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Design of species-specific primers for rapid detection and identification of *Candida parapsilosis sensu stricto*

Zasnova vrstno-specifičnih oligonukleotidnih začetnikov za hitro zaznavanje in identifikacijo kvasovke *Candida parapsilosis sensu stricto*

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Abstract: *Candida* species are the cause of approximately two million cases of candidiasis yearly worldwide, and are frequently involved in life-threatening infections. After *Candida albicans*, the *Candida parapsilosis* complex is the second most common cause of *Candida* infections, particularly in patients in intensive care units and in neonates. Contrary to many *Candida* species, *C. parapsilosis sensu stricto* is frequently present in water, and on surfaces made of plastic, rubber, and silicone, where it acts as a primary coloniser for biofilm establishment. Identification methods for the *C. parapsilosis* complex include culture-dependent methods, MALDI-TOF, and multiplex PCR using ITS region, but remains amongst the most frequently misidentified species, due to the genetic similarity and lack of species-specific primers. In the present study, we developed novel species-specific primers for detection and identification of *C. parapsilosis sensu stricto* using locus CPAR2_105320, as template for easily accessible and widely used conventional PCR method. Using these primers, we successfully detected and identified *C. parapsilosis sensu stricto* in pure cultures isolated from clinical specimens and indoor environments. Additionally, this method enables detection of *C. parapsilosis sensu stricto* in biofilms and tap water samples from which DNA was extracted, and directly from suspensions of washed swab samples. All positive cases showed single clear band with 574 base pairs. Sequencing of the amplicon proved designed primers to be species-specific. In the future, primers can serve as a tool for rapid detection of *C. parapsilosis sensu stricto* in the environment and clinical settings.

Keywords: *Candida parapsilosis*, detection methods, emerging pathogen, novel primers, conventional PCR, species specific

Izvleček: Vrste rodu *Candida* so vzrok za približno dva milijona kandidiaz letno po vsem svetu in so pogosto povzročiteljice življenjsko nevarnih okužb. Po pogostosti so okužbe s kompleksom vrst *Candida parapsilosis* na drugem mestu, takoj za *Candida albicans*. Najpogosteje okužijo bolnike na oddelkih intenzivne nege in nedonošenčke. V nasprotju s številnimi vrstami iz rodu *Candida*, je vrsta *C. parapsilosis sensu stricto* pogosto prisotna v vodi in površinah iz plastike, gume in silikona, kjer se pojavlja tudi kot začetna naseljevalka pred vzpostavitvijo biofilma. Metode identifikacije te kvasovke vključujejo gojenje, MALDI-TOF in multipleks-PCR, vendar je ta vrsta

zaradi genetske sorodnosti znotraj kompleksa vrst in pomanjkanja vrstno sprecifičnih oligonukleotidnih začetnikov še zmeraj med najpogosteje napačno identificiranimi vrstami rodu *Candida*. V študiji smo na podlagi lokusa CPAR2_105320 razvili nove, vrstno specifične oligonukleotidne začetnike za klasični PCR in hitro odkrivanje ter identifikacijo vrste *C. parapsilosis sensu stricto*. Z opisanimi oligonukleotidnimi začetniki smo uspešno zaznali in identificirali *C. parapsilosis sensu stricto* v čistih kulturah, pridobljenih iz kliničnega materiala in različnih okolij. Metoda dodatno omogoča odkrivanje te vrste v vzorcih celokupne DNA, ekstrahirane iz biofilmov in pitne vode ter direktno v vzorcih brisov površin, brez predhodne DNA ekstrakcije. Po sekvenciranju smo pri vseh pozitivnih vzorcih pridobili sekvenco dolžine 574 baznih parov, ki je bila tudi vrstno specifična. Novi oligonukleotidni začetniki lahko v prihodnosti služijo kot orodje za hitro odkrivanje *C. parapsilosis sensu stricto* v naravnem in kliničnem okolju.

Ključne besede: *Candida parapsilosis*, klasičen PCR, metode zaznavavanja, novi oligonukleotidni začetniki, porajajoči patogen, vrstna specifičnost

