Acquired multidrug antifungal resistance in *Candida lusitaniae* during therapy

S. A. Asner[1,2] MD, MSc; S. Giulieri[2] MD; M. Diezi MD[3]; O. Marchetti[2] MD; PhD; D. Sanglard[4] PhD

[1] Unit of Pediatric Infectious Diseases and Vaccinology, Department of Paediatrics; [2] Service of Infectious Diseases, Department of Internal Medicine ;[3] Unit of Pediatric Haematology and Oncology, Department of Pediatrics; [4] Institute of Microbiology, Department of Laboratory ; University of Lausanne and Hospital Center, Lausanne, Switzerland.

Background

- *Candida lusitaniae (Cl)* is a rare cause of candidemia.
- Echinocandins are used as first-line therapy for candidemia due to *Cl*.
- beta-1,3-glucan synthase encoded by *FKS* genes is the target of echinocandins.
- Missense mutations in the *Cl FKS1* hot spots (HS1) resulting in increased MICs of echinocandins have been reported in isolates of *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei* (1).
- Cross-resistance between echinocandins and other agents (multidrug resistance) is emerging (2) but has been still rarely reported in *Cl.*

Objectives

- We report the case of clinical isolates of *Cl* with rapid emergence of multidrug resistance following different antifungal treatments for persistent candidemia and document molecular analyses involved in caspofungin and fluconazole resistance.

Case Report

- A 3 year–old female with relapsed acute myeloid leukemia (AML) and 3 months of profound neutropenia, presented with severe enterocolitis and visceral adenoviral disease (adenovirus 41 F).
- Persistent *Cl* candidemia **during ongoing profound neutropenia** despite various antifungal treatments **and repeated central line removal**. No endovascular source nor hepato-splenic, pulmonary lesions were noted. The *Cl* isolates were related and exhibited 5 susceptibility profiles (P) **(Figs 1 and 2)**. Established clinical breakpoints for *C. albicans* were used for *Cl* MIC interpretations (**Tables 1 and 2**). *FKS* genes sequencing and qRT-PCR of *Cl* genes potentially involved in azole resistance were performed **(Figs 3 and 4).**

- **P1** *Cl* (recovered from stools, blood cultures (BC) negative while being on amphotericin B (AMB) for 3 months) -> SUSCEPTIBLE to all antifungals: caspofungin (CAS) (MIC: 0. 5 µg/ml); fluconazole (FLC) (MIC: 0.25 µg/ml).

- **P2** *Cl* (recovered from BC while being on caspofungin for 1 month -> RESISTANT CAS (MIC: 4 µg/ml), micafungin (MICA: MIC: 16 µg/ml) and AMB (2 µg/ml) but susceptible to FLC (MIC: 0.25 µg/ml) and 5-FC (MIC: 0.5 µg/ml).

- **P3** *Cl* (recovered from BC while being on CAS and FLC for 3 weeks followed by FLC monotherapy for 1 week)-> RESISTANT FLC (MIC: 32 µg/ml) and 5-FC (MIC: 64 µg/ml) but susceptible to CAS (MIC: 0.5 µg/ml), MICA (MIC: 0.03 µg/ml) and AMB (MIC: 0.25 µg/ml).

- **P4** *Cl* (recovered from BC while being on CAS and azoles for 3 weeks) -> RESISTANT to FLC (MIC: 8 µg/ml), 5-FC (MIC: 32 µg/ml), CAS (MIC: 8 µg/ml), MICA (MIC: 16 µg/ml) but susceptible to AMB (MIC: 0.06 µg/ml).

- **P5** *Cl* (recovered while being on CAS and azoles for 3 weeks) -> RESISTANT to CAS (MIC: 8 µg/ml), MICA (MIC: 16 µg/ml) and AMB (2 µg/ml) but susceptible to FLC (MIC: 0.25 µg/ml) and 5-FC (MIC: 2 µg/ml).

- Further evolution: Probable invasive pulmonary aspergillosis (EORTC). New positive BC while being on a 2 week regimen of AMB and voriconazole (VORI), isolate susceptible to VORI and AMB (MICs not available).

- Allogeneic hematopoeitic stem cell transplantation -> progressive fulminant hepatitis, renal dysfunction followed by death.

