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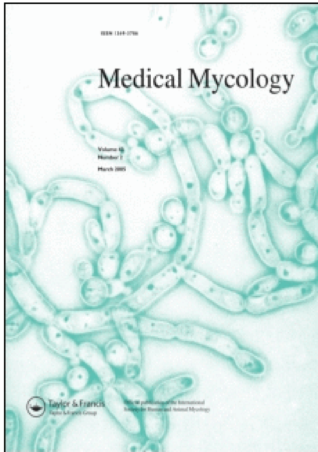

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Review

DNA and the classical way: Identification of medically important molds in the 21st century

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The advent of the 21st century has seen significant advances in the methods and practices used for identification of medically important molds in the clinical microbiology laboratory. Historically, molds have been identified by using observations of colonial and microscopic morphology, along with tables, keys and textbook descriptions. This approach still has value for the identification of many fungal organisms, but requires expertise and can be problematic in determining a species identification that is timely and useful in the management of high-risk patients. For the increasing number of isolates that are uncommon, atypical, or unusual, DNA-based identification methods are being increasingly employed in many clinical laboratories. These methods include the commercially available GenProbe assay, methods based on the polymerase chain reaction such as single-step PCR, RAPD-PCR, rep-PCR, nested PCR, PCR-RFLP, PCR-EIA, and more recent microarray-based, Luminex technology-based, and real-time PCR-based methods. Great variation in assay complexity, targets, and detection methods can be found, and many of these methods have not been widely used or rigorously validated. The increasing availability of DNA sequencing chemistry has made comparative DNA sequence analysis an attractive alternative tool for fungal identification. DNA sequencing methodology can be purchased commercially or developed in-house; such methods display varying degrees of usefulness depending on the breadth and reliability of the databases used for comparison. The future success of sequencing-based approaches will depend on the choice of DNA target, the reliability of the result, and the availability of a validated sequence database for query and comparison. Future studies will be required to determine sequence homology breakpoints and to assess the accuracy of molecular-based species identification in various groups of medically important filamentous fungi. At this time, a polyphasic approach to identification that combines morphologic and molecular methods will ensure the greatest success in the management of patients with fungal infections.

Keywords mold identification, PCR, DNA sequencing

Introduction

The accurate identification of medically important fungi remains a fundamentally important activity in the clinical mycology laboratory. Identification of fungi to species is important to determine etiology of the disease, to detect novel agents of disease, to predict

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Table 1 Molecular methods for mold identification: non-DNA sequence based

Test	Target organism(s)	Reference(s)
Gen-Probe *Accuprobe* test	*H. capsulatum*, *B. dermatitidis*, *Coccidioides* species	[12,13]
Single-step PCR	Dematiaceous fungi, dermatophytes zygomycetes, *S. commune*, *Coccidioides* species, *Fusarium*	[21–31]
RAPD-based	Dermatophytes, *H. capsulatum*, *Rhizomucor* species	[36–39]
Rep-PCR	*Fusarium*, *Aspergillus*, *H. capsulatum*, *B. dermatitidis*, *Coccidioides* species, dermatophytes	[40–43]
Nested PCR	*P. marneffei*, *Aspergillus*	[44–49]
PCR-RFLP	*Stachybotrys*, *Penicillium*, *Aspergillus*, *Cladosporium*	[50,51]
PCR-EIA	*Aspergillus*, *H. capsulatum*, *B. dermatitidis*, *Coccidioides* spp., *P. marneffei*, *Pneumocystis*	[52–54]
Microarrays	Airborne molds, *Candida*/ *Aspergillus*, *Fonsecaea, Phialophora*, *Cladosporium, Sporothrix*, *Mucor*, dermatophytes	[56–59]
Luminex	*Trichosporon*/*Cryptococcus, Fusarium*	[62–64]
LightCycler PCR	*Aspergillus fumigatus*, *H. capsulatum*, *Coccidioides* spp., *Fusarium*	[67–79]
TaqMan assay	*Aspergillus*, *Penicillium, Paecilomyces*	[80–82]

intrinsic resistance to antifungal agents, and to detect clusters of nosocomial infection among hospitalized patients. This information can be critically important in the management of fungal infection in the high-risk patient. On the other hand, it is also important to recognize that many fungal isolates recovered from clinical samples do not represent significant disease [1,2], and that it can be inappropriate to devote resources to species identification without a clear understanding of the clinical relevance of that isolate.

In many cases, classical phenotype-based methods can be used successfully to identify fungi. These methods generally rely upon colony growth on solid media and the detection of reproductive structures (conidiophores and conidia, asci and ascospores, pycnidia, etc.) elicited during incubation of an isolate on appropriate sporulation media. Recognition of characteristic structures, as well as their shape, size, form, color, and mode of attachment, is used to identify fungi. In addition, various biochemical and physiologic tests and thermotolerance studies can be used as adjuncts to morphological methods. Several textbooks and atlases have been published to aid the laboratorian in this task [3–6]. Many pathogenic fungi can be quickly and accurately identified based on recognition of these characteristic structures.

However, in some circumstances, phenotype-based identification is not helpful. If the isolate displays atypical morphology, fails to sporulate, requires lengthy incubation or incubation on specialized media not available to the laboratory, or if the phenotypic results are nonspecific or confusing, the isolate may be a candidate for DNA-based identification. Furthermore, recent advances in phylogenetic taxonomy have revealed cryptic species within morphologically indistinguishable isolates. For example, the *Fusarium solani* species complex now contains at least 50 different species [7], and *Pseudallescheria boydii* is now recognized as a species complex [8]. Based on molecular methods, several new *Aspergillus* species have been identified and validly published within the section *Fumigati* which includes the human pathogen *A. fumigatus* [9]. Precise identification of such isolates may be important in outbreak investigations or in other studies of the epidemiologic significance of particular species or groups. In these situations, DNA-based methods will be required, as members of such closely related complexes can be distinguished from one another only by genotype-based methods.

