Case-controlled Study

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Assessment of species distribution and virulence factors of oral fungal carriage among hospitalized patients with COVID-19: a case-control study

Zahra Ramezanalipour, MSc[a], Seyed Jamal Hashemi, PhD[a,*], Roshanak Daie Ghazvini, PhD[a], Mohammad Shenagari, PhD[b], Meysam Sharifdini, PhD[c], Hamid Salehiniya, PhD[h], Mohammad-Hossein Keivanlou, MD[e,f], Keyhan Ashrafi, PhD[c], Davoud Roostaei, PhD[d], Fariborz Mansour Ghanaei, MD, AGAF[f,g], Elahe Sasani, PhD[i], Zahra Rafat, PhD[c,*]

Background: The COVID-19 pandemic highlighted the need to study oral fungal carriage and its potential impact. In oral fungal environments, factors like changes in respiratory epithelium, increased pathogen attachment, local inflammation, and virulence factors could influence COVID-19 severity. The authors conducted a study to explore oral fungal carriage in COVID-19 patients and compare it to a healthy control group.

Methods: The authors executed a case-control investigation including 144 COVID-19 patients and an equivalent number of 144 healthy controls. The matching criteria encompassed age, sex, body mass index, and the history of antibiotic and antiviral medication intake. This research was performed over a span of 12 months from May 2021 to May 2022. The mouth area was sampled with a cotton-tipped swab. Subsequently, all the samples underwent fungal culture and PCR-sequencing procedures.

Results: In COVID-19 patients, oral fungal carriage was three times higher compared to healthy controls. Candida was the exclusive genus found in both groups, with Candida albicans being the most frequently isolated species (90.79%). Among COVID-19 patients, Candida species showed significantly higher esterase, proteinase, and hemolysin activity compared to healthy individuals. Both groups exhibited elevated levels of C. albicans virulence factors compared to non-albicans species.

Conclusions: It is crucial to understand the way that virulence factors of oral fungal carriage act in COVID-19 patients in order to come up with novel antifungal medications, identify the contributing factors to drug resistance, and manage clinical outcomes.

Keywords: Candida species, COVID-19, hemolysin factor, oral cavity, proteinase

[a]*Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Departments of* [b]*Medical Microbiology,* [c]*Medical Parasitology and Mycology,* [d]*Pharmacology,* [e]*Student Research Committee, School of Medicine,* [f]*Gastrointestinal and Liver Diseases Research Center,* [g]*GI Cancer Screening and Prevention Research Center, Guilan University of Medical Sciences, Rasht,* [h]*Social Determinants of Health Research Center, Birjand University of Medical Sciences, Birjand and* [i]*Infectious and Tropical Diseases Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran*

S.J.H., Z.R. contributed equally to this work as corresponds.

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**Corresponding author. Address: Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Tel.: +98 214 293 3141, fax: +982 1 88 951 392. E-mail: Sjhashemi@tums.ac.ir (S.J. Hashemi); Department of Medical Parasitology and Mycology, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran. Tel.: +981 333 690 921, Fax: +981 333 690 036. E-mail rafat.zahra2015@gmail.com, dr.zahra-rafat@gums.ac.ir (Z. Rafat).*

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Introduction

The oral microbiome, consisting of symbiotic bacteria (bacteriome) and fungi (mycobiome), along with viruses, archaea, and protozoa, is the second-largest microbiome in the human body after the gastrointestinal tract. It profoundly affects human health by regulating the immune system, metabolism, allergies, inflammation, and combating various diseases[1].

The human oral cavity starts as a sterile environment at birth. However, in newborns, elements of the oral mycobiome are acquired from the mother's vaginal microbiome during delivery or through breastfeeding, while others are gathered from the surrounding environment. As a result, the oral fungal composition of each person can be up to 80–90% distinct from that of others[2,3].

COVID-19 is a notable factor affecting the oral fungal population[1]. As of July 2022, the ongoing COVID-19 pandemic, stemming from the SARS-CoV-2, had a profound global impact, affecting an estimated 551 million individuals and resulting in over 6 million fatalities. In Iran, 7 241 648 people were affected by the virus, with 141 408 losing their lives to the disease[4].

In healthy individuals, fungal species belonging to the genera *Candida*, *Trichosporon*, *Geotrichum*, *Rhodotorula*, *Cryptococcus*, and *Aspergillus* form the main population of fungi in the oral cavity[5]. Oral fungal carriage refers to the harmless presence of

fungi, such as *Candida* species, as a part of the normal oral microbiome[6]. The oral mycobiome and fungal carriage in COVID-19 patients can differ from healthy individuals due to factors like invasive treatments, the virus's impact on the immune system, and multidrug therapies[7].

Studies have shown that the state of oral mycobiome and the severity of COVID-19 are directly related[8,9]. Studies also investigated the gastrointestinal mycobiome in patients with COVID-19[10]. However, to our knowledge, there were no prior investigations into the oral fungal carriage in COVID-19 patients compared to healthy individuals up to the point of this research.

During COVID-19 infection, physiological changes in the oral cavity, stemming from factors like medication misuse, compromised immunity, vascular issues, inflammation, and poor oral hygiene during treatment, contribute to oral fungal colonization. This disruption can lead to oral fungal infections[7,11]. The virulence characteristics of these organisms, such as the release of esterase, proteinases, and haemolytic abilities that are necessary to colonize and invade human organs, might nevertheless create oral mycoses in COVID-19 patients in addition to host-related causes[11].

