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Short Communication

Molecular detection of *Candida krusei*

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Abstract

The species identification of fungi belonging to genus *Candida* is an important issue as this genus becomes the emerging problem of nosocomial infections. As *Candida krusei* presents intrinsic resistance to the fluconazole that is the drug of the first choice in case of invasive candidiasis the PCR identifying the DNA of *C. krusei* was elaborated. The analytical sensitivity of the assay on spiked blood samples was estimated at 3-5 CFU/ml. The possibility of performing the reaction in both end-point and real-time detection format broadened the potential of its application in small and medium microbiology laboratories.

Keywords: *Candida krusei*, PCR, real-time PCR, diagnostics.

INTRODUCTION

The fungi belonging to genus *Candida* are increasingly important nosocomial pathogens in patients with disfunction of the immune system. The 60% of invasive candidiasis cases on intensive care units are candidemia. Candidemia cause prolonged hospitalization time and the raise of mortality (Pfaller *et al.*, 2008). Nowadays, the prevalence of particular species has changed from dominant *C. albicans* infections to non-albicans *Candida* species which reached almost half of the *Candida* isolates (Fiddel et al., 1999; Pfaller et al., 2001; Lyon et al., 2010). As the 17% of *Candida* isolates present reduced susceptibility or resistance for azoles, probably wide used of fluconazole is the main reason of drift in prevalence of *Candida* species (*Leroy et al., 2006*). One of the species that present intrinsic resistance for fluconazole is *Candida krusei* that is an etiological agent of 10-35% of all NAC infections, especially on oncological and hematological units (*Pfaller et al., 2007*).

Candida krusei is one of the species with intrinsic resistance for fluconazole, lowered susceptibility for amphotericin B, and there were few described cases of failure of the treatment with caspofungin. The voriconazol is presently regarded as the drug of choice in *C. krusei* infections.

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There are few assays identifying DNA of *C. krusei* described in the literature. Probably, the best known is the system SeptiFast (Yanagihara et al., 2010; Lutz et al., 2011). However it works correctly, it could be an example of the assay requiring the specific equipment that could be a bareer for its application in the small and medium laboratories (Turrenne et al., 1999). Another bareer for application of the described elsewhere tests is the format of the PCR *e.g.* multistep PCR, that increase the risk of the contamination, resulting in false positive results or need for repeating the identification reaction (Lau et al., 2008). The sensitivity of the other assays is not satisfactory, as the biggest need of the species identification of *C. krusei* concerns the specimen with very limited number of CFU equivalent (Schabereiter-Gurtner et al., 2007). Some of them, however presenting high sensitivity, were tested on a very limited number of non *C. krusei* species, so the risk of misidentification still remains unknown (Innings et al., 2007).

We describe the method for detection of *C. krusei* DNA by application both of real-time PCR and PCR reaction that broaden possibility of its application in the routine laboratories with varying equipment.

MATERIALS AND METHODS

Fungal strains

A total number of 22 reference fungal strains including

10 *C. albicans*, 1 *C. dublinensis*, 2 *C. glabrata*, 2 *C. guillermondi*, 1 *C. kefyr*, 2 *C. krusei*, 2 *C. parapsilosis*, 1 *C. tropicalis*, 1 *S. cerevisiae* and 63 clinical isolates including 12 *C. albicans*, 1 *C. arbea*, 1 *C. dublinensis*, 1 *C. famata*, 9 *C. glabrata*, 1 *C. guillermondi*, 1 *C. kefyr*, 10 *C. krusei*, 1 *C. lipolytica*, 1 *C. lusitaniae*, 10 *C. parapsilosis*, 1 *C. pseudotropicalis* , 1 *C. rugosa*, 1 *C. sake*, 1 *C. stenatoidea* and 11 *C. tropicalis* was tested in the study. Additionally 35 environmental isolates – 2 *Alternaria alternata*, 3 *A. brassicae*, 1 *Alternaria* sp., 5 *Aspergillus niger*, 5 *A. fumigates*, 10 *A. flavus*, 1 *Cladosporium cladosporioides*, 1 *Mucor racemosus*, 1 *Ochrocladosporium elatum*, 1 *Penicillium chrysogenum*, 1 *Penicillium commune*, 1 *P. melinii*, 1 *Penicillium* sp., 1 *Pleospora papaveracea*, 1 *Ulocladium tuberculatum* were included in the examination on presented assays. All of the clinical specimen were preliminary identified in the routine laboratory of microbiology.

DNA purification

DNA from pure culture of yeast was purified with Genomic Mini AX Yeast (A&A Biotechnology, Poland). DNA from molds was purified by mean of previously described method – fragment of mycelia was incubated 10 min in 95°C in buffer consists of 60 mM sodium bicarbonate [$NaHCO_3$], 250 mM potassium chloride [KCl] and 50 mM Tris, pH 9.5 and 100 µl of 2% bovine serum albumin was subsequently added. After vortex mixing, this DNA-containing solution was used for PCR (Brillowska-Dabrowska et al., 2007).

