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Development of differential media for *Candida dubliniensis* and identification of a novel clade of *Candida dubliniensis* predominant in the Middle East

A thesis submitted to the University of Dublin in fulfillment of the requirements for the degree Doctor of Philosophy by

Asmaa Al Mosaid

May 2005

Microbiology Research Unit, Department of Oral Medicine and Pathology
Dublin Dental School and Hospital,
University of Dublin,
Trinity College

Declaration

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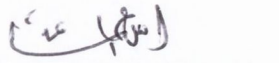

Asmaa Al Mosaid, BDS, MSc

This thesis is dedicated to a loving man
who never stopped giving of himself in countless
ways, the man who first led me through life with his
light of hope, unconditional love and support...to my father

And when ye are told to rise up, rise up: Allah will raise up,
to (suitable) ranks (and degrees), those of you who believe and who
have been granted Knowledge. And Allah is well-acquainted with all ye do.

Al-Mujadila
Verse no. 11

Summary

Candida dubliniensis is a newly described *Candida* species originally associated with oral colonisation and candidosis in Human Immunodeficiency Virus (HIV)-infected, and acquired immunodeficiency syndrome (AIDS) patients. More recently, *C. dubliniensis* has been isolated from cases of infection in other groups of immunocompromised individuals and in patients with severe underlying diseases. However, the close genotypic relationship between *C. dubliniensis* and *C. albicans* results in their sharing a broad range of phenotypic characteristics which hampers the accurate and rapid differentiation of the two species. Therefore, the phenotypic methods currently available for the identification of *C. dubliniensis* all have disadvantages in that they are time consuming, expensive or in some cases, unreliable.

The first part of the present study investigated the usefulness of a variety of culture media including; Staib, caffeic acid-ferric citrate (CAF), Pal's, tobacco and casein agar media to effectively differentiate between isolates of *C. dubliniensis* and *C. albicans* based on colony morphology and/or chlamydospore production. The results showed that all *C. albicans* tested, apart from nine isolates (7.5%) grown on casein agar, did not produce chlamydospores when cultured on the different media. In contrast, 111/ 130 (85.4%) 109/130 (83.8%) 52/53 (98%) and 106/ 109 (97.2%) of the *C. dubliniensis* isolates tested produced chlamydospores on Staib, CAF, tobacco and casein agars, respectively. When colony morphology was examined it was found that all of the 166 *C. albicans* isolates grew as smooth, shiny colonies on Staib agar after 48-72 h incubation at 30°C while 127/ 130(97.7%) of the *C. dubliniensis* isolates grew as rough colonies, many (65%) with a hyphal fringe. In contrast, 145/166 (87.4%) of the *C. albicans* and 122/130 (93.8%) of the *C. dubliniensis* isolates yielded rough colonies on CAF. On tobacco agar, 51/53 (96.2%) yielded rough, yellowish-brown colonies with a hyphal fringe around the colonies after 48 to 72 h. In contrast, 31/35 (88.5%) *C. albicans* isolates yielded smooth, white-creamy colonies with no hyphal fringe after 48 to 72 h. However, it was demonstrated that the production of a hyphal fringe around colonies grown on Pal's agar (sunflower seed agar) at 30°C for 48 to 72 h provides a simple means of discriminating between isolates of *C. dubliniensis* and *C. albicans* with 100% accuracy. Of 128 *C. dubliniensis* isolates tested on this medium, all produced a hyphal fringe. In contrast, none of the 124 *C. albicans* isolates tested produced a hyphal fringe. Pal's medium has the added

advantage of being simple, readily applicable to the high-volume throughput of isolates in many routine diagnostic laboratories and is prepared from inexpensive, readily available seeds.

DNA fingerprinting of *C. dubliniensis* isolates using the species-specific probe Cd25 previously showed that this species consists of two distinct groups termed Cd25-group I and Cd25-group II. The present study investigated the population structure of 30 *C. dubliniensis* oral isolates from Saudi Arabia and Egypt using Cd25-fingerprinting and rDNA gene internal transcribed spacer region-based genotyping. Cd25 fingerprinting analysis of these isolates revealed two distinct populations, the first of which consisted of 10 closely related genotype 1 isolates (average similarity coefficient (S_{AB}) value 0.86). The second population of 20 isolates was much more heterogenous (average S_{AB} value 0.35) and consisted of two distinct subpopulations, one of which consisted of genotype 3 (n=13) and the other genotype 4 isolates (n=7). A mixed dendrogram generated from the fingerprint data from the 30 Saudi Arabian and Egyptian isolates, 5 Israeli isolates and 51 previously characterised international isolates (32 Cd25-group I and 19 Cd25-group II) revealed the presence of three distinct main clades. The first corresponded to the previously described Cd25-group I and contained all the Saudi Arabian, Egyptian and Israeli genotype 1 isolates mixed with international isolates. The second clade corresponded to the previously described Cd25-group II and contained three Israeli isolates, one genotype 2, one genotype 3 and a genotype 4 variant isolate, mixed with international isolates. The third clade has not been described before and consisted solely of the 20 Saudi Arabian and Egyptian genotype 3 and 4 isolates identified in this study and a previously described genotype 4 Israeli isolate. All Saudi Arabian and Egyptian 20 Cd25-group III isolates exhibited high-level resistance to 5-flucytosine (5-FC) (MIC $\geq$ 128 μg/ml), whereas all Cd25 group I and Cd25-group II isolates tested (10 Saudi Arabian and Egyptian, 16 Israeli and 24 international) were susceptible to 5-FC (MIC $\leq$ 0.125 μg/ml).

In addition, to investigate the molecular mechanisms of primary resistance to 5-FC, the complete ORFs of the *C. dubliniensis FUR1* and *FCY1* genes together with flanking sequences were amplified by PCR with primers specific for the *C. dubliniensis-FUR1* and *FCY1* genes. Amplimers of both genes were obtained and sequenced from the three 5-FC-resistant Saudi Arabian isolates SA100, SA107 (both genotype 3) and SA113 (genotype 4), from three 5-FC-susceptible Saudi Arabian isolates SA101, SA102 and SA105 (all genotype 1) and from the 5-FC-resistant Egyptian isolate Eg200 (genotype 4). These sequences were compared with the

corresponding sequences obtained from selected 5-FC-susceptible reference isolates from the *Gee et al.* study (2002), including CD36 and CM6 (genotype 1), CD506, Can 6 and CD541 (genotype 2), CD519 and p6265 (genotype 3) and p7718 (genotype 4). The results showed that the sequences of the *FCY1* gene and flanking sequences from all of the 5-FC-susceptible reference isolates tested and the 5-FC-susceptible Saudi Arabian genotype 1 isolates SA102 and SA105 were identical to the *FCY1* consensus sequence of the *C. dubliniensis* type strain CD36 obtained from the *C. dubliniensis* genome sequence database. The 5-FC-resistant Saudi Arabian isolates SA100, SA107 and SA113 and the 5-FC-resistant Egyptian isolate Eg200 each contained a single identical nucleotide polymorphism from (transition from A to T) at position 258 in the *FCY1* gene sequence that did not result in an amino acid residue change in the predicted protein. Comparison of the nucleotide sequences of the *FUR1* gene from the 5-FC-susceptible reference isolates and the 5-FC-susceptible and 5-FC-resistant Saudi Arabian and Egyptian isolates revealed the presence of several single nucleotide polymorphisms, none of which resulted in changes in amino acids in the predicted protein. These results demonstrated that polymorphisms in the *FCY1* and *FUR1* genes could not be associated with 5-FC resistance in the *C. dubliniensis* isolates tested.

In conclusion, the results of the present study report for the first time the presence of a novel 5-flucytosine-resistant clade of *C. dubliniensis* (Cd25 group III) that is predominant among isolates from Saudi Arabia and Egypt and absent from a previously characterised international collection of 98 isolates from 15 countries. Consequently, this study has helped to develop a more extensive understanding of the complexity and genetic diversity of *C. dubliniensis*. Furthermore, based on the comparative colony morphology and chlamydospore production of *C. dubliniensis* and *C. albicans* isolates on Pal's agar, it was shown that differential growth on Pal's agar is a 100% accurate, simple, inexpensive, rapid, easily applicable test that allows to distinguish *C. dubliniensis* isolates from *C. albicans* isolates.

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I wish to thank Dr. Derek Sullivan for his guidance, constructive comments, and for his important support throughout this work. I also wish to thank Dr. Sarah Gee; this work could not have been completed without her help and expertise. I am deeply indebted to Dr. Gary Moran and to Dr. Emmanuelle Pinjon, who spared much valuable time to provide me with technical help, stimulating suggestions and valuable hints. A big thank you also to Mary O'Donnell for her dedication to the well being of everyone in the laboratory and for making sure that the laboratory operated efficiently and to full capacity.

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Abbreviations

AIDS	acquired immunodeficiency syndrome
A405, A600	absorption at 450nm
ATP	adenosine 5' -triphospate
Azole	synthetic *N*-substituted azoles, including the imidazole and triazole antidungal antibiotics
BMD	broth micordilution
bp	base pair
BSA	bovine serum albumin
Bq	becquerel
cfu	colony forming units
Ci	curie
cm	centimetre
Δ	deletion (of a gene)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	dideoxynucleoside
EDTA	ethylenediamine tetraacetic acid
e.g.	for example
et al.	and others
g	gram
g	gravitational force
h	hour
HIV	human immunodeficiency virus
IC	inhibitory concentration
IPTG	isopropyl-ß-D-thiogalactopyranoside
i.e.	that is

kb	kilobase pair
L agar	Luria agar
L broth	Luria broth
l	litre
M	molar
Mb	megabase
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
MIC	minimum inhibitory concentration
min	minute
NCCLS	National Committee for Clinical laboratory Standards
nm	nanometre
no.	number
$O.D_X$	optical density at x nm
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDA	potato dextrose agar
PFGE	pulsed-field gel electrophoresis
p.f.u.	plaque forming units
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm.	revolutions per minute
s	second
sarcosyl	*N* - lauroylsarcosine, sodium salt
S_{AB}	simiarlity coefficient

SDS	sodium dodecyl sulphate
SSC	salt, sodium citrate
TBE	tris-borate EDTA
Tris	tris (hydroxymethyl) aminoethane
U.K.	United Kingdom
U.S.A.	United States of America
U.V.	ultraviolet
v/v	% "volume in volume" expresses the number of millilitres of an active constituent in 100 millilitres of solution
w/v	% "weight in volume" expresses the number of grams of an active constituent in 100 grams of solution or mixture
X-gal	5-bromo-4-chloro-indoyl-ß -D-galactoside
~	approximately
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to

Publications

Much of the work presented in this thesis has been published in international journals as listed below. Offprints of published papers are included at the end of the thesis.

1. **Al Mosaid, A. A., Sullivan, D., Polacheck, I., Shaheen, F. A., Soliman, O., Al Hedaithy, S. S., Al Thawad, S., Kabadaya, M. & Coleman, D.C. (2005)**. A novel 5-flucytosine resistant clade of *C. dubliniensis* from Saudi Arabia and Egypt identified by Cd25-fingerprinting. *J Clin Microbiol* **43**, 4026-4036.

2. **Sullivan, D. J., Moran, G. P., Pinjon, E., Al Mosaid, A. A., Stokes, C., Vaughan,C. & Coleman, D. C. (2004).** Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans. FEMS Yeast Res* **4**, 369-376.

3. **Al Mosaid, A. A., Sullivan, D. J. & Coleman, D. C. (2003).** Differentiation of *Candida dubliniensis* from *Candida albicans* on Pal's agar. *J Clin Microbiol* **41**, 4787-4789.

4. **Mosca, C. O., Moragues, M. D., Llovo, J., Al Mosaid, A. A., Coleman, D. C. & Pontón. J. (2003).** Casein agar: a useful medium for differentiating *Candida dubliniensis* from *Candida albicans. J Clin Microbiol* **41**, 1259-1262.

5. **Bagg, J., Sweeney, M. P., Lewis, M. A., Jackson, M. S., Coleman, D. C., Al Mosaid, A. A., Baxter, W., McEndrick, S. & McHugh, S. (2003).** High prevalence of non-albicans yeasts and detection of anti-fungal resistance in the oral flora of patients with advanced cancer. *Palliat Med* **17**, 477-481.

6. **Al Mosaid, A. A., Sullivan, D., Salkin, I. F., Shanley, D. & Coleman, D. C. (2001).** Differentiation of *Candida dubliniensis* from *Candida albicans* on Staib agar and caffeic acid-ferric citrate agar. *J Clin Microbiol* **39**, 323-327.

Publications

Chapter 1
General Introduction

1.1 *Candida* species and candidosis

Since the 1980s there has been a significant increase in the incidence of fungal infections of humans. Fungal species are now responsible for ~10% of all nosocomial bloodstream infections in the United States (Fridkin & Jarvis, 1996). More than 75% of these infections are caused by *Candida* species which now represent the fourth most common cause of bloodstream infections (Beck-Sague & Jarvis, 1993; Jarvis *et al.*, 1995). Although *Candida* species normally colonise mucosal surfaces or skin as harmless commensals, they are provided with opportunities to cause infection by changes in the host immune system or by disturbance of the oral commensal microflora. They remain one of the most common and versatile human fungal pathogens.

Candida species can cause superficial infections such as oropharyngeal and vaginal candidiasis as well as nosocomially acquired disseminated candidosis or candidaemia, during which multiple organs can be infected. Candidaemia frequently results in prolonged hospitalisation and has an attributable mortality estimated to be as high as 38% (Wey *et al.*, 1988). In one study based on hospital discharge data from the United States, the incidence of oropharyngeal candidosis was seen to increase by 4.7-fold, while the incidence of disseminated candidosis increased by a factor of 11 (Fisher-Hoch & Hutwagner, 1995). Oropharyngeal candidosis is the most common mycosis in AIDS patients and is often the first clinical manifestation of HIV infection (Ruhnke *et al.*, 2002). Indeed, more than 90% of individuals with progressive HIV disease develop oropharyngeal candidosis at some time during the course of their disease (Garber *et al.*, 2001). Vaginal carriage of *Candida* species and other yeasts is reported to range from 10 to 20% in healthy women (Ruhnke *et al.*, 2002). Although vulvovaginal candidosis is more common in HIV-infected women, it is estimated that as many as 75% of women, regardless of their HIV status, will experience at least one episode of the disease during their life time (Ruhnke *et al.*, 2002).

The increased significance of fungi is related to a variety of predisposing factors such as the increase in the number of immunocompromised individuals, especially those infected with HIV, and those receiving cancer chemotherapy and immunosuppressive treatments. The use of invasive medical procedures, prosthetic devices including dentures, indwelling venous lines and the widespread use of broad-spectrum antibiotics and corticosteroids have also added to the problem.

Candida albicans is the species of *Candida* most frequently associated with disease, and the most pathogenic *Candida* species (Coleman *et al.*, 1997a). However, in recent years there has been a significant shift in the *Candida* species identified as the aetiological agents of candidiasis (Moran *et al.*, 1998; Sullivan *et al.*, 2004). The incidence of infections caused by non-*C. albicans Candida* species such as *Candida tropicalis, Candida krusei,* and *Candida glabrata* has increased dramatically during the last decade (Coleman *et al.*, 1997a & b; Sullivan *et al.*, 2004). In a recent surveillance study of candidemia based on the general population, it was found that non-*C. albicans Candida* species were responsible for 55% of all bloodstream infections compared to 45% caused by *C. albicans* (Hajjeh *et al.*, 2004). The reason(s) behind this epidemiological shift are not clear. However, evidence is accumulating that the widespread therapeutic and prophylactic use of antifungal medications may have been a significant factor in the selection of these organisms as they can exhibit reduced susceptibility to commonly used antifungal drugs relative to *C. albicans* (Coleman *et al.*, 1997; Ponton *et al.*, 2000). It is generally accepted that *C. krusei* is inherently resistant to fluconazole (Rex *et al.*, 1995 & 1997; White *et al.* 1998). Also, several studies have reported that a significant proportion of *C. glabrata* isolates are resistant to fluconazole and itraconazole (Fidel *et al.*, 1999). Resistance to azole drugs has also been described in other non-*C. albicans Candida* species, including isolates of *C. dubliniensis, C. tropicalis* and *C. guilliermondii,* which are frequently recovered from HIV-infected patients following protracted azole therapy. In addition, the increased longevity of severely immunocompromised individuals has resulted in a larger population of extremely debilitated patients, allowing many of these species to cause disease.

1.2 Characterisation of *C. dubliniensis*

1.2.1 Emergence of *C. dubliniensis*

The identification and classification of fungal species has depended to a large extent on the analysis of a limited number of physiological and morphological traits, particularly those structures involved in sexual reproduction. The vast majority of species contained within the genus *Candida* are asexual and of simple morphology and subsequently *Candida* taxonomy is inherently problematic (Odds, 1988). Classically, species of the genus *Candida* were differentiated on their ability to form germ tubes, chlamydospore production

and by their carbohydrate- and nitrogen-source assimilation patterns (Sullivan *et al.*, 1996). However, these criteria have proved unreliable in defining species barriers, partly due to the variability of phenotypic properties between isolates of the same species. It is not surprising, therefore, that coinciding with the dramatic increase in fungal opportunistic infections in the immunocompromised patient group came reports of the isolation of unusual *Candida* species that were referred to as "atypical *C. albicans*".

In the early 1990s, several reports described the recovery of atypical *Candida* strains from HIV-infected individuals and AIDS patients in Australia, Ireland, Switzerland, and the UK (Schmid *et al.* 1992; Sullivan *et al.* 1993; Boerlin *et al.*, 1995; McCullough *et al.*, 1995). Schmid *et al.* (1992) described two atypical *C. albicans* oral isolates recovered from AIDS patients which yielded unusual DNA fingerprint patterns with the *C. albicans*-specific probe Ca3. Sullivan *et al.* (1993) also reported the isolation of atypical *Candida* isolates from a group of HIV-infected individuals with recurrent oral candidosis. Another research group while studying oral *C. albicans* strains from 60 HIV-infected patients over a 2.5-year period described 18 atypical *Candida* isolates among the 295 *C. albicans* isolates studied. These 18 isolates produced greater amounts of extracellular proteinase, adhered more to buccal epithelial cells, and were less susceptible to the antifungal drug 5-flucytosine (5-FC) (McCullough *et al.*, 1995). Further reports of similar isolates appeared in the literature from other countries such as Switzerland and the UK (Schmid *et al.*, 1992; Boerlin *et al.*, 1995). These reports showed that the atypical *Candida* isolates shared similar features with *C. albicans* and the closely related species *C. stellatoidea,* which merited further studies to analyse the taxonomic relationship between theses species.

The atypical *Candida* isolates were phenotypically very similar to *C. albicans* in that they produced germ tubes and chlamydospores, features originally diagnostic for *C. albicans*. However, many of these isolates were found to yield atypical carbohydrate- and nitrogen-source substrate assimilation profiles when analysed with commercially available yeast identification systems such as the API 20C AUX and API ID 32C systems, which did not correspond to any known *Candida* species (Sullivan *et al.*, 1995). *Candida stellatoidea*, however, has been divided into two types termed type I and type II, respectively. Both types react with antisera raised against *C. albicans* serotype B antigenic factor but unlike *C. albicans* are incapable of assimilating sucrose (Kwon-Chung *et al.*, 1989). The atypical *C. albicans* isolates- referred to above, were sucrose-positive and have the ability to

agglutinate to *C. albicans* serotype A antiserum which distinguished them from *C. stellatoidea.*

Furthermore, *C. stellatoidea* type I isolates are generally accepted to be a subgroup of *C. albicans* since they generate distinct karyotype profiles from those of *C. albicans* or type II *C. stellatoidea.* They also produce different fingerprint patterns with the *C. albicans* fingerprinting probes 27A and Ca3 from those of *C. albicans* or type II *C. stellatoidea* (Kwon-Chung *et al.*, 1988 & 1989). In addition, fingerprinting profiles of *C. stellatoidea* type II obtained with the 27A probe are similar to those exhibited by *C. albicans* and subsequently type II are now referred to as "sucrosed-negative *C. albicans*"(Kwon-Chung *et al.*, 1990). However, DNA fingerprint patterns of atypical *C. albicans* isolates generated with the *C. albicans*-specific mid-repeat sequence DNA fingerprinting probe 27A and Ca3, karyotype analysis and random amplified polymorphic DNA analysis (RAPD) yielded different fingerprint profiles, in each case, to those obtained with *C. albicans* or type I or type II *C. stellatoidea* (Schmid *et al.*, 1992; Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995). Fingerprint profiles of the atypical *Candida* isolates hybridized with *C. albicans*-specific fingerprinting probes 27A contained only 4 to 7 weak bands, ranging in size between 500 bp and 20 kb compared to that of *C. albicans*, which yielded 10 to 15 stronger hybridising bands ranging in size between 500 bp and 20 kb (Sullivan *et al.*, 1995).

In 1995, when Sullivan *et al.* performed detailed phenotypic, genotypic and phylogenetic studies on these atypical isolates from Ireland, Australia and the UK, they concluded that they constitute a novel species for which the name *C. dubliniensis* was proposed.

1.2.2 Phenotypic properties of *C. dubliniensis*

Unlike other *Candida* species, the ability of *C. albicans* to produce germ tubes and chlamydospores were traits previously considered diagnostic for this species. However, *C. dubliniensis* is also capable of germ tube production upon incubation in serum (Sullivan *et al.*, 1995), although, unlike *C. albicans*, it does not produce germ tubes when incubated in N-acetylglucosamine-containing medium (Gilfillan *et al.*, 1998). *Candida dubliniensis* also produces chlamydospores when grown on media such as rice Tween 80 agar (RAT), Tween 80-oxgall-caffeic acid (TOC) or cornmeal agar (Sullivan *et al.*, 1995; Jabra-Rizk *et al.*, 1999a; Koehler *et al.*, 1999). In contrast to *C. albicans* isolates, which tend to produce single chlamydospores attached terminally to pseudohyphae by a single suspensor cell, isolates of *C. dubliniensis* tend to produce abundant chlamydospores, frequently arranged in contiguous pairs, triplets or larger groups attached to a single suspensor cell (Sullivan, 1995). However, this unusual feature has not been shown to be reproducible for every *C. dubliniensis* isolate in a number of laboratories (Kirkpatrick *et al*, 1998; Schoofs *et al.*, 1997). A characteristic feature of *C. dubliniensis*, in contrast to *C. albicans*, is that the vast majority of *C. dubliniensis* isolates produce chlamydospores on Staib agar. *Candida dubliniensis* isolates also form rough colonies when incubated at 30°C on Staib agar, whereas *C. albicans* do not (Staib & Morschhäuser, 1999).

Candida albicans and type II *C. stellatoidea* can be serotyped as either serotype A or serotype B on the basis of agglutination reactions with antisera raised against *Candida* antigenic factor number 6 (Odds, 1988; Kwon-Chung *et al.*, 1989). In contrast, type I *C. stellatoidea* which is genetically distinct from *C. albicans* as demonstrated by karyotype analysis and by DNA fingerprinting with the *C. albicans*-specific probe Ca3, always belong to serotype B (Kwon-Chung *et al.*, 1989). To date, all isolates of *C. dubliniensis* tested have been found to react with *C. albicans* serotype A antiserum as determined by agglutination reactions with antibodies raised against *Candida* antigenic factor No. 6, and with serotype A-specific antisera using flow cytometry (Sullivan *et al.*, 1995; Mercure *et al.*, 1996). On this basis isolates of *C. dubliniensis* can be distinguish from type I *C. stellatoidea* isolates.

Isolates of *C. dubliniensis* grow well at 30°C and 37°C on all mycological culture media used for the culture of *Candida* species (Sullivan *et al.*, 1995). However, *C. dubliniensis* isolates were observed to grow poorly or not at all at 42°C and not at all at

45°C, unlike the majority of *C. albicans* isolates which grow well at this temperatures (Sullivan *et al.*, 1995; Pinjon *et al.*, 1998). Growth at a temperature of 45°C was suggested as the basis of a simple test to distinguish between *C. dubliniensis* and *C. albicans* (Pinjon *et al.*, 1998), however, some isolates of *C. albicans* do not grow at this elevated temperature (Kirkpatrick *et al.*, 1998; Gales *et al.*, 1999).

Isolates of the closely related species *C. dubliniensis* and *C. albicans* produce white creamy colonies on conventional mycological media such as Sabouraud dextrose agar or potato dextrose agar (PDA) thus the two species are indistinguishable on such media. However, CHROMagar® Candida medium can be used for the preliminary identification of several clinically important *Candida* species including *C. dubliniensis* (Odds & Bernaerts, 1994; Sullivan & Coleman, 1998). Colonies of *C. dubliniensis* appear dark green in colour when cultured on the chromogenic medium CHROMagar Candida® on primary isolation from a clinical specimen following growth at 37°C for 48 h. In contrast, *C. albicans* colonies are a light blue-green colour when grown under the same conditions on CHROMagar Candida medium (Schoofs *et al.*, 1997; Sullivan & Coleman, 1998; Koehler *et al.*, 1999; Coleman *et al.*, 1997b). However, this medium is only useful for the preliminary isolation of *C. dubliniensis* following primary culture from clinical specimens as its distinctive colony colouration may be lost following storage and subculture (Schoofs *et al.*, 1997; Tintelnot *et al.*, 2000).

Yeast identification systems are most commonly used for identifying *Candida* species and are used routinely in diagnostic laboratories. These systems make use of the ability of different species to assimilate a variety of compounds that can be used as the sole source of carbon or nitrogen or on the detection of different enzyme activities. The pattern of substrate assimilation yields a numerical code, which is then compared to a database and leads to the identification of the isolate (Sullivan *et al.*, 1995; Gales *et al.*, 1999; Pincus *et al.*, 1999). Initially, Sullivan *et al.* (1993) noted that the codes given by *C. dubliniensis* isolates were similar to those obtained with isolates of type I *C. stellatoidea*, the difference being due to the ability of *C. dubliniensis* isolates to assimilate sucrose. Accordingly, they suggested that their atypical isolates might be sucrose positive variants of type I *C. stellatoidea*, although they possessed a different serotype (Sullivan *et al.*, 1993). Prior to the inclusion of *C. dubliniensis* in the databases of commercially available yeast identification systems such as the bioMérieux API ID 32C and API 20C AUX systems, *C.*

dubliniensis profiles did not match any known profiles in the databases or gave profiles corresponding to poor identification of species such as *C. stellatoidea*, *C. sake* and *C. colliculosa* (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995; Coleman *et al.*, 1997a; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Kirkpatrick *et al.*, 1998; Salkin *et al.*, 1998; Gales *et al.*, 1999; Jabra-Rizk *et al.*, 1999a; Tintelnot *et al.*, 2000). However, despite the introduction of a limited number of *C. dubliniensis* profiles in the databases in 1998, it has been reported that, in order to allow reliable identification, the databases should be further modified to include more *C. dubliniensis* profiles (Pincus *et al.*, 1999).

Many of the phenotypic properties of *C. dubliniensis* described above have been used for distinguishing isolates of *C. dubliniensis* from *C. albicans*. However, the majority of these phenotypic properties are not definitive for *C. dubliniensis* and therefore in order to achieve definitive identification of *C. dubliniensis* molecular methods based on genotypic characteristics (discussed below) need to be used.

1.2.3 Genotypic properties of *C. dubliniensis*

Candida dubliniensis possesses distinct genetic characteristics that originally led to it being designated as a separate species. When *Eco*RI-digested genomic DNA from *C. albican, C. dubliniensis* and *C. stellatoidea* were probed with the moderately repetitive *C. albicans* fingerprinting probes 27A and Ca3, the first evidence that suggested that *C. dubliniensis* isolates were genetically distinct from other *Candida* species was elucidated (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995). While the 27A probe generates fingerprint patterns consisting of 10-15 strongly hybridising bands with *C. albicans* DNA and with type I and type II *C. stellatoidea* DNA (Scherer & Stevens, 1988) the fingerprint patterns obtained for the *C. dubliniensis* isolates consisted of 4-7 weakly hybridising bands (McCullough *et al.*, 1995; Sullivan *et al.*, 1995). Similarly, the Ca3 fingerprinting probe hybridised weakly to *C. dubliniensis* genomic DNA while it gives strong hybridisation bands with *C. albicans* genomic DNA (Boerlin *et al*, 1995).

Other distinct fingerprint profiles were generated when the *C. dubliniensis* genomic DNA was digested with the restriction endonuclease *Hin*fI. These profiles were different from the corresponding *Hin*fI RFLP patterns of *C. albicans*, or type I and type II *C. stellatoidea* (Sullivan *et al.*, 1995, 1996 and 1997; Sullivan & Coleman, 1998; Kirkpatrick

et al., 1998; McCullough *et al.*, 1999a). Furthermore, *C. dubliniensis* can be readily distinguished from *C. albicans* and type I *C. stellatoidea* on the basis of the fingerprinting patterns obtained with the five oligonucleotide probes $(GGAT)_4$, $(GACA)_4$, $(GATA)_4$, $(GT)_8$ and $(GTG)_5$ and by RAPD analysis (Sullivan *et al.*, 1993 & 1995).

Multilocus enzyme electrophoresis (MEE) studies on *C. dubliniensis* isolates further confirmed the taxonomic position of the novel species as genetically very divergent from other species examined including *C. albicans* or type 1 *C. stellatoidea* (Boerlin *et al.*, 1995; Pujol *et al.*, 1997). Furthermore, very little intraspecies divergence was observed by MEE with *C. dubliniensis* (Boerlin *et al.*, 1995; Pujol *et al.*, 1997).

In addition, *C. dubliniensis* isolates have distinctive karyotype patterns consisting of nine or ten chromosome sized DNA bands detected by pulsed-field gel-electrophoresis (PFGE), usually with one or more bands less than 1 Mb in size, a feature shared with isolates of type I *C. stellatoidea.* In contrast *C. albicans* isolates and type II *C. stellatoidea*, typically yield seven distinct chromosome sized DNA bands by PFGE (Sullivan *et al.*, 1995).

In 1999 Joly *et al.* developed a fingerprinting probe (Cd25) for *C. dubliniensis* having identified a species-specific repetitive DNA element in this species. Computer-assisted analysis of Cd 25-fingerprint profiles from 57 isolates collected from 11 countries revealed that the isolates could be separated into two distinct groups termed group I and group II isolates. Group I isolates were very closely related (average similarity coefficient (S_{AB}) of 0.80) whereas group II isolates were less closely related (S_{AB}=0.47). The isolation of a species-specific repetitive element provides further evidence that *C. dubliniensis* is indeed a distinct species (Joly *et al.*, 1999). Later, Gee *et al.* (2002) carried out a more exhaustive study with 94 isolates from 15 different countries and confirmed the separation of the *C. dubliniensis* population into two different groups, Cd25 group I and II (Gee *et al.*, 2002). In addition, Gee *et al.* (2002) analysed and sequenced the internal transcribed spacer regions (ITS) of the rRNA gene cluster of 19 *C. dubliniensis* isolates which led to the identification of four distinct genotypes among *C. dubliniensis* (Gee *et al.*, 2002). All *C. dubliniensis* isolates belonging to Cd25 group I were found to belong to genotype 1 while the Cd25 group II isolates comprised of three distinct genotypes (genotypes 2, 3 and 4) (Gee *et al.*, 2002). To facilitate epidemiological studies, Gee *et al.* (2002) developed a genotype-specific PCR assay based on sequence differences in the ITS region between the four

genotypes (Gee *et al.*, 2002). The results of this study should facilitate future investigations of the epidemiological relevance of each genotype group of *C. dubliniensis.*

1.2.4 Phylogenetic analysis of *C. dubliniensis*

Although molecular analysis has demonstrated that *C. dubliniensis* is in fact a separate species, many researchers analysed ribosomal, non-ribosomal and mitochondrion nucleotide sequences in order to determine the phylogenetic relationships between *C. dubliniensis* and *C. albicans* or other *Candida* species.

In the first study describing *C. dubliniensis*, Sullivan *et al.* (1995) compared the sequence of 500 bp of the V3 variable region of the large rRNA subunit genes from nine isolates of *C. dubliniensis* and from reference strains of *C. albicans*, *C. stellatoidea*, *C. tropicalis*, *C. glabrata*, *C. kefyr* and *C. krusei*. A phylogenetic tree was produced based on these sequences in which *C. dubliniensis* isolates formed a homogeneous cluster which were 100% identical and significantly different from the other *Candida* species analysed (Sullivan *et al.*, 1995). The *C. dubliniensis* sequence was 2.3% divergent from the corresponding *C. albicans* sequence. This study also indicated that *C. albicans* and *C. stellatoidea* were so closely related (0-0.02 % sequence divergence) as to be considered a single species (Sullivan *et al.*, 1995*)*. These results were later confirmed by the analysis of the V3 region from a further five *C. dubliniensis* isolates from Ireland, the UK, Argentina and Switzerland (Sullivan *et al.*, 1997). The unique phylogenetic position of *C. dubliniensis* was later analysed further when Gilfillan *et al.* (1998) compared the complete nucleotide sequence of the entire small rRNA gene (SSU rRNA) from the type strain of *C. dubliniensis* with the SSU rRNA genes from *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. lusitaniae*, *C. krusei* and *Saccharomyces cerevisiae*. *Candida dubliniensis* was found to exhibit 1.4% sequence divergence from *C. albicans* (Fig. 1.1). Furthermore, Kurtzmann & Robnett, (1997) analysed the sequence of the 5' end of the large rRNA subunit genes from a wide variety of different yeasts. The sequence difference between *C. dubliniensis* and *C. albicans* was found to be about 2.2%. Another phylogenetic study which complements previous studies on rRNA gene sequences was the work of Boucher *et al.*, (1996) who analysed the D1/D2 variable region of the large subunit rRNA gene from all known clinically significant yeasts. The authors showed that *C. dubliniensis* harbours a group I

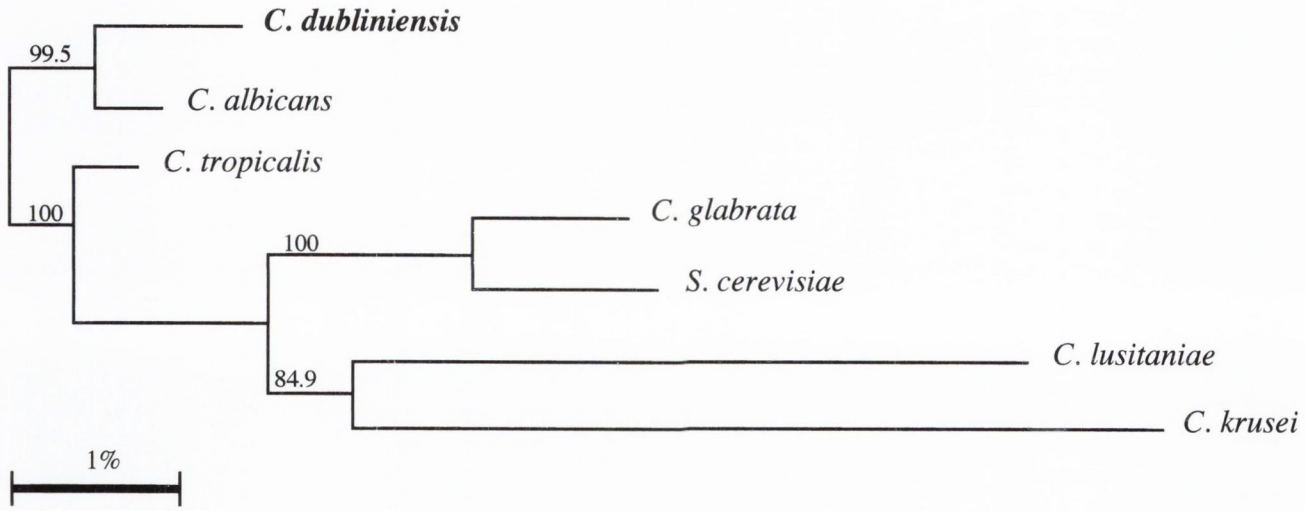


Figure 1.1 An unrooted phylogenetic neighbour joining tree generated from the alignment of the sequences encoding the small rRNA genes from *C. dubliniensis* CD36 and different non-*C. albicans* species. The scale bar represents a 1% difference in nucleotide sequence. The numbers at each node were generated by bootstrap analysis and represent the percentage of times the arrangement occurred in 1,000 randomly generated trees. Reproduced from Gilfillan *et al.* (1998).

self-splicing intron in the large ribosomal RNA as observed in about 40% of *C. albicans* strains and all *C. stellatoidea* strains. However, the *C. dubliniensis* group I intron has two widely divergent stem-loop regions when compared to the *C. albicans* intron and the corresponding sequences from a variety of yeast species. Furthermore, the sequence of the group I intron from a number of *C. dubliniensis* isolates revealed an intraspecies sequence conservation which was also observed in the introns sequenced from different isolates of *C. albicans* and *C. stellatoidea* (Boucher *et al.*, 1996).

Other researchers have reached similar conclusions on the basis of comparisons between the nucleotide sequences of a wide range of genes from *C. dubliniensis* and from other *Candida* species. Donnelly *et al.* (1999) was able to design *C. dubliniensis*-specific PCR primers based on significant divergence between the *C. dubliniensis* and *C. albicans ACT1* intron sequences. Comparison of the *CdACT1* sequence with the *C. albicans* homologue (*CaACT1*) revealed that although the exons are 97.9 % identical the introns are only 83.4 % identical (Donnelly *et al.*, 1999). Moran *et al.* (2002), on the other hand, cloned and sequenced the *C. dubliniensis* homolog of *CaMDR1*, termed *CdMDR1* which was found to be 92% identical to the corresponding *CaMDR1* sequence (Moran *et al.*, 1998). This was equivalent (92%) to the divergence observed between the two species when the *C. dubliniensis CdCDR1* and the corresponding homolog in *C. albicans* (*CDR1)* were sequenced (*Moran et al.*, 2002).

In an attempt to develop a reliable molecular-based discriminatory method to differentiate between *C. dubliniensis* and *C. albicans*, Kurzai *et al.* (1999) analysed the *PHR1* and *PHR2* structural genes in both species. The investigators found that the nucleotide sequences of *C. dubliniensis* and the corresponding *C. albicans* sequences, were 90.2%, 91.2% identical, respectively.

Moran *et al.*, (2004) used *C. albicans* whole genome DNA microarrays to perform comparative genomic hybridization study between *C. albicans* and *C. dubliniensis* in order to identify genomic differences that might explain the difference in virulence between both species. The results indicated that *C. dubliniensis* genomic DNA demonstrated a significant degree of nucleotide sequence homology (> 60 %) with the *C. albicans* gene-specific sequences. However, 4.4 % of the sequences (representing 247 genes) exhibited significant sequence divergence (< 60 % homology) or absence in *C. dubliniensis* (Moran *et al.*, 2004)

Indeed, all of these phylogenetic studies provided convincing evidence of the unique taxonomic position of *C. dubliniensis* within the genus *Candida* and for its designation as a separate species.

1.2.5 Identification of *C. dubliniensis* from clinical specimens

It is imperative that the epidemiology and clinical importance of *C. dubliniensis* be elucidated, especially with the emergence of antifungal drug resistant clinical isolates which pose a clinical challenge. In order to achieve this, simple, reliable, and inexpensive identification tests, which meet the demands of standard clinical laboratories need to be developed and introduced. Finding such a method is complicated by the fact that *C. dubliniensis* shares many phenotypic traits with *C. albicans*.

1.2.5.1 Phenotypic based tests

1.2.5.1.1 Growth on different agar media

Many studies have described a variety of culture media for differentiating isolates of *C. dubliniensis* from *C. albicans*, but many of these have been described as being unreliable. However, these media can be used for the presumptive identification of *C. dubliniensis* isolates. Perhaps the most important aid in the analysis of *Candida* populations in clinical specimens has been the development of CHROMagar® Candida medium (Odds & Bernaerts, 1994). On CHROMagar Candida medium isolates of *C. albicans*, *C. tropicalis, C. krusei, C. glabrata*, and. *C. parapsilosis* usually form light green, blue, pale pink with rough hyphal fringe, dark pink with lighter hyphal fringe and white-purple colonies, respectively (Bernal *et al.*, 2004). However, as mentioned previously, although *C. dubliniensis* produces distinctive dark green colonies on this agar on primary isolation, this property can be lost following subculture or storage at -70°C (Schoofs *et al.*, 1997). Furthermore, incubation conditions when using this medium are critical as prolonged growth of *C. dubliniensis* on this medium results in an appearance similar to *C. albicans* (Schoofs *et al.*, 1997; Pfaller *et al.*, 1999a). Others have reported that *C. albicans* colonies may produce a similar dark colour to *C. dubliniensis* (Schoofs *et al.*, 1997; Tintelnot *et al.*, 2000). Tintelnot *et al.* (2000) studied 170 light and dark green colonies following primary

isolation on CHROMagar® from a total of 150 oral rinse samples and only 30 of the 53 *C. dubliniensis* isolates recovered showed the dark green pigmentation. They concluded that both species can produce light green coloured colonies on CHROMagar® Candida medium following primary culture indicating this medium was not reliable for the differentiation of *C. dubliniensis* and *C. albicans* (Tintelnot *et al.*, 2000). Tintelnot *et al.* (2000) concluded that if CHROMagar® is used for the primary identification of *C. dubliniensis* the actual prevalence of *C. dubliniensis* will be underestimated. For these reasons, it has been recommended that this agar be used only for the presumptive identification of *C. dubliniensis* on primary isolation from clinical specimens (Sullivan *et al.*, 1999).

On Methyl blue-Sabouraud agar, *C. albicans* colonies fluoresce with a yellow colour under Woods lamp illumination (UV), whereas colonies of *C. dubliniensis* do not. However, an absence of fluorescence has been observed in some isolates of *C. albicans* (Schoofs *et al.*, 1997).

Velegraki & Logotheti, (1998) reported that *C. dubliniensis* isolates reduce the compound 2,3,5-triphenyltetrazolium chloride from red to maroon colonies on Pagano-Levin agar. In contrast, the colour of *C. albicans* colonies on this medium varies from whitish to pale pink. However, examination of the colonial morphology of 50 isolates each of *C. dubliniensis* and *C. albicans* on agar containing 2,3,5-triphenyltetrazolium chloride revealed that the colour of the *C. dubliniensis* colonies ranged from white through pink to purple, and were indistinguishable from *C. albicans* colonies (S. Donnelly & D. Coleman, personal communication).

Tween 80 medium is another differential agar that has been used to differentiate *C. dubliniensis* from *C. albicans.* It indicates whether an isolate produces esterase when grown on the medium by the presence of a hyphal fringe around an inoculated site on the Tween medium 2 to 3 days post-inoculation. Slifkin (2000) reported that *C. albicans* isolates (n= 15) tested produced a hyphal fringe on this medium but *C. dubliniensis* isolates (n=16) did not.

1.2.5.1.2 Chlamydospore production, germ tube production and serotyping of C. dubliniensis

The ability of both *C. dubliniensis* and *C. albicans* to produce germ tubes and chlamydospores has contributed to the misidentification of some *C. dubliniensis* isolates as

C. albicans (Sullivan *et al.*, 1995; Odds *et al.*, 1998; Jabra-Rizk, 2000). Prior to the first description of *C. dubliniensis* in 1995, chlamydospores and germ tubes formation were commonly used to for the definitive identification of *C. albicans* clinical isolates. However, *C. dubliniensis* also produces chlamydospores often in contiguous pairs, triplets or larger groups when grown on media such as rice Tween 80 agar (RAT), Tween 80-oxgall-caffeic acid (TOC) or cornmeal agar, whereas *C. albicans* isolates usually produce single chlamydospore attached to a single suspensor cell (Sullivan *et al.*, 1995; Jabra-Rizk *et al.*, 1999a; Koehler *et al.*, 1999). However, the formation of multiple chlamydospores attached to a single suspensor cell has not been found to be reproducible with all *C. dubliniensis* isolates tested and, therefore, chlamydospore production as a single phenotypic test, can not be used to differentiate between *C. albicans* and *C. dubliniensis* (Schoofs *et al.*, 1997; Kirkpatrick *et al.*, 1998).

All isolates of *C. dubliniensis* tested to date have been found to react with *C. albicans* serotype A antiserum as determined by agglutination reactions with antibodies raised against *Candida* antigenic factor No. 6, and with serotype A-specific antisera using flow cytometry hence classifying them as serotype A (Sullivan *et al.*, 1995; Mercure *et al.*, 1996). Furthermore, several studies have shown that *C. dubliniensis,* like *C. albicans,* is also capable of germ tube production upon incubation in serum (Sullivan *et al.*, 1995), although, unlike *C. albicans*, it does not produce germ tubes when incubated in N-acetyl glucosamine-containing medium (Gilfillan *et al.*, 1998).

1.2.5.1.3 Lack of growth at 42˚C and 45˚C

The inability of *C. dubliniensis* strains to grow at 42˚C compared to *C. albicans* strains was first thought to be a potentially useful test for differentiating *C. dubliniensis* from *C. albicans* (Sullivan *et al.*, 1995). However, reports of *C. dubliniensis* isolates that were able to grow poorly or well at 42˚C soon appeared in the literature (Coleman *et al.*, 1997a & 1997b; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Sullivan & Coleman, 1998; Kirkpatrick *et al.*, 1998; Pinjon *et al.*, 1998). Subsequently, a study that investigated the growth of *C. dubliniensis* at 45˚C suggested that *C. dubliniensis* isolates could be discriminated from *C. albicans* isolates based on the inability of the former to grow at 45˚C (Pinjon *et al.*, 1998). Although to date no *C. dubliniensis* isolates that can grow at 45˚C have been reported, Kirkpatrick *et al.* (1998) described *C. albicans* isolates that were

unable to grow at 45°C. In another study, Gales *et al.* (1999) studied 100 *C. albicans* isolates out of which 23 (23%) yielded poor or no growth at 45°C. Therefore growth of *C. dubliniensis* isolates at 45°C can not be used as a reliable method for the differentiation of *C. dubliniensis* from *C. albicans*.

1.2.5.1.4 Carbohydrate assimilation profiles

A variety of commercially available yeast identification systems including the API ID 32C, the API 20C AUX, the RapID Yeast Plus, the VITEK YBC and the VITEK 2 ID-YST systems have been used to discriminate between isolates of *C. dubliniensis* and other yeast species. However, prior to updating the respective system databases to include *C. dubliniensis* profile, these systems provided numerical codes that did not correspond with any known species. In addition, some isolates generated codes that gave low discrimination profiles which corresponded to poor identification of species such as *C. stellatoidea*, and *C. sake* (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995; Coleman *et al.*, 1997a; Schoofs *et al.*, 1997; Sullivan *et al.*, 1997; Kirkpatrick *et al.*, 1998; Salkin *et al.*, 1998; Gales *et al.*, 1999; Jabra-Rizk *et al.*, 1999a; Tintelnot *et al.*, 2000). Pincus *et al.* (1999) studied the efficacy of the API 20C AUX, ID 32 C, RapID Yeast Plus, VITEK 2 ID-YST and VITEK YBC systems to provide rapid, accurate and reproducible identification of *C. dubliniensis*. The substrate reactivity profiles of 80 *C. dubliniensis* isolates were compared with that of *C. albicans* profiles in each system database and the results were expressed as percent *C. dubliniensis* and percent *C. albicans*. Any substrate that showed >50% difference in reactivity was considered useful in differentiating the species. In general, Pincus *et al.* (1999) found that assimilation of methyl-alpha-D-glucoside (MDG), trehalose (TRE), and D-xylose (XYL) proved to be the most useful for species differentiation by the majority of the commercial systems they tested. Using the API 20C AUX and the API ID 32 C systems, Pincus *et al.* (1999) found that 15% and 30%, respectively, of the 80 *C. dubliniensis* isolates they tested were TRE positive. This finding is in contrast to what is provided in the databases of these systems which indicates that *C. dubliniensis* is TRE negative. In addition, the data indicated that XYL assimilation was the most effective test in separating *C. dubliniensis* from *C. albicans*. Using the API 20C AUX and the API ID 32 C systems, Pincus *et al.* (1999) found that 100% and 100%, respectively, of the 80 *C. dubliniensis* isolates they tested were XYL negative while 88% and 98%,

respectively, of the *C. albicans* were XYL positive. This finding is in contrast to what is provided in the databases of these systems which indicates that *Calbicans* is XYL negative Therefore, Pincus *et al.* (1999) concluded that the incorporation of this variability in a future update of the databases would correctly identify all isolates that were misidentified or unidentified. As indicated in section 1.2.2, the API yeast identification system databases were updated in 1998 to include a limited number of *C. dubliniensis* isolates profiles. However, clearly there is still a need for more extensive *C. dubliniensis* profiles to be included in the system databases to correctly and reliably identify *C. dubliniensis* isolates. This need was confirmed in subsequent studies; Tintelnot *et al.* (2000) who tested the assimilation profiles of 53 *C. dubliniensis* isolates with the API ID 32C yeast identification system but only two isolates were correctly identified as *C. dubliniensis*, whereas the remaining isolates were incorrectly identified as *C. albicans*.

1.2.5.1.5 intracellular β-glucosidase test

Atypical isolates which were subsequently identified as *C. dubliniensis* were characterised as lacking intracellular β-glucosidase activity (Boerlin *et al.*, 1995). Subsequently an assay was developed to distinguish *C. dubliniensis* from *C. albicans* based on this feature (Schoofs *et al.*, 1997). Odds *et al.* (1998) reported that 12.4% (67/537) of *C. albicans* isolates tested did not express β-D-glucosidase activity (Odds *et al.*, 1998). However, Tintelnot *et al.* (2000) showed that 13% of *C. albicans* tested showed no β-glucosidase activity and hence this phenotypic trait can not be used to differentiate between *C. dubliniensis* and *C. albicans*.

1.2.5.1.6 Pyrolysis-mass spectrometry, Fourier transform-infrared spectroscopy and gas liquid chromatography

Sophisticated techniques such as Fourier transform infrared spectroscopy (FT-IR), Pyrolysis mass spectrometry (PyMS) and fatty acid methyl ester analysis using gas liquid chromatography are not widely available in routine clinical diagnostic laboratories and are therefore not suitable for the routine identification of *C. dubliniensis* (Timmins *et al.*, 1998; Tintlenot *et al.*, 2000; Peltroche-Llacsahuanga *et al.*, 2000a). Tintelnot *at al.* (2000) used FT-IR to successfully discriminate isolates of *C. dubliniensis* from *C. albicans*. However, since this technique is based on detection of the composition and structures of all cell

compounds within an intact cell environment, the discrimination is achieved only when multiple statistical studies are applied to the analysis of a very complex spectral data (Tintelnot *et al.*, 2000). Fourier transform infrared spectroscopy (FT-IR) and Pyrolysis mass spectrometry (PyMS) were also used to analyze a group of 29 clinical and reference *Candida* isolates. Both techniques were shown to be capable of successfully discriminating between isolates of *C. albicans*, *C. dubliniensis*, and *C. stellatoidea* in a relatively short time (2 min for PyMS and 10 s for FT-IR) (Timmins *et al.* 1998).

1.2.5.1.7 Other phenotypic tests

Jabra-Rizk *et al.* (1999b) showed that only *C. dubliniensis* and not *C. albicans* coaggregates with *Fusobacterium nucleatum* when grown at 37°C. *Fusobacterium nucleatum* and other fusobacteria constitute the largest portion of the microbial population in dental plaque. Therefore, *C. dubliniensis* aggregation with *Fusobacterium nucleatum* may contribute to the ability of *C. dubliniensis* to colonise the oral cavity. Subsequently Jabra-Rizk *et al.* (1999b) suggested a test to discriminate between both species based on this characteristic. The test was proven to be simple, fast, inexpensive and suitable for use in **routine** diagnostic laboratories. Another test based on immunological detection and differential localisation of antigens on *C. dubliniensis* blastospores and *C. albicans* germ tubes was reported by Bikandi *et al.* (1998). This method is very fast, it takes less than 2 h to perform and has been tested on 85 and 49 clinical isolates of *C. dubliniensis* and *C. albicans*, respectively (Bikandi *et al.*, 1998). The anti-*C. dubliniensis* sera, which was raised in rabbits, reacted with specific components of 25, 28, 37, 40, 52, and 62 kDa in the *C. dubliniensis* extract and was able to differentially label all tested *C. dubliniensis* isolates in an indirect immunofluorescence test.

More recently an immunochromatographic membrane (ICM) test assay was developed to aid in the differentiation of *C. dubliniensis*. The advantages of this assay included speed, easy interpretation of results and no complex instruments were needed to carry out the test (Marot-Leblond *et al*, 2004).

1.2.5.2 Polymerase chain reaction (PCR)-based methods

As described above, a wide variety of phenotypic tests for *C. dubliniensis* have been described in the literature, however, to date no single phenotypic characteristic is sufficient for the definitive identification of this species. This is due to the fact that *C. dubliniensis* shares many phenotypic characteristics with *C. albicans*. Yet both species differ significantly at the genetic level. As a result, the most discriminatory methods available to date are molecular based methods such as PCR.

Since the first description of *C. dubliniensis* as a distinct species within the genus *Candida,* several PCR based techniques have been developed for the identification of *C. dubliniensis* and for its differentiation from *C. albicans*. PCR techniques are usually easy to perform, rapid, specific, and amenable to automation and to a large sample volume throughput. It is also a technique that is increasingly available to diagnostic laboratories world-wide.

Donnelly *et al.* (1999) developed a *C. dubliniensis*-specific PCR test using primers based on the *ACT1*-associated intron sequence of *C. dubliniensis* which shares 83.4% identity with the *C. albicans* sequence. These primers were used to amplify a DNA **fragment** of 288 bp from *C. dubliniensis* template DNA. An extensive evaluation of this PCR method carried out using 122 *C. dubliniensis* isolates, 53 *C. albicans* isolates and a number of isolates from other *Candida* species showed that positive identification of *C. dubliniensis* could be obtained in 4 h (Donnelly *et al.*, 1999).

A number of other PCR methods have also been developed based on sequence differences between *C. albicans* and *C. dubliniensis* in the internal transcribed spacer region (ITS) of the ribosomal gene cluster. Elie *et al.* (1998) tested 5 *C. dubliniensis* using *C. dubliniensis*-specific primers that target the internal transcribed spacer region (ITS2) of the ribosomal gene cluster in different yeast species. Their method, however, was time consuming since it involved a PCR-ELISA. Similarly, the PCR-based line probe assay of Martin *et al.* (2000) is complex and would not be routinely available in a diagnostic laboratory. In one study, the sequence of the ITS2 region was used to develop a molecular beacon probe for identification of *C. dubliniensis* as well as *C. albicans* (Park *et al.*, 2000). The designed probes were 100% accurate in identifying both species correctly. However,

the technique was applied to an unknown collection of 23 strains that largely contained *C. albicans* and a smaller number of *C. dubliniensis* (Park *et al.*, 2000)

In other studies, restriction fragment length polymorphism of the amplified ITS1 and ITS2 regions was used as a means for distinguishing *C. albicans* and *C. dubliniensis* (Irobi *et al.*, 1999; McCullough *et al.*, 1999a; Williams *et al.*, 2001; Graf *et al.*, 2004). This method is relatively simple to apply but further manipulation of the PCR products are required thereby adding to the time before a positive identification for *C. dubliniensis* can be made.

Other PCR based methods for identifying *C. dubliniensis* and for distinguishing *C. albicans* from *C. dubliniensis* have also been developed. Mannarelli & Kurtzman (1998) designed primers based upon the D1/D2 region of the large subunit rRNA gene. However, the primers were only tested against seven *C. dubliniensis* isolates. Kurzai *et al.* (1999) designed primers based upon sequence differences in the pH-regulated *PHR1* and *PHR2* genes of the two species. These primers were designed specifically to identify *C. albicans* and hence a negative test will require additional experiment for definitive identification of *C. dubliniensis*.

1.2.5.3 Molecular typing tests for identification of *C. dubliniensis*

As previously discussed, the most reliable methods for identification of *C. dubliniensis* are molecular methods based upon genetic differences e.g hybridisation with the repeat sequence probes Ca3 or 27A, hybridisation with the *C. dubliniensis* species-specific probe Cd25, hybridisation with the oligonucleotide probes $(GGAT)_4$, $(GACA)_4$, $(GATA)_4$, $(GT)_8$ and $(GTG)_5$, karyotype analysis, RAPD, PCR fingerprinting with the M13 primer and RNA sequence analysis (Boerlin *et al.*, 1995; McCullough *et al.*, 1995; Sullivan *et al.*, 1995; Gilfillan *et al.*, 1998; Joly *et al.*, 1999; Meyer *et al.*, 2001). In general these methods are not suitable for large-scale epidemiological investigation because they are labour-intensive, require specialised equipments and are expensive. However, PCR-based techniques are potentially valuable tools for the rapid and unequivocal differentiation of *C. dubliniensis*. It would seem, therefore, that the most suitable methods for identifying *C. dubliniensis* in a diagnostic laboratory would be to presumptively identify the organism on a differential medium followed by PCR with *C. dubliniensis*-specific primers.

