# Molecular Diagnosis of Aspergillus Spp Species from Cancer Patients who are suffering from Chest Respiratory Disease

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**Abstract**
The present study was designed to Isolation and diagnosis of Aspergillus species from cancer patients. A total of two hundred fifty-six (256) were collected from patients (sputum sample), aged between (11-<61 years) who visited the tumor center in Kirkuk city at a period from November 2020 to November 2021. In the current study, the sputum specimens were collected from patients with respiratory tract cancer and infection. 256 samples were directly examined by using microscopic with 10% KOH solution from which a total of 143 samples were found positive while other, 113 samples were negative. The results showed that the highest age group exposed to fungal infection was 41-50 years, while the lowest age group was 11-20 years old. The infection with Aspergillus fumigates 37(43.02%) which represented the highest record detected. A. fumigatus showed a percentage of 100%, followed by A. niger with a percentage of 92.3%. While, the lowest percentage of urease production was by A. parvisclerotigenus, that reached 50%. A. fumigatus showed a percentage of 70.3%, followed by A. niger with a percentage of 53.8%. While, the lowest percentage of protease production was by A. oryzae, that reached 20%. For the purpose of diagnosing DNA purified from local isolates of Aspergillus, global primers were adopted to amplify the ITS1 target region anterior (5' – TCCGTAGGTGAACCTGCGG - 3) and posterior (5' – TCCTCCGCTTATTGATATGC - 3') as an approved taxonomic and genetic index to study genetic data between different fungal isolates in this region. 5.8S) ITS1 of Aspergillus isolates. The polymerization products were of one molecular size (550) for all bundles. Finally, Infection with A. parvisclerotigenus was recorded, in Gene-bank, for the first time in Iraq through this study.
Keywords: Aspergillus spp.; cancer; A. parvisclerotigenus; molecular diagnosis.

## 1. Introduction

A fungus is any member of the group of eukaryotic organisms that includes microorganisms such as yeasts and molds [1]. Fungi were divided on the basis of the size of the fruiting bodies into two groups: minute fungi, which produce fruiting bodies that can only be seen using a microscope, and large fungi that form fruiting bodies above or below the soil, which can be seen with the naked eye, meaning that the dimensions of the fruit body are not less than 0.5 cm [2-3]. Respiratory tract diseases are globally responsible for one-third of infectious disease-associated mortality, accounting for 4.3 million annual deaths [4]. Despite treatment, most invasive pulmonary fungal infections are associated with high mortality rates of > 50% [5]. The air breathe is filled with thousands of fungal spores (conidia). After inhalation these tiny elements, hosts may have no symptoms or may cough up blood or have a fever or chest pain or may have symptoms ranging from allergies to life-threatening invasive mycoses [6-7]. The outcome depends on the immune status of the host. Pulmonary fungal diseases consist of fungal colonization, allergy, and infection of the pulmonary tract and lungs and in most cases, colonization is the first step in the progression to pulmonary fungal infection. During recent decades, pulmonary fungal diseases are being diagnosed with increasing frequency [8], largely because of the increasing size of the population at risks, such as patients receiving immunosuppressive therapy, that undergoing bone marrow transplantation or solid-organ transplant (SOT) and those with HIV infection, tuberculosis or cystic fibrosis [6,9]. The incidence and etiology of pulmonary fungal diseases can vary in various types of patient's hospital settings, and geographical locations. Fungi which affect immunosuppressed individuals are frequently species of Aspergillus, Candida, Cryptococcus, geographically restricted agents, and newly emerging fungal pathogens [10-11]. In one study, Aspergillus spp. was isolated from 33% (86/251 cases) of lung-transplantation recipients, which involved colonization (n = 50), tracheobronchial lesions (n = 17) or invasive aspergillosis (n = 19) [12]. Also, invasive pulmonary aspergillosis mortality of neutropenic patients was 40 to 60% in early reports [13].

