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**Original Article**

# Single Strand Conformation Polymorphism analysis of PCR-amplified rDNA to differentiate medically important *Aspergillus* Species

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## Abstract

**Background:** *Aspergillus* species are associated with allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis and invasive infection. In this study, we developed a PCR-Single Strand Confomational Polymorphism method to identify the most common *Aspergillus* species and we showed some advantages of this method comparing a PCR-Restriction Fragment Length Polymorphism with our designed restriction enzyme.

**Methods:** We selected ITS2, as a short fragment within the rDNA region (length size: 330 bp) to be amplified as small size PCR product. We mixed 5 ml of the PCR product with an equal volume of loading buffer and followed by incubation for 5 min at 95º C and quenching in an ice bath. The mixture was applied to a 6%-12% Gradient Poly acryl amide gel to run in a vertical electrophoresis, then gel was stained with ethidium bromide and silver nitrate which followed by an ethidium bromide staining.

**Results:** Our results of restriction digestion showed a fine identification of 7 tested *Aspergillus* species during 5-6 hours after an overnight mycelial growth. As our results some of tested *Aspergillus* species: *A. nidulans*, *A. fisheri, A. quadricincta, (A. fumigatus and A. niger)* as a group and (*A. flavus, A. tereus and A. ochraceus)* as another group, can be discriminated. Moreover SSCP analysis enabled us to identify above *Aspergillus* species within 8-12 h after an over night growth without using an expensive restriction enzyme.

**Conclusion:** It is concluded that Single Strand Conformational Polymorphism is a simple and rapid method for identification of some medically important *Aspergillus*.

**Keywords:** *SSCP,* Aspergillus*, Identification*, *PCR*

## Introduction

Invasive filamentous fungal infections have emerged as important human diseases in recent years, particularly in patients receiving aggressive chemotherapy regimens and immunosuppressive agents (1). Even with intensive medical treatment and antifungal medications, Invasive aspergillosis (IA) has become a leading infectious cause of death for bone marrow and organ transplant recipients. *Aspergillus* species are most abundant and widely distributed in soil, water, air, seed and food (2) and some of them are associated with allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis and invasive infection. Although *A. fumigatus* remains the most frequent cause of IA, 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 (3). 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, *A. 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 (4).

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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. Unfortunately, the number of morphological markers is generally low, which makes the classification and identification of isolated colonies difficult. Several days of culture maybe required for the development of the specific phenotypic characteristics and conidia formation that guide identification. Failure to form conidia on ordinary culture media may require colonies to be further subcultured on specialized media to induce spore formation. Conventional diagnoses of infections reliant on pathogen identification by means of morphological characters are sometimes unsuccessful because of the atypical features of some isolates. Thus, rapid, precise and safe techniques for mold identification are essential for the clinical diagnosis of *Aspergillus* species (5). 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 (6). Several methods have been reported for DNA-based identification of *Aspergillus* spp. including PCR-sequencing of the ITS region (5), repetitive-sequence based PCR (7), PCR-Enzyme Immuno Assay (EIA) (3), nested PCR targeting rDNA ITS regions (8) direct PCR/sequence (d-PCRs) (9) as well as PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) for identification of *Aspergillus* at the generic level (7) and also the species level (10). In this point, what is critical for identification of Aspergillus isolates is duration time. SSCP as a PCR based method, which is digestion free, has been our preference to use for identification of Aspergillus species isolated from clinical sources. Because of its simplicity and sensitivity, SSCP is one of the most useful techniques to detect mutations in oncogenes (7) and allelic variants (8, 9) in the human genome. Briefly, DNA material denatured to single-stranded DNA, which is subjected to polyacrylamide gel electrophoresis. The mobility of single-stranded DNA in the gel is dependent on its secondary structure as determined by the nucleotide sequence (11).
In this study, we developed the PCR based method of single strand conformational polymorphism SSCP to identify medically important *Aspergillus* species by detecting the changes in the nucleotide polymorphism.

