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Acquired multi-azole resistance in *Candida tropicalis* during persistent urinary tract infection in a dog

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ABSTRACT

Multi-azole resistance acquisition by *Candida tropicalis* after prolonged antifungal therapy in a dog with urinary candidiasis is reported. Pre- and post-azole treatment isolates were clonally related and had identical silent mutations in the *ERG11* gene, but the latter displayed increased azole minimum inhibitory concentrations. A novel frameshift mutation in *ERG3* was found in some isolates recovered after resistance development, so it appears unlikely that this mutation is responsible for multi-azole resistance.

1. Introduction

Mounting evidence suggests that acquired resistance to azoles in *Candida* species may be an emerging and underdiagnosed threat [1]. The extensive use of antifungals in medical institutions could be a major driver of this changing epidemiology, but infection by resistant isolates in azole-naïve patients has also been reported [1,2].

Candida tropicalis is a leading agent of nosocomial yeast infection in humans, although its relative importance with respect to other *Candida* species varies widely among geographical areas and patient groups [3,4]. An extensive array of virulence factors contribute to the high mortality rate attributed to this yeast [3,4]. The pathogenicity of *C. tropicalis* in diverse animal species is also well documented, and even healthy individuals can serve as a reservoir of virulent and antifungal-resistant strains [5].

In this article we report the acquisition of multi-azole resistance by *C. tropicalis* during the course of a chronic infection in a dog with persistent urinary tract infection (UTI) which underwent prolonged antifungal therapy. A previously unknown frameshift mutation in the *ERG3* gene, which encode for sterol $\Delta^{5,6}$-desaturase (essential for ergosterol biosynthesis), was found in some post-treatment isolates.

2. Case

In April 2011, 3-year-old male Yorkshire terrier dog was referred to the Clinical Veterinary Hospital of our institution (Universidad Complutense de Madrid, Spain) with a diagnosis of relapsing UTI. The dog had a history of congenital portosystemic shunt and ammonium urate bladder stones, and had been treated with marbofloxacin for a previous episode of bacterial UTI caused by *Serratia marcescens*. Microscopic examination and culturing of urine specimens obtained by cystocentesis during a first consultation revealed the presence of yeasts, so antifungal treatment with fluconazole (5 mg/kg/12 h PO) and periodic mycological surveillance was prescribed. Prolonged fluconazole therapy (three courses in 2011: from April 12 to May 5, from May 10 to June 20, and from June 30 to October 5) did not result in clinical improvement, so a five-day course of bladder irrigation with amphotericin B (1 h/day with 20 mL of a 200 mg/L solution) was administered in April 2012. Amphotericin B treatment was also unsuccessful and subsequent urine cultures remained positive for yeasts. In June 2012 the animal developed a urethral stone obstruction that was surgically removed, but soon after the procedure it entered into severe hepatic encephalopathy. Due to the

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Table 1
In vitro antifungal susceptibility of the *Candida tropicalis* isolates characterized in this study.

Isolate no.	Origin	Date of recovery	CLSI MICs (μg/mL)[a]							Sensititre YeastOne MICs (μg/mL)[a]								
			AMB	FLC	ITC	KTC	PSC	VRC	CAS	AMB	5FC	FLC	ITC	PSC	VRC	ANF	CAS	MCF
1	Urine	Nov. 12, 2010	0.5	0.125	0.031	0.004	0.063	0.016	0.125	1	< 0.06	4	0.5	0.25	0.25	< 0.015	0.015	0.015
2	Urine	May 5, 2011	0.5	0.125	0.016	0.004	0.031	0.016	0.063	1	< 0.06	1	0.125	0.125	0.06	0.03	0.03	0.015
3	Urine	Jun. 30, 2011	0.5	0.125	0.016	0.004	0.063	0.016	0.125	1	< 0.06	1	0.125	0.06	0.06	< 0.015	0.03	0.015
4	Urine	Oct. 20, 2011	0.5	0.125	0.031	0.004	0.031	0.016	0.125	0.5	< 0.06	0.5	0.06	0.03	0.03	0.06	0.03	0.015
5	Urine	Apr. 12, 2012	0.5	> 64	> 16	8	> 16	> 16	0.063	0.5	< 0.06	> 256	> 16	> 8	> 8	0.03	0.06	0.015
6	Urine	May 23, 2012	0.5	> 64	> 16	16	> 16	> 16	0.25	1	< 0.06	> 256	> 16	> 8	> 8	0.06	0.06	0.03
7	Urine	Jun. 7, 2012	0.5	> 64	> 16	> 16	> 16	> 16	0.125	1	< 0.06	> 256	> 16	> 8	> 8	0.125	0.06	0.03
8	Bladder calculus	Jun. 11, 2012	0.5	> 64	> 16	> 16	> 16	> 16	0.25	1	< 0.06	> 256	> 16	> 8	> 8	0.06	0.06	0.03

[a] Minimum inhibitory concentrations (MICs) were determined after 24 h of incubation at 35 °C. Antifungals tested: 5FC, flucytosine; AMB, amphotericin B; ANF, anidulafungin; CAS, caspofungin; FLC, fluconazole; ITC, itraconazole,; KTC, ketoconazole; MCF, micafungin; PSC, posaconazole; VRC, voriconazole.

guarded prognosis and clinical worsening of the dog's condition the owners elected euthanasia and refused necropsy.

