Identification of pathogenic *Aspergillus* species by a PCR-restriction enzyme method

Aspergillus fumigatus remains the most frequent cause of invasive aspergillosis; however, other species, including *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus nidulans* and *Aspergillus ustus* have been reported to cause human infection (Henry *et al.*, 2000). Rapid and accurate identification of *Aspergillus* species is necessary for successful clinical management of infection and for epidemiological purposes. Identification of *Aspergillus* species based on morphological methods requires adequate growth time for evaluation of colony characteristics and microscopic features. A culture time of 5 days or more is generally required for the development of anamorphic forms of *Aspergillus*. Failure to form conidia on ordinary culture media may require colonies to be further subcultured on specialized media to induce spore formation (Henry *et al.*, 2000; Hinrikson *et al.*, 2005). In addition, morphology tests are usually labour intensive and need expert mycology personnel. Due to these limitations, various molecular approaches have been used for the identification of *Aspergillus* species isolated from clinical samples,

including PCR amplification of targets followed by either fragment length analysis or DNA probe hybridization or sequence analysis (Hinrikson *et al.*, 2005). The aim of this study was to compare the internal transcribed spacer 1 (ITS1)–ITS2 nucleotide sequences of common *Aspergillus* species and design a PCR-RFLP profile for differentiation of the most medically important *Aspergillus* species.

Standard strains were provided by Teikyo University Institute of Medical Mycology, Tokyo, Japan. The ITS region of the strains was sequenced using the universal fungal primers ITS1 and ITS4 for reconfirmation of the species, and for using in enzyme selection for RFLP analysis. In addition, 33 clinical and environmental isolates were also used (Table 1). The isolates were phenotypically identified according to the macroscopic and microscopic morphology of their colonies on Czapek–Dox agar (Difco). All moulds were also cultured on Sabauroud's dextrose agar and incubated at 30 °C for 2 days. Genomic DNA was extracted using single-tube rapid glass-bead disruption (Yamada *et al.*, 2002). Total DNA in the supernatant was precipitated with 2-propanol, resuspended

in 100 µl deionized water, and preserved at –20 °C until use. Glass-bead preparation has been used repeatedly for DNA extraction from yeasts (Mirhendi *et al.*, 2006; Yamada *et al.*, 2002). We found that the method is easy to perform and inexpensive tool for rapid extraction of DNA with good recovery.

Each PCR reaction contained 1 µl template DNA, each forward (ITS1, 5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4, 5'-TCC TCC GCT TAT TGA TAT GC-3') primer at 0.20 µM, each dNTP at 0.10 mM, 10 µl 10 × PCR buffer and 2.5 U *Taq* DNA polymerase in a final reaction volume of 100 µl. An initial denaturation step at 94 °C for 5 min was followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s and extension at 72 °C for 1 min, with a final extension step of 72 °C for 7 min. Amplified products were visualized by 1.5 % agarose gel electrophoresis in TBE buffer (0.09 M tris, 0.09 M boric acid and 2 mM EDTA, pH 8.3), stained with 0.5 µg ethidium bromide ml⁻¹, and photographed.

The ITS sequences of *Aspergillus* species obtained from the GenBank database

Table 1. *Aspergillus* strains and GenBank ITS sequences used in this study

Species	Source				Total no.
	JCM reference code	No. of clinical isolates	No. of environmental isolates	ITS sequence GenBank accession no.	
<i>A. fumigatus</i>	10253	7	1	AJ853744, AB185273, AB197939, AF078892, AF078891, AF078890, AF078889, AB055971	17
<i>A. flavus</i>	2061	5	7	AJ871014, AJ853764, AJ853743, AJ876522, AB074994, AB074993, AB074992, AB074991	21
<i>A. niger</i>	10254	2	8	AJ853742, AJ876876, AF078895, AJ280006, AJ223852, U65306	17
<i>A. nidulans</i>	2728	0	1	AB243115, AM158231, AM158200, AJ937756, AM158228, AJ000933, AM176708, AM158202, AM158232, AM158201	12
<i>A. terreus</i>	10227	0	2	AJ001368, AJ413985, AF078897, AF078896, AJ001338, AJ001335, AJ001334, AJ001333, AJ001331	12

JCM, Japan Collection of Microorganisms.

Table 2. Sizes of ITS1–5.8S rDNA–ITS2 PCR products for *Aspergillus* species before and after digestion with *HhaI*

Species	Size of ITS (bp) before digestion	Cutting sites for <i>HhaI</i> *	Size of fragments after digestion (bp)
<i>A. fumigatus</i>	595	131, 145, 331, 509	14, 88, 130, 178, 186
<i>A. flavus</i>	595	144, 328, 507	89, 143, 179, 184
<i>A. niger</i>	600	124, 331, 509	90, 124, 179, 207
<i>A. nidulans</i>	562	55, 300, 476	54, 87, 176, 245
<i>A. terreus</i>	607	333, 512	96, 179, 332

*The cutting site position is relative to the ITS1–5.8S rDNA–ITS2 fragment with the forward primer sequence being the starting end.

