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Assessment of Two New Molecular Methods for Identification of *Candida parapsilosis* Sensu Lato Species▽

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***Candida parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* replaced *C. parapsilosis* groups I, II, and III in 2005. Since then, an increased interest in studying their epidemiology has arisen based on the observed antifungal susceptibilities of and virulence differences between the three species. A strict differentiation of these species cannot be achieved by phenotypic methods. We evaluate two new molecular methodologies to differentiate among these species by the use of a collection of 293 bloodstream infection isolates of *C. parapsilosis* sensu lato. For the first method, the isolates were studied using PCR amplification of a fragment of the *C. parapsilosis* sensu lato *FKS1* gene and a universal primer pair followed by EcoRI enzyme digestion. The other method used the allele discrimination ability of molecular beacons in a multiplex real-time PCR format. Both methods of identification showed 100% concordance with internal transcribed spacer 1 (ITS1)/ITS2 sequencing and proved to be effective for clinical applications, even with mixed-species DNAs.**

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Early reports showed that *Candida parapsilosis* strains are more heterogeneous than other *Candida* spp. First, *C. parapsilosis* was divided into three groups based on differences of randomly amplified polymorphic DNA (RAPD), DNA sequencing of different genes, and morphotyping (5, 12–14, 18, 20, 22). In 2005, Tavanti et al. suggested that the 3 groups of *C. parapsilosis* should be replaced by 3 different related species named *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* (25). Since then, an increased interest in studying the epidemiology has arisen based on the observed antifungal susceptibilities of and virulence differences between the three species (4, 6, 7, 10, 15, 17, 19, 23, 24, 28, 29). Differentiating among the three species of the *C. parapsilosis* sensu lato group cannot be achieved by phenotypic methods. Several PCR and molecular methodologies have previously been proposed (1–3, 10, 21, 26, 27). Each methodology has different advantages and drawbacks, and none of them are ideal in terms of speed and/or cost. The aim of this study was to evaluate two new molecular methodologies, PCR-based restriction endonuclease analysis (PCR-REA) and multiplex real-time PCR with molecular beacons (MBs), to differentiate these species by the use of a retrospective collection of blood culture isolates obtained at La Fe University Hospital of Valencia (Valencia, Spain) during a 12-year period.

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MATERIALS AND METHODS

Strains. A total of 293 strains were included in this study. All the isolates were recovered from bloodstream infections from 288 patients with proven candidemia admitted to different clinical care units of La Fe University Hospital (Valencia, Spain) between January 1995 and July 2007.

Strain identification. All isolates were identified at La Fe Hospital as *C. parapsilosis* sensu lato species by the use of Auxacolor (Bio-Rad, Spain) and Vitek (bioMérieux, Spain) and were conserved at −86°C. Upon arrival at the Public Health Research Institute (Newark, NJ), the isolates were plated onto CHROMagar *Candida* (Becton Dickinson and Company, Sparks, MD) to ensure purity. *C. parapsilosis* sensu lato genomic DNA was extracted as described before (9). The molecular identification was done by sequencing the 5.8S RNA gene and adjacent internal transcribed spacer 1 (*ITS1*) and *ITS2* regions (25, 30). This methodology has been considered the "gold standard" technique to establish the specificity of the newly proposed molecular identification techniques. The sequence analysis was performed by comparisons to the *ITS1* and *ITS2* sequences obtained from *C. parapsilosis* ATCC 22019, *C. orthopsilosis* ATCC 96139, and *C. metapsilosis* ATCC 96144 and to the *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis ITS1* and *ITS2* sequences displayed in NCBI (http://www.ncbi.nlm.nih.gov) (GenBank accession no. AB109275, EU557373 and EU557369, respectively). Phylogenetic analyses were conducted using a BioEdit sequence alignment editor (Ibis Therapeutics, Carlsbad, CA) and a maximum-parsimony clustering methodology. Phylograms were rooted to outgroup *ITS1* and *ITS2* sequences obtained from *C. albicans* ATCC 90028 and *C. glabrata* ATCC 90030.

