Acquired echinocandin resistance in a *Candida krusei* blood isolate confirmed by mutations in the *fks1* gene

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SUMMARY

We describe a case of bloodstream infection caused by a *Candida krusei* strain that developed echinocandin resistance during caspofungin therapy. Three mutations were found in the HS1 region of the *fks1* gene, two of them have never been reported either in *C. krusei* nor in *C. albicans*.

KEY WORDS: Candida krusei, Echinocandin resistance, Gene mutation.

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Candidaemia remains the most common life-threatening fungal disease despite the widespread use of antifungal prophylaxis. A number of studies conducted in Europe and the USA have reported a shift towards non-*albicans* species, particularly *C. glabrata* and *C. krusei,* as a cause of bloodstream infection (BSI) (Tortorano *et al.,* 2006; Tortorano *et al.,* 2013; Pfaller *et al*., 2007).

The introduction of echinocandins (caspofungin, anidulafungin, and micafungin) in the late 1990s represented an important advance in the treatment of candidaemia, providing an alternative to azoles and polyenes (Castanheira *et al*., 2010). This class of antifungals has a broad spectrum of anti-*Candida* activity being active also against species characterised by resistance or reduced susceptibility to other antifungal compounds such as *C. glabrata* and *C. krusei*. (Katiyar *et al*., 2006). In addition, due to the low toxicity, few drug-drug interactions and a favourable pharmacokinetic profile, echinocandins are strongly recommended as the initial treatment for disseminated candidosis (Pappas *et al.,* 2009; Cornely *et al*., 2012).

Echinocandins are lipopeptides that inhibit cell wall synthesis by targeting the 1,3-β-D-glucan synthase complex encoded by one or several *fks* genes, depending on the species (Douglas *et al.*, 1997; Desnos-Ollivier *et al.,* 2008). Resistance to echinocandins has been associated with mutations in two conserved regions of *fks1* and *fks2*, and amino acid substitutions in the proteins encoded by these genes have been observed only within two hot spot (HS) regions on each gene (Desnos-Ollivier *et al.,* 2008; Castanheira *et al*., 2010). The majority of *Candida* spp. strains displaying reduced echinocandin susceptibility have mutations in the HS1 region of *fks1*.

We describe a case of bloodstream infection caused by a *C. krusei* strain that developed echinocandin resistance during caspofungin therapy. The patient, a 74-year-old man with diffuse large B-cell lymphoma with retroperitoneal and splenic involvement, developed candi-

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TABLE 1 - *MICs of the first and last C. krusei blood isolates tested with YeastOne and broth microdilution according to EUCAST.*

	MIC, mg/L		
	1st isolate (Dec 31)	*Last isolate (Feb 14)*	
Anidulafungin	nd*	nd*	0.25#
Caspofungin	1*	16*	4#
Micafungin	nd*	2§	4#
Flucytosine	32*	16*	16#
Posaconazole	1*	0.5*	<0.03#
Voriconazole	0.5*	0.5*	1#

Data obtained with YeastOne (*), EUCAST (#), Etest (§)

daemia (septic fever) after fourth line chemotherapy (liposomal doxorubicin, 50 mg/mq and etoposide, 100 mg/mq) administered for disease progression. *C. krusei* was cultured from three blood samples drawn from central vascular catheter on December 31, January 8 and January 13, while cultures of peripheral blood remained negative. The isolate, tested using YeastOne (Trek Diagnostic System, East Grinstead, England), was susceptible to amphotericin B and caspofungin, and resistant to flucytosine and fluconazole according to the breakpoints proposed by the manufacturer (Table 1). On January 9, caspofungin therapy (50 mg/day) was started even though the patient was afebrile. The vascular catheter was not removed due to the disappearance of fever and the patient's critical condition. After one month of antifungal treatment the patient again became febrile and *C. krusei* was again isolated from blood drawn from the vascular catheter. This last isolate was resistant to caspofungin and micafungin. The patient died a few days later due to enlargement of abdominal masses and progressive deterioration of his clinical condition.

The last isolate was sent to the Laboratory of Medical Mycology of the Department of Biomedical Sciences for Health, Milan University in the framework of the FIMUA candidaemia survey. This isolate (IUM 09-0233) was tested for susceptibility to antifungal drugs [anidulafungin (Pfizer Central Research, Sandwich, UK), caspofungin (Merck & Co., Whitehouse Station, NJ, USA), micafungin (Astellas Pharma, Tokyo, Japan), flucytosine (Molekula Ltd, Wimborne Dorset, UK), posaconazole (Merck & Co.) and voriconazole (Molekula Ltd)] by broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology (Rodriguez-Tudela *et al.*, 2008).