1.2.6 Epidemiology of *C. dubliniensis*

It is believed that *C. dubliniensis* predates the AIDS pandemic since the oldest confirmed isolates of *C. dubliniensis* were found in yeast culture collections that were deposited prior to the advent of AIDS. One of these was originally misidentified as *Candida stellatoidea* and deposited in the British National Collection for Pathogenic Fungi (Bristol, U.K.) as a reference strain for this species in 1957 (Sullivan *et al.* 1995) and another was deposited in the Centraalbureau voor Schimmelcultures collection (Baarn, the Netherlands) as *C. albicans* in 1952 (Meis *et al.* 1999). It has been suggested that the AIDS pandemic and the widespread use of fluconazole as a therapeutic and prophylactic agent during the mid 1980s have allowed this species to emerge as a significant agent of disease and hence it's discovery in the mid 1990s (Sullivan *et al.,* 2004)

Candida dubliniensis was initially recovered from the oral cavities of HIV-infected patients from Ireland, Australia and the U.K. Subsequently, *C. dubliniensis* isolates have been identified from every continent and a large number of countries all over the world (Pujol *et al* 1997; Schoofs *et al.*, 1997; Sullivan *et al* 1997; Kirkpatrick *et al.*, 1998; Odds *et al* 1998; Pinjon *et al.*, 1998; Salkin *et al* 1998; Jabra-Rizk *et al* 1999a; Meiller *et al* 1999; Redding *et al* 2001; Gee *et al* 2002; Blignaut *et al.*, 2003; Fotedar & Al Hedaithy 2003 & 2004).

The vast majority of *C. dubliniensis* isolates have been recovered from the oral cavities of HIV-infected individuals or, more frequently, from patients with AIDS including haemophiliacs, intravenous drug users and homosexuals, most often in combination with *C. albicans* and /or other *Candida* species (Coleman *et al.*, 1997a & 1997b; Ponton *et al.*, 2000). One of the first studies in the literature that investigated the prevalence of this organism in the oral cavity comes from a study of Irish individuals (Table 1.1) (Coleman *et al.*, 1997b). According to this study, *C. dubliniensis* was recovered from 32% of Irish AIDS patients with clinical symptoms of oral candidosis (n=76) and 25% of asymptomatic Irish AIDS patients (n=28). In contrast, *C. dubliniensis* was only recovered from 3% of Irish HIV-negative healthy individuals. This evidence suggests that *C. dubliniensis* can exist as part of the normal oral flora as well as an opportunistic organism which can infect the oral cavities of individuals with immunodeficiency. The association between *C. dubliniensis* and the oral cavity of HIV-positive and AIDS patients

Table 1.1. Epidemiology of *C. dubliniensis* from different subject cohorts in Ireland.[a]

Group	Number of subjects[b]	% of subjects yielding *C. dubliniensis* and other *Candida* spp.	% of subjects yielding *C. dubliniensis* only
HIV+	185 [+]	26	6
	216 [-]	18	3
AIDS	82 [+]	32	10
	36 [-]	25	8
Denture stomatitis	72 [+]	14	4
Diabetic	318 [-/+]	18	4
Normal healthy	202 [-]	3.5	0.5
Vaginitis	110 [+]	3	1

[a] Modified from Ponton *et al.* (2000).

[b] [+], patient presented with clinical symptoms of oral candidosis, [-], patient presented without clinical symptoms of oral candidosis.

was also confirmed by the work of Tintelnot *et al.*, (2000) who found *C. dubliniensis* in 34% of yeast-colonised HIV-infected patients. Other studies, predominantly from the USA, found the prevalence of oral *C. dubliniensis* in HIV-infected individuals to be between 11% and 25% (Kirkpatrick *et al.*, 1998; Jabra-Rizk *et al.*, 1999a; Meiller *et al.*, 1999; Brown *et al.*, 2000). In contrast, other studies have reported significantly lower prevalence rates of *C. dubliniensis* colonising the oral cavities of HIV-positive patients (Vargas & Joly 2002; Blignaut *et al.*, 2003). Sullivan *et al.*, 2004 suggested that the divergence in the prevalence of *C. dubliniensis* in the reported studies could be due to differences in methods of sampling and identification, geographical locations and differences in the cohorts of HIV-infected individuals included in theses reports (Sullivan *et al.*, 2004).

A limited number of *C. dubliniensis* isolates have also been recovered from the vaginal tracts of HIV-negative women with vaginitis (Sullivan & Coleman, 1998, Polacheck *et al.*, 2000). Similarly, *C. dubliniensis* isolates have been recovered from specimens from a variety of other anatomical sites, including blood, faeces, sputum, urine, wounds and the respiratory tract (Odds *et al.*, 1998; Polacheck *et al.*, 2000; Gee *et al.*, 2002). More recently, the recovery of *C. dubliniensis* from immunocompromised patients affected with different conditions such as malignant diseases, diabetes and others has questioned its prevalence in these groups of patients and the role *C. dubliniensis* may play in the pathogenesis of disease and in the selection of the appropriate treatment for these patients (Meis *et al.*, 1999; Willis *et al.*, 2000; Gottlieb *et al.*, 2001; Davies *et al.*, 2002)

In all studies to date, the prevalence of *C. dubliniensis* isolates was higher in HIV-infected individuals than in non-HIV-infected individual (Coleman *et al.*, 1997a & b; Ponton *et al.*, 2000). *Candida dubliniensis* isolates from the oral cavity are most commonly recovered in mixed culture with other *Candida* species, especially *C. albicans*. However, pure cultures of *C. dubliniensis* have been recovered from individuals exhibiting clinical symptoms of oral candidosis, suggesting that this organism can be responsible for oral infections (Coleman *et al.*, 1997a; Moran *et al.*, 1997 & 1998). Indeed, epidemiological studies so far show cause for concern as this organism is prevalent in immunocompromised groups, particularly HIV-infected and AIDS patients. *Candida dubliniensis* has also been shown to rapidly develop resistance to fluconazole following exposure to the drug *in vitro*, and it has also been shown to develop resistance *in vivo* following fluconazole therapy (Moran *et al.*, 1997 & 1998; Ruhnke *et al.*, 2000).

1.2.7 Virulence of *C. dubliniensis*

Although *C. dubliniensis* and the closely related species *C. albicans* share many significant phenotypic and genotypic characteristics, *C. albicans* seem to be the more successful pathogen. *Candida dubliniensis* was recovered less frequently from the human oral cavity and vagina in healthy individuals compared to *C. albicans* (Ponton *et al.* 2000). In addition, the epidemiological data on the prevalence of candidaemia showed that *C. dubliniensis* cause infections less frequently than *C. albicans* (Kibbler *et al.*, 2003; Sullivan *et al.*, 2004). In a mouse model of infection, Gilfillan *et al.* (1998) studied the virulence of four *C. dubliniensis* isolates and demonstrated that they were less virulent than *C. albicans* isolates when an inoculum size of 2x 10^6 cells per mouse was used. When a higher inoculum was used (1 x 10^7) there was a wide variation in the mean survival times amongst the mice infected with *C. dubliniensis*. Competitive studies between the two species indicate that *C. albicans* has an advantage over *C. dubliniensis* in broth culture *in vitro* (Kirkpatrick *et al.*, 2000). However, the presence of a supporting structure for biofilm formation enables *C. dubliniensis* to tolerate more successfully the competitive pressures from *C. albicans*. It was also suggested that the slower kinetics of hyphal formation of *C. dubliniensis* may contribute to its apparently lower virulence (Gilfillan *et al.*, 1998). In this later study, the investigators used limited number of isolates to study the kinetics of hyphal formation in both species. *C. dubliniensis* isolates production of hyphae appeared to be slower in *C. dubliniensis* than in a reference strain of *C. albicans*. In addition, *C. dubliniensis*, unlike *C. albicans,* did not produce hyphae following growth in N-acetyl-D-glucosamine medium (Gilfillan *et al.*, 1998).

One of the features which is usually associated with virulence in *C. albicans* is its ability to adhere to different human cells. McCullough *et al.* (1995) reported that *C. dubliniensis* were more adherent than *C. albicans* to buccal epithelial cells. However, few other studies are in agreement with this report (Gilfillan *et al.*, 1998; Jabra-Rizk *et al.*, 2001). Gilfillan *et al.* (1998) demonstrated that *C. dubliniensis* isolates were more adherent to buccal epithelial cells than *C. albicans* when the organisms were grown in glucose-rich medium. In contrast, when different assay conditions were used Gilfillan *et al.* (1998) and Borg-von Zepelin *et al.* (2002) reported that *C. albicans* isolates were more adherent to buccal epithelial cells than *C. dubliniensis.* However, in the presence of fluconazole *C.*

dubliniensis isolates showed increased adherence when compared with controls which might explain the high prevalence of *C. dubliniensis* amongst AIDS patients receiving fluconazole therapy (Borg-von Zepelin *et al.*, 2002).

Cell surface hydrophobicity expression varies greatly between *C. albicans* and *C. dubliniensis*. Hydrophobic *C. albicans* cells may be induced by growth at 23°C which are less susceptible to phagocytic killing than hydrophilic cells which result from growth at 37°C (Hazen & Glee, 1995). In contrast, *C. dubliniensis* cells have been reported to be hydrophobic under all environmental conditions tested (Hazen *et al* 2001; Jabra-Rizk *et al.*, 2001). However, despite this difference in hydrophobicity, there was no difference in the levels of phagocytosis and induced oxidative burst and killing by human nuetrophils for both species (Peltroche-Llacsahuanga *et al.*, 2000b).

Secreted aspartyl proteinases (Saps) are believed to be involved, amongst other factors, in adherence of *C. albicans* to epithelial mucosa (Ray & Payne, 1988; Borg & Rüchel, 1988; Ollert *et al.*, 1993; Klotz *et al.*, 1994; Watts *et al.*, 1998). McCullough *et al.* (1995) reported that oral *C. dubliniensis* isolates exhibited greater proteolytic activity than *C. albicans* isolates. Gilfillan *et al.* (1998) also demonstrated that *C. dubliniensis* possess homologues of seven *C. albicans SAP* genes. Using *C. albicans* DNA microarrays, Moran *et al.* (2004) performed comparative genomic hybridisation between *C. albicans* and *C. dubliniensis* in order to identify genomic differences that might account for the difference in virulence between the two species. In their work, the authors were able to identify a set of *C. albicans* genes which may play a role in the increased prevalence and virulence of this species. Interestingly, the secreted aspartyl proteinase-encoding gene *SAP5,* among others, was found to be absent in *C. dubliniensis.* However, gene homologous to *SAP4* and *SAP6* of *C. albicans* was found in this species (Moran *et al.*, 2004).

Another factor which may contribute to *C. albicans* virulence is phenotypic switching. This phenomenon of phenotypic switching has been documented in *C. albicans* (Slutsky *et al.*, 1985 and 1987; Gallagher *et al.*, 1992). However, it has also been reported that *C. dubliniensis* isolates exhibited phenotypic switching more frequently than *C. albicans* which may contribute to its virulence (Hannula *et al.* 2000).

1.3 Molecular typing of *Candida* species using complex DNA fingerprinting probes

Phenotypic analysis was first used as a method reflecting the genetic difference between fungal pathogens. Over the years, however, investigators have realised the shortcomings of biotyping and the fundemental problems that render them in some cases inadequate for discriminating among strains within a species (Sullivan *et al.*, 1996). In contrast, strain typing using DNA fingerprinting techniques provides investigators and clinicians with tools for tracking strains, identifying the sources of particular infections and the detection of the same or different strains from recurrent episodes of infection in individual patients. Above all, strain biotyping is essential for epidemiological investigations since it gives valuable population information about isolates of the same species from separate individuals and patient groups (Sullivan *et al.*, 1996; Soll 2000; Sullivan *et al.*, 2004).

Soll, (2000) proposed a number of criteria for assessing the effectiveness of a DNA fingerprinting system. Firstly, the method should be resistant to environmental perturbations and high frequency genomic reorganisation. Secondly the data should reflect genetic distance at the resolution necessary to answer the questions posed. Thirdly, for some purposes, for example fingerprinting independent isolates, fingerprints must be stable over time. However, for studies on microevolution this is not a requirement. Fourth, fingerprints generated must be reproducible. Finally, the data generated should be amenable to computer-assisted analysis for retrospective analysis and for comparisons between laboratories. A number of fingerprinting methods that are in common use are discussed below, however, not all of these methods fulfil all of the requirements (Soll, 2000).

1.3.1 Multilocus enzyme electrophoresis (MLEE)

MLEE method uses changes in amino acid composition of proteins as a result of nucleotide polymorphisms which can result in a change in the charge of the protein and thus affect the mobility of the protein. Therefore, when cell extracts are separated on starch gels and stained with enzyme-specific stains, strain-specific staining profiles can be obtained. For diploid organisms one or two bands are observed for each enzyme. Complex data profiles can be obtained by using several enzymes and subsequently analysis using

computer-assisted methods. MLEE has been used to fingerprint many pathogens including; *C. albicans* (Caugant & Sandven 1993; Boerlin *et al.*, 1995 & 1996; Le Guennec *et al.*, 1995) *C. tropicalis* (Lehmann *et al.*, 1989), *Candida parapsilosis* (Lin *et al.*, 1995), *Cryptococcus neoformans* (Brandt *et al.*, 1993 & 1996). However, one of the drawbacks of the method is that it is time-consuming since data collected from at least 10 enzymes that reflects the variation among isolates must be used (Soll, 2000).

1.3.2 Restriction fragment length polymorphism analysis

Restriction fragment length polymorphism (RFLP) analysis involves the digestion of chromosomal DNA by restriction endonucleases and the subsequent separation of the fragments by agarose gel electrophoresis. This technique was one of the first DNA fingerprinting methods used to assess strain relatedness in fungi and has been applied to a variety of fungi including *C. albicans* (Scherer & Stevens, 1987; Clemons *et al.*, 1997). However, RFLP can result in the generation of complex patterns of fragments that are ambiguous and difficult to interpret objectively. Nevertheless, studies using a variant of this technique have shown improvement in the efficiency of discrimination between isolates. DNA is digested by restriction endonucleases that cleave DNA infrequently, thereby giving rise to large DNA fragments that can be subjected to RFLP analysis (Doebbeling *et al.*, 1993; King *et al.*, 1995). However the inability of this method to assess the relatedness of moderately related isolates coupled with the low precision of RFLP analysis meant that this technique does not lend itself to studies in which cluster analyses of moderately related isolates are necessary and is not suitable for species identification (Sullivan *et al.*, 1996; Soll, 2000).

1.3.3 Complex DNA fingerprinting probes

Another molecular approach that has been used to type isolates of *C. albicans* and non-*C. albicans Candida* species is Southern blot hybridisation of restriction enzyme-digested DNA with a complex DNA probe. It involves fingerprint analysis with species-specific DNA probes homologous to repetitive DNA sequences which are dispersed throughout the genome. The most commonly used *C. albicans* probes are the 27A and Ca3 probes which contain the repetitive element RPS (Scherer & Stevens, 1988; Schmid *et al.*, 1990). While the 27A probe hybridises strongly to *C. albicans Eco*RI-digested DNA

producing complex patterns of bands (Scherer & Stevens, 1988), the fingerprint patterns obtained for *C. dubliniensis* isolates consist of a small number of weakly hybridising bands (McCullough *et al.*, 1995, Sullivan *et al.*, 1995). Similarly, the Ca3 fingerprinting probe hybridises weakly to *C. dubliniensis* genomic DNA while it gives strong hybridisation bands with *C. albicans* genomic DNA (Boerlin *et al.*, 1995). Complex probes have also been developed for *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. dubliniensis* (Joly *et al.*, 1996 & 1999; Lockhart *et al.*, 1997; Enger *et al.*, 2001). The hybridisation patterns generated by these probes must be accommodated for computer-assisted analysis, storage and interpretation. The final interpretation must involve a measurement of similarity of the data collected for every possible pair of isolates analysed. DNA fingerprinting methods using complex fingerprinting probes are now considered the gold standard method for fingerprinting *Candida* species for studying populations of isolates (Soll, 2000). However, this technique is considered labour-intensive and time-consuming and therefore it is not amenable for the rapid analysis of a large number of strains.

1.3.4 Random amplified polymorphic DNA analysis

Random amplified polymorphic DNA analysis (RAPD) uses PCR technology to rapidly differentiate between individual *Candida* strains belonging to a particular species. However, RAPD differs from PCR in that it makes use of random primers of short length (approximately 10-15 bp) in a PCR reaction with low annealing temperatures. Amplified products are separated on an agarose gel and stained with ethidium bromide. The RAPD method of DNA fingerprinting has been popular for all infectious fungi including *C. albicans* (Del Castillo *et al.*, 1997; Clemons *et al.*, 1997), *C. dubliniensis* (Coleman *et al.*, 1997b), *C. parapsilosis* (Lott *et al.*, 1993) and others (King *et al.*, 1995; Boekhout *et al.*, 1997). The technique, however, has the problem of poor reproducibility between laboratories. Reproducibility is usually affected by the methodological aspects of the PCR including primer to template ratio, differences in the brand of PCR machine, temperature during amplification reaction and concentration of magnesium in the reaction mixture (Soll, 2000). Variations within laboratories have also been observed due to differences in batches of *Taq* polymerase (Loudon *et al.*, 1995). On the other hand, since little sample DNA is needed for RAPD and that PCR is rapid and easy to perform, RAPD has been used widely in the analysis of *Candida* populations.

1.3.5 Electrophoretic karyotyping

During karyotyping, yeast chromosome-sized DNA fragments are separated according to size through agarose gels using an alternating electric field. Pulsed field gel electrophoresis (PFGE) can be used to discriminate between individual strains because the sizes of individual chromosomes can vary widely within different *Candida* species. This technique has been used successfully in *Candida* epidemiology including *C. albicans* (Bart-Delabesse *et al.*, 1993; Lott *et al.*, 1993; Vazquez *et al.*, 1993; Barton *et al.*, 1995). However, it was demonstrated that the karyotype patterns varied among unrelated isolates of *C. albicans.* Using Southern blot hybridisation with cloned genes as probes, Thrash-Bingham & Gorman, (1992) demonstrated that in spite of karyotypic variability among strains of *C. albicans*, the general genomic organisation was maintained and that translocations contributed to karyotypic variability (Thrash-Bingham & Gorman, 1992). Nevertheless, Soll (2000) indicated that PFGE is not a technique that fulfil the necessary requirements for fingerprinting independent isolates of *Candida* species since the method is unsuitable. Soll, (2000) suggested a modified method to increase the resolution by digesting chromosome-length DNA with endonucleases prior to pulsed-field gel electrophoreses (Soll, 2000).

1.3.6 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was introduced in 1998 by Maiden *et al.*, (1998) as a molecular typing method for *Neisseria meningitidis*. It has now been extended to involve a wide variety of bacterial species and some pathogenic fungi such as *C. albicans*, *C. glabrata*, *Histoplasma capsulatum*, *Aspergillus flavus*, *Coccidoides immitis* (Bougnoux *et al.*, 2004). Sequencing of a variety of housekeeping genes is the preferred method for measuring relationships between species and within species. Furthermore, these genes are usually selected based on the assumption that they are under stabilising selective pressure (Soll, 2000).

Multilocus sequence typing (MLST) is a highly discriminatory method based on the analysis of nucleotide polymorphisms within the sequences of the internal fragments (400–500 bp) of housekeeping genes (loci). For each housekeeping locus, the different sequences are assigned as distinct alleles, and for each isolate, the alleles at each of the sequenced loci define an allelic profile or sequence type. Each isolate of a species is

therefore characterised by a series of integers that correspond to the alleles at the houskeeping loci studied (Bougnoux *et al.*, 2002). MLST is a highly reproducible method with the major advantage of generating standardised data. However, so far no DNA region has been selected for sequencing to provide a highly accurate genotype within different species and it is only been exploited mainly for the analysis of haploid microorganisms. In this regard, the application of MLST to diploid organisms including *C. albicans* is subject to several constraints (Soll, 2000).

1.4 Antifungal agents used in the treatment of *Candida* infections

The need for antifungal agents has risen dramatically over the last decade due to the increase in the number of immunocompromised patients, the emergence of refractory fungal species and the development of resistance (Pfaller *et al.*, 1994a & b; Redding *et al.*, 1994; Ruhnke *et al.*, 1994; Ghannoum & Rice, 1999; Harbarth *et al.*, 1999). Most of the antifungal agents used clinically, except for 5-FC, target the ergosterol biosynthetic pathway and this mechanism include azoles, polyenes, allylamines and morpholine derivatives (White *et al.*, 1998).

1.4.1 Azole derivatives

Azole derivatives are a class of antifungal agents which are characterised into N-1 substituted imidazoles (ketoconazole, clotrimazole, miconazole) and triazoles (fluconazole, itraconazole) and the new generation of azoles (posaconazole, ravuconazole and voriconazole). Azole derivatives inhibit the synthesis of lanosterol 14α-demethylase (Erg 11 p), an enzyme which is encoded by the *ERG11* gene. This enzyme involved in the synthesis of ergosterol which is the major sterol in the fungal plasma membrane and is important for the survival and integrity of the membrane and cell growth and division (Ghannoum & Rice, 1999).

Other enzymes which were reported to be a target for azole antifungals were the enzyme sterol C22-desaturase, involved in the last step of ergosterol biosynthesis and the enzyme NADPH-dependent 3-ketosteroid reductase (Vanden Bossche *et al.*, 1994; Kelly *et al.*, 1997).

Azoles are active against wide range of *Candida* species and each azole antifungal agent has distinct pharmacokinetics. Some azoles derivatives are, however, more active than others. For example, fluconazole is totally ineffective against *C. krusei* (Zhao & Calderone, 2002; Sanglard, 2002; Sanglard & Bille, 2002). Azole antifungal agents are only fungistatic against most yeast species, with the exception of *C. neoformans.*

1.4.2 Polyenes

Polyene antifungals are a class of natural antifungal compounds discovered in the early1950s. Amphotericin B and nystatin are considered the most commonly used polyene antifungals. Amphotericin B is a broad spectrum fungicidal compound primarily used in the treatment of systemic fungal infections (Zhao & Calderone, 2002), whereas nystatin has proven a useful medication for topical therapy of superficial candidosis (Ghannoum & Rice, 1999). The primary mode of action of polyenes is achieved through the binding of the polyene antifungal agents to ergosterol, the major sterol component of the fungal cell membrane, which results in aqueous pores in the membrane that are composed of aggregates of drug and ergosterol. This results increased membrane permeability, leakage of vital cytoplasmic components and finally death of the organism (Ghannoum & Rice, 1999). It has also been suggested that amphotericin B causes oxidative cell damage which could contribute to its fungicidal effect (Ghannoum & Rice, 1999). However, amphotericin B therapy is associated with renal toxicity; long-term kidney damage may occur if renal function is not carefully monitored during the course of intravenous therapy. In order to reduce the toxicity associated with amphotericin B treatment, new liposomal formulations of amphotericin B have recently been introduced that have been associated with less severe toxic side effects (Richardson & Kokki, 1998; Ghannoum & Rice, 1999). The inherent toxicity of polyenes to mammalian cells is thought to lie in their affinity for cholesterol, the principal sterol of mammalian cell membranes (Zhao & Calderone, 2002).

1.4.3 5-fluocytosine

5-FC was first introduced in the 1950s to treat cancerous tumors. It is a fluorinated pyrimidine that has been used to treat infections caused by *Candida* species, *Aspergillus* species and *C. neoformans* (Ghannoum & Rice, 1999). When 5-FC enters the cell of a susceptible yeast, it is converted to to 5-fluorouracil (5FU) by the enzyme cytosine

deaminase. 5-fluorouracil is then converted by uridine monophosphate pyrophosphorylase (UMPP) into 5-fluorouridylic acid, which is incorporated into RNA resulting in aberrant protein synthesis and inhibition of fungal cell growth (Ghannoum & Rice, 1999; White et al., 1998). Thus, 5-FC acts by interfering with pyrimidine metabolism, RNA, DNA and protein synthesis in the fungal cell. 5-FC is water soluble so it can be administered orally and intravenously. However, the use of 5-FC as monotherapy is not recommended due to the high incidence of resistance to this compound in *Candida* species (Vanden Bossche *et al.*, 1994). It is therefore used in combination with other antifungal agents, mainly amphotericin B. In addition, many clinically important fungal species exhibited high prevalence of primary resistance to 5-FC (e.g. *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. neoformans*) (Coleman *et al.*, 1998; Ghannoum & Rice, 1999; Sanglard & Bille, 2002). Since mammalian cells lack the enzyme cytosine deaminase, 5-FC show little toxicity (Sanglard & Bille, 2002). However, it has been reported that intestinal bacteria can convert 5-FC into 5FU, thus leading to possible clinical toxicity of oral formulations (Sanglard & Bille, 2002).

1.4.4. Allylamine

Allylamine compounds target squalene epoxidase, the first postsqualene enzyme of the ergosterol biosynthetic pathway. Allylamine compounds are reversible, noncompetitive inhibitors of the epoxidase and their inhibitory effect is thought to be caused by ergosterol depletion and squalene accumulation. The latter is thought to increase membrane permeability, leading to disruption of cellular organisation (Ghannoum & Rice, 1999). The main allylamine compounds in clinical use are terbinafine, which can be used topically or systemically, and the topical agent, naftifine. Terbinafine is the antifungal drug of choice for the treatment of superficial mycoses caused by dermatophytes (Ryder *et al.*, 1998). Although terbinafine is fungicidal against dermatophytes and filamentous fungi, it is only fungistatic against the majority of *Candida* species including *C. albicans* and *C. dubliniensis* (Ryder *et al.*, 1998; Sanglard & Bille, 2002).

1.4.5 Morpholine derivatives

The only morpholine derivative used clinically is amorolfine. The morpholine derivatives are totally synthetic compounds that inhibit two enzymes of the ergosterol biosynthetic pathway, the C-14 sterol reductase and C-8 sterol isomerase. These enzymes

are encoded by the *ERG24* and *ERG2* genes, respectively. Since amorolfine-mediated inhibition of growth results in the formation of ignosterol, a sterol by-product which was shown to accumulate in *erg24* null mutants of *S. cerevisiae*, it has been suggested that C-14 sterol reductase is the primary target of amorolfine (Crowley *et al.*, 1996). Clinically, amorolfine is restricted to topical use in the treatment of dermatophyte infections and vulvovaginal candidosis (Sanglard & Bille, 2002).

1.4.6 Cyclic lipopeptides

Cyclic lipopeptides antifungal agents of the echinocandin class are targeting the fungal cell wall as an essential component of the cell. The development of the echinocandins has helped to fill the need for more efficacious antifungals that are useful across different patient populations and have a good safety profile. It has been suggested that these antifungal agents block cell wall synthesis by inhibiting the enzyme β-1,3 glucan synthase which is part of the glucan polymers of most pathogenic fungi (Kurtz & Douglas, 1997). The echinocandin chemical family includes three antifungal agents, caspofungin, micafungin and anidulafungin. Anidulafungin is an intravenousl administered echinocandin being developed to treat mucosal and invasive fungal infections. Caspofungin, however, has been proven to be effective in the treatment of oropharyngeal and oesophageal candidosis and is particularly indicated in the treatment of invasive aspergillosis. Micafungin is fungicidal to susceptible yeast species, fungistatic against *Aspergillus* species but has no activity against *C. neoformans*, *Trichosporon* species or *Fusarium solani* (Boucher *et al.*, 2004).

1.5 Antifungal drug resistance

1.5.1 Definition of antifungal drug resistance

In a clinical setting, antifungal drug resistance is defined as the failure of treatment and the progression or persistence of infection despite appropriate antifungal drug therapy (White *et al.*, 1998). There are many reasons why a fungal infection does not respond to antifungal agents, including the immune status of the patients, the characteristics of the drug, patient compliance, presence of a protected or persistent focus of infection and the susceptibility of the pathogen to the drug (white *et al.*, 1998; Sanglard *et al.*, 2002). Fungal

species can be classified as either possessing primary resistance to drugs which is an intrinsic resistance present before exposure to antifungal drugs or a secondary resistance (aquired) which is a resistance that develops during the course of therapy and may be stable or reversible (Vanden Bossche *et al.*, 1994; white *et al.*, 1998; Sanglard & Bille, 2002). In *Candida* species, primary resistance to 5-FC is common, whereas primary resistance to amphotericin B is considered rare. Both primary and secondary resistance to azole drugs has been described in *Candida* species.

1.5.2 Resistance to azoles

Primary and secondary resistance to azole antifungals in *Candida* species, particulary resistance to fluconazole, is well documented in the literature. The first reported case of resistance to an azole antifungal was in *C. albicans* against miconazole and ketoconazole (Johnson *et al.*, 1995). Prophylaxis and prolonged treatment with fluconazole have been linked with the emergence of fluconazole-resistant *Candida* isolates especially among HIV-positive and AIDS patients with oral candidosis (Bille, 2000). Laguna *et al.* (1997) have reported the incidence of *C. albicans* isolates with reduced susceptibility to fluconazole in HIV-infected patients to be as high as 50% (Laguna *et al.*, 1997). Following the use of azole antifungals for a wide variety of clinical settings and the development of antifungal resistance, the recovery of isolates of non-*C. albicans Candida* species with elevated azole MICs has increased (Price *et al.*, 1994; Nguyen *et al.*, 1996; White *et al.*, 1998). Interestingly, initial reports of fluconazole-resistant *Candida* isolates involved non-*C. albicans* species such as *C. glabrata* and *C. krusei* (Chavanet *et al.*, 1994).

Fluconazole resistance is often associated with cross-resistance to other azoles (Johnson & Warnock, 1995; Rex *et al.*, 1995 & 1997; Klepser *et al.*, 1997). While *C. albicans* isolates are generally susceptible to fluconazole, *C. krusei* isolates are considered to be intrinsically resistant to this drug (White, *et al.*, 1998; Moran, *et al.*, 2002). Acquired fluconazole resistance in *C. glabrata* generally develops more rapidly than in *C. albicans* isolates because of the haploid nature of *C. glabrata* (Warnock, 1992).

1.5.3 Resistance to polyene antifungals

Aquired resistance in *Candida* species has remained rare despite more than 30 years of clinical use. However, intrinsic resistance to amphotericin B is common for *C. lusitaniae*

isolates (Sanglard, 2002). The development of amphotericin B resistance in clinical isolates of *C. albicans* has also been reported (Kelly *et al.*, 1997; Nolte *et al.*, 1997). Theses *C. albicans* clinical isolates were recovered from HIV-infected and leukaemic patients and exhibited cross-resistance to fluconazole.

Vazquez *et al*, (1998) demonstrated that amphotericin B-susceptible *C. albicans* cells exposed overnight to sub-inhibitory levels of fluconazole or itraconazole *in vitro* lead to the development of amphotericin B resistance. However, this potential *in vitro* selection of resistance to amphotericin B following exposure to fluconazole is not thought to have a significant clinical impact.

Amphotericin B requires the presence of ergosterol in the fungal cell membranes.for it to function. However, most polyene-resistant isolates have reduced ergosterol content in their membranes (Vanden Bossche *et al.*, 1994; white *et al* 1998). Since amphotericin B requires the presence of ergosterol to cause damage to fungal cells, the replacement of ergosterol in fungal cell membranes by other sterols is thought to mediate resistance to this drug. It has also been suggested that resistance to amphotericin B can be mediated by increased catalase activity which leads to a decreased susceptibility to oxidative damage caused by this agent (Sanglard & Bille, 2002).

1.5.4 Resistance to 5-FC

Microbiological resistance to 5-FC can be intrinsic or aquired. Acquired resistance to 5-FC is a common development in patients receiving 5-FC monotherapy (White *et al.*, 1998). As a result, 5-FC is best used in combination with other antifungal agents such as amphotericin B rather than as a single therapeutic agent (Ghannoum & Rice, 1999; White *et al.*, 1998). Investigations on the molecular mechanisms of resistance have shown that primary resistance to 5-FC is usually the result of a mutation in cytosine deaminase as observed in *S. cerevisiae* and *C. glabrata*. Secondary resistance to 5-FC in *C. albicans* is due primarily to a decrease in the activity of uracil phosphoribosyl transferase (UPRTase), which is involved in the synthesis of 5-fluorouridine monophosphate (5-FUMP) (White *et al.*, 1998). Bille, (2002) estimated that 10% of *C. albicans* clinical isolates are intrinsically resistant to 5-FC and that 30% will develop secondary resistance.

1.5.5 Azole susceptibility in C. dubliniensis

The relationship between fluconazole usage and the emergence of *C. dubliniensis* remains controversial. It has been suggested that the increased use of fluconazole treatment during the 1990s lead to a shift toward non-*C. albicans Candida* species which have the ability to successfully colonise the oral cavities of HIV-infected individuals receiving long term therapy with this drug. Evidence to support this suggestion was provided by Borg-Von Zepelin, *et al.* (2002) who showed that in the presence of fluconazole, the adherence of *C. dubliniensis* to epithelial cells increased while the adherence of *C. albicans* was decreased under the same conditions. However, the majority of *C. dubliniensis* isolates are susceptible to the most commonly used antifungals (Moran *et al.*,1997; Kirpatrick *et al.*, 1998; Odds *et al.*, 1998; Meiller *et al.*, 1999; Pfaller *et al.*, 1999a & b; Jabra-Rizk *et al.*, 1999b and 2000; Brandt *et al.*, 2000; Polacheck *et al.*, 2000). Pfaller *et al.* (1999a) in an exhaustive study, showed that 69/71 (97%) of the *C. dubliniensis* isolates tested were susceptible to fluconazole. In the study, resistance was defined as MIC $\geq$ 64 μg/ml, as recommended by the NCCLS (1997). However, isolates with dose-dependent susceptibility have also been reported in other studies (Moran *et al.*, 1997; Kirkpatrick *et al.*, 1998; Odds, 1998). Compared to *C. albicans, C. dubliniensis* geometric mean MICs for fluconazole, itraconazole and ketoconazole tend to be significantly and consistently higher than those of *C. albicans* isolates (Odds, 1998). Furthermore, sequential exposure of fluconazole-susceptible clinical isolates of *C. dubliniensis* to increasing concentrations of fluconazole in agar medium resulted in the recovery of fluconazole-resistant derivatives (MIC range 16-64 μg/ml) (Moran *et al.*, 1997 & 1998). Moran *et al.* (1998) analysed the mechanism of resistance to fluconazole in both *in vitro* -generated fluconazole resistant derivatives and in fluconazole-resistant clinical isolates of *C. dubliniensis.* Overexpression of the *MDR1* homologue, *CdMDR1* in *C. dubliniensis* has been shown to be involved in mediating reduced accumulation of drug in fluconazole-resistant clinical isolates and *in vitro*-generated derivatives (Moran *et al.*, 1998). The importance of *CdMDR1* in fluconazole resistance was later confirmed when both alleles of the gene *CdMDR1* were deleted in the fluconazole-resistant strain CM2 which rendered the deletion derivative susceptible to fluconazol (Wirsching *et al.*, 2001). Upregulation of *CdCDR1,* the *CDR1* homologue from *C. dubliniensis* has been observed in fluconazole-resistant clinical isolates and *in vitro*-generated derivatives (Moran *et al.*, 1998). However, in a subsequent study,

Moran *et al.* (2002) demonstrated that while CdCdr1p is important for mediating reduced susceptibility to itraconazole and ketoconazole, it is not required for fluconazole resistance. In contrast, in *C. albicans*, resistance to fluconazole is mainly associated with overexpression of *CDR1* (Moran *et al.*, 1998). Recently, the molecular mechanisms responsible for reduced susceptibility to azole drugs in *C. dubliniensis* genotype 3 isolates obtained from an AIDS patient with fluconazole-resistant oral candidiasis was investigated (Pinjon *et al.*, 2005). In contrast to previous studies, results showed that the reduced susceptibility to azole drugs in these isolates was associated with increased energy-dependent efflux mechanisms mediated by the overexpression of the *CdCDR1 and CdCDR2* genes and not with overexpression of the multidrug transporter *CdMDR1* (Pinjon *et al.*, 2005). Furthermore, Moran *et al.* (2002) investigated the reasons for the differential regulation of *CDR1* expression in *C. albicans* and *C. dubliniensis.* The investigators reported the high prevalence (14/24, 58%) of a nonsense mutation in the *CdCDR1* gene encoding a non-functional CdCdr1p protein amongst *C. dubliniensis* isolates (Moran *et al.*, 2002). Resistance to other antifungal drugs such as ketoconazole, amphotericin B has not yet been reported in *C. dubliniensis* (Ryder *et al.*, 1998; Pfaller *et al.*, 1999a). However, itraconazole resistance in *C. dubliniensis* has been reported *in vitro* by serial exposure to itraconazole (Pinjon *et al.*, 2003). It was found that the resistant derivatives had mutations in *CdERG3* alleles encoding the sterol C5,6-desaturase enzyme which results in altered membrane permeabilities in itraconazole-resistant derivatives (Pinjon *et al.*, 2003).

Aims of the study

Candida dubliniensis is a recently defined species which was originally identified in cases of recurrent oral candidosis in HIV-infected and AIDS patients (Coleman *et al.*, 1997a & b; Ponton *et al.*, 2000). Recently it has been reported in other immunocompromised groups such as diabetics and in patients with cancer (Sebti *et al.*, 2001; Manfredi *et al.*, 2002). Most of the studies on *C. dubliniensis* were from isolates recovered in North America and Europe. Therefore, there is very little information available on the epidemiology or population structure of *C. dubliniensis* for other part of the world including the Middle East. In addition, in-depth epidemiological analyses of *C. dubliniensis* has been hampered by the lack of a simple, accurate, inexpensive phenotypic test capable of differentiating *C. dubliniensis* from *C. albicans* in most clinical mycology laboratories.

- The first aim of this study was to evaluate the potential use of differential culture media, including bird seed agar (Staib agar), sunflower seeds agar (Pal's agar), tobacco agar and casein agar to distinguish isolates of *C. dubliniensis* from *C. albicans* using large collections of well characterised isolates of both species from different geographical locations.
- The second aim of this study was to investigate the oral mycological flora in a cohort of patients receiving palliative care for advanced cancer with a particular reference to the prevalence of *C. dubliniensis* in this population. Susceptibility to fluconazole and itraconazole was also investigated by standard methods.
- The third aim of the present study was to investigate the population structure of *C. dubliniensis* oral isolates from Saudi Arabia and Egypt recovered from HIV-negative patients using Cd25-generated fingerprint profile analysis and genotyping based on the nucleotide sequence of the ITS region of the rDNA gene cluster. In addition, since two recent studies reported an unusually high prevalence of 5-FC resistance among *C. dubliniensis* isolates from Saudi Arabia and Kuwait (Fotedar & Al Hedaithy, 2003; Ahmad *et al.*, 2004), it was decided to investigate the susceptibility of the *C. dubliniensis* isolates recovered form Saudi Arabia and Egypt to 5-FC and further investigate the molecular mechanism involved in resistance of the isolates tested.

Chapter 2
Materials and Methods

2.1 General Microbiological Methods

2.1.1 Culture media and growth conditions

All *Candida* strains and clinical isolates were routinely cultured on Potato Dextrose Agar (PDA) medium (Oxoid, Basingstoke, Hants., UK) at pH 5.6 at 37°C for 48 h. For liquid culture, isolates were routinely grown in Yeast Peptone Dextrose (YPD) broth (per litre: 10 g yeast extract [Oxoid], 20 g peptone [Difco, Detroit, MI, USA], 20 g glucose, pH 5.5) at 37°C for 18 h in a Gallenkamp (Model G25) orbital incubator (New Brunswick Scientific Company Inc., Edison, New Jersey, USA) set at 200 rpm.

The *Escherichia coli* strain XL2-Blue MRF' (D[*mcrA*]*183* Δ[*mcrCB-hsdSMR-mrr*]*173 endA1 supE44 thi-1 recA1 gyrA96* r*elA1 lac*[F'*proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^{r}) Amy Cam^{r}]^{c,d}; Sambrook *et al.*, 1989) was routinely cultured on Luria-Bertani agar (LA), pH 7.4 (Lennox, 1955), at 37°C, and for liquid culture, in Luria-Bertani broth (LB), pH 7.4 (Lennox, 1955), at 37°C for 18 h in an orbital incubator (Gallenkamp) at 200 rpm. *Escherchia coli* XL2-Blue MRF' was used as the host strain for plasmid pBluescript II KS (-) (Stratagene, La Jolla, California, USA) and its recombinant derivatives and was maintained on LA containing 100 μg ampicillin/ml.

Escherichia coli strain LE 392 (*supE*44, *supF*58, *hsdR*514, *galK*2, *galT*22, *metB*1, *trpR*55, *lacY*1) was used as the host for recombinant Lambda bacteriophage and was cultured on LA medium supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose as described by Sambrook *et al.*, 1989). For liquid culture, organisms for phage infection were routinely grown in LB medium containing 0.01 M $MgSO_4$ and 2% (w/v) maltose at 37°C for 18 h in an orbital incubator set at 200 rpm.

2.1.2 Chemicals, enzymes, radioisotopes and antifuungal drugs

All chemicals used were of analytical-grade or molecular biology-grade and were purchased from Sigma-Aldrich Ltd. (Tallaght, Dublin, Republic of Ireland), BDH (Poole, Dorset, U.K.) or from Roche Diagnostic Ltd. (Lewes, East Sussex, UK). Enzymes for molecular biology procedures were purchased from the Promega Corporation (Madison, Wisconsin, USA.), Roche Diagnostics Ltd. or New England Biolabs Inc. (Beverley, Massachusetts, USA). DNA molecular weight markers were purchased from Gibco BRL

Life Technologies (Gaithersburg, Maryland, USA) and Promega. [α-^{32}P]dATP (6,000 Ci mmol; 222 TBq /mmol) was purchased from Amersham International Plc. (Little Chalfont, Buckinghamshire, UK). Custom-synthesised oligonucleotides were purchased from Sigma-Genosys Biotechnologies Europe Ltd. (Pampisford, Cambridgeshire, UK).

Fluconazole powder was a gift from Pfizer Central Research (Sandwich, Kent, UK) and was dissolved in 10% (v/v) dimethyl sulfoxide at a concentration of 1 mg/ml. Itraconazole was a gift from Janssen Pharmaceuticals (Cork, Republic of Ireland) and was dissolved in dimethyl sulfoxide with the aid of heating to 70°C at a concentration of 1 mg/ml. 5-fluorocytosine (Sigma-Aldrich Ltd.) was dissolved in water at a concentration of 1 mg/ml.

Zymolyase 20T (21,600 U/g) was purchased from the Seikagaku Corporation (Tokyo, Japan). RNase solutions were prepared by dissolving pancreatic RNase (RNase A, Roche) at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl. This solution was boiled for 15 min to inactivate any DNases, allowed to cool to room temperature and stored at -20°C. Proteinase K (Roche) solutions were prepared in sterile distilled water at a concentration of 20 mg/ml and also stored at -20°C. DNA molecular weight markers were purchased from Gibco BRL Life Technologies (Gaithersburg, Madison, USA) and from Promega.

2.1.3 Buffers and solutions

Tris-EDTA (TE) buffer was used routinely in many experiments and consisted of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. TBE buffer was prepared at 5x concentration and consisted of 0.45 M Trizma base, 0.45 M boric acid, 0.01 M EDTA. This was diluted in distilled water to 0.5x concentration and was used as the buffer for agarose gel electrophoresis. DNA loading dye was also prepared at 10x concentration and consisted of 30% (v/v) glycerol. 0.25% (w/v) bromophenol blue and 3.8% (w/v) EDTA.

SSC buffer was prepared at 20x concentration and consisted of 3.0 M NaCl, 0.3 M tri-sodium citrate, pH 7. Liquefied phenol washed in Tris-buffer was purchased from Fisher Scientific Ltd. (Bishop Meadow Road, Loughborough, UK) and used in the preparation of phenol chloroform (1:1), which was prepared by mixing an equal volume of liquefied phenol and chloroform. This solution was stored at 4°C for up to two months in the dark.

Phosphate buffered saline (PBS) was prepared at 10x concentration and contained 1.45 M NaCl, 0.33 M NaH_2PO_4, 0.95 M Na_2HPO_4, pH 7.5.

RNase solutions were prepared by dissolving pancreatic RNase (RNase A) at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl. This solution was boiled for 15 min to inactivate any DNases, allowed to cool to room temperature and stored at -20°C.

2.1.4 Storage of *Candida* strains and isolates

Candida strains and clinical isolates were maintained on plastic beads in Protect cryo-vials (Technical Service Consultants Ltd., Lancashire, UK) at -70°C. Cells were activated by sub-culture onto PDA plates and incubated for 48 h at 37°C.

2.2 Identification of *Candida* isolates

2.2.1 Chlamydospore production

Candida dubliniensis isolates were tested for their ability to produce chlamydospores on rice-agar-Tween medium (RAT medium, bioMérieux, Marcy l'Etoile, France) as described by Sullivan *et al.* (1995). Test isolates were cultured on PDA for 24-48 h at 37°C. Single colonies (3-4 mm diameter) were removed from PDA plates with a sterile wire loop and used to inoculate the RAT medium by cutting shallow grooves in the surface of the agar medium. A glass coverslip was then placed over the inoculated area to create semi-anaerobic conditions and the plate was incubated at room temperature for 2-3 days in the dark. Plates were stained by spotting lactophenol cotton blue stain directly onto an inoculated RAT agar plate to enhance the detection of chlamydospores. Lactophenol cotton blue preferentially stains chlamydospores more intensely than suspensor cells, pseudomycelium, and blastospores (Larone, 1993). The stained area was recovered by replacing the coverslip. Plates were then examined microscopically (x 40 objective lens) 30 min after staining for the presence of pseudohyphae, hyphae and chlamydospores. The *C. albicans* oral reference strain 132A (Gallagher *et al.*, 1992) and the *C. dubliniensis* type strain CD36 (Sullivan *et al.*, 1995) were used as positive controls for chlamydospore production in all tests.

2.2.2 Carbohydrate assimilation profiles

Biotyping was carried out using the API ID32C yeast identification system (bioMérieux) which identifies *Candida* isolates to the species level using a series of standard substrates contained in 32 separate cupules on a plastic strip with a specially adapted database (Pincus *et al.*, 1999). Tests were carried out according to the manufacturer's instructions. For each test isolate, an inoculum was prepared from 24-48 h-old colonies cultured on PDA medium. Four colonies of 3-4 mm in diameter were resuspended in sterile water to a turbidity equivalent to a 2 McFarland standard. This suspension was then used to inoculate an aliquot of 'C medium', which was supplied by the manufacturers. Each of the cupules in the strip was then inoculated with 135 µl of the C medium suspension and incubated for 48 h at 30°C. Readings were made at 24 h and 48 h by visually assessing the growth of the test isolate in each of the cupules compare to that in the negative control cupule. The presence or absence of growth was recorded for each cupule on a result sheet supplied by the manufacturers, and the substrate assimilation profile of the isolate was converted into an eight-digit numerical profile. These profiles were then cross-referenced in the APILAB ID32C software package (version 3.3.3) (bioMérieux). In the database, each profile is listed along with a percentage of identification (% id), which is an estimate of how closely the profile corresponds to that of a particular taxon, relative to all the other taxa in the database and the T index, which is an estimate of how closely the profile corresponds to the most typical set of reactions for a particular taxon. Based on these parameters, a set of reactions which closely resemble those of a particular taxon will be classed as an 'excellent' or 'good' identification, and will yield an identification to the species level, whereas atypical results will be classed as having 'poor' or 'low' discriminatory powers and are usually unable to yield a reliable identification.

2.2.3 Growth at 45°C

All isolates were tested for the ability to grow on PDA at 37°C and 45°C. Most *Candida albicans* isolates grow at both temperatures, whereas *C. dubliniensis* isolates were found to grow well at 37°C, but not at 45°C (Pinjon *et al.*, 1998).

2.2.4 Growth on CHROMagar® Candida medium

CHROMagar® Candida is a commercially available agar medium containing chromogenic substrates, which allow colonies of several medically important *Candida* species to be presumptively identified on the basis of colony colour and morphology. Colonies of *C. albicans* (light green colonies), *C. glabrata* (pink colonies), *C. krusei* (rough, spreading colonies with pale pink centers and a white edge) and *C. tropicalis* (purple) can easily be distinguished from each other upon primary isolation, and the medium has been shown to be clinically useful in the presumptive identification of these species (Odds & Bernaerts, 1994). *Candida dubliniensis* colonies following primary isolation from clinical samples are dark green on this medium but can lose the ability to yield dark green colonies following subculture or storage (Schoofs *et al.*, 1997; Coleman *et al.*, 1997a; Kirkpatrick *et al.*, 1998).

2.2.5 PCR identification of C. dubliniensis

A single colony from a culture grown for 48 h at 37°C on PDA or CHROMagar Candida medium was suspended in 50 µl sterile distilled water. Cell suspensions were boiled for 10 min and the lysed cells subjected to a clearing spin for 5 min at 20, 000 x g in an Eppendorf (model 5417C) microfuge (Eppendorf, Hamburg, Germany). Template DNA contained in 25 µl supernatant was used for PCR amplification (Donnelly *et al.*, 1999).

PCR identification of *C. dubliniensis* using the *C. dubliniensis*-specific primer pair DUBF/DUBR (Donnelly *et al.*, 1999) was carried out in a 50 µl final volume containing 10 pmol each of the forward and reverse primers, 2.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 9.0 at 25°C), 10 mM KCl, 0.1 % (v/v) Triton X-100, 2.5 U *Taq* DNA polymerase (Promega) and 25 µl template DNA. Each reaction mixture also contained 10 pmol each of the universal fungal primers RNAF/RNAR (Fell, 1993), which amplify approximately 610 bp from all fungal large-subunit rRNA genes and were used as an internal positive control. Cycling conditions consisted of 6 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 58°C,

30 s at 72°C, followed by 72°C for 10 min. Amplification products were separated by electrophoresis through 2.0 % (w/v) agarose gels containing 0.5 μg ethidium bromide/ml and were visualised on a UV transilluminator.

2.3 Isolation of genomic DNA and DNA fingerprinting

2.3.1 Extraction of genomic DNA from Candida *species*

Genomic DNA was prepared from cells grown in 50 ml of YPD broth in a 250 ml flask (Erlenmyer) at 37°C in an orbital incubator at 200 rpm. for 18 h. Cultures were decanted into 50 ml Falcon tubes (Beckton Dickinson, New Jersey, USA) and centrifuged in a bench top centrifuge (Sepatech Megafuge 1.0, Heraeus, Germany) at 2,500 x *g* for 5 min. The supernatant was decanted and the pellet was resuspended in 500 μl of a solution consisting of 1 M sorbital, 20 mM phosphate buffer and transferred to 1.5 ml microfuge tube. Cell walls were digested by the addition of 15 mg Zymolyase 20T and incubation at 37°C for 30 min in a shaking waterbath. The resulting protoplasts were harvested by centrifugation at 2,500 x *g* for 10 min and the pellet was resuspended in 500 μl of 1x TE containing 1 % (w/v) sodium dodecyl sulphate (SDS). Proteinase K was added to a final concentration of 2 mg/ml and the cell lysates were incubated for 18 h at 55°C. Protein was precipitated by the addition of 250 μl of 5 M potassium acetate and incubated on ice for 30 min. The cell lysates were centrifuged at 2,500 x *g* for 10 min. The cleared supernatant was extracted three times using an equal volume of a mixture of phenol:chloroform (1:1) and precipitated with the addition of two volumes of ice-cold isopropanol. The resulting precipitate was spooled from the solution with a sterile loop and transferred to a fresh microfuge tube. The DNA precipitate was then washed in 500 μl ice-cold 70 % (v/v) ethanol, dried briefly at room temperature and resuspended in 40 μl sterile distilled water. DNA suspensions were stored at 20°C.

The concentration of DNA samples were assessed by measuring their absorbance at 260 nm using a spectrophotometer (model Genosys 2, ThermoSpectronic, supplied by AGB, Dublin, Ireland) and calculating the concentration using the following formula: 1 unit of A_{260} = 50 μg DNA.

2.3.2 Restriction endonuclease digestion of genomic DNA and agarose gel electrophoresis

Large scale restriction endonuclease digestions of genomic DNA for Southern analysis were carried out in a 40 µl volume containing 20 U of restriction enzyme and the appropriate restriction enzyme buffer according to the manufacturer's instructions. Horizontal 0.65 % (w/v) agarose gels made up in 0.5x TBE buffer containing 0.5 µg/ml ethidium bromide per ml were cast into horizontal gel trays. DNA loading dye was added to the restriction enzyme-digested DNA samples at a final concentration of 1x and the samples were loaded into the gel wells. A DNA size standard was loaded on each gel. Electrophoresis was carried out at 64 V with constant current. Gels were visualised on a UV transilluminator (wavelength 345_{nm}) and photographed using an ImageStore 7500 Version 7.22 Gel Documentation System (Ultra-Violet products Ltd (UVP), Cambridge, UK).

2.3.3 Southern transfer of DNA from agarose gels

Following the separation of restriction endonuclease digested DNA fragments by agarose gel electrophoresis, DNA was transferred to nylon membranes by capillary action using the method of Southern (1975). Reference size standards were marked on the agarose gels using sterile Pasteur pipettes The DNA was then depurinated by soaking the gels in 0.02 M HCl with gentle shaking. Following depurination, the DNA was denatured by soaking the gel in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 45 min with gentle agitation, after which the gels were placed in a neutralisation solution (1 M Tris-HCl, pH 7.5, 1.5 M NaCl) for a further 45 min with shaking.

DNA fragments were transferred to MagnaGraph nylon membranes (MSI, Wesborough, Massachusetts, USA) by capillary transfer using 20x SSC as the transfer buffer according to the method of Southern (1975). Following transfer, the positions of DNA reference size standards were then marked on the membrane using a ball-point pen. The membrane was then rinsed in 2x SSC, dried and baked at 80°C for 1 h to fix the DNA or the DNA was fixed using a crosslinker (CL-508, UVI tec, Cambridge, UK) set at 0.120 J/cm^2.

2.3.4 Random primer labelling of DNA fragments with [α-^{32}P]dATP

DNA fragments were labelled with [α-^{32}P]dATP by random primer labelling using the Prime-a-gene kit purchased from Promega. DNA fragments were denatured by boiling for 2-3 min. Denatured DNA was then added to a reaction mixture containing 1x labelling buffer, dNTP's (dTTP, dCTP and dGTP), bovine serum albumin (BSA), 2 µl of [α-^{32}P]dATP (6,000 Ci/mmol; 220 TBq/mmol) and 5 U of Klenow DNA polymerase. The mixture was incubated at room temperature for 1-2 h. Unincorporated nucleotides were removed prior to hybridisation by passing the reaction mixture through a column containing a size exclusion matrix (SigmaSpin™ post-reaction columns, Sigma-Aldrich Ltd.) following the protocol outlined by the manufacturer.

2.3.5 Southern hybridisation

Hybridisation reactions were carried out in a rotary oven (Hybaid, Teddington, Middlesex, UK) in 25 x 3.5 cm bottles (Hybaid) by the method of Sambrook *et al.* (1989). Nylon membranes were rinsed in 6x SSC prior to hybridisation to remove excess salt. Membranes were then prehybridised at 65°C in 15 ml of a solution containing 1x Denhardt's solution (1 % [w/v] Ficoll, 1 % [w/v] polyvinylpyrrolidone, 1 % [w/v] BSA), 6x SSC, 100 µg/ml denatured salmon sperm DNA and 0.5 % (w/v) SDS for 2 h.

Radiolabelled probe (> 2 x 10^6 d.p.m.) was denatured by boiling for five min followed by incubation on ice. The denatured probe was then added to the prehybridisation solution and incubated with the membrane at 65°C for 18 h. Unbound probe was removed from the membranes following hybridisation by washing the membrane in the bottle with a solution of 2x SSC, 0.1 % (w/v) SDS at room temperature for 5 min, followed by a wash at room temperature in 0.1x SSC, 0.5 % (w/v) SDS for 15 min, and finally a high stringency wash at 65°C in 0.1x SSC, 0.5 % (w/v) SDS for 30 min. After washing, the membranes were wrapped in Saran wrap (Dow Chemical Co., Stade, Germany) and placed in an autoradiography cassette with a Kodak BioMax intensifying screen (Eastman Kodak Company, Rochester, New York, USA) and exposed to Kodak Biomax MS-1 X-ray film for 24 to 72 h at-70°C. Autoradiograms were developed using Kodak GBX developer and fixed in Kodak GBX fixer according to protocols supplied by the manufacturer.

