International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.12, pp 501-510, 2016

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

Kawther Mohammed Ali Hasan

Science College for Women/ Babylon University/Hilla/ Iraq.

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 tow antifungal caspofungin (CAS) and fluconazole (FLU) for candidal isolates were shown the antifungal agent caspofungin good activity against *Candida* species. Primerpairs forwared 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 inimmunocompetent 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 animalinfections[1].Nearly 90- 95% of infections caused bythese 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. Therefor we need emergence of a standard antifungal susceptibility testfor best results of antifungal treatment[3,4]. Moreover, Tortorano[5] 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 susceptibility[6].

Cycloheximide (actidione) isan 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-Asadi[10] 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-Shukry[11] were diagnosed Candida spp from patients with vaginal candidiasis by using RAPD-PCR techniques. Moreover, the sequencing of the ITS region of the *Candida*r DNA gene has appeared as a tool was more discriminatory for species identification, addition to other molecular identification techniques[12-15]

The aim of this study is selective *Candida* species has been cyclohximide 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 were25 (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 Sabouraude'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 Epsilometer 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˚Cafter 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 zoon and the degree of concentration on MIC strip. The results recorded according to the sensitivity and resistance standards, isolates with MIC $\leq$ 8 µg/ml for fluconazole and $\leq$ 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 $\geq$ 64 µg/ml for fluconazole and $\geq$ 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-Land reverse CA-INT-R for the region of transposable intron of 25S rDNA[19]. 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 were7 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 and1 min of extension at 72˚C followed by7 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 PCRof the previous step were run on 1.5% agarose gel (Bio Basic, origin of Canada Inc.). Electrophoresis were completed in TBE bufferat 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* and4 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.(%)
C.albicans	Light green	6	8	10	24 (60)
C. tropicalis	Blue- pink	2	5	5	12 (30)
C. dubliniensis	Dark green	-	1	3	4 (10)

Antifungal susceptibility (E-test):

Table 2 showed the percentage of antifungal resistance and sensitive of *Candida* species for tow 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
C.albicans	24	24 (100%) S 0 (%) R	18 (75%) S 2 (8.3%) SDD 4(16.6%) R
C. tropicalis	12	9 (75%) S 3 (25%) R	8 (66.6%) S 2 (16.6%) SDD 2 (16.6%) R
C. dubliniensis	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
C.albicans (24)	0.125-1	0.5	0.5-32	8
C. tropicalis (12)	0.25-0.5	0.38	32-64	64
C. dubliniensis (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 and1080 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 analysisfor 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%.

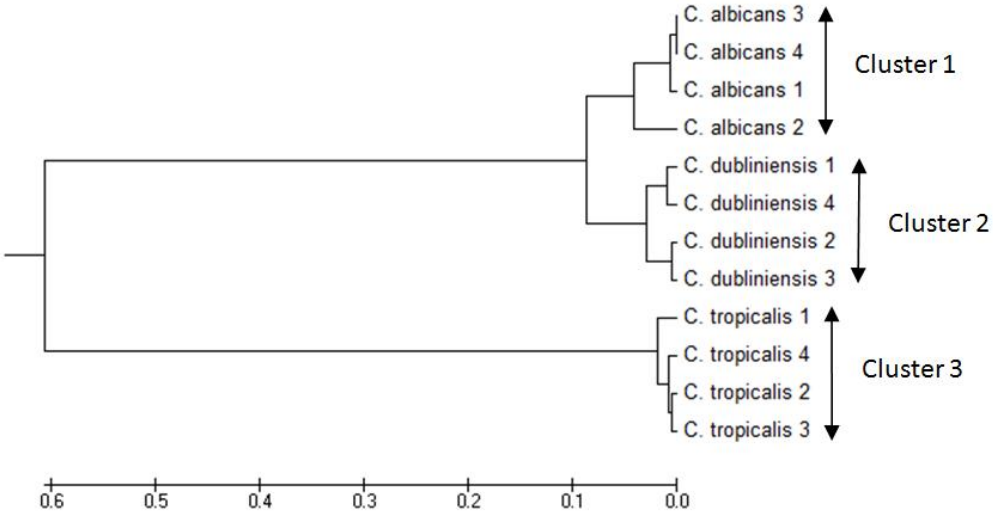

Fig. 1: Phylogenetic tree based on transposable intron region sequences of 25S rDNA for *Candida* spp.

