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Utility of Random Amplified Polymorphic DNA PCR and TaqMan Automated Detection in Molecular Identification of *Aspergillus fumigatus*

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We developed a method for the identification of *Aspergillus fumigatus* fungal isolates by using random amplified polymorphic DNA (RAPD) PCR (RAPD-PCR) cloning and the TaqMan LS50B fluorogenic detection system (Perkin-Elmer Corp., Applied Biosystems, Foster City, Calif.). DNA from seven clinically important *Aspergillus* species was screened by RAPD-PCR to identify section- or species-specific amplicons. With the OPZ19 RAPD primer a 1,264-bp product was amplified from all *A. fumigatus* strains initially examined but not from other species. A partial DNA sequence of this product was used to design a specific primer pair, which generated a single 864-bp fragment with DNA from 90 of 100 *A. fumigatus* isolates when a "touchdown" (65→55°C) annealing protocol was used. The TaqMan system, a fluorogenic assay which uses the 5′→3′ endonuclease activity of *Taq* DNA polymerase, detected this 864-bp product with DNA from 89 of these 90 *A. fumigatus* strains; 1 DNA sample generated an indeterminate result. With DNA from three morphologically typical *A. fumigatus* isolates, six white ("albino") *A. fumigatus* isolates, and five of six *Neosartorya* species (non-*A. fumigatus* members of the section *Fumigati*), the 864-bp product was amplified differentially at an annealing temperature of 56°C but not with the touchdown annealing format. No amplicon was detected with DNA from 56 isolates of heterologous *Aspergillus*, *Penicillium*, and *Paecilomyces* species or from *Neosartorya fennelliae*; TaqMan assay results were either negative (51 isolates) or indeterminate (5 isolates) for all isolates. This RAPD-PCR and TaqMan assay offers promise as a nucleic acid-based system that can be used for the identification of filamentous fungal isolates and that requires no postamplification sample manipulations.

Filamentous fungal isolates are typically identified by microscopic demonstration of characteristic morphologic structures after growth on appropriate media. Identification may be delayed if the isolate fails to form the diagnostically appropriate structures. Furthermore, inexperience in microscopy may lead to misidentification.

These problems may be obviated by using DNA-based methods for identification and species assignment of isolates. One approach to probe design uses random amplified polymorphic DNA (RAPD) PCR (RAPD-PCR) to generate markers for any specific genome (8; for a review, see reference 10). The major advantage of this approach is that no prior DNA sequence information is required. Likewise, several methods that detect amplified products exist (for a review, see reference 22), but most require extensive post-PCR sample manipulation and additional incubation time before results are available.

The automated TaqMan detection system (Perkin-Elmer Corp., Applied Biosystems, Foster City, Calif.) is a novel fluorogenic assay for the detection of PCR products. It takes advantage of the endonuclease activity of *Taq* polymerase and Förster-type energy transfer of a fluorescence-labeled probe (4, 14, 19, 26). The TaqMan probe consists of a 5′ reporter dye (6-carboxyfluorescein [FAM]), a 3′ quencher dye (6-carboxytetramethylrhodamine [TAMRA]), and a 3′ blocking phosphate group. The fluorescence emission of the reporter dye is suppressed in the intact probe by Förster-type energy transfer (9). During PCR, the probe is cleaved by the 5′ nuclease activity of *Taq* polymerase only when it is hybridized to a complementary target. When cleavage between the reporter and quencher occurs, 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 an LS50B luminescence detector (Perkin-Elmer Corp., Applied Biosystems).

The goal of this study was to explore the utility of the RAPD-PCR screening and TaqMan assay approaches in the identification and taxonomy of filamentous fungi by using *Aspergillus fumigatus*, an agent of invasive aspergillosis, as the target organism. A RAPD-PCR technique was used to screen *A. fumigatus* DNA for species-specific amplicons. A specific 1,264-bp band was amplified from *A. fumigatus* DNA but not from the DNA of other species. The DNA sequence of this fragment was determined and was used to design a specific primer pair, which amplified a single 864-bp fragment from morphologically typical *A. fumigatus* by a "touchdown" (65→55°C) annealing protocol. We then evaluated the usefulness of these primers in identifying *A. fumigatus* and the TaqMan assay for detection of the amplified product.

(These data were presented in part at the 96th General Meeting of the American Society for Microbiology, New Orleans, La., 19 to 23 May 1996 [5a].)

