

Research Paper

Development of RFLP-PCR method for the identification of medically important *Aspergillus* species using single restriction enzyme *MwoI*

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Abstract

In this study we attempted to modify the PCR-RFLP method using restriction enzyme *MwoI* for the identification of medically important *Aspergillus* species. Our subjects included nine standard *Aspergillus* species and 205 *Aspergillus* isolates of approved hospital acquired infections and hospital indoor sources. First of all, *Aspergillus* isolates were identified in the level of species by using morphologic method. A twenty four hours culture was performed for each isolates to harvest *Aspergillus* mycelia and then genomic DNA was extracted using Phenol-Chloroform method. PCR-RFLP using single restriction enzyme *MwoI* was performed in ITS regions of rDNA gene. The electrophoresis data were analyzed and compared with those of morphologic identifications. Total of 205 *Aspergillus* isolates included 153 (75%) environmental and 52 (25%) clinical isolates. *A. flavus* was the most frequently isolate in our study (55%), followed by *A. niger* 65(31.7%), *A. fumigatus* 18(8.7%), *A. nidulans* and *A. parasiticus* 2(1% each). *MwoI* enabled us to discriminate eight medically important *Aspergillus* species including *A. fumigatus*, *A. niger*, *A. flavus* as the most common isolated species. PCR-RFLP method using the restriction enzyme *MwoI* is a rapid and reliable test for identification of at least the most medically important *Aspergillus* species.

Key words: *Aspergillus*, identification, *MwoI*.

Introduction

Invasive filamentous fungal infections have emerged as important human diseases in recent years, particularly in the patients who receiving aggressive chemotherapy regimens and immunosuppressive agents (Walsh and Francesconi, 1995). *Aspergillus* species are most abundant and widely distributed in soil, water, air, seeds and foods (Anaissie and McGinnis, 2003). These molds are associated with allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis and invasive infection. *Aspergillus fumigatus* remains the most frequent cause of

invasive aspergillosis (IA), although at least 30 other species, primarily *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, *A. ustus* and *A. versicolor* have been associated with human disease (De Aguirre *et al.*, 2004). In some institutions, *A. terreus* is becoming more common and is of concern because it is less susceptible to antifungal drugs such as amphotericine-B than *A. fumigatus*. Similarly *Aspergillus nidulans* has also been reported as less susceptible to amphotericine-B than *A. fumigatus* and although it rarely causes invasive disease, *A. ustus* is less susceptible than *A. fumigatus* to antifungal medications such as voriconazole (Zhao and Kong, 2001). Therefore, accurate and timely

species identification is important to manage IA as well as for surveillance and epidemiological purposes.

Identification of *Aspergillus* species has traditionally relied on macroscopic colony characteristics and microscopic morphology (Diba *et al.*, 2007). Recently, comparatively simpler and more efficient molecular techniques have been adapted to detect and identify pathogenic fungi including *Aspergillus* species. In particular, because of its speed, reproducibility, high sensitivity and specificity, Polymerase Chain Reaction (PCR)-based tests have increasingly been used in laboratories to identify numerous fungal species (Canbe *et al.*, 2002). Several methods have been reported for DNA-based identification of *Aspergillus* spp. including PCR-sequencing of the ITS region (Moody and Tyler, 1990) repetitive-sequence based PCR (Healy and Koutoianis, 2004), PCR-Enzyme Immune Assay (EIA) (De Aguirre and Choi, 2004), nested PCR targeting rDNA ITS regions (Zhao and Kong, 2001) and PCR-restriction fragment length polymorphism (PCR-RFLP) for identification of *Aspergillus* at the generic level (Mirhendi and Diba, 2007), as well as direct PCR/sequence (d-PCRs) (Moody and Tyler, 1990). In a study, we also introduced the molecular, PCR based method of single strand conformational polymorphism (SSCP) for the identification of medically important *Aspergillus* species (Diba and Mirhendi, 2008). In this study we attempted to modify the PCR-RFLP method by using restriction enzyme *MwoI* for the identification of most common hospital isolated *Aspergillus* species.

