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Experience with the MicroSeq D2 Large-Subunit Ribosomal DNA Sequencing Kit for Identification of Commonly Encountered, Clinically Important Yeast Species

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Experience with a MicroSeq D2 large-subunit (LSU) ribosomal DNA (rDNA) sequencing kit for identification of yeast species commonly encountered in the mycology laboratory at Mayo Clinic is described here. A total of 131 isolates of yeasts recovered from clinical specimens were included in the study. Phenotypic methods used for initial identification included germ tube formation, urease production, microscopic morphological features on cornmeal agar, and an API 20C AUX system; all isolates were sequenced using a MicroSeq D2 LSU rDNA sequencing kit. Nucleic acid sequencing identified 93.9% of the isolates to the correct genus and species. A total of 100 of the isolates (representing 19 species of *Candida*) were sequenced, and 98% gave results concordant with identifications made by the API 20C AUX system; distance scores ranged from 0 to 1.88%, with an average value of 0.23%. *Candida dubliniensis* was not included in the MicroSeq database and was identified as *Candida albicans*. A total of 32 isolates representing 9 other genera (including *Cryptococcus*, *Filobasidium*, *Kloeckera*, *Malassezia*, *Pichia*, *Sporidiobolus*, *Rhodotorula*, *Zygosaccharomyces*, and *Trichosporon*) were included, and 81.3% showed concordant results when phenotypic and sequencing results were compared. Most discrepancies were attributed to the lack of inclusion of the species in the MicroSeq or API 20C AUX database. The MicroSeq D2 LSU rDNA sequencing kit appears to be accurate and useful for the identification of yeasts that might be seen in a clinical laboratory.

The identification of clinically important yeast species in the laboratory has been greatly simplified by the introduction of commercially available products. Accompanying databases have provided laboratories with the capability of identifying most of the commonly encountered species. Traditionally the identification of yeast species has been performed on the basis of their phenotypic characteristics, primarily microscopic morphology and utilization of specific substrates. An excellent review of phenotypic methods and commercially available identification systems was provided by Freydiere et al. (7).

The significance of yeast species as a cause of human disease, particularly in the immunocompromised host, has become even more important in recent years. Factors responsible for the dramatic increase in the number of cases currently seen include the following: AIDS, hematological malignancies, organ transplantation, increased use of corticosteroids, antineoplastic drug treatment, complex surgical procedures, and long-term indwelling vascular catheters, as well as other factors. For the first time in history, a choice of antifungal drugs active against yeast species is available, and more is known about species that exhibit or develop resistance to specific agents. In addition, many of the uncommon species and also newly described species are now being seen as a cause of disease in the immunocompromised patient.

The commercially available yeast identification methods (1, 2, 4, 5, 9, 13–15, 17) have easily identified the common yeast species; however, uncommon and newly described species present a significant challenge. Phenotypic characteristics often cannot distinguish between species that have overlapping phenotypic characters. Currently, the trend in microbiology is to classify organisms on the basis of molecular taxonomy; this allows for more objective separation of species. As newer species are described and associated with human infection, their identifications will become more important, particularly when resistance or susceptibility to certain antifungal drugs becomes evident. Phenotypic characteristics of yeast species probably will not be adequate for the identification of these organisms in the clinical laboratory due to the limitations of commercially available products and their cost. Nucleic acid sequencing has already become an important tool that is useful for the identification of aerobic and anaerobic bacteria, mycobacteria, and fungi, including yeast species. Kurtzman and Robnett (11) studied approximately 500 ascomycetous yeast species for divergence in the D1/D2 region of the large-subunit (LSU) ribosomal DNA (rDNA). This study indicated that sequencing the D1/D2 region could identify most of the ascomycetous yeast species, including those of *Candida* and other anamorphic genera. Fell et al. (6) studied 337 strains of basidiomycetous yeast species and yeast-like fungi and determined that the majority of the species could be identified by sequencing the D1/D2 region and that separation of closely related species required sequencing of the intergenic spacer (ITS) region. Scorzetti et al. (16) further confirmed that sequencing of the D1/D2 region could be used for the identification of most basidiomycetous yeast species and that closely related species or strains required sequencing of the ITS region. Chen et al. (3) and Turenne et al. (19) determined that the ITS1 and ITS2 regions were useful for identifying many of the clinically important yeast species. Sequencing allows for better differenti-

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TABLE 1. Comparison of yeast identifications made using the API 20C AUX method and the MicroSeq D2 LSU rDNA sequencing kit

