Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

Oral Candida Overgrowth in most Thalassemia Major Patients, Yeast Identification by CDA, VITEK 2, and PCR, and Evaluating their Virulence Factors in Thi-Qar, Iraq

Salih Jabbar[1,2*], Furdos Nouri Jafar[1], Shereen Al-Ali[3], Afaq Hameed[4]
[1]. Department of Biology, Colleg of Science, University of Basrah, Basrah, Iraq.
[2.] Health Directorate of Thi_Qar, Thi_Qar, Iraq.
[3.] Department of Pathological Analyses, College of Science, University of Basrah, Basrah, Iraq.
[4.] Department of Bio-Medical Engineering, College of Engineering, University of Thi_Qar, Thi_Qar, Iraq

Email: pgs2096@uobasrah.edu.iq

Abstract. Candida, an opportunistic fungus in immunocompromised patients, can cause an infection like oral candidiasis which usually starts with Candida overgrowth. Thalassemia major (TM) patients are known to suffer from immune abnormalities that may lead to a condition of immune deficiency. So, we aimed to estimate the prevalence and intensity of Candida growth in oral cavity of TM patients, determine the accuracy of Candida identification, and assess some virulence factors. Our study was conducted on 150 TM patients and 80 controls between (1-40 years). Oral swabs were used for microscopic examination and culturing on SDA. The identification was done by candida differential agar (CDA), VITEK 2, and conventional PCR. Also, the virulence was estimated by measurement of proteinase, phospholipase, lipase, hemolysin, and biofilm. Candida species were orally isolated from 70% of TM patients with mean colony count (124±94) significantly more than 42.5% of control with (11±7), so a significant oral Candida overgrowth was observed in most (TM) patients compared with the control, referring to an increased probability of developing to oral candidiasis. Significantly, in total TM patients, male or female patients, the age group (11–20) showed a higher prevalence of Candida than other age groups, at 52.4%, 49%, and 55.56%, respectively. PCR identified 105 isolates from TM patients: C. albicans constituted the most common species with (61.9%), C. dubliniensis (35.2%) and C. glabrata (2.86%). Generally, in comparison with results of PCR, the accuracy of identification by CDA was (95.2%) more than (87.6%) by VITEK 2, but both were typical methods for identifying C. albicans with (100%). Significantly, the higher production of proteinase and lipase was by (92.3%) and (90.7%) of C. albicans isolates, respectively. While the majority of phospholipase and biofilm production was noted by (70.3%) of C. dubliniensis and (100%) of C. glabrata, respectively. All Candida species were hemolysin producers with 100%.

Highlights:

1. Samples: 150 TM patients, 80 controls; oral Candida prevalence 70%.
2. Identification: PCR, CDA, VITEK 2; C. albicans most common (61.9%).
3. Virulence: High proteinase, lipase (C. albicans); biofilm (C. glabrata).

Keywords: accuracy of Candida identification, biofilm, extracellular enzymes, oral Candida overgrowth, thalassemia major patients

Published : 2025-05-01

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

Introduction

Candida is a polymorphic yeast, and part of the normal microbiota, living on the human mucosal, oral cavities, gastrointestinal tracts, and vaginl [1]. However, Candida switches from the normal flora to the opportunistic pathogenic form and causes infection like oral candidiasis in patients who have immunosuppressive therapy, chemotherapy, and broad-spectrum antibiotics, in addition to diabetes, AIDS, and anemic patients [2]. Some Candida species can cause oral candidiasis like Candida albicans which is the most common, C. glabrata, C. dubliniensis, C. stellatoidea, C. krusei, C. parapsilosis, C. tropicalis and C. kefyr [3,4]. Pathogenicity of Candida is enhanced by several virulence determinants including some extracellular enzymes like (proteinase, phospholipase, lipase, and hemolysin) which may perform critical functions in Candida overgrowth (colonization) by facilitating the adherence and tissue penetration and then the invasion of the host, also the biofilm has essential roles in Candida virulence [5,6].

Candida differential agar and VITEK 2 system are techniques used for the identification of Candida spp. but sometimes these techniques give misidentification, so it is necessary to follow the molecular techniques for accurate identification and treatment.

Thalassemia is a genetic disorder of hemoglobin production. Thalassemia major is the most serious form of β-thalassemia and these patients suffer from severe hemolytic anemia from the first months of life, requiring repeated blood transfusions [7]. Infections are a frequent complication of thalassemia and consider the second cause of death (after heart failure) in these patients resulting from the defective host defense which is because of the chronic immune stimulation by repeated blood transfusions, iron overload, splenectomy, and immune deficiency [8].

In concerning susceptibility to Candida infection, Dwyer et al. [9], tried to stimulate the thalassemic patients with antigens from C. albicans by skin test and noted a depression in lymphocyte proliferation, thereby thalassemic patients exhibited less immune responsiveness for C. albicans, especially in splenectomized patients, and this less responsiveness can increase the susceptibility to Candida infection. Very few cases of Candida infection were recorded in thalassemia patients in previous years. But recently, we noticed in the dental clinic at the Center of Genetic Blood Diseases in Thi-Qar governorate, that many thalassemic patients suffering from ulcers or redness or

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

white patches on the tongue, soft and hard palate, cheek, and even the corners of the mouth, and these signs are somewhat similar to those of oral candidiasis which usually starting with the Candida overgrowth. Therefore, we aimed to evaluate the prevalence of Candida species and the intensity of their growth in the oral cavity of TM patients as well as determine the accuracy of candida differential agar and VITEK 2 system to identifying Candida species in comparing with molecular methods. In addition, assessment for the important virulence factors of Candida species such as some extracellular enzymes (proteinase, phospholipase, lipase, and hemolysin) and biofilm formation.

Methods

Patients and sample collection

Two oral swabs (one for gram staining and the second for culturing) were collected from each of 150 thalassemia major patients in the Center of Genetic Blood Disease in Thi-Qar governorate and 80 healthy persons. The oral swabs were collected from the tongue, inner surface of the cheek, and roof of the mouth (soft and hard palate).

Isolation of Candida spp.

Oral swabs were cultured on sabouraud dextrose agar (SDA) medium (HiMedia, India) supplemented with 250 mg/L of chloramphenicol and 250 mg/L of Gentamicin, then incubated at 37℃ for (24-48 h).

Identification of Candida spp.