## 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), *A. ochraceus* (JCM 10255), *A. fischeri* (JCM 1740), *A. quadricincta* (JCM 1855), *A. clavatus* (JCM 10080) and *A. tereus* (JCM 02728). Our major subjects were Iranian *Aspergillus* strains isolated from clinical specimens were obtained from laboratory of Medical Mycology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Iran.

### *Culture preparation and morphological study*

All of the specimens inoculated on Sabouraud Dextrose Agar and incubated at 28° C until full sporolation. An inoculum's medium including the suspension of spores in distilled water with 2% agar, 0.5% tween 80 was prepared, and a loop of the medium transferred to four differentiating media: Czapek Dox Agar, Czapek Yeast Extract Agar, Czapek Yeast 20% sucrose Agar and Malt Extract Agar. All of the *Aspergillus* isolates were identified by using morphological characteristics of colonies and microscopically features. For the molecular steps fungal cultures was performed following the needle inoculation of 50 ml of Sabouraud Glucose Broth (Difco,) with conidia from a 7 d culture in Sabouraud Glucose Agar, then

incubated for 72 h at 30° C on a shaking platform. The hyphal mass were recovered on a 0.45 µm pore, size filter and washed with Tris-EDTA buffer and sterile dionized water aliquots of the fungal hypha were stored frozen at -20° C until use.

***DNA Extraction***

The hypha was grinded by a micro multi mixer (IEDA Trading corporation, Japan) and suspended in 300 µl of DNA extraction buffer, (1mM EDTA [pH 8.0], 1% SDS, 10 mM Tris-HCL [pH 8.0], 100 mM NaCl, 2%, Triton X-100), 300 mg Glass beads 0.5 mm (d) and 300 µl of phenol chloroform solution. Micro centrifuge tubes (1.5ml) containing hypha and buffer and phenol chloroform were shaken for 5 min, followed by centrifugation at 5000/min for 5 min, the supernatant transferred to the new tubes suspended with equal volume of chloroform. The supernatant DNA was precipitated with twice volume of 2-Propanol and 0.1 volume of sodium acetate followed by incubation in freezer for 10 min. The pellets were washed by ethanol 70° C and dissolved in 50 µl of Tris-EDTA buffer. The extracted DNA was stored at 4° C until used.

***PCR amplification***

We selected ITS2, as a short fragment within the rDNA region (length size: 330 bp) to be amplified as small size PCR product, and used the primers, Forward-P (ITS3): 5'-TCC GTA GGT GAA CCT GCG -3' and reverse Reverse-P (ITS-4): 5-TCC TCC GCT TAT TGA TAT GC-3 (synthesized by the UNMC, Eppley molecular biology lab). The PCR protocol included briefly: 5 µl of the DNA template in total reaction volume of 50 µl consisting of 5µl of PCR buffer (20 mM Tris- HCL [pH 8.0, MgCl2], 0.5µl of dNTPs, 0.5µl (0.1 mM) of each primers, 0.25µl (1.5 U) of Taq DNA polymerase and Double Dionized Water. Thirty cycles of amplification were performed in a thermal cycler after initial denaturation of DNA at 95° C for 4.5 min each cycle consisted of a denaturation step at 95° C for 30 s, an annealing step at 55° C for 30 s, and an extension step at 72° C for 1min, with a final extension at 72° C for 3 min following the last cycle. After amplification, the products were stored at 4° C until used.

***Restriction Enzyme Digestion***

ITS1-ITS4 sequences of various tested fungal species were derived from Gene Bank. Based on those sequences the restriction enzyme was determined by DNASIS software and the best enzyme was selected. For restriction digestion, 13 µl of PCR products were digested directly and individually by 5 U of the restriction enzyme *Mwo*I, 1.5 micro liter of the related buffer, and incubated at 37 °C for 180 min. Digested PCR products were subjected to electrophoresis in a 2% agarose gel and visualized with a UV system transilluminator.