MATERIALS AND METHODS

Strains and growth conditions. A total of 164 isolates were tested in this study. Seventy-one clinical *A. fumigatus* isolates and 1 isolate reidentified at the Centers for Disease Control and Prevention (CDC) as a *Neosartorya* sp. were received from San Francisco, Calif., as part of CDC's fungal active surveillance (1992 to 1994). Nineteen other clinical and environmental *A. fumigatus* strains, including

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six nonpigmented ("albino") *A. fumigatus* strains, were obtained from the CDC reference culture collection. Five clinical isolates representing morphologic variants of *A. fumigatus* were supplied by L. J. R. Milne, Regional Mycology Reference Laboratory, Western General Hospital, Edinburgh, United Kingdom. Five *A. fumigatus* and seven *Neosartorya* strains (23) were provided from the stock collection of S. Peterson, Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill. Fifty-six isolates consisting of *Penicillium notatum* ($n = 1$), *Penicillium spinulosum* ($n = 1$), *Penicillium citrium* ($n = 1$), *Penicillium funiculosum* ($n = 1$), *Penicillium marneffei* ($n = 3$), *Penicillium* (undetermined) species ($n = 1$), *Paecilomyces variotii* ($n = 1$), *Aspergillus flavus* ($n = 26$), *Aspergillus niger* ($n = 9$), *Aspergillus nidulans* ($n = 3$), *Aspergillus glaucus* ($n = 1$), *Aspergillus oryzae* ($n = 1$), *Aspergillus terreus* ($n = 4$), *Aspergillus ustus* ($n = 1$), and *Aspergillus versicolor* ($n = 2$) were also obtained from the CDC culture collection; 34 of these came from the San Francisco active surveillance. Isolates were stored at −80°C. Working stocks were maintained on Czapek Dox medium at 4°C, and species identifications were confirmed by conventional methods (3).

DNA isolation. A spore suspension from a 7-day culture on Czapek Dox medium was inoculated into 15 ml of YPD (yeast-peptone-dextrose) medium in a petri dish, and the dish was incubated at 30 or 37°C for 48 to 72 h. DNA was then prepared from the hyphal mat by the Puregene plant tissue protocol (Puregene; Gentra Systems, Inc., Research Triangle Park, N.C.) according to the manufacturer's instructions, except that the final DNA pellet was solubilized in 10 mM Tris–1 mM EDTA–0.7 M NaCl, made to 1% in cetyltrimethylammonium bromide, and incubated at 60°C for 15 min. The solution was then extracted with $CHCl_3$, and DNA was reprecipitated with 2 volumes of ethanol. The DNA concentration of each sample was determined by using a fluorometer (Hoefer, San Francisco, Calif.) with calf thymus DNA as a standard. A portion of each DNA sample was also checked for integrity on an agarose gel before use.

DNA sequencing and analysis. RAPD was performed with decamer primers (Operon Z kit; Operon Technologies, Alameda, Calif.) as described previously (5). The DNA bands of interest were excised from Tris-acetate gels, purified with GeneClean (Bio 101, Inc., La Jolla, Calif.), and cloned with the PCR-Script SK+ cloning kit (Stratagene, La Jolla, Calif.). Plasmid DNA was purified on a QIAprep spin column (Qiagen, Chatsworth, Calif.). DNA sequencing was performed on an ABI model 373 automated DNA sequencer by using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Corp., Applied Biosystems) with double-stranded plasmid DNA templates.

DNA sequences were analyzed by using DNAStar software (DNAStar, Inc., Madison, Wis.). PCR primer sequences were selected by using the Oligo program (24) (version 5; National Biosciences, Plymouth, Minn.). Two primers, primer Z19-276 (5′-TTGATCTGGCCCTGGCTTGGG) and primer Z19-660 (5′-CAACATTGAAATCCAAGAGGC), were chosen to amplify the 864-bp *A. fumigatus*-specific fragment. The universal fungal primers NS7 and NS8 (27) were used to coamplify a 377-bp fungal 18S rRNA gene fragment in the same reaction tube.

Touchdown PCR. DNA amplifications were performed in a model 9600 DNA thermal cycler (Perkin-Elmer Corp., Applied Biosystems). Reagents were obtained from Boehringer Mannheim (Indianapolis, Ind.); AmpliTaq was obtained from Perkin-Elmer Corp. PCR was performed in a 50-μl reaction mixture with final concentrations (per reaction) of 1× PCR Core Buffer, 2.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 10 pmol of each primer, 1 U of AmpliTaq, and 50 ng of template. Initial amplifications were performed as 35 cycles of 94°C for 1 min (denaturation), 56°C for 1 min (annealing), and 72°C for 1 min (extension), followed by a 10-min final extension at 72°C. Further amplifications were performed by using a touchdown protocol (7, 17) from 65 to 55°C. Briefly, the annealing temperature was programmed at 65°C for the first three cycles. Then, the annealing temperature was lowered in 1-increments every three cycles until 61°C and was then lowered in 2-degree increments from 59 to 55°C, at which 10 final cycles were performed (total of 31 cycles). No-template controls were included with every experiment.