The purposes of this review are to describe genotype-based methods that are available today for identification of medically important molds within the clinical microbiology laboratory, and to discuss the promises and pitfalls associated with each method, currently available DNA databases, commercially available systems and other electronic resources. The methods discussed are summarized in Table 1, and published molecular targets for DNA sequencing have been summarized in Table 2. The detection and identification of fungal DNA from clinical samples such as body fluids or tissues will not be addressed, nor will the identification of medically important yeasts. It is important to appreciate that many of the assays described in this review have not been used outside one or only a few laboratories and few of these assays have been rigorously validated with a large representative collection of isolates [10].

Molecular methods available for fungal identification: Non-DNA sequence based

GenProbe Accuprobe test

The single commercially available method for mold identification that does not require the polymerase chain

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Table 2 Molecular targets for DNA sequencing of molds

Organism	Target	References
Molds, undifferentiated		
Filamentous fungi (81 genera/species)	D2 region of LSU[1]	[106]
Fungal species (28 species)	D2 region of LSU	[108]
Fungi (44 species)	ITS[2]	[30]
Onygenalean molds		
Dermatophytes	ITS	[115]
Dermatophytes	D2 region of LSU	[107]
Chrysosporium and related onygenales	ITS	[116]
Onygenales (dimorphic fungi and dermatophytes)	D1/D2 region of LSU[3]	[117,118]
Other hyaline molds Aspergillus species	ITS	[93,94]
Penicillium marneffei	ITS	[45]
Fusarium species	EF-1[4]	[7,92,119]
Paecilomyces lilacinus	ITS	[82]
Trichoderma species	ITS	on line database at www.isth.info/tools/molkey/?ver_id = 1
Dematiaceous molds		
Black yeast	ITS	[120,121]
	D1/D2 region of LSU	[21]
Agents of black-grain mycetoma	ITS	[98]
Alternaria/Ulocladium species	ITS	[100]
Fonsecaea species	ITS	[122]
Phaeoacremonium species	β-tubulin	[95]
Pseudallescheria boydii	ITS	[123]
	Calmodulin/β-tubulin	[8]
Scedosporium prolificans	ITS	[124]
Other medically important molds Zygomycetes	D1/D2 region of LSU	[27]
	ITS	[99]
Filamentous basidiomycetes	V9 domain of SSU[5]	[125]
	LSU[6]	[126]
	ITS	[25]
Coelomycetes (selected)	ITS	[127–130]
Smut fungi (*Ustilago/Tilletia*)	ITS	[131]

[1] The ~ 300 nucleotide D2 region of the large ribosomal subunit.
[2] The internal transcribed spacer region of the ribosomal DNA region.
[3] The ~600 nucleotide D1–D2 region of the large ribosomal subunit.
[4] Elongation factor-1 or EF-α.
[5] The V9 domain of the small (18S) ribosomal subunit.
[6] Large ribosomal subunit.

reaction (PCR) is the GenProbe Accuprobe test (Gen Probe, San Diego, CA). This test can be used to confirm isolates of *Histoplasma capsulatum, Coccidioides* species, or *Blastomyces dermatitidis.* The test uses a DNA probe that hybridizes to ribosomal RNA in the sample. This test requires actively growing cultures (mold-phase cells not more than 4 weeks of age, or yeast cells no older than one week), and cannot be performed on formalin-killed cultures [11]. Isolates can be taken from solid media or broth cultures. A 1–2 mm portion of the agar isolate, or the broth culture, is added to lysis and hybridization buffers in a tube containing glass beads. After sonication and denaturation steps, the mixture is incubated with the acridinium ester-labeled specific hybridization probe, which will bind to complementary ribosomal RNA if present in the sample. Following a short incubation to degrade unbound probe, formation of specific DNA-RNA hybrids is quantitated in relative light units (RLU) using a luminometer. Extracts that display RLU values greater than 50,000 are considered positive.

The GenProbe test has replaced exoantigen testing for identification of the dimorphic fungi. Several studies have shown that the GenProbe method is sensitive and specific for identification of the target fungi [12,13]; however, false-positive results can be obtained when testing isolates of related fungi [14,15] within the Onygenales [16,17]. The GenProbe test does not identify *Coccidioides* isolates to the species level; *C. posadasii* and *C. immitis* are both identified as *C. immitis* [18].

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Single step PCR methods

Fungal species identification methods have exploited the enormous resolving power of the polymerase chain reaction (PCR). With PCR, only a few picograms of input DNA can be amplified so that, after 30–40 cycles, the resulting product can be easily visualized on an agarose gel or a capillary electrophoresis instrument. Furthermore, amplification occurs in a specific manner that is determined by the sequence(s) of the complementary primers (short DNA segments that initiate the PCR elongation step upon annealing or attaching to the input DNA), and by the temperature selected for the primer annealing step. The basic elements of PCR can be reviewed at www.pcrlinks.com and www.genetics.nbii.gov/images. A variety of PCR protocols have been utilized for identification of fungal isolates and for clinical samples suspected to contain fungal pathogens [19,20].

In a single step PCR method, target fragment (s) of interest are amplified directly. The PCR primers will amplify only DNA of the targeted genus or species, generating DNA product(s) only if DNA from that 'target' taxon is in the sample. If the unknown DNA is not a member of that particular genus or species, the DNA will not be amplified and no product will be obtained. Single-step PCR assays have been described for the dematiaceous pathogens *Cladophialophora carrionii* [21], *Fonsecaea pedrosoi* [22], and *Hortaea werneckii* [23], where target-specific amplicons are obtained exclusively for these organisms. Similarly, species-specific primers based on the ITS regions have been evaluated for identification of the onygenalean fungus *Paracoccidioides brasiliensis* [24] and the filamentous basidiomycete *Schizophyllum commune* [25]. A specific primer based on microsatellite sequences of *Microsporum audouinii* is also available [26]. Voigt *et al*. used 28S ribosomal sequences to design specific PCR primer pairs for the 13 zygomycetes most commonly involved in human infections [27], where each primer specifically amplified DNA of the corresponding species in a PCR, but did not amplify DNA from related species. Nagao explored the ITS sequences of five *Rhizopus* species (*R. oryzae*, *R. microsporus*, *R. stolonifer*, *R. schipperae*, and *R. azygosporus*) to produce species-specific primers that could be used to distinguish four of these five species. *R. azygosporus* and *R. microsporus* could not be distinguished by PCR using these primers [28].

