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Rapid Identification of *Candida* Species with Species-Specific DNA Probes

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Rapid identification of *Candida* species has become more important because of an increase in infections caused by species other than *Candida albicans*, including species innately resistant to azole antifungal drugs. We previously developed a PCR assay with an enzyme immunoassay (EIA) format to detect amplicons from the five most common *Candida* species by using universal fungal primers and species-specific probes directed to the ITS2 region of the gene for rRNA. We designed probes to detect seven additional *Candida* species (*C. guilliermondii*, *C. kefyr*, *C. lambica*, *C. lusitaniae*, *C. pelliculosa*, *C. rugosa*, and *C. zeylanoides*) included in the API 20C sugar assimilation panel, five probes for species not identified by API 20C (*C. haemulonii*, *C. norvegica*, *C. norvegensis*, *C. utilis*, and *C. viswanathii*), and a probe for the newly described species *C. dubliniensis*, creating a panel of 18 *Candida* species probes. The PCR-EIA correctly identified multiple strains of each species tested, including five identified as *C. albicans* by the currently available API 20C database but determined to be *C. dubliniensis* by genotypic and nonroutine phenotypic characteristics. Species identification time was reduced from a mean of 3.5 days by conventional identification methods to 7 h by the PCR-EIA. This method is simple, rapid, and feasible for identifying *Candida* species in clinical laboratories that utilize molecular identification techniques and provides a novel method to differentiate the new species, *C. dubliniensis*, from *C. albicans*.

Rapid identification of *Candida* isolates to the species level in the clinical laboratory has become more important because the incidence of candidiasis continues to rise in proportion to a growing number of patients at risk for infection with *Candida albicans* and, recently, with innately azole-resistant non-*albicans Candida* species (5, 7, 29). This patient population has increased as a result of more intensive regimens of cancer therapy, complications of abdominal or cardiothoracic surgery, organ transplantations, burns, and trauma. Affected patients may be immunocompromised or not, and common risk factors include prolonged broad-spectrum antibiotic therapy, invasive devices such as indwelling Hickman catheters, and/or prolonged hospital stays (5, 7, 26). Under these conditions, an antibiotic-resistant replacement flora, including *Candida* species, can proliferate in the gut and invade deep tissues from mucosal foci. This is especially the case when mucosal integrity has been disrupted as a result of chemotherapy or surgery. In addition, as the number of risk factors increases, the odds of developing candidiasis multiply (26). Some *Candida* species, including *C. glabrata* and *C. krusei*, are emerging, possibly because they are innately less susceptible to azole drugs (16, 18, 29). In the case of *C. parapsilosis*, its ability to survive in the hospital environment, i.e., on the hands of healthcare workers, on intravenous devices, and in solutions, increases the possibility of its nosocomial transmission (5, 16). Consequently, rapid identification to the species level is necessary for more timely, targeted, and effective antifungal therapy and to facilitate hospital infection control measures.

Identification of *Candida* species by conventional morphology and assimilation tests can require 3 to 5 days or even longer for more difficult or unusual species (25). We previously employed universal fungal primers, multicopy gene targets, and species-specific probes directed to the ITS2 region of the rRNA-encoding gene (rDNA) to develop a rapid (1-day) PCR assay to detect candidemia (6, 20). Amplicons were detected in an enzyme immunoassay (EIA) format, and the method was referred to as PCR-EIA. Since the API 20C carbohydrate assimilation panel is limited to the identification of only certain species, DNA probes were designed to detect a total of 18 *Candida* species. Of these, the following 12 species can be identified by the current API 20C panel: *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lambica*, *C. lusitaniae*, *C. parapsilosis*, *C. pelliculosa*, *C. rugosa*, *C. tropicalis*, and *C. zeylanoides*. Five other, newly emerging *Candida* species, not identified by API 20C but readily identified by molecular probes, are *C. haemulonii*, *C. norvegensis*, *C. norvegica*, *C. utilis*, and *C. viswanathii*. A species-specific probe for the newly described *Candida* species, *C. dubliniensis*, was also designed. The resulting PCR-EIA identification matrix is simple, rapid, sensitive, and feasible for identifying *Candida* species in clinical laboratories.

