RESEARCH ARTICLE

Molecular Identification of *Aspergillus nigar* Using Randomly Amplified Polymorphic Deoxyribonucleic Acid Polymerase Chain Reaction Technique

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ABSTRACT

The study group was chosen from the five patients, reviewed at the Consulting Department of Otorhinolaryngology in Samarra Hospital and private clinics in Samarra, who numbered five samples from December 2018 and swabs were taken from the ear with external otitis. All samples were examined directly and planted on the SDA medium and after its growth it was examined microscopically and the use of several tests to confirm the type. The polymerase chain reaction (PCR) technique and the *PSK* primers *PKS* F CCCAGTTCGGTTTTGCACTG, *PKS* and *PKS* R GCCCGTCAGTAACATGGGAA (307bp) and PCR amplification were used for molecular identification of *Aspergillus nigar*. The results demonstrate the degree of genetic dimension between the five *A. nigar* isolates. The genetic dimension values identified the genetic connection between the different genotypes of the Aspergillus spp., with the investigated species separated into one group. When they were isolated from the same source, the first and second samples were genetically closer to the remainder of the samples in this group (0.890). The genetic distance between the third and fourth samples is 0.734 and 0.647, respectively. However, molecular analyses utilizing random primers and the RAPD-PCR technique revealed some differences between the samples in this group, attributable to DNA sequencing alterations.

Keywords: *Aspergillus nigar*, Fungi, Molecular identification, Randomly Amplified Polymorphic DNA Polymerase Chain Reaction Random amplified polymorphic DNA - polymerase chain reaction (RAPD-PCR).

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INTRODUCTION

An inflammation of the outside section of the ear caused by a fungus is known as a fungal external ear infection[1] Otomycosis is difficult for both patients and otolaryngologists since it often necessitates long-term therapy and monitoring. The recurrence rate remains high despite good treatment and follow-up[2] Otomycosis is frequently associated with the existence of a predisposing disease in immunocompetent persons.[3] Individuals with compromised immune systems, such as those with diabetes, HIV, or lymphoma, as well as those undergoing chemotherapy or radiotherapy for cancer, are at a higher risk of having otomycosis problems[4] The most common fungi in fungal external ear infection are Aspergillus.[5] Aspergillus has a physiological ability and an external appearance that enables it to produce ferocious factors such as mycotoxins.[6] Mycotoxins are one of the most important and harmful types of toxins. Secondary compounds of fungi enhance adhesion and cause hydrolysis of host cells, adding to the pathogenicity of the fungus.[7] Aflatoxins can directly damage hepatocytes or affect the expression of genes involved in lipid metabolism. Increased cholesterol, triglyceride, and lipoprotein production may induce hepatocyte injury due to increased metabolic demand and anaerobic cell metabolism.[8] Aflatoxin B1 is the most potent carcinogen generated by Aspergillus species, and it is classified as (a group 1 carcinogen) by the International Agency for Research on Cancer.[9] The *AflD-Nor* gene is one of the diagnostic genes of *Aspergillus flavus*, as the protein-encoding for this gene enters the metabolic chain of synthesis of aflatoxin toxin, so it is counted as the most important indicator of the production of that fungal type of aflatoxin toxins.[10] The toxic effects of ochratoxins lie with toxicity to the kidneys, congenital malformations, carcinogenic effects of the kidneys and liver, and toxic effects on the nervous system and the immune system.[11] Protein synthesis and energy production are

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inhibited by ochratoxin, which also causes oxidative stress, cell apoptosis and necrosis, and DNA adduct formation.[12] Research indicates that the polykitid enzyme encoded by the *PKS* gene has a primary role in the biosynthesis of Ochratoxin.[13,14]

MATERIALS AND METHODS

Diagnosis of Fungi of Different Otomycosis

The study group was chosen from five patients reviewed by the Consulting Department of Otorhinolaryngology in Samarra Hospital and private clinics in Samarra, who numbered five samples from December 2018 and swabs were taken from the ear with external otitis. All samples were examined directly and planted on the SDA medium, and after its growth, it was examined microscopically, and used several tests to confirm the type. The recurrence percentage of the fungal species and the species that appeared due to transplantation were calculated with equations 1-2.

$$\text{Fungal growth rate} = \frac{\text{The number of isolates with fungal growth}}{\text{Total isolates number}} \times 100 \quad (1)$$

$$\text{The incidence of Fungal Species} = \frac{\text{The number of isolates per spp}}{\text{The number of isolates with fungal growth}} \times 100 \quad (2)$$

Molecular Identification of *A. niger*

A kit from Promega Corporation was used for DNA extraction and purification, and it included solutions such as DNA rehydration solution, cell lysis solution, RNase solution, protein precipitation solution and nuclei lysis solution. Liquid nitrogen, isopropanol, lyticase, and ethanol were also utilized in the DNA extraction process (70%).

