

# Azole susceptibility and resistance in *Candida dubliniensis*

E. Pinjon<sup>1</sup>, G.P. Moran, D.C. Coleman and D.J. Sullivan

Microbiology Research Unit, Division of Oral Biosciences, School of Dental Science and Dublin Dental Hospital, University of Dublin, Trinity College, Dublin 2, Republic of Ireland

## Abstract

*Candida dubliniensis* is a recently described species of pathogenic yeast that shares many phenotypic features with *Candida albicans*. It is primarily associated with oral colonization and infection in HIV-infected individuals. Isolates of *C. dubliniensis* are generally susceptible to commonly used azole antifungal agents; however, resistance has been observed in clinical isolates and can be induced by *in vitro* exposure. Molecular mechanisms of azole resistance in *C. dubliniensis* include increased drug efflux, modifications of the target enzyme and alterations in the ergosterol biosynthetic pathway.

## Introduction

Several species of the genus *Candida* form part of the normal oropharyngeal and gastrointestinal flora; however, they can become opportunistic pathogens and establish infections when host defences are impaired. Such infections range from superficial infections of the skin and mucous membranes to life-threatening invasive infections of the blood and/or organs. Having emerged as significant human pathogens during the past 2 decades, *Candida* species are frequently quoted as the fourth most common cause of nosocomial bloodstream infection in the U.S.A., with an associated mortality rate of 40–60%. The emergence of *Candida* species as significant human pathogens has been part of a general increase in the number of infections caused by fungal pathogens during the last 20 years. This shift in the epidemiology of fungal infections has been correlated with the increase in the number of immunocompromised and immunosuppressed patients, as well as with an increase in the use of invasive medical procedures, in-dwelling central venous catheters and broad-spectrum antibiotics. *Candida albicans* is the most pathogenic *Candida* species and is the most common cause of *Candida* infections. However, over the last 2 decades, since the introduction and widespread use of the azole drugs fluconazole and itraconazole, other *Candida* species have emerged as significant pathogens of clinical importance [1]. This review focuses on one of these species, *Candida dubliniensis*, with particular emphasis on its resistance mechanisms to azole antifungal agents.

## A recently described species: *Candida dubliniensis*

In the early 1990s, several authors reported the recovery of atypical isolates of *C. albicans* from HIV-infected individuals [2,3]. Although these isolates shared many features characteristic of *C. albicans*, they exhibited a number of atypical properties. These atypical isolates possessed the ability to form germ tubes and abundant chlamydospores [4]. However, they grew poorly or not at all at 42°C, unlike *C. albicans* isolates which grow well at this temperature. An extensive study of the phenotypic and genotypic characteristics of atypical isolates recovered from the oral cavities of HIV-infected patients in Ireland and Australia, together with a phylogenetic analysis of nucleotide sequences of the V3 region of the large rRNA subunit gene, demonstrated that the atypical isolates constituted a novel distinct taxon within the genus *Candida* for which the name *C. dubliniensis* was proposed [4].

## Epidemiology of *C. dubliniensis*

Since its identification in 1995, numerous studies have reported the recovery of *C. dubliniensis* isolates from a wide range of anatomical sites and clinical settings all over the world [5–8]. Despite its phenotypic similarities with *C. albicans*, *C. dubliniensis* appears to be a rare constituent of the normal oral and vaginal microbial flora. In a study of an Irish population, only 3.5% of normal healthy individuals were found to carry *C. dubliniensis* in the oral cavity [9]. However, *C. dubliniensis* is commonly associated with oropharyngeal candidosis in HIV-infected and AIDS patients [10,11]. A high prevalence (15–30%) of *C. dubliniensis* in the oral cavities of HIV-infected and AIDS patients has been reported in several studies [9,11,12]. In a study on the prevalence of *C. dubliniensis* in the oral cavity in an Irish population, 26% of HIV-infected and 32% of AIDS patients

**Key words:** azole, *Candida dubliniensis*, epidemiology, fluconazole, susceptibility, resistance mechanism.

**Abbreviations used:** Cd, *Candida dubliniensis*; Cdr1p, *Candida* drug resistance protein 1; Mdr1p, multidrug resistance protein 1; MIC, minimum inhibitory concentration.

<sup>1</sup>To whom correspondence should be addressed (email emmanuelle.pinjon@dental.tcd.ie).

with symptoms of oral candidosis harboured *C. dubliniensis*, while, in asymptomatic patients, the levels were 18% and 25% respectively [9]. However, some studies have reported a significantly lower prevalence in HIV-infected and AIDS patients [13,14], although the reasons for this disparity are unclear. A relatively high prevalence of *C. dubliniensis* in the oral cavities of patients with denture stomatitis [9], diabetes [15] and cystic fibrosis [16] has also been reported.