MATERIALS AND METHODS

Microorganisms. Clinical isolates or cultures obtained from the American Type Culture Collection (ATCC) were used in this study (see Tables 2 and 5). Isolates of *Candida* spp., *Cryptococcus humicolus*, *Stephanoascus ciferrii*, and *Trichosporon cutaneum* were grown in 50-ml Erlenmeyer flasks by seeding one 10-μl loopful of growth from an agar slant into 10 ml of YPD broth (1% yeast extract, 2% Bacto Peptone, 2% dextrose; Difco Laboratories, Detroit, Mich.). *Cryptococcus neoformans* serotypes A, B, C, and D were grown similarly; however, YPD broth was supplemented with 2.9% NaCl to reduce capsule formation. All broth cultures were grown at 35°C for 18 h in a rotary shaker set at 150 rpm prior to DNA extraction for prototype testing.

DNA isolation. DNA was extracted from all yeast species by using the Puregene DNA Isolation Kit (Gentra Systems Inc., Minneapolis, Minn.). This kit facilitates the rapid recovery of sufficient DNA for PCR amplification and allows multiple samples to be extracted in parallel. For example, multiple yeast isolates could be extracted at the same time so that a large number of samples could be processed quickly and efficiently on a given day. DNAs from filamentous and dimorphic fungi were obtained as previously described (6) or were a gift from

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TABLE 1. Synthetic oligonucleotides used in PCR and hybridization analyses

Primer or probe[a]	Nucleotide sequence (5′ to 3′)	Chemistry and source
ITS3	GCA TCG ATG AAG AAC GCA GC	5.8S rDNA universal 5′ primer
ITS4	TCC TCC GCT TAT TGA TAT GC	28S rDNA universal 3′ primer
BP	CA TGC CTG TTT GAG CGT C(GA)T T	5′-end-labeled biotin probe; generic 5.8S rDNA probe
CA	AT TGC TTG CGG CGG TAA CGT CC	5′-end-labeled digoxigenin probe; ITS2 region of *C. albicans*
DB	AA GGC GGT CTC TGG CGT CGC CC	5′-end-labeled digoxigenin probe; ITS2 region of *C. dubliniensis*
CG	TA GGT TTT ACC AAC TCG GTG TT	5′-end-labeled digoxigenin probe; ITS2 region of *C. glabrata*
CGE	TT TAC CAA CTC GGT GTT GAT CT	5′-end-labeled digoxigenin probe; ITS2 region of *C. glabrata*
GU	CC CGG CCT TAC AAC AAC CAA AC	5′-end-labeled digoxigenin probe; ITS2 region of *C. guilliermondii*
CH	CC GTT GGT GGA TTT GTT TCT AA	5′-end-labeled digoxigenin probe; ITS2 region of *C. haemulonii*
KF	GA GAC TCA TAG GTG TCA TAA AG	5′-end-labeled digoxigenin probe; ITS2 region of *C. kefyr*
CK	GG CCC GAG CGA ACT AGA CTT TT	5′-end-labeled digoxigenin probe; ITS2 region of *C. krusei*
LA2	AA AGC GAG GGG CCT TCT GCG CG	5′-end-labeled digoxigenin probe; ITS2 region of *C. lambica*
LA4	GC GAG GGG CCT TCT GCG CGA AC	5′-end-labeled digoxigenin probe; ITS2 region of *C. lambica*
LU	CT CCG AAA TAT CAA CCG CGC TG	5′-end-labeled digoxigenin probe; ITS2 region of *C. lusitaniae*
NS	AC TGA GCG AAG TAC ACA ACA CT	5′-end-labeled digoxigenin probe; ITS2 region of *C. norvegensis*
NC	AC GAG CGT CTG CTG GCT CCA CA	5′-end-labeled digoxigenin probe; ITS2 region of *C. norvegica*
CP	AC AAA CTC CAA AAC TTC TTC CA	5′-end-labeled digoxigenin probe; ITS2 region of *C. parapsilosis*
PL	AT CAG CTA GGC AGG TTT AGA AG	5′-end-labeled digoxigenin probe; ITS2 region of *C. pelliculosa*
CR	AG TTA AGC TTG TTA CAG ACT CA	5′-end-labeled digoxigenin probe; ITS2 region of *C. rugosa*
CT	AA CGC TTA TTT TGC TAG TGG CC	5′-end-labeled digoxigenin probe; ITS2 region of *C. tropicalis*
CU2	AC TCG TTA TTT TCC AGA CAG AC	5′-end-labeled digoxigenin probe; ITS2 region of *C. utilis*
VS	CT ACC AAA ACG CTT GTG CAG TC	5′-end-labeled digoxigenin probe; ITS2 region of *C. viswanathii*
CZ	TC GTT GAC CAG TAT AGT ATT TG	5′-end-labeled digoxigenin probe; ITS2 region of *C. zeylanoides*

