

Genotypic identification of cycloheximide resistance yeasts isolated from clinical cases with superficial mycosis

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Abstract : Most of *Candida* species cause many opportunistic infections, so that identification of *Candida* spp is indispensable to know the type of pathogen and determine the appropriate drug especially those with wide range antifungal resistance. Three species of *Candida* were isolated from clinical specimens on SDA with chloramphenicol and Cycloheximide *C. albicans* (60%), *C. tropicalis* (30%), and *C. dubliniensis* (10%). The study antifungal susceptibility of two antifungal caspofungin (CAS) and fluconazole (FLU) for candidal isolates were shown the antifungal agent caspofungin good activity against *Candida* species. Primerpairs forward CA-INT-L and reverse CA-INT-R was used to amplify a transposable intron region of the 25S DNA gene by using conventional PCR and used phylogenetic tree and sequencing analysis to study the genotypic relation among these species. In all isolates of *Candida* species appeared a sequence likeness between >99.4%. The results showed a close relationship by similarity between sequences of nucleotide of all isolates which belong to the same of species or different species.

Keywords: Antifungal agent, *Candida* spp,(PCR), Transposable intron region, Sequencing.

Introduction

Yeasts such as *Candida* spp. are a normal flora on and within human body and it unsatisfactory in immunocompetent hosts, but in moist conditions and immunocompromised patients, *Candida* spp. are converted to opportunistic fungi cause different diseases such as superficial infections. There are about more than one hundred species of the genus *Candida*, but just a few species cause human and animal infections¹. Nearly 90- 95% of infections caused by these five species are *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei* and *C. parapsilosis*².

Recently there have been important changes due to dynamic triangle: the infecting fungi, the host factors, and the antifungal agents. Some of these changes include the increase of the immunocompromised patients number, so the increase in invasive fungal infections of these patients, and the emergence and development of new antifungal drugs and antifungal resistance. Therefore we need emergence of a standard antifungal susceptibility test for best results of antifungal treatment^{3,4}. Moreover, Tortorano⁵ mentioned that infections with some *Candida* spp such as *C. glabrata* are associated with larger death rate than those associated with other pathogenic species of *Candida*. But maybe that correlated with types of infected patients more than reduced antifungal susceptibility⁶.

Cycloheximide (actidione) is an antibiotic used in a large number of culture media for the isolation of fungi from medical samples, especially in the cases of dermatophytoses and in same the time, it inhibits non-pathogenic fungi such as saprophytic fungi (moulds and yeasts)⁷. Often yeasts such as *Candida*, especially *C. albicans* and *C. tropicalis* can grow in culture media with Cycloheximide and the mechanism of cycloheximide

and other antifungal resistance in these yeasts happens by either change of ribosomal proteins (rps) or contain a drug efflux pump coding with *Candida* drug resistance genes (*CDR*), as well as multidrug resistance genes (*MDR*) which responsible for resistance of yeast to a number of antifungal agents^{8,9}.

The diagnoses of *Candida* spp. by using traditional methods is sometimes problematic because the taxonomy of the most *Candida* spp. have undergone changes that due to confuse in some taxonomic characters in CHROMagar medium or biochemical reactions. So far, most research using molecular techniques as well as traditional diagnosis. Recently, several studies have demonstrated that sequence analysis of different regions of rDNA is the golden method for *Candida* spp. identification. Imran and Al-Asadi¹⁰ were compared among different species of *Candida* (35 isolates) from patients with conjunctivitis and diagnosed according to the CHROMagar medium in eight isolates were *C. albicans* with green color and 27 isolates were non-albicans with white to the pink color colony as well as a molecular method with pair primer CAI microsatellite, which that amplified all *C. albicans* isolates without others While Imran and Al-Shukry¹¹ were diagnosed *Candida* spp from patients with vaginal candidiasis by using RAPD-PCR techniques. Moreover, the sequencing of the ITS region of the *Candida* DNA gene has appeared as a tool was more discriminatory for species identification, addition to other molecular identification techniques¹²⁻¹⁵

The aim of this study is selective *Candida* species has been cycloheximide resistant and identifying by using molecular methods as conventional PCR, phylogenetic tree and sequencing analysis.

2- Materials and methods

Sampling and fungal culture:-

A overall of 40 candidal isolates from clinical specimens were collected from patients with dermatomycoses that clinically diagnosed by the dermatologist from the Unit of Mycology in Al- Marghan Hospital of Hilla (Iraq). Samples were 25 (62.5%) from female and 15 (37.5%) from male, which including 8 hair fragments, 15 skin scrapings and 17 nail clippings, all samples were identified depending on culture with Sabouraud's Dextrose Agar (SDA) with tow antibiotic of Cycloheximide and chloramphenicol. The identification of *Candida* species was on CHROMagar *Candida* medium (Liofilchem Lab, Italy), in addition, other tests such as germ tube production, micromorphology on corn meal agar and grow in 45°C and identification according to the Ellis *et al.*¹⁶.