## 2. Materials and Methods

### Sample collection

A total of two hundred fifty-six (256) were collected from patients (sputum sample), aged between (11-<61 years) who visited the tumor center in Kirkuk city at a period from November 2020 to November 2021. The collection was started first by sterilizing the mouth and gargling with saline solution in the early morning and the sample was taken from each patient and placed in sterile glass bottles, and the investigation was conducted the initial examination for the presence of fungi was followed by direct

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microscopy using an amount of potassium hydroxide 10% KOH, at the same time the samples were cultured using Swabs by passing them on the surface of sterile plastic or glass Petri dishes containing Dextrose Sabouraud Agar , after which it was incubated at 37 0C for 24–72 hours.

### Direct examination

Specimens were placed on microscopic slide, with few drops of 10% KOH, (cover slip added and warmed up over a light flame just under boiling point). The slide was examined under the low power 40x and high power 100x objectives to detect fungi and their septet hyphae [14].

### Sample Culturing

Sputum Samples were cultured on SDA supplemented with 0.04 mg/mL chloromphenicol to inhibit the growth of bacteria, then incubated at 28 °C and 37°C and examined for 10 days [15].

### Laboratory diagnosis of fungal isolates

The diagnosis was based on the phenotypic characteristics of the colonies, including the colony's shape, color, size, and texture. As for the microscopic characteristics, they included the shape and color of the fungal thread and the coids. This was done by transferring part of the fungal colony using a sterile vector on a glass slide and using the cotton blue lactophenol dye, then examined under the microscope for observation Microscopic traits of mycelium [16].

### Genetic study

#### Extraction of Genomic DNA

Aspergillus spp. cultures were grown in 250 ml conical flasks containing 100 ml potato dextrose broth at room temperature (28±2) °C for (5-7) days. Mycelium was harvested by filtration and then freeze-dried. DNA was extracted from ground, freeze-dried mycelium following the method of [17]. DNA extraction kit (Quick DNA TM Fungal Miniprep Kit) was used and according to the procedure mentioned and advised by manufacturer.

### Reaction Chain Polymerase

General initiator used in the molecular diagnosis of fungi The initiator and its sequence shown in Table (1) were used to identify the isolates that selected to ITS1 region.

Table (1): the general primers that were used in the study of the barcode index with its sequences

| No. | Primer Name | Sequence 5´→→→ 3´ | Size (bp) | Annealing |
|---|---|---|---|---|
| 1. | ITS1 (forward) | 5' – TCCGTAGGTGAACCTGCGG - 3 | 600 | 58 |
| 2. | ITS4 ( reverse) | 5' – TCCTCCGCTTATTGATATGC - 3' | | |

## 3. Statistical analysis

Statisticians used the SPSS 15.01 Statistical Package for Social Sciences and Excel 2003 to conduct the statistical study. For tables with frequencies, we used the chi-square test, and for tables with averages and standard deviations, we used the independent sample t-test. The level of significance was set at a 0.05 p value. The mean and standard error were used as descriptive statistics for results.

## 4. Results and Discussion

### Isolation of fungi from the lower respiratory tract (LRT) of infected cancer patients

In the current study, the sputum specimens were collected from cancer patients with respiratory tract infection. 256 samples were directly examined by using microscopic with 10% KOH solution from which a total of 143 samples were found positive while other, 113 samples were negative (table 2). In the absence of metulae, hyphae and dichotomous were observed as branching, conidial heads and chains basipetally from phialides, whilst chains of conidia and vesicles were born directly. Using the above Potassium hydroxide alkaline solutions (KOH) the fungus samples would remain unaffected and easily recognizable from other mixed substances [14]. Both, microscopic examination and culturing methods had produced same result.

Table (2): Distribution of positive and negative cultured cases according to both diagnosing procedure adopted.

| Procedures | Samples | Positive samples | | P value |
|---|---|---|---|---|
| | | No. | % | |
| Direct examined by 10% KOH | 256 | 143 | 55.85 | 0.183 |
| Culturing Procedure | 256 | 143 | 55.85 | 0.152 |

### The distribution of infection according to the cancer type

The table (3) shows that the highest type group of lung cancer was Squamous cell carcinoma, while the lowest type group was Adenosequamous.