[a] Patents have been issued or are pending for all *Candida* species probes.

Liliana de Aguirre, Instituto Investigaciones Veterinarias, Maracay, Venezuela. Quantification of DNA was performed by using a fluorometer and Hoechst 33258 Dye (Dyna Quant 200; Pharmacia Biotech, Piscataway, N.J.). DNA was diluted in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) so that a total of 1 ng of template DNA was added to each PCR vial.

Oligonucleotide synthesis of primers and probes. Oligodeoxyribonucleotide primers and probes were synthesized as described previously (6). Universal fungal primers ITS3 and ITS4 (28) were used to amplify the ITS2 region. Oligonucleotide probes were designed from sequence data for the ITS2 region of the *Candida* sp. rDNA (13, 14).

PCR amplification. The reaction mixture (100 μl) contained 10 μl of 10× PCR buffer (100 mM Tris-HCl, 500 mM KCl [pH 8.3]; Boehringer Mannheim, Indianapolis, Ind.), 6 μl of 25 mM $MgCl_2$, 8 μl of a deoxynucleotide triphosphate mixture (1.25 mM each dATP, dCTP, dGTP, and dTTP), 1 μl of each primer (20 μM), 2.5 U of *Taq* DNA polymerase (TaKaRa Shuzo Co., Ltd., Shiga, Japan), 2 μl of template DNA (0.5 ng/μl), and sterile distilled water to bring the total volume to 100 μl. Vials were placed in the heating block of a model 9600 thermal cycler (Perkin-Elmer, Emeryville, Calif.) equilibrated at 95°C. PCR amplification conditions were 5 min of denaturation at 95°C, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. A final extension step of 72°C for 5 min was then conducted. Appropriate positive and negative controls were included, and PCR contamination precautions were followed (6, 9).

Agarose gel electrophoresis. Electrophoresis was conducted in TBE (0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA [pH 8.4]) buffer at 76 V for approximately 1 h in gels composed of 1% (wt/vol) agarose (Boehringer Mannheim) and 1% (wt/vol) NuSieve (FMC Bioproducts, Rockland, Maine). Gels were stained with 0.5 μg of ethidium bromide per ml of deionized water for 30 min, followed by a 30-min wash in deionized water. DNA bands confirming a positive PCR were visualized with a UV transilluminator and photographed.

PCR-EIA. PCR-amplified DNA was hybridized to species-specific digoxigenin-labeled probes and to a generic biotinylated probe, and then the complex was added to streptavidin-coated microtitration plates and captured as previously described (6, 20). A colorimetric EIA was then conducted to detect captured DNA by using horseradish peroxidase-conjugated anti-digoxigenin antibodies (6, 20) in a manner very similar to that of other EIAs performed routinely in many clinical microbiology laboratories. All probes were tested in a matrix format against DNA from other *Candida* species, as well as against DNAs from other fungi (see Table 3). In this manner, all probes were tested against all of the target DNAs so that fungi could be identified by a discrete pattern of reactivity. When a probe cross-reacted with heterologous DNA, probes specific to the heterologous DNA were designed. Therefore, use of both probes as part of the matrix allows species-specific identification by a process of elimination and does not require additional steps or retesting of samples because all probes and all targets are included in the complete matrix from the beginning.

Statistical analyses. Student's *t* test was used to determine significant differences between mean absorbance values of homologous and nonhomologous probe reactions. Differences were considered significant when the value of *P* was less than or equal to 0.05.