In order to reuse membranes in subsequent hybridisation experiments, bound probe was removed from membranes by immersing them in boiling distilled water, followed by a brief rinse in 2x SSC.

2.4 Recombinant DNA Techniques

2.4.1 Small scale isolation of plasmid DNA from E. coli

Small scale preparations of plasmid DNA from *E. coli* were prepared by the method of Sambrook *et al.* (1989). Briefly, *E. coli* cultures were grown overnight in LB medium in the presence of selective antibiotic (100 µg ampicillin/ml in the case of pBluescript II KS [-]). A 2 ml aliquot of this culture was pelleted at 10,000 x *g* for 30 s in a microfuge (Centrifuge 5417C, Eppendorf) and resuspended in 100 µl ice cold solution 1 (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0). Cell were lysed by the addition of 200 µl solution 2 (0.2 N NaOH, 1% [w/v] SDS) and left on ice for 5 min. Protein was then precipitated by the addition of 150 µl solution 3 (5 M potassium acetate, 11.3% (v/v) acetic acid). The mixture was vortexed and centrifuged at 10,000 x *g* for 5 min in a microfuge. The supernatant was transferred to a fresh microfuge tube and extracted once with an equal volume of phenol:chloroform (1:1), and the DNA precipitated by the addition of 2 volumes of ice-cold ethanol. The precipitate was pelleted again at 10,000 x *g* for 5 min and resuspended in 50 µl sterile distilled water. RNA was removed by incubating the DNA solution with 0.1 mg/ml RNase A for 30 min.

2.4.2 Polymerase chain reaction (PCR) and purification of PCR amplimers from agarose gels

Oligonucleotide primers were synthesised by Sigma-Genosys Ltd. (Cambridge, UK) and stored at a stock concentration of 1 mM in sterile water at -20°C. Amplification reactions were carried out in 0.5 ml microfuge tubes (Eppendorf) in a thermal cycler (Thermo Hybaid Px2, MBS, UK) in 100 µl volumes containing 1x *Taq* reaction buffer, 1.5 mM $MgCl_2$, 200 µM (each) dATP, dTTP, dCTP, dGTP (Promega), 300 nM (each) of a forward and reverse primer, 100 ng genomic DNA template and 2.5 U *Taq* DNA polymerase (Promega).

Alternatively, if the PCR product was to be sequenced or cloned into an expression vector, the reactions were carried out using the high fidelity proof reading enzyme mix Expand High Fidelity PCR System purchased from Roche and the buffers provided by the manufacturer. Amplification conditions and specific primers are described in the relevant sections.

PCR products were purified using the GenElute PCR DNA purification kit® (Sigma-Aldrich Ltd.) or from agarose gels using the Wizard Minpreps PCR DNA Purification System® (Promega) according to the manufacturers' instructions. Occasionally restriction endonuclease-generated DNA fragments were purified from agarose gels using NA45 DEAE membranes (Schleicher and Schuell BioScience, Inc., USA).The NA45 DEAE membranes were pre-treated by soaking 1 cm strips in 2 M NaCl for 5 min, followed by 3 washes in sterile distilled water for 5 min each. The strips were then stored at 4˚C in 1 mM EDTA, pH 8.0. Fragments were electrophoresed as described in section 2.3.2 in agarose gels and viewed on a UV transilluminator (345_{nm}). Using a clean scalpel blade, a small rectangular trough was excised from the gel immediately ahead of the fragment of interest, and a piece of NA45 DEAE paper was placed in the trough and the excised fragment of gel was replaced to hold the paper in place. The electrophoresis was allowed to continue until the fragment had run onto the paper, which could be verified by the fluorescent staining of the paper with ethidium bromide. The paper was then placed in 0.5 ml 1 M NaCl and placed in a water bath at 37˚C for at least 1 h to elute the fragment. The DNA solution was then extracted twice with iso-butanol to remove the ethidium bromide, and once with phenol:chloroform (1:1). The DNA was precipitated with two volumes of ice-cold ethanol, pelleted at 10,000 x *g* in a microfuge and resuspended in 5-10 µl sterile distilled water.

2.4.3 Ligation of DNA fragments

Purified DNA fragments were ligated to pBluescript II KS (-) phagemid digested with the appropriate restriction enzyme. Ligation of PCR products to pBluescript was carried out *via* restriction sites which had been designed within the oligonucleotide primers used in the amplification reactions. Purified DNA fragments were ligated directly into the appropriate restriction enzyme-generated site in the cloning vector. Ligation reactions were carried out in a 10 µl volume, with a 3:1 ratio of insert to vector DNA in 1x ligase buffer, with 1 U of T4 DNA ligase (Promega). Reactions were carried out for 18 h at 25˚C.

2.4.4 Transformation of competent E. coli *prepared using* $CaCl_2$

Transformation of *E. coli* with $CaCl_2$ was carried out by the method of Sambrook *et al.* (1989). *Escherichia coli* DH5α was inoculated from an overnight broth culture into 100 ml LB and grown at 200 rpm in an orbital incubator at 37°C for 3 h to an A_{600} of ~0.5. The culture was then decanted into ice-cold 50 ml Falcon tubes and chilled on ice for 10 min. Cells were then pelleted by centrifugation at 5,000 x *g* in a Sorvall SS34 rotor (Dupont Co., Denver, Colorado, USA) at 4°C for 10 min. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$, and centrifuged as before. The pellets were then resuspended in a volume of 2 ml 0.1 M $CaCl_2$ for each 50 ml of original culture.

A 200 µl aliquot of this cell-suspension was transferred to a sterile microfuge tube on ice for each transformation experiment. Plasmid DNA (up to 50 ng) was added to each tube and incubated on ice for 30 min. A known amount of a standard plasmid preparation was added to a separate tube as a positive control, and a second negative control tube was also included which contained no plasmid DNA. The tubes were then heat shocked at 42°C for exactly 1.5 min and rapidly transferred to an ice bath. The cells were then incubated at 37°C in a water bath in the presence of 800 µl LB medium to allow the cells to recover and express the antibiotic resistance marker (ampicillin resistance in the case of pBluescript II KS [-]). A 100 µl aliquot of this suspension was then spread on LA plates containing antibiotic (100 µg ampicillin/ml in the case of pBluescript II KS [-]), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Boerhringer Mannheim) and 100 µg (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, Roche) and incubated for 20 h at 37°C. Recombinants were identified using blue-white selection as described by Sambrook *et al.* (1989).

2.4.5 Extraction of recombinant phage lambda DNA

Recombinant phage stocks were maintained at a titre of approximately 1 x 10^{11} pfu/ml in SM broth (50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM $MgCl_2$) supplemented with 0.01 %(w/v) gelatin. Phage lysates were prepared from these stocks by the plate method of Sambrook *et. al.* (1989). Briefly, 100 µl of phage stock were mixed with 100 µl of mid-exponential phage plating bacteria (LE 392, prepared as described in section 2.1.1) and incubated for 20 min at 37°C. To this mixture was added 3 ml of molten (47°C) TB top

agar (Tryptone 10 g/ml, NaCl 5 g/ml, Bacto Agar 8 g/ml). This was poured onto a 90 mm plate containing LM agar (LB agar supplemented with 2 % (w/v) maltose and 0.01 M $MgSO_4$). The plate was incubated at 37°C overnight until confluent lysis was achieved. Three millitres of SM broth were added to the plate and it was stored at 4°C for 1 h. The SM and the soft top agar were scraped into a tube using a sterile bent glass rod. The agar suspension was incubated with 0.1 ml of chloroform with shaking for 15 min at 37°C. The tube was then centrifuged at 4000 x *g* for 10 minutes at 4°C. Approximately 500 µl of phage stock were propagated to yield 10 ml of phage lysate. DNA was extracted from this lysate using the Wizard Lambda Preps DNA Purification System (Promega) according to the manufacturer's instructions. Ten ml of phage lysate yielded approximately 5 µg of recombinant phage DNA.

2.5 DNA sequence analysis

DNA sequencing was performed commercially by Lark (Saffron Walden, Essex, UK) using the dideoxy chain termination method of Sanger *et al.* (1977), using an automated Applied Biosystems 373A DNA sequencer (Foster City, California, USA) and dye-labelled terminators. The sequencing primers used were either the M13 forward and reverse primers or in the case of direct sequencing of PCR amplimers the PCR primers were used as sequencing primers.

Chromatograms were analysed using the 373A Data Analysis program version 1.2.0 (Applied Biosystems). Sequence analysis was carried out using the DNA Strider™ version 1.3f11 software for DNA and protein analysis (CEA/Saclay, Gif-sur-Yvette, France). Searches of the EMBL and GenBank databases for nucleotide and amino acid sequence similarities were performed using the BLAST series of computer programme (Altschul *et al.*, 1990). Alignments of nucleotide and amino acid sequences were carried out using CLUSTAL W sequence alignment computer program (Thompson *et al.*, 1994).

2.6 Antifungal drug susceptibility testing procedures

2.6.1 Rodriguez-Tudela and Martinez-Suárez (1995) modified broth microdilution (BMD) assay

The broth microdilution assay was used for susceptibility testing of *C. dubliniensis* clinical isolates to the antifungals fluconazole and 5-FC and was carried out by the method of Rodriguez-Tudela and Martinez-Suárez (1995), which is a modification of the broth macrodilution method outlined in the NCCLS document M-27A, a reference standard for antifungal susceptibility testing. In the NCCLS M-27A document, the recommended medium to be used in the test was RPMI-1640 (Sigma-Aldrich Ltd.). This medium was however modified by the addition of 2% (w/v) glucose which gives improved growth with *Candida* species and allows MIC determination following 24 h incubation at 37°C (Rodriguez-Tudela & Martinez-Suàrez, 1995). The medium was buffered with 0.165 M morpholinepropanesulphonic acid (MOPS) (Sigma-Aldrich Ltd.) and adjusted to pH 7.0 using a stock solution of 10 N NaOH. The assay was carried out in sterile 96-well microtitre plates (Corning, New York, USA).

Eight MIC determinations were carried out on each 96-well microtitre plate. Wells one and twelve of each row were dispensed with 0.1 ml of RPMI-2% (w/v) glucose and were used as a sterility control and a positive growth control, respectively. Antifungal susceptibility testing was carried out in a final volume of 100 µl in wells two to eleven, ranging in concentration from 0.125 to 64 µg/ml for fluconazole and 0.12 to 128 µg/ml for 5-FC.

The inocula were prepared from 24-48 h old PDA plates. Three to four colonies (3-4 mm in diameter) of each isolate to be tested were resuspended in 3 ml of sterile saline. The cell density of each suspension was adjusted to 1 x 10^6 cfu./ml using an Improved Neubauer haemocytometer (Neubauer bright line haemacytometer, Hausser Scientific, Horsham, USA). This suspension was then diluted in RPMI-2% (w/v) glucose and 10 µl aliquots were dispensed in wells two to twelve, yielding a final cell density of 1 x 10^4 cfu/ml and 1.5 ± 1 cfu/ml for fluconazole and 5-FC, respectively.

Test plates were covered with the lids supplied by the manufacturers, wrapped in cling film and incubated for 24 h at 37°C. Prior to spectrophotometric reading of MIC end points, the plates were agitated for 2 min using a vortex (Spinmix model, Gallenkamp). End

points were determined spectrophotometrically by measuring the turbidity in each well at 405_{nm} (A_{405}) with an automated plate reader (Spectra I; SLT-Labinstruments, Salzburg, Austria). An endpoint (IC_{50}), termed the MIC, was determined by the method of Galgiani & Stevens (1976) as the lowest drug concentration which fulfilled the criterion $\%T \geq \% T_k + 0.5(100-\%T_k)$, where T is the transmission from a microtitre plate well containing fluconazole and the organism being tested, and T_k is the transmission from the fluconazole-free growth control well containing the organism being tested. The IC_{50} represents the drug concentration that inhibited growth by 50%, as determined by transmission, compared with the growth of controls, as described by (Rodriguez-Tudela & Martinez-Suàrez, 1995). All tests were performed in duplicate on at least two separate occasions.

Chapter 3

Development and assessment of novel culture media to differentiate between *C. albicans* and *C. dubliniensis*

3.1 Introduction

Due to the increasing incidence of fungal infections and the recent emergence of novel opportunistic fungal pathogens, there is a growing need for the development of simple, rapid, and accurate identification methods for potential fungal pathogens recovered in the clinical microbiology laboratory (Reiss *et al.*, 1998). This is particularly true for the newly described yeast species *C. dubliniensis*. Although first associated with oral candidiasis in human immunodeficiency virus (HIV)-infected patients (Sullivan *et al.*, 1995), it has more recently been recognised as a cause of superficial and systemic disease in HIV-negative individuals (Sullivan *et al.*, 1997; Pinjon *et al.*, 1998; Meis *et al.*, 1999; Brandt *et al.*, 2000; Polacheck *et al.*, 2000; Willis *et al.*, 2000). The close genotypic relationship between *C. dubliniensis* and *C. albicans* results in their sharing a broad range of phenotypic characteristics, which hampers the accurate and rapid differentiation of the two species (Sullivan *et al.*, 1997). Although the majority of *C. dubliniensis* isolates are susceptible to currently used antifungal drugs, it has been shown that isolates of this species, unlike *C. albicans*, can rapidly develop stable resistance to fluconazole upon exposure *in vitro* (Moran *et al.*, 1997 & 1998). This ability, the emergence of *C. dubliniensis* world-wide, its growing importance as a cause of systemic disease, and the introduction of novel antifungal agents all indicate that a thorough investigation of the prevalence and epidemiology of *C. dubliniensis* is required. In order to be able to achieve this, simple and reliable tests for differentiating *C. dubliniensis* from *C. albicans* need to be developed. The "gold standard" methods for the identification of *C. albicans* are based on its ability to produce germ tubes and chlamydospores (chlamydoconidia) on appropriate nutrient media. However, since *C. dubliniensis* also produces these structures, many isolates of *C. dubliniensis* have been misidentified as *C. albicans* (Sullivan *et al.*, 1997; Odds, 1998; Pincus *et al.*, 1999). Therefore, in order to perform urgently required epidemiological studies of *C. dubliniensis* infections, there is a need to develop inexpensive, accurate, and easy to perform tests that will allow the differentiation of isolates of the two species which have been recovered from clinical samples. In this regard, a variety of procedures have been developed and assessed in laboratories around the world, including, among others, colony color on CHROMagar® Candida medium (Schoofs *et al.*, 1997), lack of growth at 45°C (Pinjon *et al.*, 1998), immunofluorescence (Bikandi *et al.*,

1998), carbohydrate assimilation profile analysis (Pincus *et al.*, 1999), β-glucosidase activity (Boerlin *et al.*, 1995), coaggregation with *Fusobacterium nucleatum* (Jabra-Rizk *et al.*, 1999b), and PCR tests (Donnelly *et al.*, 1999). Some of these tests (e.g., PCR) are very reliable but are not yet used routinely by many clinical microbiology laboratories, while others rely on reagents which are not widely available (e.g., immunofluorescence with anti-*C. dubliniensis* antibodies) and yet others (e.g., colony color on CHROMagar Candida medium) are unreliable.

In the original description of *C. dubliniensis*, it was noted that this species produces much higher numbers of chlamydospores than *C. albicans* when grown on rice-agar-Tween agar (RAT) (Sullivan *et al.*, 1995) but subsequent studies have shown that this trait does not provide a definitive means of differentiating between the two species (Kirkpatrick *et al.*, 1998). However, in 1999 Staib & Morschhäuser reported that *C. dubliniensis* forms abundant chlamydospore on Staib agar on which *C. albicans* grows in the yeast form only and that this trait could be used to differentiate between the two species (Staib & Morschhäuser, 1999).

The aim of this part of the present study was to investigate whether colony morphology and chlamydospore production on a variety of different culture media, including Staib agar, caffeic acid-ferric citrate agar (CAF), Pal's agar, and tobacco agar could be used to reliably differentiate between isolates of *C. dubliniensis* and *C. albicans* using a large collection of well characterised *C. dubliniensis* isolates from around the world.

3.1.1 Staib and CAF agars

Staib agar (syn. *Guizotia abyssinica* creatinine agar) is a medium originally developed for the identification of *C. neoformans.* In a recent study of 14 *C. dubliniensis* and 11 *C. albicans* strains, Staib & Morschhäuser (1999) reported that colony morphology and chlamydospore production by *C. dubliniensis* on Staib agar could form the basis of a simple and accurate test for distinguishing this species from *C. albicans.* Therefore, it was decided to investigate the usefulness of colony morphology and chlamydospore production on Staib agar using a larger collection of *C. dubliniensis* isolates recovered from individuals in 18 different countries around the world. In addition, it was decided to investigate CAF agar since it is a more defined medium containing caffeic acid extracted from *Guizotia abyssinica* seeds. CAF agar was originally developed by Strachan *et al.*

(1971) to aid in the identification of *C. neoformans* based on the production of brown pigmentation by colonies of *C. neoformans* on this medium.

3.1.2 Pal's agar

Pal's medium contains sunflower seed (*Helianthus annus*) extract which was originally used for the identification of *C. neoformans* (Staib *et al.*, 1987). Since both *Helianthus annus* and *Guizotia abyssinica* belong to the same botanical family (*Asteraceae*) and since this medium has never been used to differentiate between *C. dubliniensis* and *C. albicans*, it was decided to investigate the ability of Pal's agar to differentiate between *C. dubliniensis* and *C. albicans* by comparing colony morphology and chlamydospore production on this medium.

3.1.3 Tobacco agar

Tobacco agar is a new agar which is useful in selecting colonies of *C. neoformans* from mixed cultures in environmental and clinical samples. Colonies of this particular species produce brownish colour on this medium. Unlike some other fungi which can make pigments endogenously, pigment production in *C.neoformans* depends upon presence of exogenous substrates which accumulates in the fungal cell due to an enzyme laccase. Taking this as a basis and the fact that tobacco has substrates responsible for melanin-like pigment deposition on teeth, Tendolkar *et al.* (2003) hypothesised that the substrate found in tobacco could be used as a base for a medium to identify *C. neoformans.* However, tobacco agar has never been used before to aid in the identification of any other *Candida* species.

3.1.4 Casein agar

Casein agar is a medium which is usually used to differentiate microorganisms such as actinomycetes that can produce enzymes to break down the main two components of the agar; starch, as the only carbon source and casein (milk protein) as the only protein source. However, it has never been used before to aid in the differentiation of *C. dubliniensis.*

3.2 Materials and Methods

3.2.1 Yeast isolates

All isolates included in this part of the present study were from the culture collection of the Microbiology Research Unit, School of Dental Science, Trinity College, University of Dublin, Dublin, Republic of Ireland. Each isolate was originally recovered from one or more specimens from a separate individual, and its identity was confirmed as described in chapter 2 section 2.2. All isolates were also tested for chlamydospore formation on rice agar-Tween agar, and all the *C. dubliniensis* and *C. albicans* isolates investigated produced chlamydospores on this medium.

Stock cultures of yeast isolates were maintained on plastic beads in Protect cryovials at -80°C. For each isolate, two or three plastic beads were removed from their respective cryovials using a sterile plastic loop, allowed to thaw, and then used to inoculate PDA (Oxoid) or Sabouraud glucose agar media (Difco). Forty-eight-hour-old PDA medium cultures grown at 37°C were used as the source of inoculua for subsequent experiments with Staib agar, CAF agar, Pal's agar, casein agar and tobacco agar.

All of the isolates included in the present study were examined on Staib agar, CAF agar, Pal's agar, casein agar and tobacco agar on at least two separate occasions with different batches of media prepared from different lots of reagents. In addition, all media were used fresh and within 5 days to ensure consistent results.

3.2.2 Staib and CAF agars

The yeast isolates and reference strains used in this part of the study are listed in Table 3.1. A total of 296 isolates were studied, including 130 *C. dubliniensis* isolates and 166 *C. albicans* isolates. In addition, five *C. glabrata*, five *C. tropicalis* and six *C. parapsilosis* isolates were also examined on Staib and CAF agars (Table 3.1).

Staib agar was prepared by first making an aqueous extract of *Guizotia abyssinica* seed (Power Seeds, Kildare, Republic of Ireland) by pulverizing 50 g of seed in a domestic blender (Moulinex B57, Ireland) for 2.5 min and then adding the ground seeds to 1 liter of distilled water, followed by boiling for 30 min. The seed extract was cooled and filtered, and the following ingredients were added: glucose, 1 g; KH_2PO_4, 1 g; creatinine, 1 g. The pH was adjusted to 5.5, the volume was readjusted to 1 liter, and 15 g of agar (Difco) was

Table 3.1 Yeast isolates tested on Staib and CAF media.

Yeast species and source or country of isolation	No. of isolates	Specimen	Source/reference(s)
C. dubliniensis			
Argentina	1	Oral	Sullivan *et al.*, 1997, Gee *et al.*, 2002
Australia	1	Oral	Sullivan *et al.*, 1995, Gee *et al.*, 2002
Belgium	5	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Canada	6	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Finland	1	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
France	9	Oral	This study
Germany	4	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Greece	1	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
India	1	Oral	Gee *et al.*, 2002
Israel	2	Sputum	Polacheck *et al.*, 2000, Gee *et al.*, 2002
	1	Vagina	2000, Gee *et al*
Ireland	41	Oral	Pinjon *et al.*, 1998, Sullivan *et al.*, 1995, Gee *et al.*, 2002, this study
Japan	1	Unavailable*	Gee *et al.*, 2002
Malta	3	Oral	This study
Norway	5	Oral	Gee *et al.*, 2002
Spain	5	Oral	Bikandi *et al.*, 1998, Gee *et al.*, 2002
Switzerland	3	Oral	Sullivan *et al.*, 1998, Gee *et al.*, 2002
UK	14	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
	2	Stool	Pinjon *et al.*, 1998, Gee *et al.*, 2002
	1	Blood	Pinjon *et al.*, 1998, Gee *et al.*, 2002
	1	sputum	Pinjon *et al.*, 1998, Gee *et al.*, 2002
USA	22	Oral	Pincus *et al.*, 1999; this study
TOTAL	**130**		
C. albicans			
Australia	1	Oral	Pinjon *et al.*, 1998
Hong Kong	6	Oral	Pinjon *et al.*, 1998
Ireland	61	Oral	Pinjon *et al.*, 1998
UK	2	Oral	Pinjon *et al.*, 1998
USA	96	Oral	Pincus *et al.*, 1999, this study
TOTAL	**166**		
C. glabrata	5	Oral	This study
C. tropicalis	5	Oral	This study
C. parapsilosis	6	Oral	This study

* The original source of the isolate was not available.

Table 3.2 Yeast isolates tested on Pal's agar.

Yeast species and source or country of isolation	No. of isolates	Specimen	Source/reference(s)
C. dubliniensis			
Argentina	1	Oral	Sullivan *et al.*, 1995, Gee *et al.*, 2002
Australia	2	Oral	Sullivan *et al.*, 1995, Gee *et al.*, 2002
Brazil	7	Oral	Milan *et al.*, 2001, this study
Canada	3	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
France	6	Oral	This study
Germany	2	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Israel	9	Sputum	Gee *et al.*, 2002, Polacheck *et al.*, 2000, this study
	2	BAL	This stud
	2	RT	Gee *et al.*, 2002, Polacheck *et al.*, 2000
	2	Urine	Gee *et al.*, 2002, Polacheck *et al.*, 2000, this study
	2	Vagina	Gee *et al.*, 2002
	1	Wound	Gee *et al.*, 2002
Ireland	43	Oral	Sullivan *et al.*, 1995, Pinjon *et al.*, 1998, Gee *et al.*, 2002, This study
Malta	3	Oral	This study
New Zealand	3	Oral	This study
Spain	2	Oral	Bikandi *et al.*, 1998, Gee *et al.*, 2002, this study
Switzerland	2	Oral	Sullivan *et al.*, 1998, Gee *et al.*, 2002
The Netherlands	2	Blood	Meis *et al.*, 1999, Gee *et al.*, 2002
	1	Sputum	Meis *et al.*, 1999, Gee *et al.*, 2002
UK	22	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002, this study
	2	Stool	Pinjon *et al.*, 1998, Gee *et al.*, 2002
	1	Sputum	Pinjon *et al.*, 1998, Gee *et al.*, 2002
	1	Blood	Pinjon *et al.*, 1998, Gee *et al.*, 2002
USA	7	Oral	Pincus *et al.*, 1999, this study
TOTAL	**128**		
C. albicans			
Greece	8	Oral	This study
Hungary	5	Oral	This study
Ireland	61	Oral	Pinjon *et al.*, 1998, this study
UK	2	Oral	Pinjon *et al.*, 1998
USA	48	Oral	Pincus *et al.*, 1999, this study
TOTAL	**124**		
C. glabrata	5	Oral	This study
C. tropicalis	4	Oral	This study
C. parapsilosis	5	Oral	This study
C. krusei	1	Oral	This study

Abbreviations: RT, respiratory tract; BAL, broncheoalveolar lavage.

then added before autoclaving. The composition of CAF (per liter) was as follows: $NHSO_4$, 5 g; glucose, 5 g; yeast extract, 2 g; K_2HPO_4, 0.8 g; $MgSO_4.7H_2O$, 0.7 g; caffeic acid, 0.18 g; chloramphenicol, 0.05 g; ferric citrate, 0.002 g.

3.2.3 Pal's agar

The yeast isolates and reference strains used in this part of the investigation are listed in Table 3.2. A total of 124 *C. albicans* and 128 *C. dubliniensis* isolates were included in the study. In addition, five *C. glabrata*, four *C. tropicalis*, five *C. parapsilosis*, and a single *C. krusei* isolate were also examined on Pal's medium (Table 3.2)..

Pal's agar was prepared freshly with extract of unsalted sunflower seeds (including kernels and shells). First, an aqueous extract of sunflower seeds was prepared by pulverizing 50 g of seeds in a domestic blender for 5 min and then adding the ground seeds to 1 liter of distilled water, followed by boiling for 30 min. Next, the seed extract was cooled and filtered, and the following ingredients were added: glucose (1 g), KH_2PO_4 (1 g), and creatinine (1 g). The pH was adjusted to 5.5, the volume was readjusted to 1 liter, and 15 g of agar (Difco) was added before the mixture was autoclaved at 110°C for 20 min.

3.2.4 Tobacco agar

The yeast isolates and reference strains used in this part of the investigation are listed in Table 3.3

Fifty -three *C. dubliniensis* isolates and 35 *C. albicans* isolates were included in this part of the study. In addition, seven *C. glabrata*, five *C. tropicalis*, five *C. parapsilosis*, two *C. krusei* and a single *Saccharomyces cerevisiae* were also examined on tobacco agar (Table 3.3). For tobacco agar, 50 g of commercialy avaliable tobacco (Delux mixture, Denmark) was mixed with 1 liter of distilled water. The mixture was boiled for 30 min, filtered and 20 g of agar is added to it then the volume was made up to 1 liter. Next, the pH of the agar was adjusted to 5.4 and then autoclaved at 121°C for 15 min.

3.2.1.4 Casein agar

All clinical and reference isolates included in this part of the present study are shown in Table 3.4. One hundred and nine *C. dubliniensis* isolates and 120 *C. albicans* isolates were tested on casein agar. In addition, fifty-seven non-*C. albicans* isolates were also

Table 3.3 Yeast isolates tested on tobacco agar.

Yeast species and source or country of isolation	No. of isolates	Specimen	Source/reference(s)
C. dubliniensis			
Australia	4	Oral	Sullivan *et al.*, 1995, Gee *et al.*, 2002
Brazil	1	Oral	Milan *et al.*, 2001
Canada	2	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Germany	2	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Ireland	20	Oral	Sullivan *et al.*, 1995 & 1997, Gee *et al.*, 2002
Israel	3	CF	This study
	2	Sputum	Polacheck *et al.*, 2000, Gee *et al.*, 2002.
	2	RT	Polacheck *et al.*, 2000, Gee *et al.*, 2002.
The Netherlands	2	Blood	Meis *et al.*, 1999, Gee *et al.*, 2002.
New Zealand	2	Oral	This study
Spain	4	Oral	Pinjon *et al.*, 1998, Quindos *et al.*, 2000, Gee *et al.*, 2002,
Switzerland	3	Oral	Boerlin *et al.*, 1995, Gee *et al.*, 2002
UK	6	Oral	Schoofs *et al.*, 1997, Pinjon *et al.*, 1998, Gee *et al.*, 2002
TOTAL	**53**		
C. albicans			
Greece	3	Oral	This study
Ireland	16	Oral	Pinjon *et al.*, 1998, This study
USA	16	Oral	Pincus *et al.*, 1999, This study
TOTAL	**35**		
C. glabrata	7	Oral	This study
C. tropicalis	5	Oral	This study
C. parapsilosis	5	Oral	This study
C. krusei	2	Oral	This study
S. cerevisiae	1	Oral	This study

Abbreviations: CF, cystic fibrosis; RT, respiratory tract.

Table 3.4. Yeast isolates tested on casein agar

Yeast species and source or country of isolation	No. of isolates	Specimen	Source/reference(s)
C. dubliniensis			
Argentina	1	Oral	Sullivan *et al.*, 1997, Gee *et al.*, 2002
Australia	3	Oral	Sullivan *et al.*, 1995, Gee *et al.*, 2002
Canada	5	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Finland	5	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Germany	4	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Greece	1	Oral	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Ireland	24	Oral	Sullivan *et al.*, 1995 & 1997, Gee *et al.*, 2002
	4	Blood	Gee *et al.*, 2002, this study
	1	Vaginal	Moran *et al.*, 1997
Italy	3	Oral	This study
Israel	2	RT	Gee *et al.*, 2002; Polacheck *et al.*, 2000
	2	Sputum	Gee *et al.*, 2002; Polacheck *et al.*, 2000
	1	Urine	Gee *et al.*, 2002; Polacheck *et al.*, 2000
	2	Vaginal	Gee *et al.*, 2002
	1	Wound	Gee *et al.*, 2002
The Netherlands	2	Blood	Meis *et al.*, 1999, Gee *et al.*, 2002
	1	Sputum	Meis *et al.*, 1999, Gee *et al.*, 2002
Spain	33	Oral	Pinjon *et al.*, 1998, Quindos *et al.*, 2000, Gee *et al.*, 2002, this study
	1	Blood	Salesa *et al.*, 2001
	1	Vaginal	This study
Switzerland	7	Oral	Boerlin *et al.*, 1995, Gee *et al.*, 2002
United Kingdom	6	Oral	Schoofs *et al.*, 1997, Pinjon *et al.*, 1998, Gee *et al.*, 2002
	1	Blood	Schoofs *et al.*, 1997, Pinjon *et al.*, 1998, Gee *et al.*, 2002
	1	Faecal	Pinjon *et al.*, 1998; Gee *et al.*, 2002
Culture collection	NCPF 3108	N/A	Sullivan *et al.*, 1995
TOTAL	**109**		
C. albicans			
Argentina	9	Oral	This study
Greece	4	Oral	This study
Ireland	6	Oral	This study
Hong Kong	1	Oral	Pinjon *et al.*, 1998
Spain	45	Oral	This study
	36	Vaginal	This study
	1	Urine	This study
	1	Penis	This study
United States	9	Oral	Pincus *et al.*, 1999, Al Mosaid *et al.*, 2001
Culture collections	8[a]	N/A	N/A
TOTAL	**120**		

Continued overleaf

Table 3.4 continued

Yeast species and source or country of isolation	No. of isolates	Secimen	Sources/reference(s)
C. glabrata			
Spain	9	Oral	This study
Culture collection	NCPF 3203	N/A	N/A
C. guilliermondii			
Spain	6	Oral	This study
	1	Vaginal	This study
Culture collection	NCPF 3099	N/A	N/A
C. krusei			
Spain	3	Oral cavity	This study
Culture collection	ATCC 6258	N/A	N/A
C. lusitaniae			
Spain	1	BA	This study
	1	Faecal	This study
	1	Urine	This study
C. parapsilosis			
Spain	5	Oral	This study
C. rugosa			
Bulgaria	6	Blood	This study
	1	Urine	This study
	1	PV	This study
C. stellatoidea **type I**			
Culture collection	ATCC 11006	N/A	N/A
C. stellatoidea **type II**			
Culture collection	ATCC 20408	N/A	N/A
C. tropicalis			
Ireland	5	Oral	This study
Spain	10	Oral	This study
Culture collection	NCPF 3111	N/A	N/A

[a] Culture collection strains included; NCPF 3153 & 3156, ATCC 26555, 64385, 64548, 64550, 90028 and 90029.

Abbreviations; RT, respiratory tract; BAL, broncheoalveolar lavage; PV, prosthetic valve; BA, bronchial aspirate; N/A, not applicable; ATCC, American Type Culture Collection; NCPF, National Collection of Pathogenic Fungi (UK).

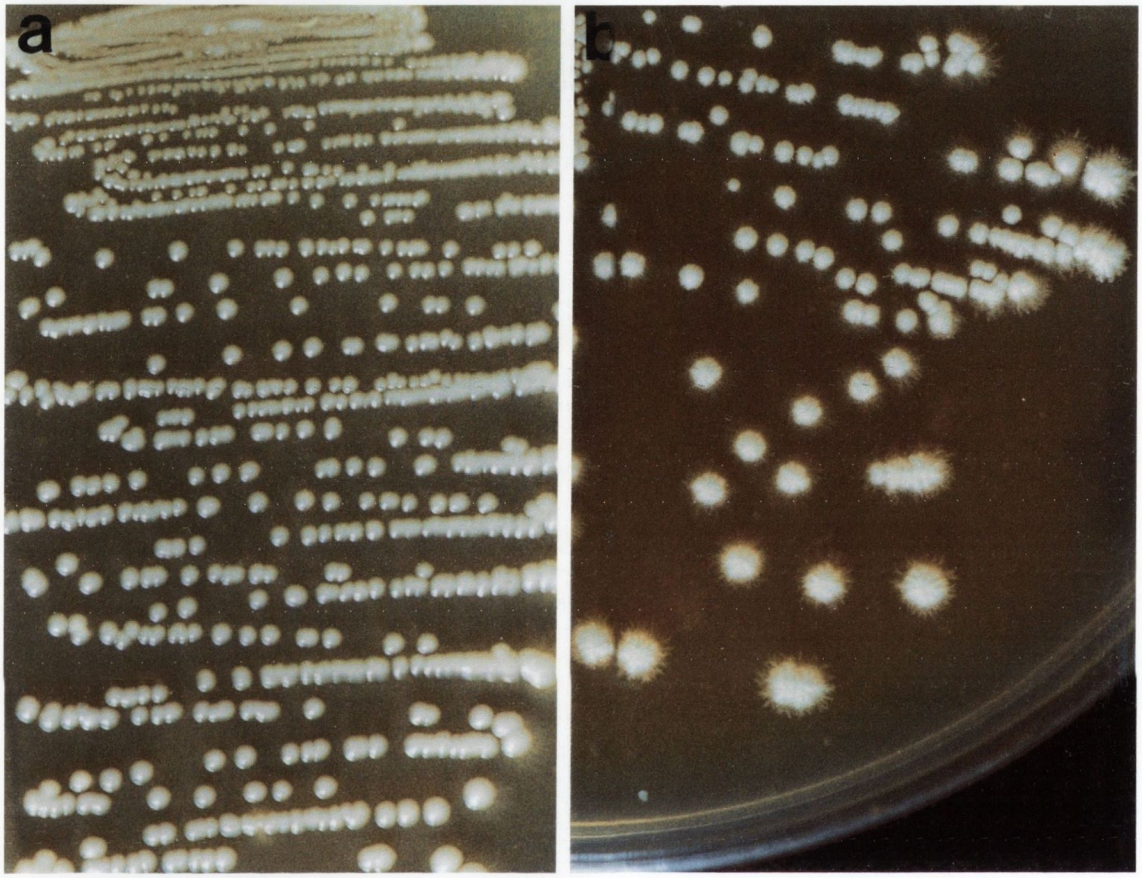
examined to determine whether they could be distinguished from isolates of *C. albicans* and *C. dubliniensis* (Table 3.4).

Casein agar was prepared as described by Larone, (1993). Briefly, 10 g of skim milk (Marvel dried skimmed milk; Premier Brands, Merseyside, UK) was dissolved in 90 ml of distilled water, and 3 g of agar was dissolved in 97 ml of distilled water. After autoclaving of both solutions separately at 121°C for 15 min, they were allowed to cool to 45 to 50°C and were then mixed together and dispensed in 25-ml amounts into 90 mm-diameter petri dishes.

A 48-h-old single colony from a PDA medium plate culture of each isolate to be tested was separately streak inoculated with a sterile wire loop onto Staib and CAF agars, Pal's, and tobacco agars, respectively, contained in 90-mm-diameter petri dishes (25 ml of agar per plate) and incubated at 30°C for 72 to 120 h. Caesin agar plates were inoculated with culture growth from a 48-h-old Sabouraud agar by cutting several shallow parallel groves in the agar with a wire loop, followed by incubation at 24°C for 48 h.

Gross colony morphologic features were examined visually on all media at 24-h intervals, and the data were recorded. Yeast colonies were also evaluated microscopically to detect the presence or absence of chlamydospore formation following 48 to 120 h of incubation. For each isolate, 10 well-separated single colonies were chosen at random and stained by the addition of 1 drop of 1% (wt/vol) lactophenol cotton blue stain (Larone, 1993) to enhance the detection of chlamydospores. Colonies were allowed to stain for 5 min and then covered with sterile glass coverslips (22 by 22 mm) and examined microscopically under bright-field illumination using a x40 objective (Sullivan *et al.*, 1995). Plates containing isolates that did not exhibit detectable chlamydospore formation within 120 h were re-examined following further incubation at intervals of 24 h for up to 3 weeks in total.

Figure 3.1 Morphological appearance of *C. dubliniensis* and *C. albicans* colonies on Staib medium following 72 h incubation at 30°C. (A) Smooth colonies exhibited by *C. albicans* isolate 132A (Gallagher *et al.*, 1992), composed exclusively of blastospores; (B) *C. dubliniensis* isolate CD36 (Sullivan *et al.* 1995) colonies displaying a hyphal fringe, containing abundant hyphae, pseudohyphae and chlamydospores.



3.3 Results

3.3.1 Growth of *Candida* isolates on Staib and CFA agars

All 296 yeast isolates grew on both Staib agar and CAF agar and yielded grey-white colonies. On Staib agar, all of the 166 *C. albicans* isolates tested produced smooth, shiny colonies after 48 h of incubation (Fig. 3.1). In all but three isolates, the colonies were found upon microscopic examination to be composed only of blastospores. Colonies of the three remaining isolates consisted mainly of blastospores with a few pseudohyphal elements after 48 and 72 h of incubation. Similar findings were observed after 96 and 120 h of incubation. In contrast, 127 (97.7%) of the 130 *C. dubliniensis* isolates tested on Staib agar yielded rough colonies, the majority (84; 64.6%) of which also exhibited a hyphal fringe around the colonies visible to the naked eye after 72 h of incubation (Fig. 3.1). The rough colonies were composed of mycelial forms (predominantly pseudohyphae) and blastospores. The three remaining *C. dubliniensis* isolates (two from Norway and one from Canada) produced smooth, shiny colonies on Staib agar that were similar in appearance and composition to those of *C. albicans* isolates, even after 2 weeks of incubation. Apart from these three isolates, the difference between the *C. dubliniensis* and *C. albicans* isolates was particularly evident on Staib agar after 48 h of incubation, and became further enhanced after 72 h of incubation, in the area of heavy culture growth where the primary inoculum was streaked (i.e., the *C. dubliniensis* culture growth appeared rough and the *C. albicans* culture growth appeared smooth and shiny). The five oral *C. glabrata* isolates tested yielded smooth, grey-white colonies similar to those of *C. albicans* on this medium. Furthermore, of the five *C. tropicalis* isolates tested, three yielded rough colonies similar to those of *C. dubliniensis* and two yielded smooth colonies similar to those of *C. albicans.* Of the six *C. parapsilosis* isolates tested, four yielded smooth, shiny colonies similar to those of *C. albicans* and two yielded rough colonies similar to those of *C. dubliniensis.*

On CAF agar, of the 166 *C. albicans* isolates included in the study, 145 (87.4%) yielded rough colonies with a mycelial halo visible to the naked eye after 5 days incubation. The remaining 21 (12.6%) isolates yielded smooth, non-shiny colonies on this medium. In comparison, 122 (93.8%) of the 130 *C. dubliniensis* isolates, including the 3 isolates which exhibited a smooth-colony phenotype on Staib agar, yielded rough colonies

with a mycelial halo after 5 days of incubation. The remaining eight (6.2%) isolates yielded rough colonies without a mycelial halo. Colonies of the *C. dubliniensis* isolates were noticeably smaller (~2 mm in diameter) than the *C. albicans* colonies (~3 mm) after 5 days of incubation on CAF.

3.2.2 Chlamydospore production on Staib and CAF agars

None of the 166 *C. albicans* isolates tested produced chlamydospores on Staib or CAF agars, even after prolonged incubation periods of up to 3 weeks (Table 3.1). In contrast, all isolates produced chlamydospores within 48 to 72 h on RAT agar. Similarly, all 130 of the *C. dubliniensis* isolates formed chlamydospores on RAT agar but not all produced chlamydospores on either Staib or CAF agars (Tables 3.1 & 3.5). A total of 111 (85.4%) of the 130 *C. dubliniensis* isolates formed chlamydospores on Staib agar, and 100 (90.1%) of these 111 produced abundant chlamydospores within 72 h, whereas 11 (9.9%) of them produced relatively few chlamydospores, which were only detected following incubation periods of up to a week. Similarly, 109 (83.8%) of the 130 *C dubliniensis* isolates produced chlamydospores on CAF, all but 5 within 120 h (Tables 3.1 and 3.2). Eighty-three (63.9%) of the *C. dubliniensis* isolates produced chlamydospores on both Staib and CAF agars, whereas the remainder produced chlamydospores on one or the other medium only (Table 3.5).

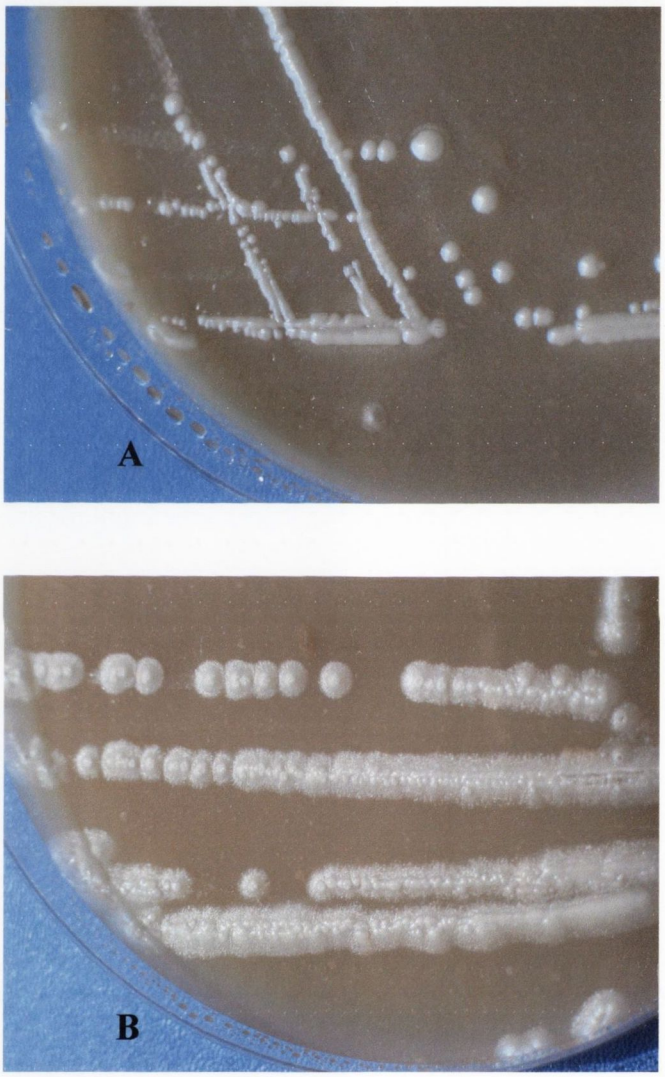
3.3.3 Growth of *Candida* isolates on Pal's agar

All of the yeast isolates tested grew well on Pal's agar (Table 3.2). Following 48 to 72 h of incubation, all 124 *C. albicans* and all 128 *C. dubliniensis* isolates tested grew as smooth creamy-gray colonies (Fig. 3.2A & B). However, all the *C. dubliniensis* isolates exhibited a hyphal fringe (Fig. 3.2B), whereas none of the *C. albicans* did (Fig. 3.2A). However, thirty-six of 124 *C. albicans* isolates (29%) were observed to produce a fringe following 10 days of incubation. Microbiological analysis revealed that the fringe surrounding *C. dubliniensis* colonies was found to be comprised of hyphae, pseudohyphae, and blastospores (Fig. 3.2A).

Table 3.5 Chlamydospre production by *C. dubliniensis* isolates on different media.

Media	No. of isolates tessted	No. of isolates Chlamydospore-positive (%)
Staib	130	111 (85.4)
CAF	130	109 (83.8)
Pal's	128	120 (93.75)
Tobbaco	53	52 (98)
Casein	109	106 (97.2)

Figure 3.2 Morphological appearance of *C. dubliniensis* and *C. albicans* colonies on Pal's medium following 72 h incubation at 37°C. (A) Smooth colonies exhibited by *C. albicans* isolate 132A (Gallagher *et al.*, 1992), composed exclusively of blastospores; (B) *C. dubliniensis* isolate CD36 (Sullivan *et al.*, 1995) colonies displaying a hyphal fringe, containing abundant hyphae, pseudohyphae and chlamydospores.



One *C. tropicalis* isolate, two *C. glabrata* isolates, and three *C. parapsilosis* isolates yielded smooth colonies similar to those of *C. albicans*. Three *C. tropicalis* isolates, two *C. parapsilosis* isolates, and the single *C. krusei* isolate formed rough colonies with a fringe similar to that formed by *C. dubliniensis*.

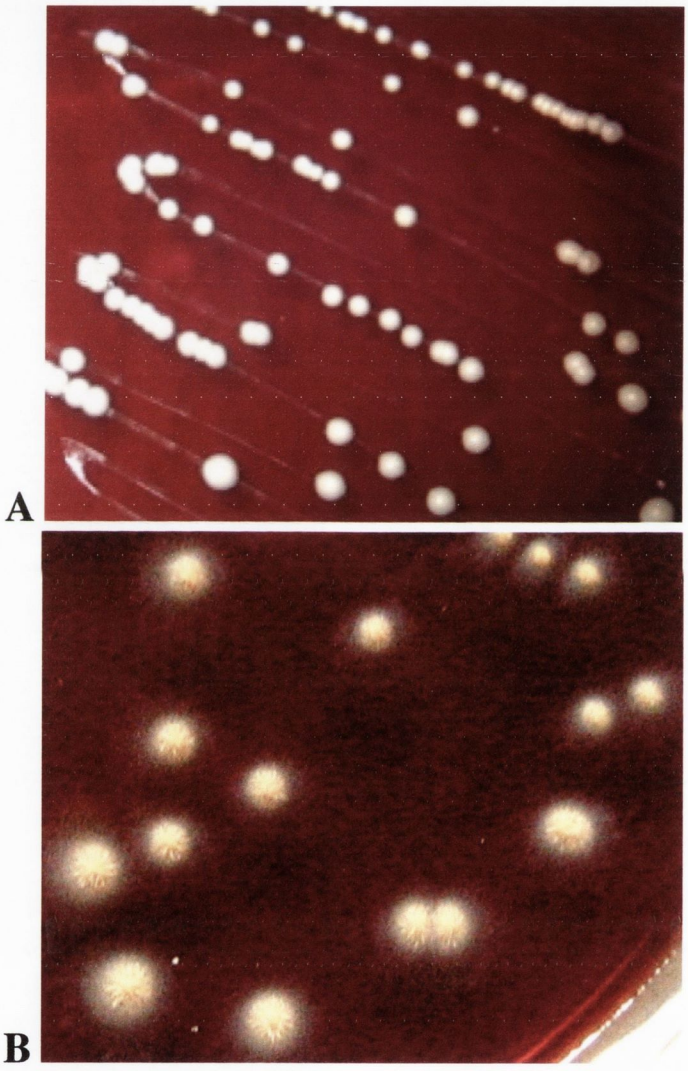
3.3.4 Chlamydospore production on Pal's agar

None of the *C. albicans* isolates produced chlamydospores on Pal's agar, even after 10 days of incubation, whereas 120/128 (93.75%) of the *C. dubliniensis* isolates tested produced chlamydospores within 48 to 72 h. The remaining 8 *C. dubliniensis* isolates (6.25%) were chlamydospore-negative even after 10 days of incubation.

3.3.5 Growth of *Candida* isolates on tobacco agar

All 107 yeast isolates grew on the medium except a single *Saccharomyces cerevisiae* isolate which did not grow at all (Table 3.3). Two oral *C. dubliniensis* Irish isolates grew very poorly on this medium. Fifty-one of the 53 (96.2%) *C. dubliniensis* isolates tested yielded rough, yellowish-brown colonies with hyphal fringe around the colonies visible to the naked eye after 48 to 72 h (Fig. 3.3A). The rough colonies were composed mainly of pseudohyphae and blastospores. The remaining two isolates (both isolates were oral isolates, one recovered from the UK and the other from Canada) produced smooth, white-creamy colonies similar to the appearance of *C. albicans* isolates on this medium. In contrast, 31/35 (88.5%) *C. albicans* isolates yielded smooth, white-creamy colonies with no hyphal fringe after 48 to 72 h (Fig. 3.3B). Of the remaining four *C. albicans* isolates, two isolates (5.7%) (both were Irish oral clinical isolates) produced smooth, white-creamy colonies with a hyphal fringe especially around the primary inoculum site. One of the remaining *C. albicans* isolates, (recovered in the USA) yielded yellow-brownish, rough colonies with a hyphal fringe around the colonies similar to those of *C. dubliniensis*, whereas the other isolat (an Irish oral clinical isolate) did not grow at all. The seven oral *C. glabrata* isolates examined produced smooth, creamy white colonies similar to those of *C. albicans* on this medium. Of the five *C. tropicalis* isolates examined, four yielded smooth, creamy white colonies with no hyphal fringes similar to those of *C. albicans* and one

Figure 3.3 Morphological appearance of *C. dubliniensis* and *C. albicans* colonies on tobacco agar following 72 h incubation at 30°C. (A) smooth, white-creamy colonies exhibited by *C. albicans* isolate 132A (Gallagher *et al.*, 1992), composed exclusively of blastospores; (B) *C. dubliniensis* isolate CD36 (Sullivan *et al.*, 1995) exhibiting yellowish-brown colonies with a hyphal fringe, containing abundant hyphae, pseudohyphae and chlamydospores.



produced smooth, creamy white colonies with a hyphal fringe. Furthermore, of the three *C. parapsilosis* isolates tested on this medium, two isolates produced smooth brownish colonies with no hyphal fringe and the third isolate exhibited smooth, white creamy colonies with a hyphal fringe. The two *C. krusei* isolates yielded rough, creamy colonies with a hyphal fringe.

3.3.6 Chlamydospore production on tobacco agar

Of the 53 *C. dubliniensis* isolates tested on tobacco agar, 52/53 (98%) produced chalmydospores after 48 to 72 h. In contrast, none of the 35 *C. albicans* isolates produced chalmydospore even after prolonged incubation of up to a week. Colonies of the *C. albicans* isolates tested consisted mainly of blastospores with a few pseudohyphal elements.

3.3.7 Chlamydospore production on casein agar

All 109 *C. dubliniensis* isolates tested produced chlamydospores on casein agar after 48 h of incubation at 24°C. Chlamydospores were stained dark blue by lactophenol cotton blue and were very abundant and arranged in groups around pseudomycelial growth (Fig. 3.4A), but in some cases isolated chlamydospores were also observed. The vast majority (111/120, 92.5%) of the *C. albicans* isolates tested did not produce chlamydospores on casein agar after 48 h of incubation. In these isolates, only blastospores stained a light blue colour by lactophenol cotton blue were observed (3.4B). However, 9/120 (7.5%) of the *C. albicans* isolates tested produced chlamydospores on casein agar. These chlamydospores, although indistinguishable from those produced by *C. dubliniensis*, were difficult to observe due to their low number. The ability to grow at 45°C was studied in an attempt to differentiate the nine *C. albicans* isolates producing chlamydospores on casein agar from the *C. dubliniensis* isolates. While no growth was found with any of the 109 *C. dubliniensis* isolates at 24 and 48 h on Sabouraud dextrose agar at 45°C, all 120 of the *C. albicans* isolates grew well at 45°C.

The production of chlamydospores on casein agar by *Candida* species other than *C. dubliniensis* and *C. albicans* was also investigated (Table 3.4). No chlamydospores were

produced by the *C. tropicalis, C. krusei, C. parapsilosis, C. guilliermondii, C. glabrata, C. lusitaniae, C. rugosa* and *C. stellatoidea* isolates tested (Table 3.4).

3.4. Discussion

As a consequence of the increasing number of reports on the isolation of *C. dubliniensis*, it is important to be able to rapidly and accurately identify this species in most clinical mycology laboratories. However, identification of *C. dubliniensis* is difficult because of its close relationship with *C. albicans*, a situation that has sometimes led to the misidentification of isolates of *C. dubliniensis* as *C. albicans* (Sullivan *et al.*, 1999). At present, the most accurate differentiation between isolates of the two species is performed in reference laboratories with the use of molecular-based techniques such as PCR or DNA fingerprinting with repetitive sequence-containing DNA probes (Tintelnot *et al.*, 2000; Sullivan *et al.*, 2004). However, these sophisticated techniques are not suitable and often not readily applicable for use in small clinical mycology laboratories where simple and rapid methods are needed. Phenotypic methods for the identification of *C. dubliniensis* isolates, including the inability of *C. dubliniensis* to grow at 45°C, colony colour on Chromagar Candida® medium, and other ancillary phenotypic tests as described in chapter 1 section 1.2.2 have been developed for discriminating between *C. dubliniensis* and *C. albicans* isolates (Coleman *et al.*, 1997a; Pinjon *et al.*, 1998; Tintelnot *et al.*, 2000; Sullivan *et al.*, 2004). However, whereas these tests are useful for the presumptive identification of *C. dubliniensis*, they are not definitive.

3.4.1 Differentiation of *C. dubliniensis* from *C. albicans* on Staib and CAF agars

Staib agar was originally developed as a means of identifying colonies of *C. neoformans*, which, unlike other members of this genus, develops dark pigmentation on this medium. Unlike all of the *C. albicans* isolates which produced smooth, shiny colonies composed of blastospores after 48 h of incubation (Fig. 3.1A), 97.7% of the 130 *C. dubliniensis* isolates tested on Staib agar exhibited rough colonies with the majority (84/130, 64.6%) showing a hyphal fringe around the colonies composed of mycelial forms and blastospores (Fig. 3.1B). These findings indicated that the different colony morphologies exhibited by isolates of *C. dubliniensis* and *C. albicans* were due predominantly to the production of mycelial forms by *C. dubliniensis* isolates. These results confirm the findings recently reported by Staib & Morschhäuser (1999) that Staib agar can

be used as a useful means to discriminate between isolates of *C. dubliniensis* and *C. albicans*. However, the method is not absolute, as a small minority (3/130, 2.3%) of the *C. dubliniensis* isolates tested were indistinguishable from *C. albicans* based on colony morphology on Staib agar.

Several isolates each of *C. tropicalis, C. glabrata* and *C. parapsilosis* were also tested on Staib agar in order to determine whether they could be distinguished from isolates of *C. albicans* and *C. dubliniensis* in the present study. The results, however, indicated that it would not be possible to identify colonies of *C. albicans* and *C. dubliniensis* based on colony morphology alone following primary isolation from a clinical specimen on Staib medium since *C. tropicalis, C. glabrata* and *C. parapsilosis* isolates exhibited colonies similar to one or both species.

Strachan *et al.*, (1971) demonstrated that *C. neoformans* produces brownish colonies when grown on a medium containing caffeic acid extracted from *Guizotia abyssinia* seeds. Since Staib agar was found to be excellent for distinguishing between *C. dubliniensis* and *C. albicans*, we investigated the usefulness of the more defined CAF agar to differentiate between isolates of these species. However, the results obtained indicated that unlike that on Staib agar, colony morphology on CAF agar could not be used to differentiate between *C. dubliniensis* and *C. albicans* isolates since the majority of both species (87.4% of the *C. albicans* isolates and 93.8% of the *C. dubliniensis* isolates) yielded rough colonies with or without a mycelial hyphal fringe. Furthermore, 111/130 (85.4%) and 109/130 (83.8%) of the *C. dubliniensis* isolates formed chlamydospores on Staib and CAF agars, respectively. In addition, 83/130 (63.9%) of the *C. dubliniensis* isolates produced chlamydospores on both Staib and CAF agars, whereas the remainder produced chlamydospores on one or the other medium only (Table 3.2). Two studies one by Lees & Barton *et al.*, (2003) and the other by Mähnß *et al.*, (2005), both published after the publication of the present work, reported a higher prevalence of *C. dubliniensis* isolates producing chlamydospres on Staib agar. However, both of these reports included a smaller number of *C. dubliniensis* isolates than the present study (nine and six *C. dubliniensis* isolates in the Lees & Barton *et al.*, (2003) and Mähnß *et al.*, (2005) reports, respectively) which very likely explains the difference in the results between the present study and the other two.

