Ph.D. thesis

MOLECULAR DETECTION AND CHARACTERIZATION OF HUMAN PATHOGENIC *CANDIDA* SPECIES

Sándor Kocsubé

Supervisor: Dr. János Varga

Biology Ph.D. program

Department of Microbiology
Faculty of Science and Informatics
University of Szeged

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INTRODUCTION

The genus *Candida* includes approximately 200 species and about 20 of them are known as potential human pathogens. In the ‘80s most infections were caused by *Candida albicans*, but from the ‘90s to present a significant epidemiological change was observed. The majority of the infections are still attributed to *C. albicans*, however, the number of cases caused by non-albicans *Candida* species (NAC) such as *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. guilliermondii* and *C. lusitaniae* is rising. Until the ‘90s NAC species represented 10-40% of all reported candidaemias, but between 1991 and 1998 this proportion grew up to 35-60% of the total number of cases studied.

The epidemiological change might have happened due to the extended use of fluconazole, against which *C. krusei* has primary, while *C. glabrata* shows secondary resistance. The virulence of the NAC species is generally lower than that of *C. albicans* but the infections caused by them can result in up to 70% mortality due to their resistance to antifungal drugs. The emergence of non-albicans *Candida* species as human pathogens might be the consequence of the increased number of surgical inventions, the extended use of chemotherapeutic agents and the growing number of HIV infected patients. Along with these changes, the development of diagnostic methods can also lead to the increase of identified infections caused by NAC species.

In the case of *Candida* infections the treatment generally starts with the use of amphotericin B and/or fluconazole. The proper identification of the causative *Candida* species is crucial in selecting the appropriate antifungal agent due to the differences in the antifungal susceptibilities of the different species. The fact that certain *Candida* species are originally insensitive or they are able to develop resistance quickly to the most

commonly used antifungal drugs, such as fluconazole or amphotericin B, makes finding the proper means of therapy even more difficult.

AIMS OF THE WORK

The number of infections caused by non-albicans *Candida* species has increased substantially during the past decades. The different *Candida* species can have different susceptibility profiles to antifungal drugs, therefore the identification of the causative agent of the infection at the species level is crucial in order to find the proper means of therapy. Therefore the following aims were addressed in our work:

1. The development of a PCR based method for the efficient identification of eight clinically important *Candida* species: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. guilliermondii* and *C. lusitaniae.*
2. The investigation of the occurrence and genetic variability of clinical isolates of *C. parapsilosis* in Hungary.
3. The examination of the effect of interactions between antifungal agents and statins on *C. parapsilosis*, *C tropicalis* and *C. guilliermondii*.

METHODS

Classical microbiological methods:

- Maintenance of the strains
- Carbohydrate assimilation tests
- *In vitro* antifungal susceptibility tests by using microdilution method
- *In vitro* antifungal susceptibility tests by using the E-test method
- Examination of the interactions between antifungal agents and statins by using the checkerboard titration method

DNA based techniques:

- DNA extraction
- Polymerase chain reaction (PCR, multiplex PCR)
- DNS sequencing
- RAPD analysis

Analysis of the nucleotide sequence data:

- Identification based on sequence data (BLAST)
- Alignment of the nucleotide sequences (CLUSTALX)
- Phylogenetic analysis
- Design of species-specific primers

RESULTS

Development of a PCR-based assay for the detection of human pathogenic *Candida* species (Beszedics *et al.*, 2008)

We developed a rapid PCR-based method that allowed us to identify the most frequent human pathogenic *Candida* species. The first group of the reactions is based on the amplification of part of the rDNA