Phenotypic identification	Sequence-based identification using D2 database	No. of isolates	No. of isolates with concordant results	Distance score range (%)
Candida albicans	*Candida albicans*	12	12	0.0–0.92
Candida ciferrii	*Stephanoascus ciferrii*[a]	3	3	0–0.31
Candida colliculosa	*Torulaspora delbrueckii*[a]	1	1	0
Candida dubliniensis[b]	*Candida albicans*	1	0	3.42
Candida famata	*Debaryomyces hansenii*[a]	1	1	0
Candida glabrata	*Candida glabrata*	11	11	0.30–0.90
Candida guilliermondii	*Yamadazyma guilliermondii/Candida xestobii*[a,c]	5	5	0–1.17
Candida kefyr	*Kluyveromyces marxianus/lactis*[a,c]	1	1	0
Candida krusei	*Issatchenkia orientalis*[a]	8	8	0
Candida lipolytica	*Yarrowia lipolytica*[a]	8	8	0–0.36
Candida lusitaniae	*Candida lusitaniae*	10	10	0–1.06
Candida norvegensis	*Candida norvegensis*	1	1	0
Candida parapsilosis	*Candida parapsilosis/Candida osornesis*[c]	14	14	0–1.88
Candida pelliculosa	*Pichia anomala–1*[a]	2	1	0
	Pichia fabianii–1			
Candida rugosa	*Candida rugosa*	2	2	0.40
Candida tropicalis	*Candida tropicalis*	13	13	0–0.31
Candida utilis	*Pichia jadinii*	1	1	1.07
Candida viswanathii	*Candida lodderae*[a]	3	3	0.47
Candida zeylanoides	*Candida zeylanoides*	2	2	0
Cryptococcus laurentii	*Cryptococcus laurentii*	2	2	0–3.48
Cryptococcus neoformans	*Filobasidiella neoformans*[a]	7	7	0–0.86
Cryptococcus uniguttulatus	*Filobasidium uniguttulatum*[a]	1	1	0
Debaryomyces hansenii	*Debaryomyces hansenii*	1	1	0
Kloeckera apis/Kloeckera apiculata[d]	*Kloeckera apiculata*	1	1	0
Malassezia pachydermatis	*Malassezia pachydermatis*	1	1	0
Pichia ohmeri	*Pichia ohmeri*	2	2	0
Rhodotorula glutinis[b]	*Sporidiobolus johnsonii*	1	0	8.72
Rhodotorula mucilaginosa	*Rhodotorula mucilaginosa*	1	1	0.31
Saccharomyces cerevisiae	*Zygosaccharomyces fermentati*	12	10	0–0.31
	Debaryomyces hansenii			
Trichosporon mucoides[b]	*Trichosporon jirovecii*	3	0	0.0–3.78

[a] Teleomorph.
[b] Not in D2 database.
[c] Indistinguishable by D2.
[d] Indistinguishable by 20C.

ation between genera and species and is reliable for the characterization of previously unidentified organisms (3, 12, 19).

This article presents our experience with the use of the commercially available MicroSeq D2 LSU rDNA fungal sequencing kit (Applied Biosystems, Foster City, Calif.) to identify commonly encountered species of yeasts.

MATERIALS AND METHODS

Yeast isolates. A total of 131 isolates of yeast species were used in the evaluation (Table 1); all were recovered from clinical specimens at Mayo Clinic or were referred to Mayo Medical Laboratories for identification by other laboratories. All were grown on Sabouraud's dextrose or inhibitory mold agars prior to nucleic acid sequencing.

Identification of cultures by phenotypic methods. All cultures of yeast species and yeast-like organisms were identified using a combination of any or all of the following: germ tube formation, urease production, colonial morphological features, microscopic morphology on cornmeal agar containing trypan blue, and the API 20C AUX identification system (bioMérieux, Hazelwood, Mo.).

LSU rDNA sequencing. The MicroSeq D2 LSU rDNA fungal sequencing kit is composed of a PCR and cycle sequencing module, identification and analysis software, and a library of fungal nucleic acid sequences.

DNA was extracted from fungal cells by placing a 1.0-μl loopful of organisms into a 2.0-ml microcentrifuge tube containing 100 μl of PrepMan Ultra sample preparation reagent (Applied Biosystems). Tubes were vortexed for 10 to 30 s followed by heating at 100°C for 10 min in a heat block. Lysates were stored at −20°C in cases in which testing was not performed immediately. Prior to testing, lysates were diluted 1:50 in deionized water.

