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Resolution of Discrepant Results for *Candida* Species Identification by Using DNA Probes

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Candida **species bloodstream isolates were collected from institutions participating in an active, population-based surveillance for candidemia. Species identifications were performed locally and then confirmed at the Centers for Disease Control and Prevention (CDC) by phenotype-based methods. Discrepancies in species identification between the referring institution and the CDC were noted for 43 of 935 isolates (4.6%). A DNA probe-based species identification system (PCR-enzyme immunoassay [EIA]) was then used to resolve these discrepancies. The PCR-EIA result was identical to the CDC phenotypic identification method for 98% of the isolates tested. The most frequently misidentified species was *Candida glabrata* (37% of all discrepant identifications). Such misidentifications could lead to the administration of inappropriate therapy given the propensity of *C. glabrata* to develop resistance to azole antifungal drugs.**

Candida species are the fourth most common cause of health care-associated bloodstream infections and are increasingly important causes of morbidity in hospitalized patients (5, 10). The emergence of non-*Candida albicans* species, including those innately or adaptively less susceptible to azole antifungals (14, 15), makes the identification of bloodstream isolates to the species level important for the implementation of appropriate antifungal therapy. Species identification is also important for an understanding of the epidemiology of candidemia, including trends in species distribution and antifungal drug susceptibility patterns.

Candida species have traditionally been identified by a combination of phenotypic tests that assess morphological characteristics and carbohydrate assimilation and fermentation patterns (9, 11). Whereas presumptive identification of *C. albicans* may be obtained in a few hours, identification of non-*C. albicans* species may require up to 72 h (8, 9, 17, 18). More recently, fungus-specific PCR primers and *Candida* species-specific DNA probes, directed to the internal transcribed spacer 2 (ITS2) region of ribosomal DNA (rDNA), have been used to detect PCR amplicons in a colorimetric enzyme immunoassay format (PCR-EIA) (6). This test has been shown to be highly specific, rapid, and easy to perform. Therefore, we used the PCR-EIA to resolve discrepancies in *Candida* species identifications between referring institutions and the Centers for Disease Control and Prevention (CDC) laboratory as part of an active, population-based surveillance for candidemia.

Collection and identification of bloodstream isolates. Bloodstream isolates were obtained from institutions in the state of Connecticut and the city and county of Baltimore, Md., from October 1998 to September 2000 (10a). All blood cultures positive for *Candida* species were identified at the referring institution according to their standard methods. In describing their blood culture identification practices, 23 of 51 responding laboratories (45%) used the germ tube formation test to identify *C. albicans*. Forty-one respondents (80%) used some type of carbon assimilation/biochemical panel to identify non-*C. albicans* species, with 20 (39%) using the API 20C system (bioMerieux Vitek, Inc., Hazelwood, Mo.) and 13 (26%) using the Vitek yeast biochemical card (bioMerieux Vitek).

A total of 935 isolates were sent to the Mycotic Diseases Branch, CDC, for confirmation of species identification. At the CDC, the isolates were first subcultured onto Sabouraud dextrose agar (BBL Difco Laboratories, Detroit, Mich.) as well as onto CHROMagar *Candida* medium (DRG International, Mountainside, N.J.). Isolates were then identified to the species level with the API 20C AUX (bioMerieux Vitek) or RapID Yeast Plus system (Innovative Diagnostics, Norcross, Ga.) and by microscopic morphology on cornmeal-Tween 80 (Dalmau) plates.

Discrepancies in the phenotypic identification between the CDC and the referring institution were resolved by using species-specific DNA probes in an EIA detection format (PCR-EIA) (6). *Candida* species isolates were grown for 18 h at 35°C in 50-ml Erlenmeyer flasks containing 10 ml of YPD broth (1% yeast extract, 2% Bacto Peptone, 2% dextrose; BBL Difco Laboratories). DNA was isolated from these cultures with the PUREGENE DNA Purification kit for yeast and gram-positive bacteria (Gentra Systems, Inc., Minneapolis, Minn.) according to the manufacturer's directions. Universal fungus-specific primers ITS3 (5′ GCA TCG ATG AAG AAC GCA GC 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (19) were then used to amplify by PCR a portion of the 5.8S rDNA region, the entire ITS2 rDNA region, and a portion of the 28S rDNA region using a Perkin-Elmer (Emeryville, Calif.) model 9700 thermal cycler and *Taq* DNA polymerase (Roche Molec-

