

Isolation and Molecular Diagnosis of Aspergillus Species Associated with Corona Virus Disease and their Sensitivity to Some Antifungal in Patients of wasit Governorate

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Abstract

Background: Respiratory infections caused by fungal pathogens present a growing global health concern and are a major cause of death in immunocompromised patients. Worryingly, corona virus disease-19 (COVID-19) resulting in acute respiratory distress syndrome has been shown to predispose some patients to airborne fungal co-infections. Material and methods: One hundred sample include nasal; throat swab and sputum sample samples were collected from patients suffering from Corona virus disease underlying different disease. This study was recorded from October 2021 to the end of April 2022, including the criteria, patients of all age groups in isolation wards in Al-Zahra Teaching Hospital in wait Governorate. Samples were cultured in sabered agar and incubated for 10 days at 25°C under aerobic conditions. The identification of molds was done depended on the shape and color of the fungus on the plate and examined under the microscope and kept for further conformation and detection of the most common virulence factor by polymerase chain reaction (PCR), as well as to measure the sensitivity of these isolates against some antifungal drugs. Results: The total positive samples for molds isolation 17(17%) out of 100 samples were collected from patients suffering from Corona virus. The most isolated species was Aspergillus fumigatus with 9/40 (22.5%), followed by Afllatus was 5/40 (12.5%) and 3/40(7.5%) was Atreus. The results in this study show Gliotoxin gene was detected 8/9 (88.8%) in Fumigates, with PCR product of this gene was approximately 512 bp. While investigate aflatoxin (far) gene was found 5/5 (%100) in Afllatus, with PCR product of this gene was approximately 588bp. And lipase gene was detected by PCR in 2/3 (66.6) samples isolation of A. terries. The PCR amplification of lipase gene was approximately 591bp. The antifungal drugs Clotrimazole and Econazole had most effect against each fungal spp. in this study. While antifungal drug Miconazole resist by Aspergillus spp., while Nystatin and Ketoconazole were resisted by Aspergillus spy only. The conclusion of this study was showed Aspergillus fumigatus were the most dominant inpatientswithpatients with Coronavirus diseases and Clotrimazole, Ketoconazole were the most antifungal drugs that have a wide effect on fungal infections.

Keywords: Corona virus disease, Aspergillus spp., Waist Governorate, Polymerase chain reaction (PCR)

1. Introduction

The common mold, Aspergillus spp., that lives indoors and outdoors. Most people are exposed to Aspergillus spores and breathe them every day without getting sick; however, individuals with compromised immune systems or lung diseases have a higher risk of developing active infection. Until the emergence of COVID-19, a number of cases with COVID 19-associated pulmonary aspergillosis (CAPA) have been reported worldwide (Salman ton-Garcia et al., 2021). Invasive pulmonary aspergillosis (IPA) is associated with high mortality rates among COVID19 patients (Apostolopoulou et al., 2020). Invasive fungal infections caused by various fungal genera, including Aspergillus, complicate and endanger lives of millions of individuals annually (Brown et al., 2012). Aspergillus genera, most frequently Aspergillus fumigatus, are ubiquitous in the environment and cause a wide range of infections in

humans, including invasive pulmonary aspergillosis (IPA), chronic pulmonary aspergillosis allergic bronchopulmonary aspergillosis chronic rhinosinusitis, fungal asthma, and Aspergillus bronchitis (Li et al., 2019). Invasive pulmonary aspergillosis (IPA), the most severe manifestation of disease from Aspergillus, associated with high mortality rates and is a prominent complication among those with profound immune suppression such as those undergoing hematopoietic transplantation as well as those with structural lung damage who receive systemic corticosteroids for their underlying condition, such as patients with chronic obstructive pulmonary diseases (Cosmides and Denning, 2015). To date, Aspergillus Co-infection in patients with coronavirus1 infections is likely to have been under-diagnosed and under-reported, most likely due to lack of clinical awareness and diagnostic screening (Thompson et al., 2020). The published literature following severe acute respiratory syndrome (SARS) produced by SARS-CoV-1 has revealed only four cases of invasive

