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Research Paper

Molecular method for the detection of resistance aspergillus fumigatus to some antifungal agents

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ABSTRACT

Background: Aspergillus fumigatus is a clinically important opportunistic fungal pathogen linked with pulmonary aspergillosis, especially in immunocompromised individuals. Antifungal resistance and genetic diversity among isolates threaten effective disease management. Rapid molecular typing methods like RAPD help monitor isolate relationships and epidemiological trends.
Objective: To determine antifungal resistance profiles and explore genetic diversity and phylogenetic relationships among clinical A. fumigatus isolates from Iraqi patients using RAPD markers.
Methods: Forty-five sputum samples from patients aged 33–82 years were collected across Iraqi governorates (August–December 2022). Of these, 41 tested positive, and 20 were selected for further analysis. Antifungal susceptibility testing and RAPD analysis using four primers (including OP-M06 and OP-R06) were performed. Genetic distances and dendrogram analysis were conducted.
Results: AFU1, AFU12, and AFU31 showed the highest antifungal resistance. RAPD produced 111 bands, including 14 common and 9 unique bands, ranging from 100–2000 bp. Polymorphism ranged from 33–100%. Genetic distances ranged from 0.228 to 0.669. Dendrogram clustering showed close phylogenetic relationships among isolates with common geographic origins.
Conclusion: Iraqi A. fumigatus isolates exhibit significant antifungal resistance and genetic diversity. RAPD analysis revealed geographic clustering, suggesting regional transmission and a link between origin and genetic relatedness.

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1. INTRODUCTION

Aspergillus spp. of the most common types offungal infections and the most frequent of mixed infections and other species are opportunistic pathogen spread abundantly in the soil and air [1], and able to grow in any living environment, and occur: due to inhalation of spores Airbone as it can access to the lung alveoli weaker Airway due to its small size, which leads to the occurrence of pulmonary Aspergillosis in the presence of factors that increase the virulence of the host infection, which enables mold to pass the main immune systemof the host They also contribute to the destruction ofthe tissue in which they exist and there are factors such as age: the injured, sex, immune regression, obesity, infection malignant diseases, injury: diabetes and the use of immunosuppressive drugs such as steroid compounds (steroid) and play some diseases of the system: chronic respiratory As factors that facilitate mold, such as tuberculosis, pneumonia (Lung abcess), asthma and bronchitis - chronic or acute. Fungi, including mold Aspergillus fumigatus take various genetic and physiological ways to avoid the host immune system where it secretes external enzymes, which is one of the most important factors of virulence in this mold [2]. In general, the strength of an injury depends on how strong it is Due to the frequent increase in allergic reactions and recent asthma and the increase in cases of Aspergillus rot, our current study aimed to isolate and diagnose the morphological, migratory and molecular diagnosis of Aspergillus fumigatus [3].

Molecular techniques based on the study of the sequence of DNA bases in the detection of the presence of mold in the external body or reproductive conidates It also reveals the presence of species that cannot be developed on the agricultural media that do not Singled out by following means of examination or traditional isolation and even residues dead molds Progress in the treatment of fungal diseases is longer in time than in bacterial infections [4]. This is because life- threatening and widespread fungal infections are a recent phenomenon that has recently started. Pathogens are few because most drugs that affect molds affect humans. Moreover, fungal drugs are toxic. In the present study, three antifungal agents (Nystatin, Fluconazole, Clotrimazol) were prepared from the General Company for Pharmaceutical and Medical Supplies/Samarra-Iraq. Due to the variety and spread of fungal infections it has been lost Drugs and antifungal drugs have been used in the treatment of mold infection, but significant progress has been observed in the resistance of these molds to antifungal agents and the lack of success of some antibiotics in the treatment of most cases. Therefore, my study tended to use the above antibodies in multiple concentrations to determine the sensitivity and resistance [5]. Antifungal resistance is a major threat to the treatment and prophylaxis of fungal infections in both immunocompetent and immunocompromised hosts. Resistance to azole can occur in patients who are using azole for long-term treatment for the management of invasive aspergillosis or may acquirefrom the environment as a consequence of exposure to azole fungicides applied in agriculture [6].

