

How to Cite:

AL-Masari, S. M. S., & Al-Obaidi, A. B. (2022). Isolation and identification of the most important aflatoxin B₁-producing species in Najaf Governorate respiratory patients. *International Journal of Health Sciences*, 6(S4), 7684–7692.
<https://doi.org/10.53730/ijhs.v6nS4.10864>

Isolation and identification of the most important aflatoxin B₁-producing species in Najaf Governorate respiratory patients

Salih M. S. AL-Masari

Department of Biology, Faculty of Science, University of Kufa, Iraq
Corresponding author email: salihmahdi782@gmail.com

Atheer B. Al-Obaidi

Department of Biology, Faculty of Science, University of Kufa, Iraq
Email: atheer.alobaidi@uokufa.edu.iq

Abstract--The purpose of this study was to evaluate the efficacy of some biological methods for removing toxins or reducing the toxic effect of aflatoxin. Initially, the genus *Aspergillus* spp was isolated from chronic lung infections, Sputum samples were collected from patients with chest and respiratory diseases who visited the consulting clinic, and the samples were subjected to direct microscopic examination and agricultural and molecular diagnosis using PCR technology. Aflatoxin B₁ chemical detection also indicated the use of some poison detection methods, such as the ammonia vapour method, a kind-layer chromatic analysis, and high performance chromatography. The tested isolation gave the same result as the ammonia test for ultraviolet rays by changing the color of the inverted dish to red-carbon and expressing one isolation as a negative result of the test.

Keywords---*A.flavus*, aflatoxin B₁, HPLC.

Introduction

Aspergilli are a type of imperfect filamentous fungus. Many of the roughly 250 known species produce beneficial secondary metabolites, such as antibiotics and other pharmaceuticals (Brakhage et al., 2008) *Aspergillus terreus*, for example, produces lovastatin, a powerful cholesterol-lowering drug. Antibiotics (penicillin and cephalosporin) and antifungals (griseofulvin) are produced by other *Aspergilli* (terrequinone A)(Hoffmeister & Keller, 2007). *Aspergillus flavus* is a saprophytic soil fungus that contaminates seed crops with the carcinogenic secondary metabolite aflatoxin before and after harvest. The fungus is also an opportunistic

animal and human pathogen that causes aspergillosis diseases, which are becoming increasingly common in immunocompromised individuals (Aspergillus Flavus - PubMed, n.d.). Aspergillus is a ubiquitous, soil-dwelling fungi that may cause a wide range of pulmonary diseases: invasive aspergillosis (IA); chronic pulmonary aspergillosis (CPA); subacute invasive aspergillosis, aspergilloma, chronic cavitary pulmonary aspergillosis (CCPA), chronic fibrotic pulmonary aspergillosis (CFPA); as well as allergic forms such as allergic bronchopulmonary aspergillosis (ABPA) or severe asthma fungal sensitization. The most relevant species are *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus* and *A. nidulans* (Vahedi-Shahandashti and Lass-Flörl, 2020); (Bongomin et al., 2020). IA normally affects immunosuppressed patients such as either those with hematologic disorders, solid organ transplantations, cirrhosis or chronic pulmonary obstructive disease (COPD), or those critically ill (Hammond et al., 2020). Furthermore, patients with viral pneumonia, i.e. due to influenza or COVID-19, face an increased risk of pulmonary aspergillosis (Koehler et al., 2021). CPA affects patients with or without little immunosuppression yet with preexisting lung disease as active or previous mycobacterial infection or COPD (Alastruey-Izquierdo et al., 2018). Allergic forms occur mainly in patients with asthma or cystic fibrosis as a result of a hypersensitive reaction to Aspergillus (Echeverria-Esnal et al., 2022). Aflatoxin-producing fungi include Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nomius, but they are also produced by other Aspergillus species and Emericella spp. (Alam et al., 2021).