Microscopic examination

A smear was prepared from the oral swab and stained with Gram stain to determine the shape and color of Candida cells.

Growth on candida differential agar medium

Cultivation at 37 °C within 24-72 hours on candida differential agar medium (HiMedia, India) was used for fast identification of some clinical Candida spp. depending on the color variation and colony morphology.

Biochemical identification by VITEK 2 system (Biomerieux, USA)

Automated VITEK 2 system using 47 biochemical tests YST card to identify yeasts. Candida isolates were activated on SDA for 24 h at 37 °C then yeast suspension

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

(4.5% NaCl) was prepared with optical density (1.8 - 2.2 McF). The test cards were automatically filled with the prepared suspension, sealed, and incubated at 25°C for 24h. Optical density was recorded automatically every 15 minutes and compared with the database of VITEK 2, then the final identification of the unknown yeast was obtained.

Molecular identification

In the beginning, Candida species have been cultured on a large area of SDA plates for 24-48 hrs at 37 °C to be used in the extraction of genomic DNA that was according to the Presto Mini gDNA yeast kit (Geneaid, Taiwan). The same PCR procedure of Abu-Mejdad et al. [10], was utilized to amplify the internal transcribed spacer (ITS 1-5.8S-ITS2) region for the studied isolates using universal primers [ITS1] F-5-TCCGTAGGTGAACCTGCGG-3 and [ITS4] R-5-TCCTCCGCTTATTGATATGC-3. The amplification of ITS region was performed in a total reaction volume (25 µl) consisting of 1 µl for each forward and reverse primer, 3 µl of template DNA, 12.5 µl of Master Mix (Bioneer, Korea), and 7.5 µl Nuclease-free water. The reaction program steps were: initial denaturation at 94 °C (5 min), 30 cycles of denaturation at 94 °C (30 sec), primer annealing at 56 °C for 45 sec., 72 °C extension for 1 min, and (7 min) final extension at 72 °C. The PCR products were visualized in 2% Agarose gel electrophoresis by UV trans-illumination system.

Sequencing

A 20 µl of (ITS 1-5.8S-ITS2) PCR product for each isolate was purified and sequenced at Macrogen Company (South Korea). By Chromas software, the sequences of studied isolates were identified when compared to matched sequences at NCBI using the BLAST tool.

Determination of virulence factors of Candida spp. (all experiments were done in Triplicate)

Measurement of proteinase activity

According to Al-laaeiby et al [11], the proteinase production test was done by using a medium of bovine serum albumin (BSA). 10 µl of (1×106 CFU/ml) suspension of each yeast strain was deposited on the plates and incubated at 37°C for 7 days, the precipitate zones around each colony represented the positive proteinase activity (Prz) which was calculated as a ratio by dividing the diameter of the colony on the total diameter of the colony and precipitate zone together. The Prz value was marked as

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

follows: when Prz = 1.0 means (no activity), Prz = 0.7–0.99 means (weak positive), Prz = 0.5–0.69 means (moderate positive), and Prz = < 0.5 means (strong positive).

Measurement of phospholipase activity

As in the procedure of Al-laaeiby et al [11], 5 µl of (1× 106 CFU/ml) yeast suspension for each isolate was inoculated on the egg yolk medium, then incubated at 37°C for 2 weeks. A transparent zone around the inoculum referred to positive activity. Phospholipase activity (Pz value) was calculated as the same pattern in the measurement of proteinase activity.

Measurement of lipase activity

As mentioned by Sunitha et al. [12], lipase activity was measured when a standard inoculum (10µl of 1×106 CFU/ml) of each Candida isolate was deposited onto the Peptone agar medium, then all plates were incubated at 37°C during 7 days. The white halo around the inoculum site indicated the positive activity of lipase. The activity of lipase (Lz) was recorded as mentioned in proteinase measurement.

Measurement of hemolytic activity

The method of Luo et al. [13] was followed and hemolysin activity was measured when a standard inoculum (10 µl of 1×106 CFU/ml) of the Candida isolates was cultured onto the blood medium plates, then all the plates were incubated in (5% CO2) at 37° C for 48 hours. The isolates that were positive for hemolysin activity produced a translucent zone around the inoculum place. Hemolysin activity (Hz) was measured as above.

Detection of biofilm formation

For detecting biofilm production, Congo red agar was prepared from a mixture of brain heart infusion broth, glucose, and Congo red stain, and the plates were inoculated by Candida spp. with a streaking pattern and incubated (2 to 3 days) at 37°C. Saxena et al. [14] referred that the positive result was black colonies with a consistency of dry crystalline defined as (strong biofilm producers), and pink colonies as (weak biofilm producers), while the negative result was white or light pink colonies as (non-biofilm producers).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 23 was employed to analyze the present results. The counts of patients and controls in the table (1) were evaluated using descriptive statistics (mean and standard deviation), and the

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

percentages were used in the rest data. The chi-squared test was utilized to detect the statistical significance at p-value ≤0.05 for all data.

Result and Discussion

Sample collection

Significantly at ($p \leq 0.05$), the prevalence of Candida spp. in oral cavity of 70% (105/150) from (TM) patients were higher than 42.5% (34/80) from healthy control subjects, also it was found: the density of Candida growth in oral cavity of (TM) patients' group with mean colonies count (124±94) were higher than mean (11±7) in healthy control group. According to the above-mentioned results, there was a significant oral overgrowth of Candida spp. in most (TM) patients when compared with the control, Table (1) and Figure (1).

Table 1. the oral prevalence rate of Candida in (TM) patients and control groups with mean colony count

Subjects	**Subjects carrying *Candida* orally**	**No. of colonies on SDA**
	No. and (%)	**Mean+SD**
Thalassemia major patients (n=150)	105 (70%)	124+94
Control (n=80)	34 (42.5%)	11+7
	p ≤ 0.05	

In this study, 54/105 (51.4 %) of females more than 51/105 (48.6 %) of males in TM patients. The age group (11-20) whether in: total thalassemia major patients or male patients or female patients, showed a significant increase in numbers at ($p \leq 0.05$) than other age groups with 52.4 %, 49%, and 55.56%, respectively, Table (2).

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

Table 2. Distribution of oral Candida isolates in thalassemia major patients according to gender and age groups.