Aspergillosis (IA), all of which were diagnosed at post-mortem (Wang et al., 2004). None of the four patients had a previous history of underlying immunocompromise, but they had received corticosteroids, which formed part of the treatment of patients with SARS in 2003. One of these patients was an intensive care physician who received several courses of methyl prednisolone. The post-mortem findings in this patient were consistent with disseminated invasive aspergillosis with abscesses in multiple organs (Wang et al., 2003). With regard to MERS-CoV, another HCoV that also causes severe respiratory infections, secondary bacterial infection have been reported (Arabi et al., 2019), but a literature search failed to reveal published evidence of Aspergillus infection. This is most likely explained by the paucity of post-mortems performed on the patients, which were generally not done either for religious and cultural reasons, or to prevent environmental contamination with the subsequent infection of health-care workers (Memes et al., 2020). Early reports from China documented Aspergillus spp. being isolated from the respiratory samples of patients with COVID-19 pneumonia, however there was no information on its clinical significance, or on the outcome of treatment of these patients (Chen et al., 2020).

2. Material and Methods

Location of study

This study has been carried out in the laboratory of department of Biology– College of Science – University of Waist, Public Health Lab in Waist, Al-

Zahra and Al-Karama Teaching Hospitals through the period from October 2021 to the end of April 2022.

Samples Collection

One hundred samples include (nasal, throat) swab and sputum samples were collected from patients suffering from Corona virus disease. This study was recorded from October 2021 to the end of April 2022, including the criteria, patients of all age groups in isolation wards in Al-Zahra Teaching Hospital. The majority of patients admitted to the hospital were from Waist and its suburbs. Nose and throat swabs from each patient were collected in sterile clean swabs with transport media and sputum sample in a sterile container. These have a code number and a name on them. All samples were delivered to the laboratory for processing and investigation. The following information was included on a questionnaire sheet on patients obtained through direct interview with patients or companions: Code number, Name, Age, gender, other disease. Sample processing took place according to standard operating procedures which included:

Cultivation on Samburu's dextrose agar plates or universal tube to isolate fungal pathogens.

Lactophenol blue cotton staining method for molds PCR (Molecular Technology) as confirmed diagnostic tool for filamentous fungi.

Detection of some virulence genes for the most common isolated molds.

Evaluation of antifungal activity

Molecular Detection

Primers

Primers		Sequence (5'-3')	PCR product size
Aspergillus fumigatus gliotoxin gene	F	GCGACCTCCGATCTTG TAG	512bp
	R	GTTCCCGGGCAAATGAG	
Aspergillus flavus aflatoxin (aflR) gene	F	TTGATTCAACTCGCGACCA	588bp
	R	TCGTCATCAGGTTGCACGAA	
Aspergillus terreus lipase gene	F	TGAAGGAGTTTCTCGCCGAC	591bp
	R	CCGCATTCCCCTTTCTCGAT	

Fungal DNA Extraction

Using the Fungi /Yeast Genomic DNA extraction Mini kit, fungal genomic DNA was isolated from isolates as follows, as per the manufacturer's instructions

Estimation of Extracted Total DNA

The extracted whole DNA was checked, by used a Nanodrop [Thermo Scientific Nano Drop Lite UV Visible Spectrophotometer. USA] that measured DNA concentration (ng/μL) & checked and purity at absorbance (260 /280nm) as following steps: It has been selected the relevant program after opening the Nanodrop program (Nucleic acid, DNA). The measuring pedestals were cleaned many times with a dry wipe. Then, using a pipette, carefully 2μl of free nuclease water onto the bottom

measurement pedestals to blank the system. The Nanodrop sampling arm was lowered and 1μl DNA sample measured.

PCR Green Master Mix Preparation

For all genes PCR master mix reactions was prepared by used (Maxime PCR Pre Mix kit) this master mix was completed in accordance with the company's specifications as shows in the (table: 2)

Components	Volume/ml
PCR Green master mix	12.5μl
Reveres primer (10pmol)	2μl
Forward primer (10pmol)	2μl
PCR water	3.5μl
DNA template	5μl
Total volume	25μL

All of the 1PCR tubes were then 1transferred to an 1Exispin vortex centrifuge at 3000 rpm for 3 minutes1 before being 1placed1 in a T100 PCR thermocycler. After that, the PCR master mix 1components 1listed in1 (table: 3) were 1placed in standard 1Maxime PCR Premix kit 1tubes 1containing1 all other components required1 for the PCR reaction, 1such as Taq 1DNA polymerase, dNTPs, 1Tris-HCl pH: 9.0, 1KCl, Mg 1 (Bio-Rad-USA).

