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Original Article

Molecular Identification of Candida albicans and C. dubliniensis Using Small Subunit rRNA Gene Sequence in Kerbala, Iraq

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Abstract

This study was conducted to confirm the phenotypic diagnosis of two *Candida* species, including *Candida albicans* (*C. albicans*) and *Candida dubliniensis* (*C. dubliniensis*). They were previously isolated in another study from cases of oral candidiasis using polymerase chain reaction and determining the nitrogenous base sequences of the 18 SrRNA product duplication using the NS1 and NS8 primers. The sequences of the multiple bases were analyzed using the Basic Local Alignment Search Tool program (BLAST), which proved that the two diagnosed *Candida* strains belong to two species, including *C. albicans* and *C. dubliniensis*, respectively. Additionally, the comparison of these sequences to the data available in the National Center for Biotechnology Information (NCBI) database showed that *C. albicans* strains in this study were 99% similar to the universal strains of *C. albicans* from Japan, Brazil, the United States, Germany, India, China, Pakistan, and Egypt. The *C. dubliniensis* strains in this study also had the highest genetic similarity rate of 99% to the *C. dubliniensis* strains isolated from the United States, Netherlands, France, and Germany. The study strains were recorded in the GenBank database with the sequence codes MZ574137 and MZ574410.1 for *C. albicans* and *C. dubliniensis*, respectively. The results of the 18 SrRNA region's duplication also showed variations between *C. albicans* and *C. dubliniensis*, represented by the presence of three mutations of the first type and two mutations in the second type at different sequence sites.

Keywords: BLAST, *Candida albicans*, *Candida dubliniensis*, NCBI

1. Introduction

Candida is found as normal flora in the skin and mucosal surface of the intestine, vagina, respiratory system, and oral cavity; however, under some conditions, such as poor oral hygiene, diabetes, random use of antibiotics, and tumors, it transforms from a commensal organism to a pathogen able to cause infection (1, 2). *Candida* has 200 species, 30% of which have been isolated from human diseases, which is increasing (3). Until recently, *Candida albicans* (*C. albicans*) was considered the most frequent opportunistic pathogen in this genus; however, other non-albicans, such as *Candida dubliniensis* (*C. dubliniensis*), *Candida tropicalis*, *Candida parapsilosis*, and *Candida glabrata*, were also recorded as causative agents of infection (4, 5). Over the past few years, there has been an increase in the incidence of infections caused by *C. dubliniensis* isolated from the mouth of patients infected with AIDS, those who frequently use antibiotics, and cases of tooth decay. It shares many phenotypic characteristics with *C. albicans*, such as the production of germ tubes in

human serum and chlamydospores in the Cornmeal Agar medium. Therefore, using traditional methods to distinguish between them leads to a false identification in fungi laboratories (6). However, many genotypic methods have been used to differentiate between these two species, such as the conventional polymerase chain reaction (PCR), Nested PCR, Multiplex PCR, RT PCR, qPCR, FISH, Microarray, Lamp, RNA interference, and RNA-seq (7). This study aimed to draw a molecular distinction between *C. albicans* and *C. dubliniensis* using the 18 SrRNA.

2. Materials and Methods

2.1. Candida Isolates

Samples taken from oral candidiasis cases were cultured on the Sabouraud Dextrose Agar medium supplemented with amoxicillin (250 mg/L) and incubated at 37°C for 24-48 h. *Candida* species were identified by germ tube production in human serum, chlamydospores, and pseudohyphae formation in the Cornmeal Agar supplemented with tween 80, and they were grown at 45°C (8).

2.2. DNA Extraction and Purification

The process of extracting and purifying the DNA from the growing colonies of *Candida* was conducted using the DNeasy Plant Kit (QIAGEN, Germany) following a few steps. First, a sample was taken from pure colonies and filled with the loop. It was then transferred to a 1.5 mL Eppendorf tube, and 400 µL of AP1 cell lysis was added. The tube was incubated in a water bath at 65°C for 10 min. Afterward, 130 µL of buffer P3 was added to the tube containing the mixture, then it was mixed and incubated for 5 min on ice. This step precipitates the detergents for buffer solutions, proteins, and polysaccharides. The tube was centrifuged at 14000 rpm for 5 min, the supernatant solution was transferred to a purple-colored QIAshredder Mini spin column tube, and a centrifugal process was conducted at the same speed (mentioned above) for 2 min. The filtrate was transferred to a new tube, 700 µL of Purification buffer (AW1) was added, and a micropipette was mixed with the contents. Subsequently, 650 µL of the mixture was transferred by a small pipette to the white DNeasy Mini spin column tube containing a special filter for purification. The DNA was centrifuged at 8000 rpm for 1 min, and the filtrate was discarded. After that, 500 µL of AW2 solution was added to the tube and centrifuged at 8000 rpm for 1 min, and the filtrate was disposed of. A total of 500 µL of AW2 solution was added to the tube and centrifuged at 14000 rpm for 2 min.

