Oral Mycotic Infections Among Covid-19 Patients in Al-Anbar Governorate West of Iraq

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1. Introduction

Coronavirus have a place with the Coronaviridae family and show up very much like spiked rings when seen through an electronic microscope. The surface looks with different spikes, which are useful to assault and tie living cells. These are the infections making the straightforward normal cold sickness extreme ailments like Middle East Respiratory Syndrome (MERS-CoV), severe acute respiratory syndrome (SARS-CoV)[1]. COVID-19, a human-to-human transmitted illness caused by the SARSCoV-2 virus, has been a devastating global public health disaster of the year. Too far, the COVID-19 has rapidly spread to 212 countries and resulted in over 5 million lab-confirmed cases and over 310,000 deaths throughout the world as of May 18th, 2020. SARS-CoV-2 has this vulnerability with SARS-CoV and the Middle East Respiratory Syndrome Coronavirus (MERS CoV). Acute respiratory discomfort may result from exposure to Tory pollution (ARDS).[2] The fungal co-infections associated with global COVID-19 pandemic are mycotic and bacterial secondary infections following COVID-19 sickness are generally being accounted for and are a region that ought to get cautious consideration. Mucor mycosis is a deadly fungal condition influencing immunocompromised patients occur due to a group of mold mucoromycetes [3]. Candida albicans (C. albicans) is an oral commensal present in very nearly 40–65% of uninfected peoples. many cases of oral candidiasis and mucor mycosis and have been accounted for recently inCOVID-19 patients, and it may raise the risks of morbidity and mortality [4-6]. Candida albicans is the most common cause of oral candidiasis, which is a common opportunistic infection of the oral cavity. Candida infection is more common in certain age groups and has been linked to a wide range of risk factors for the development of the infection [7]. These include acute candidiasis, chronic candidiasis, and the angular cheilitis. Smoking, diabetes, Cushing's syndrome, cancer and immune diseases are all variables that might increase your risk [8]. Other risk factors include drugs, a high-carbohydrate diet, dentures and extremes of life. It is important to obtain a patient's history, do an examination, and prescribe the proper antifungal medicine while also anticipating the need for further laboratory testing as part of the treatment and management process. Select high-risk groups benefit from antifungal prophylaxis by reducing the prevalence and severity of infections. In an incredible majority of cases, the prognosis is favorable.

2. Methodology

Collection of Specimens

100 samples were collected from AL-Ramadi Teaching Hospital in Ramadi City and Heet Hospital. Oral samples were taken from suspected Covid-19 patients and blood samples\. Covid-19 and oral fungal disease occurs in people of all ages and both sexes between the ages of 1and 83 years. Using sterilized cotton swaps samples were taken. The swapes was inoculation on SDA culture media for isolation of pathogenic fungi and blood analysis is done to identification of different parameters.

3. Sterilization methods

Dry heat sterilization oven was used to sterilize glassware at 160-1800C for (2-3) hours while moist thermal sterilization was employed to disinfect the media and certain solutions (not impacted by heating).

Laboratory Diagnosis

Culture Incubation

All clinical samples of suspected lesions were infected with chloramphenicol on SDA Cycloheximide supplementation intermediate using a sterile inoculation ring and incubated at 25-30°C before being assessed. Negative cultures were after 4 weeks, whereas positive cultures Fig.(.) were inspected by macroscopic and microscopic identification.

Identification of Fungal Isolates

Macroscopic and microscopic analysis of culture isolates were used to discriminate between the fungi. The thorough examination is defined by the time of growth and is one of the types that isolates pigment producing cells in the absence of pigment dependent surface morphology in the medium. The growth of fungus using lactophenol cotton blue dye is examined microscopically. Nature assisted in the formation of the fungus and conidia (large and small conidia), Distinguish between these groups.

Direct Microscope Examination

Microscopy is used to make the first diagnosis. The swap taken from growing culture and placed on a clean slide with 10% KOH added and a clean lid on top. Several times, the slide was passed through the flame of burner, then 5-30 minutes of incubation, and finally each slide was cleaned with light. Look for fungal hyphae,

Molecular Identification

DNA extraction method

Fungal DNA was extracted by using the Fungi/Yeast Genomic DNA Isolation Kit Provided by Norgen Biotek.