Table (3): the histological types regarding the sex of the study sample

| Age | Sex | | | | Total |
|---|---|---|---|---|---|
| | Male | | Female | | |
| | Number | % | Number | % | |
| Squamous cell carcinoma | 81 | 73.6 | 29 | 36.4 | 110 (42.9%) |
| Adenocarcinoma | 57 | 64.1 | 32 | 35.9 | 89 (34.8%) |
| Small cell carcinoma | 25 | 73.5 | 9 | 26.5 | 34 (13.3%) |
| Large cell carcinoma | 14 | 66.7 | 7 | 33.3 | 21 (8.2%) |
| Adenosequamous | 2 | 100 | 0 | 0 | 2 (0.8%) |
| Total | 179 | 69.9 | 77 | 30.1 | 256 (100%) |
| | CS= 4.392 P-value= 0.592 | | | | |

In order to study the clinical behavior of lung cancer in Iraq, we select 256 records of those patients proved to have this disease histo-pathologically. The male: female ratio of the current study sample is 2.3:1; this result is close to the result of Iraqi cancer registry center (2.9:1), and to other Iraqi studies conducted by Elhassani [18], Al-Alusi [19], Al-Kafaji [20] and Al-Qassir [21]. This result is also identical to other Asian countries as in China 2.6:1, Japan 2.8:1. On the other hand, El-hassani in (1987) found the ratio was 9:1, this mean a dramatic increase in the incidence and prevalence of lung cancer among Iraqi women mostly due to the invasion of smoking habit into female gender [22]. The increment in the incidence and the prevalence have been also occurred in men as lung cancer was the second most common cancer (11.9%) after bladder cancer (13.1%)in Iraqi male for the period 1980-1984 [23] and this probably due to the increase in the number of male smokers or the amount of daily consumption.

## Isolation of fungi from LRT infected cancer patients according to fungal species

The infection with A. fumigatus made up 37 (43.02%) which represented the highest record detected; followed by A. flavus 31(22.08%) as shown in table (4).

**Table (4): Distribution of positive case from infected cancer patients**

| Fungal Species | Number of positive Isolates | |
|---|---|---|
| | No. | % |
| A. fumigatus | 37 | 43.02 |
| A. flavus | 19 | 22.08 |
| A. niger | 13 | 15.1 |
| A. terreus | 8 | 9.3 |
| A. oryzae | 5 | 5.8 |
| A. parvisclerotigenus | 4 | 4.7 |
| Total | 86 | 100 |

## Biofilm

Of 78 Aspergillus spp. isolates, 23(40.3%) produced weak biofilm, 33 (57.9%) produced moderate biofilm and 1(1.8%) produced moderate biofilm as showen in table (5) and figure (1).

**Table (5): Biofilm formation by Aspergillus spp**

| Aspergillus spp | Biofilm strenght OD= 0.06 No. (%) | | |
|---|---|---|---|
| | Weak BF | Moderate | Strong |
| 57 | 23(40.3%) | 33 (57.9%) | 1(1.8%) |
| Chi | 2.593 NS | | |

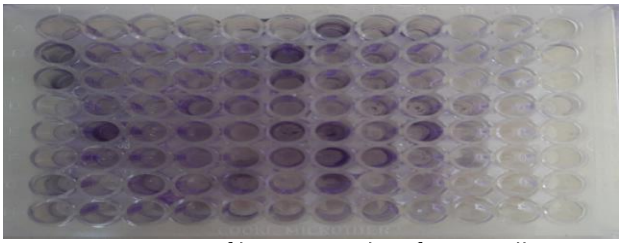

*Figure (1): Biofilm test results of Aspergillus.*

Together with gene deletion analysis and morphological studies, biofilms produced by Aspergillus spp. have been studied to a very limited extent in in vitro and in vivo model systems with microarray and RNA-sequencing. A comparison between in vitro biofilms using microarray, RNA-sequencing and proteomic analysis was already described by Muszkieta et al. [24] and Beauvais & Latgé [25]. In these studies, gene expression was compared between A. fumigatus biofilms grown on solid agar medium versus submerged planktonic growth in liquid medium conditions (PL). The results of the current study agreed that the highest rate of infection with Aspergillus was for type A. fumigatus with the results of Al- Ameri [26], which indicated that the highest percentage of infection with Aspergillus was for type A. fumigatus, which reached (29.9%), followed by A. niger by (28.9%), while it was found that the rates of infection of each of A. flavus and A. terreus reached (18.69% and 12.14%), and these results are very similar to the results of the current study. The results of the current study also agreed with the findings of Wong et al. [27], which indicated that the highest rate of infection with Aspergillus in patients with respiratory tract infection was for A. fumigatus at a rate of (33.3%), while it was followed by A. niger with a rate of infection that reached (16.7%).

