

Colony Multiplex-Tandem PCR for Rapid, Accurate Identification of Fungal Cultures[∇]

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We developed a multiplex tandem PCR (MT-PCR) assay for the rapid identification of 16 fungi directly from culture. MT-PCR results were concordant with phenotypic identification for all cultures studied ($n = 183$). The colony MT-PCR assay was rapid (<2 h), sensitive, and specific in identifying fungal pathogens directly from primary isolation plates.

The isolation of pathogenic fungi from clinical specimens has increased in recent years as a result of medical advances and the rising number of patients at risk, especially the immunocompromised. Rapid and accurate identification of fungi is important for patient management, given the increase in incidences of antifungal-resistant yeasts and molds, including *Candida glabrata*, *Aspergillus* spp. that are not *Aspergillus fumigatus*, and the Zygomycetes.

Current “gold standard” methods for the identification of yeasts involve a combination of histopathology, culture, and carbohydrate assimilation and fermentation reactions (8). These can be time consuming and expensive. Commercial phenotypic identification systems such as Vitek 2 (bioMérieux, Marcy-l’Étoile, France) and/or API ID 32C (bioMérieux) are widely used in larger diagnostic laboratories, but they require a pure culture before testing can be performed. Misidentifications or ambiguous results are not uncommon (6), as these kits cannot differentiate between closely related species, such as *Candida inconspicua* and *Candida norvegensis* (16) and *Candida guilliermondii* and *Candida famata* (18, 19) or between members of the *Candida glabrata* complex (including *Candida nivariensis* [1] and *Candida bracarenensis* [7]). For the API ID 32C system, identification is further dependent upon individual interpretation and expertise (6). While growth on second-generation chromogenic agar is useful, only three *Candida* species (*C. albicans*, *C. krusei*, and *C. tropicalis*) can be identified with confidence. Identification of molds such as *Fusarium* and *Scedosporium* species is time consuming, and considerable expertise is required for correct identification when characteristic features are not well defined.

The past decade has seen numerous groups adopt a variety of molecular identification approaches (2–5, 9–11, 13–15, 20) to improve the speed and accuracy of the identification of fungi from culture. However, routine implementation of these tests has not been established due to difficulties in performing the

assay or the need for sophisticated, expensive equipment (as in the case of microarray platforms). Moreover, DNA isolation and purification, a key step for many published assays (3, 6, 9–11, 13, 14), prolongs turnaround time. The technique of applying PCR directly to fungal cells without any DNA extraction (colony PCR) (4, 15, 17) allows for quicker identification and reduction in labor time and costs, but it has not been widely applied in the clinical setting.

Recently, multiplex tandem PCR (MT-PCR) was shown to be a simple, rapid, real-time PCR assay that allowed for the simultaneous detection and identification of numerous pathogens (up to 72 targets) (12, 21). In this study, we developed and evaluated a colony MT-PCR assay to identify 10 *Candida* species (*C. albicans*, *C. dubliniensis*, *C. famata*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lusitanae*, the *C. parapsilosis* complex, and *C. tropicalis*), *Yarrowia lipolytica*, the *Cryptococcus neoformans* complex, *Saccharomyces cerevisiae*, *Fusarium solani*, *Fusarium* sp., and *Scedosporium prolificans* directly from primary isolation culture plates. Molecular targets included the internal transcribed spacer 1 (ITS1) and ITS2 regions of the rDNA gene cluster, beta-tubulin, and elongation factor 1- α . Results were correlated with phenotypic tests.

A total of 183 culture plates containing presumptive fungal growth from clinical specimens were studied (Table 1). Phenotypic identification via the RapID Yeast Plus system (Remel Products, Lenexa, KS) and/or API ID 32C (bioMérieux) and colony MT-PCR were performed in parallel. For colony MT-PCR, a sweep of microbial growth was resuspended in 500 μ l distilled water. MT-PCR was performed on 2 μ l of this suspension with no further purification as previously described (12). Primer sequences were designed as previously published (12). The presence of an organism was determined by analysis software (AusDiagnostics) which compared the observed melt temperature with the expected melt temperature within a range of $\pm 1^\circ\text{C}$. An internal positive control was included for each specimen to monitor for PCR inhibition. The detection limit of the assay was determined by seeding distilled water with serial dilutions (a 2 McFarland standard to 10^4 CFU per ml) of *C. albicans* cells and *Fusarium* conidia, in triplicate. The concentration of the suspensions was confirmed by spreading

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TABLE 1. Results of culture and colony MT-PCR identifications