***C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* identification by PCR-based restriction endonuclease analysis (PCR-REA).** A 1,032-bp PCR fragment of the *FKS1* gene of all the strains was amplified using the primers described in Table 1. The primers were designed to align two conserved regions of the *FKS1* gene of the *C. parapsilosis* sensu lato species based on the GenBank sequences of EU350514, EU350513, and EU221325 (*C. metapsilosis FKS1*, *C. orthopsilosis FKS1*, and *C. parapsilosis* sensu stricto *FKS1*, respectively). Primers were purchased from Integrated DNA Technologies (Coralville, IA). Standard PCRs were carried out using a Hot-Start Taq Blue DNA Polymerase master mix (Denville Scientific Inc., Metuchen, NJ) following the manufacturer's instructions. PCR experiments were performed using an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) 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

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TABLE 1. Oligonucleotide primers and molecular beacon sequences used in this study

Oligonucleotide[a]	Sequence (5′ to 3′)[b]	Modification		Purpose
		3′ end	5′ end	
ITS1[c]	TCCGTAGGTGAACCTGCGG	None	None	*ITS* sequencing
ITS4[c]	GCATATCAATAAGCGGA	None	None	*ITS* sequencing
Cp1-1588F	TTTGTTCCTAGAGAATGGGCCGGT	None	None	*C. parapsilosis FKS1* sequencing
Cp1-1785F	ATGCCTTTGGGAGGTTTGTTCACC	None	None	*C. parapsilosis FKS1* sequencing
Cp1-1785R	GGTGAACAAACCTCCCAAAGGCAT	None	None	*C. parapsilosis FKS1* sequencing
Cp1-2065R	TGTTGGGATTGATGTACGCTGTCG	None	None	*C. parapsilosis FKS1* sequencing
Cp1-2243R	TTGATTTCCATTTCGGTGGTGGCC	None	None	*C. parapsilosis FKS1* sequencing
Cp1-2527R	TATCAACAGGAACAGGTTCCGGCA	None	None	*C. parapsilosis FKS1* sequencing
Cp1-2527F	TGCCGGAACCTGTTCCTGTTGATA	None	None	*C. parapsilosis FKS1* sequencing
Cp1-2868F	GGCTTCCTTGAGGTCGCAAACTTT	None	None	*C. parapsilosis FKS1* sequencing
Cp1-2969R	AAAGTTTGCGACCTCAAGGAAGCC	None	None	*C. parapsilosis FKS1* sequencing
Cp1-2969F	GGCTTCCTTGAGGTCGCAAACTTT	None	None	*C. parapsilosis FKS1* sequencing
Cp1-3147F	AGAACCGGCATTGAATGAGGACGA	None	None	*C. parapsilosis FKS1* sequencing
Cp1-3546F	TGATGTCGCTGCTGGTAAGGAACA	None	None	*C. parapsilosis FKS1* sequencing
Cp1-3546R	TGTTCCTTACCAGCAGCGACATCA	None	None	*C. parapsilosis FKS1* sequencing
Cp1-3818F	ACTTCACAACCAAGATTGGTGCCG	None	None	*C. parapsilosis FKS1* sequencing
Cp1-3998F	ACTTGAACTCATTGGCACACGAGTC	None	None	*C. parapsilosis FKS1* sequencing
Cp1-3998R	GACTCGTGTGCCAATGAGTTCAAGT	None	None	*C. parapsilosis FKS1* sequencing
Cp1-4299R	ACCGATTTAACTGTTGGTGGTGCC	None	None	*C. parapsilosis FKS1* sequencing
Cp1-4433R	TTGGAACCGTTGCTCATTGGCAAG	None	None	*C. parapsilosis FKS1* sequencing
Cm1-1F	GATTTTGATCTTTGTGGTGATTTTG	None	None	*C. metapsilosis FKS1* sequencing
Cm1-132R	TCTTTGCTGTCATGCCTTTGGGTG	None	None	*C. metapsilosis FKS1* sequencing