In the present study, none of the 166 *C. albicans* isolates tested produced chlamydospores on Staib or CAF agars. Nevertheless, when Mähnß *et al.* (2005) examined

94 *C. albicans* isolates on Staib agar, they reported that 14/94 (14.9%) of the *C. albicans* isolates tested were chlamydospores-positive at the end of the 8th day of incubation at 30°C. However, the identity of the *C. albicans* isolates included in Mähnß *et al.* (2005) study is doubtful since the formation of germ tubes in serum-free YPD-broth was observed in only 25% of the *C. albicans* strains included in their study after 1 h. In another study, Lees & Barton (2003) examined the ability of 495 clinical yeast isolates to produce chalmydospores on Staib agar. The authors reported that 419/495 (84.6%) of the yeast isolates tested were germ tube-positive and failed to produce chamydospre on staib agar. However, the investigators identified these yeast isolates as *C. albicans* without further laboratory tests to confirm the identity of these isolates. Furthermore, Lees & Barton (2003) reported that 4 *C. dubliniensis* isolates were germ tube-positive and produced chlamydospore on Staib agar. In addition, one *C. dubliniensis* isolate was found to be germ tube-negative and chlamydospore-negative on Staib agar. All *C. dubliniensis* isolates included in their study, however, were identified using API 32 C with no further tests to confirm the identity of these isolates. Therefore, the data reported in Lees & Barton (2003) study is not reliable since the identity of the yeast isolates included in their study was not confirmed. In addition, Lees & Barton (2003) examined the yeast isolates for the production of chlamydospore after 24 h of incubation at 37°C which is considered a short period of incubation when compared to other studies in the literature to evaluate chalmydospre production by *C. dubliniensis* and *C. albicans*. However, on the basis of our data, we agree with Staib & Morschhäuser, (1999) study that growth on Staib agar is an efficient means of discriminating between *C. dubliniensis* and *C. albicans*. However, our results suggest that colony morphology, rather than chlamydospore formation, is a more accurate criterion for species identification, since a significant proportion of *C. dubliniensis* isolates failed to produce chlamydospores on Staib agar. The disparity between our data and that of the Staib & Morschhäuser, (1999) could be due to either different supplies of *Guizotia abyssinica* seed, to seed of different ages, or to seed storage conditions. However, the most likely explanation lies in the larger and more diverse group of *C. dubliniensis* isolates examined in the present study.

3.4.2 Differentiation of *C. dubliniensis* from *C. albicans* on Pal's agar

In order to develop a medium with even greater discriminatory ability, we investigated the effect of replacing *Guizotia abyssinica* seed extract with extracts from the seeds of other plants. One such medium is Pal's agar, which contains sunflower (*Helianthus annus*) seed extract and is another medium originally developed for the identification of *C. neoformans* (Staib *et al.*, 1987). Since both *Helianthus annus* and *Guizotia abyssinica* belong to the same botanical family (*Asteraceae*), and since Staib agar is a good but not an absolutely reliable medium for differentiating between *C. dubliniensis* and *C. albicans*, we investigated the use of Pal's medium for this purpose. It was found that all *C. albicans* and *C. dubliniensis* isolates tested (n=252) yielded smooth creamy-gray colonies (Fig. 3.3A). In addition, all the *C. dubliniensis* isolates exhibited a hyphal fringe (Fig. 3.3B), whereas none of the *C. albicans* did. However, these findings are in contrast to previous data obtained by using Staib agar, on which 127/130 *C. dubliniensis* isolates tested (97.7%) formed rough colonies, many (65%) with a hyphal fringe, and all 166 *C. albicans* tested grew as smooth colonies. Four other *Candida* species were also tested on Pal's agar (Table 3.2). However, the results indicated that theses species can not be differentiated from *C. dubliniensis* and *C. albicans* on Pal's agar. Furthermore, all *C. albicans* isolates were chlamydospore negative while 120/128(93%) of the *C. dubliniensis* isolates produced chlamydospres within 72 h. Interestingly, in the previous study with Staib agar, none of the 166 *C. albicans* isolates tested produced chlamydospores whereas only 19 of 130 of the *C. dubliniensis* isolates examined (14.6%) were chlamydospore-negative. Twelve of these 19 chlamydospore-negative *C. dubliniensis* isolates were included in the present study; 7 were chlamydospore positive on Pal's agar, and the remaining 5 were chlamydospore negative.

In the present study reproducible results were obtained with all isolates tested on separate batches of Pal's agar prepared from the same batch of sunflower seeds, in each case with seeds purchased from two separate suppliers. However, it was found that incubation of the plates at 37°C rather than 30°C resulted in poorer discrimination between the two species.

Shortly after the publication of this part of the present study Khan *et al.* (2004) evaluated Pal's agar and another simplified form of the same agar (without creatinine and KH_2PO_4) to differentiate between isolates of *C. dubliniensis* (n=25) and *C. albicans* (n=53).

Khan *et al.* (2004) found that all *C. albicans* isolates (n = 53) yielded smooth colonies with no evidence of chlamydospore formation and that all *C. dubliniensis* isolates tested developed rough colonies, all in agreement with the result of the present study. However, Khan *et al.* (2004) reported that all *C. dubliniensis* isolates tested (25/25, 100%) formed abundant chlamydospores after incubation for 24-48 h at 28°C which is in contrast to the finding of the present study in which 6.25% of the *C. dubliniensis* isolates tested were chalmydospre-negative. The disparity between our data and that of Khan *et al.* (2004) could be due to either different supplies of sunflower (*Helianthus annus*) seed, to seed of different ages, or to seed storage conditions. However, the most likely explanation lies in the larger and more diverse group of *C. dubliniensis* isolates examined in the present study.

The results of this part of the present study demonstrated that the formation of a hyphal fringe surrounding *C. dubliniensis* colonies on Pal's medium following incubation for 48 to 72 h at 30°C provides a definitive means of discrimination between this species and *C. albicans*. However, since colonies of some isolates belonging to other *Candida* species also produce a fringe on Pal's agar, this medium should be used only to screen germ tube- and/or chlamydospore-positive isolates.

3.4.3 Differentiation of *C. dubliniensis* from *C. albicans* on tobacco agar

Tobacco agar was first used for the identification of *C. neoformans* which yields brownish colonies on this medium (Tendolkar *et al.*, 2003). During the course of the present study, Khan *et al.* (2004) reported the use of tobacco medium to differentiate *C. dubliniensis* from *C. albicans*. Khan *et al.* (2004) reported that 30/30 (100%) of the clinical *C. dubliniensis* isolates tested produced rough, yellowish-brown colonies with hyphal fringes and abundant chlamydospores while 54 *C. albicans* isolates yielded smooth, white-to-cream coloured colonies and were chlamydospores negative. In contrast, the present study showed that although 51/53 (96%) of the *C. dubliniensis* isolates tested produced yellowish-brown colonies with a hyphal fringe, 2/53 (3.7%) of the *C. dubliniensis* isolates tested yielded smooth, white creamy colonies similar to that produced by *C. albicans* after 48 h at 30°C. Furthermore, of the 35 *C. albicans* isolates examined, one isolate (2.8%) did not grow on tobacco agar, another isolate (1/35, 2.8%) exhibited yellowish-brown colonies very similar to that produced by *C. dubliniensis* and 2/35 (5.7%) *C. albicans* isolates

produced white creamy colonies with a hyphal fringe after 72 h of incubation at 30°C The remaining 31/35 *C. albicans* isolates produced smooth, white-creamy colonies with no hyphal fringe. Furthermore, 52/53 (98%) *C. dubliniensis* isolates produced abundant chlamydospores within 72 h while one (1/53, 1.8%) isolate did not. In contrast, none of the *C. albicans* isolates (35/35, 100%) examined produced chlamydospores even after prolonged incubation at 30°C. for a week.

Examination of isolates of some non-*C. albicans* species on tobacco agar revealed that these species could not be differentiated from isolates of *C. dubliniensis* and *C. albicans*. Therefore, we agree with Khan *et al.* (2004) that tobacco agar is a useful medium for the differentiation of *C. dubliniensis* from *C. albicans*. However, the agar does not identify *C. dubliniensis* with 100% accuracy as indicated in their study and therefore further identification methods are needed. The most likely explanation for the difference between our data and that of Khan *et al.* (2004) could be due to the larger and more diverse group of *C. dubliniensis* isolates examined in the present study. However, the disparity in the result is unlikely to be due to the different commercial brands of tobacco used since reproducible results were obtained in both studies, even when different commercial brands of tobacco were used.

3.4.4 Differentiation of *C. dubliniensis* from *C. albicans* on casein agar

Casein agar has been traditionally used to study the decomposition of casein by aerobic actinomycetes and dematiaceous fungi (Larone, 1993). However, results presented in the present study show, for the first time, that casein agar is a good medium to induce the production of chlamydospores by *C. dubliniensis* isolates, a feature that can be used to differentiate *C. dubliniensis* from *C. albicans.* Although 106/109 (97.2%) of the *C. dubliniensis* isolates tested produced abundant chlamydospores on casein agar, nine isolates of *C. albicans* isolates also produced a few chlamydospores. Attempts to improve discrimination between the two species by decreasing the temperature of incubation or by modifying the composition of casein agar by varying the amount of skim milk added or by incorporating 1% Tween 80 were unsuccessful (data not shown). Nevertheless, casein agar seems to be suitable for studying chlamydospore production by fresh isolates, since, when 8 fresh *C. dubliniensis* oral isolates and 10 fresh *C. albicans* oral isolates were tested, all of

the *C. dubliniensis* isolates produced abundant chlamydospores within 48 h, whereas none of the *C. albicans* isolates did. It should be noted, however, that discrimination between *C. dubliniensis* isolates and the nine *C. albicans* isolates that produced chlamydospores on casein agar was achieved by incubating the isolates at 45°C, since all nine *C. albicans* isolates grew well at that temperature, whereas the 109 *C. dubliniensis* isolates included in the study did not (data not shown). Nevertheless, although growth at 45°C alone permitted discrimination between *C. albicans* and *C. dubliniensis* isolates in the present study, it has been reported that some *C. albicans* isolates are not able to grow at 45°C (Kirkpatrick *et al.*, 1998; Pinjon *et al.*, 1998; Milan *et al.*, 2001).

3.4.5 Conclusions

Attempts to differentiate *C. dubliniensis* from *C. albicans* on the basis of chalmydospore production and/or colony morphology on the media described above proved to be useful. They are easy to prepare in routine clinical mycology laboratories and are amenable to large numbers of clinical samples. In addition, they provide a simple and inexpensive means of differentiating isolates of *C. dubliniensis* and *C. albicans.* In the vast majority of cases, isolates producing abundant chlamydospores on those media can be presumptively identified as *C. dubliniensis*. However, although casein agar was the least expensive and the easiest to be prepared, Pal's medium has an important advantage over all the media described above in that it permits an absolute discrimination between *C. dubliniensis* and *C. albicans* based on colony morphology of both species on this medium. In general, germ tube-positive oral isolates from HIV-positive and AIDS patients, as well as germ tube-positive isolates from sterile sites from other immunocompromised groups, should be tested on these readily available media in association with other phenotypic tests for *C. dubliniensis*. These media will also be of benefit for researchers interested in studying the incidence and epidemiology of this emerging pathogen. In addition, they will facilitate molecular analysis of chlamydospore development since they induce chlamydospore production by *C. dubliniensis* but not by *C. albicans*.

Chapter 4

Identification of a distinct clade of *Candida dubliniensis* from Saudi Arabia and Egypt

4.1 Introduction

4.1.1 Epidemiological analysis of *C. dubliniensis* recovered from Saudi Arabia and Egypt

Candida dubliniensis was initially associated with oral carriage and infection in Irish and Australian HIV-infected and AIDS patients (Sullivan *et al.*, 1995). It has also been found as an oral carriage organism in a small percentage of normal healthy individuals, and more recently has been implicated as an agent of oral colonisation and disease in other immunocompromised groups, such as diabetics and in patients with cancer (Polacheck *et al.*, 2000; Sebti *et al.*, 2001; Manfredi *et al.*, 2002; McMullan *et al.*, 2002; Tekeli *et al.*, 2004). Furthermore, numerous reports have confirmed the presence of *C. dubliniensis* in the oral cavities of HIV-infected individuals from widespread geographic locations (Coleman *et al.*, 1997a; Sullivan *et al.*, 1997; Sullivan & Coleman , 1998; Ponton *et al.*, 2000; Sullivan *et al.*, 2004). However, most reported isolates of *C. dubliniensis* have been recovered from individuals in Europe, the USA, South America and Australia (Polacheck *et al.*, 2000; Gee *et al.*, 2002; Sullivan *et al.*, 2004) but there is relatively little information on the prevalence or the population structure of this organism in the Middle East. There have been a few reports of the isolation of *C. dubliniensis* in Israel, Saudi Arabia and Kuwait (Polacheck *et al.*, 2000; Lefler *et al.*, 2001; Gee *et al.*, 2002; Fotedar & Al-Hedaithy, 2003 & 2004; Ahmad *et al.*, 2004; McCullough *et al.*, 2004) but little is known about the relatedness of *C. dubliniensis* isolates from this area to isolates from the rest of the world.

The development of the *C. dubliniensis* species-specific complex DNA fingerprinting probe Cd25 by Joly *et al.* (1999) has allowed the genetic relatedness of individual isolates and the population structure of *C. dubliniensis* to be investigated in detail. Computer-assisted analysis of Cd25-generated fingerprint profiles of 57 independent *C. dubliniensis* isolates from 11 different countries by Joly *et al.* (1999) revealed the existence of two distinct *C. dubliniensis* populations. The results of this study were subsequently confirmed by Gee *et al.* (2002) with 98 *C. dubliniensis* isolates recovered from 94 separate individuals from 15 different countries. In the latter study, Cd25 fingerprint group I isolates were found to be associated predominantly with HIV-infected individuals (67.6%), whereas Cd25 fingerprint group II isolates were associated predominantly with non-HIV-infected individuals (70.4%). Cd25-group I isolates were found to consist of closely related isolates

(average similarity coefficient (S_{AB}) value of 0.8), whereas Cd25-group II isolates were found to be less closely related (average S_{AB} value of 0.57) and contained a number of distantly related sub-populations and outliers (Gee *et al.*, 2002). The study of Gee *et al.* (2002) also revealed that Cd25 fingerprint group I isolates consisted of a single genotype (genotype 1) based on the nucleotide sequence of the internal transcribed spacer region (ITS) of the rDNA gene cluster, whereas Cd25 fingerprint group II isolates were found to consist of three genotypes (genotypes 2-4).

The first aim of this part of the present study was to investigate the prevalence of *C. dubliniensis* in oral-*Candida*-positive cohorts of immunocompromised patients in Saudi Arabia and Egypt.

The second aim was to investigate the relatedness of *C. dubliniensis* isolates recovered from individuals in Saudi Arabia and Egypt to isolates recovered in many different countries world-wide by analysis of genotypes and Cd25-generated fingerprint profiles.

4.1.2 Resistance to 5-Flucytosine

5-FC was discovered in 1957 during a search for novel antineoplastic agents. While devoid of anticancer properties, it became apparent that it was a potent antifungal agent. 5-FC is a water-soluble pyrimidine analogue that is related to the chemotherapeutic agent 5-fluorouracil (5-FU) (White *et al.*, 1998). The antifungal agent 5-FC is metabolised via the pyrimidine salvage pathway (Fig. 4.1). 5-FC is taken up by fungal cells via the enzyme cytosine permease. It is converted intracellularly to 5-FU by cytosine deaminase, encoded by the gene *FCY1*. The enzyme uracil phosphoribosyltransferase (UPRTase), encoded by the gene *FUR1* then converts 5-FU to 5-fluorouridine monophosphate (5-dUMP). 5-fluorouridine monophosphate is then converted to 5-fluorouridine triphosphate which disrupts protein synthesis when incorporated into RNA. 5-FU is also reduced to 5-fluorodeoxyuridine monophosphate which inhibits the enzyme thymidylate synthetase and hence DNA synthesis. Since human cells lack the enzyme cytosine deaminase, they are unable to convert 5-FC to its active metabolites (Whelan & Kerridge, 1984). 5-FC resistance however, is mediated through altered metabolism of the drug. Several previous studies have indicated that the majority of *C. dubliniensis* clinical isolates reported in the literature are susceptible to commonly used antifungal drugs, including azoles,

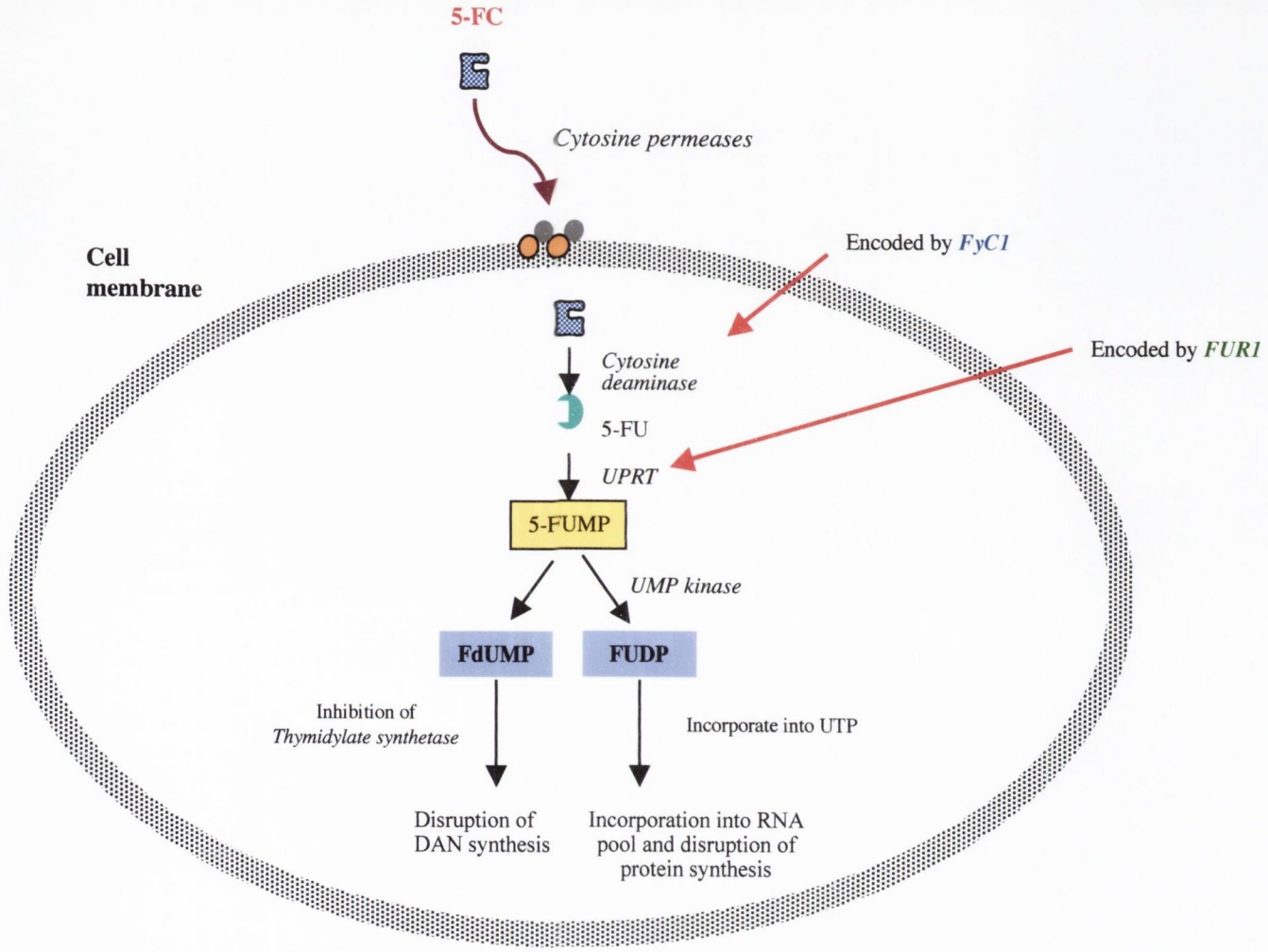


Figure 4.1. Schematic view of 5-Flucytosine metabolism.

Abbreviations; 5-Flucytosine; 5-FU, 5-fluorouracil; . 5-FUMP, 5-fluorouridine monophosphate; UPRT, uracil phosphoribosyltransferase; FUDP, 5-fluorouridine triphosphate, FdUMP, 5-fluorodeoxyuridine monophosphate

amphotericin B and 5-FC (Moran *et al.*, 1997 & 2002; Pfaller *et al.*, 1999a & 2002; Quindos *et al.*, 2004). Pfaller *et al.* (2002) assessed primary resistance to 5-FC in 8,803 clinical isolates of *Candida* species, including *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. dubliniensis, C. guilliermondii, C. lusitaniae, C. famata, C. kefyr* and others. Of the 8,803 isolates investigated, 95% were susceptible to 5-FC. A similar study to investigate 5-FC resistance in 1,021 Spanish clinical isolates of *Candida* species reported that 83.4% of the isolates studied were susceptible to 5-FC. All *C. dubliniensis* isolates included in both studies (90 isolates from the Pfaller *et al.* study and 41 isolates from the Quindos *et al.* study) were susceptible to 5-FC. In contrast, two recent studies reported an unusually high prevalence of 5-FC-resistance among *C. dubliniensis* isolates from Saudi Arabia and Kuwait (Fotedar & Al Hedaithy, 2003; Ahmad *et al.*, 2004).

Primary resistance to 5-FC in *C. albicans* was initially recognised in isolates belonging to serogroup B. More recently, resistance to 5-FC in *C. albicans* was linked to a single clade (Pujol *et al.*, 2004). A study by Dodgson *et al.* (2004) demonstrated that 5-FC resistance in *C. albicans* is linked to a point mutation at nucleotide position 301 in the *FUR1* gene encoding uracil phosphoribosyltransferase (UPRTase) activity that results in the replacement of arginine with cysteine at amino acid position 101 in the translated protein. Another recent study reported that a point mutation in the *C. albicans FCY1* gene (encoding cytosine deaminase) resulting in a glycine to aspartate substitution at position 28 in the translated protein may also be associated with 5-FC resistance (Hope *et al.*, 2004).

The third aim of the present study sought to further investigate the association between the *C. dubliniensis* isolates recovered from the Middle East and resistance to 5-FC. In addition, the molecular mechanisms of primary 5-FC resistance in *C. dubliniensis* isolates was also investigated by analysis of mutations in the *C. dubliniensis FUR1* and *FCY1* genes.

4.2 Materials and Methods

4.2.1 *C. dubliniensis* clinical isolates

The *C. dubliniensis* clinical isolates used in this study that were recovered from individual patients in Saudi Arabia, Egypt and Israel are listed in Table 4.1. A total of 309 Saudi Arabian patients at the Riyadh Medical Center and the Jeddah Kidney Center, Saudi Arabia, and 125 Egyptian patients at the National Institute of Diabetes and the National Institute of Cancer, Cairo, Egypt were tested for the presence of oral *C. dubliniensis*. Prior to sampling, the presence of oral clinical signs and symptoms indicative of oral candidiasis, including pseudomembranous candidiasis, erythematous candidiasis, median rhomboid glossitis and angular cheilitis (Coleman *et al.*, 1997a), were recorded. Swab specimens from the mid-dorsum of the tongue were taken using sterile cotton wool swabs (Venturi, Transystem, Copan, Italy). Saudi patients were sampled by myself (Asmaa Al Mosaid) under the supervision of Dr. Nisreen Khan (the Riyadh Medical Center) and Dr. Faisal Shaheen (the Jeddah Kidney Center). Egyptian patients were sampled by Dr. Motaz Kabadaya under the supervision of Dr. Osama Soliman. Following sampling, each swab was placed in a tube of sterile hydrated sodium alginate supplied with the swab, labelled, packaged and transferred to laboratory for culture. All swabs were plated onto CHROMagar Candida medium (Coleman *et al.*, 1997a; Sullivan & Coleman, 1998). After 48 h of incubation at 37°C, the number of colonies present on each plate, their colour and relative abundance were recorded.

In addition, five other *C. dubliniensis* clinical isolates (SA100 to SA104) previously recovered from various specimens from separate patients at the King Faisal and King Khalid Hospitals, Riyadh, Saudi Arabia and 16 *C. dubliniensis* isolates recovered from various specimens from patients at the Hadassah Medical Center, Jerusalem, Israel, were included in the study (Table 4.1).

The identity of all isolates was confirmed as described in chapter 2 section 2.2.

4.2.2 Preparation of yeast DNA and Southern hybridisation

The preparation of yeast genomic DNA, *Eco*RI restriction enzyme digestion and Southern hybridisation with the Cd25 probe were as described in chapter 2 sections 2.3.1 to 2.3.5.

Table 4.1. *C. dubliniensis* clinical isolates used in the study.

C. dubliniensis[a] isolate	Country[b] of origin	Yr of isolation	Underlying patient condition	Sample	5FC MIC_{50}[c] µg/ml	Cd25[d] fingerprint group	Genotype
Eg200*	Egypt	2002	Diabetes	Oral	128	III	4
Eg201	Egypt	2002	Cancer	Oral	128	III	4
Eg202	Egypt	2002	Cancer	Oral	128	III	4
Eg203	Egypt	2002	Cancer	Oral	0.125	I	1
Eg204	Egypt	2002	Cancer	Oral	0.125	I	1
Eg205	Egypt	2002	Diabetes	Oral	0.125	I	1
Eg206	Egypt	2002	Diabetes	Oral	0.125	I	1
Eg207	Egypt	2002	Diabetes	Oral	128	III	4A[f]
SA100*	S. Arabia	2002	Leukemia	Oral	128	III	3
SA101*	S. Arabia	2002	Leukemia	Oral	0.125	I	1
SA102*	S. Arabia	2002	Leukemia	Blood	0.125	I	1
SA103	S. Arabia	2002	Pneumonia	BAL	128	III	3
SA104	S. Arabia	2002	Pneumonia	Oral	128	III	4
SA105*	S. Arabia	2002	Diabetes	Oral	0.125	I	1
SA106	S. Arabia	2002	Diabetes	Oral	0.125	I	1
SA107*	S. Arabia	2002	Diabetes	Oral	128	III	3
SA108	S. Arabia	2002	Diabetes	Oral	128	III	3
SA109	S. Arabia	2002	Diabetes	Oral	128	III	3
SA110	S. Arabia	2002	Diabetes	Oral	128	III	3
SA111	S. Arabia	2002	Diabetes	Oral	128	III	3
SA112	S. Arabia	2002	Diabetes	Oral	128	III	3
SA113*	S. Arabia	2002	Diabetes	Oral	128	III	4
SA114	S. Arabia	2002	Diabetes	Oral	128	III	3
SA115	S. Arabia	2002	Diabetes	Oral	0.125	I	1
SA116	S. Arabia	2002	Diabetes	Oral	0.125	I	1
SA117	S. Arabia	2002	Diabetes	Oral	128	III	3
SA118	S. Arabia	2002	S/P renal Tx	Oral	128	III	3
SA119	S. Arabia	2002	S/P renal Tx	Oral	128	III	3
SA120	S. Arabia	2002	S/P renal Tx	Oral	128	III	3
SA121	S. Arabia	2002	S/P renal Tx	Oral	128	III	4
Is34	Israel	2000	Liver transplant	BAL	0.125	ND	3
Is35	Israel	2001	Nephritis	Oral	0.125	II	4B[f]
Is36	Israel	2001	CF	Sputum	0.125	II	3
Is38	Israel	2001	SBE	Urine	0.125	ND	1
Is39	Israel	2001	Stroke	Sputum	0.125	ND	1
Is40	Israel	2002	Renal Failure	Throat	0.125	ND	1
Is41	Israel	2002	CF	Sputum	0.125	ND	1
Is42	Israel	2002	Leukemia	Throat	0.125	ND	1
Is43	Israel	2002	CF	Sputum	0.125	ND	1
Is44	Israel	2002	CF	Sputum	0.125	ND	1
Is45	Israel	2002	CF	Sputum	0.125	I	1
Is46	Israel	2002	Trauma	BAL	0.125	ND	1
Is47	Israel	2002	Ciliary Dyskinesia	Sputum	0.125	I	1
Is48	Israel	2002	CF	Sputum	0.125	ND	1
Is49	Israel	2003	CF	Sputum	0.125	II	2
Is51	Israel	2003	Post surgery	ENT	0.125	ND	1

[a] Isolates marked with an asterisk had their *FCY1* and *FUR1* genes amplified and sequenced.

[b] Egyptian and Saudi Arabian isolates were all recovered from Arab individuals, whereas Israeli isolates were recovered from Jewish individuals apart from Is35, Is40, Is43 and Is44, which were recovered from Arab individuals in Israel.

[c] MIC values were determined according to the NCCLS document 27-A2 (NCCLS, 1997).

[d] Refers to major groups of isolates (Fig. 5.6) identified following computer-assisted analysis of Cd25-generated hybridization fingerprint profiles of *Eco*RI-digested genomic DNA using the DENDRON computer software package. Only 5 selected Israeli isolates were fingerprinted in the present study although all sixrteen were genotyped.

[e] Determined by PCR using the genotype-specific primers described by Gee *et al.* (2002).

[f] The nucleotide sequence of the ITS region of the variant genotypes 4A and 4B differed from the genotype 4 ITS consensus sequence at one and two nucleotide positions, respectively

Abbreviations: S. Arabia, Saudi Arabia; BAL, broncheoalveolar lavage; ENT, endotrachael aspirate; SBE, subacute bacterial endocarditis; S/P renal Tx, stage-post renal transplantation; CF, Cystic fibrosis; ND, not done.

4.2.3 Preparation of the Cd25 fingerprinting probe

Cd25 is a genomic sequence containing a dispersed repetitive element cloned from *C. dubliniensis* into bacteriophage Lambda EMBL3 (Joly *et al.*, 1999). Cd25 DNA was prepared as described in chapter 2 section 2.4.5.

4.2.4 Computer assisted analysis of DNA fingerprint profiles

Computer-assisted analysis of DNA fingerprint patterns of *C. dubliniensis* isolates generated with the *C. dubliniensis*-specific repetitive sequence containing probe Cd25 were performed as described previously (Schmid *et al.*, 1990; Joly *et al.*, 1999). To analyse DNA fingerprint patterns, DNA hybridisation patterns on autoradiographs were digitised using the DENDRON software package version 4.0 (Solltech, Iowa City, Iowa, USA) using an Epson scanner (Seiko Epson Corp., Japan). The fingerprint pattern of the *C. dubliniensis* isolate CM6 (Sullivan *et al.*, 1995) was used as a reference in each experiment as a universal standard to link data from different autoradiographs. Distortions in autoradiogram images were corrected using the unwarping and straightening options of DENDRON. Lanes and bands were automatically identified and analysed. Patterns of different *C. dubliniensis* isolates were compared by computing similarity coefficient values (S_{AB}) between every possible pairwise combination of isolates studied. The S_{AB} computation was based on band positions alone according to the formula S_{AB}=2E (2E +a+b), where E is the number of bands in patterns A and B sharing the same positions, a is the number of bands in pattern A with no correlates in pattern B and b is the number of bands in pattern B with no correlates in pattern A. An S_{AB} of 0.00 indicates A and B patterns with no bands in common, an S_{AB} of 1.00 indicates A and B patterns with all bands matching and S_{AB}'s from 0.01 to 0.99 represent patterns with increasing numbers of bands at the same position. The unweighted pair group method was used to create dendrograms using DENDRON based on S_{AB} values (Soll, 2000).

4.2.5 Genotyping using PCR amplification

Template DNA from each of the Saudi Arabian, Egyptian and Israeli *C. dubliniensis* isolates investigated in this study was tested in separate PCR amplification experiments with each of the primer pairs G1F/G1R, G2F/G2R, G3F/G3R, and G4F/G4R to identify the genotype of the isolates as described by Gee *et al.* (2002). Genotypes are ascribed based on

Table 4.2 International *Candida dubliniensis* isolates [a] tested for susceptibility to 5-FC.

C. dubliniensis isolates	Country of origin	Year of isolation	Body site	5 FC MIC_{50} µg/ml [b]	Genotype [c]	Reference
CM6*	Australia	1992	Oral	0.125	1	Sullivan *et al.*, 1995, Gee *et al.*, 2002
Can6*	Canada	1996	Oral	0.125	2	Pinjon *et al.*, 1998, Gee *et al.*, 2002
Can9	Canada	1996	Oral	0.125	2	Pinjon *et al.*, 1998, Gee *et al.*, 2002
CD516	Finland	1996	Oral	0.125	1	Pinjon *et al.*, 1998, Gee *et al.*, 2002
CD96.54	Germany	1996	Oral	0.125	1	Pinjon *et al.*, 1998, Gee *et al.*, 2002
CD159	Greece	1995	Oral	0.125	1	Pinjon *et al.*, 1998, Gee *et al.*, 2002
CD98923	India	1998	Oral	0.125	1	Al Mosaid *et al.*, 2001, Gee *et al.*, 2002
CD36*	Ireland	1988	Oral	0.125	1	Sullivan *et al.*, 1995, Gee *et al.*, 2002
CD506*	Ireland	1989	Oral	0.125	2	Pinjon *et al.*, 1998, Gee *et al.*, 2002
CD514*	Ireland	1995	Oral	0.125	3	Gee *et al.*, 2002
CD519*	Ireland	1997	Oral	0.125	3	Pinjon *et al.*, 1998, Gee *et al.*, 2002
CD531	Ireland	1998	Oral	0.125	2	Gee *et al.*, 2002
P7276	Israel	1999	RT	0.125	3	Polacheck *et al.*, 2000, Gee *et al.*, 2002
P6265*	Israel	1999	Sputum	0.125	3	Polacheck *et al.*, 2000, Gee *et al.*, 2002
P6785	Israel	1999	Urine	0.125	3	Polacheck *et al.*, 2000, Gee *et al.*, 2002
P7718*	Israel	1999	wound	0.125	4	Gee *et al.*, 2002
CBS 8501	Netherlands	1998	Blood	0.125	1	Meis *et al.*, 1999, Gee *et al.*, 2002
CBS2747	Netherlands	1952	sputum	0.125	2	Meis *et al.*, 1999, Gee *et al.*, 2002
CD19398	Norway	1998	Oral	0.125	1	Al Mosaid *et al.*, 2001, Gee *et al.*, 2002
CD2491	Spain	1994	Oral	0.125	1	Pinjon *et al.*, 1998, Gee *et al.*, 2002
CD75043	UK	1975	Oral	0.125	2	Pinjon *et al.*, 1998, Gee *et al.*, 2002
CD541	UK	1997	Blood	0.125	2	Pinjon *et al.*, 1998, Gee *et al.*, 2002
m26b	UK	1995	Oral	0.125	2	Pinjon *et al.*, 1998, Gee *et al.*, 2002
m196cd	UK	1995	Oral	0.125	1	Pinjon *et al.*, 1998, Gee *et al.*, 2002

[a] The *C. dubliniensis* isolates were included in a previous study by Gee *et al.*, (2002).
[b] MIC values were determined according to the NCCLS document 27-A2 (NCCLS, 1997).
[c] Determined by PCR using the genotype-specific primers described by Gee *et al.* (2002).
Isolates marked with an asterisk had their *FCY1* and *FUR1* genes amplified and sequenced.
Abbreviations: RT, respiratory tract.

the nucleotide sequence of the internal transcribed spacer 1 and 2 (ITS1 and ITS2, respectively) regions and the intervening 5.8S rRNA gene (White *et al.*, 1990). Template DNA from the four reference *C. dubliniensis* isolates (CD36, genotype 1; Can4, genotype 2; CD519, genotype 3 and p7718, genotype 4) previously described by Gee *et al.* (2002) was used in control experiments. Each PCR was carried out with one pair of genotype-specific primers and the universal fungal primers RNAF/RNAR (Fell, 1993), which amplify approximately 610 bp from all fungal large-subunit rRNA genes and were used as an internal positive control. PCR was carried out using *Taq* DNA polymerase (Promega). Each 100 µl PCR reaction with genotypes 1, 2 and 3 specific primers, respectively, contained 10 mM KCl, 0.1% (v/v) Triton X-100, dATP, 10 mM Tris/HCl (pH 9.0 at 25°C); dTTP, dCTP and dGTP at 200 µM each; 2.5 U of *Taq* polymerase; 300 nM of each primer, 1.5 mM $MgCl_2$ and either 100 ng of purified template DNA or rapidly prepared template DNA obtained by boiling yeast cells. Template DNA obtained by the latter method was prepared by boiling a single 48-h old PDA-grown colony in 50 µl of sterile ultrapure H_2O for 10 min. After boiling, the debris was pelleted by centrifugation and the DNA contained in 25 µl of the supernatant was used as template for subsequent PCR reactions. The PCR cycling conditions were as follows: initial denaturation for 3 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 50°C, 20 sec at 72°C and a final extension for 10 min at 72°C. Genotype 4-specific PCR was carried out as described above except 20 µM dNTPs were used and the annealing temperature was 55°C. Following PCR, 10 µl aliquots of the amplification mixture from genotype 1-, 2-, 3 and 4- specific PCR reactions were separated by electrophoresis through 2.4% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide and were visualised on a UV transilluminator. Genotyping experiments were performed on a minimum of two occasions with each isolate tested using separately prepared *C. dubliniensis* template DNA.

Two isolates (Eg207 and Is35, Table 4.1) failed to yield amplimers with any of the genotype-specific primer sets described above. The ITS1/ITS4 primer pair were used to amplify the internal transcribed spacer 1 and 2 (ITS1 and ITS2, respectively) regions and the intervening 5.8S rDNA gene of these *C. dubliniensis* isolates as described previously (Gee *et al.*, 2002). These primers are complementary to conserved regions of the fungal 18S (small ribosomal subunit gene) and 25S (large ribosomal subunit gene) rDNA genes, respectively, which flank the ITS1 and ITS2 regions (White *et al.*, 1990). PCR

amplifications were performed using the High Fidelity PCR System (Roche Diagnostics Ltd.) following the recommendations of the manufacturer. In brief, each 100 µl reaction contained 120 mM Tris-HCl (pH 7.5 at 25°C); 100 mM KCl; 1 mM dithiothreitol; 0.1 mM EDTA; 0.5% Tween 20 (v/v); 0.5% Nonidet P40 (v/v); 50% glycerol (v/v); dATP, dTTP, dCTP and dGTP at 200 µM each; 2.6 U of Expand High Fidelity PCR System enzyme mix; 300 nM of ITS1, 300 nM of ITS4; 1.5 mM $MgCl_2$ and 100 ng of template DNA. Amplification reactions were carried out in a thermal cycler (MBS) with initial denaturation for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 58°C, 2 min at 72°C and a final extension for 10 min at 72°C. Following amplification, the amplimers were purified as described in chapter 2 section 2.4.2 and cloned into pBluescript II KS(-) by conventional methods (Sambrook *et al.*, 1989).

4.2.6 Antifungal susceptibility testing

The 22 Saudi Arabian, the 8 Egyptian and 16 Israeli *C. dubliniensis* isolates (Table 4.1) investigated in this study were tested for susceptibility to flucytosine (5-FC) by the broth microdilution method described in the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A2 (NCCLS, 1992). In addition, 24 *C. dubliniensis* isolates (Table 4.2) from the Gee *et al.* study (2000) recovered from 12 different countries were also tested (these included 10 Cd25-group I isolates (genotype 1) and 14 Cd25-group II isolates (eight genotype 2, five genotype 3 and one genotype 4). A stock solution of 5-FC (Sigma-Aldrich Ltd.) was prepared in water at a concentration of 1 mg/ml. Serial dilutions were then made in RPMI 1640 medium (Sigma-Aldrich ltd.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid. Testing was carried out in a final volume of 100 µl in 96-well plates containing final concentrations of 5-FC ranging from 0.12 to 128 µg/ml with an inoculum density of 1.5± 1 x 10^3 cfu. Drug-free and yeast-free controls were included in each plate. After 48 h of incubation at 30°C, the cell density was measured at 450 nm with an automated plate reader (Spectra I, SLT, Austria). The MICs were determined as the lowest concentration of 5-FC that reduced turbidity by 50% (MIC_{50}) relative to the growth of the drug-free growth controls. All isolates were tested on a minimum of two occasions in separate experiments. The results were interpreted according to the criteria of the NCCLS using the following breakpoints: susceptibility, ≤4 µg per ml; intermediate resistance, 8 to 16 µg per ml; resistance, ≥32 µg per ml.

Table 4.3 Nucleotide sequence[a] of PCR primers used to amplify the *C. dubliniensis FUR1* and *FCY1* genes.

Primer	Sequence (5' to 3')	Nucleotide coordinates[b]
FCY1F	CGGCATATTAATTCCGCTTG	-145 to -164
FCY1R	CGAATTCTCTTCTGCTTCTG	+106 to +125
FUR1F	ATGTGGGTTACATCAGAAGA	-198 to -217
FUR1R	TCTCGCGACCCTCCTCTAAC	+205 to +224

[a] The nucleotide sequence of the *C. dubliniensis FUR1* and *FCY1* genes were obtained from the *C. dubliniensis* genome sequence database (http://www.sanger.ac.uk/Projects/C_dubliniensis/).
[b] Nucleotide coordinates for *C. dubliniensis* genes *FUR1* and *FCY1* are as indicated with the first base of the ATG start codon designated + 1.

4.2.7 Amplification and sequencing of *C. dubliniensis FCY1* and *FUR1* genes

The sequences of the *C. albicans* genes *FCY1* (GenBank accession no. U55194) and *FUR1* (Stanford orf6.3823) were used in a BLAST search against the *C. dubliniensis* genome sequence database (The Wellcome Trust Sanger Institute *C. dubliniensis* genome sequence project; http://www.sanger.ac.uk/Projects/C_dubliniensis/) to identify the *C. dubliniensis* homologous. The primer pairs FCY1F1/FCY1R1 and FUR1F1 /FUR1R1 were designed to amplify the complete ORFs of each of the *C. dubliniensis* genes including some upstream and downstream sequences (Table 4.3). The complete open reading frames (ORFs) of the *C. dubliniensis* genes encoding uracil phosphoribosyltransferase (*CdFUR1*) and cytosine deaminase (*CdFCY1*) were amplified by PCR from 7 selected Middle Eastern isolates, including four 5-FC-resistant (MIC_{50} ≥128 μg/ml) and three 5-FC-susceptible (MIC_{50} ≤0.125 μg/ml) isolates and their nucleotide sequences determined (Table 4.1). These were compared with the corresponding sequences of the 5-FC-susceptible reference strains CD36 and CM6 (genotype 1), CD506, Can6 and CD541 (genotype 2), CD519 and p6265 (genotype 3) and p7718 (genotype 4) (Table 4.2) which were described previously by Gee *et al.* (2002). PCRs were performed in 100 μl volumes containing 100 ng of purified template DNA, 120 mM Tris-HCl (pH 7.5 at 25°C); 100 mM KCl; 1 mM dithiothreitol; 0.1 mM EDTA; 0.5% (v/v) Tween 20; 0.5% (v/v) Nonidet; P40 50% (v/v) glycerol; dATP, dTTP, dCTP and dGTP at 200 μM each; 2.6 U of Expand High Fidelity PCR System enzyme mix; 200 nM, each of either FCY1F1/FCY1R1 or FUR1F1 /FUR1R1 and 2 mM $MgCl_2$. The reaction mixtures were subjected to initial denaturation for 7 min at 94°C; 30 cycles of 1 min at 94°C, 1 min annealing at 50°C for *FCY1* or 52.5°C for *FUR1*, and 1 min at 74°C. The final extension was for 10 min at 72°C. Amplification products were purified as described previously in chapter 2 section 2.4.2 and sequenced in both the forward and reverse directions with the primers used for their amplification. PCRs with each isolate tested were performed a minimum of three times in separate experiments with separately prepared template DNA. At least three amplimers from each separate PCR experiment were sequenced for each isolate tested.

4.2.8 DNA Sequence analysis

Sequencing was carried out as described in chapter 2 section 2.5.

Table 4.4 Recovery of oral *C. dubliniensis* from Saudi Arabian and Egyptian patients[a].

No. patients	Underlying disease	No. with oral candidiasis[a]	No. oral *Candida*-positive	No. yielding *C. dubliniensis*[b]
Saudi Arabia[c]				
160	RT	42(26.3%)	104(65%)	4(2.5%)
132	DM	9(6.8%)	78(59%)	13(9.9%)
17	HIV+	0(0%)	12(70.6%)	0(0%)
Egypt				
58	Neoplasia	12(20.7%)	39(67.2%)	4(6.9%)
67	Diabetes	6 (8.9%)	49(73.1%)	4(6.0%)
Total 432		69 (16%)	282(65.3%)	25(5.8%)

Swabs from all individuals with clinical symptoms indicative of oral candidiasis yielded semi-confluent or confluent growth of *Candida* species.

Six of the 25 *C. dubliniensis* isolates were recovered as pure cultures, whereas the remaining 19 were isolated in mixed culture with other *Candida* species, predominantly *C. albicans*.

In addition to the isolates referred to above, five additional *C. dubliniensis* isolates, recovered from various specimens from separate Saudi Arabian individuals (SA100 to SA104, Table 5.1) were included in the study but details on whether they were isolated with other yeast species was not available.

Abbreviations: RT, Renal transplant; DM, diabetes mellitus; HIV+, HIV-infected.

Table 4.4 Recovery of oral *C. dubliniensis* from Saudi Arabian and Egyptian patients[a].

No. patients	Underlying disease	No. with oral candidiasis[a]	No. oral *Candida*-positive	No. yielding *C. dubliniensis*[b]
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17	HIV+	0(0%)	12(70.6%)	0(0%)
Egypt				
58	Neoplasia	12(20.7%)	39(67.2%)	4(6.9%)
67	Diabetes	6 (8.9%)	49(73.1%)	4(6.0%)
Total 432		69 (16%)	282(65.3%)	25(5.8%)

[a]Swabs from all individuals with clinical symptoms indicative of oral candidiasis yielded semi-confluent or confluent growth of *Candida* species.

[b]Six of the 25 *C. dubliniensis* isolates were recovered as pure cultures, whereas the remaining 19 were isolated in mixed culture with other *Candida* species, predominantly *C. albicans*.

[c]In addition to the isolates referred to above, five additional *C. dubliniensis* isolates, recovered from various specimens from separate Saudi Arabian individuals (SA100 to SA104, Table 5.1) were included in the study but details on whether they were isolated with other yeast species was not available.

Abbreviations: RT, Renal transplant; DM, diabetes mellitus; HIV+, HIV-infected.

4.1 Results

4.3.1 *Candida dubliniensis* from Saudi Arabian and Egyptian patients

Oral swab samples were obtained from 434 immunocompromised patients in four hospitals in Saudi Arabia and Egypt. The proportion of individuals sampled from each country that were oral-*Candida*-positive was similar (Saudi Arabia 62.8%, Egypt 70.4%) as was the proportion of individuals that yielded *C. dubliniensis* (Saudi Arabia 5.5%, Egypt 6.4%). Details of the Saudi Arabian and Egyptian cohorts sampled and the prevalence of oral *C. dubliniensis* and other *Candida* species recovered from these cohorts are shown in Table 4.4. In total, 17 Saudi Arabian and 8 Egyptian *C. dubliniensis* isolates were recovered from the patient cohorts sampled. Five additional Saudi Arabian *C. dubliniensis* isolates were obtained from patients in two other hospitals in Saudi Arabia, resulting in a collection of 30 Saudi Arabian and Egyptian isolates for this study. An additional 16 *C. **dubliniensis*** isolates recovered in an Israeli hospital were included for comparison (Table 4.1).

4.3.2 Genotyping of *C. dubliniensis* isolates

In order to begin to investigate the population structure and diversity of *C. dubliniensis* isolates recovered in Saudi Arabia and Egypt, the 30 *C. dubliniensis* isolates recovered from separate Saudi Arabian (22 isolates) and Egyptian (8 isolates) patients were genotyped by PCR analysis. Sixteen *C. dubliniensis* isolates recovered from individual patients in Israel were used for comparison. Template DNA from each isolate was tested separately in PCR experiments with each of the genotype-specific primer pairs G1F/G1R, G2F/G2R, G3F/G3R and G4F/G4R previously described by Gee *et al.* (2002). These primers allow *C. dubliniensis* isolates to be grouped into one of four genotypes based on the nucleotide sequence of the ITS1 and ITS2 regions and the intervening 5.8S rRNA gene region of the rDNA gene cluster. While all *C. dubliniensis* isolates should produce a product of approximately 610 bp with the RNAF/RNAR primers, only *C. dubliniensis* isolates belonging to the correct genotype should yield a product of approximately 330 bp with the appropriate genotype-specific primer pair. Six (27.3%) of the Saudi Arabian and 4 (50%) of the Egyptian isolates tested were found to belong to genotype 1, whereas none of the Saudi Arabian or Egyptian isolates tested belonged to genotype 2. Thirteen (59.1%) of the Saudi Arabian isolates but none of the Egyptian isolates belonged to genotype 3. The

remaining 3 Saudi Arabian isolates and three of the four remaining Egyptian isolates belonged to genotype 4 (Table 4.1).

One Egyptian isolate (Eg207) failed to yield an amplimer with any of the genotype-specific primer pairs GIF/G1R, G2F/G2R, G3F/G3R and G4F/G4R. In order to investigate Eg207 further, the ITS region from this isolate was amplified by PCR using the ITS1/ITS2 primer pair (White *et al.*, 1990), cloned and its nucleotide sequence determined and compared with the corresponding ITS sequences of genotypes 1-4 previously reported by Gee *et al.* (2002). The ITS sequence of Eg207 was found to differ from the *C. dubliniensis* genotype 4 consensus sequence at nucleotide position 82 (T to C transition) using the numbering system of Gee *et al.* (2002). Isolate Eg207 was deemed to be a genotype 4 variant termed genotype 4A (Fig. 4.2).

Of the 16 *C. dubliniensis* isolates from Israel included in the study, 12/16 (75%) belonged to genotype 1, 1/16 (6.3%) belonged to genotype 2 and 1/16 (6.3%) belonged to genotype 3. The remaining isolate (Is35) failed to yield an amplimer with any of the genotype-specific primer pairs and had its ITS region amplified and sequenced as described above for Egyptian isolate Eg207. The ITS sequence of Is35 was found to differ from the *C. dubliniensis* genotype 4 ITS consensus sequence at nucleotide positions 63 (T to C transition) and 82 (T to C transition) and was deemed to be a genotype 4 variant termed genotype 4B (Fig. 4.3).

These results demonstrated that genotype 3 and genotype 4 isolates predominated (20/30, 66.6%) among the combined Saudi Arabian and Egyptian isolates studied. This is in contrast with the Israeli isolates, where genotype 1 isolates predominated (12/16, 75%), and with the 98 *C. dubliniensis* isolates from the Gee *et al.* study (2002) recovered in 15 different countries from around the world where genotype 1 isolates predominated (71/98, 72%).

