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Candida spp. associated with COVID-19 and its Susceptibility to some Antifungals

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ABSTRACT

The aim of this study was to conduct a survey of the *Candida* species associated with COVID-19 viral infection in 150 patients who were admitted to the intensive care unit (ICU) in Al-Diwaniyah Teaching Hospital in Al-Diwaniyah City, Iraq, for a five-month period from October 2021 to February 2022. The results indicated the dominance of *Candida* spp. over the rest of the isolated fungal species, with 97 isolates (64.66%).

C. albicans was shown to be the most abundant species with a percentage of 55.67 percent (P 0.05), compared to the other *Candida* species that were isolated (*Candida* tropicalis 13.4 percent, *Candida glabrata* 12 percent, *Candida* krusie and *Candida parapsilosis* with 9.28 percent). In addition, we brought attention to the excellent action of the antifungals amphotericin B, itraconazole, and voriconazole, all of which have a high susceptibility rate.

Introduction

World Health Organization (WHO) labeled the new coronavirus (COVID-19) outbreak a worldwide pandemic on March 11, 2020 [1]. It is a respiratory condition that has a negative impact on the overall health of the individual [2]. Fever, dry cough, weariness, dyspnea, anosmia, ageusia, or a combination of these symptoms are the most often reported clinical symptoms in patients [3]. COVID-19 infection symptoms may appear 2–14 days after exposure (based on the incubation period of COVID-19 virus).

Clinical symptoms in SARS-CoV-2 infected patients are often various, ranging from no symptoms to severe sickness. These clinical symptoms can be further classified into four groups, which are as follows: asymptomatic; mild; moderate; severe; and critical illness [4]. Critically sick COVID-19 patients had increased pro-inflammatory (IL-1, IL-2, IL-6, TNF-α) and anti-inflammatory (IL-4, IL-10) cytokine levels, fewer CD4 interferon-gamma countenance, and less CD4 and CD8 cells. This acute clinical state raises the hazard of deadly fungal infections [5].

One of the significant fungal infections linked with COVID19 is Candidiasis, which is an infection caused by any kind of *Candida* fungus, it's signs and symptoms include white spots on the tongue or other parts of the mouth and throat, while soreness and difficulty swallowing are other possible side effects[6], and invasive candidiasis (IC) has become a leading cause of illness and mortality [7].

It is common for yeasts to be solitary budding oval-shaped cells of several microns in diameter extended cells joined end-to-end Hyphal filaments are 2 µm in diameter, with parallel-sided walls and no septal constrictions. Pseudohyphal filaments, on the other hand, exhibit constrictions at septal junctions and the mother-bud neck (2.8 µm) [8].

In the last two to three decades, 5 *Candida* species have been identified as being responsible for 95 percent of all infections: *Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis,* and *Candida krusie* [9].

Infections with *Candida* can be either acute, chronic or episodic, and they are most commonly seen in the pharynx or skin. *Candida* can infect the circulation through damaged skin or mucosal barriers in immunocompromised people. When the circulation has a large number of colonies, the gastrointestinal wall can absorb them [10]. Accumulation of non-painful pustules on the skin with an erythematous base is one of the signs of invasive candidiasis or candidemia in the body, as well as fever with chills, low blood pressure, disorientation and abdominal abscesses and discomfort [11]. Furthermore, most people-to-people acquired candidiasis occur in hospitals, when patients with impaired immune systems are at risk of contracting the infection from healthcare personnel [12].

It is becoming more common knowledge that severe COVID-19 consequences are related with candidiasis. It's not known why COVID-19 patients are more susceptible to candidiasis due to a variety of causes, including weakened immune systems, anemia from iron and zinc deficiency, and infections acquired in the hospital or during a medical procedure [13]. White lesions in the mouth caused by oral candidiasis (thrush) have been documented in mild to moderately unwell COVID19 patients, particularly in those with entire dentures or prosthesis [11, 14].

Materials and Methods

Collection of the specimens and data

150 clinical samples were collected from patients infected with COVID-19, confirmed by PCR analysis, and hospitalized in the intensive care unit (ICU) at Al-Shifa Center of Al-Diwaniyah Teaching Hospital in Al-Diwaniyah governorate, Iraq, during the research period starting from October 2021 to February 2022.

