. dubliniensis* predate the AIDS epidemic. One isolate deposited in the British National Collection for Pathogenic Fungi as *C. stellatoidea* in 1957 [7] and another deposited in the Centraal Bureau voor Schimmelcultures in Holland as *C. albicans* in 1952 have recently been identified as *C. dubliniensis* [16]. This highlights the problem of misidentification of

Table 1. Recovery of oral *C. dubliniensis* isolates from different cohorts of Irish individuals.

Group	No. of subjects	Clinical symptoms of oral candidiasis	No. subjects yielding *C. dubliniensis*	Other *Candida* species co-isolated*
HIV-positive	185	Symptomatic	48 (26%)	12 *C. dubliniensis* only 36 *C. dubliniensis* & other *Candida* species
HIV-positive	216	Asymptomatic	39 (18%)	7 *C. dubliniensis* only 32 *C. dubliniensis* & other *Candida* species
AIDS	82	Symptomatic	26 (31.7%)	8 *C. dubliniensis* only 18 *C. dubliniensis* & other *Candida* species
AIDS	36	Asymptomatic	9 (25%)	3 *C. dubliniensis* only 6 *C. dubliniensis* & other *Candida* species
HIV-negative¶	72	Symptomatic	10 (13.9%)	3 *C. dubliniensis* only 7 *C. dubliniensis* & other *Candida* species
HIV-negative§	56	Symptomatic	6 (10.7%)	2 *C. dubliniensis* only 4 *C. dubliniensis* & other *Candida* species
HIV-negative#	202	Asymptomatic	7 (3.5%)	1 *C. dubliniensis* only 6 *C. dubliniensis* & other *Candida* species

Data from Coleman *et al.* [36] and [D. Coleman unpublished].
**C. albicans* was the species most commonly co-isolated with *C. dubliniensis*, followed by (in decreasing order of frequency) *C. glabrata*, *C. tropicalis*, *C. krusei* and, infrequently, several other non-*C. albicans Candida* species and other yeast species.
¶ HIV-negative subjects with denture-associated oral candidosis.
§ HIV-negative subjects with non-denture-associated oral candidosis.
# Normal healthy oral *Candida* carriers.

C. dubliniensis due to its phenotypic similarity with *C. albicans* (and *C. stellatoidea*). In two separate studies approximately 2% of germ tube- and chlamydospore-positive isolates of *Candida* originally identified as *C. albicans* were found to be *C. dubliniensis* [17,36]. When isolates recovered from HIV-infected individuals alone were taken into account the proportion of misidentified isolates assumed even greater significance.

Antifungal drug resistance and virulence

Since *C. dubliniensis* is most often associated with recurrent episodes of disease in HIV-infected individuals it has been suggested that its recent emergence as a human pathogen may have resulted from selection due to the widespread use of antifungal drug therapy [36]. However, a number of studies have revealed that the great majority of *C. dubliniensis* isolates are susceptible to commonly used and novel antifungal agents [14,17,37,38]. In the most comprehensive study performed to date 97% of the 71 *C. dubliniensis* isolates tested were susceptible to fluconazole [38] , the agent which has been used most commonly in the treatment of oral candidosis in HIV-infected individuals. In this study, resistance (e.g., the MIC interpretative breakpoint concentration) was defined as MIC ≥64 µg/ml as recommended by the NCCLS [39]. However, a number of isolates with dose-dependent susceptibility (MIC 16-32 µg/ml) have also been described in several other studies [14,17,37]. Notably, comparison of the geometric mean MICs for fluconazole, itraconazole and ketoconazole for 58 isolates each of *C. albicans* and *C. dubliniensis* revealed that the MIC values of *C. dubliniensis* were significantly and consistently higher than those of the *C. albicans* isolates [17]. Thus although the vast majority of *C. dubliniensis* isolates are susceptible to fluconazole they may be slightly less so than most *C. albicans*, perhaps allowing them a limited selective advantage in patients treated extensively with this drug. Another interesting phenomenon concerning *C. dubliniensis* is the comparative ease with which it is possible to induce stable fluconazole resistance *in vitro*. Simply growing colonies on agar medium containing sequentially increasing concentrations of fluconazole results in the development of resistance [37]. Analysis of the resistance mechanisms in both clinical and *in vitro*-generated resistant organisms has revealed that overexpression of the major facilitator protein Mdr1p appears to be largely responsible for the resistance phenotype [40]. This is in contrast to the situation in *C. albicans* where it has been suggested that overexpression of the ABC transporter protein Cdr1p is a more common mechanism of fluconazole-resistance [41,42]. To date, resistance to antifungal agents other than fluconazole (e.g., itraconazole, ketoconazole, amphotericin B, voriconazole and a range of novel agents including triazoles and echinocandins) has not been observed in *C. dubliniensis*.

