Rapid Identification of Ascomycetous Yeasts from Clinical Specimens by a Molecular Method Based on Flow Cytometry and Comparison with Identifications from Phenotypic Assays†

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This study was designed to compare the identification of ascomycetous yeasts recovered from clinical specimens by using phenotypic assays (PA) and a molecular flow cytometric (FC) method. Large-subunit rRNA domains 1 and 2 (D1/D2) gene sequence analysis was also performed and served as the reference for correct strain identification. A panel of 88 clinical isolates was tested that included representatives of nine commonly encountered species and six infrequently encountered species. The PA included germ tube production, fermentation of seven carbohydrates, morphology on corn meal agar, urease and phenoloxidase activities, and carbohydrate assimilation tests when needed. The FC method (Luminex) employed species-specific oligonucleotides attached to polystyrene beads, which were hybridized with D1/D2 amplicons from the unidentified isolates. The PA identified 81 of 88 strains correctly but misidentified 4 of Candida dubliniensis, 1 of C. bovina, 1 of C. palmioleophila, and 1 of C. bracarensis. The FC method correctly identified 79 of 88 strains and did not misidentify any isolate but did not identify nine isolates because oligonucleotide probes were not available in the current library. The FC assay takes approximately 5 h, whereas the PA takes from 2 h to 5 days for identification. In conclusion, PA did well with the commonly encountered species, was not accurate for uncommon species, and takes significantly longer than the FC method. These data strongly support the potential of FC technology for rapid and accurate identification of medically important yeasts. With the introduction of new antifungals, rapid, accurate identification of pathogenic yeasts is more important than ever for guiding antifungal chemotherapy.

Molecular methods are increasingly being used to identify cultures of pathogenic microorganisms in clinical laboratories. Advantages over standard phenotype-based tests include increased accuracy, as well as rapidity. Of the currently available DNA-based methods, nucleotide sequence analysis has proven to be the most accurate because known species, as well as undescribed species, are quickly recognized (9). However, for clinical applications, sequencing is often viewed as expensive and somewhat complex. Other DNA-based methods currently in use include species-specific primer pairs, rapid amplification of cDNA ends, amplified fragment length polymorphism, and oligonucleotide probes (2).

Flow cytometry (FC) employing Luminex DNA-based multiplexing technology allows detection of up to 100 species in a single assay well and has considerable promise for rapid species identification in a clinical laboratory setting (6). There are two basic protocols for this technology. In the first, species-specific oligonucleotides are attached to polystyrene microspheres (beads) and hybridized to amplicons of a selected gene, which have been initiated with biotinylated PCR primers for laser-based detection. Only amplicons with high sequence complementarity to the species-specific primer will hybridize to the bead and be detected by the flow cytometer. The second method employs species-specific primers that are hybridized onto complementary sites of the selected amplicon and, during the subsequent PCR-mediated extension reaction, incorporate biotin-dCTP for detection. Extension primers are then hybridized to beads, but only extended, biotinylated products are detected by the Luminex flow cytometer. Direct hybridization takes about 5 h from culture to identification, and the primer extension method takes 8 to 10 h. Diaz and Fell (4, 5) used direct hybridization to several rRNA genes to accurately identify species of *Trichosporon* and *Cryptococcus*. Page and Kurtzman (14) targeted species-specific sequences in domains 1 and 2 (D1/D2) of the large-subunit (LSU) rRNA gene to identify pathogenic species of *Candida* and other clinically important ascomycetous yeasts by direct hybridization, as well as primer extension. In the latter study, direct-hybridization probes were developed for 19 species and extension primers were developed for 34 species.

We designed the present study to determine the effectiveness of the hybridization probes developed by Page and Kurtzman (14) for identification of ascomycetous yeasts recovered from clinical specimens and to compare the accuracy of identification by both FC and phenotypic assay (PA). Major questions included whether most clinical isolates are represented in the current library of probes, whether clinical isolates exhibit nucleotide sequence variation not present in reference strains used during probe development, and whether the molecular

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assay would reduce time to identification. The strains in the present study were initially identified by standard PA, followed by identification by FC. The reference method for this study was strain identification from sequences in D1/D2 of the LSU rRNA gene.

