Conventional Morphology Versus PCR Sequencing, rep-PCR, and MALDI-TOF-MS for Identification of Clinical *Aspergillus* Isolates Collected Over a 2-Year Period in a University Hospital at Kayseri, Turkey

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Background: *Aspergillus* species cause a wide range of diseases in humans, including allergies, localized infections, or fatal disseminated diseases. Rapid detection and identification of *Aspergillus* spp. facilitate effective patient management. In the current study we compared conventional morphological methods with PCR sequencing, rep-PCR, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for the identification of *Aspergillus* strains. **Materials and Methods:** A total of 24 consecutive clinical isolates of *Aspergillus* were collected during 2012–2014. Conventional morphology and rep-PCR were performed in our Mycology Laboratory. The identification, evaluation, and reporting of strains using MALDI-TOF-MS were performed by BioMérieux Diagnostic, Inc. in Istanbul. DNA sequence analysis of the clinical isolates was performed by the BMLabosis laboratory in Ankara. **Re-**

sults: Samples consisted of 18 (75%) lower respiratory tract specimens, 3 otomycosis (12.5%) ear tissues, 1 sample from keratitis, and 1 sample from a cutaneous wound. According to DNA sequence analysis, 12 (50%) specimens were identified as *A. fumigatus*, 8 (33.3%) as *A. flavus*, 3 (12.5%) as *A. niger*, and 1 (4.2%) as *A. terreus*. Statistically, there was good agreement between the conventional morphology and rep-PCR and MALDI-TOF methods; kappa values were $\kappa = 0.869, 0.871$, and 0.916 , respectively ($P < 0.001$). **Conclusion:** The good level of agreement between the methods included in the present study and sequence method could be due to the identification of *Aspergillus* strains that were commonly encountered. Therefore, it was concluded that studies conducted with a higher number of isolates, which include other *Aspergillus* strains, are required. J. Clin. Lab. Anal. 30:745–750, 2016. © 2016 Wiley Periodicals, Inc.

Key words: *Aspergillus*; MALDI-TOF-MS; rep-PCR; sequencing

INTRODUCTION

Recently, the increasing population of susceptible patients (patients who have hematological malignancies, bone marrow and solid organ transplantation, patients

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with HIV/AIDS, patients with long stay in intensive care units, etc.) has caused an increased incidence of fungal infections (1, 2). The most common agents of fungal infections are *Candida* strains followed by *Aspergillus* strains (3). *Aspergillus* strains are opportunistic molds that can cause both allergic and systemic infections (4). Early diagnosis and treatment of *Aspergillus* strains are critical for reducing the high mortality rate, especially in immunocompromised patients (5). The standard conventional methods used for the identification of *Aspergillus* are direct microscopic examination, culture and colony morphology. The disadvantages of these methods are the long identification process, low-sensitivity level, and the variabilities of morphological characteristics depending on the culture conditions (6). Recently, novel methods have increased in number; these produce fast and accurate results, and are based on the identification of specific antibodies, fungal metabolites and DNA, and mass spectra. Identification of the clinical isolate at species level using these new methods may provide a rapid and effective contribution to treatment success due to the species-specific differences in pathogenic potentials and antifungal susceptibility patterns (7).

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a novel method that has been used for several years, and which provides a fast and final diagnosis in identifying bacterial and fungal infections (8). Although there is limited experience with MALDI-TOF-MS in mycology, some studies have shown that it may provide a benefit similar to bacterial identification, especially for identifying fungal colonies growing in agar plates (9).

The rep-PCR method, which uses short primers targeting short, repetitive, and conservative structures located in different parts of the chromosome in bacteria and many fungal species, is a semiautomated molecular typing method with fast and easy applicability that has attracted much attention (10). Several studies have demonstrated the potential of this method for quick diagnosis and species-level differentiation of fungi such as *Candida*, *Aspergillus*, *Fusarium*, dimorphic fungi, *Zygomycetes*, and dermatophytes such as *Trichophyton* (7, 11–15). The present study aims to compare conventional morphology, PCR based on repetitive DNA sequences (rep-PCR), and MALDI-TOF-MS methods with the DNA-sequencing method for the identification of *Aspergillus* strains isolated from various clinical samples.

MATERIALS AND METHODS

Study Design

The identification of *Aspergillus* strains isolated from various clinical samples using conventional methods and

rep-PCR was performed in the Mycology Laboratory, Department of Medical Microbiology, Erciyes University Medical Faculty. The identification, evaluation, and reporting of strains using MALDI-TOF-MS were performed by BioMérieux Diagnostic, Inc. in Istanbul. DNA sequence analysis of the clinical isolates was performed by the BMLabosis laboratory in Ankara.