While *C. dubliniensis* is most frequently isolated from the oral cavity, it has also been recovered from faecal, sputum, vaginal, urine and wound samples [17–22]. *C. dubliniensis* isolates have also been recovered from the blood of patients with invasive candidosis [23], particularly those with neutropenia following bone marrow or solid organ transplantation [19,24–26]. However, the incidence of *C. dubliniensis* in systemic infections is low, accounting for up to 2% of cases of candidaemia in the U.K. [18] and in the U.S.A. [25], which contrasts with the 65% of candidaemia cases accounted for by *C. albicans*. This disparity could be explained by the higher prevalence of *C. albicans* in the normal flora, but it also suggests that *C. dubliniensis* is less pathogenic than *C. albicans*.

The reasons for the emergence of *C. dubliniensis* during the last 15 years are not clear. It has been suggested that, since *C. dubliniensis* was initially isolated from the oral cavities of HIV-infected patients with recurrent oral candidosis receiving azole antifungal drug therapy, the emergence of this species may have been due to positive selection as a result of the introduction of fluconazole for the prophylaxis and treatment of oral candidosis in the early 1990s.

### Azole susceptibility in *C. dubliniensis*

Azole derivatives target the synthesis of ergosterol, the predominant sterol of the fungal cell membrane, and are currently the most widely used class of antifungal agents in the treatment of *Candida* infections. The vast majority of *C. dubliniensis* clinical isolates are susceptible to azole antifungal drugs. In a recent study, 94.6% of the 111 *C. dubliniensis* isolates tested were found to be susceptible to fluconazole [MIC (minimum inhibitory concentration) range 0.125–4 µg/ml], while 89.6% of 58 isolates were found to be susceptible to itraconazole (MIC range 0.03–0.125 µg/ml) [27]. Other recent studies reported similar findings [28,29].

While most isolates of *C. dubliniensis* are susceptible to fluconazole, a number of isolates exhibiting either decreased susceptibility ( $8 \mu\text{g/ml} \leq \text{MIC} \leq 32 \mu\text{g/ml}$ ) or resistance ( $\text{MIC} \geq 64 \mu\text{g/ml}$ ) to fluconazole have been described [12,20,21,28–32]. These isolates were mostly recovered from HIV-infected patients receiving fluconazole therapy. Furthermore, Moran et al. [30] showed that fluconazole-resistant derivatives could be generated from susceptible isolates following sequential exposure to fluconazole *in vitro*, thus indicating that *C. dubliniensis* has the ability to rapidly develop resistance to this drug. *In vitro* exposure to fluconazole has also been shown to result in increased adherence of *C. dubliniensis* to epithelial cells which correlated with

increased proteinase secretion levels, whereas the adherence of *C. albicans* was decreased under the same conditions [33]. It is thus possible that treatment with fluconazole could provide a selective advantage favouring the growth of *C. dubliniensis* over *C. albicans* isolates in the oral cavity. This could explain the high recovery rate of *C. dubliniensis* isolates from HIV-infected patients receiving fluconazole treatment. In addition, Odds et al. [20] have shown that the geometric mean MICs of *C. dubliniensis* for azole drugs are significantly higher than those of *C. albicans*.

Oral populations of *Candida* are dynamic and, in a longitudinal study, Martinez et al. [34] described the replacement of *C. albicans* with *C. dubliniensis* in HIV-infected patients with oral candidosis treated with fluconazole. The switch to *C. dubliniensis* occurred in patients initially infected with *C. albicans* strains that failed to develop fluconazole resistance. This is the only study so far to have provided evidence that *C. dubliniensis* can replace *C. albicans* following azole therapy and, although the antifungal pressure owing to prolonged fluconazole treatment may have played a role in the selection of the *C. dubliniensis* isolates, other factors may have been involved as the majority of *C. dubliniensis* isolates recovered at the end of the study did not exhibit decreased susceptibility to azoles [34].

Resistance to itraconazole has been described in clinical isolates of *C. dubliniensis* [21], and it can be induced by *in vitro* exposure to the drug [27].