PCR1Thermo-Cycler1Conditions

PCR 1thermo-cycler 1conditions 1protocol for 1each gene was computed by usingOptimase1Protocol Writer™ online application 1and done by using convention PCR thermos cycler

PCRcycle	repeat	Temp.	Time
Initial denaturation	1	95°C	5min
Denaturation	34	95°C	30sec
1Annealing		58°C	30sec
Extension1		72°C	1min
1Final extension	1	72°C	5min
Hold	-	4°C	5min

PCR1Product1Analysis

Agarose1 gel electrophoresis was used to examine the PCR results1 as 1followingsteps:

A1.5%1Agarosegel,1was prepared1by using1XTBE (Tris-Borate with EDTA) and1dissolving in microwave1for 5minutes, and 1allowed to 1cool at 50°C.

After that, 3-5µl of ethidium bromide dye was added to the agarose1 gel solution.

After placing the comb in the right position, the agarose gel solution was poured into the tray and allowed to solidify for 15 minutes at 1room temperature1 before carefully removing the comb from the tray.

The 1gel tray was placed in the electrophoresis1 chamber1 and 0.5X TBE (Tris-Borate with EDTA) buffer was1 added.

10µl PCR product were loaded in to each well1 with added 5µl (DNA marker Ladder) in1 first well. Then 1electric current was performed1 at 70/cm1 for 11.5 hour. Transilluminator was used to see the PCR results.

Statistical 1Analysis

Using SPSS V 25 for Windows, data were input and analyzed. Inferential statistics (Chi-Square Test) and descriptive statistics (frequencies, mean standard deviation and accompanying tables and graphs) were applied. A. statistically significant 1 P-value ≤ 0.

3. Results and Discussion

Isolation and Identification of Molds

Direct Examination

Directly examined microscopically for the presence of molds from (Nasal, sputum, throat swab samples) were collected from 100 patients were associated with Corona virus disease with original different diseases for detected hyphae, pseudo hyphae,

ballistoconidia and conidia.

Colonies Characteristics

The detection of molds was done depended on the shape & color of the fungus on the plate The results showed 17(17%) positive samples for molds out of 100 samples were collected from patients suffering from Corona virus. The most isolated species was *Aspergillus fumigatus* with 9 (52.9%), *afflatus* was 5 (29.4%) and 3 (17.6%) *Atreus*. (Figure:1).

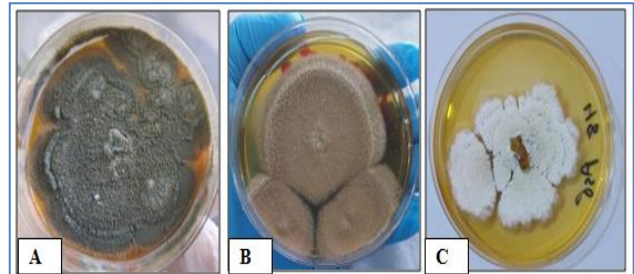


Figure (1) *Aspergillus* spp. Growth on SDA agar: A- *Aspergillus fumigatus*, B- *Aspergillus flavus*, C- *Aspergillus terreus*.

Microscopic Examination

The recognition of molds was examined under the microscope. The appearance of the fungus was determined by taking a little amount of the fungal growth, mixing it with one drop of lactose phenol cotton blue, covering it with cover slip, and examining it under a 40X microscope. This revealed septate hyphae and hyphae and pseudohyphae. (Figure:2).

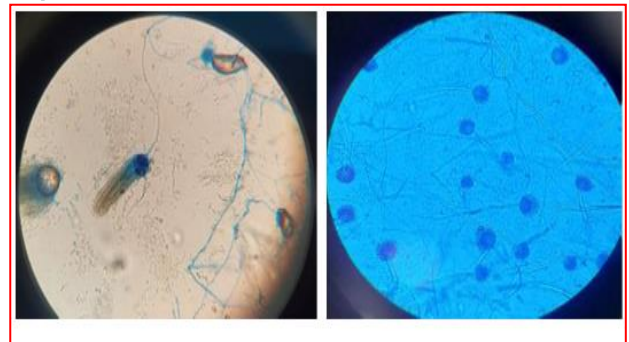


Figure (2): *Aspergillus* spp. under microscope show hyphae and pseudohyphae.