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ular Biochemicals, Inc., Indianapolis, Ind.). All other PCR reagents and thermal cycling conditions used were as previously described (6). Amplicons were captured onto a streptavidin-coated microtitration plate (Roche) with a biotinylated, all-*Candida* species DNA probe (5′ CAT GCC TGT TTG AGC GTC [GA]TT 3′) and were detected with digoxigenin-labeled species-specific DNA probes (*C. albicans*: 5′ AT TGC TTG CGG CGG TAA CGT CC 3′; *C. glabrata*: 5′ TT TAC CAA CTC GGT GTT GAT CT 3′; *C. krusei*: 5′ GG CCC GAG CGA ACT AGA CTT TT 3′; *C. lusitaniae*: 5′ CT CCG AAA TAT CAA CCG CGC TG 3′; *C. parapsilosis*: 5′ AC AAA CTC CAA AAC TTC TTC CA 3′; *C. tropicalis*: 5′ AA CGC TTA TTT TGC TAG TGG CC 3′) and horseradish peroxidase-labeled antidigoxigenin antibodies in a colorimetric EIA format (6). Oligonucleotide primers and probes were synthesized and labeled as described previously (12).

Discrepancies in species identification between the CDC's phenotypic methods and the PCR-EIA were resolved by rDNA sequencing on a Perkin-Elmer ABI Prism 310 automated capillary DNA sequencer as previously described (7). Briefly, universal fungus-specific primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) (19) and ITS4 were used to amplify by PCR a portion of the 18S rDNA region; the entire ITS1, 5.8S, and ITS2 rDNA regions; and a portion of the 28S rDNA region. Amplicons were purified with the QIAquick PCR purification kit (Qiagen, Valencia, Calif.) and sequenced on both strands with primers ITS1 or ITS4 and the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.) (7). GenBank searches and comparative sequence analyses were assisted by using BLAST search tools (1) and GeneTool, version 1.0, software (BioTools, Inc., Edmonton, Alberta, Canada), respectively.

Distribution and resolution of discrepancies in *Candida* species identification. Of the 935 isolates received at the CDC, there were discrepancies in species identification between the referring institution and the CDC laboratory for 43 (4.6%). In all but one case, the CDC identification based on biochemical and morphological criteria was validated by the PCR-EIA (Table 1). In the remaining case, an isolate reported as *C. albicans* by the referring institution and as *C. parapsilosis* or *C. lusitaniae* by the CDC was ultimately identified as *C. lusitaniae* by probe testing and DNA sequence analysis (GenBank accession number AY383555). All isolates identified at the CDC as *C. albicans* by phenotypic methods were subsequently screened by molecular identification methods (2, 10a) to differentiate isolates of *C. dubliniensis* from those of *C. albicans*. Nine cases of *C. dubliniensis* candidemia were identified. All *C. dubliniensis* isolates were reported to be *C. albicans* by the referring institutions, but these identifications were not considered to be discrepant as *C. dubliniensis* is not routinely differentiated from *C. albicans* by most clinical laboratories.

The most frequent misidentifications by the referring institutions were of *C. glabrata* (16 of 43 isolates or 37% of all discrepant identifications), followed by *C. parapsilosis* (15 of 43, 35%), *C. tropicalis* (7 of 43, 16%), *C. albicans* (3 of 43, 7%), and *C. lusitaniae* (2 of 43, 5%) (Table 1). These misidentifications represent 12 (15 of 123), 7 (16 of 226), 6 (7 of 118), and 0.7% (3 of 423) of all *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. albicans* isolates, respectively. The most common misidentifications by the referring institutions were *C. albicans* for *C. glabrata* (7 of 43 isolates or 16% of all misidentifications), *C. albicans* for *C. parapsilosis* (6 of 43, 14%), *C. tropicalis* for *C. glabrata* (5 of 43, 12%), and *C. albicans* for *C. tropicalis* (4 of 43, 9%).