Introduction

Species of the genus *Candida* have been estimated to be responsible for more than two million cases of oral, esophageal, and vulvovaginal candidiasis yearly worldwide, and an additional ~400,000 cases of invasive life-threatening fungal infections, such as candidemia and peritonitis, which have mortalities of 30% to 55% (Brown et al. 2012). About 50% of all candidemia cases are caused by *Candida albicans*, with the rest associated with some so-called 'emerging species' (Trofa et al. 2008). Among these, the second most common cause of candidemia relates to the *Candida parapsilosis* species complex (Lass-Flörl 2009). Those at the highest risk of infection are intensive care and transplantation patients, neonates, and people with cystic fibrosis (Trofa et al. 2008, Sardi et al. 2013 Cortés et al. 2019). The *Candida parapsilosis* complex consists of three closely related species: *C. parapsilosis sensu stricto*, *Candida orthopsilosis*, and *Candida metapsilosis*, with incidences in clinical specimens of 90.7%, 8.2%, and 1.1%, respectively (Cantón et al. 2011). In contrast to many *Candida* species that have been mainly related to humans and hospital environments (e.g., *C. albicans*, *Candida auris*), *C. parapsilosis sensu stricto* is widespread in nature (Novak Babič et al. 2017a, Cortés et al. 2019, Zupančič et al. 2019) and is frequently found in other organisms, in water sources (Novak Babič et al. 2017b), and on surfaces made of different natural and artificial materials. These last include, in particular, materials made of plastic, rubber, and silicone (Trofa et al. 2008, Pires et al. 2011, Novak Babič et al. 2017a, Zupančič et al. 2019), where *C. parapsilosis sensu stricto* acts as a primary colonizer, and thus represents one of the main building blocks for biofilm establishment (Raghupathi et al. 2018). The formation of stable biofilms by *C. parapsilosis sensu stricto* in patients is recognized as one of its main virulence factors, while artificial materials such as prosthetic devices and catheters represent the main route for its transmission to patients (Trofa et al. 2008, Cantón et al. 2011). The primary habitat of *C. parapsilosis sensu stricto*, has been linked to the skin and hands of health workers (Huang et al. 1998), although its increased incidence in human-made indoor environments connected to water sources, such as dishwashers, washing machines, kitchens, and bathrooms, suggest a possible route of infections in the home (Novak Babič et al. 2015, Novak Babič et al. 2016, Novak Babič et al. 2017, Zupančič et al. 2016, Zupančič et al. 2019). Rapid and accurate diagnostic tools and methods are of great importance for successful treatment and patient recovery (Liguori et al. 2010, Arastehfar et al. 2019). However, to minimize incidence of infections and prevent outbreaks, it is also important to determine the possible transmission routes of emerging pathogens that can originate from biological material and patient environments (e.g., tap water, surfaces, pros-

thetic material) (Liguori et al. 2010, Bloomfield et al. 2012).

One easily accessible and widely used diagnostic method for such detection and identification is conventional PCR. The method is amongst the fastest and cheapest of methods, and it can be carried out in Biosafety-1 laboratories, using DNA templates from pure cultures or mixed biofilms (Arastehfar et al. 2019). The aim of the present study was thus to develop novel species-specific oligonucleotides for conventional PCR to detect and identify the emerging pathogen *C. parapsilosis sensu stricto* not only in pure cultures, but also in total DNA extracted from tap water and biofilms. Additionally, we have modified the PCR cycling program to detect this yeast directly on swab samples taken from different artificial surfaces, without the need for prior culture or DNA extraction.

Materials and methods

Sampling, isolation and cultivation of Candida species from environmental samples

Samples of biofilm were obtained by sterile scrapping of a 1-cm^2 surface area of dishwasher rubber seals, and were placed in sterile 1.5-mL microcentrifuge tubes (Eppendorf, Germany). These samples were stored at -20 °C until further processing.

Five litres of groundwater and tap water samples were collected in sterile containers. These samples were kept refrigerated at 4 °C and processed within 24 hours. One litre of each groundwater and tap water sample was filtered through 0.45-µm pore diameter filters (Millipore). These filters were placed on Dichloran Rose Bengal agar prepared with chloramphenicol, to suppress the growth of filamentous fungi and bacteria. The plates were incubated at 30 °C for 3 days to 5 days.

The sampling of surfaces in bathrooms, kitchens, washing machines, dishwashers, and refrigerators was carried out using sterile cotton swabs (Golias, Slovenia) and sterile physiological saline (9% NaCl, w/v). These samples were stored at 4 °C and processed within 2 days. Swab samples of the bottom of a shower cabinet, a kitchen sink, a dish-drying rack, a washing machine rubber seal, and a refrigerator glass shelf were vortexed in 1 mL saline at maximum speed for 1 min. The resulting cell suspensions were transferred to sterile 1.5-mL microcentrifuge tubes (Eppendorf) and centrifuged at 14,000 rpm for 5 min. After discarding the supernatant, the pellet was resuspended in 50 µL TE buffer and stored at -20 °C until further use. The rest of the swab samples were wiped over the surface of malt extract agar with chloramphenicol, and incubated at 30 °C for 3 days to 5 days.

All of the yeast colonies that grew from this sampling were transferred onto malt extract agar and incubated at 30 °C for 3 days, to obtain pure cultures. The pure cultures have been deposited in the Ex Culture Collection of the Infrastructural Centre Mycosmo, of the Department of Biology, University of Ljubljana (MRIC UL) (Slovenia: http://www.ex-genebank.com/). The additional non-*C. parapsilosis* yeast strains used in this study (Tab. 1) were obtained from the Ex Culture Collection, and were also plated on malt extract agar and incubated at 30 °C for 3 days.

Table 1: Non-*Candida parapsilosis* strains and strains taxonomically related to *Candida* genus used as reference strains with their corresponding EXF numbers.

Tabela 1: Kvasovki *Candida parapsilosis* taksonomsko sorodne vrste uporabljene kot referenčni sevi in njihove EXF številke.

