Molecular detection of virulence genes for Candidiasis associated with corona virus disease in patients of Wasit Governorate

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ABSTRACT

Back ground: 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. The aims of Study are: Isolation and identification of fungal pathogens from patients with corona virus infections and Molecular documentation of the most common fungal isolate and detection some virulence genes

Material and methods: One hundred sample include nasal, throat swab and sputum sample 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 in wasit Governorate. Samples were cultured in sabouraud agar and incubated for 24-48h at 37˚C under aerobic conditions. The identification of candidiasis was done depended on growth and microscopic analysis utilizing the Gram stain staining method, Germ tube test, Api Candida well as morphological and cultural features. and kept for further conformation and detection of the most common virulence factor by polymerase chain reaction (PCR).

Results: The results were appearance of yeasts in 23/100 (23%) isolates. The total isolation of C.albicans were 10/40 (25%), followed by C. Parapsilosis 7/40 (17.5%), C.tropicals 4/40(10%) and C.kruse 2/40(5%). In the present study the nine isolates belong C. albicans were positive for ALS1 gene 9/10 (90%) with PCR product of this gene was approximately 577 bp, and eight isolates were positive for SAP1 gene 8/10 (80%), with a PCR product size of approximately 480 bp. And the hyphal formation (CPH1 gene) has been detected in all C.parapsilosis isolation with a PCR product size of approximately 546 bp. In conclusion showed Candidiasis and Aspergillosis are reported as a high incidence in patients with Coronavirus diseases .

Keywords: Corona virus disease, candidiasis, Waist Governorate, Polymerase chain reaction

Candida species are one of the most common fungal pathogens causing invasive infections at a global scale[1].*Candida albicans, Candida glabrata, Candida parapsilosis, Candida*

tropicalis, and *Candida krusei* are some of the more common yeast species that can be found on mucosal surfaces, including the skin and the respiratory, digestive, and urinary systems [2].). Although being commensal within the human host, Candida species are equipped with virulence attributes, enabling them to invade when opportunities arise and cause various infections in humans, especially when the immune system is impaired [3]. Superficial infections, such as skin disorders; mucosal infections include oropharyngeal or vulvovaginitis candidiasis; and invasive candidiasis are established clinical entities of candidiasis. The incidence of fungal infections ,including candidiasis ,has increased world wide overthe past few decades [4]. *C.albicans* remains the most common causative agent for candidiasis, leading to a range of life-threatening invasive ornonlife-threatening superficial conditions [5]. During invasive infections, *Candida* have the ability to enter the blood and infect every organ in the host [6].Candida represents the fourth leading cause of nosocomial infections and the third most common cause of catheter- related blood stream infections, with amorality rate

exceeding 50% [7].Prolonged Among the most significant factors contributing to invasive yeast infections in COVID-19 patients are hospital stays, usage of broad-spectrum antibiotics, and central venous catheters.Studies have shown high prevalence of Candida infections among patients with COVID-19, so Candida species should be considered as potential pathogens in these patients [8].On the mucosal surfaces of the skin, in the digestive, respiratory, and urinary tracts, as well as on the skin, Candida species are found. Members of the genus *Candida* are the most frequently recovered pathogens in ICUs, affecting between6% and 10% of patients.The most common species *C.albicans*, is found in 17% of hospitalized ICU patients. Infection with *C. albicans* is linked to considerable morbidity and mortality [9]. Infections by other species of the genus *Candida* are becoming more common, especially among neutropenic patients and patients receiving azole therapy [10].

Materials and Methods

Location of study

This study has been carried out in the 1laboratory of department of Biology– College of Science – University of Wasit, Public Health Lab In Wasit, Al-Zahra and Al-Karama Teaching Hospitals through the period from October 2021 to the end of April 2022.

Samples Collection

One hundred sample 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 Wasit 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.