Cm1-392F	GATGTTGTCTGTAGGCATCAAGCC	None	None	*C. metapsilosis FKS1* sequencing
Cm1-392R	GGCTTGGATGCCTACAGACAACATC	None	None	*C. metapsilosis FKS1* sequencing
CminsHS1F[d]	CAGAGAACATTTGTTAGCC	None	None	*C. metapsilosis FKS1* sequencing
Cm1-1108F	TGAGGATGCCGAGAAAGCATCTGA	None	None	*C. metapsilosis FKS1* sequencing
Cm1-1108R	TCAGATGCTTTCTCGGCATCCTCA	None	None	*C. metapsilosis FKS1* sequencing
Cm1-1336F	TGGTGGTGATCCAGAAGGATTGGA	None	None	*C. metapsilosis FKS1* sequencing
Cm1-1336R	TCCAATCCTTCTGGATCACCACCA	None	None	*C. metapsilosis FKS1* sequencing
Cm1-1838F	TCGCCATCTTGGGTGCTAGAGAAT	None	None	*C. metapsilosis FKS1* sequencing
Cm1-1838R	ATTCTCTAGCACCCAAGATGGCGA	None	None	*C. metapsilosis FKS1* sequencing
Cm1-2279F	TACGGTCACCCAGGTTTCCACATT	None	None	*C. metapsilosis FKS1* sequencing
Cm1-2279R	AATGTGGAAACCTGGGTGACCGTA	None	None	*C. metapsilosis FKS1* sequencing
Cm1-2643R	CAACAGTCATATCAGTAAAGAC	None	None	*C. metapsilosis FKS1* sequencing
Co1-1F	ATATCATCACACACTTTCACGG	None	None	*C. orthopsilosis FKS1* sequencing
CoinsHS1F[d]	GGTATGGTGATATTGTCTG	None	None	*C. orthopsilosis FKS1* sequencing
Co1-406F	GCCACTACCGAAATGGAGATCAAG	None	None	*C. orthopsilosis FKS1* sequencing
Co1-406R	CTTGATCTCCATTTCGGTAGTGGC	None	None	*C. orthopsilosis FKS1* sequencing
Co1-698F	TGCCCGAACCTGTTCCTGTTGATA	None	None	*C. orthopsilosis FKS1* sequencing
Co1-698R	TATCAACAGGAACAGGTTCGGGCA	None	None	*C. orthopsilosis FKS1* sequencing
Co1-979F	ATTGGTTTCAAATCGGCTGCTCCC	None	None	*C. orthopsilosis FKS1* sequencing
Co1-979R	GGGAGCAGCCGATTTGAAACCAAT	None	None	*C. orthopsilosis FKS1* sequencing
Co1-1311F	ACCAGCTTTGAATGAGGACGAGGA	None	None	*C. orthopsilosis FKS1* sequencing
Co1-1311R	TCCTCGTCCTCATTCAAAGCTGGT	None	None	*C. orthopsilosis FKS1* sequencing
Co1-1703F	TGGGTGATGTTGCTGCTGGTAAAG	None	None	*C. orthopsilosis FKS1* sequencing
Co1-1703R	CTTTACCAGCAGCAACATCACCCA	None	None	*C. orthopsilosis FKS1* sequencing
Co1-1996F	GGTGCTGGTATGGGTGAACAAATG	None	None	*C. orthopsilosis FKS1* sequencing
Co1-1996R	CATTTGTTCACCCATACCAGCACC	None	None	*C. orthopsilosis FKS1* sequencing
Co1-2444R	CTGTTTTCACTGATTTGACTGTTG	None	None	*C. orthopsilosis FKS1* sequencing
REA-F	GATGACCAATT**Y**TCAAGAGT	None	None	PCR-REA
REA-R	GTCAACATAAATGTAGCATTCTAGAAATC	None	None	PCR-REA
ITS-F	GTCGTAACAAGGTTTCCGTAGG	None	None	Real-time PCR
ITS-R	GATGAAGAACGCAGCGAAATG	None	None	Real-time PCR
Co-ITS-MB	CGCGATCTTAACTGCATTTTTTTACACAATCGCG	DABCYL	HEX	*C. orthopsilosis ITS* probe
Cp-ITS-MB	CGCGATCTTTGATAGGCCTTCTATATGGATCGCG	DABCYL	FAM	*C. parapsilosis ITS* probe
Cm-ITS-MB	CGCGATTTACAGAAATAGGAGAAAGGGCATCGCG	BHQ	CFR	*C. metapsilosis ITS* probe

[a] The letters F and R in the primer names represent the 5′-to-3′ orientation of the primer. F, forward (sense); R, reverse (antisense); Co-ITS-MB, *C. orthopsilosis*-ITS-MB; Cp-ITS-MB, *C. parapsilosis*-ITS-MB; Cm-ITS-MB, *C. metapsilosis*-ITS-MB.