It is noteworthy that each of the fungal species that make up the oral mycobiome has different virulence factors[12]. However, these determinants of pathogenicity remain mainly unexplored for oral fungal carriage.

Our primary objective in this case-control study was to fill this knowledge gap by investigating the prevalence and species distribution of oral fungal carriage in individuals with COVID-19. Additionally, we aimed to analyze the virulence factors exhibited by these isolated fungal agents in comparison to those from healthy control subjects.

HIGHLIGHTS

- To investigate the prevalence of oral fungal carriage among individuals with COVID-19.
- To investigate the distribution of oral fungal carriage among individuals with COVID-19.
- Evaluating the virulence factors of isolated fungal agents in comparison to a healthy control group.

Materials and methods

Ethics statement

We have announced the ethics information at the end of the article. All participants provided informed consent before taking part in the study. In addition, we reported the bases of our manuscript in line with the STROCSS criteria[13].

Patients, sampling and data collection

This study was designed as a matched-pair case-control investigation involving adult patients who were hospitalized. Over a duration of one year, from Thursday, 27 May 2021 to Friday, 27 May 2022, a total of 144 hospitalized patients with confirmed positive results for COVID-19 through real-time polymerase chain reaction (PCR) testing were carefully matched with 144 control individuals who tested negative for COVID-19 using real-time PCR. Age (±5 years), gender, BMI, underlying health conditions such as the history of treatment with broad-spectrum antibiotics, treatment with antivirals, history of ICU admission, mechanical ventilation, and corticosteroid therapy were the factors that they were matched for, and all the necessary information was extracted from patient's health records. At the time of sample collection, all patients had received solely the first shot of their COVID-19 vaccine ,ranging from a minimum of two months to a maximum of four months prior to hospitalization.

Patients admitted to the hospital who tested positive for COVID-19 using real-time PCR on the third day of hospitalization were eligible for inclusion in the case group. Additionally, for each COVID-19-positive patient (case), one of their family members residing with them (to ensure similar oral hygiene condition) and having a confirmed negative COVID-19 result based on real-time PCR was selected as a participant in the control group. To avoid potential confounding factors, patients who had received systemic antifungal drugs for treating fungal infections within the last month were excluded from the study. This exclusion criterion was implemented to prevent the interference of antifungal medication with the analysis of oral fungal carriage. Furthermore, patients presenting with oral lesions suggestive of oral candidiasis were excluded from the study to focus solely on the normal oral fungal carriage and minimize the influence of existing oral infections on the research outcomes.

Both the case and control groups comprised individuals without dentures, dental implants, orthodontic wires, or other prosthetic devices. Additionally, all participants had optimal oral and dental hygiene.

The mouth area was sampled by a cotton-tipped swab moistened with sterile serum physiology. The samples were taken from cases on the third day after hospitalization. All swabs were immediately transported to the laboratory in sterile tubes, cultured on Sabouraud Chloramphenicol Agar less than an hour from collection (SC, Merck, Germany), and incubated at 30 °C for 3 weeks. Any growth obtained from the fungal cultures was further identified by colony morphology, its rate of growth, and Lactophenol cotton blue (LCB) mounts. Consequently, molecular methods were performed on isolated colonies.

(Performing fungal culture before molecular analysis allows for the isolation and identification of specific fungal genera and species. By obtaining pure cultures, we could perform a more targeted and in-depth analysis of the isolated fungal species since the primary samples might get destroyed quickly. So this would allow repeated analysis. While it provides valuable insights into the oral cavity, it may not capture the entire fungal diversity in it. Eventually, combining both fungal culture and molecular analysis provides a comprehensive understanding of the oral fungal carriage in the study's context.)

Molecular technique

DNA extraction

Genomic DNA was extracted from colonies cultivated on Sabouraud dextrose agar (SDA) utilizing the high pure PCR template purification kit (GeneAll, Seoul, South Korea), following the manufacturer's suggested guidelines.

PCR conditions and sequencing

For each isolate, PCR amplification was carried out using universal primers, namely ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′), which binds to the end of 18S rDNA, and ITS4 (5′TCC

TCC GCT TAT TGA TAT GC 3), which binds to the beginning of 28S rDNA (Life Technologies, Barcelona, Spain). PCR products were subjected to single-direction sequencing using a forward primer (Bioneer, South Korea). The identification of each isolate's species was accomplished by comparing their sequences to reliable sequences from GenBank through the basic local alignment search tool available at the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To enhance traceability, all sequences were deposited in GenBank and are associated with accession numbers, as detailed in Table 1.