Antifungal susceptibility Epsilonometer test (E-test):-

Antifungal susceptibility by E-test method was done on Muller- Hinton agar medium (MHA) of tow antifungal caspofungin (CAS) and fluconazole (FLU) (Liofilchem Lab, Italy) for candidal isolates was determined according to the Clinical and Laboratory Standards Institute (CLSI, 2002)¹⁷. The minimum inhibitory concentrations (MICs) were determined following incubation at 35°C after 24 h. and results were confirmed by second reading after 84 h., breakpoint values have been read at the point of intersection between the edge of the inhibition zone and the degree of concentration on MIC strip. The results recorded according to the sensitivity and resistance standards, isolates with MIC ≤ 8 µg/ml for fluconazole and ≤ 1 µg/ml for caspofungin were considered susceptible (S), isolates with MIC between 16 to 32 µg/ml for fluconazole only were considered susceptible dose- dependent (SDD) and isolates with MIC ≥ 64 µg/ml for fluconazole and ≥ 2 µg/ml for caspofungin were considered resistant (R)¹⁷.

Yeasts genomic DNA extraction:

The DNA of each isolate was extracted according to Imran (18), and fungal isolates DNA is stored at -20 °C until use .

PCR assay:

The morphological results we're confident by using conventional PCR with primer pairs forward CA-INT-L and reverse CA-INT-R for the region of transposable intron of 25S rDNA¹⁹. DNA (1 µL) from *Candida* isolates were mixed with PCR mixture, with a volume of the final reaction (25 µL) consisted of 2x Master Mix (Promega) (12 µL) and primers (10 pmole) (2 µL) with free molecular- water. PCR condition were 7 min of

initial denaturation at 94°C followed by 32 cycles with 40 sec of denaturation at 94°C, 45 sec of annealing at 55°C and 1 min of extension at 72°C followed by 7 min of the final extension at 72°C. Mixture of PCR product was amplified by using the thermal cycle tool for PCR (Labnet, origin of USA).

The products for PCR of the previous step were run on 1.5% agarose gel (Bio Basic, origin of Canada Inc.). Electrophoresis was completed in TBE buffer at 100 V, the gel was stained with 0.05% of ethidium bromide. The DNA bands were exposed by using Desktop Gel documentation ultraviolet (Korea Com.).

Sequencing assay:

To study the relationship and similarity at the molecular level, the PCR products for isolates of *Candida* were sequenced by sending to the Microgene Company, USA. The sequence alignment was compared with sequences from NCBI Blast database to get the highest percentage of the match in the genus and species name of asexual phase of each isolate.

Sequencing analysis:

To reach the degree of genetic convergence among these isolates, multiple transposable intron region of 25S rDNA, nitrogen base sequences were performed by using the Mega 6 software program. Phylogenetic tree analysis applied by using an unweighted pair group method with arithmetic mean (UPGMA) software. Alignment of *Candida* spp. sequences was conducted by using BioEdit software.

3- Results

Fungal isolates:

Three species of *Candida* were isolated from clinical specimens on SDA with chloramphenicol and Cycloheximide (Table 1). Twenty four isolates of *C.albicans*, 12 isolates of *C.tropicalis* and 4 isolates of *C.dubliniensis* were identified by CHROMagar.

Table 1: Shows No. of *Candida* spp isolated from clinical specimens.

Candida spp	Colony color on CHROMagar	hair fragment	skin scrapings	nails clipping	Total No.(%)
<i>C.albicans</i>	Light green	6	8	10	24 (60)
<i>C. tropicalis</i>	Blue- pink	2	5	5	12 (30)
<i>C. dubliniensis</i>	Dark green	-	1	3	4 (10)

Antifungal susceptibility (E-test):

Table 2 showed the percentage of antifungal resistance and sensitive of *Candida* species for two antifungal agents caspofungin and fluconazole. While table 3 showed antifungal susceptibility MIC mg/L values of caspofungin and fluconazole tested against candidal isolates.

Table 2: Percentage of antifungal resistance and sensitivity of *Candida* species.

Candida spp	No. of isolates	Caspofungin	Fluconazole
<i>C.albicans</i>	24	24 (100%) S 0 (%) R	18 (75%) S 2 (8.3%) SDD 4 (16.6%) R
<i>C. tropicalis</i>	12	9 (75%) S 3 (25%) R	8 (66.6%) S 2 (16.6%) SDD 2 (16.6%) R
<i>C. dubliniensis</i>	4	4 (100%) S 0 (%) R	0 (%) S 0 (%) SDD 4 (100%) R

Table 3: Shows antifungal susceptibility MIC mg/L values of caspofungin and fluconazole tested against candidal isolates.