TaqMan detection assay. The TaqMan LS50B detection system (Perkin-Elmer Corp., Applied Biosystems) was used for automated fluorogenic detection of the products amplified in the touchdown PCR. TaqMan probes were designed by following the general rules outlined by the manufacturer (15) and were synthesized in the Biotechnology Core Facility, CDC. A probe detecting the *A. fumigatus*-specific 864-bp amplicon (5′-CTCAACAGTGGATTGGACGTAATCA) contained the reporter dye FAM covalently attached to the 5′ end and the quencher TAMRA attached to the thymidine at position 23. A 3′ phosphate was added to block amplification of the probe by *Taq* polymerase. The 377-bp rRNA gene control amplicon was detected with another TaqMan probe (5′-CCTTGG CCGAGAGGTCTGGG) containing the reporter dye hexachloro-6-carboxyfluorescein (HEX) linked to the 5′ end and TAMRA linked to the 3′ end via a linker arm thymidine (Amino-Modifier C6 dT; Glen Research, Sterling, Va.).

The TaqMan assay was carried out in a 50-μl volume reaction as described above, with the additions of FAM- and HEX-labeled probes at 5 pmol each per reaction. All samples except the controls were tested in duplicate; the controls were tested in triplicate. Following touchdown PCR, a 40-μl aliquot from each well was transferred to a 96-well flat-bottom white microtiter plate (Perkin-Elmer Corp.). The emission intensities of FAM (518 nm), HEX (556 nm), and TAMRA (582 nm) were read in a Perkin-Elmer LS-50B luminescence spectrophotometer equipped with a microtiter plate reader. An increase in emission intensity at 518 nm (FAM) or 556 nm (HEX) was seen in the presence of specific template because of the reporter dye released as the probe was digested by *Taq* polymerase.

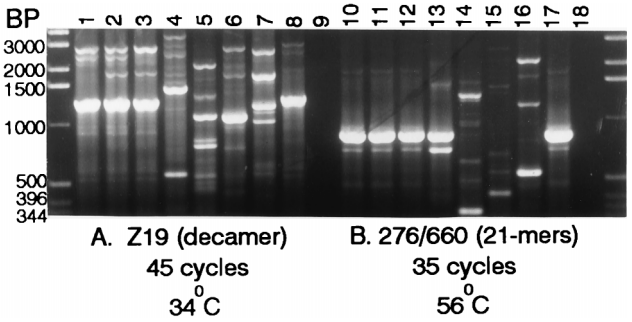

FIG. 1. DNA from *Aspergillus* spp. amplified in duplicate either by RAPD-PCR with the OPZ19 primer (A) or by conventional PCR with the derived primers Z19-276 and Z19-660 (B). Lanes 1 and 10, *A. fumigatus* CDC MAS92-229; lanes 2 and 11, *A. fumigatus* CDC MAS92-564; lanes 3 and 12, *A. fumigatus* NRRL 163 (Thom 1911 ex type); lanes 4 and 13, *A. fumigatus* CDC-B5051; lanes 5 and 14, *A. flavus* MAS92-30; lanes 6 and 15, *A. terreus* MAS92-640; lanes 7 and 16, *A. niger* MAS92-52; lanes 8 and 17, *A. fumigatus* MAS93-678; lanes 9 and 18, no-template control. BP, base pairs.

Data acquisition and analysis were performed by using the Fluorescence Data Manager 2-Reporter Multicomponent spreadsheet and software according to the manufacturer's instructions (Perkin-Elmer Corp.). For each reporter, its emission intensity was divided by the emission intensity of the quencher to give a ratio defined as RQ^+ for samples. The baseline ratio of emission intensities with the intact TaqMan probe (RQ^-) was determined similarly with a series of three no-template controls. Controls containing the FAM probe or the HEX probe alone were also incorporated to correct for spillover in the emission intensity between the two reporters. Finally, for each reporter the average RQ^- value for the three no-template controls was subtracted from the RQ^+ value for each sample to give a value defined as ΔRQ for that reporter. Nonspecific fluctuations in fluorescence were normalized in this calculation by using the quencher as an internal standard. Final ΔRQ results were expressed as normalized values for FAM and HEX fluorescence, respectively. Each normalized value represented the magnitude of signal generated during PCR with that probe.

An "indeterminate" category was created to define TaqMan results that consistently registered outside the positive and negative clusters. Indeterminate FAM standards were defined as FAM results from dilutions of two control templates (*A. versicolor* and *A. nidulans*) that consistently fell between the two clusters and that displayed only faint amounts of the 868-bp product on agarose gels. For each experiment, the means of the positive, negative, and indeterminate standards were calculated. Positive and negative cutoffs were then calculated by using the maximum-likelihood approach as the point halfway between the means of the positive and indeterminate standards and halfway between the means of the indeterminate and negative standards, respectively (2).

