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Rapid identification of clinically important *Aspergillus* species by Polymerase Chain Reaction: Restriction Fragment Length Polymorphism

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ABSTRACT

Introduction: Making a diagnosis of aspergillosis is complex and requires a combination of clinical, radiological and serological findings for interpretation. Current conventional methods are time consuming with low sensitivity. Management of aspergillosis requires rapid and accurate identification of *Aspergillus (A.)* species in order for treatment to be started as soon as possible. The objective of this pilot study was to evaluate a novel rapid method for discrimination of the most clinically relevant pathogenic *A.* species. **Materials and Method:** A total of 33 *A.* isolates including *A. fumigatus*, *A. niger*, *A. flavus* and *A. terreus* were studied. DNA extraction from fungal strains was performed using the phenol/ chloroform method. Primers internal transcribed spacer 1(ITS1) and *internal transcribed spacer 2 (ITS2)* were used in this study to generate polymerase chain reaction (PCR) product of about 600 bp for each *A.* species. The PCR amplicons from each species were incubated with 10 unit of Bacillus globigii I (*Bg/*I) enzyme and subsequently electrophoresed in 2.5% gel agarose. Restriction enzyme patterns of the *A.* species sequences were predicted for restriction endonucleases. **Result:** The ITS1 and ITS4 primers were able to amplify the ITS1–5.8S rDNA–ITS2 region of the tested isolates, providing a single PCR product of about 600 bp for each *A.* species. The digested products with *Bg/*I restriction enzyme produced smaller varying fragment sizes with different restriction enzyme patterns on gel electrophoresis, allowing for the discrimination of the four most clinically relevant pathogenic *A.* species, within a day. Repeated experiment carried out for the 33 *A.* isolates yielded similar results. **Conclusion:** Our novel method is the first to describe using ITS region nucleotide sequences of common *A.* species and a *Bg/*I enzyme digested PCR-restriction fragment length polymorphism profile can allow for rapid discrimination of the most clinically important *A.* species.

Keywords: polymerase chain reaction-restriction fragment length polymorphism, *Aspergillus* species, internal transcribed spacer region, diagnosis

INTRODUCTION

Aspergillus (A.) genus is commonly associated with life-threatening infections in immunocompromised hosts. While invasive aspergillosis is mostly caused by *A. fumigatus* and *A. flavus*, some species such as *A. niger*, A*. terreus*, *A. nidulans*, *A. ustus*, and *A. ochraceus* have been increasingly reported. [1-3] Diagnosis of aspergillosis is difficult and often requires a combination of clinical, radiological and serological finding for interpretation. The conventional methods of serological testing are both time consuming and the results have low sensitivity. The clinical man-

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agement of aspergillosis requires rapid and accurate identification of the *A.* species. The relatively low sensitivity of conventional methods frequently leads to significant delays in diagnosis and start of the treatment.

The availability of several genomes from the *A.* genus enables the discrimination of *A.* species at genomic level. Because of the limitations of conventional serological testing, different molecular approaches have been developed for detection of *A.* species which are isolated from clinical samples, including Polymerase Chain Reaction (PCR) of target sequences followed by restriction fragment length polymorphism (RFLP) [4] or sequencing [5, 6] or DNA probe hybridisation. [7, 8] In this pilot study, the Internal Transcribed Spacer (ITS) region nucleotide sequences of common species of *A.* were compared and a PCR-RFLP profile was designed for discrimination of the most clinically important and pathogenic *A.* species.

MATERIALS AND METHODS

A total of 33 *A.* isolates were studied. The isolates were maintained in our laboratory and this work did not involve sampling from humans or animals. These isolates included *A. fumigatus* (n=11), *A. flavus* (n=12), *A. niger* (n=5) and *A. terreus* (n=5). *A.* isolates were grown in Sabouraud dextrose agar (Merck, Germany) at 37° C for three days. Identification to the species level was carried out based on the *A.* species taxonomic features, as described by Raper and Fennell, 1973. [9] One ml from each *A.* isolate was transferred to an Erlenmeyer flask containing 100 ml yeast extract peptone dextrose medium (YEPD). The Erlenmeyer flasks were kept at 200 rpm under agitation at 37°C for 72 hrs to obtain mycelium growth. Using filters, the mycelia were harvested and washed with sterile distilled water (dH2O) and 0.5 M ethylenediamine tetra-acetic acid (EDTA). Then, with liquid nitrogen, the mycelia were ground into a fine powder using a pestle and mortar.

