Identification of Clinical Mold Isolates by Sequence Analysis of the Internal Transcribed Spacer Region, Ribosomal Large-Subunit D1/D2, and β-Tubulin

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Background: The identification of molds in clinical laboratories is largely on the basis of phenotypic criteria, the classification of which can be subjective. Recently, molecular methods have been introduced for identification of pathogenic molds in clinical settings. Here, we employed comparative sequence analysis to identify molds.

Methods: A total of 47 clinical mold isolates were used in this study, including Aspergillus and Trichophyton. All isolates were identified by phenotypic properties, such as growth rate, colony morphology, and reproductive structures. PCR and direct sequencing, targeting the internal transcribed spacer (ITS) region, the D1/D2 region of the 28S subunit, and the β-tubulin gene, were performed using primers described previously. Comparative sequence analysis by using the GenBank database was performed with the basic local alignment search tool (BLAST) algorithm.

Results: For Aspergillus, 56% and 67% of the isolates were identified to the species level by using ITS and β-tubulin analysis, respectively. Only D1/D2 analysis was useful for Trichophyton identification, with 100% of isolates being identified to the species level. Performances of ITS and D1/D2 analyses were comparable for species-level identification of molds other than Aspergillus and Trichophyton. In contrast, the efficacy of β-tubulin analysis was limited to genus identification because of the paucity of database information for this gene.

Conclusions: The molecular methods employed in this study were valuable for mold identification, although the different loci used had variable usefulness, according to mold genus. Thus, a tailored approach is recommended when selecting amplification targets for molecular identification of molds.

Key Words: Molds, Sequencing, Internal transcribed spacer, D1/D2, Tubulin

INTRODUCTION

The epidemiology and etiology of invasive fungal infections have changed over recent decades. Mold infections are more frequently encountered in association with increasing numbers of immunocompromised patients, and molds other than Aspergillus fumigatus, including species not previously recognized as pathogens, have emerged [1, 2]. The appearance of organisms, such as the Fusarium species and the Zygomycetes, with variable susceptibilities to conventional antifungal agents underscores the importance of correct identification [3].

Clinical laboratories are often challenged with mold identification. In contrast with bacterial or Candida species, which are identified on the basis of biochemical properties, mold identification is largely based on phenotypic criteria. Related species or phenotypic variants may be misidentified and rare species may remain unidentified. As a result, molecular methods have been developed to overcome these problems, and comparative se-
sequence analysis is now considered the gold standard identification technique [4, 5].

The internal transcribed spacer (ITS) region is the most commonly used target for sequencing in clinical laboratories because of the following benefits: (i) multiple copies of the ribosomal gene are present in all organisms, enabling sensitive detection by PCR, and (ii) the ITS region contains both highly conserved and variable regions, and is therefore, the optimal target for developing specific PCR primers that discriminate among closely related species [6-9]. However, the ITS region might not provide species-level resolution for all species. In such cases, other targets such as the D1/D2 region of the 28S subunit, the β-tubulin gene, or the translation elongation factor gene may prove useful, depending on the genus in question. In this study, we used molecular methods for mold identification and compared the performances of the ITS region, the D1/D2 region, and the β-tubulin gene as amplification targets for comparative sequence analysis.

METHODS

Forty-seven preserved isolates, which were previously obtained from clinical specimens, were cultivated to evaluate the usefulness of each locus for mold identification. Isolate selection criteria were as follows: (i) isolates most frequently recovered in our laboratory, (ii) medically important isolates recovered infrequently, or (iii) isolates unidentifiable by phenotypic methods. The genera included in this study were Absidia (1), Acremonium (1), Aspergillus (9), Cladosporium (2), Cunninghamhamella (1), Exophiala (1), Fusarium (2), Paecilomyces (3), Microsporum (1), Penicillium (3), Rhizomucor (1), Rhizopus (1), Scopulariopsis (1), Scedosporium (1), Sporothrix (2), Trichophyton (9), and unidentifiable molds (8).