Materials and Methods

Fungi isolation and identification from a sputum samples

The tested fungi were isolated from cases of lower respiratory tract infection, where sputum samples were collected from 85 patients of the consultative clinic for chest and respiratory diseases, beginning in the early morning with mouth sterilization and gargling with saline solution. Fungi were found after using direct microscopy and a small amount of potassium hydroxide (KOH 10 percent). Using the direct method of isolation, the samples were planted using sterile wooden sticks (Swabs) by passing them on the surface of sterile plastic dishes containing the food medium Sabouraud Dextrose Agar (SDA) and sterilized with sterilizer at (121 °C) for 15 minutes. (The dishes were incubated at 28±2 °C. For a seven-day period. The dishes were incubated at 25 °C ± 2 for five day.

Examine some fungal isolates' ability to produce aflatoxins B₁, B₂ Ammonia Vapor Test for Detection of Aflatoxin

Coconut Extract Agar The medium was made and poured into Petri dishes (9 cm). The dishes were inoculated with 5 mm discs grown on isolates of *A. flavus* at the age of (7) days and placed in the center of each dish after solidification. The process was repeated four times for each fungal species, then incubated at 25 ± 2 °C for a week, after which the dishes were treated with ammonia (20 %) by turning the dishes so that the base of the plate was upwards, placing a filter paper saturated with ammonia in the lid of each plate, then incubating the dishes in the incubator at 25 °C for (24) hours and watching for a change in the color of the colonies bases. The fungal color changes from transparent white to red,

indicating that the isolate can produce aflatoxins. (Saito & Machida, 1999);(Belludi et al., 2022).

Aflatoxin detection using thin-layer chromatography (TLC)

Aflatoxin B1 is detected using thin-layer chromatography (TLC), which consists of 20x20 cm plates that are activated in an electric oven at a temperature of 120 °C for 60 minutes before use. In addition, (98:2) chloroform:methanol was used. A straight line has been used below the plate (TLC) that is away from a distance of (2) cm, and taking (20) micro letters by Micropipette from standard Aflatoxin B1 that is placed on the line with a distance of (2) cm from the left edge of the plate and a distance of (2) cm from the special spot, The first measurement placed the same amount from the extraction of serum each sample on the right of standard Aflatoxin B1 with a distance of (2) cm between a sample and other, then left the spots for dry then put in Jar that contains mobile phase and it was monitored until the solution arrived at a distance of approximately (2) cm of the up end for plate, Exhumed, plate removed from the Jar, dried, and diagnosed under UV light with wave length (360) nm and have been discovered the Aflatoxin B1 with matching Relative factor (RF), $RF = \text{distance of AFB1} / \text{distance of the solvent}$, site, and bright to serum content of individuals with hepatitis diseases with a standard Aflatoxin B1 material (Sobolev and Dorner, 2002;Al-kelabe, 2021).

Detection of deoxyribonucleic acid (DNA) by PCR

The primers were used to detect DNA according to the method of work proposed by the manufacturer.

Results and Discussion

Isolation and identification of fungi present in sputum samples

The results of isolating the fungi showed the presence of (10) types of fungi belonging to (6) genera, namely *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Rhizopus*, and *Trichothecium*. The findings revealed the emergence of species from the genus *Aspergillus* SPP, where the fungus *fumigatus* lives. A had the highest percentage. The infection rate was (28.9%), followed by the fungus *A. niger* (26.3%), and *A. flavus* and *A. terreus* ratios of (23.68%) and (10.52%), respectively). And that the dominance of these four types was consistent with research (Jasim.A.N, and Aziz.F.Z,2015). Many studies conducted around the world have revealed that the fungus Thrush predominates in the respiratory system, owing to the nature of this growth. Neighborhoods where the respiratory tract is a suitable place for its growth, as it is a humid place and an appropriate temperature with the presence of Nutrients as well as predisposing factors such as chronic obstructive pulmonary disease, diabetes, and excessive antibiotic use.