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Methods of PCR for detection of specific genes

Primers Solutions

The primers were designed based on the National Center for Biotechnology Information NCBI and provided by the bioneer Company as a lyophilized product of various concentrations of picomol. Solution Final concentration of 10 pmol/µl was prepared separately by dissolving 10µl of stock solution for each primer and added to 90µl free nuclease distilled water un-ionic(ddH2O), mixed well and kept in (-20oC). They were mixed by vortex to homogenize before use. The sequences used in the study for (ITS gene) listed in Table (1).

Table (1): Sequence of PCR primer and molecular size of PCR products

Gene		Sequence of forward and reverse (Primer 3/-5/)	TM(C0)	Product (bp)	Reference
ITS	F	TCCGTAGGTGAACCTGCGG	52	550	[74]
	R	TCCTCCGCTTATTGATATGC			

Preparation PCR mixture

25µl of PCR reaction was composed of green master mix (Promega), primer solution, deionized water and template DNA with the following volume the PCR mixture used in the study

Table:(2) PCR mixture used in the current study.

NO: Content of reaction mixture	Volume of reaction mixture for a single tube.	
1	Green master mix	12.5 µl
2	DNA template	3.5µl
3	Forwar primer (10 Picomol)	1 µl
4	Reverse primer (10 Picomol)	1µl
5	Nuclase free water	7 µl
	Total volume	25µl

4. Results and Discussion

Primary Culture Results

The whole 100 of the clinical samples, which was collected from the oral area of covid-19 patient, the grew fungi in the SDA culture, including 87 *Candida spp.,* 13 *Aspergillus spp.*Figar(4.1) According to the study, the highest relative percent occurrence was Candida sp., followed by *Aspergillus spp*.Figar(1), and our results go with the outcomes of the researcher

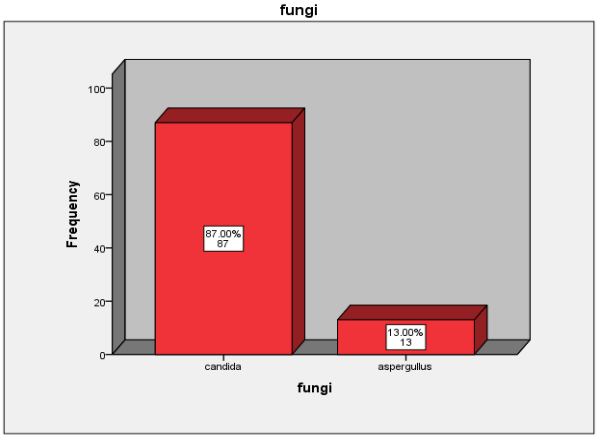
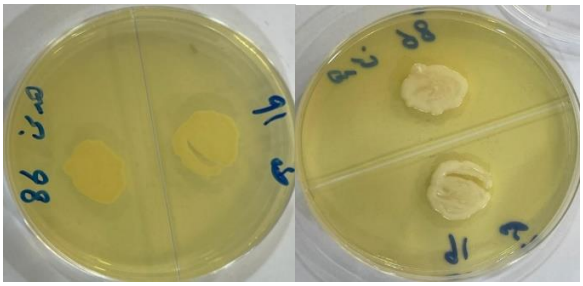


Figure 1: Frequency and Percentage of candida spp. and aspergillus spp. Infection in Covid-19 Patients.



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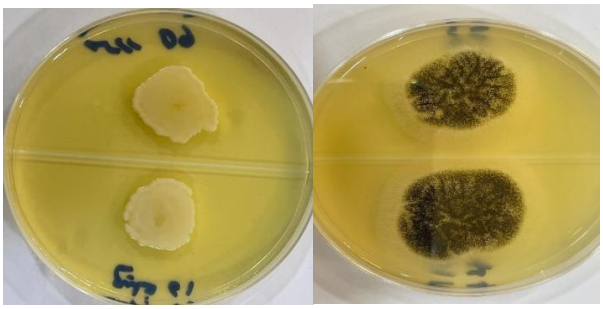

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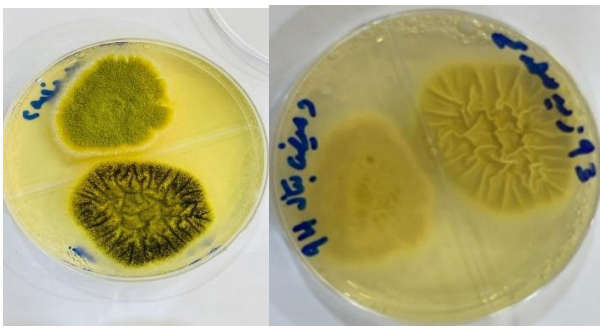


