

Determination of Genetic Relationships and Pathogenicity of Oral Candidiasis Etiological Agents in Pediatric Malignant Patients in Basrah Province, Iraq

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Abstract

The incidence of yeast infections increased coinciding with the medical advancement and rising of the immunocompromised patient's community. Few studies were conducted to identify the pathogenic agents that caused oral infections in the pediatric cancer patient in Iraq. This study was designed to identify pathogenic yeasts causing oral infections in the pediatric cancer patients and evaluate the genetic relationship among isolates by constructing the phylogenetic tree. The capacity of isolates to express two important virulence factors (biofilm formation and hydrolytic enzymes, proteinase and phospholipase) was investigated. To achieve these goals, 24 swab samples were obtained from patients and identified by both conventional and advance methods. The majority of isolates were *Candida albicans*, while *Candida non-albicans* was minor. The identified isolates of *Candida non-albicans* were classified as *C. dubliniensis*, *C. glabrata*, and *C. tropicalis*, in addition to *Rhodotorula mucilaginosa*, and *Kluyveromyces marxianus* respectively by analyzing the sequences of ITS regions. *R. mucilaginosa* was enrolled for the first time in Iraq by sequencing ITS region. The phylogenetic tree was constructed by Maximum Likelihood (ML) analyses, isolates were categorized into five clusters comparing with reference strains and public database. The results found that the majority of isolates produced enzymes and formed biofilm strongly. In conclusion, identification of etiological agents employing comparison between ITS region sequence and public database was a simple, precise and accurate method.

Keywords: *Candida* species, Cancer patients, Candidiasis, ITS region, Phylogenetic tree.

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1. INTRODUCTION

Accurate characterization of yeasts species revealed that yeasts comprise various species described as commensalism harmless organisms (Microbiota) or malicious etiological agents, meanwhile the beneficial products (secondary metabolites) have been produced as a pharmaceutical agent by yeast [1, 2, 3]. Few species have been diagnosed as etiological agents to the mankind disease, such as *Candida* spp. and *Cryptococcus* species as a superficial to systemic candidiasis and pulmonary infections called *Cryptococcosis*, respectively [1].

The incidence of *Candida* infections either *Candida albicans* or non-*albicans* has increased dramatically [4]. Most yeasts infections belonged to *Candida* species, commonly *C. albicans* and non-*albicans* spp. Immunocompromised patients with a malignant tumor, solid organ transplant or AIDS, diabetes and other patients with impaired immune defenses are at major risk of *Candidiasis* [5, 6, 7].

Yeast cells possess elements that enhance the virulence of cells called virulence factors such as biofilm, pigments and extracellular hydrolytic enzymes [8]. These factors enhance the ability of the pathogen to survive, penetrate and disseminate to other organs [8]. *Candidiasis* was varied depending on the site of infection; the most prevalence *candidiasis* is oral *candidiasis* [9].

The colonization of *Candida* species in the immunocompromised patients is higher than the healthy individual [10]. Oral *candidiasis* has been developed in the cancer patients with colonization of about 30% and 50% [11]. Uncommon *Candida* spp. were diagnosed as etiological agents of invasive *candidemia* in the cancer patients when isolated from bloodstream such as *C. kefyr* and *C. lusitanae* [12].

The extracellular hydrolytic enzymes (proteinase, phospholipase and lipase) and biofilm formation have been investigated extensively [8]. They significantly associated with yeast pathogenicity by enhancing the adherence, penetration, modulated of host defenses or colonization [8,13]. On the other hand, yeast cells assemble in the form of thin layer that adheres to solid surfaces called biofilm [14]. This layer, however, shows a thin strong appearance and possesses traits associated with pathogenicity and resistance to antifungal agents [15,8,14].

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Four categories of antifungal drugs were developed, however two obstacles have been emerged during fungal treatment[16]. Firstly, fungal and human cells are eukaryotic, therefore the influence of drug toxicity disturbs both[17]. The emergence of pathogenic strains resists the antifungal agents either intrinsically or acquired considered a significant problem in the treatment of fungal infections[17]. Pathogenic cells have been noticed alter their susceptibility by modifying their genetic material to develop different mechanisms that circumvent the lethal effect of the drug [6].

