ISSN: 0067-2904

Molecular identification of some *candida* spp. isolated from intensive care unit patients and evaluation of their sensitivity to antifungals

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Received: 26/5/2024 Accepted: 24/1/2025 Published: 30/1/2026

Abstract

Candida species found in intensive care units (ICU) exhibit a higher incidence and greater resistance compared to those unrelated to the ICU. Both phenotypic and genotypic methods were employed to identify strains of oral candidiasis, including drug-resistant variants commonly present in ICU settings. The molecular identification of multi-drug-resistant strains in the intensive care unit utilized the ITS-ribosomal RNA genes. The species identified included *Nakaseomyces glabratus*, *Candida dubliniensis*, and *Candida albicans*. These strains have been registered in the Genbank database. Identification of these strains was conducted using the VITEK2 AST-YS08 Card. The results indicate that the predominant isolate was *C. albicans*, accounting for 56.67% of the isolates, while Non-albicans isolates included *Nakaseomyces glabratus* (33.33%) and *C. dubliniensis* (10%). Investigation has been conducted on the formation of chlamydospores and the examination of temperature tolerance. Susceptibility testing of isolates using the VITEK2 AST-YS08 card revealed that *Nakaseomyces glabratus* and *C. dubliniensis*, isolated from the intensive care unit, exhibited higher resistance to six antifungal drugs. On the other hand, *C. albicans* demonstrated sensitivity to all six antifungal drugs tested, namely amphotericin B, caspofungin, flucytosine, fluconazole, micafungin, and voriconazole.

Keywords: Phenotypic assays, Intensive care unit, *Nakaseomyces*, ITS, 5.8S, Micafungin.

التشخيص الجزيئي لبعض أنواع *candida* spp المعزولة من مرضى وحدة العناية المركزة و تقييم حساسيتها لبعض المضادات الفطرية.

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الخلاصة

تظهر أنواع *Candida* الموجودة في وحدات العناية المركزة (ICU) معدل حدوث أعلى ومقاومة أكبر مقارنة بتلك غير المرتبطة بوحدات العناية المركزة. تم استخدام كل من الطرق الظاهرية والجينية لتحديد سلالات داء المبيضات الفموي، بما في ذلك العزلات المقاومة للأدوية التي توجد عادة في وحدات العناية المركزة. استخدمت عملية التشخيص الجزيئي للسلالات المقاومة المتعددة للأدوية في وحدة العناية المركزة

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جينات الحامض النووي الرايبوسومي ، الأنواع المشخصة شملت *Nakaseomyces glabratus* و*Candida dubliniensis* و*Candida albicans*. تم تسجيل هذه السلالات في قاعدة بيانات البنك الجيني. تم تشخيص هذه السلالات باستخدام بطاقة VITEK2 AST-YS08. تشير النتائج إلى أن العزلة السائدة كانت *C. albicans*، إذ شكلت 56.67% من العزلات، بينما شملت العزلات غير الألبكانية *Nakaseomyces glabratus* (33.33%) و *C. Dubliniensis* (10%). تم إجراء دراسة حول تكوين السبورات الكلاميدية و تحمل درجات الحرارة. أظهر اختبار الحساسية للعزلات باستخدام بطاقة VITEK2 AST-YS08 أن *Nakaseomyces glabratus* و*C. dubliniensis*، المعزولين من وحدة العناية المركزة، أظهرا مقاومة أعلى لستة أدوية مضادة للفطريات. من ناحية أخرى، أظهر *C. albicans* حساسية لجميع الأدوية المضادة للفطريات الستة التي تم اختبارها، وهي amphotericin B ، caspofungin ، flucytosine ، fluconazole ، micafungin ، و voriconazole .

1.Introduction

Candida spp is an opportunistic fungal pathogen that causes infections in the skin, mucosal membranes, and systemic infections [1] *Candida* spp possesses virulence characteristics, including adhesion, biofilm formation capacity, hydrolytic enzymes, and resistance to antifungal drugs [2-3-4]. Oral candidiasis presents in various forms, including oropharyngeal candidiasis, pseudomembranous candidiasis, erythematous candidiasis, and juxta vermillion candidiasis. [5]. It can spread to the bloodstream, and upper gastrointestinal tract, and it is a major cause of death in the ICU. [6-7-8-9-10]. Several factors contribute to an increased risk of fungal infections in the ICU, including the use of broad-spectrum antifungal medicines, corticosteroid drugs, vascular catheters, renal replacement therapy, and surgery [10-11].