Genus	Species	EXF number*
Candida	*orthopsilosis*	8409
Candida	*metapsilosis*	9927
Candida	*intermedia*	8410
Candida	*albicans*	9090
Candida	*atlantica*	10733
Candida	*boidinii*	5115
Candida	*boleticola*	7973
Candida	*californica*	9219
Candida	*caryicola*	12358
Candida	*ethanolica*	4926
Candida	*friedrichii*	13549
Candida	*glabrata*	7104
Candida	*glaebosa*	11071
Candida	*haemulonis*	4875
Candida	*inconspicua*	8157
Candida	*intermedia*	9462
Candida	*kanchanaburiensis*	8534
Candida	*norvegica*	6311
Candida	*pseudointermedia*	9894
Candida	*pseudolambica*	7966
Candida	*railenensis*	6414
Candida	*saitoana*	10054
Candida	*sake*	13529
Candida	*sophiae-reginae*	7981
Candida	*tropicalis*	9247
Candida	*tsuchiyae*	11007
Candida	*vartiovaarae*	7970
Candida	*zemplinina*	5068
Candida	*zeylanoides*	11878
Kluyveromyces	*marxianus*	9072
Meyerozyma	*guilliermondii*	8448
Pichia	*fermentas*	8401
Saccharomyces	*cerevisiae*	9914
Lodderomyces	*elongisporus*	9536
Debaryomyces	*hansenii*	8416
Peterozyma	*toletana*	6840
Priceomyces	*carsonii*	6753
Scheffersomyces	*spartinae*	6323
Schwanniomyces	*vanrijiae*	12083
Yamadazyma	*triangularis*	8408
Yarrowia	*lipolytica*	8413

*EXF number, Deposition number of fungal strains in the Ex Culture Collection of the Infrastructural Centre Mycosmo, MRIC UL, Slovenia.

Genomic DNA extraction from pure cultures, and from water and biofilm samples

Reference strains isolated from clinical specimens (N=7; 17.5%), groundwater (N=2; 5%), tap water (N=5; 12.5%), bathrooms (N=5; 12.5%), washing machines (N=6; 15%), kitchen surfaces (N=6; 15%), dishwashers (N=5; 12.5%) and refrigerators (N=4; 10%) previously identified as *Candida parapsilosis* sensu stricto were taken from the IC Mycosmo Culture Collection, Ljubljana, Slovenia (Tab. 3). Additionally, 41 taxonomically close relatives of *C. parapsilosis* were used as reference strains (Tab. 1). DNA from the 3-day-old grown on malt extract agar was extracted using PrepMan Ultra reagent (Applied Biosystems), following the manufacturer instructions. Three-liter water samples were filtered through 0.45-mm filters (Millipore, Merck), and total environmental genomic DNA was extracted using PowerWater DNA isolation kits (MO BIO Laboratories Inc.), according to the manufacturer instructions. Total environmental genomic DNA from biofilm samples was obtained from 1 g biofilm mass and extracted using PowerBiofilm DNA isolation kits (MO BIO Laboratories Inc.), according to the manufacturer instructions. Concentrations of DNA were measured with the use of Qubit 4 Fluorometer (Thermo Fisher Scientific). All of the DNA samples obtained were stored at -20 °C until further processing.

Design of species-specific primers

The annotated contig 005569 of the whole genome of *C. parapsilosis sensu stricto* strain CDC317, locus CPAR2_105320 (putative *URA6* gene, an ortholog of the *Saccharomyces cerevisiae* protein *URA6*, involved in for *de-novo* synthesis of pyrimidines), with a length of 843 base pairs (Guida et al. 2011) was obtained from the NCBI website and was used as the template for designing the novel species-specific primers. These were designed using the online bioinformatics tools Primer3 (Koressaar and Remm 2007, Untergasser et al. 2012) and Primer-BLAST (Ye et al. 2012), and were obtained in a desalted form from Microsynth AG (Austria).

Development of the conventional PCR method for detection of Candida parapsilosis sensu stricto

Preparation of PCR mixture: The *C. parapsilosis URA6* ortholog of all of the samples obtained was amplified with the use of conventional PCR. Each reaction had a final volume of 34 µL, and contained the following: 26.82 µL ultraclean water (MilliQ), 3.5 µL 10× Dream Taq buffer (Thermo Fisher Scientific), 0.7 µL 10 mM dNTP mix (AB, Warrington, UK), 1.4 µL of each oligonucleotide (CPAR2_105320-F, CPAR2_105320-R; Microsynth AG, Austria; final concentration, 10 pmol/µL), and 0.18 µL Dream Taq polymerase (Thermo Fisher Scientific, final concentration, 5 U/µL). Finally, 1 µL of each DNA sample was added to each reaction, with 1 µL MilliQ water added into the control reactions.

Modifications of the PCR program: Three different PCR programs were modified according to the origin of the DNA samples used as the matrix in the conventional PCR (Fig. 1, Tab. 2). PCR program #1 was developed for the genomic DNA matrix extracted from the pure yeast cultures, PCR program #2 was developed for the total environmental DNA matrix, and PCR program #3 was developed for the detection of *Candida parapsilosis sensu stricto* directly from swab samples without the need for prior DNA extraction.