The precise diagnosis of etiologic agents associated with successful treatment [6]. Conventional and advance diagnostic methods were invented, however the accuracy of molecular advance methods were confirmed[18].

This work was set out to investigate the common species infect the oral cavity of immunocompromised patients (particularly cancer patients). The conventional method was used for primitive diagnosis and the results confirmed by molecular genetic approaches. The production of hydrolytic enzymes (proteinase and phospholipase) as well as the formation of biofilm were screened as significant virulence factors. Moreover, drug sensitivities of isolates were scrutinized.

2. MATERIAL AND METHODS

2.1 Patients and Samples Isolation

Samples were obtained from 32 children with malignant tumors, who hospitalized in Basrah Specialist Children's Hospital (Basrah province\ Iraq) between October to December 2018). Agreements were obtained from the University of Basrah\ Faculty of Science \ Biology departments to perform this work. The aim of this study was illustrated to the patients and their parents to obtain their consents (Oral consents). Duplicate samples were obtained from oral thrush with a sterile pre-moistened swab. All samples were transferred to the clinical mycology laboratory in the University of Basrah for yeast isolation.

The isolation process was summarized by striking the swab samples on Sabouraud dextrose agar with chloramphenicol (SDA+C) in the aseptic condition and incubating at 37 °C for 48-72 h. Purified isolates were maintained in the SDA slocs for further analysis. The primitive examination to the swabs was performed microscopically with 10% potassium hydroxide (KOH) to identify yeast cells and / or pseudohyphae and true hyphae[11], (Figure 1).

2.2 Conventional Identification

2.2.1 Plate-Base Method

Chromogenic medium Brilliant Candida agar (BCA) was selected for primitive identification. Isolates were sub-cultured onto media and the identification was carried out following manufacture's instruction[19]. Sub-cultured isolates were incubated at 37 °C for 48 h. The identification was based on yeast colony color onto BCA plates. The growth was examined daily at over 48 h.

2.3 Germ Tube Test

All yeast isolates were subjected for germ tube test to distinguish *Candida albicans* and closely related species from other species of *Candida*. The analysis was accomplished by adding small portion of each activated isolate to 0.5 ml serum test tube, then all tubes were incubated for 3h at 37°C. After completing the incubation period, 1-2 drops of yeast inoculum were mixed with 10% KOH and explored under a magnification of 40x and 100x of light microscope to observe the attendance / lack of germ tube.

2.4 Biochemical Identification by Vitek2 and Drug Sensitivity

Commercial kits vitek2 AST and ID YS01 were obtained from BIOMERIEUX company (Biomerieux, USA). The process was involved both *Candida* spp. identification and Antifungal Susceptibility Tests (AST). The procedure was carried out according to the manufacturer's instructions.

2.5 Molecular Genetic Characterization

2.5.1 ITS Regions Sequencing

For accurate differentiation, sequencing of amplified ITS region was performed. To obtain genomic DNA, the Presto™ Mini gDNA Yeast Kit (Geneaid) was used for DNA isolation according to the manufacturer's instructions [20]. Later, isolated DNA samples were electrophoresed in 0.5% agarose gel stained with ethidium bromide and preserved in the -80 °C for PCR amplification. The ITS conservative regions of rDNA were amplified using the universal primer sequences of ITS1: F-5-TCC GTA GGT GAA CCT GCG G-3 and ITS4: R-5-TCC TCC GCT TAT TGA TAT GC-3[21]. The PCR process was carried out following Mirhendi et al. (2006). The mixture of PCR reaction composed of 25 µl of master mix (Promega), 10 µM of forward and reverse primer, 5 µL of genomic DNA (GDNA) and nuclease free water to a total volume of 50 µL. ITS region amplification condition: 3 min at 95 °C for initial denaturation, followed by 35 cycles including three steps of denaturation at 95 C for 45 sec, an annealing step at 55 C for 45 sec and an elongation step at 72 C for 1 min, one more cycle at 72 °C for 10 min as final elongation. PCR products were separated into agarose gel and fragments size determined by comparison with a 1-kb ladder (Promega). Sequencing process was achieved in the MacroGen Inc [22].

