GENETIC AND MOLECULAR INSIGHTS OF *CANDIDA KRUSEI* ANTIFUNGAL RESISTANCE

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À minha família

“Sem sonhos a vida não tem brilho. Sem metas, os sonhos não têm alicerces. Sem prioridades, os sonhos não se tornam reais. Sonhe, trace metas, estabeleça prioridades e corra riscos para executar seus sonhos. Melhor é errar por tentar do que errar por omitir.”

Augusto Cury; médico psiquiatra, psicoterapeuta e escritor. 1958

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"Ensinar não é transferir conhecimento, mas criar as possibilidades para a sua produção ou a sua construção. Quem ensina aprende ao ensinar e quem aprende ensina ao aprender."
Paulo Freire; educador, pedagosista e filósofo. 1921-1997.

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LIST OF PUBLICATIONS

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Manuscripts

I. **Ricardo E**, Silva AP, Gonçalves T, Costa de Oliveira S, Granato C, Martins J, Rodrigues AG, Pina-Vaz C. *Candida krusei* reservoir in a neutropaenia unit: molecular evidence of a foe? Clinical Microbiology and Infection. 2010. 17: 259-63.

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Abstracts

I. Pina-Vaz C, **Ricardo E**, Silva A, Granato C, Berganti R, Gonçalves T, Rodrigues AG. "Typing analysis of consecutive *C. krusei* isolates among Hemato-oncology patients." Clinical Microbiology and Infectious. 2009. 15 (S4): S636.

II. Lisboa C, **Ricardo E**, Azevedo F, Costa AR, Goncalves T, Rodrigues AG, Pina-Vaz C. "Typing of genital Candida isolates from couples using mitochondrial DNA typing". Mycoses. 2009. 52 (S1): 118.

III. **Ricardo E**, Costa-de-Oliveira S, Silva AP, Gonçalves T, Pina-Vaz C, Rodrigues AG. "Candidaemia: antifungal susceptibility and molecular typing profiles of concomitant isolates from blood and other biological products". Mycoses. 2009. 52 (S1): 97.

IV. **Ricardo E**, Faria-Ramos I, Costa de Oliveira S, Silva AP, Rodrigues AG, Pina-Vaz C. "Fungaemia by *C. krusei*: acquisition of voriconazole resistance *in vivo*." Clinical Microbiology and Infection. 2010. 16 (S2): S691-2.

V. **E Ricardo**, Faria-Ramos I, Miranda IM, Rodrigues AG, and Pina-Vaz C. "*In vivo* and *in vitro* acquisition of resistance to voriconazole by *C. krusei*: the role of efflux mechanism". Mycoses. 2011. 54 (S2): 177.

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LIST OF ABBREVIATIONS

ABC	Adenosine triphosphate Binding Cassette
AMB B	Amphotericin B
AND	Anidulafungin
ATP	Adenosine triphosphate
BBE	Basal Expression Element
BSI	Bloodstream Infections
CCCP	Carbonyl cyanide 3-chlorophenyl hydrazone
CDR	*Candida* Drug Resistance
CKRS-1	*Candida krusei* Repeated Sequence – 1
CLSI	Clinical Laboratory Standards Institute
CSF	Caspofungin
DNA	Deoxyribonucleotide Acid
dNTPs	Deoxynucleoside Triphosphates
DRE	Drug-Responsive Element
DTT	Dithiothreitol
ECMM	European Confederation of Medical Mycology
EUCAST	European Committee on Antimicrobial Susceptibility Testing
5-FC	5-Flucytosine
FLC	Fluconazole
5- FU	5 – flurouracil
5 – FUdRMP	5 – fluorodeoxyuridine monophosphate
HS	Hot Spot
IC	Invasive Candidiasis
ICU	Intensive Care Unit
ID	Identification
IDSA	Infectious Diseases Society of America
IGRs	Intergenic regions
ITC	Itraconazole
IV	Intravenous

KTC	Ketoconazole
MCF	Micafungin
MCZ	Miconazole
MDR	Multidrug-Resistant
MFS	Major Facilitator Superfamily
MIC	Minimal inhibitory Concentration
MLEE	Multi-locus Enzyme Electrophoresis
MLST	Multi-locus Sequence Typing
mtDNA	Mitochondrial DNA
NBDs	Nucleotide Binding Domains
NCCLS	National Clinical Collaborative Laboratory Standards
NRE	Negative Regulatory Element
NRS	Nourseothricin
PCR	Polymerase Chain Reaction
PFGE	Pulsed- field Gel Electrophoresis
PSC	Posaconazole
RAPD	Random Amplified Polymorphic DNA
REA	Restriction Endonuclease Analysis
SPSS	Statistical Package for Social Sciences
SREs	Steroid Responsive Element
TBE	Tris- Borate –EDTA
TE	Tris - EDTA
TMS	Trans – membrane α-helical Segments
TMDs	Trans – membrane Domains
UTI	Urinary Tract Infections
VRC	Voriconazole

LIST OF TABLES AND FIGURES

List of Tables

Chapter II. Introduction

Table 1 – Risk factors predisposing individuals to disseminated candidiasis.

Chapter IV. Mechanisms of resistance in *Candida krusei*

i. *In vivo* and *in vitro* acquisition of resistance to voriconazole by *Candida krusei*

Table 1 - MIC and susceptibility profile of *C. krusei* clinical isolates.

Table 2 - MIC and susceptibility profile to voriconazole, alone and in combination with FK506 of *C. krusei* strains.

ii. Candiduria due to *Candida krusei*: a case of induction of resistance *in vivo*

Table 1 – MIC, susceptibility profile, *ABC1*, *ABC2* and *ERG11* relative gene expression profile and *ERG11* gene sequence alterations of *C. krusei* strains induced *in vitro*.

Table 2 - MIC and susceptibility profile to VRC alone and in association with FK506.

List of Figures

Chapter II. Introduction

Figure 1 – Antifungal drugs mechanisms of action. Azoles antifungals inhibit the enzyme lanosterol 14α- demethylase, involved in the synthesis of ergosterol, a main component of the cell membrane. This inhibition leads to the production of toxic compounds altering cell membrane structure and permeability. Polyenes bind to ergosterol molecules forming pores in the membrane. Pyrimidine analogues induce the production of toxic compounds that interfere with the nucleic acid metabolism and consequently protein synthesis. Echinocandinds inhibit the enzyme β (1,3) – glucan synthase leading to alterations in the cell wall structure.

Figure 2 – Fluconazole chemical structure.

Figure 3 – Voriconazole chemical structure.

Figure 4 – Posaconazole chemical structure.

Figure 5 – Amphotericin B chemical structure.

Figure 6 – Echinocandins chemical structure. Each antifungal drug belonging to the class of the echinocandin presents a general chemical structure (A), differing in their side chains, namely, CSF, AND and MCF (B).

Figure 7 – 5-Flucytosine chemical structure.

Chapter III. *Candida krusei* reservoir in a neutropenic unit: molecular evidence of a foe?

Figure 1 – Restriction Endonuclease patterns of Hinf I-digested DNA obtained after agarose gel electrophoresis. Each pattern corresponds to *C. krusei* isolates from patients A, B, C and D (upper case letters), from different biological products (BC – Blood cultures; CVC- central venous catheter; BS - bronchial secretions; S – stools), and to *C. krusei* isolates from the room environment of patients A and C (A_E and C_E, respectively); M- Molecular Weight Marker (1-kb DNA ladder, Metabion).

Chapter IV. Mechanisms of resistance in *Candida krusei*

i. *In vivo* and *in vitro* acquisition of resistance to voriconazole by *Candida krusei*

Figure 1 - Induction of resistance to voriconazole *in vivo*. The timeline of antifungal therapy and the recovery of *C. krusei* clinical strains from the leukemia patient, with the respective susceptibility profiles and relative gene expression levels is shown. ID, identification; S, susceptible; R, resistant. ✞ Patient death. *$P \leq 0.05$.

Figure 2 - Induction of resistance to voriconazole *in vitro*. The timeline of the development of VRC resistance in a susceptible *C. krusei* clinical isolate that was exposed daily to VRC at 1 µg/ml, with the respective susceptibility profiles and relative gene expression levels, is shown. ID, identification; S, susceptible, R, resistant. *$P \leq 0.05$.

Figure 3 - Genotyping of clinical isolates. (A) Restriction endonuclease pattern of HinfI-digested DNA of *C. krusei* clinical isolates in agarose gel electrophoresis; (B) Random Amplification Polymorphic DNA (RAPD) of *C. krusei* clinical isolates with primers OPA-18 and OPE-18. Lanes M, 1-kb DNA ladder (Metabion); lane 1, S_{D0}, lane 2, $R_{clinD24}$, lane 3, $R_{clinD35}$, lane 4, $R_{clinD84}$, lane 5, Non related *C krusei* control strain; lane NC, PCR negative control.

Figure 4 - FK506 disk diffusion assay. Resistant *C. krusei* strains were grown on YPD medium and paper disks impregnated with serial 10-fold dilutions of FK506, ranging from 1000 to 1 µg/ml, without VRC (A and C), or VRC at 4 µg/ml (B and D). $R_{clinD24}$ (A and B) and R_{indD30} (C and D) are shown as representative examples of *C. krusei* strains with *in vivo* and *in vitro* induced resistance, respectively. S1, 100%, S2, 10%: disks impregnated with DMSO at different concentrations.

Figure 5 - *ABC1*, *ABC2* and *ERG11* relative gene expression levels in resistant *C. krusei* clinical isolates (A) and resistant strains induced *in vitro* (B). *ABC1*, *ABC2* and *ERG11* gene expression levels were quantified and normalized relative to the housekeeping gene, *ACT1*; relative gene expression levels were calculated as ratios between each *C. krusei* resistant strain and the S_{D0} isolate; * $P \leq 0.05$.

ii. Candiduria due to *Candida krusei*: a case of induction of resistance *in vivo*

Figure 1 - *In vivo* induction of resistance to voriconazole. (a) Timeline of the renal transplant procedure, antifungal therapy and *C. krusei* clinical isolates recovered from the kidney transplant patient and their respective susceptibility profile; (b) Genotyping of *C. krusei* clinical isolates. 1- $Ck_{B.VRC}$, 2- $Ck_{D.VRC9}$, 3- $Ck_{D.VRC16}$; 4- $Ck_{A.VRC10}$, $Ck_{A.VRC18}$, NRS 1-3 – Non related isolates, M – Molecular weight; (c) Relative gene expression profile of *ABC1*, *ABC2* and *ERG11* genes for the *C. krusei* clinical isolates; *$p \leq 0.05$.

Figure 2 - FK506 agar disk diffusion assay**:** resistant *C. krusei* strains were grown on YPD medium and paper disks impregnated with serial 10-fold dilutions of FK506, ranging from 1000 to 1 μg/ml (FK506 disks - left column) and DMSO (S1 – 100%, S2 – 10%) and in the presence of VRC 4 μg/ml (VRC 4 μg/ml + FK506 disks- right column). (a) *C. krusei* resistant clinical isolates recovered from the kidney transplant patient; (b) *C. krusei* resistant strains induced *in vitro*, after incubation with VRC.

Figure 3 - *ABC1*, *ABC2* and *ERG11* relative gene expression level in *C. krusei* resistant clinical isolates (A) and resistant *in vitro* induced strains (B). *ABC1*, *ABC2* and *ERG11* gene expression level was quantified and normalized relative to the housekeeping gene, *ACT1*; relative gene expression level was calculated as a ratio between each *C. krusei* resistant strain and the respective susceptible isolate; * $p \leq 0.05$.

ABSTRACT

Candida krusei is an agent of opportunistic fungal infections, presenting intrinsic resistance to one of the first line therapy antifungals, fluconazole. It is also considered an emerging pathogen specially associated with patients presenting hematological malignancies. The hypothesis of an outbreak was considered due to the consecutive isolation of *C. krusei* strains from different patients staying in the Neutropenic Unit of hospital S. João, Porto, Portugal, within only a two month period. *Candida krusei* strains were also collected from the room´s surfaces, such as bed and bedside table. From one of the patients it was available a *C. krusei* isolate recovered two years before this episode, since this patient had a long history of admission to the hospital S. João. The hypothesis of an outbreak was excluded, after comparing the mitochondrial DNA restriction profiles using *restriction endonuclease analysis*: the *C. krusei* isolates from the different patients presented distinct molecular typing patterns. *Candida krusei* strains recovered from the same patient and from its respective bedside table or bed presented the same typing pattern. Although the hypothesis of an outbreak was excluded this work represented a landmark in the implementation of hygiene measures among the health care workers when dealing with the patients and in the maintenance of the surrounding environment of the patients.

From one of the patients involved in the hypothetical outbreak, a leukemia patient, several *C. krusei* isolates were recovered from different biological products, therefore he was prescribed voriconazole therapy. The first isolates, recovered before the voriconazole therapy, presented a susceptible phenotype but after three weeks of voriconazole therapy, resistant isolates to voriconazole were recovered. After performing the molecular typing of the isolates using *restriction endonuclease analysis* and *random amplified polymorphic DNA* analysis, it was proved the acquisition of resistance to voriconazole *in vivo*. The first susceptible *C. krusei* isolates presented the same molecular typing pattern than the latter resistant *C. krusei* isolates. Moreover, the acquisition of resistance *in vivo* was replicated by incubating one of these susceptible *C. krusei* isolates with a sub-inhibitory concentration of voriconazole. The aim of the work was accomplished since a minimal inhibitory concentration of 4 μg/ml was

achieved after only 5 day of exposure to voriconazole, which was maintained stable even after withdrawn of the antifungal.

Another case with common characteristics was studied: a patient submitted to a kidney transplant was diagnosed candiduria due to *C. krusei*, and therefore began treatment with voriconazole. Although not usually considered a risk situation, after the determination of the susceptibility profile we were faced with two resistant isolates recovered during voriconazole therapy. The *C. krusei* isolates recovered before and after voriconazole therapy ended presented a susceptible phenotype. Moreover, the transient acquisition of resistance *in vivo* was confirmed: all the clinical isolates presented similar amplification patterns after their genotyping by PCR amplification of the intergenic repeat-PCR (CKRS-1) sequence. It was also evaluated how voriconazole at low concentrations (similar to the concentration achieved in the bladder) could influence the susceptibility profile of eight independent clinical strains susceptible to voriconazole from different backgrounds. Therefore, the acquisition of resistance *in vivo* was successfully reproduced *in vitro*.

At this point, the associated mechanisms of resistance of all the previously referred resistant strains were evaluated. Different approaches were used: a functional assay, using the efflux pump blocker Tacrolimus (FK506), and molecular assays, such as the quantification of gene expression and nucleotide sequencing. A synergistic effect was registered in the presence of Tacrolimus and voriconazole in both the microdilution assay and the test disk assay. The quantification of resistance gene expression showed that *ABC1* gene was significantly overexpressed in the majority of the *C. krusei* strains induced both in vivo and *in vitro* in the two clinical cases. *ABC2* gene presented no significant gene expression alteration in the resistant *C. krusei* strains therefore played a minor role in conferring resistance. The only exception was one of the strains obtained after the incubation *in vitro* with low concentrations of voriconazole which presented a sixteen fold increase in *ABC2* gene expression. Overall, the different clinical isolates incubated *in vitro* with very small concentrations of voriconazole developed different *ABC1* and *ERG11* gene expression profiles, and different alterations in *ERG11* gene nucleotide sequence. *ERG11* gene sequencing showed different types of mutations: several heterozygous alterations at positions T1389T/C

and C642C/T, corresponding to a synonymous mutation and heterozygous alterations resulting in non-synonymous single nucleotide polymorphisms at positions G364T/G (Ala→Ser) and T418T/C (Tyr→His). Since these alterations were found in the susceptible and resistant *C. krusei* strains they are not directly associated to voriconazole resistance. The most important alteration described was the homozygous missense mutation, at position T418C, in different resistant *C. krusei* strains yielding a Tyr→His amino acid change. Therefore, this mutation was associated to voriconazole resistance in those strains. Due to the lack of information concerning the *C. krusei* genome most probably other mechanism of resistance are yet to uncover.

Overall this work highlights the relevance of the ATP-dependent efflux pumps activity, namely Abc1p, in voriconazole antifungal resistance and the description of new mutations in *ERG11* gene among resistant *C. krusei* clinical isolates.

Resumo

Candida krusei é um agente patogénico relacionado com infeções fúngicas oportunistas, apresentando resistência intrínseca a um dos fármacos mais utilizados em terapia antifúngica, o fluconazole. *Candida krusei* é considerado um agente emergente sendo frequentemente isolado de doentes com doença hematológica. Foi colocada a hipótese de ocorrência de um surto devido ao isolamento consecutivo de várias estirpes de *C. krusei* de diferentes doentes internados na unidade de doentes neutropénicos do Hospital de S. João, Porto, Portugal, durante um período de 2 meses. Estirpes de *C. krusei* foram também isoladas das respetivas cama ou mesa-de-cabeceira dos doentes. Foi ainda incluída neste estudo uma estirpe isolada de um dos doentes, dois anos antes, uma vez que este apresentava um longo historial de internamento no Hospital de S. João. A hipótese de surto foi excluída após a análise comparativa dos perfis de restrição de DNA mitocondrial através da técnica de "*restriction endonuclease analysis*", uma vez que isolados clínicos de *C. krusei* de diferentes doentes apresentavam também padrões de restrição molecular distintos. Por outro lado, os isolados clínicos do mesmo doente e da respetiva cama ou mesa-de-cabeceira apresentavam o mesmo padrão de restrição molecular. Mesmo tendo sido excluída a hipótese de surto, o presente trabalho representa uma chamada de atenção para a implementação de medidas de higiene rigorosas entre os profissionais de saúde. Estes cuidados devem ser considerados não só na manipulação de doentes mas também na manutenção de um ambiente hospitalar adequado e seguro à permanência de doentes imunocomprometidos sem correrem riscos de infeção.

De um dos doentes envolvido no hipotético surto, um doente com leucemia, vários isolados clínicos de *C. krusei* foram obtidos a partir de diferentes produtos biológicos, pelo que iniciou tratamento com voriconazole. Os primeiros isolados clínicos obtidos antes da terapia antifúngica apresentavam um fenótipo suscetível, mas após 3 semanas de terapia, foram detetados isolados resistentes ao voriconazole. Após a tipagem molecular dos diferentes isolados, recorrendo às técnicas de "*restriction endonuclease analysis"* e *"random amplified polymorphic DNA"*, verificou-se que os primeiros isolados suscetíveis de *C. krusei* apresentavam o mesmo padrão molecular

que os isolados resistentes obtidos posteriormente. Desta forma, confirmou-se a aquisição de resistência *in vivo*. Por outro lado, pretendeu-se mimetizar *in vitro* a aquisição de resistência ao voriconazole *in vivo* por incubação de uma estirpe suscetível isolada desse mesmo doente, com uma concentração sub-inibitória de voriconazole. O objetivo foi alcançado uma vez que após 5 dias de incubação com o antifúngico as estirpes apresentavam uma concentração inibitória mínima ao voriconazole de 4 μg/ml. Mesmo após a remoção do antifúngico a estirpe manteve o fenótipo resistente.

Outro caso clínico com alguns aspectos em comum foi também estudado: foi diagnosticada candiduria por *C. krusei* num doente submetido a transplante de rim. O doente foi submetido a terapia antifúngica com voriconazole. A candiduria não é normalmente considerada uma situação de risco mas após avaliação do perfil de suscetibilidade verificou-se a existência de isolados de *C. krusei* resistentes ao voriconazole. Os isolados clínicos obtidos antes e depois da terapia antifúngica apresentavam fenótipo suscetível enquanto os isolados obtidos durante a terapia antifúngica apresentavam um fenótipo resistente. Comprovou-se a aquisição transitória de resistência *in vivo*: após a tipagem dos vários isolados clínicos por amplificação por PCR da sequência repetitiva intergénica (CKRS-1) verificou-se que estes apresentavam padrões moleculares semelhantes. Também se pretendeu avaliar o efeito da presença de uma dose mínima de voriconazole (semelhante à dose atingida na bexiga) no perfil de suscetibilidade de oito isolados clínicos suscetíveis de *C. krusei* provenientes de diferentes origens. Desta forma, a aquisição de resistência *in vivo* foi mimetizada *in vitro*.

Nesta fase, pretendeu-se estudar os mecanismos de resistência nas estirpes de *C. krusei* incluídas neste estudo. Diferentes abordagens foram realizadas: ensaios funcionais usando um bloqueador de bombas de efluxo, e estudos moleculares tais como a quantificação da expressão de genes associados a resistência e sequenciação nucleotídica. Foi registado um efeito sinérgico na presença do tacrolimus (FK506) e voriconazole quer nos ensaios de microdiluição e nos ensaios de difusão em disco. A quantificação da expressão dos genes de resistência mostrou que o gene *ABC1* é significativamente sobre-expresso na maioria das estirpes de *C. krusei* induzidas *in*

vivo em ambos os casos clínicos e *in vitro*. O gene *ABC2* não apresenta uma variação significativa na expressão em estirpes resistentes pelo que não será um gene relevante na resistência ao voriconazole. A única exceção foi uma das estirpes obtidas após incubação com concentração baixa de VRC que apresentou um aumento de 16x na expressão do gene *ABC2*. No geral, os diferentes isolados clínicos incubados *in vitro* com concentração baixa de voriconazole desenvolveram diferentes perfis de expressão dos genes *ABC1* e *ERG11* e diferentes alterações na sequência nucleotídica do gene *ERG11*. A sequenciação do gene *ERG11* levou à deteção de diferentes tipos de alterações na sua sequência nucleotídica: alterações heterozigóticas sinonimas nas posições T1389T/C e C642C/T e alterações heterozigóticas não-sinonimas nas posições G364T/G (Ala→Ser) e T418T/C (Tyr→His). Uma vez que estas alterações foram encontradas em estirpes suscetíveis e resistentes não foram nestes casos associadas a resistência ao voriconazole. No entanto, algumas estirpes resistentes de *C. krusei* apresentaram uma mutação "missense" homozigótica na posição T418C (Tyr→His), pelo que esta mutação está associada a resistência. Devido à falta de informação relativamente ao genoma de *C. krusei*, outros mecanismos de resistência ainda não descritos podem estar presentes nestas estirpes.

Em conclusão, este trabalho salienta a importância da atividade de bombas de efluxo dependentes de ATP, nomeadamente Abc1p, na resistência ao voriconazole e a descrição de novas mutações no gene *ERG11* em estirpes clinicas de *C. krusei* resistentes aos azoles.

CONTENTS

Acknowledgments/ Agradecimentos ____ xiii

List of Publications ____ xvii

List of Abbreviations ____ xix

List of Tables and Figures ____ xxi

Abstract ____ xxv

Resumo ____ xxviii

Contents ____ xxxi

CHAPTER I: AIMS AND OUTLINE OF THE THESIS ____ 1

CHAPTER II: INTRODUCTION ____ 9

The genus Candida ____ 13

Candida krusei ____ 14

Epidemiology of Candidiasis ____ 15

Identification and Typing Methods ____ 20

Restriction enzyme analysis (REA) ____ 21

Random Amplified Polymorphic DNA (RAPD) analysis ____ 22

PCR-Based Amplification of the Repetitive Polymorphic Sequence CKRS-1 ____ 22

Antifungal therapy and susceptibility assays ____ 23

The Azoles ____ 26

The Polyenes ____ 30

The Echinocandins ____ 31

The Pyrimidine analogues ____ 34

Antifungal Susceptibility Assays ____ 35

Antifungal Resistance Mechanism ____ 37

Ergosterol biosynthesis pathway: an unsuitable antifungal target for *C. krusei* ____ 38

Efflux mediated by ABC transporters ____ 39

Acquisition of mutations to echinocandins ____ 41

CHAPTER III: *CANDIDA KRUSEI* RESERVOIR IN A NEUTROPAENIA UNIT: MOLECULAR EVIDENCE OF A FOE? ____ 43

Abstract ____ 47

Background ____ 48

Patients and Methods ____ 50

Results and Discussion ____ 54

CHAPTER IV: MECHANISMS OF RESISTANCE IN *CANDIDA KRUSEI* ____ 57

i. *In vivo* and *in vitro* acquisition of resistance to voriconazole by *Candida krusei* ____ 61

Abstract ____ 62

Background ____ 63

Material and Methods ____ 65

Results ____ 73

Discussion ____ 79

ii. Candiduria due to *Candida krusei*: a case of induction of resistance *in vivo* ____ 83

Abstract ____ 84

Background ____ 86

Material and Methods ____ 88

Results ____ 95

Discussion ____ 102

CHAPTER V: OVERALL DISCUSSION AND CONCLUSIONS ____ 105

CHAPTER VI: FUTURE PERSPECTIVES ____ 115

CHAPTER VII: REFERENCES ____ 121

CHAPTER VIII: PUBLICATIONS ____ 155

Chapter I

AIMS AND OUTLINE OF THE THESIS

“As grandes ideias surgem da observação dos pequenos detalhes.”

Augusto Cury; médico psiquiatra, psicoterapeuta e escritor. 1958

AIMS OF THE STUDY

The aims of the present work were to genotype *C. krusei* strains from different sources and to clarify the molecular mechanisms of resistance to voriconazole in *C. krusei* strains. Susceptibility profile evaluation and molecular studies were performed in order to understand the overall process of acquisition and development of resistance both *in vivo* and *in vitro*.

Overall, the global aims were:

i. Identification and genotyping of *C. krusei* clinical isolates;

ii. Determination of the susceptibility profile of *C. krusei* clinical isolates to different classes of antifungals;

iii. Understand the evolution of the susceptibility profile *in vivo*, namely, the acquisition of antifungal resistance;

iv. Replicate *in vitro* the acquisition of resistance *in vivo*, in order to mimic the clinical conditions concerning the antifungal therapeutic;

v. Understand which mechanisms of resistance were present in the *C. krusei* strains involved in the study.

vii. Establish the importance of efflux activity in antifungal resistance.

viii. Search for point mutations or heterozygous polymorphisms in the target gene *ERG11*.

OUTLINE OF THE THESIS

The present work is divided in chapters, which include the different works presented throughout the years in international conferences as poster presentations or abstracts published online and as published manuscripts in international indexed journals.

Chapter I includes the aims of the work and an explanation of the structure of the thesis, in order to facilitate the reading and understanding of the overall work herein presented.

Chapter II refers to the introduction section. After a large survey throughout the literature in the field, introduction presents the most important facts published in the last decades related to the present work. It constitutes a theoretical base to understand and discuss the work presented in the following chapters.

Chapter III includes the first work published in an international indexed journal. It describes the case of several patients with candidiasis by *C. krusei* in the Neutropenic Unit of Hospital S. João, within a short period of time. The hypothesis of occurrence of an outbreak was evaluated. Fortunately, that hypothesis was discarded but another serious situation was encountered in one of the patients studied: the acquisition of resistance to voriconazole *in vivo*.

Chapter IV involves two different works but with the same aims: the study of the acquisition of resistance *in vivo* and *in vitro*. The first case, also already published in an international journal, describes a leukemia patient with several clinical isolates of *C. krusei* recovered during a long period of voriconazole treatment. The second case is a patient submitted to a kidney transplant, diagnosed with candiduria and also submitted to voriconazole treatment but for a shorter period of time. In this case, *C. krusei* isolates were recovered before, during and after antifungal therapy. This work is submitted for publication.

Chapter V includes an overall discussion of the different works presented in the two previous chapters. General conclusions are presented in this chapter.

Chapter VI describes the future perspectives, i. e., intends to point new directions based on the current findings for a more profound study of the molecular mechanisms of resistance.

Chapter VII lists the bibliography accessed throughout the development of the work and thesis.

Chapter VIII includes the publications associated to the present work.

Chapter II

INTRODUCTION

“Existem muitas hipóteses em ciência que estão erradas.

Isso é perfeitamente aceitável, elas são a abertura para achar as que estão certas”.

Carl Sagan, astrobiólogo, astrônomo, astrofísico, cosmólogo, escritor e divulgador científico.

1934- 1996.

The genus *Candida*

The genus *Candida* was first discovered by Langenbeck in 1839 from buccal aphthae in a patient with typhus [1]. The Candida name has its origin in the ancient Rome tradition of a *Candidatus*, i. e., a candidate for public office to dress in white. The genus *Candida* comprises heterogeneous anamorphic yeasts, which are able to grow in three different morphologies: yeast, pseudohyphae and hyphae. They are ubiquitous and opportunistic commensal organisms of the human host. There are approximately 200 species of *Candida*, however only a small number is pathogenic to the human host, since more than 65% do not grow at the human body temperature, 37ºC [2]. *Candida* species are mostly isolated from the genital area, oral cavity, gastrointestinal tract, blood, normally sterile body fluids, urine, respiratory tract, skin and soft tissue [3]. When there is a shift in the balance of the fungus-host interaction, in favor of the fungus, an infection process can occur, ultimately leading to a disease condition. The classification of fungal infections is dependent on the degree of tissue involvement and the mode of entry of the pathogen: (i) superficial infections are localized to the skin, hair, and nails and are the most common yeast cell's infections; (ii) subcutaneous infections are confined to the dermis, subcutaneous tissue, or adjacent structures; and (iii) systemic infections are confined to deep infection of the internal organs, entering the blood stream and causing disseminated infection. The systemic infections usually occur only in immunocompromised patients.

Complementing functional and phenotypic studies, molecular studies of *Candida* spp. have increased exponentially in the last decades due to the sequencing of several genomes of pathogenic yeast [4-6]. This fact allowed the scientific community involved in the yeast research to uncover the different mechanism of interaction between host and pathogen, to describe in detail several molecular mechanisms of resistance to antifungals or the development of molecular tools to manipulate pathogenic yeasts genomes.

Candida krusei

Candida krusei was first described by Castellani in 1910 as *Sacharomyces krusei*. Several other names were proposed: *Endomyces krusei* in 1912, *Monilia krusei* in 1913, by Chalmers and eighteen other synonyms were proposed before Berkhout - renamed it in 1923 as *Candida krusei* [7-8]. In 1960, *Issatchenkia* was proposed by Kudryavtsev for the ascosporic state of *C. krusei*. This genus was characterized by the formation of spherical, possibly roughened ascosporous formed in a persistent ascus. Kurtzman et al in 1980 assigned additional species to this genus as *Issatchenkia orientalis* [9]. *Candida krusei* is the asexual (anamorphic) form, not producing ascosporous and considered the imperfect form, while *Issatchenkia orientalis* or *Pichia kudriavzevii* is the teleomorph (non-spore) form with a complete meiotic sexual cycle, considered as the perfect form [9]. The transformation between these 2 forms is a complicated matter and usually depends on the environmental conditions [10, 11]. *Candida krusei* as it will be referred from this point is usually found in 2 basic morphological forms (dimorphism): yeast and pseudohyphae [12]. Both are frequently present in growing cultures and are not easily separated. The pseudohyphae state makes *C. krusei* invasive conversely to the non-invasive yeast form [13].

Candida krusei displays 8 chromosomes with a total size of about 20Mb estimated by pulsed- field gel electrophoresis (PFGE) [14]. It is likely diploid, as it was already demonstrated in different studies: *C. krusei* is heterozygous at a *URA* gene [15], rDNA and TUB2 probes hybridized to two chromosomes bands [14] and the presence of 2 alleles for *ERG11* gene were described [16]. Considering its genetic traits, *C. krusei* is more closely related to *Candida glabrata* and *Sacharomyces cerevisiae* than to *Candida albicans*. For example, *C. krusei* translates the codon CUG as leucine, similarly to *C. glabrata* and *S. cerevisiae* [17, 18].

Similar to other *Candida* species, *C. krusei* presents several virulence attributes, namely adherence to host surfaces, production of phospholipases and proteinases, antigenic variability, dimorphic transition (yeast to pseudohypha), phenotypic switching or capability of switching among different cells phenotype and modulation of the host's immune response [13]. Nevertheless, several virulence comparative studies performed among Candida species had concluded that *C. krusei* is relatively less virulent than

other commonly pathogenic *Candida* spp. such as *C. albicans* [19-22].

The study of *C. krusei* is always a challenge since the available information concerning this species is scarce when compared to other pathogenic yeasts.

Epidemiology of Candidiasis

In the end of the 20th century a dramatic increase in mortality rates due to mycoses was registered in the United States: from 1557 deaths in 1980 to 6534 deaths in 1997 [23]. These deaths were mostly related to *Candida*, *Aspergillus* and *Cryptococcus* spp infections, being *Candida* spp in the top of list as the most important cause of opportunistic mycoses worldwide [24]. *Candida* species are the fourth leading cause of healthcare related bloodstream infections (BSI) in the United States representing for 8% to 10% of all BSI acquired in the hospital [25-28]. Invasive *Candida* infections are usually healthcare-associated infections and may be either nosocomial (onset after 48h of hospitalization) or community-onset (occurring within 2 days of admission) [24, 29, 30]. The most frequently isolated pathogenic species of *Candida* spp., accounting for more than 90% of invasive candidiasis (IC) in humans are *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei* [24, 31-33]. A study by Pfaller and Diekema showed that the distribution of species responsible for invasive infections has changed in the last years: the rate of isolation of *C. albicans* decreased from 73.3% in the period 1997-1998 to 62.3% in 2003 [3]. Also, a more recent study by Pfaller et al, 2012 involving 23 medical centers in the USA and 2 in Canada, registered a new distribution of Candida species: *C. albicans* (42.1%), *C. glabrata* (26.7%), *C. parapsilosis* (15.9%), *C. tropicalis* (8.7%), and *C. krusei* (3.4%) [33]. Overall, the proportion of candidemia caused by non-*albicans* Candida spp. (57.9%) was higher than that caused by *C. albicans* (42.1%) solely [33]. This alteration in species distribution can be associated with different factors such as the reduced susceptibility of *Candida* species to fluconazole (FLC) due to its extensive use throughout the 1990s, the increase in the number of immunocompromised patients vulnerable to infection with less virulent species of *Candida* or even as a consequence of the recent progress in isolation and identification methods for *Candida* species [34].

In Europe, according to the European Confederation of Medical Mycology

(ECMM) survey presented in 2004, *C. albicans* was responsible for more than one-half of the Candida infection cases in all the patient populations, except in patients with hematological malignancies. In these patients, *C. albicans* was isolated in 35% of the cases, *C. tropicalis* in 17% and other Candida species in 24%, with *C. krusei* involved in 12% of these cases [35]. Similar to what happens in the United States, Candida species frequency varies among different European countries [35]. The first Portuguese study performed at a large Portuguese University Hospital, Hospital S. João, EPE, involved 117 patients with the diagnosis of fungaemia, therefore not limited to infection by *Candida* spp. Yeasts were the fourth most common agent isolated from blood. *Candida albicans* ranked on the top of the list corresponding to 35% of the yeast isolates, followed by *C. parapsilosis* (25.6%), *C. tropicalis* (12.8%), *C. neoformans* (10.3%) and *C. glabrata* (7.7%) [36].

The risk factors associated to infection by *Candida* spp have been well established and have not changed substantially in the past 2 decades (detailed in Table1).

Table 1. Risk factors predisposing individuals to disseminated candidiasis

Risk factor	References
Cancer chemotherapy	37; 38
Prior colonization and the use of intravenous catheters	36; 39; 40
Therapy with broad-spectrum antimicrobial agents	38; 39; 41;
Metabolic dysfunction such as Diabetes	42; 43
Immunosuppression (including neutropenia)	44-46
Mucosal colonization by *Candida* spp	47
Total parenteral nutrition (TPN)	36, 48; 49
Surgery (including organ transplantation	36; 47; 49
Renal failure or hemodialysis	47; 49-53

Additionally, patients with hematological malignancies are more prone to BSI caused by *non-albicans Candida* species, namely, *C. tropicalis*, *C. parapsilosis* and *C. krusei* [44, 45].

According to the ECMM survey, candidemia is associated to a high mortality rate (38%) [51, 54, 55]. Although less frequently isolated, *C. krusei, C. glabrata* and *C. tropicalis* BSIs, appeared particularly severe, with an unfavorable outcome in more than 40% of the patients [51]. In the Portuguese study, the mortality rate associated with fungemia was 39.3%; the highest values were found in patients yielding *C. glabrata* (78%), *C. tropicalis* (53%) and *C. albicans* (46%) infection. Seventy-five per cent of the fungemia episodes were nosocomial, with 48% mortality rate [36]. The high crude mortality rate of BSIs caused by these non-albicans species may be due to their occurrence in patients with underlying life-threatening conditions. Death has been considered to be directly attributable to candidemia in 8% of cancer patients [56] and 20–31% of patients staying in intensive care units (ICU) [57-59]. This makes the study of these non-*albicans Candida* species such an important issue.

Besides candidemia the occurrence of candiduria is also observed in hospitalized patients. A survey of urine cultures obtained from hospitalized patients throughout European hospitals, showed that *Candida* species were the third most common organism isolated from urine after *Escherichia coli* and *Enterococcus* species [60]. Most of the fungal infections of the urinary tract are due to *C. albicans*, accounting for 50% to 70% of the isolates [60-63]. *C. glabrata* and *C. tropicalis* are usually the second most common cause of urinary tract infections [63-65]. Other *Candida* species such as *C. parapsilosis*, 1% to 7%, or *C. krusei*, 1% to 2% are less commonly found in the urine [64]. Although few studies are available, several risk factors were already described: urinary drainage devices, urinary tract obstruction, surgery or instrumentation, *diabetes mellitus*, antibiotic use and older age [61, 62, 66]. They also concluded that these risk factors are also valid not only for hospitalized patients but also for the community in general. There is the general question if candiduria can lead to candidemia. In a large prospective surveillance study, only 7 out of 530 candiduric patients, corresponding to 1.3%, developed candidemia [61]. In another study performed in an ICU setting from France, only 5 patients developed candidemia from a total of 233 patients with candiduria, due to the same species 2 to 15 days later [67]. However, other studies indicate candiduria as a well-established risk factor for IC which

in turn is associated with significant morbidity, mortality and cost in ICU patients [68, 69]. Candida species cause urinary tract infection (UTI) by two different routes: the hematogenous route and the most common, the ascending route. The pathogenesis of ascending infection has not been studied in depth and the data are scarce [70]. For example, some studies show that the spread from the perineum into the bladder leads to colonization and the retrograde spread occurs into the collecting system of the kidney [71]. Also, the presence of an indwelling catheter allows for biofilm and fungus balls formation, i.e., mass of hyphae and yeast cells, and consequently the persistence of the organism in the host [70].

One of the largest concerns when dealing with a patient with candiduria is deciding whether this finding represents a contaminated urine sample, an organism colonizing the bladder and/or catheter, or an infection of the urinary tract. Additionally, candiduria may be a manifestation of candidemia rather than UTI; therefore it is important to understand the meaning of the presence of *Candida* in the urine [72]. Urine analysis and culture are the first laboratory studies that should be performed. The techniques routinely used in most clinical microbiology laboratories for the detection of bacteria are adequate for detecting yeasts in urine. The management of candiduria remains controversial, mainly due to clinicians' uncertainties when to initiate antifungal therapy for candiduria [73-76]. Among the different classes of antifungal drugs none of them presents a perfect therapeutic outcome. Fluconazole (FLC) is normally the first line treatment option as it is excreted unchanged by the kidneys, ketoconazole (KTC) and itraconazole (ITC) are poorly excreted in the urine, voriconazole (VRC) and posaconazole (PSC) achieve minimal urinary excretion, amphotericin B (AMB B) presents high renal toxicity, leading to decreased glomerular filtration rate; all the echinocandins are extremely poorly glomerularly filtrated or tubularly secreted *in vivo*, as 2–3% of active drug is eliminated in the urine, resulting in subtherapeutic concentrations in the urine [77-80]. Nevertheless, some antifungal therapies present positive outcomes: there are observations about several successful outcomes for the treatment of symptomatic candiduria using caspofungin (CSF) [81]. Usually, FLC achieves 10-fold higher concentrations in the urine than in serum [75]. A single 400 mg dose of FLC leads a concentration in excess in the urine of 100 mg/ml in patients with

normal renal function [77]. Overall, the treatment of candiduria involves many issues: asymptomatic candiduria should not be treated with antifungal agents, except in certain population at risk of candidemia, antifungal susceptibilities and concentrations of antifungal agents in the urine should be taken into account in the choice of the appropriate antifungal agent, the persistence of indwelling urinary catheters can interfere with the therapy and the maintenance of the obstruction in the urinary tract can interfere with the antifungal therapy efficacy.

The first case of *C. krusei* fungemia and systemic infection was described by Young *et al* in 1974, when isolated from a patient from the National Cancer Institute (Bethesda, MD, USA) [82]. Few studies have addressed solely the epidemiology of *C. krusei* in the latest century [12, 83]. Recently, the single study by Pfaller *et al*, 2008, addressed the global epidemiology of *C. krusei* alone: *C. krusei* ranked in fifth place among 22 different species of *Candida*, accounting for 3.3% of all candida spp. isolated in both Europe and North America [84]. In detail, isolation rates of *C. krusei* represented 3.5% in the United States and 1.8% in Canada; the Eastern European countries presented the highest frequency of *C. krusei* isolation, namely, the Czech Republic, 7.6%, Poland, 6.0% and Slovakia, 5.1%. Conversely in the Asia-Pacific region was 1.3% and Latin America 1.7%. In some other countries the frequency of isolation was even lower: the Netherlands 0.5%, South Korea 0.3%, Taiwan 0.6%, Thailand 0.3%, and Ecuador 0.5% [84]. In the Portuguese epidemiological study only one isolate of *C. krusei* was registered [36].

The clinical services reporting the isolation of *C. krusei* from patient specimens include the hematology-oncology service, medical and surgical services, intensive care units (medical, surgical, and neonatal), dermatology services, obstetrics and gynecology services, urology services, and the outpatient services. *Candida krusei* is mostly frequent isolated among patients with hematologic malignancies than from patients with other pathological conditions combined and among blood and bone marrow transplant recipients [84-86]. In a more recent study by Pfaller *et al* 2012, the patients infected with *C. krusei* were frequently neutropenic (51.3%), recipients of an hematopoietic stem cell transplantation (19.4%) or suffering from a hematologic

malignancy (16.0%); another interesting fact is that 79.6% of the patients with *C. krusei* infection had received prior antifungal therapy [33].

Behind thousands and thousands of epidemiological studies worldwide, accurate and specific identification and characterization of yeast species have to be performed. Therefore, several different identification and typing methods have been developed and upgraded throughout the decades of yeast research.

Identification and Typing Methods

In the microbiology laboratory routine, biochemical assays based on panels of sugar assimilation are the most commonly used techniques for yeast identification. In recent years, chromogenic agars and automated biochemical systems such as the API series and Vitek systems from bioMerieux or Auxacolor from Sanofi-Pasteur/ Biorad have been developed for yeast identification. Chromogenic and fluorogenic agars type medium incorporate compounds that when hydrolyzed by enzymes produced by yeast cells, form colored or fluorescent products, respectively [87]. These biochemical reactions allow the detection of mixtures of different yeasts species and their presumptive identification. However, when the aim is to type different strains within specie, it is necessary to use more discriminatory techniques. In early years, traditional typing systems based on phenotype, such as serotype, biotype, phage-type or antibiogram, have been used. Later, when presenting extensive epidemiological surveillance studies, molecular methods overcame previously used phenotypic tests due to its higher discriminatory power [88, 89]. Moreover, DNA sequence differences are usually more stable than phenotypic differences [90, 91]. The specificity of the molecular methods has revealed the existence of new species among previously considered homogeneous species, such as *C. bracariensis* often identified as *C. glabrata* or *C. dubliniensis* originally identified as *C. albicans* [92-93]. The identification of epidemiologically related isolates, allows to the clinicians to locate the origin of an infecting agent in different situations: clarify transmission pathways in health care facilities related to health care associated outbreaks, recurrent infections or provide information concerning the population structure of the isolates.