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).

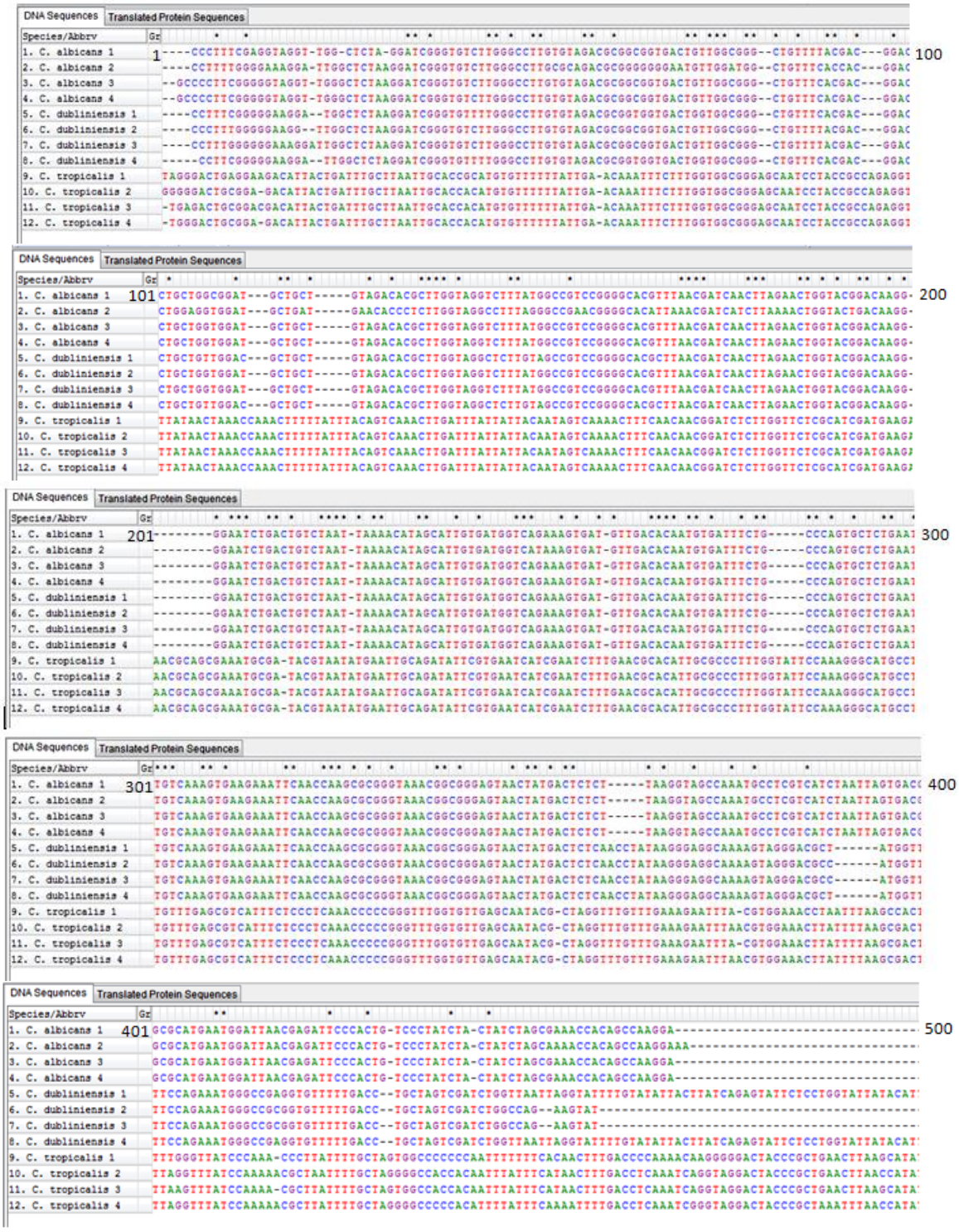

Fig. 2: sequence data for *Candida* species by using Mega 6 software, the star is showed similarity of nitrogen base of all *Candida* species isolates. Number of nucleotide sequence is shown on the left and right.

