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ORIGINAL ARTICLE

Genotypic discrimination of *Aspergillus fumigatus* strain from related species within section fumigati

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Abstract

Introduction and objective. The aim was to make an exact diagnosis of 20 strains using molecular biological methods which were isolated from the atmosphere of the inpatient rooms in the Oncology and other departments of the Ege University Medical Faculty Hospital, and identified as *Aspergillus fumigatus* through phenotypic tests, and to determine their antibiotic susceptibility patterns.

Materials and method. It was confirmed that the 20 phenotypically-identified *A. fumigatus* strains belonged to the section Fumigati after they were tested by the ITS-PCR method. Their sequence analysis was performed and the results sent to the NCBI GenBank, and their accession numbers were obtained. For their exact diagnosis at the species level, the *β-tub* (β-tubulin) and *rodA* (RodletA) genes were examined with the multiplex PCR. Anti-fungal susceptibility of the 20 strains was determined according to the M38-A2 standards.

Results. As a result of ITS-PCR, it was confirmed that 19 of the 20 strains identified as *A. fumigatus* through the phenotypic methods belonged to the section Fumigati. However, after the detection of *β-tub* and *rodA* genes, all 20 strains were identified as *A. fumigatus*.

Conclusion. Although the results of the phenotypic and molecular biological tests applied to filamentous fungi do not often overlap, in this study, the results obtained from the molecular analysis confirmed the results of the phenotypic tests. However, 1 of the 20 strains phenotypically-identified as *A. fumigatus* was identified as *Penicillium spp.* as a result of ITS-PCR and sequence analysis. On the other hand, the profile obtained from *β-tub* and *rodA* tests indicated that the strain was *A. fumigatus*. Based on these results, this strain is thought to belong to the *Aspergilloides* genus which has the features of both genera.

Key words

rodA, βtub, A. fumigatus, ITS-PCR, antifungal susceptibility testing

INTRODUCTION

Aspergillosis is the most common type of invasive fungal infection worldwide, and the most widespread species observed in these infections is *Aspergillus fumigatus* [1, 2]. *A. fumigatus* strains cause life-threatening invasive aspergillosis in patients with haematologic tumors, cystic fibrosis patients and immune-compromised patients who have had an organ transplant [3]. Of the airborne fungi, *A. fumigatus* infection developed in a patient who was staying in the intensive care unit after undergoing liver transplantation. It was also observed that 2 other patients staying in the same unit developed *A. fumigatus* infection. After the molecular studies, it was determined that Aspergillus spores were firstly transmitted into the air while wound dressings were changed, and then indirectly to other humans as airborne spores [4]. It has been reported that Aspergillus infections have increased by 2–26% in haematopoietic stem cell patients, by 1–15% in organ transplant recipients and by 10–57% in patients with cystic fibrosis, and that the mortality rates in these patients have also increased [5, 6]. In order to reduce the high mortality rates in such patients, early diagnosis and immediate initiation of anti-fungal therapy are extremely important [5]. Species of *Aspergillus fumigatus* are microscopically characterized with blue-green conidia borne on uniseriate conidiophores with subclavate vesicles, and defined as phenotypic due to their macroscopic properties [7]. However, species of *A. fumigatus* may differ morphologically and, based on minor phenotypic differences, a variety of new species are defined within this group [5, 8]. Identification of a species only on the basis of morphological characteristics is highly controversial because morphology depends on the growth conditions, and the time required for the identification of anamorphic forms of Aspergillus species is 5 days or more [8]. On the other hand, some studies have indicated that the strains phenotypically identified as *A. fumigatus* may be genetically different [5, 8]. Recently, with multilocus sequence analysis, various slow-spore-forming *A. fumigatus* isolates have been identified as a new species within the section Fumigati [7]. Since the species within the genus Aspergillus have very different antibiotic sensitivities, their identification at the species level is extremely important [5, 9]. Therefore, for the diagnosis of *A. fumigatus* infections, accurate species identification, and classification of the species within the section Fumigati, molecular methods which contribute to obtaining both phenotypic and more sensitive and exact results should be used [2, 10].

Molecular methods widely used for the identification of fungi isolated from environmental and clinical specimens

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are PCR-based, and include the analysis of genes such as 18srRNA, mitochondrial DNA, rodlet A (*rodA*), βeta-tubulin (*βtub*), actin and calmodulin and internal transcribed spacer (ITS) regions [2, 5, 11,12,13]. Therefore, this study was aimed to polyphasically classify 20 strains which were isolated from the atmosphere of the inpatient departments where the patients who presented to the Ege University Medical Faculty Hospital Newborn Unit and environment were treated, and the strains identified as *A. fumigatus* through phenotypic methods by analyzing ITS regions and *rodA* and *βtub* genes of these strains.

