Laboratory Exercise

Molecular Tools for Cryptic *Candida* Species Identification with Applications in a Clinical Laboratory

Soledad Gamarra†‡
Catiana Dudiuk†
Estefanía Mancilla‡
María Verónica Vera Garate‡
Sergio Guerrero‡§
Guillermo Garcia-Effron†‡

From the †Laboratorio de Micología y Diagnóstico Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, CONICET, Santa Fe (CP 3000), Argentina, ‡Cátedra de Parasitología y Micología, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe (CP 3000), Argentina, §Laboratorio de Bioquímica Microbiana, Instituto de Agrobiotecnología del Litoral, Universidad Nacional del Litoral, CONICET, Santa Fe (CP 3000), Argentina

Abstract

Candida spp. includes more than 160 species but only 20 species pose clinical problems. *C. albicans* and *C. parapsilosis* account for more than 75% of all the fungemias worldwide. In 1995 and 2005, one *C. albicans* and two *C. parapsilosis*-related species were described, respectively. Using phenotypic traits, the identification of these newly described species is inconclusive or impossible. Thus, molecular-based procedures are mandatory. In the proposed educational experiment we have adapted different basic molecular biology techniques designed to identify these species including PCR, multiplex PCR, PCR-based restriction endonuclease analysis and nuclear ribosomal RNA amplification. During the classes, students acquired the ability to search and align gene sequences, design primers, and use bioinformatics software. Also, in the performed experiments, fungal molecular taxonomy concepts were introduced and the obtained results demonstrated that classic identification (phenotypic) in some cases needs to be complemented with molecular-based techniques. As a conclusion we can state that we present an inexpensive and well accepted group of classes involving important concepts that can be recreated in any laboratory.

Keywords: *Candida spp.; molecular taxonomy*

Introduction

Candida spp. is the most common fungal pathogen and the fourth most common cause of hospital acquired blood stream infections [1]. The genus *Candida* (Berkhout) includes more than 160 species but only 20 species pose clinical problems [2–5]. Out of these species *C. albicans* and *C. parapsilosis* account for more than 75% of all the fungemias worldwide. In 1995, Sullivan *et al.* described a *C. albicans*-related species named *C. dubliniensis* [6]. More recently, Tavanti *et al.* demonstrated that the three groups of *C. parapsilosis* were in fact three different related species (*C. parapsilosis sensu stricto*, *C. orthopsilosis,* and *C. metapsilosis*) [7]. Since then, an increased interest in studying the epidemiology of these newly described species has arisen based on the observed antifungal susceptibilities and virulence differences [6,8–18].

Candida spp. identification has relied largely on phenotypical traits such as carbon auxotrophy and on microscopic examination (e.g., chlamydoconidia formation). However, the identification of these newly described species by phenotypic methods is frequently inconclusive or impossible. Thus, molecular-based procedures are mandatory for the taxonomic differentiation between *C. albicans* and *C. dubliniensis* and between *C. parapsilosis* sensu lato species [6,7,19].

The differentiation between *C. albicans* and *C. dubliniensis* using molecular techniques can be performed by

**Address for correspondence to: Laboratorio de Micología y Diagnóstico Molecular, CONICET, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria UNL (Ruta 168), C.C. 242-S3000ZAA, Santa Fe (Santa Fe), Argentina; E-mail: ggarcia@unl.edu.ar.*

Received December 2012; accepted 16 February 2013

DOI: 10.1002/bmb.20692

Published online 27 April 2013 in Wiley Online Library (wileyonlinelibrary.com)

