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Evaluation of Nested PCR assay for the Detection *Aspergillus* Species

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ABSTRACT

In this research, we investigated the application of nested PCR assay for the identification of *Aspergillus* species. We obtained amplified products from fungal genera other than *Aspergillus*, showing the presence of cross-reactions with fungal DNA.

Key Words*:* *Aspergillus* species, Nested-PCR

INTRODUCTION

The genus *Aspergillus* is a ubiquitous saprophytic soil fungus which colonizes the respiratory tract in humans and is responsible for opportunistic infection in immunocompromised patients [1]. Because of cancer treatment, the increasing numbers of immunocompromised patient with infected HIV (human immunodeficiency virus), individuals receiving immunosuppressive therapy, administration of broad-spectrum antibiotics have led to a dramatic rise in the number of cases of *Aspergillus* species infections [2]. Indeed, invasive aspergillosis is responsible for up to 41 % of the deaths of patients with acute leukemia [3].

Early diagnosis and initiation of antifungal therapy are essential to reduce the high rate of mortality. Traditional diagnosis methods used in the clinical laboratory include microscopy, culture, or antigen detection. The sensitivity of these methods is very low at the early stages of the infections. Consequently, rapid, sensitive and specific tests are needed to aid in the diagnosis of fungal infections [4,5]

Among non-culture methods under investigation, PCR (polymerase chain reaction) offers advantages over classical approaches, because low-level fungal infections can be detected from minimal volumes of clinical samples and DNA from both dead and viable organisms could serve as a target template for the amplification reaction [6].

In our study, we aimed identification of *Aspergillus species* by using ASP5, ASP8, ASP1 and ASP7 primers which were used by Yamakami et. al., were derived from variable region (V7/V9 region) at the small subunit (18S) ribosomal DNA gene of *Aspergillus* species [7].

MATERIALS AND METHODS

Microorganisms: *Aspergillus fumigatus* NRLL 2999, 6 clinical isolates and 2 environmental isolates of *A. fumigatus*; additionally, *Aspergillus fla vus* (clinical isolate), *A. j anus* (environmental isolate), *A . fischerii* (environmental isolate), *A. terreus* (environmental isolate), *C.albicans* CDC B385, *Alternaria alternata* NRRL 20593, *Penicillium cylindrosporum* NRRL 2673, , *Trichoderma viride* NRRL 1698, DNA extraction was made from these microorganisms [8, 9)] Human DNA was available.

Extraction of Mycelial Fungi DNA: DNA was extracted as previously described [9], with slight modification. Briefly, each mycelial fungus was inoculated into 10 ml Malt Extract Broth (MEB) and incubated at room tempature for 2 days. Then, DNA extraction was made from these cultures. Suspension cultures were filtered and washed twice with sterile distilled water and mycelium was suspended in 400 µl (20 mM Tris-HCl pH 7, 10 mM EDTA, % 0.5 SDS w/v) and added 500 µl phenol/chloroform/pentanol (25:24:1 w/v/v) and mixed with a vortex mixer for 30 seconds. After tubes were centrifuged at 10000 rpm for 30 minutes, supernatant was collected and DNA was precipitated with ethanol. DNA was dissolved in 100 µl (Tris-EDTA). Genomic DNAs were electrophoresed (Consart HU13) in an agarose gel and control of purity and concentrations (Varian 50 spectrophotometer) were made as described by Olgun et. al. [10].

Standardization of PCR

-Concentrations of $MgCl_2$: For the optimization of PCR different $MgCl_2$ concentrations were experimented (1.5 mM, 1.75 mM and 2.0 mM).

-PCR Reaction: PCR was performed in 25 µl reaction volumes containing 2.5 µl of a 10X PCR buffer (Fermantas), 1.5 µl of 1.5 mM $MgCl_2$ (Fermantas), 1.2 µl of a mixture of 20 mM each deoxynucleoside triphoshate (Sigma), 2µl (5 pmol) of each primer (outer primers: ASP5, ASP8) and 0.5 µl of 2.5 U Taq polymerase (Fermantas) and 5µl of 40-50 µg/ml DNA with the remaining volume made up with sterile distilled water.