Candida spp (No. of isolates)	Caspofungin		Fluconazole	
	Range	MIC	Range	MIC
<i>C.albicans</i> (24)	0.125-1	0.5	0.5-32	8
<i>C. tropicalis</i> (12)	0.25-0.5	0.38	32-64	64
<i>C. dubliniensis</i> (4)	0.125-0.5	0.5	0.5-12	12

Molecular identification:

All isolates of *Candida* species were tested at a molecular level to confirm the identification of cychloheximide resistance *Candida*. By using conventional PCR technique with primer pairs forward CA-INT-L and reverse CA-INT-R for transposable intron region of 25S rDNA. In gel electrophoresis, the amplicons of transposable intron region for *C. albicans*, *C. tropicalis* and *C. dubliniensis* were around 480, 520-600 and 1080 bp respectively. Satisfactory sequencing results were obtained, the isolates had been identified by comparison those isolates sequencing with sequences from the NCBI Blast databases, the identification for all isolates in the intron region was 99-100%. Our results of the sequencing analysis for candidal isolates was obtained depending on the basis of their transposable intron region sequences by using the MEGA6 software program. The results of the phylogenetic tree analysis for twelve isolates of *Candida* spp. were observed three clusters of *Candida* spp. *C. albicans* in clusters 1, *C. dubliniensis* in cluster 2 and *C. tropicalis* in cluster 3. Phylogenetic tree was showed high sequence similarity in the transposable intron region of rDNA between 99.9-100% similarities for the isolates belong to one species (Fig. 1). Phylogenetic tree of *C. albicans* cluster 1 and *C. dubliniensis* cluster 2 showed high similarity of about 99.9%, but this group with *C. tropicalis* cluster 3 showed the similar of about 99.4%.

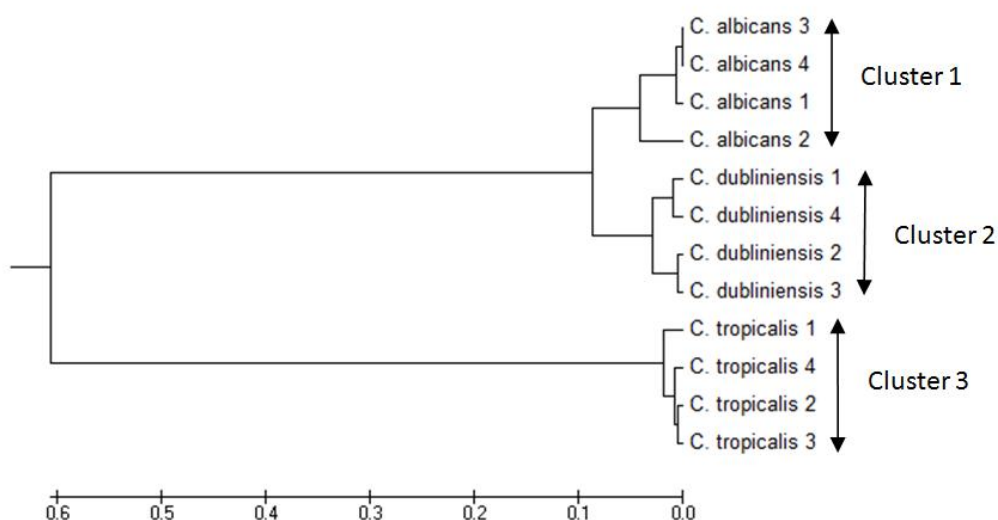
**Fig. 1: Phylogenetic tree based on transposable intron region sequences of 25S rDNA for *Candida* spp.**

Fig. 2 showed sequencing analysis data for all species were employed by using Mega 6 software, sequences of all *C. albicans* isolates were approximately similar to the sequences of all *C. dubliniensis* isolates. While *C. tropicalis* is differenced from last two species in often nitrogen bases. The final alignment of the *Candida* spp. sequences were obtained by using BioEdit software. The sequence of nitrogen base analysis of twelve of isolates of *Candida* spp. showed high similarity among the isolates which belong to the same species. Dark regions in the sequence analysis show high similarity between the isolates, while light regions showing differences in one or more of nitrogen base among the isolates (fig. 3, 4, and 5).