MATERIALS AND METHOD

Bacterial strains. In this study, the 20 *A. fumigatus* strains which were isolated in different seasons from the atmosphere of the inpatient departments where the patients who presented to the Ege University Medical Faculty Newborn Unit and environment. The isolation and phenotypic identification of these strains were conducted through macroscopic and microscopic methods. The colony colour and properties of the strains were evaluated after 7 days of incubation on CYA25 (35), MEA (30), CY20S (30), CYA37 (57) and CZ agars, and their microscopic properties were investigated [14].

Anti-fungal susceptibility testing. Anti-fungal susceptibility tests of 19 *A. fumigatus* strains were made according to the National Committee for Clinical Laboratory Standarts (CLSI) document M38-A [15], and following drugs were tested: Itraconazole (ITZ, Merck & Co., Inc., Rathway, NJ), Voriconazole (VRZ, Pfizer Pharmaceuticals, New York, NY), Caspofungin (CAS, Merck & Co., Inc., Rathway, NJ), and Posaconazole (PSZ, Merck &Co., Inc., Rathway, NJ). *A. fumigatus* CBS 133.61 was used as the type strain.

DNA extraction, ITS gene detection and sequencing. DNA extraction by a modified version of the rapid mini preparation procedure was carried out [16]. Briefly, phenotypically-identified 20 *A. fumigatus* strains were inoculated into 50 ml of Sabourod Dextrose Broth media and the samples incubated in a shaking incubator at 25–27 °C for 3–4 days. After incubation, the resultant pellets were placed in Falcon tubes and centrifuged twice. The samples were then placed in liquid nitrogen for some time and placed in a lyophilizator. The lyophilized samples were fragmented with a spatula. Some of the samples were placed in 2-ml Eppendorf tubes and 500 µl of lysis buffer (400 mM Tris HCl (pH 8.0), 60 mm EDTA pH (8.0), 150 mM NaCl, 1% SDS) added on them. The mixture was then shaken with vortex for a few seconds. 20 µl of lysozyme was added to this mixture and incubated at 65 °C for 10 min. Then, 10 µl of proteinase K was added and the mixture incubated at 65 °C for 15 min. 150 µl of 5M potassium acetate was added to the samples and they were centrifuged at 12,000 rpm for 5 min. The supernatants were placed in clean eppendorf tubes and centrifuged for 2 min after 500 µl of isopropyl alcohol had been added on them. The supernatants were then discarded and the samples again centrifuged for 1 minute after 300 µl of cold 70% ethanol was added. Following this process, the supernatants were again discarded. After the alcohol had evaporated, DNAs isolated with 50 µl of ultra-pure water added to the ependorf tubes were stored at -20 °C until the study was carried out.

The 20 *A. fumigatus* isolates were tested for ITS region with ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT G-3') primers. This region consists of intervening 5.8S gene and the ITS1 and ITS2 non-coding regions [3]. The conventional PCR method was used as described previously [17]. PCR conditions were as follows: predenaturation 5 min at 95 °C; 40 cycles of 1 min at 95 °C, 2 min at 53 °C, and 3 min at 72 °C; and a final extension of 10 min at 72 °C. Products of the PCR were electrophoresed in 1.5% agarose gel with 0.1 g saber save dye and stored at 4 °C until sequencing.

The products of ITS PCR were sent to the Izmir Institute of Technology (İzmir Yüksek Teknoloji Enstitüsü) for sequencing, and the results submitted to the GenBank database.

Nucleotide sequence Accession Numbers. The ITS1–5.8S-ITS2 gene complex sequences of the *A. fumigatus* strains were submitted to GenBank. The assigned sequence Accession Numbers are as follows: *A. fumigatus* strain eAP1, KF177398; *A. fumigatus* strain eAP2, KF177399; *A. fumigatus* strain eAP3, KF177400; *A. fumigatus* strain eAP4, KF177401; *A. fumigatus* strain eAP6, KF177402; *A. fumigatus* strain eAP7, KF177403; *A. fumigatus* strain eAP8, KF177404; *A. fumigatus* strain eAP9, KF177405; *A. fumigatus* strain eAP10, KF177406; *A. fumigatus* strain AP11, KF177407; *A. fumigatus* strain eAP12, KF177408; *A. fumigatus* strain eAP13, KA177409; *A. fumigatus* strain eAP14, KF177410; *A. fumigatus* strain eAP15, KF177411; *A. fumigatus* strain eAP16, KF177412; *A. fumigatus* strain eAP17, KF177413; *A. fumigatus* strain eAP18, KF177414; *A. fumigatus* strain eAP19, KF177415; *A. fumigatus* strain eAP20, KF177416.