The D2 LSU rDNA fragment was amplified by adding 25 μl of diluted genomic DNA to 25 μl of master mix consisting of forward and reverse primers in the PCR module. PCR conditions were as follows: 95°C for 10 min; 35 cycles at 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and a final step at 72°C for 10 min.

The amplicon (10 μl) was loaded onto a 2% E-Gel, subjected to electrophoresis, and viewed according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, Calif.) to determine whether PCR products were present.

Purification of the PCR product to remove excess primers and nucleotides was performed using shrimp alkaline phosphatase (2.0 U/μl) and exonuclease I (10.0 U/μl) from USB Corporation, Cleveland, Ohio. The enzymes were activated for 15 min at 37°C followed by inactivation at 80°C for 15 min.

Cycle sequencing was performed using the sequencing module; after removal of dyes, labeled amplicon was placed on an ABI 3100 16-capillary genetic analyzer (Applied Biosystems).

Sequence data analysis. All sequence sample files were assembled, edited, and compared to those in the MicroSeq D2 fungal library that contained 1,072 entries, including 289 yeast species (version 1.4.2 [February, 2002]). A distance score of from 0.00% to less than 1.00% was used as a guide for species identity, since no cutoff value has yet been determined. The organism choice giving the closest match was considered to be the most likely correct identification. Organisms having a distance score of greater than 1.00% were considered to be unique isolates that were most closely related to the closest database match present in the library. Sequencing results for the yeast species were available within 24 h (8).

RESULTS

A single isolate of *Candida glabrata* was sequenced repeatedly (10 times), and the sequencing results were always the same. Further, this same isolate (or a control) was included for each sequencing run and the results were always reproducible.

As shown in Table 1, 93.9% (123/131) of all yeast isolates included in this evaluation were correctly identified (using the MicroSeq D2 library) with respect to the appropriate genus and species (there were three instances in which two choices were given). A total of 100 isolates (representing 19 species of *Candida*) were sequenced, and 98% (97/99) gave results concordant with the identifications made by the API 20C AUX method. The average distance score for species of *Candida* having concordant identifications was 0.23%, and values ranged from 0.00 to 1.88%. Only one isolate each of *Candida guilliermondii*, *Candida utilis*, *Candida lusitaniae*, and *Candida parapsilosis* had distance scores of greater than 1.00%.

One isolate of *Candida dubliniensis* was identified as *Candida albicans* (the distance score was 3.42% [96.58% similarity]); the sequence for this species was not found in the D2 library. It is well known that this organism is phenotypically very similar to *C. albicans* and that the routine clinical laboratory cannot distinguish between them using the usual methods. It has been shown that nucleic acid sequencing using the D1/D2 region allows for differentiation between *C. albicans* and *C. dubliniensis* (18). Two isolates of *Candida pelliculosa* (*Pichia* [*Hansenula*] *anomala*) had a perfect match (0.00% distance score), one for *Pichia fabianii* and the other for *Pichia anomala*. *P. fabianii* and *P. anomala* are phenotypically very similar and would not be distinguishable using the methods available to most clinical laboratories (10). However, nucleic acid sequencing of the D1/D2 regions allows for separation of the two species (11); *P. fabianii* is likely the correct name for these strains.

As shown in Table 1, 32 isolates that represented 9 other genera (*Cryptococcus*, *Filobasidium*, *Debaryomyces*, *Kloeckera*, *Malassezia*, *Pichia*, *Sporidiobolus*, *Rhodotorula*, *Zygosaccharomyces*, and *Trichosporon*) and 11 species were included in this evaluation. Of these, 81.3% (26/32) showed concordant results when the API 20C AUX method and the MicroSeq D2 LSU rDNA sequencing kit were compared. The isolates whose results were identified as concordant had an average distance score of 0.41% (range, 0.00 to 3.48%). Only a single isolate of *Cryptococcus laurentii* had a distance score greater than 1.00%; this was not unexpected, since this species is known to exhibit intraspecies diversity and represents a complex of species.

The six discordant results represented one isolate of *Rhodotorula glutinis*, three isolates of *Trichosporon mucoides*, and two isolates of *Saccharomyces cerevisiae*. The single isolate of *R. glutinis* was identified as *Sporidiobolus johnsonii*; this had a distance score of 8.72% (91.28% similarity). The MicroSeq library of sequences did not include a sequence for *R. glutinis*, and the API 20C AUX database did not include a sequence for *S. johnsonii*; however, the results of a GenBank search suggested that the organism belongs to the genus *Sporidiobolus*.