Determining hemolysin factor

Hemolysin assay for *Candida* strains was carried out based on a former validated protocol by Luo *et al*.[14] "In brief, SDA supplemented with 6% human blood and 3% glucose (pH = 5.6) was

Table 1

The results of molecular identification and GenBank accession numbers of DNA sequences included in this study

	Molecular identification			Molecular identification	
Isolate	(IT'S gene)	GenBank accession number	Isolate	(ITS gene)	GenBank accession number
1	*Candida tropicalis*	MT772038	77	*Candida albicans*	MT773003
2	*Candida glabrata*	MT772039	78	*Candida albicans*	MT773004
3	*Candida albicans*	MT772040	79	*Candida albicans*	MT773005
4	*Candida tropicalis*	MT772041	80	*Candida albicans*	MT773006
5	*Candida glabrata*	MT772042	81	*Candida albicans*	MT773007
6	*Candida albicans*	MK793223	82	*Candida albicans*	MT773008
7	*Candida albicans*	MT772043	83	*Candida albicans*	MT773009
8	*Candida albicans*	MT772044	84	*Candida albicans*	MT773010
9	*Candida glabrata*	MT772045	85	*Candida albicans*	MT773011
10	*Candida albicans*	MT772046	86	*Candida albicans*	MT773012
11	*Candida tropicalis*	MK793225	87	*Candida albicans*	MT773013
12	*Candida albicans*	MT772047	88	*Candida albicans*	MT773014
13	*Candida albicans*	MT772048	89	*Candida albicans*	MT773015
14	*Candida albicans*	MT772049	90	*Candida albicans*	MT773016
15	*Candida albicans*	MT772050	91	*Candida albicans*	MT773017
16	*Candida albicans*	MT772051	92	*Candida albicans*	MT773018
17	*Candida albicans*	MT772052	93	*Candida albicans*	MT773019
18	*Candida albicans*	MT772053	94	*Candida albicans*	MT773020
19	*Candida albicans*	MH545928	95	*Candida albicans*	MT773021
20	*Candida albicans*	MT772054	96	*Candida albicans* MW573050	
21	*Candida albicans*	MT772055	97	*Candida albicans*	MT773022
22	*Candida albicans*	MT772056	98	*Candida albicans*	MT773023
23	*Candida glabrata*	MT772057	99	*Candida albicans*	MT773024
24	*Candida albicans*	MT772058	100	*Candida albicans*	MT773024
25	*Candida albicans*	MT772059	101	*Candida albicans*	MT773026
26	*Candida albicans*	MT772060	102	*Candida albicans*	MT773027
27	*Candida albicans*	MT772061	103	*Candida albicans*	MT773028
28	*Candida albicans*	MK547223	104	*Candida albicans*	MT773029
29	*Candida albicans*	MT772062	105	*Candida albicans*	MT773030
30	*Candida albicans*	MT772063	106	*Candida albicans*	MT773031
31	*Candida glabrata*	MT772064	107	*Candida albicans*	MT773032
32	*Candida albicans*	MT772065	108	*Candida albicans*	MT772033
33	*Candida albicans*	FN652301	109	*Candida albicans*	MT772034
34	*Candida albicans*	MT772066	110	*Candida albicans*	MT772035
35	*Candida tropicalis*	MT772067	111	*Candida albicans*	MT772036
36	*Candida albicans*	MT772068	112	*Candida albicans*	MT772037
37	*Candida albicans*	MK138363	113	*Candida albicans*	MT772038
38	*Candida albicans*	KC905069	114	*Candida albicans*	MT772039
39	*Candida glabrata*	MT772072	115	*Candida albicans*	MT772040
40	*Candida albicans*	MT772073	116	*Candida albicans*	MT772041
41	*Candida albicans*	MT772074	117	*Candida albicans*	MT772042
42	*Candida albicans*	MT772075	118	*Candida albicans*	MT772043
43	*Candida glabrata*	MT772076	119	*Candida albicans*	MT772044
44	*Candida albicans*	MT772077	120	*Candida albicans*	MT772045
45	*Candida albicans*	MT772078	121	*Candida albicans*	MT772046
46	*Candida albicans*	FJ515204	122	*Candida albicans*	MT772047
47	*Candida glabrata*	KU992392	123	*Candida albicans*	MT772048
48	*Candida albicans*	MT772079	124	*Candida albicans*	MT772049
49	*Candida albicans*	MT772080	125	*Candida albicans*	MT772050
50	*Candida albicans*	MT772081	126	*Candida albicans*	MT772051
51	*Candida albicans*	MT772082	127	*Candida albicans*	MT772052
52	*Candida albicans*	AM492797	128	*Candida albicans*	MT772053
53	*Candida albicans*	MT772083	129	*Candida albicans*	MT772054
54	*Candida albicans*	MT772084	130	*Candida albicans*	MT772055
55	*Candida albicans*	MT772085	131	*Candida albicans*	MT772056
56	*Candida albicans*	MT772086	132	*Candida albicans*	MT772057
57	*Candida albicans*	MT772087	133	*Candida albicans*	MT772058
58	*Candida albicans*	MT772088	134	*Candida albicans*	MT772059
59	*Candida albicans*	MT772089	135	*Candida albicans*	MT772060
60	*Candida albicans*	MF614723	136	*Candida albicans*	MT772061
61	*Candida albicans*	AY939810	137	*Candida albicans*	MT772062
62	*Candida albicans*	MT772090	138	*Candida albicans*	MT772063
63	*Candida glabrata*	MT772091	139	*Candida albicans*	MT772064
64	*Candida albicans*	MT772092	140	*Candida albicans*	MT772065
65	*Candida albicans*	MF614725	141	*Candida albicans*	MT772066
66	*Candida albicans*	MT772093	142	*Candida albicans*	MT772067
67	*Candida albicans*	MT772094	143	*Candida albicans*	MT772068
68	*Candida albicans*	MT772095	144	*Candida albicans*	MT772069
69	*Candida albicans*	MT772096	145	*Candida albicans*	MT772070
70	*Candida albicans*	MT772097	146	*Candida albicans*	MT772071
71	*Candida albicans*	MT772098	147	*Candida albicans*	MT772072
72	*Candida albicans*	MT772099	148	*Candida albicans*	MT772073
73	*Candida albicans*	MT773000	149	*Candida albicans*	MT772074
74	*Candida glabrata*	LC317498	150	*Candida albicans*	MT772075
75	*Candida albicans*	MT773001	151	*Candida albicans*	MT772076
76	*Candida albicans*	MT773002	152	*Candida albicans*	MT772077