different procedures [6,20–24]. The one used by us in these classes is the method proposed by Donnelly *et al.* [20]. It is based on the sequence differences between the *C. dubliniensis* actin 1 gene (*CdACT*1) and the *C. albicans* actin 1 gene (*CaACT*1). The intron and exon sequences of these genes are 83.4 and 97.9% identical, respectively. These sequence differences reflect the differential evolutionary pressure that introns and exons suffer. The method is a multiplexed PCR that uses two pair of primers [20]. One of the pairs (DUBF/DUBR) is designed to hybridize the *CdACT1* gene. The sense primer (DUBF) hybridizes one conserved region of both *CaACT1* and *CdACT1* exon I. On the other hand, the antisense primer (DUBR) was designed to hybridize specifically with the *CdACT1* gene intron. Thus, using these primers, a 288 bp. PCR product should be obtained only if a *C. dubliniensis* DNA is used as a template. The second pair of oligonucleotides (RNAF/RNAR) primes a conserved sequence from fungal nuclear ribosomal RNA used for fungal molecular identification [25]. The PCR product obtained using these primers (of 610 bp. approximately for *Candida* spp.) serve as an internal positive control. When both primers pairs were combined in a single PCR reaction (multiplexing), all *Candida* spp. should yield the ≈610 bp. amplimer while only *C. dubliniensis* strains should produce both the ≈610 bp. and the 288 bp. bands.

Turning to the molecular differentiation between the *C. parapsilosis* sensu lato species, multiple molecular-based methodologies have been proposed [26–31]. In our teaching experiment we decided to use a two-step method that include a PCR amplification of a *C. parapsilosis* sensu lato *FKS1* gene fragment followed by an *Eco*RI digestion [19]. The oligonucleotide pair REAF/REAR primes a conserved region of the *C. parapsilosis* sensu stricto *FKS1* (CpFKS1), *C. metapsilosis FKS1* (CmFKS1) and *C. orthopsilosis FKS1* (CoFKS1) genes. The PCR amplification would produce a 1032 bp. fragment that yield none, one and two *Eco*RI restriction sites for CpFKS1, CmFKS1 and CoFKS1, respectively. These *Eco*RI digestions allow the differentiation between these species.

What is described herein are laboratory practice classes aimed to introduce the students to fungal molecular taxonomy and to show that classic fungal taxonomy (phenotypic identification) in some cases needs to be complemented with molecular-based.

Material and Methods

Classes and Students

The classes described here are named "molecular taxonomy" and are part of the courses "clinical mycology" and "molecular biology techniques applied to the study of fungi and parasites." These courses are offered to the undergraduate students of Biochemistry and to the postgraduate students of the Biological Sciences PhD program, respectively. The Molecular taxonomy classes followed two classes named "classic yeast taxonomy" were students received the training for yeast phenotypic identification (e.g., carbon- and nitrogen-source assimilation techniques, germ tube induction, etc.). One week before the laboratory classes, a 2 days 3-h meetings were scheduled. In the first one, a concise explanation of the molecular-based methodologies for differentiation between *C. albicans* and *C. dubliniensis* and between the *C. parapsilosis sensu lato* species was given. Moreover, a description of the general aspect of the laboratory classes was imparted with emphasis in the methodologies to be used. Also, a brief reminder of how to use a freeware bioinformatics software named BioEdit Sequence Aligment Editor [32] and the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) was given. In the second 3-h meeting, the mentioned software and database were used by the students to obtain the *ACT1* and *FKS1* genes nucleotide sequences and to design oligonucleotides. We recommended students to read the paper by Donnelly *et al.* and by Garcia-Effron *et al.* were equivalent experiments were firstly described [19,20]. Afterwards, with the assistance of laboratory teachers, the students compared their designed primers and the ones used by these authors and a discussion was stimulated [19,20].

The experiments and result analysis were completed in a 2 days 5-h laboratory sessions. The students were asked to study a class guide which included a brief theoretical introduction and detailed protocols. Both classes started with a brief explanation of the main concept, the steps of the protocols to be used that day and how to analyze the results. Each laboratory group typically has 12 students which were divided into four groups (named group 1 to 4).

Experimental Procedures

The first laboratory class, each group received three *Candida* spp. strains grown overnight in YPD broth (2% yeast extract, 4% Bacto peptone, 4% dextrose). These strains were phenotypically identified by students in the "classic yeast taxonomy" classes as: *C. albicans/C. dubliniensis*, *C. parapsilosis sensu lato* or *C. tropicalis* strain (Table I).