To confirm the TaqMan assay results, 25 μl of each touchdown PCR product was electrophoresed through a 1.4% agarose gel, stained with ethidium bromide (0.5 mg/ml), and photographed.

Nucleotide sequence accession number. The complete 1,264-bp sequence of the product amplified by the Z19-RAPD primer has been deposited with GenBank and has been given the accession no. AF022238.

RESULTS

RAPD analysis. DNAs from a set of isolates comprising medically important *Aspergillus* species were screened with 20 decamer primers to identify amplicons that appeared to possess specificity at the species or section level. *A. fumigatus* ($n = 3$ isolates), *A. flavus* ($n = 4$), *A. glaucus* ($n = 1$), *A. nidulans* ($n = 1$), *A. terreus* ($n = 1$), *A. ustus* ($n = 1$), and *A. niger* ($n = 5$) were screened (Fig. 1A and data not shown). The primer OPZ19 was selected for further study. A band of 1,264 bp in length was amplified from 66 randomly selected *A. fumigatus* strains with the Z19 primer (Fig. 1A and data not shown). DNAs in this band from isolates CDC MAS92-229 and CDC MAS92-564 were cloned, generating the plasmids 19B1 and 19B2, respectively, and sequenced. The sequence information was used to design a specific primer pair, Z19-276 and Z19-

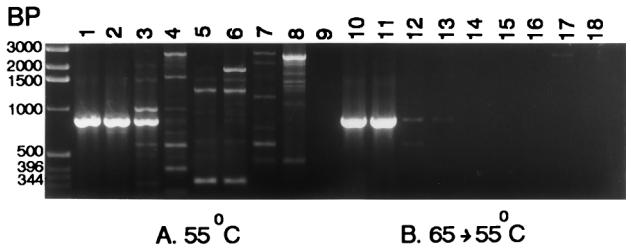

FIG. 2. DNA from *Aspergillus* spp. amplified with primers Z19-276 and Z19-660 by either a conventional annealing protocol (A) or a touchdown protocol (B). Lanes 1 and 10, *A. fumigatus* CDC MAS92-229; lanes 2 and 11, *A. fumigatus* NRRL 163; lanes 3 and 12, *N. spinosa* NRRL 5034; lanes 4 and 13, *N. fennelliae* NRRL-A22212; lanes 5 and 14, *A. flavus* MAS92-30; lanes 6 and 15, *A. flavus* MAS92-230; lanes 7 and 16, *A. niger* MAS92-641; lanes 8 and 17, *A. niger* MAS92-243; lanes 9 and 18, no-template control. BP, base pairs.

660, which amplified a DNA fragment of 864 bp from *A. fumigatus* (Fig. 1B).

A search with the BLAST algorithm shows significant sequence similarity of the full 1,264-bp sequence with two other sequences. The 1,264-bp *A. fumigatus* sequence has 65% identity with YSCH9332 (*Saccharomyces cerevisiae*) at bases 33,685 to 34,043 or 60% identity at bases 33,685 to 34,440 and has 67% identity with SPAC1F5 (*Schizosaccharomyces pombe*) at bases 26,835 to 27,179. These sequences contain open reading frames encoding hypothetical products of 3,744 and 3,655 amino acids.

Touchdown PCR. The Z19-276 and Z19-660 primer pair was evaluated for sensitivity and specificity of amplification with DNAs isolated from members of the genus *Aspergillus*. After 35 cycles at an annealing temperature of 56°C, a variety of nonspecific bands was amplified from heterologous DNA (Fig. 2A). A touchdown protocol (annealing temperatures, 65 to 55°C) was implemented to improve the specificity of PCR (7), and it nearly eliminated nonspecific amplicons, as determined by visualization of ethidium bromide-stained gels (Fig. 2B). A panel of DNAs from *Aspergillus* and *Neosartorya* strains comprising members of the section *Fumigati* were then tested with the original OPZ19 primer and the Z19-derived primer pair (Fig. 3). Under lower-stringency conditions (annealing temperature, 56°C), an 864-bp band could be amplified from all tested members of the section *Fumigati* except *Neosartorya fennelliae* (Fig. 3B). However, when identical reagents were used with the touchdown protocol, an intense 864-bp band was amplified only from strains of *A. fumigatus* (including *A. fumigatus* var. *ellipticus*, *A. fumigatus* var. *helvola*, and *A. fumigatus* var. *acolumnaris*) (Fig. 3C). Two *Neosartorya fischeri* strains and *Neosartorya glabra* produced a weaker but detectable band of the same size. Two nonpigmented (albino) strains of *A. fumigatus* and a strain of *A. fumigatus* var. *sclerotiorum* showed faint or no reactivity under these conditions.