E F

Fig. (2 Fungal macroscopic examination on SDA (A,B,C: Candida; D,E,: Aspergillus; and F: Dorsal view for Aspergillus)

Secondary culture on CHROM Agar result

The positive culture on the SDA with candida was 87 sample, and when cultured on CHROOM AGAR to determine the species of candida, the result was 64 sample is *Candida albicans*, which had 73.56% of the total number, and 23 sample *Candida tropicalis*, which had 26.44% of the total number of candida, and our results go with the outcomes of the Iranian researcher), Hallur et al. [8] Which found the highest percentage is the Candida albicans while the Candida tropicalis with low percentage, But he found more other species of Candida

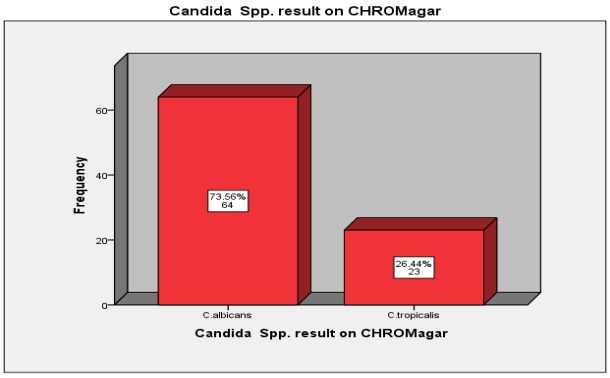


Figure 3 Frequency and percentage of candida spp. In covid-19 patients

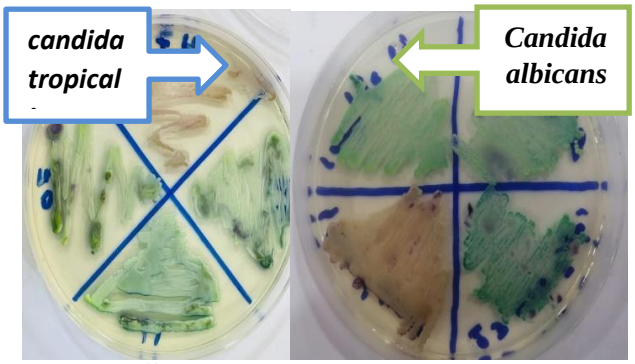
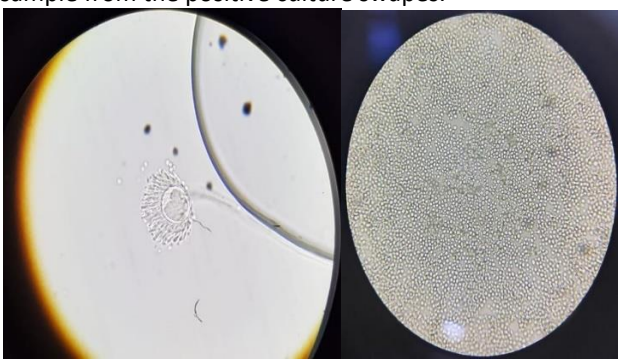


Fig.(4) Differentiation Between candida Spp. on the basis of the specific color colonies.

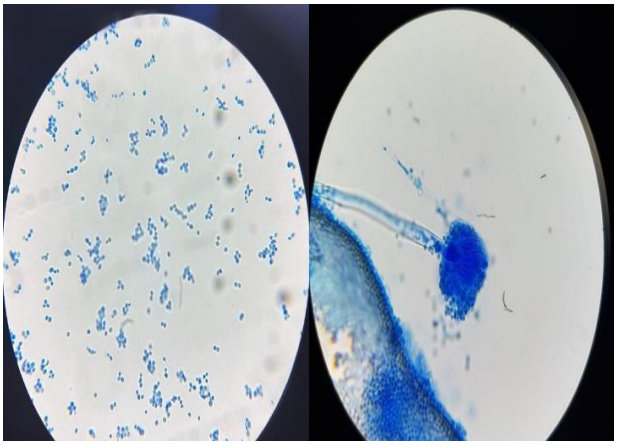
Microscopic Examination of Specimens

Culture's positive examples There were 100 positive cases (*Aspergillus spp.* and *Candida spp.*) also with 100 positive cases using a direct KOH 10% and also with Lactophenol Cotton Blue, which this sample was taken directly from the positive culture fungi, why? Because they not examine directly from the mouth, they examine after taken the sample from the positive culture swapes.



A B

Fig. (5 Direct Microscope Examination using 10% KOH diagnosis (A: Aspergillus, B: Candida).