βtub and rodA gene detection with multiplex PCR. To investigate the presence of *βtub* and *rodA* gene exclusively in *A. fumigatus*, specific primers were used. These regions were amplified with multiplex PCR by using *βtub* F (5'-TGACGGGTGATTGGGATCTC-3') and *βtub* R (5'-CGTCCGCTTCTTCCTTGTTT-3') primers for the *βtub* region, and rodA F (5'-ACATTGACGAGGGCATCCTT-3') and *rodA* R (5'-ATGAGGGAACCGCTCTGATG-3') primers for the *rodA* gene region [13]. *A. fumigatus* CBS 133.61 was used as the type strain. Multiplex PCR amplification was performed in a 25 µl final volume containing 5 microliters of genomic DNA, 2.5 µl of buffer, 0.25 µl of fermantase taq polymerase, 0.5 µl of dNTP, 3.6 µl of $MgCl_2$ and 0.5 µl of each primer. After pre-incubation at 95 °C for 5 min, the amplification was performed for a total of 35 cycles as follows: denaturation at 95 °C for 1 min, annealing at 63 °C for 90 sec, extension at 72 °C for 2 min, and a final extension step of 10 min at 72 °C [17]. Products of the multiplex PCR were electrophoresed in 2 % agarose gel with 0.1 g saber save dye.

RESULTS

Antifungal susceptibility test results are shown is Table 1. It was proved with molecular methods that 19 of the phenotypically-identified 20 *A. fumigatus* strains were included in the section Fumigati with ITS PCR (Figs. 1, 2). The fact that these strains belong to the *A. fumigatus* species was verified with the work on *βtub* and *rodA* genes in addition to ITS PCR (Fig. 3).

Table 1. Origin, MICs, and MECs of different antifungal against *Aspergillus fumigatus* strains

Isolate	Origin	MIC (µg/ml)			MEC (µg/ml)
		*ITC	*VCZ	*POS	*CAS
A.fumigatus eAP1	Hematology	0.5	0.125	0.25	2
A.fumigatus eAP2	Hematology	>16	>16	>16	4
A.fumigatus eAP3	Hematology	0.125	0.25	0.25	1
A.fumigatus eAP4	Oncology	0.25	0.25	<0.03	2
A.fumigatus eAP6	Newborn Unit	<0.03	0.25	<0.03	8
A.fumigatus eAP7	Environment	0.25	0.25	<0.03	2
A.fumigatus eAP8	Newborn Unit	<0.03	0.25	<0.03	1
A.fumigatus eAP9	Newborn Unit	<0.03	0.25	<0.03	1
A.fumigatus eAP10	Newborn Unit	8	<0.03	0.25	1
A.fumigatus eAP11	Newborn Unit	0.125	0.03	0.25	1
A.fumigatus eAP12	Newborn Unit	0.125	<0.03	0.25	1
A.fumigatus eAP13	Environment	0.5	<0.03	0.25	2
A.fumigatus eAP14	Newborn Unit	0.25	<0.03	0.25	1
A.fumigatus eAP15	Newborn Unit	<0.03	<0.03	0.25	2
A.fumigatus eAP16	Newborn Unit	<0.03	<0.03	0.5	1
A.fumigatus eAP17	Newborn Unit	0.125	<0.03	0.25	2
A.fumigatus eAP18	Newborn Unit	0.25	4	<0.03	1
A.fumigatus eAP19	Newborn Unit	0.5	4	<0.03	1
A.fumigatus eAP20	Newborn Unit	0.25	2	<0.03	1
A.fumigatus CBS 133.61	Chicken	0.06	0.25	0.03	–

*ITZ: Itraconazole , VRZ:Voriconazole, POS: Posaconazole , CAS: Caspofungin

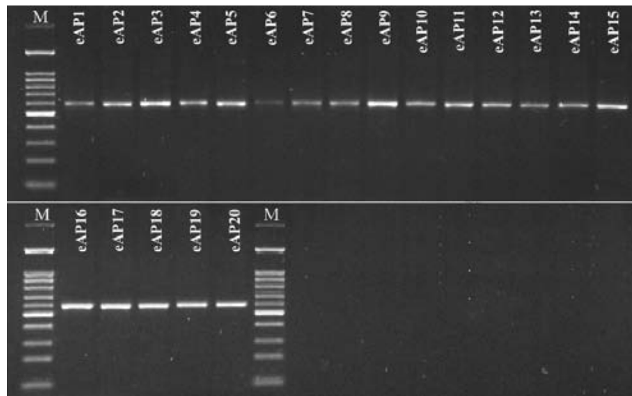

Figure 1. ITS regions of *A. fumigatus* strains.
M – Marker
eAP5 – ITS region of Penicillium spp.