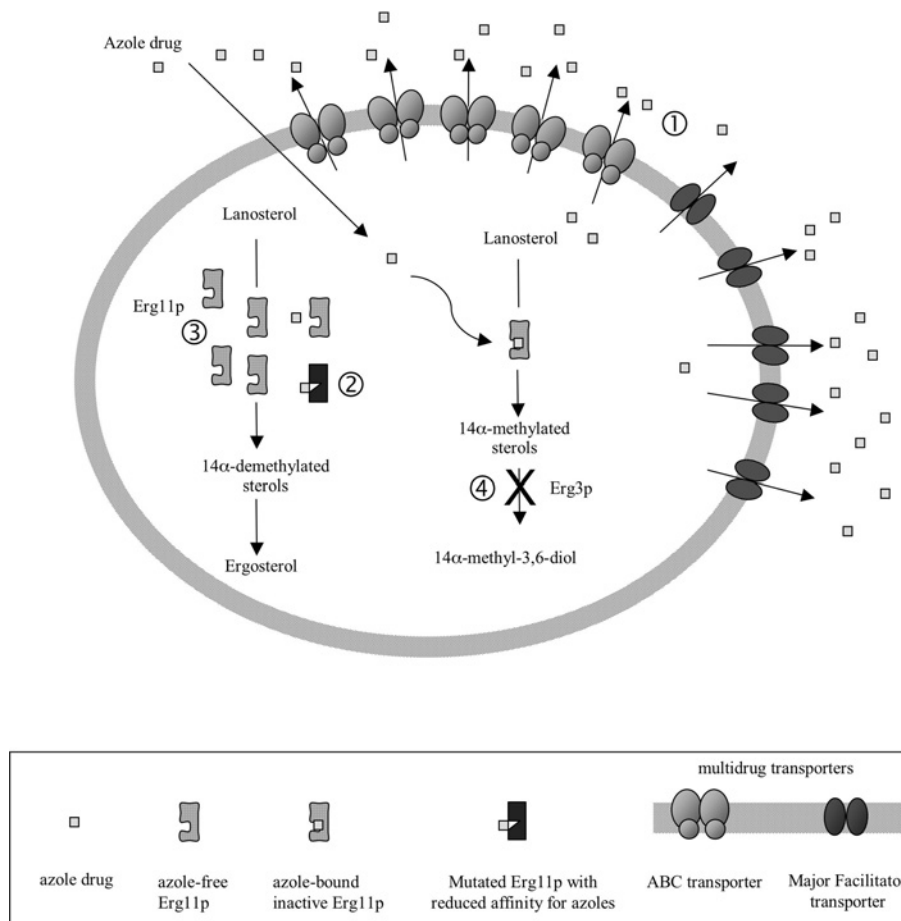
### Mechanisms of azole resistance in *C. dubliniensis*

Azole drugs target an enzyme of the ergosterol biosynthetic pathway known as lanosterol 14 $\alpha$ -demethylase, which is encoded by the *ERG11* gene. Exposure of fungal cells to azoles causes depletion of ergosterol and accumulation of 14 $\alpha$ -methylated sterols, such as lanosterol and 14 $\alpha$ -methyl-3,6-diol, which disrupt the structure of the membrane, alter its fluidity and the activity of membrane-bound enzymes. Several molecular mechanisms by which *Candida* cells can develop resistance to azole antifungal agents have been described: cells can fail to accumulate these agents owing to increased drug efflux, mutations can alter the affinity of the target enzyme for these agents, the cellular content of the target enzyme can be elevated, and other enzymes of the ergosterol biosynthetic pathway, such as the sterol C5,6-desaturase, can be inactivated by mutation (Figure 1).

Failure to accumulate azole antifungals has been shown to be a major factor involved in azole resistance in clinical *C. albicans* isolates, and numerous studies have reported the association of azole drug resistance with the up-regulation of genes encoding multidrug efflux transporters [35–37]. Two types of efflux transporters have been shown to contribute to azole resistance in *Candida*: the ABC (ATP-binding cassette) transporters Cdr1p (*Candida* drug resistance protein 1) and Cdr2p, encoded by the *CDR1* and *CDR2* genes respectively, and the major facilitator protein Mdr1p (multidrug resistance protein 1), encoded by the *MDR1* gene. While Cdr1p

**Figure 1** | Schematic representation of resistance mechanisms to azole antifungal agents in *Candida*

- ① Decreased accumulation of drug due to up-regulation of ABC (ATP-binding cassette) and major facilitator multidrug transporter genes. ② Decreased affinity to azoles of the target enzyme Erg11p. ③ Increased cellular content of Erg11p. ④ Alteration of the ergosterol biosynthetic pathway by inactivation of Erg3p (sterol C5,6-desaturase).



and Cdr2p can transport a broad range of azole drugs, including itraconazole and ketoconazole, Mdr1p can only transport fluconazole. Homologues of the genes encoding the *C. albicans* multidrug transporters have been identified in *C. dubliniensis* (termed *CdMDR1*, *CdCDR1* and *CdCDR2* respectively), and their up-regulation has been associated with azole resistance [31]. Overexpression of *CdMDR1* has been shown to be involved in mediating a reduced accumulation of drug in fluconazole-resistant clinical isolates and *in-vitro*-generated derivatives [38]. Furthermore, the role of *CdMDR1* in fluconazole resistance has been confirmed by the disruption of both alleles of the gene in a fluconazole-resistant clinical isolate overexpressing *CdMDR1* [39].