ITS, internal transcribed spacer.

used to determine the hemolysin production. Suspension of yeast (10^6 cells/ml) was prepared in saline solution and 10 µl was spot inoculated on human blood agar plates, incubated at 37 °C in 5% CO2 for 5 days. Then, we examined the plates and determined the haemolytic index (Hz value) as the ratio of the diameter of the colony to that of the translucent zone of haemolysis (mm). The interpretation of the results was as follows: high activity less than or equal to 0.59; medium activity 0.6–0.79; low activity 0.8–0.99; no activity 1[15]. *C. albicans* (ATCC 14053) was used as the positive control, while *Candida parapsilosis* (ATCC 22019) was used as the negative control. The assay was performed in duplicate on three separate occasions for each isolate."

Determining proteinase activity

The proteinase activity of the isolates was assessed, according to Staib *et al*.[16] "The fungal suspension was prepared from overnight cultures, of which 10 µl containing 1×10^6 *Candida* cells/ml was used to inoculate the bovine serum albumin (BSA) agar plate, composed of BSA solution 1%, dextrose 2%, KH2PO4 0.1%, MgSO4 0.05%, and agar 2%. We incubated the plates at 37 °C for 72 h. Thereafter, we used 20% trichloroacetic acid to fix the plate for 15 min and then stained it with 1.25% amido black for 30 min. Finally, we used 15% acetic acid to decolorize the setup before determining the zone (Pz) around the colonies. The classification of proteinase activity was represented for the hemolysin activity. *C. albicans* (ATCC 14053) was used as the positive control, while *Candida glabrata* (ATCC 90030) was used as the negative control. The assay was performed in duplicate on three separate occasions for each isolate."

Determining esterase activity

The esterase activity of the isolates was measured using the Tween 80 opacity test, according to Slifkin *et al*.[17] "To 1000 ml of distilled water, 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2$, and 15 g agar were dissolved; pH adjusted to 6.8 and then autoclaved. Then, 5 ml of sterile Tween 80 was added to the media after cooling (at 50 °C) and dispensed into 90 mm plates. Ten microliters of fungal suspension (1×10^6 cells / ml) were spotted on each plate and incubated for two days at 37 °C. The classification of esterase activity was represented by the hemolysin activity. *C. albicans* (ATCC 14053) was used as a positive control. The assay was performed in duplicate on three separate occasions for each isolate."

Statistical analysis

The data analysis was performed using SPSS software (V.24). The study was assessed using standard χ^2 and 95% CI. Statistically, *P* value less than 0.05 was considered as a significant difference or correlation. We used simple frequencies to describe the virulence factors of the isolates. Continuous variables were compared using the student's t test. Data analysis was conducted using SPSS software (V.24). The study's evaluation utilized standard χ^2 tests and 95% CI. A statistically significant difference or correlation was considered when the *P* value was less than 0.05. To define the virulence factors of the isolates, simple frequencies were employed. Additionally, continuous variables were compared using the student's *t* test.

Results

This study enroled 144 cases with positive COVID-19 real-time PCR tests, including 62 (43.05%) men and 82 (56.95%) women, and 144 matched controls. The mean (SD) age of cases and controls were 52 (14.913) and 50 (14.791) years, respectively ($P = 0.474$) (Table 2). Cases and controls were matched for age, sex, BMI, underlying conditions, and a record of taking antibiotics, systemic steroids, immunosuppressive, and antiviral medications. Analyzing the isolated fungal species from COVID-19 patients, *C. albicans* was the most frequently isolated species, accounting for 90.91% ($n = 100$) of the cases, followed by *C. glabrata* at 5.45% ($n = 6$), and *C. tropicalis* at 3.64% ($n = 4$). Similarly, in the healthy control group, *C. albicans* dominated as the most frequently isolated species, representing 90.48% ($n = 38$), with *C. glabrata* as the second most common at 9.52% ($n = 4$). The results highlighted a substantial difference between the COVID-19 patients and the control group in terms of the prevalence of oral fungal carriage. The prevalence was found to be three times higher in COVID-19 patients in comparison with healthy controls ($P < 0.01$, not demonstrated in Table).