To stimulate a discussion about the different quantity and quality of the fungal genomic DNA obtained, each group extracted yeast genomic DNAs by different procedures. All the groups used a phenol-based DNA purification [33]. Moreover, groups 1 and 2 extracted the DNA using the Accuprep Genomic DNA Purification Kit (Bioneer-Genbiotech, Buenos Aires, Argentina) following manufacturer's instructions. On the other hand, groups 3 and 4 were asked to extract the yeast DNAs using the Accuprep kit with a previous glass beads disruption step (Table I). Extracted genomic DNAs were subjected to electrophoresis and spectroscopy analysis. At the end of the first laboratory class, students set two PCR reactions per strain. The first (named PCR1) is the reaction proposed by Donelly *et al.* to

TABLE 1 *Fungal strains and activities given to each laboratory group*

	Group 1	Group 2	Group 3	Group 4
Strains	*C. dubliniensis* NCPF 3949	*C. albicans* ATCC 90028	*C. albicans* ATCC 90028	*C. dubliniensis* NCPF 3949
	C. parapsilosis sensu stricto ATCC 22019	*C. parapsilosis sensu stricto* ATCC 22019	*C. orthopsilosis* ATCC 96139	*C. orthopsilosis* ATCC 96139
	C. tropicalis ATCC 750	*C. metapsilosis* ATCC 96144	*C. tropicalis* ATCC 750	*C. metapsilosis*
DNA extraction[a]	DEK	DEK	DEK+P	DEK+P
	PB	PB	PB	PB

[a] *DEK: DNA extraction Kit (Accuprep), PB: Phenol-based extraction procedure, EKD+P: DNA Extraction Kit (Accuprep) with a physical disruption step (with glass beads).*

differentiate *C. dubliniensis* from *C. albicans* [20]. The PCR2 is the PCR reaction used as the first step of the *C. parapsilosis* sensu lato species identification [19] (Table II). Students were guided by a laboratory teacher to know how to program the thermocycler and they run the PCR reaction. After the PCR program finished, the PCR tubes where frozen until the next class. At the beginning of class 2, each group of students received one agarose gel ready to be seeded and the frozen PCR reactions were subjected to electrophoresis analysis. The groups would obtain the results showed in Table II. A result's discussion was stimulated to help the students to select the PCR tube in which the 1-h *Eco*RI digestion would be performed. The selection was performed based in the PCR band size. The restriction enzyme digestion was performed following the manufacturer's instructions. Digestion products were analyzed by electrophoresis on agarose gels and results were discussed. At the end of the second laboratory class the students answered a questionnaire in which their evaluation was based and filled out two poll forms which evaluated the quality of the classes and teachers. Objectives of each of the questions are listed in Table III.

TABLE 2 *PCR reactions*

Strains	*PCR1*[a]	*PCR2*[b]
C. albicans	+ (one band: ≈ 600 pb.)	–
C. dubliniensis	+ (two bands: ≈ 600 pb. and a 288 pb.)	–
C. parapsilosis sensu lato	+ (one band: ≈ 600 pb.)	+ (one band: 1032 pb.)
C. tropicalis	+ (one band: ≈ 600 pb.)	–
Oligonucleotide primers[c]	DUBF: GTATTTGTCGTTCCCCTTTC	REA-F: GATGACCAATTYTCAAGAGT[d]
	DUBR: GTGTTGTGTGCACTAACGTC	
	RNAF: GCATATCAATAAGCGGAGGAAAAG	
	RNAR: GGTCCGTGTTTCAAGACG	REA-R: GTCAACATAAATGTAGCATTCTAGAAATC

[a] *Designed to differentiate* C. albicans *and* C. dubliniensis *[20].*
[b] *Used to identify* C. parapsilosis sensu lato *species [19].*
[c] *Sequence (5′ to 3′) [19,20,25].*
[d] *Y represent a degenerate sequence (C or T).*

TABLE 3 *Evaluation questions*

A) Student's evaluation (Average points obtained, maximum of 10).