2.6 Detection of Virulence Factor

2.6.1 Biofilm Activities and Hydrolytic Enzymes (Phospholipase and Proteinase)

Congo red agar medium was used to investigate the potency of the isolates to form a biofilm. The method was described by Saxena et al (2014) [23]. Pure isolates were sub-cultured onto Congo red agar medium (brain heart infusion broth BHI (37 gm/L), glucose (80 gm/L), agar (10 gm/L) and Congo red (0.8 gm/L)) and incubated for 48 h at 37°C. Colonies color indicated to the potency of isolates to form a biofilm. The formation of biofilm was organized to three classes (weak, moderate and strong) according to the colonies' color degree. Red and pink colonies referred to

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strong and weak activity respectively, while white or light pink indicated to a negative result.

The proficiency of yeast isolates to secrete the hydrolytic enzymes (phospholipase and proteinase) were investigated using Egg yolk media and bovine serum albumin (BSA) agar, respectively. Following Samaranyake et.al. (1984), media were prepared and the results determined by calculating the precipitation zone (Pz)[24]. The value of precipitation zone (Pz) was referred to the potential activity of phospholipase secretion, no activity (Pz=1), weak positive (Pz= 0.7- 0.99), moderate activity (Pz= 0.5-0.69) and strong activity (Pz< 0.5).

On the other hand, the potentiality of isolates to synthesize and secrete proteinase was studied using bovine serum albumin (BSA) agar as described previously. The clear zone was visualized around the colonies and revealed proteinase activity. Similarly, to biofilm activity measurement was done.

wet smear with 10% KOH, while only 12.5% (4 of samples) revealed negative result of direct investigation. However, 85.7% (24 of samples) of positive sample swabs were shown growth onto SDA after incubation interval and the rest were failed to grow (Figure 1). All the 24 isolates were subjected for morphological identification including examining of germ tube formation and colony appearance onto Brilliant

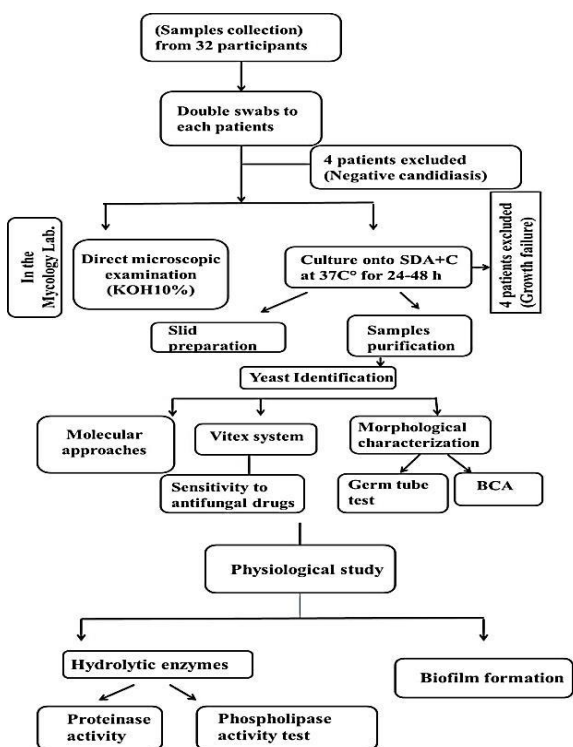


Figure 1. A diagram summarized the sequential steps of workflow.

3. RESULTS

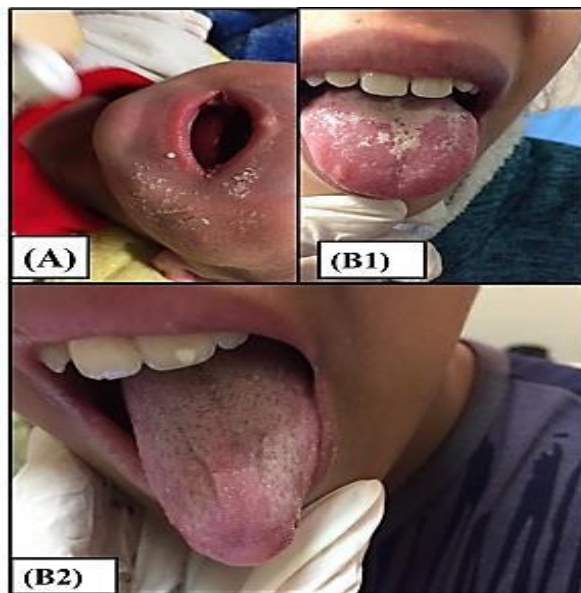
3.1 Characterization of Patients and Demography

