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Molecular Characterization, Phylogenetic Analyses and Antifungal Susceptibility Profiles, of Three Fungal Species Isolated from COVID-19 Recovered Individuals in Iraq

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ABSTRACT

Objective: *The coronavirus disease 2019 (COVID-19) pandemic has rapidly spread globally. Mycobiota is naturally present and may primarily contribute in COVID-19 coinfections rising a risk of disease severity in humans. After COVID-19, several studies showed a significant increase in antifungal resistance. The current study aimed to carefully isolate, effectively identify and phylogenetically analyzed some fungal species from recovered individuals with various COVID-19 infection and vaccination backgrounds for the first time in Iraq. The isolates of three interesting species recovered from study groups were involved in antifungal susceptibility testing.* ***Method****: Some fungal species were isolated from eighty recovered individuals with various COVID-19 infection and vaccination backgrounds. These species were identified morphologically and conformed molecularly. The evolution-nary relations between Iraqi isolates of three species and some global isolates were conducted using molecular phylogenetic analyses. Their antifungal susceptibility profiles were also detected using disk diffusion method.* ***Results****: The results showed that Aspergillus neoniger Cladosporium limoniforme, and Penicillium crustosum were commonly distributed between groups investigated. The first two species are new records in Iraq. The antifungal susceptibility results of the current species isolates have showed significantly differences in their sensitivity to seven antifungal agents with each other's regardless the study groups. Although the antifungal susceptibilities were vary based on the species, antifungals, and groups investigated, fluconazole showed the highest diameters of inhibition of fungal growth.* ***Novelty****: The study is first study in Iraq reported some mycobiota species found in recovered COIVD-19 individuals. The first report's antifungal susceptibility testing for the first records and most interesting species. This type of a study is necessary to primarily develop a comprehensive mycobiota profile and antifungal susceptibility pattern to assist in the early detection of disease and in preparing for future pandemics in Iraq.*

INTRODUCTION

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or coronavirus disease (COVID-19) pandemic emerging in late 2019 has placed an unprecedented strain on healthcare systems. During infection, COVID-19 attacks the respiratory systems resulting significant immune dysregulation in patients that creates a favor opportunity for the development of fungal disease [1]. Mycobiota is naturally present in human body, may contribute in Covid-19 coinfections and increases risk of disease severity in humans [2]. It is considered a major path of secondary infection [3]. Several fungal species have been described as COVID-19 associated fungi including *Aspergillus*, Mucorales, and *Candida* infections [4]. It has been reported 8% of individuals with coronavirus disease 2019 might have bacterial and fungal coinfections during hospital admission [5]. Among these secondary infections, fungal co-infections have

emerged as significant concerns, including COVID-19-associated pulmonary aspergillosis (CAPA) and COVID-19-associated mucormycosis (CAM) [6, 7]. For example, aspergillosis, caused by *Aspergillus* spp., such as *A. flavus*, and *A. fumigatus* is a common opportunistic fungal co-infection affecting COVID-19 patients [8, 9], leading to mortality rates exceeding 80% even with systemic antifungal therapy after several days of invasive growth [10].

Vaccination has been widely acknowledged as the most effective means of controlling COVID-19 pandemic which extremely affected civilization [11]. The race for developing an active and harmless vaccine started resulting to declare more than 200 vaccine candidates all over the world in early 2020. The unprecedented global vaccination effort against SARS-CoV-2 may present a unique chance to examine potential effects on human indigenous microbial communities [12]. With prescribing antibiotics for hospitalized COVID-19 patients, that may promote increase multiple drug-resistant microbial species [5].

Antifungal drug resistance is a growing concern in the treatment of fungal infections. Several investigational antifungals have been evaluated in preclinical and clinical trials, offering potential advantages over existing drugs [13]. Regarding the impact of COVID-19 on the antifungal profiles, several studies showed a significant increase in antifungal resistance. Based on results of the meta-analysis, 69% of COVID-19 patients with fungal co-infection have prevalence of drug-resistance strains [14]. Azole resistance has significantly accelerated in *A. fumigatus* isolates recovered from respiratory samples [15]. However, a study revealed isolates of *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus terreus* showed good susceptibility to Amphotericin B, Itraconazole and Voriconazole [16]. The prevalence of antimicrobial drug resistance is more likely to be geographic dependent. 81% prevalence of antifungal resistance was recorded in Asia [14].

To our knowledge, there is no such study investigate the impact of COVID-19 on distribution of some fungal species and antifungal susceptibility profiles in Iraq after pandemic. Thus, the current study aimed to investigate fungal species in the external auditory canals of recovered individuals with different COVID-19 and vaccination background as well as from individuals who have not infected and vaccinated. The most interesting species were prudently selected and morphologically and molecularly identified. Their evolutionary relations with other world isolates were phylogenetically detected. Antifungal susceptibility testing for isolates of these species was performed.

RESAERCH METHODS

Study design

A cross-sectional study involved 80 healthy adult volunteers with various COVID-19 infection and vaccination backgrounds. The involved volunteers had attended the hospital of Imam Hussain in Karbala, Iraq, during a period from November 2022 to January 2023. Based on questionnaire and the results of COVID-19 antibody titer tests, recovered participants were divided into four groups as described by Chyad and Al

Anbagi (2024). These included infected COVID-19 and vaccinated (IV), infected COVID-19, non-vaccinated (INV), vaccinated, non-infected COVID-19 (NIV) and non-infected COVID-19 and non-vaccinated (NINV) individuals. The main features of healthy individuals with various COVID-19 infection and vaccination histories and their immunoglobins were described in [17]. The ethical approval was granted in accordance with the research committee's letter 20220180 dated 30/10/2022.

