

PH.D. THESIS

**EXAMINATION OF THE CONNECTION BETWEEN
ANTIFUNGAL RESISTANCE AND VIRULENCE IN
*CANDIDA PARAPSILOSIS***

PAPP CSABA GERGŐ

**SUPERVISOR: PROF. DR. GÁCSEK ATTILA
PROFESSOR**

PH.D. SCHOOL OF BIOLOGY



**DEPARTMENT OF MICROBIOLOGY
FACULTY OF SCIENCE AND INFORMATICS
UNIVERSITY OF SZEGED**

**SZEGED
2018**

Introduction

Fungal diseases are becoming increasingly important as their numbers and the size of the affected geographic areas are increasing. Among the *Candida* genus there are several opportunistic pathogens, of which *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. tropicalis* are the most significant. Among the listed species, *C. albicans* is the most important, but in the last decades, the number of infections with other non-*albicans* *Candida* species has also increased to a high extent at the expense of *C. albicans*. The most likely reason for this is the widespread use of antifungal agents and the different antifungal sensitivity of different *Candida* species. *C. glabrata* and *C. parapsilosis* are the two species that are most gain relevance at the expense of *C. albicans* caused diseases. The importance of *C. parapsilosis* is further enhanced by the fact that it is one of the most common cause of nosocomial infections, which is contributed by the fact that *C. parapsilosis* is the most commonly isolated yeast from the hands of hospital workers.

In the case of *Candida* species, the number of antifungal agents used is relatively limited, basically polyenes (amphotericin B), triazoles (fluconazole, voriconazole, posaconazole) and echinocandins (caspofungin, anidulafungin, micafungin) are applied in treatment of invasive candidiasis. The effectiveness of antifungal therapy is reduced when the patient is infected with a strain resistant to the applied antifungal agent. The mechanism of resistance is typically characteristic of the given antifungal agent group, so these mechanisms often cause cross resistance to the members of the specific group of agents, but certain mechanisms may also contribute to cross resistance between different groups. The various mechanisms are known primarily from studies with *C. albicans* and *C. glabrata*.

In our study we aimed to investigate the development of resistance mechanisms against various triazoles and echinocandins in *C. parapsilosis* and dissect the effect of these mechanisms on fitness and virulence.

Methods

Cultivation and infection of the applied host and fungal cells: Isolation, breeding and differentiation of PBMC, culture of J774.2 cell line, infection of host cells with yeasts, direct selection procedure.

In vivo infection: *G. mellonella* (wax moth) larval infection and mouse infection.

Microscopy and flow cytometry: Fc-Dectin-1-FITC and WGA-FITC staining, image analysis.

Molecular methods: DNA isolation from fungal cells, PCR, gel electrophoresis, Southern hybridization, restriction digestion and ligation of DNA, targeted gene deletion in *C. parapsilosis*, Illumina sequencing (in collaboration)

Sequencing in silico analysis (in collaboration): combining reads to contigs, SNP analysis.

Analytical methods (in collaboration): examination of the cell wall composition of yeast cells by HPLC-MS, sterol composition of fungal membrane by LC-MS.

Fungal fitness experiment: growth tests in the presence or absence of stressors.

Protein structure modeling: *in silico* modeling of the enzyme topology of glucan synthase.

Results

Characterization of *C. parapsilosis cdr1-2* deletion mutant

We generated a *C. parapsilosis cdr1-2* (ABC transporter) deletion mutant strain. We investigated the sensitivity of the deletion and parental strains to amphotericin B, fluconazole and caspofungin. The deletion strain proved to be slightly more susceptible to fluconazole and caspofungin, however susceptibility of this strain did not change to amphotericin B. The deletion strain showed elevated virulence *in vitro* and *in vivo*. This phenotype may be allelic dose-dependent as the heterozygous and homozygous deletion strain has become increasingly virulent in *in vitro* infection model in parallel with the loss of alleles, although this phenomenon was not observed during the *in vivo* mouse experiment.