***SSCP***

We Set up microfuge tubes with 5 µl of DNA (PCR products) and Added equal volume of loading dye (10mM NaOH, 95% Formamide, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol) into microfuge tubes then shortly spined and heated in a water bath at 95º C for 5 min and followed by quenching in an ice bath for 5 min. The single stranded DNAs were saved at -20º C until use. We used a 6%-12% gradient poly acryl amide gel electrophoresis (PAGE) to run the single stranded DNAs. The electrophoresis buffer included 1X TBE buffer (89 mM Tris-89 mM boric acid and 2 mM EDTA) and was cooled in advance. The PAGE system was connected to power supply (voltage adjusted to 150v) and was left at -4º C in cold room until the DNA bands migrated to the middle of the gel. It usually takes 4-6 h. After dissembling the PAGE system, the gel was stained in ethidium bromide (1 mg/ml) solution for 20 min and then destained in distilled water for 15 min. Post staining with Silver Nitrate was used as following protocol: briefly the gel was floated in 500 ml of Solution 1 (50 ml of ethanol 96º C and 2 ml of acetic acid adjusted volume with distilled water to 500 ml) for 5 min on a shaking platform, the rinsed in distilled water. Solution 2 (250 ml of 1% Silver Nitrate) was added into tray including the gel and covered completely to provide a dark situation then we washed the gel three time with distilled water for 5 min.

Finally the gel was treated with 250 ml of Solution 3 (0.37% NaOH) while it was adding slowly 1ml of formaldehyde to solution 3 to appear the reddish brown DNA bands on the gel.

## Results

All PCR products was run on a 8% poly acryl amide gel electrophoresis that resulted a major single band with 1-3 extra bands because of a non specific amplification (Fig.1) and it was proved to be low variations in length between the *Aspergillus* species; the range was approximately 560-580 bp. Before using SSCP method for identification of *Aspergillus* isolates, the PCR products were digested with our designed restriction enzyme *Mwo*I. The RFLP patterns produced for each *Aspergillus* species was specific so that seven of ten tested standard species were discriminated in this method. Digestion of PCR products with *Mwo*I resulted the identification of several important *Aspergillus* species: *A. flavus, A. niger, A. tereus, A. nidulans, A. clavatus, A. ochraceus* and *A. amsteloidami* (Table 1). Our findings of SSCP assay varied by using some different electrophoresis and staining procedures. When electrophoresis was performed in a 8% PAGE without a denaturant, the resulting patterns showed no difference between the species. Addition of SSCP loading buffer and denaturation at 95º C, SSCP products showed some differences between *Aspergillus* species. The different concentrations of polyacryamide gel including 6%, 8%, 10% and 12% resulted some differentiations between the species and the most discrimination were seen on 8% PAGE. However, a huge number of non-specific extra bands around the main bands was confusing to correctly identify the *Aspergillus* species (Fig 2). In the end, the optimal SSCP condition was found included; a Gradient gel contained 6%-12% Poly acryl amide in four phases (length of each phase 3cm)**.** Using gradient (6%-12%) PAGE at a full time quenching at 4º C up to end of electrophoresis which followed by an ethidium bromide staining allowed the differentiation of six species of medically important Aspergilli; (Fig 3). As our findings *A. flavus, A. tereus and A. ochraceus* showed three pairs SS bands and it seems the bands arrangement is not different. With a punctually observation it could be seen A. *fumigatus and A. niger* formed two pairs of similar SS bands. *A. nidulans* has formed four pairs of SS bands. At last, *A. fisheri* and *A. quaricincta* showed two pairs SS bands. Furthermore, different size SS bands are shown among *A. fumigatus, A. niger and A. ficsher*i. Using number and size discrimination, we identified Aspergillus species; *A. nidulans*, *A. fisheri, A. quadricincta, (A. fumigatus and A. niger)* in a group and (*A. flavus, A. tereus and A. ochraceus)* in a group. The results were repeated with Silver Nitrate staining which was with ambiguity in diagnosis because of creating a huge number of non specific bands so that it was confusing for identification so it withdrawn as a part of the method.