Figure 1: Summary of the susceptibility profiles of *Cl* isolates.
MIC values were obtained using the EUCAST method. Antifungal susceptibility testing was carried out on the basis of EUCAST protocols with slight modifications (3). Briefly, were diluted to a density of 0.5-2 x 10e5 cells per ml in RPMI 1640 medium (Sigma) with L-glutamine and without bicarbonate. Phenol red was included as a pH indicator. The RPMI 1640 medium was buffered to pH 7 and glucose to a final concentration (w/v) of 2%. All compounds were dissolved to obtain final concentrations ranging from 128 µg/ml to 0.0162 µg/ml. The microtiter plates were incubated at 35°C for 24 h, and the MICs were determined with a spectrophotometer plate reader set at 450 nm. The MIC was defined as the drug concentration at which the optical density was ≤ 50% of that of the drug-free culture. NA: Not available.

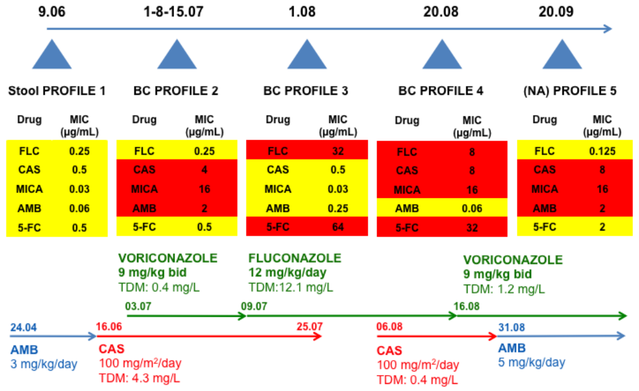
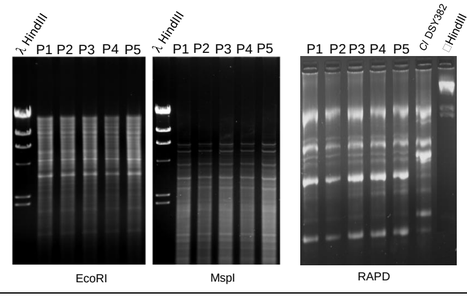
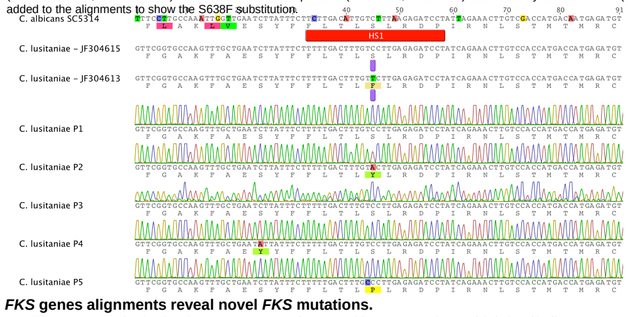


Figure 2: Restriction fragment length polymorphism (RFLP) and RAPD analyses of *Cl* isolates P1-P5.
Typing of *C. lusitaniae* isolates were performed by RFLP and RAPD as recommended (4). Genomic DNA was isolated from each isolate and digested by *Eco*RI and *Msp*. RAPD was performed with primer OPE-18 (GGACTGCAGA). Gel electrophoresis was carried out with 0.8% agarose. A picture was recorded after GreenPeq staining.



- Identical patterns stained profiles suggest a high relationship between isolates

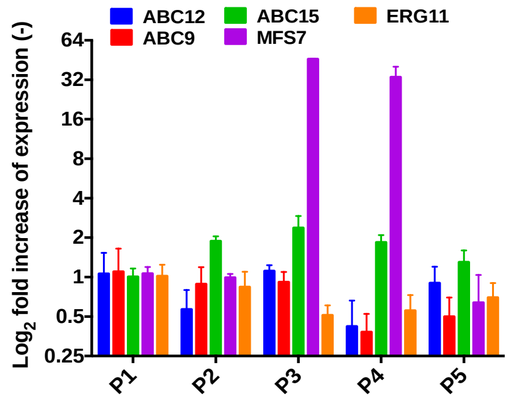
Figure 3: *FKS* genes sequencing
Primers designed in the work of Desnos *et al.* (5) were used to amplify *FKS1* alleles encoding β−glucan synthase from *C. lusitaniae* isolates. These primers were designed to amplify conserved HS1 (hot spot region 1) and HS2 regions (MDO002: GCCTTTGGGTGGTTTGTTTA and MDO003: TCGGAATCTCTTGGGAAGAA for HS1; MDO004: TGCTGGTATGGGTGAACAGA and MDO005: CGAACACTTCGAAGAATGGAG for HS2). Sequencing procedures were performed with the same primers and are described (6). Sequence alignments were performed with Geneious Software (Biomatters Ltd., New Zealand). Known *FKS1* HS1 sequences (JF304615, JF304613) described by Desnos *et al.* (5) were added to the alignments to show the S638F substitution.