Materials and Methods

Microorganisms

Referenced cultures of *Aspergillus* species were obtained from the Japanese Collection of Microorganisms (JCM) including: *A. flavus* (JCM 2061), *A. fumigatus* (JCM 10253), *A. niger* (JCM 10254), *A. nidulans* (JCM 10227) and *A. terreus* (JCM 02728). Test samples were collected from clinical specimens including; sputum, sinus discharge, broncho alveolar lavage, nail scrapings; the environmental samples were included aerosols precipitated on Sabouraud medium (Difco) and surface swabs of indoor and outdoor environments, in a University hospital, Urmia, Iran.

Culture preparation and morphological study

Morphologic identification of all standard and hospital isolated *Aspergillus* spp. was performed by using four enrichment media: Czapek dox agar, Czapek yeast extract agar, Czapek yeast 20% sucrose agar and malt extract agar (Diba and Mirhendi, 2007). For the molecular study, *Aspergillus* mycelia were harvested from sabouraud glucose broth (Difco) cultures after 24-48 h at 30 °C on a shaking platform. The mycelia were recovered on a 0.45 µm pore size filter and washed with TRIS- EDTA buffer and

sterile deionised water. Aliquots of the fungal hypha were stored frozen at -20 °C until use.

DNA extraction

Aspergillus freeze mycelia were ground using a Micro-Multimixer (IEDA Trading Corporation, Japan) or shaking with same volume of 0.3 to 0.4 mm (d) glass beads. DNA isolation of *Aspergillus* cells was performed using phenol-chloroform method (Mirhendi *et al.*, 2007; Diba *et al.*, 2008).

PCR amplification

PCR was performed using 5 µL of the DNA template to the PCR master mix including PCR buffer (20 mM Tris-HCL at pH 8.0), 50 mM MgCl₂, 0.1 mM each of forward (ITS: 5-TCC GTA GGT GAA CCT GCG G-3) and reverse (ITS-4 5-TCC TCC GCT TAT TGA TAT GC-3) primers (the universal primers synthesized by the UNMC, Eppley molecular biology lab), and 1.5 U of Taq DNA polymerase. Thermal protocol was use as our last studies on Aspergilli (8, 6). After amplification the products were stored at 4 °C until used.

Restriction Enzyme Digestion

Studying the literature and using molecular analyzer software (DNASIS Japan), we selected *MwoI* which was a desirable restriction enzyme with differential cutting patterns for the identification of medically important *Aspergillus* species. For restriction digestion, 13 µL of each PCR product was directly digested with by 5 U (0.5 µL) of the restriction enzyme *MwoI*, 1.5 µL of the related buffer, and incubated at 37 °C for 180 min (Diba and Mirhendi, 2008). Digested PCR products were subjected to electrophoresis in a 2% agarose gel and visualized by gel doc UV system.

Results

Studying a lot of well known restriction enzymes with various digestion patterns, we chose *MwoI*, as appropriate restriction enzyme with a differential power for nine of the 18 most medically important *Aspergillus* species tested in the present study. Amplification of rDNA in ITS1-5.8-ITS2 region for standard strains of *Aspergillus* species including *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus* resulted PCR bands ranging 570-598 bp. Against PCR produced bands, digestion with *MwoI* in RFLP resulted different size bands of agarose gel electrophoresis (Figure 1). The *MwoI* cutting patterns for each *Aspergillus* species was completely specific, so that none of the eight *Aspergillus* species as mentioned Table 1 was mistaken from another. The method was tested for more 52 *Aspergillus* clinical isolates of nail scraping and sinus discharge specimens, sputum, biopsy materials and ear