Age range	Female		Male		Total	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
0-10	15	27.77	20	39.2	35	33.4
11-20	30	55.56	25	49	55	52.4
21-30	6	11.12	4	7.84	10	9.5
31-40	3	5.55	2	3.96	5	4.7
Total	54	100	51	100	105	100
$p \leq 0.05$						

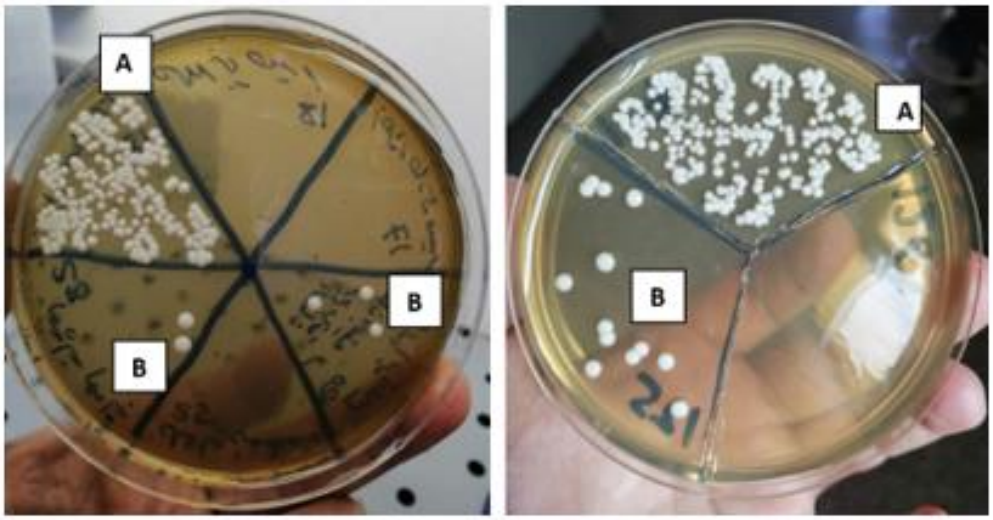

Figure 1. Candida growth on SDA from swabs of (A) thalassemia major patients and (B) control subjects

Isolation and identification of Candida spp.

The Candida isolates appeared on (SDA) as white to cream, glossy, smooth, soft, and circular colonies with yeast odor (Figure 1), and the microscopic examination for oral swabs showed two Gram-positive forms of Candida spp.: yeast and pseudohyphae. Figure (2) showed four various colors of Candida species colonies on candida differential agar: the mucous (blue-green) color of colonies was an indicator for C. albicans and the pale cream color indicated C. parapsilosis. Non-mucous dark green colonies were observed for C. dubliniensis and purple color has been seen in colonies of C. glabrata.

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

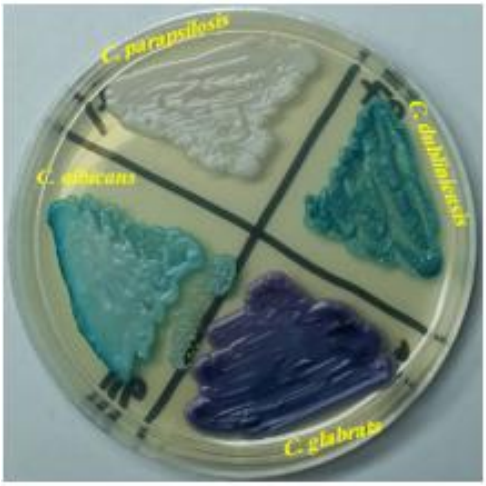

Figure 2. Candida spp. growth on candida differential agar, (blue-green color = C. albicans; dark green color = C. dubliniensis; purple color = C. glabrata; pale cream color = C. parapsilosis)

In the present study, molecular identification of 105 Candida isolates of (TM) patients was achieved by ITS1-ITS2 5.8S rDNA gene sequencing, Figure (3) showed gene size (≈500 bp) for C. albicans and C. dubliniensis and (≈ 800 bp) for C. glabrata. According to molecular identification, significantly C. albicans was the most frequent species with (61.9%) in cases of oral Candida overgrowth in thalassemia major patients followed by C. dubliniensis (35.2%) and less number for C. glabrata (2.86%), Table (3).

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

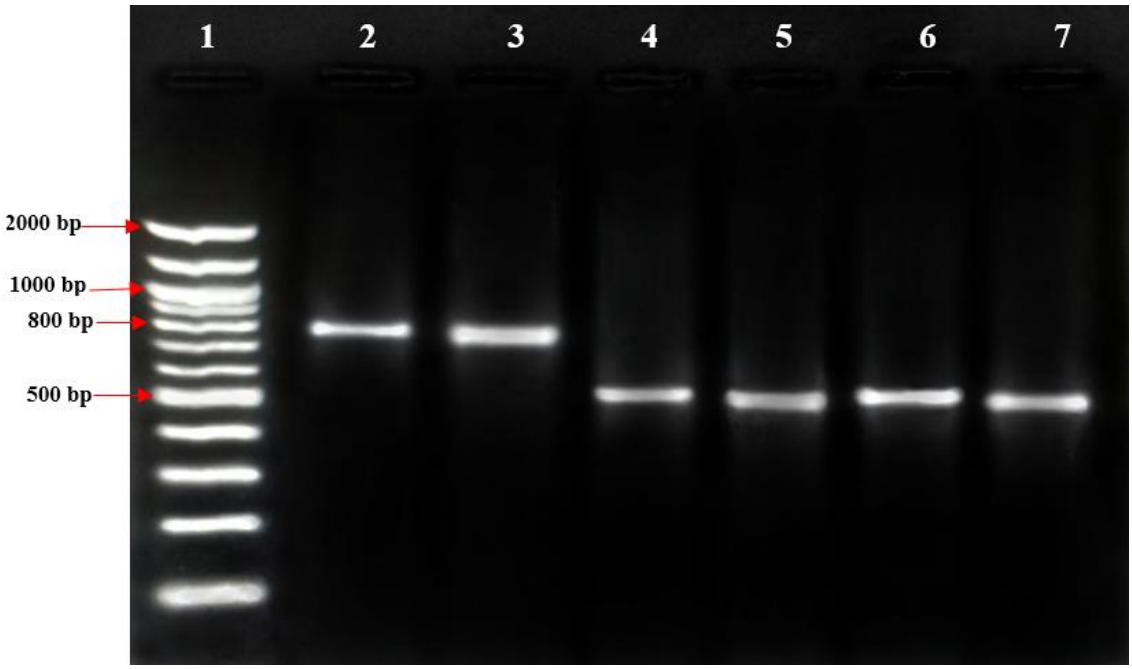

Figure 3. (2%) agarose gel electrophoresis for PCR products of the amplified (ITS1-ITS2) region of Candida spp.: Lane 1: DNA ladder (100 bp); Lane 2 & 3: C. glabrata (≈800 bp); Lane 4 & 5: C. albicans (≈500 bp); Lane 6 & 7: C. dubliniensis (≈500 bp).

