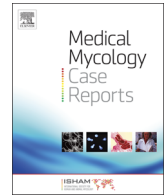




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Two missense mutations, E123Q and K151E, identified in the *ERG11* allele of an azole-resistant isolate of *Candida kefyr* recovered from a stem cell transplant patient for acute myeloid leukemia

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ABSTRACT

We report on the first cloning and nucleotide sequencing of an *ERG11* allele from a clinical isolate of *Candida kefyr* cross-resistant to azole antifungals. It was recovered from a stem cell transplant patient, in an oncohematology unit exhibiting unexpected high prevalence of *C. kefyr*. Two amino acid substitutions were identified: K151E, whose role in fluconazole resistance was already demonstrated in *Candida albicans*, and E123Q, a new substitution never described so far in azole-resistant *Candida* yeast.

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1. Introduction

Candida kefyr is an ubiquitous yeast that is usually considered as making part of the yeast microflora in dairy products and cheeses [1]. Accordingly, most of *C. kefyr* were isolated from gastrointestinal tract in humans, and it can be supposed that its carriage tightly depends on eating habits. *C. kefyr* has been described also as an emerging opportunistic pathogen, particularly in patients with oncohematological diseases [2–4]. Intriguingly, the prevalence of *C. kefyr* seems to be unexpectedly high in some oncohematology units: for example, in three French teaching hospitals, its frequency was reported to be twice that of all other wards [3]. Even though such a high prevalence remains rationally unexplained, it may pose specific problems of resistance in oncohematology where antifungal prophylaxis and empirical treatment are commonly used. It is probable that clinical isolates of *C. kefyr* can exist majoritarily under the form of haploid cells, as do its teleomorphic form *Kluyveromyces marxianus* [5]. Haploidy could contribute to the development of antifungal resistance,

notably by mutations in the genes encoding the target of anti-fungals [6]. Recently, the first clinical isolate of *C. kefyr* resistant to caspofungin was described [4]. In this study, we report the isolation of a clinical isolate cross-resistant to azole-antifungals in a patient who had undergone a stem cell transplantation for an acute myeloid leukemia. Taking advantage of the recent nucleotide sequencing of the genome of *K. marxianus* [7], we retrieved the sequence of the *ERG11* gene, and designed primers for the cloning and sequencing of the *ERG11* allele from the azole-resistant isolate. This allowed us to identify two missense mutations leading to the amino acid substitutions E123Q and K151E, whose combination could account for the high level of resistance to fluconazole and voriconazole, and to azole cross-resistance phenotype in *C. kefyr*.

2. Case

A 32-year-old woman was diagnosed in 1997 with JAK2 mutation-negative essential thrombocythemia (ET) treated by pipobroman the first 7 years, then hydroxyurea and anagrelide for 4 and 3 years, respectively. In 2012, the patient began complaining about asthenia, with a gradual decrease in hemoglobin level. Anagrelide was stopped and a bone marrow biopsy was performed in December 2012, which showed the occurrence of

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a grade 2 myelofibrosis. A few weeks later, hematologic tests revealed more than 20% of blastic cells in peripheral blood, indicating that ET with myelofibrosis had evolved into acute myeloid leukemia.

The patient had one HLA-identical sister, and received an allogeneic bone marrow hematopoietic stem-cell transplantation (HSCT) on 27 March 2013. On Day 0 (D0) of HSCT, the patient was admitted to intensive care unit (ICU) for an acute respiratory distress syndrome (RDS). Four blood cultures (BacT/Alert 3D, BioMérieux, Organon Teknika, USA) yielded *Staphylococcus aureus* and *Escherichia coli*. A broad-spectrum antibiotherapy (tazobactam/piperacillin, amikacin and vancomycin) associated with furosemide for the acute pulmonary edema led to a rapid improvement of the patient condition. An oral fluconazole prophylaxis (400 mg/day) was also started. On Day 12, the patient was readmitted to ICU for an acute RDS and a septic shock with no microbiological evidence of infection. The patient became afebrile after an empirical antibiotherapy (ceftazidime and linezolid).

On Day 37, the patient presented with the first symptoms of a Graft-versus-Host Disease (GvHD), that developed to the skin, the gastro-intestinal tract and the liver. She received corticosteroids, inolimomab, sirolimus and basiliximab but digestive disorders remained chronic with hemorrhagic manifestations.