PCR amplification is undoubtedly the technique mostly used within the different molecular typing techniques due to its high sensitivity and quickness: PCR with specific primers at the genus or species level, multiplex PCR, PCR amplification and subsequent southern blot, PCR followed by restriction analysis of amplicons, nested PCR, Real Time PCR, PCR followed by nucleotide sequencing [94–97]. Different molecular typing methods have been applied to *C. albicans*, namely, multi-locus enzyme electrophoresis (MLEE) and multi-locus sequence typing (MLST), restriction enzyme analysis (REA), karyotype analysis and randomly amplified polymorphic DNA (RAPD) analysis (98-104). Nowadays, the analysis of sequence polymorphisms is the gold standard for strain typing and the MLST is the most used molecular technique. It has already been published for the most important and frequently isolated pathogenic Candida species: *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. glabrata* [100-104].

Restriction enzyme analysis (REA) has been described in the last decade as a valuable tool for *Candida* spp. characterization. The digestion of genomic DNA with specific restriction endonucleases has been widely used in several different techniques [105-107]. However, the differences between the nuclear DNA and the mitochondrial DNA (mtDNA) such as the mtDNA small size, the high number of mtDNA molecules by cell and one single mitochondrial karyotype in each wild dikaryotic isolate, has lead the use of mtDNA in yeast taxonomy to a top position [108]. Nevertheless, the different GC content of nuclear DNA (40%) vs. the mtDNA (20%) is the most important feature in this methodology. When total fungal DNA is digested with restriction enzymes that only recognize GC rich regions, as for example MspI, HaeIII, or HinfI all with 50% GC target site, the nuclear DNA is overdigested originating a high number of short fragments, that are not detected by conventional agarose gel electrophoresis. Conversely, the restriction of mtDNA will originate fragments with higher molecular weight and those will be the only ones detected in the electrophoresis gel.

REA of the mtDNA was first applied in the biotechnology industry in order to characterize yeast strains used for wine fermentation [109-110] and, more recently, to discriminate between *Candida* clinical strains [111-114]. The data obtained demonstrate

the relevance of using molecular genetics methods in many different areas, including taxonomic, ecological and clinical surveys.

In Random Amplified Polymorphic DNA (RAPD) analysis, genomic DNA is amplified at a low, non-stringent annealing temperatures (30–38ºC) with short arbitrary sequences of oligonucleotide (usually 10 nucleotides). These short primers target unspecific genomic sequences, such as sequence length polymorphisms in the genome, generating multiple PCR products of different electrophoretic mobility. When comparing strains within species, the RAPD fingerprints obtained are combinations of different numbers of amplicons with different sizes, since the number and positions of primer binding sites are unique to a particular strain [115-117]. Similarities in banding profiles among strains (i.e. the number and mobility) can be calculated and used to infer epidemiological relationships. When multiple primers are employed, the RAPD fingerprints are sufficiently sensitive to detect variation among isolates, being a typing technique with a high discriminatory power yielding feasible results. Despite being technically fast and simple, there are some disadvantages in RAPD analysis. The major drawback is its low intra-laboratory reproducibility. Small differences in PCR conditions, such as reagents, protocols and even the technical equipment, may affect binding of the primers. This problem is minimized when the protocol and reagents are carefully standardized. For this reason, the RAPD fingerprints of individual strains are generally specific to each laboratory but not interlaboratory. RAPDs can also be problematic because bands with the same electrophoretic mobility may not share the same sequence [118]. Nevertheless, for comparing the similarities among strains and developing fingerprints for molecular epidemiology, RAPD analyses have been widely applied to a large number of medical fungi [119- 122].

PCR-Based Amplification of the Species-Specific Repetitive Polymorphic Sequence CKRS-1 was developed by Carlotti et al, as a simple and fast PCR method to identify and fingerprint specifically *C. krusei* strains. The polymorphic species-specific repetitive sequence, designated as CKRS-1 (*C. krusei* repeated sequence 1) was first identified in the nontranscribed intergenic regions (IGRs) of rRNA genes in *C. krusei*

and it is used in this technique as the template sequence [111]. First, DNA probe CkF1, 2 labeled with peroxide enzyme, was designed based on two cloned *Eco*RI restriction fragments, F1 and F2 which are polymorphic forms of the nontranscribed IGRs. Both F1 and F2 contain the repeated sequence, CKRS-1 which is made up of eight and seven shorter tandemly repeated sequences of about 165 bp, respectively. This methodology conjugates both REA and PCR amplification in order to increase the discriminatory power of the typing technique. Additionally, it combines the identification and fingerprinting of *C. krusei* since specificity assays were performed with other *Candida* species and no amplification was detected [111]. Another advantage of combining REA with the specific probe CkF1, 2 is the high degree of resolution of the fragments. This improved the rapid comparison of patterns as the number of fragments to consider was lower than in direct REAs but the number and sizes were sufficiently varied [111]. Later, based on the same polymorphic region in *C. krusei*, new specific primers were designed, Arno1 and Arno2, for an even faster, reliable and sensitive typing of *C. krusei* strains, using only DNA amplification by PCR. With this technique, results were achieved in less than 6 h and allowed the simultaneous typing of the isolates. Besides, this method showed 100% typeability, more than 98% in vitro reproducibility and a discriminatory power of 1 [111].

Several case reports of *Candida* spp. outbreaks involving the same strains have been described throughout the years, corroborated by the molecular identification and typing. However, in many cases those same strains might present different susceptibility profiles. Therefore, whether isolated from outbreaks or solely from patients, it is crucial to characterize the clinical isolates phenotype and understand their behavior towards the antifungal drugs.

Antifungal therapy and susceptibility assays

Different classes of antifungals with different mechanisms of action are available for the treatment of fungal infections, being the azoles the most used drugs. However,

the antifungal therapy is very limited due to biosafety problems, development of antifungal resistance, bioavailability and bioefficacy.

There are 4 classes of antifungals available, divided according to their mechanism of action: the azoles, i. e., FLC, VRC, PSC; the polyenes, i. e., lipossomic and deoxicolate AMB B, nystatin; the echinocandins, i.e., CSF, micafungin (MCF), anidulafungin (AND) and the pyrimidine analogues, i. e., 5-Flucytosine (5-FC). Antifungal agents have different cellular targets, important for the growth and survival of fungal pathogens. Azoles and AMB B interfere with fungal sterol, echinocandins impair the cell wall structure and pyrimidine analogues interfere with nucleic acid metabolism and protein synthesis.

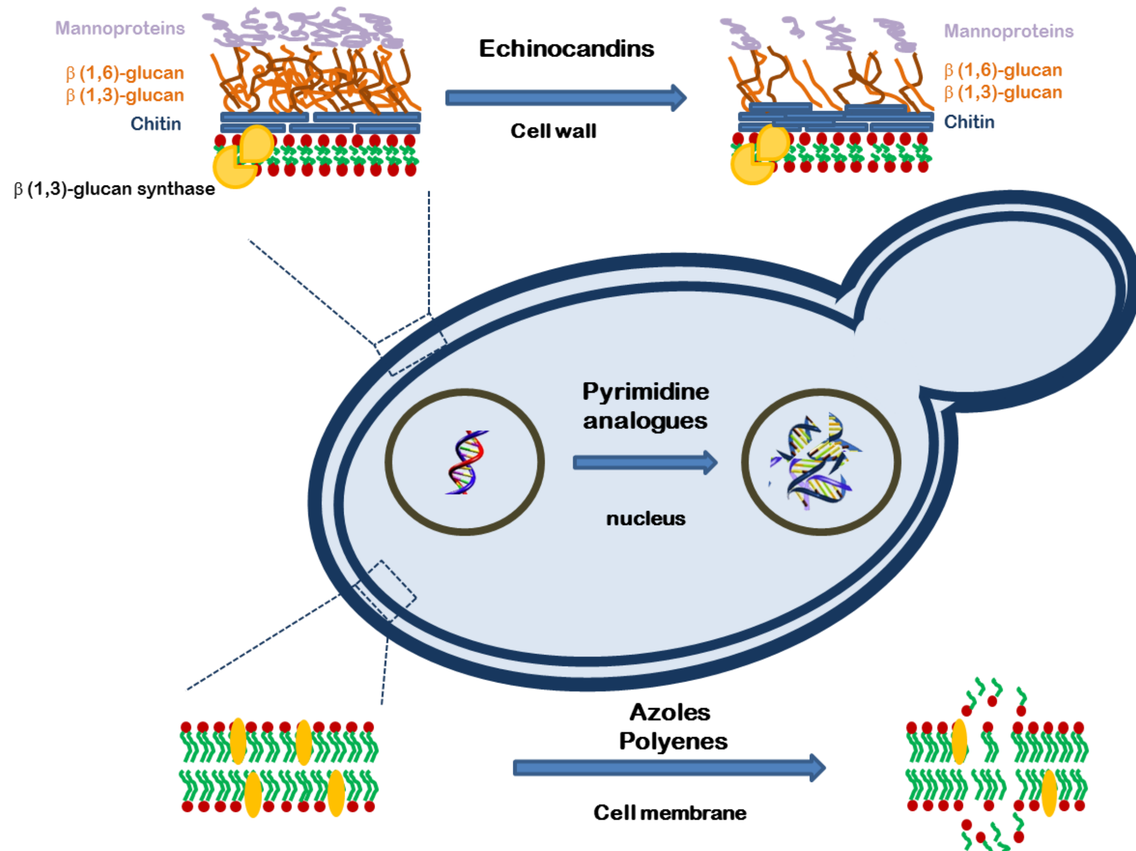


Figure 1 – Antifungal drugs mechanisms of action. Azoles antifungals inhibit the enzyme lanosterol 14α-demethylase, involved in the synthesis of ergosterol, a main component of the cell membrane. This inhibition leads to the production of toxic compounds altering cell membrane structure and permeability. Polyenes bind to ergosterol molecules forming pores in the membrane. Pyrimidine analogues induce the production of toxic compounds that interfere with the nucleic acid metabolism and consequently protein synthesis. Echinocandinds inhibit the enzyme β (1,3) – glucan synthase leading to alterations in the cell wall structure.

A recent study by Pfaller *et al*, 2012, showed that FLC was the most frequently administered antifungal agent (66.0%) followed by the echinocandins (55.1%) [33]. Neither VRC (7.8%) nor AMB B deoxycholate (2.3%) were used very often for the treatment of candidemia in the participating centers. Combination/sequential therapy, generally with an echinocandin and FLC were reported in 44.8% of patients with candidemia [33].

Antifungal activity may differ between different species of *Candida*, as is the case of *C. krusei* which is intrinsic resistant to FLC [123-125]. *Candida krusei* is considered a potentially multidrug-resistant (MDR) fungal pathogen, due to its susceptibility profile to different classes of antifungals: intrinsic resistance to FLC and decreased susceptibility to AMB and 5-FC [24, 126-130]. In the epidemiological study by Pfaller *et al*, all the isolates were considered clinically resistant to FLC and overall 7.8% were resistant to VRC [84]. However, considerable differences in the susceptibility pattern to VRC were registered among the countries studied. In North America 92.3% of the isolates were susceptible to VRC while in Latin America only 74.8% were susceptible to VRC, comprising countries like Brazil 65.9%, Colombia 61.8% and Mexico 63.6% [84]. Conversely, susceptibility rates to VRC were extremely high in countries such as Australia 100%, Taiwan 94.7%, Thailand 100%, Belgium 97.3%, Germany 90.9%, The Netherlands 100%, Poland 98.4%, Portugal 96.1%, Slovakia 90.1%, Switzerland 93.6%, Argentina 93.8%, Israel 94.7% and Canada 93.8% [84]. Considering the AMB and 5- FC susceptibility profiles, the global study corroborated results previously registered: decreased susceptibilities to AMB, i. e., 90% of the isolates were inhibited only for a MIC of 4 μg/ml, and only 8% were considered susceptible to 5- FC for a MIC of 16 μg/ml. Concerning the echinocandins, all *C. krusei* isolates were susceptible to CSF, AND and MCF at the CLSI breakpoints concentration of $\leq$ 2 μg/ml [84]. In another worldwide study, the SENTRY data, from 29 isolates of *C. krusei* tested, 3.5% of isolates were susceptible, 79.3 fell in the susceptible dose dependent range, and 17.2% were resistant to FLC [131]. In the same study, 100% of the 29 isolates were susceptible to AND and 97% to CSF; 93% were susceptible to

AMB and 93% susceptible to VRC. Only 3.4% were susceptible to 5-FC, which is a common characteristic of most studies reporting on *C. krusei* susceptibility [131].

It should also be stressed that, conversely to what would be expected, FLC exposure alone cannot be the only factor associated with an increase in infections caused by this species, since the increase in the prevalence of *C. krusei* predated the use of FLC in some institutions [132 – 134].

The Azoles

The target of the azole drugs is the cytochtome P450-dependent enzyme lanosterol 14α- demethylase - Erg11p, encoded by *ERG11* gene. This enzyme promotes the sterol 14α-demethylation reaction in ergosterol biosynthesis [135-137]. Ergosterol is the main sterol in most yeast and fungi, indispensable component of cellular membranes and an irreplaceable requisite for cell proliferation. Azoles bind to the protein active site through an heme domain, containing a free nitrogen atom in the pyrrolic rings and the iron atom from heme group, impairing the activation of the oxygen atom and consequently the demethylation of lanosterol (removal of methyl group) [138-139]. The fungistatic effect of azoles is not due to an impairment of ergosterol biosynthesis pathway but instead, due to the production and accumulation of 14-methylated toxic compounds in the yeast cells such as 14-methylergosta-8,24(28)-dien-3,6-diol, ignasterol, or 4,4- dimethyl zymosterol, and other compounds such as eburicol, lanosterol, obtusifoliol, 14a-methyl-fecosterolobtusifoliol or 14a-methyl fecosterol, at the expense of ergosterol within the cell [137, 140]. The accumulation of the toxic compounds together with the lack of ergosterol, alters the function and fluidity of the plasmatic membrane [141]. Although all azoles bind to the 14α – demethylase enzyme inhibiting its activity, they differ in their binding affinity. This difference is responsible for their varying antifungal potency and varying spectrum of activity [142-145]. For example, the imidazole derivatives such as miconazole (MCZ) and ketoconazole (KTC), inhibit besides the 14α – demethylase enzyme, several membrane bound enzymes and membrane lipid biosynthesis; conversely the triazoles derivatives such as ITC and FLC exert their antifungal activity only by inhibition of the cytochrome P450 dependent Erg11p [145].

One of the major advantages of using azoles when compared to AMB administration is their lower toxicity to the patients and oral and intravenous availability [146 - 147]. However, azole therapy can induce antifungal resistance in the pathogenic yeasts very rapidly, which might lead to an increasing in the frequency of infections [148-150]. On the other hand, some of the azoles such as FLC or ITC can interact with other drugs, namely benzodiazepines, cyclosporines, antihistamines, antibiotics or HIV protease inhibitors [151-153]. Another problem associated to the azole antifungal therapy is the homology between enzymes of the ergosterol biosynthesis pathway and the target enzymes of the cholesterol biosynthesis pathway in the liver cells of mammals, being therefore also toxic to the host in some extent. The newer azoles like VRC and PSC have been developed to overcome the limited efficacy of FLC against *Aspergillus* spp. and other moulds and to improve the absorption, tolerability, and drug interactions profile of ITC [154].

Fluconazole (figure 1) is available in both, oral suspension, presenting good absorption rate and not affected by food or gastric pH and intravenous (IV) formulations. Fluconazole is the drug of election for first line antifungal treatment of clinically stable patients and for empirical treatment in cases of suspected disseminated candidiasis in febrile non-neutropenic patients in countries where the rate of resistance species like *C. krusei* and *C. glabrata* is below 10–15%. It has been effective in treating both superficial infections such as vaginal candidiasis, oropharyngeal and esophageal candidiasis. It is also recommended for prophylaxis in bone marrow transplant patients to decrease the development of disseminated candidiasis. Fluconazole spectrum of activity includes *C. albicans*, most strains of *C. tropicalis*, and *C. parapsilosis*; conversely, *C. krusei* is intrinsically resistant and *C. glabrata* demonstrates reduced susceptibility to FLC. Fluconazole is most often considered a yeast agent and not a mould agent.

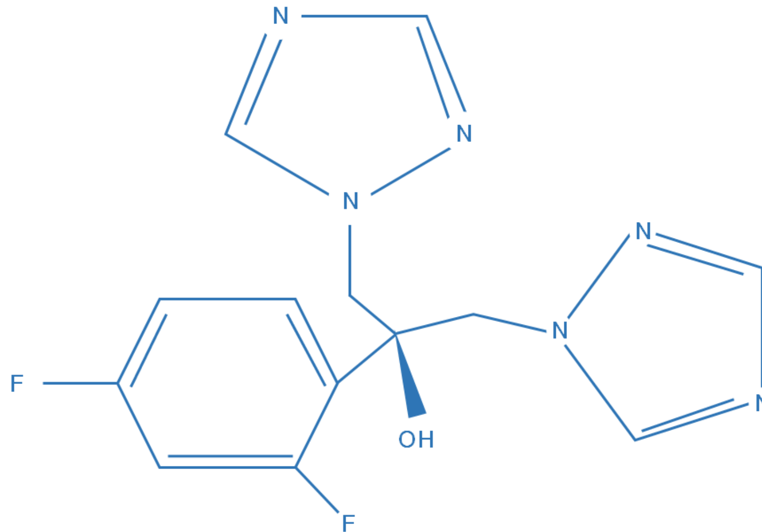


Figure 2 – Fluconazole chemical structure.

Voriconazole (figure 2) chemical structure is very similar to FLC, being the most significant difference the presence of an additional methyl group. It is available in both oral and IV formulations and is normally well tolerated. It is indicated for the primary treatment of aspergillosis, for salvage therapy of serious fungal infections due to *Fusarium sp.* and *Scedosporium* sp. (*P. boydii*) in patients refractory or intolerant to other therapies. Voriconazole presents a large spectrum of activity against *Candida* spp., including *C. krusei* and *C. glabrata*, intrinsically resistant to FLC. Importantly, VRC has been pointed out as a good alternative not only to FLC in the treatment of candidemia in the non-neutropenic patients, but also when the causative organism is resistant to other azole antifungal agents; also, in disseminated *Candida* skin infections or *Candida* infections of abdomen, kidney and bladder wall and in esophageal candidiasis [155]. The minimal inhibitory concentration (MIC) of VRC for *C. albicans* is usually 1 to 2 log lower than the MICs for FLC. However, some highly fluconazole-resistant strains of *C. glabrata* may demonstrate higher MICs to VRC [156]. Besides Erg11p, VRC also inhibits 24 – methylene dihydro-lanosterol demethylase exerting increased activity against moulds. The action is often fungicidal for moulds, increasing the sensitivity of the fungus to oxygen-dependent microbicidal systems of the host. However, there have been some reports of increased incidence of infections with *Zygomycetes* in association with increased use of VRC [157, 158].

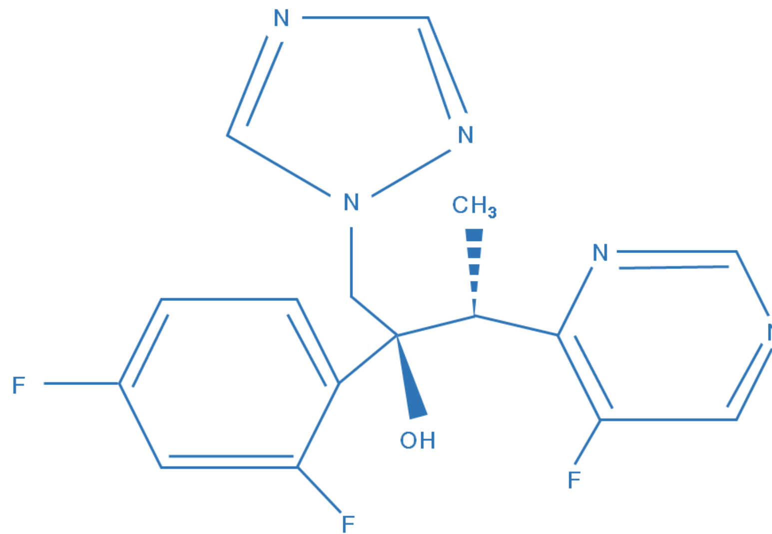


Figure 3 – Voriconazole chemical structure.

Posaconazole (Figure 3) is only available as an oral suspension. It was approved by the FDA for the prophylaxis of invasive *Aspergillus* and *Candida* spp. infections in high risk, severely immunocompromised patients older than 13 years old. The targeted patients include hematopoietic stem cell transplant recipients with graft vs. host disease and patients with hematologic malignancies with prolonged neutropenia; it is also recommended for the treatment of oropharyngeal candidiasis, including cases that are intolerant or refractory to ITC and/or FLC therapy [159-160]. Posaconazole has a broad spectrum of activity towards yeasts, filamentous and dimorphic fungi, namely *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., *Rhizopus* spp., *Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis*, dermatophytes, and dematiaceous fungi. Posaconazole is less active *in vitro* against FLC resistance in *Candida* spp., especially *C. glabrata* and *C. krusei*. It presents lower nephrotoxicity, hepatic toxicity and ocular toxicity than other triazoles. Since it is metabolized in the liver, PSC has fewer effects on patients with renal impairment.

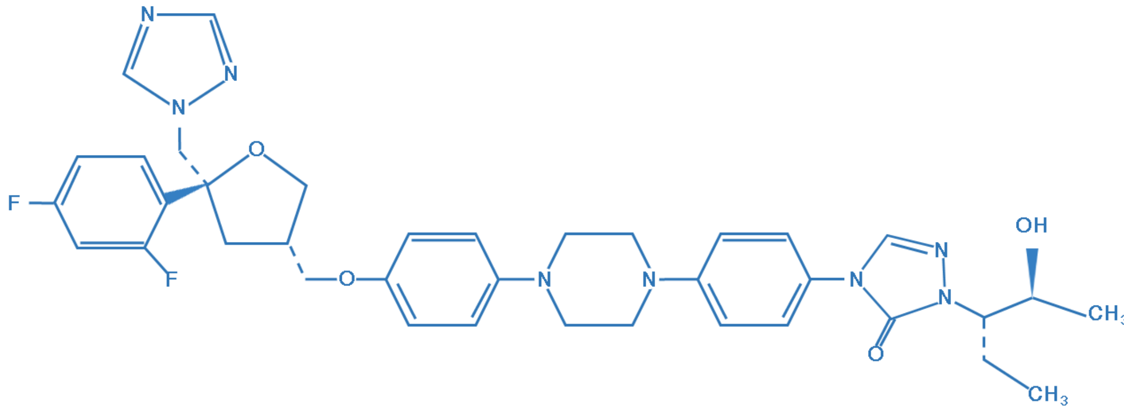


Figure 4 – Posaconazole chemical structure.

The Polyenes

The polyenes are amphipathic molecules, lipophilic and insoluble in water being the most known representatives AMB B (figure 4) and nystatin. AMB is produced naturally by *Streptomyces nodosus* and was first isolated in 1955 by Gold *et al*. It has been one of the most widely used antifungal drug worldwide, in the clinical practice due to its broad spectrum of action, fungicidal activity and low risk for the development of resistance. Previously, it was used as the first – line agent for invasive aspergillosis but nowadays, VRC has taken its place for the majority of the cases.

The target of polyenes is the ergosterol, the most abundant sterol in the plasmatic membrane of fungi, involved in the maintenance of the fluidity, asymmetry and membrane integrity. The antifungal binds to the membrane ergosterol molecules forming aqueous pores in the membrane [161, 162]. These pores alter the permeability of the membrane, promoting the leakage of the citoplasmatic organelles, potassium ions, disrupting the membrane potential and consequently leading to cellular death [135; 162]. Amphotericin B also causes an oxidation-dependent stimulation of macrophages either due to autoxidation of the drug in conjunction with the formation of free radicals or due to an increase in the membrane permeability [163]. Amphotericin B presents poor oral absorption therefore all systemic preparations are IV formulations. Due to the similarity of ergosterol to the cholesterol of mammal cellular membranes, AMB B is even more toxic to the host than azole drugs. It is particularly nephrotoxic, impairing the renal function. It leads to a medical condition characterized by abnormal high levels of nitrogen-containing compounds in the blood, mostly related to a reduction

in the glomerular filtration of the blood in the kidneys, and loss of the ability to concentrate urine [161, 164]. At high levels AMB B can also inhibit the enzyme chitin synthase, involved in the synthesis of the cell wall [161]. The conventional AMB B deoxicolate formulation is more toxic to the host when compared to the more recently developed liposomal formulation. Amphotericin B is indicated for several different clinical situations: prophylaxis in patients with risk for serious fungal infections, empiric therapy in patients who have neutropenia and fever; treatment of candidiasis including candidal esophagitis and hepatosplenic candidiasis; severe candidiasis such as candidemia, endophthalmitis, and osteomyelitis; and for the treatment of aspergillosis, cryptococcosis, and the dimorphic mycoses [163]. It is also recommended for IC in neutropenic patients and for the treatment of infections due to *Candida* isolates either intrinsically resistant to azoles or after the development of resistance under azole therapy [165-166].

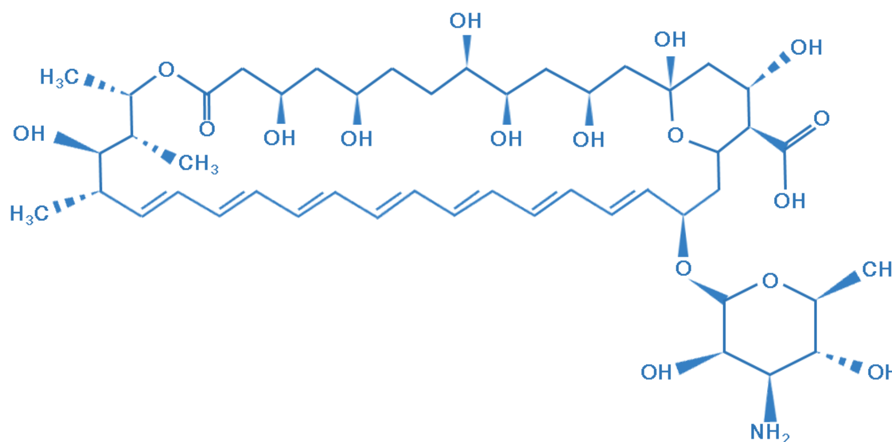


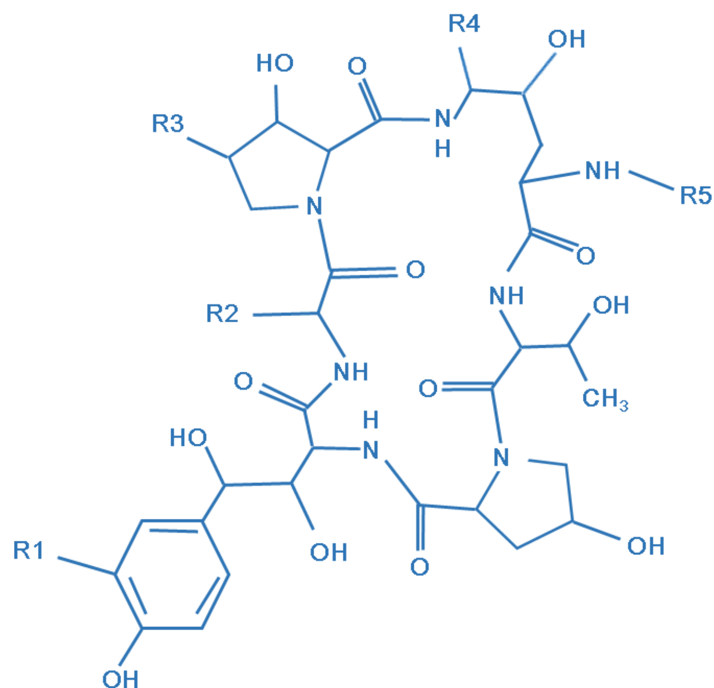
Figure 5 – Amphotericin B chemical structure.

The Echinocandins

Echinocandins (figure 5) are the most recent class of antifungal agents available. The most used drugs in the clinical practice belonging to this class of antifungals are CSF (the first approved echinocandin), AND or MCF, which are molecules isolated from fungi and chemically modified. There is poor oral absorption of the echinocandins, and thus only IV formulations are available. Their target is the 1,3-β-D-glucan synthase, encoded by the *FKS* gene, an enzyme necessary for synthesis of 1, 3-β-D-glucan, one of the most important constituents of the fungi cell wall. The 1,3-β-D-

glucan synthase is constituted by two subunits, Fksp and Rho1p. Fksp is the active site of the enzyme and is encoded by the genes *FKS1*, *FKS2* and *FKS3* [167, 168]. The cell wall plays a crucial role in the protection, morphology and rigidity of the cell, metabolism, ionic exchanges, antigen expression, primary interactions with the host and resistance to the immunological response of the host. The inhibition of the target enzyme leads to alterations in the cell wall structure, increased chitin content, reduction of the ergosterol and lanosterol content of the cellular membrane, ultimately leading to cellular lyses [135, 136, 161, 162]. Therefore, this class of antifungals is mostly considered fungicidal and demonstrates good *in vitro* and *in vivo* activity against a range of *Candida* species and is recommended as an alternative therapy for *Aspergillus* infections. However, against *C. parapsilosis*, *C. guilliermondii* and *Aspergillus* spp. these antifungals are only fungistatic [163, 168]. The echinocandins do not present major adverse side effects since the glucan target is not found in the mammalian cells. Echinocandins present no cross-resistance with other antifungal drugs [168, 169]. When clinical isolates present resistance to azoles, echinocandins are the first-line alternative antifungals to treat deep infections due to *Candida* spp., first CSF and more recently MCF and AND [170].

A.



B.

	R1	R2	R3	R4	R5
Caspofungin	H	$NH_2(CH_2)_2CHOH$	H	$NH(CH_2)_2NH_2$	O; H_3C; CH_3
Anidulafungin	H	CH_3CHOH	CH_3	OH	O; O
Micafungin	$HOSO_3$	$NH_2(CO)CH_2CHOH$	CH_3	OH	O; N–O; O

Figure 6 – Echinocandins chemical structure. Each antifungal drug belonging to the class of the echinocandin presents a general chemical structure (A), differing in their side chains, namely, CSF, AND and MCF (B).

The Pyrimidine analogues

5- Flucytosine (figure 6), a fluorinated pyrimidine, analogue of cytosine, was one of the first antifungals available for the treatment of fungal infections from 1960. It is always used in combination with other antifungal agents such as AMB B, for example, for the treatment of cryptococcal meningitis. Its mechanism of action interferes with both the synthesis of nucleic acids and proteins. A cytosine permease enzyme drives the 5-FC molecule inside the cell, where it is metabolized to 5 – flurouracil (5- FU). This product is later metabolized in 5 – fluorouridine triphosphate which substitutes the uracil in fungal RNA, altering pyrimidine metabolism and consequently protein synthesis. 5 - FU can also be converted in 5 – fluorodeoxyuridine monophosphate (5 – FUdRMP) a strong inhibitor of the enzyme thymidylate synthetase and thus interfering with fungal DNA synthesis [135, 162, 163, 171].

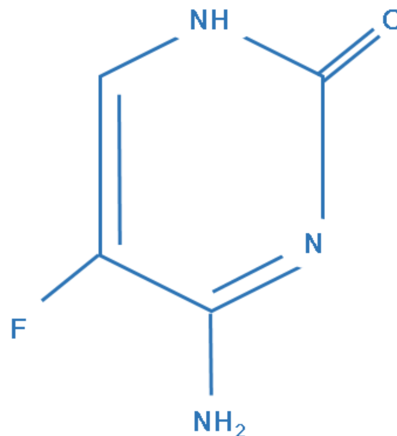


Figure 7 – 5-Flucytosine chemical structure

Other antifungal drugs belonging to different classes have been studied during the last years, namely, rapamycin, aureobasidin A or natural peptides derivatives such as hystatin among others [137].

First line antifungal treatment for candidemia and deep candidiasis remains controversial. It is usually recommended the use of echinocandins, FLC, or lipid formulation of AMB, but not AMB deoxycholate formulation due to its toxicity. Several factors are associated with the choice of the antifungal therapy namely, the clinical

status of the patient, the knowledge of the antifungal susceptibility profile of the infecting isolate, the drug toxicity and the patient's prior exposure to antifungal agents [165].

Mortality rates of IC are still very high, despite the different classes of antifungals available with different mechanism of action, therefore alternative therapies are in development. The ideal antifungal should present a large spectrum of activity and should not be toxic to the host. To find this ideal antifungal, a specific target in the pathogen organism with no similar target in the host cells should be found, such as the unique fungi cell wall, and for example, presenting a low rate of development of resistance associated [162].

Antifungal Susceptibility Assays

Several different types of antifungal susceptibility assays have been developed throughout the years, reaching a turning point in the 1990s. Over the past 2 decades the determination of the susceptibility profiles changed from a nonstandardized procedure with no clinical importance, to a standardized technique, well controlled and of major importance to the physicians when dealing with fungal infections. Before the development of these standardized techniques, the determination of the MICs were inconsistent and could vary between laboratories up to 50000-fold [172]. The US Clinical Laboratory Standard Institute (CLSI), former National Committee for Clinical Laboratory Standards (NCCLS), first published in 1992 the standard macrobroth dilution protocol M27–P for susceptibility determination [173]. Reference methods for testing yeast and moulds have been developed and improved over the years due to the intensive research and global multi-laboratory collaborations. Different protocols were approved and breakpoints were established in order to interpret antifungal susceptibility assays. The latest versions of the different protocols developed are: the M27 – A3, including the supplemental protocol M27-S4 for macrobroth and microtiter yeast tests [174, 175], M38-A2 for microtiter mould testing [176], M44-A for yeast disk diffusion testing [177] and M51-P for mould disk diffusion testing [178]. To date, interpretive guidelines have only been established for 5-FC, some azoles such as FLC or VRC, and the currently available echinocandins such as AND, CSF and MCF.

Although the reference techniques are extensively used worldwide with validated results, they present some flaws, such as the lack of reliable detection of resistance to AMB B, the poor growth of non- fermentative yeasts such as *Cryptococcus neoformans*, lack of reproducibility when testing echinocandins and the need for physicians to establish breakpoints to interpret the susceptibility results of the filamentous fungi [179, 180]. Another problem is the implementation of these standard reference procedures in the clinical laboratories since they recommend complex methods that are time and labor intensive. Therefore, the CLSI committee has approved an agar diffusion technique for yeast testing, the protocol M44-A [177], in 2004. Voriconazole and FLC disks were already approved to test the susceptibility *in vitro* of *Candida* species and echinocandin disks are under study for standardization. Techniques such as Etest® (AB Biodisk), Sensititre YeastOne® (Trek Diagnostic Systems Ltd), Neo-Sensitabs® (A/S Rosco), Fungitest® (Bio-Rad), Vitek® (bioMerieux) and others can be used to detect the resistance *in vitro* to FLC and other azoles in *Candida* isolates. These tests are a good alternative to the microdilution protocol, since the discrepancies obtained when compared with the reference MIC values of azole agents are less than 5% [181, 182]. Additionally, some of these tests have also presented very reliable results in the detection of resistance to echinocandins and Etest has proven to be the most reliable method to detect isolates with high MIC values to AMB B.

While new protocols were being revised and improved by the CLSI committee, the European community involved in the antifungal susceptibility research field also began to work on a standard method. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) developed reference protocols in order to determine the susceptibility profiles of *Candida* species and filamentous fungi [183-186]. The aim of the EUCAST is to standardize the antimicrobial breakpoints and the susceptibility testing in Europe in order to establish reproducible methods, comparable results and interpretation. It distinguishes between clinical breakpoints and epidemiological cut – off values. Clinical breakpoints define the organism as susceptible, intermediate, and resistant to antifungal drugs. Susceptible and resistant categories are related to a higher probability of clinical success or clinical failure, respectively. The clinical outcome associated with an intermediate category is uncertain. Clinical breakpoints should be

used every day in the clinical laboratories to advise the physician concerning the therapy to be applied. Epidemiological cut off values are based on the wild-type MIC distribution of each microorganism and are used as the most sensitive measure of development of resistance in the hospitals and in the community. Additionally, they are quite important in the implementation of strategies to stop the development of resistance.

Recently, breakpoints for some antifungal compounds have been set in the EUCAST protocols. Although the CLSI and EUCAST reference methodologies are different, the results obtained are equivalent [187, 188]. As more drugs reach the market, the CLSI and EUCAST committees will be challenged to expand the existing documents, especially when new classes of drugs are introduced.

One of the major concerns for microbiologists is the poor relationship between *in vitro* susceptibility profiles and the response of clinical isolates to antifungal treatments. Several factors may be associated to these differences namely pharmacogenomic, pharmacokinetics and interactions with other drugs/concomitant therapies [189]. On the other hand, when resistance is detected *in vitro* most certainly the clinical isolates present a resistant phenotype *in vivo*, escaping the antifungal action of the drugs due to the presence of resistance mechanisms.

Antifungal Resistance Mechanism

Fungal pathogens can suffer positive selection due to the presence of antifungal drugs such as the development of antifungal resistance. Different mechanisms of resistance to different classes of antifungals have been described for *Candida* spp.: mutation or overexpression of *ERG11* gene encoding for the drug target enzyme lanosterol 14α – demethylase- Erg11p, mutations in genes encoding for other enzymes of the ergosterol biosynthesis pathway, such as *ERG3* gene, compensating for the accumulation of the toxic ergosterol intermediates in the cells, overexpression of efflux pumps belonging to the ATP Binding Cassette (ABC) and Major Facilitator Superfamily (MFS) of transporter proteins, mutations in the FKS1/ 2 genes or even the presence of

complex multicellular structures such as biofilms [137, 190]. Some resistant phenotypes to echinocandins have been described in *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei*, isolated from patients under a long period of echinocandins therapy [191-194]. Also, resistance to 5 –FC develops very rapidly in most fungi especially if used alone. Most of these resistant isolates present mutations in the cytosine permease, cytosine deaminase or FUR1 gene (C301T) [137, 195]. The enzymes and genes are crucial for the entrance of the drug into the cell and to the conversion of 5 – FC into 5 –fluorouracil acid. On the other hand, some pathogens present intrinsic resistance to some antifungals due to specific traits in their genomes, such as *C. krusei* intrinsic resistance to FLC.

Ergosterol biosynthesis pathway: an unsuitable antifungal target for *C. krusei*

The ergosterol biosynthesis pathway converts acetyl-CoA into ergosterol, similar to what happens in the cholesterol biosynthetic pathway in mammals. The enzyme Erg11p is one of the most important enzymes of the biosynthetic pathway, and is one of the main targets of the azole drugs. When the azoles bind to this enzyme, inhibiting its activity, toxic precursors 14-methylated accumulate in the yeast cells. Several resistance mechanisms associated to the ergosterol biosynthesis pathway, have been described in different strains of *Candida* spp.: mutations in *ERG11* gene sequence [196] and *ERG11* gene overexpression [197], mutations in the *ERG2* gene and *ERG3* gene encoding for the enzymes sterol $D^{8,7}$ - isomerase and $\Delta^{5,6}$ –desaturase, respectively [161, 198]. The enzyme $\Delta^{5,6}$ –desaturase is involved in the final stage of ergosterol biosynthesis and converts non-toxic 14-methylated intermediates which accumulate due to the azoles inhibitory effect in the toxic sterol 14-metilergosta–8, 24(28)-dien-3, 6-diol. Organisms deficient in this enzyme produce 14- methylfecosterol and remain viable when 14α – demethylase activity is inhibited [161, 199]. All these alterations in gene sequence lead to a protein with an altered structure, reducing the affinity of the azoles to the binding site of the enzyme, therefore not exerting its antifungal effect.

Candida krusei is a peculiar case since its intrinsic resistance to FLC is associated to a diminished affinity of the drug to the target enzyme Erg11p, but not due

to mutations or polymorphisms conversely to what is described for other *Candida* sp. [196]. Radioactivity studies have shown a reduced inhibitory effect of FLC in the synthesis of ergosterol, when compared to ITC or KTC [200]. Additionally, it was also demonstrated through interaction studies, between different azoles and the cytochrome P450 enzyme, a lower affinity of FLC to the binding site of the enzyme, followed by KTC and with ITC presenting the highest affinity, therefore explaining in part the intrinsic resistance to FLC of C*. krusei* strains [200]. Comparative studies between *C. krusei* and *C. albicans* suggest that there are qualitative differences in the Erg11p in the two different species of *Candida*. It was described that the concentration of FLC required to inhibit the synthesis of ergosterol by 50% was 16 to 46-fold higher in cell extracts of *C. krusei* than in cell extracts of *C. albicans* [201].

Efflux mediated by ABC transporters

There are two types of multidrug transporter families, the ABC transporters encoded by *CDR* genes (Candida Drug Resistance), which use adenosine triphosphate (ATP) as energy source and the MFS transporters, such as *MDR1* gene (multidrug resistance), which uses a proton gradient across the membrane as the driving force for transport [202].

ABC proteins are composed by two trans – membrane domains (TMDs) and two cytoplasmic nucleotide binding domains (NBDs). The NBDs of all ABC transporters have in common the amino acid sequence identity and typical motifs, regardless the origin and nature of the substrate transported. Studies indicate that the TMDs are associated to the formation of substrate binding sites; however this is not enough for the transport of substrates across the phospholipids bilayer of the membrane [203, 204]. The transport requires energy from the hydrolysis of ATP carried out at the NBDs located at the periphery of the cytoplasm. The substrate specificity of ABC transporters is quite variable due to the high divergence of primary sequences. The most frequent mechanism of resistance to azole drugs in *Candida* spp. and in filamentous fungi are associated to efflux pumps, i. e., the alteration of the drug transport due to overexpression of efflux pumps encoded by CDRs genes or the increase of the efflux pumps activity [205-208].

The MFS transporter proteins are involved in the symport, antiport, or uniport of various substrates. MFS transporters obtain the necessary energy for the transport of substrates from an electro-chemical proton-motive force, composed by an electrical potential ($\Delta\Psi$) and a chemical proton gradient ($\Delta pH = pH_{external} - pH_{internal}$). In yeasts, the MFS-MDR transporters promote a proton antiport and are divided in two groups: the drug: H+ antiporter-1 (12 TMS) DHA1 family, and the drug: H+ antiporter-2 (14 TMS) DHA2 family [209, 210]. MDR1 (formerly *BEN^r* for benomyl resistance) and *FLU1* (fluconazole resistance) genes were identified in *C. albicans* as belonging to MFS transporter family and to be related to resistance.

ABC1 and *ABC2* genes were the first genes described in *C. krusei* encoding for the efflux pumps, Abc1p and Abc2p, and to be associated to azole resistance [211]. These genes were expressed at low constitutive levels in log phase cultures but upregulated in the stationary phase. It was suggested that FLC and other azoles are substrates for the transporter protein Abc1p therefore FLC resistance could be associated not only to the low affinity of the antifungal to the enzyme Erg11p but also to efflux activity [211]. In a previous study by Venkateswarlu K, *et al*, it was already demonstrated that resistance to different azoles other than FLC such as ITC could be associated to transporter proteins promoting drug efflux [200]. More recently, *ABC1* gene from *C. krusei* was expressed in *S. cerevisiae* and it conferred resistance to azole drugs [16].