Numerous studies have identified *Candida dubliniensis* in patients diagnosed with acute respiratory distress syndrome (ARDS) in the intensive care unit (ICU), alongside *Pneumocystis jiroveci* and Methicillin-susceptible *Staphylococcus aureus* is an infection caused by bacteria commonly found on the skin [12–13]. Invasive yeast infections (IYIs) can occur in both immunocompetent individuals and those with a weakened immune system experiencing acute COVID-19 in the intensive care unit [14–15].

Candidemia caused by *Candida glabrata* and *Candida parapsilosis* in the ICU is linked to a higher occurrence, greater resistance, and increased mortality rate compared to candidemia unrelated to the ICU [16-17-18]. Accurate identification of *Candida* species is crucial for improving treatment efficacy, and minimizing side effects and the development of resistance [19]. While phenotypic assays are commonly employed for yeast identification; however, the traditional approach necessitates a considerable amount of time and has limited accuracy [20]. In contrast, genotypic assays offer accurate, trustworthy identification, and exhibit greater stability and sensitivity compared to phenotypic assays, which are influenced by environmental circumstances [21].

Different classes of antifungal medications selectively inhibit multiple cellular processes. Antifungals can either inhibit the growth of pathogens (fungistatic) or eliminate them (fungicidal). Polyenes and azoles act as inhibitors of cell membrane biosynthesis, whereas echinocandins specifically target cell wall synthesis. Additionally, the synthesis of RNA is inhibited by 5-flucytosine [22].

The rising cases of resistance to antifungal drugs such as azoles, polyenes, and echinocandins in *C. glabrata* and *C. dubliniensis* may be attributed to DNA mutations. Therefore, studying *Candida* strains resistant to antifungal drugs is essential for better understanding, effective treatment of patients and reducing misdiagnosis and unnecessary use of antifungal drugs [23-24-25]. This study aims to isolate, phenotypically, and molecularly

diagnose *Candida* species from the oral cavity of patients in an intensive care unit and evaluate the susceptibility and resistance of these strains to various antifungal medications.

2. Materials and Methods:

Specimens' collection

A total of 30 specimens were collected from male patients in the ICU at Hilla Educational Hospital. The age range of these patients was between 20 and 60 years. Specimens were taken from individuals exhibiting clinical manifestations of oral candidiasis, such as the presence of white patches on the surfaces of the oral cavity and red patches on the palate [26]. The specimens were obtained using transport medium swabs and then transported to the laboratory. They were then cultured on Sabouraud dextrose agar (Accumix/India) supplemented with 10 mL of chloramphenicol and incubated at a temperature of 37 °C for 24 to 48 hours [20].

Phenotypic identification

Corn meal agar supplemented with Tween 80 was employed to examine the presence of pseudohyphae, plastospores, and chlamydospores. A loop was used to create three parallel lines of yeast cells on the agar surface. Subsequently, a coverslip is placed on top. The petri dish was incubated at 28 °C, and the microscopic morphology was examined after 48 hours. Additionally, a temperature tolerance test was conducted at a temperature of 45 °C [27].

Antifungal susceptibility testing and identification of *Candida* isolates using the VITEK2 AST-YS08 card

The VITEK2 AST-YS08 Card is used for identifying *Candida* spp and testing their antifungal susceptibility. The Vitek 2 AST-YS01 card contains fluconazole ranging from 2 to 64 µg/ml, flucytosine ranging from 1 to 32 µg/ml, voriconazole ranging from 0.5 to 8 µg/ml, amphotericin B ranging from 1 to 32 µg/ml, micafungin ranging from 0.06 to 4 µg/ml, and caspofungin ranging from 0.12 to 8 µg/ml. Preparation of a suspension of pure culture and adjustment of its turbidity to 1.8-2 McFarland standards. The Yeast Identification and Antimicrobial Susceptibility Testing (ID/AST) cards are placed in a separate tube, one for the antifungal susceptibility test and the other for identification, before being transferred into the Vitek-2 equipment. The cards were placed in an incubator at 37 °C for 18–24 hours. The results are analyzed by evaluating the optical density values recorded at 15-minute intervals [28–29].