The contemporary study was subjected with 32 pediatric patients who admitted in the Basrah specialist children Hospital, all of them were suffering from malignant diseases with chemo and radiotherapy period. The percentage of females was recorded at 18.75% (6/32), while 81.25% (26/32) was for males. In addition, patient ages were ranged from 6 months -11 years. Moreover, the most cases were showed inflammatory symptoms that indicated to oral candidiasis extended from acute pseudomembranous candidiasis, oropharyngeal candidiasis and angular cheilitis (Figure 2).

3.2 Conventional Identification Methods

3.2.1 Morphological and Phenotypical Analysis

Microscopic direct examinations of all oral specimens showed that 87.5% (28 of samples) were yeast infections (oral candidiasis) by presence of true and pseudo-hyphae in



Candida agar plates (BCA).

Figure 2. shows pediatric cancer patients with oral candidiasis. White layer covers the patient's tongue and mouth edge refer to yeast infection. The oral agreement was obtained from patients and their parents to capture photos.

3.3 Germ Tube Test

All the 24 isolates were induced to form germ tube by incubating into the human serum at 37 °C for 3 h. Twenty-two isolates formed germ tube as elongated structure from the mother cell in the absence of reduction at their root, the positive result were referred to rapid and presumptive identification technique, which differentiated *C. albicans*/*C. dubliniensis* from other *Candida* species.

3.4 Brilliant Candida Detection

Brilliant Candida agar was used to identify the subjected isolates depending on the colony appearance. Identification was performed according to manufactures instructions [19]. After 48 h of incubation at 37 °C, most isolates appeared in various degrees of green color, while only one isolate was dark blue and two isolates were purple. The interpretation of results based on the manufacture instructions is that green color represents both *C. albicans* and *C. dubliniensis*, dark

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blue refers to *C. tropicalis* and pink strain distinguishes by unchanged color.

The obtained results confirmed that the conventional (morphological) methods were insufficient for accurate and decisive identification due to the intersection between isolate appearances.

3.5 Identification and Antifungal Susceptibility Evaluation by Vitek Technique

Table 1 displays the analysis of Vitek system for yeast identification to species level. The susceptibility of isolates was measured against fluconazole, voriconazole, caspofungin, micafungin, and flucytosine. The results revealed that all isolates were susceptible to the above antifungal drugs except some isolates that identified as *Candida spherica*, which showed resistance to all antifungals (> 100 µg/ml).

Table 1. Antifungal sensitivity patterns of clinical isolates against Micafungin, Amphotericin B and Flucytosine by Vitek.

Isolate No.	MIC (µg/ml)						Interpretation
	Vitek results	Fluco	Vorico	Caspo	Micaf	Flucy	
1	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
3	<i>C. dubliniensis</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
6	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
7	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
8	<i>C. dubliniensis</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
10	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
11	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
12	<i>C. spherica</i>	< 100	< 100	< 100	< 100	< 100	R
14	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
15	<i>C. dubliniensis</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
16	<i>C. glabrata</i>	16	0.25	≤0.25	≤0.06	≤1	S
18	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
19	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
20	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
21	<i>C. glabrata</i>	8	0.25	≤0.25	≤0.06	≤1	S
22	<i>C. glabrata</i>	8	≤0.12	≤0.25	≤0.06	≤1	S
23	<i>C. albicans</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
24	<i>C. tropicalis</i>	≤1	≤0.12	≤0.25	≤0.06	≤1	S
26	<i>C. spherica</i>	< 100	< 100	< 100	< 100	< 100	< 100
39	<i>C. spherica</i>	< 100	< 100	< 100	< 100	< 100	< 100

Fluco= Fluconazole; Vorico= Voriconazole; Caspo= Caspofungin; Micaf= Micafungin; Flucy= Flucytosine; S= Sensitive; R= Resistant; MIC= Minimum inhibitory concentration

3.6 Molecular Identification

3.6.1 ITS Region Sequencing

All the 24 isolates were subjected for molecular identification. The process was achieved by amplifying and sequencing of ITS region for sequencing. The results of ITS region sequences were revealed that 54.16% of isolates were *C. albicans*, 12.5% *C. dubliniensis*, 12.5% *C. glabrata*, 8.33% for both *R. mucilaginosa* and *K. marxianus*, while *C. tropicalis* was at 4.16% as shown in table 2.