For both treatment and prophylaxis, triazole antifungals such as itraconazole, voriconazole, posaconazole, and isavuconazole are often used for frontline therapy. Triazole antifungals target the enzyme lanosterol 14α-demethylase encoded by the gene cyp51A. This enzyme is required for the biosynthesis of ergosterol, an essential sterol in the cytoplasmic membrane of fungal cells. Resistance to triazoles is commonly conferred by mutations withinthe cyp51A gene, inhibiting triazole binding and/or causing overexpression of the enzyme [7].

2. RELATED WORK

Over the past 30 years, there have been rising incidences of triazole resistant A. fumigatus infections worldwide, including the identifications of triazole-resistant strains in triazolenaïve patients [8]. These results suggest the importance of environmental populations of A. fumigatus to patients and to the

clinical populations of this species. Consequently, it is extremely important to understand the environmental populations of A. fumigatus. Indeed, an increasing number of environmental populations from different geographic regions have been surveyed to aid in monitoring drug resistance rates and identifying/tracking resistant A.fumigatus genotypes. The results so far suggest that agricultural use of triazole fungicides can contribute to the development of triazole resistant strains, which subsequently infect patients [9]. The CYP51A gene, also known as the lanosterol 14-alpha-demethylase gene, is a well-studied gene involved in the synthesis of ergosterol, an essential component of fungal cell membranes. It'sa target for many antifungal drugs, including clotrimazole. These drugs inhibit the activity of the CYP51A enzyme, disrupting ergosterol synthesis andultimately leading to fungal cell death [10].

Azole Resistance in Aspergillus fumigatus: Several studies have explored the growing issue of resistance in Aspergillus fumigatus to azole antifungals, which are a primary treatment for aspergillosis. Resistance is often associated with mutations in the CYP51A gene, which encodes an enzyme crucial for ergosterol synthesis in the fungal cell membrane. A key study by found that mutations such as TR34/L98H and TR46/Y121F/T289A significantly reduce the efficacy of azole treatment. Molecular methods like PCR (Polymerase Chain Reaction) and DNA sequencing are commonly employed to detect these specific mutations, offering a rapid diagnostic approach compared to traditional culture-based sensitivity testing [11]. PCR and DNA Sequencing for Rapid Detection: A study by demonstrated the utility of molecular techniques such as real-time PCR in detecting azole-resistant strains of Aspergillus fumigatus. The study emphasized that PCR can be used to directly target the most common mutations in the CYP51A gene, thus enabling early and accurate detection of resistance, which is crucial for appropriate antifungal therapy. This molecular approach significantly reduces the time required to identify resistant strains compared to conventional culture and susceptibility testing methods [12]. Environmental Contribution to Azole Resistance: Research by highlighted that the widespread use of azole fungicides in agriculture could contribute to the development of resistant Aspergillus fumigatus strains. The study utilized molecular methods, including PCR and genotyping, to show that environmental exposure to azole-like compounds promotes the selection of resistant mutations, particularly in areas with high agricultural activity. This study emphasized the need for environmental surveillance and the use of molecular tools to monitor the spread of resistance from environmental to clinical settings [13].

Whole Genome Sequencing and Multidrug Resistance: In addition to azole resistance, there has been increasing concern over multidrug-resistant Aspergillus fumigatus strains. A study by utilized whole-genome sequencing (WGS) to investigate multiple resistance mechanisms in A. fumigatus isolates resistant to both azoles and echinocandins. The study identified mutations in multiple genes, including FKS1 (associated with echinocandin resistance) and CYP51A. Whole-genome sequencing provided comprehensive insights into the genetic landscape of antifungal resistance, offering a robust tool for tracking the evolution and spread of resistant strains [14]. Real-Time PCR for Detecting Environmental Resistance: A study by explored the use of real-time PCR for detecting azole-resistant A. fumigatus in environmental samples. This study demonstrated the feasibility of applying molecular diagnostics not only in clinical settings but also for environmental monitoring. The real-time PCR assay targeted specific CYP51A mutations and allowed for rapid detection of resistant strains in agricultural and urban environments, where the prevalence of azole resistance has been increasing due to fungicide use [15]. Increased Resistance in Clinical Isolates: A study conducted by focused on the increasing frequency of azole resistance in clinical isolates of Aspergillus fumigatus across Europe. The researchers used DNA sequencing of the CYP51A gene to identify the presence of resistance-associated mutations in azole-resistant clinical isolates. The study highlighted the growing public health threat posed by azole-resistant aspergillosis and underscored the importance of molecular diagnostic techniques in managing patient care [16].

