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Molecular Detection of *Candida tropicalis* Isolated from Immunocompromised Patients with Otitis Media

Aymen Nazar Khalil*, Abbas M. Ammari

Department of Biology, College of Science, University of Diyala, Iraq.

* ayman.nazar997@gmail.com

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Abstract

Otomycoses are fungal infections that affect the external ear canal and, in some cases with perforated tympanic membranes, the middle ear. *Candida* are the most common fungal genera causative agents of otomycoses. *Candida tropicalis* is a species of yeast in the genus *Candida*. One hundred swaps were collected from patients with otitis media who attended the ENT consulting clinic at Baquba Teaching Hospital for a period extending from the 15th of December 2022 to the 15th of March 2023. The specimens were collected from immunocompromised and immunocompetent patients (50 each). Clinical diagnosis was done by a consultant Otolaryngologist. Cultural methods including (Sabouraud's dextrose agar medium (SDA) and Hi-Chrom Candida agar) media incubating at 35±2°C for 48 hrs, and Molecular methods (Conventional PCR and gene sequencing) for ITS and *Als2* genes were used for the identification of *C. tropicalis*. The susceptibility of isolated *C. tropicalis* was tested toward fluconazole (10 mg) and miconazole (10 mg) antifungals by using the disk diffusion method. The results were males have the highest rate among immunocompromised patients with otitis media (58.0%). According to the rate of *C. tropicalis* identification, 14.0% (7 out of 50) isolates were collected from otitis media immunocompromised patients. Antibiotic susceptibility results revealed that only 3 isolates of *C. tropiclais* (25.0%) were resistant to both antibiotics (Fluconazole and Miconazole). PCR results showed that the molecular weight of ITS and *Als2*

genes were 520 bp and 80 bp., respectively. The mutant isolate, has more than one substitution mutation.

Key words: *Candida tropicalis*, *Als2* gene, Otomycosis, Immunocompromised patients.

Introduction

Otitis media (OM) is an inflammatory infection that affects the middle canal of the ear and leads to deafness [1]. Otomycoses are fungal infections that affect the external ear canal, and in some cases, they can affect the middle ear when the tympanic membrane is perforated. Some of the symptoms are inflammation, itching, scaling, and a hole in the tympanic membrane, hearing loss, and discharge from the ear. The otomycosis frequency depends on the tropical and subtropical climatic conditions, with hot, humid regions (as high as 54%) and dusty areas [2]. Aspergillus, Candida, is the most common fungal causative agent of otomycoses. [3] *Candida tropicalis* is a species of yeast in the genus *Candida*. It is a typical pathogen in neutropenic hosts, where it can disseminate to peripheral organs through circulation [4]. Traditional and molecular methods are used to identify fungal species, such as morphological characteristics (macroscopic and microscopic examination) and PCR sequencing of the ITS gene [5]. Azole antifungals such as miconazole and clotrimazole work by inhibiting the cytochrome P450-dependent enzyme lanosterol 14-α-demethylase that is necessary for the conversion of lanosterol to ergosterol, which is a vital component of the cellular membrane of *Candida* species [6]. As an important step in pathogenesis, adhesion leads to tissue damage and invasive infections, and the agglutinin-like sequence (ALS) protein family forms important adhesion molecules. The composition of the ALS family of *C. tropicalis* includes 16 genes that encode cell-surface glycoproteins that contribute to adhesion [7, 8]. Because of *Candida* is the commonest fungal causes of otomycoses, this study was aimed to detect the substitution mutations in in *Als2* gene of *C. tropicalis* isolated from immunocompromised patients with otitis media.

Materials and Methods

Clinical Examinations and Specimens Collection

One hundred swaps were collected from patients with otitis media who attended the ENT consulting clinic at Baquba Teaching Hospital for a period extended from the 15th of December

2022 to the 15th of March 2023. The specimens were collected from immunocompromised and immunocompetent patients (50 each). Clinical diagnosis was done by a consultant Otolaryngologist.

Identification of *C. tropicalis*

For initial identification, a single colony of *C. tropicalis* isolate was subjected by staining with lacto phenol cotton blue on a sterile glass slide and for additional identification, a single colony of Sabouraud's dextrose agar medium (SDA) was subcultured on Hi-Chrom Candida agar medium with incubating at 35±2°C for 48 hrs [9].

Antifungal susceptibility test

The susceptibility of isolated *C. tropicalis* using an SDA medium was tested toward fluconazole (10 mg) and miconazole (10 mg) antifungals by using the disk diffusion method as described by the researchers [10].

Molecular detection of ITS region and *Als2* gene of *C. tropicalis* using PCR method [11]

Extraction of *Candida tropicalis* DNA

The genomic DNA of *C. tropicalis* was isolated according to the protocol of ABIO-pure Extraction kit for fungal DNA extraction.