1) Name the limitations of the phenotypic methods designed to differentiate *C. albicans* from *C. dubliniensis*? (9.0)

2) Which tests are available to identify *C. orthopsilosis* and *C. metapsilosis*? (9.5)

3) Why did the *C. albicans ACT1* and *C. dubliniensis ACT1* exons share a higher homology than the observed in introns? (9.0)

4) How many bands do you expect to observe in an agarose gel after a multiplex PCR reaction using primers DUBF/DUBR and RNAF/RNAR and the DNA obtained from: *C. dubliniensis*, *C. albicans*, *C. parapsilosis*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Rhodotorula mucilaginosa* and *C. tropicalis*? Why? (6.5)

Objectives:

Questions 1 and 2: Know if the students understand the limitations of classical and molecular identification procedures.

Question 3: Know if the students understand the concept of differential evolutionary pressure.

Questions 4: Know if the students understand the difference between specific (DUBF and DUBR/REAF and REAR) and panfungal (RNAF and RNAR) primers.

B) Laboratory class' evaluation[a] (Modified from [34,35])

1) The laboratory equipment and material were:

Available Easy to find Hard to find Unavailable

2) The help received from the laboratory staff was:

Ample Limited Insufficient Minimal

3) The time allotted for the experiments was:

Too short Adequate Rather Long Long

4) The work load was:

Heavy Reasonable Light Very light

5) The lab work taught me some basic skils:

Agree Neutral Disagree

6) The purpose of each experiment was clear:

Agree Neutral Disagree

7) The lab class trained me to interpret the data:

Agree Neutral Disagree

8) In the experiments I was only following instructions (like a cooking recipe):

Agree Neutral Disagree

9) The lab manual was easy to follow and organised

Agree Neutral Disagree

10) I understand each step of the protocols.

Agree Neutral Disagree

11) I found easy to make deductions from my observations.

Agree Neutral Disagree

12) The class content makes me feel near the reality of a research laboratory.

Agree Neutral Disagree

13) The class motivate me to check the results.

Agree Neutral Disagree

14) Overall evaluation of the classes:

Very Good Good Satisfactory Poor Very Poor

C) Teacher's evaluation[b]:

1) Organization of the theoretical content ().

2) Clarity of the explanations ().

3) Teaching staff overall availability to answer questions ().

4) Stimulation for student's participation ().

D) Self-evaluation[b].

1) Interest in the experiments.

2) Participation in the laboratory class.

3) Acquired knowledge:

- Theoretical:
- Practical:
- Psychomotor (pipetting, handling, etc.):

4) Overall score:

[a] *Students have to circle one of the options.*
[b] *Students rated teachers using a 1–5 scale (1: very good and 5: very poor)*

PCR Reactions

PCRs were carried out in a 50 μl volume containing 1X PCR buffer; 2 mM $MgCl_2$; 250 μM each of dATP, dGTP, dCTP,

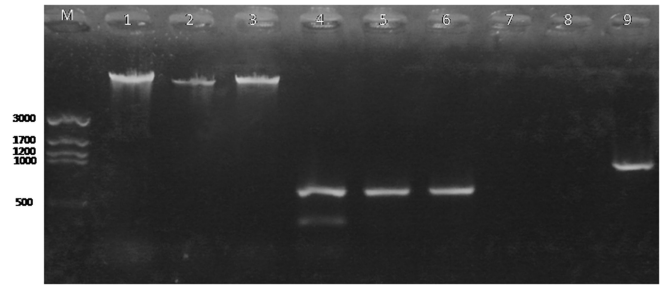

FIG 1

Electrophoresis in A 1% Agarose Gel. Line M, molecular size marker; Lanes 1 To 3, C. dubliniensis *DNA extracted using different procedures (Line 1: phenol-based DNA extraction, line 2: The Accuprep DNA extraction kit, Lane 3: Accuprep DNA extraction kit with A previous physical disruption step). Lanes 4 To 6, Pcr 1 using as template the DNA extracted from* C. dubliniensis *(Lane 4),* C. tropicalis *(Lane 5) and* C. parapsilosis Sensu Stricto *(Lane 6). Lanes 7 To 9, Pcr 2 using DNA obtained from* C. dubliniensis *(Lane 7),* C. tropicalis *(Lane 8) and* C. parapsilosis Sensu Stricto *(Lane 9).*