The specific genus or species can also be identified by the characteristic length and/or pattern of fragments generated during PCR. This pattern of fragments can be detected and/or quantified in various ways. A pair of primers was developed that distinguishes between *Coccidioides immitis* and *C. posadasii*, the agents of Valley fever, by comparing the amplicon sizes on an agarose gel [29]. Length differences can be distinguished with great precision using capillary electrophoresis, and has been used to distinguish among fungal isolates [30,31]. Similarly, fluorescent primers were designed for the ITS regions of five toxigenic and pathogenic *Fusarium* species where these primers were used to amplify species-specific DNA fragments [32].

Single-step PCR protocols may be helpful in situations where the identification of an unknown isolate is already suspected and confirmation by full-DNA sequencing is not needed, and in laboratories that work primarily on a particular taxonomic entity. These protocols may not be practical for laboratories that work on many different types of unknown organisms.

Randomly amplified polymorphic DNA (RAPD)-based methods

First developed in 1990 [33,34], RAPD analysis or arbitrarily-primed PCR (AP-PCR) uses random ten-mer oligonucleotide primers to amplify DNA. The technique yields a specific array of amplification products that are resolved using gel electrophoresis, and the resultant banding pattern is analyzed. The advantage of this method is that no prior knowledge of the genome or DNA sequences of interest is required. Species-specific patterns and/or fragments can be demonstrated using appropriate primer sets and control organisms. This technique has been used for identification of several species of dermatophytes [35,36], *Rhizomucor* species [37], and *Histoplasma capsulatum* [38]. However, due to poor inter-laboratory reproducibility, this method has found limited utility in clinical microbiology laboratories. A new approach based on rapid melting curve analysis of RAPD products has recently been shown to be a reproducible method for species identification in *Candida* [39]; the utility of this approach for identification of filamentous fungal species remains to be seen.

The repPCR method

The 'rep-PCR' (Bacterial BarCodes, Athens, GA) involves amplification of repetitive element DNA, which is present in multiple copies throughout the genomes of bacteria and fungi. Repetitive element DNA is also analyzed in ERIC-PCR, a similar method used in bacteriology and mycology. The resultant rep-PCR pattern is analyzed on a microfluidics chip in an Agilent analyzer. Each unknown DNA generates a pattern of fragments, which are compared to patterns

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in a library of known species patterns. An unknown DNA can be identified to species by its match to a pattern of a known species in the library. This system has been used for the identification of medically important *Fusarium* species [40], *Aspergillus* [41], the dimorphic fungi *Histoplasma capsulatum, Blastomyces dermatitidis*, and *Coccidioides* species [42], and common dermatophytes [43]. The potential utility of this method for fungal identification will be apparent after analysis of a broader panel of species and larger numbers of geographically representative isolates.

Nested PCR methods

In nested PCR, a DNA product of broad specificity (for example, the ITS region) is amplified in the first round of PCR, and then used as the template in a second round of PCR with a second set of species-specific internal primers [44]. The product that is generated after the second-round PCR is specific for the intended target from the species of interest only. Nested PCR [45,46] or semi-nested PCR [47] have been used to identify *Penicillium marneffei* DNA from unknown isolates or from patient materials. For identification of common *Aspergillus* species, the DNA topoisomerase II gene was targeted in a nested PCR that used a common primer set for the first round followed by a specific set of 5 species-specific primer pairs [48]. In another nested PCR, the ITS-1 region was targeted for the identification of *A. fumigatus*, *A. flavus* and *A. niger* [49].

PCR-RFLP

The first-round PCR product is digested with restriction enzyme(s) and species identification can be made by the specific restriction digest fragment pattern (restriction fragment length polymorphism or RFLP). This strategy was used to identify various species within the genera *Stachybotrys*, *Penicillium*, *Aspergillus*, and *Cladosporium*, in an assay to identify species that were involved in impacting indoor air quality [50]. Each unknown DNA was amplified with universal primers to generate a 550–600 bp fragment, which was then digested with the enzymes *EcoRI*, *Hae III*, *Msp I* and *Hinf I* restriction enzymes. Each of the fungal taxa displayed a characteristic restriction digest pattern when displayed on an agarose gel. A similar assay targeting the DNA topoisomerase II gene was designed for the identification of major dermatophytes [51]. This assay can be useful in laboratories that do not have access to sophisticated instrumentation for analysis or sequencing of PCR products. A constraint of the method is that presence of introns may influence the banding patterns resulting in misleading interpretations.

PCR-enzyme immunoassay

In the PCR enzyme immunoassay (EIA), the first-round PCR product is allowed to hybridize with various short probes that are specific for the species of interest. The probes are pre-labeled with colorimetric or fluorescent molecules so that the specific amplicon-probe complex that is formed can be detected using an appropriate assay. In one of the earliest applications of this technique, 21 specific probes were developed, targeting the 28S ribosomal subunit [52], which enabled the identification of a variety of medically important species. The PCR-EIA method builds on this principle and utilizes PCR to amplify a universal fragment, which is then allowed to bind to a species-specific probe in the second step. The probe is labeled with the colorimetric molecule digoxigenin, thus allowing detection of the target fragment-probe complex in an enzymatic assay (PCR-EIA). PCR-EIA methods have been described for identifying *Aspergillus* species [53], dimorphic fungi, including *Coccidioides immitis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Penicillium marneffei*, *Sporothrix schenckii*, and *Pneumocystis carinii* [54]. This first-generation method has now been largely supplanted by microarray and Luminex formats (see below).

Microarray-based methods

The DNA microarray can be used for high throughput identification of multiple microorganisms [reviewed in 55]. In this method, the unlabeled DNA probe is fixed on a solid support, such as a glass slide or nylon membrane. The DNA target sample is simultaneously amplified and labeled with a fluorescent nucleotide during PCR. The probe and target DNA are then allowed to hybridize on the solid support. Unbound probe is washed away, and the fluorescence from the bound target spots is read by a laser. Positive and negative spots are determined. A microarray chip can accommodate many thousands of probes, thus allowing an unknown DNA to be analyzed with many taxa simultaneously and rapidly on one chip. In a modification of this technique, a colorimetric molecule is substituted for the fluorescent molecule, and the chip is incubated with detection reagents after formation of the target-probe complex. Colored spots can be read mechanically to denote positive and negative reactions.