MATERIALS AND METHODS

Organisms tested. A total of 88 selected yeast isolates recovered from clinical specimens submitted to the Clinical Mycology Laboratory of The Johns Hopkins Hospital were tested in this evaluation. The isolates were selected as ascomycetes on the basis of PA including absence of urease, sugar fermentation reactions, and final identification by PA as described below. The commonly encountered species included strains of *Candida albicans*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis*, and *Saccharomyces cerevisiae*, as well as a small set of less commonly encountered species initially appearing to be *C. inconspicua*, *Debaryomyces hansenii*, and *Pichia anomala*. For the strains examined and their identification by sequence analysis, PA, and oligonucleotide probes, see Table S1 in the supplemental material. All of the strains examined in this study have been deposited in the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL.

Phenotypic identification. The yeasts were identified phenotypically in The Johns Hopkins Clinical Mycology Laboratory by standard mycological assays. Initially, strains were tested for germ tube production with Remel germ tube solution (Remel, Lenexa, KS) and all positive yeasts were finalized as *C. albicans* within 24 h. Germ tube-negative yeasts were then tested with a seven-carbohydrate (glucose, maltose, sucrose, lactose, galactose, trehalose, and cellobiose) fermentation assay on Christensen's urea agar, and morphology determination and the phenoloxidase assay were done with corn meal agar containing caffeic acid. These assays were read at 24 h, but a few required 48 h. Carbohydrate assimilation patterns were performed when needed for the identification of the less commonly acquired species with the API 20C Aux kit (bioMérieux, Hazelwood, MO). The latter assay added an additional 24 to 48 h for the final identification.

DNA isolation and identification by D1/D2 sequences. Growth of cultures and DNA isolation and sequencing techniques were those reported by Kurtzman and Robnett (9, 10). In addition, for rapid identification under clinical conditions, amplicons of D1/D2 were also generated by simply adding live cells from a 2-day YM agar (17) culture directly to the PCR mixture. Cells of an approximately 0.1-mm³ volume were transferred by inoculation needle to 40 μl of PCR mixture. Species were identified by a BLAST search of the D1/D2 sequences deposited in the GenBank database.

FC protocols. Procedures for probe design, DNA amplification, and the Luminex direct-hybridization method were given in detail by Page and Kurtzman (14). Briefly, biotinylated D1/D2 amplicons from the clinical isolates were each incubated with a mixture of beads with each bead type having an attached species-specific oligonucleotide probe. Following incubation, the beads were scanned in a Luminex flow cytometer, which detected whether a species-specific probe had annealed with the D1/D2 amplicon.

Development of new probes. Probes developed in the present study for differentiating *C. sojae* from *C. tropicalis* and the identification of *S. cerevisiae* were designed by aligning the D1/D2 sequences of known ascomycetous yeast species with ARB software (13) and identifying nucleotide differences unique to each species.

RESULTS AND DISCUSSION

Species identification by sequence analysis and by PA. The 88 clinical isolates examined in this study were identified by PA and by a BLAST search of the D1/D2 sequences maintained in the GenBank database. Not surprisingly, a majority of the strains were represented by *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis*. Additionally, seven strains of *S. cerevisiae* were identified (Table 1). There was little or no sequence variation between the clinical isolates tested in this study and previously used reference strains. The most variation was found among isolates of *C. glabrata* (zero to two substitutions) and *C. albicans* (zero to four substitutions). Genetic divergence

TABLE 1. Identification of 88 ascomycetous clinical yeast strains by PA, FC, and D1/D2 LSU rRNA gene sequencing

Organism[a] or parameter	No. of strains identified by:		
	D1/D2 LSU rRNA gene sequencing	PA	FC[b]
C. albicans	9	9	9
C. bovina	1	0[c]	ND
C. bracarensis	1	0[d]	ND
C. dubliniensis	4	0[e]	4
C. fermentati	1	0[f]	0[f]
C. glabrata	30	30	30
C. guilliermondii	2	2	2
C. kefyr	1	1	ND
C. krusei	4	4	4
C. lusitaniae	2	2	2
C. lusitaniae AF538871	3	0[g]	ND
C. palmioleophila	1	0[h]	ND
C. parapsilosis	9	9	9
C. tropicalis	12	12	12
P. anomala	1	1	ND
S. cerevisiae	7	0[i]	7
Total no. (%) correctly identified	88 (100)	81 (92)	79 (90)

[a] Strains designated *C. lusitaniae* AF538871 represent isolates of *C. lusitaniae* with atypical D1/D2 sequences. Although they are phenotypically identical to other *C. lusitaniae* strains in commonly used tests, the D1/D2 sequence divergence of these strains prevents their detection with the standard *C. lusitaniae* probe. See text for discussion.