The filtrate was then disposed of. The DNeasy Mini spin column tube was placed inside a 2 mL Eppendorf tube, and 100 µL of Elution Buffer TE solution was added directly to it and incubated for 5 min at room temperature. A centrifugal process was then conducted at 8000 rpm for 1 min to obtain the filtrate containing the DNA. The tube was kept at -20°C until use .

2.3. Polymerase Chain Reaction

2.3.1. Preparation of the Stock Primers Solution

The primers (QIAGEN, Germany), shown in table 1, were used and prepared according to Sarhan (8) and the manufacturer's instructions to obtain a final concentration of 100 picomol/µl. The working solution was prepared for each initiator by taking 10 µl of the stock solution each separately and adding 90 µl to it with 1 µl of deionized water to get a concentration of 10 picomol/µl.

Table 1. The primers used in the study

Primer	Sequence 5' → 3'	PCR product size(bp)
NSI	F GTA GTC ATA TGC TTG TCT C	1645-1639
NS8	R TCC GCA GGT TCA CCT ACG GA	

2.3.2. Preparation of the Ready-To-Go PCR Beads Kit

The PCR test was carried out using the Ready-To-Go PCR Beads kit (GE Healthcare, UK), which included 2.5 units of PuRE Taq DNA polymerase, 10 mM Tris HCI(PH9), 50 mM KCL, 1.5 mM MgCl2, 200 µM of each dNTP, stabilizers, and BSA. The final volume of the reaction was 25 µL, so 2 µL of the DNA was added to each tube, 1 µL from the front and 1 µL from the back primer. The volume was supplemented to 25 µL by adding deionized water.

2.3.3. Polymerase Chain Reaction Conditions

The DNA of the isolated fungi was doubled using the steps shown in table 2.

Table 2. Conditions of polymerase chain reaction

Steps	Temperature (c)	Time	Number of cycle
Initial Denaturation	95	5 minute	1
Denaturation Final	95	40 second	
Annealing	55	40 second	35
Extention Initial	72	1 minute	
Final Extension	72	5 minute	1

2.4. Electrophoresis

Agarose gel was prepared by dissolving 1.5 g in 100 mL of buffer solution (10×TBE prepared by adding 90 mL of sterile distilled water to 10 mL of TBE solution) and mixing until a clear solution was formed. After that, 5 µL of ethidium bromide dye solution was added after decreasing the temperature of the solution to 40-45°C. The mold was prepared for pouring the agarose gel containing a comb to make holes inside the agarose layer. The dissolved agarose containing the dye was poured and left at room temperature to solidify. The replication products were detected by loading 5 µL of the replicated DNA into each hole of the agarose gel layer. In addition, 5 µL of the volume guide (2000 bp DNA ladder) was added to the left side of the added samples to determine the size of the replicated DNA. The electrophoresis of the samples was carried out, and the reaction results were examined under ultraviolet rays.

The DNA replication products isolated from the PCR were sent to the Korean company Macrogen to determine the sequence of nitrogenous bases. The nitrogenous sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program to find the similarity between the studied fungi and those registered globally. They were also compared with the data recorded in the National Center for Technical Information (NCBI).

3. Results and Discussion

The results of the phenotypic and physiological diagnosis of the study samples revealed the diagnosis of two *Candida* species, which were *C. albicans* and *C. dubliniensis*. A PCR operation was performed to distinguish between them.

The results of the PCR showed the possibility of complete duplication of the 18 SrRNA region, which is vital in the diagnosis of fungi. This result agrees with what was mentioned by Ban Taha, Zakra Mohammad Kazem (9), (10-12 The selection of the 18 SrRNA in the diagnosis of fungi to the level of genus and species is due to several reasons, including the presence of many universal primers for fungi based on the conserved regions of the 18 SrRNA gene, which makes it possible to obtain the PCR products. In addition, the deposit of a large number of 18 SrRNA sequences in the Genbank database makes the similarity search convenient, and the repetitive nature of this gene has more than 100 copies per fungi genome, which makes rRNA-dependent amplification easier. Therefore, this gene is suitable for finding diverse and conserved regions within a group of fungi to establish phylogenetic relationships between closely related fungi (10).