Microarrays have been used to identify clinical and environmental isolates. Wu used the 18S rRNA gene and a series of probes to detect and identify 31 species

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of various airborne molds [56]. Hsiao et al later used ITS-1 and ITS-2 sequences to develop a microarray on a nylon membrane that identified 64 species of clinically important filamentous fungi [57]. In most cases, a single probe detected a single species; however, some group-specific probes detected two or more closely related species indistinguishably. In cases where the ITS sequences displayed high divergence, multiple probes targeting different areas of the ITS region were needed to identify a single species. These probes were labeled with digoxigenin, allowing each specific probe-template reaction to be visualized as a colored spot developed after incubation with horseradish peroxidase-labeled anti-digoxigenin antibodies and substrate solution. Hsiao et al tested these probes with a panel of 71 clinical isolates, and were able to identify 54 of 57 unique strains correctly. An additional 14 isolates, which were species not included in the microarray, correctly displayed no hybridization signals with any probes. This system could detect at least 10 pg of fungal DNA. Other investigators developed a microarray for identification of 9 common species of *Candida* and 4 of *Aspergillus* (*A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*) using ITS primers and fluorescently labeled probes [58]. Most recently, a microarray system was developed that included fluorescent probes targeted to the ITS-2 region [59] for the identification of 20 common species belonging to the genera *Aspergillus*, *Fonsecaea*, *Phialophora*, *Cladosporium*, *Sporothrix*, *Mucor*, and the dermatophytes. Results from the hybridization assay were consistent with results obtained by conventional methods for all 86 clinical isolates examined.

Further investigation, validation and refinement will determine the utility of this method for clinical laboratory use. Microarrays are being developed based on a syndromic approach to disease. In one study, a 'respiratory diseases' chip was developed that contains probes specific for over 26 respiratory pathogens [60], to enable diagnosis of a broad range of respiratory pathogens using one technique. Similar chips can be developed for sepsis, enteric disease and other syndromes. It remains to be seen how these chips can facilitate the diagnosis of fungal infections, but it has been suggested that by the year 2025 such systems may even be useful in diagnosing fungal infection rapidly in the outpatient setting [61].

Luminex technology

Another recent development based on the principle of probe-template binding is the multianalyte profiling (xMAP) system (Luminex Corp., Austin, TX). This assay utilizes a novel flow cytometer and tiny beads called microspheres. Each microsphere is color-coded with a unique dye that enables each bead to be distinguished from all others by the laser reader. Up to 100 different dyes can be distinguished in a single assay. PCR is first performed using universal primers, where the target region of interest is amplified and concurrently labeled with biotin. The amplicons are then mixed with a series of specific capture probes immobilized on different color-coded microspheres. The capture probes can be genus-specific, species-specific, or group-specific. The microspheres are then incubated with streptavidin, the biotin reporter molecule, and analyzed in a flow cytometer containing a two-channel laser, which distinguishes among the beads and allows the species identity of each bead to be determined. In addition, the positive biotin-streptavidin reaction identifies the bead(s) where the probe has hybridized to a specific amplicon. In this manner, DNA from an unknown organism can be scanned with up to 100 different probes simultaneously in one tube. At this time, Luminex assays have been described for the detection and/or identification of a variety of bacteria, viruses, and several fungi (reviewed at www.luminexcorp.com). The earliest studies adapting this technology to fungi were reported by Diaz and Fell with the basidiomycetous yeasts *Trichosporon* [62] and *Cryptococcus* [63]. Luminex assays for the identification of various medically important molds are in preparation at this writing [64].

Real-time PCR-based methods

Real-time PCR is a technique that enables PCR to be performed in a quantitative manner because the PCR can be monitored in 'real time' or as the reaction proceeds, using a reporter molecule that binds to product DNA as it accumulates during each PCR cycle. The amount of product DNA can then be extrapolated back to the amount of input DNA in the original sample. Non-specific detection of PCR product can be performed using dyes such as SYBR Green that bind preferentially to double-stranded DNA. For more specific detection, the PCR is performed using target-specific probes. Several examples of this chemistry include FRET (fluorescence resonance energy transfer) hybridization probes, TaqMan probes, and molecular beacons. Assays based on each of these chemistries have been developed to detect and identify fungi using PCR. Real-time PCR has been reviewed recently in greater detail [65].

The LightCycler system (Roche Applied Science, Indianapolis, IN) uses the FRET system, involving

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two hybridization probes, a 'donor' and an 'acceptor', each labeled with a different fluorescent molecule [reviewed in 66]. The PCR is performed using universal primers, and the two probes are simultaneously allowed to hybridize to this single-stranded DNA product. During this reaction, resonance energy is transferred from the 'donor' fluorescent molecule to the 'acceptor' fluor, a process that will occur only when the two molecules are in close proximity to each other. This proximity will result only if the 'donor' and 'acceptor' probes can hybridize in a specific manner to the PCR product, which occurs only when the sequence of the unknown target DNA is complementary to the sequence of the species probe. This process can be used to identify unknown DNA molecules to the genus or species level, depending on the specificity of the probe-primer sets. An alternative form of real-time PCR uses a single labeled probe (Idaho Technology, Salt Lake City, UT).

There are several advantages to using real-time PCR. These include the high specificity of the assay, as well as the utility of the melting curve kinetics to identify unknown species. Melting curve kinetics measure the specific temperature at which the two strands of DNA will 'melt' or dissociate; the melting temperature is based on the GC content, length and sequence of the particular fragment. This process can be monitored in a real-time PCR instrument using fluorescent dyes. The melting curve can be used to differentiate multiple amplification products separated by as little as 2°C in melting temperature, and can identify the corresponding organism based on the melting temperature of the fragment generated by PCR. By allowing determination of the total fungal burden (or load) contained in a particular clinical sample, the real-time PCR may result in the ability to distinguish between colonization and infection if it is assumed that 'true infection' produces a greater fungal burden than does 'colonization' with the same fungus. Further research is needed to validate this hypothesis. Finally, the assay is rapid, allowing a result to be obtained within one to two hours of DNA preparation.