The MFS transporter proteins are not described to be associated to resistance to azoles in *C. krusei* conversely to other *Candida* species such as *C. albicans* [209]. J Guinea *et al*, evaluated the effect of carbonyl cyanide 3-chloro-phenylhydrazone (CCCP), in the MIC values of different antifungals in *C. krusei* clinical isolates. CCCP is a protonophore which collapses the transmembrane ΔpH [213, 214] and hence inhibits the enzymes belonging to the MFS transporter family. No significant difference was registered in the MIC values in the presence/absence of the protonophore therefore they concluded that this family of proteins was not involved in *C. krusei* azole resistance.

Acquisition of mutations to echinocandins

Two different mechanisms have been associated to echinocandin resistance in *Candida* spp.: mutations in the “hot – spot” regions (HS1 and HS2) of *FKS1* and *FKS2* genes encoding for the major and presumed catalytic subunit of 1, 3 – ß – D- glucan synthase and a compensatory increase in the production of chitin, the second structural cell wall polysaccharide [215]. Point mutations associated to echinocandins resistance have been described in different species of *Candida* namely, *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis* [193, 216-219]. Specifically, the acquisition of resistance to echinocandins has been described in three clinical cases involving *C. krusei* infection [194, 220, 221]. However, in only two of the cases was described an alteration in the *FKS1* gene in the hot spot region (T2080K, resulting in a Phe655 – Cys substitution) after antifungal therapy [194, 221]. In the other clinical case involving an acute myelogenous leukemia patient, administered CSF during 17 days, 50 mg/day, no mutation in *FKS* genes was described, therefore other resistance mechanism would be associated [220]. There is also some evidence that resistance may be due to efflux pumps activity in the fungal cell wall and over expression of cell wall transporter proteins [222].

Chapter III

CANDIDA KRUSEI RESERVOIR IN A NEUTROPENIC UNIT: MOLECULAR EVIDENCE OF A FOE?

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“Só aqueles que têm paciência para fazer coisas simples com perfeição é que irão adquirir habilidade para fazer coisas difíceis com facilidade.”

Johann Von Schiller, Poeta, filósofo e historiador. 1759 – 1805.

Abstract

Candida krusei has been documented as an emerging pathogen causing nosocomial outbreaks. The consecutive isolation of *C. krusei* strains in three patients admitted to the same hospital department within 2 months lead us to consider the possibility of an outbreak. Additionally, *C. krusei* isolates were collected from the room surfaces, whereas another isolate had been recovered from the blood of one patient 2 years before. HinfI DNA restriction endonuclease-based analysis of all *C. krusei* isolates was performed and restriction profiles were compared. Surprisingly, isolates from different patients were unrelated, whereas isolates from biological products of the same patient showed indistinguishable HinfI restriction patterns and were similar to those obtained from the surrounding environment of the respective patients. The study approach revealed the endogenous origin of the *C. krusei* infectious episodes observed and demonstrated that, subsequent to colonizing a patient, *C. krusei* can be involved in infectious episodes distant in time. The hypothesis of an outbreak was excluded, although we believe that the methodology employed in the present study represents a valuable tool for diagnostic and epidemiological surveys.

Background

In the last two decades, invasive fungal infections in hospitalized patients have increased significantly worldwide. According to data obtained from USA and Europe, *Candida* species represent, respectively, the fourth and sixth most frequent cause of invasive healthcare-related infections [223, 224], accounting for 8-15% of all episodes of sepsis episodes acquired in hospital settings [35]. Inherent to these types of infection are the extremely high morbidity and mortality rates, particularly among immunocompromised patients [24, 36].

Fluconazole is one of the antifungal agents mostly used in both prophylactic and therapeutic protocols. Fluconazole prophylaxis has been associated with a decrease in the prevalence of *Candida* species such as *C. tropicalis* and *C. albicans,* and to an increase in that of *C. krusei* and *C. glabrata* [35]. *C. krusei* presents intrinsic resistance to FLC and, to some extent, reduced susceptibility to AMB B [225].

Infectious outbreaks in hospitals, especially in ICU, represent a serious health problem and are mainly due to *C. lusitaniae*, *C. albicans*, *C. parapsilosis*, and *C. krusei* [226-230]. Many factors may account for their occurrence (e.g. barrier loss, lack of proper infection control measures by health care workers when managing patients, resistance to prescribed antifungal drugs as well as insufficient drug levels.

Molecular methods represent a powerful tool to clarify transmission pathways in health care facilities, (i.e. to investigate the occurrence of possible outbreaks). Techniques such as karyotyping, restriction fragment length polymorphism (RFLP) analysis by PFGE, southern blot hybridization, PCR fingerprinting and RAPD fingerprinting have been extensively used for *Candida* typing [98, 231-234]. Restriction endonuclease analysis has been described in the last decade as a valuable tool for *Candida* spp. characterization. Restriction endonuclease analysis of the mtDNA was first applied in biotechnology industry in order to characterize yeast strains used for wine fermentation [109, 110] and, more recently, to discriminate between *Candida* clinical strains [111 -114]. The data obtained demonstrate the relevance of using molecular genetic methods in many different areas, including taxonomic, ecological and clinical surveys.

Recently, we were challenged by a hypothetical outbreak as a result of *C. krusei* in the neutropenia unit of the Hematology Department of Hospital S. João, Porto, Portugal. Within a short period of time, several *C. krusei* isolates were cultured from biological products of three patients. In addition, *C. krusei* was found in the surrounding environment of the patients. All isolates were compared using mtDNA REA, which is a convenient and powerful tool that allows valid comparisons between isolates of the same yeast species.

Patients and Methods

Patients

Patient A was a 41-year-old male with acute lymphoblastic leukemia (T/NK) diagnosed in July 2006. In August 2008, the patient was initially treated with amoxicillin and ciprofloxacin because of undetermined fever. When symptoms remained unchanged, a myelogram was performed; a first relapse of the hematological disease was diagnosed. He was admitted to the Neutropenia Unit for salvage intensive chemotherapy with fludarabine, idarubicin and ara-C, followed by growth factor (G-CSF), administered through a central venous catheter. Upon the onset of the aplastic period (28 August) he received antimicrobial treatment for 2 weeks (ciprofloxacin, amoxicillin-clavulanic acid, imipenem, acyclovir and FLC 200 mg/day). At the time of fungemia detection he displayed the following hematologic parameters: white blood cells $0.03x10^9$cell/L, hemoglobin level 8.5 g/dl and blood platelets levels $11x10^9$cells/L. As soon as *C. krusei* was identified, the patient was started on CSF for 12 days without improvement; the treatment was then changed to AMB B. This patient had previously developed a fungemia episode as a result of *C. krusei* during chemotherapy in 2006 and a corresponding isolate had been stored at -70ºC. The patient remained in room number 1 from August to October 2008.

Patient B was a 53-year-old mal with non-Hodgkin´s lymphoma, who was admitted to room number 2 for 3 weeks in September 2008. He was administered cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) followed by G-CSF. On September 12 2008, he was started on CSF but as a result of sustained fever on day 5, treatment was changed to VRC, completing 14 days of antifungal therapy. He never received FLC. At that fungemia episode he displayed the following hematologic parameters: white blood cells $2.41x10^9$cell/L, hemoglobin level 9.3 g/dl and blood platelets levels $73x10^9$cells/L.

Patient C was a 60-year-old male with acute myeloid leukemia, secondary to myelodisplastic syndrome; he was admitted to room number 2 for 3 weeks in October 2008, subsequent to patient B. He received Ara-C, Etoposide and doxorubicin for 5 days, followed by another 5 days of Ara-C; he was also prescribed amoxicillin,

ciprofloxacin and allopurinol. In addition, prophylaxic treatment included imipenem, vancomycin, FLC and acyclovir from 29 September to 20 October 2008. On that latest date he displayed the following hematologic parameters: white blood cells 0.6x10^9cell/L, hemoglobin level 9.8 g/dl and blood platelets levels 24x10^9cells/L.

Patient D served as a typing control; one single *C. krusei* strain was recovered from the patient´s bronchial secretions in September 2008 upon his admission to the Internal Medicine Department.

Clinical strains

Eighteen *C. krusei* isolates were collected from different clinical specimens (blood, urine, stools, and bronchial secretions) of the above mentioned patients admitted to the neutropenia unit within a 2-month period. Additionally, a previous *C. krusei* isolate (2006) from a blood culture of patient A was included in the study, as well as a *C. krusei* isolate obtained from the control patient D. All isolates were identified using the automatic system Vitek2 YBC identification cards (BioMérieux, Paris, France), stored at -70ºC in Brain Heart infusion (Merck KGaA, Darmstadt, Germany) with 10% glycerol and sub-cultured twice in Sabouraud agar (Merck KGaA) to ensure purity prior to experimental assays.

Environmental strains

Several environmental samples were collected from the patients' rooms (1 and 2) using Sabouraud agar contact plates (Merck KGaA, Germany); two *C. krusei* isolates were cultured: one from the bedside table of patient A (room 1) and another from the bed of patient C (room 2). The isolates were characterized and stored as described above for clinical samples. Air samples were collected by filtration with a MAS-100 Eco instrument (Merck Eurolab, Switzerland), containing Sabouraud agar plates (Merck KGaA); no *C. krusei* isolates were obtained.

Antifungal Susceptibility Testing

Voriconazole (Pfizer), PSC (Schering- Plough; New Jersey, USA), AMB B (Bristol Meyers Squibb, NY, USA), CSF (Merck, Rahway, NJ, USA) and AND (Pfizer)

stock solutions were prepared according to CLSI protocols M27-A3 [174], and maintained in stock solution at -70°C until use. Minimal inhibitory concentration of each antifungal drug was determined according to CLSI protocol [174].

Total genomic DNA extraction

Yeast cells were cultured overnight at 30ºC in 10 ml of YPD liquid medium, with continuous orbital shaking at 180 rpm, and subsequently collected by centrifugation. Total DNA was extracted using Phenol:Chloroform:Isoamyl alcohol 25:24:1, precipitated using 100% ice-cold ethanol and redissolved in 200 μl of Tris-EDTA (TE) buffer. The DNA was treated with 20 μg of RNase (Applichem, Darmstadt, Germany), incubated at 37ºC for 30min to 1h. For final precipitation, 20 μl of 4 M Ammonium acetate pH 4.8 (Sigma –Aldrich, Germany) and 600 μl of ice-cold 100% ethanol (Applichem) were added and samples were incubated overnight at -20ºC. The DNA was re-dissolved in TE buffer 1x, assessed in a biophotometer 6131 (Eppendorf®, Hamburg, Germany) and adjusted to a final concentration of 2.0-2.5 μg/μl. To assay DNA integrity, about 3-5 μg of DNA was run in agarose gel (1%w/v) (Sigma –Aldrich) in Tris- Borate-EDTA (TBE) buffer 1x and stained with ethidium bromide 0.5 mg/ml. DNA samples were stored at -20ºC for subsequent use.

Restriction Endonuclease Analysis (REA)

For each sample, a reaction mixture was prepared containing 1x Hinf I enzyme reaction buffer (Metabion, Germany), 1μg/μl RNase, 0.5U/μl Hinf I restriction enzyme (Metabion), approximately 25 to 30 μg of total DNA and DNAse-RNAse free water up to 20 μl final volume; reaction tubes were incubated overnight at 37ºC. Restriction was ended upon HinfI inactivation by incubating 20 min at 80ºC. The total reaction mixture was run on a 1% agarose gel (20cmx24cm), 120mv, for 3-5h, stained with ethidium bromide solution (0.5mg/ml) and the DNA visualized under UV light.

Results analysis

Restriction patterns were analyzed using the UVIDOC 12.6 software for Windows (Topac Inc., Cohasset, MA, USA) and the resulting groups of strains were compared.

Results and Discussion

There have been an increased number of reports describing non-*albicans Candida* hospital outbreaks. Given that *C. krusei* is not the main pathogen causing nosocomial infections, the detection of simultaneous episodes at the neutropenia unit of our hospital lead us to consider the possibility of an outbreak. The *C. krusei* isolates were all susceptible to all the antifungals assayed (FLC was not tested since *C. krusei* presents intrinsic resistance to this agent). Variations in the antifungal MICs for different isolates were not significant. The MICs of amphotericin B were from 0.06 to 1µg/ml, for CSF from 0.125 to 1µg/ml, for AND 0.06µg/ml; for VRC from 0.25 to 2µg/ml and for PSC from 0.03 to 0.5µg/ml.

The routine biochemical identification protocols or antifungal susceptibility profiles are usually not sufficient to either corroborate or exclude an outbreak hypothesis. REA for *Candida* species was first described by Scherer and Stevens (1987) who considered this method to be an extremely valuable tool for epidemiological studies [235]. Fujita et al (2000) described HinfI restriction patterns exhibiting a superior discriminatory power among distinct *Candida* isolates compared to patterns with other enzymes such as EcoRI or MspI [113]. Additionally, Sancak et al (2004) established a correspondence of almost 100% between the results obtained with HinfI REA and PCR methodologies [114]. In the present study, a total of 22 *C. krusei* isolates (20 clinical, two environmental) were analyzed using REA. A high number of *C. krusei* isolates was obtained from different biological products of patient A in distinct periods of time. All of them showed the same restriction pattern, including the isolate recovered in 2006 from a blood culture (A_{BC1}) (figure 1, lanes A_{BC1}, A_{BC2} and A_{CVC}), indicating that they are the same strain.

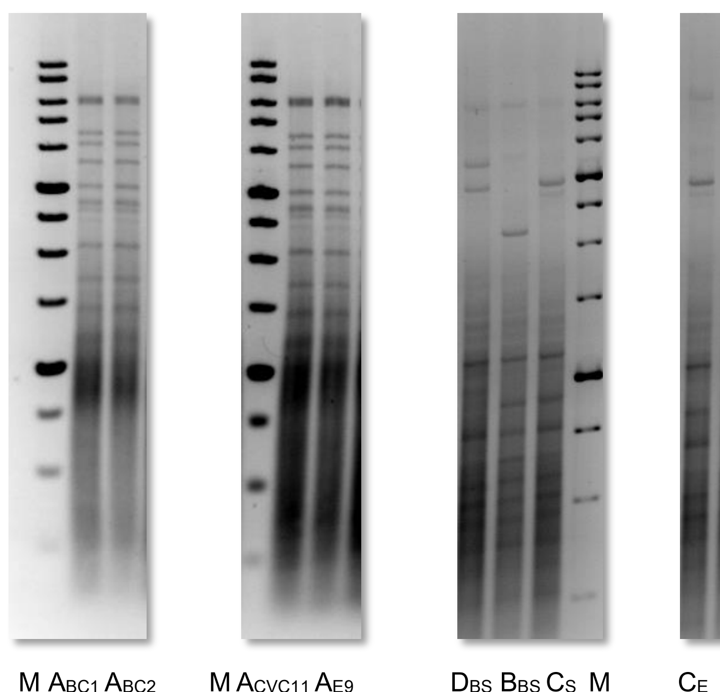

Figure 1 – Restriction Endonuclease patterns of Hinf I-digested DNA obtained after agarose gel electrophoresis. Each pattern corresponds to *C. krusei* isolates from patients A, B, C and D (upper case letters), from different biological products (BC – Blood cultures; CVC- central venous catheter; BS - bronchial secretions; S – stools), and to *C. krusei* isolates from the room environment of patients A and C (A_E and C_E, respectively); M- Molecular Weight Marker (1-kb DNA ladder, Metabion).

Most certainly, this patient harbors a reservoir of *C. krusei* and was colonized throughout a long period of time, as described for *Acinetobacter* [236] and *Pseudomonas* [237]. This is the first report, to our knowledge, describing a long-lasting colonization by *C. krusei.* These results have implications in terms of prophylactic measures (i. e. FLC is not recommended in a patient with previous isolation of *C. krusei*). Other antifungals such VRC or AMB B are more likely to be efficient in these cases [238, 239].

The *C. krusei* isolates from each patient yielded distinct Hinf I restriction patterns suggesting that the isolates were different strains (figure 1, lanes A_{BC2}, B_{BS}, C_S), at the same time discarding the hypothesis of an outbreak in the neutropenia unit where the patients were admitted. The *C. krusei* strain isolated from patient D showed a pattern distinct from those isolated from the remaining patients (figure 1, lane D_{BS}), as expected.

The two environmental *C. krusei* isolates collected from the surfaces of the rooms where patient A (A_E) (room 1) and patients B and C (C_E) (room 2) had stayed were different, as depicted in figure 1. This excludes the possibility of different *C. krusei* strains being transmitted as a result of patient handling by health care workers. However, both the environmental and the clinical *C. krusei* isolates associated with the same patient displayed an undistinguishable restriction pattern, as shown in figure 1 (lanes A_{CVC} and A_E vs. C_S and C_E), suggesting a putative environmental reservoir and the possibility of subsequent transmission from it to other patients. Our results emphasize the need to enhance preventive infection control measures, both when handling the patients directly and when cleaning patients´ facilities particularly those admitting neutropenic patients. Indeed, a study by Berrouane *et al*. described the relatedness among *C. krusei* clinical isolates obtained from different biological products from patients and health care workers [126].

As described in the present study, we were able to exclude the hypothesis of an outbreak occurring in the neutropenia unit throughout the time period considered, supporting the usefulness and suitability of the REA methodology. Moreover, the present study provided very useful information concerning *C. krusei* reservoirs existing in patients and their surrounding environment.

Chapter IV

MECHANISM OF RESISTANCE IN *CANDIDA KRUSEI*

“Acredite que você pode, assim você já está no meio do caminho”

Teodore Roosevelt; 26º Presidente dos Estados Unidos da América, historiador, naturalista, explorador, escritor, militar. 1858-1919.

- i -

In vivo and *in vitro* acquisition of resistance to voriconazole by *Candida krusei*

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Abstract

Candida krusei is an important agent of opportunistic infections that often displays resistance to several antifungals. We describe here the *in vivo* acquisition of resistance to voriconazole by *C. krusei* isolates recovered from a leukemia patient under a long period of VRC therapy. In order to mimic the *in vivo* development of voriconazole resistance, a susceptible *C. krusei* isolate was exposed daily to 1μg/ml of voriconazole *in vitro*. Interestingly, after 5 days of exposure to voriconazole, a MIC of 4 μg/ml was achieved; this value remaining constant after 25 additional days of treatment with VRC, and also after 30 consecutive days of incubation in voriconazole -free medium. Our objective was to determine the associated molecular resistance mechanisms, such as expression of efflux pumps genes and *ERG11* gene mutations, among the resistant strains.

Synergistic effects between the efflux blocker Tacrolimus (FK506) and voriconazole were found in all the resistant strains. Moreover, *ABC1* gene expression increased over time in both the *in vivo*- and *in vitro* - induced resistant strains, in contrast to the *ABC2* and *ERG11* genes, whose expression was invariably lower and constant. *ERG11* gene sequencing showed two different types of mutations, i.e., heterozygosity at T1389T/C, corresponding to a synonymous mutation, in *C. krusei* strains and a missense mutation, at position T418C, resulting in a change from Tyr to His, among resistant *C. krusei* clinical isolates. This study highlights the relevance of ATP-dependent efflux pump (namely, Abc1p) activity in voriconazole antifungal resistance and describes new mutations in the *ERG11* gene among resistant *C. krusei* clinical isolates.

Background

In recent years, we witnessed the emergence of low-pathogenicity non-*albicans* *Candida* species, such as *C. krusei*. It is an opportunistic pathogen especially among patients with hematologic malignancies and those undergoing bone marrow transplantation [56, 240]. Mortality rates among such patients with *C. krusei* fungemia are unacceptably high, ranging from 60 to 80% [56]. Nevertheless, few studies concerning the epidemiology and antifungal susceptibility profile of *C. krusei* are available. *C. krusei* ranked in fifth place among 22 different species of *Candida*, accounting for 3.3% of all *Candida* isolates both in Europe and North America [84, 130].

Candida krusei is often described as a multidrug-resistant (MDR) fungal pathogen due to its intrinsic resistance to FLC and decreased susceptibility to 5-FC, AMB B and KTC [127, 128]. Resistance to FLC is a major problem among neutropenic and critically ill patients since this drug is frequently used for prophylaxis [241]. *Candida krusei* resistance to echinocandins was also described among patients with acute myelogenous leukemia [220, 221].

Two major mechanisms of resistance to azoles are observed in *C. krusei*, i. e., reduced intracellular drug accumulation due to the activity of efflux pump proteins Abc1p and Abc2p and alterations in the target enzyme, cytochrome P450 lanosterol 14α-demethylase, encoded by Erg11p, which is involved in the ergosterol biosynthesis pathway [201, 211]. Such efflux pumps belong to the ATP Binding Cassette (ABC) transporter family of proteins encoded by *ABC1* and *ABC2* genes. Venkateswarlu *et al* described a group of clinical isolates of *C. krusei* resistant to ITC due to activity of efflux pumps [242]. However, the scarce information available regarding efflux pump activity and gene expression following azole exposure makes *C. krusei* resistance to azoles poorly understood [16]. On the other hand, it is not yet possible to explain the reduced affinity of azoles, namely FLC, for the target, the Erg11 protein binding site [201]. No resistance-conferring alterations in the *ERG11* gene sequence have yet been described for *C. krusei*, in comparinson with findings for *C. albicans* [196].

We addressed the molecular mechanisms of resistance acquired *in vivo*, i.e. in voriconazole (VRC)- resistant *C. krusei* strains isolated from a leukemia patient undergoing VRC therapy, or following induction *in vitro*, i.e., in VRC-resistant *C. krusei* strains repeatedly incubated with VRC at sub-inhibitory concentrations. We concluded that repeated exposure to VRC results in the development of concomitant resistance mechanisms, namely, enhanced activity of efflux pumps encoded by the *ABC1* gene and single-point mutations in *ERG11* gene.

Material and Methods

Patient clinical data

A 41-year-old male patient, admitted to Centro Hospitalar S. João (Porto, Portugal), was diagnosed with acute lymphoblastic leukaemia (T/natural killer [NK] cells) in August 2008. Due to protracted fever that persisted after antibacterial treatment, a myelogram was performed and a first relapse of the hematological disease was diagnosed. The patient was admitted at once at the Neutropenic Unit for salvage chemotherapy with fludarabine, idarubicin and 1-ß-D-arabinofuranosyl cytosine (Ara-C), followed by the growth factor granulocyte colony-stimulating factor (G-CSF), administered through a central venous catheter (CVC). Due to the persistent fever, the patient began treatment with 200 mg/daily of FLC. *Candida krusei* was first isolated from a blood culture after 9 days of FLC therapy; a second blood culture was positive for *C. krusei* at day 14. Fluconazole treatment was suspended and the patient began treatment with VRC at 200mg/day. During the initial 24 days of VRC therapy, several *C. krusei* isolates were recovered from blood cultures and from other biological samples; subsequently, *C. krusei* was recovered only from bronchial secretions and stools samples (figure 1). The patient had undergone VRC therapy for 216 days upon his death in April 2009.

All of the clinical isolates were identified using Vitek 2 YBC identification cards (bioMérieux, Marcy l'Etoile, France) and were stored at -70ºC in brain heart broth (Merck KGaA, Darmstadt, Germany) with 40% glycerol. Prior to experiments, isolates were subcultured twice in Sabouraud agar (Merck KGaA, Darmstadt, Germany) to ensure the purity of cultures.

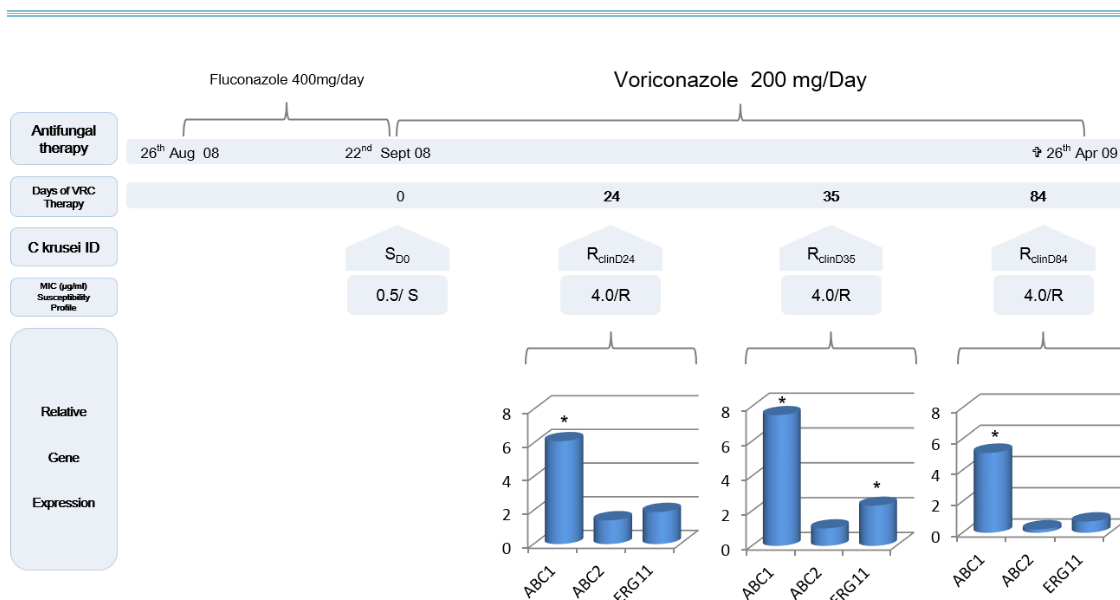


Figure 1 - Induction of resistance to voriconazole *in vivo*. The timeline of antifungal therapy and the recovery of *C. krusei* clinical strains from the leukemia patient, with the respective susceptibility profiles and relative gene expression levels is shown. ID, identification; S, susceptible; R, resistant. ✞ Patient death. *$P \leq 0.05$

Antifungal Drugs and Susceptibility Testing

Stock solutions of VRC (Pfizer, Groton, USA), PSC (Schering- Plough; New Jersey, USA), FLC (Pfizer, Groton, USA), AMB B (Bristol Meyers Squibb, New York, USA), CSF (Merck, Rahway, USA), AND (Pfizer, Groton, USA) and MCF (Astellas Pharma Inc., Tokyo, Japan) were prepared according to the M27-A3 and M27-S4 protocols of the Clinical and Laboratory Standards Institute (CLSI) and were maintained at -70°C until use [174, 175]. Minimal inhibitory concentrations were determined for all *C. krusei* isolates according to the same protocols. The VRC susceptibility profiles of the *C. krusei* isolates were determined in accordance with MICs of $\leq$0.5 µg/ml (susceptible), 1.0 µg/ml (susceptible dose dependent) and $\geq$ 2.0 µg/ml (resistant). Visual readings were performed after 24h and 48h of incubation, according to the same protocols. *C. krusei* type strain ATCC 6258 from the American Type Culture Collection was used as a control, as recommended [174, 175].

Molecular typing

All of the clinical isolates recovered from the leukemia patient were evaluated for their genetic relatedness, in order to determine whether the patient was colonized by the same *C. krusei* strain.

Total genomic DNA extraction. *C. krusei* isolates were cultured overnight in 10 ml of yeast extract-peptone-dextrose (YPD) liquid medium at 35ºC, at 150 rpm, and subsequently collected by centrifugation at 1610xg for 10min at room temperature (Universal 320 R, Hettich). Total DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1; Sigma-Aldrich, Munich, Germany), precipitated with 100% ice-cold ethanol (Applichem, Darmstadt, Germany), and redissolved in 200 µl of TE buffer. The DNA was treated with 20 µg of RNase (Applichem, Darmstadt, Germany) and incubated at 37ºC for 1 h. For final precipitation, 20 µl of 4 M ammonium acetate (pH 4.8) (Sigma-Aldrich, Munich, Germany) and 600 µl of ice-cold 100% ethanol (Applichem, Darmstadt, Germany) were added and samples were incubated overnight at – 20ºC.

DNA samples were resuspended in 1x TE buffer, the concentration was adjusted to 2.0-2.5 µg/µl and samples were stored at -20ºC for later use.

Restriction Endonuclease Analysis (REA). For each sample, a reaction mixture containing: 1x HinfI enzyme reaction buffer (Metabion, Martinsried, Germany) 1µg/µl RNase (Applichem, Darmstadt, Germany), 0.5 U/µl HinfI restriction enzyme (Metabion, Martinsried, Germany), approximately 25 to 30 µg of total DNA and DNAse/RNAse-free water up to a final volume of 20 µl was prepared as described by Ricardo et al [243]; reaction tubes were incubated overnight at 37ºC. Total reaction mixture was run on a 1% agarose gel (20cm by 24cm, 120 mv) for 3 to 5 h, stained with ethidium bromide solution (0.5mg/ml; Applichem, Darmstadt, Germany) and visualized with UV with a Chemidoc XRS+ imaging system (Bio-Rad, Hercules, USA). Restriction patterns were analyzed using the Image Lab Software (version 4.0.1; Bio-Rad, Hercules, USA) and the different isolates were compared for restriction pattern similarities. A nonrelated *C. krusei* isolate recovered from stools from a different patient was used as a control.

Random Amplification Polymorphic DNA. Random amplification polymorphic DNA (RAPD) analysis was performed as described previously [244], with some alterations. Briefly, the reaction mixture for RAPD analysis contained 1x Dream Taq enzyme reaction buffer, 0.04 U/μl DreamTaq DNA Polymerase, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2 mM $MgCl_2$ (all from Fermentas, Vilnius, Lithuania), 0.4 μM Primers OPA-18 (5' – AGCTGACCGT- 3') or OPE-18 (5' - GGACTGCAGA- 3') (STABVida, Lisbon, Portugal), 200 ng total DNA and DNase/RNase-free water up to a final volume of 25 μl. All PCRs were performed in an Eppendorf RealPlex2 Mastercycler (Eppendorf, Hamburg, Germany) and the reaction parameters were one cycle of 95ºC for 2min; 38 cycles of 95ºC for 1 min, 36ºC for 1 min and 72ºC for 2 min; and one cycle of 72ºC for 10 min. The amplified DNA fragments were analyzed by electrophoresis in a 2% agarose gel at 120 mv, for 2 h. The agarose gel was stained with ethidium bromide solution (0.5 mg/ml; Applichem, Darmstadt, Germany) and visualized with UV light with the Chemidoc XRS+ imaging system. Electrophoresis patterns were analyzed using the Image Lab Software (version 4.0.1).

In vitro induction of resistance

A VRC-susceptible *C. krusei* isolate, recovered from the leukemia patient before VRC antifungal therapy, was repeatedly incubated with VRC. Briefly, a single, randomly selected colony from a fresh 24-h culture on Sabouraud agar was suspended in 10 ml of RPMI 1640 medium buffered to pH 7.0 with 0.165M MOPS (morpholinepropanesulfonic acid) (both from Sigma-Aldrich, Munich, Germany), in the presence of VRC at 1 µg/ml, and was incubated at 35°C at 150 rpm. Every 24 h, 1 ml of the culture was suspended in 9 ml of fresh 1 µg/ml VRC-containing RPMI 1640 medium, for a 30-day period. Subsequently, in order to evaluate the stability of the susceptibility profile, this strain was subcultured daily in fresh RPMI 1640 medium without VRC for an additional 30 days; again, every 24 h, 1ml of the culture was suspended in 9 ml of fresh RPMI 1640 medium. At each daily subculture, an aliquot was stored at -70°C in 40% glycerol; every 2 days, a 10-µl, loopful of yeast cells was cultured in Sabouraud agar to check for culture contamination. Every 5 days during the 60 days of the assay, VRC

MIC values were determined according to the CLSI M27-A3 and M27-S4 protocols [174, 175].

Effect of the efflux blocker Tacrolimus (FK506) on *C. krusei* susceptibility profile

The VRC MIC values for all of the resistant *C. krusei* strains (clinical isolates or induced *in vitro*) were redetermined according to the CLSI M27-A3 and M27-S4 protocols in the presence of 100 µg /ml FK506, a recognized ATP-dependent efflux pump inhibitor [245]. An agar disk diffusion assay was also performed using blank paper disks impregnated with FK506, to corroborate the results obtained with the CLSI protocols. Yeast suspensions of resistant C. *krusei* strains were prepared to an optical density of 0.5 McFarland standard (Densimat; bioMérieux, Marcy l'Etoile, France) and spread onto YPD agar plates with VRC at 4 µg/ml or without VRC. Blank paper disks (BBL, 6 mm; Becton, Dickinson) were impregnated with serial 10-fold dilutions of FK506 solutions, ranging from 1,000 to 1 µg/ml, or with its solvent, dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany); paper disks were applied to the inoculated agar plates. A plate containing only VRC at supra-MIC values was used as a control for cell viability for each strain. Results were read after 24 h and 48 h of incubation at 37°C.

Resistance gene expression analysis

Total RNA extraction: *C. krusei* strains were incubated in YPD broth at 35°C, at 150 rpm, until the exponential growth phase. Yeast cells were harvested by centrifugation at 1,610x*g* for 5 min at room temperature (Universal 320 R; Hettich) and were immediately frozen in liquid nitrogen. Total RNA was extracted using the hot acid-phenol method, as described by Köhrer and Domdey [246]. RNA samples were resuspended in DNase/RNase-free water, adjusted to a final concentration of 100 ng/µl, and stored at -70°C for later use.

Reverse transcriptase PCR (RT-PCR). Two-step real-time PCRs were performed. Reverse transcriptase reactions were performed as recommended by the manufacturer; 50 ng of RNA sample, 0.015 ng/µl random primers (Invitrogen, Carlsbad, CA), 0.5 mM dNTPs (Fermentas, Vilnius, Lithuania), and RNase-free water up to 13 µl

were incubated at 65°C for 5 min and then placed on ice for 1 min. Subsequently, 1x reverse transcriptase enzyme buffer (Invitrogen, Carlsbad, CA), 5 mM dithiothreitol (DTT) (Invitrogen), 1 U/μl RNasin enzyme (Promega, Madison, WI), 5 U/μl reverse transcriptase enzyme (Invitrogen), and RNase-free water were added up to a final volume of 20 μl. The reaction tubes were incubated at 25°C for 5 min, at 50°C for 60 min, and at 70°C for 15 min for enzyme inactivation. Reactions were carried out in a Mastercycler ep gradient RealPlex2 system. The cDNAs were kept at -20°C.

Quantitative Real-Time PCR (qRT-PCR): Genes were amplified using the following primers *ABC1* (GenBank accession number DQ903907) (forward, 5' – GAT AAC CAT TTC CCA CAT TTG AGT – 3' and reverse, 5' – CATATGTTGCCATGTACA CTTCTG – 3'), *ABC2* (GenBank accession number, AF250037) (forward, 5' – CCTTTTGTTCAGTGCCAGATTG – 3' and reverse, 5' – GTAACC AGGGACACCAGCAA– 3'), *ERG11* (GenBank accession number FJ445756) (forward, 5' – ATTGCGGCCGATGTCCAGAGGTAT – 3' and reverse, 5' – GCGCAGAGTATAAGAAAGGAATGGA – 3'), *ACT1* (GenBank accession number AJ389086) (forward, 5' - TGGGCCAAAAGGATTCTTATG -3' and reverse, 5'-AGATCTTTTCCATATCATCCCAG- 3') (*STABVida*, Lisbon, Portugal). The quantitative real-time (qRT)-PCR mixture contained 1x PerfeCTa SYBR green FastMix (Quanta Biosciences, Gaithersburg, MD), forward and reverse primers (*ABC1*, *ABC2*, and *ERG11*, 0.9 μM; *ACT1*, 0.5 μM), 2 μl of cDNA, and RNase-free water, up to a final reaction volume of 20 μl. MgCl2 was used in the *ABC2* gene reaction mixture at a final concentration of 1 mM. All reactions were performed in the Mastercycler ep gradient RealPlex2 system; parameters were chosen according to the manufacturer's recommendations except for primer annealing temperatures, which were as follows: *ABC1*, 54°C; *ABC2*, 56°C; *ERG11*, 60°C; *ACT1*, 54°C. To check for PCR product specificity, a melting curve was established, with temperatures ranging from 60°C to 95°C, for 20 min.

Data Analysis: A standard curve, containing serial 5-fold dilutions ranging from 500 ng to 0.8 ng of RNA transcribed to cDNA, was determined in triplicate for

quantification and assessment of reaction efficiency for each gene. Assays were validated for reaction efficiencies ranging from 80% to 100%, and with a standard curve presenting a mean squared error higher than 0.99. The results were analyzed using RealPlex software (version 1.5.474; Eppendorf). Relative gene expression levels were calculated using REST 2009 software (Qiagen GmbH, Munich, Germany) [247], with the susceptible *C. krusei* strain as the reference sample and each resistant *C. krusei* strain as the target sample; the *ACT1* gene was used to normalize levels of gene expression. Genes exhibiting 2-fold increases in expression were considered overexpressed.

Statistical analysis

Analyses of results were performed using SPSS version 19.0. Continuous and paired-sample Student's *t* tests were used to analyze significant differences in gene expression displayed by the distinct *C. krusei* strains; *P* values of $\leq$0.05 were considered statistically significant.

Sequencing data analysis of ERG11 gene

Total genomic DNA was extracted as described above. The *ERG11* gene (1,890 bp) was amplified by PCR; the reaction mixture contained 1 U/µl Dream Taq DNA Polymerase enzyme, 1x Dream Taq DNA Polymerase enzyme buffer, 0.2 mM dNTPs (all from Fermentas, Vilnius, Lithuania), forward_1 (5' – GGTTGTTTGTTCATTTAATGTGTGT – 3') and reverse (5' – GAAGGGGGAAAGAAAGGGAA – 3') primers at 0.8 µM (*STABVida*, Lisbon, Portugal) and RNAse-free water up to a final volume of 25 µl. All reactions were performed in a Mastercycler ep gradient RealPlex2 system, and reaction parameters involved an initial 2-min denaturation step at 95ºC, 30 cycles of 95ºC for 30 s, 52ºC for 30 s and 72ºC for 1 min, and a final 10-min extension step at 72ºC. PCR products were treated with ExoSAP-IT (USB Corp., Cleveland, OH) and used as templates for the sequencing reactions. Sequencing was performed with the BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Carlsbad, USA) using the forward_1, forward_2 (5' – AACTACTGGAAAAGAGATGCTGC – 3') and forward_3 (5' –

CACTCGTGATTTACCCGTTCC – 3') primers, at 0.8 μM final concentration. DNA products were purified with Sephadex G-50 Fine (GE Healthcare, Buckinghamshire, United Kingdom) and sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Foster city, CA). Results were analyzed with Sequencing Analysis software (version 5.2; Applied Biosystems). The *ERG11* gene coding sequences of the resistant strains were aligned with the susceptible *ERG11* gene coding sequence using MUSCLE software/ClustalW [248]. Alignments were analyzed with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)/ ClustalW.

Results

Nomenclature and susceptibility profile of *C. krusei* strains

Four *C. krusei* clinical isolates were selected according to the VRC MIC values and the collection date (more than 10 days apart), i.e., a susceptible isolate with a VRC MIC of 0.5 µg/ml, that was recovered from a blood culture before initiation of VRC therapy, designated strain S_{D0}, and three resistant isolates with VRC MICs of 4 µg/ml. The latter were designated $R_{clinD24}$, $R_{clinD35}$, and $R_{clinD84}$ according to the day of recovery during VRC therapy. $R_{clinD24}$ was the first VRC-resistant isolate and was recovered from stools, while $R_{clinD35}$, and $R_{clinD84}$ were collected from bronchial secretions (figure 1). The susceptibility profile of the 4 *C. krusei* clinical isolates with the different classes of antifungals are detailed in table 1. As expected, *C. krusei* revealed intrinsic resistance to FLC.

Table 1 - MIC and susceptibility profile of *C. krusei* clinical isolates.

Strain identification	Recovery site	MIC (µg/ml)/ Susceptibility profile						
		AMB B	FLC	VRC	PSC	AND	CSF	MCF
S_{D0}	Blood	0.5/S	64/R	0.5/S	0.5/S	≤0.06/S	0.5/I	0.5/S
$R_{clinD24}$	Stools	1.0/S	64/R	4.0/R	≤0.03/S	≤0.06/S	0.25/S	0.5/S
$R_{clinD35}$	Bronchial Secretions	1.0/S	64/R	4.0/R	0.06/S	≤0.06/S	0.125/S	0.25/S
$R_{clinD84}$	Bronchial Secretions	0.125/S	64/R	4.0/R	0.5/S	≤0.06/S	0.5/S	0.25/S

NOTE: Antifungals: AMB B – Amphotericin B; CSF – Caspofungin; AND- Anidulafungin; MCF – Micafungin; FLC – Fluconazole; VRC – Voriconazole; PSC – Posaconazole; Susceptibility phenotype: S-susceptible, I- intermediate, R-resistant.

The VRC MIC turning point to resistance of strain S_{D0} during the *in vitro* induction assay occurred at day 5 of exposure to VRC, corresponding to a MIC of 4 µg/ml. The VRC MIC for all *C. krusei* strains remained unchanged during the 55 subsequent days of the assay (Table 2; figure 2). Selected strains were designated according to the day of incubation with VRC; strains obtained at days 5, 10, and 30 of

the induction assay were named R_{indD5}, R_{indD10}, and R_{indD30}, respectively. The resistant strain obtained after 30 days of culture in VRC-free medium was named R_{D60} (figure 2).

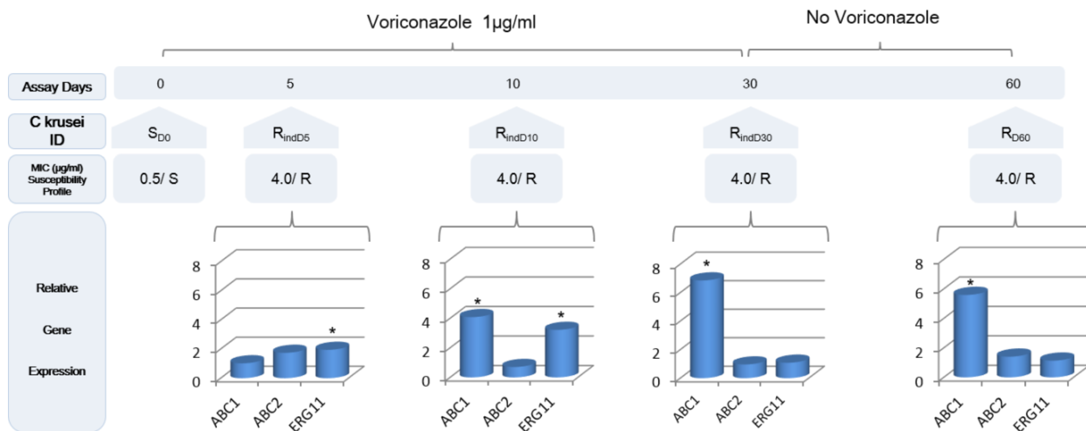


Figure 2 - Induction of resistance to voriconazole *in vitro*. The timeline of the development of VRC resistance in a susceptible *C krusei* clinical isolate that was exposed daily to VRC at 1 µg/ml, with the respective susceptibility profiles and relative gene expression levels, is shown. ID, identification; S, susceptible, R, resistant. *$P \leq 0.05$

Table 2 - MIC and susceptibility profile to VRC, alone and in combination with FK506 of *C krusei* strains.

