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Molecular Method for the Detection of Resistance Aspergillus Fumigatus to Some Antifungal Agents

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Abstract:** Twenty samples were obtained from Aspergillus fumigatus, out of a total of 45 phlegm samples 41 samples were positive for A. fumigatus patients aged (33 to 82 years of both sexes), from August 2022 to December 2022 from the Specialist Center Samples were collected from several Iraqi governorates from the north, center and south. The results indicated that the isolation of AFU1, AFU12 and AFU31 was the highest resistance to the antihistamines. Anti-fungal resistance, most of which have allergic reactions to these antibiotics in a clear reference to the ability of these molds to adapt to the ocean up this adaptation to the occurrence of genetic mutations Genetic diversity and relationships were studied using the RAPD technique. It produced four primers (111) major pandas, including 9 unique pandas, common pandas (14) and polymorphic forests. Inflated ranges ranged from 100 to 2,000 basis points. The value of the genetic polymorphism of each primer ranged between 33-100%. In terms of unique band patterns, the most characteristic band pattern for the number isolated with the primer OP-M06 and OP-R06 was given 37 pandas of which 3 were unique in each. Genetic distances ranged from 0.22805 to 0.66905 between A. fumigatus isolates. Cluster analyzes were conducted to construct a dendrogram tree showing the interconnection of isolates. 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.

***Keywords:** A. Fumigatus, RAPD, Antifungal Agent. Clotrimazole, Fluconazole.*

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1. INTRODUCTION

Aspergillus spp. Of the most common types of fungal 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 system of the host They also contribute to the destruction of the 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 acquire from 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 within the cyp51A gene, inhibiting triazole binding and/or causing overexpression of the enzyme [7].

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2. RELATED WORKS

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 triazole naïve 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's a target for many antifungal drugs, including clotrimazole. These drugs inhibit the activity of the CYP51A enzyme, disrupting ergosterol synthesis and ultimately 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 Verweij et al. (2009) 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 Mellado et al. (2007) 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 Snelders et al. (2012) 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 Zhang et al. (2017) utilized whole-genome sequencing (WGS) to investigate multiple resistance mechanisms in A. fumigatus isolates resistant to both azoles and echinocandins. The

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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 Bader et al. (2019) 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].

6. Increased Resistance in Clinical Isolates: A study conducted by Van der Linden et al. (2013) 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

Materials and Methods

Sputum samples were collected from 45 patients aged (33 to 82 years) and from both sexes suspected of developing aspergillosis (as clinically determined 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 and compared with the taxonomic characteristics of the device; the object is given a probability ratio and the level of confidence; for example if the probability ratio 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 a positive result was examined for the diagnosis of VITEK® System conducted using low concentrations of 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 the minimum inhibitory concentration and the minimum lethal concentration of the four antibodies mentioned

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using 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

Aspergillus Fumigatus Resistance to Fluconazole

The following table 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 ($P<0.05$).

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Study of the effect of clotrimazole on isolates of the genus *Aspergillus fumigates*
Table shows the rate of inhibition diameters for 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, 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 1: The number and location of isolated sampl

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**

Technical results PCR -RAPD

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.

Primer OP-M05
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).

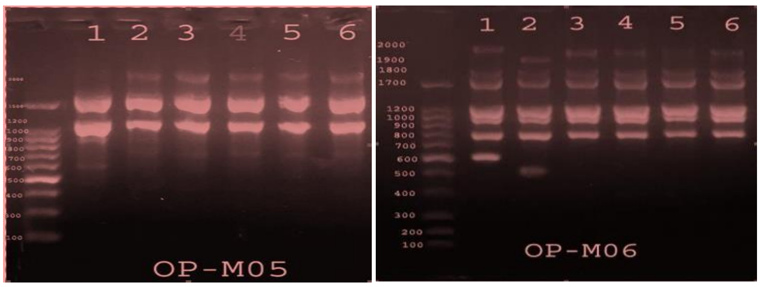

Figure 1: Electrophoresis of the DNA packs by RAPD-PCR primers for different A. fumigatus isolates