Of all isolates reidentified at the CDC that could be associated with a given type of institution, 618 (66%) were from nonacademic institutions and 316 (34%) were from academic institutions. Comparison of the misidentification rate between academic (university- or medical school-associated) and nonacademic institutions showed that, for the 41 of 43 isolates that could be associated with a particular category of institution, 34 (83%) of the misidentifications were from nonacademic institutions whereas 7 (17%) were from academic institutions. As a percentage of the total number of isolates received from academic versus nonacademic institutions, the overall misidentification rate for academic institutions was roughly one-half that for the nonacademic institutions (i.e., 2.2 versus 5.5%, respectively). Misidentifications were not associated with any one particular institution.

The vast majority (15 of 16, 94%) of misidentified isolates that were reidentified as *C. glabrata* in the CDC laboratory had typical biochemical profiles and morphologies on Dalmau plates and gave the expected colony color and appearance on CHROMagar *Candida* medium (Table 1). In contrast, 53% of *C. parapsilosis* isolates and 71% of *C. tropicalis* isolates showed profiles interpreted as "acceptable" to "low discrimination" by the API 20C AUX system; these isolates were differentiated from the alternative species choices listed in the API 20C AUX profile index by microscopic morphology and colony color on CHROMagar *Candida* medium (Table 1). *C. albicans* isolates could also be identified by their distinctive colony color on CHROMagar *Candida* medium and by their capacity to form chlamydospores (Table 1).

Specificity of DNA probes to identify *Candida* species. The PCR-EIA generated results that were highly specific, the DNA did not cross-react with DNA from other *Candida* species tested, and the results were easy to interpret (Table 2). Mean positive EIA values ± standard errors (SE) ranged from 0.95 ± 0.10 for DNA from *C. tropicalis* to 0.38 ± 0.02 for DNA from *C. lusitaniae*. Inherent differences in absolute EIA values obtained for each of the probes may reflect differences in their G+C compositions and, as a result, their rate of denaturation and annealing during thermal cycling or their rate of hybridization during probe attachment to the PCR product. Nonetheless, the EIA values reported here were very similar to those reported previously for the identification of these same species (6) and were approximately 200 times above background values after subtraction of the water blank (Table 2). Testing of heterologous-species DNA gave no significant background reactivity (mean A_{650} ± SE = 0.002 ± 0.0001), making discrimination of a positive from a negative result unequivocal (Table 2). All *Candida* species DNAs were also tested with a probe specific for *C. krusei* DNA, and no reactivity with heterologous DNA was observed (mean A_{650} ± SE for the *C. krusei*-specific probe versus *C. krusei* DNA and versus all other *Candida* species DNAs, 0.49 ± 0.05 and 0.0014 ± 0.0004, respectively; $n = 72$).

Conclusions. Several population-based and sentinel surveillance studies have noted an increase in the proportion of *Candida* bloodstream infections caused by species other than

TABLE 1. Phenotypic identifications of *Candida* species by the referring hospital and by CDC compared to those by the PCR-EIA