[b] ND, species identity not determined because there was no probe for this species. Present probes do not distinguish between the closely related species *C. fermentati* and *C. guilliermondii* (16).

[c] Isolate identified as *C. inconspicua*.

[d] Isolate identified as *C. glabrata*.

[e] Isolates identified as *C. albicans*.

[f] Isolate identified as *C. guilliermondii*.

[g] Isolate identified as *C. lusitaniae*.

[h] Isolate identified as *D. hansenii*.

[i] Isolates identified as *Saccharomyces* sp.

among populations of *C. albicans* has been previously reported (7), and these divergent strains may represent closely related species or subspecies.

Five strains of *C. lusitaniae* were identified by PA. Sequence analysis showed two to be typical *C. lusitaniae* strains, but three of the strains matched an isolate of *C. lusitaniae* with a divergent D1/D2 sequence (GenBank accession no. AF538871). Lachance et al. (12) reported 10 different versions of D1/D2 sequences among isolates of *Clavispora* (*Candida*) *lusitaniae*. These isolates were interfertile, as assessed by mating and ascospore formation, and the actin gene sequences for two of the strains with divergent D1/D2 sequences were reported to show no variation greater than that found among conspecific strains of other species. Consequently, although these strains were correctly identified by both PA and a BLAST search of GenBank sequences, they were not recognized by our current library of oligonucleotide probes because of their divergent sequences.

On the basis of sequence comparisons, PA correctly identified 84 of the 88 strains examined, if one allows that there is no major phenotypic distinction between *C. albicans* and *C. dubliniensis* and that the seven *S. cerevisiae* strains were identified as *Saccharomyces* sp. Otherwise, 73 of 88 strains were correctly identified by PA. Four species were not recognized by PA but were identified by D1/D2 sequence analysis as the following:

TABLE 2. Characteristics of direct hybridization oligonucleotide probes for detection of *C. tropicalis* and *S. cerevisiae*

Probe[a]	Species identified	Negative range	Positive range	Minimum ratio[b]
Scrvcld1	*S. bayanus, S. cariocanus, S. cerevisiae, S. kudriavzevii, S. mikatae, S. paradoxus, S. pastorianus*	0–377	1554–2288	4.1
Saclade2	*S. cerevisiae*	NA[c]	1115–2672	NA
Trop11	*C. tropicalis, C. ergastensis, C. glucosophila, C. maltosa*	NA	949–2224	NA
Soj9	*C. sojae, C. neerlandica, C. parapsilosis, Candida* sp. strain NRRL Y–17456, *L. elongisporus, P. triangularis*	NA	1047–1880	NA

[a] Nucleotide sequences of probes: *Scrvcld1*, 5′ GACTGCGACGTAAGTCAAGG; *Saclade2*, 5′ GGACTGAGGACTGCGACGTA; *Trop11*, 5′ ACAGTTTATCG GGCCAG; *Soj9*, 5′ ACAGTTTACCGGGCCAG.
[b] The minimum ratio is the lowest signal from a positive probe divided by the highest signal detected with a negative probe. A minimum ratio of 2 or greater has been recommended for clear recognition of positive reactions (6).
[c] NA, not applicable.

(i) *C. bracarensis* (3), a newly described species phenotypically similar to *C. glabrata*; (ii) *C. fermentati*, which is phenotypically almost indistinguishable from *C. guilliermondii* (16); (iii) *C. bovina*, identified phenotypically as *C. inconspicua* and; and (iv) *C. palmioleophila*, identified phenotypically as *D. hansenii*. *C. palmioleophila* appears to be a novel clinical isolate, but *C. fermentati* (15) and *C. bovina* (11) are known human pathogens. *C. nivariensis* (1), another newly described species that is phenotypically similar to *C. glabrata*, was not detected among the clinical isolates in the present study.

Species identification by the FC-oligonucleotide probe method. Of the 88 clinical isolates examined in this study, 69 were unambiguously identified to the species level with the direct-hybridization probes developed by Page and Kurtzman (14). Of the remaining 19 isolates, 15 reacted with none of the probes and included *S. cerevisiae* (seven strains), *C. bovina* (one strain), *C. bracarensis* (one strain), *C. kefyr* (teleomorph *Kluyveromyces marxianus*) (one strain), *C. lusitaniae* (GenBank accession no. AF538871) (three strains), *C. palmioleophila* (one strain), and *P. anomala* (one strain). The remaining four strains reacted weakly with probes for both *C. sojae* (probe *SojC*) and *C. tropicalis* (probe *Trop5B*). Sequence analysis revealed that the four weakly reacting strains were *C. tropicalis* and that there were no species-specific probes for the other 15 strains. From these results, it was apparent that a probe for *S. cerevisiae* was needed and it was imperative to determine why four strains of *C. tropicalis* were not unambiguously detected by our previously successful probes.