Table 1
represents the total and percentage of *Aspergillus* species isolated from
respiratory tract patients

Isolated species	Aspergillus	total number of isolates	percentage of frequency (%)
A.fumigatus		11	28.9
A.niger		10	26.3
A.flavus		9	23.68
A.terreus		4	10.52
A.nidulans		2	5.26
A.ochraceus		1	2.63
A.parasiticus		1	2.63
Total number of isolates		38	100

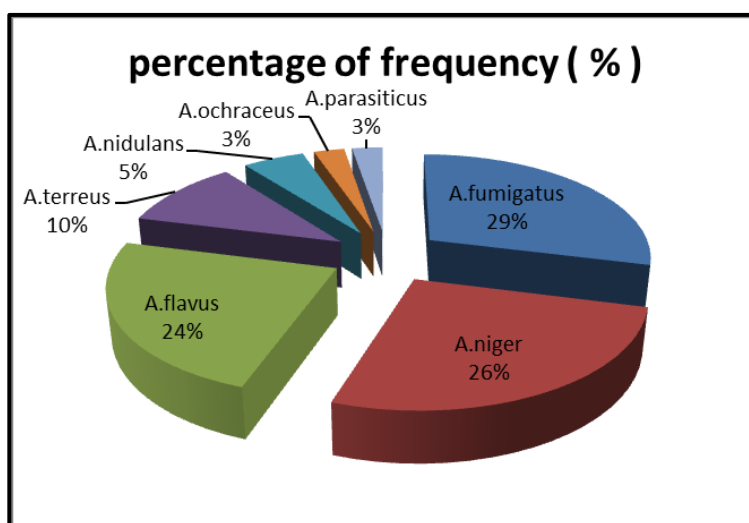


Figure 1. The percentage frequency of *Aspergillus* species in sputum samples Test the ability of some fungal isolates to produce aflatoxins B₁, and B₂ by ammonia test

The results of this test showed the ability of 14 out of 15 isolates of *A.flavus* to produce aflatoxin by changing the colour of the base of the P.D.A medium on which the fungus isolates are grown, with a percentage of 93.33%, while only one isolate gave a negative test result and the isolates differed in the number of aflatoxins, depending on the severity of The colour of the colony base changed as the isolates AF1, AF2, AF3, AF4, AF6, and AF7 were the most aflatoxin producing isolates. Whereas AF8 and AF9 isolates showed a medium ability to produce aflatoxins, the rest of the isolates were weak in their production of aflatoxins. This result is consistent with what was indicated by (Al-Salami, 2019) The results of this test showed the ability of 7 isolates out of a total of 10 isolates of *A. flavus* to produce aflatoxin B₁ by changing the colour of the base of the centre of the PDA on which the fungus isolates were growing at 70%, whereas only three isolates were given negative test results. The isolates differed in the amount of aflatoxin B₁ Due to the intensity of the colony colour change. This result is also in line with

the findings of (Kteo & Mohammed, 2019) The results of this test showed the ability of 24 isolates out of 38 isolates of *A.flavus* isolated from infected wheat grains and body of *T.granarium* larvae to produce aflatoxin by changing the colour of coconut media that they were cultured on, while 14 isolates gave negative test results. The difference in the ability of the isolates to produce aflatoxin quantitatively and qualitatively may be due to the difference in the genetic susceptibility of the strains and this explains the gradient in the red colour.

Table 2
Testing the ability of some fungi isolates to produce aflatoxin in the medium of Coconut Extract Agar (CEA)

isolation symbol	Colour intensity on medium(CEA)
AF1	+++
AF2	+++
AF3	+++
AF4	+++
AF5	-
AF6	++ +
AF7	+++
AF8	++
AF9	++
AF10	+
AF11	+
AF12	+
AF13	+
AF14	+
AF15	+

- (+) change of culture media to pink colour with isolate which produced aflatoxins.
 (++) change of culture media to light red colour with isolate which produced aflatoxins.
 (+++) change of culture media to dark red colour with isolate which produced aflatoxins.
 (-) no change of culture media colours with isolated fungi.