TaqMan assay system screening of homologous and heterologous clinical isolates. The TaqMan assay system was adapted for automated fluorogenic detection of the 864-bp *A. fumigatus*-specific amplicon and the 377-bp rRNA control amplicon simultaneously. Seventy-one clinical isolates of *A. fumigatus* from San Francisco were simultaneously screened with the Z19-derived primer pair and with the control primers NS7 and NS8 (Fig. 4 and Table 1). In order to rule out the possibility that the 864-bp amplicon represented a geographic variant unique to San Francisco, 29 clinical, environmental, and stock collection isolates of *A. fumigatus* from a variety of additional geographic locations were tested.

DNAs from 89 isolates of *A. fumigatus* were detected by the

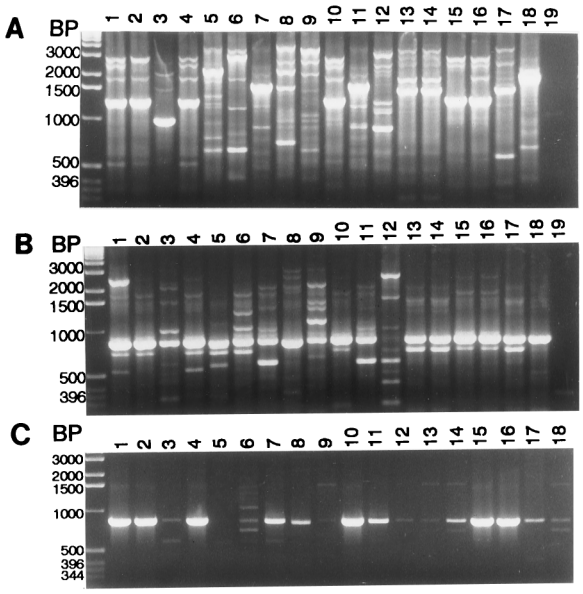

FIG. 3. DNA from members of *Aspergillus* section *Fumigati* (*A. fumigatus* or *Neosartorya* spp.) amplified by RAPD-PCR with the OPZ19 primer (A), by conventional PCR with primers Z19-276 and Z19-660 (B), or by touchdown PCR with primers Z19-276 and Z19-660 (C). Lanes 1, *A. fumigatus* var. *ellipticus* NRRL 5109; lanes 2, *A. fumigatus* NRRL 163; lanes 3, *N. spinosa* NRRL 5034; lanes 4, *A. fumigatus* var. *helvola* NRRL 2244; lanes 5, *N. aureola* NRRL 2244; lanes 6, *A. fumigatus* var. *sclerotiorum* NRRL 6137; lanes 7, *N. fischeri* NRRL 181; lanes 8, *N. glabra* NRRL 2163; lanes 9, *N. pseudofischeri* NRRL 3496; lanes 10, *A. fumigatus* var. *acolumnaris* NRRL 5587; lanes 11, *N. fischeri* NRRL A7223; lanes 12, *N. fennelliae* NRRL A22212; lanes 13, *A. fumigatus* CDC 95-11279 (albino); lanes 14, *A. fumigatus* CDC 95-11277 (albino); lanes 15, *A. fumigatus* CDC MAS92-229; lanes 16, *A. fumigatus* CDC MAS92-564; lanes 17, *A. fumigatus* CDC B5051; lanes 18, *A. fumigatus* CDC 1809; lanes 19, no-template control. BP, base pairs.

TaqMan assay as an elevation in FAM fluorescence that correlated with the presence of an 864-bp band that intensely stained with ethidium bromide after agarose gel electrophoresis (Fig. 4 and Table 1). These isolates included 85 of 89 morphologically typical clinical *A. fumigatus* strains, the type strain (*A. fumigatus* NRRL 163), *A. fumigatus* var. *acolumnaris*, *A. fumigatus* var. *ellipticus*, and *A. fumigatus* var. *helvola*. Data