3. METHODOLOGY

3.1 Materials and Methods

Sputum samples were collected from 45 patients aged (33 to 82 years) and from both sexes suspected of developing aspergillosis (as clinicallydetermined by the doctor) from 1 August 2019 to 1

December 2019 at the Specialized Center for Respiratory Diseases, Ministry of Health, Baghdad Governorate This specialist center generally accepts patients from different Iraqi provinces. Samples were examined directly under a microscope using 10%KOH and cultured on agar.

Isolates Identification by VITEK® System Identification Levels: The level of diagnosis of the object is determined by the map of its tests andcompared with the taxonomic characteristics of the device, the object is given a probability ratio and the level of confidence; for example if the probabilityratio is 96-99% is at the level of confidence is excellent.

Determination of minimal inhibition concentration (MICs): The minimal inhibition concentrations (MICs) of the test agents were established using the agar dilution method, described by and modified by. Study of the effect of antifungal agents on the

Aspergillus fumigatus: The sensitivity of the isolates that gave apositive result was examined for the diagnosis of VITEK® System conducted using low concentrationsof all antibiotics used in the present study and found that the total (41) isolation showed a variation in antifungal resistance Where (20) isolation showed significant resistance to antibiotics while some did not show any resistance, but it showed a clear sensitivity to the initial concentrations used for that was not included in the study, but the study was limited to (20) other isolation that showed different variation in resistance to antibiotics Used. The base material for the antifungal was obtained in the form of powder Pure (Powder Pure) from the General Company for the manufacture of medicines and medical supplies SDI / Samarra-Iraq, where theminimum inhibitory concentration and the minimumlethal concentration of the four antibodies mentionedusing half (dilutions) concentrations were prepared and a basic solution) Storage solution (concentration of (100 µg/ml) for each of the antibiotics used and from which the rest of the concentrations were prepared (75.50.25.15.10.5) µg/ml.

4. RESULTS AND DISCUSSION

4.1 Aspergillus Fumigatus Resistance to Fluconazole

The following Table 1 shows the significant effect of concentration of 75, 15 and 10 compared to other concentrations and control on the first day of the lap. On the third day of the lap, there was a significant effect of damsel control compared to the control and other concentrations. It was also found that on the seventh day there was no significant effectof different concentrations compared with the control and this is a clear indication of the presence of fungus resistance to the antibiotic on the seventh day The results of (MIC) of Fluconazole ranged from (200-600µg/ml) for Fluconazole resistance The original values (64-128µg/ml) and this result are no longer identical to the findings of studies.

Table 1. Shows the Effect of the Mean ± Risk for Different Concentrations of Fluconazole and Clotrimazole on Aspergillus Fumigatus

Fluconazole	
Incubation Seventh Day	**The Influence Concentration**
78.61± 1.66 A	Control
77.59 ± 2.01 AB	DMSO/Control
77.59 ± 2.01 AB	2.5 concentration
73.95 ± 1.81 AB	5 concentration
74.85 ± 1.71 AB	10 concentration
74.57 ± 1.77 AB	15 concentration
70.83 ± 3.46 B	25concentration
74.37 ± 1.74 AB	50 concentration
75.45 ± 2.05 AB	75concentration
Clotrimazole	
Incubation Seventh Day	**The Influence Concentration**
71.79 ± 1.71 A	Control