(Table 2) were aligned and restriction patterns of the sequences for each species were predicted for each of the known restriction enzymes, using DNASIS software (Hitachi Software Engineering). Predicted restriction fragments were compared in order to select those with the best discrimination. Digestion was performed by incubating a 20 µl aliquot of PCR product with 10 U enzyme in a final reaction volume of 25 µl at 37 °C for 2 h, and the digested DNA electrophoresed in 2 % agarose gel according to the manufacturers' instructions.

Fungus-specific universal primer pairs (ITS1 and ITS4) were able to successfully amplify the ITS1–5.8S rDNA–ITS2 region of all tested *Aspergillus* isolates, providing a single PCR product of about 570 bp for *A. nidulans* and about 600 bp for the other species (Fig. 1a). After analysis of various restriction enzymes, *HhaI* was selected as the best enzyme for differentiation between five medically important *Aspergillus* species. The actual size of the PCR

products and the predicted size of *HhaI*-RFLP products for each species are shown in Table 2. The products of digestion with *HhaI* in Fig. 1(b), show that the bands generated corresponded to the predicted sizes (Table 2). The restriction enzyme pattern for *A. fumigatus* is relatively close to *A. flavus*, yet easily distinguishable. The enzyme was also used on PCR products of 33 clinical and environmental isolates of *Aspergillus*. No variation was seen in RFLP patterns of each species among different strains (Fig. 2). The results of PCR-RFLPs for identification of the clinical isolates were completely identical to those obtained by conventional morphological methods.

Rapid and accurate differentiation of pathogenic species of *Aspergillus* has become particularly important for selecting effective antifungal therapy. In addition, information about species identity is important for epidemiological and control purposes, such as for outbreaks of invasive aspergillosis in

hospitals, for surveillance of the emergence of new species and accurate determination of incidence rates (Henry *et al.*, 2000; Hinrikson *et al.*, 2005).

In order to overcome the limitation of phenotypic methods some genetic targets have been investigated, including the mitochondrial cytochrome *b* gene (Wang *et al.*, 2001), DNA topoisomerase II gene (TOP II) (Kanbe *et al.*, 2002), and various rDNA regions, such as the small-subunit (18S) and large-subunit (28S) rDNA (Iwen *et al.*, 2002), ITS1 and ITS2 (Henry *et al.*, 2000). Many PCR-based methods have also been used for detecting the target DNA, such as DNA sequence analyses (Henry *et al.*, 2000), DNA probe hybridization (Martinez-Culebras & Ramon, 2007; Meletiadiis *et al.*, 2003), PCR enzyme immunoassay (de Aguirre *et al.*, 2004) and single strand conformational polymorphism (Kumeda & Asao, 1996; Rath & Ansorg, 2000). Although there are a few reports of the application of RFLP for identifying some *Aspergillus* species (Kumeda & Asao, 1996; Martinez-Culebras & Ramon, 2007; Rath & Ansorg, 2000; Somashekar *et al.*, 2004), as far as we know, there is no report about the application of RFLP analyses to identify common pathogenic species of *Aspergillus*.

In the present study we assessed the utility of the ITS region as a target for an easy-to-perform one-enzyme DNA-based PCR-RFLP method to discriminate the five most common pathogenic *Aspergillus* species. The profile is based on various ITS sequences belonging to the *Aspergillus* species deposited in GenBank (Table 1). The ITS region as a DNA target for PCR amplification has some advantages over

Fig. 1. Agarose gel electrophoresis of ITS PCR products before (a) and after (b) digestion with *HhaI*. Lanes 1–5, *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus*, respectively. M, 100 bp ladder molecular size marker (sizes in bp).

Fig. 2. Agarose gel electrophoresis of *HhaI*-RFLP of ITS-PCR products for the clinical and environmental isolates of *A. fumigatus* (a), *A. flavus* (b) and *A. niger* (c). M, 100 bp ladder molecular size marker (sizes in bp).

other molecular targets, e.g. increased sensitivity due to the existence of approximately 100 copies per genome, and sequence variability for distinguishing individual species and stability among strains belonging to a given species. We analysed data and selected an inexpensive restriction enzyme, *HhaI*, that was capable of the discrimination of the above-mentioned organisms. Our RFLP clearly differentiated the five *Aspergillus* standard strains. This PCR-restriction enzyme profile is applicable for reference medical microbiology laboratories. Identification and differentiation of the most medically important *Aspergillus* species from short-term culture using the profile can be a quick, reliable and economical method that provides faster identification than standard culture methods. DNA extraction, PCR and restriction digestion for a dozen samples, can be carried out within 1 working day, compared with morphological procedures that take several days.

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