Polymerase chain reaction (PCR) technique

The polymerase chain reaction (PCR) combination of 25 µL final volume was prepared from Go Taq Green Master Mix (2X) 12.5 µL, forward primer 1 µL, reverse primer 1 µL, DNA template 3 µL, and nuclease-free water 7.5 µL. The primer sequences for ITS region and *Als2*gene are listed in (Table 1). The PCR program was performed using a PCR thermal cycler under the conditions mentioned in (Table 2).

Table 1: The name, sequence and product size of primers for ITS region and *Als2* gene of *C. tropicalis*.

Name primer	Sequence of primer	Product size (bp)	Reference
ITS-F	5`-TCCGTAGGTGAACCTGCGG-3` 5`-	520	[9]
ITS-R	TCCTCCGCTTATTGATATGC-3` 5'-		
Als2 –F	ACTCGTGCCTATACCTAC -3'	80	[8]
Als2 –R	5'- TTGTTGCCGTAATGGTGG -3'		

Table 2: PCR program for ITS region and *Als2* gene amplification of *C. tropicalis* isolates.

Step	Temperature (°C)	Time	No. of Cycles
Initial denaturation	95	10 min	1
Denaturation	94	30 sec	40
Annealing	55	60 sec	
Extension	72	90 sec	
Final extension	72	7 min	1

Agarose Gel electrophoresis

After PCR. carrying out, agarose gel electrophoreses was done to detect the existence and integrity of the PCR. product. (0.5) grams of agarose powder were dissolved in fifty mLs of buffered Tris Borate EDTA (TBE) to create agarose gel (pH = 8). Next, the mixture was dissolved in a microwave. Then, two microliters of ethidium bromide (5 mg/ ml) were added to the agarose solution stirred to mix and the mixture was cooled at 45°C. After the comb was fixed one cm away from the margin, the agarose solution was put in a tray of gel. After solidifying of gel, the comb was sided and the gel tray was put in the tank which was filled with 0.5X buffered TBE. Three microliters of *C. tropicalis* DNA were disordered with 2 µl of bromophenol blue dye (loading buffer). Specimens were put onto the gel wells, the electrical power was turned on 5 volt/cm2 for 30 min. DNA mobile from (-) cathode pole to (+) anode. Stained bands visible by UV transiluminator at 350nm.

Molecular detection of *Als2* gene of *C. tropicalis* using Gene sequencing method

Two isolates of *C. tropicalis* for *Als2* gene sequencing in both direction were sent to Microgen Inc., South Korea. The sequencing data of targeted gene that received from Microgen Inc. were assembled and translated to contig formate and text document using Contig Express module of Vector NTI 9.0 program. All the reference nucleotide sequences of targeted gene of *A. niger* from (www.ncbi) and aligned using ClustalW method of MEGA4 program. To identify the nucleotide sequences of *Als2* gene of *C.tropicalis*, the reference nucleotide sequences of *Als2* gene of *C. tropicalis* which prepared at previous step was aligned together with nucleotide sequences of *Als2* gene samples of *C. tropicalis* for this study using ClustalW method of MEGA4 program.

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Results and Discussion

Among immunocompromised patients with otitis media, the highest rate was in males, which was 58.0% (29 out of 50). While among immunocompetent patients with otitis media, the highest rate was in females, which was 54.0% (27 out of 50). According to the rate of *C. tropicalis* identification, 14.0% (7 out of 50) and 10.0% (5 out of 50) isolates were collected from immunocompromised and immunocompetent patients with otitis media, respectively. In addition to *C. albicans* (21.1%), *C. parapsilosis* (11.4%) has been established as a common cause of otomycosis, particularly in Europe, where its prevalence is high. Other Candida species such as *C. guilliermondii*(1.1%), *C. tropicalis*(7.8%), *C. krusei* (0.6%), and *C. glabrata* (10.3%) have also been isolated and identified as etiological agents of the EAC infection, although with relatively low incidence [13]. Figure [1] shows different colonies of *C. tropicalis* on SDA which appeared as cream-colored with a slightly yeasty border. Whereas, the appearance of colonies cultured on Hi-Chrom *Candida* agar medium was Dull blue, to purple color that diffused into surrounding agar with pale pink edges.

(A) (B)

Figure1: *Candida tropicalis* cultured on: (A) Sabouraud′s dextrose agar incubated at 37°C for 7 days and (B) Hi-Chrom Candida agar medium incubated at 37°C for 48-72 hrs.