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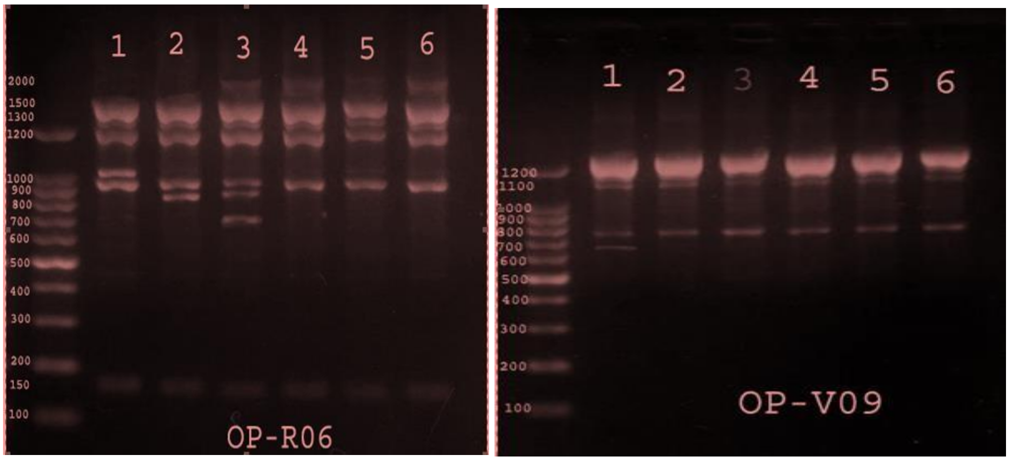

The product was electrophoresis on 2% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).
The first sample: when using the OPM-05 primer showed two unique bands at the molecular weights (600, 900) pb.
When using the OPM-06 primer, it showed a unique band at the molecular weight (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 2: The random primers used to amplify the DNA for different A. fumigatus 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 (2) shows that the OPR-06 primer gave the 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.

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Table 3: 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 (3) 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 an D sixth samples. This is what we see in Figure (2).

Figure 2: Dendrogram illustrated the genetic fingerprint and the relationship between A. fumigatus isolates developed from RAPD data

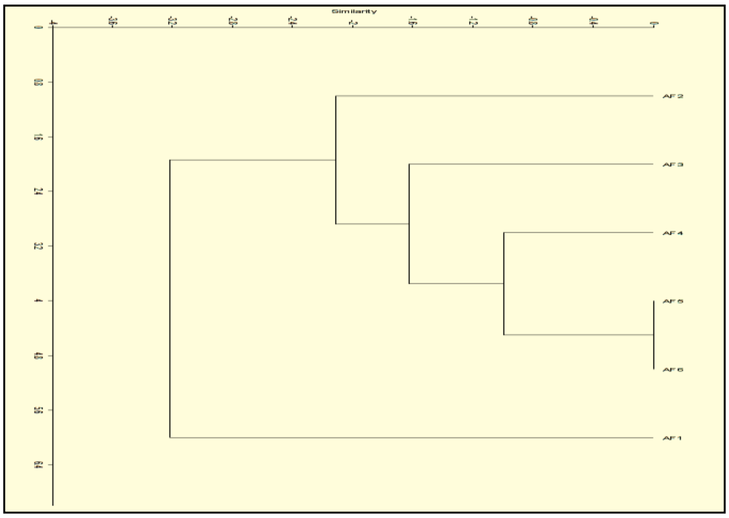

Figure (2) shows the genetic dimension and the extent of the relationship between the six samples of mushrooms.

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.

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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 infecting that area of the body and show actual resistance. The indiscriminate 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 these antibiotics 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 fungi in 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 the natural bacterial communities and allowing the opportunistic colonization of the fungus [19].

Study of the effect of nystatin on isolates of the genus

Aspergillus Fumigatus

The study showed that the lowest inhibitor concentration (MIC) of nystatin varied from one sample to another according to the sample in addition to 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 inhibitory concentration 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.

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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