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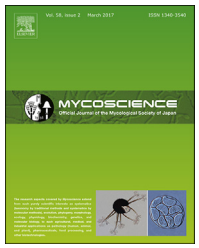

Short communication

ERG11 mutations are associated with high-level azole resistance in clinical *Candida tropicalis* isolates, a Singapore study

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ABSTRACT

Candida tropicalis is a commonly isolated non-albicans *Candida* in Asia. In this clinical microbiology laboratory study, a total of 1579 *C. tropicalis* were isolated during 2009–2014 and of these 348 isolates were tested for azole susceptibility. We show that the current rates of fluconazole resistance in *C. tropicalis* increased from 2.9% in 2009–2011 to 9% in 2012–2014 ($P = 0.03$). High fluconazole minimum inhibitory concentrations (MICs) were strongly associated with the presence of Y132F and S154F mutations in Erg11p. No amino acid changes were observed in Erg3p or Erg11p in sensitive isolates. This suggests that Y132F and S154F mutations were responsible for azole resistance. Multilocus sequence typing (MLST) based phylogenetic analysis performed for azole-resistant isolates suggested possible clonal clustering of antifungal-resistance.

Candida spp. are part of human flora on skin, in the gastrointestinal tract, and on oral and genital mucosa. Although usually benign, they have the potential to cause invasive infections. Breaches of physical barriers to infection such as use of central intravenous catheters, administration of total parenteral nutrition, and use of broad-spectrum antibiotics that disturb normal flora predispose patients to candidaemia (Pfaller 2012). Although *C. albicans* is traditionally the most common cause of invasive candidiasis, there is increasing evidence that non-albicans *Candida*, *C. tropicalis* included, is contributing to invasive infections both locally and globally (Tan et al. 2010, 2015a; Guinea 2014; Fernández-Ruiz et al. 2015).

Fluconazole, an azole antifungal, was previously recommended as a first-line agent for treatment of candidaemia for non-neutropaenic adult patients. Although current guidelines suggest the use of echinocandins as first-line agents, fluconazole is still recommended as a step-down agent (Pappas et al.

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2015). Azole drugs inhibit the enzyme lanosterol 14α-demethylase (Erg11p), interfering with the biosynthesis of ergosterol, an essential component of the fungal cell membrane (Pappas et al. 2015). However, multiple mechanisms conferring azole resistance have been elucidated in major *Candida* species (Pfaller 2012). Resistance can arise from; (i) the overexpression of the multidrug efflux transporters; (ii) the overexpression of *ERG11* gene, which encodes the azole enzyme target, Erg11p; (iii) amino acid substitutions in ERG11p, decreasing azole affinity; (iv) amino acid substitutions in Erg3p, an enzyme of the ergosterol biosynthesis pathway encoded by *ERG3*. These amino acid changes result in Erg3p inactivation allowing the cells to bypass accumulation of toxic sterols (Pfaller 2012). Little is known about the molecular aspects of fluconazole resistance in non-albicans *Candida*. There are a handful of studies describing mechanisms of azole resistance in *C. tropicalis* (Barchiesi et al. 2000; Eddouzi et al. 2013; Jiang et al. 2013) although we are not aware of any local reports to date. In this study, we assessed the contribution of Erg3p and Erg11p polymorphisms to azole resistance in clinical *C. tropicalis*.

A total of 1579 *C. tropicalis* were isolated during 2009–2014 at the clinical microbiology laboratory. Isolates with likely clinical significance (based on specimen type) were tested for azole susceptibility hence 348 isolates subjected to susceptibility testing (Table 1). Susceptibility testing was performed prospectively. Non-duplicate azole-resistant and sensitive isolates that were molecularly characterized in this study (Table 2), were obtained from the time period of 2012–2014 and these were retrieved retrospectively. Isolates were identified using Microflex LT Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-ToF) instrument (Bruker Daltonik GmbH, Leipzig, Germany). Minimum inhibitory concentrations (MICs) of fluconazole, voriconazole, amphotericin B and caspofungin were determined using the Etest method (bioMérieux, Marcy l'Etoile, France) on RPMI agar. This method has been validated to be a viable alternative to the Clinical and Laboratory Standards Institute (CLSI, Wayne, USA) reference method for determining *Candida* susceptibilities to fluconazole (Pfaller et al. 1998; Maxwell et al. 2003; Ranque et al. 2012). The trailing endpoints phenomenon of *Candida* with azole antifungals was taken into account (Revankar et al. 1998) with fluconazole and voriconazole Etest MICs interpreted as recommended by manufacturer (http://www.biomerieux-diagnostics.com/sites/clinic/files/etest-reading-guide-antifungal.pdf). The MICs of Etests were determined after 24 h with an 80% growth inhibition used as the MIC cutoff (microcolonies were ignored). The MIC values for fluconazole, voriconazole, and amphotericin B, were interpreted based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints, whilst the Clinical and Laboratory Standards Institute (2012) breakpoints were used for caspofungin.