All three isolates of *T. mucoides* were identified by nucleic acid sequencing (distance score, 0.00%; 100% similarity) as *Trichosporon jirovecii*; the former species was not included in the MicroSeq library of sequences and the latter was not included in the API 20C AUX database. The two discrepant isolates of *S. cerevisiae* were identified as *Zygosaccharomyces fermentati* and *Debaryomyces hansenii*. Both were perfect matches; however, *Z. fermentati* was not included in the API 20C AUX database. D1/D2 sequencing can be used to separate *T. mucoides* from *T. jirovecii*; however, the two are closely related (6, 16).

DISCUSSION

The initial process for the identification of yeast species in most clinical laboratories commonly utilizes phenotypic methods including (among others) the germ tube test, urease production, and perhaps a screening test for *Candida glabrata*. When an organism cannot be identified using these methods, a commercially available yeast identification system is generally used in combination with the microscopic morphological features of the organism observed on cornmeal agar. Phenotypic test results are often variable, with identification requiring subjective interpretation; the accuracy of the final result often depends on the expertise of the person performing the testing.

The clinical microbiology laboratories of Mayo Clinic use nucleic acid sequencing to identify selected isolates of mycobacteria and aerobic and anaerobic bacteria. The clinical mycology laboratory has the D2 rDNA fungal identification system component available for use. As described previously, the accompanying library of fungal sequences contains 1,072 entries that represent 289 yeast species, including the type cultures for most.

The yeast species included in this evaluation were representative of those seen routinely in the clinical mycology laboratory at Mayo Clinic and also included those sent by other laboratories for identification. In the latter instance, the organisms sent usually represented those that could not be readily identified by the referring laboratories. We sought to determine how well nucleic acid sequencing would perform for the identification of yeast species seen in our clinical mycology laboratory.

Overall, nucleic acid sequencing identified 93.9% of the clinical isolates of yeast species to the correct genus and species. However, when the sequences for discrepant isolates were matched with those in GenBank, 99.2% of identifications agreed with the identification provided by the MicroSeq database and only 94.7% of the phenotypic identifications were concordant. Since (due to costs) discordant results were not resolved by further phenotypic methods, one cannot definitely establish the accuracy of these results. Our clinical mycology laboratory usually reports the common anamorphic name for organisms, but the MicroSeq D2 library uses the name of the teleomorph (when available); however, this did not affect the interpretation of reported results. No standard cutoff point was available for interpreting the distance score, but most of the species having concordant identifications had a distance score of less than 1.00%. Additional studies will perhaps determine that this value is valid but are more likely to show some genetic diversity within some species.

As has been seen in other areas of clinical microbiology, molecular methods have allowed for better differentiation between organisms and, in some instances, rearrangement of the taxonomic classification. It appears that molecular taxonomy

will soon be the standard. Additional work is needed to determine which gene or combination of genes will allow for complete separation of genera and species. Some genera and species may require sequencing of more than one target before a definitive identification can be made.

The API 20C AUX system has been a very useful tool in the laboratory for many years; however, the yeast species whose sequence information is contained within the database likely have not been classified by nucleic acid sequencing and discrepancies between phenotypic and molecular methods are to be expected. Commercially available databases often contain type cultures from the American Type Culture Collection for the species, and even these have not been subjected to sequencing to determine whether or not they were accurately identified when they were initially placed in the American Type Culture Collection. The API 20C AUX system remains a reliable method to identify common yeast species seen in the clinical laboratory.

Nucleic acid sequencing will probably provide the greatest benefit to the laboratory by identifying those organisms whose identities are questionable or cannot be determined by phenotypic methods. A cost analysis comparing phenotypic testing to nucleic acid sequencing showed that the cost of sequencing was $29.50 higher than the cost of using the API 20C AUX system for identification. That the MicroSeq D2 library is somewhat limited and is not inclusive of all clinically important species should be recognized. However, it allows each laboratory to construct a custom database; this makes the system even more useful and complete. Our clinical mycology laboratory is in the process of constructing a database of species not already included in the MicroSeq library and of isolates that show some genetic diversity among their sequences. Sequences for these may be found in GenBank and are listed sequentially as AY234870 to AY235033. It is important to document and publish information related to infections caused by the new and unusual species recognized by nucleic acid sequencing so that their clinical significance may be determined.

The MicroSeq D2 LSU rDNA sequencing kit appears to be accurate, reliable, and useful for the identification of yeast species that might be seen in a clinical laboratory, some of which are relatively uncommon. The most economical use for the system is that of the identification of organisms that cannot fully be identified by commercially available systems.

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