Table 2. The incidence (%) of identified yeast genera by molecular genetic technique

Genus	Species	Incidence of strains (%)
Candida	albicans	54.16
	dubliniensis	12.5
	glabrata	12.5
	tropicalis	4.16
Rhodotorula	mucilaginosa	8.33
Kluyveromyces	marxianus	8.33

3.7 The Agreement of Identification Methods

The agreement of three identification methods (morphological appearance, Vitek and Molecular genetics) were compared to investigate the harmony and accuracy of the results. 29.16% (7/24) of identified isolates were

corresponded to the results of morphological appearance and Vitek, 6/7 isolates were *C. albicans* and one was *C. dubliniensis*. The agreement between morphological approach (applying BCA) and Vitek was 83.33%, while 33.33% was between Vitek and molecular methods (Figure 3).

3.8 Evaluation of Virulence Factor

Three virulence factors were investigated in this study including the ability of isolates to form biofilm and produce phospholipase and proteinase. The potential facility of biofilm formation was classified into three categories, strong, moderate and weak. The results revealed that 79% of the

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isolates formed biofilm strongly, while weak formation was spotted out at 21%.

On the other hand, the enzymatic activity of studied isolates was characterized. All yeast isolates produced phospholipase and the production was distributed into three categories: 83% strong, 4.2% moderate and 12.5% weak. Meanwhile, the measurement of proteinase was revealed the strong ability of 58% of isolates to produce proteinase, while 21% and 17%

of the isolates showed moderate and weak activity, respectively. Only 4% of the isolates showed an inability to produce proteinase (Figure 4).

3.9 Phylogenetic Tree

Phylogenetic relationship among 23 isolated *Candida* species was scrutinized by constructing a phylogenetic tree (Figure5).

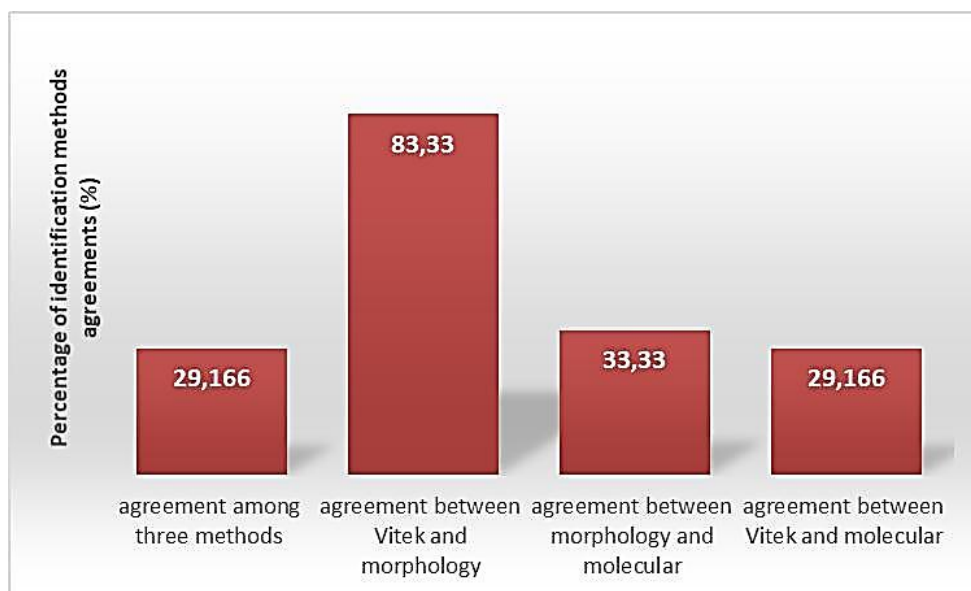


Figure 3. The percentage of agreements of three identification methods (morphological method (BCA), Vitek and genetic molecular).