Detection of Aflatoxin B₁ in PDA medium by TLC method

In light of the results of the first test (ammonia test), Seven isolates of the fungus *A. flavus* were selected, which showed a response to the detection of ammonia and gave pink and red colour. The results of this test showed the ability of the Seven isolates of *A. flavus* to produce aflatoxin B₁ (Figure 2). These results are similar to what was indicated by one of the studies about the ability of 75% of the isolates of *A.flavus* to produce aflatoxin B₁ (Yu et al., 2004; Al-Khalidi, 2017). Also similar to the study(Ajmal et al., 2022) result of aflatoxigenic isolates, 88.3% of the isolates were reported to produce AFB₁. The difference in the ability of isolates to produce aflatoxins B₁ and B₂ may be attributed to the different metabolic pathways of the moulds strain (Lee, 1991).

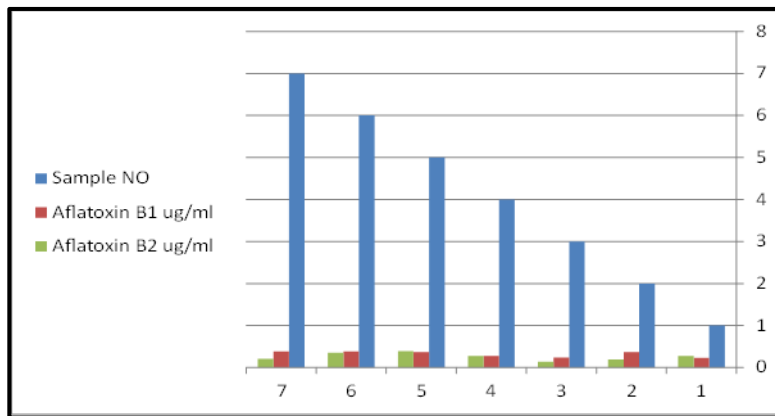


Figure 3. The graph depicts the concentrations of aflatoxin B1 and B2 in serum (ug/ml) in various serum samples

P.C.R technology was used to detect the ability of some *A. flavus* isolates to produce aflatoxins

Afl-R

Forward primer 5'-CAACTCGGCGACCATCAGAG-3' 650 bp

Reverse primer 5'-GGGAAGAGGTGGGTCAGTGT-3'

Afi-s

Forward primer 5'-GTGGAGGATACGCTCACTCG-3' 356bp

Reverse primer 5'- GCGACTCGCATGGTGATTTC-3'

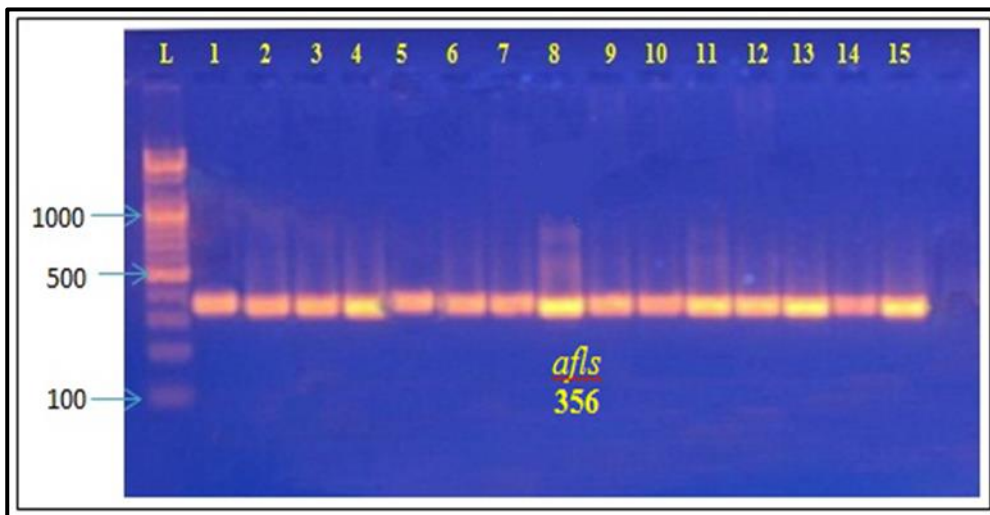


Figure 4. PCR amplification products of *Aspergillus* isolates that amplified with *afls* gene primers with product 356 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1, 2, 3, 4, 5, 6,7,8, 9, 10, 11, 12, 13, 14, 15) show positive results with the *afls* gene. (Liao et al., 2020);(Uka et al., 2020)