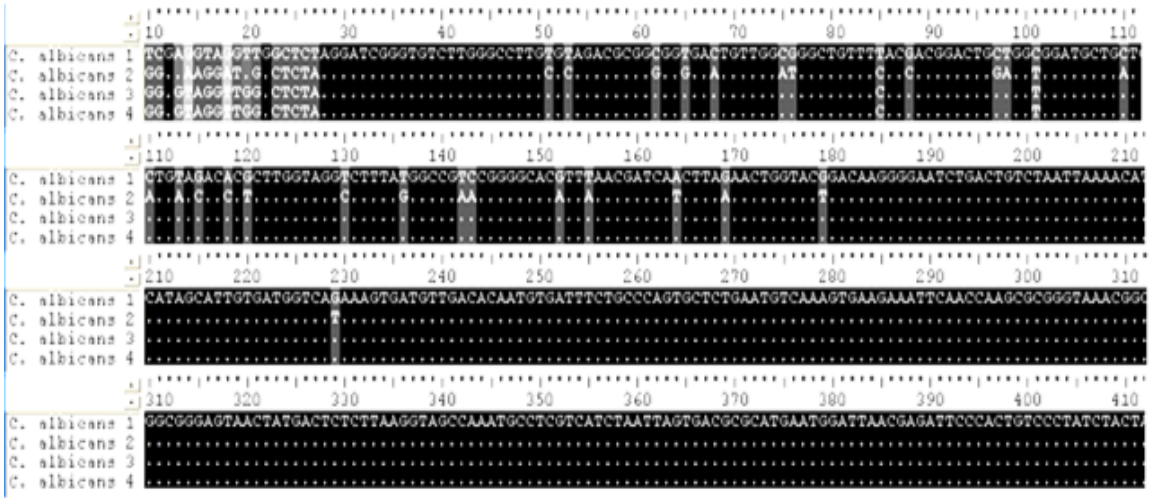

Fig. 3: Alignment of transposable intron region sequences for *C. albicans* by using BioEdit software. Nucleotide sequences are present in all intron regions are shaded in dark color.

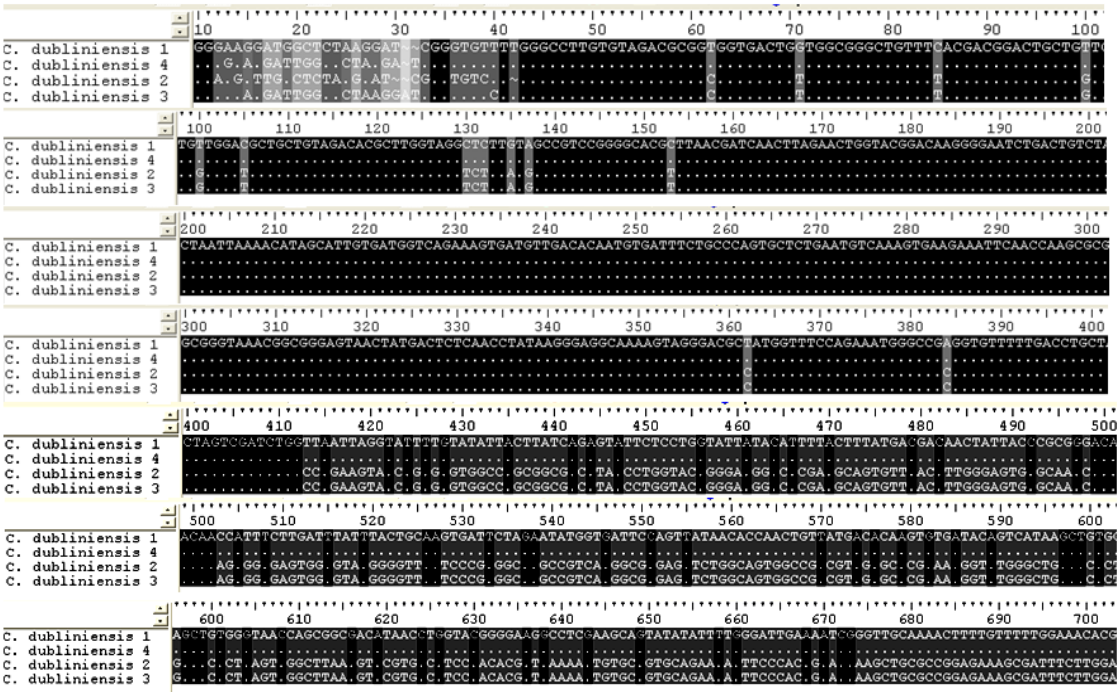

Fig. 4: Alignment of transposable intron region sequences for *C. dubliniensis* by using BioEdit software. Nucleotide sequences are present in all intron regions are shaded in dark color.