Nucleotide sequence accession numbers. The GenBank accession numbers for *C. dubliniensis* and *C. pelliculosa* sequences are U96719 and U96720, respectively. The accession numbers for the DNA sequences of the other *Candida* species used in this study are published in references 13 and 14.

RESULTS

Specificity of digoxigenin-labeled probes. Eighteen *Candida* species probes (Table 1) were designed and tested in the PCR-EIA against the DNAs from the fungi listed in Table 2. The absorbance value obtained for each probe tested against its homologous DNA was significantly greater than that obtained when probes were tested against nonhomologous DNA ($P \leq 0.05$), with two exceptions (Table 3). In the first instance, the probe for *C. guilliermondii* (GU) cross-reacted with DNA from *C. zeylanoides* (CZ). However, the probe for *C. zeylanoides* (CZ) did not cross-react with DNA from *C. guilliermondii* (GU). Therefore, by using both probes as part of the complete matrix, species-specific identification could be achieved by a process of elimination (Table 3). In the second instance, as previously described (6), the probe for *C. glabrata*, CG, cross-reacted with *Saccharomyces cerevisiae* DNA (mean absorbance, 0.882). In the present study, the CG probe also cross-reacted with *C. pelliculosa* and *C. utilis* DNAs, which had not been tested previously (mean absorbance ± standard deviation [SD], 0.810 ± 0.197 and 0.800 ± 0.648, respectively). Therefore, the CG probe was redesigned, resulting in the elimination of cross-reactions with all of the species tested except *S. cerevisiae* while retaining positive reactivity with homologous *C. glabrata* DNA (new CG probe named CGE, Table 3). Preliminary testing of an *S. cerevisiae* probe indicates that it does not cross-react with *C. glabrata* DNA (data not shown), allowing the differentiation of *C. glabrata* DNA from *S. cerevisiae* DNA.

All of the negative controls tested, except *S. cerevisiae* and *C. zeylanoides*, as mentioned above, gave mean optical density OD values (± SD) ranging from 0.001 ± 0.001 to 0.009 ±

TABLE 2. Microorganisms tested against all probes

Organism	Strain	Source
Candida albicans	B311	CDC[a] Mycology Reference Laboratory, human
Candida dubliniensis	CBS 7987	Type culture, human tongue
Candida glabrata	Y-65	Type culture, feces
Candida guilliermondii	ATCC 6260	Type culture, bronchitis
Candida haemulonii	ATCC 22991	Type culture, gut contents of fish
Candida kefyr	ATCC 46764	Clinical isolate
Candida krusei	CDC 259-75	CDC Mycology Reference Laboratory
Candida lambica	ATCC 24750	Type culture, beer
Candida lusitaniae	ATCC 34449	Type culture, pig
Candida norvegensis	ATCC 22977	Type culture, sputum
Candida norvegica	ATCC 36586	Type culture, sputum
Candida parapsilosis	ATCC 22019	Type culture, sprue
Candida pelliculosa	ATCC 8168	Type culture
Candida rugosa	ATCC 10571	Type culture, human feces
Candida tropicalis	CDC 38	CDC Mycology Reference Laboratory
Candida utilis	ATCC 22023	Type culture, factory
Candida viswanathii	ATCC 22981	Type culture, cerebrospinal fluid
Candida zeylanoides	ATCC 7351	Type culture, blastomycotic macroglossia
Aspergillus flavus	ATCC 11497	Environmental isolate
Aspergillus fumigatus	ATCC 36607	Clinical isolate
Aspergillus nidulans	ATCC 10074	Unspecified
Aspergillus niger	ATCC 16404	Environmental isolate
Aspergillus terreus	ATCC 7860	Unspecified
Blastomyces dermatitidis	CDC B4478	CDC Mycology Reference Laboratory, dog
Candida catenulata	ATCC 18812	Perleche
Candida catenulata	ATCC 10565	Type culture, human feces
Candida famata	ATCC 36239	Type culture
Cryptococcus humicolus	ATCC 14438	Type culture, soil
Cryptococcus humicolus	ATCC 38294	Human leg
Cryptococcus neoformans serotypes A, B, C, D	A, 9759-MU-1; B, BIH409; C, K24066TAN; D, 9375	Reference strains from R. Cherniak, Georgia State University
Histoplasma capsulatum	G217B	CDC Mycology Reference Laboratory
Penicillium marneffei	CDC B3420	CDC Mycology Reference Laboratory, human lymph node
Saccharomyces cerevisiae	AB972	Baker's yeast
Stephanoascus ciferrii	ATCC 22873	Type culture of *Candida ciferri*, neck of cow
Trichosporon cutaneum	ATCC 34148	Clinical isolate