Table 3. Distribution of Candida spp. according to results of Candida differential agar, VITEK 2 and Molecular techniques

Species	Candida diff. agar		VITEK 2		Molecular	
	No.	%	No.	%	No.	%
C. albicans	65	61.9	65	61.9	65	61.9
C. dubliniensis	32	30.5	27	25.7	37	35.2
C. glabrata	5	4.75	-	-	3	2.86
C. parapsilosis	3	2.85	-	-	-	-
Hanseniaspora opuntiae	-	-	4	3.8	-	-
Unidentified Organism	-	-	9	8.6	-	-
Total	105	100 %	105	100 %	105	100 %

Comparison between Candida differential agar, VITEK 2 system, and Molecular Identification

When the comparison with molecular identification, the candida differential agar correctly identified all C. albicans isolates (65/65) with (100%), Table (3) and (4). Also in recent study, this agar correctly identified 32/37 of C. dubliniensis isolates with

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

(86.4%), and 3 isolates as C. glabrata, Table (3). Misidentified results can be seen in Table (4). The correct identification (65 albicans + 32 dubliniensis + 3 glabrata = 100), then 100/105 = (95.2%) which was the accuracy of candida differential agar for identification of Candida species in this study.

In comparison with the molecular identification, VITEK 2 correctly diagnosed all 65/65 isolates of C. albicans (100%), Table (3) and (4), Also in our study, VITEK 2 correctly diagnosed 27/37 isolates of C. dubliniensis with (72.9%), Table (3) and (4). The correct identification (65 albicans + 27 dubliniensis = 92), then 92/105 = (87.6%) which was the accuracy of the VITEK 2 system in our study. Whereas, VITEK 2 reported 9/105 isolates as (Unidentified Organisms) and misidentified 4/105, Table (4).

Table 4. A comparison between Candida differential agar, VITEK 2, and molecular identification (ITS1-ITS2 rDNA gene).

No.	**Candida Differential Agar identification**	**VITEK 2 identification**	**ITS1-ITS2 rDNA gene identification**	
92	65 isolates as a *C. albicans*	65 isolates as a *C. albicans*	65 isolates as a *C. albicans*	Similar identification by the three methods for 92 isolates
	27 isolates as a *C. dubliniensis*	27 isolates as a *C. dubliniensis*	27 isolates as a *C. dubliniensis*	
93	*C. dubliniensis*	*Hanseniaspora opuntiae*	*C. dubliniensis*	Different identification between the three methods for 13 isolates
94	*C. dubliniensis*	*Hanseniaspora opuntiae*	*C. dubliniensis*	
95	*C. dubliniensis*	*Hanseniaspora opuntiae*	*C. dubliniensis*	
96	*C. dubliniensis*	*Hanseniaspora opuntiae*	*C. dubliniensis*	
97	*C. dubliniensis*	Unidentified Organism	*C. dubliniensis*	
98	*C. parapsilosis*	Unidentified Organism	*C. dubliniensis*	
99	*C. parapsilosis*	Unidentified Organism	*C. dubliniensis*	

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

100	*C. parapsilosis*	Unidentified Organism	*C. dubliniensis*
101	*C. glabrata*	Unidentified Organism	*C. dubliniensis*
102	*C. glabrata*	Unidentified Organism	*C. dubliniensis*
103	*C. glabrata*	Unidentified Organism	*C. glabrata*
104	*C. glabrata*	Unidentified Organism	*C. glabrata*
105	*C. glabrata*	Unidentified Organism	*C. glabrata*

New global strains recording in BLAST

When the ITS1-5.8S-ITS2 rDNA gene sequences of yeast isolates were compared with the sequences in GenBank of NCBI, it was noted that isolates 26A, 29A, and 32A were similar to C. albicans strain m9b, C. albicans n45a and C. albicans B90B, respectively with 99.8%. The three isolates were published as new global strains in the National Center for Biotechnology Information (NCBI), Table (5).

Table 5. The new Candida albicans strains recorded in GenBank of NCBI

No.	No. of isolate	Name of the new strain in NCBI	Accession No.	Homology (%)
1	A26	*Candida albicans* strain BU22S1	OM662276.1	99.80
2	A29	*Candida albicans* strain BU22S2	OM665396.1	99.80
3	A32	*Candida albicans* strain BU22S3	OM665397.1	99.80

Study of Virulence Factors

Figure (4-A) and (4-B) showed the proteinase and phospholipase activity for the Candida isolates, respectively. In Table (6), proteinase activity (positivity) was observed in 92.3% of C. albicans, 64.8% of C. dubliniensis, and 66.7% of C. glabrata at significant differences ($p \leq 0.05$).

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

Table 6. positive activity of extracellular enzymes and biofilm formation according to Candida species

Candida Species	Positive isolates									
	Proteinase		Phospholipase		Lipase		Hemolysin		Biofilm	
	No.	%	No.	%	No.	%	No.	%	No.	%
C. albicans (n = 65)	60	92.3	36	55.4	59	90.7	65	100	39	60
C. dubliniensis (n =37)	24	64.8	26	70.3	19	51.3	37	100	26	70.2
C. glabrata (n =3)	2	66.7	2	66.7	1	33.3	3	100	3	100
Total (n = 105)	**$p \leq 0.05$**									

At significant differences ($p \leq 0.05$), Table (6) demonstrated 55.4% of C. albicans, 70.3% of C. dubliniensis and 66.7% of C. glabrata secreting phospholipase, Figure (4-C) showed the lipase activity of the Candida isolates, and according to Table (6), at significant differences ($p \leq 0.05$), the highest lipase activity was in 90.7% of C. albicans, followed by 51.3% of C. dubliniensis and 33.3% of C. glabrata. Figure (4-D) referred to the levels of hemolysin activity for the studied Candida isolates, hemolysin activity (positivity) was showed with 100% in all Candida isolates, and no significant differences at ($p \leq 0.05$) among C. albicans, C. dubliniensis, and C. glabrata,Table (6).