On Day 63, during another admission to ICU for similar respiratory symptomatology, a new antibiotic regimen (imipenem, ciprofloxacin and subsequently ceftriaxone) associated to intravenous caspofungin (50 mg/day) was started. Routine microbiological surveillance cultures were performed weekly. During two months, June and July, *Candida albicans* was isolated from oropharynx swab, rectal swab or stools and urine. Then, from 12 August, *C. albicans* was no longer isolated but cultures documented a colonization of the gastrointestinal and urinary tracts by *C. kefyri* (telemorph, *K. marxianus*), with an increasing fungal load.

Caspofungin stopped after 2 months and switched to oral fluconazole (400 mg/day) was restarted on 17 August. The neutropenic patient suffered from an unexplained fever that led to perform a thoracic computed tomography (CT) on 24 September. CT imaging showed pulmonary nodules suspected to be a pulmonary aspergillosis. Antifungal therapy with caspofungin was switched to voriconazole. Serum galactomannan (GM) assay (Platelia[®] Aspergillus Ag Kit, Bio-Rad), performed twice a week, was constantly negative. On 26 September, GM assay index measured on bronchoalveolar lavage (BAL) was negative. The BAL culture yielded no *Aspergillus* but heavy growth of *C. kefyri*. Blood cultures remained negative. No other pathogen was found.

On 2 October, measurement of (1→3)-β-D-glucan antigenemia (Fungitell[®] Assay, Associates of Cape Cod, Inc., East Falmouth, MA) revealed a significantly elevated serum concentration of 411 pg/mL (positive cutoff ≥ 80 pg/mL). A possible deep candidiasis was suspected by the clinicians and treatment with caspofungin was resumed [8]. Follow-up CT showed the increase in size and number of pulmonary lesions. The patient was readmitted to ICU with an acute renal failure and died of multiple organ failure one week later.

Identification of *C. kefyri* was performed with matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Microflex, Bruker Daltonik). Identification was confirmed by amplifying and sequencing the internal transcribed spacer (ITS) region of rDNA and a segment of the 18S rDNA gene, using the universal primer pair ITS1 and ITS4, and the primer pair NS3 and NS4, respectively (Table 1). PCR products were synthesized using HiFidelity Taq-polymerase (QIAGEN, Hamburg, Germany), and purified with QIAquick PCR purification kit (QIAGEN). ITS1, ITS4, NS3, NS4 were used individually as primers to sequence both strands of each PCR product with ABIPrism Dye Terminator Cycle Sequencing Ready Reaction v1.1 Kit (Applied

Biosystems, Foster City, CA, United States) according to the manufacturer's instructions. The nucleotide sequences of ITS (GenBank accession number KF964549) and of the 18S rDNA gene segment (GenBank accession number KF964550) were compared with those of the databases of the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) using the Basic Local Alignment Search Tool [9].

Antifungal susceptibility testing was performed by *E*-test (BioMérieux) on RPMI medium. The MICs were measured after 24 h and 48 h of incubation, and tested twice from different *C. kefyri* isolates. The MIC values of azole, amphotericin B and echinocandin antifungals for the strain isolated in September, so called the *C. kefyri* PAZ isolate, are given in Table 2. The PAZ isolate exhibited high MIC values for all azole antifungals tested, and was fully resistant to fluconazole and voriconazole. The MIC values of two other strains were determined as control: one routinely recovered from an unrelated patient (isolate TEM) and the reference strain *K. marxianus* CBS 6556, the telemorph of *C. kefyri*. Both strains were fully susceptible to all antifungals tested.