Table 2

Demographic characteristics, underlying conditions, medications, and clinical symptoms in the two groups studied

Variable	Cases ($n = 144$)	Controls ($n = 144$)	P[a]
Age (years), Mean ± SD	52 ± 14.913	50 ± 14.791	0.474
Sex, *n* (%)			
Male	62 (43.05)	65 (45.1)	0.615
Female	82 (56.95)	79 (54.9)	
BMI			
Normal (18.5–24.9 kg/m^2)	63	54	
Overweight (25–29.9 kg/m^2)	40	32	0.912
Obese—Class 1 (30–34.9 kg/m^2)	21	29	
Obese—Class 2 (35–39.9 kg/m^2)	20	29	
Obese—Class 3 ($\geq$ 40 kg/m^2)			
Underlying conditions, *n* (%)			
Diabetes	36 (0.25)	20 (13.88)	0.391
Malignancy	24 (16.66)	4 (2.77)	0.085
Smoking	24 (16.66)	16 (11.11)	0.643
Heart disease	20 (13.88)	4 (2.77)	1.000
Chronic kidney disease	16 (11.11)	12 (8.33)	0.347
ICU admission	12 (8.33)	4 (2.77)	0.404
Pregnancy	8 (5.55)	0	0.560
Intubation	4 (2.77)	0	0.316
Medications, *n* (%)			
Systemic steroids	74 (51.38)	20 (13.88)	0.733
Broad-spectrum antibiotics	8 (5.55)	3 (2.08)	0.052
Systemic antifungals	1 (0.69)	0	0.316
Immunosuppressive drugs	74 (51.38)	20 (13.88)	0.408
Clinical symptoms			
Dry cough	144 (100)	48 (33.33)	0.00
Fatigue	128 (88.88)	60 (41.66)	0.00
Persistent fever	124 (86.11)	56 (38.88)	0.00
Chills	104 (72.22)	52 (36.11)	0.00
Body aches	88 (61.11)	56 (38.88)	0.01
Sweating	72 (50)	44 (30.55)	0.02
Abdominal pain	56 (38.88)	44 (30.55)	0.02
Diarrhoea	40 (27.77)	20 (13.88)	0.40
Sore throat	32 (22.22)	24 (16.66)	0.08
Loss of taste	24 (16.66)	28 (19.44)	0.01
Nasal congestion	20 (13.88)	28 (19.44)	0.01
Runny nose	12 (8.33)	8 (5.55)	0.03

[a]Statistically significant difference between groups ($P < 0.05$).

Table 2 shows the distribution of clinical symptoms, underlying health conditions, and medications in case and control groups. Statistical analysis of the results showed that there was no significant relationship between the prevalence of oral fungal carriage and the ICU admission ($P = 0.064$), intubation ($P = 0.13$), diabetes ($P = 0.07$), chronic kidney disease ($P = 0.37$), heart disease ($P = 0.09$), malignancy ($P = 0.08$), pregnancy ($P = 0.52$), or smoking ($P = 0.64$) in case group and these underlying conditions had no effect on the prevalence of oral fungal carriage in COVID-19 patients (*P* values not demonstrated in Table). Furthermore, the usage of systemic steroids ($P = 0.93$) and immunosuppressive medicines ($P = 0.80$) during the hospitalization, had no significant effect on the prevalence of oral fungal carriage in cases (*P* values not shown in Table). Also, there was no statistical difference between cases and controls based on the studied underlying conditions (for each $P > 0.05$) (Table 2).

Findings show that in the case group, the most prevalent symptoms were dry cough ($n = 144$, 100%), fatigue ($n = 128$, 88.88%), persistent fever ($n = 124$, 86.11%), and chills ($n = 104$, 72.22%) and the severity of these symptoms required hospitalization. When compared to the healthy controls, the prevalence of these clinical symptoms in COVID-19 patients was significantly higher (for each $P < 0.05$) (Table 2). However, the results indicated that there was no statistically significant correlation between the clinical symptoms and the prevalence of oral fungal carriage in COVID-19 patients ($P > 0.05$, not demonstrated in Table).

The association between the produced virulence factors and their varying degrees of activity in 152 clinical isolates of *Candida* spp. is illustrated in Table 3. Among the 138 *C. albicans* isolates from cases and controls, 76 isolates (55.07%) exhibited esterase activity. Notably, among these, 70 isolates (92.10%) were from cases, and only 6 isolates (7.89%) were from control subjects. On the other hand, 62 *C. albicans* isolates (44.93%) did not demonstrate esterase activity. For the 10 *C. glabrata* isolates from cases and controls, only 2 isolates (20%) from cases exhibited esterase activity. Similarly, among the 2 *C. tropicalis* isolates from cases, both showed esterase activity. The esterase enzyme's activity level was determined to be high in all *Candida* isolates with positive results for esterase activity. Furthermore, the mean activity of this enzyme was measured to be 0.54 in COVID-19 patients and 0.9 in the control group, and a statistically significant difference between the two groups was observed ($P = 0.000$) (Table 3). As indicated in Table 3, of the 138 *Candida* species isolates from cases and controls, the highest esterase activity was observed in *C. albicans* (n = 76, 50%), followed by *C. glabrata* and *C. tropicalis* (each $n = 2$, 1.31%).

Among the 68 *C. albicans* isolates that exhibited positive hemolysin activity, 56 isolates (82.35%) were associated with cases, and 12 isolates (17.65%) were from controls. Of the 6 *C. glabrata* isolates from cases, 5 isolates (83.33%) demonstrated hemolysin activity. However, in this study, no hemolysin activity was detected for *C. glabrata* species (0%) isolated from controls. Additionally, all 4 *C. tropicalis* isolates from cases (100%) displayed hemolysin activity. The hemolysin enzyme activity level was found to be high in all *Candida* isolates with positive results for hemolysin activity. In terms of mean Enzyme Activity Index (EAI) for hemolysin, patients with COVID-19 had a mean EAI of 0.56, while healthy controls had a mean EAI of 0.72 (Table 3). The results showed that the COVID-19 patients' hemolysin enzyme activity was substantially higher than that of the control group ($P = 0.033$).