This quantitative PCR system has been used for simultaneous detection, identification, and quantification of *Aspergillus fumigatus* and *Candida albicans* directly from blood and bronchial lavage fluid [67–72], and of dermatophyte fungi directly from skin specimens [73]. The role of real-time PCR in the diagnosis of invasive aspergillosis has been reviewed recently [74,75].

Comparatively fewer real-time assays have been described for the identification of unknown fungal DNA from cultured isolates. A Light Cycler assay was described for the detection and identification of *Histoplasma capsulatum* in fungal isolates, culture extracts, and from clinical patient specimens [76]. This assay targeted the ITS region, and distinguished 34 isolates of *H. capsulatum* from 73 other clinically important molds and yeasts. Furthermore, *H. capsulatum* DNA was identified in lung biopsy, bronchial lavage, and an EDTA blood sample from patients known to have histoplasmosis. Interestingly, the corresponding culture of the EDTA blood sample failed to detect the organism. In a similar study, DNA from the ITS-2 region of *Coccidioides* species was detected in clinical isolates, respiratory specimens and fresh or paraffin-imbedded tissues [77]. *Coccidioides* DNA could be detected directly from respiratory specimens (sputum, bronchial washings, pleural fluid) with a sensitivity of 100% and specificity of 98.4%. Four specimens were positive by PCR but negative by culture. Analysis of fresh tissue samples showed 92% sensitivity, while paraffin-imbedded tissues showed a sensitivity of 73%. The detection limit was less than 50 copies of target DNA per reaction. Another study used a single-color multiplexed PCR with seven primers and the binding dye LCGreen PLUS to distinguish four species of *Aspergillus* in culture by analysis of melting temperatures (T_m) of the ITS-1 region [78]. This assay can be useful if each species has a characteristic and distinct melting temperature. Finally, a Light Cycler assay with SYBR Green was established for the detection of *Fusarium* species producing tricothecene toxins [79]. Melting point analysis was used to identify the amplicons as members of major *Fusarium* species.

Another fluorogenic assay for the detection of PCR products, the TaqMan assay (Roche Molecular Systems, Pleasanton, CA) takes advantage of the 5′ endonuclease activity of *Taq* polymerase and the Förster-type energy transfer of a fluorescent-labeled probe. The TaqMan probe consists of a 5′ reporter dye, a 3′ quencher dye, and a 3′ blocking phosphate group. The fluorescence emission of the reporter dye is suppressed in the intact probe. During PCR, the probe is cleaved by the 5′ endonuclease activity of *Taq* polymerase only when it is hybridized to a complementary target. When cleavage between the reporter and quencher occurs, the suppression of fluorescence is reversed and an increase in reporter dye fluorescence occurs, indicating that the probe-specific PCR product has been generated. Repeated cycles of probe annealing and cleavage result in exponential amplification of the PCR product and of reporter fluorescence. Fluorescence intensity is measured in a luminescence detector (Roche Molecular Systems).

An early report described the utility of PCR and TaqMan detection for identification of *Aspergillus*

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fumigatus [80]. More recently, 65 quantitative PCR assays utilizing TaqMan chemistry were used for the detection and identification of various species of *Aspergillus*, *Penicillium*, and *Paecilomyces* species in HVAC (heating, ventilation and air-conditioning systems) dust samples [81]. A TaqMan probe was also developed for the identification of human and environmental isolates of *Paecilomyces lilacinus* [82].

Finally, molecular beacons have been used in diagnostic assays. Molecular beacons are single stranded hairpin-shaped oligonucleotide probes. One end of the beacon is labeled with a fluorophore and the other end with a quencher molecule. In the presence of the target DNA sequence, the beacon unfolds, the 'stem' portion hybridizes to the target, and fluorescence occurs as the fluor is no longer constrained by the quencher. In a quantitative assay, the resulting signal is proportional to the amount of target DNA. A molecular beacon assay has been reported for the identification of *Candida dubliniensis* [83], but no assays have been described for identification of medically important molds.

Molecular methods available for mold identification: DNA sequence-based

Recent improvements in technology and the availability of whole genome sequences for many fungi have made DNA sequence-based methods useful for both research and clinical microbiology applications. Several systems utilizing various chemistries for DNA sequencing (BigDye system, Applied Biosystems, Foster City, CA; CEQ system, Beckman Coulter, Fullerton, CA) allow PCR-based sequencing to be performed at reasonable cost. Today it is possible to obtain a sequence-based identification of an unknown fungal species within 24 h of growth in culture and at a cost of under 10 US dollars [84]. Given that this method is rapid, fairly economical and yields robust results, DNA sequencing is fast becoming an attractive method for identification of medically important fungi. In fact, given the excellent specificity of this technique, it has been recently designated as the gold standard for fungal molecular identification [85].

The success of a sequencing strategy in a clinical microbiology laboratory for identification of a wide range of clinical fungi lies in: (i) the choice of locus, (ii) the amenability of the region to PCR amplification and sequencing, (iii) the reliability of interpretation of the results, and (iv) the availability of a sequence database for comparison. The choice of locus depends on the type of fungi studied and the level of identification required. In a study using pyrosequencing where 32 bases in the conserved 18S region were sequenced, *Aspergillus* could not be reliably distinguished from *Penicillium* [86]. In general, the conserved 18S or 28S regions are appropriate for analyses at the genus level and above, while the ITS regions and the variable D1/D2 domains are used for analyses at the clade or species level and below [14,84,87,88, reviewed in 89]. Although the ITS regions and the D1/D2 domains remain the most commonly sequenced fungal loci, they suffer from several disadvantages, including failure to distinguish closely related species due to few variable nucleotide sites [90], existence of paralogous (duplicate) copies of the ITS region in some genera, and inability to clearly delineate individuals at low taxonomic levels (see below). Alternate markers that have been evaluated as possible "universal loci" for use with a wide range of fungi include translation elongation factor 1 α (EF-1), β-tubulin, and *RPB2*, the gene that encodes the second largest RNA polymerase subunit. In the genus *Fusarium*, for example, the ITS region is an inappropriate locus due to the existence of paralogues [91]; hence protein coding genes such as translation elongation factor 1α (EF-1) are sequenced for reliable species/clade identification [92]. In contrast, in the genus *Aspergillus* the ITS region can discriminate species such as *A. terreus*, *A. flavus* and *A. fumigatus* and can be employed in microbiology laboratories for this purpose [93,94]. However, the ITS region is inadequate for intra-section species identification within some sections of the aspergilli; for example, it does not offer clear resolution among various medically important species within the section *Fumigati* such as *Neosartorya pseudofischeri, A. lentulus* and *N. udagawae.* These species can be delineated clearly by sequence analysis using protein coding genes such as β tubulin and Rodlet A [90]. Preliminary studies also indicate that the *RPB2* gene may be useful for intra-species level identification within the section *Fumigati* (Carla Rydholm, personal communication). In the future, it is expected that multiple targets will need to be assessed in order to provide correct species identification within some closely related groups, as has been shown for groups such as the *Aspergillus* section *Fumigati,* the *Fusarium solani* species complex, *Phaeoacremonium* species [95] and others.