The *ERG11* gene encoding lanosterol 14-α-demethylase of *C. kefyri* was identified from the raw database of the genome sequence of the strain CBS 6556 (ATCC 26548) of *K. marxianus* [7]. The nucleotide sequence of *ERG11* was retrieved from the scaffold 2 (Genbank accession number JH924897) by the dot-plot DNA matrix analysis tool [10] embedded in the MacVector software (v12.7), using as query the *ERG11* nucleotide sequence of the *Kluyveromyces lactis* strain NRRL Y-1140 (Genbank accession CR382125, locus tag KLLA0_E03653g, [11]). The *ERG11* gene was then amplified by PCR from the clinical isolates PAZ and TEM, and from the reference strain CBS 6556, using the primer pair FergCk0 and RergCk0 (Table 1). The nucleotide sequence of the 1581 bp coding region was determined for the three alleles with the aim to identify mutations that could be responsible for the resistance to azole antifungals in the isolate PAZ. The sequence data were assembled and compared each other, allowing the identification of several single nucleotide polymorphisms (SNP) (Table 3). Most SNP corresponded to silent mutations with no amino acid changes. Interestingly, the PAZ *ERG11* allele possessed two missense mutations, E123Q and K151E that were not present in the *ERG11* alleles of the azole susceptible control strains. Genbank accession numbers for the nucleotide sequences of the TEM, PAZ and CBS 6556 *ERG11* alleles are KF964548, KF964547 and KF964546, respectively.

3. Discussion

C. kefyri may be considered as an emerging pathogen, particularly in patients of oncohematology units [2–4]. In Bordeaux University Hospital Center (Bordeaux, France), during an 8-year period (2005–2012), a total of 3701 *Candida* spp. were isolated from routine mycological cultures performed from oncohematological patients. Non-*albicans Candida* represented 1858 isolates (50.2%), of which 359 isolates of *C. kefyri* (9.7% of total *Candida* isolates). This unexpected prevalence of *C. kefyri* is two-fold higher than that previously reported (4.8%) from oncohematology wards of three other French teaching hospitals [3]. The incidence of *C. kefyri* in the 612 candidemia diagnosed over the period 2005–2012 from all wards of our institution was 1.8%. This is similar to the data reported from the YEASTS surveillance program in another French area during the period 2002–2010 (1.7% of candidemia caused by *C. kefyri* [12]). However, in Bordeaux, two-third of candidemia due to *C. kefyri* were diagnosed in adult patients hospitalized in oncohematology, making *C. kefyri* responsible for 15% of the candidemia in the adult oncohematology ward.

The reasons of the high incidence of *C. kefyri* in the gastrointestinal flora of patients with hematologic malignancies are not

Table 1
Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'–3')	Use
ITS1	TCCGTAGGTGAACCTGCGG	Amplifying and sequencing the <i>C. kefyri</i> ITS region
ITS4	TCCTCCGCTTATTGATATGC	
NS3	GCAAGTCTGGTGCCAGCAGCC	Amplifying and sequencing part of the <i>C. kefyri</i> 18S rDNA gene
NS4	CTCCGCAAITCCITTAAG	
FergCk0	CACGATAATAGATCATGCTACGCTGAATCG	Amplifying and sequencing the <i>C. kefyri</i> <i>ERG11</i> gene
RergCk0	TAATTGATTTACTTCCTCAAAGTCCACTCAA	
FergCk2	AAGTTCGTTAAGGGTGCTTTGACT	Sequencing the <i>C. kefyri</i> <i>ERG11</i> gene
FergCk3	GCTGCTACCTCCGCTTGGGCT	
RergCk5	GATGTAATCTTAGGGTTTCTTGATAG	
RergCk6	TGAGTGACCATGACGTTGATCTTACC	

Table 2
MIC values of azole, echinocandin and amphotericin B antifungals for *C. kefyri* clinical isolates and reference strain CBS 6556.

Strain/isolate	Antifungal MIC (µg/mL)						
	Fluconazole	Voriconazole	Itraconazole	Posaconazole	Caspofungin	Micafungin	Amphotericin B
PAZ	> 256	> 32	1	1	0.19	0.19	0.75
TEM	0.047	0.004	0.016	0.012	0.094	0.094	0.25
CBS 6556	0.016	0.004	0.016	0.016	0.006	0.023	0.25

Table 3
SNP occurring in the different *ERG11* alleles of the *C. kefyri* clinical isolates and the reference strain CBS 6556.