1. Conventional identification

Clinical specimens were cultured on universal media (Sabouraud dextrose agar) and/or selective media (Mycogel agar) for a maximum of 3 weeks, according to the type of specimen. Specimens were incubated at 30°C for the first 2 days and then at 25°C. Isolates were sub-cultured for identification on Sabouraud dextrose agar or potato dextrose agar. Presumptive thermally dimorphic fungi were grown on Sabouraud dextrose agar at 25°C and brain heart infusion agar at 37°C. Conventional identification was made according to macro- and micro-morphologic criteria.

2. DNA extraction

DNA was extracted using the MagNA Pure LC DNA Isolation Kit according to macro- and micro-morphologic criteria. Conventional identification was made after isolates were grown on Sabouraud dextrose agar at 25°C and brain heart infusion agar at 37°C. Conventional identification was made according to macro- and micro-morphologic criteria.

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Table 1. Phenotypic and molecular identification data for 47 isolates

| No. | Specimen       | Phenotypic identification | ITS Identification | Accession No. | Identity (%) | Molecular identification | Accession No. | Identity (%) | B-tubulin Identification | Accession No. | Identity (%) |
|-----|----------------|--------------------------|--------------------|---------------|--------------|--------------------------|---------------|--------------|--------------------------|---------------|--------------|
| 1   | Sputum         | Absidia sp.              | A. corymbifera     | EU330179.1    | 538/538 (100%) | A. corymbifera           | FJ719444.1    | 462/471 (98%) | A. terreus               | GQ76132.1     | 490/490 (100%) |
|     |                |                          |                    |               |              |                          |               |              |                          |               |              |
| 2   | Skin (Plantar) | Acremonium sp.           | A. strictum        | GU595023.1    | 505/505 (100%) | Acremonium sp.           | AB294802.1    | 591/591 (100%) | Cercospora piaorpi       | AF146117.1    | 323/323 (100%) |
| 3, 4 | TTA/sputum     | Aspergillus fumigatus    | A. flavus          | AY214444.1    | 560/560 (100%) | A. terreus               | GQ461911.1    | 537/537 (100%) | A. terreus               | GQ76130.1     | 538/539 (99.8%) |
|     |                |                          |                    |               |              |                          |               |              |                          |               |              |
| 5-7 | Sputum         | Aspergillus fumigatus    | A. fumigatus       | HQ026746.1    | 559/559 (100%) | A. fumigatus             | AY216670.1    | 560/560 (100%) | A. fumigatus             | HQ285593.1    | 545/545 (100%) |
|     |                |                          |                    |               |              |                          |               |              |                          |               |              |
| 8, 9 | TTA           | Aspergillus terreus      | A. terreus         | GQ461911.1    | 537/537 (100%) | A. tubingenesis          | EF661209.1    | 570/570 (100%) | A. terreus               | GQ76130.1     | 538/539 (99.8%) |
|     |                |                          |                    |               |              |                          |               |              |                          |               |              |
| 10  | Skin (Plantar) | Aspergillus sp.          | A. ustus           | AF455532.1    | 559/559 (100%) | A. pseudodeflectus       | EF652507.1    | 539/539 (100%) | A. pseudodeflectus       | HM060842.1    | 423/424 (99.7%) |
|     |                |                          |                    |               |              |                          |               |              |                          |               |              |
| 11  | Closed pus     | Aspergillus sp.          | A. vesicolor       | GU232767.1    | 569/569 (100%) | A. sydowii              | EF652473.1    | 569/569 (100%) | A. sydowii              | FR775355.1    | 335/335 (100%) |

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Table 1. (Continued from the previous page) Phenotypic and molecular identification data for 47 isolates