In this study, replications were obtained with sizes ranging from 1639 bp for *C. albicans* to 1643-1645 bp for *C. dubliniensis* after DNA extraction from the fungi in the presence of the forward and reverse primers, NS1 and NS8 (13) (Figure 1). It was deposited in the GenBank database of the NCBI. The isolate was identified as *C. albicans* with the sequence code MZ574137.1 and *C. dubliniensis* with the sequence code MZ574410.1.

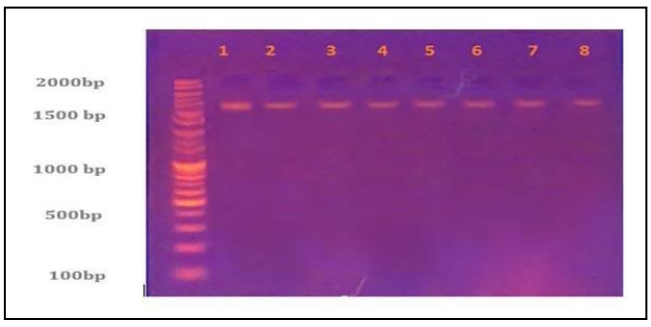

Figure 1. Electrophoresis of the PCR product of C. albicans and C. dubliniensis. M=2000bp DNA ladder, 2= C. albicans, 6= C. dubliniensis, 1,3,4,5 = other yeasts. 7,8 = no addition.

The analysis of the nitrogenous bases of the DNA replication products using the BLAST program proved that the studied strains belong to *C. albicans* and *C. dubliniensis*. The results also showed variations between the two diagnosed species, as *C. albicans* was distinguished by the presence of two mutations (Transition G/A at sites 995 and 1026 and Transition (C/G) at site 1040). On the other hand, *C. dubliniensis* showed two mutations of Transition (C/G) at sites 1079 and 1084 (Table 3).

The comparison of the sequence of nitrogenous bases for the study strains showed a 99% match between the isolates of our study and the global ones. The MEGA evolutionary genetic analysis program (version 7) was used. One of the programs was designed to analyze and compare the sequences of identical genes, evolutionary relationships, as well as the pattern of DNA and protein evolution. The program also provides many facilities through serial data, which can be presented as an evolutionary tree (14). The studied strain of *C. albicans* showed a similarity rate of 99% with global isolates of *C. albicans* from Japan, Brazil, the United States, Germany, India, China, Pakistan, and Egypt. The studied strain also showed a genetic similarity rate of 98% with isolates from Iraq (Wasit) and Australia and a genetic similarity rate of 97% with the isolates from Holland (Table 4).

The results of the phylogenetic analysis confirmed this diagnosis by grouping the fungal isolate within groups of isolates belonging to the same species shown in figure 2. The genetic distance was calculated using the neighbor-joining method.

In addition, the comparison of the sequence of the nitrogenous bases of the doubled NS1 and NS8 regions of *C. dubliniensis* isolated in this study revealed that it has a genetic similarity rate of 99% with *C. dubliniensis* isolates from the United States, Holland, France, and Germany, as well as a 98% similarity with those from Ireland, Holland, and the United States, while its percentage of similarity with the Thai isolates was 96% (Table 5 and Figure 3).

Table 3. Variations between *C. albicans* and *C. dubliniensis* isolates

Name of strain	Accession number	Type of substitution	Location	*Nucleotide*
C.albicans	MZ574137.1	Transition	995	G\A
		Transition	1026	G\A
		Transvertion	1040	C\G
C.dubliniensis	MZ574410.1	Transition	1079	G\A
		Transition	1084	G\A

Table 4. Comparison of the sequence of nitrogenous bases of C. albicans isolated in this study with other isolates of the same fungus registered globally at NCBI

Accession number	Origin	Strain name	similarity
MZ574137.1*	Iraq	*Candida albicans*	99%
CP032012.1	Japan	*Candida albicans*	99%
CP025165.1	Brazil	*Candida albicans*	99%
HQ876034.1	USA	*Candida albicans*	99%
M60302.1	Framingham	*Candida albicans*	99%
AJ005123.1	Germany	*Candida albicans*	99%
KJ095700.1	India	*Candida albicans*	99%
JN940584.1	Philippines	*Candida albicans*	99%
MN826341.1	China	*Candida albicans*	99%
KX557293.1	Pakistan	*Candida albicans*	99%
AB013586.1	Japan	*Candida albicans*	99%
MG851796.1	Egypt	*Candida albicans*	99%
X53497.1	Belgium	*Candida albicans*	99%
AF114470.1	Australia	*Candida albicans*	98%
MW736400.1	Iraq:Wasit	*Candida albicans*	98%
MW281685.1	Netherlands	*Candida albicans*	97%