Out of the 138 *C. albicans* isolates, 124 isolates (89.85%) showed positive proteinase activity, with 94 of these isolates (68.12%) originating from COVID-19 patients. However, none of the *C. glabrata* and *C. tropicalis* isolates from both patients and controls exhibited any activity of the proteinase enzyme. The mean EAI for proteinase in COVID-19 patients was 0.38, while in healthy controls, it was notably higher at 0.82 (Table 3). Compared to healthy controls, COVID-19 patients had considerably greater proteinase activity ($P = 0.001$).

Table 3

The relationship between the virulence factors produced and different levels of their activities among the *Candida* species isolated in the present study[a]

		COVID-19 patients (cases)[b]		Normal individuals (controls)[b]		Total[c]		Activity level[c]		
Virulence factor	*Candida* species	Positive	Negative	Positive	Negative	Positive	Negative	High	Medium	Low
Esterase activity	*C. albicans*	70	30	6	32	76 (55.07)	62 (44.93)	76 (100)	0 (0)	0 (0)
	C. glabrata	2	4	0	4	2 (20)	8 (80)	2 (100)	0 (0)	0 (0)
	C. tropicalis	2	2	0	0	2 (50)	2 (50)	2(100)	0(0)	0(0)
Mean EAI (mm)		0.54		0.9		*P*: 0.000				
Haemolytic activity	*C. albicans*	56	44	12	26	68 (49.27)	70 (50.72)	68 (100)	0 (0)	0 (0)
	C. glabrata	5	1	0	4	5 (50)	5 (50)	5 (100)	0 (0)	0 (0)
	C. tropicalis	4	0	0	0	4 (100)	0 (0)	4 (100)	0 (0)	0 (0)
Mean EAI (mm)		0.56		0.72		*P*: 0.033				
Proteinase activity	*C. albicans*	94	6	30	8	124 (89.85)	14 (10.15)	124 (100)	0 (0)	0 (0)
	C. glabrata	0	6	0	4	0 (0)	10 (100)	0 (0)	0 (0)	0 (0)
	C. tropicalis	0	4	0	0	0 (0)	4 (100)	0 (0)	0 (0)	0 (0)
Mean EAI (mm)		0.38		0.82		*P*: 0.001				

EAI, enzymatic activity index.

[a]*P* value < 0.5.

[b]Values are expressed as No.

[c]Values are expressed as No. (%).

Discussion

For more than a century, fungi have been recognized as commensal residents in the human oral cavity. The oral environment stands out as one of the most common colonization sites for fungi, and it's worth noting that the states of the oral and pulmonary mycobiome are closely interconnected[1,18]. Therefore, a change in the oral fungal carriage can be a fair sign to pay more attention to the lungs. Studies showed that physiological and microbial gradients exist along the oral cavity, oropharynx, trachea, and lungs[19,20]. The COVID-19 pandemic has emphasized the importance of studying the oral cavity's microbiome and its alterations. While many studies have predominantly delved into the bacterial microbiome of the oral cavity in COVID-19 patients or have concentrated on the gut microbiome, the role of the oral microbiome, including fungi, has frequently been sidelined or underestimated[1,10,11]. Thus, the objective of our study was to assess the species distribution of oral fungal carriage among individuals with COVID-19 and characterize the virulence factors of the isolated fungal agents. This allowed us to gain insight into the fungal composition within COVID-19 patients and compare it with that of healthy controls.

The choice of swabs to collect samples was based on their ease of use, non-invasiveness, and ability to precisely obtain samples from specific areas in the oral cavity[21]. Regarding alternative collection methodologies, several approaches could have been considered for sampling the oral cavity. One such method is the use of mouthwash with a saline solution[22]. Nevertheless, it is worth acknowledging that this method may dilute the concentration of microorganisms, potentially affecting the sensitivity of the analysis. Additionally, collecting saliva[23], another alternative, poses challenges due to the unique conditions of COVID-19 patients, such as dry mouth, which may necessitate stimulation to produce saliva. This could cause discomfort for the patients and increase the collection time. Ultimately, the decision to use a swab-based sampling method in this study appears to have balanced practicality, accuracy, and specific research objectives.

In this study, the prevalence of oral fungal carriage was found to be three times higher in COVID-19 patients in comparison with healthy controls, highlighting an increase in the fungal species richness in COVID-19 patients compared to healthy controls. Furthermore, in a research conducted by Mukherjee *et al.*[24], a significant difference in oral mycobiome was detected in HIV-infected patients compared to healthy controls. Also, the results of a previous investigation on the gut mycobiome in COVID-19-infected patients described a boost in the fungal gut component in COVID-19 infection[25]. As previously discussed regarding the oral microbiome, our findings on the more prevalent oral fungal carriage in COVID-19 patients can also be interpreted in the same way that the SARS-CoV-2 can trigger an exaggerated immune response in some individuals, leading to immune dysregulation[26]. This dysregulation may affect the balance of the oral microbiome, allowing certain opportunistic fungi, such as *Candida* species, to overgrow and colonize the oral cavity. Also, severe cases of COVID-19 are associated with a cytokine storm, where there is an excessive release of pro-inflammatory cytokines[1]. These cytokines can create a favourable environment for fungal growth and colonization.