71.54 ± 1.57 AB	DMSO/Control
64.09 ± 2.52 C	2.5 concentration
58.80 ± 2.90 C	5 concentration
65.35 ± 2.22 ABC	10 concentration
64.94 ± 2.14 BC	15 concentration
59.41 ± 2.11 C	25concentration
63.32 ± 2.60 C	50 concentration
59.41 ± 2.10 C	75concentration

The different characters within one column show a significant difference at the probability level (P<0.05). Study of the effect of clotrimazole on isolates of the genus Aspergillus fumigates

Table 2 shows the rate of inhibition diametersfor all isolates used under the present study and according to each concentration. The lowest killer concentration (MFC) for all isolates varies depending on the type and source of the disease. The results showed that the values of MIC and MFC for clotrimazole ranged from (2.5-100) which is shown in Table 2, where there was resistance the quality of some hardships without others. These results were also close to my study who concluded that the minimum inhibitory concentration of clotrimazole ranged from 8-16 µg/ml a suitable environment for fungus activity and increased virulence and consequently gaining resistance against fungi.

Table 2. The Number and Location of Isolated Sample Technical results PCR-RAPD

Samples A. Fumigatus	Place	Number	Injury Rate %
AFU1	Samarra center	5	20.83
AFU2	Al-Qalaa	1	4.16
AFU3	Tigris district (Abbasia)	5	20.83
AFU4	Al-Mu'tasim district	4	16.66
AFU5	Al-Tharthar district (Al Jazeera)	5	20.83
AFU6	Banat Al-Hasan	4	16.66
Total		24	100

4.2 Results of Interactions

RAPD indices were used in this study to analyze the genetic variation between resistant and non-resistant antigen and detect the genetic relationship to determine the genetic dimension between the studied samples, and then use the results to find the fingerprint of these samples. DNA variations between the studied samples were recorded in four formats.

- The presence or absence of multiple DNA bundles.
- Differences in molecular weights between beams.
- Differences in the number of packets.

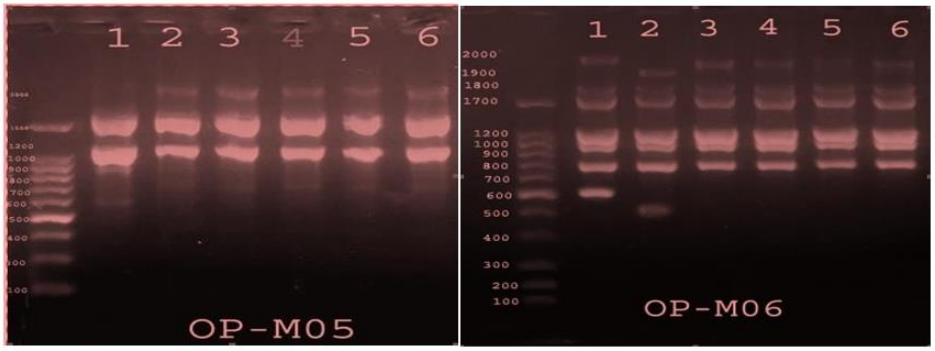

Figure 1. PCR Product of Primer OP-M05 the Product was Electrophoresis on 2% Agarose at 5 Volt/Cm2. 1x TBE Buffer for 1:30 Hours. N: DNA Ladder (100)

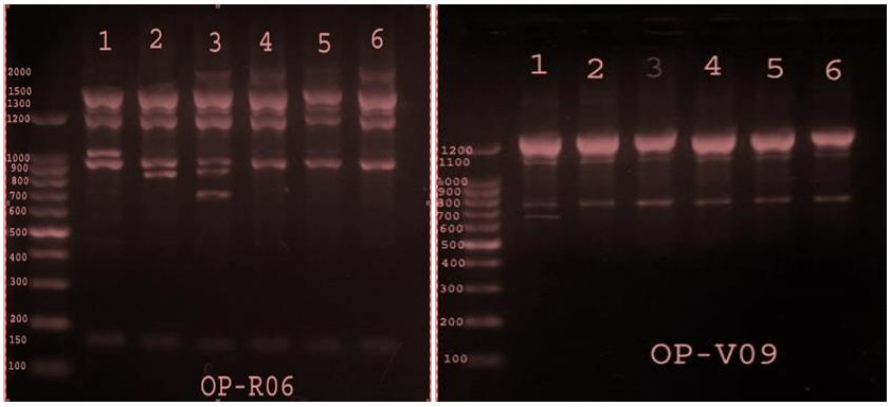

Figure 2. Electrophoresis of the Dna Packs by Rapd-Pcr Primers for Different A. Fumigatus Isolates