Visualization of PCR products: The PCR products obtained were transferred to 1% (w/v) agarose (Sigma Aldrich) gels that had previously been stained with SYBR Safe (Thermo Fisher Scientific). GeneRuler DNA Ladder (Thermo Fisher Scientific) was used as the marker for comparison of base-pair lengths. The electrophoresis in 1X TAE buffer was run at 120 V for 20 min, with the products visualized using the G:BOX and Gene Tools software (Syngene, UK).

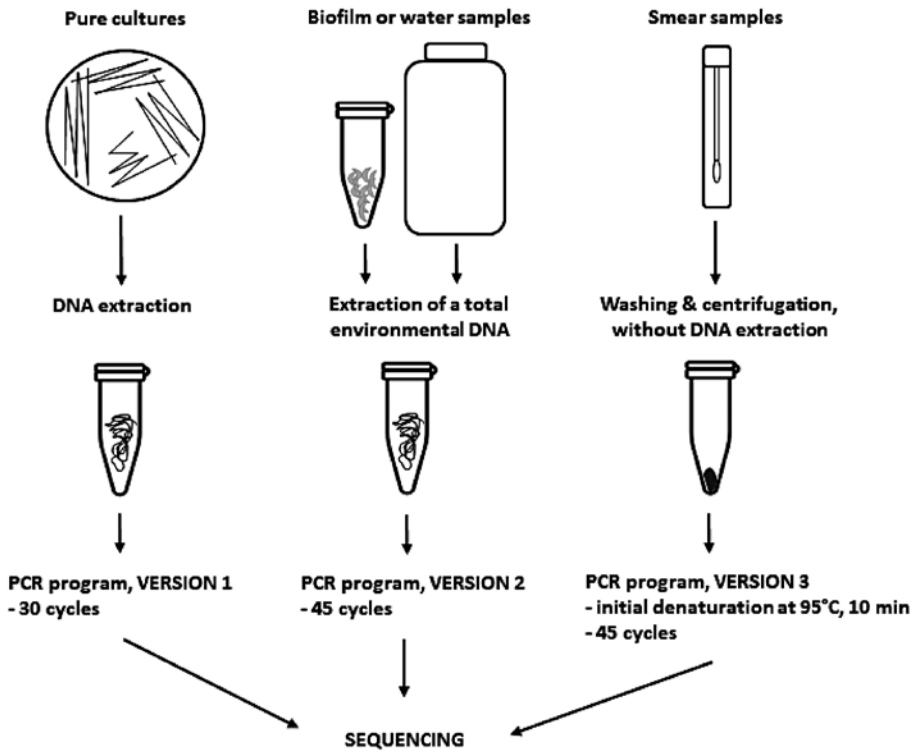

Figure 1: The three PCR programs for use of the novel primers, as designed according to the different DNA matrices. PCR program #1 is designed for DNA samples obtained from pure cultures; PCR program #2 is designed for total environmental DNA samples; and PCR program #3 is designed for swab samples, without the need for prior DNA extraction.

Slika 1: Programi za uporabo novih oligonukleotidnih začetnikov s klasičnim PCR glede na različne DNA matrice. Program #1 je zasnovan za DNA vzorce pridobljene iz čistih kultur, program #2 za vzorce celokupne DNA iz okolja in program #3 za vzorce brisov prez predhodne DNA ekstrakcije.

Table 2: Details of the three programs for the novel primers for conventional PCR detection of *Candida parapsilosis sensu stricto*.

Tabela 2: Podrobnosti programov za uporabo novih oligonukleotidnih začetnikov za hitro odkrivanje *Candida parapsilosis sensu stricto* z metodo PCR.

	PCR program		
PCR step	**#1**	**#2**	**#3**
Sample type	**Pure cultures**	**Total DNA samples**	**Swab samples**
Initial denaturation	95 °C, 2 min	95 °C, 2 min	95 °C, 10 min
Number of cycles	30	45	45
Denaturation	95 °C, 35 s	95 °C, 35 s	95 °C, 35 s
Annealing	55 °C, 35 s	55 °C, 35 s	55 °C, 35 s
Elongation	70 °C, 1 min	70 °C, 1 min	70 °C, 1 min
Final elongation	70 °C, 5 min	70 °C, 5 min	70 °C, 5 min

Identification of Candida parapsilosis sensu stricto strains

The PCR products were further processed to obtain the DNA sequences of the fragments. These sequences were obtained through Microsynth AG (Austria) and assembled using FinchTV 1.4 (Geospiza, PerkinElmer Inc.). Additionally, the Molecular Evolutionary Genetics Analysis version 7.0 software (Tamura et al. 2011) was used for the alignments. The strains were identified using the BLAST algorithm from the NCBI website (Altschul et al. 1990). The identified sequences were further processed with the DNA sequence submission tool Sequin, version 15.50 (NCBI, USA), for assignment of the GenBank numbers.