The oral mycobiome could potentially contribute to the severity of COVID-19 infection through various mechanisms, including modifying the respiratory epithelium, facilitating the adhesion of respiratory pathogens, promoting local inflammation, and engaging specific virulence factors[27]. The influence of fungi on immune development is mediated indirectly through ecological changes in the bacterial microbiome and the facilitation of macrophage infiltration, cytokine levels, and T cells. Considering the significance of the oral mycobiome, further research is warranted, particularly in conditions like COVID-19, where a cytokine storm serves a crucial part in the pathophysiology[28]. The bidirectional influence of COVID-19 and the oral cavity highlights the importance of comprehending the interplay between oral fungal carriage and immune responses. Such understanding holds the potential to offer valuable insights into disease outcomes and guide the development of effective therapeutic strategies to manage both COVID-19 and its impact on oral health.

In the present study, the genus *Candida* was the sole fungal genus isolated from the oral fungal carriage of both individuals with COVID-19 and the control group. The timing of sample collection and the specific conditions of the COVID-19 patients might have influenced the predominance of *Candida*. Different fungal genera could be more prevalent at different stages of the disease or under varying patient conditions[5]. Also, the oral cavity has a dynamic and diverse ecosystem, and the presence of different fungal genera can vary significantly between individuals. It's possible that in the specific study population *Candida* was more prevalent than the other genera. Furthermore, there is always room for selection bias or handling errors.

Among *Candida* species, *C. albicans* was the most common (90.91 in COVID-19 patients and 90.48 in healthy controls) isolated species. *C. albicans* is a commensal yeast typically found in minimal quantities within the oral flora of a significant portion of individuals. Studies showed that the difference between *C. albicans* commensalism and disease in the oral cavity is related to a delicate equilibrium between fungal virulence and the host's defense mechanisms[29]. Of all *Candida* species, *C. albicans* is the most prevalent causative species of mucosal infections, thanks to its virulence factors and compatibility attributes[29].

Results of a recent study indicated that the production of interleukin (IL)-17 is guided by dendritic cells that have the unique property to co-produce the three major IL-17-instructing cytokines IL-23, IL-1, and IL-6 in oral mucosa in the presence of *C. albicans*[30]. On the other hand, IL-6, along with IL-1 beta (β) inflammatory chemokines, can notably lead to fever, lymphopenia, coagulation, lung injury, and multi-organ failure in COVID-19 patients[31].

The present study showed that in COVID-19-infected patients dry cough was the predominant clinical symptom, and compared with the healthy controls the prevalence of dry cough in patients with COVID_19 was significantly higher. This finding may suggest that there might be a correlation between COVID-19, its clinical symptoms, and oral fungal carriage. A dry cough is an unproductive cough and a natural reflex of removing irritants from the upper (throat) and lower (lungs) airways. The cough reflex can take part in the transmission of the mycobiome from the lungs to the oral cavity and vice versa[32,33].

Most *Candida* species isolated from humans secrete hemolysin, proteinase, and esterase, which might make their active invasion into host cells easier[15,34]. In this work, we found that the greatest esterase activity was related to *C. albicans* (55.07%). This finding is in line with the results that Pakshir *et al.*[35] had obtained.

However, in research conducted by Noori *et al.*[36], the highest esterase level was found in *Candida krusei* (75%). Additionally, according to the findings, the esterase activities of *Candida* isolates from COVID-19 patients varied from those of isolates from the control group, and esterase secretion was higher in the cases than it was in the normal oral flora of healthy participants. Contrary to the result of our study, Maheronnaghsh *et al.*[37] reported that compared to chemotherapy patients, the mean esterase activity of *Candida* isolates in healthy people was considerably higher. The differences in findings might be attributed to variations in geographical locations, diagnostic methodologies, study demographics, and sample sizes. Moreover, considering the nature of the study and the bidirectional influence of COVID-19 and oral fungal carriage on each other, there was a discrepancy in the number of isolates obtained from patients with COVID-19 compared to the control group. This disparity could potentially impact the study results and should be noted when interpreting the findings about the activity of the virulence factors.

In accordance with the research by Manns *et al.*[38] and Watanabe *et al.*[39], our investigation showed that the *Candida* species showed considerably higher haemolytic activity in COVID-19 patients than in the control group. They stated that different strains of *Candida* may release iron from red blood cells through hemolysin enzymes, and the free iron in saliva is essential for the pathogenicity of different strains of *Candida*.

The results of this study corroborate earlier investigations that reported among *Candida* species, the highest proteinase production is related to *C. albicans* isolates[40,41]. Nevertheless, Andreola *et al.*[42] found that about 97% of *Candida* spp. were positive for proteinase. Furthermore, in a study by da Silva *et al.*[43], 50% of the yeasts demonstrated proteinase activity. In the present study, in comparison to the healthy group, enzyme activity in the patient group was noticeably higher. In a study conducted by Tsang *et al.*[44], which compared oral *Candida* isolates between diabetic patients and controls, it was observed that both groups showed proteinase secretion; however, the patient group produced more of it. Also, Price and colleagues found that the enzyme can produce a persistent attachment of the yeast cell to the host and ultimately result in infection by digesting the host cell membrane. They demonstrated that the level of enzyme activity influences how various strains of *Candida* cause disease[45].