## Genetic study for Aspergillus Spp.

### DNA extraction and amplification

Fungal growth was transferred from the surface of a single agar plate into a pre-cooled (-208C) sterile ceramic mortar, overlaid with liquid nitrogen, and ground with a sterile ceramic pestle into a fine powder. Two ml of buffer G-2 (Genomic DNA buffer set; Qiagen, Valencia, Calif.) 6-+containing RNase (200 mg/ml; Sigma Chemical Company, St. Louis, Mo.) was added to suspend the powder and the suspension was transferred into a clean test tube. Forty-five microliters of proteinase K solution (20 mg/ml stock solution; Sigma) was added and the suspension was incubated with intermittent agitation for 3 h at 558C.

The suspension was centrifuged at 21,500/g for 10 min, the supernatant was transferred into a clean test tube, and DNA was extracted and purified using Genomictip 20/G columns (Qiagen) according to the manufacturer's instructions. Two and one-half microliters of glycogen solution (20 mg/ml; Gentra Systems, Minneapolis, Minn.) was added to the eluted DNA which was then precipitated by standard methods using isopropanol and ethanol. DNA was resuspended in 60 ml of DNA rehydration buffer (PureGene kit, Gentra Systems) and stored at -208C until used.

## Determination of DNA Concentration

### Procedure

Added 200µl from Tris-EDTA (TE) to 3,800 from D. water the mix 4000 µl, pull 10 µl ignore it and add 10 µl from dye (DNA Dye)

Pull 200 µl of the mix for each sample.

| The series of the following tubes are prepared as follows: | | | |
|---|---|---|---|
| | Blank | Standard | Sample |
| Mix | 200 µl | 200 µl | 200 µl |
| DNA Extraction | | 2 µl | 2 µl |

Make vortex for second to mix.

Leaves on rake at room temperature for 5 min. Extracted the value from the device immediately.

| Nanodrop of DNA | | |
|---|---|---|
| 260/280 purity | Nucleic acid conc (ng/ml) | sample ID |
| 1.81 | 112 | 1 |
| 1.90 | 77 | 2 |
| 1.90 | 95 | 3 |
| 1.86 | 75 | 4 |
| 1.90 | 78 | 5 |
| 1.88 | 90 | 6 |
| 1.89 | 95 | 7 |
| 1.95 | 100 | 8 |
| 1.94 | 110 | 9 |
| 1.83 | 99 | 10 |

The results of the extraction of Aspergillus using the special equipment for this purpose and the electrophoresis by agarose gel and its detection using the safe red dye and examination under ultraviolet rays showed during which one molecular size, as shown in figure (2).

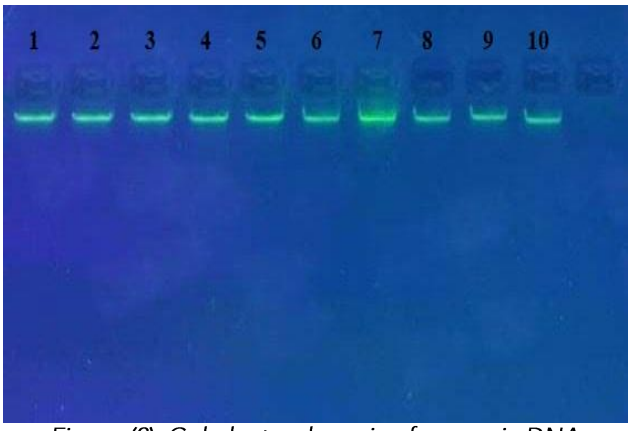


*Figure (2): Gel electrophoresis of genomic DNA extraction from bacteria 1% agarose gel at 1houre*

For the purpose of diagnosing DNA purified from local isolates of Aspergillus, global primers were adopted to amplify the ITS1 target region anterior (5' – TCCGTAGGTGAACCTGCGG - 3) and posterior (5' – TCCTCCGCTTATTGATATGC - 3') as an approved taxonomic and genetic index to study genetic data between different fungal isolates in this region. 5.8S) ITS1 of Aspergillus isolates. The polymerization products were of one molecular size (550) for all bundles as in the figure (3).