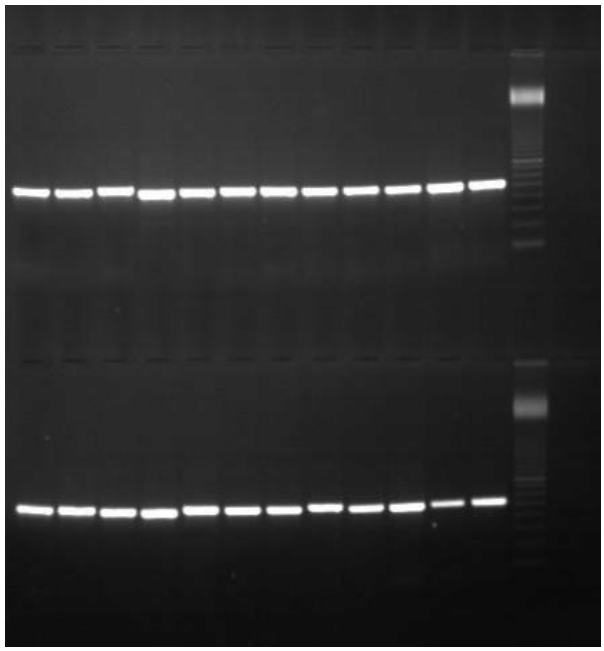

**Fig. 1:** PCR products loaded on poly acrylamide gel electrophoresis with size 560-580 bp.

**Table 1:** The fragment sizes of ITS1-ITS2 PCR products after digestion with the enzyme *Mwo*I for various *Aspergillus* species, as it shown there are different cutting (digestion) patterns among the first seven *Aspergillus* species , including: *A. flavus, A. niger, A. tereus, A. nidulans, A. clavatus, A. ochraceus* and *A. amsteloidami*

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

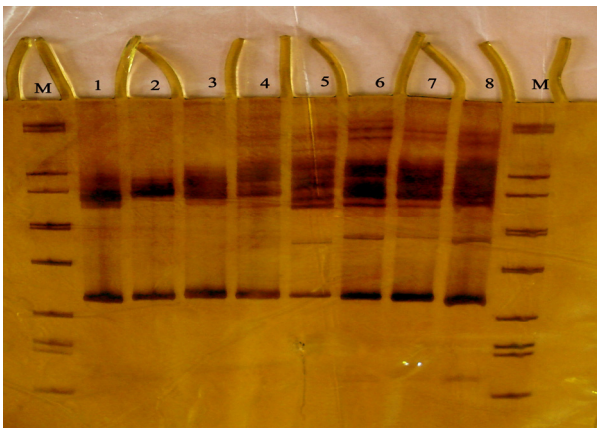


**Fig. 2:** Poly acrylamide gel electrophoresis of PCR products after denaturation and quenching that stained with silver nitrate resulted the most discrimination between the species in 8% PAGE. Lane 1-8: *A. fumigatus, A. flavus, A. niger, A. nidulans, A. terreus, A. ochraceus, A. clavatus, A. fischeri* and M: marker.