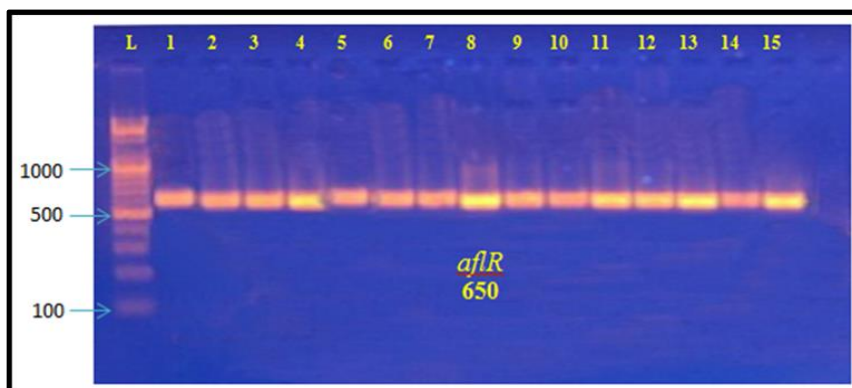


Figure 5. PCR amplification products of *Aspergillus* isolates that amplified with *aflR* gene primers with product 650 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15) show positive results with the *aflR* gene (Liao et al., 2020; Uka et al., 2020)

References

- Ajmal, M., Alshannaq, A. F., Moon, H., Choi, D., Akram, A., Nayyar, B. G., Gibbons, J. G., & Yu, J.-H. (2022). Characterization of 260 Isolates of *Aspergillus* Section Flavi Obtained from Sesame Seeds in Punjab, Pakistan. *Toxins*, 14(2), 117.
- Alam, P., Shakeel, F., Alqarni, M. H., Foudah, A. I., Faiyazuddin, M., & Alshehri, S. (2021). Rapid, sensitive, and sustainable reversed-phase HPTLC method in comparison to the normal-phase HPTLC for the determination of pterostilbene in capsule dosage form. *Processes*, 9(8), 1305.
- Alastruey-Izquierdo, A., Cadranet, J., Flick, H., Godet, C., Hennequin, C., Hoenigl, M., Kosmidis, C., Lange, C., Munteanu, O., & Page, I. (2018). Treatment of chronic pulmonary aspergillosis: current standards and future perspectives. *Respiration*, 96(2), 159–170.
- Al-kelabe, H. F. H. (2021). *The relationship between Aflatoxin B1 and some Immunological parameters in hepatitis patient in Najaf city*.
- Al-Khalidi, B. O. H. (2017). Detection of aflatoxin B1 in the food of primary school students. *Al-Kufa University Journal for Biology*, 9(1).
- Al-Salami, I. (2019). Aflatoxin produced by toxigenic fungi isolated from feedstuff of animal diets. *Plant Archives*, 19(2), 1953–1957.
- Aryani, L. N. A., & Lesmana, C. B. J. (2019). Neuropsychiatric factor and polymorphism gene in internet addiction. *International Journal of Health & Medical Sciences*, 2(1), 39-44. <https://doi.org/10.31295/ijhms.v2n1.90>
- Aspergillus flavus* - *PubMed*. (n.d.). Retrieved April 4, 2022, from <https://pubmed.ncbi.nlm.nih.gov/21513456/>
- Belludi, R., Sandhu, P. S., Sharma, P., & Sekhon, A. S. (2022). Diversity analysis of aflatoxigenic isolates of *Aspergillus* sp. from major groundnut growing states of India. *Indian Phytopathology*, 1–10.
- Bongomin, F., Asio, L. G., Baluku, J. B., Kwizera, R., & Denning, D. W. (2020). Chronic pulmonary aspergillosis: notes for a clinician in a resource-limited setting where there is no mycologist. *Journal of Fungi*, 6(2), 75.
- Brakhage, A. A., Schuemann, J., Bergmann, S., Scherlach, K., Schroeckh, V., & Hertweck, C. (2008). Activation of fungal silent gene clusters: a new avenue to