complex. With these reactions we were able to detect and identify eight *Candida* species: *C. krusei*, *C. guilliermondii*, *C. lusitaniae*, *C. glabrata C. albicans*, *C. dubliniensis C. tropicalis* and *C. parpapsilosis sensu lato*. Our aim was to design a multiplex PCR that yields identical amplicons from every *Candida* species. We divided the eight species into two groups and designed two common and eight species specific primers. The first common primer was designed to bind to the 5.8S region of the rDNA complex and targeted *C. krusei*, *C. guilliermondii*, *C. lusitaniae* and *C. glabrata.* The second common primer was developed on the basis of sequences of the 18S rRNA gene and targeted *C. albicans*, *C. dubliniensis C. tropicalis,* and *C. parpapsilosis sensu lato* group. We tested the applicability of the reaction with the involvement of 14 reference strains of clinically relevant *Candida* species: *C. albicans* ATCC 10231, *C. lusitaniae* CBS 6936, *C. krusei* CBS 573, *C. inconspicua* CBS 180, *C. dubliniensis* CBS 7987, *C. tropicalis* CBS 94, *C. norvegensis* SZMC 198, *C. pulcherrima* CBS 5833, *C. glabrata* CBS 138, *C. parapsilosis* CBS 604, *C. guilliermondii* CBS 566, *C. zeylanoides* CBS 619, *C. lipolytica* CBS 6124, *C. norvegica* CBS 4239. Following the optimization of the reaction no false positive bands were obtained with any of the reference strains. By the optimization of the cycles of the reaction we were able to design a nearly one–hour-long PCR.

Our second aim was to develop a control reaction. Two single copy genes, *FTR* (high affinity iron permease) and *PLD* (phospholipase D) were chosen as targets. The examined part of the *FTR* gene failed to be suitable for the development of a reliable detection system, while the analyzed region of the *PLD* gene allowed us to design species specific primers. We were not able to design a control reaction suitable for the detection of all species, detected by the previous PCR targeting the rDNA complex. Seven of the eight species were identical except *C. lusitaniae*, and we developed specific primer for *C. kefyr*, which is also an emerging human pathogen. We

designed four common primers for *C. albicans*/*C. dubliniensis*, *C. glabrata*/*C. kefyr*, *C. tropicalis*/*C. guilliermodii* and *C. parapsilosis*/*C. krusei*, as well as eight species specific primers. The developed primer sets were used dependably for the detection of the eight species in single or multiplex PCR.

We investigated the sensitivity of the reactions using colony PCR. A six-step tenfold serial dilution was prepared in distilled water from a suspension with a concentration of 10^8 cells/ml. One µl from each step of the dilution was added to each reaction. The sensitivity of the reactions fell into the range of 10^4 and 10^5 CFU/ml, while in the case of the reaction based on the *PLD* gene it was one step lower (10^5-10^6 CFU/ml).

Examination of the genetic variability of *C. parapsilosis* isolates (Kocsubé *et al*., 2007)

We examined the prevalence and genetic variability of Hungarian clinical isolates of *C. parapsilosis*. The genetic variability of *C. parapsilosis sensu lato* isolates was examined using random amplified polymorphic DNA (RAPD) analysis and the sequence analysis of the internal transcribed spacer (ITS) region of the rDNA gene cluster. Altogether, 209 *Candida* isolates derived from blood samples from two Hungarian hospitals, located in Debrecen and Pécs were examined. Among the previously identified 26 *C. parapsilosis* isolates 20 were found to belong to the *C. parapsilosis sensu lato* group using the species-specific primer pair developed by Luo and Mitchell (2002). The sequence analysis of the ITS region revealed the identity of the rest of the isolates as *C. lusitaniae*, *C. krusei*, and *C. albicans* (2, 3 and 1 strains, respectively). RAPD analysis of the isolates was carried out by using 21 random decamer primers. The genetic variability observed among the isolates was low: the majority of the isolates exhibited highly

similar or identical RAPD patterns with most primers tested. Two of the isolates showed higher variability compared to the main group, and could also be distinguished from other *C. parapsilosis* isolates using the *C. parapsilosis* group I-specific primer pair, developed by Pontieri *et al.* (2001). According to the ITS sequence analysis the two isolates were identified as *C. metapsilosis*.The two species were also distinguishable by the API 20C AUX kit. The *C. metapsilosis* isolates were found to be able to utilize D-xylitol. We examined the antifungal susceptibility profiles of isolates belonging to the *C. parapsilosis sensu lato* group using the Etest method. The *C. metapsilosis* isolates were found to be more susceptible to amphotericin B and voriconazole than the *C. parapsilosis* strains. Among the examined isolates 9.6% of *Candida* infections were found to be caused by *C. parapsilosis sensu lato* in our survey at two Hungarian hospitals. Two of these isolates were found to belong to the recently described species *C. metapsilosis*. This is believed to be the first report on the identification of *C. metapsilosis* from bloodstream infection.