As recently pointed out by Summerbell and colleagues [85] and anecdotally known for several years, several fungi (for reasons not yet known) are not amenable to PCR amplification and/or cycle sequencing. For such fungi, additional steps such as redesigning primers or cloning may result in a sequence product. Thus, in spite of the promise shown by sequencing strategies, standardization needs to be

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done both in terms of choice of targets and the methodologies to be employed.

Interpretation of DNA sequence data

Once the target locus is decided and a sequencing product is obtained, the next crucial step is to compare DNA sequence of the unknown with DNA sequences in a database. This is usually done using the BLASTn (basic local alignment search tool) algorithm to find regions of homology between two sequences [96]. Such BLAST searches yield a pair-wise alignment of the queried sequences matched with sequences in the database and represented as ranked scores. The species identification of the unknown isolate can be determined if there is a high sequence similarity (99–100%) to one or more reliable sequences of a known species in the database. Ideally these sequence(s) will be derived from the ex-type culture or other reliably identified isolates. Based on this output, the species identification of the unknown isolate can be determined by comparison to the similarity scores of the database sequences. However, there can be problems in interpreting the results when the percentage similarity is lower. A still unresolved dilemma exists regarding the definition of a genetically distinct species, or, in other words, how many nucleotide differences are required to call an isolate a new species, and, conversely, how many nucleotide similarities are needed to call two isolates members of the same species. In their study of ascomycetous yeasts using the D1–D2 domains, Kurtzman and Robnett demonstrated that isolates of the same species showed differences of 1% or less (6 base pairs or less) in this region, but that isolates of different species showed differences of >1% (more than 6 differences) [97]. Even so, there were exceptions found to this rule among some yeasts. Many investigators have extrapolated this rule to molds; however, few studies have actually validated such a rule using large collections of phenotypically identified isolates and type strains. Several investigators have begun to address these issues by assessing the accuracy of molecular identification of various groups of medically important filamentous fungi [94,99,100]. It has been argued that identification and description of new species should not be done by single locus sequence analysis [101]; however in already well described species, it is not clear if multigene sequence analysis should be the norm for species identification. An additional consideration is the fact that such multigene analysis could prove cumbersome and expensive for high-throughput laboratories. At this time, no specific rules or guidelines for species identification of molds exist based on sequence information. As a significant first step, a working group for the genus *Aspergillus* has been created under the sponsorship of ISHAM (the International Society for Human and Animal Mycoses). One of the goals of this working group is to develop practical species definitions for members of this genus [www.isham.org/working_group_ISHAM_Aspergillus.pdf].

Publicly available DNA sequence databases

It is critical to appreciate that the reliability of any sequence-based identification scheme is dependent on the phylogenetic breadth and taxonomic accuracy of the database being consulted [14]. A reliable database should contain not only sequences of known pathogenic fungi, but also sequences of genetically close relatives and morphologically similar saprophytic fungi. The publicly available GenBank sequence database (http://www.ncbi.nlm.nih.gov/ BLAST), maintained by the US National Library of Medicine in collaboration with EMBL (the European Molecular Biology Laboratory), is most commonly used. GenBank contains a web-based interface where users can both submit sequences to be added to the database and/ or interrogate the database(s) with an unknown sequence using the BLASTn search tool. Any investigator can submit a new DNA sequence to GenBank for no cost, thus continually expanding the number of sequences available in the database. It is now mandatory that novel sequences be submitted to GenBank prior to publication in many scientific journals. The advantage of GenBank is that it is a repository of an ever increasing number of sequences from diverse sources. At the same time, its availability as an open source in itself appears to be GenBank's Achilles heel. The ultimate responsibility for sequence identity and quality rests with the investigator or laboratory group depositing the sequence. Incorrect entries into the database are not challenged nor can they be corrected by anyone other than the original depositor. Unfortunately this has led to many entries with incorrect fungal names or inaccurate and /or 'dirty' sequences. A recent study found that up to 14% of the GenBank sequences for the genera *Alternaria* and *Ulocladium* were incorrect entries [100]. Another *in silico* study using about 51,000 fungal ITS sequences demonstrated that the taxon sampling of fungi in GenBank is incomplete, with 20% of the entries erroneously identified to species level, and that the majority of entries lacked descriptive and up-to-date annotations [102].

Complicating this scenario are two factors peculiar to the field of mycology: (i) as taxonomic research

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progresses, many species names become obsolete and these are not updated in GenBank; (ii) many fungal species may be represented by two names – one indicative of their asexual stage (anamorph) and the other their sexual stage (teleomorph), with GenBank preferentially displaying the teleomorph name. A result of the first situation is that, during sequence query, GenBank can return several high-level matches that appear to refer to multiple different species, but which, in fact, actually all refer to sequences deposited under obsolete names. A recent study illustrates the problem [103]. In a BLAST search the D1–D2 sequence of an unknown yeast matched with 99% homology to sequences from *Candida versatilitis* NRRL Y-6652 and with 99% homology to three additional sequences from *Debaromyces tamari* NRRL Y-6665, *Candida mannitofaciens* NRRL Y-7226, and *Candida halophila* NRRL Y-2483. Consultation with the most recent comprehensive yeast taxonomy manual, which includes both physiological and molecular data [104], showed that all three species had been listed as synonyms of *Candida versatilitis*, thus allowing the choice of this name for the final identification of the unknown isolate. It is the user's responsibility to check the validity of the search results by assessing the taxonomic validity of each high-matching sequence that is returned. The user must also recognize that search results may list unfamiliar names because sequence deposits are often placed under the teleomorph name whereas the anamorph name is more familiar to most clinical laboratorians. For example, most *Histoplasma capsulatum* or *Blastomyces dermatitidis* sequences will match in GenBank to their respective teleomorphs *Ajellomyces capsulatus* and *A. dermatitidis* [16].