4.3.3 DNA fingerprint analysis of Saudi Arabian and Egyptian *C. dubliniensis* isolates

In order to investigate the unexpectedly high prevalence of genotype 3 and genotype 4 isolates and the range of genetic diversity among *C. dubliniensis* isolates from Saudi Arabia and Egypt the 30 isolates included in the study were fingerprinted with the *C. dubliniensis*-specific complex DNA fingerprinting probe Cd25. Only 5 of the Israeli

```
Genotype 1  CTGATTTGCTTAATTGCACCACATGTGTTTTGTTCTGGACAAACTTGCTTTGGCGGTGGG   60
Genotype 2  CTGATTTGCTTAATTGCACCACATGTGTTTTGTTTTGGACAAACTTGCTTTGGCGGTGGG
Genotype 3  CTGATTTGCTTAATTGCACCACATGTGTTTTGTTCTGGACAAACTTGCTTTGGTGGTGGG
Genotype 4  CTGATTTGCTTAATTGCACCACATGTGTTTTGTTTTGGACAAACTTGCTTTGGCGGTGGG
EG207       CTGATTTGCTTAATTGCACCACATGTGTTTTGTTTTGGACAAACTTGCTTTGGCGGTGGG
            ********************************** ****************** ******

Genotype 1  CCCCTGCCTGCCGCCAGAGGACATAAACTTACAACCAAATTTTTTATAAACTTGTCACGA  120
Genotype 2  CCTCTACCTGCCGCCAGAGGACATAAACTTACAACCAAATTTTTTATAAACTTGTCACGA
Genotype 3  CTTCTGCCTGCCGCCAGAGGACATAAACTTACAACCAAATTTTTTATAAACTTGTCACGA
Genotype 4  CCTCTGCCTGCCGCCAGAGGATATAAACTTACAACCAAATTTTTTATAAACTTGTCACGA
EG207       CCTCTGCCTGCCGCCAGAGGACATAAACTTACAACCAAATTTTTTATAAACTTGTCACGA
            *  ** *************** ************************************

Genotype 1  GATTATTTTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAG  180
Genotype 2  GATTATTTTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAG
Genotype 3  GATTATTTTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAG
Genotype 4  GATTATTTTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAG
Eg207       GATTATTTTTAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAG
            ************************************************************

Genotype 1  AACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTT  240
Genotype 2  AACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTT
Genotype 3  AACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTT
Genotype 4  AACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTT
Eg207       AACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTT
            ************************************************************

Genotype 1  GAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCC  300
Genotype 2  GAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCC
Genotype 3  GAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCC
Genotype 4  GAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCC
Eg207       GAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCC
            ************************************************************

Genotype 1  CTCAAACCCCTAGGGTTTGGTGTTGAGCAATACGACTTGGGTTTGCTTGAAAGATGATAG  360
Genotype 2  CTCAAACCCCTAGGGTTTGGTGTTGAGCAATACGACTTGGGTTTGCTTGAAAGATGATAG
Genotype 3  CTCAAACCCCTAGGGTTTGGTGTTGAGCAATACGACTTGGGTTTGCTTGAAAGATGATAG
Genotype 4  CTCAAACCCCTAGGGTTTGGTGTTGAGCAATACGACTTGGGTTTGCTTGAAAGATGATAG
Eg207       CTCAAACCCCTAGGGTTTGGTGTTGAGCAATACGACTTGGGTTTGCTTGAAAGATGATAG
            ************************************************************

Genotype 1  TGGTATAAGGCGGAGAT-GCTTGACAATGGCTTAGGTGTAACCAAAAACATTGCTAAGGC  420
Genotype 2  TGGTA--AGGCGGAGAT-GCTTGACAATGGCTTAGGTGTAACCAAAAACATTGCTAAGGC
Genotype 3  TGGTA--AGGCGGAGATTGCTTGACAATGGCTTAGGTGTAACCAAAAACATTGCTAAGGC
Genotype 4  TGGTA--AGGCGGAGATTGCTTGACAATGGCTTAGGTGTAACCAAAAACATTGCTAAGGC
Eg207       TGGTA--AGGCGGAGATTGCTTGACAATGGCTTAGGTGTAACCAAAAACATTGCTAAGGC
            *****  ********** ****************************************

Genotype 1  GGTCTCTGGCGTCGCCCATTTTATTCTTCAAACT                            454
Genotype 2  GGTCTCTGGCGTCGCCCATTTTATTCTTCAAACT
Genotype 3  GGTCTCTGGCGTCGCCCATTTTATTCTTCAAACT
Genotype 4  GGTCTCTGGCGTCGCCCATTTTATTCTTCAAACT
Eg207       GGTCTCTGGCGTCGCCCATTTTATTCTTCAAACT
            **********************************
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Figure 4.2 Alignment of the sequences of the ITS1, 5.8S rRNA gene, and ITS2 regions of *C. dubliniensis* strains representative of genotypes 1 (CD33), 2 (CD520), 3 (CD519), 4 (p.7718) and the 4A variant Egyptian isolate Eg207 generated using the Clustal W software package. The central 5.8S rRNA gene sequence is shown in italics, whereas the ITS1 and ITS2 sequences located 5' and 3', respectively, are shown in plain text. Identical nucleotides are indicated by asterisks and gaps are indicated by hyphens. Sequences of the *C. dubliniensis* genotype-specific primers G1F/G1R, G2F/G2R, G3F/G3R, and G4F/G4R are underlined.

isolates were fingerprinted because the majority (12/16) belonged to genotype 1 and a previous study from this laboratory demonstrated that genotype 1 Israeli *C. dubliniensis* isolates belong to Cd25 fingerprint group I (Gee *et al.*, 2002). The Israeli isolates fingerprinted included the two genotype 1 isolates Is45 and Is47, the genotype 2 isolate Is49, the genotype 3 isolate Is36 and the genotype 4B variant isolate Is35 (Table 4.1). The Cd25 DNA fingerprint profiles were subjected to computer-assisted analysis with the fingerprint profile analysis software package DENDRON. S_{AB} values were computed for every possible pairwise combination of isolates and these data were used to construct dendrograms showing the relationships between the isolates. Examination of the dendrogram constructed from fingerprint data obtained with the Saudi Arabian and Egyptian isolates revealed that the 30 isolates could be clearly divided by a node at an S_{AB} value of 0.05 into two distinct main populations of isolates, the first corresponding to genotype 1 and the second to genotypes 3 and 4, respectively, (Fig.4.4). The total collection of Saudi Arabian and Egyptian isolates had an average S_{AB} value of 0.28 ± 0.27. The first sub-population (genotype 1) of isolates had an average S_{AB} value of 0.86 ± 0.27, whereas the second subpopulation (genotype 3 (average S_{AB} value 0.47 ± 0.15) and genotype 4 (average S_{AB} value 0.53±0.22) had an average S_{AB} value of 0.35±0.19.

In order to investigate the relationships between the two populations of Saudi Arabian and Egyptian isolates identified in this collection and the two groups (Cd25-group I and Cd25-group II, respectively) identified in isolates from countries around the world by Joly *et al.* (1999) and verified by Gee *et al.* (2002), a mixed dendrogram was generated from the 30 Saudi Arabian and Egyptian isolates, the 5 Israeli isolates and 51 independent isolates from 13 countries from the Gee *et al.* study (2002) (which included 32 isolates from Cd25-group I and 19 isolates from Cd25-group II. Examination of the mixed dendrogram revealed that the isolates could be clearly divided at an S_{AB} node of 0. 05 into three distinct populations (Fig. 4.5). The first group of isolates (average S_{AB} value of 0.63 ± 0.12) consisted solely of genotype 1 isolates and corresponded to Cd25-group I previously described by Joly *et al.* (1999) and Gee *et al.* (2002). This group contained the 6 Saudi Arabian (SA101, SA102, SA105, SA106, SA115 and SA116), the 4 Egyptian (Eg203, Eg204, Eg205 and Eg206) and the 2 Israeli (Is45 and Is47) genotype 1 isolates fingerprinted in this study mixed with isolates from the Gee *et al.* (2002) study. The second group of isolates (average S_{AB} value of 0.37 ± 0.30) corresponded to Cd25-group II

Average S_{AB} value

Genotype 1 = 0.86 ±0.27
Genotype 3 = 0.47 ±0.15
Genotype 4 = 0.53 ±0.22

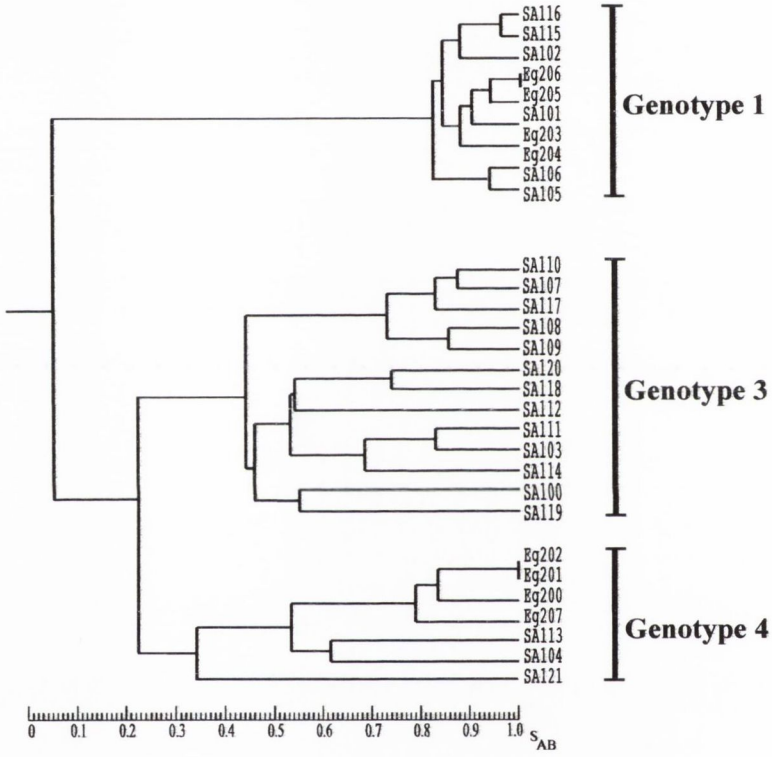

Figure 4.4 Dendrogram generated from the similarity coefficients (S_{AB}s) computed for every possible pairwise combination of 30 *C. dubliniensis* isolates recovered from individual patients in Saudi Arabia and Egypt fingerprinted with Cd25. The provenance of the isolates is shown in Tables 1 and 2. At an S_{AB} node of 0.05, the isolates are divided into two main populations. The first of these populations consists solely of genotype 1 isolates and are closely related with an average S_{AB} value of 0.86±0.27. The second population consists of genotype 3 and genotype 4 isolates that are less closely related to each other than genotype 1 isolates with an average S_{AB} value of 0.35±0.19. At an S_{AB} node of 0.22 the second population is divided into two sub-populations consisting of genotype 3 and genotype 4 isolates, respectively.

previously described by Joly *et al.* (1999) and Gee *et al.* (2002) and did not contain any Saudi Arabian or Egyptian isolates, but contained the other three Israeli isolates fingerprinted in this study including Is49 (genotype 2), Is36 (genotype 3) and Is35 (genotype 4B) mixed with isolates from the Gee *et al.* (2002) study. The third group of isolates (average S_{AB} value of 0.35 ± 0.19) consisted of the 20 genotype 3 and genotype 4 Saudi Arabian and Egyptian isolates included in this study together with the genotype 4 Israeli isolate p7718 from the study of Gee *et al.* (2002). This latter isolate was a distinct outlier within Cd25-group II in the study of Gee *et al.* (2002). None of the Saudi Arabian or Egyptian genotype 3 or genotype 4 isolates mixed with any of the Cd25-group I or Cd25-group II isolates from the Gee *et al.* (2002) study. These findings demonstrated that genotype 3 and genotype 4 Saudi Arabian and Egyptian *C. dubliniensis* isolates consist of a hitherto undescribed clade of isolates that is prevalent in Saudi Arabia and Egypt, but apart from the Israeli isolate p7718, was absent from the international collection of isolates from the study of Gee *et al.* study (2002). The third group of *C. dubliniensis* isolates forming the novel Saudi Arabian and Egyptian clade was termed Cd25-group III (Fig. 4.5).

The Cd25-generated fingerprinted patterns of *C. dubliniensis* isolates within the Cd25-group III clade are very distinct from the corresponding patterns of Cd25-group I and Cd25-group II isolates and can be easily distinguished by direct visual comparison (Fig. 4.6). In particular, Cd25-group III isolates yield fingerprints with far fewer bands (i.e. between 8-13 bands) compared with Cd25-group I and Cd25-group II fingerprint profiles (i.e. 15-20 bands). More strikingly, the majority of Cd25-group III isolates investigated in this study have no or very few bands below 3.5 kb (Fig. 4.6 & 4.7).

Examination of the dendrogram shown in Fig. (4.5) revealed the presence of closely related clusters of isolates (e.g. S_{AB} values >0.9) are evident among the Saudi Arabian and Egyptian isolates. In some cases clusters correspond to the hospital of isolation and the country of origin of the isolates. For example, there are two pairs of Saudi isolates, SA116 and SA115 and SA105 and SA106, respectively, that are comprised of isolates from the same hospital in Riyadh- Saudi Arabia. There is also another cluster of Saudi isolate (SA101) with two Egyptian isolates (Eg206 and Eg205), the later recovered from two different patients in the same hospital. The two pairs of identical isolates (Eg206, Eg205 and Eg202, Eg201) were collected from different patients attending the same hospital.

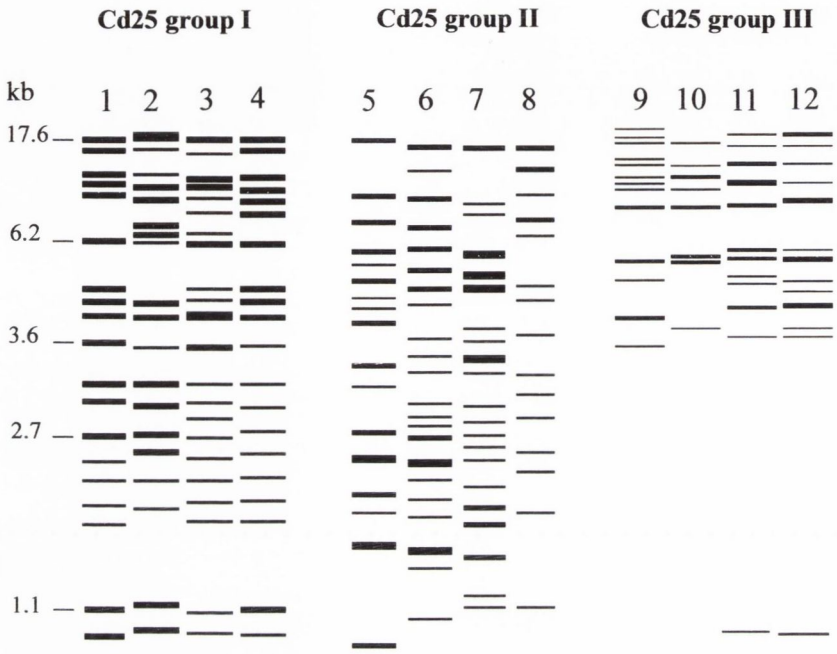
Cd25 group I
Cd25 group II
Cd25 group III
kb
1 2 3 4
5 6 7 8
9 10 11 12
17.6
6.2
3.6
2.7
1.1

Figure 4.6 Model generated using the DENDRON software package showing band position and intensity of Cd25-generated hybridization fingerprint patterns of *Eco*RI-digested genomic DNA of *C. dubliniensis* isolates belonging to the major clades Cd25-group I, Cd25-group II and the novel 5-FC-resistant Saudi Arabian and Egyptian clade Cd25-group III identified in this study. Molecular sizes in kilobases are shown on the left. The *C. dubliniensis* isolates from which the corresponding patterns in the lanes were obtained with the genotype (G) shown in parenthesis are as follows: lane 1, CM6 (G1); lane 2, CD518 (G1); lane 3, SA103 (G1); lane 4, Eg204 (G1); lane 5, Can9 (G2); Lane 6, CD514 (G2); lane 7, Is49 (G2); lane 8, p6265 (G3); lane 9, SA103 (G3); lane 10, SA121 (G4); lane 11, SA119 (G3); lane 12, Eg201 (G4).

Whether both patients in each case harboured the same strain which may be endemic to the respective hospital was not investigated.

4.3.4 Flucytosine resistance in *C. dubliniensis* isolates

All of the Saudi Arabian, Egyptian and Israeli *C. dubliniensis* isolates included in the study (Table 4.1) were tested for susceptibility 5-FC using the Rodriguez-Tudela and Martinez-Suárez modified broth microdilution method (1995).

A selection of 24 independent *C. dubliniensis* isolates from 12 different countries from the Gee *et al.* study (2002) were also included for comparison (which included 10 Cd25-group I isolates (genotype 1) and 14 Cd25-group II isolates (eight genotype 2, five genotype 3 and one genotype 4) (Table 4.2). All 10 Cd25-group I (genotype 1) Saudi Arabian and Egyptian isolates, all 16 Israeli isolates and all 24 reference isolates from the Gee *et al.* study (2002) were found to be 5-FC susceptible (MIC_{50} ≤0.125 μg/ml). In contrast, all 20 of the Saudi Arabian and Egyptian isolates belonging to Cd25-group III (genotypes 3 and 4) were 5-FC-resistant (MIC_{50}≥ 128 μg/ml) (Table 4.1). Interestingly the Israeli isolate p7718 which belongs to this group was susceptible to 5-FC (MIC_{50} ≤0.125 μg/ml). These findings demonstrated that the Saudi Arabian and Egyptian isolates forming the novel CD25-group III clade can be distinguished from *C. dubliniensis* isolates belonging to other groups on the basis of resistance to 5-FC.

4.3.5 Analysis of *C. dubliniensis FCY1 and FUR1* gene sequences.

The complete ORFs of *C. dubliniensis FUR1* and *FCY1* genes together with flanking sequences were amplified by PCR with primers specific for the *C. dubliniensis-FUR1* and *FCY1* genes (Table 4.3). The *C. dubliniensis FUR1* gene is 841 bp in length and exhibited 93.3% homology with the *C. albicans* gene (Fig. 4.8). The *C. dubliniensis FCY1* gene is 529 bp in length and exhibited 100% homology with the *C. albicans* gene (Fig. 4.9). Amplimers of both genes were obtained and sequenced from the three 5-FC-resistant (MIC_{50} ≥128 μg/ml) Saudi Arabian isolates SA100, SA107 (both genotype 3) and SA113 (genotype 4), from three 5-FC-susceptible (MIC_{50} ≤0.125 μg/ml) Saudi Arabian isolates SA101, SA102 and SA105 (all genotype 1) and from the 5-FC-resistant (MIC_{50} ≥128 μg/ml) Egyptian isolate Eg200 (genotype 4). These sequences were compared with the corresponding sequences obtained from selected 5-FC-susceptible (MIC_{50} ≤0.125 μg/ml)

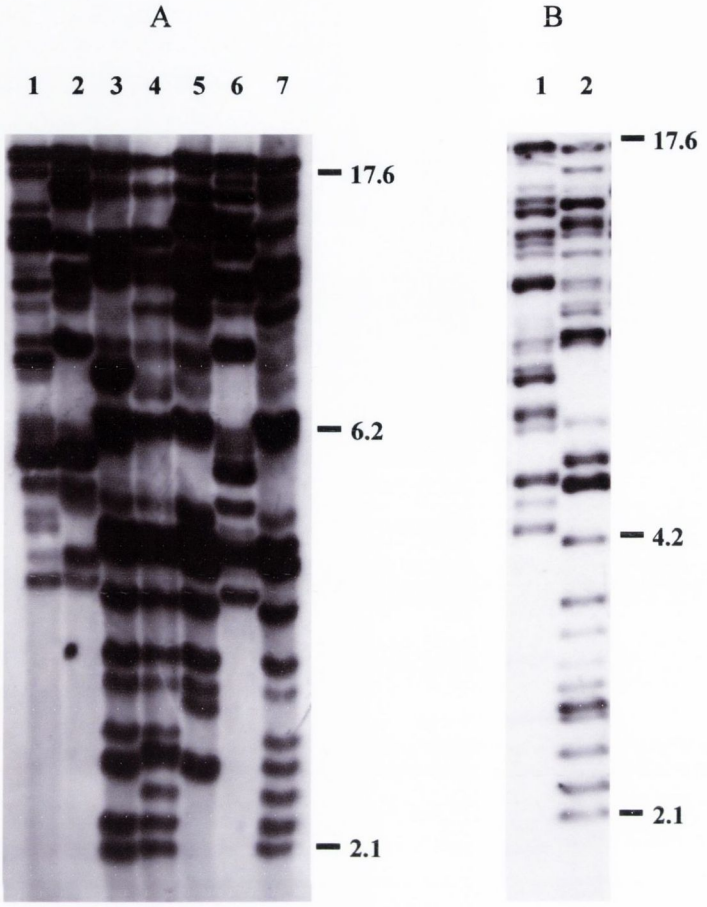

Figure 4.7 Southern blot hybridisation fingerprinting profiles of Saudi Arabian (panel A) and Egyptian (panel B) *C. dubliniensis* isolates generated with the Cd25 probe. (A) Lane 1, SA100 (genotype 3); lane 2, SA113 (genotype 4); lane 3, SA115 (genotype 1); lane 4, SA106 (genotype 1); lane 5, SA105 (genotype 1); lane 6, SA103 (genotype 3) and lane 7 the *C. dubliniensis* reference isolate CM6 (genotype 1). (B) Lane 1, Eg202 (genotype 4) and lane 2, Eg203 (genotype 1). The relative positions of molecular weight markers (in kilobases) are indicated to the right of the panels.

```
C. dubliniensis      ATGTCTGTTGCCAAAGCTGTGAGCAAGAACGTTATTTTATTACCACAAACTAACCAATTA
C. albicans          ATGTCTGTTGCCAAAGCTGTGAGCAAAAACGTTATTTTATTACCGCAAACCAACCAATTA
                     ************************** ********** ***** *********

C. dubliniensis      ATTGGTTTGTATTCAATCATTCGTGATCAGCGTACTAAACGTGGAGATTTTGTTTTCTAT
C. albicans          ATTGGTTTATACTCAATCATTCGTGATCAACGTACTAAACGTGGAGATTTTGTATTTTAT
                     ******** ** ********** ********** ** ***

C. dubliniensis      TCAGATAGAATCATTCGTTTATTAGTCGAAGAAGGTTTGAACCAATTACCAGTTGAAGAA
C. albicans          TCAGATAGAATCATTCGTTTATTAGTTGAAGAAGGTTTGAACCAATTACCAGTTGAAGAA
                     ************************** **********

C. dubliniensis      GCAATTATCAAGTGTCATGGTGGATACGAATACAAGGGGGCAAAGTTTTTAGGTAAAATT
C. albicans          GCAATTATAAAATGCCATGGTGGATATGAATACAAGGGAGCCAAATTTTTAGGTAAAATT
                     ******** ** ** ********** ********** ** ** **********

C. dubliniensis      TGTGGAGTATCTATTGTCAGAGCTGGAGAATCCATGGAAATGGGGTTAAGGGATTGTTGT
C. albicans          TGTGGTGTATCTATTGTTCGAGCTGGGGAATCAATGGAAATGGGATTAAGGGATTGTTGT
                     ***** **********  ******* ***** ********** **********

C. dubliniensis      CGTTCAGTACGAATTGGGAAAATCTTGATTCAAAGAGATGAAGAAACTGCATTACCAAAA
C. albicans          CGTTCTGTAAGAATTGGGAAAATCTTGATTCAAAGAGATGAAGAAACTGCATTACCAAAA
                     ***** *** **********

C. dubliniensis      TTGTTTTATGAAAAATTACCTGAAGATATCAGTGAACGTTATGTATTTTTATTAGACCCA
C. albicans          TTGTTTTATGAAAAATTACCTGAAGATATCAGTGAACGTTATGTATTTTTATTAGATCCA
                     ********** ***

C. dubliniensis      ATGTTGGCAACAGGAGGTTCAGCAATGATGGCCGTTGAGGTTTTATTGGCCAGAGGAGTG
C. albicans          ATGTTGGCCACAGGAGGATCAGCAATGATGGCTGTTGAAGTTTTATTGGCAAGAGGAGTG
                     ******** ******** ********** ***** ********** *********

C. dubliniensis      AAAATGGACAGAATTTTATTCTTAAACTTATTGGCAGCACCAGAAGGTATCAAAGCATTC
C. albicans          AAAATGGACAGAATTTTATTTTTGAATTTATTAGCAGCACCAGAAGGTATTAAAGCATTC
                     ********** ** ** ***** ********** *********

C. dubliniensis      CATGAAAAATACCCTGATGTCAAAATAATCACCGGTGGAATTGATGAAAAATTAGATGAA
C. albicans          CAGGATAAATACCCAGATGTCAAAATAATCACTGGTGGAATTGACGAAAAATTAGATGAA
                     ** ** ******** ********** ********** **********

C. dubliniensis      GACAAATACATTGTTCCAGGTCTAGGAGATTTCGGTGACAGATATTACTGTATTTAA
C. albicans          AATAAATACATTGTTCCAGGTCTAGGTGATTTCGGTGATAGATATTACTGTATTTAA
                      ********** ********** **********
```

Figure 4.8 Alignment of the *C. albicans* and *C. dubliniensis FUR1* genes. The *C. dubliniensis* sequence was from strain CD36 (this study) and the *C. albicans* sequence was from isolate SC5314 (Stanford orf6.3823). Asterisks indicate identical nucleotides.

```
C. dubliniensis      ATGACATTTGACGACAAAAAAGGTTTACAAATTGCTCTTGATCAAGCAAAGAAAAGTATG
C. albicans          ATGACATTTGACGACAAAAAAGGTTTACAAATTGCTCTTGATCAAGCAAAGAAAAGTATG
                     ************************************************************

C. dubliniensis      TGGATATCGTCACAGAGGTGTGAACATTCAAACAACTATATTTCAGTTTTACTAACACAC
C. albicans          TGGATATCGTCACAGAGGTGTGAACATTCAAACAACTATATTTCAGTTTTACTAACACAC
                     ************************************************************

C. dubliniensis      TTGCTGTTTAGGTTACTTTGAAGGTGGCATACCTATTGGATCATGTATTATTTCATCTGA
C. albicans          TTGCTGTTTAGGTTACTTTGAAGGTGGCATACCTATTGGATCATGTATTATTTCATCTGA
                     ************************************************************

C. dubliniensis      CGGTACAGTATTAGGTCAAGGACATAACGAACGAATCCAAAAACATTCATCTATTTTACA
C. albicans          CGGTACAGTATTAGGTCAAGGACATAACGAACGAATCCAAAAACATTCATCTATTTTACA
                     ************************************************************

C. dubliniensis      TGGGGAAATGTCAGCATTAGAAAACGCAGGAAGATTGCCAGGCAAAACCTATAAGGATTG
C. albicans          TGGGGAAATGTCAGCATTAGAAAACGCAGGAAGATTGCCAGGCAAAACCTATAAGGATTG
                     ************************************************************

C. dubliniensis      TACCATATATACTACTTTGTCACCATGTAGTATGTGTACAGGGGCCATTTTATTATATGG
C. albicans          TACCATATATACTACTTTGTCACCATGTAGTATGTGTACAGGGGCCATTTTATTATATGG
                     ************************************************************

C. dubliniensis      GTTCAAACGAGTTGTTATGGGGGAAAATGTCAATTTCTTGGGTAACGAAAAGTTATTGGT
C. albicans          GTTCAAACGAGTTGTTATGGGGGAAAATGTCAATTTCTTGGGTAACGAAAAGTTATTGGT
                     ************************************************************

C. dubliniensis      TGAAAATGGTGTTGAAGTTGTGAATTTGAATGATCAAGCATGTATTGATTTGATGGCCAA
C. albicans          TGAAAATGGTGTTGAAGTTGTGAATTTGAATGATCAAGCATGTATTGATTTGATGGCCAA
                     ************************************************************

C. dubliniensis      ATTTATTAAAGAGAAACCTCAAGATTGGAATGAAGATATTGGAGAATAA
C. albicans          ATTTATTAAAGAGAAACCTCAAGATTGGAATGAAGATATTGGAGAATAA
                     **************************************************
```

Figure 4.9 Alignment of the *C. albicans* and *C. dubliniensis FCY1* genes. The *C. dubliniensis* sequence was from the strain CD36 (this study) and the *C. albicans* sequence was from strain Sc5314 (GenBank accession no. U55194; Erbs *et al.*, 1997). Asterisks indicate identical nucleotides.

reference isolates from the Gee *et al.* study (2002), including CD36 and CM6 (genotype 1), CD506, Can 6 and CD541 (genotype 2), CD519 and p6265 (genotype 3) and p7718 (genotype 4).

The sequences of the *FCY1* gene and flanking sequences from all of the 5-FC-susceptible reference isolates tested and the 5-FC-susceptible Saudi Arabian genotype 1 isolates SA101, SA102 and SA105 (fig. 4.10) were identical to the *FCY1* consensus sequence of the *C. dubliniensis* type strain CD36 obtained from the *C. dubliniensis* genome sequence database (http://www.samger.ac.uk/Projects/*C_dubliniensis*/). The 5-FC-resistant Saudi Arabian isolates SA100, SA107 and SA113 and the 5-FC-resistant Egyptian isolate Eg200 each contained a single identical nucleotide polymorphism (transition from A to T) at position 258 in the *FCY1* gene sequence that did not result in an amino acid residue change in the predicted protein (Fig. 4.11, Fig. 4.12 & Fig 4.13). Comparison of the nucleotide sequences of the *FUR1* gene from the 5-FC-susceptible reference isolates and the 5-FC-susceptible and 5-FC-resistant Saudi Arabian and Egyptian isolates revealed the presence of several single nucleotide polymorphisms, none of which resulted in changes in amino acids in the predicted protein.

These findings suggested that *C. dubliniensis* encoded homologues of both the *C. albicans FUR1* and *FCY1* genes, termed *CdFUR1* and *CdFCY1*, respectively. However, polymorphisms in the *FCY1* and *FUR1* genes could not be associated with 5-FC resistance in the *C. dubliniensis* isolates tested.

```
C. dubliniensis     ATGACATTTGACGACAAAAAAGGTTTACAAATTGCTCTTGATCAAGCAAAGAAAAGTATG
SA101               ATGACATTTGACGACAAAAAAGGTTTACAAATTGCTCTTGATCAAGCAAAGAAAAGTATG
SA102               ATGACATTTGACGACAAAAAAGGTTTACAAATTGCTCTTGATCAAGCAAAGAAAAGTATG
SA105               ATGACATTTGACGACAAAAAAGGTTTACAAATTGCTCTTGATCAAGCAAAGAAAAGTATG
                    ************************************************************

C. dubliniensis     TGGATATCGTCACAGAGGTGTGAACATTCAAACAACTATATTTCAGTTTTACTAACACAC
SA101               TGGATATCGTCACAGAGGTGTGAACATTCAAACAACTATATTTCAGTTTTACTAACACAC
SA102               TGGATATCGTCACAGAGGTGTGAACATTCAAACAACTATATTTCAGTTTTACTAACACAC
SA105               TGGATATCGTCACAGAGGTGTGAACATTCAAACAACTATATTTCAGTTTTACTAACACAC
                    ************************************************************

C. dubliniensis     TTGCTGTTTAGGTTACTTTGAAGGTGGCATACCTATTGGATCATGTATTATTTCATCTGA
SA101               TTGCTGTTTAGGTTACTTTGAAGGTGGCATACCTATTGGATCATGTATTATTTCATCTGA
SA102               TTGCTGTTTAGGTTACTTTGAAGGTGGCATACCTATTGGATCATGTATTATTTCATCTGA
SA105               TTGCTGTTTAGGTTACTTTGAAGGTGGCATACCTATTGGATCATGTATTATTTCATCTGA
                    ************************************************************

C. dubliniensis     CGGTACAGTATTAGGTCAAGGACATAACGAACGAATCCAAAAACATTCATCTATTTTACA
SA101               CGGTACAGTATTAGGTCAAGGACATAACGAACGAATCCAAAAACATTCATCTATTTTACA
SA102               CGGTACAGTATTAGGTCAAGGACATAACGAACGAATCCAAAAACATTCATCTATTTTACA
SA105               CGGTACAGTATTAGGTCAAGGACATAACGAACGAATCCAAAAACATTCATCTATTTTACA
                    ************************************************************

C. dubliniensis     TGGGGAAATGTCAGCATTAGAAAACGCAGGAAGATTGCCAGGCAAAACCTATAAGGATTG
SA101               TGGGGAAATGTCAGCATTAGAAAACGCAGGAAGATTGCCAGGCAAAACCTATAAGGATTG
SA102               TGGGGAAATGTCAGCATTAGAAAACGCAGGAAGATTGCCAGGCAAAACCTATAAGGATTG
SA105               TGGGGAAATGTCAGCATTAGAAAACGCAGGAAGATTGCCAGGCAAAACCTATAAGGATTG
                    ************************************************************

C. dubliniensis     TACCATATATACTACTTTGTCACCATGTAGTATGTGTACAGGGGCCATTTTATTATATGG
SA101               TACCATATATACTACTTTGTCACCATGTAGTATGTGTACAGGGGCCATTTTATTATATGG
SA102               TACCATATATACTACTTTGTCACCATGTAGTATGTGTACAGGGGCCATTTTATTATATGG
SA105               TACCATATATACTACTTTGTCACCATGTAGTATGTGTACAGGGGCCATTTTATTATATGG
                    ************************************************************

C. dubliniensis     GTTCAAACGAGTTGTTATGGGGGAAAATGTCAATTTCTTGGGTAACGAAAAGTTATTGGT
SA101               GTTCAAACGAGTTGTTATGGGGGAAAATGTCAATTTCTTGGGTAACGAAAAGTTATTGGT
SA102               GTTCAAACGAGTTGTTATGGGGGAAAATGTCAATTTCTTGGGTAACGAAAAGTTATTGGT
SA105               GTTCAAACGAGTTGTTATGGGGGAAAATGTCAATTTCTTGGGTAACGAAAAGTTATTGGT
                    ************************************************************

C. dubliniensis     TGAAAATGGTGTTGAAGTTGTGAATTTGAATGATCAAGCATGTATTGATTTGATGGCCAA
SA101               TGAAAATGGTGTTGAAGTTGTGAATTTGAATGATCAAGCATGTATTGATTTGATGGCCAA
SA102               TGAAAATGGTGTTGAAGTTGTGAATTTGAATGATCAAGCATGTATTGATTTGATGGCCAA
SA105               TGAAAATGGTGTTGAAGTTGTGAATTTGAATGATCAAGCATGTATTGATTTGATGGCCAA
                    ************************************************************

C. dubliniensis     ATTTATTAAAGAGAAACCTCAAGATTGGAATGAAGATATTGGAGAATAA
SA101               ATTTATTAAAGAGAAACCTCAAGATTGGAATGAAGATATTGGAGAATAA
SA102               ATTTATTAAAGAGAAACCTCAAGATTGGAATGAAGATATTGGAGAATAA
SA105               ATTTATTAAAGAGAAACCTCAAGATTGGAATGAAGATATTGGAGAATAA
                    ************************************************
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C.dubliniensis      ATGACATTTGACGACAAAAAAGGTTTACAAATTGCTCTTGATCAAGCAAAGAAAAGTATG
SA107               ATGACATTTGACGACAAAAAAGGTTTACAAATTGCTCTTGATCAAGCAAAGAAAAGTATG
                    ************************************************************

C. dubliniensis     TGGATATCGTCACAGAGGTGTGAACATTCAAACAACTATATTTCAGTTTTACTAACACAC
SA107               TGGATATCGTCACAGAGGTGTGAACATTCAAACAACTATATTTCAGTTTTACTAACACAC
                    ************************************************************

C. dubliniensis     TTGCTGTTTAGGTTACTTTGAAGGTGGCATACCTATTGGATCATGTATTATTTCATCTGA
SA107               TTGCTGTTTAGGTTACTTTGAAGGTGGCATACCTATTGGATCATGTATTATTTCATCTGA
                    ************************************************************

C. dubliniensis     CGGTACAGTATTAGGTCAAGGACATAACGAACGAATCCAAAAACATTCATCTATTTTACA
SA107               CGGTACAGTATTAGGTCAAGGACATAACGAACGAATCCAAAAACATTCATCTATTTTACA
                    ************************************************************

C. dubliniensis     TGGGGAAATGTCAGCATTAGAAAACGCAGGAAGATTGCCAGGCAAAACCTATAAGGATTG
SA107               TGGGGAAATGTCAGCATTAGAAAACGCAGGAAGATTGCCAGGCAAAACCTATAAGGATTG
                    ************************************************************

C. dubliniensis     TACCATATATACTACTTTGTCACCATGTAGTATGTGTACAGGGGCCATTTTATTATATGG
SA107               TACCATATATACTACTTTGTCACCATGTAGTATGTGTACTGGGGCCATTTTATTATATGG
                    ************************************ ********************

C. dubliniensis     GTTCAAACGAGTTGTTATGGGGGAAAATGTCAATTTCTTGGGTAACGAAAAGTTATTGGT
SA107               GTTCAAACGAGTTGTTATGGGGGAAAATGTCAATTTCTTGGGTAACGAAAAGTTATTGGT
                    ************************************************************

C. dubliniensis     TGAAAATGGTGTTGAAGTTGTGAATTTGAATGATCAAGCATGTATTGATTTGATGGCCAA
SA107               TGAAAATGGTGTTGAAGTTGTGAATTTGAATGATCAAGCATGTATTGATTTGATGGCCAA
                    ************************************************************

C. dubliniensis     ATTTATTAAAGAGAAACCTCAAGATTGGAATGAAGATATTGGAGAATAA
SA107               ATTTATTAAAGAGAAACCTCAAGATTGGAATGAAGATATTGGAGAATAA
```

Figure 4. 11 Alignment of the *C. dubliniensis FCY1* gene sequence from the 5-FC resistant genotype 3 Saudi Arabian isolate SA107 generated using the Clustal W software package. A single nucleotide polymorphism is highlighted in colour (transition from A to T) The *C. dubliniensis FCY1* gene sequence of the *C. dubliniensis* type strain CD36 (this study) was used for comparison. Identical nucleotides are indicated by asterisks.

Figure 4.12 Nucleotide and deduced amino acid sequences of the *C. dubliniensis FCY1* gene from the Saudi Arabian isolate SA107 as a representative of 5-FC resistant Saudi Arabian and Egyptian isolates.

```
1/1                                                 31/11                                                61/21                                                91/31
atg aca ttt gac gac aaa aaa ggt tta caa att gct ctt gat caa gca aag aaa agt gaa ggt ggc ata cct att gga tTa tgt att att tca tct gac ggt aca gta tta ggt caa gga
M   T   F   D   D   K   K   G   L   Q   I   A   L   D   Q   A   K   K   S   E   G   G   I   P   I   G   L   C   I   I   S   S   D   G   T   V   L   G   Q   G
121/41                                              151/51                                               181/61                                               211/71
cat aac gaa cga atc caa aaa cat tca tct att tta cat ggg gaa atg tca gca tta gaa aac gca gga aga ttg cca ggc aaa acc tat aag gat tgt acc ata tat act act ttg tca
H   N   E   R   I   Q   K   H   S   S   I   L   H   G   E   M   S   A   L   E   N   A   G   R   L   P   G   K   T   Y   K   D   C   T   I   Y   T   T   L   S
241/81                                              271/91                                               301/101                                              331/111
cca tgt agt atg tgt act ggg gcc att tta tta tat ggg ttc aaa cga gtt gtt atg ggg gaa aat gtc aat ttc ttg ggt aac gaa aag tta ttg gtt gaa aat ggt gtt gaa gtt gtg
P   C   S   M   C   T   G   A   I   L   L   Y   G   F   K   R   V   V   M   G   E   N   V   N   F   L   G   N   E   K   L   L   V   E   N   G   V   E   V   V
361/121                                             391/131                                              421/141
aat ttg aat gat caa gca tgt att gat ttg atg gcc aaa ttt att aaa gag aaa cct caa gat tgg aat gaa gat att gga gaa taa
N   L   N   D   Q   A   C   I   D   L   M   A   K   F   I   K   E   K   P   Q   D   W   N   E   D   I   G   E   *
```

Nucleotide sequences are numbered in the 5' to 3' direction from the first base (+1) of the ATG translation start codon. Amino acid residue are numbered from the initial methionine. The single base change between the 5-FC resistant and the 5-FC susceptible *C. dubliniensis* isolates which did not result in an amino acid change is located at position 258 and is underlined. The corresponding amino acid is highlighted in bold.

Figure 4.13 Nucleotide and deduced amino acid sequences of the *C. dubliniensis FCY1* gene from the CD36 isolates as a representative of the 5-FC susceptible *C. dubliniensis* isolates.