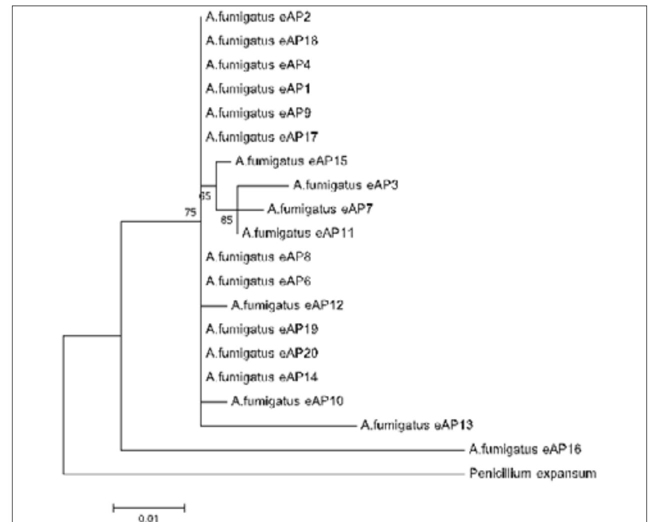

Figure 2. Phylogenetic tree obtained by maximum-likelyhood phylogenetic analysis with 1,000 bootstrap on the basis of ITS sequences from 19 *A. fumigatus* strains

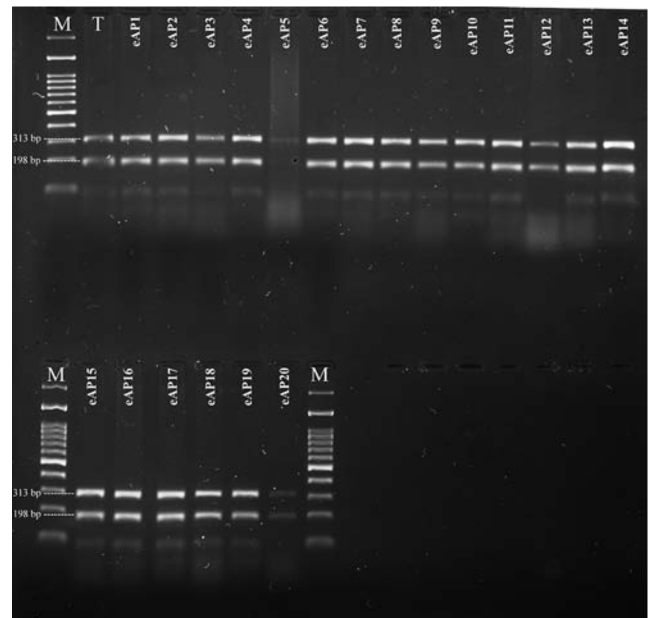

Figure 3. *βtub* and *rodA* gene regions of *A. fumigatus* strains

DISCUSSION

Recent increases in invasive fungal infections and in mortality rates associated with this disease make it essential to quickly identify the moulds obtained from clinical specimens and to initiate the treatment as early as possible [2, 3- 13]. In clinical laboratories, inaccurate identification of fungal species belonging to the section Fumigati is increasing day-by-day due to their morphological similarities. Therefore, in many cases, species such as *Aspergillus lentulus, Aspergillus viridinutans, Aspergillus fumigatiaffinis, Aspergillus fumisynnematus, Neosartorya pseudofischeri, Neosartorya hiratsukae and Neosartorya udagawae* are mistakenly reported as *A. fumigatus*, which leads to the inaccurate treatments of infections and increased mortality rates, especially in immunocompromised patients [13, 18]. The International Society for Human and Animal Mycology-sponsored Aspergillus Working Group has recommended the use of a comparative sequencing-based identification method that uses the ribosomal internal transcribed spacer (ITS) region for identification to the species complex level, and a protein-coding locus such as the βtubulin region, for the identification of species within the complex for identification of the Aspergillus species [18]. Therefore, 20 strains isolated from the hospital atmosphere and identified phenotypically were subjected to molecular biologic investigation. Based on the results of phenotypic tests, some species, such as *A. lentulus* and Neosartorya, have been reported as *A. fumigatus* in several studies conducted to-date [13, 19, 20]. However, the results obtained in the presented study through phenotypic and genotypic diagnoses support each other. Only one isolate identified phenotypically as *A. fumigatus* was determined

as Penicillum, according to the results of ITS sequencing (data not shown). However, in some studies, some species belonging to the genera Penicillium and Aspergillus were identified as Aspergillloides since they have the properties of both genera [21]. The isolate identified as Penicillium in this study is highly likely to be Aspergilloides.

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