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 cychloheximide 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 dermatophytes[20]. 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 tineacapitis in Egypt.*C. tropicalis* was isolated from 19 systemic candidiasis patients (35%) with skin lesions[24].

Three species of *Candida* were tested by using E-test strip with tow 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 Arikan[3]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 agentand 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. was had been used successfully such as amphotericin B, azoles especially fluconazole and echinocandins especially caspofungin[33-36]. 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 tothe 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 patient[38]. Pfaller and Diekema[39] 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 treeo f 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. albecans* and non- *C. albecans* isolates, while *Bfa*I can be differentiated among non- *C. albecans* species. Xu*et al*.,[13] were compared the API20C technique for identification of *C. albicans* and sequencing of the ITS2 region for 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.

References

1. Ho, K. and Cheng, T. (2010). Common superficial fungal infections a short review. Medical bulletin, 15(11): 23-27.
2. Pfaller, M.A. and Diekema, D.J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. ClinMicrobiol Rev, 20(1): 133-163.
3. Bille, J.; Marchetti, O. and Calandra, T. (2005). Changing face of health-care associated fungal infections. CurrOpin Infect Dis 18: 314-319.
4. Arikan, S. (2007). Current status of antifungal susceptibility testing methods. Med Mycol, 45: 569-587.
5. Torttorano, A.; Penman, J.; Bemhardt, H.; Klingspor, L.; Kibbler, C.C. and et al. (2004). Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital based surveillance study. Eur J ClinMicrobiol Infect Dis, 23(4): 317-322.
6. Johnson, E.M. (2008). Issues in antifungal susceptibility testing. J antimicrobchemother, 61(1): 13-18.
7. Sutton, D.A. . (2003). Specimen collection, transport, and processing: mycology. In: Murray, P.R.; Baron, E.J.; Jorgensen, J.H.; Pfaller, M.A. and Yolken, R.H. Manual of clinical microbiology. 8th ed. Washington DC: American Society for Microbiology.