Up-regulation of *CdCDR1* has been observed in fluconazole-resistant clinical isolates of *C. dubliniensis* and *in-vitro*-generated derivatives [31,38]. However, Moran et al. [40] have shown that, while CdCdr1p is important for mediating reduced susceptibility to itraconazole and ketoconazole, it is not required for fluconazole resistance in isolates that exhibit increased *CdMDR1* expression. In contrast, in

*C. albicans*, resistance to fluconazole is mainly associated with overexpression of *CDR1*. In a study that investigated the reasons for the differential regulation of *CDR1* expression in *C. albicans* and *C. dubliniensis*, Moran et al. [40] reported the high prevalence amongst *C. dubliniensis* isolates of a nonsense mutation in the *CdCDR1* gene. *CdCDR1* genes that harbour the nonsense mutation encode a non-functional CdCdr1p protein and correction of the mutation by site-directed mutagenesis has been shown to restore function [40]. All isolates that harbour the nonsense mutation belong to *C. dubliniensis* genotype 1, a group of very closely related isolates that have mainly been recovered from HIV-infected individuals, many of whom have received fluconazole treatment [17]. In these isolates, cross-resistance to other azole drugs has not been described, and this observation is consistent with up-regulation of *CdMDR1* as the primary mechanism of fluconazole resistance, since this transporter can only transport fluconazole and not any other azoles. In contrast, in a recent study of genotype 3 *C. dubliniensis* isolates, decreased susceptibility to fluconazole was associated

with up-regulation of *CdCDR1* and *CdCDR2*, while expression of *CdMDR1* was not detected [32]. In these isolates, decreased susceptibility to fluconazole was associated with decreased susceptibility to other azoles. While this was the first report of *CdCDR2* overexpression in clinical isolates of *C. dubliniensis*, the exact contribution of *CdCDR2* up-regulation to the phenotype of decreased azole susceptibility is unclear because of the concomitant up-regulation of *CdCDR1* [32].

In *C. albicans*, mutations that affect the affinity of the target enzyme Erg11p for azole antifungal drugs have been well documented as a drug-resistance mechanism [41–46]. In *C. dubliniensis*, Perea et al. [31] have described mutations in the *CdERG11* gene which were associated with fluconazole resistance. Two of the mutations described by Perea et al. [31] (F126L and G464S) are identical with mutations that have been shown previously to be involved in fluconazole resistance in *C. albicans*. The other mutations identified by Perea et al. [31] have not yet been conclusively shown to be involved in azole resistance.

Although up-regulation of *ERG11* has been associated with azole resistance in several clinical isolates of *C. albicans* [47,48], the contribution of *ERG11* overexpression to azole resistance has been difficult to assess because it has always been found in combination with other alterations that are associated with azole resistance, such as decreased accumulation of drug or the presence of mutations in Erg11p. Similarly, in *C. dubliniensis*, up-regulation of *CdERG11* has been observed, but always in conjunction with other resistance mechanisms, and thus the relevance of increased levels of the target enzyme in mediating azole resistance in *C. dubliniensis* is not known [27,31].

In *C. albicans*, resistance to azole drugs has also been associated with modifications of the ergosterol biosynthetic pathway, such as defects in the sterol C5,6-desaturation step that avoid the accumulation of the toxic 14 $\alpha$ -methyl-3,6-diol metabolite and circumvent azole-mediated growth arrest [49,50]. Loss of function mutations in the *C. dubliniensis* homologue of the gene encoding this enzyme (*CdERG3*) have been shown to be associated with the development of azole resistance in *C. dubliniensis* following sequential exposure to itraconazole *in vitro* [27]. Although increased expression of *CdCDR1* and *CdERG11* was also observed in the azole-resistant derivatives generated by itraconazole exposure, this was thought to be a consequence of alterations in membrane composition owing to the defective C5,6-desaturation step of the ergosterol biosynthetic pathway, rather than the cause of azole resistance [27]. Thus the study highlighted the fact that, if not thoroughly investigated, azole resistance can be wrongly attributed to the apparent overexpression of multidrug resistance genes and the target enzyme. Sterol C5,6-desaturase loss-of-function mutations provide a mechanism which is often overlooked and its contribution to clinical azole resistance remains to be investigated further, not only in *C. dubliniensis*, but in all *Candida* species.

In *C. albicans*, azole resistance is often multifactorial, sometimes involving the up-regulation of more than one

multidrug transporter in conjunction with point mutations in Erg11p and up-regulation of the *ERG11* gene [47,48]. Similarly, in *C. dubliniensis*, the development of azole resistance appears to be a complex phenomenon that can involve multiple molecular mechanisms working in combination [31].

In summary, the molecular mechanisms of azole resistance in *C. dubliniensis* are similar to those previously described in *C. albicans*, although genotype-specific combinations of mechanisms have been described.

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