Results

Specifics of the novel primers

The newly designed primers had a length of 20 oligonucleotides and contain 55% guanine–cytosine (GC) nucleobases. The sequence of the forward primer (CPAR2_105320-F: 5'-CAC CAC GCA AGC CAA TAC AC-3') starts at the 42nd base and ends at the 61st base, and the sequence of the reverse primer (CPAR2_105320-R: 5'-GGC AAC AAC AGG GAT AGG GT-3') starts at the 615th base and ends at the 596th base of the putative *URA6* template sequence used. The PCR product has a predicted length of 574 base pairs.

Performance of designed PCR programs

Three different programs for conventional PCR were designed to test the performance of the novel primers on different DNA templates.

PCR program #1 was developed for application to the DNA matrix extracted from pure yeast cultures where the concentrations of DNA were >50 ng/µL. The 30 cycles proposed are sufficient to obtain single-band products of predicted length and good quality for direct sequencing. PCR program #1 was used to test the species specificity of the novel primers on strains of *C. parapsilosis sensu stricto* obtained from clinical material, groundwater, tap water, and different indoor environments, all of which were particularly related to water sources (Tab. 3). As the control groups, non-*C. parapsilosis* strains and strains taxonomically related to *Candida* genus were used (Tab. 1). Of these tested strains, all *C. parapsilosis sensu stricto* yielded single-band products of the predicted length, while the strains in the control group showed no bands of any size.

PCR program #2 was used to detect and amplify the possible present of *C. parapsilosis sensu stricto* in the total DNA matrix extracted from environmental samples (e.g., biofilm, tap water), where the DNA concentrations of the whole extracted biomass varied between 2-60 ng/µL. The only differences in comparison to PCR program #1 was in the number of cycles, which was set to 45 instead of 30 (Tab. 2). With PCR program #2 applied to these total environmental DNA samples, single-band products of the predicted length were successfully obtained in 50% (five out of 10) of the tap water samples and in 100% (five out of five) of the biofilm samples. *C. parapsilosis sensu stricto* was also confirmed in all of the positive environmental samples using culture-dependent methods, while *C. parapsilosis sensu stricto* was not isolated from the five tap water samples that showed negative PCR results.

PCR program #3 was developed for detection and identification of *C. parapsilosis sensu stricto* directly from swab samples, when only washed in physiological saline. This was carried out without previous DNA extraction, and was thus the most rapid and reliable indicator of the presence of *C. parapsilosis sensu stricto*. Instead of DNA extraction, the initial denaturation of the samples at 95 °C was prolonged to 10 min, and the number of cycles was extended to 45 (Tab. 2). All of the swab samples tested that were taken from plastic, rubber, metal, and glass surfaces yielded positive results here, which were also confirmed using culture-dependent methods.

Table 3: Details of *Candida parapsilosis sensu stricto* strains used in the present study, with corresponding EXF and GenBank numbers.
Tabela 3: Podrobnosti sevov *Candida parapsilosis sensu stricto*, z EXF- in GenBank številkami.

Sample source	Isolation method	DNA extraction	EXF* or sample number	PCR program	GenBank accession number (MK-)
Clinical sample	Unknown	Pure culture – reference strains	EXF-10095	#1	782836
			EXF-10096		782837
			EXF-10097		782838
			EXF-10098		782839
			EXF-10099		782840
			EXF-10192		782841
			EXF-10193		782842
Groundwater	Filtration	Pure culture – reference strains	EXF-8460	#1	782843
			EXF-8247		782844
Tap water	Filtration	Pure culture – reference strains	EXF-10058	#1	782845
			EXF-10067		782846
			EXF-10048		782847
			EXF-5670		782848
			EXF-9623		782849
Bathroom	Swabbing	Pure culture – reference strains	EXF-9696	#1	782850
			EXF-12765		782851
			EXF-9692		782852
			EXF-6998		782853
			EXF-8149		782854
Washing machine	Swabbing	Pure culture – reference strains	EXF-5730	#1	782855
			EXF-5731		782856
			EXF-9782		782857
			EXF-9781		782858
			EXF-8239		782859
			EXF-6334		782860
Kitchen	Swabbing	Pure culture – reference strains	EXF-9952	#1	782861
			EXF-9955		782862
			EXF-9976		782863
			EXF-9925		782864
			EXF-12806		782865
			EXF-9928		782866
Dishwasher	Swabbing	Pure culture – reference strains	EXF-9112	#1	782867
			EXF-9092		782868
			EXF-9099		782869
			EXF-9088		782870
			EXF-9395		782871

Refrigerator	Swabbing	Pure culture – reference strains	EXF-9596	#1	782872
			EXF-11755		782873
			EXF-12203		782874
			EXF-12266		782875
Tap water	Filtration	Total environmental DNA	VOD-POM-3	#2	782821
			VOD-POM-30		782822
			VOD-KUH-5		782823
			VOD-KUH-10		782824
			VOD-KUH-12		782825
Biofilm (dishwasher rubber)	Scraping	Total environmental DNA	BF POM-3	#2	782826
			BF POM-13		782827
			BF POM-27		782828
			BF POM-30		782829
			BF POM-33		782830
Bottom of shower cabinet	Swabbing	Without prior DNA extraction	ShowerC-1	#3	782831
Kitchen sink	Swabbing	Without prior DNA extraction	KitchenS-1	#3	782832
Dish drying rack	Swabbing	Without prior DNA extraction	Dish-rack-1	#3	782833
Washing machine rubber	Swabbing	Without prior DNA extraction	WashingM-1	#3	782834
Glass shelf in refrigerator	Swabbing	Without prior DNA extraction	RefrigeratorS-1	#3	782835

*EXF number, Number given to fungal strains for deposition in the Ex Culture Collection of the Infrastructural Centre Mycosmo, MRIC UL, Slovenia.