DNA extraction from fungal strains was performed using the phenol/chloroform method. ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers were used in this study. Each PCR reaction contained 100 ng of genomic DNA, 2.2 mM MgCl, 200 µM each Deoxynucleotide (dNTP) (CinnaGen, Iran), 50 pmol for each primer and 2.5 U Taq polymerase (CinnaGen, Iran) in a total volume of 50 µl. PCR amplification conditions were: 5 mins initial step, followed by 35 cycles at 94 °C for 2 mins, 53 °C for 2 mins and 72 °C for 2 mins and a final extension step at 72 °C for 30 mins. Amplification products were electrophoresed in agarose gels (1% w/v) in 1× Tris -acetate-EDTA (TAE) and stained with ethidium bromide.

A BLAST search demonstrated that the PCR products from *A.* species were 99% homologous with the relevant sequences in the GenBank. Also the ITS region sequences of the studied *A.* species derived from GenBank database (NCBI). Digestion of PCR products was carried out by incubating a 10 ml of PCR product with 10 Unit *Bacillus globigii* I (*Bgl*I) enzyme in a final reaction volume of 20 ml at 37° C for 3 hrs, and the digested DNA electrophoresed in 2.5% gel agarose. The size of digested fragments was estimated with 100 bp ladders. The digestion patterns were compared with reference strain of each species of *A* (Table 1). Molecular diagnosis including extraction of DNA, PCR and RFLP can be performed in one day comparing with conventional methods which take several days.

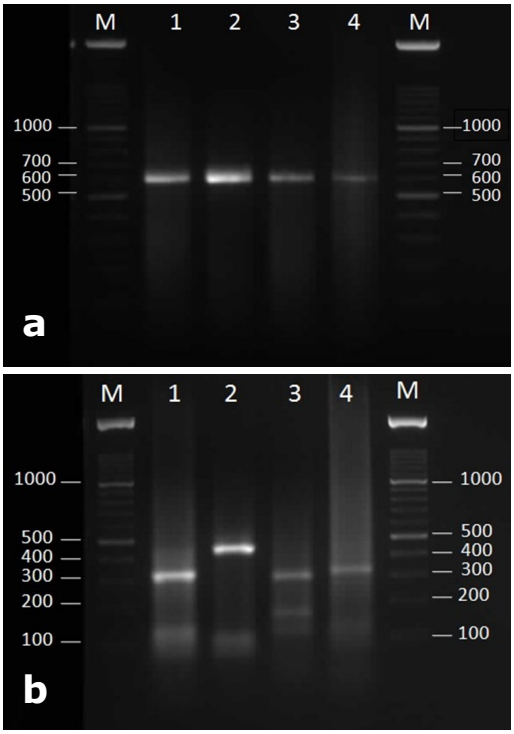
Restriction enzyme pattern of the sequences for each species were predicted for restriction endonucleases, using Restriction Mapper version 3software. The whole experiment was repeated twice for all 33 *A.* isolates to assess reproducibility of the method.

RESULTS

The ITS1 and ITS4 primers were able to am-

Table 1: Sizes of PCR products from ITS region of *Aspergillus* species before and after cut with *Bgl*I.

Aspergillus (A.) species	ITS size before digestion (bp)	Fragment size after digestion (bp)
A. fumigatus	595	315, 146, 68, 65
A. flavus	**595**	**433, 99**
A. niger	600	300, 164, 122, 12
A. terreus	**607**	**328, 112, 70, 68**



Figs. 1: a) Agarose gel electrophoresis of ITS region products (bp) of *Aspergillus* species before digestion with *Bg*/I, showing fairly similar patterns which is impossible to discern any differences. Lane M, 100 bp Ladder; Lane 1, *A. fumigatus*; Lane 2*, A. flavus*; Lane 3, *A. niger*, Lane 4, *A. terreus*,
b) Agarose gel electrophoresis of ITS region products (bp) of *Aspergillus* species after digestion with *Bgl*I, showing differing patterns for the 4 *Aspergillus* species. Lane M, 100 bp Ladder; Lane 1, *A. fumigatus*; Lane 2*, A. flavus*; Lane 3, *A. niger*, Lane 4, *A. terreus*.

plify the ITS1–5.8S rDNA–ITS2 region of the tested isolates, providing a single PCR product of about 600 bp for each *A.* species, which are too close to differentiate the aspergillus species. Figure 1a showed the electrophoresed banding of the amplicons obtained from one strain for each species of *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, which clearly showed the closeness of the pattern.

Digestion of the PCR products of the 4 clinically relevant aspergillus species with restriction endonuclease *Bgl*I, produced digested products with fragment sizes ranging from 12 to 433 bp. Gel electrophoresis of the digested products clearly showed that the bands generated for each of the four *A.* species have different restriction enzyme patterns (Figure 1b). Although the restriction patterns for the larger fragment banding of *A. fumigatus, A. niger* and *A. terreus* are relatively close, the smaller fragment banding easily differentiate the three species. There was not variation in restriction patterns of each *A.* species among various isolates. Repeated experiment for all 33 *A.* species yielded similar results.