Clinical Samples and Conventional Method

The identification of *Aspergillus* strains isolated from various clinical samples (bronchoalveolar lavage [BAL]; lung, ear, and eye tissues; and wound site) included both macroscopic and microscopic characteristics. The colonies growing in Sabouraud dextrose agar (SDA) were inoculated into potato dextrose agar to increase conidium and pigment productions and into Czapek-Dox Agar for the identification of species. For each of the growing species, the colony color, morphology and growth characteristics at 25°C, 35°C, and 44°C were recorded. For microscopic examination, lactophenol cotton blue was used for making a preparation, and the form and color of conidia, the number of sterigmata, shape of vesicles, structure of conidiophores, and presence and shape of Hülle cells were examined.

MALDI-TOF-MS

All isolates were tested in duplicate. Fungal material was gently removed with a sterile swab, which was pre-moistened with medium suspension (BioMérieux Ref. 20150), and suspended in medium suspension. Ethanol absolute (0.9 ml; Sigma-Aldrich, Lyon, France) was added, samples were mixed and centrifuged for 2 min at $15,000 \times g$, and the supernatant was discarded. Next, 50 μ l trifluoroacetic acid and 40 μ l acetonitrile (both Sigma-Aldrich) were added and samples were mixed and centrifuged for 2 min at $15,000 \times g$. The supernatant was spotted on a single-use target (VITEK-MS, BioMérieux, Marcy l'Etoile, France) and the samples were allowed to dry. One microliter of α -cyano-4-hydroxycinnamic acid (CHCA; BioMérieux) was added as a matrix and the targets were allowed to dry. Then the slide was loaded into the VITEK-MS machine. As with the Microflex MS system, the sample was submitted to multiple laser shots. The matrix absorbs the laser light and vaporizes along with the sample in the process of ionization. A VITEK-MS was used for generating spectra from the bacterial suspension and Biotyper software (version 2.00) was used for analyzing the results. Both systems were calibrated immediately before the analysis according to the manufacturer's instructions (16).

rep-PCR

Samples were subcultured to SDA plates and incubated at 30°C for 2–5 days. DNA was extracted from a 10 µl loop of each *Aspergillus* isolate using the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Solano Beach, CA). Next, the fungal DNA was amplified using a DiversiLab Fungal kit (BioMérieux, Inc., Durham, NC) in accordance with the manufacturer's instructions. Detection and analysis of rep-PCR products were performed using the DiversiLab system (BioMérieux). The amplified fragments of various sizes and fluorescence intensities were separated and detected using a microfluidics chip with an Agilent 2100 Bioanalyzer. Further analysis was performed with the web-based DiversiLab software, version 3.4.4 (17).

DNA-Sequencing Analysis

As a reference method for species designation, sequencing of the internal transcribed spacer ITS1 and ITS2 regions flanking 5.8S ribosomal DNA (rDNA; ITS1–5.8S–ITS2) was performed for all of the isolates studied, followed by sequencing of partial portions of the β -tubulin (β -TUB) and/or calmodulin (CAM) genes for *Aspergillus* isolates; the elongation PCR products were sequenced and species identification was performed by searching databases with the BLAST sequence analysis tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). The isolate was assigned to a species if it had $\geq 99\%$ sequence homology with a sequence entry available in the searched databases (6).

Statistical Analysis

The data obtained in the present study were statistically analyzed using SPSS 15 (Statistical Package for Social Sciences, Chicago, IL). The agreement of the sequence method with conventional diagnosis, rep-PCR, and MALDI-TOF-MS methods was measured using Cohen's kappa analysis. In kappa analysis, a value close to 0 indicates no agreement, whereas a value close to 1 indicates high agreement. Additionally, the sensitivity and specificity values were calculated for all methods based on species.