[a] CDC, Centers for Disease Control and Prevention.

0.025 (Table 3). Minor cross-reaction was observed with the VS probe against *C. tropicalis* DNA (0.175 ± 0.093); however, the CT probe did not cross-react with *C. viswanathii* DNA (0.001 ± 0.001). Similarly, minor cross-reactions were observed when the CT and CU2 probes were used versus *Aspergillus terreus* (0.114 ± 0.052 and 0.147 ± 0.035, respectively), but an *A. terreus*-specific probe has been developed and has been reported separately (3). Finally, the CK probe demonstrated a minor cross-reaction with *C. lambica* DNA (0.114 ± 0.042), but the LA probe gave no cross-reaction with *C. krusei* DNA (0.002 ± 0.003). Therefore, all species could be differentiated by a process of elimination. In addition, compared to the absorbance values for their respective positive controls, the significantly weaker cross-reactions of the negative controls could be easily discriminated visually.

In addition, the ITS2 regions from seven strains of *Candida famata* were sequenced. Although all of the strains tested were obtained from the ATCC as *C. famata* or its teleomorph *Debaryomyces hansenii*, each strain showed sequence heterogeneity in this region (data not shown), suggesting that this species is a taxonomic complex of more than one species. An ITS2 probe designed for specificity to one of these strains was found to hybridize only with its own DNA and not to the DNA from any of the other four *C. famata* strains tested. None of the other non-*C. famata Candida* sp. probes reacted with DNA from the type culture of *C. famata* (ATCC 36239), indicating

TABLE 3. Specificities of DNA probes

Species	Probe	Specificity for:	
		Same species[a]	Other species/genera[b]
C. albicans	CA	0.426 ± 0.275	0.002 ± 0.002
C. dubliniensis	DB	0.322 ± 0.119	0.004 ± 0.007
C. glabrata	CGE	1.034 ± 0.343	0.006 ± 0.011 (0.755 ± 0.229)[b]
C. guilliermondii	GU	0.821 ± 0.303	0.014 ± 0.026 (0.639 ± 0.172)[b]
C. haemulonii	CH	0.841 ± 0.332	0.002 ± 0.002
C. kefyr	KF	1.184 ± 0.384	0.003 ± 0.006
C. krusei	CK	0.389 ± 0.136	0.009 ± 0.025
C. lambica	LA	0.640 ± 0.175	0.002 ± 0.002
C. lusitaniae	LU	0.483 ± 0.162	0.002 ± 0.001
C. norvegensis	NS	0.716 ± 0.242	0.006 ± 0.004
C. norvegica	NC	0.258 ± 0.102	0.004 ± 0.004
C. parapsilosis	CP	0.493 ± 0.231	0.003 ± 0.004
C. pelliculosa	PL	0.624 ± 0.320	0.003 ± 0.004
C. rugosa	CR	0.222 ± 0.098	0.002 ± 0.001
C. tropicalis	CT	0.870 ± 0.354	0.006 ± 0.020
C. utilis	CU2	1.161 ± 0.193	0.006 ± 0.024
C. viswanathii	VS	0.988 ± 0.419	0.006 ± 0.028
C. zeylanoides	CZ	0.361 ± 0.170	0.001 ± 0.001

[a] Mean A_{650} value ± SD for 22 to 86 test samples.

[b] No significant ($P < 0.05$) cross-hybridization of probe with DNAs from 37 other fungi which are listed in Table 2, except for the CGE probe with DNA from *S. cerevisiae* and the GU probe with DNA from *C. zeylanoides* (values in parentheses).