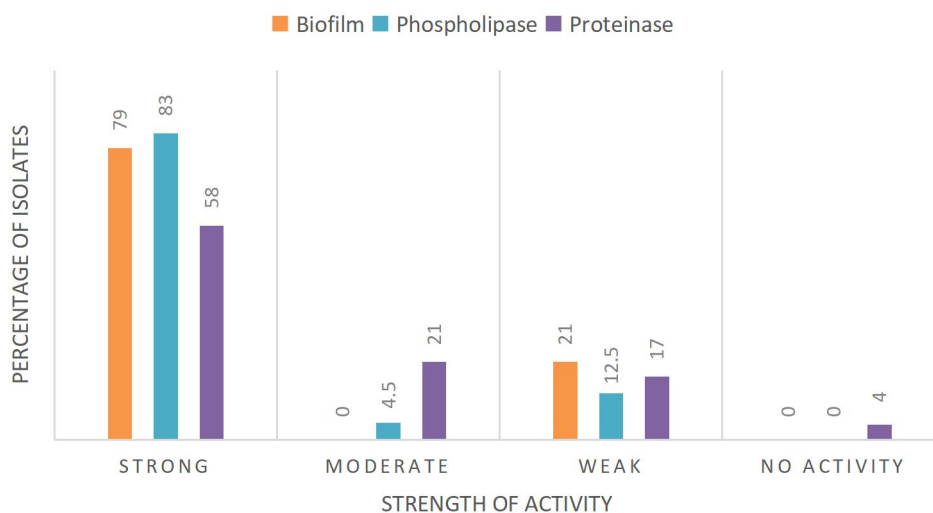


Figure 4. The percentages of yeast isolates to form biofilm and synthesize phospholipase and proteinase or have no activities.

The construction was achieved using Maximum Likelihood (ML) analyses based on the ITS1 and ITS2 region sequences of rDNA of isolates. The sequence of six reference strains (*C. albicans*, *C. tropicalis*, *C. dubliniensis*, *C. glabrata*, *K. marxianus* and *R.mucilaginosus*) were recovered from the public database in the NCBI. The results found that the isolates were classified into 6 clades. 12 *C. albicans* were categorized in the first clade closing to the reference strain

KJ651874.1, while the second clade includes *C. dubliniensis* corresponding to KY6731961.1. *C. tropicalis* related to third clade and similar to MT539196.1. Two strains of *K. marxianus* associated with the fourth clade, which showed

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similarity to MN062966.1. Clades fourth and fifth represented by *C. glabrata* and *R. mucilaginosa* isolates respectively and corresponded to the two reference strains MK343435.1 and MT465994.1, respectively. Bootstrap values were based on 1100 replicates. ITS sequence of *Streptococcus salivarius* (NR 042776.1) was used as the out-group species.

4. DISCUSSION

In this study, conventional (morphological), biochemical (Vitek) and molecular methods were applied to identify yeast

isolates. The consent of three identification methods was investigated. It was found that morphological and Vitek methods results were identical by 83.3%. It was reported previously morphological methods are time consuming, inaccurate comparing with the reliable and precise molecular approach[25]. Simplicity and reliability of molecular identification owing to the availability of ITS region sequences in the public database are proven to be optimal, therefore decisive interpretation was based on molecular