```
1/1                                   31/11                                 61/21                                 91/31
atg aca ttt gac gac aaa aaa ggt tta caa att gct ctt gat caa gca aag aaa agt gaa ggt ggc ata cct att gga tca tgt att att tca tct gac ggt aca gta tta ggt caa gga
M   T   F   D   D   K   K   G   L   Q   I   A   L   D   Q   A   K   K   S   E   G   G   I   P   I   G   S   C   I   I   S   S   D   G   T   V   L   G   Q   G
121/41                                151/51                                181/61                                211/71
cat aac gaa cga atc caa aaa cat tca tct att tta cat ggg gaa atg tca gca tta gaa aac gca gga aga ttg cca ggc aaa acc tat aag gat tgt acc ata tat act act ttg tca
H   N   E   R   I   Q   K   H   S   S   I   L   H   G   E   M   S   A   L   E   N   A   G   R   L   P   G   K   T   Y   K   D   C   T   I   Y   T   T   L   S
241/81                                271/91                                301/101                               331/111
cca tgt agt atg tgt acA ggg gcc att tta tta tat ggg ttc aaa cga gtt gtt atg ggg gaa aat gtc aat ttc ttg ggt aac gaa aag tta ttg gtt gaa aat ggt gtt gaa gtt gtg
P   C   S   M   C   T   G   A   I   L   L   Y   G   F   K   R   V   V   M   G   E   N   V   N   F   L   G   N   E   K   L   L   V   E   N   G   V   E   V   V
361/121                               391/131                               421/141
aat ttg aat gat caa gca tgt att gat ttg atg gcc aaa ttt att aaa gag aaa cct caa gat tgg aat gaa gat att gga gaa taa
N   L   N   D   Q   A   C   I   D   L   M   A   K   F   I   K   E   K   P   Q   D   W   N   E   D   I   G   E   *
```

Nucleotide sequences are numbered in the 5' to 3' direction from the first base (+1) of the ATG translation start codon. Amino acid residue are numbered from the initial methionine. The single base change between the 5-FC resistant and the 5-FC susceptible *C. dubliniensis* isolates which did not result in an amino acid change is located at position 258 and is underlined. The corresponding amino acid is highlighted in bold.

4.4 Discussion

Our understanding of the epidemiology and population structure of the yeast pathogen *C. dubliniensis* was greatly enhanced by the development of *C. dubliniensis*-specific DNA fingerprinting probes by Joly *et al.* in 1999 which was particularly useful for strain discrimination. Using this specific probe, two recent studies on *C. dubliniensis* isolates recovered from different countries world-wide demonstrated that the *C. dubliniensis* isolates could be divided into two distinct populations (Joly *et al.*, 1999; Gee *et al.*, 2002). In both studies the majority of isolates belonged to a major clade referred to as Cd25-group I, which consisted of a population of closely related isolates (S_{AB} values of 0.8 in each case). Furthermore, in both studies, isolates belonging to a second major clade termed Cd25-group II exhibited significantly greater diversity and were less closely related than Cd25-group I isolates (S_{AB} values of 0.47 in the Joly *et al.* (1999) study and 0.57 in the Gee *et al.* (2002)) study. A more recent study by Blignaut *et al.* (2003) reported similar findings with a collection of 15 *C. dubliniensis* isolates recovered from individuals in South Africa. The study of Gee *et al.* (2002) also revealed that *C. dubliniensis* isolates could be sub-divided into one of four genotypes on the basis of the nucleotide sequence of the ITS region of the rDNA gene cluster. Cd25-group I consisted solely of genotype 1 isolates, whereas Cd25-group II consisted of three genotypes (genotypes 2-4). This latter finding reflects the greater diversity observed among Cd25-group II isolates. To date, the majority of *C. dubliniensis* isolates that have been subjected to Cd25 DNA fingerprinting analysis have been recovered in Europe, Australia and the USA. There have been several reports describing the recovery of *C. dubliniensis* isolates in a variety of Middle Eastern countries (Gee *et al.*, 2002; Fotedar & Al Hedaithy, 2003; Ahmad *et al.*, 2004; McCullough *et al.*, 2004), but little is known about the population structure or the epidemiology of these isolates, or their relationship to isolates recovered from the rest of the world. Gee *et al.* (2002) included eight independent isolates of *C. dubliniensis* recovered in Israel in their study, four of which belonged to Cd25-group I (genotype 1) and four to Cd25-group II. Interestingly, three of these four Cd25-group II isolates belonged to genotype 3 and one to genotype 4. In fact, of the five genotype 3 isolates identified by Gee *et al.* (2002) out of a total of 98 isolates investigated from 15 different countries, three (60%) were from Israel and two (40%) from Ireland. The single genotype 4 isolate identified among the Gee *et al.*

(2002) isolate collection was also from Israel. These findings suggested that distinct sub-populations of *C. dubliniensis* may be present in Israel and possibly in neighbouring countries. The purpose of the present study was to investigate this possibility further by analysing additional isolates of *C. dubliniensis* from Saudi Arabia, Egypt and Israel.

4.4.1 *Candida* species in Saudi Arabian and Egyptian patients

In the present study, isolates of oral *Candida* species were recovered from 64% (127/199) of the Saudi and Egyptian diabetic patients included in the study. This oral *Candida* prevalence rate was in accordance with previous studies of diabetic patients; Fisher *et al.* (1987) found candidal species in 51% of the diabetic patients they tested (Fisher *et al.*, 1987). In another study, Willis *et al.* (2000) reported that 77% (318/414) of type-1 diabetes mellitus patients they tested were oral *Candida*-positive (Willis *et al.*, 2000). In contrast, Tekeli *et al.* (2004) reported a relatively lower prevalence rate of oral *Candida* (35%) among 230 type-1 diabetes mellitus patients investigated. A number of relevant factors have been associated with differences in the prevalence of oral *Candida* carriage in diabetic patients including smoking, denture wearing, degree of glycaemic control, insulin-dependent or non insulin-dependent diabetic patients and age (Willis *et al.*, 2000, Manfredi *et al.*, 2002; Tekeli *et al.*, 2004). However, Manfredi *et al.*, (2002) observed that local oral factors, such as the presence of dentures, seemed to have a greater influence than diabetic status on the density and species of *Candida* isolated from the oral cavities of diabetic patients (Manfredi *et al.*, 2002). Although diabetes mellitus is known to predispose to oral candidal infection (Lamey *et al.*, 1988), only 15 (7.5%) diabetic patients included in the present study showed clinical signs and symptoms of oral candidiasis.

In addition, a total of 104/160 (65%) renal transplant patients harboured oral candidal species in the present study. This finding is consistent with that of Al-Mohaya *et al.*, (2002) who investigated the prevalence of oral *Candida* species in 43 Saudi Arabian patients and found that 65% (28/43) of their patients were *Candida*-positive. However, in the present study more renal transplant patients had oral candidiasis than that reported by Al-Mohaya *et al.*, (2002) study (26.3% vs. 15.5%). The prevalence of oral candidiasis among renal transpalnt patients investigated in the present study was also higher than that of King *et al.*, (1994) (10%) and Gupta *et al.*, (1994) (10.5%). The disparity between our data and that of

the others may be due to differences in the general medical conditions and medications of the patients investigated. On the other hand, 39/58 (67%) patients who had cancer were colonised with *Candida* species. A comparable study involving cancer patients reported similar finding (66%) (Davies *et al.*, 2002).

4.4.2 *C. dubliniensis* in Saudi Arabian and Egyptian patients

Recently, there has been an increase in the reporting of the isolation of non-*Candida albicans* yeast species, including the newly described species *C. dubliniensis*, particularly from immunocompromised patients (Coleman *et al.*, 1997a; Davies *et al.*, 2002 Sullivan *et al.*, 2004). Twenty-five independent isolates of *C. dubliniensis* were recovered from Saudi Arabian and Egyptian patients with severe underlying conditions, including cancer, diabetes, and organ transplantation, yielding a *C. dubliniensis* oral prevalence of 5.8% (25/432) (Tables 4.1). A further five independent *C. dubliniensis* clinical isolates from other patients were obtained from patients in two other hospitals in Saudi Arabia.

The overall proportion of diabetic patients from whom oral *C. dubliniensis* was isolated in the present study was 8.5%. However, this finding is in contrast to previously published reports where oral *C. dubliniensis* colonisation was found to be higher. Willis *et al.* (2000) and Ponton *et al.* (2000) demonstrated a higher incidence of *C. dubliniensis* recovery (14% in each case) when they tested 318 and 58 diabetic patients, respectively. However, a much lower prevalence rate was reported by Manfredi *et al.* (2002) who found that only 3.6% (5/137) of the diabetic patients they sampled harboured *C. dubliniensis* in their oral cavities. Furthermore, Tekeli *et al.* (2004) failed to recover oral *C. dubliniensis* in the 81 *Candida*-postive diabetic patients they included in their study (Tekeli *et al.*, 2004). The reason (s) for the disparity in the prevalence of *C. dubliniensis* in these is not known. However, it is likely that the sampling and identification methods used, the geographical locale and the cohort of diabetic patients examined could have contributed to the differences in the epidemiological data (Davies *et al.*, 2002; Sullivan *et al.*, 2004).

Interestingly, no *C. dubliniensis* isolates were recovered from the 17 HIV-infected patients included in the present study. This finding is rather unusual since the majority of *C. dubliniensis* have been recovered from the oral cavities of HIV-infected individuals (Sullivan *et al.*, 1995; Coleman *et al.*, 1997a & b; Sullivan & Coleman, 1998). A high incidence of recovery of *C. dubliniensis* was recorded for Irish HIV-infected individuals

both symptomatic (27 %) and asymptomatic (19 %) (Coleman *et al.*, 1997b). Furthermore, a study carried out on an Irish archival culture collection found that 16.46 % of *C. albicans* isolates recovered from HIV-positive individuals were in fact *C. dubliniensis* (Sullivan *et al.*, 1997). Odds *et al.* (1998) found that of a stock collection of 2,588 yeasts originally identified as *C. albicans*, 2.1 % of these were isolates of *C. dubliniensis* that had been misidentified. Furthermore, a significant proportion (24.7 %) of *C. dubliniensis* isolates in Odds *et al.* (1998) study were recovered from HIV-infected individuals. A prospective study by Jabra-Rizk and colleagues (1999) also found that 5/25 (20%) HIV-positive individuals were found to harbour *C. dubliniensis* in their oral cavities. However, the low incidence of oral carriage of *C. dubliniensis* found in the Saudi Arabian HIV-infected patients may be due to the small number of HIV-infected patients included in the present study. A large-scale study is needed to reveal the prevalence and importance of *C. dubliniensis* in this particular group of patients in Saudi Arabia.

4.4.3 Genotyping of Saudi Arabian and Egyptian *C. dubliniensis* isolates

Genotyping of the *C. dubliniensis* isolates demonstrated that the majority (12/16, 75 %) of the sixteen Israeli isolates tested belonged to genotype 1 (Table 4.1). Similarly, previous studies by Gee *et al.* (2002) and Brena *et al.* (2004) reported that the majority of *C. dubliniensis* isolates genotyped belonged to genotype 1. One of the remaining Israeli isolates belonged to genotype 2, one to genotype 3 and the last was a genotype 4 variant termed genotype 4B that harboured two single nucleotide base substitutions compared with the *C. dubliniensis* genotype 4 ITS consensus sequence. In contrast, 4/8 (50%) of the Egyptian Isolates and 6/22 (27.2%) of the Saudi Arabian isolates belonged to genotype 1 (Table 4.1). None of the Egyptian or Saudi Arabian isolates belonged to genotype 2. Interestingly, the majority (13/22, 59%) of the Saudi Arabian isolates belonged to genotype 3 and the remainder (3/22, 13.6%) to genotype 4 (Table 4.1). None of the Egyptian isolates belonged to genotype 3 and 3/8 (37.5%) belonged to genotype 4. The remaining Egyptian isolate was found to be a genotype 4 variant, termed 4A, that harboured a single nucleotide base substitution compared with the *C. dubliniensis* genotype 4 ITS consensus sequence (Fig. 4.2). These findings demonstrated that the prevalence and distribution of *C. dubliniensis* genotypes among the isolates from Saudi Arabia and Egypt compared to Israel is significantly different ($p<0.003$), at least among the subject cohorts from whom the

isolates were recovered. Interestingly, of the 30 combined Saudi Arabian and Egyptian *C. dubliniensis* isolates included in the study, 20/30 (66.6%) belonged to genotypes 3 and 4 and none were of genotype 2. In contrast, in the previous study by Gee *et al.* (2002) only 6/94 (6.4%) independent *C. dubliniensis* isolates from 15 different countries belonged to genotypes 3 and 4. These results indicated that the *C. dubliniensis* population present in Saudi Arabia and Egypt is quite different from the corresponding populations present in other countries around the world.

4.4.4 DNA fingerprint analysis of Saudi Arabian and Egyptian *C. dubliniensis* isolates

In order to further investigate the population structure of *C. dubliniensis* isolates from the Middle East, all 30 Saudi Arabian and Egyptian *C. dubliniensis* isolates and 5 selected Israeli isolates (two genotype 1, one genotype 2, one genotype 3 and the genotype 4B variant) were subjected to DNA fingerprint analysis with the *C. dubliniensis*-specific fingerprinting probe Cd25. Computer-assisted analysis of the fingerprint patterns obtained from the combined Saudi Arabian and Egyptian isolate collection revealed that the isolates separated at a deep-rooted S_{AB} node of 0.05 into two distinct main populations (Figs. 5.4 and 5.5). The first main population consisted solely of genotype 1 isolates that were closely related with an average S_{AB} value of 0.86. (Fig. 4.4). The second main population was much more diverse with an average S_{AB} value of 0.35 and could be separated at an S_{AB} node of 0.22 into two equally diverse sub-populations, one of which consisted solely of genotype 3 isolates and the other solely of genotype 4 isolates (Fig. 4.4). In order to investigate the relationships between the two main populations identified among this isolate collection and the Cd25-group I and Cd25-group II clades identified in previous studies by Joly *et al.* (1999) and Gee *et al.* (2002), a mixed dendrogram was generated from the Cd25-generated fingerprint patterns of the 30 Saudi Arabian and Egyptian isolates and the corresponding patterns of 51 independent isolates from 13 different countries from the Gee *et al.* (2002) study. The fingerprint profiles of the five Israeli isolates fingerprinted in the present study were also included. Examination of the mixed dendrogram revealed that the isolates separated clearly into three distinct major populations or clades, one of which corresponded to Cd25-group I, one to Cd25-group II and the third to a hitherto undescribed major clade which we have termed CD25-group III (Fig. 4.5). In agreement with the

findings of Gee *et al.* (2002), all genotype 1 isolates were grouped within Cd25-group I and all genotype 2 isolates were grouped in a sub-clade within Cd-25 group II (Fig. 4.5). The Israeli genotype 3 isolate Is36 and the genotype 4B variant isolate Is35 fingerprinted in this study grouped within a second sub-clade within Cd25-group II in agreement with the findings of a previous study reported by Gee *et al.* (2002). The Cd25-group III clade contained all of the Saudi Arabian and Egyptian genotypes 3 and 4 isolates and the single genotype 4 isolate (p7718) from the Gee *et al.* (2002) study (Fig. 4.5). The Cd25-group III clade also contained two sub-clades at an S_{AB} node of 0.22, the first of which contained only Saudi Arabian isolates of genotype 3 and the second both Saudi Arabian and Egyptian genotype 4 isolates. All of these findings demonstrated that the population structure of *C. dubliniensis* isolates in Saudi Arabia and Egypt is significantly different to previously investigated populations from other countries around the world, possibly reflecting a more homogenous ethnic population in Saudi Arabia and Egypt.

4.4.5 5-Flucytosine resistance in *C. dubliniensis* isolates and analysis of *C. dubliniensis FCY1* and *FUR1* gene sequences

All 20 Saudi Arabian and Egyptian *C. dubliniensis* isolates forming the Cd25-group III clade were found to be 5-FC-resistant (MIC > 125 μg/ml). The majority of *C. dubliniensis* clinical isolates reported to date are susceptible to commonly used antifungal drugs including azoles, polyenes and 5-FC (Pfaller *et al.*, 1999a & 2002; Moran *et al.*, 2002; Quindos *et al.*, 2004). However, two recent studies reported that 18/32 (56%) and 2/7 (29%) *C. dubliniensis* isolates tested from Saudi Arabia (Fotedar & Al Hedaithy, 2003) and Kuwait (Ahmad *et al.*, 2004), respectively, were 5-FC-resistant (MIC > 32 μg/ml). These two studies by Fotedar & Al Hedaithy (2003) and Ahmad *et al.* (2004) and the present study are the only reports to describe 5-FC-resistance in *C. dubliniensis*. Based on the results of the present study, it is interesting to speculate that the 5-FC-resistant *C. dubliniensis* isolates described by Fotedar & Al Hedaithy (2003) and Ahmad *et al.* (2004) may also belong to the CD25-group III clade. In support of this suggestion, a recent study demonstrated that 5-FC resistance in *C. albicans* is restricted to a single genetic clade (Pujol *et al.*, 2004).

A recent study by Dodgson *et al.* (2004) demonstrated that clade-specific 5-FC resistance in *C. albicans* is due to a single nucleotide change in the *FUR1* gene that results

in the substitution of arginine by cysteine at amino acid position 101 in the translated protein. Another recent study by Hope *et al.* (2004) reported that a point mutation in the *C. albicans FCY1* gene resulting in a glycine to aspartate substitution at position 28 in the translated protein may also be associated with 5-FC-resistance in this species. In order to determine whether mutations in the *C. dubliniensis* homologs of the *FUR1* and *FCY1* genes were associated with 5-FC-resistance in Cd25-group III clade isolates, the *CdFUR1* and *CdFCY1* ORFs and flanking sequences were cloned and sequenced from 5-FC-resistant and 5-FC-susceptible *C. dubliniensis* isolates (Tables 4.1 & 4.2). No mutations were observed in the ORFs that result in amino acid changes in 5-FC-resistant isolates (Fig. 4.10-Fig. 4.13) demonstrating that mutations in these genes are not responsible for the 5-FC-resistant phenotype exhibited by these isolates.

The identification of a novel clade of *C. dubliniensis* predominant in Saudi Arabia and Egypt, and possibly in other Arab countries, further extends our knowledge about the population structure of this yeast species.

Chapter 5
General Discussion

5.1 Development of culture media to differentiate *C. dubliniensis* from *C. albicans*

Candida dubliniensis was first described as a distinct taxon in 1995 (Sullivan *et al.*, 1995). Since then, a number of other studies have reported the recovery of this species as part of a general increase in the number of infections caused by non-*C. albicans* species during the last two decades. However, it is essential to be able to accurately identify *C. dubliniensis* in a clinical sample in order to determine the clinical importance of this species and its role in human disease. A number of studies have suggested that the increase in reports describing this organism may reflect the development of improved methods for identifying and typing pathogenic yeasts including *C. dubliniensis* (Pujol *et al.*, 1997). Nevertheless, routine discrimination between *C. dubliniensis* and the closely related species *C. albicans* has been problematic (Coleman *et al.*, 1997a & b; Sullivan *et al.*, 2004). The most accurate means of identifying *C. dubliniensis* and discriminating it from *C. albicans* requires PCR-based tests, however, these are not readily applicable for the high-volume throughput of isolates in clinical laboratories and they are labour-intensive, expensive and require special equipment which make them unsuitable to many routine diagnostic laboratories (Donnelly *et al.*, 1999; Tintelnot *et al.*, 2000). In contrast, phenotype-based tests, as described in chapter 2 section 2.2, may lead to ambiguous results because of the many phenotypic similarities between *C. albicans* and *C. dubliniensis* (Coleman *et al.*, 1997b; Schoofs *et al.*, 1997; Pinjon *et al.*, 1999).

Candida albicans and *C. dubliniensis* share the ability to produce chlamydospores. However, it was recently found that there are species-specific differences in the regulation of this developmental process in *C. dubliniensis*. In a recent study of 14 *C. dubliniensis* isolates and 11 *C. albicans* isolates, Staib & Morschhäuser, (1999) reported that the ability of *C. dubliniensis* to produce rough colonies and chalmydospores on Staib agar provided a simple means of differentiating it from its close relative *C. albicans*.

The initial aim of this project was to assess and develop a rapid and dependable phenotypic methods for the differentiation of *C. dubliniensis* from *C. albicans* using colony morphology and chlamydospore. For this purpose, the efficacy of Staib agar, CAF agar, Pal's agar, tobacco agar and casein agar to differentiate between *C. albicans* and *C. dubliniensis* based on colony morphology and the ability of *C. dubliniensis* isolates to

produce chlamydospore on these media was investigated. In the present investigation the colony morphology and chlamydospore production of 130 *C. dubliniensis* and 166 *C. albicans* isolates on Staib was examined (Table 3.1) and the results were compared to the related defined CAF medium. All *C. dubliniensis* and *C. albicans* isolates produced chlamydospores on the control medium, i.e., rice-agar-Tween agar. However, while none of the *C. albicans* isolates produced chlamydospores on either Staib or CAF, 85.4% and 83.8% of the *C. dubliniensis* isolates produced chlamydospores on Staib and CAF, respectively. All of the *C. albicans* isolates grew as smooth, shiny colonies on Staib after 48-72 h incubation at 30°C while 97.7% of the *C. dubliniensis* isolates grew as rough colonies, many (65%) with a hyphal fringe (Fig. 3.1). In contrast, 87.4% of the *C. albicans* and 93.8% of the *C. dubliniensis* isolates yielded rough colonies on CAF. Although the results of this study confirm that Staib agar is good medium for distinguishing between *C. dubliniensis* and *C. albicans,* the discrimination between the two species is best achieved on the basis of colony morphology rather than chlamydospore production.

Production of chlamydospores on casein agar at 24°C for 48 h was also investigated in the present study (Table 3.4). Of 109 *C. dubliniensis* isolates tested on this medium, 106 (97.2%) produced abundant chlamydospores and three produced few chlamydospores. In contrast, of the 120 *C. albicans* isolates tested, 111 (92.5%) failed to produce any chlamydospores, whereas the remaining nine isolates produced few chlamydospores (Fig. 3.4). These findings indicate that abundant chlamydospore production on casein agar is a useful test for discriminating between *C. dubliniensis* and *C. albicans*. However, this method was not absolute. On tobacco agar, fifty-one *C. dubliniensis* isolates 51/53 (96.2%) yielded rough, yellowish-brown colonies with a hyphal fringe around the colonies visible to the naked eye after 48 to 72 h. The other two isolates 2/53 (3.7%) produced smooth, white-creamy colonies similar to the appearance of *C. albicans* isolates on this medium. In contrast, 31/35 (88.5%) *C. albicans* isolates yielded smooth, white-creamy colonies with no hyphal fringe after 48 to 72 h. Of the remaining four *C. albicans* isolates, two isolates (5.7%) produced smooth, white-creamy colonies with a hyphal fringe especially around the primary inoculum site, one isolate (2.8%) yielded yellow-brownish, rough colonies with a hyphal fringe around the colonies similar to those of *C. dubliniensis* and the other one (2.8%) did not grow at all (Fig. 3.3). However, although the agar does not identify *C.*

dubliniensis with 100% accuracy, it provides a simple means for differentiating *C. albicans* from *C. dubliniensis*.

Of all the media investigated in the present study, Pal's (sunflower seed) agar proved to be 100% accurate in distinguishing *C. dubliniensis* from *C. albicans*. Of 128 *C. dubliniensis* isolates tested on this medium, all produced a hyphal fringe at 30°C for 48 to 72 h. In contrast, none of the 124 *C. albicans* isolates tested produced a hyphal fringe (Fig. 3.2). Pal's medium is prepared from seeds which are readily available and inexpensive, which makes it an ideal medium for large-scale epidemiological studies. Indeed, this simple assay has been successfully used in two subsequent studies to differentiate *C. dubliniensis* from *C. albicans* (Adou-Bryn *et al.*, 2003; Khan *et al.*, 2004). Pal's agar is useful for the presumptive identification of *C. dubliniensis* in a previously identified germ tube-positive isolates. However, it could not be used as a medium for the primary isolation of *C. dubliniensis* since both *C. albicans* and *C. dubliniensis* isolates grown on Pal's agar can not be differentiated from other non-*C. albicans Candida* species. Nevertheless, the use of Pal's agar to differentiate *C. dubliniensis* and *C. albicans* isolates has a number of advantages over other phenotypic methods. For example, testing germ tube- or chlamydospore-positive isolates on Pal's agar has an advantage over the use of carbohydrate assimilation profile analysis because the use of Pal's agar is considerably less expensive and amenable to the analysis of large numbers of isolates. In addition, whereas the databases used with many of the commonly used commercial yeast identification systems (e.g., the bioMérieux API 20C AUX and ID 32C systems) have been updated in recent years to include *C. dubliniensis* profiles, the profile range they contain are far from comprehensive. In this regard, recent studies have highlighted the necessity to revise the databases to improve the accuracy of identification of *C. dubliniensis* (Pincus *et al.*, 1999; Tintelnot *et al.*, 2000).

In conclusion, Pal's agar is an excellent method for discriminating between *C. dubliniensis* and *C. albicans* and can be used for the definitive identification of *C. dubliniensis* if the isolates examined are germ-tube positive and produced rough colonies on this medium.

It is interesting to speculate why *C. dubliniensis* produces chlamydospores on Staib and Pal's media. The seed extract contained in both these media (*G. abyssinica* and *H. annus*) and belong to the same botanical family (*Asteraceae*). Therefore, Pal's and Staib agars may have specific compounds in common which induce the production of

chlamydospores in *C. dubliniensis*. However, Khan *et al.*, (2004) did not report any decline in the efficacy of Pal's agar to differentiate *between C. dubliniensis* and *C. albicans* when simplified form (by eliminatig creatinine and KH_2PO_4) of Pal's medium was used in their study (Khan *et al.*, 2004). In addition, *C. dubliniensis* isolates produced chlamydospores when grown on casein and tobacco agars as well, results that favour the involvement of other factors in the differential regulation of chlamydospore development on these agars. In general, it is believed that chlamydospores are a dormant growth forms which arise under conditions of nutrient depletion. Few studies have been undertaken to investigate the biological role of chlamydospore production and the molecular basis behind this phenomena which should provide new insights into the regulation of developmental processes and pathogenicity mechanisms of *C. dubliniensis* and *C. albicans*. In this regard, Staib & Morschhäuser (2005a) constructed a *C. albicans* genomic library in *C. dubliniensis* to identify genes that control chlamydospore formation on Staib agar, thereby partially elucidating the genetic basis of this species-specific phenotypic trait in *C. albicans* and *C. dubliniensis*. Their results showed that differential regulation of a single gene, *NRG1*, in *C. albicans* and *C. dubliniensis* is responsible for their species-specific response to environmental changes (*i.e* growth on Staib agar) that induce chlamydospore development. However, overexpression of *CdNRG1* suppressed pseudohyphal growth and production of chlamydospores in *C. dubliniensis*. In contrast, deletion of *CaNRG1* in *C. albicans* resulted in chlamydospore formation on Staib agar, confirming its central role in the regulation of this morphogenetic process (Staib & Morschhäuser, 2005a). In another study, Staib & Morschhäuser (2005b) demonstrated that liquid growth culture medium containing *Helianthus annuus* (sunflower) extract or *Guizotia abyssinica* seed will also induce chlamydospore formation in *C. dubliniensis* which will also be highly useful for the analysis of gene expression during chlamydospore development and has an advantage over a solid media which lead to submerged mycelial growth of the cells and prevent separation of chlamydospores from the growth medium which makes their analysis problematic. Therefore, comparative analysis of gene expression on these media in subsequent studies could prove helpful in increasing our understanding of dimorphism in these species.

5.2 Molecular epidemiology of *C. dubliniensis* in Saudi Arabia and Egypt

Candida dubliniensis has emerged at a time when significant changes in the epidemiology of fungal infections have occurred. This shift in the epidemiology of fungal infections has been explained, at least in part, by a change in the patient population susceptible to fungal infections. In particular, the increase in the number of immunocompromised patients such as HIV-infected patients and immunosuppressed patients undergoing chemotherapy, bone marrow or solid organ transplantation and the increasing use of invasive medical procedures have been associated with the increase in fungal disease (Coleman *et al.*, 1995 and 1998; Pfaller *et al.*, 1999b & 2000; Krcmery & Barnes, 2002). Since then *C. dubliniensis* has been recovered from every continent and in different patient cohorts and clinical samples (Sullivan *et al.*, 1997; Pinjon *et al.*, 1998; Jabra-Rizk *et al.*, 1999a; Kamei *et al.*, 2000; Polacheck *et al.*, 2000; Quindos *et al.*, 2000; Fisher *et al.*, 2001). However, much of the literature pertaining to *C. dubliniensis* has been on isolates recovered from Europe and the USA and little is known about the molecular epidemiology of *C. dubliniensis* isolates recovered from the Middle East, particularly the Arabs countries. One of the aims of the present study was to further investigate the genetic diversity of *C. dubliniensis* by fingerprint and genotype analysis of a collection of isolates recovered from individuals in Saudi Arabia and Egypt. The present work is the first work in which the *C. dubliniensis* isolates recovered from specific groups of immunocompromised patients including diabetics, renal transplant and cancer patients were examined by Cd25 fingerprinting. In order to investigate the population structure and diversity of *C. dubliniensis* isolates recovered in Saudi Arabia and Egypt compared to isolates recovered in other countries around the world, the 30 *C. dubliniensis* isolates recovered from separate Saudi Arabian (22 isolates) and Egyptian (8 isolates) patients were genotyped by PCR analysis according to Gee *et al.* (2002) (Table 4.1). The genotypes of sixteen *C. dubliniensis* isolates recovered from individuals in Israel were also assessed for comparison. The genotype results of the 16 Israeli isolates (12/16, 75%) re-confirm the findings of previous studies which reported that the majority of *C. dubliniensis* isolates belonged to genotype 1 (Gee *et al.*, 2002; Brena *et al.*, 2004). However, the majority of the 30 combined Saudi Arabian and Egyptian *C. dubliniensis* isolates included in the study, 20/30 (66.6%) belonged to genotypes 3 and 4 while only 6/94 (6.4%) *C. dubliniensis*

isolates from 15 different countries around the world belonged to genotypes 3 and 4 in the previous study by Gee *et al.* (2002) (Table 4.1). However, the majority of the genotype 3 isolates (60%) identified by Gee *et al.* and the single genotype 4 isolate were originally recovered in Israel. The significance of these genotypes is not yet known. However, anatomical selection of strains of *Candida* species in healthy individuals has been reported previously (Soll *et al.*, 1991). In the Gee *et al.* (2002) study, isolates of *C. dubliniensis* belonging to genotypes 1 and 2 were predominantly recovered from the oral cavity (87.3% and 81% of genotype 1 and genotype 2, respectively). In contrast, the single genotype 4 isolate and 71% of the genotype 3 isolates studied were from non-oral sites which suggest the possibility that there is anatomical selection of particular *C. dubliniensis* genotypes. However, this was not seen in the present study since all Saudi Arabian and Egyptian *C. dubliniensis* isolates apart from two (one belonged to genotype 1 and the other belonged to genotype 3) (Table 4.1), were oral isolates. Therefore, it is possible that the high prevalence of genotypes 3 and 4 isolates in the combined Saudi Arabian and Egyptian isolates population reflects ethnic or racial selection of a particular *C. dubliniensis* genotypes.

The two isolates (Eg207 & Is35) which failed to yield an amplimer with any of the genotype-specific primer pairs differ from the *C. dubliniensis* genotype 4 sequence at one and two nucleotide positions, respectively (Fig. 4.2 & Fig. 4.3). However, in the Gee *et al.* (2002) study the *C. dubliniensis* genotypes differed from one another by approximately 5 bp in the entire ITS region. Hence, in the present study it appears that there is no substantial sequence difference observed between the *C. dubliniensis* genotypes even with the inclusion of more diverse *C. dubliniensis* isolates from different parts of the world. A larger study of *C. dubliniensis* isolates needs to be carried out with isolates from different geographic locations that have never been studied such as India, China, Africa, South America and the other Middle Eastern countries to further examine the genetic diversity of *C. dubliniensis* and to determine if there are other genotypes of *C. dubliniensis*. In particular, it would be interesting to examine more *C. dubliniensis* isolates from Arabs, including oral isolates from subgingival and peritonsillar areas and non-oral sites. It is possible that more genotypes would be observed in a larger collection of isolates from Arabs. McCullough *et al.* (1999b) reported differences in the geographic distribution of *C. albicans* genotypes in which *C. albicans* isolates from Israel belong predominantly to genotype C, in contrast to isolates from the United States which belong predominantly to

genotypes A and B (McCullough *et al.*, 1999b).

A total of 30 Saudi Arabian and Egyptian *C. dubliniensis* isolates and 5 Israeli isolates from separate individuals were subjected to DNA fingerprinting analysis using the Cd25 fingerprinting probe and the complex hybridisation profiles were subjected to computer-assisted analysis with the fingerprint profile analysis software DENDRON which was used to compute similarity coefficient (S_{AB}) values for every possible pairwise combination of isolate patterns and these data were used to generate a dendrogram (Fig. 4.5). The dendrogram showed that the isolates separated into two populations of isolates, the first corresponding to genotype 1 and the second to genotypes 3 and 4, respectively. To investigate the relatedness between the isolates recovered from the Middle East in the present study and the *C. dubliniensis* isolates investigated in a previous study by Gee *et al.* (2002) from 15 countries around the world, a mixed dendrogram was generated from the Cd25 generated fingerprint patterns of the 30 Saudi Arabian and Egyptian isolates, from 51 independent isolates from 13 different countries from the Gee *et al.* (2002) and the five Israeli isolates included in the present study. Examination of the mixed dendrogram revealed that the isolates could be clearly divided at an S_{AB} node of 0.05 into three distinct populations (Fig. 4.6). The first group corresponded to Cd25 group I previously described by Joly *et al.* (1999) and Gee *et al.* (2002) and consisted of all genotype 1 isolates which were very closely related with an average S_{AB} value of 0.63±0.12. In addition to three of five Israeli isolates fingerprinted in the present study including Is49 (genotype 2), Is36 (genotype 3) and Is35 (genotype 4B), all genotype 2 and genotype 3 isolates tested from the Gee *et al.* (2002) study grouped within the second group of isolates and corresponded to Cd25-group II isolates previously described by Joly *et al.* (1999) and Gee *et al.* (2002). No Saudi Arabian or Egyptian isolates belonged to Cd25-group II. As observed by Joly *et al.* (1999) and Gee *et al.* (2002) Cd25-group II isolates were much more diverse than the Cd25-group I population with an average S_{AB} value of 0.37±0.30. Significantly, all 20 Saudi Arabian and Egyptian genotype 3 and 4 formed the third group of isolates, termed Cd25-group III (average S_{AB} value of 0.35±0.19), together with a single genotype 4 Israeli isolate from the Gee *et al.* (2002) study. Previous studies suggested that because Cd25-group I is a more homogeneous subgroup of *C. dubliniensis* they may represent a recent group of isolates which has become predominant world-wide due to the HIV-pandemic (Joly *et al.*, 1999; Gee *et al.* 2002). Unfortunately no *C. dubliniensis* isolates were

recovered from the HIV-positive patients investigated in the present study to further examine this possibility (Table 4.4). Of particular interest is the fact that the Cd25-generated fingerprinted patterns of *C. dubliniensis* isolates within the Cd25-group III clade are very distinct from the corresponding patterns of Cd25-group I and Cd25-group II isolates and can be easily distinguished by direct visual comparison (Fig. 4.6 & 4.7). This suggests that there are significant differences in the genomic organisation of the Cd25-group III population. The uniqueness of Cd25-group III isolates was further confirmed by the finding that they were all resistant to 5-FC a part from the single Israeli isolate p.7718. The majority of *C. dubliniensis* clinical isolates reported in the literature to date are susceptible to 5-FC (Moran *et al.*, 1997 & 2002; Pfaller *et al.*, 1999 & 2002; Quindos *et al* 2004). Interestingly, an examination of the records revealed that p7718 was recovered from an Arab individual in Jerusalem in 1999. If our speculation that the 5-FC-resistant isolates reported by Fotedar and Al Hedaithy (2003) and Ahmad *et al.* (2004) from Saudi Arabia and Kuwait, respectively, also belong to Cd25-group III is correct, this would suggest that Cd25-group III isolates may be predominant in and/or possibly restricted to individuals of Arab demographic/ethnic groups. Associations between certain *Candida* species and particular geographical regions and racial groups have been reported previously. A recent study by Blignaut *et al.* (2003) demonstrated a higher relative prevalence of *C. dubliniensis* among white healthy individuals (16%) than among HIV-positive white individuals (9%), black healthy individuals (0%), and black HIV-positive individuals (1.5%) in South Africa. The results of this study suggested that in South Africa, *C. dubliniensis* colonisation is influenced more by race than HIV infection status. However, no South African-specific clade was identified; 14/15 (93%) *C. dubliniensis* isolates investigated in this study were shown to belong to Cd25-group I and the remaining isolate to Cd25-group II (Blignaut *et al.*, 2003). Another recent study by Blignaut *et al.* (2002) also identified a novel clade of *C. albicans* in black and white South Africans. Furthermore, a study by McCullough *et al.* (2004) reported that the genotype distribution of *C. albicans* isolates from Arabs and Druze ethnic groups in Israel were significantly different from global populations. Further studies are required to investigate how widespread Cd25-group III isolates are in the Middle East and to determine their prevalence in other Arab populations including Jordanians, Syrians, Iraquis and others. Furthermore, it would be interesting to investigate whether additional clades are present among specific racial groups. Such studies may be facilitated by the

application of MLST analysis to *C. dubliniensis* epidemiological studies. As described in chapter 1 section 1.3.6, MLST is a recently proposed method for typing *C. albicans* (Bougnoux *et al.*, 2002). It has been used frequently in the analysis of many *Candida* species (Lin *et al.*, 1995; Pujol *et al.*, 1997). When multilocus sequence types was applied to 42 *C. albicans* isolates it allowed the identification of 39 unique genotype combinations (Bougnoux *et al.*, 2002). Similarly, It would therefore be interesting to examine *C. dubliniensis* isolates by MLST to further examine the genetic diversity of *C. dubliniensis*.

In conclusion, the present study has demonstrated the considerable genetic diversity of *C. dubliniensis* and reported for the first time the presence of a novel 5-flucytosine-resistant clade of *C. dubliniensis* (Cd25 group III) that is predominant among isolates from Saudi Arabia and Egypt and absent from a previously characterised international collection of 98 isolates from 15 countries. The present study has also provided a simple, inexpensive method to differentiate between *C. dubliniensis and C. albicans* isolates which is essential if the epidemilogy of *C. dubliniensis* is to be elucidated.

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isolates were found to be less closely related (average S_{AB} value, 0.57) and included a number of distantly related subpopulations and outliers (18). The close relatedness of Cd25 group I isolates was further confirmed in a recent study by Moran et al. (29) that showed that 14/24 (58%) of Cd25 group I isolates tested from 11 different countries contained identical nonsense mutations within the *CdCDR1* gene encoding the ABC transporter protein CdCdr1p, suggesting that many of these isolates were clonal in origin. None of 16 Cd25 group II isolates tested from five different countries contained the nonsense mutation. The study of Gee et al. (18) also revealed that Cd25 fingerprint group I isolates consisted of a single genotype (genotype 1) based on the nucleotide sequence of the internal transcribed spacer region (ITS) of the rRNA gene cluster, whereas Cd25 fingerprint group II isolates were found to consist of three genotypes (genotypes 2 to 4).

Most reported isolates of *C. dubliniensis* have been recovered from individuals in Europe, the United States, South America, and Australia (18, 45, 47), but there is relatively little information on the prevalence or population structure of this organism in the Middle East. There have been a few reports of the isolation of *C. dubliniensis* in Israel, Saudi Arabia, and Kuwait (1, 16–18, 24, 26, 36), but little is known about the relatedness of *C. dubliniensis* isolates from this area to isolates from the rest of the world. The purpose of the present study was to investigate the relatedness of *C. dubliniensis* isolates recovered from individuals in Saudi Arabia and Egypt by analysis of genotypes and Cd25-generated fingerprint profiles. The results of the study identified the presence of a novel flucytosine (5FC)-resistant *C. dubliniensis* clade predominant among Saudi Arabian and Egyptian isolates that, apart from a single related isolate originally recovered from an Arab individual in Israel, was completely absent from a previously characterized group of 98 *C. dubliniensis* isolates recovered from 94 separate individuals from 15 different countries.

MATERIALS AND METHODS

***C. dubliniensis* isolates.** The *C. dubliniensis* clinical isolates used in this study that were recovered from individual patients in Saudi Arabia, Egypt, and Israel are listed in Table 1. A total of 309 Saudi Arabian patients at the Riyadh Medical Center and the Jeddah Kidney Center, Saudi Arabia, and 125 Egyptian patients at the National Institute of Diabetes and the National Institute of Cancer, Cairo, Egypt, were tested for the presence of oral *C. dubliniensis*. Prior to sampling, the presence of oral clinical signs and symptoms indicative of oral candidiasis, including pseudomembranous candidiasis, erythematous candidiasis, median rhomboid glossitis, and angular cheilitis (12), were recorded. Swab specimens from the mid-dorsum of the tongue were taken and plated onto CHROMagar Candida medium (11, 45). After 48 h of incubation at 37°C, the number of colonies present on each plate and their colors and relative abundances were recorded. Single-colony isolates were presumptively identified as *C. dubliniensis* or *Candida albicans* on the basis of their dark green or light green coloration, respectively (11, 21, 23, 45), and subsequently by their colony morphology following growth on Staib agar and Pal's agar as described previously (2, 3, 44). In addition, 5 other *C. dubliniensis* clinical isolates (SA100 to SA104) previously recovered from various specimens from separate patients at the King Faisal and King Khalid Hospitals, Riyadh, Saudi Arabia, and 16 *C. dubliniensis* isolates recovered from various specimens from patients at the Hadassah Medical Center, Jerusalem, Israel, were included in the study (Table 1). Ten of the 16 Israeli *C. dubliniensis* isolates were recovered as pure cultures, 4 in mixed culture with non-*C. albicans Candida* species and 2 in mixed culture with *Aspergillus* species.

Definitive identification of all *C. dubliniensis* isolates was confirmed by the inability or ability of the isolates to grow at 45°C (35), by their substrate assimilation profiles determined with the API ID 32C yeast identification system (bioMérieux, Marcy l'Etoile, France) (9, 34), and by PCR analysis with *C. dubliniensis*-specific primers as described previously (14).

Isolates were routinely cultured on potato dextrose agar (PDA; Oxoid) medium, pH 5.6, at 37°C. For liquid culturing, isolates were grown overnight in yeast extract-peptone-dextrose (YPD) broth at 37°C in an orbital incubator (Gallenkamp, Leicester, United Kingdom) at 200 rpm.

Chemicals, enzymes, radioisotopes, and oligonucleotides. Analytical-grade or molecular biology-grade chemicals were purchased from Sigma-Aldrich Ireland Ltd. (Tallaght, Dublin, Ireland), BDH (Poole, Dorset, United Kingdom), or Roche Diagnostics Ltd. (Lewes, East Sussex, United Kingdom). Enzymes were purchased from the Promega Corporation (Madison, Wis.) and from New England Biolabs Inc. (Beverly, Mass.) and used according to the manufacturers' instructions. [α-^{32}P]dATP (6,000 Ci mmol^{-1}; 222 TBq mmol^{-1}) was purchased from Amersham International Plc. (Little Chalfont, Buckinghamshire, United Kingdom). Custom-synthesized oligonucleotides were purchased from Sigma-Genosys Biotechnologies (Europe) Ltd. (Pampisford, Cambridgeshire, United Kingdom).

Southern blot hybridization and computer-assisted analysis. Southern blot hybridization of restriction endonuclease-digested genomic DNA from *C. dubliniensis* isolates was performed as described previously (48). EcoRI-digested DNA was electrophoresed through 0.65% (weight/volume) agarose gels for 16 h at 65 V and transferred by capillary blotting to nylon membrane filters (Osmonics, Westborough, Mass.) as described previously (48). Hybridization reactions were carried out under high-stringency conditions with the Cd25 probe labeled with ^{32}P by random primer labeling (18).

Computer-assisted analyses of hybridization patterns were performed using the DENDRON software package version 2.4 (Solltech, Iowa City, Iowa) as described previously (18, 22, 43). DNA from the *C. dubliniensis* isolate CM6 was used as a reference on each gel used for computer-assisted analysis as described previously by Joly et al. (22) and Gee et al. (18). S_{AB}s based on band position alone were calculated for each pairwise combination of isolate patterns according to the formula $S_{AB} = 2E/(2E + a + b)$, where E is the number of bands shared by strains A and B, a is the number of bands unique to A, and b is the number of bands unique to B (43). An S_{AB} of 1.00 represents identical patterns, and S_{AB}s ranging from 0.01 to 0.99 represent patterns with increasing proportions of bands at the same positions. A selection of Cd25-generated fingerprint patterns from EcoRI-digested DNA from an international collection of *C. dubliniensis* isolates from the study of Gee et al. (18) from this laboratory were used with DENDRON to generate mixed dendrograms with the fingerprint pattern data generated with the Saudi Arabian, Egyptian, and Israeli isolates as described previously (5, 43).

Genotyping. Template DNA from each of the Saudi Arabian, Egyptian, and Israeli *C. dubliniensis* isolates investigated in this study was tested in separate PCR amplification experiments with each of the primer pairs G1F/G1R, G2F/G2R, G3F/G3R, and G4F/G4R to identify the genotype of the isolate as described by Gee et al. (18). Genotypes are ascribed based on the nucleotide sequences of the internal transcribed spacer 1 (ITS1) and ITS2 regions and of the intervening 5.8S rRNA gene (51). Template DNAs from the four reference *C. dubliniensis* isolates (CD36, genotype 1; Can4, genotype 2; CD519, genotype 3; and p7718, genotype 4) previously described by Gee et al. (18) were used in control experiments. Each PCR was carried out with one pair of genotype-specific primers and the universal fungal primers RNAF/RNAR (15), which amplify approximately 610 bp from all fungal large-subunit rRNA genes and were used as an internal positive control. Two isolates (Eg207 and Is35 [Table 1]) failed to yield amplimers with any of the genotype-specific primer sets described above. The ITS region from each of these isolates was amplified using the ITS1/ITS2 (51) primer pair and cloned, and the nucleotide sequence was obtained and compared as described previously (18). pBluescript II KS(−) was used to clone the purified amplimers using standard procedures (40). Genotyping experiments were performed on a minimum of two occasions with each isolate tested with separately prepared *C. dubliniensis* template DNA.

Antifungal susceptibility testing. The 22 Saudi Arabian, 8 Egyptian, and 16 Israeli *C. dubliniensis* isolates investigated in this study were tested for susceptibility to 5FC by the broth microdilution method described in the Clinical and Laboratory Standards Institute (CLSI) (formerly National Committee for Clinical Laboratory Standards [NCCLS]) document M27-A2 (31). In addition, 24 *C. dubliniensis* isolates from the study of Gee et al. (18), which were recovered from 12 different countries, were also tested (these included 10 Cd25 group I isolates [genotype 1] and 14 Cd25 group II isolates [8 of genotype 2, 5 of genotype 3, and 1 of genotype 4]). A stock solution of 5FC (Sigma-Aldrich) was prepared in water at a concentration of 1 mg/ml. Serial dilutions were then made in RPMI 1640 medium (Sigma-Aldrich) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid. Testing was carried out in a final volume of 100 μl in 96-well plates

TABLE 1. *C. dubliniensis* clinical isolates used in the study[a]

C. dubliniensis isolate[b]	Country of origin[c]	Yr of isolation	Underlying patient condition	Sample	5FC MIC_{50} (μg/ml)[d]	Cd25 fingerprint group[e]	Genotype[f]
Eg200*	Egypt	2002	Diabetes	Oral	128	III	4
Eg201	Egypt	2002	Cancer	Oral	128	III	4
Eg202	Egypt	2002	Cancer	Oral	128	III	4
Eg203	Egypt	2002	Cancer	Oral	0.125	I	1
Eg204	Egypt	2002	Cancer	Oral	0.125	I	1
Eg205	Egypt	2002	Diabetes	Oral	0.125	I	1
Eg206	Egypt	2002	Diabetes	Oral	0.125	I	1
Eg207	Egypt	2002	Diabetes	Oral	128	III	4A
SA100*	S. Arabia	2002	Leukemia	Oral	128	III	3
SA101*	S. Arabia	2002	Leukemia	Oral	0.125	I	1
SA102*	S. Arabia	2002	Leukemia	Blood	0.125	I	1
SA103	S. Arabia	2002	Pneumonia	BAL	128	III	3
SA104	S. Arabia	2002	Pneumonia	Oral	128	III	4
SA105*	S. Arabia	2002	Diabetes	Oral	0.125	I	1
SA106	S. Arabia	2002	Diabetes	Oral	0.125	I	1
SA107*	S. Arabia	2002	Diabetes	Oral	128	III	3
SA108	S. Arabia	2002	Diabetes	Oral	128	III	3
SA109	S. Arabia	2002	Diabetes	Oral	128	III	3
SA110	S. Arabia	2002	Diabetes	Oral	128	III	3
SA111	S. Arabia	2002	Diabetes	Oral	128	III	3
SA112	S. Arabia	2002	Diabetes	Oral	128	III	3
SA113*	S. Arabia	2002	Diabetes	Oral	128	III	4
SA114	S. Arabia	2002	Diabetes	Oral	128	III	3
SA115	S. Arabia	2002	Diabetes	Oral	0.125	I	1
SA116	S. Arabia	2002	Diabetes	Oral	0.125	I	1
SA117	S. Arabia	2002	Diabetes	Oral	128	III	3
SA118	S. Arabia	2002	S/P renal Tx	Oral	128	III	3
SA119	S. Arabia	2002	S/P renal Tx	Oral	128	III	3
SA120	S. Arabia	2002	S/P renal Tx	Oral	128	III	3
SA121	S. Arabia	2002	S/P renal Tx	Oral	128	III	4
Is34	Israel	2000	Liver transplant	BAL	0.125	ND	3
Is35	Israel	2001	Nephritis	Oral	0.125	II	4B
Is36	Israel	2001	CF	Sputum	0.125	II	3
Is38	Israel	2001	SBE	Urine	0.125	ND	1
Is39	Israel	2001	Stroke	Sputum	0.125	ND	1
Is40	Israel	2002	Renal failure	Throat	0.125	ND	1
Is41	Israel	2002	CF	Sputum	0.125	ND	1
Is42	Israel	2002	Leukemia	Throat	0.125	ND	1
Is43	Israel	2002	CF	Sputum	0.125	ND	1
Is44	Israel	2002	CF	Sputum	0.125	ND	1
Is45	Israel	2002	CF	Sputum	0.125	I	1
Is46	Israel	2002	Trauma	BAL	0.125	ND	1
Is47	Israel	2002	Ciliary dyskinesia	Sputum	0.125	I	1
Is48	Israel	2002	CF	Sputum	0.125	ND	1
Is49	Israel	2003	CF	Sputum	0.125	II	2
Is51	Israel	2003	Postsurgery	ENT	0.125	ND	1

[a] The provenance of the isolates is detailed in Materials and Methods. Abbreviations: S. Arabia, Saudi Arabia; BAL, broncheoalveolar lavage; ENT, endotracheal aspirate; SBE, subacute bacterial endocarditis; S/P renal Tx, stage post-renal transplantation; CF, cystic fibrosis; ND, not done.

[b] Isolates marked with an asterisk had their *FCY1* and *FUR1* genes amplified and sequenced.

[c] Egyptian and Saudi Arabian isolates were all recovered from Arab individuals, whereas Israeli isolates were recovered from Jewish individuals, apart from Is35, Is40, Is43, and Is44, which were recovered from Arab individuals in Israel.

[d] MIC_{50} values were determined according to NCCLS document M27-A2 (31).

[e] Refers to major groups of isolates (Fig. 2) identified following computer-assisted analysis of Cd25-generated hybridization fingerprint profiles of EcoRI-digested genomic DNA using the DENDRON computer software package. Only 5 selected Israeli isolates were fingerprinted in the present study, although all 16 were genotyped.

[f] Determined by PCR using the genotype-specific primers described by Gee et al. (18). The nucleotide sequences of the ITS regions of the variant genotypes 4A and 4B differed from the genotype 4 ITS consensus sequence at one and two nucleotide positions, respectively.

containing final concentrations of 5FC ranging from 0.12 to 128 μg/ml with inoculum densities of $1.5 \times 10^3 \pm 1 \times 10^3$ CFU. Drug-free and yeast-free controls were included in each plate. After 48 h of incubation at 30°C, the cell density was measured at 450 nm with a Spectra I automated plate reader (SLT Labinstruments, Grödig, Austria). The MICs were determined as the lowest concentrations of 5FC that reduced turbidity by 50% (MIC_{50}) relative to the growth of the drug-free growth controls. All isolates were tested on a minimum of two occasions in separate experiments. The results were interpreted according to the criteria of the NCCLS using the following breakpoints: susceptibility, ≤4 μg per ml; intermediate resistance, 8 to 16 μg per ml; resistance, ≥32 μg per ml.

Amplification and sequencing of *C. dubliniensis FCY1* and *FUR1* genes. The complete open reading frames (ORFs) of the *C. dubliniensis* genes encoding uracil phosphoribosyltransferase (*FUR1*) and cytosine deaminase (*FCY1*) were amplified by PCR from seven selected Middle Eastern isolates, including four

TABLE 2. Nucleotide sequences of PCR primers used to amplify the *C. dubliniensis FUR1* and *FCY1* genes[a]

Primer	Sequence (5′ to 3′)	Nucleotide coordinates[b]
FCY1F	CGGCATATTAATTCCGCTTG	−145 to −164
FCY1R	CGAATTCTCTTCTGCTTCTG	+106 to +125
FUR1F	ATGTGGGTTACATCAGAAGA	−198 to −217
FUR1R	TCTCGCGACCCTCCTCTAAC	+205 to +224

[a] The nucleotide sequences of the *C. dubliniensis FUR1* and *FCY1* genes were obtained from the *C. dubliniensis* genome sequence database (http://www.sanger.ac.uk/Projects/C_dubliniensis/).

[b] Nucleotide coordinates for *C. dubliniensis* genes *FUR1* and *FCY1* have the first bases of the ATG start codons designated +1.

5FC-resistant ($MIC_{50} \geq 128$ μg/ml) and three 5FC-susceptible ($MIC_{50} \leq 0.125$ μg/ml) isolates (Table 1), and their nucleotide sequences were determined. These were compared with the corresponding sequences of the 5FC-susceptible reference strains CD36 and CM6 (genotype 1), CD506, Can6, and CD541 (genotype 2), CD519 and p6265 (genotype 3), and p7718 (genotype 4) described previously by Gee et al. (18). The sequences of the *C. albicans* genes *FCY1* (GenBank accession no. U55194) and *FUR1* (Stanford orf6.3823) were used in a BLAST search against the *C. dubliniensis* genome sequence database (the Wellcome Trust Sanger Institute *C. dubliniensis* genome sequence project; http://www.sanger.ac.uk/Projects/C_dubliniensis/) to identify the *C. dubliniensis* homologs. The *C. dubliniensis FUR1* gene is 841 bp in length and exhibited 93.3% homology with the *C. albicans* gene. Similarly, the *C. dubliniensis FCY1* gene is 529 bp in length and exhibited 100% homology with the *C. albicans* gene (data not shown). The primer pairs FCY1F1/FCY1R1 and FUR1F1/FUR1R1 were designed to amplify the complete ORFs of each of the *C. dubliniensis* genes, including some upstream and downstream sequences (Table 2). Reaction mixtures contained 100 ng of purified template DNA, 1× Expand High Fidelity buffer (Roche, Lewes, East Sussex, United Kingdom), deoxynucleoside triphosphates at concentrations of 0.2 mM each, 2.5 U of Expand High Fidelity PCR system enzyme mix (Roche), and 0.2 μM concentrations of either FCY1F1/FCY1R1 or FUR1F1/FUR1R1. The reaction mixtures were subjected to an initial denaturation for 7 min at 94°C and 30 cycles of 1 min at 94°C, 1 min of annealing at 50°C for *FCY1* or at 52.5°C for *FUR1*, and 1 min at 74°C. The final extension was for 10 min at 72°C. Amplification products were purified using a Promega PCR extraction kit (Promega) and sequenced in both the forward and reverse directions with the primers used for their amplifications. PCRs with each isolate tested were performed a minimum of three times in separate experiments with separately prepared template DNA. At least three cloned amplimers from each separate PCR experiment were sequenced for each isolate tested.

DNA sequence analysis. Nucleotide sequence analysis was performed by the dideoxy chain-terminating method of Sanger et al. (41) by use of an automated Applied Biosystems 377 DNA sequencer and dye-labeled terminators (Applied Biosystems, Foster City, Calif.). The sequencing primers used for ITS1 and ITS2 and the intervening 5.8S rRNA gene of *C. dubliniensis* were the M13 forward and reverse primers. To sequence the *FCY1* and *FUR1* genes, the FCY1F1/FCY1R1 and FUR1F1/FUR1R1 primer pairs were used (Table 2). Sequence alignments were carried out using the CLUSTAL W sequence analysis computer software package (50).

RESULTS

***Candida dubliniensis* from Saudi Arabian and Egyptian patients.** Oral swab samples were obtained from 434 immunocompromised patients in four hospitals in Saudi Arabia and Egypt (Table 3). The proportions of oral *Candida*-positive individuals sampled from the two countries were similar (Saudi Arabia, 62.8%; Egypt, 70.4%), as were the proportions of individuals that yielded *C. dubliniensis* (Saudi Arabia, 5.5%; Egypt, 6.4%) (Table 3). Details of the Saudi Arabian and Egyptian cohorts sampled and the prevalence of oral *C. dubliniensis* and other *Candida* species recovered from these cohorts are shown in Table 3. In total, 17 Saudi Arabian and 8 Egyptian *C. dubliniensis* isolates were recovered from the patient cohorts sampled. Five additional Saudi Arabian *C. dubliniensis* isolates were included in the study, resulting in a collection of 30 Saudi Arabian and Egyptian isolates for this investigation (Table 1). An additional 16 *C. dubliniensis* isolates recovered in an Israeli hospital were included for comparison (Table 1).

Genotyping of *C. dubliniensis* isolates. In a previous study in which 98 *C. dubliniensis* isolates from 15 different countries were investigated, it was found that *C. dubliniensis* consists of four separate genotypes based on the nucleotide sequence of the ITS region of the rRNA gene cluster (18). Interestingly, 4/6 genotype 3 and genotype 4 isolates included in this study were from Israel. In order to begin to investigate the population structure and diversity of *C. dubliniensis* isolates recovered in Saudi Arabia and Egypt compared to those of isolates recovered in other countries around the world, the 30 *C. dubliniensis* isolates (Table 1) recovered from separate Saudi Arabian (22 isolates) and Egyptian (8 isolates) patients were genotyped by PCR analysis. Sixteen *C. dubliniensis* isolates recovered from individual patients in Israel were used for comparison (Table 1). Template DNA from each isolate was tested separately in PCR experiments with each of the genotype-specific primer pairs GIF/G1R, G2F/G2R, G3F/G3R, and G4F/G4R previously described by Gee et al. (18). These primers allow *C. dubliniensis* isolates to be grouped into one of four genotypes based on the nucleotide sequences of ITS1 and ITS2 regions and of the intervening 5.8S rRNA gene region of the rRNA gene cluster. Isolates belonging to a particular genotype yield a single PCR amplimer only with the corresponding genotype-specific primers. Template DNA from each of the reference *C. dubliniensis* isolates, i.e., CD36 (genotype 1), Can4 (genotype 2), CD519 (genotype 3), and p7718 (genotype 4), was used in control experiments.