C. tropicalis probes. In our previous work, the *SojC* and *Trop5B* probes unambiguously identified 15 *C. tropicalis* strains and 2 *C. sojae* strains (14), and in the present study, 8 *C. tropicalis* strains were clearly identified but 4 were not. Sequencing demonstrated that the four ambiguously identified strains had sequences identical to all of the other strains tested in the region complementary to the probe. One difference from our earlier study, which used purified DNA, was that amplicons in the present study were generated by adding live cells directly to the PCR mixture. Consequently, we compared the quantity and quality of amplicons synthesized from purified DNA and from cells added to the PCR. Gel electrophoresis showed that purified DNA often gave stronger PCR products, but amplicons generated from cells were usually sufficient for

strain identification. Whether the concentration of amplicons derived from cells was overestimated by the all-species positive control probe (*UniD*) for the four strains of *C. tropicalis* giving ambiguous results is uncertain, but gel electrophoresis may give a more definitive estimate of amplicon concentration. Nonetheless, two additional probes were developed for differentiating *C. sojae* from *C. tropicalis*. The original *Trop5B* and *SojC* probes were still effective for detecting *C. sojae* and *C. tropicalis* from all other species, so probe design focused on the resolution of just these two species. *Trop11* and *Soj9* were designed on the basis of D1/D2 to achieve maximum resolution of the two species. *Trop11* and *Soj9* were added to the assay, which was tested with 27 *C. tropicalis* strains and 2 *C. sojae* strains both with purified DNA preparations and by PCR amplification directly from living cells. All 29 strains were clearly differentiated from both DNA preparations with a probe combination consisting of *SojC*, *Trop5B*, *Trop11*, and *Soj9*.

S. cerevisiae probes. Because of the high frequency of *S. cerevisiae* isolates among the strains examined, a probe for *S. cerevisiae* was developed in this study. Evaluation of the sequence of *S. cerevisiae* when aligned with those of other ascomycetous yeast species showed that it would not be possible to design a single *S. cerevisiae* probe on the basis of D1/D2. A probe designated *Scrvcld1* identified known species of the *S. cerevisiae* clade, i.e., *S. cerevisiae, S. mikatae, S. cariocanus, S. pastorianus, S. bayanus, S. kudriavzevii*, and *S. paradoxus*. A second probe (*Saclade2*) was designed that identified *S. cerevisiae*, as well as a number of other species, but none of these species included other members of the *S. cerevisiae* clade. Used in combination, these two probes effectively discriminate *S. cerevisiae* from all other known ascomycetous yeast species, as demonstrated in the following validation.

Validation of newly designed probes. With the development of four new probes, the probe composition of our original assay was changed and it was necessary to test the effectiveness of each probe when combined in a new direct-hybridization assay. A mixture of 28 probes, including the 4 newly designed probes, was hybridized to PCR-amplified products from 539 strains representing 402 species that originally were used to develop and validate the Luminex FC technique for ascomycetous yeasts (14). The results of this validation experiment are summarized in Table 2. *Scrvcld1* was positive for *S. cerevisiae, S.*

TABLE 3. Oligonucleotide probes used for identification of clinically isolated ascomycetous yeast species

Species	Probe(s)[a]	High negative value	Positive value range	Minimum ratio[b]
C. albicans	Alb7	25–133	649–1217	4.9
C. bovina	NA	NA	NA[c]	NA
C. bracarensis	NA	NA	NA	NA
C. dubliniensis	Dub5	14–166	567–1595	3.4
C. glabrata	Glab5	33–111	420–1127	3.8
C. guilliermondii	GG2	NA	1026–1570	NA
C. guilliermondii	GG3	24–448	977–1871	2.2
C. kefyr	NA	NA	NA	NA
C. krusei	KrusF	29–78	1542–1855	19.8
C. lusitaniae	Lusit2	19–101	421–732	6.8
C. lusitaniae AF538871	NA	NA	NA	NA
C. palmioleophila	NA	NA	NA	NA
C. parapsilosis	Parap2B	23–1221	1408–2622	NA
C. parapsilosis	LPWA	24–441	2059–2674	4.7
C. parapsilosis	Soj9	27–368	676–1549	1.8
C. tropicalis	Trop11	NA	628–1549	NA
C. tropicalis	Trop5B	25–69	242–1743	2.5
P. anomala	NA	NA	NA	NA
S. cerevisiae	Scrvcld1, Saclade2	28–59	373–1724	6.3