- drug discovery. *Natural Compounds as Drugs*, 1–12.
- Echeverria-Esnal, D., Martín-Ontiyuelo, C., Navarrete-Rouco, M. E., Barcelo-Vidal, J., Conde-Estévez, D., Carballo, N., De-Antonio Cusco, M., Ferrández, O., Horcajada, J. P., & Grau, S. (2022). Pharmacological management of antifungal agents in pulmonary aspergillosis: an updated review. *Expert Review of Anti-Infective Therapy*, 20(2), 179–197.
- Hammond, E. E., McDonald, C. S., Vestbo, J., & Denning, D. W. (2020). The global impact of *Aspergillus* infection on COPD. *BMC Pulmonary Medicine*, 20(1), 1–10.
- Hoffmeister, D., & Keller, N. P. (2007). Natural products of filamentous fungi: enzymes, genes, and their regulation. *Natural Product Reports*, 24(2), 393–416.
- Jassim, N. A., & Aziz, Z. F. (2015). Isolation and identification of genus species. *Aspergillus* spp from the respiratory tract of people with chronic lung disease. *Journal of University of Babylon*, 23(1).
- Koehler, P., Bassetti, M., Chakrabarti, A., Chen, S. C. A., Colombo, A. L., Hoenigl, M., Klimko, N., Lass-Flörl, C., Oladele, R. O., & Vinh, D. C. (2021). Defining and managing COVID-19-associated pulmonary aspergillosis: the 2020 ECMM/ISHAM consensus criteria for research and clinical guidance. *The Lancet Infectious Diseases*, 21(6), e149–e162.
- Kteo, B. Q., & Mohammed, A. A. (2019). ASPERGILLUS FLAVUS ISOLATES ASSOCIATED WITH WHEAT GRAINS INFECTED WITH KHAPRA BEETLE TROGODERMA GRANARIUM AND THEIR ABILITY TO PRODUCE AFLATOXINS B1. *Plant Archives*, 19(2), 1765–1770.
- Lee, Y. J. and H. W. M. (1991). • Lee, Y.J. and Hagler W.M. (1991). Aflatoxin and cyclopiazonic acid production by *A. flavus* isolated from contaminated maiza . *J. of Food In.:an official-publication of institute of food technologists,USA*.
- Liao, J., He, Z., Xia, Y., Lei, Y., & Liao, B. (2020). A review on biosynthesis and genetic regulation of aflatoxin production by major *Aspergillus* fungi. *Oil Crop Science*, 5(4), 166–173. <https://doi.org/10.1016/j.ocsci.2020.11.001>
- Saito, M., & Machida, S. (1999). A rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus* by ammonia vapor. *Mycoscience*, 40(2), 205–208. <https://doi.org/https://doi.org/10.1007/BF02464300>
- Sobolev, V. S., & Dorner, J. W. (2002). Cleanup procedure for determination of aflatoxins in major agricultural commodities by liquid chromatography. *Journal of AOAC International*, 85(3), 642–645.
- Suryasa, I. W., Rodríguez-Gámez, M., & Koldoris, T. (2022). Post-pandemic health and its sustainability: Educational situation. *International Journal of Health Sciences*, 6(1), i-v. <https://doi.org/10.53730/ijhs.v6n1.5949>
- Uka, V., Cary, J. W., Lebar, M. D., Puel, O., De Saeger, S., & Diana Di Mavungu, J. (2020). Chemical repertoire and biosynthetic machinery of the *Aspergillus flavus* secondary metabolome: A review. *Comprehensive Reviews in Food Science and Food Safety*, 19(6), 2797–2842.
- Vahedi-Shahandashti, R., & Lass-Flörl, C. (2020). Novel antifungal agents and their activity against *aspergillus* species. *Journal of Fungi*, 6(4), 213.
- Yu, J., Chang, P.-K., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Payne, G. A., Linz, J. E., Woloshuk, C. P., & Bennett, J. W. (2004). Clustered pathway genes in aflatoxin biosynthesis. *Applied and Environmental Microbiology*, 70(3), 1253–1262.