Molecular diagnosis using PCR technology

DNA extraction

The DNA of a pure culture of *Candida* isolates was extracted and purified using the DNeasy Plant Kit (QIAGEN, Germany). The Eppendorf tube contains yeast colonies and 400µL of AP1, incubated in a water bath at 65°C for 10 minutes. The Precipitation procedure included adding 130 µL of buffer P3 to the sample, followed by incubation on ice. The resulting mixture was subsequently centrifuged at 14000 rpm for 5 minutes. Finally, the supernatant was transferred to a purple-coloured QIA shredder tube. After centrifugation for 2 minutes, the filtrate was moved to a new tube, and 700 µL of AW1 buffer was added. Transfer 650 µL of the mixture into the white DNeasy tube and perform its centrifugation at 8000 rpm. After additional centrifugation at both 8,000 rpm and 14,000 rpm for 2 minutes, 500 µL of AW2 solution was used to remove biomolecules. The elution buffer employed was TE solution, and the extracted DNA was stored at a temperature of -20 °C [30].

Polymerase Chain Reaction in this study

Study primers provided by QIAGEN, Germany, are listed in Table 1. The prepare a primer solution with a concentration of 100 picomol/µl, 10 µl of stock solution was mixed with 90 µl of de

Table 1: Primers used in this study ionized water, following the instructions provided by the manufacturer.

Primer	→ 5' 3'	PCR product size(bp)
ITS1	5' (TCC GTA GGT GAA CCT GCG C) 3'	1645-1639
ITS4	5' (TCC TCC GCT TAT TGA TAT GC) 3'	

Ready-To-Go PCR Beads

A ready-to-use PCR beads kit (GE Healthcare, UK) was used to conduct PCR tests. To achieve a final volume of 25 µL, 2 µL of DNA was added to two tubes, and then deionized water was added to get a total volume of 25 Ml [30].

Polymerase Chain Reaction Conditions

The PCR amplification process consists of an initial denaturation cycle at 95 °C for 5 minutes and 35 cycles for the final denaturation for 40 seconds. The temperature at which the annealing process occurs is 55 °C, and it lasts for 40 seconds. This process was repeated 35 times. Within each cycle, the initial extension was set at 72°C and lasted for one minute, while the final extension was set at 72° and lasted for 5 minutes.

Electrophoresis

The agarose gel was prepared by dissolving 1.5 grams of agarose in 100 mL of TBE buffer (10x TBE). Additionally, 5 µL of ethidium bromide dye was incorporated into the mixture. After pouring the agarose gel was poured and allowed to harden. The DNA was loaded, and a 2000 bp DNA ladder was utilized to determine the size of the duplicated DNA. The Macrogen Company determined the DNA product sequences. We analyzed and compared the isolated sequences using the National Center for Biotechnology Information (NCBI) database. We used the NCBI database to identify similarities and differences between the study and global isolates. We employed molecular evolutionary genetics analysis (MEGA) to construct a genetic tree for the isolates.

3. Results and Discussion

Phenotypic identification

C. albicans and *C. dubliniensis* can undergo phenotypic switching, which refers to a reversible change between different sets of morphologies [31]. The cornmeal agar test result reveals that *C. albicans* produces single-terminal chlamydospores, while *C. dubliniensis* generates branched pseudohyphae with terminal chlamydospores. The formation of Chlamydospore is influenced by various factors, including oxygen level, pH value, and genetic factors. *Nakaseomyces glabratus* did not exhibit chlamydospores; these results correspond to Böttcher *et al*. [32] and Ravinder *et al* [33], as shown in Figure 1. Temperature tolerance test results revealed that *C. albicans* can grow at 45°C, but *C. dubliniensis* exhibits poor growth under the same conditions. Incubating strains at a temperature of 45°C allows for the differentiation between *C. albicans* and *C. dubliniensis*. During this temperature, *C. dubliniensis* does not exhibit growth, whereas *C. albicans* quickly established colonies; these results correspond to those of Ryberg *et al.* [34]. Additionally, high-density growth of Nakaseomyces glabratus was observed at 45 °C, as shown in Figure 2.