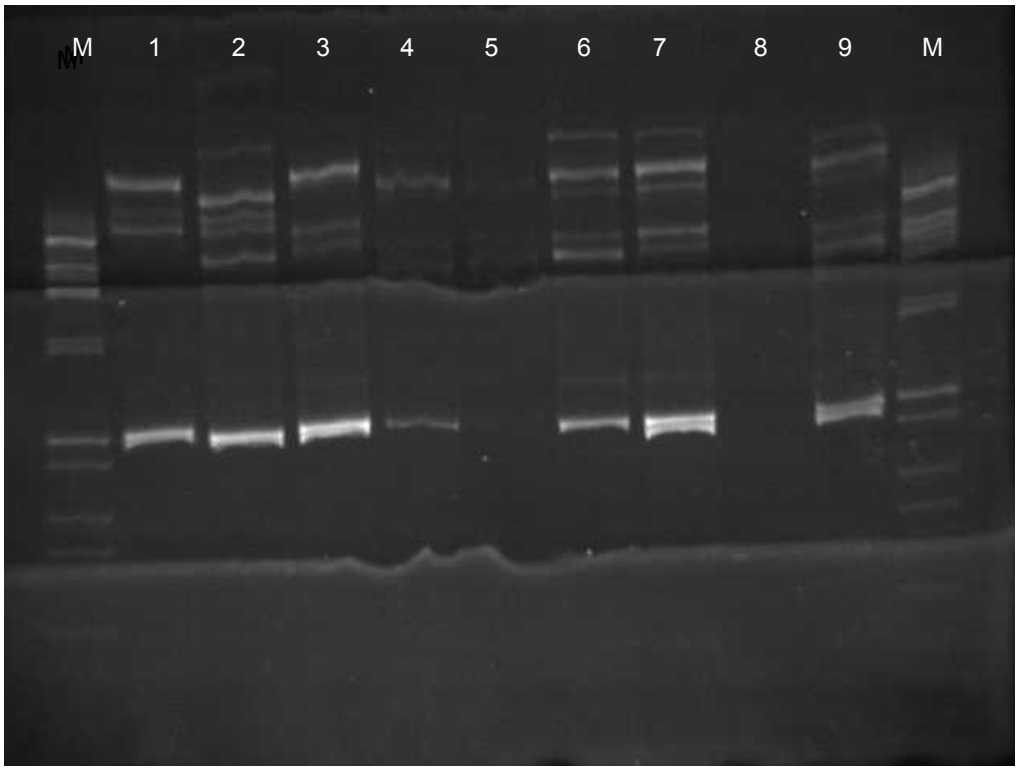


**Fig. 3:** Poly acrylamide gel electrophoresis of ITS1-ITS2 PCR products from standard *Aspergillus* species after denaturation and quenching. Lanes 1-9: *A. fisheri, A. nidulans, A. quadricincta, A. fumigatus, A. niger, A.flavus, A. tereus, A. clavatus*, *A. ochraceus*, *and.* Lanes M: 100bp ladder

## Discussion

It has been shown that *Aspergillus* species exhibit different levels of resistance to amphotericin B and the other antifungal drugs in vitro (13) so it is necessary to find a discriminative test for identification of *A. spesies*. Until recent years, the identification of *Aspergillus* species has been going on the basis of macroscopic and microscopic characteristics. The development of fruiting structures, which are necessary for morphological identification, requires 2 to 10 d of culture on different media at room temperature (12). Therefore, a rapid, simple and correct identification of infection agent is critical for diagnosis and then treatment of patients. In the present study we under took a method for discrimination between the medically important *Aspergillus* species, such as: *A. fumigatus*, *A. flavus*, *A. (Emericella) nidulans*, *A. niger, A. terreus* and *A. clavatus* which is independent of the cultural expression of macroscopic and microscopic characteristics but it is completely PCR based.

It is considered that the rRNA gene is one of the most conserved regions of fungal genome and several workers to identify fungi and explore biologic diversities (5) have used it. In the present study, we selected the most variable region located inside of rRNA (ITS region) to detect the small variations in the sequence of these regions for differentiation of Aspergilli up to the species level. Using the universal fungal primers ITS 1 and ITS 4, all our investigated *Aspergillus* species were amplified by PCR that resulted the same size fragments which approximately 580 bp. This finding is similar to others' result, which was approximately 600 bp and found in Aspergilli of the section *Flavi* (14, 15). Therefore, the band size in PCR products cannot be an accurate factor to differentiate Aspergilli in the level of species. To identify the changes in the nucleotide polymorphism we used two methods including RFLP and single strand conformational polymorphism (SSCP). In other studies the discriminatory power of RFLP analysis for PCR product of reference strains was tested [unpublished data] by using different enzymes (*Bam* HI, *Dde* I, *Dra* I, *Eco* RI, *Hae* III, *Hinf* I, *Ksp* I, *Sma* I and the other restriction enzymes) (1). In their study, the endonuclease (restriction enzyme) with the discriminatory power was *Sma*I clearly discriminating *A. fumigatus*, *A. flavus*, and *A. nidulans*. However, the *A. fumigatus* strains could not be discriminated from the *A. tereus* strains just as *A. flavus* not from *A. niger*. In our Study, digestion of PCR products with the restriction enzyme (*Mwo*I that designed before) resulted the identification of seven of ten tested *Aspergillus* species. Fig. 2 shows that the species *A. flavus, A. niger, A. tereus, A. nidulans, A. clavatus, A. ochraceus* and *A. amsteloidam* were discriminated from each other. Use of RFLP techniques had some problems, included the expensive restriction enzyme and the other, a sufficient digestion of the PCR product was obtained only after an overnight incubation of the enzyme mixture, making this method time consuming.