[b] Bold letters represent a degenerate sequence (C or T). This difference was found in the *C. orthopsilosis FKS1* sequence (C at nucleotide position 801 in GenBank accession no. EU350513), whereas in the *C. metapsilosis* and *C. parapsilosis* sensu stricto sequences, there is a T (at nucleotide positions 997 and 2640 in GenBank accession no. EU350514 and EU221325, respectively). The MB probe domains are underlined.

[c] White et al. (30).

[d] Garcia-Effron et al. (9).

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of 10 min at 72°C. Afterward, restriction enzyme digestion was performed using 10 units of EcoRI enzyme (New England BioLabs, Beverly, MA) following the manufacturer's protocol. Digestion products were analyzed by electrophoresis on agarose gels.

Candida parapsilosis **sensu lato *FKS1* sequence analysis.** *FKS1* gene fragments of 2,869-, 2,467-, and 2,663-bp lengths of *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*, respectively, were sequenced using *C. parapsilosis* sensu lato *FKS1*-specific primers (9) (Table 1). These *FKS1* fragments included the hot spot regions linked with echinocandin resistance and the regions used for identification by PCR-REA (for *C. parapsilosis* sensu stricto, nucleotide [nt] 1588 to nt 4433; for *C. metapsilosis*, nt 1 to nt 2663; and for *C. orthopsilosis*, nt 1 to nt 2467). The sequencing analyses were designed to achieve two objectives: (i) identification of mutations that could interfere with PCR-REA identification and (ii) study of the echinocandin resistance hot spot regions. Sequencing reactions were performed as previously described (9).

C. parapsilosis **sensu lato DNA sequencing.** DNA sequencing was performed with a CEQ dye terminator cycle sequencing Quick Start kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's recommendations. Sequence analysis was performed using CEQ 8000 genetic analysis system software (Beckman Coulter, Fullerton, CA).

C. parapsilosis **sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* identification by multiplex real-time PCR using MBs.** Specific MBs for the *ITS1* regions of *C. metapsilosis*, *C. orthopsilosis*, and *C. parapsilosis* sensu stricto were designed on the basis of published sequences (GenBank accession no. EU564207.1, EU564208, and EU564209, respectively). These sequences were confirmed by sequencing the 5.8S RNA gene and adjacent *ITS1* and *ITS2* regions of *C. metapsilosis* ATCC 96144, *C. orthopsilosis* ATCC 96139, and *C. parapsilosis* ATCC 22019. MBs were designed with Beacon Designer software (version 2.12; PREMIER Biosoft International, Palo Alto, CA). A *C. metapsilosis*-specific MB was labeled with Cal Fluor Red 610 (CFR) (5′ end) and with Black Hole Quencher (BHQ) (3′ end). *C. parapsilosis* sensu stricto- and *C. orthopsilosis*-specific MBs were labeled with benzoic acid succinimidyl ester (DABCYL) at the 3′ ends and with 5-carboxyfluorescein (FAM) and with 2′,4,4′,5′,7,7′-hexachlorofluorescein (HEX) at the 5′ ends, respectively. MBs were purchased from Biosearch Technologies, Inc. (Novato, CA). MB thermal denaturation profiling and target primer design was done as previously described (8). Real-time PCR primers were designed by using the oligonucleotide design tool of the IDT SciTools software suite (Integrated DNA Technologies, Coralville, IA) and were purchased from Integrated DNA Technologies. Each MB and the corresponding primer set were validated in real-time assays using DNA isolated from control *C. parapsilosis* sensu lato species. All the MBs, targets, and primers are listed in Table 1.