8. Vandeputte, P.; Ferrari, S. and Coste, A. (2012). Antifungal resistance and new strategies to control fungal infections.Internat J Microbiol, 26pp.
9. Ali, G.F.; Al-Hamadani, A.H. and Mohammad, A.W. (2015). Detection of cyclohximide resistant gene in selected pathogenic fungi. J Contemp Med Sci, 1(4): 23-26.
10. Imran, Z.K. and Al-Asadi, Y.F. (2014). Multiple molecular markers for diagnosis of conjunctivitis caused by *Candida* spp. in Iraq.Afr J Microbiol Res, 8(38):3482-3488.
11. Imran, Z.K. and Al-Shukry, H.N. (2014). Molecular diagnosis of vaginal candidiasis by polymerase chain reaction (PCR) and random amplification polymorphism DNA (RAPD-PCR) in Babylon Province, Iraq.Afr J Microbiol Res, 8(6):496-502.
12. Elie, C.M.; Lott, T.J.; Reiss, E. and Morrison, C.J. (1998). Rapid identification of Candida species with species- specific DNA probes. J ClinMicrobiol 36(11): 3260-3265.
13. Xu, J.; Millar, B.C.; Moore, J.E.; McClurg, R.; Walker, M.J.; Evans, J.; Hedderwick, S. and McMullan, R. (2002). Comparison of API20C with molecular identification of Candida spp isolated from bloodstream infections. J ClinPathol, 55: 774-777.
14. Cornet, M.; Sendid, B.; Fradin, C.; Gaillardin, C.; Poulain, D. and Nguyen, H. (2011). Molecular identification of closely related Candida species using two ribosomal intergenic spacer fingerprinting methods. J Molecular Diagnostics, 13(1): 12-22.
15. Mohammadi, R. and Abdi, S. (2015). Molecular identification of *Candida* species isolated from gastro-oesophageal candidiasis in tehran, Iran. GastroenterolHepatol bed Bench, 8(4): 288-293.
16. Ellis, D.; Davis, S.; Alexiou, H.; Handke, R. and Bartley, R. (2007). Description of medical fungi.2nd ed. Mycology unit.Women´s and Children´s Hospital. North Adelaide, Australia.
17. Clinical and Laboratory Standard Institute CLSI (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts, Approved standard- second edition. CLSI document M27-A2. Pennsylvania, USA.
18. Imran, Z.K. (2015). *Candida albicans* ssp. *dubliniensis stat.et comb.Nov*., a new combination for *Candida dubliniensis* based on genetic criteria.Afr J Microbiol Res, 9(17): 1205-1214.
19. McCullough, M.J.; Clemons, K.V. and Stevens, D.A. (1999). Molecular and phenotypic characterization of genotype C. albicans subgroups and comparsion with Candida dubliniensis and Candida stellatoidea. J ClinMicrobiol, 37: 417-421.
20. Aggarwal, A.; Arora, U. and Khanna, S. (2002). Clinical and mycological study of superficial mycoses in Amritsar. Indian J Dermatol, 47(4): 218-220.
21. Prasad, N.; Mahapatra, A. and Chayani, N. (2013). Changing trends in the fungal isolates from clinical specimens of suspected superficial mycosis.Inaian Medical Gazette, pp: 60-62.
22. Abdel-Fatah, B.E.; Moharram, A.M.; Moubasher, A.A.H. and Al-Ryani, M.A. (2013). Genetic relationships and isozyme profile of dermatophytes and *Candida* strains from Egypt and Libya. Afr. J. Biotechnol., 12(29): 4554-4568.
23. Younes, A.E.H.; Mohamed, E.E.M.; Tawfik, K. M. and Ezzat, A.A. (2012). Tineacapitis in Assiut (Egypt).AA M J, 10(1): 45-54.
24. Bae, G.Y.; Chang, S.E.; Moon, K.C.; Lee, M.W.; Choi, J.H. and Koh, J.K. (2005). Clinicopathologic review of 19 patients with systemic candidiasis with skin lesions.Int J Dermatol, 44(7): 550-555.
25. Lozano-Chiu, M.; Nelson, P. W.; Lancaster, M.; Pfaller, M.A. and Rex, J.H. (1997). Lot-to- lot variability of antibiotic medium 3 when used for susceptibility testing of Candida isolates to amphotericin B. J ClinMicrobiol, 35:270-272.
26. Rubio, M.C.; Gil, J.; de Ocáriz, I.R.; Benito, R. and Rezusta, A. (2003). Comparison of results obtained by testing with three different agar media and by the NCCLS M27-A method for in vitro testing of fluconazole against *Candida* spp. J ClinMicrobiol, 41(6): 2665-2668.
27. Capoor, M.R.; Rawat, D.; Nair, D. Deb, D. and Aggarwal, P. (2007). Evaluation of glucose-methylene-blue-Mueller-Hinton agar for E-test minimum inhibitory concentration determination in *Candida* spp. Indian J Med Microbiol, 25(4): 432-433.
28. Espinel-Ingroff, A.; Canton, E.; Gibbs, D. and Wang, A. (2007). Correlation of neo-sensitable tablet diffusion assay results on three different agar media with CLSI broth microdilution M27-A2 and disk diffusion M44-A results for testing susceptibilities of *Candida* spp. and *Cryptococcus neoformans* to amphotericin B, caspofungin, fluconazole, Itraconazole, and voriconazole. J ClinMicrobiol, 45(3): 858-864.
29. Liao, R.S.; Rennie, R.P. and Talbot, J.A. (2001). Novel fluorescent broth microdilution method for fluconazole susceptibility testing of Candida albicans. J ClinMicrobiol, 39:2708-2712.