DNA Sequences		Translated Protein Sequences	
Species/Abbrv	Gc		
1. C. albicans 1	1	----CCCTTTTCGAGGTAGGT-TGG-CTCTA-GGATCGGGTGTCTTGGGCTTGTGTAGACCGGGCGGTGACTTGGCGGG--CTGTTTACGAC--GGAC	100
2. C. albicans 2		----CCCTTTTCGAGGTAGGT-TGGGCTTAAGGATCGGGTGTCTTGGGCTTGTGTAGACCGGGCGGTGACTTGGCGGG--CTGTTTACGAC--GGAC	
3. C. albicans 3		----CCCTTTTCGAGGTAGGT-TGGGCTTAAGGATCGGGTGTCTTGGGCTTGTGTAGACCGGGCGGTGACTTGGCGGG--CTGTTTACGAC--GGAC	
4. C. albicans 4		----CCCTTTTCGAGGTAGGT-TGGGCTTAAGGATCGGGTGTCTTGGGCTTGTGTAGACCGGGCGGTGACTTGGCGGG--CTGTTTACGAC--GGAC	
5. C. dubliniensis 1		----CCCTTTTCGAGGTAGGT-TGGGCTTAAGGATCGGGTGTCTTGGGCTTGTGTAGACCGGGCGGTGACTTGGCGGG--CTGTTTACGAC--GGAC	
6. C. dubliniensis 2		----CCCTTTTCGAGGTAGGT-TGGGCTTAAGGATCGGGTGTCTTGGGCTTGTGTAGACCGGGCGGTGACTTGGCGGG--CTGTTTACGAC--GGAC	
7. C. dubliniensis 3		----CCCTTTTCGAGGTAGGT-TGGGCTTAAGGATCGGGTGTCTTGGGCTTGTGTAGACCGGGCGGTGACTTGGCGGG--CTGTTTACGAC--GGAC	
8. C. dubliniensis 4		----CCCTTTTCGAGGTAGGT-TGGGCTTAAGGATCGGGTGTCTTGGGCTTGTGTAGACCGGGCGGTGACTTGGCGGG--CTGTTTACGAC--GGAC	
9. C. tropicalis 1		TAGGGACTGAGGAGACATTAAGTATGCTTAATGACCCGATGTGTTTTTATTTGA-ACAAATTTCTTTGGTGGCGGAGCAATCTACCCGAGAGG	
10. C. tropicalis 2		GGGGGACTGCGGA-GACATTACTGATTTGCTTAATGACCCGATGTGTTTTTATTTGA-ACAAATTTCTTTGGTGGCGGAGCAATCTACCCGAGAGG	
11. C. tropicalis 3		TGAGACTGCGGA-GACATTACTGATTTGCTTAATGACCCGATGTGTTTTTATTTGA-ACAAATTTCTTTGGTGGCGGAGCAATCTACCCGAGAGG	
12. C. tropicalis 4		TGAGACTGCGGA-GACATTACTGATTTGCTTAATGACCCGATGTGTTTTTATTTGA-ACAAATTTCTTTGGTGGCGGAGCAATCTACCCGAGAGG	
DNA Sequences		Translated Protein Sequences	
Species/Abbrv	Gc		
1. C. albicans 1	101	CTGCTGGCGAT---GCTGCT---GTAGACACGCTTGGTAGGCTTTATGGCCGTCCGGGGCACGTTTAAAGATCAACTTAGAACTGGTACGGACAAGG	200
2. C. albicans 2		CTGAGGGTGGAT---GCTGAT---GAACACCCCTTTGGTAGGCTTTAGGGCCGAAACGGGGCACATTAACGATCATCTTAAACTGGTACTGACAAAGG	
3. C. albicans 3		CTGCTGGTGGAT---GCTGCT---GTAGACACGCTTGGTAGGCTTTATGGCCGTCCGGGGCACGTTTAAAGATCAACTTAGAACTGGTACGGACAAGG	
4. C. albicans 4		CTGCTGGTGGAT---GCTGCT---GTAGACACGCTTGGTAGGCTTTATGGCCGTCCGGGGCACGTTTAAAGATCAACTTAGAACTGGTACGGACAAGG	
5. C. dubliniensis 1		CTGCTGGTGGAT---GCTGCT---GTAGACACGCTTGGTAGGCTTTATGGCCGTCCGGGGCACGTTTAAAGATCAACTTAGAACTGGTACGGACAAGG	
6. C. dubliniensis 2		CTGCTGGTGGAT---GCTGCT---GTAGACACGCTTGGTAGGCTTTATGGCCGTCCGGGGCACGTTTAAAGATCAACTTAGAACTGGTACGGACAAGG	
7. C. dubliniensis 3		CTGCTGGTGGAT---GCTGCT---GTAGACACGCTTGGTAGGCTTTATGGCCGTCCGGGGCACGTTTAAAGATCAACTTAGAACTGGTACGGACAAGG	
8. C. dubliniensis 4		CTGCTGGTGGAT---GCTGCT---GTAGACACGCTTGGTAGGCTTTATGGCCGTCCGGGGCACGTTTAAAGATCAACTTAGAACTGGTACGGACAAGG	
9. C. tropicalis 1		TTATAACTAAACCAAACTTTTATTTACAGTCAAACTGTATTTATTTATCAATAGTCAAAACTTTCAACCAAGGATCTCTTGGTCTCGCATCGATGAAG	
10. C. tropicalis 2		TTATAACTAAACCAAACTTTTATTTACAGTCAAACTGTATTTATTTATCAATAGTCAAAACTTTCAACCAAGGATCTCTTGGTCTCGCATCGATGAAG	
11. C. tropicalis 3		TTATAACTAAACCAAACTTTTATTTACAGTCAAACTGTATTTATTTATCAATAGTCAAAACTTTCAACCAAGGATCTCTTGGTCTCGCATCGATGAAG	
12. C. tropicalis 4		TTATAACTAAACCAAACTTTTATTTACAGTCAAACTGTATTTATTTATCAATAGTCAAAACTTTCAACCAAGGATCTCTTGGTCTCGCATCGATGAAG	
DNA Sequences		Translated Protein Sequences	
Species/Abbrv	Gc		
1. C. albicans 1	201	-----GGAACTGACTGCTTAAT-TAAACATAGCATTTGATGGTCAGAAAGTGAT-GTTGACCAATGTGATTTCTG-----CCAGTGCTCTGAA	300
2. C. albicans 2		-----GGAACTGACTGCTTAAT-TAAACATAGCATTTGATGGTCAGAAAGTGAT-GTTGACCAATGTGATTTCTG-----CCAGTGCTCTGAA	
3. C. albicans 3		-----GGAACTGACTGCTTAAT-TAAACATAGCATTTGATGGTCAGAAAGTGAT-GTTGACCAATGTGATTTCTG-----CCAGTGCTCTGAA	
4. C. albicans 4		-----GGAACTGACTGCTTAAT-TAAACATAGCATTTGATGGTCAGAAAGTGAT-GTTGACCAATGTGATTTCTG-----CCAGTGCTCTGAA	
5. C. dubliniensis 1		-----GGAACTGACTGCTTAAT-TAAACATAGCATTTGATGGTCAGAAAGTGAT-GTTGACCAATGTGATTTCTG-----CCAGTGCTCTGAA	
6. C. dubliniensis 2		-----GGAACTGACTGCTTAAT-TAAACATAGCATTTGATGGTCAGAAAGTGAT-GTTGACCAATGTGATTTCTG-----CCAGTGCTCTGAA	