The samples varied between oral swabs, throat swabs and sputum samples. As for the swabs, a sterile cotton swabs were rotated inside the patient's mouth cavity and then kept in plastic containers until use. While sputum samples were collected using a container with a diameter of 5 cm. After washing the mouth with water to minimize oral bacteria and dilute saliva, samples were taken. Sputum should not be swallowed but should be quickly spat out into a sterile container.
Data on demographics (age, gender, residence location), and comorbidities are collected.

Specimens' cultivation

For each sputum sample, 0.1 mL of the specimen was removed and streaked onto Sabouraud dextrose agar medium [15] . The swabs streaked directly onto SDA; three replications of the culture were made to ensure that the fungal growth was not contaminated during the culture process. The dishes were incubated at a temperature of 37°C for 24 hours [16]. Then for subculture we used another culture media like, Corn Meal Agar, and Chrom *Candida* Agar for diagnostic reasons. Also, we conducted some additional tests for detection the virulence factors in *Candida* sp.

Identification of Fungal species

Depending on the culture and microscopic properties of the fungus as stated in [17].

Germ tube formation

This test was done by vaccinating 37 ml of human blood serum with *Candida* albicans colonies and incubating them at 37°C for 2-3 hours. In the case of the formation of the germ tube, this means that the examination is positive and distinct for *Candida albicans*, as it is observed that the germ tube protrudes from one side of the cell [18].

Chlamydospore formation

In this test, the corn flour was inoculated by making parallel incisions on it by a sterile wire immersed in a part of the fungal colonies. Then the slide cover was placed on the place of inoculation. Then the dishes were incubated at a temperature of 28 °C for 48-72 hours, after which the plate was examined under a microscope to observe the fungal hyphae and Chlamydospores.[19]

Phospholipase Production

The medium for this test was prepared by mixing the components together well, then sterilized, inoculated and incubated at 37°C. The appearance of a sedimentation zone around the colonies means that the result is positive [20]

Biochemical test by using Hi*Candida* Identification for *Candida* sp*.*

A ready-made diagnostic kit was used in the biochemical diagnosis to verify the isolated species according to the manufacturer's instructions. The system consists of a tape containing 12 wells containing basic materials. This kit was used to determine the patterns of the sugar's assimilation and urease production [21], where the fungal suspension, after regrowth on PDA, is added to each well as follow :

1- The kit was opened in a sterile way. Then the packaging foil is removed.
2- The surface inoculation method was used to inoculate each well with 50 µl of the aforesaid inoculum.
3- Incubation, Temperature of incubation: 22.5 C ± 2.5 C. Duration of incubation: 24-48 hours.
4- Interpretation of results, the results was interpreted as per the standards given in the identification index.

Sensitivity test by disc diffusion method

According to [22]Disk tests are affordable and simple to do, making them an ideal screening tool. For *Candida* spp. isolates, the disk diffusion method to evaluate antifungals has only been developed and validated for azoles and echinocandins. It suggests using Mueller-Hinton agar supplemented with 2% glucose, which is ideal for the growth of the majority of yeasts, and 0.5 mg/L methylene blue dye medium (which increases the zone boundary delineation) to minimize the trailing effect. The inoculum is standardized to 0.5 McFarland, and plates should be incubated at 350C for 24 hours; certain strains exhibit inadequate growth and may require 48 hours of incubation. In addition, parameters for quality control have been created in accordance with the CLSI standard processes. The susceptibility test according to the zone diameter interpretative criteria for ketoconazole, fluconazole, voriconazole,

itraconazole and AmphotericinB for fungi species enables classification of the isolate into one of the following categories: susceptible, resistant.

Statistical analysis

Statistical analysis of the data was carried out using one-way ANOVA with the least significance difference (LSD) using the statistical analysis software program (Special Package for Statistical Science SPSS version 26), with a significant value $P \leq 0.05$.

Results and Discussion

Cultural Characteristics

The colonies growing on Sabouraud Dextrose Agar (SDA) appeared in the form of white to creamy and smooth colonies, and in the form of circular colonies (Fig. 4-1). In this regard, Hamid, *et al*. [23] indicates that the *Candida* colonies possess such phenotypic characteristics when growing on SDA, and this result is consistent with what Alkhuzaie [24] mentioned about the appearance of colonies of creamy, shiny, smooth, circular shape due to the availability of appropriate cultivation conditions.