RESULTS

Samples consisted of 18 (75%) respiratory tract specimens (BAL: 11, lung tissue: 7), 3 (12.5%) ear tissues, 1 eye tissue, and 1 wound specimen. Of the samples with *Aspergillus* growth detected based on the results of DNA sequence analysis, 12 (50%) were identified as *A. fumigatus*, 8 (33.3%) as *A. flavus*, 3 (12.5%) as *A. niger*, and 1 (4.2%)

as *A. terreus*. For 18 (75%) of 24 strains, similar results were obtained with all the three methods (Table 1). Three strains (nos. 2, 9, and 24) could not be identified with MALDI-TOF-MS. The strains nos. 2 and 24 were identified as *A. niger* and *A. terreus*, respectively, by conventional method, rep-PCR and DNA sequence analysis, whereas strain no. 9 was identified as *A. niger* by conventional method and *A. flavus* by rep-PCR and DNA sequence analysis. Sequence analysis assay was accepted as gold standard and good agreement was detected among the conventional method, rep-PCR, and MALDI-TOF methods; the kappa values were $\kappa = 0.869, 0.871, 0.916$, respectively ($P < 0.001$). When the conventional identification method was compared to the sequence method, the sensitivity values were 91.7%, 87.5%, and 100%, and the specificity values were 100%, 93.8%, and 95.2% for *A. fumigatus*, *A. flavus*, and *A. niger*, respectively. When rep-PCR was compared to the sequence method, the sensitivity values were 91.7%, 87.5%, and 100%, and the specificity was 100%, 100%, and 90.5% for *A. fumigatus*, *A. flavus*, and *A. niger*, respectively. When MALDI-TOF-MS was compared to the sequence method, the sensitivity values were 91.7%, 100%, and 100%, and the specificity values were 100%, 92.9%, and 100% for *A. fumigatus*, *A. flavus*, and *A. niger*, respectively.

DISCUSSION

Due to the widespread use of wide spectrum antibacterial agents, the long hospital stay of intensive care patients and increased number of immunocompromised people, molds, and especially *Aspergillus* strains have become more common (18). The species most commonly isolated in hospital-acquired aspergillosis are *A. fumigatus* and *A. flavus*. Additionally, there have been increasing reports of infections caused by nonfumigatus species such as *A. niger* and *A. terreus* in recent years (19, 20). Species-level identification of *Aspergillus* grown in culture during antifungal use is very important in order to differentiate the strains clinically resistant to amphotericin B such as *A. terreus*, *A. nidulans*, *A. lentulus*, *A. ustus*, and *A. glaucus* (21, 22). Of the samples with *Aspergillus* growth detected based on the results of DNA sequence analysis, 12 (50%) were identified as *A. fumigatus*, 8 (33.3%) as *A. flavus*, 3 (12.5%) as *A. niger*, and 1 (4.2%) as *A. terreus* in the present study. Additionally, *A. fumigatus* was reported to be the dominant species in respiratory tract samples compared to *A. flavus* in wound site infections and *A. niger* in ear samples (23, 24). The present study obtained similar results.

Identification of molds in clinical laboratories is based on direct microscopic examination, culture and colony morphology. This is a slow and complex process requiring the presence of very experienced mycologists; inaccurate

TABLE 1. Identification of Clinical *Aspergillus* Isolates by Conventional Methods, rep-PCR, MALDI-TOF-MS, and Sequencing

Patient no./ department	Specimen	Conventional methods	rep-PCR	MALDI- TOF-MS	Sequencing
1/INF	BAL	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>
2/ENT	Ear debris	<i>A. niger</i>	<i>A. niger</i>	ND	<i>A. niger</i>
3/DRD	BAL	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
4/DRD	BAL	<i>A. niger</i>	<i>A. niger</i>	<i>A. niger</i>	<i>A. niger</i>
5/ICU	BAL	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>
6/ONC	BAL	<i>A. flavus</i>	<i>A. niger</i>	<i>A. flavus</i>	<i>A. flavus</i>
7/DRD	BAL	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>
8/PHO	Wound	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>
9/ENT	Ear debris	<i>A. niger</i>	<i>A. flavus</i>	ND	<i>A. flavus</i>
10/INF	BAL	<i>A. flavus</i>	<i>A. fumigatus</i>	<i>A. flavus</i>	<i>A. fumigatus</i>
11/PHO	Lung tissue	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>
12/PHO	Lung tissue	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>	<i>A. flavus</i>
13/DO	Eye tissue	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
14/DRD	BAL	<i>A. niger</i>	<i>A. niger</i>	<i>A. niger</i>	<i>A. niger</i>
15/ICU	BAL	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
16/ICU	Lung tissue	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
17/PHO	Lung tissue	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
18/ICU	BAL	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
19/DO	Eye tissue	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
20/ICU	Lung tissue	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
21/ICU	Lung tissue	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
22/ENT	Ear debris	<i>A. fumigatus</i>	<i>A. niger</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
23/DRD	Lung tissue	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>	<i>A. fumigatus</i>
24/ONC	BAL	<i>A. terreus</i>	<i>A. terreus</i>	ND	<i>A. terreus</i>