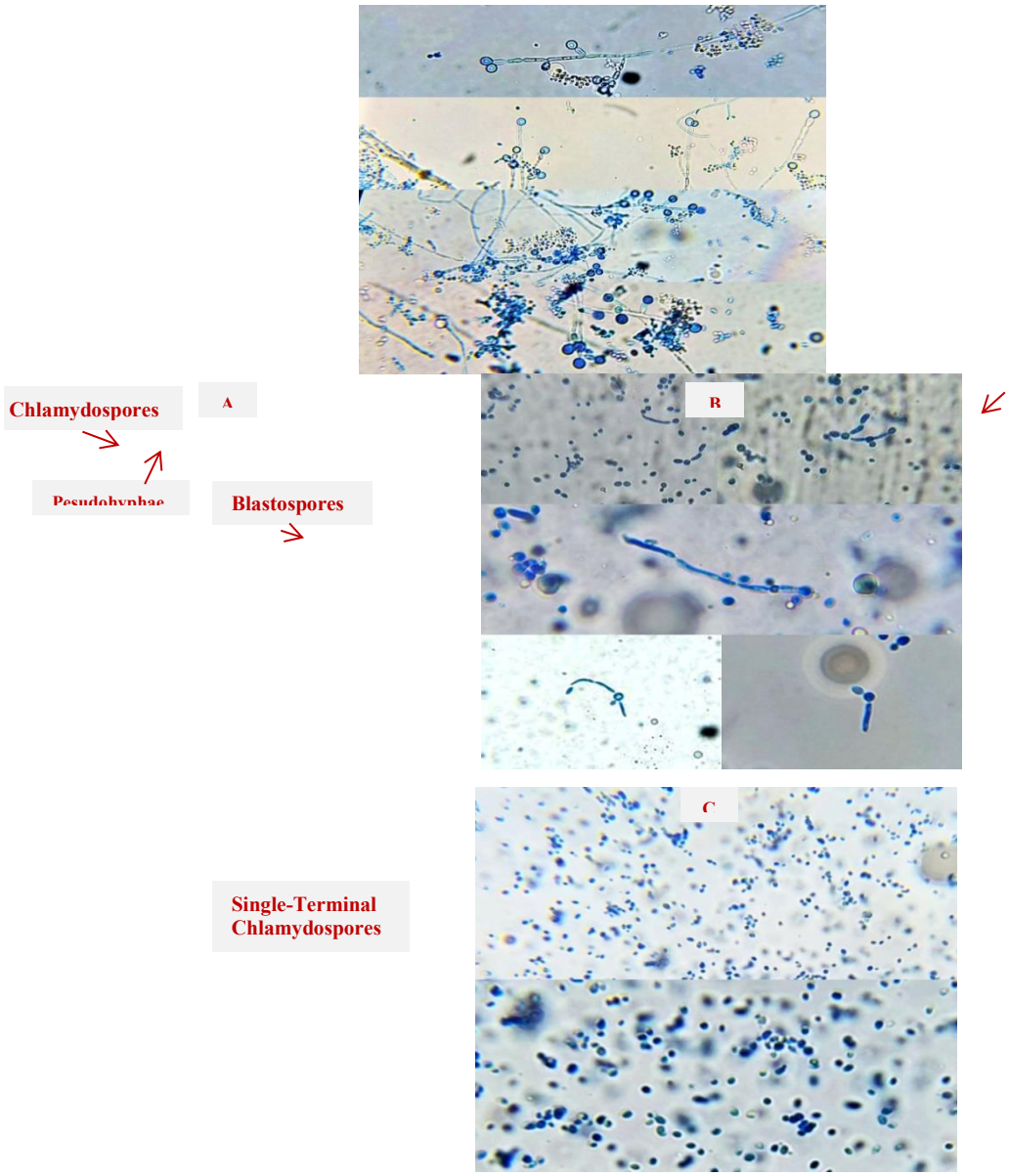

Figure 1 Cornmeal agar medium with Tween 80: Production of Chlamydospores and Pesudohyphae : A*: C. dubliniensis* , B: *C. albicans* and C: *Nakaseomyces glabratus*. (40X)