| No. | Specimen          | Phenotypic identification | Molecular identification |
|-----|-------------------|---------------------------|--------------------------|
| 12  | Brain abscess     | Cladosporium sp.          |                          |
| 13  | Toe nail          | Cladosporium sp.          | Unidentifiable           |
| 14  | Sputum            | Cunninghamella bertholletiae | Unidentifiable           |
| 15  | Open wound        | Exophiala sp.             | E. pisciphila            |
| 16  | Cornea            | Fusarium sp.              | F. soloni                |
| 17  | Voided urine      | Fusarium sp.              | F. dimerum               |
| 18  | Microsporum sp.   | M. canis                  | M. candida               |
| 19  | TTA               | Paecilomyces sp.          | Geosmithia argillacea    |
| 20  | Finger nail       | Paecilomyces sp.          | P. lilacinus             |
| 21  | Abscess           | Paecilomyces sp.          | P. lilacinus             |
| 22  | Throat swab       | Penicillium sp.           | P. glabrum               |
| 23  | Sputum            | Penicillium sp.           | P. purpurens             |
| 24  | Urine             | Penicillium sp.           | P. marneffei             |
| 25  | TTA               | Rhizopus morchii          | R. pusillus              |
| 26  | TTA               | Rhizopus sp.              | R. microsporus           |
| 27  | Scopulariopsis sp.| Microascus sp.            | Scopulariopsis sp.       |

|    | Specimen          |identification     | Accession No. | Identity  | Accession No. | Identity  | Accession No. | Identity  |
|----|-------------------|-------------------|---------------|-----------|---------------|-----------|---------------|-----------|
| 12 | Brain abscess     | Cladosporium sp.  | GU017501.1    | 549/549 (100%)| C. sphaerosperm | AB100654.1 | 568/568 (100%)| A. terreus | GQ376127.1 | 548/548 (100%) |
| 13 | Toe nail          | Cladosporium sp.  | Unidentifiable |            | Unidentifiable |           |              |           |
| 14 | Sputum            | Cunninghamella    | AJ557801.1    | 585/585 (100%)| C. bertholletiae | FJ345351.1 | 540/540 (100%)| No PCR    |           |           |
|    |                   | mauritiicola      |               |           |               |           |              |           |
| 15 | Open wound        | Exophiala sp.     | AF050273.1    | 612/612 (100%)| E. pisciphila | AF050273.1 | 600/600 (100%)| ND        |           |           |
| 16 | Cornea            | Fusarium sp.      | GU866321.1    | 543/543 (100%)| F. soloni     | FJ345352.1 | 596/596 (100%)| F. soloni  | AB426619.1 | 594/594 (100%) |
| 17 | Voided urine      | Fusarium sp.      | EU862841.1    | 518/518 (100%)| F. dimerum    | EU926284.1 | 533/533 (100%)| F. dimerum | EU926417.1 | 284/284 (100%) |
| 18 | Microsporum sp.   | M. canis          | GU291265.1    | 605/605 (100%)| M. candida    | AY213708.1 | 573/575 (100%)| Unidentifiable |           |           |
|    |                   | M. distortum      | EF631608.1    | 605/605 (100%)| M. candida    | EF078482.1 | 573/575 (100%)|           |           |           |
|    |                   | M. ferrugineum    | EF581133.1    | 605/605 (100%)| M. candida    |           |              |           |
| 19 | TTA               | Paecilomyces sp.  | GU165726.1    | 436/439 (99.3%)| G. argillacea | EU862338.1 | 414/415 (99.7%)| Unidentifiable |           |           |
| 20 | Finger nail       | Paecilomyces sp.  | GU968671.1    | 430/430 (100%)| P. lilacinus  | AB363751.1 | 424/424 (100%)| P. lilacinus | FJ15301.1 | 316/316 (100%) |
| 21 | Abscess           | Paecilomyces sp.  | HQ842838.1    | 549/549 (100%)| P. lilacinus  | AY213717.1 | 549/549 (100%)| ND        |           |           |
|    |                   |                   |               |           |               |           |              |           |
| 22 | Throat swab       | Penicillium sp.    | EF502172.1    | 559/559 (100%)| P. glabrum    | EF200097.1 | 558/558 (100%)| P. glabrum  | EU128574.1 | 436/439 (99.3%) |
| 23 | Sputum            | Penicillium sp.    | DQ861325.1    | 558/558 (99.8%)| P. purpurens  | A033408.1  | 558/558 (100%)|           |           |           |
|    |                   |                   | AY373934.1    | 558/559 (99.8%)| P. spinulosum | FJ403781.1 | 556/556 (99%) |           |           |           |
| 24 | Urine             | Penicillium sp.    | FJ009566.1    | 481/481 (100%)| P. marneffei  | AB363755.1 | 594/594 (100%)| ND        |           |           |
| 25 | TTA               | Rhizopus morchii  | AB369914.1    | 610/610 (100%)| R. pusillus   | A134751.1  | 556/556 (100%)| No PCR    |           |           |
| 26 | TTA               | Rhizopus sp.      | AB381937.1    | 678/679 (99.9%)| R. microsporus| AB250181.1 | 615/616 (99.8%)|           |           |           |
| 27 | Scopulariopsis sp.| Microascus sp.     | HQ649982.1    | 512/518 (98.8%)| Scopulariopsis sp. | HQ676488.1 | 566/566 (100%)| Unidentifiable |           |           |