A B

Fig (6) microscopically examination fungal spores and other fungal structures with Lactophenol Cotton Blue. (A: candida, B: Aspergillus)

Germ tube test for diagnosis for candida

The 86 sample of candida was tested with gem tube test to confirm the diagnosis of the fungi, the results were positive for all samples as confirmation of the culture results and the results of the microscopic examination.

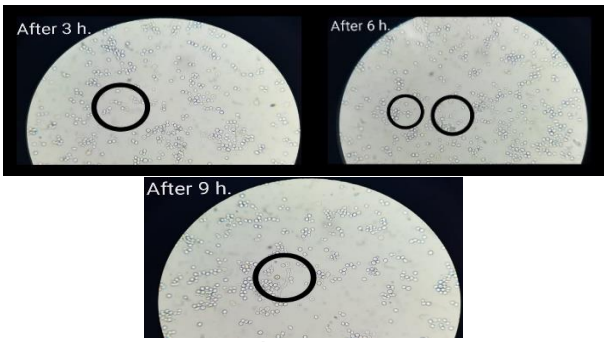


Fig.7) Germ tube test for candida diagnosis, it tested after 3 h and tested again after 6 h and finally after 9 h

The Percentage and frequency of oral fungal infection in Males and Females. males had a positive infection rate of 56 percent, while females had a positive infection rate of 44%, according to the findings. Males had an 87.5 percent of candida infections in and 12.5 percent of aspergillus cultural examination rate, while females had an 85.71 percent of candida and 14.29 of aspergillus of cultural examination rate. In this research, the incidence of oral fungi between females and males was close, our results go with the outcomes of researcher

Table 3 The Percentage and frequency of oral fungal infection in Males and Females

		Frequency	Percent
gender	female	44	43.6
	male	56	55.4
	Total	100	99
Fungal infection in female	candida	36	85.71
	aspergillus	6	14.29
	Total	42	100.0
Fungal infection in male	candida	49	87.5
	aspergillus	7	12.5
	Total	56	100.0

The frequency of fungi depending on Rural and Urban Settings

The oral fungal infections were 43 cases in rural areas and their communities, and 57 cases of infection in urban areas, as shown in Table 4.4, The percent of aspergillus was 14% in rural and 11.6% in urban while the percent of candida was 86% in rural and 88.4% in urban. The results were close between rural and urban Covid-19 patients.

Table(4) Fungal infection among rural and urban

		Frequency	Percent
Fungal infection depending on residence	Rural	43	43.0
	Urban	57	57.0
	Total	100	100.0
Fungal infection in rural	Aspergillus	8	14.0
	Candida	49	86.0
Fungal infection in urban	Aspergillus	5	11.6
	Candida	38	88.4

Table 5 Frequency of Mycotic Infections Depending on Age Group

Age Group	Frequency	Percent
<= 20	40	40.0
21-41	33	33.0
42-62	15	15.0
63+	12	12.0
Total	100	100.0

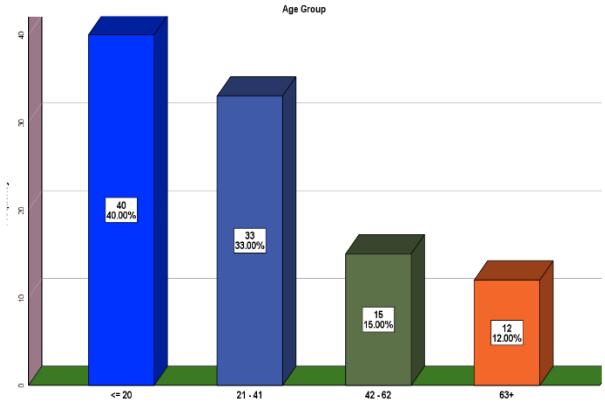


Figure 8. Frequency of Mycotic Infections Depending on Age Group.