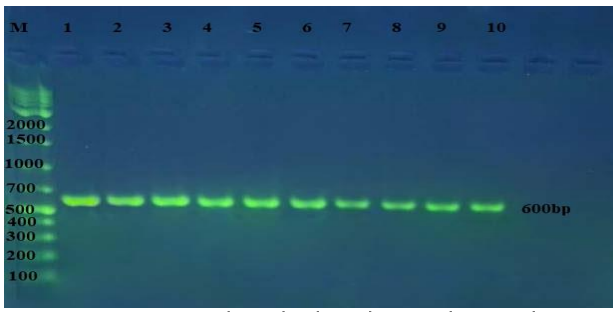


*Figure (3): PCR product the band size. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100).*

The emergence of this result is evidence of the success of a primer in amplifying the target region (5.8S) ITS1 of the ribosomal rDNA chromosome, and this was reflected in the appearance of bundles of one molecular weight as a result of the presence of common and similar sequences in the sequence of nitrogenous bases in the DNA of the fungal isolates that were able to complement those in the primers and monomers Specialized interaction and generation of DNA packets of similar sizes. These results directly contributed to the diagnosis between closely related Aspergillus species and the distinction between them, and this diagnosis is of medical importance in the early diagnosis of different species of the same genus [28]. The species A. oryzae1 in current study showed similarity with the A. oryzae isolates recorded in gene bank with No: MT406750.1. Also, the species A. oryzae2 in current study showed similarity with A. oryzae isolates recorded from El-Sharqia Governorate, Egypt with No: MT406750.1. The species A. niger3, 4 in current study showed similarity with the A. niger isolates recorded in gene bank with No MK263026.1. The isolates of A. flavus5, 6 had the sequence of nitrogenous bases of the gene in the studied regions by 99% with that of A. flavus isolates in Iraq recorded in the gene bank with the number: MN856403.1. Otherwise, the two isolates whose species was not determined by microscopic and phenotypic examination were found to be A. parvisclerotigenus7, 8 and the similarity rate was 99% with A. parvisclerotigenus isolate in Nigeria and registered in the gene bank with the number: MH270568.1. The species A. terreus9 in current study showed similarity with the A. niger isolates recorded from Saudi Arabia with No: MN856403.1. Also, the species A. terreus10 in current study showed similarity with A. niger isolates recorded from El-Sharqia Governorate, Egypt with No: MT558939.1. The species A. fumigatus 11, 12 in current study showed similarity rate was 97% with the A. fumigatus isolates recorded in gene bank with No MK070012.1. While, A. fumigatus 13, 14 in current study showed similarity rate was 97% with the A. fumigatus isolates recorded in gene bank with No MN634626.1. The species A. niger 15, 16 in current study showed similarity rate was 100% with the A. fumigatus isolates recorded in gene bank with No MT447497.1. The isolates of A. tubingensis 17, 18 had the sequence of nitrogenous bases of the gene in the studied regions by 99% with that of A. tubingensis isolates in India recorded in the gene bank with the number: MN818622.1. The isolates of A. oryzae 19, 20 had the sequence of nitrogenous bases of the gene in the studied regions by 99% with that of A. oryzae isolates in Mafikeng Campus, South Africa recorded in the gene bank with the number: MH270563.1.