Fig.1: *Candida* sp. growth on SDA at 37 for 24 h

4.1.1.2 Microscopic Characteristics

After being stained with lactophenol cotton blue, the yeast cells were blacker and oval in shape, in contrast to the typical *C. albicans* cells, which are bright and spherical in shape as in the figure 2. This result is in agreement with Wibawa, *et al*. [25]

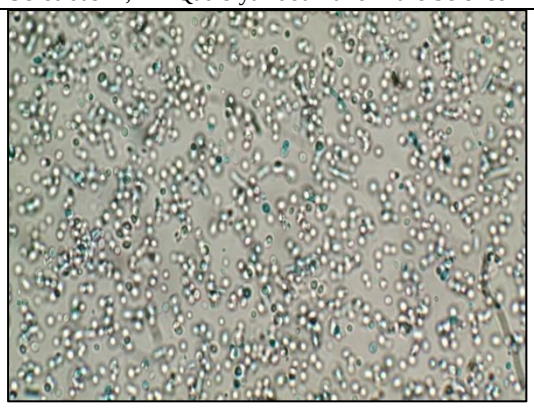

Fig.2: *Candida* sp. cells under microscope (40X)

Growth at 37°C

The isolates under test showed positive results at a temperature of 37°C, as the isolated species were grown on SDA. The growth was monitored daily, and it was found that the grown species had the ability to grow at this temperature. These results are in agreement with the findings of Alidami [26], as the phenotypic characteristics of the growing colonies were observed in terms of color, colony shape, height from the surface of the medium and its strength.

Growth on HiCrome *Candida* Differential Agar

When *Candida* species were grown on the aforementioned medium for 24-48 hours at 37°C, results of distinct and different colors appeared. The species *Candida albicans* grew in green color, while *Candida tropicalis* grew in blue color, *Candida parapsilosis* in pale cream color, and *Candida krusie* in pink color. And *Candida glabrata*, its colonies were violet in color. as in the figure (3). This medium was used as a differential medium with accurate results in the diagnosis of *Candida* species. This finding is consistent with what was stated by Jain, *et al*. [27] , It also came in accordance with the study of Khadka, *et al*. [28] regarding the isolation and quick identification of *Candida* species, as one of the most essential media utilized in the field of fungal diagnostics, since the diagnosis is dependent on the medium's coloring.

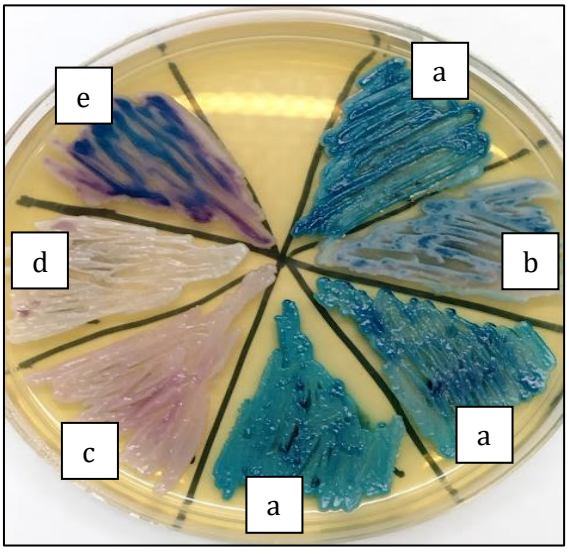

Fig.3) Candida sp. growth on HiCrome™ Candida Differential Agar at 37°C for 24h,
a) *Candida albicans* b) *Candida tropicalis* c) *Candida krusie*
d) *Candida parapsilosis* e) *Candida glabrata*

Germ tube formation

The findings of the test indicated that a germinal tube was generated by every isolate of *Candida albicans* when it was incubated at 37°C for two to three hours in 0.5 milliliters of human blood serum as in the (figure 4). However, the germinal tube was not formed by the other species under the same conditions. These outcomes are consistent with Alkhuzaie [24] and was likewise similar to what was stated by Matare, *et al*. [29]. It has been hypothesized that it plays a role in the pathogenesis of *Candida albicans* as a contributing virulence factor Ganguly, *et al*. [30]. The physiological circumstances of an immunocompromised host can cause *C. albicans* to dimorph into a hyphal stage of development Raghunath, *et al*. [31].