and dTTP; 0.5 μM of each primers for PCR1 (DUBF, DUBR, RNAF, and RNAR) and 1 μM of each primers for PCR2 (REA-F and REA-R) (primer sequences are displayed in Table II); 2.5 U of Taq DNA polymerase; and 25 to 50 ng of *Candida* spp. genomic DNA. PCR1 and PCR2 experiments were performed using a thermal cycler for one cycle of 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 45 s at 58°C, and 90 s at 72°C, followed by one final cycle of 10 min at 72°C.

Equipment

During this laboratory exercise the following equipment was used: microcentrifuges, vortex mixers, thermocycler, gel electrophoresis equipment, heating block, pipets, microspectrophotometer and a gel documentation system.

Laboratory Supplies

Each group of three students received: gloves, lab glasses, disponsable lab coats, sterile pipette tips, ice boxes, sterile tubes (1.5 ml and 0.2-mL PCR tubes) and different aliquots of enzymes and solutions. The aliquots were provided to avoid cross contamination and enzyme degradation since students have little or no experience in handling these solutions.

Results and Discussion

Bioinformatics

Using BioEdit software and the GenBank nucleotide database, students were able to easily find the *CaACT1*, *CdACT1*, and *FKS1* genes sequences. Moreover, the primers for *CaACT1* and *CdACT1* were designed by the students and were consistent or very similar with those designed by Donnelly *et al*. [20]. On the other hand, the *C. parapsilosis sensu lato FKS1* primers designed by students showed important discrepancies with the published primers [19]. Students aligned CpFKS1, CoFKS1 and CmFKS1 sequences using the bioedit software and choose conserved regions to design the primers. However, they did not consider that *FKS* genes are conserved among fungal species [36]. Thus, student's primers would hybridize different fungal species *FKS1* genes and not only *C. parapsilosis sensu lato* species *FKS1* genes.

Experimental Results

DNA extractions qualities and quantities were better when using the phenol-based procedures. However, interesting conclusions were obtained. Students realized the biohazard inconvenience due to the phenol. Moreover, the majority thought that the phenol-base protocol was more laborious than the commercial kit. The extractions performed with the glass beads pretreatment (Groups 3 and 4) yielded better DNA quantities and qualities. The reasons for these results were discussed with the students. The conclusion was that a physical disruption step followed by chemical disruption is better to break the fungal cell wall than the one disruption step (chemical) used by the commercial kit (Fig. 1).

The PCR1 and PCR2 experiments from all the groups achieved the awaited results, as can be seen in Fig. 1. As expected, students choose the PCR tubes containing the biggest band (1032 bp) to perform the *Eco*RI digestion. The restriction enzyme digestions were performed correctly and students were able to discriminate between the *C. parapsilosis sensu lato* species (Fig. 2).

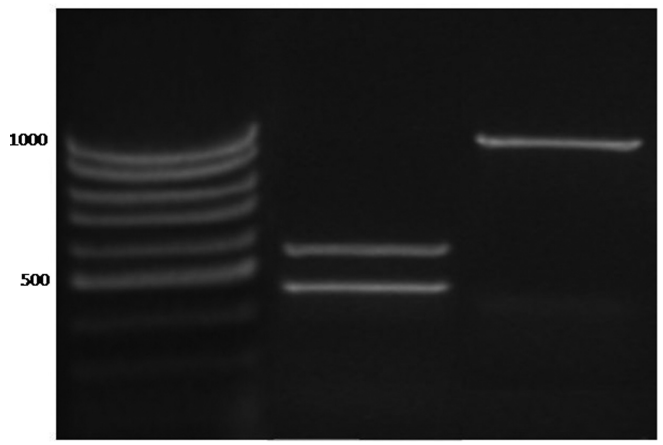

FIG 2

2% Agarose gel electrophoresis of the C. parapsislosis Sensu Lato Fks1 *fragment digested by* Ecor*1. Lane M, 100-Bp molecular size marker; Lane 1,* C. Metapsilosis*; Lane 2,* C. Parapsilosis Sensu Stricto.