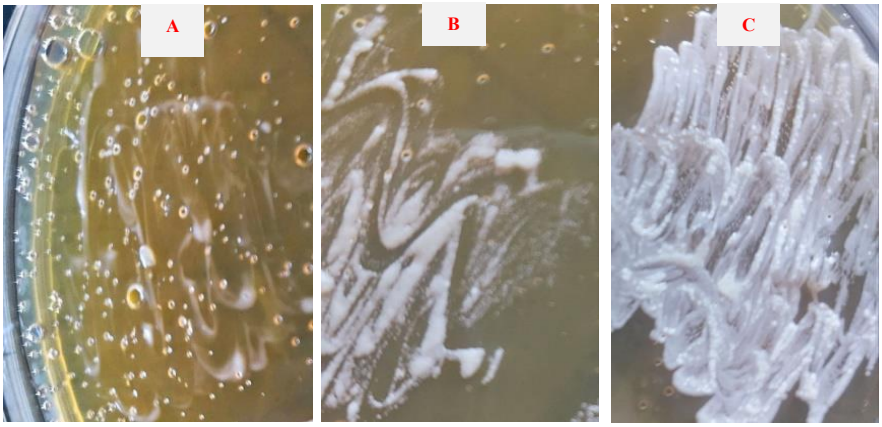

Figure 2: The growth at 45°C on sabouraud dextrose agar medium: A: C. dubliniensis , B: C. albicans and C: Nakaseomyces glabratus.

Antifungal susceptibility testing and Identification of *Candida* isolates using VITEK2 AST–YS08 Card

The study isolates identified using the VITEK2 AST-YS08 Card showed that *C. albicans* was the most common isolate, while non-albican isolates were *Nakaseomyces glabratus* and *C. dubliniensis*. Isolating *C. glabrata* independently is uncommon due to its adhesion to C. *albicans* hyphae, leading to its common identification with *C. albicans*. The invasive ability of *C. glabrata* may be attributed to co-infection with other microbes, as this yeast is frequently isolated with *C. albicans* [35], as listed in Table 2.

Table 2: Candida species distribution

Study strains	Number of Isolates	Strains percentage
Candida albicans	17	56.67%
Nakaseomyces glabratus	10	33.33%
Candida dubliniensis	3	10%

In the susceptibility test, *C. albicans* has shown sensitivity to all six antifungals, whereas *Nakaseomyces glabratus* and *C. dubliniensis* demonstrated resistance to the antifungal agent under investigation. *Nakaseomyces glabratus* exhibited resistance to all six antifungals, while *C. dubliniensis* displayed sensitivity solely to flucytosine and amphotericin B *Candida* spp. Intensive care unit isolates exhibit higher levels of resistance to antifungals compared to other isolates, particularly due to multi-drug resistance [16–17] Energy-dependent drug efflux is the primary cause of high-level fungal azole resistance. However, the majority of oral fungi exist in multi-species biofilms. These biofilms are resistant to antifungals because they have efflux pumps, matrix permeability, and stress responses [36], as listed in Table 3.

Studies have demonstrated that *C. glabrata* has higher resistance and mortality rates in intensive care units than non-ICU isolates; therefore, the delay in diagnosing *C. glabrata* in ICU patients results in clinical deterioration and death [18-36]. In Iraq, the use of non-culture and culture-based diagnostic methods in the ICU is limited, leading to misdiagnosis or delays in initiating antifungal treatment. The likelihood of acquiring infection with antibiotic-resistant organisms increases with greater age, severity of illness and debility, prolonged stay in the intensive care unit, prior antibiotic use, and exposure to indwelling prosthetic devices [37].

Studies have shown that the prevalence of *C. dubliniensis* resistance to azole drugs is on the rise due to gene MDR1p overexpression [38–39].