All of the *C. dubliniensis* isolates tested yielded the 610-bp product resulting from amplification with the fungal universal primers. Six (27.3%) of the Saudi Arabian and four (50%) of the Egyptian isolates tested were found to belong to genotype

TABLE 3. Recovery of oral *C. dubliniensis* from Saudi Arabian and Egyptian patients

Country and no. of patients	Underlying condition	No. (%) with oral candidiasis[a]	No. (%) oral *Candida* positive	No. (%) yielding *C. dubliniensis*[b]
Saudi Arabia[c]				
160	Renal transplant	42 (26.3)	104 (65)	4 (2.5)
132	Diabetes	9 (6.8)	78 (59)	13 (9.9)
17	HIV-infected	0 (0)	12 (70.6)	0 (0)
Egypt				
58	Neoplasia	12 (20.7)	39 (67.2)	4 (6.9)
67	Diabetes	6 (8.9)	49 (73.1)	4 (6.0)
Total, 432		69 (16)	282 (65.3)	25 (5.8)

[a] Swabs from all individuals with clinical symptoms indicative of oral candidiasis yielded semiconfluent or confluent growth of *Candida* species.

[b] Six of the 25 *C. dubliniensis* isolates were recovered as pure cultures, whereas the remaining 19 were isolated in mixed culture with other *Candida* species, predominantly *C. albicans*.

[c] In addition to the isolates referred to above, five additional *C. dubliniensis* isolates, recovered from various specimens from separate Saudi Arabian individuals (SA100 to SA104 in Table 1), were included in the study, but details on whether they were isolated with other yeast species were not available.

1 (Table 1), whereas none of the Saudi Arabian or Egyptian isolates tested belonged to genotype 2. Thirteen (59.1%) of the Saudi Arabian isolates but none of the Egyptian isolates belonged to genotype 3. The remaining three Saudi Arabian isolates and three of the four remaining Egyptian isolates belonged to genotype 4 (Table 1). One Egyptian isolate (Eg207) failed to yield an amplimer with any of the genotype-specific primer pairs, i.e., GIF/G1R, G2F/G2R, G3F/G3R, and G4F/G4R. In order to investigate Eg207 further, the ITS region from this isolate was amplified by PCR using the ITS1/ITS2 primer pair (51) and cloned, and its nucleotide sequence was determined and compared with the corresponding ITS sequences of genotypes 1 to 4 previously reported by Gee et al. (18). The ITS sequence of Eg207 was found to differ from the *C. dubliniensis* genotype 4 consensus sequence at nucleotide position 82 (T-to-C transition) according to the numbering system of Gee et al. (18). Isolate Eg207 was deemed to be a genotype 4 variant, termed genotype 4A. Of the 16 *C. dubliniensis* isolates from Israel included in the study, 12/16 (75%) belonged to genotype 1, 1/16 (6.3%) belonged to genotype 2, and 2/16 (12.5%) belonged to genotype 3 (Table 1). The remaining isolate (Is35) failed to yield an amplimer with any of the genotype-specific primer pairs and had its ITS region amplified and sequenced as described above for Egyptian isolate Eg207. The ITS sequence of Is35 was found to differ from the *C. dubliniensis* genotype 4 ITS consensus sequence at nucleotide positions 63 (T-to-C transition) and 82 (T-to-C transition) according to the numbering system of Gee et al. (18) and was deemed to be a genotype 4 variant, termed genotype 4B.

These results demonstrated that genotype 3 and genotype 4 isolates predominated (20/30; 66.6%) among the combined Saudi Arabian and Egyptian isolates studied. This is in contrast with the Israeli isolates, where genotype 1 isolates predominated (12/16; 75%), and with the 98 *C. dubliniensis* isolates from the study of Gee et al. (18), which were recovered in 15 different countries from around the world where genotype 1 isolates predominated (71/98; 72%).

DNA fingerprint analysis of Saudi Arabian and Egyptian *C. dubliniensis* isolates. In order to investigate the unexpectedly high prevalence of genotype 3 and genotype 4 isolates and the range of genetic diversity among *C. dubliniensis* isolates from Saudi Arabia and Egypt, the 30 isolates included in the study were fingerprinted with the *C. dubliniensis*-specific complex DNA fingerprinting probe Cd25. Only 5 of the Israeli isolates were fingerprinted, because the majority (12/16) belonged to genotype 1 and a previous study from this laboratory demonstrated that genotype 1 Israeli *C. dubliniensis* isolates belong to fingerprint group Cd25-I (18). The Israeli isolates fingerprinted included the two genotype 1 isolates Is45 and Is47, the genotype 2 isolate Is49, the genotype 3 isolate Is36, and the genotype 4B variant isolate Is35 (Table 1). The Cd25 DNA fingerprint profiles were subjected to computer-assisted analysis with the fingerprint profile analysis software package DENDRON. S_{AB} values were computed for every possible pairwise combination of isolates, and these data were used to construct dendrograms showing the relationships between the isolates. Examination of the dendrogram constructed from fingerprint data obtained with the Saudi Arabian and Egyptian isolates revealed that the 30 isolates could be clearly divided by a node at an S_{AB} value of 0.05 into two distinct main populations of isolates, the first corresponding to genotype 1 and the second to genotypes 3 and 4 (Fig. 1). The total collection of Saudi Arabian and Egyptian isolates had an average S_{AB} value of 0.28 ± 0.27. The first subpopulation (genotype 1) of isolates had an average S_{AB} value of 0.86 ± 0.27, whereas the second subpopulation (genotype 3 [average S_{AB} value, 0.47 ± 0.15] and genotype 4 [average S_{AB} value, 0.53 ± 0.22]) had an average S_{AB} value of 0.35 ± 0.19. In order to investigate the relationships between the two populations of Saudi Arabian and Egyptian isolates identified in this collection and the two groups (Cd25 group I and Cd25 group II) identified in isolates from countries around the world by Joly et al. (22) and verified by Gee et al. (18), a mixed dendrogram was generated from the 30 Saudi Arabian and Egyptian isolates, the 5 Israeli isolates, and 51 independent isolates from 13 countries from the study of Gee et al. (18) (which included 32 isolates from Cd25 group I and 19 isolates from Cd25 group II). Examination of the mixed dendrogram revealed that the isolates could be clearly divided at an S_{AB} node of 0. 05 into three distinct populations (Fig. 2). The first group of isolates (average S_{AB} value of 0.63 ± 0.12) consisted solely of genotype 1 isolates and corresponded to Cd25 group I, previously described by Joly et al. and Gee et al. (18, 22). This group contained the six Saudi Arabian (SA101, SA102, SA105, SA106, SA115, and SA116), the four Egyptian (Eg203, Eg204, Eg205, and Eg206), and the two Israeli (Is45 and Is47) genotype 1 isolates fingerprinted in this study mixed with isolates from the study of Gee et al. (18). The second group of isolates (average S_{AB} value of 0.37 ± 0.30) corresponded to Cd25 group II, previously described by Joly et al. and Gee et al. (18, 22), and did not contain any Saudi Arabian or Egyptian isolates but did contain the other three Israeli isolates fingerprinted in this study, including Is49 (genotype 2), Is36 (genotype 3), and Is35 (genotype 4B) mixed with isolates from the study of Gee et al. (18). The third group of isolates (average S_{AB} value of 0.35 ± 0.19) consisted of the 20 genotype 3 and genotype 4 Saudi Arabian and Egyptian isolates included in this study together with the genotype 4 Israeli isolate p7718 from the study of Gee et al. (18). This latter isolate was a distinct outlier within Cd25 group II in the study of Gee et al. (18). None of the Saudi Arabian or Egyptian genotype 3 or genotype 4 isolates mixed with any of the Cd25 group I or Cd25 group II isolates from the study of Gee et al. (18). These findings demonstrated that genotype 3 and genotype 4 Saudi Arabian and Egyptian *C. dubliniensis* isolates consist of a hitherto-undescribed clade of isolates that is prevalent in Saudi Arabia and Egypt but, apart from the Israeli isolate p7718, was absent from the international collection of isolates from the study of Gee et al. (18). The third group of *C. dubliniensis* isolates forming the novel Saudi Arabian and Egyptian clade was termed Cd25 group III (Fig. 2).

The Cd25-generated fingerprinted patterns of *C. dubliniensis* isolates within the Cd25 group III clade are very distinct from the corresponding patterns of Cd25 group I and Cd25 group II isolates and can be easily distinguished by direct visual comparison (Fig. 3). In particular, Cd25 group III isolates yield fingerprints with far fewer bands (i.e., 8 to 13 bands) compared with Cd25 group I and Cd25 group II fingerprint profiles (i.e., 15 to 20 bands). More strikingly, the majority of Cd25 group III isolates investigated in this study have no or very few bands below 3.5 kb (Fig. 3).

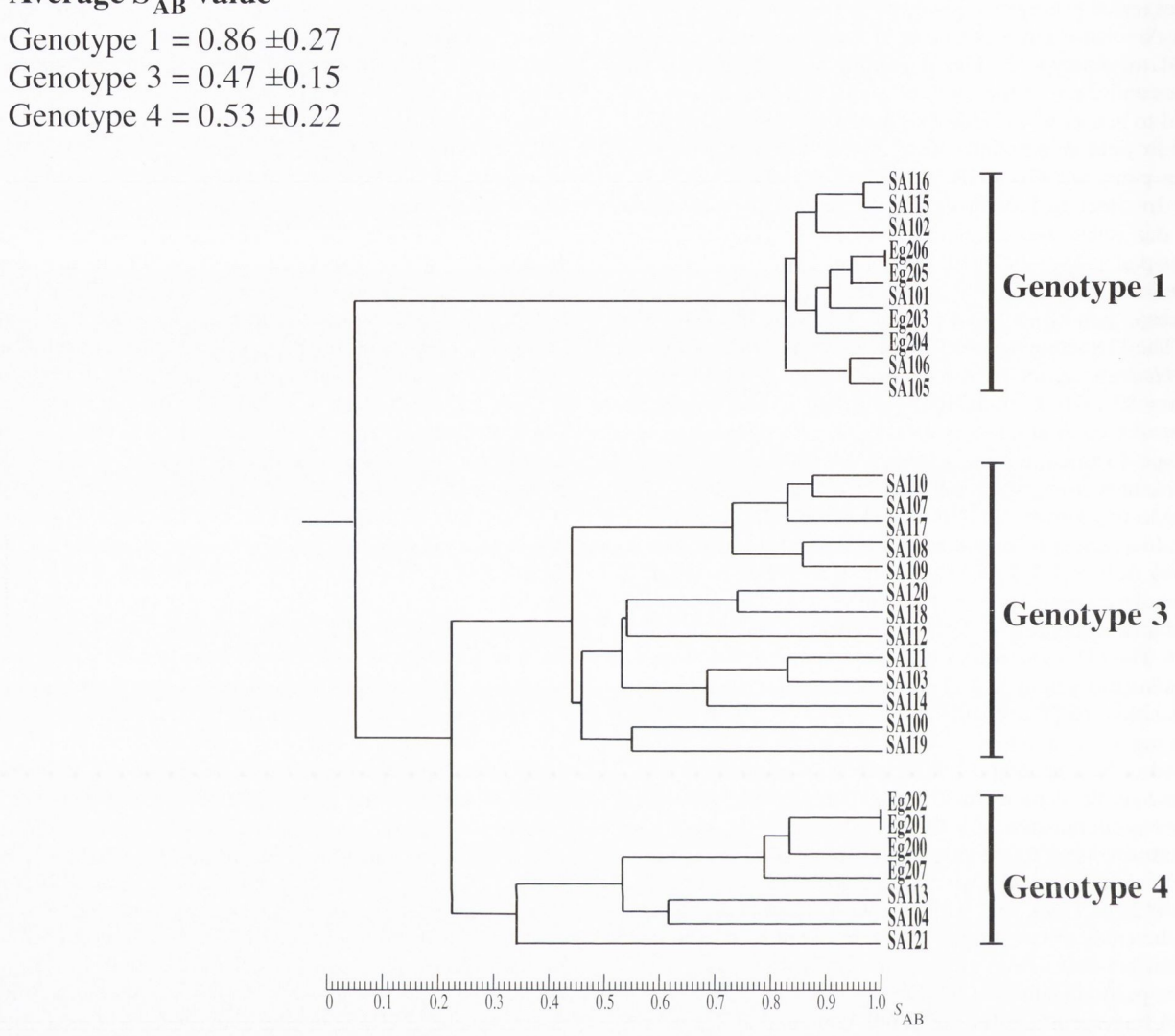
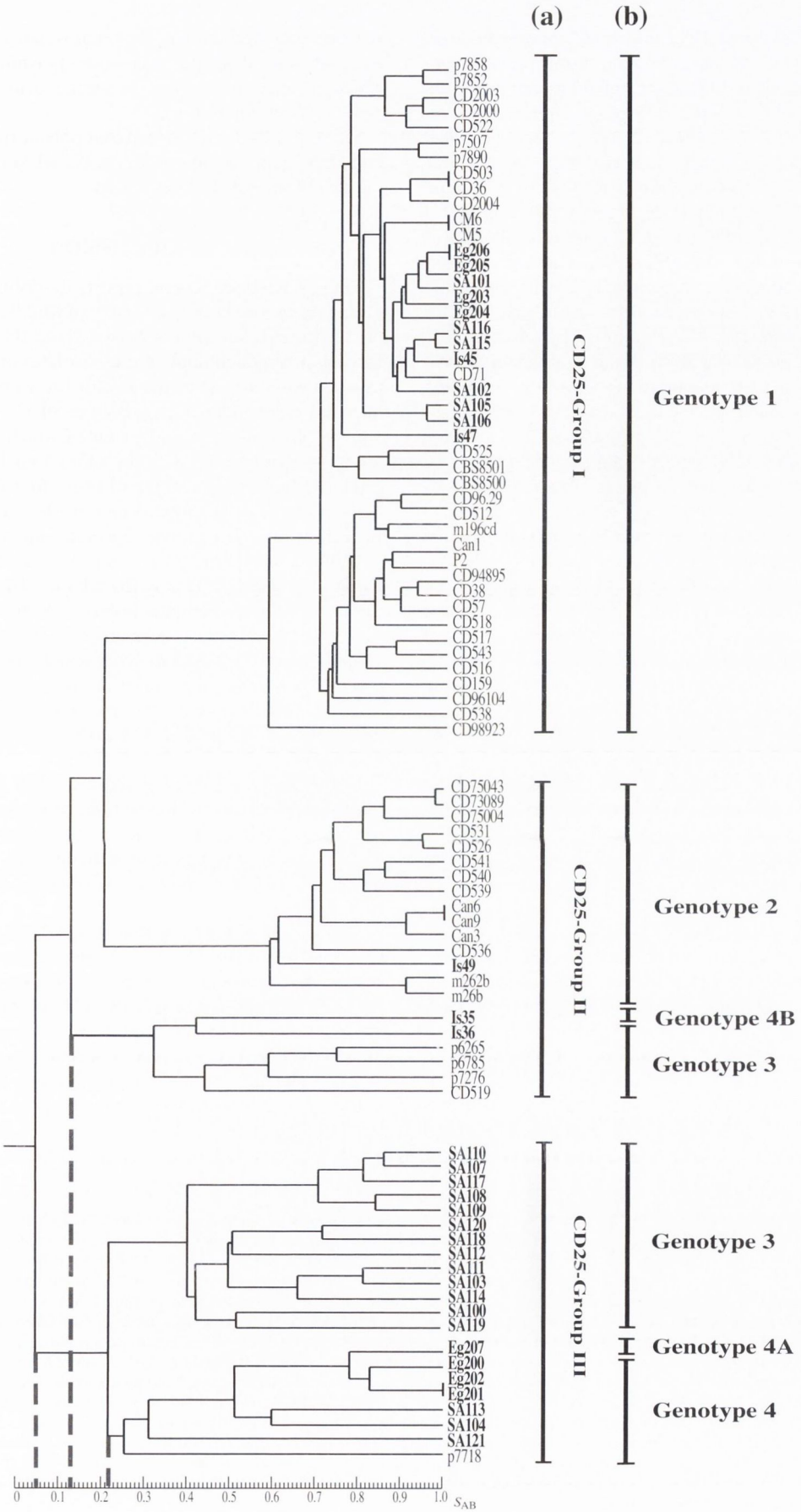


FIG. 1. Dendrogram generated from the S_{AB}s computed for every possible pairwise combination of 30 *C. dubliniensis* isolates recovered from individual patients in Saudi Arabia and Egypt fingerprinted with Cd25. The provenances of the isolates are shown in Tables 1 and 3. At an S_{AB} node of 0.05, the isolates are divided into two main populations. The first of these populations consists solely of genotype 1 isolates and are closely related, with an average S_{AB} value of 0.86 ± 0.27. The second population consists of genotype 3 and genotype 4 isolates that are less closely related to each other than are genotype 1 isolates and have an average S_{AB} value of 0.35 ± 0.19. At an S_{AB} node of 0.22, the second population is divided into two subpopulations consisting of genotype 3 and genotype 4 isolates, respectively.

Flucytosine resistance in Cd25 group III isolates. Several previous studies have reported that the majority of *C. dubliniensis* clinical isolates reported in the literature are susceptible to commonly used antifungal drugs, including azoles, amphotericin B, and 5FC (29, 30, 32, 33, 39). However, two recent studies reported an unusually high prevalence of 5FC resistance among *C. dubliniensis* isolates from Saudi Arabia and Kuwait (1, 17). In order to investigate whether there is an association between the Cd25 group III isolates identified in the present study and resistance to 5FC, all of the Saudi Arabian, Egyptian, and Israeli *C. dubliniensis* isolates included in the study were tested for susceptibility to 5FC with the NCCLS-approved broth microdilution method (31). A selection of 24 independent *C. dubliniensis* isolates from 12 different countries from the study of Gee et al. (18) were also included for comparison (which included 10 Cd25 group I isolates [genotype 1] and 14 Cd25 group II isolates [8 of genotype 2, 5 of genotype 3, and 1 of genotype 4]). All 10 Cd25 group I (genotype 1) Saudi Arabian and Egyptian isolates, all 16 Israeli isolates, and all 24 reference isolates from the study of Gee et al. (18) were found to be 5FC susceptible ($MIC_{50} \leq 0.125$ μg/ml) (Table 1). In contrast, all 20 of the Saudi Arabian and Egyptian isolates belonging to Cd25 group III (genotypes 3 and 4) were 5FC resistant ($MIC_{50} \geq 128$ μg/ml) (Table 1). Interestingly, Israeli isolate p7718, which belongs to this group, was susceptible to 5FC ($MIC_{50} \leq 0.125$ μg/ml). These findings demonstrate that the Saudi Arabian and Egyptian isolates forming the novel CD25 group III clade can be distinguished from *C. dubliniensis* isolates belonging to other groups on the basis of resistance to 5FC.


(a)
(b)
p7858
p7852
CD2003
CD2000
CD522
p7507
p7890
CD503
CD36
CD2004
CM6
CM5
Eg206
Eg205
SA101
Eg203
Eg204
SA116
SA115
Is45
CD71
SA102
SA105
SA106
Is47
CD525
CBS8501
CBS8500
CD96.29
CD512
m196cd
Can1
P2
CD94895
CD38
CD57
CD518
CD517
CD543
CD516
CD159
CD96104
CD538
CD98923
CD25-Group I
Genotype 1
CD75043
CD73089
CD75004
CD531
CD526
CD541
CD540
CD539
Can6
Can9
Can3
CD536
Is49
m262b
m26b
Is35
Is36
p6265
p6785
p7276
CD519
CD25-Group II
Genotype 2
Genotype 4B
Genotype 3
SA110
SA107
SA117
SA108
SA109
SA120
SA118
SA112
SA111
SA103
SA114
SA100
SA119
Eg207
Eg200
Eg202
Eg201
SA113
SA104
SA121
p7718
CD25-Group III
Genotype 3
Genotype 4A
Genotype 4
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
S_{AB}

Analysis of *C. dubliniensis FCY1* and *FUR1* gene sequences. Resistance to 5FC in *C. albicans* has been shown previously to be linked to a single clade (38). A recent study demonstrated that 5FC resistance in *C. albicans* is linked to a point mutation at nucleotide position 301 in the *FUR1* gene encoding uracil phosphoribosyltransferase activity that results in the replacement of arginine with cysteine at amino acid position 101 in the translated protein (13). Another recent study reported that a point mutation in the *C. albicans FCY1* gene (encoding cytosine deaminase) resulting in a glycine-to-aspartate substitution at position 28 in the translated protein may also be associated with 5FC resistance (20). In order to investigate whether mutations in the *C. dubliniensis FUR1* and *FCY1* genes could be associated with 5FC resistance in the Saudi Arabian and Egyptian Cd25 group III isolates identified in the present study, the complete ORFs of these genes together with flanking sequences were amplified by PCR with primers specific for the *C. dubliniensis FUR1* and *FCY1* genes (Table 2). Amplimers of both genes were obtained and sequenced from three 5FC-resistant ($MIC_{50} \geq 128$ μg/ml) Saudi Arabian isolates, i.e., SA100, SA107 (both genotype 3), and SA113 (genotype 4), from three 5FC-susceptible ($MIC_{50} \leq 0.125$ μg/ml) Saudi Arabian isolates, i.e., SA101, SA102, and SA105 (all genotype 1), and from the 5FC-resistant ($MIC_{50} \geq 128$ μg/ml) Egyptian isolate Eg200 (genotype 4). These sequences were compared with the corresponding sequences obtained from selected 5FC-susceptible ($MIC_{50} \leq 0.125$ μg/ml) reference isolates from the study of Gee et al. (18), including CD36 and CM6 (genotype 1), CD506, Can 6, and CD541 (genotype 2), CD519 and p6265 (genotype 3), and p7718 (genotype 4).

The sequences of the *FCY1* gene and flanking sequences from all of the 5FC-susceptible reference isolates tested and the 5FC-susceptible Saudi Arabian genotype 1 isolates SA102 and SA105 were identical to the *FCY1* consensus sequence of the *C. dubliniensis* type strain CD36 obtained from the *C. dubliniensis* genome sequence database (http://www.sanger.ac.uk/Projects/C_dubliniensis/). The 5FC-resistant Saudi Arabian isolates SA100, SA107, and SA113 and the 5FC-resistant Egyptian isolate Eg200 each contained a single identical nucleotide polymorphism (transition from A to T) at position 258 in the *FCY1* gene sequence that did not result in an amino acid residue change in the predicted protein. Comparison of the nucleotide sequences of the *FUR1* genes from the 5FC-susceptible reference isolates and the 5FC-susceptible and 5FC-resistant Saudi Arabian and Egyptian isolates revealed the presence of several single nucleotide polymorphisms, none of which resulted in changes in amino acids in the predicted protein (data not shown).

These results demonstrated that polymorphisms in the *FCY1* and *FUR1* genes could not be associated with 5FC resistance in the *C. dubliniensis* isolates tested.

DISCUSSION

There have been several reports describing the recovery of *C. dubliniensis* isolates in a variety of Middle Eastern countries (1, 17, 18, 26), but little is known about the population structure or the epidemiology of these isolates or their relationship to isolates recovered in the rest of the world. Gee et al. (18) included eight independent isolates of *C. dubliniensis* recovered in Israel in their study, four of which belonged to Cd25 group I (genotype 1), with the other four belonging to Cd25 group II. Interestingly, three of these four Cd25 group II isolates belonged to genotype 3 and one belonged to genotype 4. In fact, of the five genotype 3 isolates identified by Gee et al. (18) out of a total of 98 isolates investigated from 15 different countries, three (60%) were from Israel. The single genotype 4 isolate identified among the isolate collection of Gee et al. (18) was also from Israel. These findings suggested that distinct subpopulations of *C. dubliniensis* may be present in Israel and possibly in neighboring countries. The purpose of the present study was to investigate this possibility further by analyzing additional isolates of *C. dubliniensis* from Saudi Arabia, Egypt, and Israel.

In this study, we investigated a total of 46 independent *C. dubliniensis* isolates recovered from three countries in the Middle East, including 30 *C. dubliniensis* isolates recovered from Saudi Arabian and Egyptian patients with severe underlying conditions and 16 *C. dubliniensis* isolates from Israeli patients (Table 1).

Genotyping of the *C. dubliniensis* isolates demonstrated that the majority (12/16; 75%) of the sixteen Israeli isolates tested belonged to genotype 1 (Table 1), similar to what has been found in previous studies (8, 18). One of the remaining Israeli isolates belonged to genotype 2, two belonged to genotype 3, and the last was a genotype 4 variant, termed genotype 4B, which harbored two single nucleotide base substitutions compared with the *C. dubliniensis* genotype 4 ITS consensus se-

FIG. 2. Dendrogram generated from the S_{AB}s computed for every possible pairwise combination of independent *C. dubliniensis* isolates from Saudi Arabia and Egypt ($n = 30$), Israel ($n = 5$), and 13 other countries ($n = 51$) from the study of Gee et al. (18) fingerprinted with Cd25. At an S_{AB} node of 0.05 (short dashed vertical line), the isolates are divided into three main populations, the first and second of which correspond to the major clades Cd25 group I and Cd25 group II described previously (18, 22). The third main population (Cd25 group III) corresponds to a third major clade identified in this study and contains 20/30 of the Saudi Arabian and Egyptian isolates investigated. The Cd25 group I major clade consists solely of closely related genotype 1 isolates with an average S_{AB} value of 0.63 ± 0.12. At an S_{AB} node of 0.13 (short dashed vertical line), the Cd25 group II major clade can be divided into two minor clades, the first of which consists solely of genotype 2 isolates. The second minor clade consists of genotype 3 isolates and the genotype 4B variant isolate Is35. At an S_{AB} node of 0.22 (short dashed vertical line), the Cd25 group III major clade can also be divided into two minor clades, the first of which consists solely of genotype 3 isolates and the second solely of genotype 4 isolates, including the genotype 4A variant isolate Eg207. Within the Cd25 group III major clade, all of the isolates were from Saudi Arabia or Egypt and were resistant to 5FC ($MIC_{50} \geq 128$ μg/ml), apart from the Israeli isolate, p7718 (18), which was susceptible to 5FC ($MIC_{50} \leq 0.125$ μg/ml). All of the other Saudi Arabian, Egyptian, and Israeli isolates investigated in this study and a selection of 24 independent isolates from the study of Gee et al. (18) (10 Cd25 group I isolates and 14 Cd25 group II isolates) were susceptible to 5FC ($MIC_{50} \leq 0.125$ μg/ml). The Saudi Arabian, Egyptian, and Israeli isolates investigated in this study are highlighted in boldface. The Cd25 groups (a) and the ITS genotypes (b) of the isolates are shown to the right of the dendrogram.

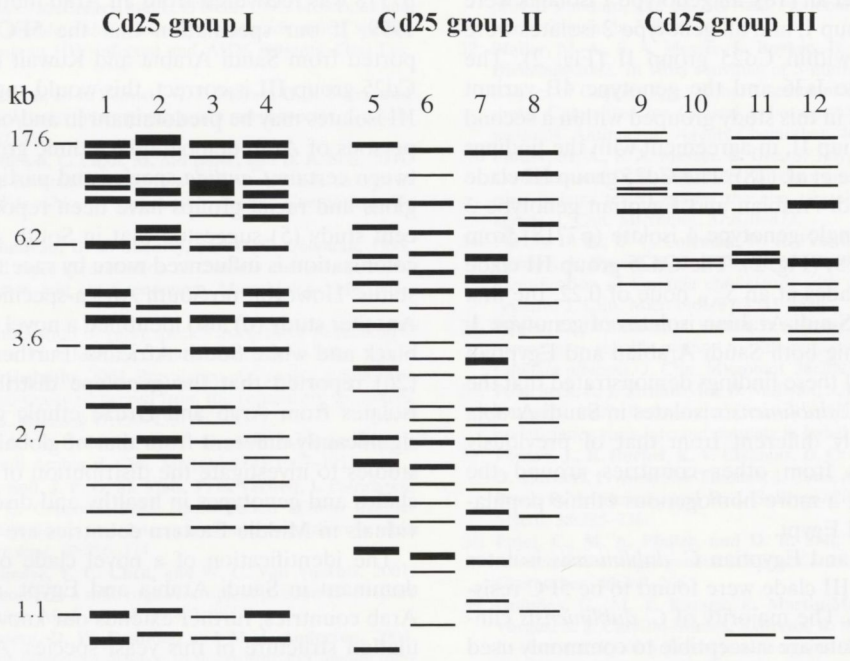


FIG. 3. Model generated using the DENDRON software package showing band positions and intensities of Cd25-generated hybridization fingerprint patterns of EcoRI-digested genomic DNA of *C. dubliniensis* isolates belonging to the major clades Cd25 group I, Cd25 group II, and the novel 5FC-resistant Saudi Arabian and Egyptian clade Cd25 group III identified in this study. Molecular sizes in kilobases are shown on the left. The *C. dubliniensis* isolates from which the corresponding patterns in the lanes were obtained are as follows (genotypes are shown in parentheses): lane 1, CM6 (1); lane 2, CD518 (1); lane 3, SA102 (1); lane 4, Eg204 (1); lane 5, Can9 (2); lane 6, CD514 (2); lane 7, Is49 (2); lane 8, p6265 (3); lane 9, SA103 (3); lane 10, SA121 (4); lane 11, SA119 (3); lane 12, Eg201 (4).

quence. In contrast, 4/8 (50%) of the Egyptian isolates and 6/22 (27.2%) of the Saudi Arabian isolates belonged to genotype 1 (Table 1). None of the Egyptian or Saudi Arabian isolates belonged to genotype 2. Interestingly, the majority (13/22; 59%) of the Saudi Arabian isolates belonged to genotype 3, with the remainder (3/22; 13.6%) belonging to genotype 4 (Table 1). None of the Egyptian isolates belonged to genotype 3, and three out of eight (37.5%) belonged to genotype 4. The remaining Egyptian isolate was found to be a genotype 4 variant, termed 4A, which harbored a single nucleotide base substitution compared with the *C. dubliniensis* genotype 4 ITS consensus sequence. These findings demonstrated that the prevalence and distribution of *C. dubliniensis* genotypes among the isolates from Saudi Arabia and Egypt compared to those of Israeli isolates are significantly different, at least among the subject cohorts from whom the isolates were recovered. Interestingly, of the 30 combined Saudi Arabian and Egyptian *C. dubliniensis* isolates included in the study, 20/30 (66.6%) belonged to genotypes 3 and 4 and none were of genotype 2. In contrast, in the previous study by Gee et al. (18), only 6/94 (6.4%) independent *C. dubliniensis* isolates from 15 different countries were identified as belonging to genotypes 3 and 4. These results indicated that the *C. dubliniensis* population present in Saudi Arabia and Egypt is quite different from the corresponding populations present in other countries around the world.

In order to further investigate the population structure of *C. dubliniensis* isolates from the Middle East, all 30 Saudi Arabian and Egyptian *C. dubliniensis* isolates and 5 selected Israeli isolates (two of genotype 1, one of genotype 2, one of genotype 3, and the genotype 4B variant) were subjected to DNA fingerprint analysis with the *C. dubliniensis*-specific fingerprinting probe Cd25. Computer-assisted analysis of the fingerprint patterns obtained from the combined Saudi Arabian and Egyptian isolate collection revealed that the isolates separated at a deep-rooted S_{AB} node of 0.05 into two distinct main populations (Fig. 1 and 2). The first main population consisted solely of genotype 1 isolates that were closely related and had an average S_{AB} value of 0.86. (Fig. 1). The second main population was much more diverse, with an average S_{AB} value of 0.35, and could be separated at an S_{AB} node of 0.22 into two equally diverse subpopulations, one of which consisted solely of genotype 3 isolates and the other consisting solely of genotype 4 isolates (Fig. 1). In order to investigate the relationships between the two main populations identified among this isolate collection and the Cd25 group I and Cd25 group II clades identified in previous studies by Joly et al. (22) and Gee et al. (18), a mixed dendrogram was generated from the Cd25-generated fingerprint patterns of the 30 Saudi Arabian and Egyptian isolates and the corresponding patterns of 51 independent isolates from 13 different countries from the study of Gee et al. (18). The fingerprint profiles of the five Israeli isolates fingerprinted in the present study were also included. Examination of the mixed dendrogram revealed that the isolates separated clearly into three distinct major populations or clades, the first of which corresponded to Cd25 group I, the second to Cd25 group II, and the third to a hitherto-undescribed major clade which we have termed CD25 group III (Fig. 2). In agreement

with the findings of Gee et al. (18), all genotype 1 isolates were grouped within Cd25 group I, and all genotype 2 isolates were grouped in a subclade within Cd25 group II (Fig. 2). The Israeli genotype 3 isolate Is36 and the genotype 4B variant isolate Is35 fingerprinted in this study grouped within a second subclade within Cd25 group II, in agreement with the findings of a previous study by Gee et al. (18). The Cd25 group III clade contained all of the Saudi Arabian and Egyptian genotype 3 and 4 isolates and the single genotype 4 isolate (p7718) from the study of Gee et al. (18) (Fig. 2). The Cd25 group III clade also contained two subclades at an S_{AB} node of 0.22, the first of which contained only Saudi Arabian isolates of genotype 3, with the second containing both Saudi Arabian and Egyptian genotype 4 isolates. All of these findings demonstrated that the population structure of *C. dubliniensis* isolates in Saudi Arabia and Egypt is significantly different from that of previously investigated populations from other countries around the world, possibly reflecting a more homogenous ethnic population in Saudi Arabia and Egypt.

All 20 Saudi Arabian and Egyptian *C. dubliniensis* isolates forming the Cd25 group III clade were found to be 5FC resistant (MIC > 125 μg/ml). The majority of *C. dubliniensis* clinical isolates reported to date are susceptible to commonly used antifungal drugs, including azoles, polyenes, and 5FC (29, 32, 33, 39). However, two recent studies reported that 18/32 (56%) and 2/7 (29%) *C. dubliniensis* isolates tested from Saudi Arabia (17) and Kuwait (1), respectively, were 5FC resistant (MIC > 32 μg/ml). On the basis of the results of the present study, it is interesting to speculate that the 5FC-resistant *C. dubliniensis* isolates described by Fotedar and Al Hedaithy (17) and Ahmad et al. (1) may also belong to the CD25 group III clade. In support of this suggestion, a recent study demonstrated that 5FC resistance in *C. albicans* is restricted to a single genetic clade (38).

A recent study by Dodgson et al. (13) demonstrated that clade-specific 5FC resistance in *C. albicans* is due to a single nucleotide change in the *FUR1* gene that results in the substitution of arginine by cysteine at amino acid position 101 in the translated protein. Another recent study by Hope et al. (20) reported that a point mutation in the *C. albicans FCY1* gene resulting in a glycine-to-aspartate substitution at position 28 in the translated protein may also be associated with 5FC resistance in this species. In order to determine whether mutations in the *C. dubliniensis* homologs of the *FUR1* and *FCY1* genes were associated with 5FC resistance in Cd25 group III clade isolates as reported for *C. albicans* (13, 20), the *CdFUR1* and *CdFCY1* ORFs and flanking sequences were cloned and sequenced from 5FC-resistant and 5FC-susceptible *C. dubliniensis* isolates (Tables 1 and 2). No mutations were observed in the ORFs that resulted in amino acid changes in 5FC-resistant isolates, demonstrating that mutations in these genes are not responsible for the 5FC-resistant phenotypes exhibited by these isolates (data not shown). Additional studies are required to elucidate the mechanism(s) of 5FC resistance in Cd25 group III *C. dubliniensis*.

The results of the present study demonstrate that *C. dubliniensis* isolates belonging to the Cd25 group III clade appear to be restricted to Saudi Arabia and Egypt, and with the exception of isolate p7718 from Israel (18), all are 5FC resistant. Interestingly, an examination of the records revealed that p7718 was recovered from an Arab individual in Jerusalem in 1999. If our speculation that the 5FC-resistant isolates reported from Saudi Arabia and Kuwait (1, 17) also belong to Cd25 group III is correct, this would suggest that Cd25 group III isolates may be predominant in and/or possibly restricted to persons of Arab demographic/ethnic groups. Associations between certain *Candida* species and particular geographical regions and racial groups have been reported previously. A recent study (5) suggested that in South Africa, *C. dubliniensis* colonization is influenced more by race than by HIV infection status. However, no South Africa-specific clade was identified. Another study (6) also identified a novel clade of *C. albicans* in black and white South Africans. Furthermore, a recent study (26) reported that the genotype distribution of *C. albicans* isolates from Arab and Druze ethnic groups in Israel were significantly different from that of global populations. Further studies to investigate the distribution of *C. dubliniensis* major clades and genotypes in healthy and diseased cohorts of individuals in Middle Eastern countries are in progress.

The identification of a novel clade of *C. dubliniensis* predominant in Saudi Arabia and Egypt, and possibly in other Arab countries, further extends our knowledge about the population structure of this yeast species. Additional studies are required to investigate how widespread the Cd25 group III clade is and whether additional clades are present among specific racial groups; such studies may be facilitated by the application of multilocus sequence typing analysis to *C. dubliniensis* epidemiological studies.

ACKNOWLEDGMENTS

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MiniReview

Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*

Derek J. Sullivan *, Gary P. Moran, Emmanuelle Pinjon, Asmaa Al-Mosaid, Cheryl Stokes, Claire Vaughan, David C. Coleman

Microbiology Research Unit, Department of Oral Medicine, Oral Surgery and Oral Pathology, Dublin Dental School and Hospital, University of Dublin, Trinity College, Dublin 2, Ireland

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bstract

Candida dubliniensis is a pathogenic yeast species that was first identified as a distinct taxon in 1995. Epidemiological studies have nown that *C. dubliniensis* is prevalent throughout the world and that it is primarily associated with oral carriage and oropharyngeal ıfections in human immunodeficiency virus (HIV)-infected and acquired immune deficiency syndrome (AIDS) patients. However, unlike *'andida albicans*, *C. dubliniensis* is rarely found in the oral microflora of normal healthy individuals and is responsible for as few as 2% of ıses of candidemia (compared to approximately 65% for *C. albicans*). The vast majority of *C. dubliniensis* isolates identified to date are ısceptible to all of the commonly used antifungal agents, however, reduced susceptibility to azole drugs has been observed in clinical olates and can be readily induced in vitro. The primary mechanism of fluconazole resistance in *C. dubliniensis* has been shown to be verexpression of the major facilitator efflux pump Mdr1p. It has also been observed that a large number of *C. dubliniensis* strains express non-functional truncated form of Cdr1p, and it has been demonstrated that this protein does not play a significant role in fluconazole ·sistance in the majority of strains examined to date. Data from a limited number of infection models reflect findings from ɔidemiological studies and suggest that *C. dubliniensis* is less pathogenic than *C. albicans*. The reasons for the reduced virulence of *C. ubliniensis* are not clear as it has been shown that the two species express a similar range of virulence factors. However, although *C. ubliniensis* produces hyphae, it appears that the conditions and dynamics of induction may differ from those in *C. albicans*. In addition, *. dubliniensis* is less tolerant of environmental stresses such as elevated temperature and NaCl and H_2O_2 concentration, suggesting that *. albicans* may have a competitive advantage when colonising and causing infection in the human body. It is our hypothesis that a enomic comparison between these two closely-related species will help to identify virulence factors responsible for the far greater ırulence of *C. albicans* and possibly identify factors that are specifically implicated in either superficial or systemic candidal infections.

eywords: *Candida dubliniensis*; *Candida albicans*; Epidemiology; Virulence; Resistance

. Introduction

Fungi are important agents of human disease. Amongst ıe most important fungal pathogens are yeast species elonging to the genus *Candida*. These species can cause wide range of human diseases ranging from superficial ıucosal infections, such as vulvovaginal (VVC) and oro-haryngeal candidosis (OPC), to life-threatening invasive ıfections. In the majority of cases OPC and systemic infections occur only in individuals who are severely ill and/or immunocompromised. In particular, oropharyngeal infections are very commonly diagnosed in human immunodeficiency virus (HIV)-infected individuals and individuals with acquired immune deficiency syndrome (AIDS), while deep-seated systemic infections are frequently associated with patients with neutropenia, for example as a result of antineoplastic therapy or immunosuppressive therapy associated with organ transplantation. The most common cause of candidosis is the polymorphic species *Candida albicans*, which can grow as yeast cells, pseudohyphae and hyphae. It also produces chlamydospores which are refractile spore-like structures that are mainly produced at the termini of hyphae under specific environmental condi-

* Corresponding author. Tel.: +353 (1) 612 7275; ax: +353 (1) 6127295.
E-mail address: dereksullivan@dental.tcd.ie (D.J. Sullivan).

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ons in vitro. However, other *Candida* species, including *Candida glabrata* and *Candida parapsilosis*, are currently lso significant human pathogens. Indeed, it has been sug-ested that the incidence of infections caused by non-*C. lbicans Candida* species is increasing [1].

While performing an in-depth epidemiological analysis f the *Candida* species associated with oral candidal infec-ons in the Irish HIV-infected and AIDS population in ıe early 1990s, we identified an unusual group of isolates]. These were originally identified as *C. albicans* because ıey were germ tube- and chlamydospore-positive (two aits previously recognised as being specific for this spe-es only), however, when they were fingerprinted using ıe *C. albicans*-specific DNA fingerprinting probe 27A, ıe fingerprint patterns obtained were observed to be atyp-al and clearly distinct from those of known *C. albicans* rains. Following a thorough comparison of the pheno-pes and genotypes of these atypical isolates and repre-ntative strains of *C. albicans* (and *Candida stellatoidea*), became increasingly clear that there were many differ-nces between these organisms. This was confirmed by erforming a phylogenetic analysis comparing the nucleo-de sequences of the V3 variable region of the large sub-nit ribosomal RNA gene from the atypical isolates and om *C. albicans*. The results of this led us to suggest that ıe atypical organisms belonged to a novel taxon, which e called *Candida dubliniensis* (after the city and Univer-ty of Dublin) [2]. The phylogenetic relationship between *. dubliniensis* and the rest of the *Candida* genus has since een established on the basis of comparisons between the ucleotide sequences of a wide range of other genes, in-uding the small ribosomal RNA gene [3], *ACT1* [4], *1DR1* [5], *CDR1* [6] and *ERG3* [7].

Clinical significance of *C. dubliniensis*

In order to determine the clinical importance of *C. dub-niensis* and to determine its role in human disease it is ssential to be able to accurately identify the species in inical samples. However, due to the phenotypic similar-ies between *C. dubliniensis* and *C. albicans* this can be roblematic. Since its identification, a large number of henotypic and genotypic tests have been developed with view to investigating the prevalence of *C. dubliniensis* in ıe human population. Many of these tests have been escribed in detail in earlier reviews [8–10] and are sum-arised in Table 1. Very few of these phenotype-based entification tests are 100% accurate. However, it has cently been reported that all 128 *C. dubliniensis* isolates sted produced hyphal fringes when incubated at 30°C for 3–72 h on Pal's agar (a medium containing bird seed tract), whereas all of the 124 *C. albicans* isolates tested roduced smooth colonies when grown under the same ılture conditions (Fig. 1), thus indicating that this is a ry accurate and reliable differentiation test [11]. How-ever, the most definitive methods of identifying *C. dubliniensis* are based on differential amplification of species-specific sequences using the polymerase chain reaction (PCR) and *real-time* PCR [4,12–16]. Ideally, in the clinical laboratory more than one method should be used to definitively identify *C. dubliniensis* isolates.

C. dubliniensis was originally identified because DNA from this species hybridised very poorly to *C. albicans* species-specific DNA fingerprinting probes and had unusual karyotype patterns, suggesting that the genomes of the two species have very distinctive differences [2]. This suggestion has since been confirmed by a study that identified *C. dubliniensis*-specific semi-repetitive sequences [17]. These sequences (i.e. Cd1, Cd24 and Cd25), have been fully characterised and developed for use as DNA fingerprinting probes in the analysis of *C. dubliniensis* populations. The interesting finding from this study and from an extended follow-up study that compared fingerprints and sequences derived from the ITS region of the rRNA operon is that *C. dubliniensis* is comprised of four separate genotypes [18]. The significance of these genotypes has yet to be established. However, isolates belonging to the predominant clade (genotype 1) are primarily associated with carriage and infection in HIV-infected individuals. It is also apparent that the *C. dubliniensis* genome undergoes microevolutionary genomic rearrangements at a far higher rate than that of *C. albicans* [18]. In a thorough investigation of this phenomenon, Joly et al. [19] have determined that the *C. dubliniensis* genome contains more than twice the number of RPS sequences than that of *C. albicans*, and that these could act as recombination hotspots leading to non-homologous recombination between chromosomes. This might explain the unusual karyotypes with small supernumary chromosomes that are typical of *C. dubliniensis*. What effects this increased level of mitotic recombination might have on *C. dubliniensis* is not known. It could have a beneficial effect by facilitating adaptation to changing environments by modulating phenotypic traits, such as drug resistance. However, it could also have a detrimental effect by leading to a loss of heterozygosity.

The development of improved methods for identifying *C. dubliniensis* during the past five years has resulted in a large volume of published data describing the epidemiology of this species. It has been identified in studies from every continent and has been found in a wide range of anatomical sites and clinical samples [20–23]. Interestingly, despite the phenotypic similarities between *C. dubliniensis* and *C. albicans*, the former appears to be only a minor constituent of the normal oral and vaginal microbial flora. In a study on an Irish population of normal healthy individuals only 3.5% of individuals were found to carry *C. dubliniensis* in the oral cavity while the prevalence of this species in the vagina was found to be even lower [24]. *C. dubliniensis* is most commonly associated with OPC in HIV-infected and AIDS patients. In a study on oral can-

Table 1
Comparison of specific phenotypic traits of *C. albicans* and *C. dubliniensis*

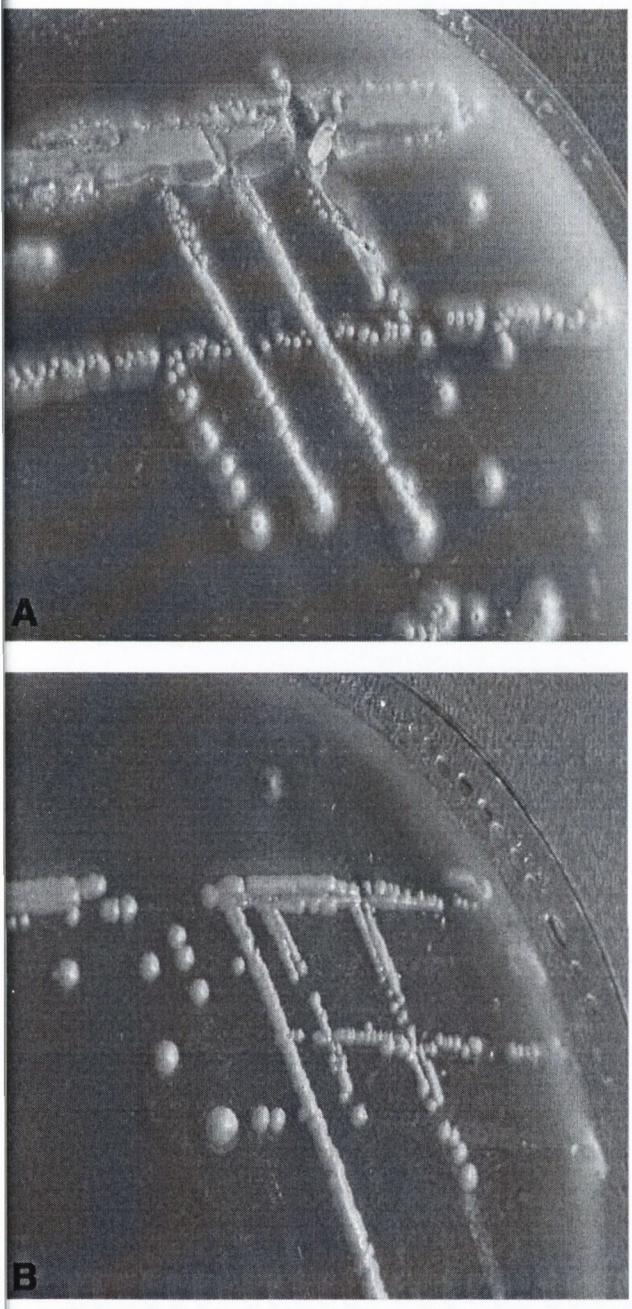
Trait	*C. albicans*	*C. dubliniensis*	Ref.
Production of germ tubes	++	+	[2]
Production of chlamydospores	+	++	[2]
Growth at 37°C[a]	++	++	[2]
Growth at 42°C[a]	++	−	[2]
Growth at 45°C[a]	+	−	[59]
Growth in broth containing 6.5% (W/V) NaCl	+	−	[60]
Growth on xylose[b]	+	−	[62]
Growth on lactate[b]	+	−	[62]
Growth on α-methyl-D-glucoside[b]	+	−	[62]
Growth on trehalose[b]	+	±	[62]
Colony colour on CHROMagar Candida	light blue/green	dark green	[63]
Colony morphology on Pal's agar	smooth	rough+hyphal fringe	[11]

[a]Strains grown on potato dextrose agar.
[b]Based on data obtained using the ID32C yeast identification system (bioMérieux, France)

idosis in our own laboratory, we have shown that in an rish cohort, 26% of HIV-infected and 32% of AIDS pa-ients with symptoms of OPC harboured *C. dubliniensis*, vhile in patients without symptoms of OPC the levels were 8 and 25%, respectively [24]. *C. dubliniensis* was usually ound in combination with other yeast species, especially *C. albicans*. However, in up to 10% of cases it was the only *Candida* species detectable, indicating that *C. dubliniensis* vas very likely to be responsible for the symptoms of isease observed in these cases. *C. dubliniensis* carriage nd infection were also particularly prevalent in recurrent ases of infection, following prior treatment with azole ntifungal drugs, such as fluconazole. A high prevalence f *C. dubliniensis* in the oral cavities of HIV-infected and AIDS patients has also been reported in studies from Ger-nany [25] and the USA [26,27]. However, in a number of ther studies, the levels of *C. dubliniensis* found were sig-ificantly lower [28,29]. The reasons for the disparity in he prevalence of *C. dubliniensis* in these studies are not lear. However, it is possible that the sampling and iden-ification methods used, the geographical locale and the ohort of HIV-infected individuals examined (e.g. intra-enous drug user, homosexual, etc.) could have contrib-ted to the differences in the epidemiological data. In ad-ition, a relatively high prevalence of *C. dubliniensis* has lso been observed in the oral cavities of patients with enture stomatitis [24], diabetes [30] and cystic fibrosis 31].

C. dubliniensis has also been recovered from blood sam-les obtained from cases of invasive disease, particularly in atients who were neutropenic following bone marrow or olid organ transplantation [32–35]. However, despite the igh prevalence of *C. dubliniensis* in cases of OPC in HIV-nfected individuals, this species is only rarely associated vith systemic infections. In two recent studies, *C. dublin-ensis* accounted for approximately 2% of cases of candi-emia in the UK [36] and in the USA [34]; this is in stark ontrast with *C. albicans*, which accounts for approxi-nately two thirds of all cases of candidemia. It should also be noted that in many cases *C. dubliniensis* isolates were recovered from blood samples that also yielded bacterial species such as *Staphylococcus aureus* and *Escherichia coli*, thus in these cases it is impossible to attribute the symptoms of disease to *C. dubliniensis* alone. These epidemiological data suggest that despite the phenotypic similarities between the two species, *C. albicans* has a competitive advantage in colonising and infecting humans.

In summary, *C. dubliniensis* is a rare constituent of the human normal microbial flora, and this is reflected in the low prevalence of *C. dubliniensis* in invasive infections. However, *C. dubliniensis* is a very significant contributor to cases of OPC in HIV-infected and AIDS patients and is clearly pathogenic and present at high levels in these patients. The reasons for the puzzling disparity between the levels of *C. dubliniensis* in HIV- and non-HIV-infected individuals are not understood. One possible explanation is that the methods currently used for routinely isolating oral yeasts (e.g. swabs and rinsing) do not adequately sample all of the microniches that *C. dubliniensis* might inhabit in the oral cavity (e.g. the gingivae). Another possibility is that *C. dubliniensis* strains could be acquired from exogenous sources following sufficient depletion of an individual's T cell count as HIV infection proceeds. Another interesting question concerns whether *C. dubliniensis* has only recently emerged as a human pathogen or whether it has always been associated with human colonisation and infection. From the published epidemiological studies it is clear that the vast majority of *C. dubliniensis* strains identified to date have been found in collections from HIV-infected patients dating from the early 1990s onwards, possibly correlating with the introduction of fluconazole for the treatment of oral candidosis in these patients. However, in several studies of archival strain collections a small number of *C. dubliniensis* strains have been identified that predate the emergence of HIV [37,38], with one strain dating as far back as the 1950s [9]. This suggests that *C. dubliniensis* has been associated with human colonisation (and possibly infection), albeit at a



ig. 1. Photograph of colonies of *C. dubliniensis* (A) and *C. albicans* (B) rown on Pal's agar at 30°C for 48–72 h. The *C. dubliniensis* colonies re rough and are surrounded by a hyphal fringe, whereas the *C. albi-ans* colonies are smooth and have no hyphal fringe.

ower level than *C. albicans*, for a long time and has very kely been misidentified as *C. albicans*. However, the HIV andemic and antifungal therapy appears to have led to its election and ultimately to its identification as a novel pecies in 1995.

. Antifungal drug resistance in *C. dubliniensis*

As *C. dubliniensis* was initially isolated from the oral avities of HIV-infected patients with recurrent oral candidosis, many of whom had previously received azole antifungal drug therapy, it was originally suggested that the apparent emergence of *C. dubliniensis* during the early 1990s may have been due to positive selection as a result of the introduction of novel therapeutic strategies, in a manner similar to that suggested for the emergence of *C. glabrata*. However, this hypothesis may not be correct as several studies have since shown that the great majority of *C. dubliniensis* isolates are inherently susceptible to azole, polyene and echinocandin antifungals [37,39–42]. Despite this, it is worth noting that one study has reported that the geometric mean minimal inhibitory concentration (MIC) for fluconazole in a group of *C. dubliniensis* isolates was significantly higher than that of a matched cohort of *C. albicans* isolates [37].

Resistance to fluconazole in *C. dubliniensis* was first described by Moran et al. in a group of isolates recovered from HIV-infected and AIDS patients [40]. In addition, this study showed that fluconazole-resistant derivatives could be generated from susceptible isolates following exposure to fluconazole in vitro, indicating that this species can rapidly develop resistance. Resistance to fluconazole in clinical isolates of *C. dubliniensis* has been reported by others, and as with *C. albicans*, resistance is primarily associated with isolates recovered from HIV-infected patients [39,42,43]. Exposure of *C. dubliniensis* isolates to fluconazole in vitro not only results in the selection of derivatives with reduced susceptibility, but also increases the adherence of *C. dubliniensis* to epithelial cells and results in increased levels of proteinase secretion [44]. Thus, it is possible that fluconazole therapy could provide a selective pressure that favours the growth of *C. dubliniensis* over *C. albicans* under some conditions in the oral cavity. In one longitudinal study, Martinez et al. [39] described the replacement of *C. albicans* with *C. dubliniensis* in HIV-infected patients receiving fluconazole therapy. All 42 patients included in the study harboured *C. albicans* at the outset, 12 of whom went on to develop infections with fluconazole-resistant *C. albicans*. However, by the end of the study, in another eight patients *C. albicans* was replaced by *C. dubliniensis* in the oral cavity. Surprisingly, in only two of these eight cases were the *C. dubliniensis* isolates resistant to fluconazole in vitro. These results suggest that factors in addition to antifungal drug resistance might play a role in the positive selection of *C. dubliniensis* in the oral cavities of HIV-infected individuals. Clearly, the effects of fluconazole on oral *Candida* population dynamics are complex and further epidemiological analysis and in vitro studies on the potential virulence-modulating effects of fluconazole are required before definitive conclusions can be reached.

The molecular mechanisms of azole resistance in *C. dubliniensis* have been investigated in a number of studies [5,6,40,42,43]. Homologues of the genes encoding *C. albicans* drug efflux pumps, *CDR1* and *MDR1*, have been described in *C. dubliniensis* (termed *CdCDR1* and

CdMDR1, respectively) and have been implicated in the development of resistance to azole drugs. Moran et al. [5] analysed the resistance mechanisms in a group of fluconazole-resistant isolates of *C. dubliniensis* and in vitro generated fluconazole-resistant derivatives and found that in each case, fluconazole resistance was associated with upregulation of *CdMDR1*. Similarly, Perea et al. [42] described increased *CdMDR1* expression in fluconazole-resistant clinical isolates of *C. dubliniensis* recovered from two separate AIDS patients. The importance of *CdMDR1* in the development of fluconazole resistance in clinical isolates of *C. dubliniensis* was confirmed by gene deletion studies in the fluconazole-resistant strain CM2, which was rendered fluconazole-susceptible following deletion of both alleles of *CdMDR1* [45].

In contrast to *C. dubliniensis*, where *CdMDR1* is the principle fluconazole efflux mechanism identified so far, in most isolates of *C. albicans* increased expression of *CaCDR1* is more commonly identified as the main mediator of fluconazole resistance. Unlike the fluconazole-specific Mdr1 pumps, Cdr1 pumps can transport a broad range of azole drugs, including ketoconazole and itraconazole. Cross-resistance to these azoles is relatively rare in fluconazole-resistant *C. dubliniensis* isolates, and this may reflect the high prevalence of *MDR1*-mediated drug resistance in *C. dubliniensis*. The reason(s) for the apparent reduced contribution of *CdCDR1* overexpression to fluconazole resistance in *C. dubliniensis* is not clear. However, Moran et al. [6] recently provided a likely explanation for the lack of *CdCDR1* expression seen in many fluconazole-resistant isolates. Approximately 58% of genotype 1 *C. dubliniensis* isolates (genotype 1 is the predominant genotype recovered from HIV-infected patients) were found to harbour a nonsense mutation in the *CdCDR1* gene, resulting in the translation of a truncated, non-functional protein. But fluconazole-resistant isolates with functional *CdCDR1* alleles may still exhibit increased *CdCDR1* mRNA expression in conjunction with *CdMDR1*. Deletion of both *CdCDR1* copies in an isolate coexpressing both pumps resulted in increased susceptibility to ketoconazole and itraconazole. However, no effect on fluconazole MIC was observed. This was most likely due to the high levels of *CdMDR1* still expressed in this mutant [6].

Point mutations in the *ERG11* gene which result in specific amino acid substitutions in the enzyme 14α-lanosterol demethylase have also been shown to be important in the development of fluconazole resistance in *C. albicans*. To date, only one study, by Perea et al. [42], has described mutations in the *C. dubliniensis CdERG11* gene associated with fluconazole resistance. Two of the mutations described are identical to mutations previously shown to be involved in fluconazole resistance in *C. albicans*, namely G307A and G464S. It has yet to be experimentally verified whether the remaining *CdERG11* mutations described affect fluconazole susceptibility. These mutations occurred in isolates which also exhibited increased drug efflux pump expression, indicating that fluconazole resistance in *C. dubliniensis*, as in *C. albicans*, is multifactorial.

Resistance to itraconazole has not yet been described in clinical isolates of *C. dubliniensis*, but itraconazole-resistant derivatives (that are also cross-resistant to other azoles) can be generated in vitro following serial subculture of susceptible isolates on agar medium containing increasing concentrations of drug [7]. Itraconazole-resistant derivatives were found to have altered membrane permeabilities compared to susceptible isolates. Analysis of their membrane sterol contents revealed profiles lacking ergosterol which is consistent with a mutation in the sterol C5,6-desaturase enzyme encoded by *CdERG3*. Although increased expression of *CdCDR1* and *CdERG11* was also noted in these derivatives, loss-of-function mutations in *CdERG3* were found to be responsible for the high levels of azole cross-resistance observed. Mutations in the *ERG3* gene have also been associated with azole resistance in *C. albicans* [46], however, the contribution of this resistance mechanism to azole drug resistance in *Candida* species has not been fully investigated.

In summary, as in *C. albicans*, the majority of *C. dubliniensis* isolates are susceptible to a wide range of antifungal agents, thus it seems unlikely that the emergence of *C. dubliniensis* in HIV-infected patients has been due to selection by antifungal therapy. When resistance does emerge the molecular mechanisms are broadly similar in the two species. However, there appear to be differences in the relative roles of the efflux proteins Cdr1p and Mdr1p, probably due to the fact that in a large number of *C. dubliniensis* isolates the *CdCDR1* gene is defective.

4. Virulence of *C. dubliniensis*

The epidemiological data on the prevalence of *C. dubliniensis* described above clearly show that this species is only relatively rarely encountered in the normal oral flora of immunocompetent individuals, but is apparently enriched selectively in the oral cavities of immunocompromised patients, particularly HIV-infected and AIDS patients who have received antifungal therapy with fluconazole. It is also clear that while *C. dubliniensis* may in some cases cause systemic infections, it does so far less frequently than *C. albicans*. Confirmation of this apparent reduced virulence of *C. dubliniensis* has been obtained in comparative studies using a mouse model of systemic infection [3,47]. Therefore, despite the significant phenotypic and genotypic similarities shared between *C. albicans* and *C. dubliniensis*, current evidence from epidemiological and virulence studies indicates that *C. albicans* is better adapted to colonise and cause disease in vivo. This begs the question, what differences are there between the two species that render *C. albicans* more pathogenic?

Candida infections involve a very complex interaction between a wide range of host factors and yeast virulence

leterminants that may be differentially expressed depend-
ng on the prevailing environmental conditions. The iden-
ification and determination of the contribution of specific
irulence factors to various *C. albicans* infections is cur-
ently a topic of major interest to medical mycologists.
)ne of the most important virulence factors of *C. albicans*
s its ability to adhere to certain human tissues. However,
iven the range of host tissues which *Candida* species can
olonise and infect this suggests that these organisms pos-
ess a large number of surface adhesins, very few of which
ave actually been characterised. There have been several
tudies to date which have compared the adherence of *C.*
lbicans and *C. dubliniensis* to a range of human cells.
Iowever, it is difficult to meaningfully compare the results
f these studies as different cells and culture conditions
ere used in each case. Depending on the conditions
sed, *C. dubliniensis* was sometimes found to be more
dherent than *C. albicans* [3,48,49], while using different
ssay conditions the opposite was also found [3,44]. Inter-
stingly, in one study, inclusion of fluconazole in the assay
nedium led to an increase in adherence of *C. dubliniensis*
o Vero cells, suggesting that this might contribute to the
igh prevalence of *C. dubliniensis* in HIV-infected and
AIDS patients receiving fluconazole therapy [44]. It has
lso been shown that *C. dubliniensis*, unlike *C. albicans*,
s hydrophobic at 37°C and that *C. dubliniensis* expresses
reatly reduced levels of acid-labile mannans in cell sur-
ace *N*-glycans [49,50]. Cell surface hydrophobicity has
reviously been associated with enhanced adherence and
esistance to phagocytosis [51], however, this hydrophobic-
ty does not appear to confer resistance to phagocytosis on
C. dubliniensis [52]. The only study that investigated the
dherence of *C. dubliniensis* to a specific host protein com-
ared the adherence of a range of *Candida* species to mu-
in, to which *C. dubliniensis* was found to adhere as
trongly as *C. albicans* [53]. A gene family encoding pro-
eins homologous to the ALS family of surface adhesion
lycoproteins in *C. albicans* has also been identified in *C.*
ubliniensis, but it appears that these genes are regulated
ifferently in the two species [54].

Another trait of *C. albicans* that has been proposed as
n important virulence factor is its ability to grow either in
east or in hyphal form (i.e. dimorphism). *C. dubliniensis*
s the only *Candida* species other than *C. albicans* able to
roduce germ tubes and true hyphae. However, the dy-
amics of production of hyphae in both species differ de-
ending on the culture conditions used; in most cases in-
uction of hyphae is more efficient in *C. albicans* (e.g. in
he presence of *N*-acetyl-D-glucosamine [3]), but con-
ersely, under certain circumstances *C. dubliniensis* produ-
es hyphae, while *C. albicans* does not (e.g. on Pal's agar
1]) (Fig. 1). There also appears to be a disparity in the
roduction of hyphae in vivo, as histopathological analy-
is of infected kidneys in a systemic mouse infection model
howed that the *C. dubliniensis* cells were predominantly in
he yeast phase, while the *C. albicans* strains produced far
higher levels of hyphae and pseudohyphae [47]. These data suggest that there are clearly differences in the regulation and dynamics of induction of hyphae in the two species. Phenotypic switching is another important *C. albicans* virulence factor, however, it has been reported that *C. dubliniensis* can undergo phenotypic switching more frequently than *C. albicans* [55]. The production of a range of extracellular hydrolases, such as the secretory aspartyl proteinases (Saps), has been implicated in the pathogenicity of *C. albicans*. Southern hybridisation analysis has shown that *C. dubliniensis* encodes a similar range of genes as the *C. albicans SAP* family. However, phenotypic studies designed to determine the levels of proteinase produced by *C. dubliniensis* have yielded contradictory results [44,48, 55]. Interestingly, analysis of phospholipase production in the two species suggests that *C. dubliniensis* may produce lower levels than *C. albicans* [55,56].

While *C. dubliniensis* grows well in vitro at 37°C and produces biofilm under specific conditions [57], it has been reported that its growth rate is less than that of *C. albicans* [47,58], and that in mixed cultures, *C. albicans* out-grows *C. dubliniensis* [58]. This suggests that *C. albicans* has a competitive growth advantage under the conditions tested. The different growth characteristics of the two species are even more pronounced under conditions of environmental stress and it has been shown that *C. dubliniensis* is significantly less tolerant of elevated temperature (e.g. >42°C [59]), osmotic pressure (e.g. 6.5% (w/v) NaCl [60]) and oxidative stress (e.g. 10 mM H_2O_2 [47]).

The comparative virulence of *C. dubliniensis* and *C. albicans* is clearly a very complex topic. While some differences in virulence factors have been identified there are also numerous contradictory data. A full understanding of the reasons for the greater capacity of *C. albicans* to cause infection will require a concerted effort to compare the genomes and phenotypes of the two species. In particular, there is an urgent need to compare the effects of a greater number of strains belonging to each of the two species in a wider range of animal models of infection. The results of these studies should provide valuable information concerning the molecular mechanisms of how *Candida* species cause disease.

5. Conclusions

C. dubliniensis is now firmly recognised as a significant human pathogen. Since the introduction of highly active anti-retroviral therapy (HAART) the incidence of oral candidosis in HIV-infected and AIDS patients has decreased dramatically [61]. Consequently, the incidence of *C. dubliniensis* in OPC has decreased since it first emerged in the early 1990s. However, the prevalence of this species should continue to be monitored in case of changes in the epidemiology of AIDS (e.g. due to emerging resistance to HAART or to lack of compliance) and due to the intro-

duction of novel antifungal agents such as new azole derivatives and the echinocandins. In addition, comparative genomic analyses using microarrays and genome sequence data will aid the identification of genetic differences between the two species and contribute to the elucidation of the reasons for the differences in the capacity of these species to colonise and infect humans, thus improving our understanding of candidal pathogenicity.

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Differentiation of *Candida dubliniensis* from *Candida albicans* on Pal's Agar

Asmaa Al Mosaid, Derek J. Sullivan, and David C. Coleman*

Microbiology Research Unit, Department of Oral Medicine and Oral Pathology, School of Dental Science and Dublin Dental Hospital, Trinity College, University of Dublin, Dublin 2, Republic of Ireland

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Production of a hyphal fringe around colonies grown on Pal's agar (sunflower seed agar) at 30°C for 48 to 72 h provides a simple means of discriminating between isolates of *C. dubliniensis* and *C. albicans* with 100% accuracy. Of 128 *C. dubliniensis* isolates tested on this medium, all produced a hyphal fringe. In contrast, none of the 124 *C. albicans* isolates tested produced a hyphal fringe. Pal's medium has the added advantage of being prepared from inexpensive, readily available seeds.