[a] The nucleotide sequences of these probes are given in Table 2, footnote a, and in reference 14. The probes for C. guilliermondii also detect C. fermentati.
[b] See Table 2, footnote b, for an explanation of the minimum ratio.
[c] NA, not applicable.

mikatae, S. paradoxus, S. bayanus, S. pastorianus, S. cariocanus, and S. kudriavzevii but negative for all of the other species tested. Saclade2 was positive for S. cerevisiae and a number of other species but was negative for all other species in the S. cerevisiae clade, making the combination of the two probes effective for distinguishing S. cerevisiae from other known ascomycetous yeasts. Of the other two newly developed probes, Trop11 was positive for all C. tropicalis strains, as well as C. glucosophila, C. maltosa, and C. ergatensis. Soj9 was positive for all C. sojae strains, as well as C. parapsilosis, C. neerlandica, Candida sp. strain NRRL Y-17456 (near C. parapsilosis), Lodderomyces elongisporus, and Pichia triangularis. Trop5B was negative for the species detected by Trop11, with the exception of C. tropicalis, and SojC was negative for the species Soj9 detected, with the exception of C. sojae (Table 2). After establishing unambiguous results for these probe combinations, the new assay, which included Soj9, Trop11, Scrvcld1, and Saclade2, was retested and it unambiguously identified 79 of the 88 clinical strains, i.e., all of the species for which probes had been developed (Tables 2 and 3).

An interesting advantage of probe technology became apparent during this study when one of the clinical isolates was identified as C. lusitaniae by PA as well as by sequence analysis, but the hybridization assay reported both C. lusitaniae and C. fermentati-C. guilliermondii. Reexamination of the culture by streak plate revealed the presence of infrequent colonies that differed from the majority. This small population of C. fermentati was not readily apparent from the initial sequence analysis but was subsequently verified by sequence analysis of individual colonies. Diaz and Fell (4) reported that 10^2 genome molecules in a PCR produced detectable levels of a biotinylated amplification product. Consequently, multiple species are likely to be detected in a mixed culture if the correct species-specific probes are present.

Conclusions. These results confirm that phenotypic methods, when used by experienced personnel, are effective for the identification of many commonly encountered pathogenic yeasts, although identification of less frequently encountered species is problematic. In addition, the time required to identify species by PA ranges from 2 h to 5 days. DNA probe technology, in contrast, offers a much more rapid (within 1 working day) and accurate means for identifying species, but its effectiveness is limited if uncommon species are encountered for which probes have not been developed. However, the presence of the unknown would be revealed by the positive control probe and additional probes can be developed for unknowns as they are detected and added to the assay. Currently, gene sequencing offers the most accurate method for strain identification and it permits recognition of previously unknown species. One exception is the presence of mixed cultures in which the population of one species is sufficiently low that it escapes detection by sequencing or PA. This was the case for the mixed culture of C. lusitaniae and C. fermentati reported above, which was detected by FC.

The results of this study are consistent with data from an evaluation of a commercially available molecular kit (Micro-SeqD2 LSU rRNA gene sequencing kit) for the identification of commonly encountered yeasts (8). The accuracy of the kit was 93.9% with a panel of 131 yeast strains representing both ascomycetous and basidiomycetous species. With the Candida spp., the accuracy was 98%. Similar to our experience, the most common identification failure was due to lack of particular sequences in the database supplied.

The cost of strain identification by molecular methods is a concern for many clinical laboratories. We estimate that reagent costs for FC are comparable to those for PA. However, additional costs include purchase of a thermal cycler for PCR and a flow cytometer. Because up to 96 strains can be identified in one assay plate, larger clinical laboratories will find that the savings in personnel time will soon pay for the additional equipment. Smaller laboratories may find FC more expensive that PA, but the increased speed and accuracy of strain identification will outweigh these additional costs.

The data presented here strongly support the potential of FC technology for rapid, accurate identification of medically important yeasts. With the introduction of new antifungal drugs, changes in the epidemiology of invasive candidiasis, and the recognition that ordinarily nonpathogenic species may become etiologic agents of human infections, the need for rapid, accurate identification is more important than ever to aid in appropriate antifungal decisions.

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