Specimen processing and Fungal isolation

The sterile cotton swabs were gently inserted into the external part of the ear canal and rotated fully for 30 seconds to collect the specimen for each individual. Then, each swab was placed into sterile tube, stored in cold condition, and transfer to the laboratory for further process. These samples were cultured independently on the surface of Sabouraud dextrose agar (SDA) plates and incubated at 25±2°C for three to seven days [18]. Later, the fungal colonies were purified cautiously and morphologically identified. Based on their distribution across the four investigated sets, three clinical species including *Aspergillus neoniger, Cladosporium limoniforme* and *Penicillium crustosum* were selected.

Molecular species identification and phylogenetic analysis

The genetic material was extracted from previous purified colonies of isolates of previous three species using DNA extraction kits (Favorgen) following the manufacturer instructions. After being quantified of the extracted DNA and determined the DNA quality, the obtained DNA from fungal isolates was amplified using primer pair ITS1 and ITS4 and the cycling conditions were employed [19]. The PCR products were sent for sequencing (Macrogen, Korea) The raw sequences were assembled into consensus sequences and blast using a BLASTn analysis (https://blast.ncbi.nlm.nih.gov/). The newly obtained sequences of Iraqi isolates were deposited in GenBank nucleotide sequence database under accession numbers OR673619-OR673623 for *Aspergillus neoniger*, OR673635- OR673659 for *Cladosporium limoniforme*, R673667 and R673670 for *Penicillium crustosum*. The phylogenetic analyses of sequence data were performed using Genious (Swofford 2000). Statistical support was measured by Maximum Likelihood (ML) analysis using the RAxML (randomized accelerated maximum likelihood) software [20]. The maximum parsimony was run with 1000 bootstrap replicates to evaluate the robustness of the tree topology.

Inoculum preparation of selected isolates and inoculation of test plates

The fungal suspensions and following process were prepared according to [21] with some modifications. To achieve the desired turbidity, the spore suspension's optical density was adjusted spectrophotometrically to a 0.5 McFarland standard at a wavelength of 530-nm. A sterile cotton swab was immersed in the inoculum suspension of the selected isolates. Then, the swabs were uniformly spread the fungal inoculum on the surface of sterile Mueller-Hinton.

Antifungal susceptibility testing of fungal isolates

In the current study, the disk diffusion method was performed for selected filamentous fungi using commercially equipped disks containing set concentrations of

two classes of antifungals. These include polyene exemplified by amphotericin-B (Am-B) 100 unites and nystatin (NYS) 50 µg and azoles represented by clotrimazole (CLO) 10 µg, fluconazole (FLC) 10 µg, itraconazole (ITZ)10 µg, ketoconazole (KT) 10 µg, miconazole (MIC) 30 µg (HiMedia, India). The antifungal disks were placed on the surface agar plates that had been previously inoculated by fungal isolates. The plates were turned upside down and incubated for 48 h at 35 °C [21]. Following a period of 48 hours within the incubator, the plates underwent examination. The diameters of the zone inhibition around the antifungal disks were observed and measured to the closest millimeter. Each assay was achieved in triplicate.

Statistical analysis

The collected data were analyzed. Two-way analysis of variance (ANOVA) test was used to identify any significance differences in antibiotic activity among groups. The Tukey HSD (Honest Significant Difference) test was used for pairwise comparison between groups. Results were considered statistically significant when the p-value was less than 0.05.

RESULTS AND DISCUSSION

Phenotyping and molecular identification

The recovered species from the external canal ears included 28 species sampled from four clinical investigated groups; non-infected non-vaccinated (NINV), infected non-vaccinated (INV), non-infected vaccinated (NIV), infected vaccinated (IV) groups. However, in this study, *Aspergillus neoniger, Cladosporium limoniforme,* and *Penicillium crustosum* were focused on and their identification were performed morphologically and confirmed molecularly. *Aspergillus neoniger*, (5 sequences) is the first record in Iraq. The isolates of species were sampled from NINV, and NIV groups. The molecular identification showed more than 99% similarity with the sequence of the accession number OL711719.1, USA. *A. neoniger* was isolated earlier from desert sand in Namibia, mangrove water in Venezuela, and Eucalyptus leaf in Australia [22]. Also, *Cladosporium limoniforme* (25 sequences) is the first record in Iraq and first sequencing submitted in the GenBank database. The species was the most dominant and isolated from IV, INV, NIV and NINV groups. Its sequences were 98-100% similar to the Egyptian sequence, KT600397. The species has been isolated from plant substates and hypersaline water [23]. Globally, the isolates of *C. limoniforme* has been isolated from house dust, kitchen sink, indoor air, water, plants, vegetables and clinical samples [23]. The isolates of *Penicillium crustosum* (4 sequences) were sampled from IV and NIV groups with NR 077153, USA. The species has been isolated and identified based on morphological methods from indoor dust of Mosul city Buildings during October 2003 and February 2004 [24]. This species is airborne and saprobes and a biologically active metabolite of a wide range of secondary metabolites including several mycotoxins. These specific secondary metabolites adaptive the species to survive under diverse stress factors to endure the on-going defense attacks by the host organisms, like humans [25].