DNA Extraction

Based on other researches,[15] after the passage of 15–20 growth was taken with sterile forceps and placed in a petri dish containing sterile filter paper, and then weighed 2–3 grams of fungal growth and transferred to a sterile ceramic bouquet and then added liquid nitrogen to it with rapid grinding until it is obtained. Each tube received 7.5 liters (20 g L^{-1}) of lyticase, which was mixed for 5 minutes with a vortex. The tubes were incubated for one hour at 37°C in a water bath. Then the tubes were centrifuged for 2 minutes at 1400 rpm, and the supernatant was transferred to new Eppendroff tubes. Later, 200 liters of nuclei lysis were added to these tubes and mixed with a vortex, followed by 70 liters of protein precipitation, which was added and mixed in the tubes but kept on ice for 5 minutes before centrifugation at 13000–14000 rpm for 3 minutes.

The supernatant was transferred to new (Eppendroff tubes) containing 300 mL Isopropanol, gently mixed, and centrifuged for 2 minutes at 13000–14000 rpm. The tubes were then dried after the supernatant was removed. After that, each tube was filled with 70% ethanol and gently mixed, then centrifuged for 2 minutes at 13000-14000 rpm, the ethanol was removed, and the tubes were dried for 10–15 minutes. A total of 50 liters of DNA rehydration solution was added to the tubes, followed by 1.5 liters of RNase to purify the DNA, vortexed for 1 second. It was then incubated for 15 minutes at 37°C. Following that, the tubes were centrifuged for 5 seconds at 13000 to 14000 rpm before being incubated at 65°C for 1 hour. Finally, the genomic DNA was analyzed on an agarose gel and stored for future use at -20°C.

For *A. niger* molecular identification. PCR amplification with PSK primers PKS F CCCAGTTCGGTTTTGCACTG, PKS, and PKS R GCCCGTCAGTAACATGGGAA (307bp). The PCR reactions took a total of 25 liters to complete, with 12.5 liters of master mix, 5 liters of DNA, 1.5 liters of forward primer, and 1.5 liters of reverse primer, with the volume brought up to 25 liters by free water. To amplify DNA for the PKS primer, the following program was used: 5 minutes at 95°C (1 cycle), 5 seconds at 95°C, 30 seconds at 58°C, 1-minute at 72°C (30 cycles), and 5 minutes at 72°C (1 cycle). After that, electrophoresis was carried out.[16-18]

RESULTS

The study group was chosen from five patients reviewed by the Consulting Department of Otorhinolaryngology in Samarra Hospital and private clinics in Samarra, numbered five samples from December 2018, and swabs were taken from the ear with external otitis. All samples were examined directly and planted on the SDA medium and after its growth, it was examined microscopically and used several tests to confirm the type. RAPD-PCR technique was used for molecular detection for *A. nigar*. In addition, knowledge of the efficacy of molecular testing against biochemical accuracy tests in diagnosing *A. nigar*. A randomized secretion of A. nigar DNA was carried out using 12 random primers (Table 1) under ideal circumstances to get reliable findings.[19]

Images from electrophoresis regarding the primers OP A-01, OP A-06, OP C-08, Image 1 gave packs of different *A. nigar* and the other primers in Images (2–5) (Table 2).

The degree of genetic dimension between the five *A. nigar* isolates is shown in Table 3. The molecular connection between the genotypes of the *A.* genus was established using the genetic dimension values shown in Figure 1, with the investigated species grouped. This group contained *A. nigar*

Table 1: RAPD primers and their sequences

No.	*Primer*	*Primer code*	*Nucleotide sequence 5 to 3*
1	P1	OP A-01	CAGGCCCTTC
2	P2	OP A-06	GGTCCCTGAC
3	P3	OP B-14	TCCGCTCTGG
4	P4	OP B-20	GGACCCTTAC
5	P5	OP C-08	TGGACCGGTG
6	P6	OP C-16	CACACTCCAG
7	P7	OP D-03	GTCGCCGTCA
8	P8	OP D-18	GAGAGCCAAC
9	P9	OP E-03	CCAGATGCAC
10	P11	OP F-05	CCGAATTCCC
11	P12	OP F-20	GGTCTAGAGG
12	P15	OP H-08	GAAACACCCC

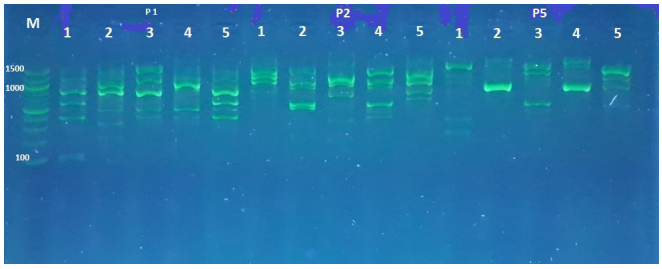

Image 1: Electrophoresis of the primer OP A-01, OP A-06, OP C-08 (packs) by RAPD-PCR of different *A. nigar*