At this point, what is critical is simplicity and duration time of for identification of *Aspergillus* isolates. SSCP as a PCR based and digestion free method has been our preference to use for identification of *Aspergillus* species isolated from clinical sources. One of the most successful methods for mutation detection and identification of microorganisms is the SSCP technique (16). As in fact SSCP method for discrimination of species is based on the polymorphism in a discrete region within the ITS locus (ITS-2). The SSCP analysis of the entire amplified ITS failed to differentiate some microorganisms (streptococcal species) due to high molecular mass of the amplified fragment and the limited level of sequences present among analyzed species (17). Nevertheless, PCR SSCP with a multiplex PCR based on three regions of the 16S rRNA was tested on 178 bacterial isolates (18) which belonged to 51 species resulted in 47 different PCR SSCP patterns. SSCP method has been used as some literatures for molecular identifications of ectomycorrhizal fungi (19), *Phytophtora* species (20), medically important opportunistic fungi (14) and

other infection caused fungi (6) included the species of *Aspergillus* section *Flavi* (21).

In this study, SSCP method enabled us to identify some medically important *Aspergillus species* from each other. One of the advantages of the SSCP analysis is that definitive results can be obtained within 8-10 h after the first mycelial growth. Our results of SSCP for identification of Aspergilli are in agreement with study of Walsh et al. (15) so that the SSCP patterns of *A. fumigatus* and *A. niger* were similar in that study (14) confirming our results (Fig 3). Nonetheless, these authors investigated another PCR product (197-bp fragment from the 18S rDNA) and studied few number of *A. species* (*A. fumigatus, A. flavus, A. niger*). Other researchers used the primers ITS1 and ITS4 to identify medically important *Aspergillus* species (13); also, Kumeda (21) used the same primers to investigate *Aspergillus* section *Flavi* and showed that the SSCP techniques allow a clear separation of species within this section. Attempts made to find the optimal conditions for SSCP after PCR, It was shown that adding formamide to the acryl amide gels resulted in an improved separation of the single strands (21). In the present study, formamide was added to loading dye that mixed with PCR products before denaturation, also 10X TBE added to the acryl amide gel as other researches (13). Because of large size fragment of ITS2 region (350 bp) used in our study, poly acryl amide gel electrophoresis (PAGE) of single stranded DNAs resulted some non-specific extra bands around the major bands, this problem was overcome by using a gradient poly acryl amide gel so that most of SS bands appeared on the second phase of PAGE.

As our results of Single Strand Conformational Polymorphism method, some of tested *Aspergillus* species: *A. nidulans*, *A. fisheri, A. quadricincta, (A. fumigatus and A. niger)* in a definite group and (*A. flavus, A. tereus and A. ochraceus*) in another group, can be discriminated. Moreover SSCP analysis enabled us to identify above *Aspergillus* species within 8-12 h after an over night growth without using an expensive restriction enzyme, although the sensitivity should be increased by optimizing the thermal conditions, gel composition, ionic strength and additives.

It is concluded that Single Strand Conformational Polymorphism method is a simple and rapid method for identification of some medically important *Aspergillus.* We recommend that this test is useful as a complement test with PCR-restriction digestion test (RFLP) to cover identification of more *Aspergillus* species.

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The authors declare that they have no conflict of interests.

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