Learning Results and Discipline Achievements

The answers to the questionnaire showed that the students meet the objectives of the laboratory class (Table III). Students learned that there is no phenotypic method able to differentiate *C. albicans* from *C. dubliniensis* and *C. parapsilsosis sensu stricto* from *C. metapsilosis* and *c. orthopsilosis*. Thus, using a molecular method is mandatory (questions 1 and 2). Moreover, 11 out of 12 answered that it is necessary to first use a classical method to identify *C. parapsilosis sensu lato* complex. These answers demostrate that students undestood that clasical and molecular-based identification are complementary and both are essential to arrive to a definitive identification. The second objective of the classes was to understand the concept of differential evolutionary pressure in introns and exons. The answers to question three demonstrate that this objective was also achieved by students. With their answers, students confirmed that they have a clear notion that exons could not mutate freely while introns have no such restriction. Moreover, the students understand how molecular taxonomy uses these concepts considering that two related species has to have higher homology in exons than in introns. Analyzing the answers to question 4 it was clear that the majority of the students understand the panfungal nature of the PCR2 (RNAF/RNAR primers). However, 3 out of the 12 students thought that this PCR was in fact a pancandida reaction (positive for all *Candida* spp. but negative for *Aspergillus* spp. *Cryptococcus* spp. and *Rhodotorula* spp.) while 2 believed that the PCR was only positive for *C. albicans* and/or *C. dubliniensis*. Therefore, in future classes, we will dwell on the panfungal nature of the RNAF and RNAR primers.

The evaluation of student's participation was performed in two ways: by a self-evaluation test (Table III) and by following the attitude of the students by taking notes of the questions raised along the laboratory exercises and the answers given to open questions. Also, the way students handle samples and followed the protocols were evaluated. The class and teachers were evaluated by the questions listed in Table III.

As a conclusion we can state that we present an inexpensive and well accepted group of classes involving important concepts that can be recreated in any laboratory.

References

[1] Walsh, T. J. and Groll, A. H. (1999) Emerging fungal pathogens: Evolving challenges to immunocompromised patients for the twenty-first century. Transpl. Infect. Dis. 1, 247–261.

[2] Barret, J. A., Payne, R. W., and Yarrow, D., in Barret, J. A., Payne, R. W., and Yarrow, D. Eds. (1990) Descriptions of the Species, Arranged Alphabetically. Yeasts: Characteristics and Identification, Cambridge University Press, Cambridge, pp.79–695.

[3] Fridkin, S. K. (2005) The changing face of fungal infections in health care settings. Clin. Infect. Dis. 41, 1455–1460.

[4] Nucci, M. and Marr, K. A. (2005) Emerging fungal diseases. Clin. Infect. Dis. 41, 521–526.

[5] Pfaller, M. A. and Diekema, D. J. (2007) Epidemiology of invasive candidiasis: A persistent public health problem. Clin. Microbiol. Rev. 20, 133–163.

[6] Sullivan, D. J., Westerneng, T. J., Haynes, K. A., Bennett, D. E., and Coleman, D. C. (1995) *Candida Dubliniensis* Sp. Nov.: Phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology 141 (Part 7),1507–1521.

[7] Tavanti, A., Davidson, A. D., Gow, N. A., Maiden, M. C., and Odds, F. C. (2005) *Candida Orthopsilosis* and *Candida Metapsilosis* Spp. Nov. to replace *Candida Parapsilosis* groups II and III. J. Clin. Microbiol. 43, 284–292.