Identification of isolated Yeast

Swab sticks collected from patients with covid19 were streaked on the well labeled Sabouraud's dextrose agar (SDA) plates incubated at 35-37 for 24-48 hours. The growth was recognized by microscopic analys was utilizing the Gram stain staining method, Germ tube test, Api Candida as well as morphological and cultural features.

Gram stain

According to the manufacturer's directions, make the gram stain [11].After being stained with Grams stain and studied under a microscope on the slide, cultural features on SDA that include form, color, and size are used to identify the morphology of the Candida [12].

Germ tube test

To distinguish *C. albicans* from other yeast, this test was employed. Candida forms a germ tube when it is cultured in human or sheep serum at 37 degrees for three hours. Put 0.5 ml of human serum in a tiny tube. Using a Pasteur pipette, gently emulsify a yeast colony in the serum. Incubate the tube at 37 degrees for two to three hours. The serum is then placed to

a slide to be inspected, covered with a coverslip, and viewed under low- and high-power microscopes **[13].**

Molecular Detection
Primers

PCR primers were designed in this study using NCBI-Genbank database and primer3 plus online. These primers were provided by Macrogen company from Korea as following Table (1)

Table (1): PCR detection gene primers with their nucleotide sequence and product size for *candida* spp. [14].

Primers		Sequence(5'-3')	PCR product size
C. albicans Adhesion like protein*ALS1*gene	F	GCGACCCTCCGATCTTGTAG	577bp
	R	GACGACTGCCAGCACAAGTA	
C. albican sproteinase*SAP1*gene	F	GCTTTTGCTGGTTGATGCCA	480bp
	R	TGCTGATTGACCAGGACGAG	
C. parapsilosis hyphal formation*CPH1* gene	F	CAGGTTCTTTGGGCAGCAAC	546bp
	R	GCTGCTGCATTTGCCTCTTT	

Fungal DNA Extraction
Using the Fungi /Yeast Genomic DNA extraction Mini kit, which was described in the table (2–5), 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 Nanodrop [Thermo Scientific Nano Drop Lite UV Visible Spectrophotometer. USA] that measured DNA concentration (ng/µL) & checked a DNA purity at absorbance (260 /280nm)as following steps:

- ❖ It has been selected the relevant program after opening the Nanodrop program(Nucleicacid,DNA).
- ❖ The measuring pedestals were cleaned many times with a dry wipe. Then, usinga pipette, carefully 2µl of free nuclease water onto the bottom measurementpedestals toblankthe system.
- ❖ TheNanodrop samplingarm waslowered a nd1µlDNA samplemeasure

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PCR Green Master Mix Preparation
For all genes PCR master mix reactions was prepared by usied (Maxime PCRPre Mix kit) and this master mix was completed in accordance with the company's specifications as show in the Table (2).

Table(2):Standard PCR master mix protocol

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 PCR tubes were then transferred to an Exispin vortex centrifuge at 3000 rpm for 3 minutes before being placed in a T100 PCR thermo cycler. After that, the PCR master mix components were placed in standard Maxime PCR PreMix kit tubes containing all other components required for the PCR reaction, such as Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, Mg (BioRad-USA).

PCR Thermo-Cycler Conditions

PCR thermo-cycler conditions protocol for each gene was computed by usingOptimase Protocol Writer™ online application and done by using convention PCR thermo cycler Table(3).

Table(3):PCR thermo-cycler conditions protocol

PCRcycle	repeat	Temp.	Time
Initial denaturation	1	95˚C	5min
Denaturation	34	95˚C	30sec
Annealing		58˚C	30sec
Extension		72˚C	1min
Finalextension	1	72˚C	5min
Hold	-	4˚C	5min

PCR Product Analysis

Agarose gel electrophoresis was used to examine the PCR results as followingsteps:

- A1.5% Agarosegel, wasprepared byusing1 XTBE (Tris-Borate with EDTA) & dissolving in microwave for 5minutes, and allowed to cool at50°C.
- After that, 3-5µl of ethidium bromide dye was added to the agarose gelsolution.
- 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 room temperature before carefully removing the comb from the tray.
- The gel tray was placed in the electrophoresis chamber and 0.5X TBE(Tris-Borate with EDTA) bufferwas added.
- 10µl PCR product were loaded in to each well with added 5µl (DNA markerLadder) in first well.Then electriccurrent wasperformed at 70/cm for 1.5 hour.
- UV Tran illuminator 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 significant1 P-value ≤ 0.