Phylogenetic analyses

The phylogenetic trees were analysis based on the molecular sequencing analysis for the most widespread isolated species of the present study (Figures 1-3). The phylogenetic analysis of the ITS region successfully confirmed the blast search and clearly discriminated the Iraqi isolates of the genus *Aspergillus* which assigned to the section *Aspergillus* s. str in agreement with previous studies. The first cluster contains isolates of Iraqi *A.neoniger*, the first recording species which are homologues with *A.neoniger* OL711719 with the bootstrap value 100%. This species is a sister group to *Aspergillus niger* which are both related to the *Aspergillus niger* aggregate clade belonging to *Aspergillus* section *Nigri* also called "the black aspergilli" [26]. This section covers the most unclear and multifaceted because of the delicate differences between the species [27]. Hereditarily, the were some differences between isolated strains of *A. neoniger* but with low bootstrap values > 70 % between Iraqi strains and a high bootstrap value 95% with *A. neoniger* OL711719 impaling high genetic diversity in the investigated population.

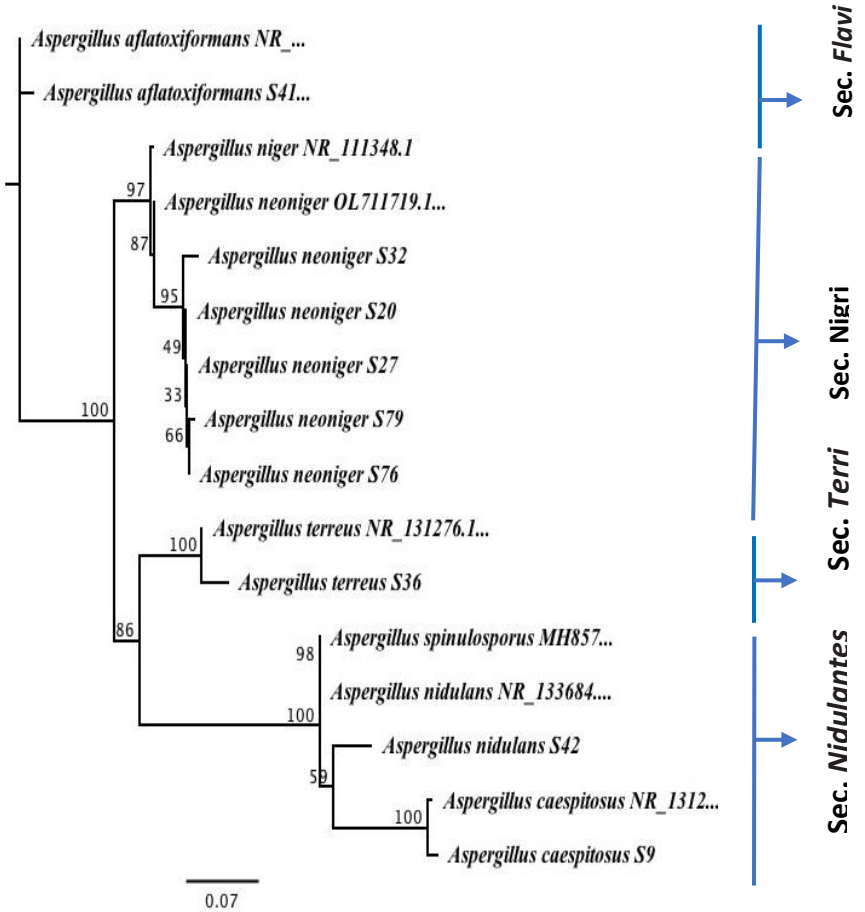

Figure 1. Maximum likelihood phylogenetic tree of *Aspergillus* section inferred from the ITS sequences of 32 strains. Bootstrap values are indicated at nodes. Branch lengths are proportional to distance. Colored blocks represent the species complex affinity of the Iraqi described isolate here. Names of Iraqi species are indicated in S with numbers. 0.07 subs per site.

The second phylogenetic tree was related to the genus *Cladosporium* (Cladosporiaceae, Capnodiales) and the different partitions were congruent as determined by the partition homogeneity test (P = 0.05, Figure 2). The isolates of the first recorded *C. limoniforme* species were related to *C. herbarum* complex. The Iraqi isolates of *C. limoniforme* were grouped together with other Iraqi isolates of *C. variables* and both were grouped phylogenetically relative to the Egyptian isolate (KT600397) and to USA isolates (MH863131) respectively. The predominant isolates of *C. limoniforme* were distributed differently between the four investigated sets in the phylogenetic tree. In this study, the phylogenetic tree confirmed the molecular identification and agreed with previous studies [23, 28, 29].