[8] Canton, E., Espinel-Ingroff, A., Peman, J., and Del, C. L. (2010) In vitro fungicidal activities of echinocandins against *Candida Metapsilosis*, *C. Orthopsilosis*, and *C. Parapsilosis* evaluated by time-kill studies. Antimicrob. Agents Chemother. 54, 2194–2197.

[9] Diekema, D. J., Messer, S. A., Boyken, L. B., Hollis, R. J., Kroeger, J., Tendolkar, S., and Pfaller M. A. (2009) In vitro activity of seven systemically active antifungal agents against a large global collection of rare *Candida* species as determined by CLSI broth microdilution methods. J. Clin Microbiol 47, 3170–3177.

[10] Gomez-Lopez, A., Alastruey-Izquierdo, A., Rodriguez, D., Almierante, B., Pahissa, A., Rodriguez-Tudela, J. L., and Cuenca-Estrella, M. (2008) Prevalence and susceptibility profile of *Candida metapsilosis* and *Candida orthopsilosis*: Results from population-based surveillance of candidemia in Spain. Antimicrob. Agents Chemother. 52, 1506–1509.

[11] Lockhart, S. R., Messer, S. A., Pfaller, M. A., and Diekema, D. J. (2008) Geographic distribution and antifungal susceptibility of the newly described species *Candida orthopsilosis* and *Candida metapsilosis* in comparison to the closely related species *Candida parapsilosis*. J. Clin. Microbiol. 46, 2659–2664.

[12] Moran, G. P., Sullivan, D. J., Henman, M. C., McCreary, C. E., Harrington, B. J., Shanley, D. B., and Coleman, D. C. (1997) Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. Antimicrob. Agents Chemother. 41, 617–623.

[13] Orsi, C. F., Colombari, B., and Blasi, E. (2010) *Candida metapsilosis* as the least virulent member of the '*C. Parapsilosis*' complex. Med. Mycol. 48, 1024–1033.

[14] Silva, A. P., Miranda, I. M., Lisboa, C., Pina-Vaz, C., and Rodrigues, A. G. (2009) Prevalence, distribution, and antifungal susceptibility profiles of *Candida parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* in a tertiary care hospital. J. Clin. Microbiol. 47, 2392–2397.

[15] Szabo, Z., Szilagyi, J., Tavanti, A., Kardos, G., Rozgonyi, F., Bayegan, S., and Majoros, L. (2009) In vitro efficacy of 5 antifungal agents against *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* as determined by time-kill methodology. Diagn. Microbiol. Infect. Dis. 64, 283–288.

[16] Van Asbeck, E., Clemons, K. V., Martinez, M., Tong, A. J., and Stevens, D. A. (2008) Significant differences in drug susceptibility among species in the *Candida parapsilosis* group. Diagn. Microbiol. Infect. Dis. 62, 106–109.

[17] Varga, I., Soczo, G., Kardos, G., Borbely, A., Szabo, Z., Kemeny-Beke, A., and Majoros, L. (2008) Comparison of killing activity of caspofungin against *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis*. J. Antimicrob. Chemother. 62, 1466–1468.

[18] Gilfillan, G. D., Sullivan, D. J., Haynes, K., Parkinson, T., Coleman, D. C., and Gow, N. A. (1998) *Candida dubliniensis*: Phylogeny and putative virulence factors. Microbiology 144 (Part 4),829–838.

[19] Garcia-Effron, G., Canton, E., Peman, J., Dilger, A., Roma, E., and Perlin, D. S. (2011) Assessment of two new molecular methods for identification of *Candida parapsilosis* Sensu Lato species. J. Clin. Microbiol. 49, 3257–3261.

[20] Donnelly, S. M., Sullivan, D. J., Shanley, D. B., and Coleman, D. C. (1999) Phylogenetic analysis and rapid identification of *Candida*

dubliniensis based on analysis of *Act1* intron and exon sequences. Microbiology 145 (Part 8),1871–1882.

[21] Sullivan, D. and Coleman, D. (1998) *Candida dubliniensis*: Characteristics and identification. J. Clin. Microbiol. 36, 329–334.