Resistance	Strain ID	VRC MIC (µg/ml)/ susceptibility profile when used with:	
		No FK506	100 µg/ml FK506
None	S_{D0}	0.5/S	0.125/S
In vivo acquired	$R_{clinD24}$	4.0/R	0.5/S
	$R_{clinD35}$	4.0/R	0.5/S
	$R_{clinD84}$	4.0/R	0.25/S
In vitro acquired	R_{indD5}	4.0/R	0.5/S
	R_{indD10}	4.0/R	0.5/S
	R_{indD30}	4.0/R	0.5/S
	R_{D60}	4.0/R	0.25/S

Molecular typing

Both REA and RAPD techniques exhibit high discriminative power, as described by Sancak *et al* and Bautista-Muñoz *et al,* respectively [114, 244]. All of the *C. krusei* clinical strains exhibited the same restriction pattern in REA, and the same amplification pattern in RAPD analysis, as shown in figure 3. Therefore, these results strongly indicate that the strains colonizing the leukemia patient were genetically related and arose from the common ancestor susceptible strain S_{D0}.

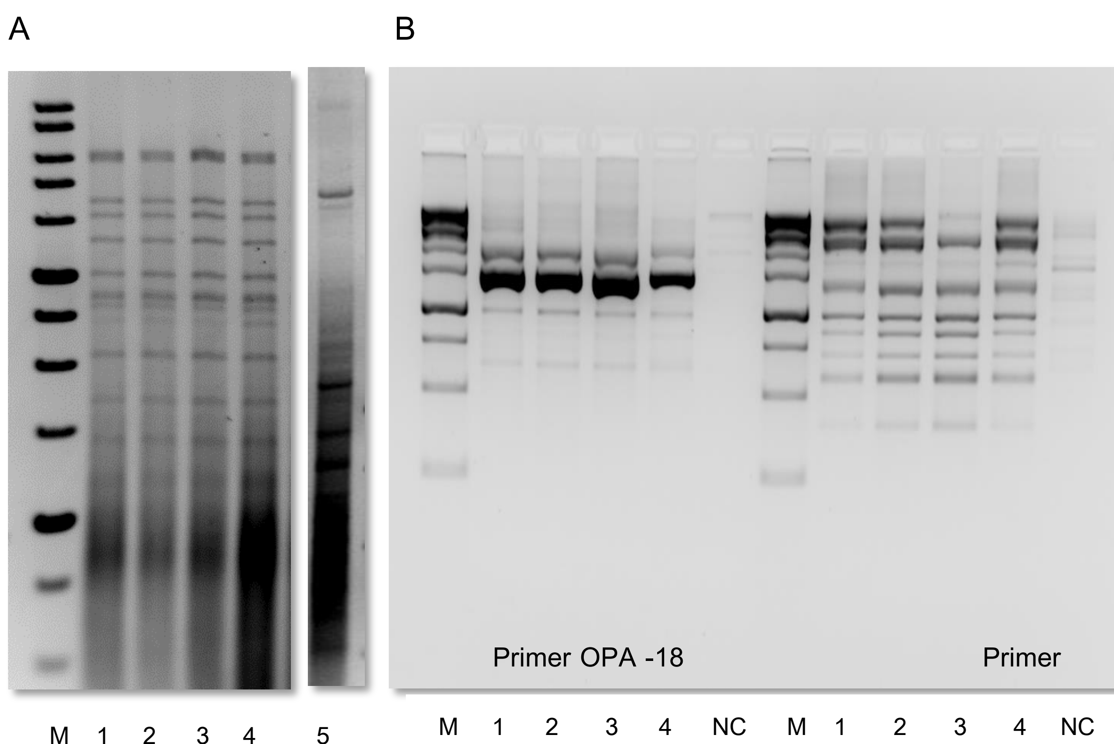


Figure 3 - Genotyping of clinical isolates. (A) Restriction endonuclease pattern of HinfI-digested DNA of *C. krusei* clinical isolates in agarose gel electrophoresis; (B) Random Amplification Polymorphic DNA (RAPD) of *C. krusei* clinical isolates with primers OPA-18 and OPE-18. Lane M, 1-kb DNA ladder (Metabion); lane 1, S_{D0}, lane 2, $R_{clinD24}$, lane 3, $R_{clinD35}$, lane 4, $R_{clinD84}$, lane 5, Non related *C krusei* control strain; lane NC, PCR negative control.

Effect of FK506 upon *C. krusei* susceptibility profile

In the presence of FK506 all of the resistant strains changed to a VRC susceptible phenotype (Table 2). The agar disk diffusion assay confirmed the microdilution results; growth inhibition was found for all the resistant strains around

disks containing the two highest FK506 concentrations (100 and 1000 μg/ml) in the presence of VRC at 4 μg/ml (figure 4, B and D). Susceptible strain S_{D0} was unable to grow in the presence of VRC at 4 μg/ml. The FK506 solvent, DMSO, did not impair the growth of the strains, and neither did FK506 alone (figure 4, A and C).

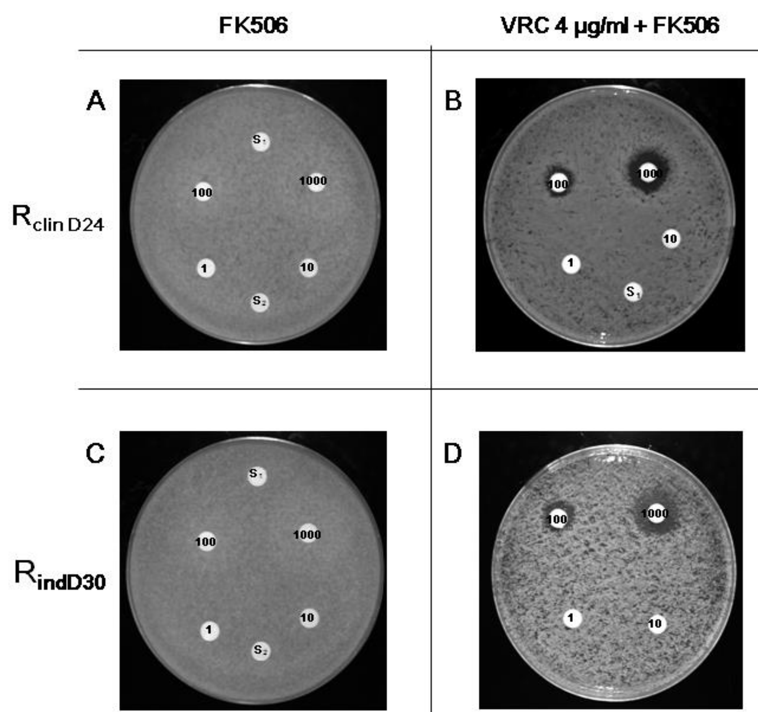


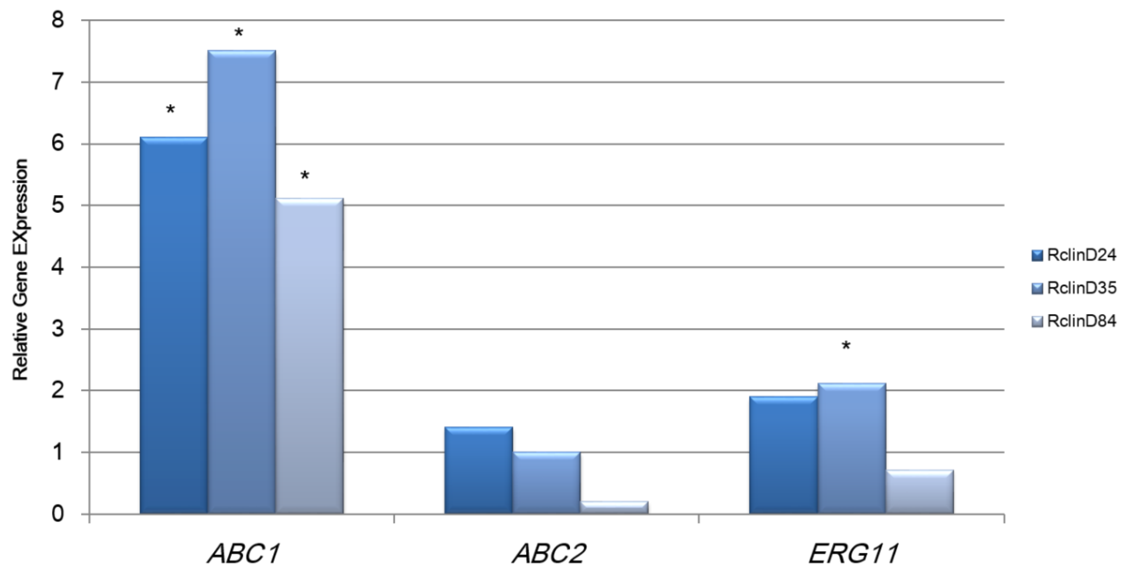
Figure 4 - FK506 disk diffusion assay. Resistant *C. krusei* strains were grown on YPD medium and paper disks impregnated with serial 10-fold dilutions of FK506, ranging from 1000 to 1 μg/ml, without VRC (A and C), or VRC at 4 μg/ml (B and D). $R_{clinD24}$ (A and B) and R_{indD30} (C and D) are shown as representative examples of *C. krusei* strains with *in vivo* and *in vitro* induced resistance, respectively. S1, 100%, S2, 10%: disks impregnated with DMSO at different concentrations.

Resistance gene expression analysis

The analysis of gene expression in R_{clin} strains with real-time PCR showed significant overexpression of the *ABC1* gene, in comparison with the susceptible S_{D0} strain (figure 1; figure 5A). This was not the case for the other two associated resistance genes, *ABC2* and *ERG11*. Only the $R_{clinD35}$ strain presented a significant increase in *ERG11* gene expression. Similar *ABC1* gene expression profiles were registered for all of the R_{ind} strains and the R_{D60} strain, i.e., during the induction protocol, significant increases in the relative expression of the *ABC1* gene were documented (figure 1;

figure 5B). Variations in *ABC2* gene expression were not significant for any of these strains. *ERG11* relative gene expression levels demonstrated significant increases in the R_{indD5} and R_{indD10} strains.

A



B

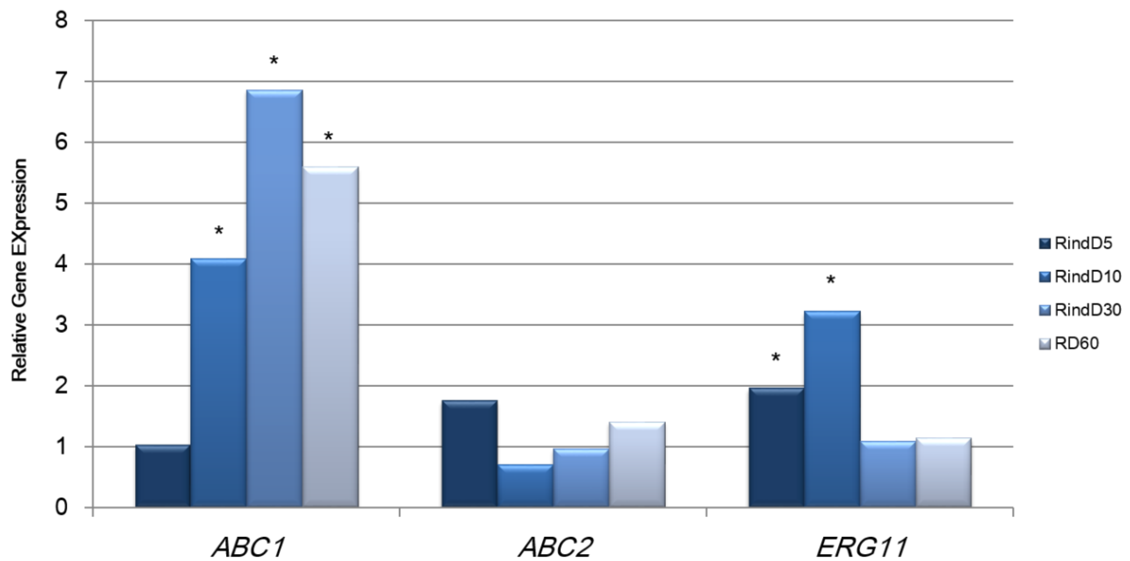


Figure 5 - *ABC1*, *ABC2* and *ERG11* relative gene expression levels in resistant *C. krusei* clinical isolates (A) and resistant strains induced *in vitro* (B). *ABC1*, *ABC2* and *ERG11* gene expression levels were quantified and normalized relative to the housekeeping gene, *ACT1*; relative gene expression levels were calculated as ratios between each *C. krusei* resistant strain and the S_{D0} isolate; * $P \leq 0.05$.

ERG11 gene sequencing analysis

Several *ERG11* gene mutations were reported previously to be associated with azole resistance in *C. albicans* [196]; therefore, the *C. krusei ERG11* gene was sequenced in our strains. Two different types of mutations were found. All of the susceptible and resistant *C. krusei* strains presented a heterozygous alteration at 1,389 bp (T→C) (overlapping signals in the electropherogram data), resulting in synonymous single-nucleotide polymorphisms (SNPs). Notably, $R_{clinD35}$ and $R_{clinD84}$ presented a missense mutation at position 418 bp (T→C), yielding a Tyr→His amino acid change. This point mutation had not been described previously for *C. krusei* strains.

Discussion

C. krusei is one of the leading agents of candidemia among patients with hematologic malignancy. In this study, the 4 consecutive *C. krusei* clinical isolates obtained from the leukemia patient during VRC therapy were evaluated for their susceptibility profiles and genetic relationships. Although they displayed distinct susceptibility profiles, these isolates were genetically related (figure 3). The initial colonizing strain S_{D0} changed from a VRC-susceptible phenotype to a stable VRC-resistant phenotype during a long period of VRC therapy. Thus, we were confronted with a case of resistance to VRC acquired *in vivo*. It should be stressed that the bioavailability and concentrations of the bioactive drug *in vivo* are highly variable due to several factors, such as different infection sites, concomitant therapies, and the status of the host immune system [152]. The *C. krusei* clinical isolates might have been in contact with subinhibitory concentrations of the antifungal agent for a long period, which were not sufficient to eliminate the organism but were enough to stimulate stress adaptation mechanisms leading to resistance. Therefore, a susceptible *C. krusei* isolate (strain S_{D0}) was later continuously exposed to VRC *in vitro*, in order to compare the development of resistance *in vivo* and *in vitro*.

FK506 was used as a first approach to explore the mechanisms of resistance to VRC displayed by *C. krusei* strains. It was previously described as being able to reverse multidrug resistance in different types of eukaryotic cells, due to the blockade of ATPdependent efflux pumps, namely, human P-glycoprotein, *C. albicans* Cdr1p/Cdr2p, and more recently *C. krusei* Abc1p [16, 245, 249]. The synergistic effects of FK506 and VRC that occurred with all of the resistant *C. krusei* strains, in both the microdilution and test disk assays, clearly showed that efflux pumps contributed to the VRC-resistant phenotype in both R_{clin} and R_{ind} strains.

Several genes have been reported to be involved in *C. krusei* resistance to azoles. For example, according to Katiyar and Edlind, efflux pumps from the ABC family of proteins are crucial for resistance to azoles [211]; in contrast, Guinea et al. concluded that the MDR family of proteins plays a minor role in *C. krusei* resistance to azoles [212]. *ERG11* gene upregulation in *C. krusei* strains in response to azole treatment (3 h

of incubation with FLC at 9 µg/ml) was described [250]. Taken this into account, molecular insights were needed to corroborate the hypothesis that VRC resistance is mostly associated with the activity of efflux pumps and to exclude other resistance mechanisms.

This is the first work to address the quantification of target gene expression by real-time PCR in wild-type resistant isolates, i.e., not genetically manipulated strains. The gene expression profile described herein for all of the resistant *C. krusei* strains (figure 5) strongly suggests that the acquisition of long-term resistance is mostly associated with the *ABC1* gene. Similar findings were previously described by Holmes et al. for *C. albicans* and by Bennett et al. for *Candida glabrata*, i.e., Cdr1p efflux activity contributes more to FLC resistance than Cdr2p [251, 252]. However, our *C. krusei* R_{indD5} strain is a controversial case since neither *ABC1* nor *ABC2* genes are overexpressed. Initially, exposure to VRC induces increases in *ABC2* gene expression, overcoming *ABC1* gene expression. We hypothesize that *ABC2* can be activated more rapidly, although transiently. For long-term VRC tolerance, yeasts clearly prefer to activate the Abc1p efflux pump, which seems to be more efficient in antifungal expulsion. On the other hand, other genes encoding ATP-dependent efflux transporters may be present in C. *krusei*, such as a *CgSNQ2* homologous gene that was described as an azole-associated resistance gene in *C. glabrata* [253]. Although it has been sequenced, the *C. krusei* genome is not yet completely annotated; thus, other transporter genes were not assessed. In addition, Lamping *et al*. incubated *C. krusei* strains for up to 4 h with different antifungals, including VRC, and no significant increases in *ABC1* mRNA levels were recorded [16]. In our case, the *ABC1* gene was significantly overexpressed in *C. krusei* strains after only 10 days of *in vitro* exposure (strain R_{indD10}) or 24 days of VRC therapy (strain $R_{clinD24}$). Together, these facts clearly show that the *ABC1* gene is upregulated after an extended period of antifungal exposure, playing a late role in the development of resistance. After being triggered, however, *ABC1* gene overexpression correlates with a stable resistant phenotype, playing a definite role in long-term VRC resistance even in the absence of azoles (strain R_{D60}) (figure 5B). *ERG11* gene overexpression seems to be relevant in the development of VRC resistance only at an early stage, as an initial adaptation mechanism (figure 5). Later, other distinct

mechanisms, such as the acquisition of point mutations, predominate. The point mutation described herein is definitely associated with VRC resistance in *C. krusei*, since the same type of mutation was already reported to be associated with azole resistance in *C. albicans* [254]. Thus, we were confronted with the fact that *C. krusei* acquired multiple resistance mechanisms not previously described for this fungal pathogen. Such a finding is of medical relevance in considering therapeutic protocols; a susceptible isolate can develop resistance to VRC during a therapeutic regimen. On the other hand, the heterozygous alteration detected (T1389C), which is located outside the azole binding site, according to previously published data, was found in both susceptible and resistant strains [255]. This alteration corresponds to synonymous SNPs, whose repercussions remain to be determined. These findings are in accordance with previous results by Lamping *et al.*, who found several synonymous SNPs in the *ERG11* gene sequence, including the one detected by us [16]. The results presented emphasize that prolonged therapy with azole antifungals can lead to resistant clones, which can ultimately spread and colonize other susceptible hosts.

In this study, we elucidated for the first time the presence of multiple concomitant resistance mechanisms in resistant *C. krusei* strains induced in both *in vivo* and *in vitro* assays. We demonstrated the relevant role that efflux activity plays as a mechanism of resistance to VRC, as well as the acquisition of a missense point mutation in the target enzyme Erg11p. The set of *C. krusei* strains described herein depicts the evolutionary process of resistance acquisition both *in vitro* and *in vivo*, being a valuable tool for the study of antifungal resistance in *C. krusei*.

- ii -

Candiduria due to *Candida krusei*: a case of induction of resistance *in vivo*

Authors: Elisabete Ricardo, Frédéric Grenouillet, Isabel M. Miranda, Raquel M Silva, Nadège Devillard, Laurence Millon, Acácio Gonçalves Rodrigues, Cidália Pina-Vaz.

Abstract

The treatment of candiduria is always a concern in the clinical practice, due to the low level of antifungal drug reaching the urinary tract. A patient submitted to a kidney transplant was diagnosed with candiduria involving *C. krusei* and treated with VRC 200mg/2x day for 20 days. Five *C. krusei* isolates recovered from the urine were selected: one susceptible before VRC therapy (MIC 0.25 μg/ml), two resistant during VRC therapy (MIC 4.0 μg/ml) and two susceptible after VRC discontinuation (MIC 0.25 μg/ml). Clinical isolates were all genotyped using both intergenic repeat-PCR patterns (CKRS-1) and CKTNR microsatellite analysis, confirming their genetic relationship. Therefore, we were dealing with a case of development of transient resistance *in vivo*. Based on these results, we intended to corroborate the hypothesis that exposure to suboptimal VRC concentrations could lead to VRC resistance. We incubated eight independent clinical strains of *C. krusei* with VRC 0.001 μg/ml, during 30 days. The MICs to VRC of these strains ranged from 2 to 8 μg/ml, corresponding to a resistant phenotype. In the presence of VRC and the efflux pump blocker FK506, the susceptible phenotype was restored in the microdilution assay and inhibition of growth of all the VRC-resistant isolates occurred in the agar disk diffusion
assay. In order to uncover the mechanisms of resistance to VRC, efflux pumps encoded by *ABC1* and *ABC2* genes and the target enzyme of azoles Erg11p, encoded by *ERG11* gene, were investigated. *ABC1* and *ERG11* genes were overexpressed in the clinical isolates resistant to VRC and decreased to a basal level of expression in post-therapy isolates. The *C. krusei* strains incubated *in vitro* with small doses of VRC presented at least one of the resistance genes significantly overexpressed, mostly *ABC1* and *ERG11* genes and different alterations in *ERG11* gene sequence. Interestingly, the strain presenting the lowest level of gene expression associated to resistance genes displayed a homozygous nonsynonymous mutation at position 418bp (T →C), translating into a different aminoacid Tyr→His.

In conclusion, although the effective therapeutic concentration of antifungal reaching the bladder is usually low, the acquisition of resistance to antifungal drugs can

be achieved. The mechanisms of resistance studied in the *C. krusei* strains resistant strains were all present and in some strains complement each other.

Background

Candida species appear to be unique in their ability to both colonize and cause invasive disease in the urinary tract [256]. Candiduria is defined as a superficial infection with *Candida* spp., therefore does not belong to the group of IC [31, 257, 258]. Candiduria is asymptomatic in up to 96% of the patients and it is not considered a serious clinical condition [61]. However, it can be problematic in patients admitted to ICU namely, immunocompromised patients with underlying serious diseases, in patients with permanent urinary catheters, with diabetes or with altered bacterial flora due to intensive use of broad spectrum antibiotics [61, 62, 75, 259, 260]. *Candida albicans* is the most important and commonly isolated yeast among *Candida* species followed by *C. glabrata* and *C. tropicalis* [61, 76, 261, 262]. In this non-*albicans* group, *C. krusei* is not frequently isolated [263]. Colonization and invasion of the urinary tract can occur in either an antegrade fashion from the bloodstream or retrograde via the urethra and bladder, being the latter the most common route of infection. The presence of *Candida* species in the urine may be due to different clinical conditions: pyelonephritis or cystitis, hematogenous seeding of the kidney cortex due to disseminated candidiasis or colonization of the bladder, perineum or indwelling urinary catheter. However, there is some concern about candiduria since it can be the spark to candidemia in critically ill patients. Also, when fungus ball form, there is a strong probability of biofilm formation allowing the persistence of the organism in the host [70]. One of the big concerns related to candiduria is the therapeutic decision of the physicians [76]. This is probably due to the fact that no diagnostic tools are available to readily differentiate between candidal UTI, colonization or contamination in the ICU setting. The IDSA (Infectious Diseases Society of America) has defined indications for therapy of candiduria in late 2003 in the following groups: infants with very low birth weights, patients undergoing genitourinary procedures, patients with neutropenia, renal transplant recipients and symptomatic patients. Several classes of antifungals are available but none of them is the best suited antifungal. Fluconazole is the first choice as it is excreted unchanged by the kidneys, KTC and ITC are poorly excreted in the urine and VRC and PSC achieve minimal urinary excretion, AMB B is very nephrotoxic, and all echinocandins are

associated to extremely poor glomerular filtration or tubular secretion *in vivo*, as 2–3% of active drug is eliminated in the urine, resulting in subtherapeutic concentrations in the urine [61, 77, 78, 80].

We describe in this work a case of candiduria by *C. krusei* in a patient submitted to a kidney transplant and under VRC therapy. It was registered the transient acquisition of resistance to VRC *in vivo*. Two major mechanisms associated to azole resistance have been described in *C. krusei*: the presence of efflux pump proteins (Abc1p and Abc2p), and a diminished sensitivity of the azole antifungals to the enzyme cytochrome P450 lanosterol 14α-demethylase, Erg11p, encoded by *ERG11* gene. Efflux pump proteins in *C. krusei* belong to the ABC transporter family, encoded by *ABC1* and *ABC2* genes which promote the extrusion of the antifungal drug from the cell, reducing the cellular drug accumulation. *Candida krusei* is intrinsically resistant to FLC due to alterations in Erg11p binding site [201, 211]. Recently, one mutation associated to resistance was described by our team in *ERG11* gene in *C. krusei* strains resistant to VRC at position 418 bp (T →C) translating into a Tyr→His amino acid change [264]. Acquisition of resistance *in vivo* was replicated *in vitro* in the presence of very low concentrations of VRC. We intended to uncover the mechanisms of resistance that developed in *C. krusei* strains *in vitro* and *in vivo*.

Material and Methods

Case report and clinical *C. krusei* strains isolation

A 20-year-old woman was hospitalized in December 2003, for acute renal failure secondary to rhabdomyolisis, induced by atorvastatine (Tahor®). She presented with a probable evolution of a previously undiagnosed chronic renal insufficiency, associated with urological malformation and familial hypercholesterolemia. In November 2005, renal transplantation was performed and immunosuppressive treatment (tacrolimus, mycophenolate mofetil and corticoids) was given. Twelve days after transplantation, bacteraemia due to *Escherichia coli* occurred probably due to a contamination of the solution for graft kidney preservation. She was administered ceftriaxone and ciprofloxacine. At day 18 of transplantation, she presented with an aneurysm dissection of renal artery associated with hematoma. Therefore, the surgeons had to perform hypogastric autograft and distal anastomosa in hilus of renal graft, resulting in the preservation of the graft. Pre-renal hematoma was progressively resolved with antibiotics. At day 25th after transplant, the first episode of candiduria occurred. At day 44th after transplant, oral FLC 200 mg/day was implemented. However, at day 44 of kidney transplantation, FLC stopped and began to take oral VRC 200 mgx2/day, prescribed for 20 days mainly for graft preservation (prophylaxis), despite probable low urinary diffusion associated with low expected efficacy against candiduria. After 82 days of renal transplant she was admitted to the emergency with fever, probable dissecting aneurysm of renal artery at CT-scan. Therefore, she was submitted to resection of kidney graft and prescribed antibiotics and intravenous AmB 1 mg/kg/day for 3 weeks. Since the first episode of *C. krusei* candiduria several *C. krusei* isolates were recovered and five were selected. The *C. krusei* isolates recovered before the VRC treatment presented a susceptible phenotype, those recovered during VRC treatment presented a resistant phenotype and those isolates recovered after the withdraw of the antifungal turned to a susceptible phenotype.

Candida krusei clinical isolates genotyping

All the clinical isolates recovered from the patient submitted to kidney transplant were evaluated for their genetic relatedness, in order to confirm whether the patient harbored the same *C. krusei* strain or not during her follow-up.

Total genomic DNA extraction. The protocol was performed as next described. Briefly, *C. krusei* isolates were cultured in 10 ml of YPD liquid medium, overnight at 35ºC, 150 rpm, and subsequently collected at room temperature by centrifugation (Hettich, Universal 320 R, 1610xg, 10min). Total DNA was extracted using phenol:chloroform:isoamyl alcohol 25:24:1 (Sigma-Aldrich, Munich, Germany), precipitated with 100% ice-cold ethanol (Applichem, Darmstadt, Germany), and redissolved in 200 µl of TE buffer. The DNA was treated with 20 µg of RNase (Applichem, Darmstadt, Germany), incubated at 37ºC for 1 h. For final precipitation, 20 µl of 4 M ammonium acetate, pH 4.8 (Sigma-Aldrich, Munich, Germany) and 600 µl of ice-cold 100% ethanol (Applichem, Darmstadt, Germany) were added and samples were incubated overnight at – 20ºC. DNA samples were resuspended in TE buffer 1x, concentration adjusted to 2.0-2.5 µg/µl and stored at -20ºC for later use.

Genotyping analysi*s*: (i) *PCR CKRS-1 analysi*s. Amplification was performed on 10 ng of genomic DNA with primers Arno1 (5'-GCCAACACATACATACCTT-3') and Arno2 (5'-GGTAGGATACTAACCACAGC-3') as described by Carlotti *et al* [265]. Reaction were performed in 50-µL mixtures containing with 100 µM of each dNTP (dNTPset, MBI Fermentas, Vilnius, Lithuania), 0,2 µM of each primer (Genset SA, Paris, France), 1X Buffer and 1,5 U REDTaq-Polymerase™ (Sigma™, St Louis, MO, USA). The amplified fragments were visualized after separation by agarose gel electrophoresis with ethidium bromide (0.5µg/mL) staining. PCR amplification parameters included an initial denaturation step for 4 min at 92°C, 32 cycles of annealing for 30s at 55°C, extension for 2 min at 72°C, and denaturation for 30s at 92°C followed by final extension at 72°C for 10 min [265].PCR products were visualized and compared for amplification pattern similarities using electrophoresis at 140 V for 4 h on a 1% agarose gel, and staining with ethidium bromide solution 0.5 mg/ml

(Applichem, Darmstadt, Germany). PCR amplification products were analyzed using the Gel Logic 100 Imaging System Software (Kodak). Three unrelated *C. krusei* clinical isolates of distinct patients were used as a *control.*

(ii) Microsatellite CKTNR analysis: Assessment of CKTNR polymorphism was performed by fragment size analysis, using primers CKTNR3 and CKTNR5 as described by Shemer *et al* [266]. Primers CKTNR3 was 5' labelled with hexachlorocarboxyfluorescein (HEX). Reaction were performed in 20-µL mixtures containing 30 ng of genomic DNA, 200 µM of each dNTP (dNTPset, MBI Fermentas, Vilnius, Lithuania), 0,5 µM of each primer (Genset SA, Paris, France), 1X Buffer and 0,5 U REDTaq-Polymerase™ (Sigma™, St Louis, MO, USA). Amplicons were sized using capillary electrophoresis on ABI Prism 3130. Results were expressed with respect of haplotype denomination described by Shemer *et al.,* using *C. krusei* strain CBS 573 as control [266].

In vitro generation of VRC-resistant derivatives of *C. krusei* after VRC exposure

Eight independent clinical strains of *C. krusei* susceptible to VRC were isolated from several biological products of different patients with different underlying diseases, not submitted to VRC therapy (table 1). The strains were grown in brain-heart infusion (BHI) broth medium containing 0.001 µg/ml of VRC, with daily subcultures in fresh BHI-VRC for 30 days, in order to obtain derivatives resistant to VRC. Briefly, every 24 h, 1 ml of the culture was suspended into 9 ml of fresh BHI broth medium; every 2 days a 10 µl loopful of yeast cells was cultured in Sabouraud agar plates to check for culture contamination.

Susceptibility Testing and Effect of the efflux blocker FK506 (Tacrolimus)

Voriconazole (Pfizer, Groton, CT), PSC (Schering- Plough; New Jersey, USA) and ITC (Sigma Aldrich, Saint-Quentin, France) antifungals stock solutions were prepared according to the M27-A3 protocol and M27-S4, the fourth informational supplement, by the CLSI and maintained in stock solution at -70°C until use. Susceptibility profile of the *C. krusei* clinical isolates and the strains obtained before (D0) and after 30 days (D30) of incubation with VRC were performed for the antifungals

VRC, PSC and ITC, according to CLSI M27-A3 protocol and M27-S4, the fourth informational supplement, by the Clinical Laboratory for Standards Institute (CLSI)) [174, 175].

The susceptibility profiles to VRC of the *C krusei* isolates were in accordance to the following MIC values: susceptible whenever MIC $\leq$ 0.5 μg/ml, susceptible dose dependent whenever MIC=1.0 μg/ml and resistant whenever MIC $\geq$ 2.0 μg/ml. The susceptibility profiles to PSC of the *C krusei* isolates were in accordance to the following MIC values: susceptible whenever MIC $\leq$ 0.5 μg/ml, susceptible dose dependent whenever MIC=1.0 μg/ml and resistant whenever MIC $\geq$ 2.0 μg/ml. The susceptibility profiles to ITC of the *C krusei* isolates were in accordance to the following MIC values: susceptible whenever MIC $\leq$ 0.125 μg/ml, susceptible dose dependent whenever MIC ranges between 0.25-0.5μg/ml and resistant whenever MIC $\geq$ 1.0 μg/ml. Visual readings were performed after 24h and 48h of incubation, according to the same protocols. *C. krusei* type strain ATCC 6258 from the American Type Culture Collection was used as control, as recommended [174, 175].

Microdilution Assay: The reversion of multidrug resistance was described in different types of cells, including *Candida* spp when in the presence of antifungals azoles and FK506 (Tacrolimus), a blocker of ATP dependent efflux pumps [245]. Therefore, MICs of VRC for *C. krusei* strains were re- determined, according to CLSI M27- A3 and M27-S4 protocols, in the presence of Tacrolimus (FK506) 100 μg/ml.

Agar disk diffusion assay: An agar disk diffusion assay was also performed using FK506 to corroborate the results obtained with the CLSI protocol: yeast suspensions of the distinct resistant strains (0.5 McFarland standard; Densimat, Biomerieux, France), were spread each onto YPD agar plates containing VRC at a supra-MIC value, according to the MIC value of each strain and without VRC. Blank paper disks, 6mm (BBL, Becton Dickinson France S.A.), were impregnated with serial 10-fold dilutions of FK506 solutions, ranging from 1000 to 1 μg/ml, and with its solvent, dimethyl sulfoxide (DMSO, Merck). Dried paper disks were applied onto the inoculated agar plates. A plate containing only VRC at the supra-MIC values was used as a control

for cell viability for each strain. The agar plates were incubated at 37ºC, and the results were registered after 24h and 48h.

Resistance gene expression analysis

Total RNA extraction: Selected *C. krusei* strains were incubated in YPD broth at 35ºC, 150 rpm, until exponential growth phase; yeast cells were harvested by centrifugation at room temperature, (Hettich, Universal 320 R, 1610xg, 5 min), immediately frozen in liquid nitrogen. Total RNA was extracted using the hot acid phenol method, as described by Köhrer & Domdey [246]. RNA samples were resuspended in DNase/ RNase-free water, concentration adjusted to a final concentration of 100 ng/μl and stored at -70ºC for later use.

Reverse transcriptase PCR (RT-PCR). Two-step real-time PCR reactions were performed. Reverse transcriptase reactions were performed as previously described by Ricardo *et al* [264]. The cDNAs were kept at -20ºC until later use.

Quantitative Real-Time PCR (qRT-PCR): *ABC1*, *ABC2* and *ERG11* genes were amplified using the primer pairs described by Ricardo et al [264]. qRT-PCR reaction mixture contained: SensiFAST SYBR No-Rox Mix 1x (Bioline, Taunton, MA, USA), primers forward and reverse (*ABC1*, *ABC2*, *ERG11* 0.9 μM and *ACT1* 0.5 μM), 2 μl of cDNA and RNAse-free water, up to a 20 μl final reaction volume. $MgCl_2$ was used in *ABC2* gene reaction mixture at a final concentration of 1mM. All reactions were performed in the *Mastercycler epgradient Realplex2*; parameters were chosen according to the manufacturer's recommendations, including primer annealing temperature, 60ºC. To check for PCR product specificity, a melting curve was established, i. e., temperature ranging from 60ºC to 95ºC, for 20 min.

Data Analysis: A standard curve was inserted, in triplicate, containing serial five-fold dilutions ranging from 500 ng to 0.8 ng of RNA transcribed to cDNA, as previously described by Ricardo *et al* [264]. The results were analyzed using the program software Realplex version 1.5.474, from Eppendorf. Relative target gene expression levels were

calculated using the software REST 2009 (QIAGEN GmbH, Munich, Germany), being the susceptible *C. krusei* strain the reference sample and each resistant *C. krusei* strain the target sample; *ACT1* gene was used to normalize gene expression levels [247]. Target genes exhibiting a 2-fold increase in expression and with a *p* value $\leq$0.05 associated were considered significantly overexpressed.

Statistical analysis

Result analysis was performed using the software SPSS (Statistical Package for Social Sciences) version 19.0. Continuous and paired sample Student's t-test was used to analyse significant differences between target gene expression displayed by the distinct *C. krusei* strains; a *p* value $\leq$0.05 was considered statistical significant.

Sequencing data analysis of ERG11 gene

ERG11 gene was sequenced in all selected *C. krusei* strains in order to look for mutations that could be associated to VRC resistance.

Total genomic DNA extraction was performed as previously described in this section.

ERG11 gene sequencing reactions and analysis. *ERG11* gene (1890 bp) was amplified by PCR according to the protocols previously described by Ricardo *et al* [264]. PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, USA) and used as template for the sequencing reactions. Sequencing was performed with a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Carlsbad, USA) using primers forward_1, forward_2 and forward_3, as previously described by Ricardo *et al* [264]. DNA products were purified with Sephadex G-50 Fine (GE Healthcare, Buckinghamshire, United Kingdom) and sequenced in an ABI Prism 3130 genetic analyser (Applied Biosystems, Foster city, USA). Results were analysed with Sequencing Analysis software, version 5.2 from Applied Biosystems. The *ERG11* gene coding sequences of the resistant strains were aligned with the susceptible *ERG11*

gene coding sequence using MUSCLE software/ClustalW [248]. Alignments were analyzed in BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)/ ClustalW.

Results

Candida krusei strains designation and susceptibility profiles

Five *C. krusei* clinical isolates were selected and designated according to their susceptibility profile and the time of recovery, respectively, during VRC therapy of the patient: susceptible isolate $Ck_{B.VRC}$ (isolated Before VRC therapy), resistant isolates $Ck_{D.VRC9}$ and $Ck_{D.VRC16}$ (isolated During VRC therapy, 9th and 16th day), susceptible isolates $Ck_{A.VR10}$ and $Ck_{A.VRC18}$ (isolated After VRC discontinuation, 10th and 18th days). Timeline of isolation and susceptibility profiles of each of the *C. krusei* clinical isolates are detailed in figure 1.

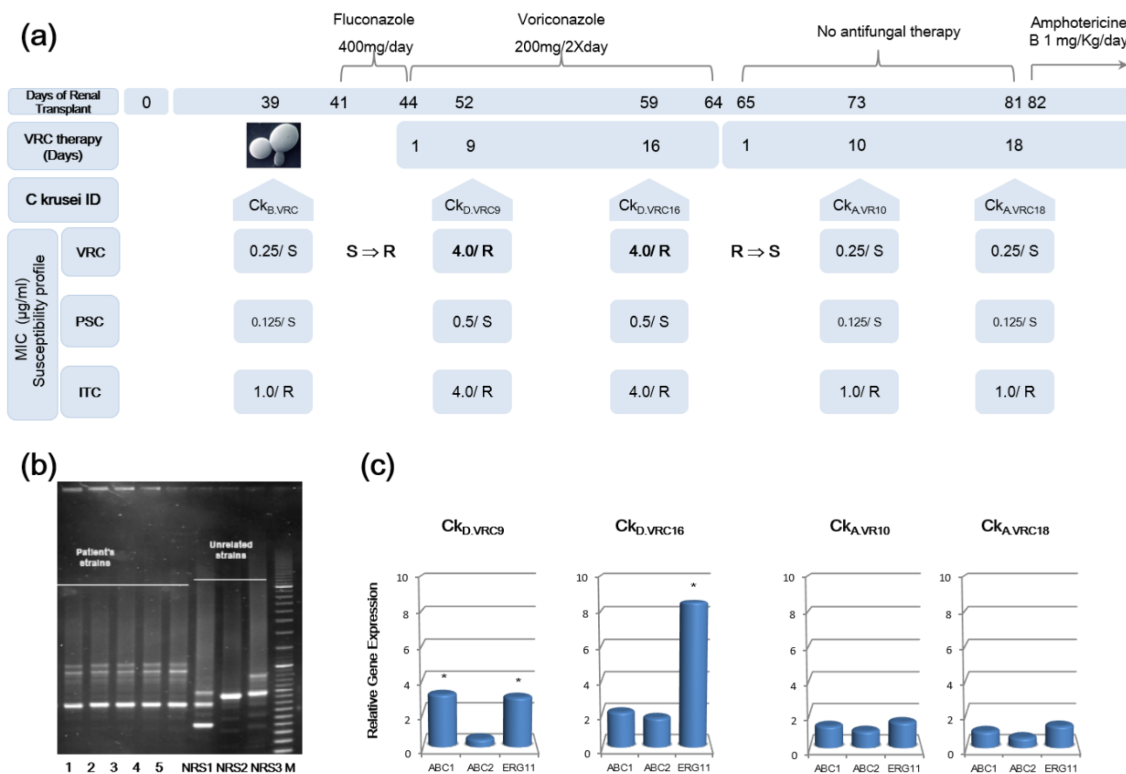


Figure 1 - *In vivo* induction of resistance to voriconazole. (a) Timeline of the renal transplant procedure, antifungal therapy and *C. krusei* clinical isolates recovered from the kidney transplant patient and their respective susceptibility profile; (b) Genotyping of *C. krusei* clinical isolates. 1- $Ck_{B.VRC}$, 2- $Ck_{D.VRC9}$, 3- $Ck_{D.VRC16}$; 4- $Ck_{A.VRC10}$, $Ck_{A.VRC18}$, NRS 1-3 – Non related isolates, M – Molecular weight; (c) Relative gene expression profile of *ABC1*, *ABC2* and *ERG11* genes for the *C. krusei* clinical isolates; $*p \leq 0.05$.

An increase in the MIC to VRC was registered after 9 days of VRC therapy, the isolates achieving a resistant phenotype ($Ck_{D.VRC9}$ and $Ck_{D.VRC16}$); however when the drug was withdraw the MIC value turned to the initial values and consequently the initial susceptible phenotype ($Ck_{A.VR10}$ and $Ck_{A.VRC18}$). In the case of ITR the first isolates were already resistant but in the presence of VRC the MIC value increased even more but decreased after its removal. No significant variation occurred in the MIC value of PSC which only increased to values related to a SDD phenotype. The hypothesis that the same *C. krusei* strain was colonizing our transplanted patient was corroborated by the genotyping technique. All the isolates presented with the same CKRS-1 electrophoretic pattern (Fig. 1, panel b) and the same allelic CKNTR microsatellite profile, i.e. profile e-f/d, according previously described nomenclature [266]. Therefore, all *C. krusei* isolates from our patient were clonal, despite presenting different susceptibility profiles.

Concerning the strains used for experimental VRC exposure and their *in vitro* induced derivatives, they were randomly numbered and designated according to the day of incubation with VRC, i.e., the initial susceptible *C. krusei* strains obtained before incubation with VRC were referred as D0 and their derivatives after 30 days of incubation with VRC were referred as D30. Therefore, we have available the initial strains and their derivatives: $Ck1_{D0}$ and $Ck1_{D30}$, $Ck8_{D0}$ and $Ck8_{D30}$, $Ck21_{D0}$ and $Ck21_{D30}$, $Ck24_{D0}$ and $Ck24_{D30}$, $Ck32_{D0}$ and $Ck32_{D30}$, $Ck34_{D0}$ and $Ck34_{D30}$, $Ck40_{D0}$ and $Ck40_{D30}$, $Ck42_{D0}$ and $Ck42_{D30}$. Susceptibility profiles are detailed in Table 1. At day 30 of incubation with VRC, all the derivatives presented a resistant phenotype to VRC, with the MIC values ranging from 2 μg/ml to 8μg/ml and ITC with the MIC values ranging from 1 μg/ml to 16 μg/ml.