Despite the fact that *C. dubliniensis* is a significant cause of human disease, very few studies have been performed to investigate virulence factors in this species. Given the close phenotypic similarity between *C. dubliniensis* and *C. albicans* it might be expected that they may share the ability to produce certain putative virulence factors. Both species are dimorphic, although in one limited study, it has been suggested that the kinetics of hyphal production in *C. dubliniensis* is slower than that observed for reference *C. albicans* strains [30]. This may have a bearing on the ability of *C. dubliniensis* isolates to invade tissue and may contribute to the apparent lower virulence of this species. In the same study it was also shown that *C. dubliniensis* possesses homologues of seven *C. albicans* secretory aspartyl proteinase genes (*SAP*). Contrary to expectation, an early study on five atypical *Candida* isolates, which were later identified as *C. dubliniensis*, suggested that these isolates produced higher levels of proteinase activity than reference isolates of *C. albicans* [15]. Both of these studies also suggested that *C. dubliniensis* isolates are more adherent to buccal epithelial cells than the *C. albicans* strains tested [15,30]. Interestingly, SAPs have been proposed to play a role in adherence to tissue. Clearly the pathogenicity of *C. dubliniensis* is a complex subject and the data from these two studies have yet to be confirmed. The only available published data from an animal model is also equivocal. In a limited study, the *in vivo* virulence of four *C. dubliniensis* isolates (one vaginal and three oral) and one reference *C. albicans* isolate was tested in a systemic mouse model of infection. With an inoculum size of 2×10^6 cells per mouse the *C. dubliniensis* strains were clearly less virulent than the reference *C. albicans* isolate, however, when the inoculum was increased to 1×10^7 cells per mouse the results were less clear cut [30]. These data are clearly very preliminary and are based on limited numbers of strains. In addition, a systemic infection model is not ideal for the analysis of virulence of organisms implicated in superficial infections.

Conclusions

C. dubliniensis has emerged as a significant cause of candidosis. Although it is primarily associated with recurrent oral infections in HIV-infected individuals, it has also been implicated in cases of superficial and systemic disease in non-HIV-infected individuals. In order to confirm the true clinical significance of *C. dubliniensis* there is a clear need for a thorough investigation of its epidemiology. This should be facilitated by the recent development of a number of reliable identification tests. We recommend the use of CHROMagar Candida medium as a primary means for the presumptive identification of *C. dubliniensis* in clinical samples following primary culture. Any colonies showing a dark green colour should be examined using one or more of the following simple tests; carbohydrate assimilation (particularly xylose, α-methyl-D-glucoside and lactate), absence of growth at 45˚C, fluorescence with methyl-umbelliferyl-β-glucoside or PCR using species-specific primers. In the future, further studies should also be performed to determine the frequency of antifungal drug resistance in clinical isolates and the mechanisms of resistance used by this species. Such studies should help to determine some of the reasons for the recent emergence of *C. dubliniensis* as a cause of human disease. Finally, the analysis of virulence mechanisms in *C. dubliniensis* and their comparison with those of *C. albicans* should help our understanding of how both of these organisms cause disease.

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