[22] Neppelenbroek, K. H., Campanha, N. H., Spolidorio, D. M., Spolidorio, L. C., Seo, R. S., and Pavarina, A. C. (2006) Molecular fingerprinting methods for the discrimination between *C. Albicans* and *C. Dubliniensis*. Oral Dis. 12, 242–253.

[23] Joly, S., Pujol, C., Rysz, M., Vargas, K., and Soll, D. R. (1999) Development and characterization of complex DNA fingerprinting probes for the infectious yeast *Candida dubliniensis*. J. Clin. Microbiol. 37, 1035–1044.

[24] Kurzai, O., Heinz, W. J., Sullivan, D. J., Coleman, D. C., Frosch, M., and Muhlschlegel, F. A. (1999) Rapid PCR test for discriminating between *Candida albicans* and *Candida dubliniensis* isolates using primers derived from the Ph-regulated Phr1 and Phr2 genes of *C. Albicans*. J. Clin. Microbiol. 37, 1587–1590.

[25] Haynes, K. A., Westerneng, T. J., Fell, J. W., and Moens, W. (1995) Rapid detection and identification of pathogenic fungi by polymerase chain reaction amplification of large subunit ribosomal DNA. J. Med. Vet. Mycol. 33, 319–325.

[26] Asadzadeh, M., Ahmad, S., Al Sweih, N., and Khan, Z. U. (2009) Rapid molecular differentiation and genotypic heterogeneity among *Candida parapsilosis* and *Candida orthopsilosis* strains isolated from clinical specimens in Kuwait. J. Med. Microbiol 58, 745–752.

[27] Borman, A. M., Linton, C. J., Oliver, D., Palmer, M. D., Szekely, A., Odds, F. C., and Johnson, E. M. (2009) Pyrosequencing analysis of 20 nucleotides of internal transcribed spacer 2 discriminates *Candida parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis*. J. Clin. Microbiol. 47, 2307–2310.

[28] Borst, A., Theelen, B., Reinders, E., Boekhout, T., Fluit, A. C., and Savelkoul, P. H. (2003) Use of amplified fragment length polymorphism analysis to identify medically important *Candida* Spp., including *C. Dubliniensis*. J. Clin. Microbiol. 41, 1357–1362.

[29] Sabino, R., Sampaio, P., Rosado, L., Stevens, D. A., Clemons, K. V., and Pais, C. (2010) New polymorphic microsatellite markers able to distinguish among *Candida parapsilosis* Sensu Stricto isolates. J. Clin. Microbiol. 48, 1677–1682.

[30] Tavanti, A., Hensgens, L. A., Ghelardi, E., Campa, M., and Senesi, S. (2007) Genotyping of *Candida orthopsilosis* clinical isolates by amplification fragment length polymorphism reveals genetic diversity among independent isolates and strain maintenance within patients. J. Clin. Microbiol. 45, 1455–1462.

[31] Tay, S. T., Na, S. L., and Chong, J. (2009) Molecular differentiation and antifungal susceptibilities of *Candida parapsilosis* isolated from patients with bloodstream infections. J. Med. Microbiol. 58, 185–191.

[32] Hall, T. A. (1999) Bioedit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/Nt. Nucl. Acids. Symp. Ser. 41, 95–98.

[33] Holden, D. W., in Maresca, B. and Kobayashi, G. S., Eds. (1994) DNA Mini Prep Method for *Aspergillus fumigatus* (and Other Filamentous Fungi). Molecular Biology of Pathogenic Fungi, A Laboratory Manual, Telos Press, New York, pp.3–4.

[34] Jhonstone, A. (2005) Evaluation of Teaching, Higher Education Academyphysical Sciences Centre, Hull, UK.

[35] Malago, W., Jr., Soares-Costa, A., and Henrique-Silva, F. (2009) DNA as genetic material: Revisiting classic experiments through a simple, practical class. Biochem. Mol. Biol. Educ. 37, 290–295.

[36] Perlin, D. S. (2007) Resistance to echinocandin-class antifungal drugs. Drug Resist. Updat. 10, 121–130.