***Candida krusei* specific PCR**

The following specific *Candida krusei* primers were designed based on FKS gene alignment of species belonging to *Candida* sp.: CkFKSfor359 5' CAT TGG CCG TTT CCA TTG TGT TC 3' and CkFKSrev359 5' CAT CAA ACC AAG CGT GAT TCT TGC 3'. A 1 µl of 10 mM solution of each primer was applied with the 2xPCR Master Mix Plus High GC in the 20 µl reaction mixture according to manufacturer protocol (A&A Biotechnology, Poland). The amplification profile consisted of an initial incubation of 3 min at 94°C followed by 40 cycles of 45 s at 94°C, 30 s at 59°C and 45 s at 72°C, with a final 10 min hold at 72°C. The PCR mixture consisted of the 359 bp *C. krusei* product was separated by agarose gel electrophoresis (2% agarose containing 0.5 µg/ml ethidium bromide in 0.5 × TBE buffer) and analysed.

***Candida krusei* specific real-time PCR**

The 20 µl real-time PCR mixture consisted of 1 µl 10 mM solution of CkFKSfor359 and 1 µl of 10 mM solution of CkFKSrev359 primers, real-time 2xPCR Mix EvaGreen (A&A Biotechnology, Poland), water and extracted DNA. The reaction was performed in Light Cycler 1,5 (Roche) in the following conditions: preincubation 10 min in 94°C, amplification: 10 sec in 94°C, 10 sec of primers annealing at 65°C and 10 sec of product elongation 72°C. The reaction was followed by a melting curve analysis (1 min at 95°C, cooling for 1 min at 65°C, and ramp to 95°C, at a ramp rate of 0.1°C/s). The samples with products, which melting curve indicated temperature 91°C were considered as positive (containing DNA of *C. krusei*).

Determination of sensitivity of designed primers

In order to estimate a sensitivity of DNA extraction procedure and the designed PCR assay from blood specimen dilution of *Candida krusei* 1 ml of EDTA-anticoagulated whole blood sample from healthy donor was spiked with 30 µl of 24-hours *C. krusei* culture. Serial dilutions of the inoculated blood samples were made and the fungal suspensions were concurrently serially diluted in phosphate buffered saline. A 200 µl of each dilution of saline and the corresponding blood specimen was used for total DNA extraction according to modified Genomic Mini AX Yeast (A&A Biotechnology, Poland) protocol – 0.2 ml spheroplast buffer, 50 µl of chitinase and 15 µl 1 M DTT were added to the tubes containing spiked samples, mixed and incubated 30 min at 50˚C. Next steps of purification were performed according to manufacturer protocol. Precipitated DNA was re-suspended in various volumes of TB buffer. Fungal counts for each dilution in blood and saline were performed in triplicate and enumerated on Sabouard agar. The numbers of colony forming units (10^{12} to 10^{0} CFU/ml, in serial dilutions) were compared to the presence of PCR product exhibiting melting temperature 91°C.

RESULTS AND DISCUSSION

The alignment of available sequences of genes encoding FKS from different *Candida* species allowed the design of the pair of primers detecting *C. krusei* DNA. The specificity of the designed PCR and real-time PCR assays with DNA from pure cultures of reference and clinical strains, and human blood samples was confirmed. The 100% specificity on the group of investigated isolates was obtained - positive results were obtained for all samples containing *C. krusei* DNA and negative for the 73 isolates belonging to other *Candida* species, and 35 non-*Candida* isolates. Additionally the test was performed with negative results on the DNA from 2 healthy individuals.

To estimate detection limit of the designed set up a

Table 1. Sensitivity of the PCR and real-time PCR detecting DNA of *C. krusei*

Final volume of the extracted DNA (µl)	Volume applied in the reaction (µl)	Sensitivity of the PCR (CFU/ml)	Sensitivity of the Real-time PCR (CFU/ml)
30	5	50	50
	2	150	150
10	5	3	5
	2	20	15

known quantity of colony forming units of *C. krusei* were inoculated into whole blood. The sample was equilibrated and 10 fold serial dilutions were performed in whole blood and respectively in Sabouraud medium. Total DNA was extracted from all blood spiked samples and resuspended in 10 µl and 30 µl of TE buffer. To optimise the PCR and real-time PCR reaction 5 µl or 2 µl of extracted DNA were added to the reaction mixtures. The sensitivity of the reactions performed with different DNA concentration is presented in the Table 1. The best sensitivity of the extraction procedure and the designed PCR test estimated as 3-5 CFU/ml (Tabel 1) was achieved when DNA was resuspended in 10 µl of TB buffer and 5 µl was applied in both PCR and real-time PCR reaction.

As fungi belonging to this species are infectious agents of the increasing number of invasive fungal infections, the elaborated DNA extraction procedure and PCR diagnostic assay could be applied in routine laboratory as a confirmative test in case of probable invasive fungal infection. *C. krusei* strains are intrinsically resistant for the first choice antifungals, fast identification of *C. krusei* as an infectious agent will decrease the risk of choice of not correct therapy. However the introduction of the described test to the routine praxis requires detailed examination on the large number of patient specimens. Moreover, according to general rule the validation of assay with multicenter trials is also necessary (Khot, 2009)

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