Table 3 : Antifungal susceptibility results of Candida species

Antifungal	Concentration	*C. albicans*	*C. dubliniensis*	*Nakaseomyces glabratus*
		MIC	MIC	MIC
Fluconazole	1 - 64 µg/ml	< = 0.5	-	-
Voriconazole	0.5 - 8 µg/ml	< = 0.12	-	-
Caspofungin	0.12 - 8 µg/ml	< = 0.12	-	-
Micafungin	0.06 - 4 µg/ml	< = 0.06	-	-
Amphotericin B	1 - 32 µg/ml	0.5	<= 0.25	-
Flucytosine	1 - 32 µg/ml	< = 1	<= 1	-

Note : (-) means : Resistant

Acquired resistance, arising during treatment, is challenging to predict. Fluconazole used to treat *Candida* infections, *C. glabrata*, exhibits cross-resistance to different azoles. Echinocandins resistance is observed in prolonged treatment. *Candida* resistance to amphotericin B is uncommon but challenging to identify. *Candida glabrata* typically exhibits

elevated minimum inhibitory concentrations (MICs) for amphotericin B, and an increasing number of studies indicate its resistance to polyenes [37].

Molecular identification of *Candida* isolates

The nitrogen bases of the Internal Transcribed Spacer (ITS) function as a universal barcode that displays notable variation and conserved regions. As a result, they are well-suited for constructing universal primers. The vast number of copies of different strains' Internal Transcribed Spacer (ITS) accessible in the Genbank database significantly facilitates the comparison procedure [40]. The extraction and amplification of 5.8S ribosomal RNA genes from *Nakaseomyces glabratus* produce a 799 bp linear DNA sequence that is identical to strains from Kuwait, Brazil, Argentina, Iran, China, Saudi Arabia, and Russia (Table 4). The linear DNA of *C. dubliniensis*, consisting of 536 base pairs, exhibits a high degree of similarity to the strain from the Netherlands, which is 100%. However, the similarity to the strain from Iraq (Nasiriyah) is 98.87%, so the dissimilarity can be attributable to six distinct nucleotides Table 5. The 523 base pair linear DNA of *C. albicans* has a high degree of similarity to the strain from Saudi Arabia, with a similarity of 99.62%, and the dissimilarity is attributed to a single distinct nucleotide. However, the similarity to the strain from the Netherlands approaches 99.04% and the dissimilarity is attributed to two distinct nucleotides and three deletions (Table 6). Analyze the strains available in the GenBank database and use the BLAST tool to compare their nitrogenous bases with those of global strains.

Table 4: Comparison Nakaseomyces glabratus ITS-ribosomal RNA genes sequence study strain with other strains registered globally at NCBI

Sequence ID	Origin	Strain	Identities	Query Length
PP467556.1*	Iraq	*Nakaseomyces glabratus*	100%	799bp
LS398112.1	Kuwait	*Nakaseomyces glabratus*	100%	886 bp
KX450830.1	Brazil	*Nakaseomyces glabratus*	100%	858 bp
KX450823.1	Brazil	*Nakaseomyces glabratus*	100%	854 bp
MG241517.1	Argentina	*Nakaseomyces glabratus*	100%	832 bp
LC389276.1	Iran	*Candida glabrata*	100%	818 bp
KY963116.1	China	*Nakaseomyces glabratus*	100%	891 bp
MK560212.1	Saudi Arabia	*Nakaseomyces glabratus*	100%	894 bp
MK998697.1	Russia	*Nakaseomyces glabratus*	100%	808 bp
LC389265.1	Iran	*Candida glabrata*	100%	863 bp

Table 5: Comparison C. dubliniensis ITS-ribosomal RNA genes sequence study strain with other strains registered globally at NCBI

Sequence ID	Origin	Strain	Identities	Query Length
PP467530.1*	Iraq	*C. dubliniensis*	100%	536 bp
KY102057.1	Netherlands	*C. dubliniensis*	100%	547 bp
MK394123.1	Netherlands	*C. dubliniensis*	99.05%	2663 bp
MH545916.1	Netherlands	*C. dubliniensis*	99.05%	2804 bp
KJ451682.1	Brazil	*C. dubliniensis*	99.05%	890 bp
KP131697.1	Australia	*C. dubliniensis*	99.50%	916 bp
MZ536252.1	Netherlands	*C. dubliniensis*	98.87%	2663 bp
MZ536251.1	Iraq(Nasiriyah)	*C. dubliniensis*	98.87%	565 bp
MZ536252.1	Iraq(Nasiriyah)	*C. dubliniensis*	98.87%	568 bp