Candida dubliniensis is a novel *Candida* species first described by our laboratory in 1995 (20). Studies to date indicate hat this species is primarily associated with oral carriage and nfection in human immunodeficiency virus-infected and AIDS ›atients and has rarely been identified in blood cultures from ›atients with candidemia (3, 4, 6, 7, 10, 14, 19, 20). Routine liscrimination between *C. dubliniensis* and the closely related pecies *Candida albicans* has been problematic (4, 13, 18). The nost accurate means of identifying *C. dubliniensis* and discrimnating it from *C. albicans* requires PCR-based tests; however, hese are not readily applicable to the high-volume throughput ›f isolates in many routine diagnostic laboratories (5, 8). In ontrast, while a number of phenotype-based tests (e.g., deternination of colony color on CHROMagar Candida plates and ack of growth at 45°C and carbohydrate assimilation profiling) ıave proved to be useful for identifying *C. dubliniensis* isolates, hey do not give completely reliable results (4, 12, 13, 15, 18). n a recent study, Staib and Morschhäuser suggested that Staib gar (which contains *Guizotia abyssinica* seed extract) was a ood discriminatory medium (17). This finding was confirmed n a study from our laboratory in which 127 of 130 isolates of *. dubliniensis* (97.7%) grew as rough colonies and all 166 *C. lbicans* isolates tested grew as smooth colonies (1). Staib agar ›as originally developed for the identification of *Cryptococcus eoformans*, which produces melanin-like pigment on this agar 16). In order to develop a medium with even greater discrimnatory ability, we investigated the effect of replacing *G. abysinica* seed extract with extracts from the seeds of other plants.)ne such medium is Pal's agar, which contains sunflower (*Heianthus annus*) seed extract and is another medium originally eveloped for the identification of *C. neoformans* (16). Since oth *H. annus* and *G. abyssinica* belong to the same botanical amily (*Asteraceae*), and since Staib agar is a good but not an bsolutely reliable medium for differentiating between *C. dubniensis* and *C. albicans*, we investigated the use of Pal's meium for this purpose.

* Corresponding author. Mailing address: Microbiology Research Jnit, Department of Oral Medicine and Oral Pathology, School of)ental Science and Dublin Dental Hospital, Trinity College, Univerty of Dublin, Dublin 2, Republic of Ireland. Phone: 353 1 6127276. ax: 353 1 6127295. E-mail: dcoleman@dental.tcd.ie.

The yeast isolates used in this study are shown in Table 1. The identity of all isolates was reconfirmed using the ID 32C yeast identification system (bioMérieux, Marcy l'Étoile, France). In addition, the identities of *C. dubliniensis* and *C. albicans* isolates were confirmed by growth at 45°C and by PCR (5, 13). All isolates were also tested for chlamydospore formation on rice agar-Tween agar, and all the *C. dubliniensis* and *C. albicans* isolates produced chlamydospores on this medium (20). Pal's agar was prepared freshly with unsalted sunflower seeds (including kernels and shells) and used within 5 days to ensure consistent results. First, an aqueous extract of sunflower seeds was prepared by pulverizing 50 g of seeds in a domestic Moulinex (Dublin, Ireland) model B57 blender for 5 min and then adding the ground seeds to 1 liter of distilled water, followed by boiling for 30 min. Next, the seed extract was cooled and filtered, and the following ingredients were added: glucose (1 g), KH_2PO_4 (1 g), and creatinine (1 g). The pH was adjusted to 5.5, the volume was readjusted to 1 liter, and 15 g of agar (Difco) was added before the mixture was autoclaved at 110°C for 20 min. For each isolate included in the study, part of a single colony grown on potato dextrose agar (Oxoid) at 37°C for 48 h was streaked onto Pal's agar contained in 90-mm-diameter single-vent petri dishes (25 ml of agar per plate) and incubated at 30°C. Colony morphology was examined visually every 24 h for up to 10 days, and the data were recorded. At 48 to 72 h, colonies were examined microscopically for the presence or absence of chlamydospores: 10 to 20 well-separated single colonies were stained with 1 drop of 1% (wt/vol) lactophenol cotton blue stain (which stains chlamydospores preferentially), allowed to stain for 5 min, and then covered with sterile glass coverslips (22 by 22 mm) and examined microscopically under bright-field illumination with a 40× objective (9, 20). Chlamydospore-negative isolates continued to be examined at 24-h intervals for up to 10 days.

All of the yeast isolates tested grew well on Pal's agar. Following 48 to 72 h of incubation, all 124 *C. albicans* and all 128 *C. dubliniensis* isolates tested grew as smooth creamy-gray colonies (Fig. 1). However, all the *C. dubliniensis* isolates exhibited a hyphal fringe (Fig. 1B), whereas none of the *C. albicans* did (Fig. 1A). It should be noted that 36 of 124 *C. albicans* isolates (29%) were observed to produce a fringe following 10 days of incubation. Microbiological analysis re-

TABLE 1. *Candida* isolates[a] used in the study

Yeast species	Country of isolation	No. of isolates	Reference
C. dubliniensis[b]	Argentina	1	6, 20
	Australia	2	6, 20
	Brazil	7	11, this study
	Canada	3	1, 6, 13
	France	6	1
	Germany	2	1, 6, 13
	Israel	18	1, 6, 14, this study
	Ireland	43	1, 6, 13, 20
	Malta	3	1
	New Zealand	3	This study
	Spain	2	1, 2, 6
	Switzerland	2	1, 6, 18
	The Netherlands	3	6, 10
	United Kingdom	26	1, 6, 13
	United States	7	1, 12
Total		128	
C. albicans[b]	Greece	8	This study
	Hungary	5	This study
	Ireland	61	1, 13
	United Kingdom	2	1, 13
	United States	48	1, 12
Total		124	
C. glabrata		5	This study
C. tropicalis		4	This study
C. parapsilosis		5	This study
C. krusei		1	This study

[a] All isolates were clinical isolates from the culture collection of the Microbiology Research Unit, Department of Oral Medicine and Oral Pathology, School of Dental Science, Trinity College, University of Dublin, Ireland.

[b] Ninety-five of the *C. dubliniensis* isolates and 111 of the *C. albicans* isolates investigated here were also included in a previous Staib agar study (1).

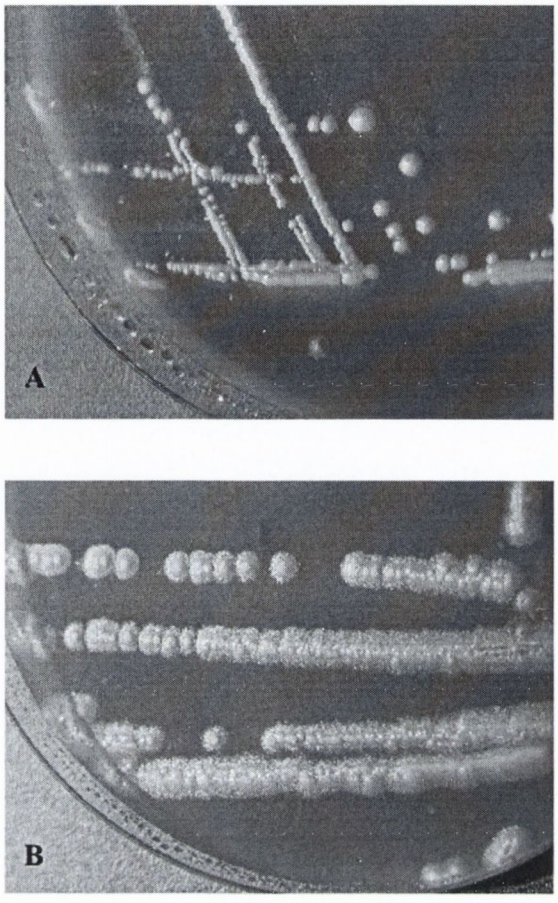

FIG. 1. Macroscopic appearance of *C. dubliniensis* and *C. albicans* colonies on Pal's medium following 72 h of incubation at 30°C. (A) Smooth colonies exhibited by *C. albicans* composed exclusively of blastospores; (B) *C. dubliniensis* colonies displaying a hyphal fringe, containing abundant hyphae, pseudohyphae, and chlamydospores.

ealed that the fringe surrounding *C. dubliniensis* colonies was ound to be comprised of hyphae, pseudohyphae, and blastopores. These findings are in contrast to previous data obtained y using Staib agar, on which 127 of 130 *C. dubliniensis* isolates ested (97.7%) formed rough colonies, many (65%) with a yphal fringe, and all 166 *C. albicans* tested grew as smooth olonies (1). In the present study, none of the *C. albicans* solates produced chlamydospores on Pal's agar, even after 10 lays of incubation, whereas 120 of 128 of the *C. dubliniensis* solates tested (93.75%) produced chlamydospores within 48 to 2 h. The remaining 8 of 128 *C. dubliniensis* isolates (6.25%) ere chlamydospore negative even after 10 days of incubation. nterestingly, in the previous study with Staib agar, none of the 66 *C. albicans* isolates tested produced chlamydospores hereas only 19 of 130 of the *C. dubliniensis* isolates examined 14.6%) were chlamydospore negative (1). Twelve of these 19 hlamydospore-negative *C. dubliniensis* isolates were included n the present study; 7 were chlamydospore positive on Pal's gar, and the remaining 5 were chlamydospore negative.

The colony morphologies of selected oral isolates of four ther *Candida* species (Table 1), including five *C. glabrata*, four *C. tropicalis*, five *C. parapsilosis*, and a single *C. krusei* isolate, were also examined on Pal's medium. One *C. tropicalis* isolate, two *C. glabrata* isolates, and three *C. parapsilosis* isolates yielded smooth colonies similar to those of *C. albicans*. Three *C. tropicalis* isolates, two *C. parapsilosis* isolates, and the single *C. krusei* isolate formed rough colonies with a fringe similar to that formed by *C. dubliniensis*.

Similar results to those described above were obtained with all isolates tested on separate batches of Pal's agar prepared from the same batch of sunflower seeds, in each case with seeds purchased from two separate suppliers. However, we found that incubation of the plates at 37°C rather than 30°C resulted in poorer discrimination between the two species. We therefore propose that the formation of a hyphal fringe surrounding *C. dubliniensis* colonies on Pal's medium following incubation for 48 to 72 h at 30°C provides a definitive means of discrimination between this species and *C. albicans*. However, since colonies of some isolates belonging to other *Candida* species also produce a fringe on Pal's agar, we suggest that this medium should be used only to screen germ tube- and/or chlamydospore-positive isolates. Pal's medium has an impor-

ant advantage over Staib agar in that it permits an absolute discrimination between *C. dubliniensis* and *C. albicans*.

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Casein Agar: a Useful Medium for Differentiating *Candida dubliniensis* from *Candida albicans*

Christian O. Mosca,[1]† María D. Moragues,[2] José Llovo,[3] Asmaa Al Mosaid,[4] David C. Coleman,[4] and José Pontón[1]*

Departamento de Inmunología, Microbiología y Parasitología, Facultad de Medicina y Odontología,[1] and Departamento de Enfermería I,[2] Universidad del País Vasco, Vizcaya, and Servicio de Microbiología, Hospital Clínico Universitario de Santiago, A Coruña,[3] Spain, and Microbiology Research Unit, Department of Oral Medicine and Oral Pathology, School of Dental Science, Trinity College, University of Dublin, Dublin 2, Republic of Ireland[4]

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Production of chlamydospores on casein agar at 24°C for 48 h provides a simple means for differentiating *Candida dubliniensis* from *Candida albicans* based on chlamydospore production. Of 109 *C. dubliniensis* isolates tested on this medium, 106 (97.2%) produced abundant chlamydospores and three produced few chlamydospores. In contrast, of the 120 *C. albicans* isolates tested, 111 (92.5%) failed to produce any chlamydospores, whereas the remaining nine isolates produced few chlamydospores. These findings indicate that abundant chlamydospore production on casein agar is a useful test for discriminating between *C. dubliniensis* and *C. albicans*.

Since its first description in 1995, *Candida dubliniensis* has been isolated from a variety of specimens from humans in countries all over the world (6, 13, 15, 18–20). As a consequence of the increasing number of reports on the isolation of *C. dubliniensis*, it is important to be able to rapidly and accurately identify this species in most clinical mycology laboratories. However, identification of *C. dubliniensis* is hampered by its close relationship with *Candida albicans*, a situation that has sometimes led to the misidentification of isolates of *C. dubliniensis* as *C. albicans* (19). At present, the most accurate differentiation between isolates of the two species is performed in reference laboratories with the use of molecule-based techniques such as PCR or DNA fingerprinting with repetitive sequence-containing DNA probes (5, 17, 19). However, these sophisticated techniques are not suitable and often not readily applicable for use in small clinical mycology laboratories, where simple and rapid methods are needed. Reliable phenotypic methods for the identification of *C. dubliniensis* isolates include carbohydrate assimilation profile analysis by using commercially available yeast identification systems and detection of differential antigen expression by immunofluorescence microscopy (2, 3, 11, 12, 20). Furthermore, a variety of other ancillary tests have been developed for discriminating between *C. dubliniensis* and *C. albicans* isolates, including the inability of *C. dubliniensis* to grow at 45°C (12). However, whereas these tests are useful for the presumptive identification of *C. dubliniensis*, they are not definitive. One of the key features employed in the initial description of *C. dubliniensis* was its ability to produce abundant chlamydospores on cornmeal agar and rice-agar-Tween-agar (20). Chlamydospore production by *C. dubliniensis* on Staib agar and caffeic acid-ferric citrate agar has also been used recently for the differentiation of *C. dubliniensis* from *C. albicans* (1, 17). In the present study, the production of chlamydospores by *C. dubliniensis* and *C. albicans* on casein agar was investigated as an additional means for differentiating the two species.

The reference and clinical isolates used in this study are shown in Table 1. Conventional morphological and physiologic methods, as well as molecular techniques, were employed to confirm the identity of all isolates (1, 2, 4, 5, 11, 19). All yeasts studied were initially grown for 48 h at 30°C on Sabouraud glucose agar (Difco, Detroit, Mich.). Casein agar was prepared as described by Larone (7). Briefly, 10 g of skim milk (Sveltesse dried skimmed milk; Nestlé España SA, Esplugues de Llobregat, Barcelona, Spain; and Marvel dried skimmed milk; Premier Brands, Merseyside, United Kingdom) was dissolved in 90 ml of distilled water, and 3 g of agar was dissolved in 97 ml of distilled water. After autoclaving of both solutions separately at 121°C for 15 min, they were allowed to cool to 45 to 50°C and were then mixed together and dispensed in 25-ml amounts into 90 mm-diameter petri dishes. Caesin agar plates were inoculated with culture growth from a 48-h-old Sabouraud agar by cutting several shallow parallel groves in the agar with a wire loop, followed by incubation at 24 C for 48 h. Following incubation, samples of culture growth were stained with lactophenol cotton blue and were examined for chlamydospore production by light microscopy (20). Yeast isolates were also tested for growth at 45°C for 48 h on Sabouraud glucose agar as described by Pinjon et al. (12).

All 109 *C. dubliniensis* isolates tested produced chlamydospores on casein agar after 48 h of incubation at 24°C. Chlamydospores were stained dark blue by lactophenol cotton blue and were very abundant and arranged in groups around pseudomycelial growth (Fig. 1A), but in some cases isolated chlamydospores were also observed. The vast majority (111 of 120 [92.5%]) of the *C. albicans* isolates tested did not produce

* Corresponding author. Mailing address: Departamento de Inmunología, Microbiología y Parasitología, Facultad de Medicina y Odontología, Universidad del País Vasco, Apartado 699, E-48080 Bilbao, Vizcaya, Spain. Phone: 94-601-2855. Fax: 94-464-9266. E-mail: oipposaj@lg.ehu.es.

† Present address: Departamento de Microbiología e Inmunología, Facultad de Odontología, Universidad de Buenos Aires, Buenos Aires, Argentina.

TABLE 1. Yeast isolates used in this study

Organism and source or country of isolation	No. of isolates	Specimen	Source or reference(s)
C. dubliniensis			
Argentina	1	Oral	5, 18
Australia	3	Oral	5, 20
Canada	5	Oral	5, 12
Finland	1	Oral	5, 12
Germany	4	Oral	5, 12
Greece	1	Oral	5, 12
Ireland	24	Oral	5, 18, 20
	4	Blood	5; this study
	1	Vaginal	5, 10
Italy	3	Oral	This study
Israel	2	Res. tr.[a]	5, 13
	2	Sputum	5, 13
	1	Urinary	5, 13
	2	Vaginal	5
	1	Wound	5
The Netherlands	2	Blood	5, 8
	1	Sputum	5, 8
Spain	33	Oral	5, 12, 14; this study
	1	Blood	15
	1	Vaginal	This study
Switzerland	7	Oral	3, 5
United Kingdom	6	Oral	5, 12, 16
	1	Blood	5, 12, 16
	1	Fecal	5, 12
Culture collection	NCPF 3108	N/A	20
C. albicans			
Argentina	9	Oral	This study
Greece	4	Oral	This study
Ireland	6	Oral	1; this study
Hong Kong	1	Oral	12
Spain	45	Oral	This study
	36	Vaginal	This study
	1	Urinary	This study
	1	Penile	This study
United States	9	Oral	1, 11
Culture collection	NCPF 3153 and 3156 and ATCC 26555, 64385, 64548, 64550, 90028, and 90029	N/A	
C. glabrata			
Spain	9	Oral	This study
Culture collection	NCPF 3203	N/A	
C. guilliermondii			
Spain	6	Oral	This study
	1	Vaginal	This study
Culture collection	NCPF 3099	N/A	
C. krusei			
Spain	3	Oral	This study
	1	Vaginal	This study
Culture collection	ATCC 6258	N/A	
C. lusitaniae			
Spain	1	Bronchial aspirate	This study
	1	Fecal	This study
	1	Urinary	This study
C. parapsilosis			
Spain	5	Oral	This study
C. rugosa			
Bulgaria	6	Blood	This study
	1	Urinary	This study
	1	Prosthetic valve	This study
C. stellatoidea type I			
Culture collection	ATCC 11006	N/A	
C. stellatoidea type II			
Culture collection	ATCC 20408	N/A	
C. tropicalis			
Ireland	5	Oral	This study
Spain	10	Oral	This study
Culture collection	NCPF 3111	N/A	

[a] Res. tr., respiratory tract; N/A, not applicable.

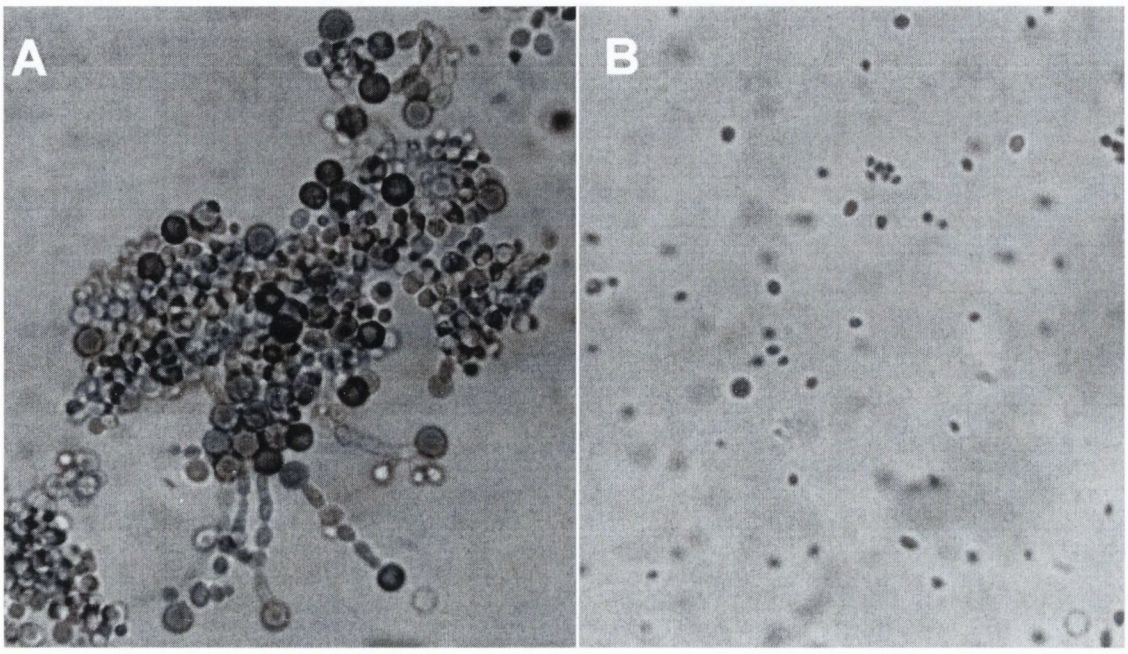

FIG. 1. Abundant chlamydospore production on casein agar by *C. dubliniensis* isolate 00131 (A) and absence of chlamydospore production by *C. albicans* isolate 00160 (B) incubated for 48 h at 24°C. Magnification, ×400.

chlamydospores on casein agar after 48 h of incubation. In these isolates, only yeast-like cells of different sizes stained a light blue color by lactophenol cotton blue were observed (Fig. 1B). However, 9 of 120 (7.5%) of the *C. albicans* isolates tested produced chlamydospores on casein agar. These chlamydospores, although indistinguishable from those produced by *C. dubliniensis*, were difficult to observe due to their low number. The identity of these isolates as *C. albicans* was reconfirmed by carbohydrate assimilation profile analysis with the API ID32 system, by lack of reactivity with a *C. dubliniensis* antiserum, and in some cases by PCR (2, 4, 11). The production of chlamydospores on casein agar by *Candida* species other than *C. dubliniensis* and *C. albicans* was also investigated (Table 1). No chlamydospores were produced by the *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida guilliermondii*, *Candida glabrata*, *Candida lusitaniae*, *Candida rugosa*, *Candida stellatoidea* type I, and *C. stellatoidea* type II isolates tested (Table 1). For all of the *Candida* strains included in the study, results similar to those described above were obtained in separate experiments with three different batches of casein agar.

Casein agar seems also to be suitable for studying chlamydospore production by fresh isolates, since, when 8 fresh *C. dubliniensis* oral isolates and 10 fresh *C. albicans* oral isolates were tested, all of the *C. dubliniensis* isolates produced abundant chlamydospores within 48 h, whereas none of the *C. albicans* isolates did.

The ability to grow at 45°C was studied in an attempt to differentiate the *C. albicans* isolates producing chlamydospores on casein agar from the *C. dubliniensis* isolates. Previous studies demonstrated that *C. dubliniensis* isolates do not grow at 45°C, whereas the majority of *C. albicans* do (12). While no growth was found with any of the 109 *C. dubliniensis* isolates at 24 and 48 h on Sabouraud dextrose agar at 45°C, all 120 of the *C. albicans* isolates grew well at 45°C.

Casein agar has been traditionally used to study the decomposition of casein by aerobic actinomycetes and dematiaceous fungi (7). However, results presented in this study show, for the first time, that casein agar is a good medium to induce the production of chlamydospores by *C. dubliniensis* isolates, a feature that can be used to differentiate *C. dubliniensis* from *C. albicans*. Although 106 of 109 (97.2%) of the *C. dubliniensis* isolates tested produced abundant chlamydospores on casein agar, nine isolates of *C. albicans* isolates also produced very few chlamydospores. Attempts to improve discrimination between the two species by decreasing the temperature of incubation or by modifying the composition of casein agar by varying the amount of skim milk added or by incorporating 1% Tween 80 were unsuccessful (data not shown). Discrimination between *C. dubliniensis* isolates and the nine *C. albicans* isolates that produced chlamydospores on casein agar was achieved by incubating the isolates at 45°C, since all nine *C. albicans* isolates grew well at that temperature, whereas the 109 *C. dubliniensis* isolates included in the study did not. Although growth at 45°C alone permitted discrimination between *C. albicans* and *C. dubliniensis* isolates in this study, it has been reported that some *C. albicans* isolates are not able to grow at 45°C (6, 9, 12).

Attempts to differentiate *C. dubliniensis* from *C. albicans* on the basis of chlamydospore production in media such as Staib agar and caffeic acid-ferric citrate agar have been made (1, 17). However, casein agar is less expensive and simpler to prepare than these media and casein agar could easily be prepared in routine clinical mycology laboratories. In conclusion, casein agar provides a simple and inexpensive means of differentiating isolates of *C. dubliniensis* and *C. albicans*. In the vast majority of cases, isolates producing abundant chlamydospores on casein agar can be presumptively identified as *C. dubliniensis*.

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High prevalence of non-albicans yeasts and detection of anti-fungal resistance in the oral flora of patients with advanced cancer

J Bagg and **MP Sweeney** Glasgow Dental Hospital and School, University of Glasgow, Glasgow, **MAO Lewis** Dental School, University of Wales College of Medicine, Cardiff, **MS Jackson** Glasgow Dental Hospital and School, University of Glasgow, Glasgow, **D Coleman** and **A Al Mosaid** Dublin Dental School and Hospital, University of Dublin, Dublin, **W Baxter** ACCORD Hospice, Paisley, **S McEndrick** Prince & Princess of Wales Hospice, Glasgow and **S McHugh** Glasgow Dental Hospital and School, University of Glasgow, Glasgow

Abstract: Oral fungal infections frequently develop in individuals with advanced cancer. This study examined the oral mycological flora of 207 patients receiving palliative care for advanced malignant disease. Demographic details and a clinical history were documented from each participant. A tongue swab was collected and cultured on CHROMAgar Candida® (CHROMAgar Paris, France). All yeasts were identified by germ tube test, API ID 32C profiles and, for *Candida dubliniensis*, by species-specific PCR. Susceptibility to fluconazole and itraconazole was determined by a broth microdilution assay according to the National Committee for Clinical Laboratory Standards (NCCLS). At time of sampling, 54 (26%) of the 207 subjects had clinical evidence of a fungal infection and yeasts were isolated from 139 (67%) individuals. In total, 194 yeasts were isolated, of which 95 (49%) were *Candida albicans*. There was a high prevalence of *Candidia glabrata* (47 isolates) of which 34 (72%) were resistant to both fluconazole and itraconazole. All nine isolates of *C. dubliniensis* recovered were susceptible to both azoles. No relationship was established between anti-fungal usage in the preceding three months and the presence of azole resistant yeasts. This study of patients with advanced cancer has demonstrated a high incidence of oral colonization with non-*C. albicans* yeasts, many of which had reduced susceptibility to fluconazole and itraconazole. The role of improved oral care regimes and novel anti-fungal drugs merits further attention, to reduce the occurrence of fungal infection in these patients. *Palliative Medicine* 2003; **17:** 477–481

Key words: azole anti-fungals; *Candida* spp; oral health; palliative care

Introduction

Oral fungal infection is a frequent finding among patients receiving palliative care for advanced cancer.[1–4] Infection is likely to be due in part to the immunocompromised state of such patients, often in conjunction with xerostomia, another well recognized oral complication in hospice patients.[5,6] Prompt and effective treatment of such infections is an important element of palliative medicine.

In view of the recurrent nature of oral fungal infections in patients with advanced cancer, many sufferers will receive repeated courses of anti-fungal medication. In other clinical settings, it has been suggested that prolonged or repeated exposure to fluconazole may be associated with the emergence of fluconazole resistance among strains of *Candida albicans*.[7,8] In addition, there is the potential for selection of non-*C. albicans* species of yeast,[9] which may have implications for treatment, since many of the non-*C. albicans* yeasts, such as *Candida glabrata*[10] and *Candida krusei*,[11] are inherently less susceptible than *C. albicans* to fluconazole. More recently, a new species of yeast named *Candida dubliniensis* has been reported.[12] This yeast was first described at a high prevalence among patients infected with HIV[13] and it was postulated that *C. dubliniensis* may have been selected for in this population through the extensive use of azole anti-fungals.

Previous studies have suggested a diverse oral mycological flora in hospice patients, with a significant proportion of non-*C. albicans* yeasts,[6,14] a result confirmed recently in a study of oral yeast carriage among 120 patients with advanced cancer.[15] A large scale, detailed study which has determined susceptibility of oral isolates to systemic anti-fungal drugs would not appear to have been reported, though data in press from another group has shown that 28% of a collection of 86

Address for correspondence: Professor J Bagg, Level 9, Glasgow Dental Hospital & School, 378 Sauchiehall Street, Glasgow G2 3JZ, UK.
E-mail: j.bagg@dental.gla.ac.uk

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yeasts isolated from the mouths of patients with advanced cancer were resistant to one or more azole anti-fungal drugs.[16] Clearly this type of information is of value in determining appropriate anti-fungal drug regimes for patients receiving palliative care.

In the light of these concerns, the aim of the present study was to examine in detail the oral mycological flora in a large series of patients with advanced cancer, receiving care in three separate hospices. The susceptibility of the yeast isolates to fluconazole and itraconazole would also be determined by use of standardized *in vitro* anti-fungal susceptibility testing.

Methods

Ethical approval for the study was gained in each of the three centres from the relevant local research ethics committees.

Patients

In total, 207 patients receiving hospice care were enrolled. The patients were attending one of three hospices: the ACCORD Hospice, Paisley, the Prince & Princess of Wales Hospice, Glasgow and the Holme Tower Hospice, Penarth, South Wales, UK. All patients gave either informed written consent or witnessed verbal consent prior to participation.

Clinical examination

Demographic details were collected for each patient, including age, sex and nature of the primary tumour. Where possible, information on anti-fungal therapy over the previous three months was elicited, usually from the patients' general medical practitioners. The oral cavity was examined by a dentally qualified author, experienced in oral medicine, (JB, MPS or MAOL) for clinical evidence of fungal infection.

Sample collection

A swab (Transwabs®, Medical Wire & Equipment Co (Bath) Ltd, Corsham, Wiltshire, UK) was used to collect a sample from the dorsal surface of the tongue of each patient. The swabs were transported to the local laboratory for processing within three hours of collection.

Laboratory processing of specimens

Swabs were inoculated directly onto CHROMAgar Candida® (CHROMAgar Paris, France) and incubated for 48 hours at 37°C. A semi-quantitative assessment of growth was made, in which confluent growth was defined as 'heavy', semi-confluent growth as 'moderate' and scattered colonies as 'light' growth. Colonies of each morphological type present on primary isolation plates were purified by subculture on Sabouraud's agar plates (Bioconnections, Leeds, UK). Small, dark green colonies on primary isolation plates were considered as possible isolates of *C. dubliniensis*[17] and four separate colonies from each plate were subcultured for subsequent analysis.

For identification purposes, germ tube tests were performed on all isolates. In addition, API ID 32C (bio-Mérieux S A, Marcy-l'Etoile, France) profiles were determined for all isolates, whether germ tube-positive or germ tube-negative. All germ tube-positive isolates were screened by PCR using *C. dubliniensis* – specific and fungal universal primers, as described by Donnelly and coworkers.[18] This procedure allowed *C. dubliniensis* isolates to be definitively identified and differentiated from *C. albicans* isolates.

Susceptibility to fluconazole and itraconazole was determined for all isolates by means of a broth microdilution assay.[19] For fluconazole, isolates with an MIC of ≤ 8 vg/mL were classified as susceptible, those with an MIC of 16–32 vg/mL were susceptible-dose dependent (S-DD) and those with an MIC of ≥ 64 vg/mL were reported as resistant. For itraconazole, isolates with an MIC of ≤ 0.125 vg/mL were classified as susceptible, those with an MIC of 0.25–0.5 vg/mL were S-DD and those with an MIC of ≥ 1 vg/mL were reported as resistant. In all cases, if results fell between categories, then the next higher category was implied.[19]

Statistical analysis

Data were entered into a Microsoft Access database and were subsequently analysed using Minitab version 11 (Minitab Inc., Pennsylivania, USA). Primary analysis of categorical data was carried out using descriptive statistics and cross tabulation. Potential differences between groups were investigated using the Chi-square test of association.

Results

Patients

Of the 207 patients included in the study, 92 (45%) were male. The age range of the patients was 25 years to 99 years (mean 67.9 years). The types of tumour present in the patient population are summarized in Table 1.

A total of 102 (50%) of the patients were edentulous, 52 (25%) were partially dentate and the remaining 53 (25%) were dentate. Of the 154 edentulous or partially dentate patients, 124 (81%) wore dentures. Ninety-seven (48%) of the patients had clinical evidence of xerostomia. At the time of sampling, 54 (26%) of the patients had clinical evidence of an oral fungal infection. Twenty-six had erythematous candidosis, eight had pseudomembranous candidosis, 10 had angular cheilitis and 10 had combinations of these forms of infection. A significantly higher proportion of patients with clinical evidence of a

Table 1 Nature of the primary tumour in 207 hospice patients

Primary tumour	Number (%)
Lung, trachea and bronchus	38 (18)
Breast	33 (16)
Prostate	15 (7)
Unknown primary	14 (7)
Colon	13 (6)
Lymphoid, haemopoietic and related tissue	11 (5)
Oral	10 (5)
Pancreas	9 (4)
Oesophagus	9 (4)
Stomach	8 (4)
Ovary	7 (3)
Other	40 (19)

fungal infection yielded growth of a least one species of yeast, compared with those who did not have a fungal infection ($P < 0.01$). There was no evidence for an association between denture wearing and fungal infection ($P = 0.15$).

Data on anti-fungal therapy during the previous three months were available for 206 patients. Forty-six (22%) had received anti-fungal treatment in the preceding month, nine with systemic, 34 with topical and three with both topical and systemic agents.

Yeast isolates

Yeasts were isolated from the mouth of 139 (67%) of the patients studied. Of these patients, 91 (65%) were colonized with one yeast species, 41 (30%) with two yeast species and seven (5%) with three yeast species, resulting in a total of 194 clinical isolates (Table 2). The density of fungal growth was recorded as light for 43 (31%) patients, moderate for 30 (22%) and heavy for 66 (47%). Of the 54 patients with clinical evidence of a fungal infection, 47 (87%) yielded growth of a yeast. Of these 47 patients, 37 (79%) were colonized with *C. albicans* and the remaining 10 patients with non-*C. albicans* yeasts.

Table 2 Identity of 194 yeast species isolated from the mouths of 207 hospice patients

Species	Number (%)
Candida albicans	95 (49)
Candida glabrata	47 (24)
Candida tropicalis	18 (9)
Saccharomyces cerevisiae	17 (9)
Candida dubliniensis	9 (5)
Candida parapsilosis	3 (2)
Candida guillermondii	2 (1)
Candida lusitaniae	2 (1)
Candida krusei	1 (<1)

The data in this table relate to isolates from the 139 patients from whom oral yeasts were recovered

Fluconazole and itraconazole susceptibilities

In vitro susceptibility to fluconazole and itraconazole was determined for all isolates. For fluconazole, 137 (71%) were susceptible, 13 (6%) were S-DD and 44 (23%) were resistant (Table 3). For itraconazole, 107 (55%) were susceptible, 41 (21%) were S-DD and 46 (24%) were resistant (Table 3). A high proportion of the isolates of *C. glabrata* and *Saccharomyces cerevisisae* recovered were found to be resistant to both drugs.

There was no evidence of a significant association between anti-fungal therapy in the previous three months and the presence of yeasts resistant to fluconazole ($P = 0.14$) or itraconazole ($P = 0.65$). However, there was evidence of significant associations between xerostomia and the presence of yeasts resistant to both fluconazole ($P = 0.01$) and itraconazole ($P = 0.02$). There was a slightly higher percentage of patients with evidence of fungal infection who had at least one yeast isolate resistant to the azole anti-fungals tested, but this was not statistically significant for either fluconazole ($P = 0.33$) or itraconazole ($P = 0.29$).

Discussion

Intraoral fungal infection continues to be a frequent finding among patients with advanced cancer. At the time of sampling, a quarter of the patients in the present study had clinical evidence of oral candidosis and yeasts were recovered from the mouth of 67% of the participants.

Resistance to fluconazole and itraconazole was rare in the isolates of *C. albicans* recovered. However, approximately half of the yeasts recovered from the cancer patients were strains of non-*C. albicans* species, a finding in keeping with previous work of this group[6] and similar to recent data reported by another group.[15]

The heterogeneity of yeast populations in these patients is well recognized. CHROMAgar Candida® is very effective for demonstrating heterogeneity between species, but heterogeneity within the same species should also be considered. This was taken into account for *C. dubliniensis*, an initial focus of this study. Thus, for isolates with a morphology suggestive of this species, four colonies were collected from the primary plates for anti-fungal susceptibility testing, all of which proved susceptible. Ideally, a selection of each morphological type, regardless of species, should be taken as a routine, though this is cumbersome in a clinical diagnostic setting. Although this procedure was not undertaken in the present study, a high prevalence of anti-fungal resistance was nevertheless detected among certain non-*C. albicans* strains.

A very significant finding in these studies has been the high level of isolation of *C. glabrata*. Whilst historically

Table 3 Fluconazole and itraconazole susceptibility of 194 yeasts isolated from the mouths of 207 hospice patients

Species	Number of isolates	Fluconazole susceptibility *			Itraconazole susceptibility *		
		Susceptible	Susceptible dose dependent	Resistant	Susceptible	Susceptible dose dependent	Resistant
Candida albicans	95	92 (97)	0	3 (3)	76 (80)	15 (16)	4 (4)
Candida glabrata	47	5 (11)	8 (17)	34 (72)	2 (4)	11 (24)	34 (72)
Candida tropicalis	18	16 (89)	0	2 (11)	13 (72)	3 (17)	2 (11)
Saccharomyces cerevisiae	17	8 (47)	5 (29)	4 (24)	3 (18)	8 (47)	6 (35)
Candida dubliniensis	9	9 (100)	0 (0)	0 (0)	9 (100)	0 (0)	0 (0)
Candida parapsilosis	3	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)
Candida guilliermondii	2	2 (100)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)
Candida lusitaniae	2	2 (100)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)
Candida krusei	1	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)

* Figures in parentheses are percentages

C. glabrata has been viewed as a comparatively non-pathogenic member of the normal flora there is now a recognition, following the increased use of immunosuppressive and anti-fungal agents, that this species is a relatively frequent cause of infection in immunocompromised individuals.[20] Compared with other *Candida* species, *C. glabrata* isolates have typically been associated with higher MICs to all azole drugs and are innately less susceptible to most anti-fungals.[20] In the present study, 72% of isolates of *C. glabrata* were resistant to both fluconazole and itraconazole. Clearly this reduced susceptibility raises important issues in relation to management of oral fungal infections for those with advanced cancer. Regular use of mycological culture for detection of such fungal infections would appear to be advisable, particularly in patients with florid oral candidosis which is refractory to standard treatment regimes.

C. dubliniensis[12] has been associated with oral candidosis in HIV-infected and AIDS patients. The ability of this yeast to rapidly develop resistance to fluconazole *in vitro*[21] has been suggested as a possible reason for the emergence of *C. dubliniensis* within HIV-infected individuals. In the light of the high prevalence of non-*C. albicans* yeasts already reported in those with advanced cancer,[6,14] and the working hypothesis that this may be due to previous exposure to fluconazole, it was clearly of interest to determine the presence of *C. dubliniensis* in this patient group. In view of the phenotypic similarities between *C. albicans* and *C. dubliniensis*, all germ tube-positive isolates were subjected to PCR screening using *C. dubliniensis*-specific primers to discriminate isolates *of C. dubliniensis* and *C. albicans*. The prevalence of *C. dubliniensis* was low (nine of 194 isolates), confirming the findings of another very recent study in which only 5% of 120 isolates were *C. dubliniensis*.[15] All of the clinical isolates of *C. dubliniensis* in the present study were susceptible to both fluconazole and itraconazole.

It has been suggested that use of fluconazole is associated with selection of *Candida* isolates that have a reduced susceptibility to fluconazole.[22] Among HIV-infected patients the number of episodes of candidosis and the duration of fluconazole therapy have been reported as independent predictors of fluconazole resistance.[23] In addition, it has been suggested that prolonged or repeated exposure to low dose fluconazole, as opposed to high dose therapeutic schedules, was associated with emergence and spread of *C. albicans* isolates with reduced susceptibility.[8] We were unable to show a significant association between use of anti-fungal drugs within the three months prior to this study and isolation of yeasts resistant to fluconazole or itraconazole. However, there are reasons why this may have been so. First, whilst strenuous efforts were made to gain accurate information from patients' medical practitioners on anti-fungal medication in the preceding three months, the quality of this retrospective information cannot be guaranteed. It is also possible that some of the patients had self-medicated with anti-fungal agents. Finally, in a recent study of the use of a single course of itraconazole for the treatment of denture stomatitis, we have shown long-term shifts in the oral mycological flora after a period of three years (submitted for publication). Therefore it is likely that the history of anti-fungal usage would need to be studied for a period much longer than three months to determine any association.

This study of patients with advanced cancer has revealed a high incidence of oral colonization with non-*C. albicans* yeasts, many strains of which had a reduced susceptibility to fluconazole and itraconazole. This finding mirrors closely the results of a recent study of oral and vaginal *Candida* isolates from women infected with, or at risk from, HIV.[9] If this shift does represent the result of multiple exposure to azole anti-fungal drugs, then it is possible that a reduction in the use of prolonged low dose or intermittent prescriptions may help to reverse the trend.[8] However, in a group of patients with the level of systemic illness studied here, oral fungal infections will continue to prove a clinical problem. Improvements in

oral care and the development of more effective treatment regimens for correction of xerostomia are also likely to be important factors that would assist this problem. This approach is reinforced by the finding in this study of a significant relationship between xerostomia and the presence of azole resistant yeasts. The potential role of novel anti-fungal agents, such as eberconazole and voriconazole,[24] also merits future attention.

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Differentiation of *Candida dubliniensis* from *Candida albicans* on Staib Agar and Caffeic Acid-Ferric Citrate Agar

ASMAA AL MOSAID,[1] DEREK SULLIVAN,[1] IRA F. SALKIN,[2] DIARMUID SHANLEY,[1] AND DAVID C. COLEMAN[1]*

Microbiology Research Unit, Department of Oral Medicine and Oral Pathology, School of Dental Science and Dublin Dental Hospital, Trinity College, University of Dublin, Dublin 2, Republic of Ireland,[1] and Wadsworth Center, New York State Department of Health, Albany, New York[2]

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The methods currently available for the identification of the pathogenic yeast *Candida dubliniensis* all have disadvantages in that they are time-consuming, expensive, and/or, in some cases, unreliable. In a recent study (P. Staib and J. Morschhäuser, Mycoses 42:521–524; 1999) of 14 *C. dubliniensis* and 11 *C. albicans* isolates, it was suggested that the ability of *C. dubliniensis* to produce rough colonies and chlamydospores (chlamydoconidia) on Staib agar (SA) provided a simple means of differentiating it from its close relative *C. albicans*. In the present investigation, we examined the colony morphology and chlamydospore production of 130 *C. dubliniensis* and 166 *C. albicans* isolates on SA and on the related defined medium caffeic acid-ferric citrate agar (CAF). All of the *C. dubliniensis* and *C. albicans* isolates produced chlamydospores on the control medium, i.e., rice-agar-Tween agar. However, while none of the *C. albicans* isolates produced chlamydospores on either SA or CAF, 85.4 and 83.8% of the *C. dubliniensis* isolates produced chlamydospores on SA and CAF, respectively. All of the *C. albicans* isolates grew as smooth, shiny colonies on SA after 48 to 72 h of incubation at 30°C, while 97.7% of the *C. dubliniensis* isolates grew as rough colonies, many (65%) with a hyphal fringe. In contrast, 87.4% of the *C. albicans* and 93.8% of the *C. dubliniensis* isolates yielded rough colonies on CAF. Although the results of this study confirm that SA is a good medium for distinguishing between *C. dubliniensis* and *C. albicans*, we believe that discrimination between these two species is best achieved on the basis of colony morphology rather than chlamydospore production.

Due to the increasing incidence of fungal infections and the recent emergence of novel opportunistic fungal pathogens, there is a growing need for the development of simple, rapid, and accurate identification methods for potential fungal pathogens recovered in the clinical microbiology laboratory (18). This is particularly true of the newly described yeast species *Candida dubliniensis*. Although first associated with oral candidiasis in human immunodeficiency virus (HIV)-infected patients (25), it has more recently been recognized as a cause of superficial and systemic disease in HIV-negative individuals (3, 11, 16, 17, 24, 26). The close genotypic relationship between *C. dubliniensis* and *C. albicans* results in their sharing a broad range of phenotypic characteristics, which hampers the accurate and rapid differentiation of the two species (23). Although the majority of *C. dubliniensis* isolates are susceptible to currently used antifungal drugs, it has been shown that isolates of this species, unlike *C. albicans*, can rapidly develop stable resistance to fluconazole upon exposure in vitro (12, 13). This ability, the emergence of *C. dubliniensis* worldwide, its growing importance as a cause of systemic disease, and the introduction of novel antifungal agents all indicate that a thorough investigation of the incidence and epidemiology of *C. dubliniensis* is required. In order to be able to achieve this, simple and reliable tests for differentiating *C. dubliniensis* from *C. albicans* need to be developed. The "gold standard" methods for the identification of *C. albicans* are based on its ability to produce germ tubes and chlamydospores (chlamydoconidia) on appropriate nutrient media. However, since *C. dubliniensis* also produces these structures, many isolates of *C. dubliniensis* have been misidentified as *C. albicans* (4, 14, 15). Therefore, in order to perform urgently required epidemiological studies of *C. dubliniensis* infections, there is a need to develop inexpensive, accurate, and easy to perform tests that will allow the differentiation of isolates of the two species which have been recovered from clinical samples. In this regard, a variety of procedures have been developed and assessed in laboratories around the world, including, among others, colony color on CHROMagar Candida medium (19), lack of growth at 45°C (16), immunofluorescence (1), carbohydrate assimilation profiles (15), β-glucosidase activity (2), coaggregation with *Fusobacterium nucleatum* (8), and PCR tests (5). Some of these tests (e.g., PCR) are very reliable but are not yet used routinely by many clinical microbiology laboratories, while others rely on reagents which are not widely available (e.g., immunofluorescence with anti-*C. dubliniensis* antibodies) and yet others (e.g., colony color on CHROMagar Candida medium) are unreliable. In the original description of *C. dubliniensis*, it was noted that this species produces much higher numbers of chlamydospores than *C. albicans* when grown on rice-agar-Tween agar (RAT; 25) but subsequent studies have shown that this trait does not provide a definitive means of differentiating between the two species (9). In a recent study of 14 *C. dubliniensis* and 11 *C. albicans* strains, Staib and Morschhäuser (21) reported that colony morphology and chlamydospore production by

* Corresponding author. Mailing address: University of Dublin, Microbiology Research Unit, Department of Oral Medicine and Oral Pathology, School of Dental Science, Trinity College, Dublin 2, Republic of Ireland. Phone: 353 1 6127276. Fax: 353 1 6127295. E-mail: dcoleman@dental.tcd.ie.

TABLE 1. *C. dubliniensis* and *C. albicans* isolates used in this study

Yeast species and country of isolation	No. of isolates	Chlamydospore production (no. of isolates)						Source or reference
		SA		CAF		RAT		
		+	−	+	−	+	−	
C. dubliniensis[a]								
Argentina	1	1	0	1	0	1	0	24
Australia	1	0	1	0	1	1	0	25
Belgium	5	5	0	5	0	5	0	16
Canada	6	4	2	5	1	6	0	16
Finland	1	1	0	1	0	1	0	16
France	9	5	4	8	1	9	0	This study
Germany	4	4	0	3	1	4	0	16
Greece	1	1	0	1	0	1	0	16
India	1	1	0	1	0	1	0	This study
Israel	3	1	2	3	0	3	0	17
Ireland	41	36	5	36	5	41	0	16, 25, this study
Japan	1	1	0	1	0	1	0	This study
Malta	3	3	0	3	0	3	0	This study
Norway	5	4	1	0	5	5	0	This study
Spain	5	5	0	4	1	5	0	1
Switzerland	3	3	0	2	1	3	0	23
United Kingdom	18	16	2	16	2	18	0	16
United States	22	20	2	19	3	22	0	15, this study
Total	130	111	19	109	21	130	0	
C. albicans[a]								
Australia	1	0	1	0	1	1	0	16
Hong Kong	6	0	6	0	6	6	0	16
Ireland	61	0	61	0	61	61	0	16, this study
United Kingdom	2	0	2	0	2	2	0	16
United States	96	0	96	0	96	96	0	15
Total	166	0	166	0	166	166	0	

[a] *C. dubliniensis* type strain CD36 (25) produced abundant chlamydospores on all three media, whereas *C. albicans* reference strains 132A (serotype B) (6) and 179A (serotype A) (6) only produced chlamydospores on RAT.

C. dubliniensis on Staib agar (SA; a medium originally developed for the identification of *Cryptococcus neoformans*) could form the basis of a simple and accurate test for distinguishing this species from *C. albicans*.

In the present study, we evaluated the usefulness of colony morphology and chlamydospore production on SA and on caffeic acid-ferric citrate agar (CAF; a defined medium also developed to aid in the identification of *C. neoformans*) as a means of differentiating *C. albicans* from *C. dubliniensis* using a large collection of *C. dubliniensis* isolates recovered from individuals in 18 different countries around the world.

MATERIALS AND METHODS

Yeast isolates. The yeast isolates and reference strains used in this investigation are listed in Table 1. A total of 296 isolates were studied, including 130 *C. dubliniensis* isolates and 166 *C. albicans* isolates. All isolates were from the culture collection of the Microbiology Research Laboratory, Department of Oral Medicine and Oral Pathology, School of Dental Science, Trinity College, University of Dublin, Dublin, Republic of Ireland. Each isolate was originally recovered from one or more specimens from a separate individual, and its identity was confirmed using the API ID 32C (bioMérieux, Marcy l'Étoile, France) yeast identification system, growth at 45°C, and PCR analysis with *C. dubliniensis*-specific primers (5, 15, 16).

Chemicals, enzymes, and oligonucleotides. Analar-grade or molecular biology grade chemicals were purchased from Sigma-Aldrich or BDH (Poole, Dorset, United Kingdom). Enzymes were purchased from Roche Diagnostics Ltd. (Lewes, East Sussex, United Kingdom) or the Promega Corporation (Madison, Wis.) and used in accordance with the manufacturer's instructions. Custom-synthesized oligonucleotides were purchased from Sigma-Genosys Biotechnologies (Pampisford, Cambridge, United Kingdom).

Culture media and growth conditions. Stock cultures of yeast isolates were maintained on plastic beads in Protect cryovials (Technical Service Consultants Ltd., Lancashire, United Kingdom) at −80°C. For each isolate, two or three plastic beads were removed from their respective cryovials using a sterile plastic loop, allowed to thaw, and then used to inoculate potato dextrose agar (PDA; Oxoid) medium. Forty-eight-hour-old PDA medium cultures grown at 37°C were used as the source of inoculua for subsequent experiments with SA and CAF. SA (20) and CAF (7) were prepared fresh as described previously and used immediately. SA was prepared by first making an aqueous extract of *Guizotia abyssinica* seed (Power Seeds, Kildare, Republic of Ireland) by pulverizing 50 g of seed in a Moulinex B57 domestic blender for 2.5 min and then adding the ground seeds to 1 liter of distilled water, followed by boiling for 30 min. The seed extract was cooled and filtered, and the following ingredients were added: glucose, 1 g; KH_2PO_4, 1 g; creatinine, 1 g. The pH was adjusted to 5.5, the volume was readjusted to 1 liter, and 15 g of agar (Difco) was then added before autoclaving. The composition of CAF (per liter) was as follows: $NHSO_4$, 5 g; glucose, 5 g; yeast extract, 2 g; K_2HPO_4, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.7 g; caffeic acid, 0.18 g; chloramphenicol, 0.05 g; ferric citrate, 0.002 g.

A 48-h-old single colony from a PDA medium plate culture of each isolate to be tested was separately streak inoculated with a sterile wire loop onto SA and CAF, respectively, contained in 90-mm-diameter petri dishes (25 ml of agar per plate) and incubated at 30°C for 72 to 120 h. Gross colony morphologic features were examined visually on both media at 24-h intervals, and the data were recorded. Yeast colonies were also evaluated microscopically to detect the presence or absence of chlamydospore formation following 48 to 120 h of incubation on SA and on CAF. For each isolate, 10 well-separated single colonies were chosen at random and stained by the addition of 1 drop of 1% (wt/vol) lactophenol cotton blue stain (10) to enhance the detection of chlamydospores. Lactophenol cotton blue preferentially stains chlamydospores more intensely than suspensor cells, pseudomycelium, and blastospores (blastoconidia; 25). Colonies were allowed to stain for 5 min and then covered with sterile glass coverslips and examined microscopically under bright-field illumination using a ×40 objective. Plates containing isolates that did not exhibit detectable chlamydospore formation within 120 h were re-examined following further incubation at intervals of 24 h for up to 3 weeks in total. All isolates were also tested for chlamydospore formation on RAT (bioMérieux) as described previously (25).

All of the isolates included in this study were examined on SA and CFA on at least two separate occasions with different batches of medium prepared from different lots of reagents.

RESULTS AND DISCUSSION

Growth of *Candida* isolates on SA and CFA. All 296 yeast isolates grew on both SA and CAF and yielded grey-white colonies. On SA, all of the 166 *C. albicans* isolates tested produced smooth, shiny colonies after 48 h of incubation (Fig. 1a). In all but three isolates, the colonies were found upon microscopic examination to be composed only of blastospores. Colonies of the three isolates consisted mainly of blastospores with a few pseudohyphal elements after 48 and 72 h of incubation. Similar findings were observed after 96 and 120 h of incubation.

In contrast, 127 (97.7%) of the 130 *C. dubliniensis* isolates tested on SA yielded rough colonies, the majority (84; 64.6%) of which also exhibited a hyphal halo or fringe around the colonies visible to the naked eye after 72 h of incubation (Fig. 1b). The rough colonies were composed of mycelial forms (predominantly pseudohyphae) and blastospores. The three remaining *C. dubliniensis* isolates (two from Norway and one from Canada) produced smooth, shiny colonies on SA that were similar in appearance and composition to those of *C. albicans* isolates, even after 2 weeks of incubation. The identity of these isolates as *C. dubliniensis* was reconfirmed using PCR with *C. dubliniensis*-specific primers, by carbohydrate assimilation profile analysis with the API ID 32C system, and by absence of

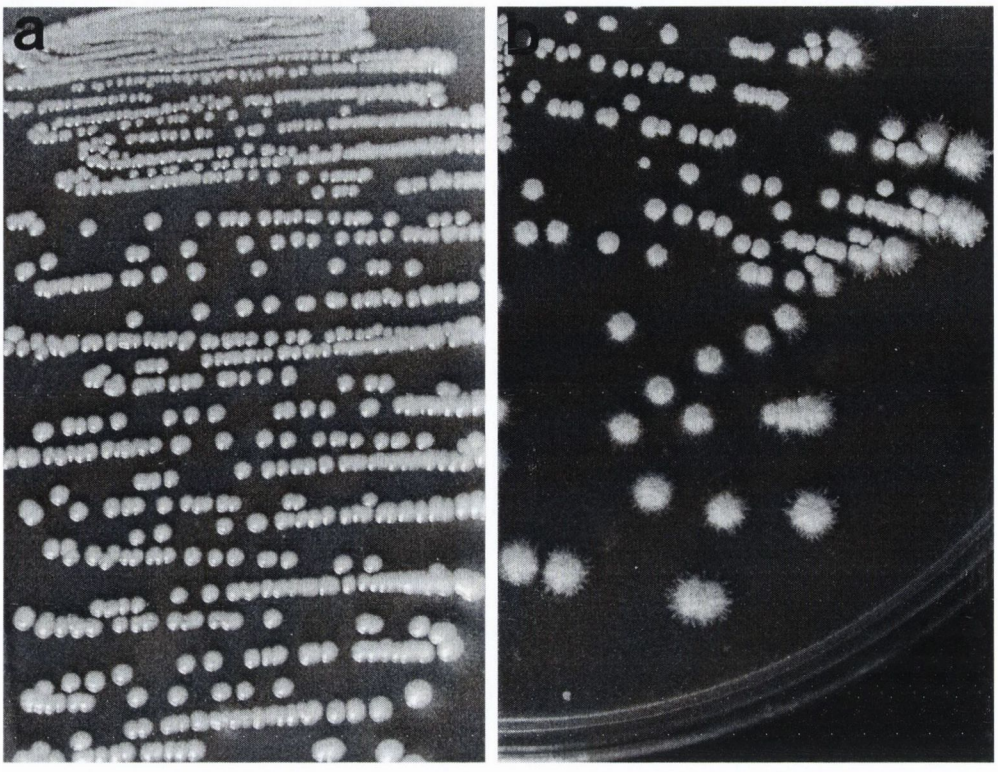

FIG. 1. Morphological appearance of *C. dubliniensis* and *C. albicans* colonies on SA following 72 h of incubation at 30°C. (a) Smooth, shiny colonies exhibited by *C. albicans* strain 132A (6). (b) Rough colonies exhibited by *C. dubliniensis* strain CD36 (25) displaying a hyphal fringe or halo.

growth at 45°C. Apart from these three isolates, the difference between the *C. dubliniensis* and *C. albicans* isolates was particularly evident on SA after 48 h of incubation, and became further enhanced after 72 h of incubation, in the area of heavy culture growth where the primary inoculum was streaked (i.e., the *C. dubliniensis* culture growth appeared rough and the *C. albicans* culture growth appeared smooth and shiny). These findings indicated that the different colony morphologies exhibited by isolates of *C. dubliniensis* and *C. albicans* were due predominantly to the production of mycelial forms by *C. dubliniensis* isolates. These results confirm the findings recently reported by Staib and Morschhäuser (21) that SA can be used as a useful means to discriminate between isolates of *C. dubliniensis* and *C. albicans*. However, the method is not absolute, as a small minority (3 [2.3%]) of the 130 *C. dubliniensis* isolates tested were indistinguishable from *C. albicans* based on colony morphology on SA.

Several isolates each of *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* were also tested on SA in order to determine whether they could be distinguished from isolates of *C. albicans* and *C. dubliniensis*. The five oral *C. glabrata* isolates tested yielded smooth, grey-white colonies similar to those of *C. albicans* on this medium. Furthermore, of the five *C. tropicalis* isolates tested, three yielded rough colonies similar to those of *C. dubliniensis* and two yielded smooth colonies similar to those of *C. albicans*. Of the six *C. parapsilosis* isolates tested, four yielded smooth, shiny colonies similar to those of *C. albicans* and two yielded rough colonies similar to those of *C. dubliniensis*. These findings indicated that it would not be possible to identify colonies of *C. albicans* and *C. dubliniensis* on SA based on colony morphology alone following primary isolation from a clinical specimen. Therefore, we propose that SA be used to assess clinical isolates which have been shown to be germ tube positive or to confirm the identity of *C. dubliniensis* isolates presumptively identified following primary isolation on CHROMagar Candida medium or by other means. The use of SA to differentiate *C. dubliniensis* and *C. albicans* isolates has a number of advantages over carbohydrate assimilation profile analysis. Firstly, the use of SA is considerably less expensive. Secondly, it is amenable to the analysis of large numbers of isolates. Finally, whereas the databases used with many of the commonly used commercial yeast identification systems (e.g., the bioMérieux API 20C AUX and ID 32C systems) have been updated in recent years to include *C. dubliniensis* profiles, they are far from comprehensive. In this regard, recent studies have highlighted the necessity to revise the databases to improve the accuracy of identification of *C. dubliniensis* (15).

SA was originally developed as a means of identifying colonies of *C. neoformans*, which, unlike other members of this genus, develops dark pigmentation on this medium. Strachan et al. (22) demonstrated that similar results could be obtained on a growth medium containing caffeic acid extracted from *G. abyssinia* seeds. Since SA was found to be excellent for distinguishing between *C. dubliniensis* and *C. albicans*, we investigated the usefulness of the more defined CAF to differentiate between isolates of these species. Of the 166 *C. albicans* isolates included in the study, 145 (87.4%) yielded rough colonies with a mycelial halo visible to the naked eye after 5 days

TABLE 2. Chlamydospore production by *C. dubliniensis* isolates[a] on SA and CAF

Medium and chlamydospore production	No. of isolates
SA	
+	111[c]
−	19[d]
CAF	
+	109[e]
−	21[f]
SA + CAF[b]	
+	83
−	3

[a] 130 isolates were studied.
[b] Refers to isolates that were chlamydospore positive on both SA and CAF or chlamydospore negative on both media.
[c] 28 isolates were negative on CAF.
[d] 16 isolates were positive on CAF.
[e] 26 isolates were negative on SA.
[f] 18 isolates were positive on SA.

incubation. The remaining 21 (12.6%) isolates yielded smooth, nonshiny colonies on this medium. In comparison, 122 (93.8%) of the 130 *C. dubliniensis* isolates, including the 3 isolates which exhibited a smooth-colony phenotype on SA, yielded rough colonies with a mycelial halo after 5 days of incubation. The remaining eight (6.2%) isolates yielded rough colonies without a mycelial halo. Colonies of the *C. dubliniensis* isolates were noticeably smaller (~2 mm in diameter) than the *C. albicans* colonies (~3 mm) after 5 days of incubation on CAF. These findings indicated that, unlike that on SA, colony morphology on CAF could not be used to differentiate between *C. dubliniensis* and *C. albicans* isolates.

Chlamydospore production on SA and CAF. None of the 166 *C. albicans* isolates tested produced chlamydospores on SA or CAF, even after prolonged incubation periods of up to 3 weeks (Table 1). In contrast, all isolates produced chlamydospores within 48 to 72 h on RAT. Similarly, all 130 of the *C. dubliniensis* isolates formed chlamydospores on RAT but not all produced chlamydospores on either SA or CAF (Tables 1 and 2). A total of 111 (85.4%) of the 130 *C. dubliniensis* isolates formed chlamydospores on SA, and 100 (90.1%) of these 111 produced abundant chlamydospores within 72 h, whereas 11 (9.9%) of them produced relatively few chlamydospores, which were only detected following incubation periods of up to a week. Similarly, 109 (83.8%) of the 130 *C. dubliniensis* isolates produced chlamydospores on CAF, all but 5 within 120 h (Tables 1 and 2). Eighty-three (63.9%) of the *C. dubliniensis* isolates produced chlamydospores on both SA and CAF, whereas the remainder produced chlamydospores on one or the other medium only (Table 2).

On the basis of these data, we agree with Staib and Morschhäuser that growth on SA is an efficient means of discriminating between *C. dubliniensis* and *C. albicans*. However, our results suggest that colony morphology, rather than chlamydospore formation, is a more accurate criterion for species identification, since a significant proportion of *C. dubliniensis* isolates failed to produce chlamydospores on SA. The disparity between our data and that of Staib and Morschhäuser could be due to either different supplies of *G. abyssinica* seed, to seed of different ages, or to seed storage conditions. However, the most likely explanation lies in the larger and more diverse group of *C. dubliniensis* isolates examined in the present study.

SA is inexpensive and readily available in many clinical mycology laboratories and provides a simple test for the accurate differentiation of *C. dubliniensis* from *C. albicans*. We propose that colony morphology on SA is a reliable and inexpensive phenotypic test for confirming the identification of *C. dubliniensis* and will be of benefit for researchers interested in studying the incidence and epidemiology of this emerging pathogen. We suggest that germ tube-positive oral isolates from HIV-positive and AIDS patients, as well as germ tube-positive isolates from sterile sites from other immunocompromised groups, should be tested on SA in association with other phenotypic tests for *C. dubliniensis*.

The molecular basis of the phenotypic differences observed between *C. dubliniensis* and *C. albicans* following growth on these media has yet to be established. However, comparative analysis of gene expression on these media could prove helpful in increasing our understanding of dimorphism in these species.

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