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Molecular diagnosis of *Candidemia* of intensive care unite patients based on sequencing analysis of ITS regions

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Abstract : Invasive candidiasis (IC) bears has high risk of morbidity and mortality in the intensive care units (ICU). The *Candidia* spp was the main cause of hospitalized bloodstream infections and it is associated with mortality highly rate. ICU receive huge number of patients exposed to multi-accidents types such asterrorist attacks; Cerebrovascular accidents(CVA), etc., most of these cases lead to penetration of *Candida*spp to blood stream. The aim of the study was prospective, accurate and rapped identification molecular methods for detection of *Candida* from blood stream and oral infections in intensive care units in Iraq.400 samples clinically diagnosed candidemia were collected over a period from November / 2015 to April / 2016 from ICU of Margan Teaching Hospital and Hilla Teaching Hospital in the province of Babylon, Iraq, the Middle East. Out of 264 blood samples (53) were culture positive (32n=male,21n=female) ,while 71 out of 136 oral swabs were culture positive. Out of which 63.8% isolates were *C. parapsilosis*20.34% were *C.albicans*12.7%,*C.membrenifaciens* 2.97% and 0.25% for *C.sake* .Our conclusion : This study gave more attention of the risk factor of candidemia, and showed many *Candida*spp were penetrate blood stream and showed unique genetic polymorphism patterns of *Candida* in Iraq. The new recorded for the *C. parapsilosis,C.sake* and *, C.membrenifaciens* from blood samples for the first time in Iraq based on sequencing analysis of the whole ITS region and the ITS2 of the rDNA emphasizes the low precise of conventional identification methods.
Keywords : Invasive candidiasis, ITS and ITS2 sequencing analysis, multiple alignment sequence.

Introduction

Candidemia and others types of Candidiasis are generally common causes of nosocomial infections through the worldwide, the incidence ,prevalence and identification pictures are attention in many countries such as USA ,European and some of Asian countries and ICU have been notarized for *C.albicans,C.sake, C.krusei, C.parapsilosis and C.glabrata* strain[1,2].

In Intensive Care Units(ICU) *Candidia* spp. was the 3rd generality common cause of hospitalized bloodstream infections and is associated with death rate of 47%[3].*C.albicans*is considered as the more serious *Candida* spp and main cause of candidiasis, oral thrush, Candiduria and vaginal candidiasis[4,5].Although the *C.albicans* has long been the most common types isolated during blood stream infection, recent reports has shown a shift to Non Candida Albicans(NCA) like *C.sake, C.krusei, C.parapsilosis*and *C.glabrata*[6-8], especially in ICU and patients[9,10]. Many studies showed the *C.albicans* and *C.glabrata* representative a major threat to the patients in hospitals worldwide[11,12]. NCAcandidemia has develop a rising significant infection and it to replace

C.albicans especially uncommon species such *C.sake* and *C.mambranifaciens* in most clinical sites any bloodstream infections[13].

Huge number of patients exposed to multi-accidents types lead to penetration of *Candida*spp to blood stream as in related to inquiries in car accident and war or terroristattacks. *Rodriguez et al*.,[14] studied the risk factors associated with invasive fungal infections in combat trauma.Blyth et al.[15] identified many *Candida*spp colonization and infection of Combat-Related Injured Patients of USA from Iraq and Afghanistan.

A conventional diagnostic methods such as CHROMagar,fermentation and other phenotypic testwere consuming time and gave bias diagnoses of *Candida* isolates to the species level[16-18] reports that the CHROMagarunreliable test for distinguish between*C.glabrata* and *C.parapsilosis*,both showed white-pink alsothe CHROMagar showed confuse color to distinguish between many species like *C.kefyr,C.utilis, C.robusta, C.famata, C.rugosa, C.guilliermondii*and *C.pelliculosa.* All make the same kind of glossy pink colonies as *C.glabrata*, consequently misidentification can happen[19,20].

Advance molecular methods especially PCR and sequencing analysis is being increasingly used for the rapid diagnose of *Candida* isolates to the species level[17]. The most widespread targets of PCR amplification are rDNA genes included typing of ITS regions and ITS2 regions of fungi were beneficial for the rapid identification of clinically important fungi[21]. DNA sequencing is reinforcement new discovery which revolt the conceptual foundations of numerous fields[22].

The main *Candida*spp was identified in nosocomial candidemia in Suadia Arabia like *C.parapsilosis ,C,tropicalis ,C.krusei*[23-25]. Shokohi et al.,[26]showed many *Candidia* spp. like*C. parapsilosis*, *C. glabrata*, *C. albicans*, *C. tropicalis*,*C.kruse*and*C.guilliermondii*in Iran. Also Vijayakumar et al.,[27] was identified *C. parapsilosis* was the common yeast isolated from IC patients.*C. glabrata, C. albicans*, *C. tropicalis*, and*C. kefyr*, were identified from candidemia patient in India based on PCR and RFLP-PCR .but few attention in the Middle East[28].

Unfortunately ,the picture of candidemiais no clear till now in Iraq due to absence of any attention and lack of real studies on candidemia except the short study ofSabeeh et al,[12] diagnosed the *C.albicans* based on conventional methods as causes agent of candidemia in leukemia in one province of Iraq. Risan[29] isolated *C.glabrata* only from blood samples of acute leukemia in Baghdad province.On the other hand,the intensive care units patients of the most hospitals in Iraq were receiving injured persons due to car accident or war or terrorist attacks. The variant human accidents lead to penetration of *Candida* spp. to blood stream and may be reflective of a population at higher risk for patients severity and death.