Table 6: Comparison C. albicans ITS-ribosomal RNA genes sequence study strain with other strains registered globally at NCBI

Sequence ID	Origin	Strain	Identities	Query Length
PP467531.1*	Iraq	*C. albicans*	100%	523 bp
MN419369.1	Saudi Arabia	*C. albicans*	99.62%	550 bp
OK267607.1	China	*C. albicans*	99.43%	545 bp
OK267806.1	China	*C. albicans*	99.43%	534 bp
OK267958.1	China	*C. albicans*	99.24%	546 bp
OK267947.1	China	*C. albicans*	99.24%	532 bp
OK267944.1	China	*C. albicans*	99.24%	532 bp
OK267983.1	China	*C. albicans*	99.24%	535 bp
KY101883.1	Netherlands	*C. albicans*	99.04%	815 bp

The BLAST program has detected certain variations among isolate sequences. *C. dubliniensis* exhibits a high degree of similarity to *C. albicans* of 91.17, with a query cover of 91%. *C. dubliniensis* closely resembles *C. albicans* either phenotypically or genetically [41]. *Nakaseomyces glabratus* shows a degree of similarity to *C. albicans* of 91.91%, with a query cover of only 37%. *Candida albicans* and *C. glabrata* are the predominant pathogenic yeasts affecting humans, yet they exhibit significant biological, genetic, and phenotypic differences [42]. The comparison aimed to identify substitution or deletion sites in the analyzed strains. A significant difference in sequence variation was observed between C. dubliniensis and C. albicans (Table 7).

Table 7 : Variations of speacies closely related to C. albicans

Query strain	Strain	Query cover	Variation type	Location	Nucleotide
C. albicans PP467531.1	***C.dubliniensis*** PP467530.1	91%	Substitution\Transversion Substitution\ Transition Deletion	90, 123, 368 91,96,189-190 98,179-180	G\T, C\A, C\G T\C', A\G , T\A- T\C A, C

By comparing the nitrogen base sequences of identical genes using molecular evolutionary genetics analysis (MEGA), it can identify evolutionary relationships among similar strains and calculate the genetic distance by neighbour-joining. The phylogenetic relationships were created based on the ITS-rDNA nitrogen bases and global ones of the same strains, as illustrated in Figures 3, 4, and 5. The phylogenetic relationships show that *Nakaseomyces glabratus* is distantly related to strains from Kuwait, Argentina, Iran, China, and Brazil, with 100% similarity. *C. dubliniensis* is distantly related to strains from the Netherlands 100%, Australia 99.05%, Brazil 99.05%, and Iraq 98.87%, with some variations in some nucleotides as noted in Table 8. Study isolate *C. albicans* is distantly related to strains from Saudi Arabia (99.62%), China (99.43%), and the Netherlands (99.23%), as shown in Table 9.

Table 8: The Dissimilar sequences among study isolate *C.dubliniensis* and closely related global ones

Query strain	Subject Strain	Sequence ID	Origin	Variation type	Location	Nucleotide
C.dubliniensis PP467530.1	*C.dubliniensis* 98.87%	MZ536251.1	Iraq	Substitution\ Transition Substitution\ Transversion Deletion	9,12 10-11 18,513	A\G G\T C
	C.dubliniensis 99.05%,	KP131697.1	Netherlands	Substitution\ Transition Substitution\Transversion Deletion	40 , 42 41 638	G\A,C\T T\A T
	C.dubliniensis 99.05%,	KJ451682.1	Brazil	Substitution\ Transition Substitution\ Transversion Deletion	11,13 12 6, 628	G\A , C\T T\A T

Table 9 : The Dissimilar sequences among study isolate *C. albicans* and closely related global ones

Query strain	Subject Strain	SequenceID	Origin	Variation type	Location	Nucleotide
C.albicans PP467531.1	*C.albicans* 99.62%	MN419369.1	Saudi Arabia	Substitution\ Transversion	527	G\C
	C.albicans 99.43%	OK267607.1	China	Substitution\ Transition Deletion	461 28	C\T T