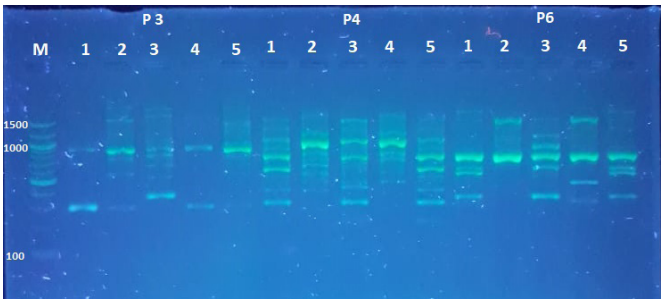

Image 2: Electrophoresis of the primer OP B-14, OP B-20, OP C-16 (packs) by RAPD-PCR of different *A. nigar*

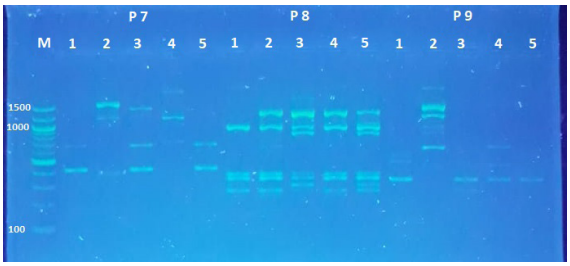

Image 3: Electrophoresis of the primer OP D-03, OP D-18, OP E-03 (packs) by RAPD-PCR of different *A. nigar*

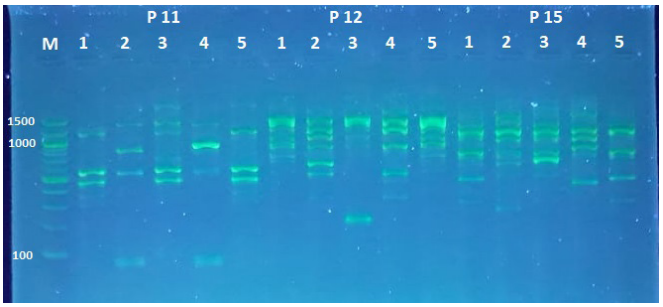

Image 4: Electrophoresis of the primer OP F-05, OP F-20, OP H-08 (packs) by RAPD-PCR of different *A. nigar*

Table 2: Steps of RAPD technique

Steps	*Temperature*	*Time (minutes)*
Initial denaturation	94	5
Denaturation	94	1
Annealing	36	2
Extension	72	1
Final extension	72	10

Table 3: The genetic dimensional values according to RAPD indicators

	1	*2*	*3*	*4*	*5*
1	0.000				
2	0.890	0.000			
3	0.365	0.805	0.000		
4	0.713	0.381	0.734	0.000	
5	0.339	0.823	0.241	0.647	0.000

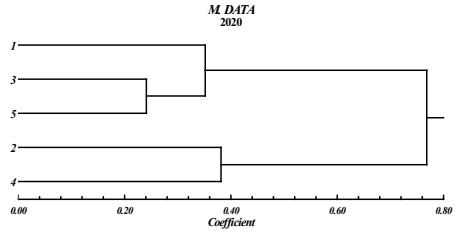

Figure 1: The genetic relationship between *A. nigar* types according to the RAPD indicators

specimens (1, 2, 3, 4, 5, 6) isolated by themselves in the tree diagram in Figure 1.

The first and second samples were genetically closer to the other samples of the studied group (0.890). The third and fourth samples have genetic distances 0.734 and 0.647, respectively. However, molecular analyses utilizing random primers and the RAPD-PCR technique revealed some differences between the samples in the studied group, which can be attributable alterations in DNA sequencing (Table 2).

DISCUSSION

The function of opportunistic fungi as a significant cause of nosocomial infections has been well documented. Aspergillus spp. became known as a cause of life-threatening infections in immunocompromised patients between 1980 and 1990.[20] Nosocomial Aspergillosis is still a major clinical issue.[21] Although PCR-based methods for fungi detection have recently been described.[22] Identifying the true source of opportunistic fungi such as Aspergillus spp. that cause nosocomial infections remains challenging. This investigation used a PCR-based approach such as RAPD-PCR to detect a link between the environment and instances identified Aspergillus.[23] Before applying the RAPD approach for epidemiologic surveys, the amount of the differences of patterns in DNA that can be obtained must be assessed.[24]

Using RAPD-PCR in this investigation was based on earlier research on the method's discriminatory power among medically significant *Aspergillus* species utilizing random primers.[25] When the results of RAPD on the cases are compared to those obtained from environmental isolates, it is clear that several *Aspergillus* isolates have identical DNA patterns.

Other research has validated our findings of identification of Aspergillus species in external ear specimens, with *A. fumigatus* and *A. flavus* being the most often identified Aspergillus spp. in individuals with established Aspergillosis

a comparable study.[23] According to various research, airborne spores of *Aspergillus* spp. have been identified from unfiltered air, ventilation systems, and dusts prevalent during hospital restorations and constructions.[26]

CONCLUSION

To summarize, RAPD-PCR is a simple, quick, and helpful approach; however, it plays a minor role in identifying clinical isolates of *Aspergillus*. However, it should be emphasized that choosing random primers is a critical step in determining the highest level of molecular similarity.

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