Antibiotics susceptibility test for *A. niger*

Disc diffusion method (Kirby-Bauer) to perform susceptibility test of two antibiotics (FLU and MCL) against 12 isolates of *C. tropicalis*, the results were compared with CLSI stander (CLSI, 2022). The results showed that only 3 isolates of *C. tropiclais* (25.0%) were resistant against both antibiotics (Fluconazole and Miconazole), Table 3. The researchers [11 and 13] whom found that the resistance of *C. tropicalis* toward fluconazole was (62% and 9%, respectively). The sampling number and the substitution mutations in virulence genes of isolated otomycoses may be play an important role in explanation these results

Antibiotics susceptibility test for *C.tropicalis*:

Disc diffusion method (Kirby-Bauer) to perform susceptibility test of two antibiotics (FLU and MCL) against 12 isolates of *C. tropicalis* (7 out of 50 for immunocompromised and 5 out of 50 for immunocompetent patients), the results were compared with CLSI standered (CLSI, 2022). The results showed that only 3 isolates of *C. tropiclais* (25.0%) were resistant against to both antibiotics (Fluconazole and Miconazole) [Table 1]. The researchers [12 and 14] whom found that the resistance of *C. tropicalis* toward fluconazole was (62% and 9%, respectively). The sampling number and the substitution mutations in virulence genes of isolated otomycoses play an important role in the explanation these results.

Table 1: Susceptibility results of twelve *C. tropicalis* isolates against studied antibiotics.

Study groups		**Fluconazole**		**Miconazole**	
		***R**	****S**	**R**	**S**
Immunocompromised patients	Co.	4	3	4	3
	%	80.0%	42.9%	44.4%	100.0%
Immunocompetent patients	Co.	1	4	5	0
	%	20.0%	57.1%	55.6%	0.0%
Total	Co.	5	7	9	3
	%	100.0%	100.0%	100.0%	100.0%

*R=resistance, **S= sensitive

Molecular identification of *Candida tropicalis*

Detection of ITS region of *C. tropicalis* by PCR

The molecular weight of ITS gene was 520 bp., this was indicated sign for successes reaction. [Figure 2].

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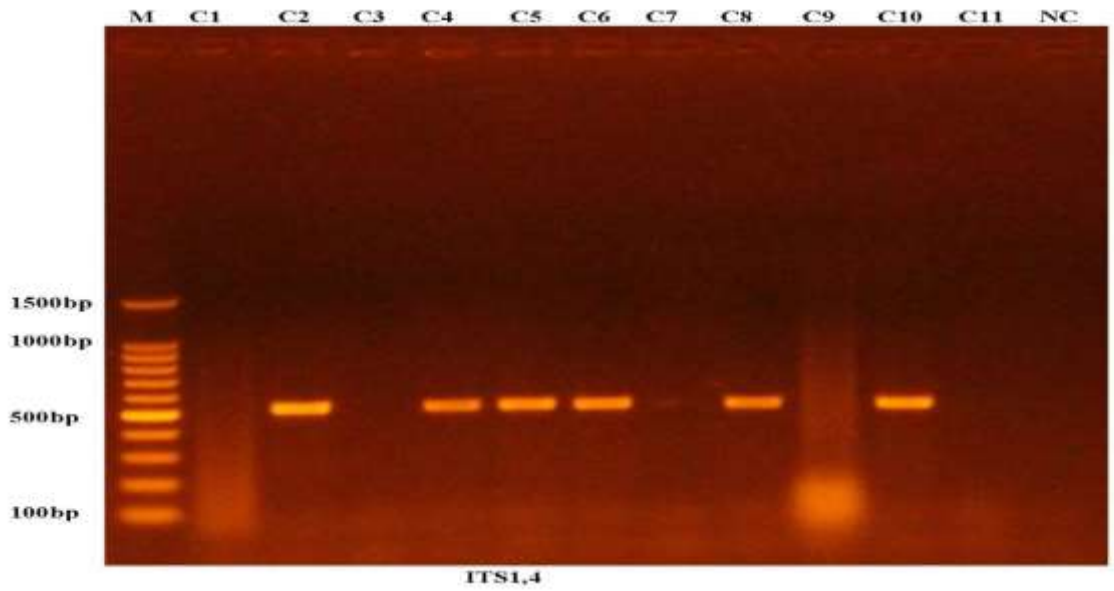

Figure 2: Agarose gel electrophoreses of PCR products of ITS region for *C tropicalis* using 1% agarose gel at 7volt/ cm for 1 hour. Lanes: (M) 100 bp.DNA ladder, (1-11) PCR products.

Detection of *Als2* gene of *C. tropicalis* using singleplex PCR:

The molecular weight of *Als2* gene was 80 bp., this was indicated sign for successes reaction. [Figure 3].