The product was electrophoresis on 2% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100). The first sample: when using the OPM-05 primer showed two unique bands at the molecular weights (600, 900) pb. When using the OPM-06 primer, it showed a unique band at the molecular weight (600) pb. When using the OPR-06 primer, it showed two unique bands at the molecular weights (600, 1050) pb. When using the OPV-09 initiator, it showed a unique band at the molecular weight (700) pb. Where the highest sample number of unique bunds was 6 bunds.

Second sample: When using the OPM-06 primer, it showed two unique bands at the molecular weights (500, 1900) pb. Third sample: When using the OPR-06 primer, it showed a unique band at the molecular weight (700) pb. As for the fourth, fifth and sixth samples, there were no unique bands or variation among them.

Table 3. The Random Primers Used to Amplify the Dna for Different A. Fumigates Isolates and the Number of Beams Produced by Each Primer

Primer Name	Sequences(5′-3′)	Size Range (bp)	Nu. of Bands Amplified in Different A. Fumigatus		
			Total	Unique	Polymorphic
OPM-05	GGGAACGTGT	2000-600	24	2	22
OPM-06	CTGGGCAACT	2000-500	39	3	36
OPR-06	GTCTACGGCA	2000-150	42	3	39
OPV-09	TGTACCCGTC	1200-700	19	1	18
Total			124	9	115

Table 3 shows that the OPR-06 primer gavethe highest number of packets, while the OPV-09 primer gave the least number of packets. The primers OPM-06 and OPR-06 gave the highest number of unique packets, while the primer OPV-09 gave the least number of unique packets.

Table 4. The Genetic Dimensional Values Depending on Indicators of RAPD

	1	2	3	4	5	6
1	0.000					
2	0.001	0.000				
3	0.210	0.032	0.000			
4	0.320	0.043	0.006	0.000		
5	0.888	0.066	0.008	0.013	0.000	
6	0.900	0.072	0.009	0.025	0.001	0.000

The Table 4 represents the genetic distancebetween the six samples of A. fumigatus that were isolated from six different sites.

It appeared that the highest genetic dimension was 0.9 between the first and sixth samples, and that the lowest genetic distance was 0.001 between the first and second samples, as well as a similar distance between the fifth a D sixth samples. This is what we see in Figure 3.

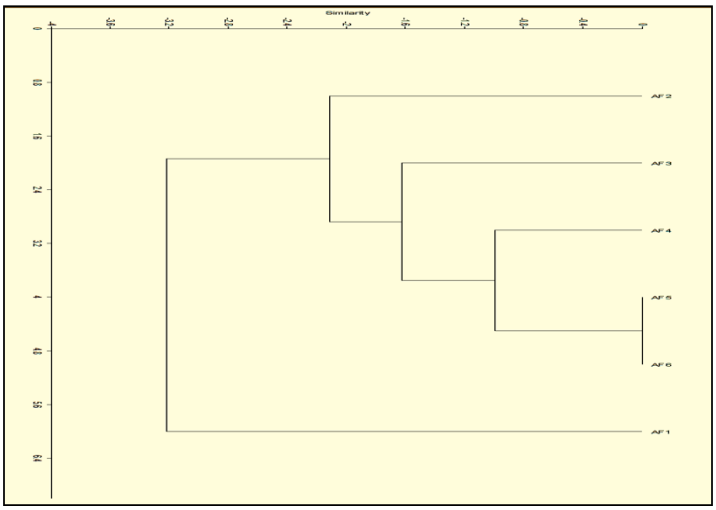

Figure 3. Dendrogram Illustrated the Genetic Fingerprint and the Relationship between A. Fumigatus Isolates Developed from Rapd Data

Figure 3 shows the genetic dimension and the extent of the relationship between the six samples of mushrooms.