7. C. dubliniensis 3		-----GGAACTGACTGCTTAAT-TAAACATAGCATTTGATGGTCAGAAAGTGAT-GTTGACCAATGTGATTTCTG-----CCAGTGCTCTGAA	
8. C. dubliniensis 4		-----GGAACTGACTGCTTAAT-TAAACATAGCATTTGATGGTCAGAAAGTGAT-GTTGACCAATGTGATTTCTG-----CCAGTGCTCTGAA	
9. C. tropicalis 1		AACGACGCGAAATGCGA-TACGTAATATGAATGCGAGATATTCGTGAATCATCGAATCTTTGAAACGACATTCGCGCCCTTTGGTATTCGAAGGGCATGGCC	
10. C. tropicalis 2		AACGACGCGAAATGCGA-TACGTAATATGAATGCGAGATATTCGTGAATCATCGAATCTTTGAAACGACATTCGCGCCCTTTGGTATTCGAAGGGCATGGCC	
11. C. tropicalis 3		AACGACGCGAAATGCGA-TACGTAATATGAATGCGAGATATTCGTGAATCATCGAATCTTTGAAACGACATTCGCGCCCTTTGGTATTCGAAGGGCATGGCC	
12. C. tropicalis 4		AACGACGCGAAATGCGA-TACGTAATATGAATGCGAGATATTCGTGAATCATCGAATCTTTGAAACGACATTCGCGCCCTTTGGTATTCGAAGGGCATGGCC	
DNA Sequences		Translated Protein Sequences	
Species/Abbrv	Gc		
1. C. albicans 1	301	TGTCAAAGTGAAAGAAATCAACCAAGCGCGGGTAAACGGCGGAGTAACATGACTCTCT-----TAAGGTAGCCAAATGCCCTGTCATCTAATTAAGTAC	400
2. C. albicans 2		TGTCAAAGTGAAAGAAATCAACCAAGCGCGGGTAAACGGCGGAGTAACATGACTCTCT-----TAAGGTAGCCAAATGCCCTGTCATCTAATTAAGTAC	
3. C. albicans 3		TGTCAAAGTGAAAGAAATCAACCAAGCGCGGGTAAACGGCGGAGTAACATGACTCTCT-----TAAGGTAGCCAAATGCCCTGTCATCTAATTAAGTAC	
4. C. albicans 4		TGTCAAAGTGAAAGAAATCAACCAAGCGCGGGTAAACGGCGGAGTAACATGACTCTCT-----TAAGGTAGCCAAATGCCCTGTCATCTAATTAAGTAC	
5. C. dubliniensis 1		TGTCAAAGTGAAAGAAATCAACCAAGCGCGGGTAAACGGCGGAGTAACATGACTCTCT-----TAAGGTAGCCAAATGCCCTGTCATCTAATTAAGTAC	
6. C. dubliniensis 2		TGTCAAAGTGAAAGAAATCAACCAAGCGCGGGTAAACGGCGGAGTAACATGACTCTCT-----TAAGGTAGCCAAATGCCCTGTCATCTAATTAAGTAC	
7. C. dubliniensis 3		TGTCAAAGTGAAAGAAATCAACCAAGCGCGGGTAAACGGCGGAGTAACATGACTCTCT-----TAAGGTAGCCAAATGCCCTGTCATCTAATTAAGTAC	
8. C. dubliniensis 4		TGTCAAAGTGAAAGAAATCAACCAAGCGCGGGTAAACGGCGGAGTAACATGACTCTCT-----TAAGGTAGCCAAATGCCCTGTCATCTAATTAAGTAC	
9. C. tropicalis 1		TGTTTGAAGCGTCAATTTCCCTCAAACCCCGGGTTTGGTGTGAGCAATACG-CTAGGTTTGTGTTGAAAGAAATTA-CGTGGAAACCTAATTTAAGGCAC	
10. C. tropicalis 2		TGTTTGAAGCGTCAATTTCCCTCAAACCCCGGGTTTGGTGTGAGCAATACG-CTAGGTTTGTGTTGAAAGAAATTA-CGTGGAAACCTAATTTAAGGCAC	
11. C. tropicalis 3		TGTTTGAAGCGTCAATTTCCCTCAAACCCCGGGTTTGGTGTGAGCAATACG-CTAGGTTTGTGTTGAAAGAAATTA-CGTGGAAACCTAATTTAAGGCAC	
12. C. tropicalis 4		TGTTTGAAGCGTCAATTTCCCTCAAACCCCGGGTTTGGTGTGAGCAATACG-CTAGGTTTGTGTTGAAAGAAATTA-CGTGGAAACCTAATTTAAGGCAC	
DNA Sequences		Translated Protein Sequences	
Species/Abbrv	Gc		
1. C. albicans 1	401	GGCGATGAATGGATTAAAGAGATTCCCACTG-TCCCTATCTA-CTATCTAGCGAAACCAAGCCAAAGG-----	500
2. C. albicans 2		GGCGATGAATGGATTAAAGAGATTCCCACTG-TCCCTATCTA-CTATCTAGCGAAACCAAGCCAAAGG-----	
3. C. albicans 3		GGCGATGAATGGATTAAAGAGATTCCCACTG-TCCCTATCTA-CTATCTAGCGAAACCAAGCCAAAGG-----	
4. C. albicans 4		GGCGATGAATGGATTAAAGAGATTCCCACTG-TCCCTATCTA-CTATCTAGCGAAACCAAGCCAAAGG-----	
5. C. dubliniensis 1		TTCCAGAAATGGGCGGAGGTGTTTTGACC--TGCTAGTGCATCTGGTTAATTAAGTATTTGTATATTACTTACAGAGTATTCCTGGTATTTATACAT	
6. C. dubliniensis 2		TTCCAGAAATGGGCGGAGGTGTTTTGACC--TGCTAGTGCATCTGGTTAATTAAGTATTTGTATATTACTTACAGAGTATTCCTGGTATTTATACAT	
7. C. dubliniensis 3		TTCCAGAAATGGGCGGAGGTGTTTTGACC--TGCTAGTGCATCTGGTTAATTAAGTATTTGTATATTACTTACAGAGTATTCCTGGTATTTATACAT	
8. C. dubliniensis 4		TTCCAGAAATGGGCGGAGGTGTTTTGACC--TGCTAGTGCATCTGGTTAATTAAGTATTTGTATATTACTTACAGAGTATTCCTGGTATTTATACAT	
9. C. tropicalis 1		TTTGGGTTATCCCAAA-CCCTTATTTTGTAGTGGGCCCCCAATTTTTCACAACTTTGACCCCAAAACAGGGGGAGTACCCGCTGAACCTTAAGCATA	
10. C. tropicalis 2		TTAGGTTTATCCCAAAACGCTAATTTTGTAGGCGCCCAACAAATTTTTCACAACTTTGACCTCAAACTCAGGTAGGAGTACCCGCTGAACCTTAAGCATA	
11. C. tropicalis 3		TTAGGTTTATCCCAAA-CCCTTATTTTGTAGTGGGCCCCCAACAAATTTTTCACAACTTTGACCTCAAACTCAGGTAGGAGTACCCGCTGAACCTTAAGCATA	
12. C. tropicalis 4		TTAGGTTTATCCCAAAACGCTAATTTTGTAGGCGCCCAACAAATTTTTCACAACTTTGACCTCAAACTCAGGTAGGAGTACCCGCTGAACCTTAAGCATA	