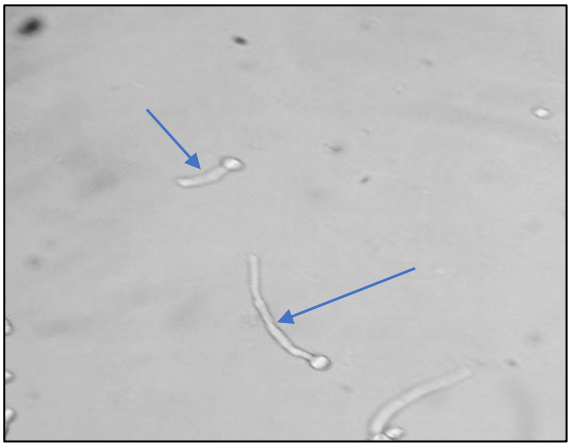

Fig.4 germtube formation in *Candida* albicans (40X)
after 3 hours incubation in human serum at 37°C

Chlamydospore formation

Using this test, it was determined that the isolates that belonging to *Candida albicans* form chlamydospores as in the figure 5. While other species of *Candida* did not form the chlamydospores under the same conditions (25°C for 24h). The results of this test were the same as those of Devi and Maheshwari [32] where the results indicated that *Candida albicans* generated chlamydial spores on Corn Meal Agar, as this medium is regarded as a diagnostic feature for *Candida albicans.* It is also agreed with Navarathna, *et al*. [33] that the fungal spores with thick, circular walls are formed at the end of the fungal hyphae, which may be single or in clusters on the maize flour medium, which is considered a nutrient-poor and suitable medium for its growth; therefore, Chlamydospore is formed as a response to the nutrient-poor medium.

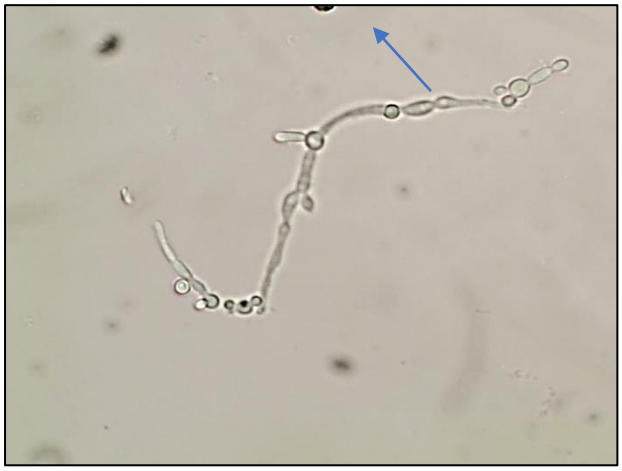

Fig.5: Chlamydospore formation in *Candida albicans* (40X) on CMA at 37°C for 48h.

Phospholipase Enzyme Production (PL)

The results of the study showed that the two species, *Candida albicans* and *Candida krusie*, have the ability to produce phospholipid enzyme, as a halo-shaped deposition area appears around the inoculum as shown in Figure (6), resulting from complex formation between calcium ions and fatty acids released from the decomposition of phosphorylated lipids present in egg yolk, while *C. parapsilosis*, *C. tropicalis* and *C. glabrata* haven't the ability to produce PL, and this explains why *Candida albicans* is able to have an adverse effect on health more than other species because it has this enzyme, which is a key part of its pathogenesis Oksuz, *et al*. [34]. This is what Alshukri [35] reached in her study on some types of *Candida* , as the two species *C. albicans* and *C. krusie* gave positive results for their secretion of this enzyme. This enzyme is one of the virulence factors in *Candida* yeast, since it is responsible for the breakdown and degradation of the cell membranes of the host, which in turn speeds up the process of tissue invasion Lahkar, *et al*. [36].

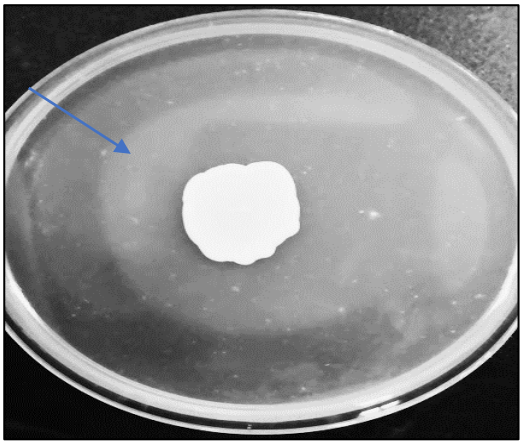

Fig.6: Phospholipase production in *Candida* albicans on Egg yolk Agar at 37°C for 48h. blue arrow: the halo-shaped deposition area