4.3 Dendrogram Divided into Two Groups

The first group: represents the first sample, which showed the highest number of unique bundles and the most genetic distance from the rest of the samples. The second group: which represents the rest of the samples, was divided into four subgroups The first sub-group: represents the second sample, which is the closest to the first sample. The second subgroup: represents the third sample. The third subgroup: represents the fourth sample. The fourth subgroup: represents the fifth and sixth samples that are genetically close.

4.4 Discussion

The different characters within one column show a significant difference at the probability level ($P<0.05$). Current results show that Aspergillus fumigatus was resistant to clotrimazole (100%) as it is characterized by low sensitivity to the azole group [17]. Some sensitivities have been shown to be sensitive to the antagonist itself. Most of the isolates that were resistant to the antibiotic used were isolated from the lower respiratory tract. This resistance is attributed to the random and irregular use of antifungals by patients without consulting specialists, which weakens the body's immune defenses [18]. Health fungi increase the chance of fungi in the event of infection, especially as these fungi are characterized by being opportunistic fungi and this was the first and important factor in infectingthat area of the body and show actual resistance. Theindiscriminate use of antibiotics by many people, without taking into account the harm caused by antibiotics and their reduced medical effect at times when the patient actually needs them, has made theseantibiotics lose their effect if used indiscriminately and irregularly; Antibiotics become useless, in addition to other effects caused by antibiotics in the body, which increases the growth and spread of fungiin the body Studies have confirmed that the use of antibiotics increases the risk of fungi from 30 - 10% due to the effect of antibiotics in suppressing thenatural bacterial communities and allowing the opportunistic colonization of the fungus [19]. Study of the effect of nystatin on isolates of the genus

4.5 Aspergillus Fumigatus

The study showed that the lowest inhibitor concentration (MIC) of nystatin varied from one

sample to another according to the sample in additionto the lack of resistance to the samples that lead to increase the ferocity of these species and resistance to the inhibitory effect of the antagonist as the inhibitory activity in determining the inhibitoryconcentration and the lowest killer of nystatin This result is comparable to that of many researchers for fungi, and the value of (MFC) ranging between (25-50) µg/ml The inhibitory activity was (8–64 µg/ml), and an approach to what many have reached The difficulty in determining its MIC values is due to the difference in therapeutic efficacy and the difference between the manufacturers [20]. Wild breeds Efficacy and duration of antimycotic need to kill the fungus depends on its concentration either in laboratory conditions and found that the value of the minimum inhibitory concentration of antimycotics MIC varies depending on the medium and brood temperature and lap duration, as the effectiveness increases with the duration of the incubation either due to the killing All samples of aspergillus are due to the high toxicity of the antagonist and not to be used frequently for aspergillosis [21].

5. CONCLUSION

Most of the isolated A. fumigatus isolates from the patient developed come from the same region (conservative) in close relationship (sub-mass) indicating a relationship between RAPD patterns and origin of isolates.

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Author Contributions Statement

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Humam Saadi Hussein		✓		✓		✓		✓	✓			✓		✓
Mohammed Sami Farhan	✓	✓		✓	✓		✓			✓		✓	✓	
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C: **C**onceptualization
M: **M**ethodology
So: **So**ftware
Va: **Va**lidation
Fo: **Fo**rmal analysis
I: **I**nvestigation
R: **R**esources
D: **D**ata Curation
O: Writing- **O**riginal Draft
E: Writing- Review& **E**diting
Vi: **Vi**sualization
Su: **Su**pervision
P: **P**roject administration
Fu: **Fu**nding acquisition

Conflict of Interest Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Informed Consent

All participants were informed about the purpose of the study, and their voluntary consent was obtained prior to data collection.

Ethical Approval

The study was conducted in compliance with the ethical principles outlined in the Declaration of Helsinki and approved by the relevant institutional authorities.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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