For second step PCR: After amplification, 5 µl removed from first PCR and added to the second step PCR for further amplification with ASP1, ASP7 inner primers.

-PCR parameters: The first cycle include 5 min of denaturation at 94 °C, 1 min of annealing at 50 °C, and 3 min of primer extension at 72 °C, followed by 30 cycles of 1 min. of denaturation at 94 °C, 1 min of annealing at 50 °C, 3 min. of extension at 72 °C. PCR was completed by 1 min of denaturation at 94 °C, 1 min of annealing at 50 °C, 5 min. of extension at 72 °C (Crocodile III, Appligene Oncor). For nested PCR: PCR was repeated as described above.

-Electrophoresis and imaging: The PCR products were electrophoresed (Consart HU13) in an agarose gel (%2.5) for 1 hour at 100 V at room temperature in TBE buffer (Tris-Borate) stained with ethidium bromide and visualized with UV light (360nm).

RESULTS

Standardization of PCR:

- Concentrations of $MgCl_2$: The best and clear DNA fragment which was 357 bp. was produced at 1.5 mM $MgCl_2$ concentration.

- Results of PCRs:

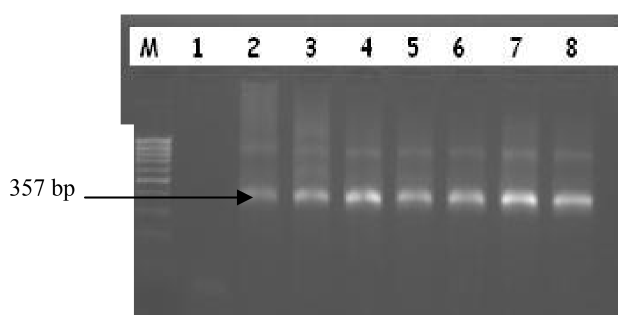

Figure 1: Agarose gel electrophoresis of PCR products from different clinical isolates of *A. fumigatus A.*: (M) Molecular weight marker (100 bp), (1) Negative control, (2) Positive control (*A. fumigatus* NRRL 2999), (3-8) *A. fumigatus* (clinical isolates).

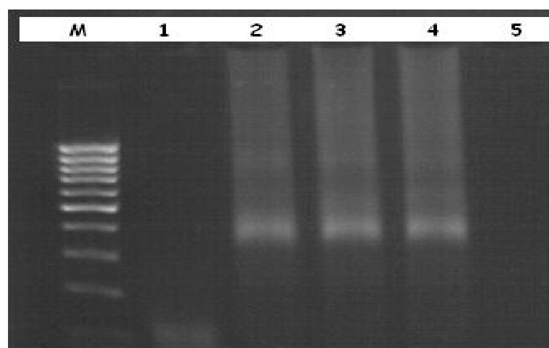

Figure 2: Agarose gel electrophoresis of PCR products from different environmental isolates of *A. fumigatus A.*: (M) Molecular weight marker (100 bp.), (1) Negative control, (2) Positive control (*A. fumigatus* NRRL 2999), (3-4) *A. fumigatus* (enviromental isolates), (5) Human DNA.

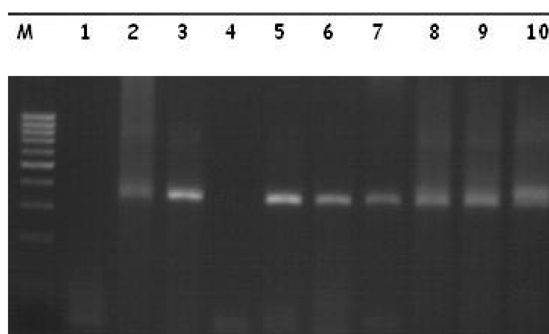

Figure 3: Agarose gel electrophoresis showing PCR products from *A. fumiga tus* NRRL 2999 and different fungi: (M) Molecular weight marker (100 bp), (1) Negative control, (2) Positive control (*A. fumigatus* NRRL 2999) (3) *C. albicans* CDC B385, (4) *Alternaria alternata* NRRL 20593, (5) *Penicillium cylindrosporum* NRRL 2673, (6) *Trichoderma viride* NRRL 1698, (7) *A. fla vus* (clinical isolate), (8) *A. janus* (enviromental isolate), (9) *A. fischerii* (environmental isolate), (11) *A. te rreus* (environmental isolate).