Our result showed increase of *Aspergillus fumigatus* isolation with 9/40 (22.5%), followed by *afflatus* was 5/40 (12.5%) and 3/40(7.5%) was *Atreus*. These results agree with (Adrastea et al.,2020) Where he found it by studying that the genus *Aspergillus*, especially *A. fumigatus*, were ubiquitous environmental pathogens and were responsible for a wide range of human fungal infections including invasive pulmonary aspergillosis, chronic pulmonary aspergillosis, allergic pulmonary aspergillosis, rhinitis, fungus, chronic asthma, and Bronchitis due to aspergillus. When someone has a damaged immune system, this illness can be lethal. The present results were also consistent with (Thompson et al., 2020) that respiratory viruses damage the airway epithelium in addition to disrupting normal ciliary filtration, causing leukopenia & lymphopenia & transient defects in cellular immunity that can

provide *Aspergillus* species a chance to penetrate tissues.

According to a recent study, *Aspergillus flavus* was isolated from the respiratory tract of One of the 99 COVID-19 patients in the first prescribed group & from 3.8% of the severely ill patients in the second group in Wuhan (Armstrong-James et al., 2020). The current study is compatible with other studies in terms of infection rate and the type of fungi that infect patients with COVID-19. In China (Chen et al., 2020) 99 patients with COVID-19 were given a fungal culture, and five (5 percent, 5/99) cases of fungal infection were discovered, including one case of *Aspergillus flavus*.

Detection of Virulence Factors to *Aspergillus* spp. isolation

Aspergillus fumigatus Virulence Factors

The results in this study show Gliotoxin gene was detected 8/9 (88.8%) in *Fumigates*, with PCR product of this gene was approximately 512 bp. as shown in the (Figure: 3). Gliotoxin gene was an immune virulence factor such as reported in the study of (Abad et al., 2010). This gene was discovered to be the most important viral agent that can escape from immune reactions and harm the host.

Aspergillus flavus Virulence Factors

In this study to investigate aflatoxin (afIR) gene was found 5/5 (%100) in *A. flavus*, with PCR product of this gene was approximately 588bp. as shown in the (Figure: 4). The results of this research proved the presence of aflatoxin gene in each of the *A. flavus* samples by polymerase chain reaction, and were compatible with (Daejeon and Stoyanova, 2020) which showed the potential to explore toxicological strains of *A. flavus* in industrial production. of alpha amylase.

Aspergillus terries Virulence Factors

In our study lipase gene was detected by PCR in 2/3 (66.6) samples isolation of *A. terreus*. The PCR amplification of lipase gene was approximately 591bp. as shown in the (Figure: 5).

The presence of the lipase enzyme was consistent with (Sethi et al., 2016) who noted that extracellular lipase was discovered to be produced in large amounts by *A. terries*. This enzyme exhibited high thermal stability, activity in a wide range of pH values, with the highest stability at slightly acidic pH and a marked trademark.

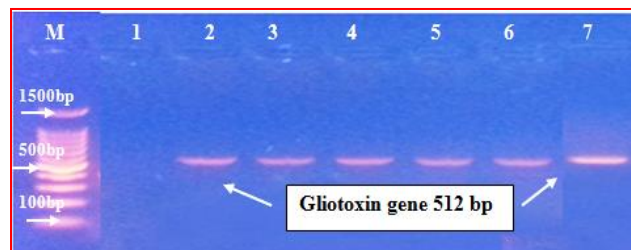


Figure (3): Gel electrophoresis (1.5% agarose, 7v/cm², 1.5hrs) of the PCR products, lane 1 (MW): One hundred base pairs DNA ladder; lane 1: Negative control; lanes (2-7): Positive results sample for *A. fumigatus* (lipase gene 512bp).