Results and Discussion

Isolation and Identification of Yeast

Direct Examination

Nasal, sputum, throat swab samples were collected from 100 patients suffering from Corona virus disease with underlying different diseases were directly examined microscopically for the presence of yeast and other molds. Hyphae, pseudohyphae, blastoconidia and conidia (Figure: 1.A).

Colonies Characteristics

The suspected Candida spp. colonies on the SDA began as smooth, cream-colored colonies at 30°C (Figure 1.B), which changed to wrinkled, whitish-creamy colonies after an additional 7 days of incubation with a yeasty odor.

Microscopic Examination and Germ tube test

By using the lactophenol cotton blue and Grams staining the yeast cells appeared budding yeast cells oval to spherical or globose to ovoid like *C. albicans*with hyphae or pseudohyphae. Germ tube formation was the aspect of *C. albicans* isolates while other Candida spp. and yeasts do not able to form it. (Figure: 1.(C,D))

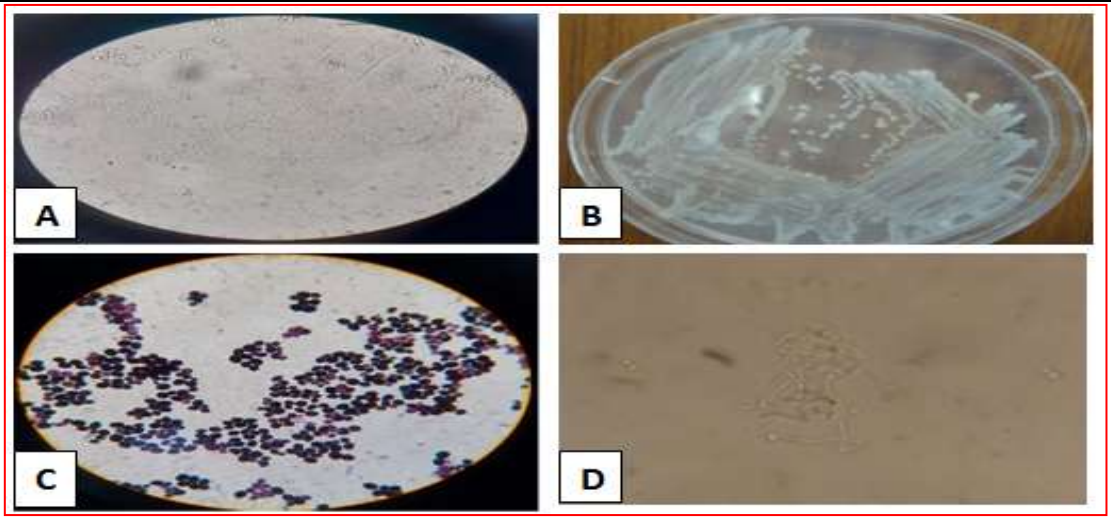


Figure (1): A- Direct examination shows hyphae and blastoconidia of yeast. (X400) : B-Colonies white creamy characteristics of *Candida* spp. on SDA:C- Grams stain, budding yeast cell: D-Round to ovoid and large swollen blastoconidia.(X400).

The results are in accordance with [15]. for perfect diagnosis of the causative agent can be prepared used mycological techniques& (Song *et al*.,2020) who found that the, Infections caused by *Aspergillus*&*Candida* in COVID-19 patients need to be detected early and thoroughly investigated.In addition to mycological techniques like direct microscopic examination & sample culture, invasive fungal infections can also be diagnosed using molecular techniques.