Table 1 – MIC, susceptibility profile, *ABC1*, *ABC2* and *ERG11* relative gene expression profile and *ERG11* gene sequence alterations of *C. krusei* strains induced *in vitro*.

		MIC (µg/ml)/Susceptibility Profile						Relative Gene Expression	*ERG11* gene sequence alterations	
		VRC		PSC		ITC				
Strains ID	Biological Sample	D0	D30	D0	D30	D0	D30	*ABC1 ABC2 ERG11*	D0	D30
Ck1	Sputum	0.25/S	**2/R**	0.125	0.5	0.25/SDD	**1/R**		-----	-----
Ck8	Mouth	0.25/S	**2/R**	0.125	1	0.5/SDD	**> 16/R**		Heterozygous synonymous A756T	Heterozygous synonymous A756T
Ck21	Digestive fistula	0.50/S	**8/R**	0.25	1	0.5/SDD	**> 16/R**		-----	Heterozygous Non synonymous G364G/T (A→S)
Ck24	Urine	0.25/S	**4/R**	0.25	0.25	0.5/SDD	**> 16//R**		-----	**Homozygous Non synonymous T418C (Y→H)**
Ck32	Sputum	0.125/S	**2/R**	0.125	1	0.25/SDD	**> 16/R**		Heterozygous synonymous A756T	Heterozygous Non synonymous C365C/T (A→V) Heterozygous synonymous A756T;
Ck34	Stools	0.50/S	**4/R**	0.25	0.5	0.5/SDD	**> 16/R**		-----	-----
Ck40	Sputum	0.25/S	**2/R**	0.25	0.5	0.5/SDD	**> 16/R**		-----	-----
Ck42	Ascitis	0.25/S	**4/R**	0.25	0.25	0.5/SDD	**> 16/R**		Heterozygous synonymous A756T; Heterozygous Nonsynonymous C1091T (A→V)	Heterozygous synonymous A756T; Heterozygous Non synonymous T418C (Y→H);

Effect of efflux blocker FK506 (Tacrolimus)

In the microdilution assay the presence of the efflux blocker FK506 lowered the MIC values to VRC in all the resistant strains up to 7 fold as it was the case of the *C. krusei* strain $Ck21_{D30}$. Therefore, all the resistant strains displayed a susceptible phenotype in the presence of FK506, with MIC values decreasing from 2-fold up to 7-fold (Table 2).

Table 2 - MIC and susceptibility profiles to VRC alone and in combination with FK506 of *C. krusei* strains.

Resistance	Strains ID	VRC MIC (µg/ml)/ susceptibility profile when used with:	
		No FK506	100 µg/ml FK506
In vivo acquired	$Ck_{D.VRC9}$	4.0/R	0.125/S
	$Ck_{D.VRC16}$	4.0/R	0.125/S
In vitro acquired (D30 strains)	Ck1	2.0/R	0.25/S
	Ck8	2.0/R	0.06/S
	Ck21	8.0/R	0.06/S
	Ck24	4.0/R	1.0/SDD
	Ck32	2.0/R	0.125/S
	Ck34	4.0/R	0.125/S
	Ck40	2.0/R	0.06/S
	Ck42	4.0/R	0.25/S

Concerning the agar disk diffusion assay, all the strains assayed presented growth inhibition (although variable in their extent of inhibition) around the disks impregnated with the highest concentration of FK506, in the presence of VRC at MIC values. Besides, neither FK506 alone nor DMSO, the FK506 solvent, inhibited the growth of the *C. krusei* strains.

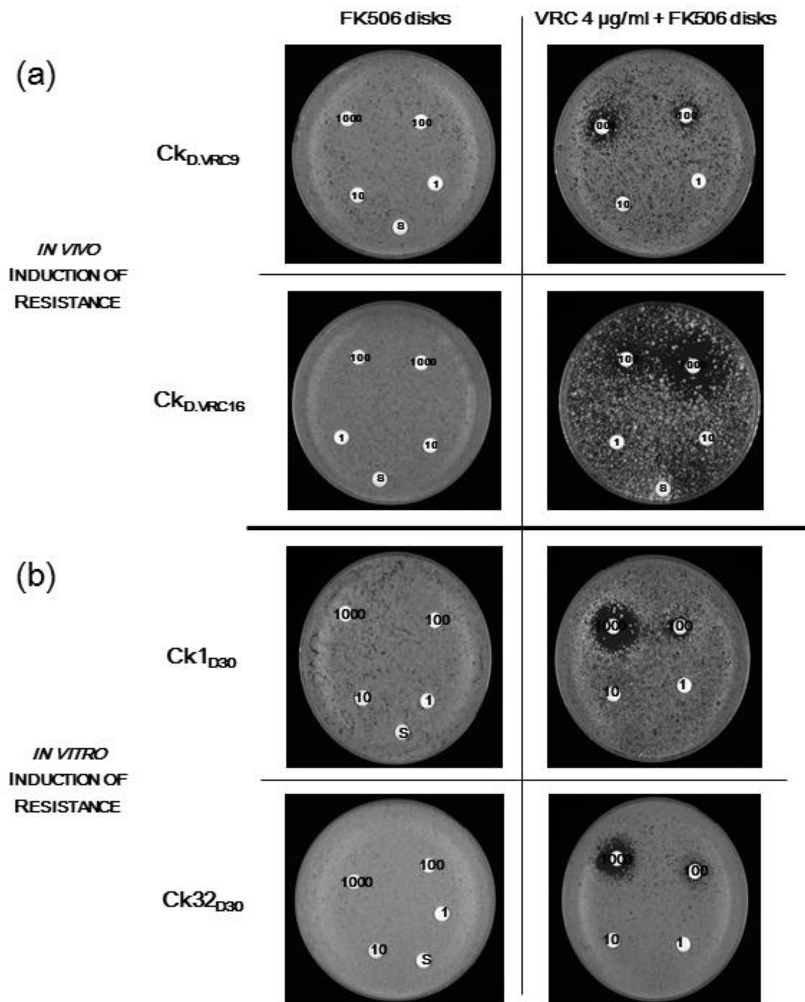


Figure 2 - FK506 agar disk diffusion assay: resistant *C. krusei* strains were grown on YPD medium and paper disks impregnated with serial 10-fold dilutions of FK506, ranging from 1000 to 1 μg/ml (FK506 disks - left column) and DMSO (S1 – 100%, S2 – 10%) and in the presence of VRC 4 μg/ml (VRC 4 μg/ml + FK506 disks- right column). (a) *C. krusei* resistant clinical isolates recovered from the kidney transplant patient; (b) *C. krusei* resistant strains induced *in vitro*, after incubation with VRC.

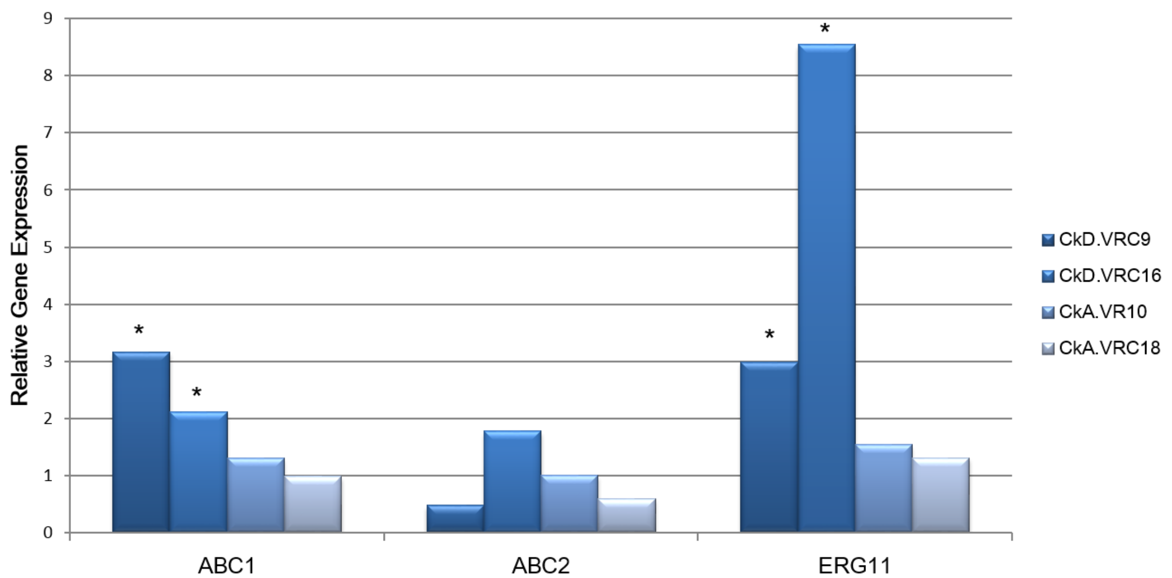
Resistance gene expression analysis

ABC1, *ABC2* and *ERG11* gene expression, for the different groups of strains is detailed in Figure 3 (see also figure 1 and table 1). The gene expression profile of the different *C. krusei* clinical isolates $Ck_{D.VRC9}$ and $Ck_{D.VRC16}$ is in accordance to their susceptibility profile, i.e. there is a significant increase in the expression of the resistance genes *ABC1* and *ERG11*: *C. krusei* strain $Ck_{D.VRC9}$ *ABC1* p= 0.007, *ERG11* p= 0.001, $Ck_{D.VRC16}$ *ABC1* p= 0.003, *ERG11* p= 0.009. Conversely, *ABC2* gene was not overexpressed in these strains. The post-therapy susceptible isolates $Ck_{A.VR10}$ and

$Ck_{A.VRC18}$ presented basal gene expression level for all genes, similar to the reference susceptible isolate $Ck_{B.VRC}$, which was in accordance to their susceptibility profile.

Interestingly, the *C. krusei* strains incubated *in vitro* with small doses of VRC presented different gene expression profiles. Except for strains, $Ck24_{D30}$, $Ck34_{D30}$ and $Ck40_{D30}$ all the remaining strains D30 present at least one of the resistance genes significantly overexpressed when compared to the respective susceptible strain - D0. The most remarkable increase in both *ABCs* genes was registered in $Ck21_{D30}$ strain, being also the strain with the highest MIC value to VRC.

A



B

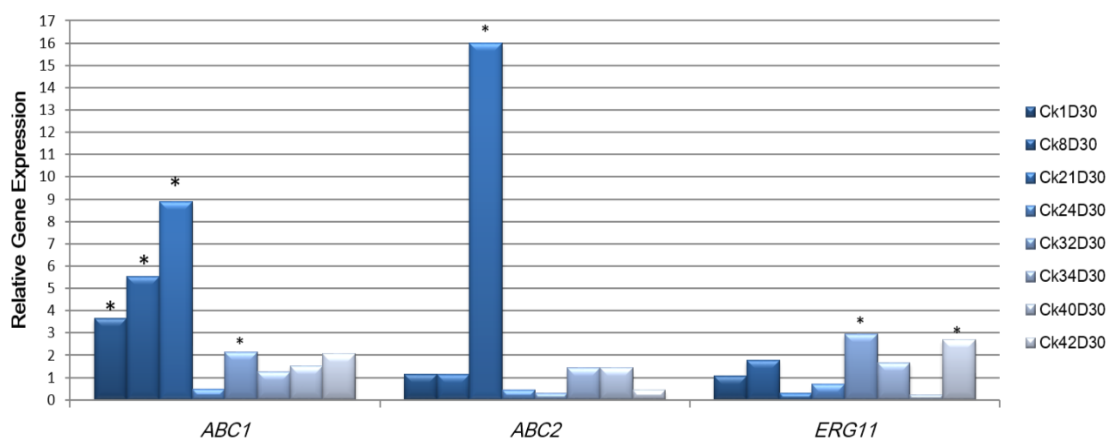


Figure 3 - *ABC1*, *ABC2* and *ERG11* relative gene expression level in *C. krusei* resistant clinical isolates (A) and resistant *in vitro* induced strains (B). *ABC1*, *ABC2* and *ERG11* gene expression level was quantified and normalized relative to the housekeeping gene, *ACT1*; relative gene expression level was calculated as a ratio between each *C. krusei* resistant strain and the respective susceptible isolate; * $p \leq 0.05$.

Sequencing analysis of *ERG11* Gene

Several *ERG11* gene mutations have been previously described to be associated to azole resistance in *C. albicans* [196] therefore, we sequenced *C. krusei* *ERG11* gene in the selected strains.

Candida krusei strains induced *in vitro* presented different alterations in *ERG11* gene sequence detailed in table 1. All the susceptible and resistant *C. krusei* strains from both groups of strains presented heterozygous alterations at 642bp (C→T) and 1389bp (T→C), all of them resulting in synonymous SNPs. It should be highlighted the homozygous nonsynonymous mutation at position 418 bp (T →C), translating a different amino acid Tyr→His in strain $Ck24_{D30}$, which presented the lowest level of gene expression associated to the resistance genes studied.

Discussion

Candiduria cases are usually not considered serious clinical situations because patient's life is not at risk. However, the particular case of the patient submitted to a kidney transplant triggered our interest once it involved an unusual acquisition of resistance as a result of VRC therapy. Management of candiduria involves many uncertainties in the indication for antifungal therapy [61, 267]. Therapeutic approaches for candiduria are very controversial due to the difficulty in evaluating its clinical importance, i.e., if the presence of *Candida* in the urine reflects infection or colonisation [31, 62, 74, 75, 76]. Empirical therapy refers to the treatment of high-risk hosts with symptoms of disease, even in the absence of positive cultures or other clinical evidences [53]. However, even when antifungal therapy is applied the concentration reaching the urinary tract is very small, not reaching an effective dose for treatment. In the clinical case presented in this study most certainly the concentration of VRC was not effective for the treatment but it was enough to induce resistance *in vivo*. Also, this clinical case was an example of therapeutic errors due to empirical therapy since FLC was administrated to the patient when *C. krusei* strains were responsible for candiduria. One possible explanation can be the time of interval between the isolation of the pathogenic yeast and its identification. As soon as it was identified the therapy was changed to another azole. It was not recommended at the moment to use AMB B due to its nephrotoxicity. VRC is not commonly used in this clinical conditions however, since the patient was a transplant recipient with a high risk of rejecting the new transplant (the 2nd transplantation), the aim of the antifungal treatment was first to protect the parenchyma of the kidney transplant from *Candida* infection and then to treat candiduria. According to figure 1, the strain colonizing the urinary tract developed a resistant phenotype after 9 days of VRC therapy and maintained it during the antifungal treatment for additional 7 days - $Ck_{D.VRC9}$ and $Ck_{D.VRC16}$, respectively. When VRC treatment was discontinued the same strain turned susceptible as shown by the genotyping assays, figure 1 panel (b). We can conclude that the *C. krusei* isolate suffered a selective pressure, developing resistance and activitating the efflux pumps. However, when the treatment stopped, along with the selective pressure the strain no

longer needed to actively maintain the efflux activity. Anyway the patient was under a risk situation because the resistant strains could spread to biological sites where antifungal drug concentration could be higher and therefore maintain the resistant phenotype permanently.

The resistant phenotype registered for both groups of *C. krusei* strains is associated to multiple resistance mechanisms, being efflux activity one of the most common mechanism as it has already been described in other studies [16, 242, 264]. This hypothesis is corroborated by the results obtained with the efflux pump inhibitor, since a synergistic effect was registered in the presence of VRC in both the microdilution assay and in the agar disk diffusion assay (table 2 and figure 2). Also, ITC resistance among several *C. krusei* clinical isolates was previously described to be mostly associated to efflux pumps activity [242]. However, concerning the resistant *C. krusei* strains induced *in vitro*, only $Ck1_{D30}$, $Ck8_{D30}$ and $Ck21_{D30}$ presented a significant increase in *ABCs* genes expression. Most probably other not yet described genes belonging to the ABC transporter family of proteins are present in these strains conferring resistance. For example, were described in *C. glabrata* three genes associated to resistance to azoles: *CgCDR1*, *CgCDR2* and CgSNQ2 and *C. krusei* is more closely related to *C. glabrata* and *Sacharomyces cerevisiae* than to *C. albicans* [17, 18, 268, 269].

ERG11 gene sequencing showed some heterozygous alterations already described in *C. krusei* by Ricardo *et al*, but not directly associated to resistance since they were present in both susceptible and resistant strains [264]. Since FLC resistance is associated with diminished affinity of the azole to the Erg11p it can be speculated that these heterozygous alterations could be associated to fluconazole intrinsic resistance. Strain $Ck24_{D30}$ presented a mutation also described by Ricardo *et al*, at position 418 bp (T $\rightarrow$C), translating into a Tyr$\rightarrow$His amino acid change [264]. This strain presented the lowest gene expression level for the three genes studied therefore this mutation is associated to resistance to VRC. Another curious fact is that in the resistant strains $Ck21_{D30}$, $Ck32_{D30}$ and $Ck42_{D30}$, heterozygous non synonymous alterations were present at position 418 bp. Since the process of evolution of resistance can occur in a stepwise way we can suggest that the occurrence of these alterations could only be a midterm

position for a more stable resistant phenotype. These alterations could evolve for homozygous nonsynonymous mutations in those strains.

In the present work we showed a case of development of transient resistance to VRC *in vivo* and *in vitro* in *C. krusei* strains from different backgrounds. Despite the antifungal concentrations reached in the urine are very low, in this case, it was enough to induce resistance. Although, normally candiduria is not considered a serious clinical situation it can lead to the development of candidemia. Taken all these facts together, candiduria might not be an innocent situation and more attention should be given by the physicians to these cases. Also, different resistance mechanisms were found in the different strains namely efflux pumps activity and *ERG11* gene mutations associated to resistance.

Chapter V

OVERALL DISCUSSION AND CONCLUSIONS

“Descobrir consiste em olhar para o que todo mundo está vendo e pensar uma coisa diferente”.

Roger Von Oech; fundador e presidente da empresa americana *Creative Think*. 1948 -

Among the genus *Candida*, *C. krusei* is one of the less studied species perhaps because it is not so commonly found as a cause of human infections. The occurrence of candidemia as a result of *C. krusei* is associated to neutropenia, a very serious condition, with very high mortality rates associated [270].

The different molecular typing techniques were applied in two distinct situations: to corroborate or discard the hypothesis of an outbreak (chapter III) and to confirm the acquisition of resistance *in vivo* since different isolates from the same patient with different phenotypes were available (chapter IV). Ideally, a typing method should discriminate very closely related isolates to reveal person-to-person strain transmission, which is important in the development of strategies to prevent further spread. At the same time it must be rapid, inexpensive, highly reproducible, and easy to perform and interpret [271, 272]. On the other hand, when consecutive isolates are recovered from different biological products of the same patient, the hypothesis that the patient could be colonized by one or more strains should be corroborated or excluded by genotyping. The different techniques used in this study complemented and corroborated each other, undoubtedly confirming or discarding the hypothesis presented throughout the present work. Several studies point for a combination of RAPDs with other methods providing optimal discrimination [273- 275].

In chapter IV two independent clinical cases of candidiasis were presented, although with many factors in common: one case of candidemia in a leukemia patient submitted to a long period of VRC treatment until his death and a case of candiduria in a patient submitted to a kidney transplant and then under treatment with VRC during 20 days. Although very different in terms of life risk to the patients, both clinical situations point for the importance of monitoring the susceptibility profile of clinical isolates recovered during an antifungal treatment. In both cases the acquisition of resistance to VRC occurred due to the antifungal therapy with VRC. In the case of the leukemia patient the initial *C. krusei* isolates recovered from blood cultures were in permanent contact with a higher dose of the antifungal drug in contrast to the isolates recovered

from the urine of the kidney transplant patient, since VRC presents low urinary diffusion [276]. Indeed, the treatment of candiduria is one of the biggest concerns among physicians. Fluconazole is the first line treatment in these cases, since other antifungals present either renal toxicity such as amphotericin B or low urinary diffusion such as VRC, PSC or the echinocandins. However, when dealing with *C. krusei* known to be intrinsic resistant to FLC, other options had to be administered. Even though low concentration was achieved in the urine, it was enough to induce resistance. Another interesting fact in the development of resistance to VRC is that although two of the patients involved in the outbreak hypothesis (chapter III) - the leukemia patient and a patient diagnosed with non-Hodgkin lymphoma - were submitted to antifungal therapy with VRC, only *C. krusei* isolates recovered from the leukemia patient were resistant. Only 2 *C. krusei* isolates were recovered from the patient diagnosed with non-Hodgkin lymphoma therefore in this case the antifungal therapy was successful. Taken all these facts together, we can discuss the fact that the presence of the antifungal is important for the development of resistance but other factors are also involved. For example, Lin et al. [277] found that patient exposure to piperacillin-tazobactam and vancomycin was more important than exposure to FLC in promoting *C. krusei* BSI. They suggested that these two antibacterial agents may promote skin and gastrointestinal tract colonization with *C. krusei* by altering the normal flora and thereby facilitating the colonization of the host by the pathogenic yeast [277]. Many factors that influence the development of resistance *in vivo* are not present in the *in vitro* assays described, such as: concomitant therapies, the underlying disease of the host or the diffusion of the antifungal drug to the site of isolation of the *C. krusei* strains. Besides, it is well known the poor relationship between the susceptibility profiles *in vivo* and *in vitro*. As a result, in contrast to what happened in the case of the patient with the kidney transplant, in the absence of the antifungal, the strains still maintained the resistant phenotype *in vitro*.

Another issue that arose from this work was the administration of empirical therapies. When *C. krusei* isolates were first recovered from the patients, some of them were receiving FLC. It is widely known the intrinsic resistance of *C. krusei* to FLC so this is an example of the major importance of the rapid identification of clinical isolates. Unfortunately, a non-directed antifungal therapy is more common in the clinical practice

than we would expect [278-281]. This type of studies should be a wakeup call for the physicians to claim for rapid identification methods to obtain results before the administration of antimicrobial therapy. A study by Abi- Said *et al.* found that infections caused by *C. krusei* were strongly associated with FLC prophylaxis among neutropenic patients at the M. D. Anderson Cancer Centre (Houston, TX) [282]. Candida BSIs have been shown to have some of the highest rates of inappropriate therapy and hospital mortality among all etiologic agents examined [41, 42, 283-287]. Overall, the treatment of candidemia is often found to be inadequate due to the use of an agent to which the organism is resistant, to insufficient dose or duration of treatment, or to the absence of any treatment at all [41, 42, 285, 286, 288, 289]. Taken together, the presented results in this work and others in the same research area emphasize the plasticity of *C. krusei* with respect to the development of resistance to a broad array of antifungals [221, 242, 251, 253]. Therefore, along with *C. glabrata*, *C. krusei* must be considered an important indicator specie that should be monitored for the development of antifungal resistance [24, 221].

One of the main goals of this work was to uncover the mechanisms of resistance present in the resistant *C. krusei* strains either induced *in vivo* and *in vitro*. Some of the mechanisms previously described in *C. krusei* strains were confirmed in the present work, namely the existence of efflux pumps proteins that promote the cellular extrusion of the antifungal. It was highlighted the role of Abc1p encoded by *ABC1* gene in both works of chapter IV and conversely to the genes *CDR2* in *C. albicans* or *C. glabrata*, it was established the minor role of *ABC2* gene in azole resistance in *C. krusei*. On the other hand, it was described for the first time a new point mutation in *ERG11* gene associated to resistance in *C. krusei*. The mutation was described in two of the resistant *C. krusei* strains recovered from the leukaemia patient, therefore from resistant strains induced *in vivo* and in a resistant strain incubated *in vitro* with very low concentration of VRC. These are completely unrelated strains, with different backgrounds, but possessing the same mutation.

One of the major drawbacks in the development of this work was the lack of information concerning *C. krusei* genome, which is already sequenced but not

annotated. For example, until now only 3 genes were associated to resistance in *C. krusei*, *ABC1*, *ABC2* and *ERG11* and we questioned the role of *ABC2* gene in resistance. Other genes were already studied in *C. krusei* such as the transporter proteins belonging to the MFS but they were not associated to resistance in *C. krusei* [212]. Moreover, in other *Candida* species, cis and trans –elements regulating the expression of multidrug transporters were described, such as the transcription factor CgPdr1p in *C. glabrata*, which is the main regulator of the genes associated with resistance to azoles, *CgCDR1*, *CgCDR2* and CgSNQ2 [290, 268, 269]. *FCR1* gene was identified as a negative regulator of FLC susceptibility in *C. albicans*, since its deletion resulted in a mutant hyperresistant to FLC [291]. *TAC1* (transcriptional activator of CDR genes) gene was described as the main mediator of ABC transporters conferring azole resistance due to upregulation of the ABC transporters in *C. albicans* [292]. The molecular dissection of the *CDR1* and *CDR2* promoters identified five distinct regulatory elements in *C. albicans*: the BBE (basal expression element) responsible for basal expression, the DRE (drug-responsive element) required for the response to drugs such as fluphenazine and oestradiol, two SREs (steroid responsive element) involved in the response to steroid hormones and the NRE (negative regulatory element) [293-295]. This is just a small example of the genes and regulators described in other species of *Candida* associated to resistance. Most probably, homologues genes are present in *C. krusei*, conferring resistance not only to azoles but also to other classes of antifungals that could explain in more detail the intrinsic resistance of *C. krusei* to FLC. For example, the two FLC-inducible ABC transporters CgCdr1p and CgPdh1p, whose upregulation is mediated by the transcription factor CgPdr1p, are the main cause for the innate FLC resistance of *C. glabrata* [296].

Some questions remained unanswered at the end of this work and that's why the future perspectives referred at the end of a thesis are the first step to begin a new work.

CONCLUSIONS

With the present work the following achievements were accomplished:

- The work presented in chapter III described a long-lasting colonization by *C. krusei,* which should be taken into account when considering the therapeutic protocols given to these patients.
- Molecular genotyping techniques were crucial for the development of the work presented in both chapters III and IV. First, in chapter III we concluded that there was no outbreak occurring in the neutropenia unit, only a misfortune coincidence. Second, in chapter IV we corroborated two cases of acquisition of resistance *in vivo* by susceptible *C. krusei* isolates in two different patients, with different underlying diseases, different concomitant therapies, and different therapeutic protocols. Therefore, we concluded that resistance can arose from a diversity of backgrounds being the treatment with VRC the common factor. Each case is unique in the process of acquisition of resistance and it is a multifactorial phenomenon. It seems that the presence of the antifungal agent is almost sufficient condition but the time of development can be influenced by other factors.
- In chapter III we also highlight the importance of good hygiene measures among health care workers not only when dealing with the patients but also concerning the surrounding environment as they can be contaminated/colonized.
- In chapter IV we confirmed efflux as one of the main mechanism of resistance to azole drugs, such as VRC, mostly associated to an increase in *ABC1* gene expression. We concluded that *ABC2* gene plays a minor role in antifungal resistance. *ERG11* gene was associated to a VRC resistant phenotype in two different ways: due to its overexpression and the description of a new point mutation in some resistant *C. krusei* strains, never described before.

Chapter VI

FUTURE PERSPECTIVES

“A ciência nunca resolve um problema sem criar pelo menos outros dez.”

George Bernard Shaw. Dramaturgo, critico, activista politico.1856-1950.

At the end of this work many questions remained unanswered and other questions arose.

We would like to corroborate the results obtained concerning the mechanisms of resistance to VRC, through the knockout of *ABC1* and *ABC2* genes in resistant *C. krusei* strains. First, we would have to develop a molecular tool for gene disruption. A plausible selectable marker would be the *C. albicans* SAT1 gene, which confers resistance to nourseothricin (NRS). Construction of homozygous null mutants directly from *C. krusei* wild-type strains by the use of dominant selection markers would eliminate all potential problems related to the use of auxotrophic markers. The first steps were given for the accomplishment of this task and some preliminary results were already obtained: in solid YPD medium containing 300 μg/ml of NRS wild type *C. krusei* strains did not grow up to 5 days of incubation. However, one major problem in the development of the molecular tool and other molecular studies is the lack of information concerning the *C. krusei* genome.

A reverse approach could also be performed. *Candida albicans* susceptible strains with deleted *CDR1* and/or *CDR2* efflux genes, (DSY 448, DSY 653 and DSY 654) available in our laboratory could be complemented with *C. krusei ABC1* and *ABC2* genes, restoring azole resistance in these strains.

Other genes besides *ABCs* and *ERG11* may be associated to resistance. We could also evaluate gene expression of other genes described to be associated to resistance, look for mutation in those genes or transcription factors.

Chapter VII

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“Quanto mais aumenta nosso conhecimento, mais evidente fica nossa ignorância”.

John F. Kennedy. Empresário, político e 35º Presidente dos Estados Unidos. 1917-1963.

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Chapter VIII

PUBLICATIONS

“Os dias prósperos não vêm por acaso; são granjeados como as searas, com muita fadiga e com muitos intervalos de desalento”

Camilo Castelo Branco. Escritor, romancista, cronista, critico, dramaturgo, historiador, poeta e tradutor. 1825-1890.

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Candida krusei reservoir in a neutropaenia unit: molecular evidence of a foe?

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Abstract

Candida krusei has been documented as an emerging pathogen causing nosocomial outbreaks. The consecutive isolation of *C. krusei* strains in three patients admitted to the same hospital department within 2 months lead us to consider the possibility of an outbreak. Additionally, *C. krusei* isolates were collected from the room surfaces, whereas another isolate had been recovered from the blood of one patient 2 years before. *HinfI* DNA restriction endonuclease-based analysis of all *C. krusei* isolates was performed and restriction profiles were compared. Surprisingly, isolates from different patients were unrelated, whereas isolates from biological products of the same patient showed indistinguishable *HinfI* restriction patterns and were similar to those obtained from the surrounding environment of the respective patients. The study approach revealed the endogenous origin of the *C. krusei* infectious episodes observed and demonstrated that, subsequent to colonizing a patient, *C. krusei* can be involved in infectious episodes distant in time. The hypothesis of an outbreak was excluded, although we believe that the methodology employed in the present study represents a valuable tool for diagnostic and epidemiological surveys.

Keywords: *Candida krusei,* haematological patients, health care related infections, molecular typing, mt DNA Restriction, Endonuclease Analysis, nosocomial outbreak

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Introduction

In the last two decades, invasive fungal infections in hospitalized patients have increased significantly worldwide. According to data obtained from the USA and Europe, *Candida* species represent, respectively, the fourth and sixth most frequent cause of invasive healthcare-related infections [1,2], accounting for 8–15% of all episodes of sepsis acquired in hospital settings [3]. Inherent to these types of infection are the extremely high morbidity and mortality rates, particularly among immunocompromised patients [4,5].

Fluconazole is one of the antifungal agents mostly used in both prophylactic and therapeutic protocols. Fluconazole prophylaxis has been associated with a decrease in the prevalence of *Candida* species such as *Candida tropicalis* and *Candida albicans*, and to an increase in that of *Candida krusei* and *Candida glabrata* [3]. *C. krusei* presents intrinsic resistance to fluconazole and, to some extent, reduced susceptibility to amphotericin B [6].

Infectious outbreaks in hospitals, especially in intensive care units, represent a serious health problem and are mainly due to *Candida lusitaniae* [7], *C. albicans* [8], *Candida parapsilosis* [9], and *C. krusei* [10,11]. Many factors may account for their occurrence (e.g. barrier loss, lack of proper infection control measures by health care workers when managing patients, resistance to prescribed antifungal drugs, as well as insufficient drug levels).

Molecular methods represent a powerful tool to clarify transmission pathways in health care facilities (i.e. to investigate the occurrence of possible outbreaks). Techniques such as karyotyping, restriction fragment length polymorphism analysis by pulsed-field gel electrophoresis, southern blot

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hybridization, PCR fingerprinting and randomly amplified polymorphic DNA fingerprinting have been extensively used for *Candida* typing [12–16]. Restriction endonuclease analysis (REA) has been described in the last decade as a valuable tool for *Candida* spp. characterization. REA of the mitochondrial DNA (mtDNA) was first applied in the biotechnology industry in order to characterize yeast strains used for wine fermentation [17,18] and, more recently, to discriminate between *Candida* clinical strains [19–22]. The data obtained demonstrate the relevance of using molecular genetic methods in many different areas, including taxonomic, ecological and clinical surveys.

Recently, we were challenged by a hypothetical outbreak as a result of *C. krusei* in the neutropaenia unit of the Haematology Department of Hospital S. João, Porto, Portugal. Within a short period of time, several *C. krusei* isolates were cultured from biological products of three patients. In addition, *C. krusei* was found in the surrounding environment of the patients. All isolates were compared using mtDNA REA, which is a convenient and powerful tool that allows valid comparisons between isolates of the same yeast species.

Patients and Methods

Patients

Patient A was a 41-year-old male with acute lymphoblastic leukaemia (T/NK) diagnosed in July 2006. In August 2008, the patient was initially treated with amoxicillin and ciprofloxacin because of undetermined fever. When symptoms remained unchanged, a myelogram was performed; a first relapse of the haematological disease was diagnosed. He was admitted to the neutropaenia unit for salvage intensive chemotherapy with fludarabine, idarubicin and ara-C, followed by growth factor (G-CSF), administered through a central venous catheter. Upon the onset of the aplastic period (28 August), he received antimicrobial treatment for 2 weeks [ciprofloxacin, amoxicillin-clavulanic acid, imipenem, acyclovir and fluconazole (200 mg/day)]. At the time of fungaemia detection, he displayed the following haematologic parameters: white blood cells 0.03×10^9 cells/L, haemoglobin level 8.5 g/dL and blood platelets 11×10^9 cells/L. As soon as *C. krusei* was identified, the patient was started on caspofungin for 12 days without improvement; the treatment was then changed to amphotericin B.

This patient had previously developed a fungaemia episode as a result of *C. krusei* during chemotherapy in 2006 and a corresponding isolate had been stored at −70°C.

The patient remained in room number 1 from August to October 2008.

Patient B was a 53-year-old male with non-Hodgkin's lymphoma, who was admitted to room number 2 for 3 weeks in September 2008. He was administered cyclophosphamide, doxorubicin, vincristine, and prednisone, followed by G-CSF. On 12 September 2008, he was started on caspofungin but, as a result of sustained fever on day 5, treatment was changed to voriconazole, completing 14 days of antifungal therapy. He never received fluconazole. At that fungaemia episode, he displayed the following haematologic parameters: white blood cells 2.41×10^9 cells/L, haemoglobin 9.3 g/dL and blood platelets 73×10^9 cells/L.

Patient C was a 60-year-old male with acute myeloid leukaemia secondary to myelodisplastic syndrome; he was admitted to room number 2 for 3 weeks in October 2008, subsequent to patient B. He received Ara-C, etoposide and doxorubicin for 5 days, followed by another 5 days of Ara-C; he was also prescribed amoxicillin, ciprofloxacin and allopurinol. In addition, prophylactic treatment included imipenem, vancomycin, fluconazole and acyclovir from 29 September to 20 October 2008. On that latest date, he displayed the following haematologic parameters: white blood cells 0.6×10^9 cells/L, haemoglobin 9.8 g/dL and blood platelets 24×10^9 cells/L.

Patient D served as a typing control; one single *C. krusei* isolate was recovered from the patient's bronchial secretions in September 2008 upon his admission to the Internal Medicine Department.

Clinical strains

Eighteen *C. krusei* isolates were collected from different clinical specimens (blood, urine, stools and bronchial secretions) of the above mentioned patients admitted to the neutropaenia unit within a 2-month period. Additionally, a previous *C. krusei* isolate (2006) from a blood culture of patient A was included in the study, as well as a *C. krusei* isolate obtained from the control patient D. All isolates were identified using the automatic system Vitek2 YBC identification cards (BioMérieux, Paris, France), stored at −70°C in Brain Heart infusion (Merck KGaA, Darmstadt, Germany) with 10% glycerol and sub-cultured twice on Sabouraud agar (Merck KGaA) to ensure purity prior to experimental assays.

Environmental strains

Several environmental samples were collected from the patients' rooms (1 and 2) using Sabouraud agar contact plates (Merck KGaA); two *C. krusei* isolates were cultured: one from the bedside table of patient A (room 1) and another from the bed of patient C (room 2). The isolates were characterized and stored as described above for clinical

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samples. Air samples were collected by filtration with a MAS-100 Eco instrument (Merck Eurolab, Dietlikon, Switzerland), containing Sabouraud agar plates (Merck KGaA); no *C. krusei* isolates were obtained.

Antifungal susceptibility testing

Voriconazole (Pfizer, New York, NY, USA), posaconazole (Schering-Plough; Kenilworth, NJ, USA), amphotericin B (Bristol Meyers Squibb, New York, NY, USA), caspofungin (Merck, Rahway, NJ, USA) and anidulafungin (Pfizer) stock solutions were prepared according to CLSI protocols (M27-A3) [23] and maintained at −70°C until use. Minimal inhibitory concentration (MIC) of each antifungal drug was determined according to CLSI protocol M27-A3 [23].

Total genomic DNA extraction

Yeast cells were cultured overnight at 30°C in 10 mL of YPD liquid medium, with continuous orbital shaking at 180 r.p.m., and subsequently collection by centrifugation. Total DNA was extracted using phenol:chloroform:isoamyl alcohol 25:24:1, precipitated using 100% ice-cold ethanol and redissolved in 200 μL of TE buffer. The DNA was treated with 20 μg of RNase (Applichem, Darmstadt, Germany), incubated at 37°C for 30 min to 1 h. For final precipitation, 20 μL of 4 M ammonium acetate, pH 4.8 (Sigma-Aldrich, Munich, Germany) and 600 μL of ice-cold 100% ethanol (Applichem) were added and samples were incubated overnight at −20°C. The DNA was re-dissolved in TE buffer 1x, assessed in a biophotometer 6131 (Eppendorf, Hamburg, Germany) and adjusted to a final concentration of 2.0–2.5 μg/μL. To assay DNA integrity, approximately 3–5 μg of DNA was run in agarose gel (1%, w/v) (Sigma-Aldrich) in TBE buffer 1x and stained with ethidium bromide (0.5 mg/mL) (Applichem). DNA samples were stored at −20°C for subsequent use.

REA of mt DNA

For each sample, a reaction mixture was prepared containing 1x *Hin*fI enzyme reaction buffer (Metabion, Planegg, Germany), 1 μg/μL RNase, 0.5 U/μL *Hin*fI restriction enzyme (Metabion), approximately 25–30 μg of total DNA and DNAse-RNAse free water up to 20 μL final volume; reaction tubes were incubated overnight at 37°C. Restriction was ended upon *Hin*fI inactivation by incubating for 20 min at 80°C. The total reaction mixture was run on a 1% agarose gel (20 cm × 24 cm) at 120 mV for 3–5 h, stained with ethidium bromide (0.5 mg/mL) and the DNA visualized under UV light.

Restriction patterns were analyzed using the UVIDOC 12.6 software for Windows (Topac Inc., Cohasset, MA, USA) and the resulting groups of strains were compared.

Results and Discussion

There have been an increasing numbers of reports describing non-*albicans Candida* hospital outbreaks. Given that *C. krusei* is not the main pathogen causing nosocomial infections, the detection of simultaneous episodes at the neutropaenia unit of our hospital lead us to consider the possibility of an outbreak.

The *C. krusei* isolates were all susceptible to all the antifungals assayed (fluconazole was not tested because *C. krusei* presents intrinsic resistance to this agent). Variations in the antifungal MICs for different isolates were not significant. The MICs of amphotericin B were from 0.06 to 1 mg/L; for caspofungin from 0.125 to 1 mg/L; for anidulafungin 0.06 mg/L; for voriconazole from 0.25 to 2 mg/L; and for posaconazole from 0.03 to 0.5 mg/L.

The routine biochemical identification protocols or antifungal susceptibility profiles are usually not sufficient to either corroborate or exclude an outbreak hypothesis. REA for *Candida* species was first described by Scherer and Stevens [24] who considered this method to be an extremely valuable tool for epidemiological studies. Fujita *et al.*[21] described *Hin*fI restriction patterns as exhibiting a superior discriminatory power among distinct *Candida* isolates compared to patterns obtained with other enzymes such as *Eco*RI or *Msp*I. Additionally, Sancak *et al.*[22] established a correspondence of almost 100% between the results obtained with *Hin*fI restriction endonuclease-based analysis and PCR methodologies. In the present study, a total of 22 *C. krusei* isolates (20 clinical, two environmental) were analyzed using REA. A high number of *C. krusei* isolates was obtained from different biological products of patient A in distinct periods of time. All of them showed the same restriction pattern, including the isolate recovered in 2006 from a blood culture (A_{BC1}) (Fig. 1, lanes A_{BC1}, A_{BC2} and A_{CVCII}), indicating that they are the same strain. Most certainly, this patient harbors a reservoir of *C. krusei* and was colonized throughout a long period of time, as described similarly for *Acinetobacter* [25] and *Pseudomonas* [26]. This is the first report, to our knowledge, describing a long-lasting colonization by *C. krusei.* These results have implications in terms of prophylactic measures (i.e. fluconazole is not recommended in a patient with previous isolation of *C. krusei*). Other antifungals, such voriconazole or amphotericin B, are more likely to be efficient in these cases [27,28].

The *C. krusei* isolates from each patient yielded distinct *Hin*fI restriction patterns, suggesting that the isolates were different strains (Fig. 1, lanes A_{BC2}, B_{BS}, C_S), at the same time discarding the hypothesis of an outbreak in the neutropaenia unit where the patients were admitted. The *C. krusei* strain isolated from

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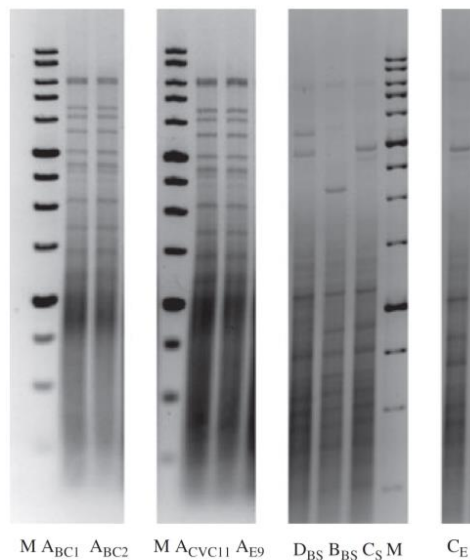

FIG. 1. Restriction endonuclease patterns of *Hin*fI-digested DNA obtained after agarose gel electrophoresis. Each pattern corresponds to *Candida krusei* isolates from patients A, B, C and D (upper case letters), from different biological products (BC, blood cultures; CVC, central venous catheter; BS, bronchial secretions; S, stools), and to *C. krusei* isolates from the room environment of patients A and C (A_E and C_E, respectively); M, molecular weight marker (1 kb DNA ladder; Metabion).

patient D showed a pattern distinct from those isolated from the remaining patients (Fig. 1, lane D_{BS}), as expected.

The two environmental *C. krusei* isolates collected from the surfaces of the rooms where patient A (A_{E9}) (room 1) and patients B and C (C_E) (room 2) had stayed were different, as depicted in Fig. 1. This excludes the possibility of different *C. krusei* strains being transmitted as a result of patient handling by health care workers. However, both the environmental and the clinical *C. krusei* isolates associated with the same patient displayed an undistinguishable restriction pattern, as shown in Fig. 1 (lanes A_{CVC11} and A_{E9} vs. C_S and C_E), suggesting a putative environmental reservoir and the possibility of subsequent transmission from it to other patients. Our results emphasize the need to enhance preventive infection control measures, both when handling the patients directly and when cleaning patients' facilities, particularly those admitting neutropenic patients. Indeed, a study by Berrouane *et al.* [29] described the relatedness among *C. krusei* clinical isolates obtained from different biological products from both patients and health care workers.