Biochemical Test

The results of this test shown the ability of *Candida* species to detect Urease enzyme and utilize carbohydrate fermentation, the interpretation of the results (color change) with identification index, attached in the box of the kit, which lists all the species that can be recognized using this technology. the result shown in the table (1) and in (Figure 6)

Table 1: Results of using Hi*Candida* Identification kit

Test Species	Urease	Melibios-	Lactose	Maltose	Sucrose	Galactos-	Cellobio--	Inositol	Xylose	Dulcitol	Raffinos-	Trehalos-
C. albicans	-	-	-	+	+	+	-	-	+	-	-	-
C. glabrata	-	-	-	+	-	-	-	-	-	-	-	+
C. krusie	+	-	-	-	-	-	-	-	-	-	-	-
C. parapsilosis	-	-	-	+	+	-	-	-	+	-	-	-
C.tropicalis	-	-	-	+	+	+*	+	-	+	-	-	+

+: positive reaction, -: negative reaction, *: strain variation

The results of this test were the same as those of Hedayati, *et al*. [37] in which the data showed that *Candida* species were accurately identified using the Hi*Candida* identification kit, which is also conforms to Singh, *et al*. [38] who stated that Identification was accomplished with the support of diagnostic by using this kit.

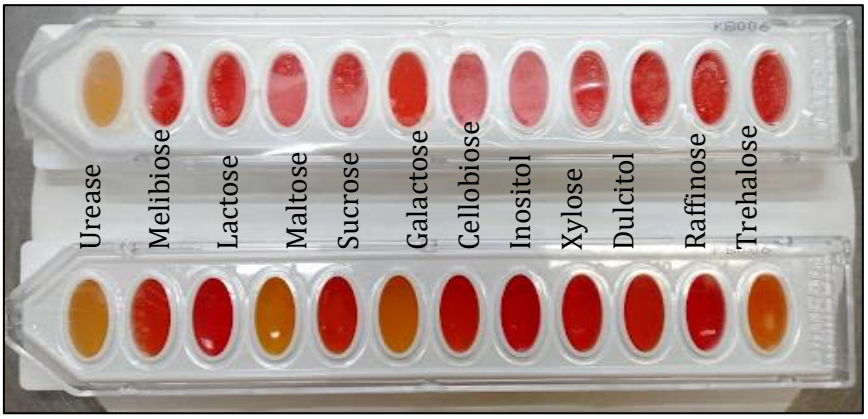

Fig.7: Hi*Candida* identification kit

Candida spp. Frequency

The current study found that *Candida* is the most prevalent fungi identified from Covid-19 patients, with a 64.66 % frequency. As seen in the table (2), *Candida albicans* was the most common (55.67%) than the other isolated *Candida* species (*Candida tropicalis* 13.4%, *Candida glabrata* 12%, *Candida krusie* and *Candida parapsilosis with* 9.28% for each.

Table 2: *Candida* spp. isolates

Candida sp.	isolates	Percentage to *Candida sp.* Isolates
Candida albicans*	54	55.67
Candida tropicalis	13	13.40
Candida glabrata	12	12.37
Candida krusie	9	9.28
Candida parapsilosis	9	9.28
total	97	100

*There are S.D. between *Candida albicans* and other groups (LSD= 5.405496), P value= 2.49E-08

Our result about the diagnosed *Candida* spp. is in similarity to what found by Salehi, *et al*. [39] that detected coinfection in COVID-19 patients with *Candida albicans* (70.7%), *Candida glabrata* (10.7%), *Candida parapsilosis* (4.6%), *Candida tropicalis* (3%), and *Candida krusei* (1.5%) in a total of 53 hospitalized COVID-19 patients.

Several observations of COVID-19-associated candidiasis (CAC) have been documented. Arastehfar, *et al*. [40] stated that *C. albicans* was the species that was detected the most frequently followed by other species of *Candida* , this is in line with Salehi, *et al*. [39] who stated that *C. albicans* accounted for 70.7 percent of the *Candida* isolates, whereas *C. tropicalis* was the least common species.

The overall *Candida* isolation percentage was found to be 20% lower than that of previously reported level by Arastehfar, *et al*. [41] who found that infection with *Candida sp*. constitutes a percentage about 85.7% of fungal infections associated with COVID-19. Similar findings were observed by Antinori, *et al*. [42] who isolate *Candida* with 60 percent and indicate that C. albicans is prevalent than other *Candida* species.