In both control and case groups of the present investigation, *C. albicans* isolates were more prevalent than other *Candida* species, and this species had the greatest levels of all three enzyme secretions in both studied groups. According to a study by Issa *et al.*[46], *C. albicans* species isolated from various regions of the human body, including the oral cavity, have demonstrated a greater capacity to secrete pathogenic enzymes compared to other *Candida* species.

The current study also demonstrated that the isolated *Candida* strains from patients and healthy individuals have the ability to produce virulence factors. It suggests that all commensal strains are opportunistic and can cause the disease in a favourable condition.

Conclusion

The oral cavity serves as a significant COVID-19 reservoir, impacting disease severity, symptoms, and transmission dynamics by modifying the interplay of local and distant mycobiomes. Notably, the study revealed a threefold higher prevalence of oral fungal carriage in COVID-19 patients compared to the control group. *Candida* was the predominant genus in both groups, with *C. albicans* as the most common species. COVID-19 patients exhibited increased esterase, proteinase, and hemolysin activity in *Candida* species compared to healthy individuals. Furthermore, *C. albicans* displayed higher virulence factors in both groups than non-albicans species, indicating its potential role in related infections. Understanding these virulence factors in COVID-19 patients is crucial for developing new antifungal medications, addressing treatment resistance, and improving patient outcomes.

Limitations

The study did not investigate aspects related to Candidalysin, biofilm formation and some other significant factors contributing to the pathogenicity of *Candida* species. The presence of these factors might have implications for oral fungal carriage dynamics in COVID-19 patients, and not addressing these aspects could be considered a limitation.

The matching of cases and controls based on certain factors (age, sex, BMI, and history of receiving antibiotic and antiviral medications) might not have accounted for other potential confounding variables, which could impact the interpretation and generalization of the results. The collection of oral samples on the third day after hospitalization might not have captured the full spectrum of oral mycobiome alterations that occur during the course of COVID-19 infection, and the methodology used in this study solely captured the oral fungal carriage. Different time points of sampling could offer a more comprehensive understanding of fungal colonization dynamics. The oral mycobiome is composed of various fungal species that could interact with each other and impact the overall colonization dynamics. The absence of analysis on the broader fungal community is a limitation.

Recommendations

Conducting larger multicenter studies with diverse populations can enhance the generalizability of the results and provide more robust insights into the association between COVID-19 and oral fungal carriage alterations. Future research should also explore the underlying mechanisms behind the observed alterations in oral fungal carriage and how these changes may influence the pathogenesis of COVID-19 and other related complications. The central question revolves around determining whether the increased prevalence of fungi is a result of their enhanced virulence properties, or if the presence of these fungi opportunistically led to an escalation in their virulence traits. Further inquiries ought to be directed towards comprehending the associations among particular virulence factors of *Candida* species and their interrelation with the severity of diseases and the clinical results in COVID-19 patients. Researches that inspect the interactions between the host and pathogen, which involves SARS-CoV-2 and oral fungal species, may offer significant enlightenment concerning the effects of the virus on the oral fungal carriage and vice versa, which could potentially lead to novel therapeutic approaches.

The findings demonstrate a substantially increased oral fungal carriage prevalence associated with COVID-19 status compared to healthy individuals. Follow-up studies incorporating

hospitalized non-COVID patients can allow comparison of outcomes to determine if the oral mycobiome changes observed are specifically linked to COVID-19 pathology, related to the effects of hospitalization itself or potential synergistic effects of both factors. Such insights can further elucidate the interplay between COVID-19, hospitalization influences, and oral microbiome equilibrium. The current study provides the foundation for expanded analyses on this aspect in future investigations.

Ethics statement

The study received approval from the Research Ethics Committee of Tehran University of Medical Sciences (with the ethics committee protocol number: IR.TUMS.SPH.REC.1400.030).

Consent

Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

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Author contribution

Z.R.: investigation, data curation, writing—review and editing. S.J.H.: conceptualization, methodology, project administration, funding acquisition. R.D.G.: conceptualization, validation, supervision. M.S.: methodology, investigation. M.S.: methodology, investigation. H.S.: software, formal analysis. M.-H.K.: investigation. K.A.: investigation. D.R.: investigation, F.M.G.: investigation. E.S.: investigation. Z.R.: conceptualization, methodology, project administration, Writing—original draft, resources, visualization, data curation, writing—review and editing. All authors contributed to the article and approved the submitted version.

Conflicts of interest disclosure

The authors have no conflict of interest to declare.

Research registration unique identifying number (UIN)

Dear Editor We understand your journal's requirement for prospective registration, but we inadvertently failed to register our study before the first subject recruitment. We apologize for this deviation. We are committed to transparency and compliance and seek your understanding in this matter. We kindly request an exception to the registration policy, with the commitment to rectify our oversight by registering our study retrospectively. We will promptly provide the registration number. We appreciate your consideration and guidance on how to proceed with our submission.

Guarantor

Zahra Rafat.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article (and/or) its supplementary materials.

Provenance and peer review

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