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| No. isolate | Specimen          | Phenotypic identification | Molecular identification | Molecular identification | Molecular identification | Molecular identification |
|------------|-------------------|---------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 28         | Eye discharge     | *Scedosporium* sp.        | *S. apiospermum*         | GQ476985.1 536/537 (99.8%) | *S. apiospermum* AB363764.1 590/590 (100%) | ND                       |
| 29         | Skin lesion       | *Sporothrix schenckii*    | *S. schenckii*           | AB128006.1 554/554 (100%) | *S. schenckii* AB363791.1 544/547 (99.4%) | *S. schenckii* AM116967.1 387/387 (100%) |
| 30         | Skin lesion       | *Sporothrix schenckii*    | Unidentifiable           | Saccharomyces *fibuligera* | AB550119.1 499/499 (100%) | Unidentifiable           |
| 31-33      | Toe nail          | *Trichophyton mentagrophytes* | *T. mentagrophytes* | HQ395066.1 549/549 (100%) | *T. interdigitale* AB363760.1 570/570 (100%) | Unidentifiable           |
|            |                   |                           | *T. interdigitale*       | FM986736.1 549/549 (100%) |                           |                          |
|            |                   |                           | *T. krajdenii*           | AF170462.1 549/549 (100%) |                           |                          |
| 34-39      | Toe nail          | *Trichophyton rubrum*     | *T. rubrum*              | FJ746657.1 509/509 (100%), *T. rubrum* AY213629.1 565/565 (100%) | Unidentifiable           |
|            |                   |                           | *T. mentagrophytes*      | AB520841.1 509/509 (100%), *T. rubrum* AY213629.1 565/565 (100%) |                           |
|            |                   |                           | *T. fischeri*            | AB430481.1 509/509 (100%), *T. rubrum* AY213629.1 565/565 (100%) |                           |
|            |                   |                           | *T. kanei*               | AB430480.1 509/509 (100%), *T. rubrum* AY213629.1 565/565 (100%) |                           |
|            |                   |                           | *T. raubitschekii*       | EU921293.1 509/509 (100%), *T. rubrum* AY213629.1 565/565 (100%) |                           |
| 40-42      | Toe nail/finger nail/CSF | Unidentifiable             | Unidentifiable           | Unidentifiable           | Unidentifiable           | Unidentifiable           |
| 43         | Toe nail          | Unidentifiable            | *Coniosporium* sp.       | EU730589.1 489/489 (100%) | *Coniosporium* sp. FJ355954.1 427/427 (100%) | Unidentifiable           |
| 44         | Lung tissue       | Unidentifiable            | *Geosmithia argillacea*  | HQ246728.1 420/420 (100%) | ND                       | *G. argillacea* GU968696.1 421/422 (99.7%) |
| 45         | Toe nail          | Unidentifiable            | *Phialophora* sp.        | HQ832998.1 627/627 (100%) | ND                       | ND                       |
| 46         | Toe nail          | Unidentifiable            | *T. rubrum*              | GU291266.1 469/469 (100%) | *T. rubrum* AY213629.1 460/460 (100%) | Unidentifiable           |
|            |                   |                           | *T. mentagrophytes*      | AB520841.1 469/469 (100%) | *T. rubrum* AY213629.1 460/460 (100%) |                           |
|            |                   |                           | *T. raubitschekii*       | EU921293.1 469/469 (100%) | *T. rubrum* AY213629.1 460/460 (100%) |                           |
|            |                   |                           | *T. fischeri*            | AB430481.1 469/469 (100%) | *T. rubrum* AY213629.1 460/460 (100%) |                           |
|            |                   |                           | *T. kanei*               | AB430480.1 469/469 (100%) | *T. rubrum* AY213629.1 460/460 (100%) |                           |
| 47         | Toe nail          | Unidentifiable            | *T. rubrum*              | AY213629.1 449/449 (100%) | Unidentifiable           |