Candida dominance in fungal coinfections linked to COVID-19 is related to a variety of factors, COVID-19 is accompanying with xerostomia or a dehydrated mouth Riad, *et al*. [11] , in addition, long term of using mechanical ventilation will lead to xerostomia Therefore, it is not completely out of the question to postulate that may cause changes to the oral flora and an increase in the likelihood of developing opportunistic infections such as candidiasis.

Table 3: Antifungal susceptibility for Yeast (disc diffusion)

Fungi. *(n)*	**Sensitivity**	**Antifungal agents**				
		Amp 20µg/ml	**FLC 25µg/ml**	**KT 10µg/ml**	**IT 10µg/ml**	**VRC 1µg/ml**
Candida albicans (54)	**S**	**50 (92)**	**22 (40.7)**	**21 (38.9)**	**52 (96.3)**	**51 (94.4)**
	S-DD		**2**	**4**		
	R	**4 (8%)**	**30 (55.6)**	**31 (57.4)**	**2 (3.7)**	**3 (5.6)**
Candida tropicalis (13)	**S**	**12 (92.3)**	**2 (15.4)**	**13 (100)**	**8 (61.53)**	**11 (84.6)**
	S-DD		**-**			
	R	**1 (7.7)**	**11 (84.6)**		**5 (38.46)**	**2 (15.4)**
Candida krusie (9)	**S**	**8 (88.9)**	**0 (0)**	**7 (77.8)**	**6 (66.7)**	**7 (77.8)**
	S-DD		**-**			
	R	**2 (11.1)**	**9**	**2 (22.2)**	**3**	**2 (22.2)**
Candida glabrata (12)	**S**	**8 (66.7)**		**12 (100)**	**12 (100)**	**12 (100)**
	S-DD	**3**	**-**			
	R	**1 (8.3)**	**12 (100)**			
Candida parapsilosis (9)	**S**	**8 (88.9)**	**1 (11.11)**	**9 (100)**	**9 (100)**	**9 (100)**
	S-DD		**-**			
	R	**2 (11.1)**	**8 (88.89)**			

TOTAL (97)	**S**	**87 (88.8)**	**25 (25.51)**	**62 (63.26)**	**88 (89.8)**	**91 (92.85)**
	R	**11 (10.2)**	**71 (72.45)**	**33 (33.67)**	**10 (10.2)**	**7 (7.15)**

It can be seen from the table (4-17) that the most effective antifungals were (Voriconazole, Itraconazole, AmphotericinB) has the highest percentage of susceptibility against yeast fungi with (92.85, 89.8,88.88 respectively) and Ketoconazole (63.26), while Fluconazole the lowest percentage of susceptibility with (25.51).

This result in accordance with Mamun, *et al*. [43] who record the sensitivity of *Candida* sp. to AMB with (88.58%) while FLC exhibited the highest degree of resistance with (60%), and this is in line with Giri and Kindo [44] where AMB has the lowest resistant with (0%). Our result in agreements with Pfaller, *et al*. [45] who verified the ability of voriconazole has the potential to yield maximum ratio of sensitivity with 98 percent against *Candida sp.* and in consistent with the findings of Kothari and Sagar [46]., amphotericin B resistance sits at 8%.

In addition, we detected the resistance of *Candida* sp. to FLC, this in accordance with study of Nasrollahi, *et al*. [47] where detected the presence (94%) of C. albicans isolates were resistant to fluconazole. The development of fluconazole resistance in *Candida* species has been extensively studied in *C. albicans* and has been described in detail. It is absolutely necessary to define fluconazole resistance in NCAC species now that the epidemiology of *Candida* infections is changing to include more instances of NCAC species [48].

Conclusion

Among patients diseased with coronavirus (COVID-19), fungal co-infection is a considerable health risk, Candidiasis is becoming increasingly common as the globe continues to fight COVID-19, Candidiasis accounting for 64.66 % of all infections and significantly C. albicans represent the most prevalent species with (55.67%) (P<0.05) than the other isolated *Candida* species (*Candida tropicalis* 13.4*%, Candida glabrata* 12%, *Candida krusie* and *Candida parapsilosis with* 9.28%). furthermore, the antifungals AmphotericinB, Itraconazole and Voriconazole have a good activity with high susceptibility ratio.

Finally, given the high proportion of infections with fungi related to COVID-19 stated in this study, patients with COVID-19 should be screened for fungal infections at the earliest possible stage of infection to minimize the risk of developing a more serious illness because timely detection is vital for effective management of fungal co-infection.

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