Identification of the sequences

PCR products were sequenced and the sequences obtained were clean and without superior signals and of the correct length. The sequences were genetically matching to the template locus CPAR2_105320 of *C. parapsilosis sensu stricto* type strain CDC317 with 100% similarity.

However, the sequences obtained from single strains isolated from bathrooms (EXF-6998, EXF-8149), a washing machine (EXF-8239), a kitchen (EXF-9925), a dishwasher (EXF-9099), and swab samples of plastic surfaces of a shower cabinet (ShowerC-1) and a dish-drying rack (Dish-rack-1) differed from the original template of the type strain by a single nucleotide. This difference was seen at the 574th nucleotide position, where the guanine (G) from the original template was replaced by an adenine (A). This difference was also reflected in the amino-acid sequences, where the valine (V) of the original type strain template was then read as isoleucine (I) in these samples.

Discussion

Although the most frequent *Candida* species that causes disease in humans remains *C. albicans*, recent trends show a remarkable increase in non-*C. albicans* species worldwide. Many *Candida* species are a part of the human microbiota, and they are carried in the gastrointestinal and genital tracts, or other mucosal epithelia, of some 60% to 75% of people (Cortés et al. 2019). However, not all *Candida* species are related solely to humans or hospital environments, as many mainly non-*C. albicans* species have been isolated from natural environments, like fruit, vegetables, soil, sand, drinking water, and waste water (Shah et al., 2011, Novak Babič et al. 2016, Kulesza et al. 2018, de Hoog et al. 2019). Also, human-made indoor environments, such as refrigerators, washing machines, dishwashers, kitchens, and bathroom surfaces, are known to harbour a variety of *Candida* species. Consequently, these environments can represent reservoirs for the propagation and disseminations of these yeast (Novak Babič et al. 2015, Novak Babič et al. 2016, Novak Babič et al. 2017, Zupančič et al. 2016, Zupančič et al. 2019).

As an emerging pathogen, *C. parapsilosis sensu stricto* is also often involved in infections of neonates and in bloodstream infections through the use of intravenous catheters (Trofa et al. 2008, Sardi et al. 2013, Arastehfar et al. 2019, de Hoog et al. 2019). The regular presence of this yeast can even lead to outbreaks if the source of the primary contamination is not detected in good time (Bloomfield et al. 2012). The time for diagnosis or detection of a pathogen in the patient environment is thus crucial for rapid and correct treatment to achieve positive outcomes, or to adopt extra safety measures (Bloomfield et al. 2012). The additional consequence of slow diagnostic methods could lead to the increasing resistance of strains due to the continued use of broad-spectrum antifungals (Sardi et al. 2013). Current well-established methods for detection and identification of *Candida* in clinical and environmental samples include culture-depended methods, biochemical assays, matrix-assisted laser desorption time of flight mass spectrophotometry (MALDI-TOF MS), and multiplex PCR amplifying ITS1 or ITS2 regions (Luo and Mitchell 2002, Liguori et al. 2010, Taira et al. 2014, Arastehfar et al. 2019, Cortés et al. 2019).

Cultures can be grown on Sabouraud and on malt extract or yeast extract agar media, where *C. parapsilosis* colonies usually grow within 2-3 days at an incubation temperature from 25 °C to 37 °C. Here they grow as smooth, cratered, creped or concentric phenotypes with white or yellowish pigmentation (Cortés et al. 2019, Zupančič et al. 2019). However, when grown on standard media alone, this does not provide accurate identification. In addition, chromogenic agar can be used as a primary isolation medium, but it usually does not distinguish well between *C. parapsilosis* and *C. glabrata* (Cortés et al. 2019) 34]. Other culture-dependent methods for identification include commercial biochemical and phenotypic systems, like API 20 C AUX, Vitek2 and RapID yeast panel, which have been shown to provide 80% to 85% accuracy for species identification (Liguori et al. 2010, Posteraro et al. 2015). A more advanced methodologies based on pure cultures is seen for MALDI-TOF MS. The advantages here include easy preparation of the samples, broad applicability, and reproducibility (Croxatto et al. 2012). However, this technique has high costs and cannot be applied directly on clinical samples. Furthermore, the isolates tested can only be correctly identified if the reference spectra of the species are already included in the required database (Clark et al. 2013, Arastehfar et al. 2019), with the chance remaining that genetically closely related microorganisms with similar spectra might not be differentiated, as is frequently the case in differentiation of *Candida parapsilosis* species complex (Clark et al. 2013).