TABLE 4. Consistency of A_{650} values for isolates within a given species

Species	Probe	No. of strains tested (group)	Mean A_{650} ± SD[a]
C. guilliermondii	GU	5	1.088 ± 0.396
C. haemulonii	CH	2 (1)	0.790 ± 0.122
C. haemulonii	CH	1 (2)	0.088 ± 0.026[b]
C. kefyr	KF	3	1.299 ± 0.270
C. lambica	LA	2	0.640 ± 0.166
C. lusitaniae	LU	2	0.658 ± 0.164
C. norvegensis	NS	2	0.588 ± 0.175
C. parapsilosis	CP	3 (I)	0.477 ± 0.209
C. parapsilosis	CP	3 (II)	0.489 ± 0.206
C. parapsilosis	CP	2 (III)	0.158 ± 0.068[b]
C. pelliculosa	PL	6	0.693 ± 0.250
C. rugosa	CR	3	0.234 ± 0.070
C. tropicalis	CT	2	1.244 ± 0.083
C. utilis	CU2	3	1.105 ± 0.399
C. zeylanoides	CZ	4	0.485 ± 0.128

[a] Mean values for 6 to 22 test samples.
[b] Each probe listed detected multiple isolates of the same species, except for the *C. haemulonii* probe against one *C. haemulonii* isolate, strain 90.00.3593 ($P < 0.001$) and for the *C. parapsilosis* probe against DNA from *C. parapsilosis* group III isolates ($P < 0.01$).

that *C. famata* (type culture) would not be misidentified as another *Candida* sp. by using these probes.

Multiple strains of several *Candida* species were tested, and some inherent variability in probe hybridization for strains of the same species was apparent in the range of standard deviations of the absorbance values observed (Table 4). However, all strains gave absorbance signals of sufficient strength to allow differentiation of truly positive from truly negative samples, with two exceptions. The CP probe tested against *C. parapsilosis* Lehmann group III DNAs (12) and the CH probe for *C. haemulonii* tested against *C. haemulonii* group 2 (strain 90.00.3593) DNA were significantly less reactive than with the respective positive control strains. These discrepant cases may indicate a finer taxonomic discrimination of isolates by genotypic than phenotypic methods (10–12, 30). Alternatively, combinations of probes could be designed for detection of all groups of *C. parapsilosis* and all groups of *C. haemulonii* in a clinical setting.

Species-specific probes were also designed to discriminate between two species that have a phenotype in common. A new species, *C. dubliniensis*, first described by Sullivan et al. (23), is typically identified as *C. albicans* by routine phenotypic methods. The probes designed in this study readily discriminated *C. albicans* from *C. dubliniensis* (Table 5). The CA probe which detected *C. albicans* DNA did not react with DNA from any *C. dubliniensis* strain tested, and the DB probe for *C. dubliniensis* identification did not hybridize with DNA from any *C. albicans* strain tested. The CA probe also detected both *C. stellatoidea* type I and II DNAs and differentiated *C. stellatoidea* DNA from *C. dubliniensis* DNA (Table 5).

DISCUSSION

Previous research in this laboratory demonstrated that five *Candida* species-specific probes could be designed and adapted to a simple PCR-EIA format to detect *Candida* species DNA (6, 20). This report extends the range of probes to include a test matrix of 18 *Candida* species that is capable of complementing species identification by the API 20C carbohydrate assimilation system. Sixteen of the probes are totally specific and can be used to identify their respective *Candida* species, including *C. dubliniensis*. In addition, *C. stellatoidea* types I and II (10) can be differentiated from *C. dubliniensis* by these probes. The *C. guilliermondii* probe (GU) cross-reacted with *C. zeylanoides* DNA, but the *C. zeylanoides* probe (CZ) did not cross-react with *C. guilliermondii* DNA, allowing species-specific identification by a process of elimination. Future studies will attempt to design a species-specific probe for each. However, at present, positive identification can still be achieved by a process of elimination by using both probes in the matrix configuration. As designed, all of the probes can be used in the matrix at the same time so that all possible combinations of probes and target DNAs can be tested in a single run. As more probes are added to the matrix, automation and chip array technologies become attractive ways to identify large numbers of different organisms simply and rapidly. The previously published probe for *C. glabrata*, CG, was found to cross-react with *C. pelliculosa* and *C. utilis* DNAs in this study. Therefore, a new probe, CGE, was designed which eliminated all cross-reactions except for that with *S. cerevisiae* DNA. Such cross-reactivity should have little impact on clinical diagnosis, however, since it is unlikely, although not impossible, that *S. cerevisiae* would be found in blood cultures or other normally sterile deep tissue sites. Also, since clinical isolates of both *C. glabrata* and *S. cerevisiae* are innately fluconazole resistant (21, 24, 31), clinical treatment decisions would most likely not be negatively impacted by the inability to discriminate *C. glabrata* from *S. cerevisiae*. An *S. cerevisiae* probe has been developed (data not shown), and preliminary testing indicates that it is specific for *S. cerevisiae* detection. Therefore, this probe could be used in conjunction with the CGE probe to differentiate the two species by a process of elimination.