Characterization of Echinokandin evolved strains

Three echinocandin resistant *C. parapsilosis* strains were generated using echinocandins by direct selection method. Of the evolved strains, the caspofungin

(CAS^{EVO}) and anidulafungin (AND^{EVO}) selected strains showed cross-resistance to all echinocandins, while the micafungin evolved strain (MIC^{EVO}) was exclusively resistant to micafungin.

The evolved strains have been susceptible to cell wall perturbing agents. This phenotype was pronounced in the MIC^{EVO} strain, since it was the most sensitive to cell wall stress and it was also sensitive to oxidative stressors. Interestingly, CAS^{EVO} and MIC^{EVO} strains showed resistance to membrane perturbing sodium dodecyl sulphate (SDS). The decreased virulence of echinokandin evolved strains were detected, as they caused reduced elimination of *G. mellonella* (wax moth) larvae and in the mice infection model, evolved strain infected mice have lower fungal burden than in the CLIB 214 infected animals.

According to examination of the composition and structure of the fungal cell wall of parental and evolved strains, the cell wall composition did not change in response to the echinokandin selection compared to the parent strain. In contrast, the structure of the cell wall was altered in the evolved strains as they expressed larger

amount of inner cell wall component, such as β -1,3-glucan and chitin on the surface of cells than the CLIB 214 strain. The decreased virulence experienced during *in vivo* infections may be due to this structural change.

However, the change in the cell wall structure did not cause *in vitro* virulence diminution of the evolved strains, as PBMC-DM primary cells isolated from human donors were equally capable to phagocytose the cells of each different strains.

According to the whole genome sequencing, amino acid change causing mutations have been identified in the *C. parapsilosis* β -1,3-glucan synthase enzyme (Fks1) coding gene in echinocandin evolved strains, which are likely to be responsible for the development of echinocandin resistance. The amino acid substitutions were characteristic of each strain, so in the CAS^{EVO} and AND^{EVO} strains a homozygous W1370R amino acid substitution was detectable and an S656P substitution in heterozygous form in AND^{EVO} strain was also identified. In the Fks1 protein of the MIC^{EVO} strain, the L703F amino acid substitution was present in homozygous form. We generated the topology model of

C. parapsilosis Fks1 protein using *in silico* methods and the identified amino acid exchange was mapped to the model. (Papp, et al., 2018)

Characterization of triazol evolved *C. parapsilosis* strains

Similarly, to the previous method, three triazole-evolved strains were also established for the investigation of triazol resistance and its effects on viability and virulence. The susceptibility of triazole-evolved strains showed a similar pattern in susceptibility of fluconazole (FLU^{EVO}) and voriconazole (VOR^{EVO}) evolved strains as both were resistant to fluconazole and voriconazole, while the MIC value of itraconazole showed only slight increase and sensitivity to posaconazole remained the same as of parental strain. In contrast, the posaconazole evolved strain (POS^{EVO}) were the resistance to all kind of azole drugs and showed the highest azole MIC values. Additionally, in the case of the POS^{EVO} strain, increased MIC values to echinocandins and even resistance to micafungin were detectable.

Triazole evolved strains were sensitive to osmotic active compounds. Interestingly, FLU^{EVO} and VOR^{EVO}

strains showed resistance to caffeine, which affects the cell wall synthesis through the TOR signaling pathway. In contrast, POS^{EVO} strain was unable to grow in the presence of caffeine. For other cell wall perturbing agents, the evolved strains proved to be sensitive. The FLU^{EVO} strain was resistant to membrane perturbing SDS, however, VOR^{EVO} and POS^{EVO} strains did not grow in the presence of this compound.

In vitro and *in vivo* infection experiments suggest decreased virulence of FLU^{EVO} and VOR^{EVO} strains. The J774.2 macrophages more effectively phagocytosed the cells of these two strains, and these strains caused less damage to *G. mellonella* larvae than the parental strain.

Sequencing the genome of parental and triazol evolved strains, point mutations in genes that may be potentially responsible for the development of a resistant phenotype has been identified. In the FLU^{EVO} and VOR^{EVO} strains, an AA change causing mutation was identified in the *MRR1* gene controlling the expression of Mdr1 efflux pump protein, so in these strains probably constitutively a high production of Mdr1 is the cause of resistance. In the POS^{EVO} strain, two potentially

resistance causing genes have been identified harboring AS substitution causing mutations. One of these is encoding the Δ^5 sterol-desaturase (Erg3), which is normally responsible for ergosterol and in the presence of azoles toxic sterol biosynthesis. The other point mutation harboring gene was the *BPH1*, the product of which has an effect on cell wall synthesis.