Real-time PCR. Real-time PCR experiments were performed using a Stratagene Mx3005P multiplex quantitative PCR system (Stratagene, La Jolla, CA) and the "quantitative PCR (multiple standards)" setting. Each real-time PCR was carried out in a 25-μl reaction volume containing 12.5 μl of 2× Brilliant multiplex QPCR Master Mix (Stratagene, La Jolla, CA), 10 pmol of each MB, 25 pmol of each primer, and 1 ng of *C. parapsilosis* sensu lato genomic DNA. Amplifications were performed according to the following protocol: 1 cycle of 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C. Fluorescence detection was performed during the 30 s of the annealing step. The Mx3005P filters were set to recover fluorescence signals by the following channels: FAM (495 nm), HEX (535 nm), and CFR (585 nm).

RESULTS

Molecular identification by sequencing of the 5.8S RNA gene and adjacent internal transcribed spacer 1 (*ITS1*) and *ITS2* regions. The *ITS* sequence of the majority of the *C. parapsilosis* sensu lato strains showed 100% similarity with the sequence each of the control strains used. The phylograms grouped all the strains studied in three groups and together with each of the control strains. Among the 218 strains grouped in the phylogram with the *C. parapsilosis* sensu stricto ATCC 22019 strain, 214 (98.2%) showed no *ITS* sequence differences (100% sequence identity) from the *ITS* sequence of the control strain. On the other hand, 3 strains showed 1 nt difference, whereas 1 *C. parapsilosis* sensu stricto strain showed 2 nt differences with the *ITS* sequence of the control strain. These four strains showed no epidemiological relationship to each other, since they were isolated in different years and departments and from different types of patients. Moreover, there were no particular differences in MIC values between them and the rest of the isolates (data not shown).

In the studies of the *C. orthopsilosis* strains, all but one of the isolates (98.6%) were shown to share the same *ITS* sequence with the ATCC 96139 *C. orthopsilosis* strain. The strain with the different *ITS* sequence had 1 nt difference. All 6 of the *C. metapsilosis* strains showed 100% sequence similarity in comparisons of the *ITS* sequences to the control strain sequence. Moreover, no strains were identified as *Lodderomyces elongisporus*, a newly described human pathogen biochemically indistinguishable from *C. parapsilosis* sensu lato species (16).

Identification of *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* based on PCR-REA with EcoRI of an *FKS1* gene fragment. PCR amplification with the primers REA-F and REA-R (Table 1) yielded an amplicon of 1,032 bp by the use of DNA from any of the *C. parapsilosis* sensu lato species as the template. No amplification bands were obtained using the REA-F and REA-R primer pair and DNAs of other *Candida* species (*C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. dubliniensis*, *C. lusitaniae*, and *C. guilliermondii* were tested) and the PCR conditions described before, demonstrating the species specificity of this primer set (data not shown), and sequence analysis of the *FKS* genes of the *Candida* spp. described (GenBank accession no. XM_716336, XM_446406, XM_448401, EF426563, EU676168, GQ342611, HQ822128, and HQ822127, respectively) revealed between 2 and 6 mismatches with primers REA-F and REA-R, explaining the lack of PCR amplification. When the *C. parapsilosis* sensu lato *FKS1* 1,032-bp fragment sequence was analyzed, each of the species had different restriction maps for EcoRI. *C. orthopsilosis* and *C. metapsilosis* showed a predicted EcoRI site at position 474. Moreover, the *C. orthopsilosis FKS1* fragment showed another EcoRI site at position 778 whereas *C. parapsilosis* sensu stricto showed no EcoRI digestion sites. Thus, electrophoresis of the EcoRI digestion of the *C. orthopsilosis*, *C. metapsilosis*, and *C. parapsilosis* sensu stricto *FKS1* fragments showed 3 bands (474 bp, 306 bp, and 258 bp), 2 bands (474 bp and 564 bp), and 1 band (no digestion), respectively, as expected (Fig. 1). When the PCR-REA results for all 293 clinical strains were compared with those obtained using the *ITS* sequencing, no discrepancies were found. The 100% concordance was corroborated when the *FKS1* sequence was analyzed, and no mutations were found in the EcoRI sites for any of the *C. parapsilosis* sensu lato strains.