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

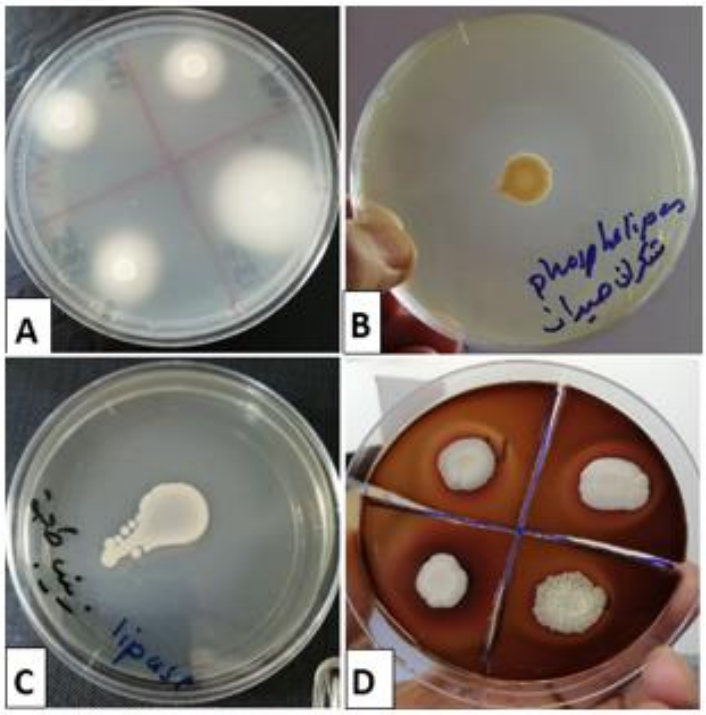

Figure 4. Enzymatic activities for the studied Candida spp.: (A) proteinase, (B) Phospholipase, (C) Lipase, (D) Hemolysin

Biofilm formation

In this study, the results of biofilm formation test for the Candida isolates were represented by different levels of biofilm by identifying different colors on Congo red agar, figure (5). Our results in table (6) demonstrated (60%) of C. albicans, (70.2%) of C. dubliniensis, and (100%) of C. glabrata were biofilm producers with significant differences ($p \leq 0.05$)

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

Figure 5. Biofilm formation of Candida isolates: Negative, Weak positive, and Strong positive

Discussion

Sample collection

In our study, there was a significant oral overgrowth of Candida spp. in most (TM) patients when compared with the control. This is attributed to some factors may increase either the overgrowth or Candida infection in the oral cavity of thalassemia patients these factors, First: some immune abnormalities such as impairment of chemotaxis and phagocytosis of PMN and macrophage against microorganisms [15], reduced levels of complement components (C3 and C4) [16], decreasing of T-helpers numbers (CD4) and increasing of T-suppressor (CD8) that resulting in reduced CD4/CD8 ratio, low activity of natural killers (NK) 8, and increase in circulating B-lymphocytes and increased levels of IgM, IgG, and IgA but with non-specificity as a result of multiple blood transfusions, all these immune events are almost because of iron overload in these patients [15]. Second: anemia could also be a contributing factor to oral fungal overgrowth [17]. Third: perhaps because of the presence of iron which is necessary for the growth of yeast, especially thalassemia tissues containing high iron concentrations.

In this study, (51.4 %) of females was more than (48.6 %) of males in TM patients, and this ratio may initially be due to the (female/male ratio) in Iraqi society as mentioned in the statistical analysis of Iraqi peoples provided by the ministry of planning in the year 2021.

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

Also, our results reveal the age group (11-20 years) whether in all thalassemia major patients or male patients or female patient group, a significant increase in numbers at ($p \leq 0.05$) than other age groups. The reason is that the death rate for thalassemia patients increases with age, so the majority of thalassemia patients are young (about ≤ 20 years), where repeated blood transfusions for a long time lead to serious iron overload-induced toxicity that causes critical problems in the heart or other organs and eventually early death [18].

Isolation and identification of Candida spp.

The current study showed the mucous (blue-green) color of colonies was an indicator for C. albicans and the pale cream color indicated C. parapsilosis, both observations in consonance with Dadhich et al. [19]. Non-mucous dark green colonies were observed for C. dubliniensis and purple color has been seen in colonies of C. glabrata, these findings were consistent with what was observed by Mathavi et al. [20]. The principle of candida differential agar to identify Candida spp. depends on the presence of chromogenic substrates that react with the specific exoenzymes for each of the different Candida spp. and form various pigmented colonies [19].

In this study generally; VITEK 2 identified (91.4%) of total Candida spp. isolates, similarly with Graf et al. [21] observed (92.1%) 222 of 241 Candida isolates, were unequivocally identified to the species level by the VITEK 2 system, but (8.6%) of our isolates haven't been identified. Low reactivity of some strains within the incubation time, could be the reason for the non-identification of isolates because when no positive reactions made unknown profile is different from that found in database of VITEK 2 software (unidentified organism) or may be because of new species [21].

According to molecular identification, significantly C. albicans was the most frequent species with (61.9%) in cases of oral Candida overgrowth in thalassemia major patients followed by C. dubliniensis (35.2%) and less number for C. glabrata (2.86%), and this was consistent with Aboualigalehdari et al. [22] in (Ahvaz city, Iran) who showed oral overgrowth of eight Candida species in HIV-infected patients, and significantly the first three most numerous species were C. albicans with (69.3%), C. dubliniensis (32.9%) and C. glabrata (20.4%). Isolation of the recent Candida species in our study may be due to the geographical area, lifestyle, and oral hygiene care 11, C. albicans is often reported as the most common isolated species in both healthy and patients, and

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

that is because this specie has a variety and strong virulence factors, in addition to production of chlamydospores which was believed it is most often forming to adapt for surviving hard environments [23, 24]. Also there is an important cause to increase chance of C. albicans growth in oral cavity of thalassemia patients, as the opportunistic microorganisms like C. albicans can adapt well to iron overload and even iron deficiency in the host body [25].