DISCUSSIONS

Increased availability of molecular tools for use in *A. genus* is a necessary step toward accelerating the identification of the pathogenic species of this genus. The ribosomal DNA holds several regions of greatly conserved sequence valuable for getting suitable sequence alignments. Ribosomal DNA sequences of ITS region for discerning of *A.* species is time consuming and costly.[11] Random amplified polymorphic DNA (RAPD) are typically very difficult for interpreting and it is not adequately reliable for recognition of clinically isolated fungi. [12] In our study restriction enzyme pattern of the ITS region products was tested to evaluate its efficiency as a rapid technique to identify different species of *A.*

isolate.

The ITS region of the four *A.* species; *A. fumigatus*, A. *flavus*, *A. niger* and *A. terreus* before digestion with *Bg*/I restriction endonucleases, have fragment sizes around 600bp which was too similar to discriminate effectively among the four species on gel electrophoresis patterns. However after digestion with *Bg*/I restriction endonuclease, the PCR-RFLP produced have smaller varying fragment sizes which were can be clearly differentiated between these four clinically important *A.* species on gel electrophoresis. Such novel technique has never been reported before and will provide clinical laboratory with a rapid test for diagnosing of A. infection within a day, allowing for initiation of clinical treatment quickly.

There are a few reports of the application of PCR-RFLP for identification of some *A.* species [10], but our study is the first to report on the use of *Bgl*I enzyme-PCR-RFLP analyses to identify common pathogenic species of *A*. Our *Bgl*I enzyme-PCR-RFLP method obviously discriminated the four medically important of *A.* species and repeated testing of the methods have produce identical results, indicating that it is reliable and reproducible.

In conclusion, our study has provided a novel technique using *Bgl*I enzyme digested PCR-RFLP for the rapid identification and differentiation of the four clinically important pathogenic species of *A,* within 24 hours. This can provide clinicians with a diagnostic results to start treatment early. As this is a pilot study, further studies are required to substantiate our findings by other laboratories.

REFERENCES

1: Fricke S, Fricke C, Oelkrug C, et al. A real-time PCR for the detection and characterisation of *Aspergillus* species. Mycoses. 2012; 55:416-25.

2: Schabereiter-Gurtner C, Selitsch B, Rotter ML, Hirschl AM, Willinger B. Development of novel real-time PCR assays for detection and differentiation of eleven medically important *Aspergillus* and *Candida* species in clinical specimens. J Clin Microbiol. 2007; 45:906-14.

3: Hao J, Wu W, Wang Y, et al. Arabidopsis thaliana defense response to the ochratoxin A-producing strain (*Aspergillus ochraceus* 3.4412). Plant Cell Rep. 2015; 34:705-19.

4: de Valk HA, Klaassen CH, Meis JF. Molecular typing of *Aspergillus* species. Mycoses. 2008; 51:463-76.

5: Sabino R, Veríssimo C, Parada H, et al. Molecular screening of 246 Portuguese *Aspergillus* isolates among different clinical and environmental sources. Med Mycol. 2014; 52:519-29.

6: Henry T, Iwen PC, Hinrichs SH. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. J Clin Microbiol. 2000; 38:1510–5.

7: Myoken Y, Sugata T, Mikami Y, Murayama SY, Fujita Y. Identification of *Aspergillus* species in oral tissue samples of patients with hematologic malignancies by in situ hybridization: a preliminary report. J Oral Maxillofac Surg. 2008; 66:1905-12.

8: Martinez-Culebras PV, Ramon D. An ITS-RFLP method to identify black *Aspergillus* isolates responsible for OTA contamination in grapes and wine. Int J Food Microbiol. 2007; 113:147–53.

9: Raper KB, Fennell DI. 1973. The Genus *Aspergillus*. Krieger Publishing Company, Huntington, New York.

10: Soleiro CA, Pena GA, Cavaglieri LR, et al. Typing clinical and animal environment *Aspergillus fumigatus* gliotoxin producer strains isolated from Brazil by PCR-RFLP markers. Lett Appl Microbiol. 2013; 57:484-91.

11: Yuan GF, Liu CS, Chen CC. Differentiation of Aspergillus parasiticus from Aspergillus sojae by random amplification of polymorphic DNA. Appl Environ Microbiol. 1995; 61:2384-7.

12: Walsh TJ, Francesconi A, Kasai M, Chanock SJ. PCR and single-strand conformational polymorphism for recognition of medically important opportunistic fungi. J Clin Microbiol. 1995; 33:3216-20.