Standardization of DNA extraction for all *Candida* species is facilitated by using a broth culture method and a commercially available extraction kit. To facilitate prototype testing, isolates were grown overnight to obtain sufficiently large quantities of

TABLE 5. Differentiation of *C. albicans* from *C. dubliniensis* by species-specific probes

Target DNA	Mean A_{650} ± SD[b]	
	C. albicans probe	*C. dubliniensis* probe
C. albicans[a]		
ATCC 11006 (*C. stellatoidea* type I)	0.485 ± 0.252	0
ATCC 36232 (*C. stellatoidea* type I)	0.409 ± 0.178	0
ATCC 20408 (*C. stellatoidea* type II)	0.370 ± 0.168	0
B36	0.568 ± 0.236	0
Q10	0.640 ± 0.192	0
Lecocq	0.464 ± 0.124	0
2730	0.631 ± 0.192	0
3153A	0.528 ± 0.068	0
Mean A_{650} for CA probe	0.512 ± 0.095	0
C. dubliniensis[c]		
M1	0	0.351 ± 0.090
M4	0	0.407 ± 0.186
P30	0	0.346 ± 0.088
1419-2	0	0.402 ± 0.132
901013	0	0.393 ± 0.101
Mean A_{650} for DB probe	0	0.380 ± 0.029

[a] Strains not obtained from ATCC were obtained from the Centers for Disease Control and Prevention Mycology Reference Laboratory.
[b] Mean values for 4 to 10 replicates for each isolate.
[c] Strains were received from S. Lockhart, D. Ahearn, and S. Meyer.

DNA for repeated analyses and probe development. In the clinical laboratory setting, sufficient DNA for routine testing, derived from primary cultures without subculturing, would further shorten the time required for species identification. Indeed, even species contained in mixed yeast cultures (*C. albicans* and *C. glabrata*) have been correctly identified from primary cultures in our laboratory by using species-specific probes (20).

All of the currently available commercial tests for species identification, such as the API 20C system, RapID, etc., require subculturing from clinical specimens to obtain pure cultures before inoculation of the test panels. Therefore, even if an overnight culture were required prior to PCR-EIA testing, the time to species identification after obtaining a pure culture is still reduced to 7 h rather than a mean of 3.5 days by conventional phenotypic identification methods. Also, species identification of unusual species such as *C. norvegensis* or *C. utilis* by conventional methods may require up to 4 or 5 weeks (25), whereas the PCR-EIA can identify these species in a single day. Since some species are innately resistant to certain drugs, e.g., *C. krusei* to fluconazole (16, 18, 29), accurate and timely species identification is important for selection of appropriately targeted therapy.

The recently described species *C. dubliniensis* was discovered when DNA from phenotypically identified "*C. albicans*" strains did not react with the *C. albicans*-specific mid-repetitive element Ca3 (2, 23). Molecular differences between *C. albicans* and *C. dubliniensis* were confirmed in the current research in that sufficiently significant sequence differences occurred in the ITS2 region to facilitate the development of species-specific probes. Although *C. dubliniensis* is not currently listed as one of the yeasts identified in the API 20C database, differences between *C. albicans* and *C. dubliniensis* in the assimilation of xylose and α-methyl-D-glucoside may prove useful for the phenotypic differentiation of these species (19). In addition, other physical (growth temperature) or chemical (β-glucosidase activity) tests may also help discriminate *C. albicans* from *C. dubliniensis* (17, 22).