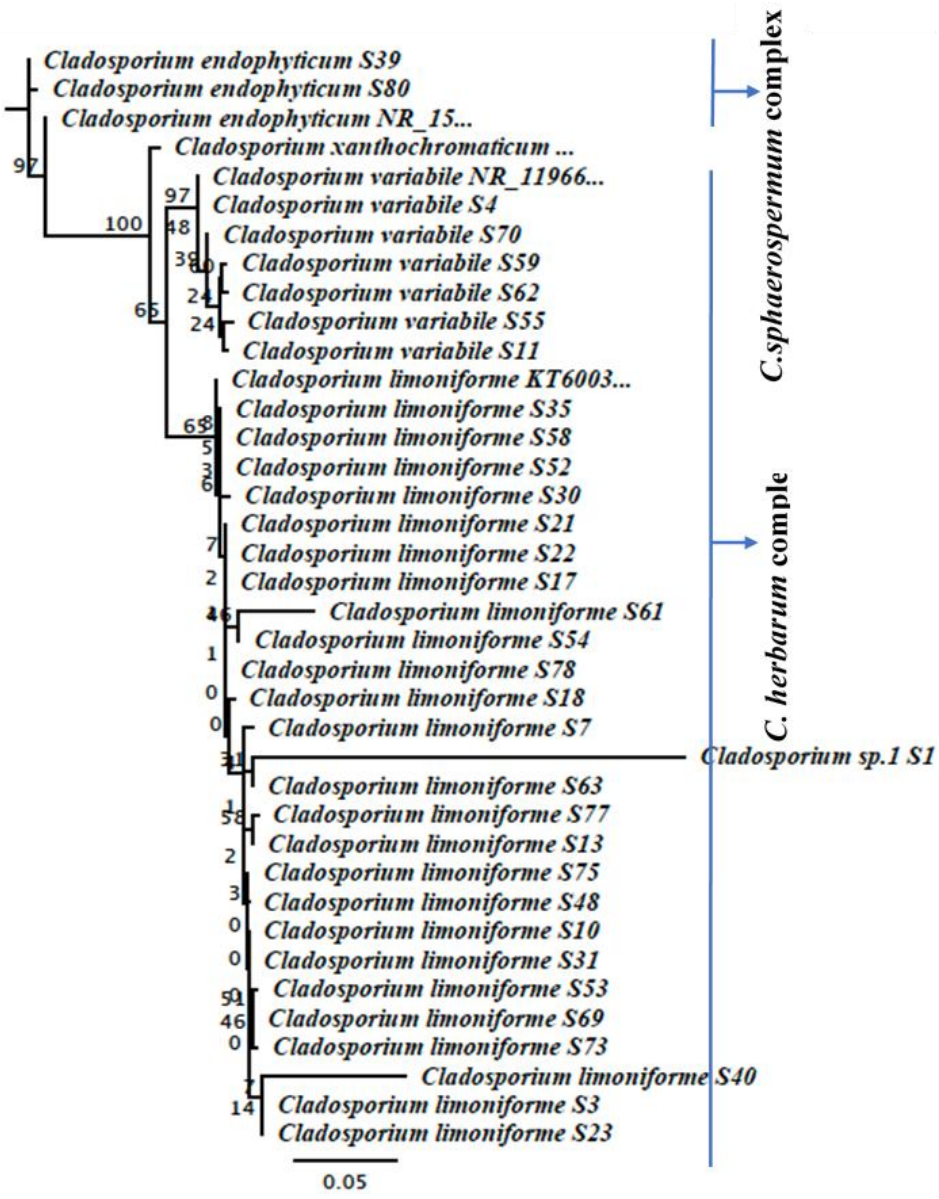

Figure 2. Maximum likelihood (ML) tree obtained from the ITS sequences of 37 strains from C*ladosporium* species. Numbers on the branches represent ML bootstrap support values. Branch lengths are proportional to distance. Colored blocks represent the species complex affinity of the Iraqi described isolate here. Names of Iraqi species are indicated in S with numbers**.**

In this study, the phylogenetic analysis of the genus *Penicillium* obtained by RAxML showed topological congruence and was polyphyletic (Figure 3). Unique site pattern values for the bootstrap values were 99, 97 and 85. The Iraqi isolates clustered into eight groups and phylogenetically relative to five sections. In the current study, the tree topology of the investigated species concurred with the previous phylogeny of *Penicillium* belonged to other genera of Trichocomaceae [30]. The present ITS tree, *P. crustosum* and *P. polonicum* (section Fasciculata) were isolated and subsequently grouped as sister groups to *P. chrysogenum*, and *P. nalgiovenese* (section Chrysogenum).