To demonstrate that the AA change in Erg3 enzyme of POS^{EVO} strain is caused by a loss of function mutation, we investigated the sterol composition of POS^{EVO} strain relative to the parental CLIB 214 strain. The absence of ergosterol or toxic sterol in the POS^{EVO} strain, and the enrichment of episterol and ergosta-dienol or in the presence of azoles the enrichment of lanosterol / episterol / obtusifoliol and 14 α -methyl-fekosterol showed that the mutation of *ERG3* gene of this strain is a loss of function mutation.

Summary

- Generation of *C. parapsilosis cdr1-2* deletion, echinocandin and triazol evolved strains
- Determination of antifungal susceptibility of established strains
- Investigating the stress tolerance of evolved strains
- Detection of increased virulence of *C. parapsilosis cdr1-2* deletion strain during *in vivo* and *in vitro* infection
- Showed the attenuated virulence of echinocandin evolved, FLU^{EVO} and VOR^{EVO} strains *in vivo*
- Determination of the composition and structure of cell wall of echinocandin evolved strains
- Topology model prediction of *C. parapsilosis* Fks1 protein
- Identification of mutations in the genome of evolved strains potentially causing resistant phenotype
- Determination of sterol composition of CLIB 214 and POS^{EVO} strains

Publications

Papp, C., Kocsis, K., Tóth, R., Bodai, L., Willis, R. J., Ksiezopolska, E., Lozoya-Pérez, E. N., Vágvölgyi, C., Mora-Montes, H. M., Gabaldón, T., Nosanchuk, J. D., Gácsér, A., Echinocandin microevolution of *Candida parapsilosis* influences virulence and abiotic stress tolerance. *mSphere*. **IF (2018): 3,575**

Zoppo, M., Lombardi, L., Rizzato, C., Lupetti, A., Bottai, D., **Papp, C.**, Gácsér, A., Tavanti, A. (2018) *CORT0C04210* is required for *Candida orthopsilosis* adhesion to human buccal cells. *Fungal Genet Biol*. **IF (2017): 3,476**

Rónavári, A., Igaz, N., Gopisetty, M. K., Szerencsés, B., Kovács, D., **Papp, C.**, Vágvölgyi, C., Boros, I. M., Kónya, Z., Kiricsi, M., Pfeiffer I. (2018) Biosynthesized silver and gold nanoparticles are potent antimycotics against opportunistic pathogenic yeasts and dermatophytes. *Int J Nanomedicine*. **IF (2017): 4,37**

Tóth, R., Cabral, V., Thuer, E., Bohner, F., Németh, T., **Papp, C.**, Nimrichter, L., Molnár, G., Vágvölgyi, C., Gabaldón, T., Nosanchuk, J. D., Gácsér, A. (2018)

Investigation of *Candida parapsilosis* virulence regulatory factors during host-pathogen interaction. Sci Rep. **IF (2018): 4,122**

Tóth, R., Tóth, A., **Papp, C.**, Jankovics, F., Vágvölgyi, C., Alonso, M. F., Bain, J. M., Erwig, L. P., Gácsér, A. (2014) Kinetic studies of *Candida parapsilosis* phagocytosis by macrophages and detection of intracellular survival mechanisms. Front Microbiol. **IF (2014): 5,12**

Németh, T., Tóth, A., Szenzenstein, J., Horváth, P., Nosanchuk, J. D., Grózer, Z., Tóth, R., **Papp, C.**, Hamari, Z., Vágvölgyi, C., Gácsér, A. (2013) Characterization of Virulence Properties in the *C. parapsilosis* Sensu Lato Species. PLoS One. **IF (2013): 4,49**

Summarized IF: 25,153

MTMT Author ID: **10038259**

This project was supported by the GINOP-2.3.2-15-2016-00035 and GINOP-2.3.3-15-2016-00006.