Fig. 2: sequence data for *Candida* species by using Mega 6 software, the star is showed similarity of nitrogen base of all *Candida* species isolates. Number of nucleotide sequence is shown on the left and right.

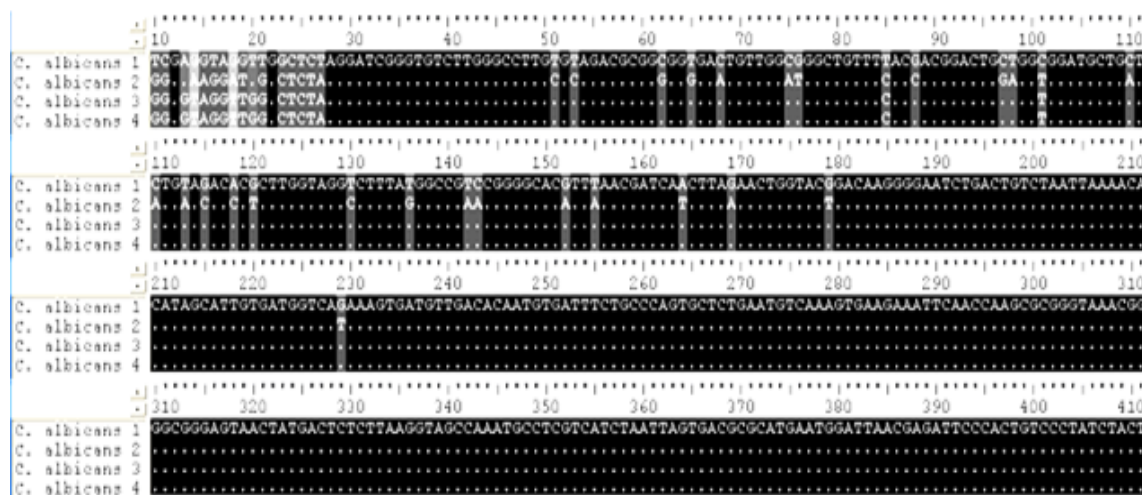


Fig. 3: Alignment of transposable intron region sequences for *C. albicans* by using BioEdit software. Nucleotide sequences are present in all intron regions are shaded in dark color.