As described in the present study, we were able to exclude the hypothesis of an outbreak occurring in the neutropaenia unit throughout the time period considered, supporting the usefulness and suitability of the REA methodology. Moreover, the present study provided very useful information concerning *C. krusei* reservoirs existing in patients and their surrounding environment.

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Transparency Declaration

The authors declare that there is no source of funding and no potential conflicts of interest.

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Antimicrobial Agents and Chemotherapy

In Vivo and *In Vitro* Acquisition of Resistance to Voriconazole by Candida krusei

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In Vivo and *In Vitro* Acquisition of Resistance to Voriconazole by *Candida krusei*

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***Candida krusei* is an important agent of opportunistic infections that often displays resistance to several antifungals. We describe here the *in vivo* acquisition of resistance to voriconazole (VRC) by *C. krusei* isolates recovered from a leukemia patient during a long period of VRC therapy. In order to mimic the *in vivo* development of VRC resistance, a susceptible *C. krusei* isolate was exposed daily to 1 μg/ml of VRC *in vitro*. Interestingly, after 5 days of exposure to VRC, a MIC of 4 μg/ml was achieved; this value remained constant after 25 additional days of treatment with VRC and also after 30 consecutive days of incubation in VRC-free medium. Our objective was to determine the associated molecular resistance mechanisms, such as expression of efflux pump genes and *ERG11* gene mutations, among the resistant strains. Synergistic effects between the efflux blocker tacrolimus (FK506) and VRC were found in all of the resistant strains. Moreover, *ABC1* gene expression increased over time in both the *in vivo*- and *in vitro*-induced resistant strains, in contrast to the *ABC2* and *ERG11* genes, whose expression was invariably lower and constant. *ERG11* gene sequencing showed two different types of mutations, i.e., heterozygosity at T1389T/C, corresponding to synonymous mutations, in *C. krusei* strains and a missense mutation at position T418C, resulting in a change from Tyr to His, among resistant *C. krusei* clinical isolates. This study highlights the relevance of ATP-dependent efflux pump (namely, Abc1p) activity in VRC resistance and describes new mutations in the *ERG11* gene among resistant *C. krusei* clinical isolates.**

In recent years, we have witnessed the emergence of low-pathogenicity non-*albicans Candida* species, such as *Candida krusei*. It is an opportunistic pathogen, especially among patients with hematological malignancies and those undergoing bone marrow transplantation (1, 2). Mortality rates among such patients with *C. krusei* fungemia are unacceptably high, ranging from 60 to 80% (1). Nevertheless, few studies concerning the epidemiology and antifungal susceptibility profile of *C. krusei* are available. *C. krusei* ranked in fifth place among 22 different species of *Candida*, accounting for 3.3% of all *Candida* isolates in both Europe and North America (3, 4).

C. krusei is often described as a multidrug-resistant (MDR) fungal pathogen, due to its intrinsic resistance to fluconazole (FLC) and its decreased susceptibility to flucytosine, amphotericin B (AMB), and ketoconazole (KTC) (5, 6). Resistance to FLC is a major problem among neutropenic and critically ill patients, since this drug is frequently used for prophylaxis (7). *C. krusei* resistance to echinocandins was also described among patients with acute myelogenous leukemia (8, 9).

Two major mechanisms of resistance to azoles are observed in *C. krusei*, i.e., reduced intracellular drug accumulation due to the activity of the efflux pump proteins Abc1p and Abc2p and alterations in the target enzyme, cytochrome P450 lanosterol 14α-demethylase, encoded by Erg11p, which is involved in the ergosterol biosynthesis pathway (10, 11). Such efflux pumps belong to the ATP-binding cassette (ABC) transporter family of proteins encoded by *ABC1* and *ABC2* genes. Venkateswarlu et al. described a group of clinical isolates of *C. krusei* resistant to itraconazole (ITC) due to the activity of efflux pumps (12). However, the scarce information available regarding efflux pump activity and gene expression following azole exposure makes *C. krusei* resistance to azoles poorly understood (13). On the other hand, it is not yet possible to explain the reduced affinity of azoles, namely, FLC, for the target, the Erg11 protein binding site (11). No resistance-conferring alterations in the *ERG11* gene sequence have yet been described for *C. krusei*, in comparison with findings for *Candida albicans* (14).

We addressed the molecular mechanisms of resistance acquired *in vivo*, i.e., in voriconazole (VRC)-resistant *C. krusei* strains isolated from a leukemia patient undergoing VRC therapy, or following induction *in vitro*, i.e., in VRC-resistant *C. krusei* strains repeatedly incubated with VRC at subinhibitory concentrations. We concluded that repeated exposure to VRC results in the development of concomitant resistance mechanisms, namely, enhanced activity of efflux pumps encoded by the *ABC1* gene and single-point mutations in *ERG11*.

MATERIALS AND METHODS

Patient clinical data. A 41-year-old male patient, admitted to Centro Hospitalar São João (Porto, Portugal), was diagnosed with acute lymphoblastic leukemia (T/natural killer [NK] cells) in August 2008. Due to protracted fever that persisted after antibacterial treatment, a myelogram was performed and a first relapse of the hematological disease was diagnosed. The patient was admitted at once to the neutropenic unit for salvage chemotherapy with fludarabine, idarubicin, and 1-β-D-arabinofuranosylcytosine (Ara-C), followed by the growth factor granulocyte colony-stim-

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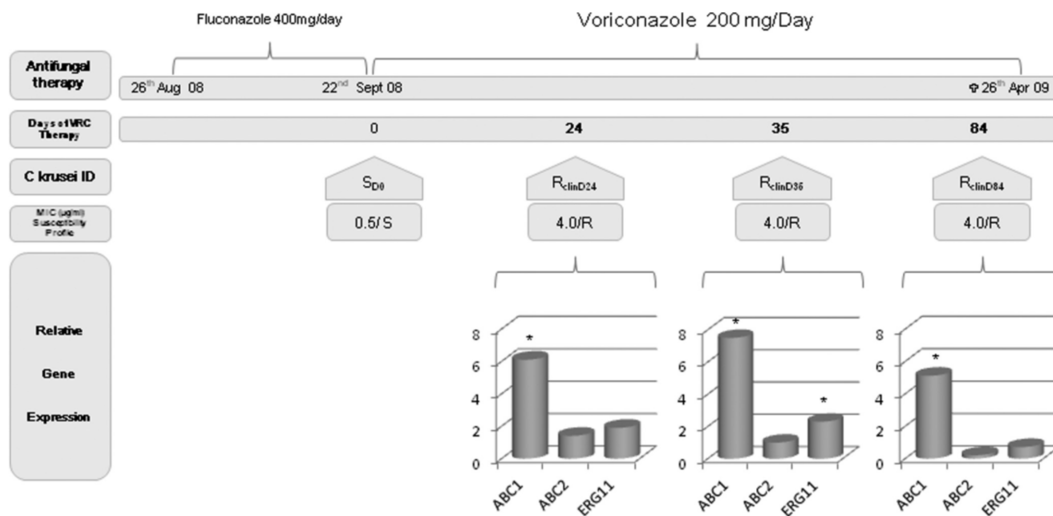

FIG 1 Induction of resistance to voriconazole *in vivo*. The timeline of antifungal therapy and the recovery of *C. krusei* clinical strains from the leukemia patient, with the respective susceptibility profiles and relative gene expression levels, is shown. ID, identification; S, susceptible, R, resistant. Cross, patient death; *, $P \leq 0.05$.

ulating factor (G-CSF), administered through a central venous catheter (CVC). Due to the persistent fever, the patient began treatment with 200 mg/day of FLC. *C. krusei* was first isolated from a blood culture after 9 days of FLC therapy; a second blood culture was positive for *C. krusei* at day 14. FLC treatment was suspended and the patient began treatment with VRC at 200 mg/day. During the initial 24 days of VRC therapy, several *C. krusei* isolates were recovered from blood cultures and from other biological samples; subsequently, *C. krusei* was recovered only from bronchial secretions and stool samples (Fig. 1). The patient had undergone VRC therapy for 216 days upon his death in April 2009.

All of the clinical isolates were identified using Vitek 2 YBC identification cards (bioMérieux, Marcy l'Etoile, France) and were stored at −70°C in brain heart infusion broth (Merck KGaA, Darmstadt, Germany) with 40% glycerol. Prior to experiments, isolates were subcultured twice in Sabouraud agar (Merck KGaA, Darmstadt, Germany) to ensure the purity of cultures.

Antifungal drugs and susceptibility testing. Stock solutions of VRC (Pfizer, Groton, CT), posaconazole (PSC) (Schering-Plough, Summit, NJ), FLC (Pfizer, Groton, CT), AMB (Bristol-Meyers Squibb, New York, NY), caspofungin (CAS) (Merck, Rahway, NJ), anidulafungin (AND) (Pfizer, Groton, CT), and micafungin (MCF) (Astellas Pharma, Inc., Tokyo, Japan) were prepared according to the M27-A3 and M27-S4 protocols of the Clinical and Laboratory Standards Institute (CLSI) and were maintained at −70°C until use (15, 16). MICs were determined for all *C. krusei* isolates according to the same protocols. The VRC susceptibility profiles of the *C. krusei* isolates were determined in accordance with MICs of ≤0.5 μg/ml (susceptible), 1.0 μg/ml (susceptible dose dependent), and ≥2.0 μg/ml (resistant). Visual readings were performed after 24 h and 48 h of incubation, according to the same protocols. *C. krusei* type strain ATCC 6258 from the American Type Culture Collection was used as a control, as recommended (15, 16).

Molecular typing. (i) Total genomic DNA extraction. All of the clinical isolates recovered from the leukemia patient were evaluated for their genetic relatedness, in order to determine whether the patient was colonized by the same *C. krusei* strain. *C. krusei* isolates were cultured overnight in 10 ml of yeast extract-peptone-dextrose (YPD) liquid medium at 35°C, at 150 rpm, and subsequently collected by centrifugation at 1,610 × *g* for 10 min at room temperature (Universal 320 R; Hettich). Total DNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1; Sigma-Aldrich, Munich, Germany), precipitated with 100% ice-cold ethanol (Applichem, Darmstadt, Germany), and redissolved in 200 μl of Tris-EDTA (TE) buffer. The DNA was treated with 20 μg of RNase (Applichem, Darmstadt, Germany) and incubated at 37°C for 1 h. For final precipitation, 20 μl of 4 M ammonium acetate (pH 4.8) (Sigma-Aldrich, Munich, Germany) and 600 μl of ice-cold 100% ethanol (Applichem, Darmstadt, Germany) were added and samples were incubated overnight at −20°C. DNA samples were resuspended in 1× TE buffer, the concentration was adjusted to 2.0 to 2.5 μg/μl, and samples were stored at −20°C for later use.

(ii) Restriction endonuclease analysis. For each sample, a reaction mixture containing 1× HinfI enzyme reaction buffer (Metabion, Martinsried, Germany), 1 μg/μl RNase (Applichem, Darmstadt, Germany), 0.5 U/μl HinfI restriction enzyme (Metabion, Martinsried, Germany), approximately 25 to 30 μg of total DNA, and DNase/RNase-free water up to a final volume of 20 μl was prepared as described by Ricardo et al. (17); reaction tubes were incubated overnight at 37°C. The total reaction mixture was run on a 1% agarose gel (20 cm by 24 cm, 120 mV) for 3 to 5 h, stained with ethidium bromide solution (0.5 mg/ml; Applichem, Darmstadt, Germany), and visualized with UV light with a Chemidoc XRS+ imaging system (Bio-Rad, Hercules, CA). Restriction patterns were analyzed using Image Lab software (version 4.0.1; Bio-Rad, Hercules, CA), and the different isolates were compared for restriction pattern similarities. A nonrelated *C. krusei* isolate recovered from stools from a different patient was used as a control.

(iii) Random amplification polymorphic DNA analysis. Random amplification polymorphic DNA (RAPD) analysis was performed as described previously (18), with some alterations. Briefly, the reaction mixtures for RAPD analysis contained 1× DreamTaq enzyme reaction buffer, 0.04 U/μl DreamTaq DNA polymerase, 0.2 mM deoxynucleoside triphosphates (dNTPs), 2 mM $MgCl_2$ (all from Fermentas, Vilnius, Lithuania), 0.4 μM primer OPA-18 (5′-AGCTGACCGT-3′) or OPE-18 (5′-GGACTGCAGA-3′) (STAB Vida, Lisbon, Portugal), 200 ng total DNA, and

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TABLE 1 MICs and susceptibilities of *C. krusei* clinical isolates

Strain identification	Recovery site	MIC (μg/ml)/susceptibility to indicated antifungal[a]: AMB	FLC	VRC	PSC	AND	CAS	MCF
S_{D0}	Blood	0.5/S	64/R	0.5/S	0.5/S	≤0.06/S	0.5/I	0.5/S
$R_{clinD24}$	Stools	1.0/S	64/R	4.0/R	≤0.03/S	≤0.06/S	0.25/S	0.5/S
$R_{clinD35}$	Bronchial secretions	1.0/S	64/R	4.0/R	0.06/S	≤0.06/S	0.125/S	0.25/S
$R_{clinD84}$	Bronchial secretions	0.125/S	64/R	4.0/R	0.5/S	≤0.06/S	0.5/S	0.25/S

[a] AMB, amphotericin B; CAS, caspofungin; AND, anidulafungin; FLC, fluconazole; VRC, voriconazole; PSC, posaconazole; MCF, micafungin; S, susceptible; I, intermediate; R, resistant.

DNase/RNase-free water up to a final volume of 25 μl. All PCRs were performed in an Eppendorf RealPlex2 Mastercycler (Eppendorf, Hamburg, Germany), and the reaction parameters were one cycle of 95°C for 2 min; 38 cycles of 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and one cycle of 72°C for 10 min. The amplified DNA fragments were analyzed by electrophoresis in a 2% agarose gel at 120 mV for 2 h. The agarose gel was stained with ethidium bromide solution (0.5 mg/ml; Applichem, Darmstadt, Germany) and visualized with UV light with the Chemidoc XRS+ imaging system. Electrophoresis patterns were analyzed using Image Lab software (version 4.0.1).

***In vitro* induction of resistance.** A VRC-susceptible *C. krusei* isolate, recovered from the leukemia patient before VRC antifungal therapy, was repeatedly incubated with VRC. Briefly, a single, randomly selected colony from a fresh 24-h culture on Sabouraud agar was suspended in 10 ml of RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid) (both from Sigma-Aldrich, Munich, Germany), in the presence of VRC at 1 μg/ml, and was incubated at 35°C at 150 rpm. Every 24 h, 1 ml of the culture was suspended in 9 ml of fresh 1 μg/ml VRC-containing RPMI 1640 medium, for a 30-day period. Subsequently, in order to evaluate the stability of the susceptibility profile, this strain was subcultured daily in fresh RPMI 1640 medium without VRC for an additional 30 days; again, every 24 h, 1 ml of the culture was suspended in 9 ml of fresh RPMI 1640 medium. At each daily subculture, an aliquot was stored at −70°C in 40% glycerol; every 2 days, a 10-μl loopful of yeast cells was cultured in Sabouraud agar to check for culture contamination. Every 5 days during the 60 days of the assay, VRC MIC values were determined according to the CLSI M27-A3 and M27-S4 protocols (15, 16).

Effects of the efflux blocker tacrolimus (FK506) on *C. krusei* susceptibility profiles. The VRC MIC values for all of the resistant *C. krusei* strains (clinical isolates or induced *in vitro*) were redetermined according to the CLSI M27-A3 and M27-S4 protocols in the presence of 100 μg/ml FK506, a recognized ATP-dependent efflux pump inhibitor (19). An agar disk diffusion assay was also performed using blank paper disks impregnated with FK506, to corroborate the results obtained with the CLSI protocols. Yeast suspensions of resistant C. *krusei* strains were prepared to an optical density of 0.5 McFarland standard (Densimat; bioMérieux, Marcy l'Etoile, France) and spread onto YPD agar plates with VRC at 4 μg/ml or without VRC. Blank paper disks (BBL, 6 mm; Becton, Dickinson) were impregnated with serial 10-fold dilutions of FK506 solutions, ranging from 1,000 to 1 μg/ml, or with its solvent, dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany); paper disks were applied to the inoculated agar plates. A plate containing only VRC at supra-MIC values was used as a control for cell viability for each strain. Results were read after 24 h and 48 h of incubation at 37°C.

Resistance gene expression analysis. (i) Total RNA extraction. *C. krusei* strains were incubated in YPD broth at 35°C, at 150 rpm, until the exponential growth phase. Yeast cells were harvested by centrifugation at 1,610 × *g* for 5 min at room temperature (Universal 320 R; Hettich) and were immediately frozen in liquid nitrogen. Total RNA was extracted using the hot acid-phenol method, as described by Köhrer and Domdey (20). RNA samples were resuspended in DNase/RNase-free water, adjusted to a final concentration of 100 ng/μl, and stored at −70°C for later use.

(ii) Reverse transcription-PCR. Two-step real-time PCRs were performed. Reverse transcriptase reactions were performed as recommended by the manufacturer; 50 ng of RNA sample, 0.015 ng/μl random primers (Invitrogen, Carlsbad, CA), 0.5 mM dNTPs (Fermentas, Vilnius, Lithuania), and RNase-free water up to 13 μl were incubated at 65°C for 5 min and then placed on ice for 1 min. Subsequently, 1× reverse transcriptase enzyme buffer (Invitrogen, Carlsbad, CA), 5 mM dithiothreitol (DTT) (Invitrogen), 1 U/μl RNasin enzyme (Promega, Madison, WI), 5 U/μl reverse transcriptase enzyme (Invitrogen), and RNase-free water were added up to a final volume of 20 μl. The reaction tubes were incubated at 25°C for 5 min, at 50°C for 60 min, and at 70°C for 15 min for enzyme inactivation. Reactions were carried out in a Mastercycler ep gradient RealPlex2 system. The cDNAs were kept at −20°C.

(iii) Quantitative real-time PCR. Genes were amplified using the primers *ABC1* (GenBank accession number DQ903907) (forward, 5′-GATAACCATTTCCCACATTTGAGT-3′, and reverse, 5′-CATATGTTGCCATGTACACTTCTG-3′), *ABC2* (GenBank accession number AF250037) (forward, 5′-CCTTTTGTTCAGTGCCAGATTG-3′, and reverse, 5′-GTAACCAGGGACACCAGCAA-3′), *ERG11* (GenBank accession number FJ445756) (forward, 5′-ATTGCGGCCGATGTCCAGAGGTAT-3′ and reverse, 5′-GCGCAGAGTATAAGAAAGGAATGGA-3′), and *ACT1* (GenBank accession number AJ389086) (forward, 5′-TGGGCCAAAAGGATTCTTATG-3′ and reverse, 5′-AGATCTTTTCCATATCATCCCAG-3′) (STAB Vida, Lisbon, Portugal). The quantitative real-time (qRT)-PCR mixture contained 1× PerfeCTa SYBR green FastMix (Quanta Biosciences, Gaithersburg, MD), forward and reverse primers (*ABC1*, *ABC2*, and *ERG11*, 0.9 μM; *ACT1*, 0.5 μM), 2 μl of cDNA, and RNase-free water, up to a final reaction volume of 20 μl. $MgCl_2$ was used in the *ABC2* gene reaction mixture at a final concentration of 1 mM. All reactions were performed in the Mastercycler ep gradient RealPlex2 system; parameters were chosen according to the manufacturer's recommendations except for primer annealing temperatures, which were as follows: *ABC1*, 54°C; *ABC2*, 56°C; *ERG11*, 60°C; *ACT1*, 54°C. To check for PCR product specificity, a melting curve was established, with temperatures ranging from 60°C to 95°C, for 20 min.

(iv) Data analysis. A standard curve, containing serial 5-fold dilutions ranging from 500 ng to 0.8 ng of RNA transcribed to cDNA, was determined in triplicate for quantification and assessment of reaction efficiency for each gene. Assays were validated for reaction efficiencies ranging from 80% to 100% and with a standard curve presenting a mean squared error higher than 0.99. The results were analyzed using RealPlex software (version 1.5.474; Eppendorf). Relative gene expression levels were calculated using REST 2009 software (Qiagen GmbH, Munich, Germany) (21), with the susceptible *C. krusei* strain as the reference sample and each resistant *C. krusei* strain as the target sample; the *ACT1* gene was used to normalize levels of gene expression. Genes exhibiting 2-fold increases in expression were considered overexpressed.

Statistical analysis. Analyses of results were performed using SPSS version 19.0. Continuous and paired-sample Student's *t* tests were used to analyze significant differences in gene expression displayed by the distinct *C. krusei* strains; *P* values of ≤0.05 were considered statistically significant.

Sequencing data analysis of the *ERG11* gene. Total genomic DNA was extracted as described above. The *ERG11* gene (1,890 bp) was ampli-

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TABLE 2 MICs and susceptibilities to voriconazole, alone and in combination with FK506, of *C krusei* strains

		VRC MIC (μg/ml)/susceptibility profile when used with[a]:	
Resistance	Strain identification	No FK506	100 μg/ml FK506
None	S_{D0}	0.5/S	0.125/S
In vivo acquired	$R_{clinD24}$	4.0/R	0.5/S
	$R_{clinD35}$	4.0/R	0.5/S
	$R_{clinD84}$	4.0/R	0.25/S
In vitro acquired	R_{indD5}	4.0/R	0.5/S
	R_{indD10}	4.0/R	0.5/S
	R_{indD30}	4.0/R	0.5/S
	R_{D60}	4.0/R	0.25/S

[a] S, susceptible; R, resistant.

fied by PCR; the reaction mixture contained 1 U/μl DreamTaq DNA polymerase enzyme, 1× DreamTaq DNA polymerase enzyme buffer, 0.2 mM dNTPs (all from Fermentas, Vilnius, Lithuania), forward_1 (5′-GGTTGTTTGTTCATTTAATGTGTGT-3′) and reverse (5′-GAAGGGGGAAAGAAAGGGAA-3′) primers at 0.8 μM (STAB Vida, Lisbon, Portugal), and RNase-free water up to a final volume of 25 μl. All reactions were performed in a Mastercycler ep gradient RealPlex2 system, and reaction parameters involved an initial 2-min denaturation step at 95°C, 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and a final 10-min extension step at 72°C. PCR products were treated with ExoSAP-IT (USB Corp., Cleveland, OH) and used as templates for the sequencing reactions. Sequencing was performed with the BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Carlsbad, CA) using the forward_1, forward_2 (5′-AACTACTGGAAAAGAGATGCTGC-3′), and forward_3 (5′-CACTCGTGATTTACCCGTTCC-3′) primers, at 0.8 μM final concentrations. DNA products were purified with Sephadex G-50 Fine (GE Healthcare, Buckinghamshire, United Kingdom) and sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Foster City, CA). Results were analyzed with Sequencing Analysis software (version 5.2; Applied Biosystems). The *ERG11* gene coding sequences of the resistant strains were aligned with the susceptible *ERG11* gene coding sequence using MUSCLE software/ClustalW (22). Alignments were analyzed with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)/ClustalW.

RESULTS

Nomenclature and susceptibility profiles of *C. krusei* strains. Four *C. krusei* clinical isolates were selected according to the VRC MIC values and the collection date (more than 10 days apart), i.e., a susceptible isolate with a VRC MIC of 0.5 μg/ml that was recovered from a blood culture before initiation of VRC therapy, designated strain S_{D0}, and three resistant isolates with VRC MICs of 4 μg/ml. The latter were designated $R_{clinD24}$, $R_{clinD35}$, and $R_{clinD84}$ according to the day of recovery during VRC therapy. $R_{clinD24}$ was the first VRC-resistant isolate and was recovered from stools, while $R_{clinD35}$ and $R_{clinD84}$ were collected from bronchial secretions (Fig. 1). The susceptibility profiles of the 4 *C. krusei* clinical isolates with the different classes of antifungals are detailed in Table 1. As expected, *C. krusei* revealed intrinsic resistance to FLC.

The VRC MIC turning point to resistance of strain S_{D0} during the *in vitro* induction assay occurred at day 5 of exposure to VRC, corresponding to a MIC of 4 μg/ml. The VRC MIC for all *C. krusei* strains remained unchanged during the 55 subsequent days of the assay (Table 2; Fig. 2). Selected strains were designated according to the day of incubation with VRC; strains obtained at days 5, 10, and 30 of the induction assay were named R_{indD5}, R_{indD10}, and R_{indD30}, respectively. The resistant strain obtained after 30 days of culture in VRC-free medium was named R_{D60} (Fig. 2).

Molecular typing. Both restriction endonuclease analysis (REA) and RAPD techniques exhibit high discriminative power, as described by Sancak et al. (23) and Bautista-Muñoz et al. (18), respectively. All of the *C. krusei* clinical strains exhibited the same restriction pattern in REA and the same amplification pattern in RAPD analysis, as shown in Fig. 3. Therefore, these results strongly indi-

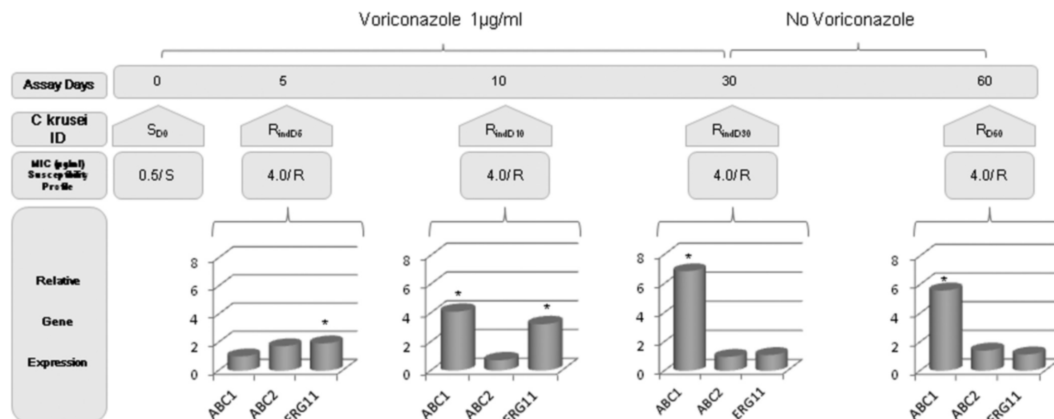

FIG 2 Induction of resistance to voriconazole *in vitro*. The timeline of the development of VRC resistance in a susceptible *C. krusei* clinical isolate that was exposed daily to VRC at 1 μg/ml, with the respective susceptibility profiles and relative gene expression levels, is shown. ID, identification; S, sensitive, R, resistant. *, $P \leq 0.05$.

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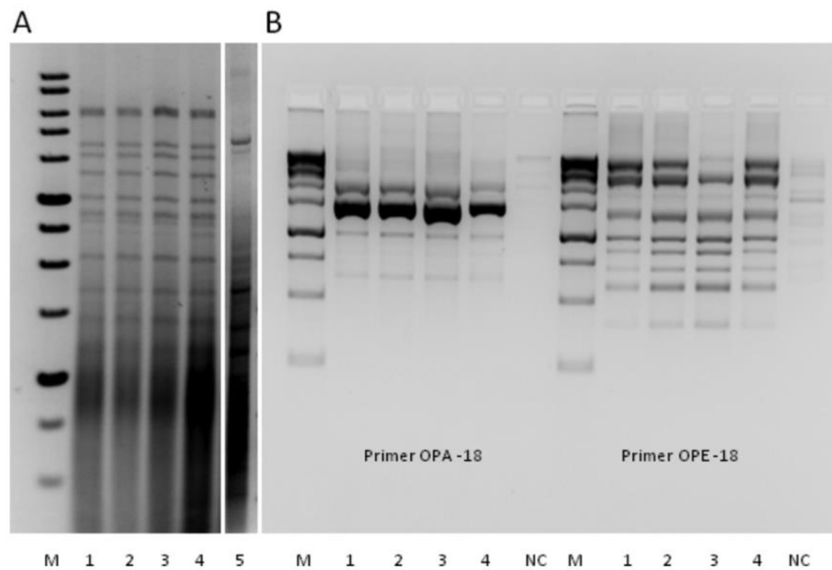

FIG 3 Genotyping of clinical isolates. (A) Restriction endonuclease pattern of HinfI-digested DNA of *C. krusei* clinical isolates in agarose gel electrophoresis. (B) Random amplification polymorphic DNA (RAPD) analysis of *C. krusei* clinical isolates with primers OPA-18 and OPE-18. Lanes M, 1-kb DNA ladder (Metabion); lanes 1, S_{D0}; lanes 2, $R_{clinD24}$; lanes 3, $R_{clinD35}$; lanes 4, $R_{clinD84}$; lane 5, nonrelated *C. krusei* control strain; NC, negative control for PCR.

cate that the strains colonizing the leukemia patient were genetically related and arose from the common ancestor susceptible strain S_{D0}.

Effects of FK506 on *C. krusei* susceptibility profiles. In the presence of FK506, all of the resistant strains changed to a VRC-susceptible phenotype (Table 2). The agar disk diffusion assay confirmed the microdilution results; growth inhibition was found for all of the resistant strains around disks containing the two highest FK506 concentrations (100 and 1,000 μg/ml) in the presence of VRC at 4 μg/ml (Fig. 4B and D). Susceptible strain S_{D0} was unable to grow in the presence of VRC at 4 μg/ml. The FK506 solvent, DMSO, did not impair the growth of the strains, and neither did FK506 alone (Fig. 4A and C).

Resistance gene expression analysis. The analysis of gene expression in R_{clin} strains with real-time PCR showed significant overexpression of the *ABC1* gene, in comparison with the susceptible S_{D0} strain (Fig. 1 and 5A). This was not the case for the other two associated resistance genes, *ABC2* and *ERG11*. Only the $R_{clinD35}$ strain presented a significant increase in *ERG11* gene expression. Similar *ABC1* gene expression profiles were registered for all of the R_{ind} strains and the R_{D60} strain, i.e., during the induction protocol, significant increases in the relative expression of the *ABC1* gene were documented (Fig. 1 and 5B). Variations in *ABC2* gene expression were not significant for any of these strains. *ERG11* relative gene expression levels demonstrated significant increases in the R_{indD5} and R_{indD10} strains.

***ERG11* gene sequencing analysis.** Several *ERG11* gene mutations were reported previously to be associated with azole resistance in *C. albicans* (14); therefore, the *C. krusei ERG11* gene was sequenced in our strains. Two different types of mutations were found. All of the susceptible and resistant *C. krusei* strains presented a heterozygous alteration at 1,389 bp (T→C) (overlapping signals in the electropherogram data), resulting in synonymous single-nucleotide polymorphisms (SNPs). Notably, $R_{clinD35}$ and $R_{clinD84}$ presented a missense mutation at position 418 bp

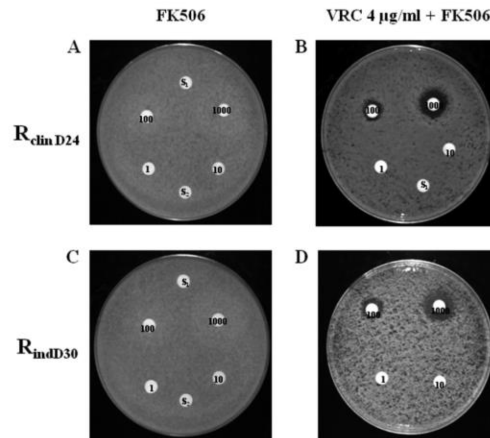

FIG 4 FK506 disk diffusion assay. Resistant *C. krusei* strains were grown on YPD medium and paper disks impregnated with serial 10-fold dilutions of FK506, ranging from 1,000 to 1 μg/ml, without VRC (A and C) or with VRC at 4 μg/ml (B and D). $R_{clinD24}$ (A and B) and R_{indD30} (C and D) are shown as representative examples of *C. krusei* strains with *in vivo* and *in vitro* induced resistance, respectively. S_1 (100%) and S_2 (10%) indicate disks impregnated with DMSO at different concentrations.

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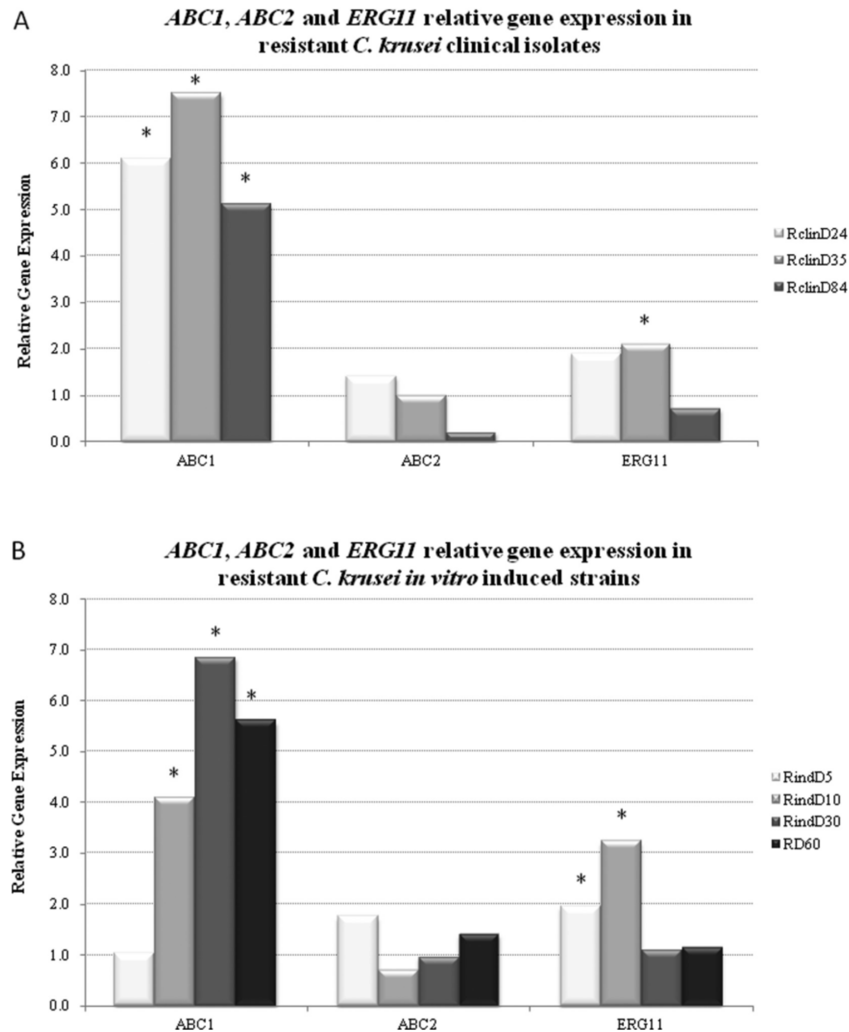

FIG 5 *ABC1*, *ABC2*, and *ERG11* relative gene expression levels in resistant *C. krusei* clinical isolates (A) and resistant strains induced *in vitro* (B). *ABC1*, *ABC2*, and *ERG11* gene expression levels were quantified and normalized relative to the housekeeping gene *ACT1*; relative gene expression levels were calculated as ratios between each resistant *C. krusei* strain and the S_{D0} isolate. *, $P \leq 0.05$.

(T →C), yielding a Tyr→His amino acid change. This point mutation had not been described previously for *C. krusei* strains.

DISCUSSION

C. krusei is one of the leading agents of candidemia among patients with hematological malignancies. In this study, the 4 consecutive *C. krusei* clinical isolates obtained from the leukemia patient during VRC therapy were evaluated for their susceptibility profiles and genetic relationships. Although they displayed distinct susceptibility profiles, these isolates were genetically related (Fig. 3). The initial colonizing strain, S_{D0}, changed from a VRC-susceptible phenotype to a stable VRC-resistant phenotype during a long period of VRC therapy. Thus, we were confronted with a case of resistance to VRC acquired *in vivo*. It should be stressed that the bioavailability and concentrations of the bioactive drug *in vivo* are highly variable due to several factors, such as different infection sites, concomitant therapies, and the status of the host immune system (24). The *C. krusei* clinical isolates might have been in contact with subinhibitory concentrations of the antifungal agent for a long period, which were not sufficient to eliminate the organism but were enough to stimulate stress adaptation mechanisms leading to resistance. Therefore, a susceptible *C. krusei* iso-

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late (strain S_{D0}) was later continuously exposed to VRC *in vitro*, in order to compare the development of resistance *in vivo* and *in vitro*.

FK506 was used as a first approach to explore the mechanisms of resistance to VRC displayed by *C. krusei* strains. It was previously described as being able to reverse multidrug resistance in different types of eukaryotic cells, due to the blockade of ATP-dependent efflux pumps, namely, human P-glycoprotein, *C. albicans* Cdr1p/Cdr2p, and more recently *C. krusei* Abc1p (13, 19, 25). The synergistic effects of FK506 and VRC that occurred with all of the resistant *C. krusei* strains, in both the microdilution and test disk assays, clearly showed that efflux pumps contributed to the VRC-resistant phenotype in both R_{clin} and R_{ind} strains.

Several genes have been reported to be involved in *C. krusei* resistance to azoles. For example, according to Katiyar and Edlind, efflux pumps from the ABC family of proteins are crucial for resistance to azoles (10); in contrast, Guinea et al. concluded that the MDR family of proteins plays a minor role in *C. krusei* resistance to azoles (26). *ERG11* gene upregulation in *C. krusei* strains in response to azole treatment (3 h of incubation with FLC at 9 μg/ml) (27) was described. Taking this into account, molecular insights were needed to corroborate the hypothesis that VRC resistance is mostly associated with the activity of efflux pumps and to exclude other resistance mechanisms.

This is the first work to address quantification of target gene expression by real-time PCR in wild-type resistant isolates, i.e., not genetically manipulated strains. The gene expression profile described herein for all of the resistant *C. krusei* strains (Fig. 5) strongly suggests that the acquisition of long-term resistance is mostly associated with the *ABC1* gene. Similar findings were previously described by Holmes et al. for *C. albicans* and by Bennett et al. for *Candida glabrata*, i.e., Cdr1p efflux activity contributes more to FLC resistance than Cdr2p (28, 29). However, our *C. krusei* R_{indD5} strain is a controversial case since neither *ABC1* nor *ABC2* genes are overexpressed. Initially, exposure to VRC induces increases in *ABC2* gene expression, overcoming *ABC1* gene expression. We hypothesize that *ABC2* can be activated more rapidly, although transiently. For long-term VRC tolerance, yeasts clearly prefer to activate the Abc1p efflux pump, which seems to be more efficient in antifungal expulsion. On the other hand, other genes encoding ATP-dependent efflux transporters may be present in C. *krusei*, such as a *CgSNQ2* homologous gene that was described as an azole-associated resistance gene in *C. glabrata* (30). Although it has been sequenced, the *C. krusei* genome is not yet completely annotated; thus, other transporter genes were not assessed. In addition, Lamping et al. incubated *C. krusei* strains for up to 4 h with different antifungals, including VRC, and no significant increases in *ABC1* mRNA levels were recorded (13). In our case, the *ABC1* gene was significantly overexpressed in *C. krusei* strains after only 10 days of *in vitro* exposure (strain R_{indD10}) or 24 days of VRC therapy (strain $R_{clinD24}$). Together, these facts clearly show that the *ABC1* gene is upregulated after an extended period of antifungal exposure, playing a late role in the development of resistance. After being triggered, however, *ABC1* gene overexpression correlates with a stable resistant phenotype, playing a definite role in long-term VRC resistance even in the absence of azoles (strain R_{D60}) (Fig. 5B). *ERG11* gene overexpression seems to be relevant in the development of VRC resistance only at an early stage, as an initial adaptation mechanism (Fig. 5). Later, other distinct mechanisms, such as the acquisition of point mutations, predominate. The point mutation described herein is definitely associated with VRC resistance in *C. krusei*, since the same type of mutation was already reported to be associated with azole resistance in *C. albicans* (31). Thus, we were confronted with the fact that *C. krusei* acquired multiple resistance mechanisms not described previously for this fungal pathogen. Such a finding is of medical relevance in considering therapeutic protocols; a susceptible isolate can develop resistance to VRC during a therapeutic regimen. On the other hand, the heterozygous alteration detected (T1389C), which is located outside the azole binding site, according to previously published data, was found in both susceptible and resistant strains (32). This alteration corresponds to synonymous SNPs, whose repercussions remain to be determined. These findings are in accordance with previous results by Lamping et al., who found several synonymous SNPs in the *ERG11* gene sequence (13), including the one detected by us. The results presented emphasize that prolonged therapy with azole antifungals can lead to resistant clones, which can ultimately spread and colonize other susceptible hosts.

In this study, we elucidated for the first time the presence of multiple concomitant resistance mechanisms in resistant *C. krusei* strains induced in both *in vivo* and *in vitro* assays. We demonstrated the relevant role that efflux activity plays as a mechanism of resistance to VRC, as well as the acquisition of a missense point mutation in the target enzyme Erg11p. The set of *C. krusei* strains described herein depicts the evolutionary process of resistance acquisition both *in vitro* and *in vivo*, being a valuable tool for the study of antifungal resistance in *C. krusei*.

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Title: Candiduria due to *Candida krusei*: a case of induction of resistance *in vivo* to voriconazole

Running title: VRC treatment for candiduria induces resistance.

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Abstract

The treatment of candiduria is always a concern in the clinical practice, due to the low level of antifungal drug reaching the urinary tract. A patient submitted to a kidney transplant was diagnosed with candiduria involving *C. krusei* and treated with VRC 200mg/2x day for 20 days. Five *C. krusei* isolates recovered from the urine were selected: one susceptible before VRC therapy (MIC 0.25 μg/ml), two resistant during VRC therapy (MIC 4.0 μg/ml) and two susceptible after VRC discontinuation (MIC 0.25 μg/ml). Clinical isolates were all genotyped using both intergenic repeat-PCR patterns (CKRS-1) and CKTNR microsatellite analysis, confirming their genetic relationship. Therefore, we were dealing with a case of development of transient resistance *in vivo*. Based on these results, we intended to corroborate the hypothesis that exposure to suboptimal VRC concentrations could lead to VRC resistance. We incubated eight independent clinical strains of *C. krusei* with VRC 0.001 μg/ml, during 30 days. The MICs to VRC of these strains ranged from 2 to 8 μg/ml, corresponding to a resistant phenotype. In the presence of VRC and the efflux pump blocker FK506, the susceptible phenotype was restored in the microdilution assay and inhibition of growth of all the VRC-resistant isolates occurred in the agar disk diffusion

assay. In order to uncover the mechanisms of resistance to VRC, efflux pumps encoded by *ABC1* and *ABC2* genes and the target enzyme of azoles Erg11p, encoded by *ERG11* gene, were investigated. *ABC1* and *ERG11* genes were overexpressed in the clinical isolates resistant to VRC and decreased to a basal level of expression in post-therapy isolates. The *C. krusei* strains incubated *in vitro* with small doses of VRC presented at least one of the resistance genes significantly overexpressed, mostly *ABC1* and *ERG11* genes and different alterations in *ERG11* gene sequence. Interestingly, the strain

presenting the lowest level of gene expression associated to resistance genes displayed a homozygous nonsynonymous mutation at position 418bp (T →C), translating into a different aminoacid Tyr→His.

In conclusion, although the effective therapeutic concentration of antifungal reaching the bladder is usually low, the acquisition of resistance to antifungal drugs can be achieved. The mechanisms of resistance studied in the *C. krusei* strains resistant strains were all present and in some strains complement each other.