Hospital ID[b]	CDC ID	PCR-EIA ID	CHROMagar result	API 20C or RapID profile result[a] (% ID[c])	Morphology[d]
C. albicans	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. albicans	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. albicans	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. albicans	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. albicans	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. albicans	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. albicans	*C. glabrata*	*C. glabrata*	Pink	Implicit CG (99.0)	BSP w/o PSH
C. krusei	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. krusei	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. krusei	*C. glabrata*	*C. glabrata*	Purple	Very good CG (99.4)	BSP w/o PSH
C. parapsilosis	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. tropicalis	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. tropicalis	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. tropicalis	*C. glabrata*	*C. glabrata*	Pink	Very good CG (99.4)	BSP w/o PSH
C. tropicalis	*C. glabrata*	*C. glabrata*	Pink	Satisfactory CG (99.2)	BSP w/o PSH
C. tropicalis	*C. glabrata*	*C. glabrata*	Pink	Low discrim. PW (70.7), CG (29.3)	BSP w/o PSH
C. albicans	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Excellent CP (99.9)	BSP and PSH
C. albicans	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Good CP (99.1)	BSP and PSH
C. albicans	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Acceptable CP (97.4)	BSP and PSH
C. albicans	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Satisfactory CP (95.7)	BSP and PSH
C. albicans	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Low discrim. CP (83.3), CN (13.3)	BSP and PSH
C. albicans	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Acceptable genus CT (57.1), CA (27.6), CP (12.2)	BSP and PSH
C. glabrata	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Good CP (97.8)	BSP and PSH
C. glabrata	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Acceptable CP (90.5)	BSP and PSH
C. glabrata	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Low discrim. CN (50.2), CP (47.5)	BSP and PSH
C. guilliermondii	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Good CP (98.0)	BSP and PSH
C. lusitaniae	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Very good CP (99.9)	BSP and PSH
C. lusitaniae	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Low discrim. CP (71.9), CN (18.9), CT (4.9)	BSP and PSH
C. tropicalis	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Very good CP (99.0)	BSP and PSH
C. tropicalis	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Good CP (95.9)	BSP and PSH
C. tropicalis	*C. parapsilosis*	*C. parapsilosis*	Pale pink	Low discrim. CN (50.2), CP (47.5)	BSP and PSH
C. albicans	*C. tropicalis*	*C. tropicalis*	Blue	Low discrim. CT (71.1), CN (18.5), CL (7.6)	BSP and PSH
C. albicans	*C. tropicalis*	*C. tropicalis*	Blue	Low discrim. CT (71.1), CN (18.5), CL (7.6)	BSP and PSH
C. albicans	*C. tropicalis*	*C. tropicalis*	Blue	Low discrim. CT (71.1), CN (18.5), CL (7.6)	BSP and PSH
C. albicans	*C. tropicalis*	*C. tropicalis*	Blue	Low discrim. CT (71.1), CN (18.5), CL (7.6)	BSP and PSH
C. glabrata	*C. tropicalis*	*C. tropicalis*	Blue	Adequate CT (97.0)	BSP and PSH
C. glabrata	*C. tropicalis*	*C. tropicalis*	Blue	Good CT (95.9)	BSP and PSH
C. glabrata	*C. tropicalis*	*C. tropicalis*	Blue	Low discrim. CT (71.1), CN (18.5), CL (7.6)	BSP and PSH
C. glabrata	*C. albicans*	*C. albicans*	Green	Low discrim. CA (58.7), CT (26.0), CN (14.4)	CHL and PSH
C. tropicalis	*C. albicans*	*C. albicans*	Green	Good genus CT (48.4), CA (44.4)	CHL and PSH
C. tropicalis	*C. albicans*	*C. albicans*	Green	Unacceptable profile	CHL and PSH
C. albicans	*C. lusitaniae*	*C. lusitaniae*	Tan	Good genus CT (60.7), CL (29.6)	BSP and PSH
C. albicans	*C. parapsilosis* or *C. lusitaniae*	*C. lusitaniae*	Pale pink	Very good genus CP (53.5), CL (38.8), CT (7.1)	BSP and PSH

[a] CA, *C. albicans*; CG, *C. glabrata*; CL, *C. lusitaniae*; CP, *C. parapsilosis*; CT, *C. tropicalis*; CN, *Cryptococcus neoformans*; PW, *Prototheca wickerhamii.* "Implicit CG" and "satisfactory CG" are RapID Yeast Plus results; all other results are from the API 20C AUX system. Low discrim., presumptive identification.
[b] ID, identification.
[c] %ID, percent identification likelihood.
[d] BSP, blastospores; PSH, pseudohyphae; CHL, chlamydospores; w/o, without.