Growth-dependent methods remain the first choice for laboratories without specific molecular biology equipment. These methods are reliable, but they also require longer times for identification. If possible, the use of molecular methods is recommended, particularly with species-specific primers (Liguori et al. 2010). PCR of DNA barcodes is now recognised as one of the standards in laboratories worldwide due to its accessibility and reproducibility (WHO 2016), although further Sanger sequencing is still limited in developing countries (Arastehfar et al. 2019). A good alternative to culture dependent methods is seen in commonly used conventional or nested

multiplex PCR, which frequently does not require additional sequencing, and can provide rapid detection and identification of different *Candida* species. However, this method is usually carried out with primers designed on a template of ITS1 or ITS2 conserved regions, which cannot always provide optimal identification due to the genetic similarity of these *Candida* yeast. The lack of new species-specific primers designed on a template other than the ITS region is one of the pressing issues when using multiplex PCR (Luo and Mitchell 2002, Trofa et al. 2008, Liguori et al. 2010, Taira et al. 2014, Arastehfar et al. 2019). Although different methods for detection and identification have developed rapidly over the last 20 years, and sophisticated multiplex PCR is becoming a suitable solution, particularly in clinical settings (Arastehfar et al. 2019), *C. parapsilosis* still remains among the most frequently misidentified species, as it is still often mistaken for *C. glabrata*, *C. tropicalis*, *C. metapsilosis*, or *C. orthopsilosis* (Luo and Mitchell 2002, Ghelardi et al. 2008, Trofa et al. 2008, Liguori et al. 2010, Taira et al. 2014, Arastehfar et al. 2019).

Sequencing of whole genomes provided the additional insight in housekeeping genes, enabling the design of new primers for conventional or multiplex PCR. One of them is locus CPAR2_105320 used in the present study. The locus is encoding life-essential protein (an orthologue of the *Saccharomyces cerevisiae* protein *URA6*) which is one in the cascade for *de-novo* synthesis of pyrimidines. Pyrimidine biosynthesis includes the six enzymatic steps occurring nearly universally in all organisms (Hermansen et al. 2015). The enzymatic organisation, structure, localization, and regulation changes due to the evolution among different organisms (Hermansen et al. 2015). The DNA sequences of the locus thus differ among the species, enabling the use of the sequence as a template for novel species-specific primers, providing a unique barcode for certain species as already proved for *Saccharomyces cerevisiae* (Hermansen et al. 2015).

In the present study we used conventional PCR, which can be easily accessed and applied also in BSL-1 laboratories. These newly designed primers and the methods described here show the important advantages of this method: low cost, little time needed, and accurate identification. The primers and programs can be applied in three different ways: to identify pure yeast cultures; to detect the yeast in the whole DNA extracted from various environments (e.g., tap water, biofilms); and to detect the yeast directly on swab samples taken from different surfaces (e.g., glass, plastic, rubber) without the need for prior cultivation. Among the three versions of the method described here, the process of species-specific identification from swab samples was the most rapid, altogether taking 3 h to 4 h, and providing 100% accuracy. To get the optimal results in further studies, we suggest the final volume of the PCR mixture being 34 µl with the addition of 1 µl DNA. Five microliters of the PCR product may be used for visualisation on the agarose gel and 30 µl is the volume needed in cases of additional Sanger sequencing. However, the exact final concentrations of chemicals are provided in the result section, enabling the successful further replication studies even if using different final volumes.

For laboratories without possible access to the Sanger sequencing we recommend the use of the agarose gel electrophoresis to visualize the obtained PCR products. Agarose gel may be prepared in any concentration, ranging from 1% to 3% (w/v). However, the fastest method still enabling an accurate result included the use of 1% (w/v) agarose gel. In any case, only one band of the proper size (~570 bp) should be observed indicating the positive outcome of the PCR.

The advantage of this method is its complete complementarity with current tools, as it can be carried out in parallel with all of the culture-depended methods, or with MALDI-TOF or multiplex PCR. To the best of our knowledge, these newly designed primers are the first primers that amplify only *C. parapsilosis sensu stricto*, and at the same time completely exclude the closely related species *C. metapsilosis* and *C. orthopsilosis* and other clinically relevant species, including *C. albicans, C. glabrata*, and *C. tropicalis.* Novel primers can be used as taxonomical barcode markers for *C. parapsilosis sensu stricto*, to resolve frequent misidentification with closely related species obtained from both clinical and environmental samples. Even when probed on a total DNA samples obtained from biofilms or tap water with total DNA concentrations below

2 ng/µl, the PCR product showed only one band here, and could thus be directly sequenced. The primers described here were successfully applied to pure cultures obtained from environmental and clinical samples, and to concentrated suspensions of washed swab samples, without prior DNA extraction. In the future, these could also be probed directly on clinical samples, like blood and sputum, to further demonstrate their efficiency for rapid diagnosis of *C. parapsilosis sensu stricto* also in clinical specimens.