Detected of *Candida spp* by Candida Chromogenic Agar

After preparation Chrome agar Candida and all white creamy colonies were subculture on Candida chromogenic agar which was used according to the manufacturer's guidelines. Green colonies were identified as *C.albicans*, Blue colonies were identified as *C. tropical*, Colonies of other *Candida* spp, including *C.krusei and C.parapsilosis* appear as pink, and white respectively. These results agree with [16].which found Candida isolates were classified according to the colors on the Chromoagar and based on a designed colored key.The results were appearance of yeasts colonies show total of 23 (23%) isolates of Candida spp out of 100 isolates. Identified as 10/23 (43.47%) *C. albicans*, 7/23 (30.43%) *C. parapsilosis*, 4/23(17.39%) *C.tropicals* and 2/23(8.69%) *C.kruse* (Figure:2,3).

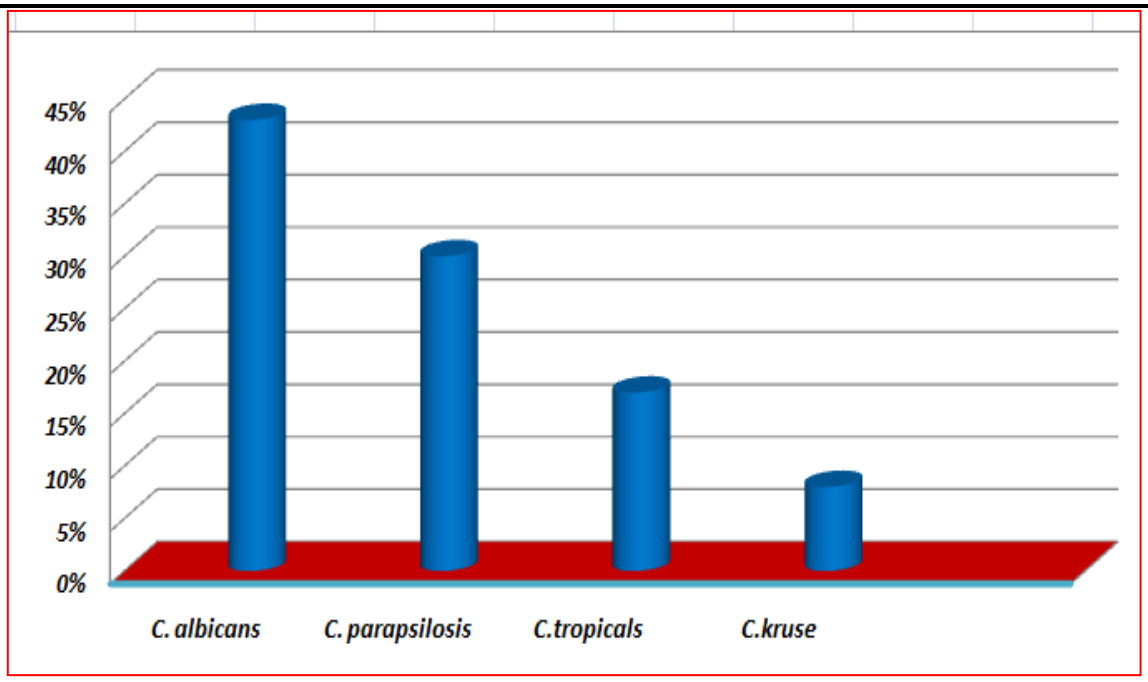


Figure (2) : Identification of *Candida spp*. by Chrome agar Candida.