BAL, bronchoalveolar lavage; DO, Department of Ophthalmology; DRD, Department of Respiratory Diseases; DPHO, Department of Medicine, Division of Pediatric Hematology Oncology; ENT, Department of Otorhinolaryngology; ICU, Intensive Care Unit; INF, Department of Infectious Diseases; ND, not detected; ONC, Department of Oncology.

identifications can be made even in reference laboratories (25). The diagnosis of *Aspergillus* is primarily based on microscopy/culture, clinical, radiological and histological methods; however, novel methods have been introduced in recent years, which produce fast and accurate results and are based on the identification of specific antibodies, fungal metabolites and DNA, and mass spectra.

MALDI-TOF-MS is a novel method that has been used for several years, which provides a fast and definitive diagnosis in identifying bacterial species in particular and, more recently, yeast species. Fewer studies have been conducted with this method in molds compared to yeasts (18, 26). Ongoing studies are aimed at establishing a combination of a standard extraction technique and matrix solution that can be applied to all species in mold identification. Panda et al. (27) conducted a study with 125 fungal isolates and reported that the correlation of MALDI-TOF-MS and conventional methods for yeast identification was 100% at both genus and species levels, whereas the correlation was more heterogeneous for mold identification when the same sample preparation protocol was used; 10.81% were accurately identified at genus level, 56.7% were accurately identified at both genus and species levels and 32.42% were identified as "Not Reliable Identification." The same study reported that there was a significant improvement when the sample preparation protocol

for molds was changed; 86.4% were accurately identified at both genus and species levels, and only 2.7% were identified as "Not Reliable Identification." Identification with MALDI-TOF-MS is based on comparing the spectrum of the microorganism with the data in the spectra frequency library, and finding the equivalent value (28). A study, which evaluated the performance of a database including 28 *Aspergillus* strains of clinical significance, reported that MALDI-TOF-MS produced an accurate identification at a rate of 98.6% and its specificity was 100% (0% inaccurate identification) (29). A study, which evaluated the use of single-deposit strategy without previous protein extraction, reported that 33 of 44 *Aspergillus* isolates were identified as *A. fumigatus*, 2 were identified as *A. flavus*, and 1 was identified as *A. niger*; six strains that were not included in the database (*A. sydowii*, *A. terreus*, *A. tubingensis*, *A. calidoustus*, *A. nidulans*, and *A. puniceus*) could not be identified, while the method had good specificity. The reason for the failure to identify was shown as the limited number of *Aspergillus* strains (*A. fumigatus*, *A. flavus*, *A. niger*, *A. versicolor*) in the available VITEK-MS database (30). In the present study, MALDI-TOF-MS could not identify an isolated *A. terreus* species; however, there was a good agreement between MALDI-TOF-MS and the sequence method for the most common *Aspergillus* species ($\kappa = 0.916$; $P < 0.001$).

The rep-PCR method is a rapid, effective, and easy-to-use genotyping method using short primers to target short, repetitive, and conservative structures located in different parts of the chromosome in bacteria and many fungal species (31). Healy et al. (7) reported that rep-PCR had an agreement with the morphology-based identification method and sequence-based identification method of 98% and 100%, respectively; it could also differentiate *A. flavus* and *A. terreus* species at the species level. Another study reported that 2 of 100 *Aspergillus* isolates studied using rep-PCR created similar sets at >85% with different species identified by conventional method, and the sequence method applied to these two isolates confirmed the rep-PCR result. The isolates identified as *A. flavus* and *A. versicolor* by conventional method were identified as *A. ustus* and *A. niger*, respectively (32). The present study found a good agreement between the rep-PCR and sequence methods ($\kappa = 0.871$; $P < 0.001$), and identified both isolates as *A. niger* with rep-PCR, which were identified as *A. flavus* and *A. fumigatus*, respectively, with the sequence method.

In conclusion, the search for a solution to the problems experienced in identifying molds by classical methods was attempted by several novel methods. There is no doubt that improved technology is assisting mycology in more rapid and accurate diagnosis of fungal disease. On the other hand, the good level of agreement between the methods included in the present study and sequence method could be due to the identification of *Aspergillus* strains that were commonly encountered; therefore, it was concluded that studies with a higher number of isolates including other *Aspergillus* strains are required.

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CONFLICT OF INTEREST

The authors have no conflicts of interest. They alone are responsible for the content and composition of the manuscript.

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