On-line databases of fungal names can be helpful in addressing these problems especially if they are up-to-date and reflect consensus opinion of mycological taxonomists. The compilers assess names for validity and legitimacy so the databases can be indispensable sources of information. However, under occasional circumstances, there can be disagreements between the taxonomy database of GenBank and other compilations. Examples include the Index of Fungi which attempts to cover all published fungal names (both extant and extinct) and is available online at www.indexfungorum.org, and the list of medically important fungi found in DoctorFungus (www.doctorfungus.org). A recent initiative is the Mycobank (www.MycoBank.org), an online registration of new fungal names and their associated descriptions and illustrations. This database will be valuable in obtaining information on newly published species, and, as older names are added, also offers an exciting and rapid way to help solve problems that arise in determining which names are validly published [105]. Here, each taxon will receive a unique accession number, analogous to a GenBank accession number, that will be issued for each sequence of that taxon submitted; this unique number can then be used by the authors for citation purposes.

As an alternative to the open sequence database(s), more restricted databases are being constructed, which contain only sequences from isolates whose identification has been well validated phenotypically and taxonomically. The users of such databases can practice greater control over the quality of the sequences and isolates in the databases, and the database can be updated periodically to reflect taxonomic changes and updates. Such species-specific searchable databases of mention are those developed for *Trichoderma* (http://www.isth.info/morphology.php) and *Phaeoacremonium sp*. (http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx)

Commercially available databases

Commercially developed fungal identification systems based on curated sequence databases are also available. One such example is the MicroSeq D2 LSU fungal sequencing system (Applied Biosystems) where the complete system provides reagents for sequencing the ~300 bp D2 region of the large ribosomal subunit, identification and analysis software, and a library of fungal sequences for comparison with unknown DNA. This system was evaluated by Hall *et al*., who were able to identify 158 of 234 medically important filamentous fungal isolates belonging to 36 genera/species of dematiaceous fungi and 45 genera/species of hyaline fungi [106]. Sequences for nearly 30% of the isolates evaluated (11 dematiaceous genera and 16 hyaline genera) were not included in the original MicroSeq library. In other study using the MicroSeq sequencing kit, investigators sequenced 133 isolates of 9 common dermatophyte species and found that all species could be easily identified based on the D2 sequence, with the interspecies differences ranging from 0.4–4.5% [107]. The MicroSeq was also evaluated using 28 different fungal species [108]. These isolates were identified to species in 12 cases and to the genus level in 11 cases. Five samples could not be identified. The authors note that in some cases no interspecies differences could be found in the portion of the D2 region studied, so that in these cases species identification using the D2 region was difficult or impossible. The utility of the D2 region in identifying all common medically important fungi remains to be determined.

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Another scheme, the SmartGene IDNS (SmartGene, Lausanne, Switzerland: www.smartgene.ch) offers a system where proofreading, sequence alignment, interpretation, phylogenetic tree, and report creation are integrated in one set of web-based modules. DNA sequences from an investigator-constructed database are complemented by downloading sequences from GenBank. This system was used to construct a database of 140 ITS sequences from medically important phenotypically validated molds [DE Ciardo *et al*., 2005, Abstr Swiss Society for Microbiology; JI Pounder, KE Simmon, CA Barton, SL Hohmann and CA Petti. Abstr Amer Soc for Microbiol 2006]. The utility of this system in a larger set of medically important fungi remains to be seen. This system may be useful in laboratories with limited experience in DNA sequencing but with the financial means to purchase the product.

User-developed databases have also been described, such as that of Rakeman *et al*. [30], who produced a phenotypically validated database that included ITS sequences from 143 clinical isolates and 58 reference or type strains. In their study, which included 44 medically important species, identifications by phenotype and genotype methods were 100% concordant. These sequences have been deposited in GenBank. Strains of the same species demonstrated >99% sequence identity at the ITS 1 and ITS 2 loci for all but two species tested (*Cladosporium cladosporioides* and *Mucor racemosus*).

Instructive case reports

With the ever increasing number or molecular tools available for fungal identification, the contemporary clinical microbiologist is faced with the conundrum – do traditional methods of fungal identification largely based on morphology continue to have a place in the laboratory? A multi-faceted approach that combines both traditional and molecular methods would be the most useful, practical, and cost-effective way forward for fungal identification and we present a few cases to illustrate this point.

Case study 1

A white mold was isolated from bronchial washings of an HIV-infected male living in an area of the US where histoplasmosis was endemic. The slide culture showed cylindrical and pyriform microconidia but no macroconidia; the organism did not convert to the yeast phase at 37°C. However, the GenProbe test for *H. capsulatum* was positive when tested several times in two laboratories. DNA sequencing of the ITS region showed one match in GenBank at a 94% homology to an isolate of *Nannizziopsis vriesii*. Further investigation identified the isolate as a (not clinically significant) *Chrysosporium* anamorph of *N. vriesii*, a genus that is taxonomically within the Onygenales and thus related to the genus *Histoplasma* [14]. This example reminds us that filamentous fungi that are genetically close relatives of human pathogens may be isolated from clinical samples, and molecular tools should be able to distinguish these from human pathogens. Furthermore, many filamentous fungi historically considered saprophytes may be causative agents of disease in the high-risk patient. The ideal system should be able to identify such opportunistic pathogens as well as more common agents. We are also reminded that morphologic and molecular data should be compared and should be consistent.