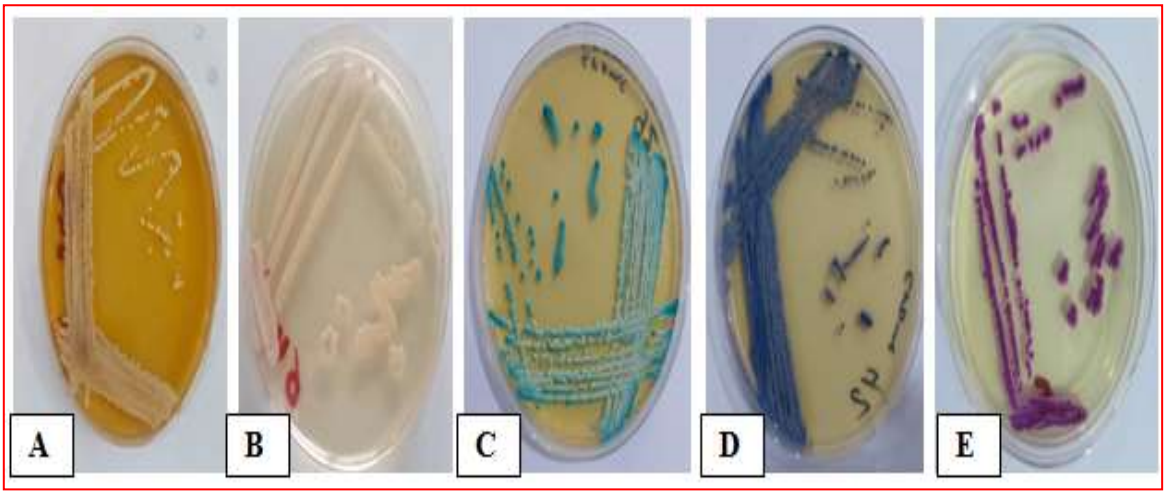


Figure (3): Colonies Characteristics of *Candida spp*. On Chrome agar. A- *Candida spp*.; B-; C*parapsilosis* -C;*C.albicans* ; D- *C.tropical* ; E- *C. kruse*.

Analytic profile index 20 for Candida

Analytic profile index 20 (API 20) is a test used as confirmatory identification test of the yeast isolates. The positive result is read after 72 hr. depending on the turbidity of each test (Figure: 4). The yeast isolates which are diagnosed by the strip were total of 23 (23%) isolates of Candida spp out of 100 isolates. Identified as 10/23 (43.47%)*C. albicans*, 7/23 (30.43%) *C. parapsilosis*,4/23(17.39%) *C.tropicals* and 2/23(8.69%) *C.kruse*. these results were matched to Candida Chromogenic Agar detected yeasts. Identification was made by referring to the list of numerical profiles and a computer program provided by the manufacturer.

Figure (4) : Identification of *Candida spp* by Api candida.

In the present results agree with description of [17], [18].Who finding Chromoagar culture brought about thedividing Candida species into two categories: C. albicans and non-albicans species. API 20 was considered as a confirmatory detection test for yeast isolates. A positive result was read after 72 hours depending on the turbidity test, and these results are consistent with the Chrome agar culture.

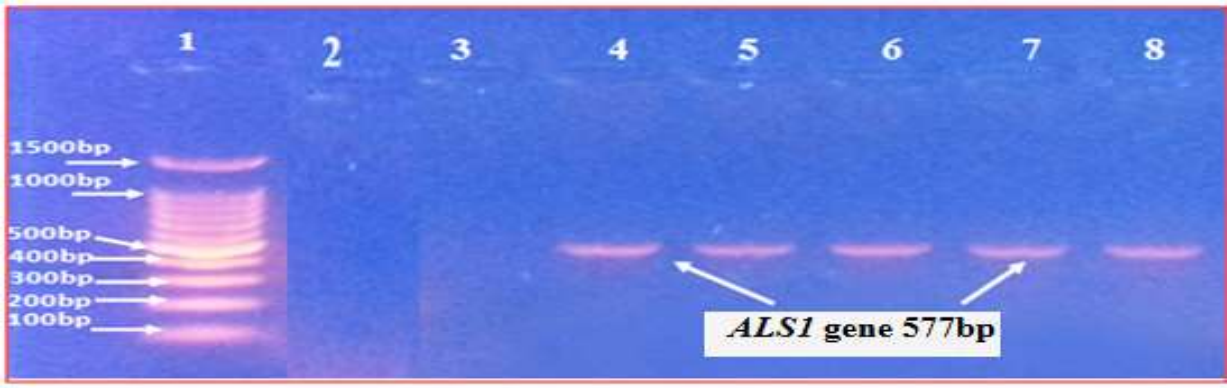
Detection of Virulence Factorsto Candida spp. isolation