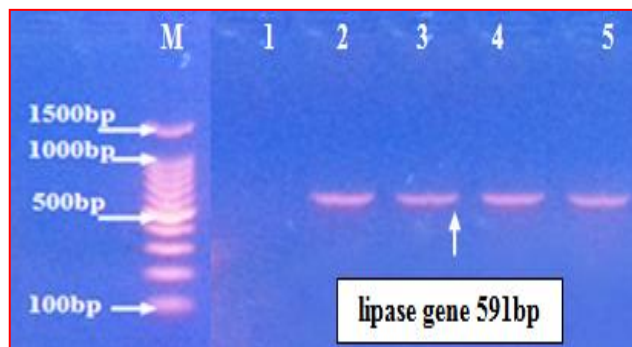


Figure (4): Gel electrophoresis (1.5% agarose, 7v/cm², 1.5hrs) of the PCR products, lane 1 (MW): One hundred base pairs DNA ladder; lane 1: Negative control; lanes (2-6): Positive results sample for *A. flavus* (aflatoxin gene 588bp).

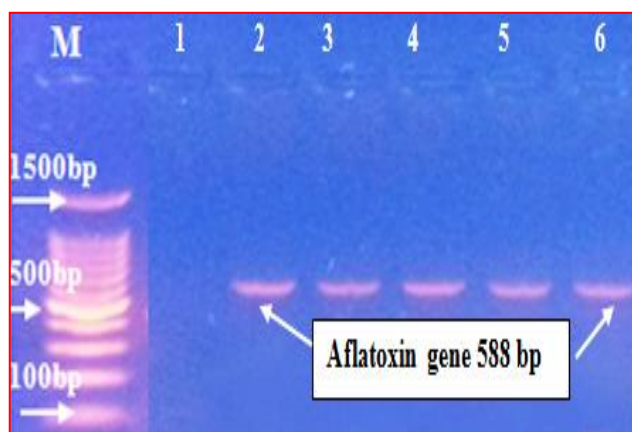


Figure (5): Gel electrophoresis (1.5% agarose, 7v/cm², 1.5hrs) of the PCR products, lane 1 (MW): One hundred base pairs DNA ladder; lane 1: Negative control; lanes (2-5): Positive results sample for *A. terreus* (lipase gene 591bp).

Antifungal susceptibility Determination Using Disk Diffusion

In the current study, in vitro the (17) fungal spp. isolates were subjected to susceptibility testing using (5) different antifungals (Nystatin, Miconazole, Clotrimazole, ketoconazole, and Econazole). The antifungal drugs Clotrimazole and Econazole had most effect against each fungal spp. in this study. Miconazole resist by *Aspergillus* spp., while Nystatin and Ketoconazole were resisted by *Aspergillus* spp only. Also, in our study show Miconazole resist by *Aspergillus* spp. According to statistical analysis's findings, significant differences ($P < 0.01$ and $P < 0.05$) regarding the studied isolates' antifungal drug susceptibility (Table: 4).

The results obtained (total mean values) revealed that, Clotrimazole and Econazole inhibited all yeasts and molds isolates, while Miconazole gave Lower inhibition zones in all yeasts and molds isolates. Nystatin and Ketoconazole resist by *Aspergillus* spp. The outcome of the statistical study revealed significant differences ($P < 0.05$) on susceptibility of the tested isolate to anti-fungal medications. The resistance of yeast to antifungal agents has been recently investigated also in the aspect of their ability of slime production and biofilm formation (Manzouri et al., 2001).

Table (4): In vitro antifungal susceptibility and resistance for fungal isolation

Fungal isolation	No isolates	Type of antifungal									
		Nystatin		Miconazole		Clotrimazole		Ketoconazole		Econazol	
		S	R	S	R	S	R	S	R	S	R
<i>A.fumigatus</i>	9	-	9	-	9	9	-	-	9	9	-
<i>A.flavus</i>	5	-	5	-	5	5	-	-	5	5	-
<i>A.terreus</i>	3	-	3	-	3	3	-	-	3	3	-
Total	17	-	17	-	17	17	-	-	17	17	-
X² = 6.97 P < 0.05											
R = Resistance, S = Sensitive											

4. Conclusions

Aspergillus fumigatus were the most dominant in patients with Coronavirus diseases. Clotrimazol and Ketoconazol are the most antifungal drugs that have a wide effect on fungal infections.

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