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F1

C. parapsilosis **sensu lato *FKS1* sequence.** *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis FKS1* fragments of 2,869-, 2,467-, and 2,663-bp-length fragments, respectively, were sequenced. These fragments included the 1,032-bp sequence used for PCR-REA identification and sequences of the echinocandin resistance hot spot regions. When the resistance hot spot regions on the *FKS1* gene were analyzed, all the *C. parapsilosis* sensu lato strains showed the characteristic naturally occurring proline-to-alanine amino acid substitution linked with the intrinsic reduced echinocandin susceptibility (9). When the Fks1p hot spot for region 2 was studied, the 218 *C. parapsilosis* sensu stricto and the 6 *C. metapsilosis* strains showed no amino acid substitutions compared with *C. albicans*

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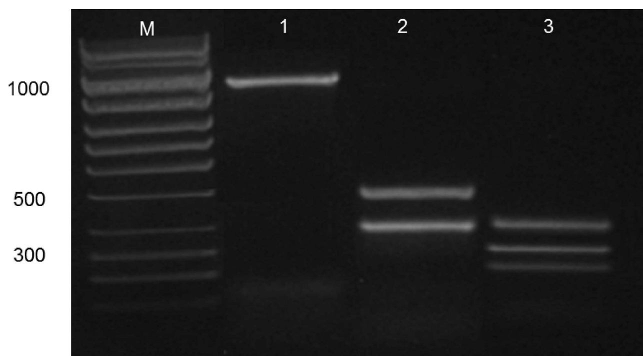

FIG. 1. Electrophoresis of the 1,032-bp *FKS1*. PCR fragments were digested by EcoRI and resolved with a 1% agarose gel. Lane M, 100-bp molecular size marker; lane 1, *C. parapsilosis* sensu stricto ATCC 22019; lane 2, *C. metapsilosis* ATCC 96144; lane 3, *C. orthopsilosis* ATCC 96139.

(GenBank accession no. XM_716336). On the other hand, 65 (94.2%) of the 69 *C. orthopsilosis* strains showed an homozygous isoleucine-to-valine amino acid substitution, as previously described (9). The other 4 isolates showed a heterozygous A/G mutation at position 2272 (following the published nucleotide number for GenBank accession EU350513.1 and equivalent to nt 4111 in the *C. parapsilosis* sensu stricto GenBank EU221325.1 *FKS1* sequence) that led to a heterozygous amino acid substitution at valine 758 to valine/isoleucine (equivalent to nt 1371 of *C. parapsilosis* sensu stricto Fks1p). As described for the *ITS* differences, the isolates showing Fks1p substitutions were isolated from patients hospitalized in different departments over several years and had no epidemiological relationship to one another. Moreover, the mutations were not linked with echinocandin MIC differences for the 3 approved drugs (data not shown). AQ: D

Identification of *C. parapsilosis* sensu lato species by multiplex real-time PCR using MBs. MBs were designed to hybridize to amplicons generated by PCR amplification of the *ITS1* regions of *C. metapsilosis*, *C. orthopsilosis*, and *C. parapsilosis* sensu stricto. The ability of each MB to specifically identify its target was first tested in a single-color simplex assay using DNA templates obtained from control strains. Later, the possibility of combining them in a multiplex assay was investigated. A single-tube assay with the three different MBs (one for each *C. parapsilosis* sensu lato species) and the DNA template corresponding to each of the species was performed. Specific hybridization was observed for each of the MB-DNA template combinations. The annealing temperature used was 56°C (as stated in Materials and Methods), but different annealing temperatures were tested to assess the robustness of the technique. All the MB-DNA template combinations worked properly at an annealing temperature of between 54°C and 57°C, and each MB was able to specifically and reliably differentiate each of the species. The specificity of the assay was determined by amplifying DNA targets from several *Candida* species (*C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, *C. kefyr*, and *C. lusitaniae*) both alone and mixed with *C. parapsilosis* sensu lato species. The utility of the MB real-time PCR assay was assessed using the 293 strains included in this study. The simplex and multiplex assays performed using control strains showed 100% specificity; more importantly, the MB typing methodology identified the 218 *C. parapsilosis* sensu stricto, the 69 *C. orthopsilosis*, and the 6 *C. metapsilosis* strains correctly. The results represented 100% concordance with the results obtained with *ITS* sequencing (our gold standard) and PCR-REA.