Abbreviations: ITS, internal transcribed spacer; TTA, transtracheal aspirate; ND, not done.
Molecular identification of molds

Jang J-H, et al.

DISCUSSION

In this study, we employed a molecular method for mold identification and compared the performance of the 3 commonly used amplification targets. Using this method, 2 genera previously unidentified in a clinical laboratory were discovered, Coniosporium and Geosmithia. The Coniosporium species isolate was a slow-growing, black pigmented fungus recovered from a toenail, and showed arthroconidia microscopically. Coniosporium, which is known to colonize plants, has been reported in the literature as a human pathogen recovered from a superficial skin lesion [10]. The isolates identified as G. argillacea grew as whitish to olive colonies and had phialides, which were difficult to distinguish from those of Penicillium or Paecilomyces. Geosmithia is a polyphyletic genus created to accommodate Penicillium species that do not produce green colonies. According to recent reports [11, 12], G. argillacea can colonize the respiratory tract of cystic fibrosis patients, although it was not found to be associated with exacerbation of the disease. One of our isolates was obtained from the trans-tracheal aspirate of an acute lymphoblastic leukemia patient with influenza H1N1 infection, and the other isolate was obtained from the lung tissue of a patient with chronic cavitory pulmonary aspergillosis. Additional research is required to determine the clinical implications of colonization with G. argillacea.

ITS and D1/D2 region analyses performed well for identification of most isolates in this study. However, D1/D2 analysis was not appropriate for species-level identification of Aspergillus species, and this finding is consistent with the results of a previous study [13]. In contrast to D1/D2 analysis, where all species yielded 100% identical sequence data for at least one molecular sibling (closely related but different taxa), the ITS analysis distinguished some of the species (A. fumigates, A. terreus) from their molecular siblings. The β-tubulin gene was also helpful for some species (A. fumigatus, A. terreus, A. sydowii). In contrast, D1/D2 analysis was more appropriate than that of ITS for identifying Trichophyton species. Interestingly, isolates morphologically identified as T. mentagrophytes were re-classified into T. interdigitale (Arthroderma vanbreuseghemii) after analysis of the D1/D2 region, according to the current taxonomy suggested by Graser et al. [14]. The sequence of these isolates was identical to the neotype of T. interdigitale, CBS 428.63 (AF506033), but not with the neotype of T. mentagrophytes, CBS 318.56 (AY185126). Although the naming of the T. mentagrophytes complex has been a topic of debate for years, use of T. mentagrophytes rather than T. interdigitale could result in confusion, an issue recently raised in the literature [15, 16]. A consensus on the taxonomy of the T. mentagrophytes complex must be reached as soon as possible.

ITS and D1/D2 analyses yielded comparable performances for identification of species other than Aspergillus and Trichophyton. β-tubulin analysis was limited to genus-level identification due to the paucity of database information available for this gene. Since there is a variety of reference sequences deposited in the public database, the ITS region may be the most appropriate primary sequencing target, except in the case of Aspergillus, as recommended by the CLSI [17]. Analysis of the D1/D2 region or β-tubulin gene could be used for further resolution, and the decision to use additional targets should be based on clinical implications and laboratory policies, since the relevance of species-level identification has only been determined for a limited number of genera [18, 19]. In summary, molecular meth-
ods are useful for mold identification, although the identification performance of each locus varied according to genus. Thus, a tailored approach is recommended when selecting amplification targets for molecular identification of molds.