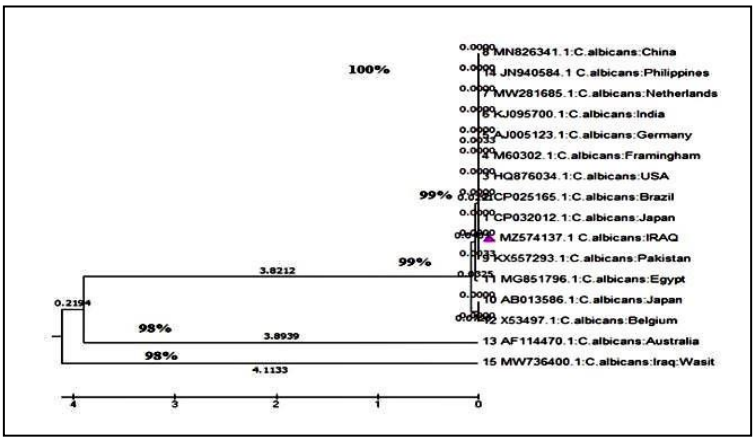

Figure 2. The genetic tree of Candida albicans (marked with a purple triangle) showing the relationship with global fungal strains

Table 5. Comparison of the nitrogenous base sequences of C. dubliniensis isolated in this study with other isolates of the same fungus registered globally in NCBI

Accession number	Country	Strain name	similarity
MZ574410.1*	Iraq	*Candida dubliniensis*	99%
FM992695.1	United Kingdom	*Candida dubliniensis*	99%
MW281616.1	Netherlands	*Candida dubliniensis*	99%
MF045510.1	Germany	*Candida dubliniensis*	99%
EU139420.1	France	*Candida dubliniensis*	99%
MW281617.1	Netherlands	*Candida dubliniensis*	98%
MW281618.1	Netherlands	*Candida dubliniensis*	98%
X99399.1	IRELAND	*Candida dubliniensis*	98%
AY669334.1	UK	*Candida dubliniensis*	98%
KF501421.1	Thailand	*Candida dubliniensis*	96%

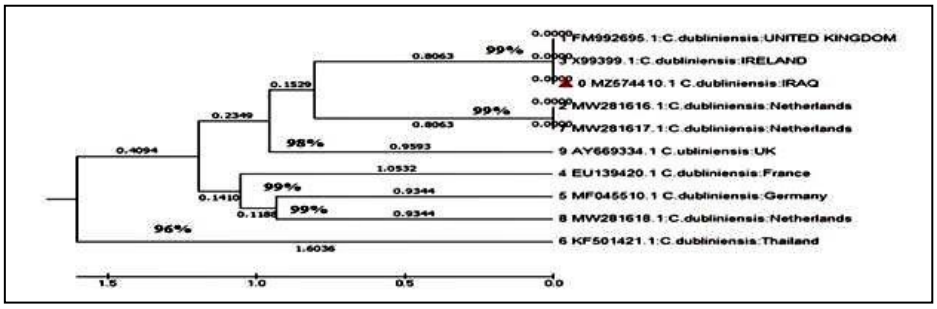

Figure 3. The genetic tree of Candida dubliniensis (marked in a red triangle) showing the relationship with global fungal strains

Despite the importance of phenotypic diagnosis in limiting the fungi under study into smaller groups, many problems accompany this diagnosis, including the researcher's need for high expertise in the diagnostic process, especially in diagnosing closely related species, as well as the need for significant time and effort. On the other hand, the diagnosis of fungi using interaction technology sequential polymerization is characterized by high accuracy, which helps the researcher to overcome diagnostic problems typical of traditional methods (7). Khot, Ko (14) indicated that fungal infections, including candidiasis, change with the increase in the use of medicine. It also changes with the increase in the use of antifungal drugs, so it is necessary to use diagnostic methods capable of diagnosing pathogens, including molecular methods,

which increase the chance of success in choosing the appropriate antifungal treatment.

Authors' Contribution

Study concept and design: M. T. A.
Acquisition of data: B. M. H.
Analysis and interpretation of data: S. A. A.
Drafting of the manuscript: B. M. H.
Critical revision of the manuscript for important intellectual content: B. M. H.
Statistical analysis: M. T. A.
Administrative, technical, and material support: M. T. A.

Conflict of Interest

The authors declare that they have no conflict of interest.

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