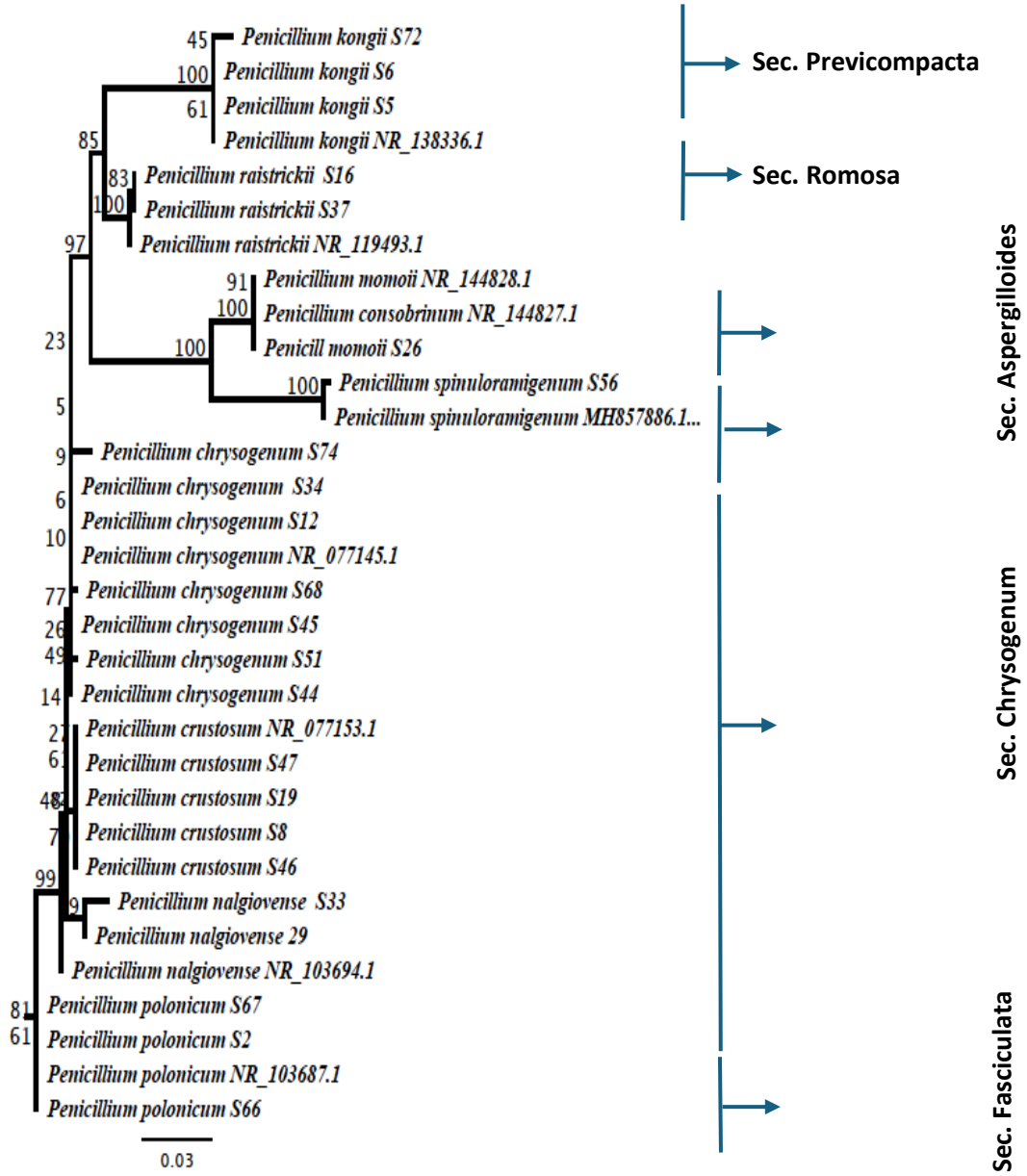

Figure 3. Maximum likelihood phylogenetic tree of *Penicillium* section inferred from the ITS sequences of 32 strains. Bootstrap values are indicated at nodes. Branch lengths are proportional to distance. Colored blocks represent the species complex affinity of the Iraqi described isolate here. Names of Iraqi species are indicated in S with numbers.

Antifungal susceptibility testing of isolated stains

The current results revealed variations in susceptibilities to seven antifungal agents among the strains being predominant recovered from the four groups; NINV, INV, NIV, and IV groups. The current results are the first *in vitro* data on previously unreported antifungal susceptibility patterns isolated from healthy individuals and individuals recovered from COVID-19 and its vaccine.

The current results of the antifungal susceptibility testing of *Aspergillus neoniger* strains showed that strains isolated from both NINV and NIV sets had different sensitivity to seven explored antifungals (Table 1, Figure 4). The significantly highest (P<0.0001) inhibition zones with values 55 and 53 mm were observed with fluconazole (FLU) followed by clotrimazole (CLO) with values 31 and 29 mm in both NIV and NINV groups respectively. However, amphotericin B (Amp-B) and miconazole (MIC) displayed the lowest inhibition zone ranging 22.5-24.3 mm. Comparing the strains from two groups, it can be inferred that the strain isolated from the NIV group showed the higher sensitivity to the tested antibiotics compared to that from the NINV group, P-value was 0.0012. That indicate significant differences between groups. Further statistical analysis comparing the differences among groups for each antifungal, the results showed that there were no significant differences between groups (P>0.05) except for ketoconazole (KT). It was significantly higher (P=0.0340) in NIV group compared to NINV group.

Limited studies about the antifungal susceptibility against *A. neoniger* strains recovered from clinical samples due to be considered cryptic species. In agreement with the current results, [31] detected a predominant resistance to Amp-B among *A. neoniger* isolates from chronic pulmonary Aspergillosis (CPA) patients. In contradiction of this study, that report observed no itraconazole (ITZ) resistant recorded. To our knowledge, the current results might be the first report of *A. neoniger* susceptibility from healthy individuals and the second report after the Indonesian isolates from CPA patients [31]. This species is related to *Aspergillus niger* complex so the complex results might be considered. The results of [32] revealed that 27.8% of isolates related to *A. niger* complex with otomycosis sources showed invariably resistance to FLU. Good susceptibility to FLU was also recorded to *Aspergillus niger* recovered from patients with otomycosis [33]. In contrast to the current results, [34] reported that *A. niger* isolated from patients with otomycosis was highest sensitivity to Amp-B followed by both ITZ and NYS, where the fungal species was resistant to FLU with the 100% resistance rate. Thus, recorded different sensitivity of isolates in the present study might be due to being isolated from healthy individuals. Many factors play essential roles in the emergence of azole and polyene resistance among filamentous fungi [35].

Table 1. Antifungal susceptibility pattern of *Aspergillus neoniger* against various antifungal agents measured in terms of the diameter of the inhibition zones (DIZ) in millimeters (mm).