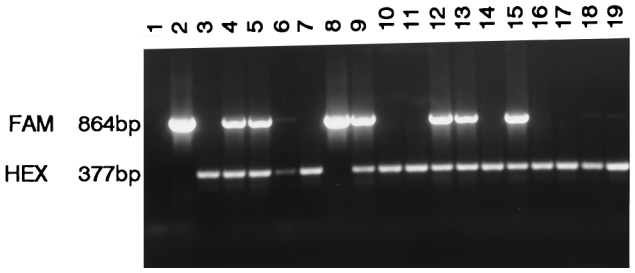

FIG. 4. PCR for detection of the 864-bp *A. fumigatus* diagnostic product (FAM-labeled probe) and the 377-bp fungal rRNA control (HEX-labeled probe). Lane 1, no-template control; lane 2, plasmid 19B1 (FAM probe-only control); lane 3, *A. flavus* CDC MAS92-30 (HEX probe-only control); lane 4, *A. fumigatus* CDC MAS92-229 (FAM-positive control); lane 5, *A. fumigatus* MAS92-564 (FAM-positive control); lane 6, *A. versicolor* MAS92-490 (FAM- and HEX-indeterminate control); lane 7, *A. flavus* MAS92-230 (FAM-negative control); lane 8, plasmid 19B1 (HEX negative); lane 9, *A. fumigatus* MAS93-805; lane 10, *A. terreus* MAS93-997; lane 11, *A. flavus* MAS93-1040; lane 12, *A. fumigatus* var. *ellipticus* NRRL 5109; lane 13, *A. fumigatus* NRRL 163; lane 14, *N. spinosa* NRRL 5034; lane 15, *A. fumigatus* var. *helvola* NRRL 174; lane 16, *N. aureola* NRRL 2244; lane 17, *A. fumigatus* var. *sclerotiorum* NRRL 6137; lane 18, *N. fischeri* NRRL 181; lane 19, *N. glabra* NRRL 2163.

TABLE 1. Comparison of TaqMan PCR and ethidium bromide staining for detection of the 800-bp *A. fumigatus*-specific amplicon in a touchdown PCR annealing format

TaqMan detection result (FAM)	Isolate (no. of isolates) with the following ethidium bromide staining result:	
	Positive	Negative
Positive	*A. fumigatus* (85) *A. fumigatus* var. *acolumnaris* (1) *A. fumigatus* var. *ellipticus* (1) *A. fumigatus* var. *helvola* (1) *A. fumigatus* ex. type (1)	
Indeterminate	*A. fumigatus* (1) *Neosartorya* sp. (1)	*A. flavus* (1) *A. versicolor* (1) *A. nidulans* (3)
Negative		*A. fumigatus* (3) *A. fumigatus* (albino) (6) *A. fumigatus* var. *sclerotiorum* (1) *A. flavus* (25) *A. glaucus* (1) *A. niger* (9) *A. oryzae* (1) *A. terreus* (4) *A. ustus* (1) *A. versicolor* (1) *N. aureola* (1) *N. spinosa* (1) *N. fennelliae* (1) *N. pseudofischeri* (1) *N. glabra* (1) *N. fischeri* (2) *Penicillium notatum* (1) *P. spinulosum* (1) *P. citrium* (1) *P. funiculosum* (1) *P. marneffei* (3) *Penicillium* sp. (1) *Paecilomyces variotii* (1)

from a representative experiment are shown in Fig. 5. Of the remaining 11 *A. fumigatus* isolates, DNA from one morphologically typical strain produced an indeterminate FAM result but showed the 864-bp band on a stained gel (scored as positive). DNAs from three morphologically typical strains, six white (albino) strains, and *A. fumigatus* var. *sclerotiorum* produced no elevated FAM signal and no 864-bp product. When these samples were retested at a lower annealing temperature (56°C), all produced both an elevated FAM result and the 864-bp band (Fig. 3 and data not shown).

Neosartorya aureola, *Neosartorya spinosa*, *N. fenneliae*, and *Neosartorya pseudofischeri* produced no FAM reactivity and no visible 864-bp amplicon under touchdown conditions. *N. glabra* and both isolates of *N. fischeri* produced faint amounts of the 864-bp amplicon (scored as negative), with no elevated FAM reactivity (Fig. 4). One San Francisco isolate originally identified as *A. fumigatus* by the referring institution was later confirmed as a *Neosartorya* sp. at the CDC Fungal Reference Laboratory. This template generated an indeterminate FAM result, and the 864-bp amplicon was present (scored as positive).

Fifty-six isolates of heterologous species (47 isolates of *Aspergillus* species and 9 isolates of morphologically similar *Penicillium* and *Paecilomyces* species) were included (Table 1). DNAs from 51 isolates demonstrated no false-positive results either in the TaqMan assay or on a stained agarose gel. DNA from one isolate of *A. flavus*, one isolate of *A. versicolor*, and three isolates of *A. nidulans* produced an indeterminate FAM result, but no 864-bp amplicon was detected. These results were interpreted as negative. When these 56 samples were retested at lower stringency (56°C), the 864-bp band still could not be detected.

The sensitivity of the TaqMan assay was comparable to that of ethidium bromide staining. When a known positive template was tested in a dilution series, the detection limit for an unequivocal positive result was approximately 10 ng of input template DNA, and 1 ng to 10 pg correlated with an indeterminate result. With less than 10 pg both TaqMan assay and staining results were negative (data not shown). TaqMan assay results with a template concentration of 50 ng per 50-μl reaction volume were consistently reproducible, so this template concentration was selected for subsequent assays.

All tested samples demonstrated an increased HEX signal that correlated with amplification of the 377-bp rRNA control target. On initial testing, two samples (one *A. versicolor* isolate and one *A. nidulans* isolate) failed to generate either an elevated HEX signal or the control band. DNA was reisolated from these cultures, and the control was successfully amplified from the new preparations.