We developed a PCR-based identification method allowing the differentiation between the members of the *C. parapsilosis sensu lato* group. The target sequence was the ITS region of the rDNA gene complex. During the examination of the sequences of the strains from our culture collection in comparison with corresponding sequences from the NCBI GeneBank database, we found high variability between *C. metapsilosis* isolates. Based on the differences in the variable ITS1 region the *C. metapsilosis* isolates could be divided into two groups. We developed a PCR-based technique specific for the distinct groups. Among the strains in our collection we found two isolates belonging to the second group of the *C. metapsilosis* isolates. We examined two further genes, the translation elongation factor alpha (TEF1-α) and the 18S rRNA genes. Although the TEF1-α sequences

were variable in certain positions none of them were able to distinguish reliably between the two groups.

Examination of the *in vitro* interactions between antifungal agents and statins (Nyilasi *et al.*, 2010a,b)

Within the frames of previous studies performed at our Department we determined the MIC values of ketoconazole, fluconazole, itraconazole, primycin, amphotericin B and nystatin, as well as lovastatin, fluvastatin, simvastatin, atorvastatin, rosuvastatin and pravastatin, then carried out also the combination tests using *Candida albicans* ATCC 90028 and *C. glabrata* CBS 138.

The concentration of ketoconazole needed for the complete inhibition appeared to be above 16 μg/ml in the case of *C. albicans*, while 100% inhibition of *C. glabrata* was achieved in the range of 0.5-1 μg/ml. The concentration of fluconazole and itraconazole required for the total inhibition of the examined *C. albicans* strain could not be determined either. The highest tested concentrations of the compounds were 64 and 16 μg/ml, respectively, while for the *C. glabrata* isolate the MIC values were found to be 8-16 and 0.5 μg/ml. Primycin resulted in 100% inhibition of *C. albicans* at a concentration of 64 μg/ml, while it could block the growth of *C. glabrata* entirely at 32 μg/ml. Both isolates were inhibited completely by amphotericin B at 1 μg/ml, whereas the concentrations of nystatin needed for the 100% inhibition of *C. albicans* and *C. glabrata* were shown to be 2 and 1 μg/ml, respectively.

Among statins simvastatin and fluvastatin appeared to be the most efficient compounds. Simvastatin inhibited *C. albicans* and *C. glabrata* entirely at 8 and 32 μg/ml, respectively. Fluvastatin was found to cause complete inhibition of the examined *C. albicans* isolate at a concentration of

32 μg/ml, while in the case of the *C. glabrata* strain the 100% inhibition was achieved at 64 μg/ml. Atorvastatin and lovastatin were found to block *C. albicans* completely at 128 and 64 μg/ml, respectively, while the concentration needed for the total inhibition of *C. glabrata* was shown to be 128 μg/ml for both compounds. Rosuvastatin resulted in 100% inhibition of both isolates at 128 μg/ml. Pravastatin was found to be the least efficient substance among statins as it could not inhibit the examined isolates even in the highest concentration (128 μg/ml) tested.

The examination of the effect of all the possible combinations revealed that every antifungal agent had good interacting partners among statins. In the case of nystatin and amphotericin B only pravastatin showed weak interactions. The antifungal effect of ketoconazole was increased significantly by statins on the examined *C. albicans* and *C. glabrata* strains. In the case of *C. albicans* only the ketoconazole/pravastatin combination was futile. Pravastatin also failed to increase the effect of fluconazole, but the other statins appeared to be good interaction partners in the case of both strains. The same observation was made in the case of the itraconazole/statin combintions. The antifungal effect of primycin was increased to the highest degree by simvastatin, fluvastatin and atorvastatin. Due to the interactions the concentration needed for the total inhibition of growth decreased by one dilution step on average. Based on these observations, we aimed to investigate further three *Candida* species

We examined the effects of interactions of antifungal agents and statins on C. *parapsilosis* CBS 604, *C. guilliermondii* CBS 566 and *C. tropicalis* CBS 94 strains. Our aim was to discover effective combinations that can be used to treat skin infections caused by *Candida* species. We determined the MIC (Minimal Inhibitory Concentration) values of eight antifungal drugs (amphotericin B, ketoconazole, fluconazole, nystatin, griseofulvin, itraconazole, primycin, terbinafine) and six statins (lovastatin,

simvastatin, fluvastatin, rosuvastatin, atorvastatin, pravastatin) for the three species. In the case of *C. parapsilosis* and *C. guilliermondii* the most effective antifungal agents were ketoconazole and itraconazole, among the statins simvastatin and fluvastatin caused the highest inhibition in the growth. In the case of *C. tropicalis* nystatin, itraconazole and primycin appeared to be the most effective antifungal drugs. Among the statins, simvastatin and fluvastatin were the most efficient.