***FKS* genes alignments reveal novel *FKS* mutations.**

- The S to Y substitution in P2 corresponds to position S638 of P1 which itself aligns to position S645 in *C. albicans*.
- The S to Y substitution in P4 corresponds to position S631 of P1 which itself aligns to position S638 in *C. albicans.*
- The S to P substitution in P5 corresponds to position S638 of P1 which itself aligns to position S645 in *C. albicans.*
- No substitutions were observed in HS2 (not shown).

Figure 4: qRT-PCR of *Cl* genes potentially involved in azole resistance.
qRT-PCR was performed as described (7). Briefly, total RNA was extracted from log phase cultures with an RNeasy Protect Mini kit (Qiagen) by a process involving mechanical disruption of the cells with glass beads and an RNase-free DNase treatment step as previously described [4]. Gene expression levels were determined by real-time qRT-PCR in a StepOne Real-time PCR System (Applied Biosystems) using the Mesa Blue qPCR Mastermix Plus for Sybr assay kit (Eurogentec). Each reaction was run in triplicate on three separate occasions. Expression levels were normalized by *ACT1* expression.
Primers were designed to amplify specific regions of genes corresponding to ABC and MFS transporters in *Cl.* Choice of primers were based on available genome sequence (Broad Institute) and based on a study published by Reboutier *et al.* (7) in which ABC and MFS transporters were categorized. ABC9, ABC12, ABC 15 and MFS7 were selected since the corresponding genes were differentially expressed in several *C. lusitaniae* isolates as reported (8).



- No expression variations in azole resistance genes belonging to the ABC transporter family or to *ERG11* (target of azoles) were identified.

- A Major facilitator gene (*MFS7*) transporter was found overexpressed in FLC-resistant isolates P3 and P4, thus raising the hypothesis that FLC resistance may be attributed to the overexpression of this gene. Cross-resistance to 5-FC may be related to *MFS7* overexpression since no mutations were detected in cytosine permease (*FCY2*) and cytosine deaminase (*FCY1*). *FUR1* mutations were excluded since no changes in 5-fluorouracil (deaminated 5-FC) suceptibility was observed.

Table 1: CLSI and EUCAST breakpoints (valid in *C. albicans*) for MIC interpretations in *Cl* (adapted from (9,10))

Drug	EUCAST valid from 11.03.13		CLSI 2011 & 2012		
	S	R	S	I	R
Amphotericin B	≤ 1	> 1	No criteria		
Caspofungin	No breakpoints yet established		≤ 0,25	0,5	≥ 1
Micafungin	≤ 0,016	> 0,016	≤ 0,25	0,5	≥ 1
Fluconazole	≤2	> 4	≤ 2	4	≥ 8
5-FC	No criteria	No criteria	≤ 4	8-16	≥ 32

Table 2. Summary of *Cl* profiles with their respective documented phenotypes/genotypes

Date	Origin	Profile	FLC µg/ml	CAS µg/ml	MICA µg/ml	AMB µg/ml	5-FC µg/ml	*FKS1* mutations	*MFS7* overexpression	AF
9.06	Stools	1	0.25	0.5	0.03	0.06	0.5	no	no	AMB
1.07	Blood	2	0.25	4	16	2	0.5	S638Y	no	CAS
1.08	Blood	3	32	0.5	0.03	0.25	64	no	yes	FLC
23.08	Blood	4	8	8	16	0.06	32	S631Y	yes	VORI/CAS
2.09	Stools	4	32	4	8	0.125	32	S631Y	yes	VORI/AMB
20.09	NA	5	0.125	8	16	2	2	S638P	no	VORI/AMB

Legend: FLC: fluconazole, CAS: caspofungin, MICA: micafungin, AMB: liposomal amphotericin B, 5-FC: 5-fluorocytosine, VORI: voriconazole, *MSF7*: major facilitator gene. Drug: minimal inhibitory concentrations are reported in each column, AF refers to the antifungal regimen (AF) of the patient, while the specific strain emerged (Profiles 1-5).