DISCUSSION

In this study, we applied nested PCR techniques to extracted DNAs. We used ASP5 and ASP8 as internal primer and used ASP1 and ASP7 as external primer. Yamakami et. al designed these primers specific to *Aspergillus* species. They designed these primers as a result of their studies in the sequence of the variable region V7- V9 of the 18S subunit and point out that they only obtained a positive result from those fungal species belonging to the genus *Aspergillus* (*A. fum igatus*, *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus*). They didn't obtain amplified product from species belonging to other fungal genera (*C. albicans*, *C. glabrata*, *C. par apsilosis*, *Cryptococcus neoformans*, *Trichosporon beigelii*, *Penicillium citr eoviridae*, *P.expansum*, and *P.crustosum*) [7]. Same researchers also investigated the possible presence of DNA specific for *Aspergillus* species in serum samples of patients with invasive aspergillosis by the nested PCR method with same primers. Target DNA was

detected by the nested PCR with as little as 50 fg of the extracted DNA of *A.fumigatus* [11].

To evaluate the sensitivity of these primers we applied the PCR to DNA samples from the above microorganisms. Firstly, for the optimization of PCR different $MgCl_2$ concentrations were experimented and the best and clear 357 bp DNA fragment was produced at 1.5 mM $MgCl_2$ concentration. All strains of the genus *Aspergillus* from which DNA was extracted and on which this nested PCR was performed *Aspergillus fu migatus* NRLL 2999, 6 clinical isolates and 2 environmental isolates of *A. fumigatus*, *Aspergillus fla vus* (clinical isolate), *A. janus* (environmental isolate), *A . fischerii* (environmental isolate), *A. te rreus* (environmental) showed the presence of the amplified product, that is, a 357 bp band in the second amplification (Figure 1 and 2). Of those strains that did not belong to the genus *Aspergillus*, amplified product was obtained the following strains, *C. albicans* CDC B385, *Penicillium cylindrosporum* NRRL 2673 ve *Trichoderma viride* NRRL 1698 (Figure 3). No bands were obtained from *Alternaria alternata* NRRL 20593.

Melchers et al. made the complete nucleotide sequence of the genes encoding the 18S rRNA of *Aspergillus* species and other clinically relevant prokaryotic and eukaryotic microorganisms. The sequence alignment data of the V7 to V9 regions of the rRNA of the different *Aspergillus* species showed extensive homology with those *Penicillium marneff ei*, *P. chrysogenum* , and *Paecilomyces variotii* but not with the other microorganisms [12].

Cruzado et al., investigated the application of this nested PCR assay for the diagnosis of invasive aspergillosis, by studying the possible cross-reactions among different fungal species and genera. They found that the sensitivity of this nested PCR can be considered adequate (1 pg of fungal DNA was detected), although they obtained amplified products from fungal genera other than *Aspergillus* (*Acremonium chrysogenum* , *Alternaria alternata*, *Cladosporium cladosporioides*, *Cunnighamella elegans*, *Geotrichum lactis* , *Mucor r acemosus*, *P.chrysogenum*, *Syncephalastrum racemosus* and *Trichophyton mentagrophytes*) [13].

Our study and those published by other authors, region V7-V9 is considered variable within the 18S subunit of ribosomal DNA, we found cross-reactions with other fungal species which do not belong to the genus *Aspergillus*. The detection of *Aspergillus* species using nested PCR assay is a valid alternative in the laboratory diagnosis of aspergillosis. Nevertheless, we must also bear in mind the possibility of cross-reactions between fungal species other than *Aspergillus.*

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