Antifungal agents	DIZ for the tested agents (mm)		*P*-value*	Mean	*P*-value†
	COVID-19 & vaccination statuses				
	NIV	NINV			
Amphotericin-B Amp-B)	23.5	22.5	0.8427	23.0^{d}	< 0.0001
Clotrimazole (CLO)	31.0	29.0	0.1391	30.0^{b}	
Fluconazole (FLU)	55.0	53.0	0.1391	54.0^{a}	
Itraconazole (ITZ)	26.5	27.5	0.8427	27.0^{c}	
Ketoconazole (KT)	26.0	23.5	**0.0340**	24.8^{d}	
Miconazole (MIC)	24.3	23.0	0.5738	23. 7^{d}	
Nystatin (NYS)	29.5	29.5	>0.9999	29.5^{b}	
Control	0.0	0.0		0.0	
Mean	30.83^{a}	29.71^{b}			
***P*-value‡**	0.0012				

Abbreviations: non-infected non-vaccinated (NINV) and non-infected vaccinated (NIV). * Compering each antifungal between the study groups; † Comparing antifungals means with each other's regardless study groups; ‡ Comparing the study groups using all antifungals. There were not significantly different between the interaction of study groups and antifungals with the *P*-value =0.0623. The values not connected by any letter or that have same letter (a) are not significantly different. Amp-B (100 U), CLO (10 µg), FLU (10 µg), ITZ (10 µg) (KT), MIC (30 µg), NYS (50 µg).

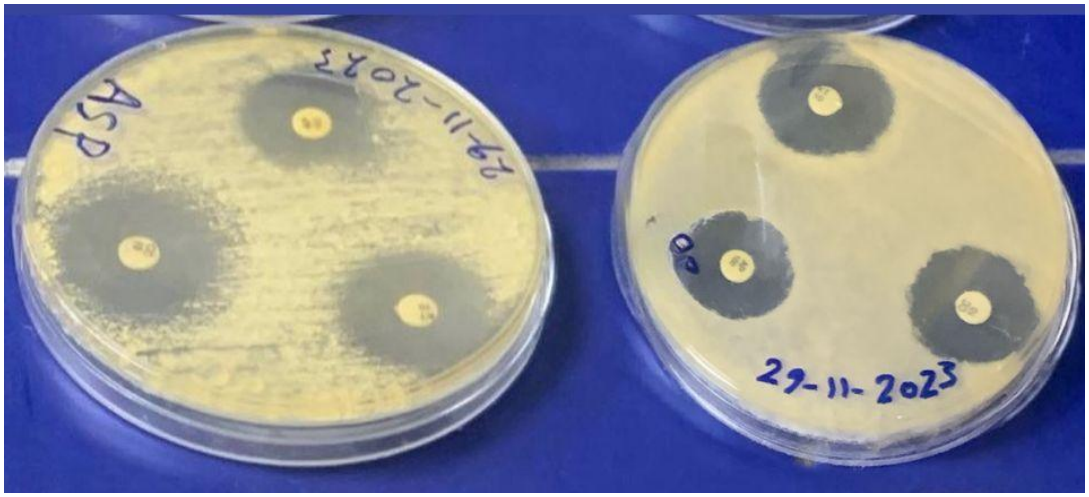

Figure Error! No text of specified style in document.. Antifungal susceptibility of *Aspergillus neoniger* against various antifungal agents.

The results of the antifungal susceptibility testing of *Cladosporium limoniforme* strains isolated from four sets (NINV, INV, NIV, IV) showed various sensitivity between isolates (Table 2, Figure 5). However, there were no significant variances

(*P*=0.0698) between the strains isolated from four sets and antifungals. Generally, the four isolated stains from four groups had not affected significantly (*P*= 0.0949) by the applied antifungals.

On the other hand, the results of antifungals on strains from four investigated groups displayed significant variance(P<0.0001) to inhibit fungal growth regardless of strains from group. Both NYS. and KT. exhibited no inhibition to slight inhibition zones across the four investigated groups. Similarly, Amp-B showed extremely small inhibition zones in these groups. These results pointed to be having a resistance to these agents by all strains of *C. limoniforme*. Generally, the FLU demonstrated the highest inhibition zone 50.88 mm regardless of the investigated groups, suggesting its effectiveness against *C. limoniforme*. Followed to the FLU, ITZ also displayed inhibition zone of 31.5 mm as an average of strains in all groups. Additionally, further analysis comparing the differences among study groups for each antifungal showed that CLO and MIC were significantly higher (*P*=0010 and 0.0243 respectively) in IV and NINV groups compared to INV and NIV groups, FLU was significantly higher in NINV group compared to other study groups, while other antifungal showed no significant differences (*P*>0.05) among study groups.

This study presented the first result report about the polyene and azole susceptibility for the first-time record of this species from clinical specimens. A scarcity of information has been regarded antifungal susceptibility patterns for *Cladosporium* species from the clinical samples. The current species is related to the *C. herbarum* complex which are considered a clinically related specie [32]. The *Cladosporium* species have been rarely reported from humans. In contrast to this study, Amp-B had exhibited more active against species of the *C. herbarum* complex compared to members of *C. cladosporioides* and *C. sphaerospermum* complexes [36]. Similar to the current results, the ITZ with the minimum inhibitory concentration (MIC90) of 1.68 µg/ml was actively affect the *Cladosporium* species complexes including the *C. herbarum* complex among the azole testing in the same previous study. The variability between isolates might be due to have different species from distinctive anatomical human sites.

Table 2. Antifungal susceptibility pattern of *Cladosporium limoniforme* against various antifungal agents measured in terms of the diameter of the inhibition zones (DIZ) in millimeters (mm).