Comparison between Candida differential agar, VITEK 2 system, and Molecular Identification

When the comparison with molecular identification, the candida differential agar correctly identified all C. albicans isolates with (100%), Also Magare and Awasthi [26] obtained an accuracy (100%) in the identification of C. albicans by CHROMagar. Also, our results demonstrated 86.4% (32/37) of C. dubliniensis isolates and 3 isolates were correctly diagnosed as C. glabrata, while only the rest 5 isolates of C. dubliniensis were misidentified. So the (95.2%) was the accuracy of Candida differential agar for diagnosis of Candida species as calculated in results of this study and that is close to the accuracy of CHROMagar (92% and 95%) that were obtained by the researchers Jose et al. [27] and (Magare and Awasthi) [26] respectively. In contrast, Abu-Mejdad and Al-Badran [28] suggested to that CHROM agar candida did not provide conclusive results in her study because most her samples were non-clinical.

By VITEK 2, all C. albicans isolates were correctly diagnosed (100%) and the same ratio was obtained by both Graf et al. [21] and Hata et al. [29] while only (72.9%) of C. dubliniensis isolates were correctly diagnosed when compared with molecular diagnosis. The (87.6%) was the accuracy of VITEK 2 system in this study and that by chance was exactly the same percentage demonstrated by Kord [30] when he identified yeast species from blood infection by VITEK 2 compared with molecular in Iran. The other 10 isolates of C. dubliniensis; 6 isolates were not diagnosed and reported as (Unidentified Organism) and the cause was mentioned above, and 4 isolates of them was incorrectly diagnosed as Hanseniaspora opuntiae, the reason for incorrect identification was also likely the low reactivity of some strains during the incubation time, resulting in (false-negative) biochemical reactions [21]. In addition, there are other causes for incorrect identification by VITEK 2: either: the inter laboratory variability (in

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

equipment, reagents, or personnel skill), or the important reason the old version of ID card VITEK 2 compact system which needs to updating periodically [28].

New recording for global strains in blast

Three new global C. albicans strains were identified in this study, and these strains had identical percentages (99.80 %) when compared with their type strains in NCBI. In general, the genome of C. albicans is extremely active and many truncations, translocations, and other mutations happen more frequent than in other microorganisms [23].

Study of Virulence Factors

Extracellular enzymes

The degree of virulence and pathogenicity of Candida species are correlated with the level of the important secreted extracellular enzymes, such as hydrolytic enzymes (proteinase, phospholipase, and lipase) these enzymes disorganize the elements of the cell membrane, leading to gaining nutrients, adhesion and destroying of the host cell membrane to induce tissue invasion and infection [31, 32] and hemolysin enzyme, is a hemolytic factor that causes hemoglobin breakdown and releasing iron that is acquired by the pathogenic organisms as a crucial element for their survival and the ability to infect the mammalian host [33].

The proteinase activity was observed in 92.3% of C. albicans, 64.8% of C. dubliniensis, and 66.7% of C. glabrata at ($p \leq 0.05$), these observations agreed with Al-laaeiby et al. [11] they noticed that C. albicans significantly produced higher amounts of proteinases than non-C. albicans, also Costa et al. [34] declared (88.1%) of C. albicans and (69.8%) of non-C. albicans isolates experimentally produced proteinase. Whereas, Mroczyńska and Brillowska-Dąbrowska [35] showed maximum proteinase production in both albicans and non-albicans Candida.

At significant differences ($p \leq 0.05$), the 55.4% of C. albicans secreting phospholipase, which is approximately the same results of Costa et al. [34] who referred to 55.9% of C. albicans produced phospholipase, and we noticed 70.3% of C. dubliniensis and 66.7% of C. glabrata secreting phospholipase, in concurrence with the report of Sachin et al 36 which showed a maximum phospholipase production for C. dubliniensis. In contrast, there is a study indicating phospholipase production in non-albicans Candida is a rare event [27].

Indonesian Journal on Health Science and Medicine
Vol 2 No 1 (2025): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

Significantly ($p \leq 0.05$), the highest lipase activity was in 90.7% of C. albicans, followed by 51.3% of C. dubliniensis and 33.3% of C. glabrata, these results were in agreement with the study of Ramesh et al [37] where they found that C. albicans had higher lipase activity than C. glabrata, and the cause behind the maximal lipase production by C. albicans, perhaps because the opportunistic C. albicans possesses a big family of genes encoding for lipase whose extracellular action that is necessary for overgrowth and later infection [38].

Our results referred to that hemolysin activity was 100% in all studied Candida isolates, and no significant differences at ($p \leq 0.05$) among (C. albicans, C. dubliniensis, and C. glabrata). Similarly, De Melo Riceto et al. [39] and Hussein et al. [6] reported that all isolates of Candida were hemolysin-producing species. On another side, a study showed the differences in hemolysin activities, the most productive species for hemolysin was C. albicans with (94.8%) and (60%) of C. dubliniensis, while C. glabrata have less production with (21.4%) [36]

Biofilm formation

Biofilm of Candida is a heterogeneous structure of multi-microorganisms like planktonic and mycelial forms, embedded in an extracellular matrix consist predominantly of carbohydrates, proteins, glycoproteins, lipids, and some of nucleic acids, protects the pathogen via increased resistance to antifungal agents and evading host immune system 40, we found (60%) of C. albicans, (70.2%) of C. dubliniensis and (100%) of C. glabrata were biofilm producers, which correlate well with the findings of Jose et al. 27 and Saxena et al. [14], they found C. albicans isolates produced biofilm but were significantly lower than that percentage of non-albicans.

Conclusion

We illustrated a significant oral Candida overgrowth (colonization) in most TM patients with (70%) and mean colony count (124±94) compared to (42.5%) of control subjects with (11±7). Significantly, in total TM patients, male or female patients, the age group (11–20) showed a higher prevalence of Candida than other age groups, at 52.4%, 49%, and 55.56%, respectively. Also significantly, the molecular methods identified 105 Candida isolates of TM patients: C. albicans which was the major specie with (61.9%), followed by C. dubliniensis (35.2%) and less number of C. glabrata (2.86%). In

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

comparison with molecular methods, C. albicans was identified 100% by candida differential agar and VITEK 2, but generally, the accuracy of Candida identification by candida differential agar was (95.2%) more than accuracy (87.6%) of VITEK 2. Hemolysin activity was shown at (100%) in all studied 105 isolates (C. albicans, C. dubliniensis, and C. glabrata). but C. albicans revealed a significant maximum production of proteinase with (92.3%) and lipase (90.7%), while the majority of phospholipase and biofilm was produced by (70.3%) of C. dubliniensis and (100%) of C. glabrata, respectively.