The *C. dubliniensis* strains listed in Table 5 were originally obtained in Europe and Australia. *C. dubliniensis* has also been isolated in North America, as reported by Kleinegger et al. (8) and Boucher et al. (1) in 1996. We confirmed the presence of *C. dubliniensis* in North America by using our probes to test DNA obtained from oropharyngeal isolates from a population of human immunodeficiency virus-positive persons in the Atlanta, Ga. area (4). To our knowledge, these are the first isolates of *C. dubliniensis* recovered from a human immunodeficiency virus-positive population in the United States (4, 19).

This genotype-based identification method has revealed the need for further taxonomic resolution of some species, for example, *C. famata* (teleomorph form, *D. hansenii*). Although the identities of seven *C. famata* strains tested in this study were confirmed by conventional phenotypic methods (data not shown), the ITS2 probe designed for one of these strains was found to hybridize only with its own DNA and not to the DNA of any of the other four *C. famata* strains tested. When the sequences of the ITS2 regions of these seven strains were determined and compared, greater differences were observed among these strains than among strains of other *Candida* species tested. Therefore, the complexity of this taxon is apparent and agrees with the DNA-DNA hybridization studies of Nishikawa et al. (15), who described a low percentage of hybridization between some DNAs from several *C. famata* strains. Because *C. famata* appears to be a taxonomic complex, further studies are needed to determine sequences which will allow the detection of clinically encountered members of this complex either by redefinition of these strains into subspecies or by the use of combinations of probes to simultaneously detect members of the complex. Additional molecular characterization is needed to clarify the taxonomy and identification of strains which appear to be *C. famata* by phenotypic criteria but differ by genotypic criteria.

Lower absorbance values were obtained for some strains of *C. parapsilosis* (group III) and *C. haemulonii* (group 2) than for their positive control strains. However, these values were still significantly greater than those for their respective negative controls. Because of the controversy surrounding *C. parapsilosis* group III and *C. haemulonii* group 2, the designation of a truly positive cutoff value for these groups awaits taxonomic resolution. The clinical significance of *C. parapsilosis* group III and *C. haemulonii* group 2 is not known, nor are the true incidence and prevalence of these groups. However, the CP probe correctly identified all clinical blood isolates of *C. parapsilosis* in a previous study (20). Therefore, it is likely that the CP probe, at least, can identify clinically relevant strains. The same is yet to be determined for the CH probe. In addition, combinations of probes, used in one reaction mixture, to identify all groups of *C. parapsilosis* and all groups of *C. haemulonii* could be designed if such a need were identified in the clinical setting.

The present method of sample preparation and PCR-EIA is amenable to automation, and the entire panel of 18 different probes can be tested against an unknown yeast in a simple microtitration plate format. Greater numbers of isolates will be tested in prospective clinical studies to validate the specificity of each probe. These probes were designed to detect 1 ng of target DNA recovered from *Candida* sp. cultures, and the limit of their sensitivity has not yet been determined in clinical samples. Previous studies using positive blood culture bottles suggest that these probes may be useful for the direct identification of *Candida* species from primary cultures (20). Minor probe cross-reactions should not be problematic, since a process of elimination with specific, non-cross-reacting, paired probes allows specific differentiation (e.g., the CK probe and *C. lambica* DNA). When sensitivity limits become an issue, such as in clinical samples containing unknown quantities of DNA, then a matrix of probes will differentiate truly positive samples from truly negative samples. Ultimately, once a sufficient panel of medically important *Candida* and/or yeast species has been constructed, the ideal assay will consist of probes attached to a nylon membrane or to wells of a microtiter plate, a single universal PCR using premixed PCR reagents, and the colorimetric development of the matrix array in a single assay. Commercially prepared, premixed PCR reagents are available, and prototype assays with such configurations have already been examined in our laboratory (27) and continue to be improved to optimize the speed and simplicity of species-specific yeast identification.

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