Antifungal agents	DIZ for the tested agents (mm)				*P*-value*	Mean	*P*-value†
	COVID-19 & vaccination statuses						
	IV	INV	NIV	NINV			
Amphotericin-B (Amp-B)	0.5	0.5	1.3	1.5	0.0542	0.9^{e}	< 0.001
Clotrimazole (CLO)	11.3^{a}	8.5^{b}	9.3^{b}	11.0^{a}	**0.0010**	10.0^{d}	
Fluconazole (FLU)	47.5^{c}	51.0^{b}	50.5^{b}	54.5^{a}	**<0.0001**	50.9^{a}	
Itraconazole (ITZ)	30.5	31.5	32.5	31.5	0.1742	31.5^{b}	

Ketoconazole (KT)	1.5	0.0	0.5	1.0	0.0770	0.8[e]
Miconazole (MIC)	13.5[a]	11.5[b]	12.3[ab]	13.5[a]	**0.0243**	14.2[c]
Nystatin (NYS)	0.5	0.0	0.0	0.0	0.1818	0.1[e]
Control	0.0	0.0	0.0	0.0		0.0
Mean	15.04	14.7	16.1	16.1		
***P*-value‡**		0.0949				

Abbreviations: non-infected non vaccinated (NINV), infected non vaccinated (INV), non-infected vaccinated (NIV), and Infected Vaccinated (IV). * Compering each antifungal between the study groups; † Comparing antifungals means with each other's regardless study groups; ‡ Comparing the study groups using all antifungals. There were not significantly different between the interaction of study groups and antifungals with the *P*-value = 0.0698. The values not connected by any letter or that have same letter (a) are not significantly different. Amp-B (100 U), CLO (10 µg), FLU (10 µg), ITZ (10 µg) (KT), MIC (30 µg), NYS (50 µg).

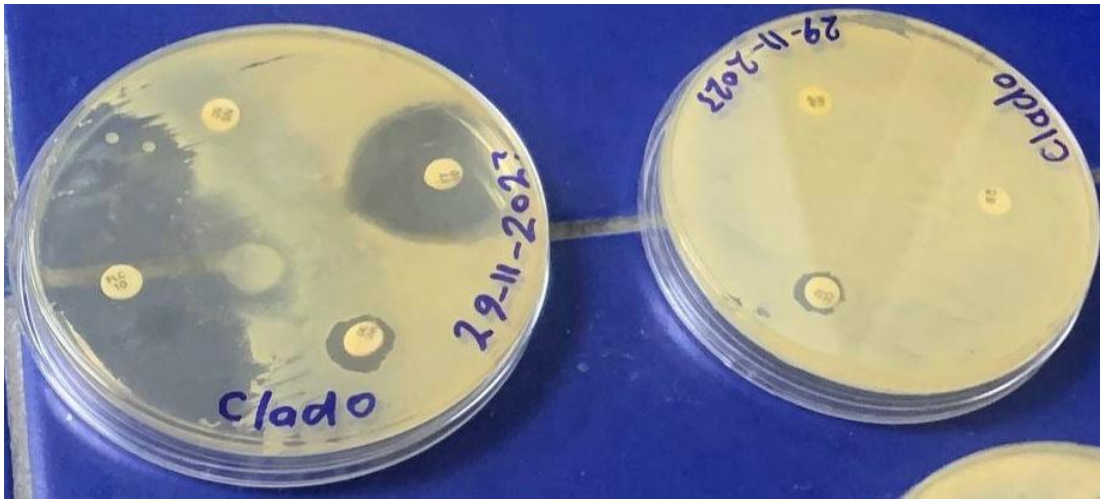

Figure 5. Antifungal susceptibility of *Cladosporium limoniforme* against various antifungal agents.

In the current results, variable activity of antifungal susceptibility testing had been reported against *Penicillium crustosum* strains of the NINV and IV groups (Table3, Figure 6). However, there were no significant differences in the interaction (*P*=0.096) between the strains isolated from both groups and antifungal agents. Overall, the two stains had not affected significantly (*P*=0.0576) by the applied antifungals. Furthermore, the results of antifungals on strains from both NINV and IV groups revealed significant variances (*P*<0.0001) to inhibit fungal growth regardless of strains from the group. KT and Amp-B exhibit extremely low inhibition zones in both groups. The FLU was significantly effective against *P. crustosum* strains in both NIV and IV with inhibition zone 53 .5 compared to other antifungals, followed by the MIC., ITZ., and NYS.

The statistical analysis also showed that Itraconazole was significantly higher (*P*=0.0203) in IV groups compared to NIV group when comparing the groups for each antifungal, while other antifungals showed no significant differences (*P*>0.05).

The current findings of *P. crustosum* partially align with those presented by [34] regarding the resistance profiles of *P. duclauxi* to a spectrum of antifungals, including Amp-B, FLU., ITZ., and KT. However, the current results do not extend this resistance to Fluconazole and Itraconazole, suggesting a potential variance in the sensitivity of both species strains or perhaps differences in the experimental conditions or methodologies.

Table 3. Antifungal susceptibility pattern of *Penicillium crustosum* against various antifungal agents measured in terms of the diameter of the inhibition zones (DIZ) in millimeters (mm).