DISCUSSION

Since the description of the new *C. parapsilosis* sensu lato species by Tavanti et al. (25), several PCR and molecular methods to differentiate the three species have been described (1–3, 10, 21, 26, 27). Secondary alcohol dehydrogenase-restriction fragment length polymorphism (SADH-RFLP) analysis (performed using BanI) is the most commonly used method, since it was the first described by Tavanti et al. (25). Yet a recent report demonstrated that this method misidentified *C. metapsilosis* as *C. orthopsilosis* because of a missing BanI restriction site in these clinical strains. AQ: E On the other hand, *ITS1*/*ITS2* sequencing is considered the gold standard, since this method can identify all the isolates without any doubt (10, 11, 17, 27). However, this methodology is time-consuming and expensive, since it is a 6-step procedure (PCR amplification, electrophoresis, purification, sequencing reaction, purification, and sequencing).

In this work, we propose two new molecular methods of identification whose results showed 100% concordance with *ITS1*/*ITS2* sequencing identifications and are both quicker and cheaper. The REA method is based on PCR amplification of an *FKS1* region followed by an EcoRI digestion. That region had no mutations at any of the EcoRI sites in any of the 293 clinical strains analyzed, demonstrating a low probability of false identification. This methodology is robust and highly reproducible. Also, it is easily available for numerous laboratories, because it uses standard PCR equipment. However, it has same drawbacks as any PCR followed by a restriction enzyme digestion, as it is time-consuming (4 h plus the DNA extraction time) and as possible enzymatic digestions problems may occur. On the other hand, the second method, based on MB technology, takes advantage of its power of allele discrimination and sensitivity. Both methods proved useful even with mixed DNAs. Of those two methods, the MB assay is the faster (1 h, starting with extracted DNA) for effective use in clinical applications.

As a potential methodological drawback, it could be argued that mutations in the MB hybridization zone could give false results. This is possible, since we detected mutations at the regions amplified by the ITS1 and ITS4 primers in 1.74% (5/288) of the strains analyzed. However, the MBs were designed to bind DNA sequences that are present in only one of the *C. parapsilosis* sensu lato species and absent in the others. For example, the *C. orthopsilosis* ITS MBs (Co-ITS-MBs) hybridize in an ITS region where *C. parapsilosis* sensu stricto and *C. metapsilosis* present a gap when the three sequences are aligned. AQ: F Finally, it was demonstrated that both methods of identification were specific for *C. parapsilosis* sensu lato species, since there were no amplicons produced when DNA from other 8 *Candida* spp. were tested. Moreover, they were designed for use after a classical identification procedure. Thus, although even other *Candida* or yeast species might be able to

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produce a positive PCR result with the described primers and MBs, this possibility is almost nil.

In conclusion, we propose two new identification methods that can differentiate these species and provide epidemiological information about these newly defined species.

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AQ: G

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 1

AQA—"Sensu stricto" and "sensu lato" have been changed throughout from italic to roman formatting per ASM style.

AQB—"Co-ITS-MBs," "Cp-ITS-MBs," and "Cm-ITS-MBs" spelled out as meant in Table 1 footnote a? If not, please clarify.

AQC—The sentence shows seven items related to eight items, respectively. Please correct as necessary. Also, please clarify what is respective to what for the three bands, two bands, and one band mentioned later in the paragraph or delete the word "respectively."

AQD—Please specify which 3 approved drugs are meant here.

AQE—Please specify which of the reference citations is meant by "a recent report." If a new reference must be added, please provide all relevant publication information; do not renumber the references.

AQF—"Co-ITS-MBs" spelled out as meant here? If not, please clarify.

AQG—Please note that per ASM's new reference style, references with 6 or more authors in the author line are abbreviated to include just the first author plus "et al."