C. albicans, and, in particular, an increase in the frequency of candidemia due to *C. glabrata* (reviewed in reference 15). Given the known propensity of *C. glabrata* to develop resistance to azole antifungals, the fact that this species was most frequently misidentified in this study is disturbing. Because each referring institution used its own method(s) for species identification, the reasons for the high rate of *C. glabrata* misidentification are not clear. However, for the 28% of non-*C. glabrata* isolates that were misidentified as *C. albicans* by the referring institution, two factors may provide some insight. First, in many referring institutions (45% of those surveyed), the germ tube test was performed as a primary screen for the identification of *C. albicans*. Second, a disproportionately greater number of misidentifications were received from nonuniversity laboratories than from university or medical school laboratories. These data suggest that the nonuniversity institutions in our study may have employed fewer specialists in mycology, who in turn had less experience in interpreting the germ tube test. This hypothesis is supported by the work of others (4) who found that, when the germ tube test was performed on a series of isolates tested in a blinded fashion by technicians who were not specialists in mycology, germ tube test specificity declined. Misinterpretation of results, particularly of pseudohyphal production, accounted for this drop in specificity (4). This might account for those isolates originally identified by the referring institution as *C. albicans* but ultimately identified as *C. lusitaniae*, *C. parapsilosis*, or *C. tropicalis* by the CDC. In addition, the CDC laboratory routinely employs CHROMagar *Candida* medium (16) as an adjunct to biochemical and morphological tests whereas none of the institutions polled in the surveillance area reported the use of this medium. The distinctive color of each species on this

TABLE 2. Specificity of DNA probes for species identification by PCR-EIA

DNA target species (no. of isolates tested)	Mean A_{650} ± SE[a] after reaction with probe for:				
	C. glabrata	*C. parapsilosis*	*C. tropicalis*	*C. albicans*	*C. lusitaniae*
C. glabrata (16)	0.91 ± 0.07	0	0	0	0
C. parapsilosis (15)	0	0.79 ± 0.05	0	0	0
C. tropicalis (7)	0	0	0.95 ± 0.10	0	0
C. albicans (3)	0	0	0	0.40 ± 0.05	0
C. lusitaniae (2)	0	0	0	0	0.38 ± 0.02[b]

[a] Mean A_{650} ± SE was calculated from spectrophotometric readings after target DNA was reacted with the DNA probes listed above. All samples were run in duplicate, and reagent blanks were run on each plate for each probe. Reagent blank values have been subtracted from test sample values above (mean reagent blank A_{650} = 0.038 ± 0.001; n = 72). Mean A_{650} ± SE for all control samples after subtraction of the reagent blanks for all probes was 0.002 ± 0.0001 (n = 226) and is represented in this table as 0 for ease of presentation.

[b] Includes one *C. lusitaniae* isolate identified as *C. albicans* by the referring institution and as *C. parapsilosis* or *C. lusitaniae* by CDC phenotypic methods.

medium may be helpful in cases where the biochemical results are equivocal and the expertise for distinguishing various species based on morphology on cornmeal-Tween agar is lacking.

Most clinical laboratories do not differentiate isolates of *C. albicans* from those of *C. dubliniensis.* Therefore, the nine cases of *C. dubliniensis* candidemia identified at the CDC by molecular identification methods and reported as *C. albicans* by the referring institutions were not considered to be discrepant identifications. Nonetheless, it has been demonstrated previously that the PCR-EIA can unequivocally differentiate isolates of *C. dubliniensis* from those of *C. albicans* (6, 7).

Unlike current phenotypic identification methods, which may require a series of tests to confirm the identity of a given *Candida* species (8, 9, 17), the PCR-EIA is a single test that can be used to identify the majority of medically important *Candida* species (6). In contrast to phenotype-based identification methods, the PCR-EIA can be performed in a single day and the results are very easy to interpret. Use of a commercial kit for the isolation of *Candida* species DNA (6) makes this test a fast and reliable method for *Candida* species identification. Although other PCR-based methods for the identification of *Candida* species have been described (reviewed in references 3 and 13), these methods did not use a combination of (i) an EIA detection format; (ii) universal fungal, multicopy rRNA gene targets to increase test sensitivity; (iii) a commercial kit for rapid and simple sample preparation; and (iv) probes that could detect more than one or a few *Candida* species. This study demonstrates the usefulness of the PCR-EIA for the resolution of discrepant phenotype-based species identifications. Conversion of the DNA probes described in this study into either a real-time PCR format or an automated microarray format would reduce postamplification manipulation steps and further reduce the time required for accurate species identification.

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