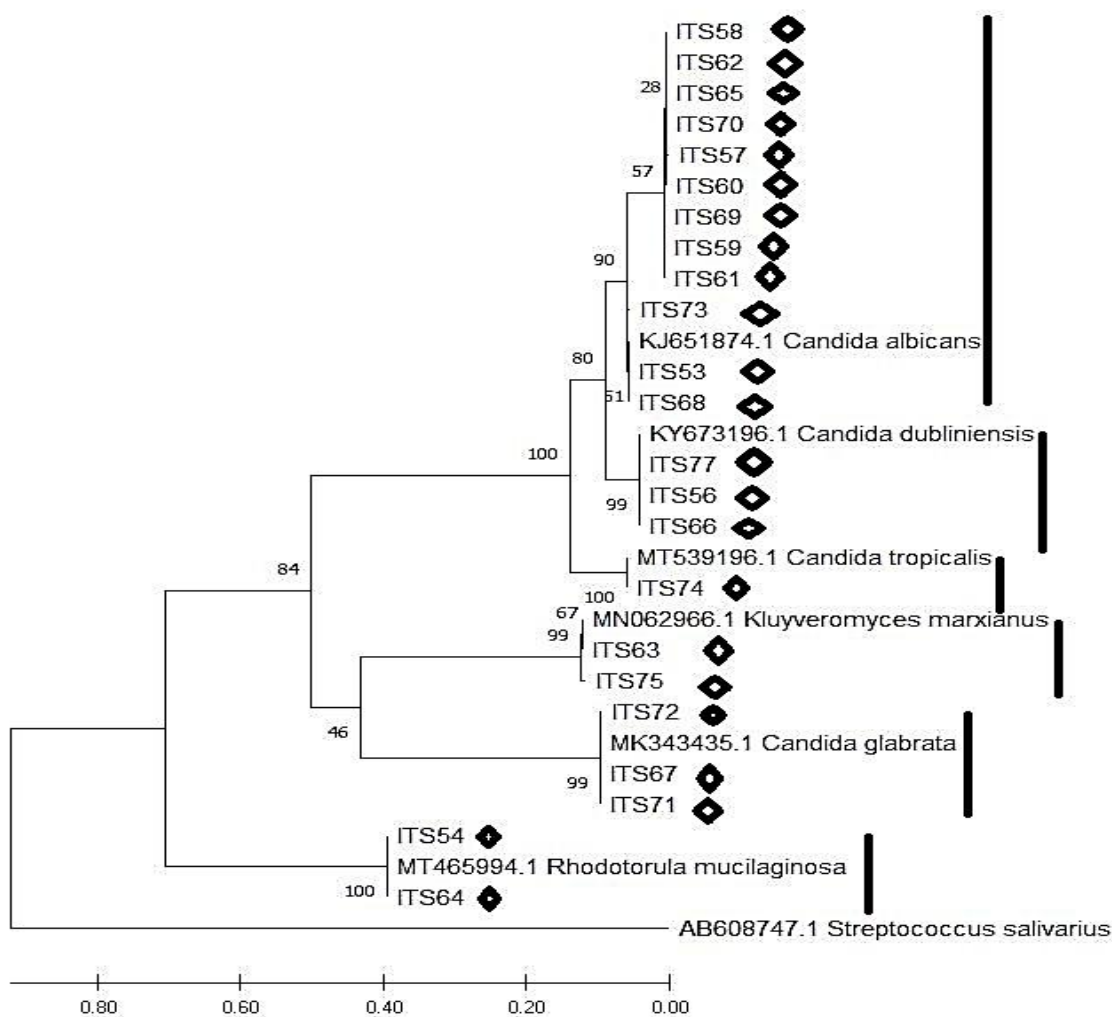


Figure 5. Collective phylogenetic tree generated with the ITS-rDNA sequences of the 23 yeast isolates, as inferred from Maximum Likelihood (ML) analyses, isolated from different cancer patients in this study (determined by black squares) and related species belonging to the *Candida albicans*, *Candida tropicalis*, *Candida dubliniensis*, *Candida glabrata*, *Kluyveromyces marxianus* and *Rhodotorula mucilaginosa* retrieved from GenBank.

method[26]. This result confirmed the discrepancy between molecular genetics and phenotypic characterization. Our results are in agreement with Abu-Mejdad et al., (2020) when biochemical (Vitek2) failed to identify most environmental isolates comparing with molecular approach[27]. Our result is also consent with previous findings that reported *C. albicans* is a predominant species causing oral candidiasis[11,28,29]. The most common yeast species were *C. albicans*, followed by non-albicans spp., *C. dubliniensis* and *C. glabrata*, this results are agreed with the previous finding of Aldossary et al., (2018) however the

percentage of incidence differs due to the age group of patients and treatment strategy[26]. In addition, our study and Aldossary et al., (2018) investigated the incidence of oral yeast candidiasis and colonization respectively. Jabari et al., (2016) found that *C. albicans* the common yeast isolated from pediatric immunocompromised patients followed by *C. glabrata* in Iran[29]. The variation in the results can be

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attributed many reasons such as geographical area, age groups, medical care and managements. Similarly, *C. tropicalis* and *K. marxianus* (*C. kefir*) were enrolled as colonized or infectious agents in cancer patients [30,12]. In this study, *C. albicans* recognized as an etiological agent causing candidiasis about 54.16%, while 45.8% of isolates were identified as non-*albicans*. This result compared with the previous finding of Jabari et al., (2016) recorded *C. albicans* causing infections more than non-*albicans*. An environmental isolate of *R. mucilaginosa* was identified in Iraq [31,27]. However, it was believed that in this study was recovered from clinical resource in Iraq for the first time and deposited in the Genbank with the accession number MT796852.