Case study 2

A three-year old child was admitted to a hospital one year after a liver transplant. On admission, four crusted nodules 4–12 mm in size were observed on his left shin. A biopsy showed inflammation in the superficial dermis and fungal elements on silver staining. A black mold was isolated in pure culture from the biopsy material. The colony grew quickly but displayed no sporulation on any of a variety of media under different conditions. The ITS sequence displayed 100% identity to a single *Paraphaeospheria* species (anamorph *Paraconiothyrium*) in GenBank. The sequence was then sent to the US Department of Agriculture for comparison with a large coelomycete database. The isolate was identified as *Paraconiothyrium cyclothyrioides* using the more complete USDA database. Coelomycetes are plant pathogens that produce both sexual spores in asci and asexual pycnidia (fruiting bodies) containing asexual conidia. They are usually unable to complete their life cycles under artificial conditions, and so frequently fail to sporulate in the laboratory. They are common environmental organisms, and are being seen more frequently as causative agents of infection in compromised hosts, such as this child. DNA sequencing was the only option for this isolate, which displayed no morphologic features offering a clue as to its taxonomic placement. GenBank was partially helpful, but the more complete database in a specialty laboratory was most helpful in establishing a timely identification for this isolate.

Case study 3

A 90-year-old man with a history of myelodysplastic syndrome and diabetes presented with lower leg wound

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and cellulitis about 1 month after trauma to the leg while trimming brush. The histopathology showed hyphae typical of a zygomycete and culture yielded a fungus identified as a *Mucor* species. Identification as *M. circinelloides* was suggested by morphological characteristics and confirmed by comparison of an ITS sequence which aligned with >98% similarity to multiple sequences of *M. circinelloides* [109]. However, in mating experiments, the isolate failed to produce zygospores with known tester strains, thus casting some doubt about the identification, since the production of zygospores has been considered a reliable procedure for accurate identification of heterothallic zygomycetes, including *M. circinelloides* [110]. Sequencing of additional isolates, including mating strains, revealed genetic heterogeneity among isolates identified as *M. circinelloides,* with the neotype strain being the most divergent. Although this example highlights the utility of ITS sequence comparison for the identification of difficult zygomycetes such as *Mucor* species, it also reveals the problems in interpreting conflicting results including the absence of mating competence among isolates with high% ITS sequence similarity. Results also point out that evaluation of additional loci and mating tests, including a broader array of isolates, will be needed to determine whether *M. circinelloides* is another example of a species complex among the medically important fungi.

Practical impact on the clinical microbiology laboratory

The recognition that some fungal agents of disease possess intrinsic or acquired resistance to antifungal drugs, has prompted calls for fungal identification methods that are rapid and reliable at determining the genus and/or species of potential fungal pathogens in order to prescribe appropriate antifungal therapy [111]. Many clinical laboratories are beginning to assess the usefulness of DNA-based methods for identification of isolates recovered from culture of clinical samples, in order to complement morphology-based methods, or to supplant them when culture results are delayed due to slow or absent sporulation [112]. This approach may be adopted more extensively in the future, particularly as well-validated databases become available. The discoveries of several new species and species complexes have also called into question the ability to use morphologic means to identify every fungal isolate accurately to species. In some cases, distinguishing closely related genera and many species within species complexes will require DNA-based strategies. Another instance where DNA-based methods may be helpful is when the isolate fails to sporulate or displays atypical color, features, or morphology and is therefore impossible to identify based on phenotype alone. The workflow, workload and financial resources available to each laboratory will no doubt greatly impact the ability of that laboratory to adopt DNA-based methods as part of its daily routine [113]. In cases where cost may preclude use of DNA-based methods, problem isolates may be referred to a reference laboratory for identification if needed. A reference laboratory can be useful if the result can be returned in time to make a significant impact in patient care, or if the result can be used to inform hospital staff about novel fungal pathogens. Isolates that reflect discrepancies between phenotypic and genotypic results should also be referred to a reference laboratory, where more detailed testing can be performed. At every instance, publication of well-documented case reports describing novel agents of disease and deposition of isolates in recognized culture collections should be encouraged.

In the future, well designed epidemiologic studies will be required to determine the need and clinical usefulness of identifying isolates within species complexes on a routine basis. At this time, identification to species complex is probably sufficient for most sporadic patient isolates. Timely identification of fungal isolates to species can be extremely important when recovered from high-risk patients, as fungal infections in these patients can be serious, difficult to treat, and rapidly fatal. In such high-risk patient settings, identification to species can be extremely helpful in guiding appropriate antifungal therapy. On the other hand, species level identification (based on molecular methods) may have limited importance for many fungal isolates recovered from clinical samples, as these isolates may not represent significant disease [114]. Basic clinical laboratories, especially those in hospitals caring for compromised patients, should have the proficiency to identify filamentous fungi to the genus level, and should be able to distinguish intrinsically antifungal resistant species from their susceptible counterparts. A clinical isolate should be further identified to species: (i) if it is a causative agent of disease, (ii) if species identification can predict appropriate antifungal treatment, or (iii) if the isolate is suspected of involvement in an outbreak of nosocomial infection. Phenotype and genotype-based results should be compared and interpreted together with the clinical history of the patient to arrive at a final identification. Thus, the identification of fungal pathogens requires input from both the clinician and the laboratorian for the diagnostic process to be successful, productive and cost-effective.

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Conclusion

The future of medical mycology will undoubtedly require the use of the methods described in this review. However, it is important that such methods be well validated and subject to appropriate quality assurance. Although GenBank continues to improve as more and more appropriately validated sequences are deposited as the result of competent taxonomic studies, the existence of poorly characterized or frankly incorrect GenBank entries continues to preclude its use by individuals not aware of its pitfalls. Currently available databases, both public and private, should be extended by including more validated isolates encompassing taxa of clinically relevant fungal pathogens and closely related members. Concerted research aimed at setting sequence homology based breakpoints for determining conspecificity of species should be undertaken by experts in the field jointly with clinical microbiologists. With all this in place, medical mycologists will be well-equipped to move fungal identification into the 21st century.

Disclaimer

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the CDC. The use of product names in this manuscript does not imply their endorsement by the US Department of Health and Human Services.

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