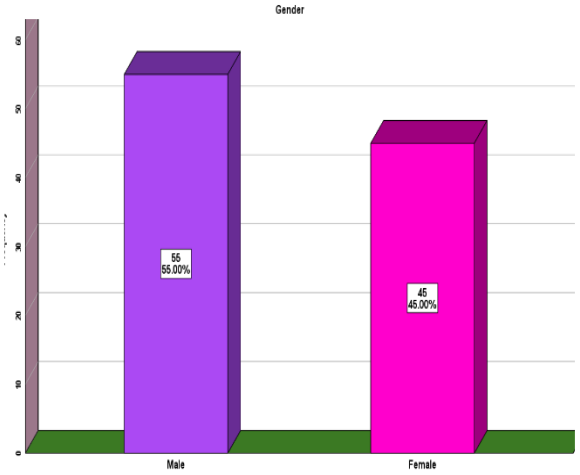


Figure 9 Frequency of Mycotic Infections Dependent on Gender

Table 6 Frequency of Mycotic Infections Dependent on Residence		
Residence	**Frequency**	**Percent**
Urban	57	57.0
Rural	43	43.0
Total	100	100.0

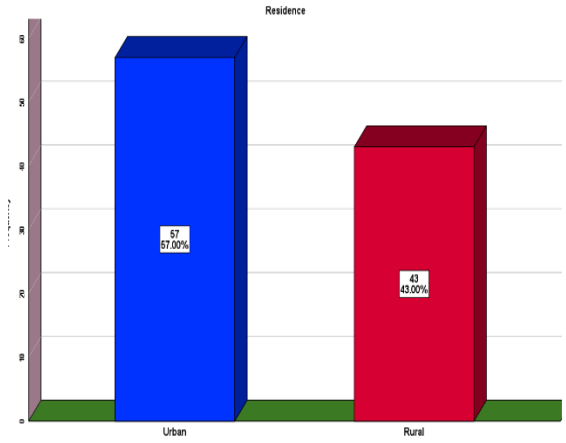


Figure 10. Frequency of Mycotic Infections Dependent on Residence

Table7 Frequency of Fungal infection depending on smoking		
Smoking	**Frequency**	**Percent**
No	77	77.0
Yes	23	23.0
Total	100	100.0

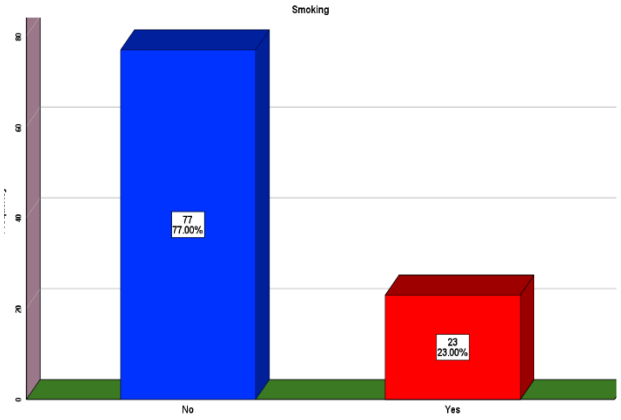


Figure 11 Frequency of Fungal infection depending on smoking

Table 12. Frequency of Mycotic Infections Dependent on D.M.		
D.M	**Frequency**	**Percent**
NO	96	96.0
Yes	4	4.0
Total	100	100.0

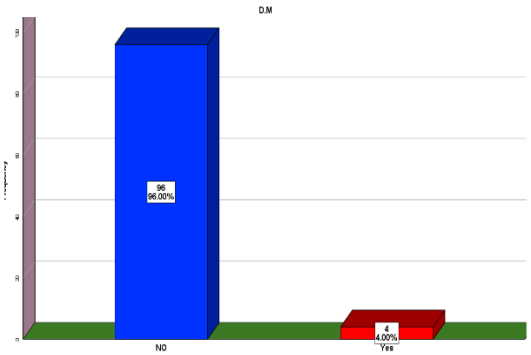


Figure 12. Frequency of Mycotic Infections Dependent on D.M.

Table13 Frequency of fungus types in covid-19 patients		
Fungus type	**Frequency**	**Percent**
Aspergillus	13	13.0
Candida	87	87.0
Total	100	100.0

Molecular study

DNA extracted from 30 samples was analyzed by gel electrophoresis (Figure 4.8) and DNA fragment coding for fungal ITS gene was amplified from the genomic DNA of candida. Gel electrophoresis analysis showed the bands of the amplified gene with the predicted size of 550 bp (Figure 4-9).