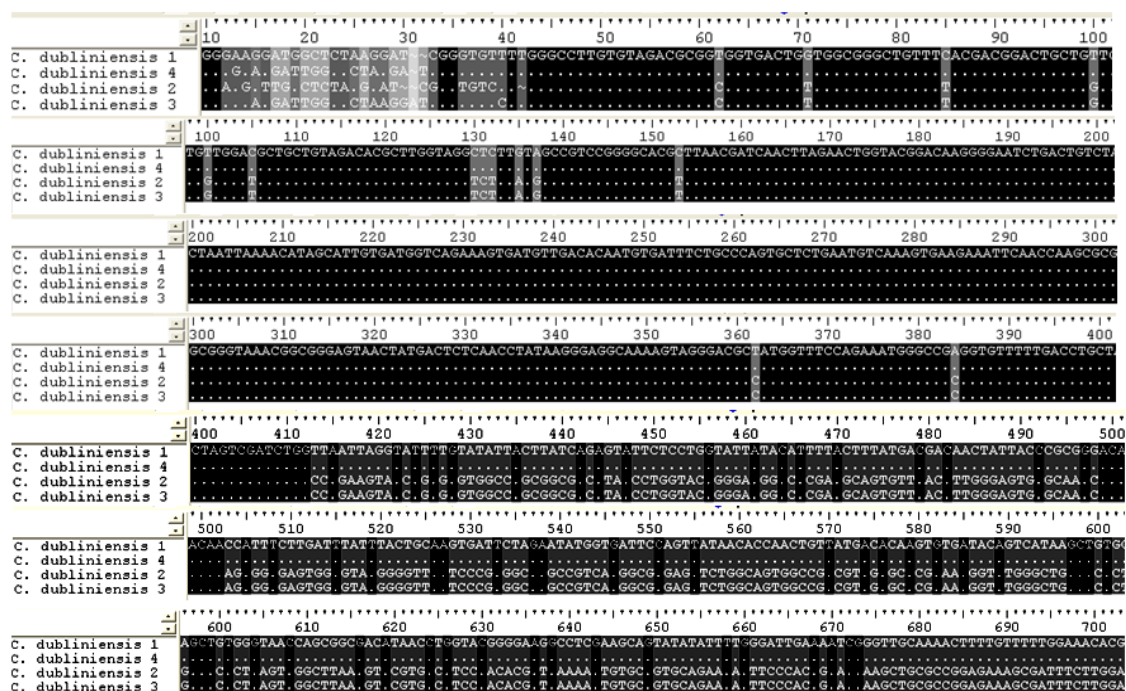


Fig. 4: Alignment of transposable intron region sequences for *C. dubliniensis* by using BioEdit software. Nucleotide sequences are present in all intron regions are shaded in dark color.



Fig. 5: Alignment of transposable intron region sequences for *C. tropicalis* by using BioEdit software. Nucleotide sequences are present in all intron region are shaded in dark color.

4- Discussion

The results showed that three species of cycloheximide resistance *Candida*, 60% were *C. albicans* and 30% *C. tropicalis* while 10% *C. dubliniensis*. In the last two decades, where the increasing number of infections with non-dermatophyte fungi as agents of nail and skin in humans, those infections producing skin lesions clinically similar to lesions caused by dermatophytes²⁰. Prasad *et al.*,²¹ reported that decrease in the frequency rate of *C. albicans* (25%) while increase in *C. tropicalis* (31.25%) were isolated from superficial infections. Abdel-Fatah *et al.*,²² were isolated three *Candida* species from onychomycosis patients are *C. albicans*, *C. tropicalis* and *C. krusei*. In a study of dermatophytes and other fungi prevalence among children of school of Nigeria, the frequency rate of *C. albicans* was 5.5%. Younes *et al.*,²³ were isolated *C. albicans* (2.5%) from tinea capitis in Egypt. *C. tropicalis* was isolated from 19 systemic candidiasis patients (35%) with skin lesions²⁴.