Acknowledgements

The authors acknowledge the Center of Genetic Blood Disease for helping in sample collection and Al-Haboby teaching hospital for giving a license to work in the laboratory to accomplish this study.

Funding

Self-Funding.

Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

FNJ have suggested the title and designed the plan of current study, and provided some important materials. SJB have done the experiments of the study, and have written the manuscript of article. SJK have done the statistic of data, and proofread the manuscript.

References

[1]. G. Moran, D. Coleman, and D. Sullivan, "An introduction to the medically important candida species," in ASM Press eBooks, 2014, pp. 9–25. doi: 10.1128/9781555817176.ch2.

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

[2]. A.-C. Bostănaru et al., "Genotype comparison of Candida albicans isolates from different clinical samples," Revista Română De Medicină De Laborator, vol. 27, no. 3, pp. 327–332, Jul. 2019, doi: 10.2478/rrlm-2019-0027.

[3]. J. W. Millsop and N. Fazel, "Oral candidiasis," Clinics in Dermatology, vol. 34, no. 4, pp. 487–494, Mar. 2016, doi: 10.1016/j.clindermatol.2016.02.022.

[4]. A. Al-Laaeiby, S. Ali, and A. H. Al-Saadoon, "Candida species: the silent enemy," African Journal of Clinical and Experimental Microbiology, vol. 20, no. 4, p. 260, Aug. 2019, doi: 10.4314/ajcem.v20i4.1.

[5]. S. Dabiri, M. Shams-Ghahfarokhi, and M. Razzaghi-Abyaneh, "Comparative analysis of proteinase, phospholipase, hydrophobicity and biofilm forming ability in Candida species isolated from clinical specimens," Journal De Mycologie Médicale, vol. 28, no. 3, pp. 437–442, May 2018, doi: 10.1016/j.mycmed.2018.04.009.

[6]. K. I. Othman, S. M. Abdullah, B. Ali, and M. Majid, "Isolation and Identification Candida spp from Urine and Antifungal Susceptibility Test," Nov. 28, 2018. https://ijs.uobaghdad.edu.iq/index.php/eijs/article/view/554

[7]. A. Bazi, I. Shahramian, H. Yaghoobi, M. Naderi, and H. Azizi, "The role of Immune System in Thalassemia Major: A Narrative review," Journal of Pediatrics Review, vol. 6, no. 2, Oct. 2017, doi: 10.5812/jpr.14508.

[8]. A. Gluba-Brzózka, B. Franczyk, M. Rysz-Górzyńska, R. Rokicki, M. Koziarska-Rościszewska, and J. Rysz, "Pathomechanisms of immunological disturbances in B-Thalassemia," International Journal of Molecular Sciences, vol. 22, no. 18, p. 9677, Sep. 2021, doi: 10.3390/ijms22189677.

[9]. J. Dwye et al., "Abnormalities in the immune system of children with beta-thalassaemia major," PubMed, Jun. 01, 1987. https://pubmed.ncbi.nlm.nih.gov/3498580/

[10]. N. M. Abu-Mejdad et al., "A new record of interesting basidiomycetous yeasts from soil in Basrah province/Iraq" Basrah Journal of Science, vol. 3, no. 37, p. 9307-34, Sep. 2019.

[11]. A. I. E. Al-Laaeiby, A. A. Al-Mousawi, I. M. N. Alrubayae, A. Al-Saadoon, and M. Almayahi, "Innate pathogenic traits in oral yeasts," Karbala International Journal of Modern Science. https://kijoms.uokerbala.edu.iq/home/vol6/iss4/5/

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

[12]. V. Sunitha, D. Nirmala Devi, C. Srinivas, "Extracellular enzymatic activity of endophytic fungal strains isolated from medicinal plants" World Journal of Agricultural Sciences, vol. 9, no. 1, p. 1-9, 2013.

[13]. G. Luo, L. P. Samaranayake, and J. Y. Y. Yau, "Candida species exhibit differential in vitro hemolytic activities," Journal of Clinical Microbiology, vol. 39, no. 8, pp. 2971–2974, Aug. 2001, doi: 10.1128/jcm.39.8.2971-2974.2001.

[14]. S. Abdulla, B. Dheeb, S. A.-D. Al-Qaysi, M. S. Farhan, M. Massadeh, and A. Diab, "Isolation and Diagnosis of Biofilms of Klebsiella pneumoniae Bacteria and Candida albicans Yeast, and Studying the Sensitivity of The Pathogens to Antibiotics and Antifungals," Egyptian Academic Journal of Biological Sciences. G, Microbiology, vol. 15, no. 2, pp. 73–88, Oct. 2023, doi: 10.21608/eajbsg.2023.320271.

[15]. C. Asadov D. and Institute of Hematology and Transfusiology, Baku, Azerbaijan, "Immunologic abnormalities in B-Thalassemia," J Blood Disorders Transf, vol. 5, no. 7, p. 224, Jul. 2014, [Online]. Available: https://www.walshmedicalmedia.com/open-access/immunologic-abnormalities-in-thalassemia-2155-9864.1000224.pdf

[16]. C. Thiengtavor et al., "Increased ferritin levels in non-transfusion-dependent β°-thalassaemia/HbE are associated with reduced CXCR2 expression and neutrophil migration," British Journal of Haematology, vol. 189, no. 1, pp. 187–198, Dec. 2019, doi: 10.1111/bjh.16295.

[17]. W. Wanachiwanawin, "Infections in E-B thalassemia," the American Journal of Pediatric Hematology/Oncology, vol. 22, no. 6, pp. 581–587, Nov. 2000, doi: 10.1097/00043426-200011000-00027.

[18]. G. M. Mokhtar, M. Gadallah, N. H. K. E. Sherif, and H. T. A. Ali, "Morbidities and mortality in Transfusion-Dependent Beta-Thalassemia patients (Single-Center experience)," Pediatric Hematology and Oncology, vol. 30, no. 2, pp. 93–103, Jan. 2013, doi: 10.3109/08880018.2012.752054.

[19]. D. Dadhich, N. Saxena, A. E. Chand, G. Soni, and S. Morya, "Detection of candida species by hichrom agar and their antimycotic sensitivity in Hadoti region," International Journal of Scientific Study, journal-article, Jul. 2016. doi: 10.17354/ijss/2016/367.