Authors’ Disclosures of Potential Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

REFERENCES

1. Nucci M and Marr KA. Emerging fungal diseases. Clin Infect Dis 2005; 41:521-6.
2. Malani AN and Kauffman CA. Changing epidemiology of rare mould infections: implications for therapy. Drugs 2007;67:1803-12.
3. Nucci M. Emerging moulds: Fusarium, Scedosporium and Zygomycetes in transplant recipients. Curr Opin Infect Dis 2003;16:607-12.
4. Summerbell RC, Levesque CA, Sefert KA, Bowers M, Fell JW, Diaz MR, et al. Microcoding: the second step in DNA barcoding. Philos Trans R Soc Lond B Biol Sci 2005;360:1897-903.
5. Balajee SA, Borman AM, Brandt ME, Cano J, Cuenca-Estrella M, Dananou E, et al. Sequence-based identification of Aspergillus, fusarium, and mucorales species in the clinical mycology laboratory: where are we and where should we go from here? J Clin Microbiol 2009;47:877-84.
6. Ciardo DE, Schar G, Altwegg M, Böttger EC, Bossiard PP. Identification of moulds in the diagnostic laboratory—an algorithm implementing molecular and phenotypic methods. Diagn Microbiol Infect Dis 2007;59:49-60.
7. Ciardo DE, Lucke K, Imhof A, Bloemberg GV, Böttger EC. Systematic internal transcribed spacer sequence analysis for identification of clinical mold isolates in diagnostic mycology: a 5-year study. J Clin Microbiol 2010;48:2809-13.
8. Iwen PC, Hinrichs SH, Rupp ME. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med Mycol 2002;40:87-109.
9. Atkins SD and Clark IM. Fungal molecular diagnostics: a mini review. J Appl Genet 2004;45:3-15.
10. Li DM, de Hoog GS, Saunte DM, van den Ende AH, Chen XR. Conioспorium epidermids sp. nov., a new species from human skin. Stud Mycol 2008;61:131-6.
11. Barton RC, Borman AM, Johnson EM, Houbraken J, Hobson RP, Denton M, et al. Isolation of the fungus Geosmithia argillacea in sputum of people with cystic fibrosis. J Clin Microbiol 2010;48:2615-7.
12. Giraud S, Pihet M, Razafimandimbry B, Camere J, Degand N, Mely L, et al. Geosmithia argillacea: an emerging pathogen in patients with cystic fibrosis. J Clin Microbiol 2010;48:2381-6.
13. Hinrikson HP, Hurst SF, Lott TJ, Warnock DW, Morrison CJ. Assessment of ribosomal large-subunit D1-D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important Aspergillus species. J Clin Microbiol 2005; 43:2092-103.
14. Graser Y, Kuijpers AF, Presber W, De Hoog GS. Molecular taxonomy of Trichophyton mentagrophytes and T. tonsurans. Med Mycol 1999;37: 315-30.
15. Sun PL, Hsieh HM, Ju YM, Jee SH. Molecular characterization of dermatophytes of the Trichophyton mentagrophytes complex found in Taiwan with emphasis on their correlation with clinical observations. Br J Dermatol 2010;163:1312-8.
16. Heidemann S, Monod M, Graser Y. Signature polymorphisms in the internal transcribed spacer region relevant for the differentiation of zoophilic and anthropophilic strains of Trichophyton interdigitale and other species of T. mentagrophytes sensu lato. Br J Dermatol 2010;162:282-95.
17. Clinical and Laboratory Standards Institute, Interpretive criteria for identification of bacteria and fungi by DNA target sequencing: approved guideline. CLSI document MM18-A. Wayne, PA: Clinical and Laboratory Standards Institute, 2008.
18. Balajee SA, Gritskov JL, Hanley E, Nickle D, Marr KA. Aspergillus lentulus sp. nov., a new sibling species of A. fumigatus. Eukaryot Cell 2005; 4:625-32.
19. Montenegro G, Sánchez Puch S, Jewtuchowicz VM, Pinoni MV, Rellioso S, Temporitti E, et al. Phenotypic and genotypic characterization of Aspergillus lentulus and Aspergillus fumigatus isolates in a patient with probable invasive aspergillosis. J Med Microbiol 2009;58:391-5.