Specimen plate no.	Culture identification ^a	Colony MT-PCR identification
1-25	<i>C. albicans</i>	<i>C. albicans</i>
26	<i>C. albicans</i>	<i>C. albicans</i> , <i>C. kefyri</i> ^{c,d}
27	<i>C. albicans</i>	<i>C. albicans</i> , <i>S. cerevisiae</i> ^{c,d}
28	<i>C. albicans</i> , <i>Aspergillus fumigatus</i>	<i>C. albicans</i> ^b
29-41	<i>C. albicans</i> , <i>C. glabrata</i>	<i>C. albicans</i> , <i>C. glabrata</i>
42	<i>C. albicans</i> , <i>C. kefyri</i>	<i>C. albicans</i> , <i>C. kefyri</i>
43	<i>C. albicans</i> , <i>C. krusei</i>	<i>C. albicans</i> , <i>C. krusei</i>
44	<i>C. albicans</i> , <i>Candida</i> sp.	<i>C. albicans</i> , <i>C. glabrata</i>
45-47	<i>C. albicans</i> , <i>Candida</i> sp.	<i>C. albicans</i> , <i>C. parapsilosis</i>
48-51	<i>C. dubliniensis</i>	<i>C. dubliniensis</i>
52-53	<i>C. dubliniensis</i> , <i>C. glabrata</i>	<i>C. dubliniensis</i> , <i>C. glabrata</i>
54	<i>C. glabrata</i>	<i>C. albicans</i> , <i>C. glabrata</i> ^c
55-120	<i>C. glabrata</i>	<i>C. glabrata</i>
121	<i>C. glabrata</i>	<i>C. glabrata</i> , <i>C. tropicalis</i> , <i>S. cerevisiae</i> ^c
122	<i>C. glabrata</i>	<i>C. glabrata</i> , <i>C. tropicalis</i> ^c
123-124	<i>C. guilliermondii</i>	<i>C. guilliermondii</i>
125	<i>C. guilliermondii</i> , <i>C. parapsilosis</i>	<i>C. guilliermondii</i> , <i>C. parapsilosis</i>
126-127	<i>C. kefyri</i>	<i>C. kefyri</i>
128-130	<i>C. krusei</i>	<i>C. krusei</i>
131-132	<i>C. lusitaniae</i>	<i>C. lusitaniae</i>
133-138	<i>C. neoformans</i> complex	<i>C. neoformans</i> complex
139-166	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
167	<i>C. parapsilosis</i> , <i>A. fumigatus</i>	<i>C. parapsilosis</i> ^b
168	<i>C. parapsilosis</i> , <i>Aspergillus terreus</i>	<i>C. parapsilosis</i> ^b
169-171	<i>C. tropicalis</i>	<i>C. tropicalis</i>
172	<i>C. tropicalis</i>	<i>C. tropicalis</i> , <i>C. albicans</i> , <i>C. glabrata</i> ^c
173	<i>C. tropicalis</i> , <i>C. albicans</i> , <i>C. glabrata</i>	<i>C. tropicalis</i> , <i>C. albicans</i> , <i>C. glabrata</i>
174	<i>C. tropicalis</i> , <i>C. glabrata</i>	<i>C. tropicalis</i> , <i>C. glabrata</i>
175-176	<i>F. solani</i>	<i>F. solani</i>
177-181	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
182	<i>S. prolificans</i>	<i>S. prolificans</i>
183	<i>Trichosporon</i> species, <i>Aspergillus flavus</i>	No MT-PCR identification ^b

^a Multiple fungal species may have been isolated from culture.

^b Not all fungal species were identified by colony MT-PCR due to the absence of a detection target on the assay.

^c Colony MT-PCR identified other yeasts in addition to the pathogen identified by culture.

^d Pathogens were identified directly from general-purpose mycological media rather than from CHROMagar Candida.

100 μ l of the 10² dilution onto Sabouraud dextrose agar plates in duplicate and counting the colonies.

Specificity was confirmed, as outlined previously (12), by testing DNA from 49 clinically relevant organisms, including fungi, bacteria, and parasites. No cross-reaction with nontargeted microorganisms was observed. The detection limit of the assay (without nucleic acid extraction) was 10⁵ CFU/ml for both *C. albicans* and *Fusarium* sp. All 183 culture plates tested returned an MT-PCR result that matched the phenotypic identification (Table 1). However, MT-PCR detected extra fungal pathogens from five (2.7%) samples (specimens 26, 27, 54, 121, and 122) that were not identified phenotypically. The presence of these extra species was confirmed by (i) reinoculating the suspensions onto CHROMagar Candida medium (CHROMagar, Paris, France) to identify *C. albicans*, *C. tropicalis*, and *C. krusei* and using the RapID Yeast Plus kit to identify other yeast species and (ii) subjecting the suspensions to panfungal PCR, amplifying the entire ITS1, 5.8S rRNA, and ITS2 regions, followed by DNA sequencing. Both methods confirmed the presence of multiple fungal species not identified on the original culture plate. Possible explanations for this include the various expertises of the laboratory staff that may overlook the presence of minute or hidden colonies and the lack of distinct morphological characteristics on general-purpose mycological media. Furthermore, negative PCR controls

proved that the detection of extra species by MT-PCR was not due to contamination.

This study is the first to combine MT-PCR with colony PCR for the rapid identification of fungi directly from culture. Fungal pathogens were easily detected and correctly identified, even in mixed fungal cultures, without the need for DNA extraction. Although the release and purity of DNA were compromised by omitting an extraction procedure (10⁵ CFU/ml detection limit for both yeasts and molds), adequate template was still available for detection by colony MT-PCR. Use of colony PCR reduced the time for identification to <2 h. This is considerably faster than biochemical identification, which requires a pure culture and an additional 4 to 48 h to reach biochemical endpoints. Furthermore, colony MT-PCR resulted in unambiguous species identification. Costs for MT-PCR are similar to those for current routine laboratory tests (AU\$18, US\$14.69, or €10.39 for consumables plus 30 min labor costs), and a robot for automated operation is available for laboratories whose staff have little or no molecular or mycology experience.

With the growing number of organisms being "genetically" reclassified and the limitations of traditional phenotypic tests, fungal identification using molecular methods has become increasingly important. We are currently implementing colony

MT-PCR into our routine laboratory tests. Work on expanding the number of fungal targets is under way.

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