Table 3. Candin MICs of *S.cerevisiae FKS1* mutants

Isolate	MIC (µg/ml) [a]		
	CAS	MICA	ANI
S. cerevisiae wild type IMX581	0.03	0.015	0.03
DSY4762 (*FKS1*^{S636Y})	1 (32) [b]	0.5 (32)	0.5 (16)
DSY4763 (*FKS1*^{S643Y})	2 (64)	4 (256)	1 (32)
DSY4764 (*FKS1*^{S643P})	8 (256)	4 (256)	1 (32)

[a] : MIC assays were performed according to the EUCAST protocol but at 30°C and with YEPD medium.
[b] : Numbers in brackets represent relative fold increase in MICs as compared to the MIC value of wild type.

Abstract

Objectives: *Candida lusitaniae* (*Cl*) is intrinsically susceptible to echinocandins. Beta-1,3-glucan synthase encoded by *FKS* genes is the target of echinocandins and few missense mutations in the *Cl FKS1* hot spots 1 (HS1) have been reported. We report here the rapid emergence of antifungal resistance in *Cl* isolated during therapy with amphotericin B (AMB), caspofungin (CAS) and azoles for persistent candidemia in an immunocompromised child with severe enterocolitis and visceral adenoviral disease. Molecular analysis of resistance mechanism was undertaken.

Results: As documented from restriction fragment length polymorphisms, the *Cl* isolates were related with each other. From antifungal susceptibilities and molecular analysis, 5 different profiles (P) were obtained. Since no clinical breakpoints exist for *Cl*, those established in *C. albicans* were used to define resistance. These profiles included P1 (CAS-, fluconazole (FLC)-susceptible) while being on AMB Tx for 3 months), P2 (FLC-susceptible but CAS-resistant while on CAS Tx for 2 weeks), P3 (CAS-susceptible but FLC-resistant while on azoles and CAS combined Tx initially followed by azoles alone for a week), P4 (CAS- and FLC-resistant while on both drugs Tx for three weeks) and P5 (FLC-susceptible but CAS-resistant). CAS resistance was associated with cross-resistance not only to micafungin but also to AMB. Analysis of CAS resistance revealed 3 novel *FKS1* mutations in CAS-resistant isolates (S638Y on P2; S631Y on P4; S638P on P5). FLC resistance could be linked with overexpression of a major facilitator gene (*MFS7*) in *Cl* P2 and P4 and was associated with cross-resistance to 5-flurocytosine.

Conclusions: This clinical report describes resistance of *Cl* to all common antifungals. While candins or azole resistance followed monotherapy, multidrug antifungal resistance emerged during combined therapy.

Conclusions

1. We document resistance of *Cl* to all common antifungals in a profoundly neutropenic patient with severe enterocolitis and report **cross resistance between 2 major candins** while exposed to CAS and **between 5-FC and FLC** while exposed to FLC (Table 1).
2. Resistance to CAS was corroborated with the identification of **three novel *FKS1* mutations.**
3. Overexpression of a **major facilitator gene *(MFS7)*** was documented in FLC-resistant strains.
4. Rapid selection of mutants under drug pressure probably resulted from the **haploid nature** of the organism and **coexistence of several populations of *Cl*** with the emergence of a dominant strain while exposed to a specific antifungal agent.
5. Rapid emergence of multidrug resistant mutants on combined therapy reinforces the need for **limiting dual therapy to exceptional situations.**

References
1. Perlin DS. Current perspectives on echinocandin class drugs. Future Microbiol **2011**; 6:441–457.
2. Arendrup MC, Perlin DS. Echinocandin resistance: an emerging clinical problem? Current Opinion in Infectious Diseases. **2014**;27(6):484–492.
3. Rodriguez-Tudela, J. L., Arendrup, M. C., Barchiesi, F., Bille, J., Chryssanthou, E., Cuenca-Estrella, M., Dannaoui, E., Denning, D. W., Donnelly, J. P., Dromer, F., Fegeler, W., Lass-Flori, C., Moore, C., Richardson, M., Sandven, P., Velegraki, A., Verweij, P., (Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST)). EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin Microbiol Infect **2008**; 14:398–405.

Acknowledgements
The authors are thankful to the providers of the following substances: Caspofungin (Merck), Micafungin (Astellas)