UltraClean® Microbial DNA Isolation Kit (Mo Bio, Carlsbad, USA) was used to extract *C. tropicalis* genomic DNA. Long-range PCR (LongRange PCR Kit, QIAGEN, Valencia, CA, USA) was performed to obtain full-length gene sequences of *ERG3* and *ERG11* using the primer sets ERG3F: 5′-ATGGATATCGTTCTTGAAATTTGTG-3′ and ERG3R: 5′-CTATTCTTCAGAGACATATTCT-3′, and ERG11F: 5′-ATGGCTATTGTTGATACTGCCAT-3′ and ERG11R: 5′-GAGAGATACTTGTATGGTTTAG-3′, respectively. For detection of mutations, sequenced amplicons were compared to reference wild-type GenBank sequences KC542320 (*ERG3*) and KC542326 (*ERG11*). Multilocus sequence typing (MLST) (Tavanti et al. 2005) was performed on azole-resistant isolates with the assignment of diploid sequence type (DST) using the *C. tropicalis* MLST sequence-type database (http://pubmlst.org/ctropicalis/).

BioNumerics software version 6.6.11 (Applied Maths, Kortrijk, Belgium) was used to generate Unweighted Pair Group Method with Arithmetic Mean (UPGMA) trees using concatenated MLST alleles and 500 bootstrap replicates for all sequences. In-frame concatenated MLST allele sequences for the DSTs were downloaded from the *C. tropicalis* MLST database (http://ukmirror2.pubmlst.org/perl/bigsdb/bigsdb.pl?page=plugin&name=Concatenate&db=pubmlst_ctropicalis_seqdef&scheme_id=1). Further, MLST clonal clusters were determined using the eBURST package (http://eburst.mlst.net/). The eBURST algorithm places all related isolates into clonal complexes and predicts the founding or ancestral DST of each complex.

The rise in fluconazole resistance was significant, from 2.9% of isolates in 2009–2011 to almost 9% in 2012–2014 (Table 1). The overall distribution of *Candida* species in institution was comparable to that observed in a recent large scale surveillance of candidaemia across Asia (Tan et al. 2015a) as well as in locally performed studies where *C. tropicalis* and *C. glabrata* were the most commonly isolated non-albicans species (Tan et al. 2010). However, fluconazole resistance rates as high as 23.2% has been reported (Fernández-Ruiz et al. 2015). Seventeen isolates of these fluconazole-resistant *C. tropicalis* collected from 2012 to 2014, along with 32 fluconazole sensitive

Table 1 – In vitro fluconazole activity against the most commonly isolated *Candida* spp. between years 2009 and 2014.

Species	2009–2011				2012–2014			
	No.	S (%)	I (%)	R (%)	No.	S (%)	I (%)	R (%)
C. albicans	264	262 (99.2)	0	2 (0.8)	334	331 (99.1)	0	3 (0.9)
C. glabrata	124	9 (7)	99 (80)	16 (13)	203	1 (0.5)	159 (78.3)	43 (21.2)
C. tropicalis	136	132 (97)	0	4 (2.9)[a]	212	190 (89.6)	3 (1.4)	19 (9.0)[a]
C. parapsilosis	94	92 (97.9)	1 (1.1)	1 (1.1)	168	158 (94)	4 (2.4)	6 (3.6)
Total	618	495 (80.1)	100 (16.2)	23 (3.7)	917	680 (74.2)	166 (18.1)	71 (7.7)

S: susceptible; I: intermediate; R: resistant.

[a] Using Fisher's exact test where a P value of <0.05 was considered statistically significant. The rates of fluconazole resistance in *C. tropicalis* were significantly raised between years 2009–2011 and 2012–2014, $P = 0.03$.