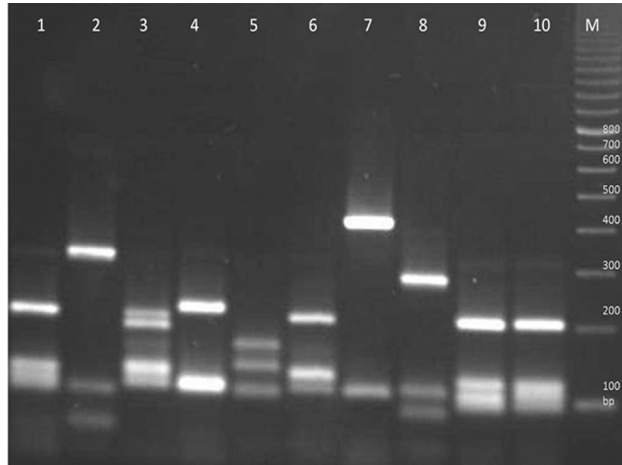


Figure 1 - Agarose gel electrophoresis of ITS1-ITS2 PCR products of standard *Aspergillus* species after digestion with the restriction enzyme *MwoI* Lanes 1-9: *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *A. nidulans*, *A. clavatus*, *A. ochraceus*, *A. amstelodami* and *A. fischeri*. Lane 10: *N. quadricincta* (Digestion pattern is similar to *A. fumigatus*). Lane M 100 bp ladder.

Table 1 - The fragment size of ITS1-ITS2 PCR products after digestion with restriction enzyme *MwoI* for some medically important *Aspergillus* species.

| Species | Cutting size |
|-----------------------|-------------------------------|
| <i>A. fumigatus</i> | 207, 125, 108, 29, 21, 9 |
| <i>A. flavus</i> | 325, 98, 65, 40, 20 |
| <i>A. niger</i> | 192, 175, 120, 108, 30, 21, 9 |
| <i>A. terreus</i> | 220, 109, 106, 96, 29, 9 |
| <i>A. nidulans</i> | 162, 135, 104, 31, 29, 9 |
| <i>A. clavatus</i> | 210, 125, 106 |
| <i>A. ochraceus</i> | 420, 90, 39, 9 |
| <i>A. amstelodami</i> | 286, 106, 100, 29, 9 |
| <i>N. fischeri</i> * | 200, 140, 120 |

*: *Neosartorya fischeri*.

Discussion

Use of molecular methods is preferred for the identification of medically important *Aspergillus* species comparing morphological tests which have some disadvantages including, delayed production or lack of characteristic morphologic features on culture media, lack of the sexual reproduction cycle in some *Aspergillus* species which makes difficult the identifications and similarity of microscopic or macroscopic characteristics in the species level and being risky for the health of laboratory personnel (Pryce and Palladino, 2006). The rDNA contains several regions of highly conserved sequence useful for obtaining proper sequence alignments. Most previous publications on PCR-based detection or identification systems were relevant to the sequencing rDNA 18S or 28S regions as target

swabs, as well as 153 environmental specimens including indoor surfaces swabs.

During 18 month of our study, totally 205 isolates including 11 *Aspergillus* species were identified (Table 2). *Aspergillus* isolates included 153 (75%) environmental and 52 (25%) clinical isolates. Among all, *A. flavus* was the most common species (55.0%) isolated from both clinical specimens followed by *A. niger* (31.7%), *A. fumigatus* (8.7%), *A. nidulans* (1.0%) and *A. terreus* (0.5%). Among environmental *Aspergillus* isolates, also *A. flavus* was found as the most frequent species followed by *A. niger*, *A. fumigatus* and the other *Aspergillus* species listed in Table 2.

Table 2 - Frequency of *Aspergillus* species isolated from clinical and environmental sources.