Yeast isolates recovered from clinical specimens (Table 1) were identified as *Candida tropicalis* on the basis of the morphology and pigmentation of their colonies on ChromAgar medium (TecLaim, Madrid, Spain), and also by sequencing the D1/D2 domains of the large subunit (LSU) rRNA gene [6].

Genotyping of isolates was performed by the MLST scheme developed by Tavanti et al. [7], which comprises the following six housekeeping genes: *ICL1*, encoding for an isocitrate lyase; *MDR1*, multidrug resistance protein; *SAPT2*, secreted aspartic protease 2; *SAPT4*, secreted aspartic protease 4; *XYR1*, D-xylose reductase I or II; and *ZWF1a*, putative glucose-6-phosphate dehydrogenase. In all cases, PCR amplification was performed in reaction volumes of 50 μL, consisting of 5 μL of buffer II (10 × Applied Biosystems, Austin, TX, USA), 1.5 mM $MgCl_2$ (Applied Biosystems), 200 μM each dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 2 U AmpliTaq Gold DNA polymerase LD (Applied Biosystems), 20 pmol of each forward and reverse primer (Integrated DNA Technologies, Leuven, Belgium; see sequences in Tavanti et al. [7]) and 20 ng of genomic DNA (obtained following the procedure described in Álvarez-Pérez et al. [8]). Amplifications were carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) and consisted of a denaturation step of 7 min at 94 °C, followed by 30 cycles of 60 s at 94 °C for, 60 s at 52 °C, and 65 s at 74 °C, and a final extension step at 74 °C for 10 min. PCR products were purified using the High pure PCR product purification kit (Roche Diagnostics), sequenced in the forward and reverse directions using the ABI Prism Big Dye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and analyzed on an ABI Prism 3730 sequencer (Applied Biosystems). DNA sequences were assembled and edited with Sequencher v.4.1.4 software (Gene Codes Inc., Ann Arbor, MI, USA). Raw DNA chromatograms were visually checked and scrutinized for heterozygosity, which was defined as the presence of overlapping peaks in the forward and reverse chromatograms. The final sequences were compared with others from the *C. tropicalis* MLST database (http://pubmlst.org/ctropicalis/). The results of these analyses revealed that all studied isolates belonged to a same diploid sequence type (DST) which was not found in the *C. tropicalis* MLST database. Therefore, the new allelic profile was submitted to the curator of the MLST database to be assigned a new accession number (DST no. 408; allelic profile: *ICL1*, 1; *MDR1*, 103 [new allele]; *SAPT2*, 4; *SAPT4*, 3; *XYR1*, 107 [new allele]; *ZWF1a*, 4).

In vitro antifungal susceptibility of isolates was determined by the reference Clinical and Laboratory Standards Institute (CLSI) broth microdilution procedure [9]. The antifungal agents (all purchased from Sigma-Aldrich, Madrid, Spain) and concentrations tested were: amphotericin B and caspofungin (0.031 to 16 mg/L), fluconazole (0.031–64 mg/L), and itraconazole, ketoconazole, posaconazole and voriconazole (0.002–16 mg/L). Assay plates were incubated at 35 °C in a humid atmosphere and read macroscopically at 24 and 48 h. The endpoint for minimum inhibitory concentration (MIC) was the antifungal concentration that produced a complete (100%) inhibition of visual growth (for amphotericin B) or prominent inhibition of growth ($\leq$ 50% inhibition, for the remaining antifungal agents). *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains and all isolates were tested at least twice on different days. In addition, antifungal susceptibility of isolates was also determined by the commercially prepared Sensititre YeastOne colorimetric antifungal panel (TREK Diagnostics Systems, East Grinstead, UK). On the day of the assay, suspensions of 1.5–8 × 10^3 cells/mL were prepared in YeastOne inoculum broth, and the dried panels were rehydrated by dispensing 100 μL of yeast suspension into each well. The panels were covered with adhesive seals and incubated at 35 °C for 24 h. The MIC endpoints were defined as the lowest concentration of antifungal drug preventing the development of a red color (i.e. first blue or purple well).

Table 2
Mutations observed in the *ERG3* and *ERG11* sequences of the isolates characterized in this study.[a]

Gene	Nucleotide change	Amino acid change	Isolates with the mutation
ERG3	2-bp insertion in positions 1130 and 1131	Frame shift from position 378 onwards[b]	#6, 7 and 8
ERG11	T225C	None	All
	G264A	None	All
	G1362A	None	All
	T1554C	None	All

[a] As determined by comparison with reference wild-type sequences retrieved from the GenBank.
[b] See Fig. 1.

The *ERG3* and *ERG11* genes, which encode for sterol $\Delta^{5,6}$-desaturase and the azole target 14α-sterol demethylase, respectively, were PCR amplified and sequenced using the panels of oligonucleotide primers described by Forastiero et al. [10] and Vandeputte et al. [11]. PCR reactions consisted of 5 μL of buffer II (10 ×), 1.5–3 mM $MgCl_2$, 200 μM each dNTP, 2 U AmpliTaq Gold DNA polymerase LD, 20 pmol of the corresponding forward and reverse primers and 50 ng of genomic DNA in a final volume of 50 μL. Amplification consisted of a denaturation step of 5 min at 94 °C, 35 cycles of 60 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C, and a final extension at 72 °C for 10 min. Purification and sequencing of PCR products was performed as described above. *ERG3* and *ERG11* sequences obtained for the isolates characterized in this study (and also the amino acid sequences deduced from them) were