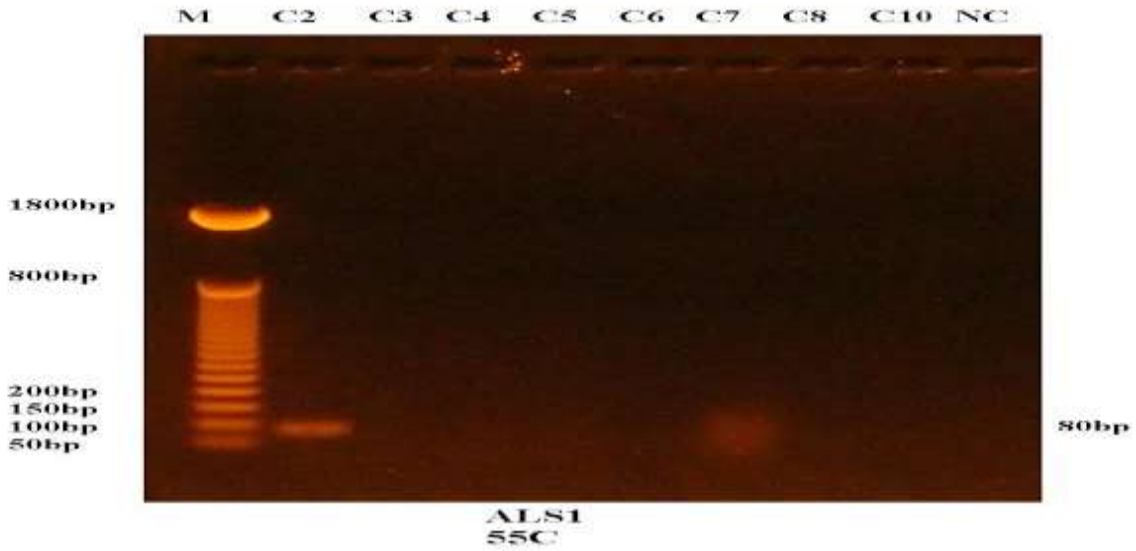

Figure 3: Agarose gel electrophoreses of PCR products of *Als2* gene for *C tropicalis* using 1% agarose gel at 7volt/ cm for 1 hour. Lanes: (M) 50 bp.DNA ladder, (1-6) PCR products.

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Detection of substitution mutation of *Als2* gene of *C. tropicalis* using gene sequencing

After performing the alignment between the amino acid reference sequences of the *Als2* gene of *C. tropicalis* and the amino acid sequences of two isolates of this study. The mutant isolate, has more than one substitution mutation. This result is close to [15] which revealed that 907 SNPs of the whole genome sequence of *ALS2* gene among 60 isolates of *C. tropicalis*. Agglutinin like sequence proteins are an important family involved in the adhesion process of *C. tropicalis* by mediating the attachment of different epithelial cells and functioning as a virulent.

Percentage of *C. tropicalis* concerning the gender and age factors

In immunocompromised patients with otitis media, *C. tropicalis* was the most frequently isolated in males, (5 out of 7). These results disagreed with [16,17 and 18] who revealed that *C. tropicalis* was the most frequently isolated in females. In orbital countries, *Candida* is capable of the most fungi-complicated cause of otomycoses [5, 19]. According to the results of *C. tropicalis*, it correlates to the age factor. *C. tropicalis* was the most frequently isolated from the age group (41-60), with a percentage of (40.0%). [Table 2]. These results are close to [20,21]. These discrepancies in the results may due to the differences in the age group involved within this study, in addition to other conditions such as weather and, treatment mechanisms all may be play a role in these varieties in the results. Also, the pH and water content change of the ear canal has led to fungal survival [22].

Table 2: Percentage of *C. tropicalis* among otitis media immunocompromised and immunocompetent patients around their genders and age factors.

Study groups	Age groups		Gender		Total
			Males	**Females**	
Immunocompromised patients	**<21 years**	Co.	1	0	1
		%	20.0%	0.0%	14.2%
	21-40 years	Co.	1	1	2
		%	20.0%	50.0%	28.6%
	41-60 years	Co.	2	0	2
		%	40.0%	0.0%	28.6%
	>61 years	Co.	1	1	2
		%	20.0%	50.0%	28.6%
Total		Co.	5	2	7
		%	100.%	100.0%	100.%
	<21 years	Co.	1	0	1

Immunocompetent patients		%	50.0%	0.0%	20.0%
	21-40 years	Co.	1	1	2
		%	50.0%	33.3%	40.0%
	41-60 years	Co.	0	1	1
		%	0.0%	33.3%	20.0%
	>61 years	Co.	0	1	1
		%	0.0%	33.3%	20.0%
Total		Co.	2	3	5
		%	100.%	100.0%	100.%

Conclusions

Candida tropicalis is reported as the most causative agent of otitis media and the diagnosis at the molecular level is necessary to avoid the misdiagnosis of otomycoses causes. *Als2* gene may play an important role in the virulence of *C. tropicalis* among immunocompromised patients with otitis media.

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