30. Ostrosky-Zeichner, L.; Rex, J.H.; Pappas. P.G.; Hamill, R.J.; Larsen, R.A. (2003). Antifungal susceptibility survey of 2,000 bloodstream Candida isolates in the United States. Antimicrob Agents Chemother 47:3149-3154.
31. Pfaller, M.A.; Chaturvedi, V.; Diekema, D.J.; Ghannoum, M.A.; Holliday, N.M.; Killian, S.B.; Knapp, C.C.; Messer, S.A.; Miskov, A. and Ramani, R. (2008). Clinical evaluation of the sensititreYeastOne colorimetric antifungal panel for antifungal susceptibility testing of the echinocandinsanidulafungin, caspofungin, and micafungin. J ClinMicrobiol, 46(7): 2155-2159.
32. Pfaller, M.A.; Espinel-Ingroff, A.; Boyken, L.; Hollis, R.J.; Kroeger, J.; Messer, S.A.; Tendolkar, S. and Diekema, D.J. (2011). Comparsion of the broth microdilution (BMD) method of the European committee on antimicrobial susceptibility testing with the 24-hour CLSI BMD method for testing susceptibility of *Candida* species to fluconazole, posaconazole, and voriconazole by use of epidemiological cutoff values. J ClinMicrobiol, 49(3): 845-850.
33. Peyron, F.; Favel, A.; Michel-Nguyen, A.; Gilly, M.; Regli, P. and Bolmstrom, A. (2001). Improved detection of amphotericin B-resistant isolates of Candida lusitaniae by Etest. J ClinMicrobiol, 39(1): 339-342.
34. Betts, R.; Glasmacher, A.; Maertens, J.; Maschmeyer, G.; Vazquez, J.A. and et al. (2006). Efficacy of caspofungin against invasive *Candida* or invasive *Aspergillus* infections in neutropenic patients. Cancer, 106(2): 466-473.
35. Park, B.J.; Arthington-skaggs, B.A.; Hajjeh, R.A.; Iqbal, N.; Ciblak, M.A. and et al. (2006). Evaluation of amphotericin B interpretive breakpoints for Candida bloodstream isolates by correlation with therapeutic outcome. Antimicrob Agents Chemother 50(4): 1287-1292.
36. Pfaller, M.A.; Diekema, D.J. and Sheehan, D.J. (2006). Interpretive breakpoints for fluconazole and *Candida* revisited: ablueprint for the future of antifungal susceptibility testing. ClinMicrobiol Rev, 19(2): 435-447.
37. Rodriguez-Tudela, J.L.; Almirante, B.; Rodriguez-Pardo, D.; Laguna, F.; Donnelly, J.P.; Mouton, J.W.; Pahissa, A. and Cuenca-Estrella, M. (2007). Correlation of the MIC and Dose/MIC ratio of fluconazole to the therapeutic response of patients with mucosal candidiasis and candidemia.Antimicrob Agents Chemother, 51(10): 3599-3604.
38. Chapeland-Leclerc, F.; Hennequin, C.; Papon, N.; Noël, T.; Girard, A.; Socié, G.; Ribaud, P. and Lacroix, C. (2010). Acquisition of fluctosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. Antimicrob Agents Chemother, 54(3): 1360-1362.
39. Pfaller, M.A. and Diekema, D.J. (2004). Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigates*. J ClinMicrobiol, 42(10): 4419-4431.
40. Pujol, C.; Daniels, K.L.; Shawn, R.L.; Srikantha, T.; Joshua, B.R.; Geiger, J. and Soll, D.R. (2004). The closely related species Candida albicans and Candida dubiniensis can mate. Eukaryot Cell, 3(4): 1015-1027.
41. Jackson, A.P.; Gamble, J.A.; Yeomans, T.; Moran, G.P.; Saunders, D.; Harris, D.; Aslett, M.; BarrellJ.F.; Butler, G.; Citiulo, F. Coleman, D.C. and et al. (2009). Comparative genomic of the fungal pathogens *Candida dubliniensis* and *C. albicans*.Genome Res 19: 2231- 2244.
42. Costa, J.M.; Garcia-Hermoso, D.; Olivi, M.; Cabaret, O.; Farrugia, C.; Lecellier, G.; Dromer, F. and Bretagne, S. (2010). Genotyping of Candida albicans using length fragment and high-resolution melting analyses together with minisequencing of a polymorphic microsatellite locus. J Microbiol Methods, 80(3): 306-309.
43. Cirak, M.Y.; Kalkanci, A. and Kustimur, S. (2003). Use molecular methods in identification of Candida species and evaluation of fluconazole resistance. MemInstOswaldo Cruz, 98(8): 1-11.
44. Turenne, C.Y.; Sanche, S.E.; Hoban, D.J.; Karlowsky, J.A. and Kabani, A.M. (1999). Rapididentification of fungi by using the internal transcribed spacer 2 genetic region and an automated fluorescent capillary electrophoresis system. J ClinMicrobiol, 37: 1846-1851.
45. Fujita, S.; Senda, Y.; Nakaguspecieschi, S. and hashimoto, T. (2001). Multiplex PCR using internal transcribed spacer 1 and 2 regions for rapid detection and identification of yeast strains. J ClinMicrobiol, 39(10): 3617-3622.

*****