Table (6): Comparison of sequence similarity ratio of nitrogenous bases of Aspergillus isolates isolated in this study with other isolates of the same fungus registered at the National Center for Biopurification Information NCBI and identifying the sites of occurrence of variations and mutations in isolates

| Gene: 18S ribosomal RNA gene | | | | | | |
|---|---|---|---|---|---|---|
| Identities | Source | Sequence ID with compare | Nucleotide | Location | Type of substitution | No. |
| 99% | A. oryzae[1] | ID: MT406750.1 | G\A | 12 | Transition | A1F |
| 99% | A. oryzae[2] | ID: MT406750.1 | C\T | 12 | Transition | A1R |
| 99% | A. niger[3] | ID: MK263026.1 | G\C | 470 | Transvertion | A5F |
| 99% | A. niger[4] | ID: MK263026.1 | C\G | 470 | Transvertion | A5R |
| 99% | A. flavus[5] | ID: MN856403.1 | G\T | 43 | Transvertion | A6F |
| 99% | A. flavus[6] | ID: MN856403.1 | C\A | 43 | Transvertion | A6R |
| 99% | A. parvisclerotigenus[7] | ID: MH270568.1 | G\A | 11 | Transition | A8F |
| | | | A\G | 536 | Transition | |
| 99% | A. parvisclerotigenus[8] | ID: MH270568.1 | T\C | 536 | Transition | A8R |
| | | | C\T | 11 | Transition | |
| 99% | A. terreus[9] | ID: MT558939.1 | C\A | 131 | Transvertion | A10F |
| | | | G\A | 301 | Transition | |
| | | | T\A | 379 | Transvertion | |
| | | | G\A | 443 | Transition | |
| | | | C\G | 452 | Transvertion | |
| | | | G\T | 453 | Transvertion | |
| | | | C\G | 455 | Transvertion | |
| 99% | A. terreus[10] | ID: MT558939.1 | G\C | 455 | Transvertion | A10R |
| | | | C\A | 453 | Transvertion | |
| | | | G\C | 452 | Transvertion | |
| | | | C\T | 443 | Transition | |
| | | | A\T | 379 | Transvertion | |
| | | | C\T | 301 | Transition | |
| | | | G\T | 131 | Transvertion | |
| 97% | A. fumigatus[11] | ID: MK070012.1 | G\A | 99 | Transvertion | A11F |
| | | | C\G | 113 | Transvertion | |
| | | | T\A | 118 | Transvertion | |
| | | | A\G | 125 | Transition | |
| | | | A\T | 155 | Transvertion | |
| | | | A\G | 171 | Transition | |
| | | | G\C | 201 | Transvertion | |
| | | | G\A | 217 | Transition | |
| | | | G\A | 378 | Transition | |
| | | | A\G | 380 | Transition | |
| | | | C\G | 384 | Transvertion | |
| | | | A\G | 385 | Transition | |
| 97% | A. fumigatus[12] | ID: MK070012.1 | T\C | 385 | Transition | A11R |
| | | | G\C | 384 | Transvertion | |
| | | | T\C | 380 | Transition | |
| | | | C\T | 378 | Transition | |
| | | | C\T | 217 | Transition | |
| | | | C\G | 201 | Transvertion | |
| | | | T\C | 171 | Transition | |
| | | | T\A | 155 | Transvertion | |
| | | | T\C | 125 | Transition | |
| | | | A\T | 118 | Transvertion | |
| | | | G\C | 113 | Transvertion | |
| | | | C\T | 99 | Transvertion | |
| 99% | A. fumigatus[13] | ID: MN634626.1 | T\A | 501 | Transvertion | A12F |
| | | | G\C | 519 | Transvertion | |
| 99% | A. fumigatus[14] | ID: MN634626.1 | C\G | 519 | Transvertion | A12R |
| | | | A\T | 501 | Transvertion | |
| 100% | A. niger[15] | ID: MT447497.1 | ----- | ------- | ------- | A15F |
| 100% | A. niger[16] | ID: MT447497.1 | ----- | ------- | ------- | A15R |
| 99% | A. tubingensis[17] | ID: MN818622.1 | G\A | 603 | Transition | A16F |
| | | | A\G | 607 | Transition | |
| 99% | A. tubingensis[18] | ID: MN818622.1 | T\C | 607 | Transition | A16R |
| | | | C\T | 603 | Transition | |
| 99% | A. oryzae[19] | ID: MH270563.1 | C\G | 546 | Transvertion | A17F |
| 99% | A. oryzae[20] | ID: MH270563.1 | G\C | 546 | Transvertion | A17R |

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