Three species of *Candida* were tested by using E-test strip with two antifungal agents fluconazole belong to the class Azole and caspofungin belong to the class Echinocandins. Antifungal agent caspofungin showed good activity against *Candida* species, it gave 100% sensitivity percentage against both *C. albicans* and *C. dubliniensis* and 75% sensitivity percentage against *C. tropicalis*. While fluconazole gave 75% and 66.6% sensitivity percentage against *C. albicans* and *C. tropicalis* respectively, high resistance of *C. dubliniensis* to fluconazole was observed, it showed 100% resistance to fluconazole. The MIC values of caspofungin were 0.5, 0.38 and 0.5 for *C. albicans*, *C. tropicalis* and *C. dubliniensis* respectively while the MIC values of fluconazole were 8, 64 and 12 for *C. albicans*, *C. tropicalis* and *C. dubliniensis* respectively. However, there are several factors that depend on them on the success of antifungal susceptibility test. First, user medium: Different authors have evaluated different media for disk diffusion and E-test, some of these media were antibiotic medium 3 (AM3), Mueller-Hinton with 2% glucose (MHG), Roswell Park Memorial Institute (RPMI) agar, Shadomy medium (SHDM) or non-supplemented Mueller-Hinton agar (MHA) (25- 28). Rubio *et al.*,²⁶ were used three different media for testing of fluconazole by the disk diffusion agar test against *Candida* spp. they MHA with 2% glucose and 0.5 µg of methylene blue per ml, RPMI agar and SHDM, the first medium was optimum because it produces inhibition zone with clear edges and with less intrazonal yeast growth.

Second, the period of incubation: According to Arian³ that some *Candida* isolates showed to be susceptible at 24h but may be appear false results as resistant at 48h because of dense growth of yeast microcolonies which observed inside the inhibition ellipse in E-test or inhibition zone in disk diffusion. This phenomenon appears in azole susceptibility testing and in case increase of the incubation period. Different references have reported this phenomenon for isolates of *C. albicans*, *C. tropicalis* and other *Candida* spp.^{29,30}. The MIC values were determined after 24h of incubation for three agents belong to echinocandins

groups (anidulafungin, caspofungin and micafungin) against 404 isolates due to six species of *Candida*³¹. And three agents belong to azole groups (fluconazole, posaconazole and voriconazole) against 1056 isolates due to five species of *Candida*³².

Third, type of antifungal agent and fungal species: The correlation between antifungal agent and fungal species is often variable. Several references reported that large number of antifungal agents against *Candida* spp. has been used successfully such as amphotericin B, azoles especially fluconazole and echinocandins especially caspofungin³³⁻³⁶. Rodriguez-Tudela *et al.*,³⁷ reported that the correlation between two groups of patients (candidemia 126 cases and oropharyngeal candidiasis 110 cases), who had been treated with different doses of fluconazole, they were concluded that an exposure increase above the dose/MIC of 35.5 has increased the cure rate in patients, and cure rate was reached 100% when the dose/MIC was higher than 100.

Sometime, we noted that one species of *Candida* having variable resistance to the multiple antifungal agent and over different periods of time, such as *C. glabrata* was acquired resistance to all amphotericin B, flucytosine, voriconazole, fluconazole and caspofungin. This resistance was readily mutated *in vivo* in a single patient³⁸. Pfaller and Diekema³⁹ mentioned that *C. glabrata* and *C. krusei* demonstrates less susceptibility to amphotericin B than other *Candida* species, especially when used E-test.

In our study, analysis of phylogenetic tree of twelve isolates of *Candida* spp. displayed high sequence similarity in the transposable intron region of 25S rDNA between 99.9-100% similarities for the isolates belong to one species (Fig. 2). While the difference in sequence between *C. albicans* and *C. dubliniensis* were shown approximately 0.1%. Our results agreed with number of studies were demonstrated a high degree of genotypic homogeneity between both *C. dubliniensis* and *C. albicans* and these studies referred to the similarity of several regions of rDNA for both *C. albicans* and *C. dubliniensis* as well as the similarity between the two species in some of the loci sequencing^{18, 40-42}. There are several studies were identified by *Candida* species by one or more of molecular methods, Cirak *et al.*,⁴³ were identified forty four isolates of *Candida* spp. due to five species by using RFLP-PCR with three restriction enzymes: *Bfa*I, *Dde*I and *Hae*III. The last enzyme can be differentiated between *C. albicans* and non- *C. albicans* isolates, while *Bfa*I can be differentiated among non- *C. albicans* species. Xu *et al.*,¹³ were compared the API20C technique for identification of *C. albicans* and sequencing of the ITS2 region for identification of *C. dubliniensis*. Because of differences of amplicon length and base sequence among different species, Turenne *et al.*⁴⁴ found that differences in three nucleotide band in size of ITS2 region of *C. Krusei* and *C. albicans*. Fujita *et al.*⁴⁵ were determined the PCR fragment lengths of ITS regions for six of *Candida* species by using both agarose gel electrophoresis and microchip electrophoresis. While Abdel-Fatah *et al.*²² were used three molecular methods RAPD, ISSR and RFLP for identification three species for *Candida* and six species of dermatophytes isolated from patients with skin mycosis from Egypt and Libya and for determination the genetic relationship among these species were analyzed phylogenetic tree for RAPD and ISSR products, while in RFLP were used ITS1 and ITS4 primers and digested the PCR product with *Hinf*I and *Hae*III enzymes.

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