DISCUSSION

A variety of filamentous fungi have been increasingly recognized as agents of serious disease in susceptible patient populations (1, 21). Accurate and timely identification of the causative agent is extremely important for the diagnosis and management of these diseases, as well as for surveillance and epidemiologic studies (6).

One activity of this laboratory is to explore the utility of novel technologies potentially useful as rapid, straightforward alternatives to traditional microscopic and phenotypic identification methods for filamentous fungi. Nucleic acid-based identification strategies have been developed for many pathogenic fungi (25; for a review see reference 18), but many of these approaches require extensive DNA sequence information, postamplification sample manipulation, radioisotope usage, or other advanced technical and interpretive skills. In this investigation, we developed PCR primers to identify *A. fumigatus* in unknown fungal isolates. These primers amplify a fragment originally identified by DNA fingerprinting with arbitrary decamer primers at low stringency.

Our goal was to develop an assay that would discriminate closely related species within the section *Fumigati* (23) while simultaneously recognizing a range of morphologic variants as members of the species *A. fumigatus* (13). Other investigators have attempted to achieve similar goals by using selected rRNA gene regions as amplification targets (12, 16, 20, 25); however, taxonomically related strains had not been tested. In this investigation, we used identical reagents in annealing formats of differential specificities: a touchdown annealing for discrimination of *A. fumigatus* and lower-stringency annealing to include *Neosartorya* spp. and nonpigmented *A. fumigatus* strains.

The TaqMan fluorescent assay represents a novel development in the detection of amplified PCR products. In our laboratory, this technique enabled samples to be analyzed rapidly (as soon as 5 to 10 min after PCR was completed) without

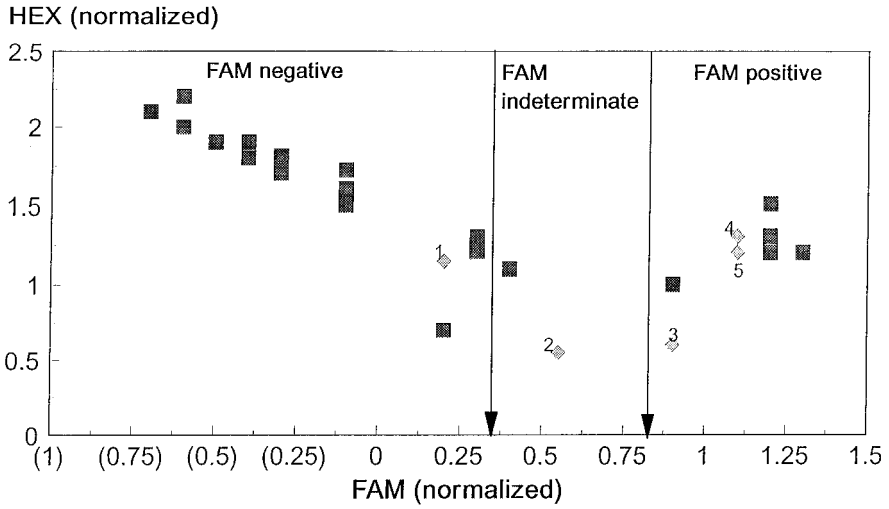

FIG. 5. Representative results from a TaqMan PCR experiment performed with the templates shown in Fig. 4. Results are expressed as normalized FAM result (*A. fumigatus*-specific probe) versus normalized HEX result (rRNA control probe). Arrows indicate the positions of the positive and negative cutoffs (0.8 and 0.35, respectively) for this experiment. Diamonds indicate TaqMan assay results for control templates: 1, *A. flavus* MAS92-230 (FAM negative, HEX positive); 2, *A. versicolor* MAS92-490 (FAM and HEX indeterminate); 3, plasmid 19B1 (FAM positive, HEX negative); 4, *A. fumigatus* CDC MAS92-229 (FAM and HEX positive); 5, *A. fumigatus* CDC MAS92-564 (FAM and HEX positive).

postamplification manipulations, which can be technically challenging and which can provide a significant source of laboratory contamination. Furthermore, use of the TaqMan assay provides an additional level of diagnostic specificity, because the fluorescent probe will not be cleaved unless it is hybridized to its complementary target sequence.