| <i>Aspergillus</i> species | Clinical isolates | | Environmental isolates | | Total | |
|----------------------------|-------------------|------|------------------------|------|--------|------|
| | Number | % | Number | % | Number | % |
| <i>A. flavus</i> | 36 | 69.2 | 76 | 49.7 | 112 | 55 |
| <i>A. niger</i> | 4 | 7.6 | 61 | 39.8 | 65 | 31.7 |
| <i>A. fumigatus</i> | 7 | 13.5 | 11 | 7.2 | 18 | 8.7 |
| <i>A. nidulans</i> | 1 | 1.9 | 1 | 0.6 | 2 | 1.0 |
| <i>A. terreus</i> | 1 | 1.9 | 0 | 0 | 1 | 0.5 |
| <i>A. parasiticus</i> | 1 | 1.9 | 1 | 1.6 | 2 | 1.0 |
| <i>A. penicilloides</i> | 0 | 0 | 1 | 0.6 | 1 | 0.1 |
| <i>A. tamarii</i> | 0 | 0 | 1 | 0.6 | 1 | 0.5 |
| <i>A. ochraceus</i> | 1 | 1.9 | 0 | 0 | 1 | 0.5 |
| <i>A. sojae</i> | 1 | 1.9 | 0 | 0 | 1 | 0.5 |
| <i>A. niveus</i> | 1 | 1.9 | 1 | 0.6 | 1 | 0.5 |
| Total | 52 | 100 | 153 | 100 | 205 | 100 |

DNA, however, the sequences in these regions looks conserved across a wide range of fungi. It is therefore difficult to design truly species-specific primers to make differentiations by PCR amplifications. Several studies have shown that ITS-2 length polymorphism and/or RFLP patterns obtained after treatment of the PCR product with restriction endonuclease might be useful for the identification of fungal pathogens (Moody and Tyler, 1990).

Determination of ribosomal DNA sequences in ITS regions for discriminating *Aspergillus* species is so expensive that makes difficult using it as a routine diagnostic method in hospital medical labs or reference laboratories. Therefore, it has been often restricted to use for research aims (Yuan and Chen, 1995). Random amplified polymorphic DNA (RAPD) has proved useful for sub-typing and discriminating between *Aspergillus* strains (Walsh and Francesconi, 1995) however, the results are usually too complicated to interpret and also it is not sufficiently reliable for identification of clinically isolated fungi. There is sufficient sequence variability in rDNA region that can serve as an identification target for *Aspergillus* isolates by PCR-RFLP and ITS1 - ITS2 sub regions have been revealed as markers for discrimination among the species if using species-specific restriction enzyme analysis. De Aguirre developed a PCR-Enzyme Immuno Assay method using ITS1 and ITS2 region oligonucleotide probes to detect seven important *Aspergillus* species, but the method requires DNA sequencing followed by analysis on an automated capillary DNA sequencer which is time consuming and relatively expensive (De Aguirre and Hurst, 2004). Identification of the pathogenic *Aspergillus* species by nested-PCR using a mixture of specific primer to the DNA topoisomerase II gene was studied by Canbe so that *A. fumigatus*, *A. flavus*, *A. nidulans* and *A. terreus* were identified using the primer mix (*PsIV*) by nested PCR as a rapid (Canbe and Kikuchi, 2002). Simple and available method using primer sets designed for amplification of conserved genes from filamentous ascomycetes to identify them to the generic level (Yuan and Chen, 1995). In the present study, amplification of the 570-598 bp fragment (ITS1-5.8 S-ITS2) of *Aspergillus* species rDNA gene followed by *MwoI* restriction in RFLP analysis allowed us to identify the most medically important *Aspergillus* species. Few investigators have reported use of RFLP with single restriction enzyme to identify *Aspergillus* to the level of species. De Aguirre (De Aguirre and Hurst, 2004) used the restriction enzyme *StyI* to show an identical pattern for *Aspergillus* species in the diagnosis of *Aspergillus* isolates at the generic level. An analysis of PCR-RFLP was used to identify five species of filamentous ascomycetes (Yuan and Chen, 1995). Dentis used RFLP to identify five *Aspergillus* species (Dentis, 2003). In this study we succeeded to differentiate at least 9 medically important *Aspergillus* species. Using this method, all tested standard strains were identified successfully except *N. fischeri* and *A. amsteloidami*.

The similarity between electrophoretic band patterns of *Neosartoria fischeri* and *A. amsteloidami* was exception as shown in the figure. Our findings of PCR-RFLP analysis on the clinical and environmental isolates were comparable with those of morphologic examinations. We recommend this method using restriction enzyme *MwoI* as simple and reliable to identify of *Aspergillus* species isolated from some sources in a short time (5-6 h after a 24 h growth) in a clinical laboratory.

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