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

[20]. S. Mathavi, G. Sasikala, A. Kavitha, and R. I. Priyadarsini, "CHROMagar as a primary isolation medium for rapid identification of Candida and its role in mixed Candida infection in sputum samples," www.academia.edu, Jul. 2016,

[21]. B. Graf, T. Adam, E. Zill, and U. B. GöBel, "Evaluation of the VITEK 2 System for Rapid Identification of Yeasts and Yeast-Like Organisms," Journal of Clinical Microbiology, vol. 38, no. 5, pp. 1782–1785, May 2000, doi: 10.1128/jcm.38.5.1782-1785.2000.

[22]. E. Aboualigalehdari, M. T. Birgani, M. Fatahinia, and M. Hosseinzadeh, "Oral Colonization by Candida Species and Associated Factors among HIV-infected Patients in Ahvaz, Southwest Iran," Epidemiology and Health, p. e2020033, May 2020, doi: 10.4178/epih.e2020033.

[23]. M. A. Kabir, M. A. Hussain, and Z. Ahmad, "Candida albicans: A Model Organism for Studying Fungal Pathogens," ISRN Microbiology, vol. 2012, pp. 1–15, Sep. 2012, doi: 10.5402/2012/538694.

[24]. B. Böttcher, C. Pöllath, P. Staib, B. Hube, and S. Brunke, "Candida species Rewired Hyphae Developmental Programs for Chlamydospore Formation," Frontiers in Microbiology, vol. 7, Oct. 2016, doi: 10.3389/fmicb.2016.01697.

[25]. R. Fourie, O. O. Kuloyo, B. M. Mochochoko, J. Albertyn, and C. H. Pohl, "Iron at the Centre of Candida albicans Interactions," Frontiers in Cellular and Infection Microbiology, vol. 8, Jun. 2018, doi: 10.3389/fcimb.2018.00185.

[26]. F. C. Odds and R. Bernaerts, "CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important Candida species," Journal of Clinical Microbiology, vol. 32, no. 8, pp. 1923–1929, Aug. 1994, doi: 10.1128/jcm.32.8.1923-1929.1994.

[27]. N. V. Jose, N. Mudhigeti, J. Asir, S. D. Chandrakesan, "Detection of virulence factors and phenotypic characterization of Candida isolates from clinical specimens". Journal of Current Research in Scientific Medicine, vol. 1, no. 1, pp. 27, 2015.

[28]. N. Abu-Mejdad, A. I. Al-Badran, "Isolation and molecular identification of yeasts, study their potential for producing killer toxins and evaluation of toxins activity against some bacteria and pathogenic fungi" Basrah: PhD thesis. PP. 1-249, 2019.

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

[29]. D. J. Hata, L. Hall, A. W. Fothergill, D. H. Larone, and N. L. Wengenack, "Multicenter evaluation of the new VITEK 2 Advanced Colorimetric Yeast Identification Card," Journal of Clinical Microbiology, vol. 45, no. 4, pp. 1087–1092, Feb. 2007, doi: 10.1128/jcm.01754-06.

[30]. M. Kord et al., "Epidemiology of yeast species causing bloodstream infection in Tehran, Iran (2015–2017); superiority of 21-plex PCR over the Vitek 2 system for yeast identification," Journal of Medical Microbiology, vol. 69, no. 5, pp. 712–720, May 2020, doi: 10.1099/jmm.0.001189.

[31]. T. Peremalo et al., "Antifungal susceptibilities, biofilms, phospholipase and proteinase activities in the Candida rugosa complex and Candida pararugosa isolated from tertiary teaching hospitals," Journal of Medical Microbiology, vol. 68, no. 3, pp. 346–354, Feb. 2019, doi: 10.1099/jmm.0.000940.

[32]. P. Czechowicz, J. Nowicka, and G. Gościniak, "Virulence Factors of Candida spp. and Host Immune Response Important in the Pathogenesis of Vulvovaginal Candidiasis," International Journal of Molecular Sciences, vol. 23, no. 11, p. 5895, May 2022, doi: 10.3390/ijms23115895.

[33]. W. T. H. Tanaka, N. Nakao, T. Mikami, and T. Matsumoto, "Hemoglobin Is Utilized byCandida albicansin the Hyphal Form but Not Yeast Form," Biochemical and Biophysical Research Communications, vol. 232, no. 2, pp. 350–353, Mar. 1997, doi: 10.1006/bbrc.1997.6247.

[34]. C. R. Costa, X. S. Passos, L. K. H. E. Souza, P. De Andrade Lucena, O. De Fátima Lisboa Fernandes, and M. D. R. R. Silva, "Differences in exoenzyme production and adherence ability of Candida spp. isolates from catheter, blood and oral cavity," Revista Do Instituto De Medicina Tropical De São Paulo, vol. 52, no. 3, pp. 139–143, Jun. 2010, doi: 10.1590/s0036-46652010000300005.

[35]. M. Mroczyńska and A. Brillowska-Dąbrowska, "Virulence of clinical candida isolates," Pathogens, vol. 10, no. 4, p. 466, Apr. 2021, doi: 10.3390/pathogens10040466.

[36]. C. Sachin, K. Ruchi, and S. Santosh, "In vitro evaluation of proteinase, phospholipase and haemolysin activities of Candida species isolated from clinical specimens," 2012. https://www.ajol.info/index.php/ijmbr/article/view/91862

ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/10.21070/ijhsm.v2i1.146

[37]. N. Ramesh, M. Priyadharsini, C. S. Sumathi, V. Balasubramanian, J. Hemapriya, and R. Kannan, "Virulence Factors and Anti Fungal Sensitivity Pattern of Candida Sp. Isolated from HIV and TB Patients," Indian Journal of Microbiology, vol. 51, no. 3, pp. 273–278, Apr. 2011, doi: 10.1007/s12088-011-0177-3.

[38]. D. A. Schofield, C. Westwater, T. Warner, and E. Balish, "DifferentialCandida albicanslipase gene expression during alimentary tract colonization and infection," FEMS Microbiology Letters, vol. 244, no. 2, pp. 359–365, Feb. 2005, doi: 10.1016/j.femsle.2005.02.015.

[39]. R. Alves, et al., Adapting to survive: "How Candida overcomes host-imposed constraints during human colonization", PLoS pathogens. Vol. 5, no. 16, pp. 1008478, 2020.