TEM versus CBS 6556			PAZ versus CBS 6556			PAZ versus TEM		
SNP	Mutation	aa	SNP	Mutation	aa	SNP	Mutation	aa
T43C	Synonymous	L15L	T43C	Synonymous	L15L	G150T	Synonymous	S50S
G270A	Synonymous	V90V	G150T	Synonymous	S50S	A270G	Synonymous	V90V
A930T	Synonymous	G310G	G367C	Missense	E123Q	G367C	Missense	E123Q
T1107C	Synonymous	T369T	A451G	Missense	K151E	A451G	Missense	K151E
C1120T	Synonymous	L374L	C753T	Synonymous	Y251Y	C753T	Synonymous	Y251Y
C1209T	Synonymous	G403G	T1371A	Synonymous	S457S	T930A	Synonymous	G310G
A1284T	Synonymous	P428P				C1107T	Synonymous	T369T
C1305T	Synonymous	A435A				T1120C	Synonymous	L374L
						T1209C	Synonymous	G403G
						T1284A	Synonymous	P428P
						T1305C	Synonymous	A435A
						T1371A	Synonymous	S457S

SNP: single nucleotide polymorphism; aa: amino acid.

fully defined. Some of the proposed hypothesis are mucositis, exceptionally frequent among this patient population, and the selection pressure exerted by empirical therapeutics as well as antifungal prophylaxis on less susceptible *C. kefyri* strains [3]. Both mechanisms could have been involved in the case reported here. Indeed, colonization of the gastro-intestinal tract of the patient by *C. kefyri* was persistent in spite of antifungal treatment based on azole and echinocandin. This was intriguing because *C. kefyri* is considered as being susceptible to all systemic antifungals [13,14].

The *C. kefyri* PAZ isolate recovered was resistant to all azole antifungals, with very high MICs to fluconazole and voriconazole, and susceptible to echinocandins and amphotericin B. Taking advantage of the publication of the genome sequence of *K. marxianus* [7], the teleomorph of *C. kefyri*, we designed oligonucleotide primers for the cloning and sequencing of the different *ERG11* alleles from the azole resistant PAZ clinical isolate, and the azole susceptible TEM clinical isolate and reference strain CBS 5665 of *K. marxianus*.

Pairwise comparison of the nucleotide variability of the 1581 bp coding region of the different *ERG11* alleles revealed from

6 to 12 SNP according to the alleles considered. Most of them were silent mutations, but two, specific to the *ERG11* allele of the azole-resistant isolate, were missense mutations (G367C and A451G) encoding the amino acid substitutions E123Q and K151E. Both substitutions were located in the hot spot region 1 thought to be involved in the conformation of a channel for access of the inhibitors to the catalytic site [15–17]. The K151E substitution of the *C. kefyri* Erg11p corresponds to the well characterized K143E substitution of the *C. albicans* Erg11p, which was demonstrated to confer resistance to fluconazole, but no significant cross-resistance to other azole [18,19]. As frequently reported, this mutation was in fact often associated to another mutation for conferring high-level resistance to several azole antifungals [19]. Interestingly, an E123Q substitution was also present in the azole-resistant *C. kefyri* Erg11p. The occurrence of a substitution at the orthologous position (E115) in the protein of *C. albicans* was never described so far [17]. Although many other mechanisms can be involved in azole resistance, mainly *ERG11* upregulation and ABC efflux transporters overexpression [6], we believe that the combination E123Q and K151E could account for the high level resistance to fluconazole

and voriconazole, and azole cross-resistance phenotype in the PAZ *C. kefyri* isolate. In particular, the precise role in azole resistance of the newly described mutation E123Q of the *C. kefyri* Erg11p, ortholog of E115 in *C. albicans*, and of the β 1-5 segment of the protein that carries the substitution [16], remains to be demonstrated by functional allele replacement.

Although serum level of β -D-glucan strongly suggested an invasive fungal infection, the causal relevance of *C. kefyri* could not be strictly demonstrated in the case reported here. However, it underlines the risk of emergence of antifungal-resistant *C. kefyri* strains in patients with oncohematological diseases, as a consequence of the high prevalence in this species combined to the antifungal selective pressure exerted on a haploid yeast. Recently, it was reported the first *C. kefyri* isolate with acquired echinocandin resistance [4]. These findings, along with the case reported here, point the importance of antifungal susceptibility testing for the surveillance of emergent antifungal-resistant *C. kefyri* in oncohematology unit.

Conflict of interest

The authors have no conflict of interest. The authors are responsible for the content and writing of the paper.

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