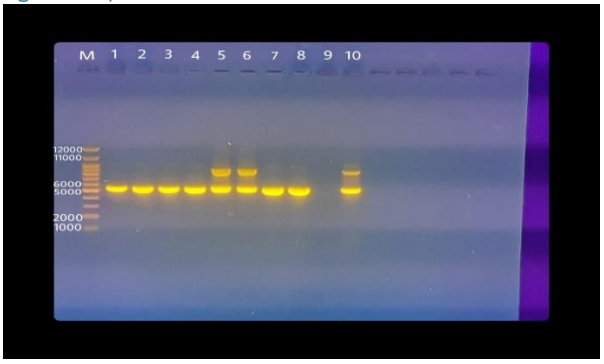


Figure 13 Gel electrophoresis of genomic DNA extraction, 1% agarose gel

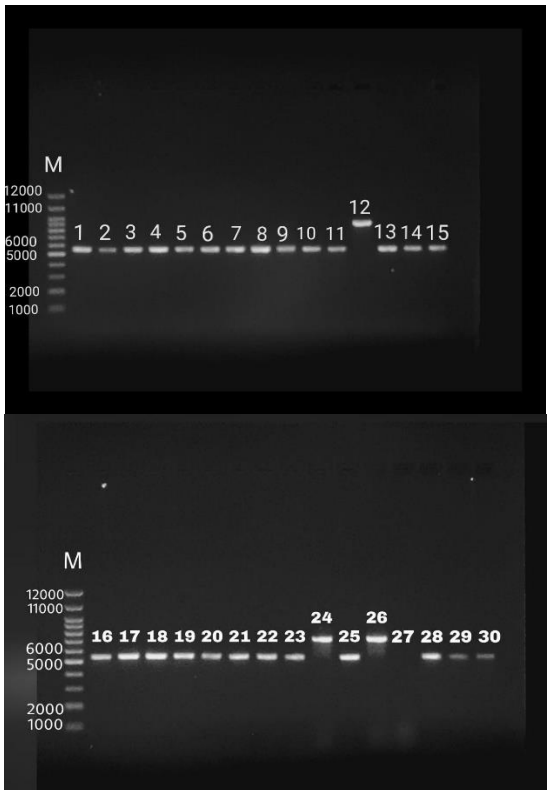


Figure (14) Gel electrophoresis showing PCR product with band size of 550 bp. The products were run on 2% agarose. N: DNA ladder (1000 plus)

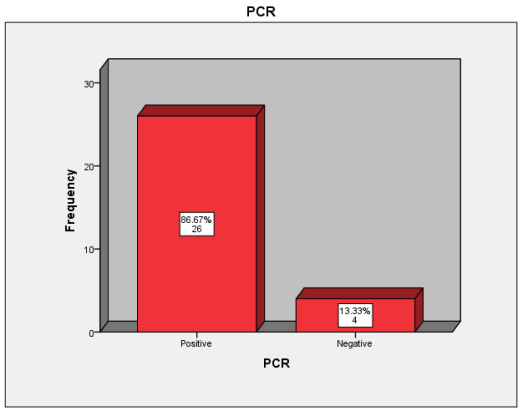


Figure 15 PCR result of candida diagnosis

Table14PCR result of candida diagnosis		
PCR	**Frequency**	**Percent**
Negative	4	4.0
Positive	26	26.0
Total	30	30.0

5. Discussion

Among various factors leading to morbidity and mortality in COVID-19 patients, opportunistic bacterial or fungal infections could deteriorate the status of patients and leading to ARDS.

In the first week of severely ill patients which infected with COVID-19 admission, we observed many cases of oral candidiasis (OC), particularly those hospitalized in (ICU) with low lymphocyte counts, and some critically ill COVID-19 patients with high level of lactate dehydrogenase (LDH), especially those undergoing immunosuppressive or corticosteroid therapies. And the same result is mentioned in other some researches. [9].

Remarkably, lymphopenia was the main laboratory finding in 85% of critically ill patients with COVID-19[10]. Since the lymphocytes play a decisive role in maintaining immune homeostasis and defensive response against microbial invasion throughout the body, it might be hypothesized that inadequate lymphocyte count may be a key factor contributing to secondary fungal infections such as OPC and PJP in COVID-19 patients [11]. corticosteroid therapy, i.e., hydrocortisone, dexamethasone, and methyl prednisolone, may raise the risk of secondary fungal infections in these patients.

Thus, it seems that, coronavirus infection itself might not increase the risk for fungal infections, but other risk factors might have. Besides, using broad-spectrum antibiotics, either empirically or targeted therapy for super-infection in patients with severe COVID-19 raises the odds of fungal infections due to endogenous fungi such as *Candida* species. Meanwhile, it comes as no surprise that frequent antibiotics usage of meropenem or moxifloxacin in severely ill COVID-19 patients are effective against wide spectrum of bacteria, undoubtedly impair the balance of microorganisms resident in mucosal area, leading to further oral health complication [12].

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