Testing was performed in RPMI 1640 supplemented with glucose to a final concentration of 2%. Microtitre plates were read spectrophotometrically at 490 nm after 24 h of incubation at 37°C. The minimal inhibitory concentration (MIC) was defined as the lowest drug concentration giving 50% growth inhibition compared to growth in control well. All tests were performed in duplicate. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included as quality controls.

The EUCAST breakpoints were used to define the resistance against anidulafungin (MIC >0.06 µg/ml) (Arendrup *et al.,* 2011a). The Clinical and Laboratory Standards Institute (CLSI) breakpoints were used to identify isolate resistant to caspofungin (MIC ≥1 µg/ml), micafungin (MIC ≥1 µg/ml) and voriconazole (MIC ≥4 µg/ml) (CLSI M27-S4, 2012) as EUCAST breakpoints are not available for these antifungals. Echinocandins were also tested by Etest (BioMérieux) methodology on RPMI 1640 agar.

The HS1 region of the *fks* gene was sequenced to verify the presence of mutations. DNA was extracted with the PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions.

The PCR products were amplified in a 2700 thermocycler (Applera, Foster City, CA, USA) using the primers CKS1f (ACTGCATCGTTTGCTCCTCT) and CKS1r (GAACATGATCAATTGCCAAC) (Desnos-Ollivier *et al.,* 2008). Amplicons were purified and sequenced using Big Dye terminators (Applera) in a 310 ABIPRISM sequencer (Applera). Nucleotide sequences were analyzed using Finch TV software Version 1.4.0. and alignments using Clustal W2 software. The consensus sequence obtained was aligned with the complete sequence *fks1* gene of *C. krusei* ATCC 6258 reported in GenBank (EF 426563).

Table 1 reports the susceptibility profiles of the first and the last isolate. The last strain resulted resistant to all three echinocandins when tested by broth microdilution according to EUCAST methodology, YeastOne and Etest.

In the *C. krusei* resistant isolate, three mutations were found in the HS1 region of *fks1* gene: G1939A, G2076A and C2101A. Two of them, G1939A and C2101A, were missense mutations resulting in A647T and L701M amino acid change. On the contrary, mutation G2076A does not involve amino acid substitution. Mutation L701M has already been reported in *C. krusei* (Desnos-Ollivier *et al.,* 2008), whereas to our knowledge, the other two have never been reported either in *C. krusei* or in *C. albicans*. (Desnos-Ollivier *et al.,* 2008). The mutation A647T is located just few aminoacids outside the *C. krusei* HS1 region and its involvement in caspofungin resistance needs to be confirmed by further genetic analyses. The third mutation might be considered a simple polymorphism with no influence on the protein structure of *fks1*.

C. krusei, as a diploid organism, can have either homozygous or heterozygous mutations in *fks1*. In our strain all three mutations were homozygous.

Homozygous mutants isolates were shown to need a higher caspofungin dosage than heterozygous mutants, to obtain a decrease in fungal burden in a mouse model of candidiasis (Park *et al.*, 2005). Therefore, it is likely that human infections by these resistant *C. krusei* isolates might also improve using caspofungin at a higher dosage.

A high degree of interlaboratory variation of caspofungin MIC values, obtained using CLSI as well as EUCAST methodologies, was recently reported, potentially leading to incorrect categorization of susceptibility results (Espinel-Ingroff *et al.*, 2013). Therefore EUCAST has already recommended the use of anidulafungin MICs as markers for the echinocandins and avoidance of caspofungin MIC results for clinical decision-making (Espinel-Ingroff *et al.*, 2013; Arendrup M.C. *et al.*, 2011a**,** Arendrup M.C. *et al.*, 2011b). An incorrect categorization of caspofungin susceptibility of a *C. krusei* isolate is particularly dangerous as the intrinsic resistance to fluconazole of this species limits the treatment options.

In conclusion, our isolate is a true caspofungin-resistant isolate as it harbours three homozygous mutations in the *fks1* gene, two of which have never been described. The case here reported highlights the need to monitor the development of resistance in patients under treatment with echinocandins using, as recently suggested, either micafungin or anidulafungin MIC endpoints as predictors of susceptibility or resistance of *Candida* to caspofungin (Espinel-Ingroff *et al.*, 2013).

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