By using touchdown annealing conditions, the Z19-derived primer-probe combination successfully amplified and detected DNAs from 89 of 100 *A. fumigatus* isolates obtained from a variety of geographic locations (including *A. fumigatus* var. *ellipticus*, *A. fumigatus* var. *helvola*, and *A. fumigatus* var. *acolumnaris*). The TaqMan assay did not detect one morphologically typical *A. fumigatus* isolate (indeterminate TaqMan assay result), but the amplicon was detected on a stained gel. DNAs from three morphologically typical clinical isolates, six nonpigmented (albino) strains, and the stock strain of *A. fumigatus* var. *sclerotiorum* did not react with either the PCR primers or the corresponding TaqMan probe under these conditions. The detection sensitivity for the primer-probe combination, with gel electrophoresis used to resolve indeterminate TaqMan results, was calculated to be 90% for all 100 isolates classified as *A. fumigatus* or 96% if only the 94 morphologically typical isolates were included. The detection sensitivity of the TaqMan assay alone (without gel confirmation) was 89% for all isolates or 94% when the six albino strains were excluded. These reagents did not react under either touchdown or lower-temperature (56°C) annealing conditions with any of 56 strains of heterologous *Aspergillus*, *Penicillium*, or *Paecilomyces* species, for a specificity of 100% when gel electrophoresis was used to resolve the results for five isolates with TaqMan assay-indeterminate results. The use of control reagents, which co-amplified and detected a 377-bp rRNA gene fragment in every sample containing fungal DNA, identified false-negative results presumably caused by PCR inhibition. In two cases, this situation was corrected by producing new template DNA and repeating the test.

Neosartorya species are taxonomically most closely related to *A. fumigatus* and are traditionally differentiated by electron microscopy (23). The reagents in this study identified *Neosartorya* species in a differential manner. DNA from one of nine isolates was amplified under touchdown annealing conditions, but it was not detected with the TaqMan probe (indeterminate result). A faint band was detected after touchdown PCR with several additional species; we have interpreted this as a negative result because the amount of 864-bp target varies in replicate experiments and consistently fails to match the abundant amounts of amplicon produced by DNA from *A. fumigatus* samples under these conditions. Furthermore, the TaqMan probe is not cleaved under touchdown annealing conditions (indeterminate or negative TaqMan results). Decreasing the annealing stringency to 56°C improved the consistency of amplification so that eight of nine isolates could be detected with these reagents. A higher percentage of nucleotide sequence mismatches between primer and target can cause poor reproducibility of reactions at comparatively higher annealing temperatures and/or when competing with a more efficient annealing reaction such as that of the 377-bp control template (Fig. 4). We are investigating this presumed differential stability of *Neosartorya* primer-template hybrids at different annealing temperatures as a possible means of discriminating among members of the section *Fumigati* and between *Fumigati* and members of other *Aspergillus* sections. We are examining these sequences directly to investigate the extent of variation (work in progress). Our results confirm previous molecular taxonomic evidence of variation among *A. fumigatus* and *Neosartorya* species. Peterson (23) showed that four strains of *A. fumigatus* had total DNA sequence similarity of 92 to 100%, while the similarity between *A. fumigatus* and the most closely related *Neosartorya* species, *N. fischeri*, was 65 to 68%. Girardin et al. (11) reported that a moderately repetitive DNA probe that hybridizes efficiently with *A. fumigatus* DNA shows only weak reactivity with *N. fischeri*, *N. glabra*, and *N. spinosa*.

DNA from 10 isolates of *A. fumigatus* also displayed differential reactivities with these reagents (amplification at the lower annealing temperature but not at the higher annealing temperature). It is interesting that 6 of the 10 strains currently classified as *A. fumigatus* but nonreactive with the Z19-derived primers at 65°C are regarded as nonpigmented (albino) *A. fumigatus* variants. Possibly, this amplicon may have a role in pigment production for strains of the section *Fumigati*. It is

also possible that the nonpigmented strains may require reclassification (work in progress).

With DNA from one *A. fumigatus* isolate, the 864-bp target was successfully amplified but not detected (indeterminate FAM signal in the TaqMan assay). Conversely, with five isolates of heterologous species, an indeterminate FAM signal was generated but the amplicon was not produced. Similar results were obtained by altering the template concentration from 1 to 100 ng, by repeat testing of the same template DNA preparation, or by preparing another template sample. However, indeterminate results could be resolved by examining a stained gel for the presence of the 864-bp amplicon. Indeterminate results presumably arise from sequence mismatch at the region of the TaqMan probe, which could lead to poor probe binding and probe cleavage failure even when the target could be amplified.

RAPD-PCR and the TaqMan PCR assay appear to be useful strategies for identifying and detecting diagnostic DNA targets. Because no prior DNA sequence information is required, RAPD-PCR allows diagnostic information to be readily obtained even for fungal species with little or no previous molecular characterization. Although we have validated this technology with DNA prepared from fungal cultures, the TaqMan assay has also been used with template DNA prepared directly from clinical materials (19, 26). These approaches appear promising in facilitating laboratory identification and taxonomic classification of filamentous fungal isolates. The clinical utility of these tools in the diagnosis and management of fungal diseases remains to be determined.

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