Introduction

Candida species appear to be unique in their ability to both colonize and cause invasive disease in the urinary tract [1]. Candiduria is defined as a superficial infection with *Candida* spp., therefore does not belong to the group of invasive candidiasis (IC) [2-4]. Candiduria is asymptomatic in up to 96% of the patients and it is not considered a serious clinical condition [5]. However, it can be problematic in patients admitted to intensive care units (ICU) namely, immunocompromised patients with underlying serious diseases, in patients with permanent urinary catheters, with diabetes or with altered bacterial flora, due to intensive use of broad spectrum antibiotics [5-9]. *Candida albicans* is the most important and commonly isolated yeast among *Candida* speciesfollowed by *C. glabrata* and *C. tropicalis* [5, 11-13]. In this non-albicans group, *C. krusei* is not frequently isolated [14]. Colonization and invasion of the urinary tract can occur in either an antegrade fashion from the bloodstream or retrograde via the urethra and bladder, being the latter the most common route of infection. The presence of *Candida* species in the urine may be due to different clinical conditions: pyelonephritis or cystitis, hematogenous seeding of the kidney cortex due to disseminated candidiasis or colonization of the bladder, perineum or indwelling urinary catheter. However, there is some concern about candiduria since it can be the spark to candidemia in critically ill patients. Also, when fungus ball form, there is a strong probability of biofilm formation allowing the persistence of the organism in the host [15]. One of the big concerns related to candiduria is the therapeutic decision of the physicians [13]. This is probably due to the fact that no diagnostic tools are available to readily differentiate between candidal urinary tract infections (UTI), colonization or contamination in the ICU setting. The IDSA (Infectious Diseases Society of America)

has defined indications for therapy of candiduria in late 2003 in the following groups: infants with very low birth weights, patients undergoing genitourinary procedures, patients with neutropenia, renal transplant recipients and symptomatic patients. Several classes of antifungals are available but none of them is the best suited. Fluconazole (FLC) is the first choice as it is excreted unchanged by the kidneys. Ketoconazole and itraconazole are poorly excreted in the urine, voriconazole and posaconazole achieve minimal urinary excretion, amphotericin B is very nephrotoxic, and all echinocandins are associated to extremely poor glomerular filtration or tubular secretion *in vivo,* as 2–3% of active drug is eliminated in the urine, resulting in subtherapeutic concentrations in the urine [5, 16-18].

We describe in this work a case of candiduria by *C. krusei* in a patient submitted to a kidney transplant and under VRC therapy. It was registered the transient acquisition of resistance to VRC *in vivo*. Two major mechanisms associated to azole resistance have been described in *C. krusei*: the presence of efflux pump proteins (Abc1p and Abc2p), and a diminished sensitivity of the azole antifungals to the enzyme cytochrome P450 lanosterol 14α-demethylase, Erg11p, encoded by *ERG11* gene. Efflux pump proteins in *C. krusei* belong to the ATP Binding Cassette (ABC) transporter family, encoded by *ABC1* and *ABC2* genes which promote the extrusion of the antifungal drug from the cell, reducing the cellular drug accumulation. *Candida krusei* is intrinsically resistant to FLC due to alterations in Erg11p binding site [19, 20]. Recently, one mutation associated to resistance was described by our team in *ERG11* gene in *C. krusei* strains resistant to VRC at position 418 bp (T →C) translating into a Tyr→His amino acid change [26]. Acquisition of resistance *in vivo* was replicated *in vitro* in the presence of very low concentrations of VRC. We intended to uncover the mechanisms of resistance that developed in *C. krusei* strains *in vitro* and *in vivo*.

Material and Methods

CASE REPORT AND CLINICAL C. KRUSEI STRAINS ISOLATION

A 20-year-old woman was hospitalized in December 2003, for acute renal failure secondary to rhabdomyolisis, induced by atorvastatine (Tahor®). She presented with a probable evolution of a previously undiagnosed chronic renal insufficiency, associated with urological malformation and familial hypercholesterolemia. In November 2005, renal transplantation was performed and immunosuppressive treatment (tacrolimus, mycophenolate mofetil and corticoids) was given. Twelve days after transplantation, bacteraemia due to *Escherichia coli* occurred probably due to a contamination of the solution for graft kidney preservation. She was administered ceftriaxone and ciprofloxacine. At day 18 of transplantation, she presented with an aneurysm dissection of renal artery associated with hematoma. Therefore, the surgeons had to perform hypogastric autograft and distal anastomosa in hilus of renal graft, resulting in the preservation of the graft. Pre-renal hematoma was progressively resolved with antibiotics. At day 25th after transplant, the first episode of candiduria occurred. At day 44th after transplant, oral FLC 200 mg/day was implemented. However, at day 44 of kidney transplantation, FLC stopped and began to take oral VRC 200 mgx2/day, prescribed for 20 days mainly for graft preservation (prophylaxis), despite probable low urinary diffusion associated with low expected efficacy against candiduria. After 82 days of renal transplant she was admitted to the emergency with fever, probable dissecting aneurysm of renal artery at CT-scan. Therefore, she was submitted to resection of kidney graft and prescribed antibiotics and intravenous AmB 1 mg/kg/day for 3 weeks. Since the first episode of *C. krusei* candiduria several *C. krusei* isolates were recovered and five were selected. The *C. krusei* isolates recovered before the VRC treatment presented

a susceptible phenotype, those recovered during VRC treatment presented a resistant phenotype and those isolates recovered after the withdraw of the antifungal turned to a susceptible phenotype.

CANDIDA KRUSEI CLINICAL ISOLATES GENOTYPING

All the clinical isolates recovered from the patient submitted to kidney transplant were evaluated for their genetic relatedness, in order to confirm whether the patient harbored the same *C. krusei* strain or not during her follow-up.

Total genomic DNA extraction. The protocol was performed as next described. Briefly, *C. krusei* isolates were cultured in 10 ml of YPD liquid medium, overnight at 35ºC, 150 rpm, and subsequently collected at room temperature by centrifugation (Hettich, Universal 320 R, 1610xg, 10min). Total DNA was extracted using phenol:chloroform:isoamyl alcohol 25:24:1 (Sigma-Aldrich, Munich, Germany), precipitated with 100% ice-cold ethanol (Applichem, Darmstadt, Germany), and redissolved in 200 µl of TE buffer. The DNA was treated with 20 µg of RNase (Applichem, Darmstadt, Germany), incubated at 37ºC for 1 h. For final precipitation, 20 µl of 4 M ammonium acetate, pH 4.8 (Sigma-Aldrich, Munich, Germany) and 600 µl of ice-cold 100% ethanol (Applichem, Darmstadt, Germany) were added and samples were incubated overnight at – 20ºC. DNA samples were resuspended in TE buffer 1x, concentration adjusted to 2.0-2.5 µg/µl and stored at -20ºC for later use.

Genotyping analysis: (i) *PCR CKRS-1 analysis*. Amplification was performed on 10 ng of genomic DNA with primers Arno1 (5'-GCCAACACATACATACCTT-3') and Arno2 (5'-GGTAGGATACTAACCACAGC-3') as described by Carlotti *et al* [21].

Reaction were performed in 50-µL mixtures containing with 100 µM of each dNTP (dNTPset, MBI Fermentas, Vilnius, Lithuania), 0,2 µM of each primer (Genset SA, Paris, France), 1X Buffer and 1,5 U REDTaq-Polymerase™ (Sigma™, St Louis, MO, USA). The amplified fragments were visualized after separation by agarose gel electrophoresis with ethidium bromide (0.5µg/mL) staining. PCR amplification parameters included an initial denaturation step for 4 min at 92°C, 32 cycles of annealing for 30s at 55°C, extension for 2 min at 72°C, and denaturation for 30s at 92°C followed by final extension at 72°C for 10 min [21].PCR products were visualized and compared for amplification pattern similarities using electrophoresis at 140 V for 4 h on a 1% agarose gel, and staining with ethidium bromide solution 0.5 mg/ml (Applichem, Darmstadt, Germany). PCR amplification products were analyzed using the Gel Logic 100 Imaging System Software (Kodak). Three unrelated *C. krusei* clinical isolates of distinct patients were used as a *control.*

(ii) Microsatellite CKTNR analysis: Assessment of CKTNR polymorphism was performed by fragment size analysis, using primers CKTNR3 and CKTNR5 as described by Shemer *et al* [266]. Primers CKTNR3 was 5' labelled with hexachlorocarboxyfluorescein (HEX). Reaction were performed in 20-µL mixtures containing 30 ng of genomic DNA, 200 µM of each dNTP (dNTPset, MBI Fermentas, Vilnius, Lithuania), 0,5 µM of each primer (Genset SA, Paris, France), 1X Buffer and 0,5 U REDTaq-Polymerase™ (Sigma™, St Louis, MO, USA). Amplicons were sized using capillary electrophoresis on ABI Prism 3130. Results were expressed with respect of haplotype denomination described by Shemer *et al.,* using *C. krusei* strain CBS 573 as control [22].

In vitro generation of VRC-resistant derivatives of *C. krusei* after VOR exposure

Eight independent clinical strains of *C. krusei* susceptible to VRC were isolated from several biological products of different patients with different underlying diseases, not submitted to VRC therapy (table 1). The strains were grown in brain-heart infusion (BHI) broth medium containing 0.001 µg/ml of VRC, with daily subcultures in fresh BHI-VRC for 30 days, in order to obtain derivatives resistant to VRC. Briefly, every 24 h, 1 ml of the culture was suspended into 9 ml of fresh BHI broth medium; every 2 days a 10 µl loopful of yeast cells was cultured in Sabouraud agar plates to check for culture contamination.

Susceptibility Testing and Effect of the efflux blocker FK506 (Tacrolimus)

Voriconazole (Pfizer, Groton, CT), PSC (Schering- Plough; New Jersey, USA) and ITC (Sigma Aldrich, Saint-Quentin, France) antifungals stock solutions were prepared according to the M27-A3 protocol and M27-S4, the fourth informational supplement, by the CLSI and maintained in stock solution at -70°C until use. Susceptibility profile of the *C. krusei* clinical isolates and the strains obtained before (D0) and after 30 days (D30) of incubation with VRC were performed for the antifungals VRC, PSC and ITC, according to CLSI M27-A3 protocol and M27-S4, the fourth informational supplement, by the Clinical Laboratory for Standards Institute (CLSI)) [23, 24].

The susceptibility profiles to VRC of the *C krusei* isolates were in accordance to the following MIC values: susceptible whenever MIC $\leq$ 0.5 µg/ml, susceptible dose dependent whenever MIC=1.0 µg/ml and resistant whenever MIC $\geq$ 2.0 µg/ml. The

susceptibility profiles to PSC of the *C krusei* isolates were in accordance to the following MIC values: susceptible whenever MIC ≤ 0.5 µg/ml, susceptible dose dependent whenever MIC=1.0 µg/ml and resistant whenever MIC ≥ 2.0 µg/ml. The susceptibility profiles to ITC of the *C krusei* isolates were in accordance to the following MIC values: susceptible whenever MIC ≤ 0.125 µg/ml, susceptible dose dependent whenever MIC ranges between 0.25-0.5µg/ml and resistant whenever MIC ≥ 1.0 µg/ml. Visual readings were performed after 24h and 48h of incubation, according to the same protocols. *C. krusei* type strain ATCC 6258 from the American Type Culture Collection was used as control, as recommended [23, 24].

Microdilution Assay: The reversion of multidrug resistance was described in different types of cells, including *Candida* spp when in the presence of antifungals azoles and FK506 (Tacrolimus), a blocker of ATP dependent efflux pumps [25]. Therefore, MICs of VRC for *C. krusei* strains were re- determined, according to CLSI M27- A3 and M27-S4 protocols, in the presence of Tacrolimus (FK506) 100 µg/ml.

Agar disk diffusion assay: An agar disk diffusion assay was also performed using FK506 to corroborate the results obtained with the CLSI protocol: yeast suspensions of the distinct resistant strains (0.5 McFarland standard; Densimat, Biomerieux, France), were spread each onto YPD agar plates containing VRC at a supra-MIC value, according to the MIC value of each strain and without VRC. Blank paper disks, 6mm (BBL, Becton Dickinson France S.A.), were impregnated with serial 10-fold dilutions of FK506 solutions, ranging from 1000 to 1 µg/ml, and with its solvent, dimethyl sulfoxide (DMSO, Merck). Dried paper disks were applied onto the inoculated agar plates. A plate containing only VRC at the supra-MIC values was used as a control for cell viability for

each strain. The agar plates were incubated at 37ºC, and the results were registered after 24h and 48h.

RESISTANCE GENE EXPRESSION ANALYSIS

Total RNA extraction: Selected *C. krusei* strains were incubated in YPD broth at 35ºC, 150 rpm, until exponential growth phase; yeast cells were harvested by centrifugation at room temperature, (Hettich, Universal 320 R, 1610xg, 5 min), immediately frozen in liquid nitrogen. Total RNA was extracted using the hot acid phenol method, as described by Köhrer & Domdey [26]. RNA samples were resuspended in DNase/ RNase-free water, concentration adjusted to a final concentration of 100 ng/µl and stored at -70ºC for later use.

Reverse transcriptase PCR (RT-PCR). Two-step real-time PCR reactions were performed. Reverse transcriptase reactions were performed as previously described by Ricardo *et al* [27]. The cDNAs were kept at -20ºC until later use.

Quantitative Real-Time PCR (qRT-PCR): *ABC1*, *ABC2* and *ERG11* genes were amplified using the primer pairs described by Ricardo et al [27]. qRT-PCR reaction mixture contained: SensiFAST SYBR No-Rox Mix 1x (Bioline, Taunton, MA, USA), primers forward and reverse (*ABC1*, *ABC2*, *ERG11* 0.9 µM and *ACT1* 0.5 µM), 2 µl of cDNA and RNAse-free water, up to a 20 µl final reaction volume. $MgCl_2$ was used in *ABC2* gene reaction mixture at a final concentration of 1mM. All reactions were performed in the *Mastercycler epgradient Realplex2*; parameters were chosen according to the manufacturer's recommendations, including primer annealing temperature, 60ºC.

To check for PCR product specificity, a melting curve was established, i. e., temperature ranging from 60ºC to 95ºC, for 20 min.

Data Analysis: A standard curve was inserted, in triplicate, containing serial five-fold dilutions ranging from 500 ng to 0.8 ng of RNA transcribed to cDNA, as previously described by Ricardo *et al* [27]. The results were analyzed using the program software Realplex version 1.5.474, from Eppendorf. Relative target gene expression levels were calculated using the software REST 2009 (QIAGEN GmbH, Munich, Germany), being the susceptible *C. krusei* strain the reference sample and each resistant *C. krusei* strain the target sample; *ACT1* gene was used to normalize gene expression levels [28]. Target genes exhibiting a 2-fold increase in expression and with a p value $\leq$0.05 associated were considered significantly overexpressed.

STATISTICAL ANALYSIS

Result analysis was performed using the software SPSS (Statistical Package for Social Sciences) version 19.0. Continuous and paired sample Student's t-test was used to analyse significant differences between target gene expression displayed by the distinct *C. krusei* strains; a p value $\leq$0.05 was considered statistical significant.

SEQUENCING DATA ANALYSIS OF ERG11 GENE

ERG11 gene was sequenced in all selected *C. krusei* strains in order to look for mutations that could be associated to VRC resistance.

Total genomic DNA extraction was performed as previously described in this section.

<u>ERG11 gene sequencing reactions and analysis</u>. *ERG11* gene (1890 bp) was amplified by PCR according to the protocols previously described by Ricardo *et al* [27]. PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, USA) and used as template for the sequencing reactions. Sequencing was performed with a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Carlsbad, USA) using primers forward_1, forward_2 and forward_3, as previously described by Ricardo *et al* [264]. DNA products were purified with Sephadex G-50 Fine (GE Healthcare, Buckinghamshire, United Kingdom) and sequenced in an ABI Prism 3130 genetic analyser (Applied Biosystems, Foster city, USA). Results were analysed with Sequencing Analysis software, version 5.2 from Applied Biosystems. The *ERG11* gene coding sequences of the resistant strains were aligned with the susceptible *ERG11* gene coding sequence using MUSCLE software/ClustalW [29]. Alignments were analyzed in BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)/ ClustalW.

Results

C KRUSEI STRAINS DESIGNATION AND SUSCEPTIBILITY PROFILES

Five *C. krusei* clinical isolates were selected and designated according to their susceptibility profile and the time of recovery, respectively, during VRC therapy of the patient: susceptible isolate $Ck_{B.VRC}$ (isolated Before VRC therapy), resistant isolates $Ck_{D.VRC9}$ and $Ck_{D.VRC16}$ (isolated During VRC therapy, 9th and 16th day), susceptible isolates $Ck_{A.VR10}$ and $Ck_{A.VRC18}$ (isolated After VRC discontinuation, 10th and 18th days). Timeline of isolation and susceptibility profiles of each of the *C. krusei* clinical isolates are detailed in figure 1.

An increase in the MIC to VRC was registered after 9 days of VRC therapy, the isolates achieving a resistant phenotype ($Ck_{D.VRC9}$ and $Ck_{D.VRC16}$); however when the drug was withdraw the MIC value turned to the initial values and consequently the initial susceptible phenotype ($Ck_{A.VR10}$ and $Ck_{A.VRC18}$). In the case of ITR the first isolates were already resistant but in the presence of VRC the MIC value increased even more but decreased after its removal. No significant variation occurred in the MIC value of PSC which only increased to values related to a SDD phenotype. The hypothesis that the same *C. krusei* strain was colonizing our transplanted patient was corroborated by the genotyping technique. All the isolates presented with the same CKRS-1 electrophoretic pattern (Fig. 1, panel b) and the same allelic CKNTR microsatellite profile, i.e. profile e-f/d, according previously described nomenclature [22]. Therefore, all *C. krusei* isolates from our patient were clonal, despite presenting different susceptibility profiles.

Concerning the strains used for experimental VRC exposure and their *in vitro* induced derivatives, they were randomly numbered and designated according to the day of incubation with VRC, i.e., the initial susceptible *C. krusei* strains obtained before

incubation with VRC were referred as D0 and their derivatives after 30 days of incubation with VRC were referred as D30. Therefore, we have available the initial strains and their derivatives: $Ck1_{D0}$ and $Ck1_{D30}$, $Ck8_{D0}$ and $Ck8_{D30}$, $Ck21_{D0}$ and $Ck21_{D30}$, $Ck24_{D0}$ and $Ck24_{D30}$, $Ck32_{D0}$ and $Ck32_{D30}$, $Ck34_{D0}$ and $Ck34_{D30}$, $Ck40_{D0}$ and $Ck40_{D30}$, $Ck42_{D0}$ and $Ck42_{D30}$. Susceptibility profiles are detailed in Table 1. At day 30 of incubation with VRC, all the derivatives presented a resistant phenotype to VRC, with the MIC values ranging from 2 μg/ml to 8μg/ml and ITC with the MIC values ranging from 1 μg/ml to 16 μg/ml.

EFFECT OF EFFLUX BLOCKER FK506 (TACROLIMUS)

In the microdilution assay the presence of the efflux blocker FK506 lowered the MIC values to VRC in all the resistant strains up to 7 fold as it was the case of the *C. krusei* strain $Ck21_{D30}$. Therefore, all the resistant strains displayed a susceptible phenotype in the presence of FK506, with MIC values decreasing from 2-fold up to 7-fold (Table 2).

Concerning the agar disk diffusion assay, all the strains assayed presented growth inhibition (although variable in their extent of inhibition) around the disks impregnated with the highest concentration of FK506, in the presence of VRC at MIC values. Besides, neither FK506 alone nor DMSO, the FK506 solvent, inhibited the growth of the *C. krusei* strains.

RESISTANCE GENE EXPRESSION ANALYSIS

ABC1, *ABC2* and *ERG11* gene expression, for the different groups of strains is detailed in Figure 3 (see also figure 1 and table 1). The gene expression profile of the different *C. krusei* clinical isolates $Ck_{D.VRC9}$ and $Ck_{D.VRC16}$ is in accordance to their susceptibility profile, i.e. there is a significant increase in the expression of the resistance

genes *ABC1* and *ERG11*: *C. krusei* strain $Ck_{D.VRC9}$ *ABC1* p= 0.007, *ERG11* p= 0.001, $Ck_{D.VRC16}$ *ABC1* p= 0.003, *ERG11* p= 0.009. Conversely, *ABC2* gene was not overexpressed in these strains. The post-therapy susceptible isolates $Ck_{A.VR10}$ and $Ck_{A.VRC18}$ presented basal gene expression level for all genes, similar to the reference susceptible isolate $Ck_{B.VRC}$, which was in accordance to their susceptibility profile.

Interestingly, the *C. krusei* strains incubated *in vitro* with small doses of VRC presented different gene expression profiles. Except for strains, $Ck24_{D30}$, $Ck34_{D30}$ and $Ck40_{D30}$ all the remaining strains D30 present at least one of the resistance genes significantly overexpressed when compared to the respective susceptible strain - D0. The most remarkable increase in both *ABCs* genes was registered in $Ck21_{D30}$ strain, being also the strain with the highest MIC value to VRC.

SEQUENCING ANALYSIS OF *ERG11* GENE

Several *ERG11* gene mutations have been previously described to be associated to azole resistance in *C. albicans* [30] therefore, we sequenced *C. krusei ERG11* gene in the selected strains.

Candida krusei strains induced *in vitro* presented different alterations in *ERG11* gene sequence detailed in table 1. All the susceptible and resistant *C. krusei* strains from both groups of strains presented heterozygous alterations at 642bp (C→T) and 1389bp (T→C), all of them resulting in synonymous SNPs. It should be highlighted the homozygous nonsynonymous mutation at position 418 bp (T →C), translating a different amino acid Tyr→His in strain $Ck24_{D30}$, which presented the lowest level of gene expression associated to the resistance genes studied.

Discussion

Candiduria cases are usually not considered serious clinical situations because patient's life is not at risk. However, the particular case of the patient submitted to a kidney transplant triggered our interest once it involved an unusual acquisition of resistance as a result of VRC therapy. Management of candiduria involves many uncertainties in the indication for antifungal therapy [5, 31]. Therapeutic approaches for candiduria are very controversial due to the difficulty in evaluating its clinical importance, i.e., if the presence of *Candida* in the urine reflects infection or colonisation [3, 8, 9, 13, 32]. Empirical therapy refers to the treatment of high-risk hosts with symptoms of disease, even in the absence of positive cultures or other clinical evidences [32]. However, even when antifungal therapy is applied the concentration reaching the urinary tract is very small, not reaching an effective dose for treatment. In the clinical case presented in this study most certainly the concentration of VRC was not effective for the treatment but it was enough to induce resistance *in vivo*. Also, this clinical case was an example of therapeutic errors due to empirical therapy since FLC was administrated to the patient when *C. krusei* strains were responsible for candiduria. One possible explanation can be the time of interval between the isolation of the pathogenic yeast and its identification. As soon as it was identified the therapy was changed to another azole. It was not recommended at the moment to use AMB B due to its nephrotoxicity. VRC is not commonly used in this clinical conditions however, since the patient was a transplant recipient with a high risk of rejecting the new transplant (the 2nd transplantation), the aim of the antifungal treatment was first to protect the parenchyma of the kidney transplant from *Candida* infection and then to treat candiduria. According to figure 1, the strain colonizing the urinary tract developed a resistant phenotype after 9 days of VRC therapy and maintained it during the antifungal treatment for additional 7

days - $Ck_{D.VRC9}$ and $Ck_{D.VRC16}$, respectively. When VRC treatment was discontinued the same strain turned susceptible as shown by the genotyping assays, figure 1 panel (b). We can conclude that the *C. krusei* isolate suffered a selective pressure, developing resistance and activitating the efflux pumps. However, when the treatment stopped, along with the selective pressure the strain no longer needed to actively maintain the efflux activity. Anyway the patient was under a risk situation because the resistant strains could spread to biological sites where antifungal drug concentration could be higher and therefore maintain the resistant phenotype permanently.

The resistant phenotype registered for both groups of *C. krusei* strains is associated to multiple resistance mechanisms, being efflux activity one of the most common mechanism as it has already been described in other studies [27, 34, 35]. This hypothesis is corroborated by the results obtained with the efflux pump inhibitor, since a synergistic effect was registered in the presence of VRC in both the microdilution assay and in the agar disk diffusion assay (table 2 and figure 2). Also, ITC resistance among several *C. krusei* clinical isolates was previously described to be mostly associated to efflux pumps activity [34]. However, concerning the resistant *C. krusei* strains induced *in vitro*, only $Ck1_{D30}$, $Ck8_{D30}$ and $Ck21_{D30}$ presented a significant increase in *ABCs* genes expression. Most probably other not yet described genes belonging to the ABC transporter family of proteins are present in these strains conferring resistance. For example, were described in *C. glabrata* three genes associated to resistance to azoles: *CgCDR1*, *CgCDR2* and CgSNQ2 and *C. krusei* is more closely related to *C. glabrata* and *Sacharomyces cerevisiae* than to *C. albicans* [36-39].

ERG11 gene sequencing showed some heterozygous alterations already described in *C. krusei* by Ricardo *et al*, but not directly associated to resistance since they were present in both susceptible and resistant strains [27]. Since FLC resistance is

associated with diminished affinity of the azole to the Erg11p it can be speculated that these heterozygous alterations could be associated to fluconazole intrinsic resistance. Strain $Ck24_{D30}$ presented a mutation also described by Ricardo *et al*, at position 418 bp (T →C), translating into a Tyr→His amino acid change [27]. This strain presented the lowest gene expression level for the three genes studied therefore this mutation is associated to resistance to VRC. Another curious fact is that in the resistant strains $Ck21_{D30,}$ $Ck32_{D30}$ and $Ck42_{D30}$, heterozygous non synonymous alterations were present at position 418 bp. Since the process of evolution of resistance can occur in a stepwise way we can suggest that the occurrence of these alterations could only be a midterm position for a more stable resistant phenotype. These alterations could evolve for homozygous nonsynonymous mutations in those strains.

In the present work we showed a case of development of transient resistance to VRC *in vivo* and *in vitro* in *C. krusei* strains from different backgrounds. Despite the antifungal concentrations reached in the urine are very low, in this case, it was enough to induce resistance. Although, normally candiduria is not considered a serious clinical situation it can lead to the development of candidemia. Taken all these facts together, candiduria might not be an innocent situation and more attention should be given by the physicians to these cases. Also, different resistance mechanisms were found in the different strains namely efflux pumps activity and *ERG11* gene mutations associated to resistance.

Acknowledgments

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Table 1 – MIC, susceptibility profile, *ABC1*, *ABC2* and *ERG11* relative gene expression profile and *ERG11* gene sequence alterations of *C. krusei* strains induced *in vitro*.

		MIC (µg/ml)/Susceptibility Profile						Relative Gene Expression	*ERG11* gene sequence alterations	
		VRC		PSC		ITC				
Strains ID	Biological Sample	D0	D30	D0	D30	D0	D30	*ABC1* *ABC2* *ERG11*	D0	D30
Ck1	Sputum	0.25/S	**2/R**	0.125	0.5	0.25/SDD	**1/R**		-----	-----
Ck8	Mouth	0.25/S	**2/R**	0.125	1	0.5/SDD	**> 16/R**		Heterozygous synonymous A756T	Heterozygous synonymous A756T
Ck21	Digestive fistula	0.50/S	**8/R**	0.25	1	0.5/SDD	**> 16/R**		-----	Heterozygous Non synonymous G364G/T (A→S)
Ck24	Urine	0.25/S	**4/R**	0.25	0.25	0.5/SDD	**> 16//R**		-----	**Homozygous Non synonymous T418C (Y→H)**
Ck32	Sputum	0.125/S	**2/R**	0.125	1	0.25/SDD	**> 16/R**		Heterozygous synonymous A756T	Heterozygous Non synonymous C365C/T (A→V) Heterozygous synonymous A756T;
Ck34	Stools	0.50/S	**4/R**	0.25	0.5	0.5/SDD	**> 16/R**		-----	-----
Ck40	Sputum	0.25/S	**2/R**	0.25	0.5	0.5/SDD	**> 16/R**		-----	-----
Ck42	Ascitis	0.25/S	**4/R**	0.25	0.25	0.5/SDD	**> 16/R**		Heterozygous synonymous A756T; Heterozygous Nonsynonymous C1091T (A→V)	Heterozygous synonymous A756T; Heterozygous Non synonymous T418C (Y→H);

Table 2 - MIC and susceptibility profiles to VRC alone and in combination with FK506 of *C. krusei* strains.

Resistance	Strains ID	VRC MIC (μg/ml)/ susceptibility profile when used with:	
		No FK506	**100 μg/ml FK506**
***In vivo* acquired**	**$Ck_{D.VRC9}$**	4.0/R	0.125/S
	$Ck_{D.VRC16}$	4.0/R	0.125/S
***In vitro* acquired (D30 strains)**	**Ck1**	2.0/R	0.25/S
	Ck8	2.0/R	0.06/S
	Ck21	8.0/R	0.06/S
	Ck24	4.0/R	1.0/SDD
	Ck32	2.0/R	0.125/S
	Ck34	4.0/R	0.125/S
	Ck40	2.0/R	0.06/S
	Ck42	4.0/R	0.25/S

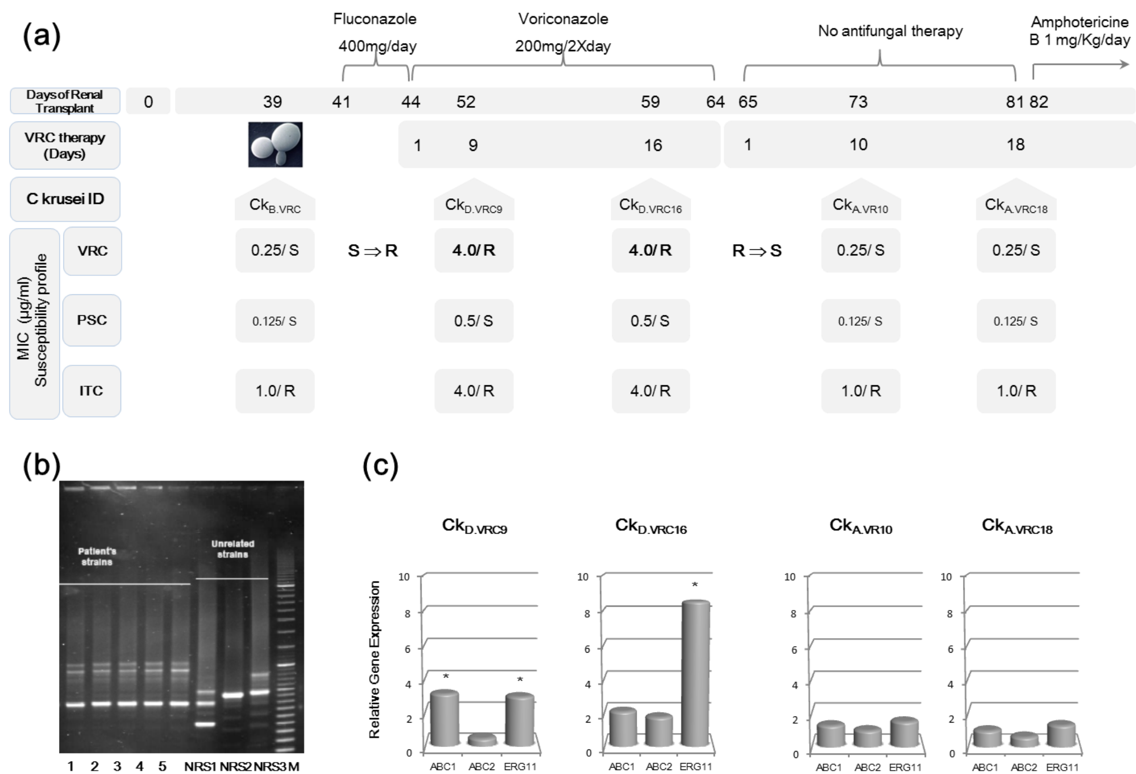


Figure 1 - *In vivo* induction of resistance to voriconazole. (a) Timeline of the renal transplant procedure, antifungal therapy and *C. krusei* clinical isolates recovered from the kidney transplant patient and their respective susceptibility profile; (b) Genotyping of *C. krusei* clinical isolates. 1- $Ck_{B.VRC}$, 2- $Ck_{D.VRC9}$, 3- $Ck_{D.VRC16}$; 4- $Ck_{A.VRC10}$, $Ck_{A.VRC18}$, NRS 1-3 – Non related isolates, M – Molecular weight; (c) Relative gene expression profile of *ABC1*, *ABC2* and *ERG11* genes for the *C. krusei* clinical isolates; $*p \leq 0.05$.

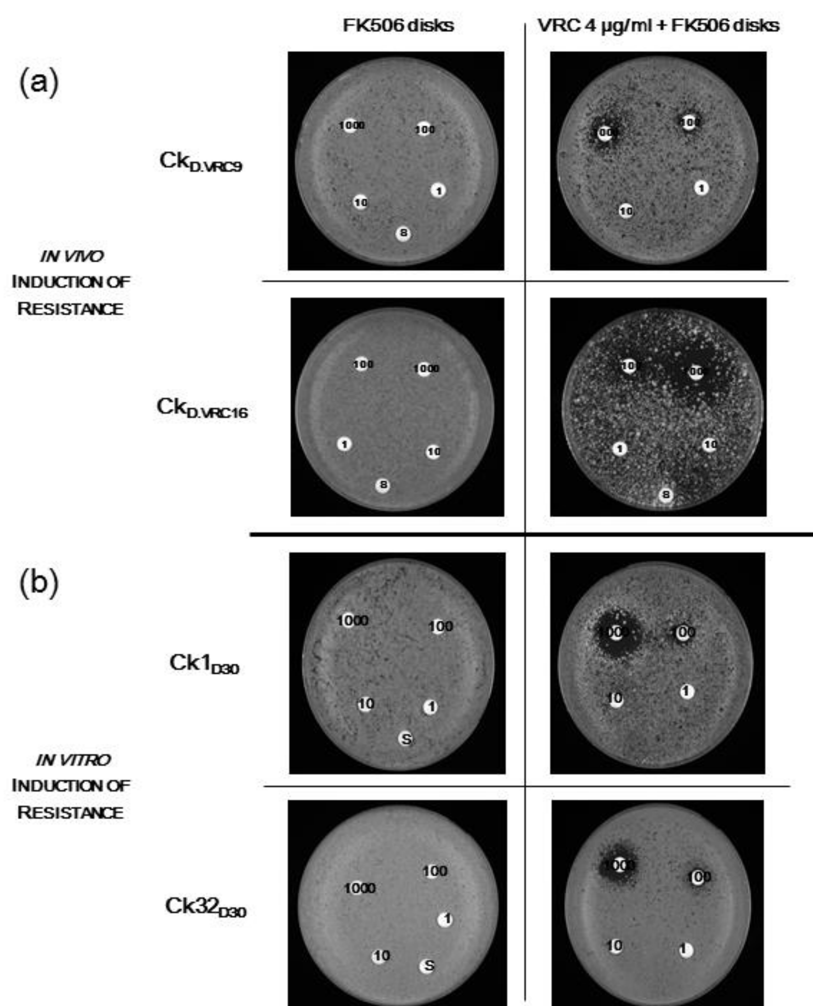
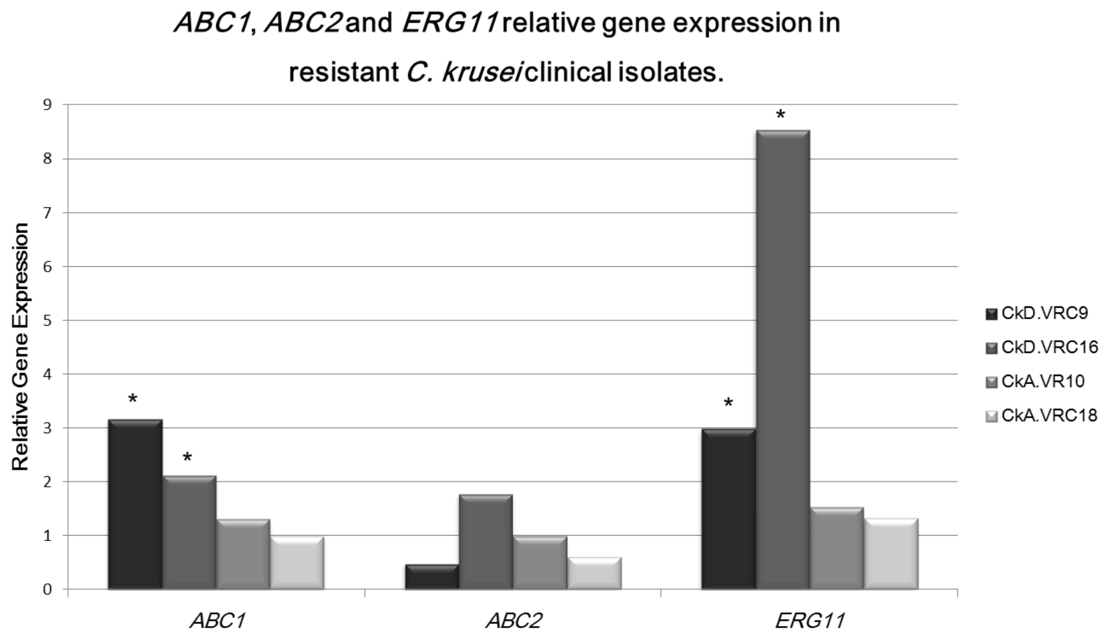


Figure 2 - FK506 agar disk diffusion assay: resistant *C. krusei* strains were grown on YPD medium and paper disks impregnated with serial 10-fold dilutions of FK506, ranging from 1000 to 1 µg/ml (FK506 disks - left column) and DMSO (S1 – 100%, S2 – 10%) and in the presence of VRC 4 µg/ml (VRC 4 µg/ml + FK506 disks- right column). (a) *C. krusei* resistant clinical isolates recovered from the kidney transplant patient; (b) *C. krusei* resistant strains induced *in vitro*, after incubation with VRC.

A



B

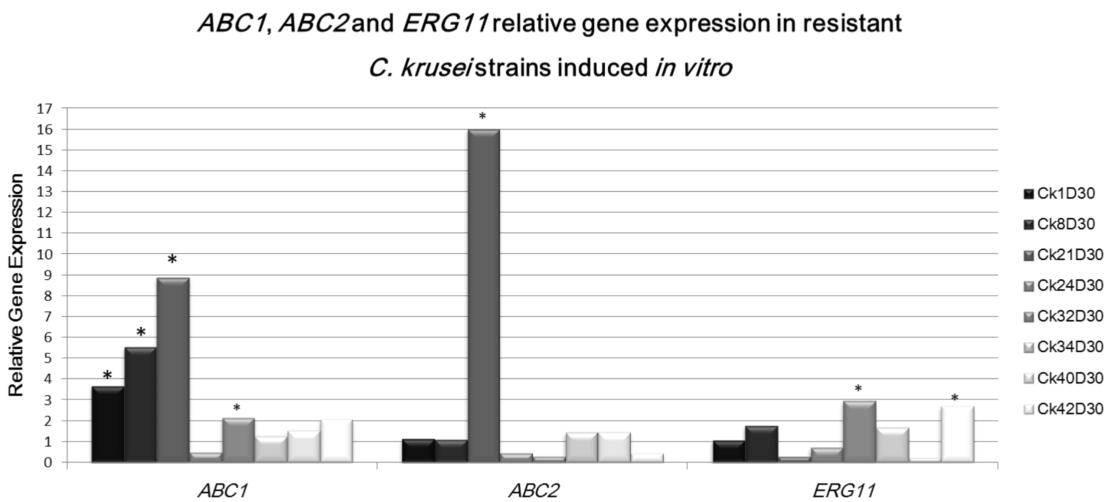


Figure 3 - *ABC1*, *ABC2* and *ERG11* relative gene expression level in *C. krusei* resistant clinical isolates (A) and resistant *in vitro* induced strains (B). *ABC1*, *ABC2* and *ERG11* gene expression level was quantified and normalized relative to the housekeeping gene, *ACT1*; relative gene expression level was calculated as a ratio between each *C. krusei* resistant strain and the respective susceptible isolate; * $p \leq 0.05$.

Results: *H. pylori* DNA was isolated from the bile and liver biopsies samples in 54% cases of chronic noncalcular cholecystitis and 72% cases of cirrhosis. Results of *H. pylori* genotyping are presented in the table.
Conclusions: The prevalent genotype of *H. pylori* isolated from the patients with hepato-biliary disorders is UreC positive VacAs1 m2 positive CagA and BabA negative. The received data indicate the possible role of CagA negative *H. pylori* in biliary tract and liver diseases.

R2157 Typing analysis of consecutive *C. krusei* isolates among haemato-oncology patients

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Many reports have documented the emergence of non-*albicans* species of *Candida* as important nosocomial pathogens, like *C. krusei*. The origin of that colonisation/infection can be exogenous, while the primary reservoir in the hospital setting is unknown, it is often linked to a prior colonisation (endogenous). Molecular typing methods, aiming to determine genetic relatedness help to clarify the origin of the strain as well of its main routes of transmission.
Objectives: In order to clarify the hypothesis of an outbreak of *C. krusei* among haemato-oncology patients, mitochondrial DNA typing was used.
Material and Methods: From three hospitalised patients admitted in single rooms at the neutropenic unit of Hospital S. João, Porto, within three weeks, *C. krusei* was isolated from distinct body sites; two of the patients had been admitted consecutively in the same room. All patients had received fluconazole-prophylaxis until the first *C. krusei* isolation. From one of these patients, an additional *C. krusei* strain, isolated from the blood two years before, was included, as well as one *C. krusei* strain obtained from a distinct patient admitted at another department. From both rooms, air and surfaces samples were collected. The identification of the yeast isolates was performed using Vitek 2 system (BioMérieux, Paris) and susceptibility profile to the main antifungals assessed accordingly microdilution CLSI protocol M-27 A2. Total DNA was extracted using a phenol-chlorophorm-isoamilic alcool protocol and quantified; 25–30 μg of DNA were digested with the restriction enzyme, Hinf I, at 37ºC, during 9–12 hours. The digestion reactions were run on 1% agarose gel, for 3–5 h, 120 mV and visualised in UV light. Restriction patterns were compared between strains.
Results: *C. krusei* were recovered from both room surfaces; the air samples were found negative. Considering all the studied isolates, only those recovered from the same patient and its respective room, showed indistinguishable electrophoretic patterns. Isolates from different patients were unrelated. Susceptibility profile was similar for all evaluated strains.
Conclusion: The molecular strategy described, showed a good discriminatory power. It did not support the hypothesis of an outbreak. Moreover, these findings highlight the possibility of an endogenous reservoir, even for long periods of time. This fact should be taken in account on future antifungal therapeutic protocols.