Following the determination of the MIC values we examined the effects of the interactions between the statins and the antifungal agents using checkerboard-titration method

In the case of the examined *C. parapsilosis* CBS 604 isolate the griseofulvin/statin combinations did not result in interactions. In the combination of itraconazole and ketoconazole with statins the antifungal agents dominated, antagonistic interactions were not observed. The nystatin/statin combinations resulted in a neutral interaction, with the dominance of the antifungal agent. The highest number of interactions were detected in the terbinafine combinations. The MIC_{50} values were lowered by the statins by one dilution step. In the case of amphotericin B the strongest interactions were achieved in the combinations with atorvastatin and fluvastatin, while in the case of fluconazole the combination with fluvastatin was the most effective. In the combinations of primycin with statins fluvastatin and simvastatin were the best interaction partners, but in the case of atorvastatin strong antagonism was observed.

In the case of *C. guilliermondii* CBS 566, the most effective interactions were observed in the itraconazole/statin combinations. Simvastatin and atorvastatin lowered the concentration needed for the total inhibition of growth by several dilution steps. For amphotericin B the best interactions were caused by atorvastatin. In the case of fluconazole fluvastatin increased the effect of the antifungal agent the most effectively.

The inhibition of growth caused by nystatin was not affected by statins significantly. The effectiveness of terbinafine was affected mostly by lovastatin, simvastatin, fluvastatin and rosuvastatin. These statins lowered the terbinafine concentration needed for the 50% inhibition by one dilution step. High antagonism was observed in the case of primycin combined with simvastatin, fluvastatin and atorvastatin.

The most significant interactions were observed in the combinations tested with the *C. tropicalis* CBS 94 isolate. The antifungal effect of fluconazole, ketoconazole and itraconazole were increased notably by almost every statins. The MIC values were lowered by several dilution steps. Pravastatin did not affect the MIC values but the MIC_{50} values were lowered by one or two dilution steps. In the case of amphotericin B the most effective interaction partners were atorvastatin and rosuvastatin. The antifungal effect of griseofulvin was increased by fluvastatin but the total inhibition was not achieved. The effect of nystatin and terbinafine was also raised by fluvastatin notably. Antagonism was not observed in the primycin/statin combinations. The interactions with simvastatin lowered the MIC values by one dilution step. Fluvastatin lowered the concentration of primycin needed for the complete inhibition by three dilution steps.

In conclusion, the most effective interaction partner was the fluvastatin. The antifungal effect of fluconazole was raised substantially by fluvastatin in the experiments carried out with *C. parapsilosis* CBS 604 and *C. guilliermondii* CBS 566. The effect of amphotericin B was increased significantly by atorvastatin on all the examined isolates. The least effective interaction partners were pravastatin and lovastatin.

SUMMARY

Our results can be summarized in the following points:

1. We developed a PCR-based method for the reliable identification of *C. krusei*, *C. guilliermondii*, *C. lusitaniae, C. glabrata, C. albicans*, *C. dubliniensis C. tropicalis, C. parpapsilosis sensu lato és C. kefyr* isolates.

2. We investigated the genetic variability and the frequency of the infections caused by Hungarian *C. parapsilosis* isolates. We developed species specific primers for the detection of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*.

3. We analyzed the susceptibility profiles of *C. albicans* ATCC 90028, *C. glabrata* CBS 138, *C. parapsilosis* CBS 604, *C. guilliermondii* CBS 566 and *C. tropicalis* CBS 94 isolates to antifungal agents and statins.

4. Using the checkerboard-titration method we examined the interactions between antifungal drugs and statins on the previously examined isolates.

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