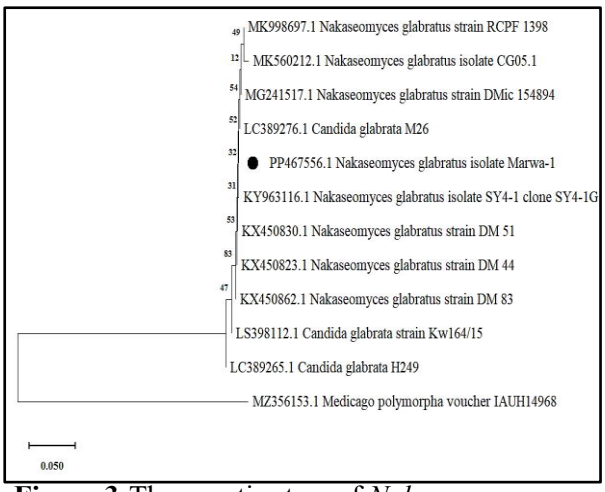

Figure 3: The genetics tree of *Nakaseomyces glabratus* isolate Marwa-1(black point)

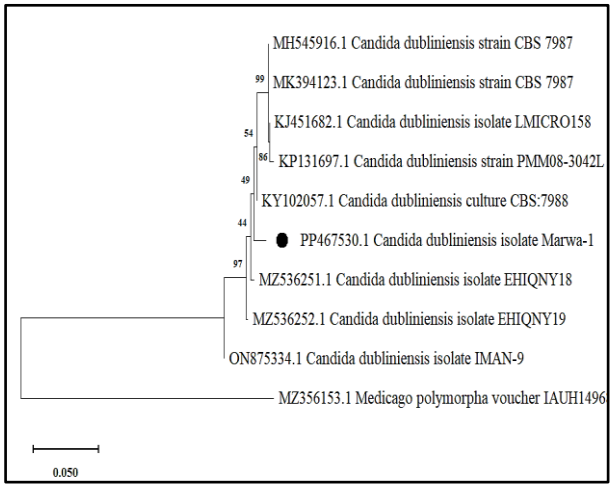

Figure 4 : The genetics tree of *Candida dubliniensis* isolate Marwa-1(black point)

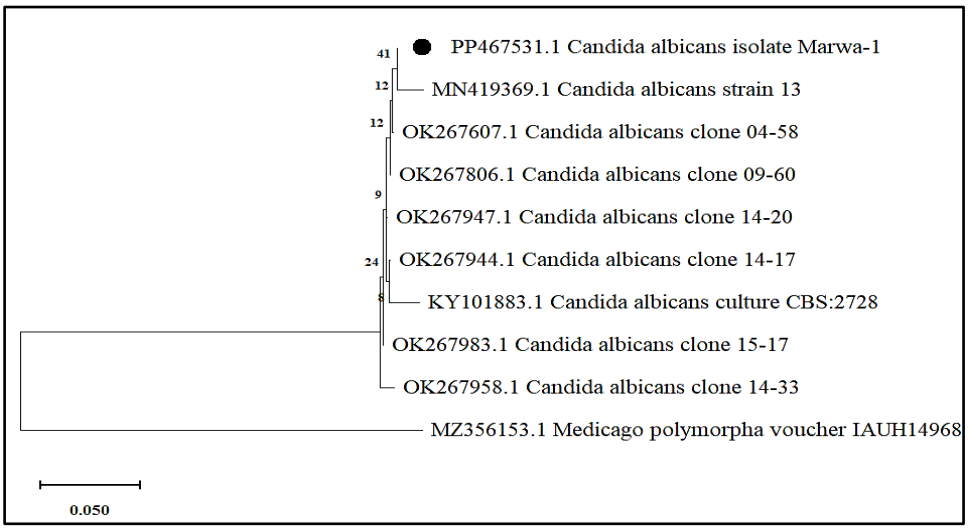

Figure 5: The genetic tree of *Candida albicans* isolate Marwa-1(black point)

Conclusion

Identification of multi-drug-resistant yeast in the ICU was conducted through molecular analysis of the ITS 1 and 2,5.8S ribosomal RNA genes. The species identified included *Nakaseomyces glabratus*, *Candida dubliniensis*, and *Candida albicans*. The findings indicate that *Candida* spp species in the ICU exhibit a higher level of resistance compared to *Candida* spp unrelated to the ICU.

Conflicts of interest

The authors declare no conflicts of interest.

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