Povzetek

Obolevnost zaradi različnih glivnih okužb v svetu v zadnjih 20 letih strmo narašča. Med najpogostejšimi oblikami bolezni je kandidemija, ki jo povzročajo glive iz rodu *Candida.* Približno 50 % vseh primerov kandidemij povzroča vrsta *Candida albicans*, pri preostale pa so povzročiteljice tako imenovane porajajoče vrste (Trofa in sod. 2008). Med temi so najpogostejši vzrok kandidemije vrste iz *Candidda parapsilosis* kompleksa vrst (Lass-Flörl 2009). Kompleks vrst sestavljajo tri, genetsko ozko sorodne vrste: *Candida parapsilosis sensu stricto, Candida orthopsilosis* in *Candida metapsilosis*. Iz kliničnega materiala je najpogosteje osamljena *C. parapsilosis sensu stricto* (90,7 %), sledi ji *C. orthopsilosis* (8,2 %), medtem ko se *C. metapsilosis* v kliničnem materialu pojavlja najredkeje (1,1 %) (Cantón in sod. 2011). Najvišje tveganje za okužbo z vrstami *C. parapsilosis* kompleksa je sicer prisotno pri bolnikih z oslabljenim imunskim sistemom, pri nedonošenčkih in ljudeh s cistično fibrozo (Trofa in sod. 2008, Sardi in sod. 2013, Cortés in sod. 2019). Za razliko od mnogih vrst rodu *Candida*, ki so povezane predvsem s človekom in bolnišničnim okoljem (npr. *C. albicans, C. auris*), se *C. parapsilosis sensu stricto* pojavlja tudi v naravi, v drugih organizmih, v vodnih virih in na površinah iz različnih naravnih in umetnih materialov (Novak Babič in sod. 2017a,b, Cortés in sod. 2019, Zupančič in sod. 2019).

Za zmanjšanje pojavnosti okužb in preprečevanje izbruhov bolezni, je pri porajajočih patogenih pomembno določiti možne poti prenosa na človeka. Le-te lahko izvirajo iz biološkega materiala ali okolja v katerem se pacient nahaja (npr. voda iz pipe, površine, protetični materiali) (Liguori in sod. 2010, Bloomfield in sod. 2012). V kolikor pa je pacient že okužen, so hitra in natančna diagnostična orodja ključnega pomena za uspešno zdravljenje in okrevanje (Liguori in sod. 2010, Arastehfar in sod. 2019). Trenutno uveljavljene metode za odkrivanje in identifikacijo *C. parapsilosis sensu stricto* v kliničnih in okoljskih vzorcih vključujejo kultivacijo, biokemijsko določanje profilov razgradnje različnih substratov, masno spektrometrijo (MALDI-TOF MS) in multipleks PCR. Naštete metode so pogosto nedostopne v manjših laboratorijih, zamudne in povezane z višjimi stroški. Naša raziskava se je zato osredotočila na uporabo klasične PCR metode, ki je dostopna tudi v večini laboratorijev z najnižjo stopnjo varnosti (Biosafety Level (BSL)-1).

Na novo opisani oligonukleotidni začetniki za določanje *C. parapsilosis sensu stricto* s klasičnim PCR pomnožujejo del regije CPAR2_105320 v velikosti 574 baznih parov, ki kodira zapis za ortolog proteina *URA6* (kot je poimenovan pri *Saccharomyces cerevisiae*). Protein *URA6* je pomemben del kaskade pri *de-novo* sintezi pirimidinov (Hermansen in sod. 2015). Kljub temu, da je zapis v genomih prisoten univerzalno, se DNA zaporedje med organizmi dovolj razlikuje, da omogoča vrstno-specifično identifikacijo (Hermansen in sod. 2015). Metode in PCR programi opisani v naši študiji, so bili zasnovani tako, da omogočajo zaznavanje in identifikacijo kvasovke na podlagi DNA čiste kulture, celokupne DNA iz vzorcev okolja kot so biofilmi in pitna voda ter direktno v vzorcih brisov odvzetih iz različnih površin površin (npr. stekla, plastike in gume), brez predhodnega gojenja. Med temi različicami je bil postopek identifikacije iz vzorcev brisov najhitrejši, skupaj je trajal 3-4 ure in zagotavljal 100 % natančnost. Kvasovko smo zaznali tudi v vzorcih z vsebnostjo celokupne DNA < 2 ng / µl, hkrati pa pri nobeni izmed ozko sorodnih vrst nismo dobili pomnožka. S tem smo potrdili vrsto-specifičnost dobljenih produktov.

Novi oligonukleotidni začetniki se lahko uporabljajo kot taksonomski označevalci za *C. parapsilosis sensu stricto*, kar bi omogočilo odpravo pogosto napačne identifikacije s tesno sorodnimi vrstami, pridobljenimi tako iz kliničnih

kot iz okoljskih vzorcev. Opisane metode in progami uporabe imajo poleg natančnosti identifikacije tudi nekatere druge pomembne prednosti, kot so nizki stroški, hitrost in združljivost z že uveljavljenimi metodami identifikacije. V prihodnosti bi opisane metode lahko preizkusili vzporedno z že obstoječimi metodami neposredno na kliničnih vzorcih, kot sta kri in sputum, s čemer bi prispevali k hitrejši diagnostiki ob sumu na *C. parapsilosis sensu stricto* v kliničnih vzorcih.

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