R2158 Molecular typing of *Klebsiella pneumoniae* in neonatal intensive care units using amplified fragment length polymorphism analysis

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Objectives: The aim of the study was to determine the population dynamics of *Klebsiella pneumoniae* in neonatal intensive care units (NICUs) of two major hospitals in Estonia, using amplified fragment length polymorphism (AFLP) analysis.
Methods: From August 2006 until December 2007, repeated perirectal and nasopharyngeal swabs were collected from all neonates admitted to two Estonian NICUs with suspected early onset neonatal sepsis. Mucosal carriage of *K. pneumoniae* was detected in 51 of 278 eligible patients. Finally, 47 of 51 patients were analysed: 38 in unit A (including 5 patients with blood stream infection) and 9 in unit B (plus 1 patient with blood stream infection but without mucosal colonisation). Depending on the length of hospitalisation, the per patient isolate number varied from 1 to 7. Alltogether, 88 perirectal, 52 nasopharyngal, 3 tracheal and 5 blood isolates were used. The AFLP analysis was implemented as described previously1. After restriction enzyme digestion, the fragments were selectively amplified and separated in agarose gel electrophoresis. The fingerprints on gel were analysed using GeneTools program (Syngene). In parallel, 10 samples of 5 patients were typed using pulse-field gel electrophoresis (PFGE) technique.
Results: After the reproducibility analysis of AFLP method, isolates with up to 3-band pattern difference were considered identical. Two predominant *K. pneumoniae* clonal groups (type A in 26 patients over 7 months and five months later, type K in 6 patients over 2 months) were detected in unit A, while in unit B all colonising *K. pneumoniae* strains were different. In all but one subject within individual concordance between nasopharyngeal and perirectal isolates was observed. All five blood stream infections in unit A occurred during the period of type A *K. pneumoniae* colonisation. On AFLP and PFGE analysis, all five invasive isolates were identical to colonising ones. Ampicillin and cefotaxime resistance was detected in 41% and 1.4% of isolates, respectively, but was not reflected on AFLP fingerprints.
Conclusion: AFLP method identified two predominant colonising clonal groups of *K. pneumoniae* in NICU setting A, whereas in setting B all patients had different strain. Vast majority of invasive disease was caused by only type A *K. pneumoniae* infection, but it is not known whether because of possibly higher virulence or as a result of widespread colonisation.

Reference(s)

[1] Van der Zee et al 2003. J. Clin. Microbiol. 41:798–802

R2159 Amplified fragment length polymorphism for high-resolution typing of *Listeria monocytogenes* from foods and the environment

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Objective: *Listeria monocytogenes* is a Gram-positive food-borne pathogen that is commonly present in the environment and occurs naturally in many foods. It is the causative agent of listeriosis, a severe food-borne disease associated with a high case fatality rate. The recent association of *L. monocytogenes* with several large food-borne disease outbreaks suggests that contaminated foods, including meat, dairy, vegetable and fish products, may be the primary source of the organism. Control of food-borne bacterial pathogens is predicated upon the identification of their sources and routes of transmission. Source tracking of *L. monocytogenes* has proved to be difficult as it is ubiquitous in the environment and also because cases are generally sporadic and outbreaks are rare. Recently, several molecular methods such as ribotyping, Pulsed-Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP), multilocus sequence typing have been used to characterise *L. monocytogenes* isolates. In this study we compared two AFLP protocols to characterise 162 *L. monocytogenes* isolates from foods and environmental sources.
Methods: Four selective primer combinations using the restriction enzymes, HindIII and HhaI, and a previously published protocol using EcoRI and MseI, were evaluated for their suitability in genotyping *L. monocytogenes*. Based on the number and distribution of the amplified bands, a protocol using HindIII and HhaI was selected for further genotyping of a collection of 162 *L. monocytogenes* isolates from different food and environmental sources.
Results: On the whole, 28 different AFLP types were identified. The differences in the fingerprinting profiles clearly distinguished two genetic lineages, recognized by serotyping, with a similarity level of 55%. This provided confirmatory evidence that there is a phylogenetic divergence between the strains of serotypes 1/2a, 1/2c, 3a, 3c that formed one cluster and the strains of serotypes 1/2b, 3b, 4b/4e and 4c forming

five years 2004–2008. These patients were examined at the Outpatient Departments of the 'A. Sygros' University Hospital for Skin and Venereal Diseases and the Army General Hospital in Athens, Greece. Both hospitals receive patients from Athens and a large area from Central Greece and the surrounding islands.

Methods: The assessment of these symptomatic patients was based on the clinical evaluation and the microbiological analysis. As tinea pedis we had defined the presence of clinical symptoms combined with a positive direct microscopical examination and a positive culture of a sample from the toe web and the surrounding skin collected by scraping with a sterile scalpel.

Results: Out of 1655 symptomatic patients 640 (38.7%) were found to fulfill the above criteria. The prevalence was higher in men (58.5%) than in women. The aetiological agents were as follows: *T. rubrum* (85.3%), *T. mentagrophytes* var.interdigitale (10.5%), *T. tonsurans* (3.1%), *E. floccosum* (1.1%) and Fusarium spp. (1.0%). *Corynobacterium minutissimum* was the prevailing bacterial pathogen, isolated in 160 cases (9.7%).

Conclusions: Dermatophytes seem to be the main cause in the aetiology of tinea pedis interdigitalis in our study. Appropriate therapeutic approach should be considered for these patients since tinea pedis is a dynamic infectious niche for tinea unguium whose diagnosis and treatment present well known difficulties.

P313

Typing of genital *Candida* isolates from couples using mitochondrial DNA typing

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Objectives: Genital candidosis is a common problem worldwide. Despite therapeutic advances, the reservoir of infection isn't yet fully elucidated. Genital candidosis can be sexually transmitted; neverthless, its contribution to the pathogenesis of *Candida* spp infection remains unknown. Molecular strain typing is a key tool in investigation. This study aims to compare *Candida* spp isolates recovered from couples suffering from genital candidosis using mitochondrial DNA.

Patients and methods: Men and women attending STD clinic suffering from genital candidosis were recruited. The respective sexual partners were offered clinical and microbiological evaluation. Participants who had taken or applied antifungal therapy within 6 weeks before enrollment were invariably excluded. Specimen collection interval between the visit of the index case and the corresponding partner's visit did not exceed 4 weeks. Specimens for yeast culture were collected from the glans penis and inner preputial layer in men and vaginal exsudate in women and cultured in CHROMagar Candida (CHROMagar Company, Paris, France). API 20C AUX galleries (BioMérieux) were additionally used to characterize Candida spp isolates. In order to compare Candida strains a restriction endonuclease analysis of mitochondrial DNA (mtREA) with the restriction enzyme HinfI followed by conventional electrophoresis was performed.

Results: From 44 heterosexual couples, the same Candida species was recovered from the genital area just in 14 (31.8%). These 28 participants were aged between 16 and 51 years (mean age 32.7), all of them assumed a single sexual partner during the last 6 months and reported sexual intercourse in the 6 days previous to specimen collection. All the men were uncircumcised. In seven couples both members had genital candidosis and in the remaining couples just the index case had signs and symptoms of candidosis. *C. albicans* was isolated from 12 couples; *C. glabrata* and *C. parapsilosis* were isolated from a couple each. Comparing the mtREA patterns for Candida spp isolates within sexual partners we found agreement of strain types in 11 couples (78.6%). *C. glabrata* and *C. parapsilosis* showed the same typing pattern. Different strain patterns of *C. albicans* were present in 3 couples; in such couples the timing of the partners visit had been four weeks apart from the visit of the index cases. Interestingly, in concordant couples the specimen collection interval had never exceed 2 weeks.

Conclusions: We found a high agreement of *Candida* isolates from sexual partners, suggesting the high possibility of sexual transmition of genital *Candida* infection. Concerning the timing of the partner visit, partners presenting within 2 weeks after the index case seem to be more likely to test concordant. Restriction endonuclease analysis of mitochondrial DNA is a method that provides interstrain differention rapidly, efficiently and reproducibly. It is easy to perform, not very expensive and is applicable to Candida spp other than *C. albicans*.

P314

Oropharyngeal candidiasis: a 21-year study in a general teaching hospital

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Objective: Oropharyngeal candidiasis is not a life-threatening condition, however, as it can impair nutrition and progress to esophagitis, specific treatment is often required. Moreover, antifungal resistant strains have been frequently reported in adult patients. We aimed to evaluate the incidence, etiology and antifungal resistance patterns of oral candidiasis episodes over a 21-year period in a large teaching institution.

Methods: From 1988 to 2008, all episodes of oropharyngeal candidiasis were collected and divided into two periods: (P1) (1988–1997) and (P2) (1998–2008). Both adult and pediatric populations were analyzed. Antifungal susceptibility patterns of amphotericin B (AMB), fluconazole (FZ), itraconazole (IZ), ketoconazole (KZ), voriconazole (VZ), flucytosine (FC), and caspofungin (CAS) were determined by Sensititre YeastOne and/or by the E-test.

Results: A total of 3626 episodes from 3116 patients (2681 adult patients and 435 pediatric patients) were recorded. The distribution of episodes and patients during the study period was as follows: P1(1920/1650) and P2 (1706/1466). The incidence of oral candidiasis, in P1 and P2 was, respectively, 3.12 and 2.01 cases per 1000 admissions. The incidence of oral candidiasis in adult and pediatric patients during both periods was as follows: 1988–1997 (2.7/0.44) and 1998–2008 (1.7/0.34). The distribution of species causing oral candidiasis in P1/P2 was *Candida albicans* (77.6%/65.8%), non-*albicans Candida*(20.7%/31.5%), and non-*Candida* yeasts (1.7%/2.7%). The MICs90 ($\mu g\ ml^{-1}$) of 460 available yeasts were as follows: AB (1), FZ (16), IZ (0.5), KZ (0.5), VZ (0.125), FC (0.25) and CS (0.125).

Conclusions: The global incidence of oral candidiasis decreased in our hospital during the study period, especially in the adult population (the incedence of oral candidiasis in HIV-infected patients, during the post-HAART period, has decreased).Most cases of oral candidiasis were caused by *C. albicans*, although the incedence of other, more antifungal-resistant yeasts increased during the second period. To our knowledge, this is the largest series of oral candidiasis reported to date.

P315

Maxillary sinus fungus ball due to *Rhizomucor pusillus* : an uncommon clinical presentation of mucormycosis

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Objectives: Mucormycosis are highly severe invasive infections occurring mostly in immunocompromised patients such as those with diabetes mellitus or with neutropenia. Non-invasive clinical manifestations due to Mucorales are rarely described. Paranasal sinus fungus ball were mainly due to *Aspergillus* species, especially *Aspergillus fumigatus*. We described a case of maxillary sinus fungus ball due to

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commonly implicated microorganisms in the pathogenesis of atherosclerosis. But, we have limited data about the fungal signatures on the cardiovascular system. In the present study, the samples of human aortic wall and left internal mammary artery (LIMA) were examined for the presence of fungal DNA.
Methods: Fungal DNA was analyzed by real-time polymerase chain reaction method in all biopsy samples. The specimens were obtained from 26 patients who underwent coronary artery bypass grafting. Biopsy samples were taken from proximal bypass punching areas of aorta (Group A), and from the LIMA which is accepted as resistant to the atherosclerosis (Group B). Primers and two probes bind to conserved regions of the fungal 18S rRNA gene. For amplicon detection, real-time PCR was performed with fluorescence resonance energy transfer (FRET) hybridization probes using the Light Cycler DNA Master Hybridization Probes Kit and the Light Cycler instrument.
Results: Fungal DNA was detected in 6 of 26 samples of group A (46.15%) and in 12 of 26 samples of Group(23.07%). Fungal DNA was found in eight samples of four patients in both tissues.
Conclusion: Results demonstrate that fungal DNA could be detected in aortic wall and LIMA speciemens which do not support this hypothesis concerning the role of fungi in atherosclerosis etiology, but may be an independent risk factor in the pathophysiology of coronary hearth diseases. The overall presence of fungi in our natural environment, the permanent fungal colonization of human microbial communities and the contact with fungi through food components make it likely that fungi cross human barriers leading to temporary fungaemia in the cardiovascular system.

P224

Retrospective screening of 164 clinical isolates for *C. orthopsilosis*, *C. metapsilosis*, *C. nivariensis* and *C. bracarensis*

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Objectives: Recent reports indicate that a minority of clinical *C. parapsilosis* isolates should be correctly re-identified to discriminate *C. orthopsilosis* or *C. metapsilosis*. Similarly, a number of *C. glabrata* isolates resistant to antifungals are actually belonging to the new species *C. nivariensis* and *C. bracarensis*. As the clinical significance and respective susceptibilities of these new species are unclear, *C. parapsilosis* and *C. glabrata* local isolates of the last 10 years were molecularly re-identified and their antifungal susceptibilities determined.
Methods: Ninety *C. orthopsilosis* isolates from blood (n = 67), skin (5), mucosa (10), chest (3) and nail (5) infections, conventionally identified by the API 32C system, were re-identified by sequencing of the ITS and the sequences were deposited to the GenBank (www.ncbi.nlm.nih.gov). Seventy-four *C. glabrata* isolates from blood (n = 44), mucosa (22), chest (6) and peritoneal (2) infections were analysed by *C. glabrata* specific PCR. Voriconazole, posaconazole, itraconazole, amphotericin B, flucytosine, caspofungin, anidulafungin and micafungin MICs were recorded by the CLSI M27-A3 microdilution method.
Results: Only one *C. orthopsilosis* isolate was recovered but no *C. metapsilosis*, *C. nivariensis* or *C. bracarensis* isolates. The *C. orthopsilosis* isolate, from a bloodstream infection, was resistant to fluconazole. *C. parapsilosis* exhibited rare resistance (MIC > 64) to fluconazole (13%) while 23% of the *C. glabrata* isolates were resistant.
Conclusion: C. orthopsilosis comprise only a small fraction of the *C. parapsilosis* Greek isolates. More clinical yeast isolates have to be studied before drawing definite conclusions on the exact *C. metapsilosis*, *C. nivariensis* and *C. bracarensis* incidence, although predictably low. *C. orthopsilosis* resistance pattern to fluconazole has to be studied more extensively.

P226

Candidaemia: antifungal susceptibility and molecular typing profiles of concomitant isolates from blood and other biological products

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Objectives: Bloodstream fungal infections have increased worldwide during the last two decades and are considered a serious public health problem. A prospective, observational study was conducted at a Portuguese University hospital, aiming to evaluate the susceptibility pattern of isolates from patients with bloodstream fungal infection and to determine the degree of similitude between distinct isolates of the same patient.
Methods: Yeasts isolated from blood cultures (89) and from other body sites (40) of patients with fungaemia admitted at a university hospital of Porto were collected and identified with VITEK 2. Minimal Inhibitory Concentrations (MIC) to fluconazole, posaconazole voriconazole, amphotericin B, Caspofungin and Anidulafungin were determined according to the protocol M27-A3 from the Clinical Laboratory for Standards Institute (CLSI). The strains were classified as S, R, susceptible-dose dependent (S-DD) or non susceptible accordingly the CLSI protocol. Sixty strains (*C. albicans*, *C. glabrata* and *C. parapsilosis*) isolated from blood cultures (40) and other biological products (20), from 16 patients were studied regarding the presence of different restriction patterns after restriction endonuclease analysis (REA) with HinfI enzyme. Restriction patterns were analyzed using the UVIDOC 12.6 software and compared among the distinct groups of strains.
Results: From a total of 89 strains isolated from blood cultures during the first fungaemia episode 43% corresponded to *C. albicans*, 26% to *C. parapsilosis*, 13% to *C. glabrata* and 7% to *C. tropicalis*. *C. albicans* (28%), *C. parapsilosis* (24%), *C. glabrata* (18%) and *C. krusei* (17%) were the most frequent yeasts isolated from other body sites. Regarding the susceptibility profile, 8% of *C. albicans*, 17% of *C. parapsilosis*, *C. tropicalis* and 58% of *C. glabrata* isolates were resistant to fluconazole. Resistance to equinochandins was detected in 8% of *C. glabrata* and 22% of *C. parapsilosis*. Regarding molecular typing, the method did not provide satisfactory results for *C. parapsilosis* since the same pattern was obtained when comparing among different patients. For the other tested *Candida* species the results obtained among the different set of isolates for each patient were very heterogeneous. From one patient yielding *C. albicans* (n = 2) and *C. parapsilosis* n = (9) strains, isolated both from blood cultures and other biological products, the differences both in the restriction pattern and susceptibility profile were only found at a interspecies level. Conversely, the *C. albicans* strains isolated only from the blood cultures of two patients (three strains of each one), were all different within each patient; in one patient the susceptibility profiles were similar but in the other major differences were registered.
Conclusion: High resistance to azoles and equinochandins was observed. Differences in susceptibility pattern do not necessarily imply differences in restriction patterns, i.e. different strains. REA is a rapid and simple technique to be used for strains typing. This technique could be of value in the follow up of patients under antifungal prophylaxis protocols to clarify strains relatedness in the case of emergence of fungal infection.
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Objectives: Antifungal activity studies on lactic acid bacteria (LAB) and Metschnikowia pulcherrima yeast strains against *Candida albicans* (Cc, C3), *Candida parapsilosis* (M6) and *Candida tropicalis* (OT4) strains isolated from vaginal and oral infection.
Enhancement of antifungal activity of *M. pulcherrima* strains using sodium bicarbonate (NaHCO3) and calcium chloride (CaCl2).
Methods: For this study 114 strains of lactic acid bacteria were isolated from sourdough, newborn faeces, fermented milk and plants. Selection of LAB strains was made by cultivation on MRS CaCO3, Gram stain and catalase test. All strains were screened for *Candida* growth inhibiting capacity by using spot agar method.
Antifungal and killer activity were tested by spoting three *M. pulcherrima* strains (SG1, SG2, CPM1) on plates flooded with the *Candida* isolates. For further tests cell suspensions were mixed with: NaHCO3 0.1%, 0.5%, 1%, 2%, and CaCl2 1%, 2%, 3%.
Results: Nine LAB strains were selected for high antifungal activity against our *Candida* isolates. API, BIOLOG and REP-PCR analysis allowed us to place LAB strains in *Lactobacillus*, Pediococcus, Weissela and *Enterococcus* genera. The antifungal activity was correlated with the biosynthesis of organic acids.
The three *M. pulcherrima* strains showed high killer activity against OT4 and Cc. The weakest action was recorded against M6. The best results for antifungal activity were obtained for all three strains on OT4, with wide halos and growth inhibition. Significant results were observed for SG1 and SG2 against Cc. In the case of M6, only SG1 formed a shallow halo. OT4 strain was sensible at SG1 and SG2 with 2% NaHCO3 or CaCl2 1%, while CPM1 was active only in 0.1% NaHCO3 mixture. Clear halos were obtained on Cc plates for all three *M. pulcherrima* strains with 2% NaHCO3 or 2% CaCl2. The weeker results were recorded against C3 strain.
Conclusions: The antifungal activity of the nine LAB selected strains was correlated with the biosynthesis of organic acids.
The most important killer and antifungal activity of the three *M. pulcherrima* strains were observed against OT4 and Cc, and was enhanced in mixture with 2% NaHCO3 or 2% CaCl2. SG1 and SG2 showed the higher antagonistic potential against all the *Candida* isolates tested.

R2308 Sensitivity of *Candida albicans* during fluconazole prophylaxis

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Objectives: Fluconazole prophylaxis in adult neutropenic leukemia patients was introduced in our hospital in 2000 and continued until 2006, with the exception of a period of about 12 months. We studied the sensitivity of *Candida albicans* to fluconazole in adult haematologic patients from 1998 to 2007 retrospectively. The species distribution of yeast isolates in all samples from the haematology ward was recorded for the study period.
Methods: 75 yeast isolates from adult haematologic patients were tested, 25 before start of prophylaxis, 25 in 2003 and 25 in 2007. Available *C. albicans* isolates from any material were selected in chronologic order from our archive. The strains were cultured on Sabouraud glucose agar and on RPMI agar for resistance testing by Etest® (AB-Biodisk and bioMérieux), according to the producer's recommendation. The plates were incubated for 24 hours, the MIC was read at 80% inhibition and the result was controlled after 48 hours of incubation. Simultaneous culture on CandidaCHROMagar® (BD Diagnostics) was made to exclude non-*albicans* and mixed infections. A search in our database for yeast isolates and species distribution in all patients admitted to the adult haematological ward was made for the years 1998 through 2007.
Results: *C. albicans* was the most prevalent yeast found. The number of samples and patients tested in the time period was increasing, in total were 404 samples and 226 patients found. *C. albicans* was isolated in 84–100% of the samples and non-*albicans* strains in 0.14–32%. The number of patients with non-*albicans* strains, mainly *C. glabrata* but also *Saccharomyces cerevisiae* was increasing. The small amount of samples may have masked a preexisting prevalence of these strains.
The MIC results demonstrated low values for all years studied. In 2007, we observed a rise in the MIC values and a higher frequency of double inhibition zones for fluconazole.
Conclusion: Fluconazole prophylaxis in haematologic patients may induce subpopulations of *C. albicans* with an elevated MIC. The prevalence of *C. albicans* was high in samples from the haematologic ward. Non-albicans species were found in addition in increasing numbers, suggesting a selection pressure induced by fluconazole prophylaxis.

R2309 *In vitro* susceptibilities of invasive samples isolates of non-*albicans Candida* sp. to antifungal agents

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Objectives: To study the susceptibility to azoles, echinocandins and amphotericin B for *Candida* species other than *C. albicans* isolates obtained from invasive samples over a period of four years in the Microbiology Service, University Hospital Virgen de las Nieves, Granada (Spain).
Methods: The susceptibility to antifungal agents for non-*albicans Candida* species isolated has been studied in invasive samples obtained between October 2005 and October 2009, generally there was a single isolated per patient. The antifungal agents investigated were fluconazole, itraconazole, voriconazole, caspofungin and amphotericin B and the method used was Sensititre Yeast One® (Trek Diagnostic Systems).
The results for fluconazole and itraconazole were interpreted according to the susceptibility criteria of CLSI (M27-A2). For the other antifungals, in which there are no breakpoints, we determined the MIC90.
Results: We detected 127 invasive samples (belonging to 95 patients) with isolates of *Candida* species other than *C. albicans*: 76 from blood cultures (59.8%), 30 from intra-abdominal samples (23.6%), 14 from respiratory tract samples (11%), 3 from skin / soft tissue (2.4%), 2 from cerebrospinal fluid (1.6), 1 from osteoarticular tissue (0.8%) and 1 from cardiac valves (0.8%). From these samples we isolated 98 strains of yeasts and their susceptibility to antifungal agents as shown in Table 1.
Conclusions:
- It has been verified that on these species fluconazole and itraconazole have a limited activity, except *C. parapsilosis*; voriconazole is the most active tested azole.
- Caspofungin appears to be very effective, including azole-resistant *Candida* species, like *C. krusei.*
- Amphotericin B is still efficient on the treatment of serious infections caused by *Candida* species other than *C. albicans*.

Table 1. Antifungal susceptibility for non-*albicans Candida* species

Candida species (n)	Fluconazole		Itraconazole		Voriconazole	Caspofungin	Amphotericin B
	% S[1]	% SDD[2]	%S	% SDD	MIC_{90}	MIC_{90}	MIC_{90}
C. parapsilosis (32)	100	–	85.2	15	1	0.12	1
C. glabrata (28)	4.17	66.7	0	33.3	1	0.25	1
C. tropicalis (21)	83.3	–	20	40	0.5	0.25	1
C. krusei (15)	0	–	16.7	83.3	0.5	0.5	2
C. kefyr (1)	100	–	100	–	0.03	0.03	1
C. pseudotropicalis (1)	100	–	100	–	0.03	0.03	2
Total (98)	55.3	22.4	40.8	36.3	1	0.5	1

[1] Percentage of susceptible strains; [2] Percentage of dose dependent susceptible strains.

R2310 Fungaemia by *C. krusei:* acquisition of voriconazole resistance *in vivo*

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Fluconazole prophylaxis has been associated to an increased prevalence of *C. krusei* and *C. glabrata* strains. *C. krusei* shows intrinsic resistance to fluconazole, but usually not to the other azoles such as voriconazole. *C. krusei* blood isolates were recovered from a leukemia patient during two months; during this period he was submitted to voriconazole therapy. An increase in minimal inhibitory concentration (MIC) values

of voriconazole was registered among consecutive isolates, which finally developed a resistant phenotype.

Objectives: Aiming to clarify the acquired resistance mechanism, we raised the hypothesis of such resistance being due to overespression of efflux pumps.

Methods: Two clinical *C. krusei* isolates were studied: one susceptible (MIC 1μg/ml) and one resistant (MIC 8μg/ml) to voriconazole. Agar disk diffusion assay was performed in order to study the synergistic effect between FK506 (Tacrolimus, described as an efflux blocker), and voriconazole, in the resistant *C. krusei* isolate, as described by Ricardo E. et al (2009). Ten-fold dilutions of FK506 ranging from 1000 to 1μg/ml were assayed and voriconazole was added to YEPD agar plates at supra-MIC value (1 μg/ml). In order to induce resistance, the susceptible strain was exposed, *in vitro*, to sub-inhibitory concentrations of voriconazole (1μg/ml). Every day 1ml of culture broth was transferred to new fresh medium, with voriconazole (1μg/ml). Susceptibility profiles to voriconazole were assayed until acquisition of resistance. Flow cytometry assays using rhodamine 6G (Rd-6G) 5μM (an efflux pump fluorescent substrate) were performed in order to compare the resistant isolates (*in vivo* and *in vitro* induced) with the susceptible isolate, regarding the role of efflux pumps in *C. krusei* resistance.

Results: Agar disk diffusion assay showed growth inhibition around the disks impregnated with the highest FK506 concentrations (100 and 1000μg/ml). At the 10th day of incubation of the susceptible strain with voriconazole the MIC value was 64μg/ml. Both resistant strains showed less intensity of fluorescence when stained with Rd-6G 5μM comparing to the susceptible isolate.

Conclusions: A synergistic effect was observed between voriconazole and the highest FK506 concentrations. The resistant strains accumulate less intracellular Rd-6G than the susceptible strain, which means that prolonged exposition to voriconazole, *in vivo* and *in vitro*, induced resistance associated to efflux pumps activity.

Fungal infections

R2311 Vulvovaginal candidiasis in a Kuwait hospital during a 2-year period

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Objectives: The purpose of this study was to determine the etiologic agent of all vulvovaginal candidiasis (VVC) isolated in our hospital during a two-year period.

Methods: 178 samples received in the hospital laboratory from July 2007 to July 2009 belonging to 89 women between ages 18 and 68 with a diagnose of VVC were reviewed. All samples were cultured on Sabouraud Dextrose Agar for 24–48 hours at 37 degree C. *Candida* spp. were identified on the basis of the macroscopic appearance of colonies, Gram-stained specimens and the identification to the species level using the API System ID 32C (bioMérieux, France).

Results: From 89 isolated strains, 38 strains (43%) were identified as *Candida albicans*, 27 strains (29.5%) identified as *Candida glabrata*, 22 strains (25%) as *Candida parapsilosis* and 2 strains (2.5%) as *Candida* crusei. When we attemped to sort the 89 cases with *Candida* by age groups, in the 13–20 years age group we included 10 samples (11.2%), in the 21–30 years group 46 samples (51.7%), in the 31–40 years group 21 samples (23.6%), in the 41–50 years group 5 samples (5.6%).

Conclusion: VVC affects female's everyday life. In the last years there has been a rise in the share of VVC attributable to non-*albicans Candida* species. It is important to know the etiological agents in each hospital and in each population in order to obtain the most precise diagnosis and treatment.

R2312 Fluconazole susceptibility testing in candidaemia isolates

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Objectives: Candidemia is a life-threatening disease, requiring early and correct treatment. Fluconazole is the standard antifungal therapy; however, not all *Candida* isolates are susceptible. Different methods for antifungal susceptibility testing are described. Therefore, we compared the E-test and disk diffusion versus broth microdilution. We also evaluated the possibility of direct susceptibility testing on positive haemocultures.

Methods: All records from patients with candidemia from January 2005 until August 2009 were analysed and the susceptibility for fluconazole was calculated. In a substudy the fluconazole E-test (on RPMI 1640 + 2% glucose agar), disk diffusion (on Shadomy agar according Neo-Sensitabs protocol) and a reference method (broth microdilution according CLSI M27-A protocol) were compared.

Results: Overview of data: The total number of patients with *Candida* septicemia decreased from 2005 till present: from 50 to 38 episodes each year. *C. albicans* was most frequently isolated at approximately 25 times a year, almost all susceptible to fluconazole (98.3%). This was followed by *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis*, all with annually decreasing trend. The susceptibility of *C. glabrata* varied most depending on the method used: 22.7% susceptible (S) and 36.6% resistant (R) with disk diffusion on Shadomy and 17.2% S and 41.4% R with broth microdilution.

Technical validation: Strains with known MIC value for fluconazole (with reference method) were used to evaluate E-test and disk diffusion. For *C. glabrata* and *C. parapsilosis* different results were found using different methods. Direct susceptibility testing was conducted on samples spiked with ATCC control strains and patient haemocultures positive for yeasts. If growth was sufficient, correct results were obtained except for *C. glabrata*. However in many cases growth on the RPMI agar was insufficient, so repeated standardized susceptibility testing was needed.

Conclusion: Correct and rapid identification of *Candida* in septicemia is more important than antifungal susceptibility testing. Inconsistent results with different methods and wide MIC distribution for wild-type *C. glabrata* makes the testing of fluconazole susceptibility a challenge in daily practice.

R2313 Effect of non-steroidal anti-inflammatory drugs on *Candida* sp.

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Objectives: The aim of the study was to investigate the action of some non-steroidal anti-inflammatory drugs (NSAIDs) sodium diclofenac, aspirin and piroxicam on cells viability of planktonic cells of *Candida albicans* and *Candida krusei* strains and possible ultra structural changes of *C. albicans* cells treated with diclofenac.

Methods: Four strains of *C. albicans* and one *C. krusei* were used in this study. Strains were isolated from pharyngeal excreta. Isolates were identified by the conventional and molecular typing (amplification of ARN ribosomal 5.8S gene and restriction with enzymes Dde I, Cfo I, and Hind III). Tests of viability of cells in the presence of three NSAIDs were done by the serial dilution method. The standard method of electronic microscopy was used to study the ultra structural changes in *C. albicans* cells, induced by the diclofenac treatment (1mM and 2mM).

Results: The results of study identified strains through conventional and molecular typing showed us that four strains were in *C. albicans* species and one strain in *C. krusei.* The cells viability tests showed us a value of this up to 38–50% for all *C. albicans* strains which were treated with sodium diclofenac to plasmatic concentration (3.6 microg/ml) and 55% for *C. krusei* strain. In the presence of aspirin 1 mM the viability of cells was 76–81% for *C. albicans* strains and up to 51–79% for piroxicam. For *C. krusei* strain the cells viability was 93% for aspirin and 80%, piroxicam respectively. The electronic microscopy study stood out the ultra structural changes of *C. albicans* cells treated with sodium diclofenac 1 mM and 2 mM. We noticed changes to the plasmalema level (separated from the cytoplasm), to outer layer of cell wall (became more electrono-transparent), the invaginations of plasmatic membrane and the disorganization of cytoplasm which appeared completely damaged in the small parts. Cells had necrotic or aged aspects.

the BioMerieux E-test and the sensitivity test against other antifungal drugs using the automated system Vitek2.
Results: The number and rate of each species are shown in table 1 and the susceptibility results to antifungal drugs are shown in table 2.
Conclusion: The number of the isolated from blood cultures yeast strains is increasing and thus the candidemia issue is considered to be a continuous challenge in the nosocomial environment (6). The development of resistance of some strains to certain antifungal agents (6) (7) (8), as shown in table 2, along with the high incidence of fungal blood infections in immunocompromised patients, have to be dealt with continuous surveillance and wisely used antifungal therapy.

P308

In vivo and *in vitro* acquisition of resistance to voriconazole by *C. krusei*: the role of efflux mechanism

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Objectives: The aim of this study was to evaluate the role of efflux pumps in the *in vivo* and *in vitro* acquired resistance to voriconazole by *C. krusei* strains.
Methods: Several *C. krusei* isolates were recovered from a patient with leukemia during voriconazole therapy. After MIC determination to voriconazole, a susceptible isolate (Ck_{D1}) was selected and exposed *in vitro* to a sub-inhibitory concentration of voriconazole (1 mg l^{-1}); susceptibility profiles were evaluated until acquisition of resistance; the stability of voriconazole resistance was also monitored.

In order to understand the role of efflux pumps in *C. krusei* voriconazole resistance, five strains were selected: one susceptible (MIC 1 mg l^{-1}; the first clinical isolate previous to antifungal administration – Ck_{D1}) and four resistant isolates (MIC 4 mg l^{-1}; one clinical induced – Ck_{D24} – and three *in vitro* induced resistance – Ck_{Ind_RD5}, Ck_{Ind_RD10}, Ck_{Ind_RD30}) using different approaches:

- microdilution susceptibility assay to voriconazole, according to CLSI M27-A3 protocol and agar disk diffusion assay, in the presence/absence of two efflux pump blockers (FK506 100 mg l^{-1} and CCCP 0.5 mg l^{-1});
- rhodamine 6G (an ATP-dependent efflux pump fluorescent substrate) staining, with and without FK506, using a flow cytometric assay.
- *ABC1* and *ABC2* genes (encode for ATP dependent efflux pumps; accession numbers DQ903907 and AF250037, respectively) expression quantification by Real Time PCR; *ACT1* gene (encoding for actin; accession number, AJ389086) was used as the housekeeping gene and SYBR Green as the florescent marker.

Results: The first voriconazole resistant isolate was recovered from the patient following 24 days of voriconazole therapy, showing a MIC to voriconazole of 4 mg l^{-1} – Ck_{D24}. *In vitro* exposure of the susceptible *C. krusei* strain to voriconazole resulted in a MIC of 4 mg l^{-1} after 5 days of incubation (Ck_{Ind_RD5}) and maintained the same MIC value throughout 30 days of incubation (Ck_{Ind_RD30}).

In all the resistant strains assayed, MIC to VRC decreased in the presence of FK506 according to the CLSI protocol M27-A3 (from 4 to 0.5 mg l^{-1}); the agar disk diffusion assay showed growth inhibition around the disks impregnated with the highest FK506 concentrations (100 and 1000 mg l^{-1}); the flow cytometric assays, revealed that the resistant induced strains showed a significant lower intensity of rhodamine-6G staining when compared to the susceptible isolate; however, this decrease was not statistically significant for the Ck_{D24} strain. *ABC1* and *ABC2* relative gene expression is detailed in figure 1; *ABC1* gene expression increased overtime while *ABC2* gene expression was relatively constant but low during the 30 days of exposure to voriconazole (Ck_{Ind_RD30} strain).
Conclusions: A synergistic effect between voriconazole and FK506 was observed in *C. krusei* resistant isolates. Repeated exposure to voriconazole, both *in vivo* and *in vitro* resulted in a resistant profile related to ATP-dependent efflux pumps promoted activity. *ABC1* gene seems to play a more active role in voriconazole resistance than ABC2 gene and *ABC1* gene relative expression depends on the exposure time. This is particularly important when considering the acquisition of azole resistance *in vivo* by clinical strains.

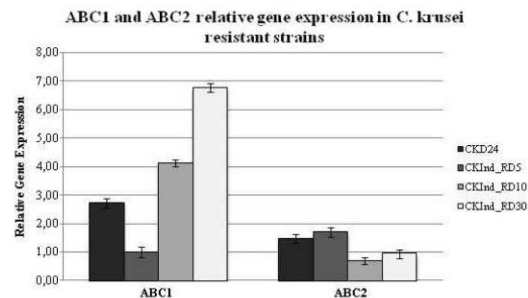

Figure 1

P309

Mechanisms of acquired azole resistance in *Candida glabrata* in treated patients – ARCANGE study

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Objectives: The major mechanisms of azole resistance in *Candida glabrata* (*Cg*) include enhanced amount and/or a modification of the target CgErg11p, and over-expression of CgCdr1p, CgCdr2p and CgSnq2p efflux pumps. The expression of these pump-encoding genes is regulated by the transcription factor CgPdr1p and single point mutations in CgPdr1p can increase the expression of at least one efflux gene. The clinical significance of these different resistance mechanisms is poorly investigated. We identified the role of these mechanisms in the emergence of resistance to azoles in clinical strains isolated from surgical intensive care unit (SICU) patients.
Methods: During a 3-year study, SICU patients were weekly screened for *Cg* colonization. For each colonized patient, the susceptibility to fluconazole (FLC) was determined in the first and last *Cg* isolates, using CLSI M27-A2 method. The emergence of FLC resistance was defined as follow: FLC MIC of the last isolate >8 μg ml^{-1} and at least fourfold higher than the first isolate. Clonality between early and late isolates was controlled by microsatellite-based MLVA method. The expression of genes encoding CgErg11p, CgCdr1p, CgCdr2p, CgSnq2p and CgPdr1p was assessed by RT-qPCR, using *URA3* as reference gene. Genes with an expression at least twofold higher in the resistant isolate than in the susceptible isolate were considered as over-expressed. *CgERG11* and *CgPDR1* genes were sequenced in both early and late isolates.
Results: Emergence of FLC resistance was detected in nine patients treated with azoles. FLC resistant isolates showed also cross-resistance to other azole antifungals. Eight resistant strains over-expressed two (CgCdr1p and CgCdr2p) or three (CgCdr1p, CgCdr2p and CgSnq2p) efflux pumps. Over-expression of these pumps was associated with mutations (five undescribed non-synonymous mutations and a seven amino acids insertion) in CgPdr1p in six isolates among which four over-expressed CgPdr1p. The target CgErg11p was over-expressed in two strains, one of which with no other gene up-regulation. The *CgERG11* sequence was conserved for each pair of strains.
Conclusion: We confirmed the diversity of the mechanisms involved in the emergence of azole resistance in *Cg*. Although the overexpression of the efflux pumps genes CgCdr1p, CgCdr2p and CgSnq2p was the more frequently implicated, an enhanced expression of the target CgErg11p can also lead to resistance to azoles. We are currently studying the impact of CgPdr1p mutations described in this study in the resistance of *Cg* to azoles.

P026

In vivo and *in vitro* resistance mechanisms to voriconazole in clinical *Candida krusei* strains

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Objectives We assessed the mechanisms of resistance to voriconazole (VOR) acquired *in vivo* and *in vitro* by clinical *C. krusei* strains.
Methods Five *C. krusei* successive isolates were recovered from the urine of a kidney transplant patient who received a 3-week VOR treatment: isolate 628 L (before therapy), isolates 633L and 640L (9th and 16th day of VOR therapy), 657L and 671L (10th and 20th days after VOR discontinuation). We genotyped the isolates using both microsatellite analysis and intergenic repeat-PCR (CKRS-1). In addition, we chose four independent clinical strains of *C. krusei* that were VOR-susceptible and isolated from VOR-free patients (P1, P21, P24, P34). They were grown in brain-heart infusion (BHI) containing 0.001 mg/l of VOR, with daily subcultures in fresh BHI-VOR for 30 days, in order to obtain VOR-resistant derivatives. VOR MICs, in the presence or not of 100 mg/l of the efflux pump inhibitor FK506, were assessed according to CLSI M27-A3 protocol. The expression of *ABC1* and *ABC2* genes (coding for ATP dependent efflux pumps) and *ERG11* gene (coding for lanosterol-14α-demethylase target enzyme) was assessed by RT-qPCR using *ACT1* gene (coding for actin) as the reference. VOR-resistant isolates with an expression of the target genes higher than 2-times that of its susceptible parent was considered as an overexpressing isolate.The expression of genes in the pre-treatment isolate was used as reference and set at 1.0.
Results All the strains isolated from the kidney transplant patient were clonal. VOR MIC increased during therapy from 0.25 (isolate 628L) to 4.0 mg/l (isolates 633L and 640L) and decreased after VOR discontinuation (isolates 657L and 671L, VOR MICs 0.25 mg/l). The efflux inhibitor FK506 restored the susceptibility of the VOR-resistant isolates (from 4.0 to 0.125 mg/l). *In vitro* exposure of VOR-free *C. krusei* clinical strains to low VOR concentrations generated VOR-resistant derivatives with MICs ranging from 4 to 16 mg/l. Here again, the efflux inhibitor FK506 restored the susceptibility to VOR. *ABC1*, *ABC2* and *ERG11* expression is detailed in Figures 1 and 2.

The expression of *ABC1* was higher than that of *ABC2* in both *in vivo* and *in vitro* VOR-resistant isolates. The expression of *ABC1* and *ERG11* was increased in *in vivo* VOR-resistant isolates (633 L, 640 L) and went back to basal level in post-therapy isolates (657 L, 671 L). Surprisingly, the four *in vitro* VOR-resistant isolates showed different gene expression profiles: two (P21, P34) overexpressed exclusively *ABC1* and *ERG11*, respectively, while the two others overexpressed none of the tested resistance genes.
Conclusions The VOR-resistant isolates of *C. krusei* overexpressed *ABC1* and/or *ERG11*. The restoration of the susceptibility to VOR by FK506 confirms the role of the efflux in the resistance to VOR. Our results highlighted the diversity of resistance mechanisms to azole in *C. krusei*. Interestingly, two VOR-resistant isolates did not overexpress *ABC1*, *ABC2* or *ERG11* suggesting yet unknown resistance mechanisms.

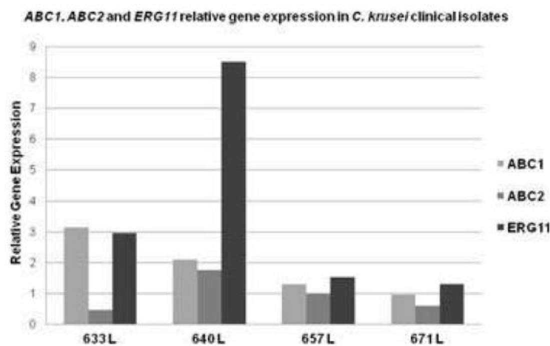

Figure 1

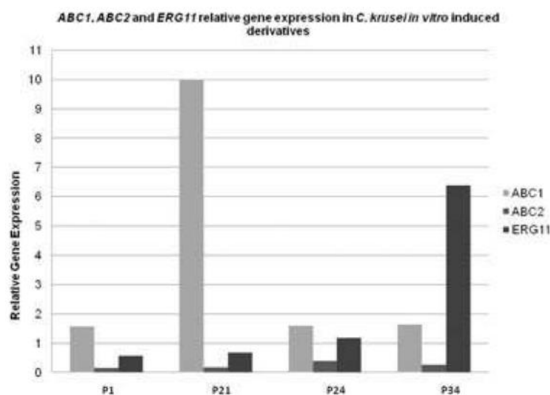

Figure 2

P027

Comparison of the 'susceptible-intermediate-resistant' categorization of fungal clinical isolates in four mycology departments using previous breakpoints and newly revised 24 h clinical breakpoints

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Context Early and optimal antifungal therapy is an essential element for a successful outcome of invasive fungal infections. The emergence of resistance to antifungal agents including the latest triazoles and echinocandins enhances necessity of a rapid and reliable determination of in vitro MIC. New CLSI clinical breakpoints (CBPs) have been proposed for an interpretation of MICs after 24 hours (Pfaller, JCM 2012).
Objectives
– Compare the interpretation of MICs of fluconazole, voriconazole and caspofungin against 18 clinical isolates in four hospital centres, using previous CLSI breakpoints (BPs) and newly revised CLSI CBPs.
– Determine if the new 24H CLSI CBPs take into account a late apparition of resistance (after 48 h incubation), particularly for azoles.
Methods 160 MICs of 18 clinical isolates were determined in four labs of Mycology from French University hospitals (Poitiers, Reims, Rennes and Toulouse).

Strains: 5 Candida species (6 *C. glabrata*, 4 *C. krusei*, 4 *C. parapsilosis*, 2 *C. albicans* and 2 *C. tropicalis*)

Antifungal agents: fluconazole (except for *C. krusei*), voriconazole (except for *C. glabrata*), caspofungin were tested.

Antifungal susceptibility profile of the strains: 8 of 18 strains (44%) were chosen because presenting a decrease susceptibility for at least one antifungal molecule.

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