***Candida albicans* Virulence Factors**

In the present study the nine isolates belong *C. albicans* were positive for *ALS1* gene 9/10 (90%) with PCR product of this gene was approximately 577 bp, and eight isolates were positive for *SAP1* gene 8/10 (80%), with PCR product size of approximately 480 bp, as shown in the Figure (5 and 6). In the results study shown Agglutinin-Like Sequence *ALS1* gene was detected in 9/10 (90%) *C. albicans* isolation , which was encodes a cell surface protein that mediates adherence of *C.alblcans* to endothelial cell such as mentioned in the study of **[19].**This gene was It was discovered that the most important virulence factor can escape immune reactions nutrients and harm the host **[20].** In this study, secreted aspartate proteinase (SAP) was detected in 8/10 (80%) cases of C. albicans infection using polymerase chain reaction (PCR) technique. This gene was one of the most discussed virulence factors produced by a human pathogen[21].

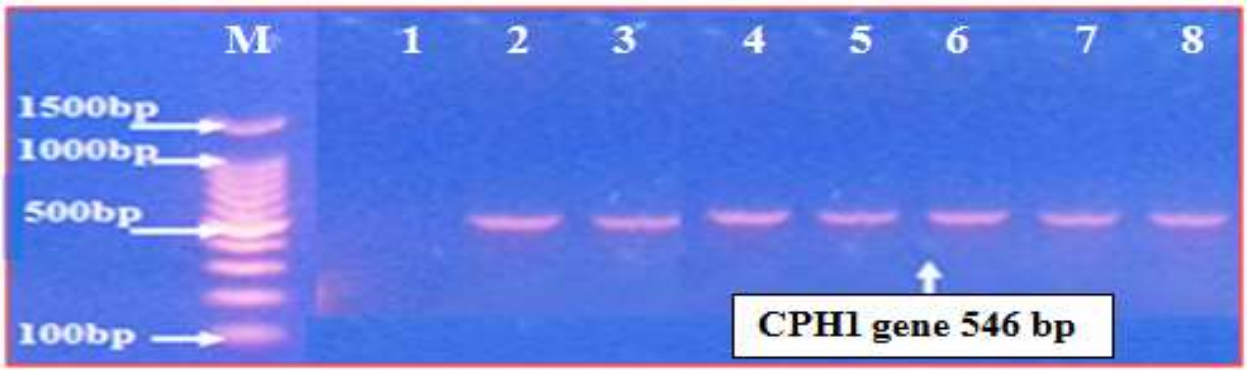
***Candida parapsilosis* Virulence Factors**

In this study, the hyphal formation (CPH1 gene) has been detected in all *C.parapsilosis* isolation with an approximate PCR product size 546 bp, as shown in the Figure (7). In this study, the hyphal formation CPH1 gene was detected in all positive samples of C. *parapsilosis* by using PCR technique. *Candida spp.* cells' capacity to attach to the mucosal surfaces of various host organ [20].



Figure(5): Gel electrophoresis(1.5% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane2: Negative control; lane(3): Negative result; lane(4-8) postive reults sample for *C.albican* (*ALS1* gene 577bp).

Figure(6): Gel electrophoresis(1.5% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane2: Negative control; lanes(4,5,7): Negative results; lanes(6,8-10) postive result postive reults sample for *C.albican* (*SAP1* gene 480bp).



Figure(7): Gel electrophoresis(1.5% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane1: Negative control; lanes(2-8): Postive reults sample for *C.parapsilosis* (CPH1 gene 546bp).

Conclusions

Candidiasis are reported as a high incidence in patients with Coronavirus diseases. *Candida albicans* are the most dominant in patients with patients with Coronavirus diseases .

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