Antifungal agents	**DIZ for the tested agents (mm)**		***P*-value***	**Mean**	***P*-value†**
	COVID-19 & vaccination statuses				
	IV	**NIV**			
Amphotericin-B (Amp-B)	0.0	2.0	0.9558	1.0[d]	
Clotrimazole (CLO)	14.8	13.5	0.9971	14.1[c]	
Fluconazole (FLU)	53.0	54.0	0.9993	53.5[a]	
Itraconazole (ITZ)	33.0	26.0	**0.0203**	29.5[b]	<
Ketoconazole (KT)	0.5	0.0	>0.9999	0.3[d]	0.0001
Miconazole (MIC)	32.5	31.0	0.9911	31.8[b]	
Nystatin (NYS)	30.5	26.5	0.4117	28.5[b]	
Control	0.0	0.0		0.0	
Mean	23.5	21.9			
***P*-value‡**	0.0576				

Abbreviations: non-infected non vaccinated (NINV), and Infected Vaccinated (IV) groups. * Compering each antifungal between the study groups; † Comparing antifungals means with each other's regardless study groups; ‡ Comparing the study groups using all antifungals. There were not significantly different between the interactions of study groups and antifungals with the *P*-value = 0.0958. The values not connected by any letter or that have same letter (a) are not significantly different**.** Amp-B (100 U), CLO (10 µg), FLU (10 µg), ITZ (10 µg) (KT), MIC (30 µg), NYS (50 µg).

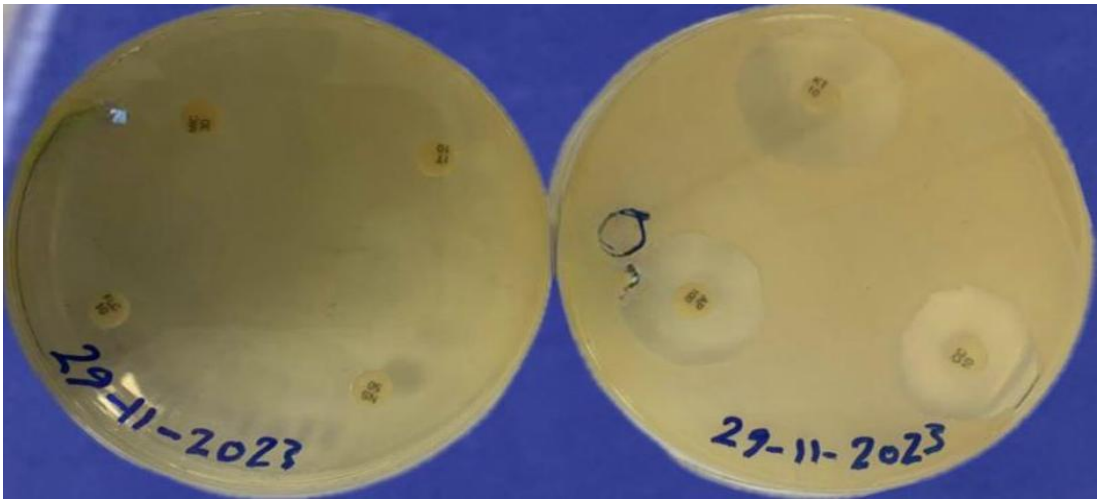

Figure 6. Antifungal susceptibility of *Penicillium crustosum* against various antifungal agents.

Regarding the effect of COVID-19 on antifungal susceptibility profiles, [37] noted negligible changes in resistance patterns among yeast and mold clinical isolates when comparing data from before and after the onset of the pandemic. Both periods showed that the majority of *Candida* and *Aspergillus* species-maintained susceptibility or exhibited wild-type (WT) profiles to common antifungals such as azoles, echinocandins, and amphotericin B. However, variations were primarily observed with azole resistance in *Candida* species. Analysis between the pre-COVID and COVID periods showed a decline in fluconazole and other azole resistances among *Candida glabrata* strains, while resistance levels for *Candida parapsilosis* and *Candida tropicalis* strains were on the rise. Presently, the azole resistance in *C. parapsilosis* and *C. tropicalis* has outstripped. This emphasizes the importance of precise species recognition and *in vitro* susceptibility testing to effectively manage invasive fungal infections during the COVID-19 pandemic. In their 2023 study at southern Thailand, [38] conducted results indicated a notable shift in MIC values alongside an increased incidence of resistance to azoles and echinocandins among *Candida* species. These findings with other results serve as a caution against the indiscriminate empirical application of some antifungals specially for emerging species of clinical isolates not only for monitoring antifungal resistance fungal species but also for contributing essential data that can enhance infection control practices within the hospital setting or during pandemics as with the COVID-19 pandemic.

CONCLUSION

Fundamental Finding : Three fungal species were identified from COVID-19 recovered individuals, including two species newly recorded in Iraq. Iraqi isolates were phylogenetically closely related to isolates reported in other countries. Antifungal susceptibility testing showed that *A. neoniger*, *C. limoniforme*, and *P. crustosum* had the highest inhibition zones with fluconazole and different sensitivities to other antifungal

agents. **Implication :** These fungal species may have potential effects on diseases associated with indoor and outdoor environments. The findings also highlight the importance of establishing comprehensive mycobiota profiling and antifungal susceptibility protocols for routine health monitoring and pandemic preparedness in Iraq. **Limitation :** The study focused only on the first reported isolates and a limited number of fungal species from COVID-19 recovered individuals in Iraq. **Future Research :** Future studies should expand fungal profiling programs and investigate broader antifungal susceptibility patterns to support early disease detection and improve responses to future pandemics.

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