In the literature, it was found that yeast infections are multifactorial. Pathogenic agent possesses elements (called virulence factors) enhance body attack and transfer to deep and systematic infection [8]. Consequently, the current study was evaluated the activities of most common virulence factors. The process of infection involves essential steps including entry and adherence to the cells, invasion, reproduce and disseminate to other organs [8]. In constant with our results, the ability of *C. albicans* and *C. dubliniensis* to form germ tube was high, which considered as distinguished characteristic [32]. On the other hand, it boosts pathogenicity to increase the ability of pathogen and to develop infection [33].

The production of extracellular hydrolytic enzymes (phospholipase and proteinase) by *Candida* species was demonstrated [34]. The pathogenicity of isolates exhibited high exoenzyme activity were screened in the animals model [13]. The activity of enzyme production was investigated. Contrary to our result, Saxena et al., (2014) found that the 78.27% of *Candida* isolates produce biofilm weakly while only 21.73% produce strong biofilm [23]. Our results found that strong and intermediate activity of biofilm formation was presented in the majority of isolates. It could be explained our finding that the isolates possess the potential ability of isolates to adhere to the tissue [35].

The genetic relatedness among the isolates was obtained by constructing the phylogenetic tree following Maximum Likelihood (ML) analyses. The construction was based on the sequence results of isolates comparing with the reference strains that retrieved from the public database in the GenBank. The isolates were distributed among five clusters and grouped in unique genera. It is obvious that some isolates were 100% identical (ITS54 & ITS64), (ITS56, ITS66 & ITS77), (ITS58, ITS60, ITS65 & ITS70), (ITS59, ITS61 & ITS69) representing *R. mucilaginosa*, *C. dubliniensis* and *C. albicans*, respectively. These identical strains isolated from different patients, it could be concluded that the infections may be acquired exogenously as nosocomial infections. This result in consent with Malek et al., (2016) who evaluated the genetic relationship among clinical *Candida* spp. isolates using RAPD assay [7].

However, our study has few drawbacks, the number of samples is few due to the difficulties to gain agreement from patients and their relatives. The investigation of sensitivities of antifungal drugs was achieved only by Vitek methods and by using other methods such as Etest.

5. CONCLUSION

In conclusion, all clinical isolates were identified by combining morphological and advance molecular methods. *Candida albicans* was the most common isolated species, while *Candida non-albicans* species are less frequent including *C. dubliniensis*, *C. glabrata*, in addition to *C.*

tropicalis, *R. mucilaginosa* and *K. marxianus*. The activity of species to produce extracellular hydrolytic enzymes (phospholipase and proteinase) was classified in three categories strong, intermediate and weak. Most isolates showed high activities of enzymes production. The difference of enzyme productions among isolates encouraged us to extend our work in the future to investigate the molecular and express the differences among three classes of enzyme production as well as to examine the most isolates for biofilm formation. Because of the clinical yeasts are multifactorial pathogenic agents thus deep analysis of the virulence factors by using molecular methods is important to investigate the mechanism of etiological agents to overcome the host defence and resist fungal drugs.

6. CONFLICT OF INTEREST

Authors declare that there is no conflict of interest regarding this work.

7. ACKNOWLEDGMENTS

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8. AUTHOR'S CONTRIBUTIONS

Dr Inaam M.N. Alrubayae designed, conceived this study and achieved virulence factor and morphological analysis. Molecular genetic identification was carried out by Dr. Ayat Al-laeiby while Dr. Mohammed Hussein Minati constructed and, analyzed phylogenetic study. Dr. Shrouk ALibraheem sent samples for Vitek analysis. The manuscript was written by Ayat Al-laeiby, edited by Dr. Inaam Al-Rubayae, revised and proofread by Dr. Mohammed Hussein Minati.

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