The aim of this study was prospective, accurate and rapped identification molecular methods for detection of *Candida* spp. from blood stream and oral infections in intensive care units in Iraq .

2.Material and methods:

Four hundreds clinical samples (246 blood,77 swab immune patients incancer 23 swab HematologyUnit, 36 swab patients in ICU; were collected based on standard methods[12,30].The samples were collected from patient.These samples collected with different age groups ranging from (4 to 97 years). The diseases associated with immune deficiency included attenuated and hospitalized Margan Teaching Hospital and Hilla Teaching Hospital in the province of Babylon, the survey covering the period from November / 2015 to April / 2016 was performed.

Two milliliters of blood were collected from each patient, blood put in EDTA tubefor keeping blood from clotting,then incubated blood cultures at 37C^{o} for 24h to 96h on Sabouraud's dextrose agar (SDA).Blood and swab were streaking on SDA (12),single colonies were subjected to species identification basedon character's color production on CHROMagar medium[31].

2.1.PCR assay

The phenotypic results were confirmedby simple PCR by specific primer pair for *Candida*.OneμLofDNA(20μg/ml)from each of 20 *Candida* isolates were mixed with PCR mixture (final

reaction volume 25 µL) consisted of 12 µL of 20x Master Mix (Promega),2 µL of primers (10 pmole) and restmolecular-gradewater. The PCR conditions for primer pairs ITS5/ITS4,ITS3/ITS4 primers were :95 ºC for 3 min followed by30 cycles 94 ºC for 1min.55ºC for 40sec. and 72ºC for 1min.and final extension 72ºC for 5min.The PCR mixture was amplified by thermal cycler PCR System (Labnet, USA)[17].

The PCR products for each target region were run on 1.2% agarose gel (Bio Basic Canada Inc.).Electrophoresis performed at 100 V. in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator (Korea Com.). The purified products for 17 isolates were subjected to sequencing. Sequence analysis was performed inMacrogeneLab., USA. The sequencealignmentof*Candida* sppwas compared with theBLAST data base and were aligned with sequences from the BLAST data base derived fromthe following reference strains. The phylogenetic tree(UPGM) based on sequencing wereconstructed employing the Mega 6 software, pairwise and multiple sequence alignment based on BioEdit software was performed[18].

3.Results

All isolates of *Candida* spp. were preliminary classified based on color on CHROMagar,later only 24 isolates were subjected for PCR assays amplified whole ITS region ITS2 region by primer pair ITS5/ITS4 and ITS3/ITS4 respectively (Figure 1).Only seventeen of the PCR products for both ITS regions of selectively isolates based on similarity and difference in amplicons sizes were sequenced (Table 1).

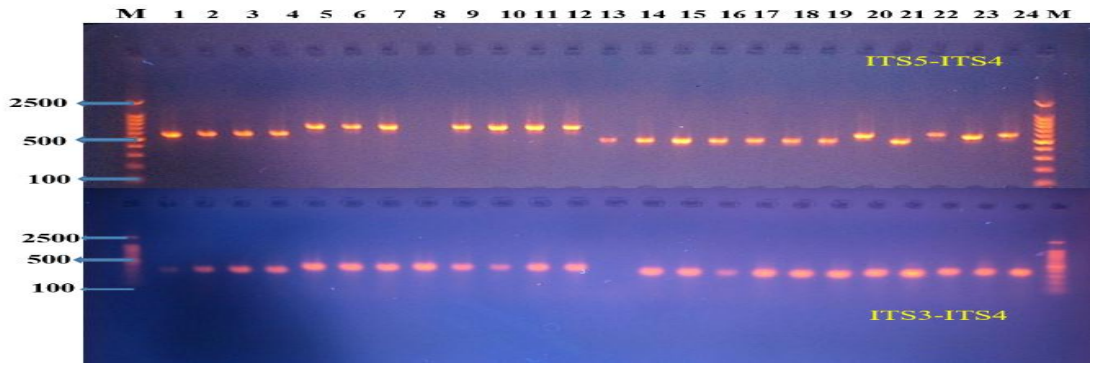

Figure (1): Agarose gel electrophoresis of PCR products for *Candida* spp isolates amplified by primer pair (ITS5-ITS4) and primer pair (ITS3-ITS4).

3.1. Sequence analysis

Five species of *Candida* out of 17 sequence were identified based on pairwis alignment with reference isolates in the gene bank ,they are *C.sake* ,*C.prapsilosis* ,*C.membrenifaciens*, *C.albicans* and the telomorphe of *C.kruse* is *Pichiakudriavzevii* for both target region (ITS and ITS2 respectively(Table 1).*C.sake* and *C.membrenifaciens* were recorded for the first time in Iraq.

3.2. Multiple alignment of 17 sequences for both ITS and ITS2 regions

The results of Multiple alignmentanalysis based on BioEdit software was performed for 17 isolates of *Candida* spp. (5n for *C.sake*,2n for *C.albicans*,4n for both *C.parapsilosis* and *C.membrenifaciens* and one isolate for *Pichiakudriavzevii*(Figures 2-3) . Each set of isolates for *Candida*sppwas showed high similarity with leading sequences with some mutation or substitutions, these sequence variation were indicated to microevolution in each set of isolates .

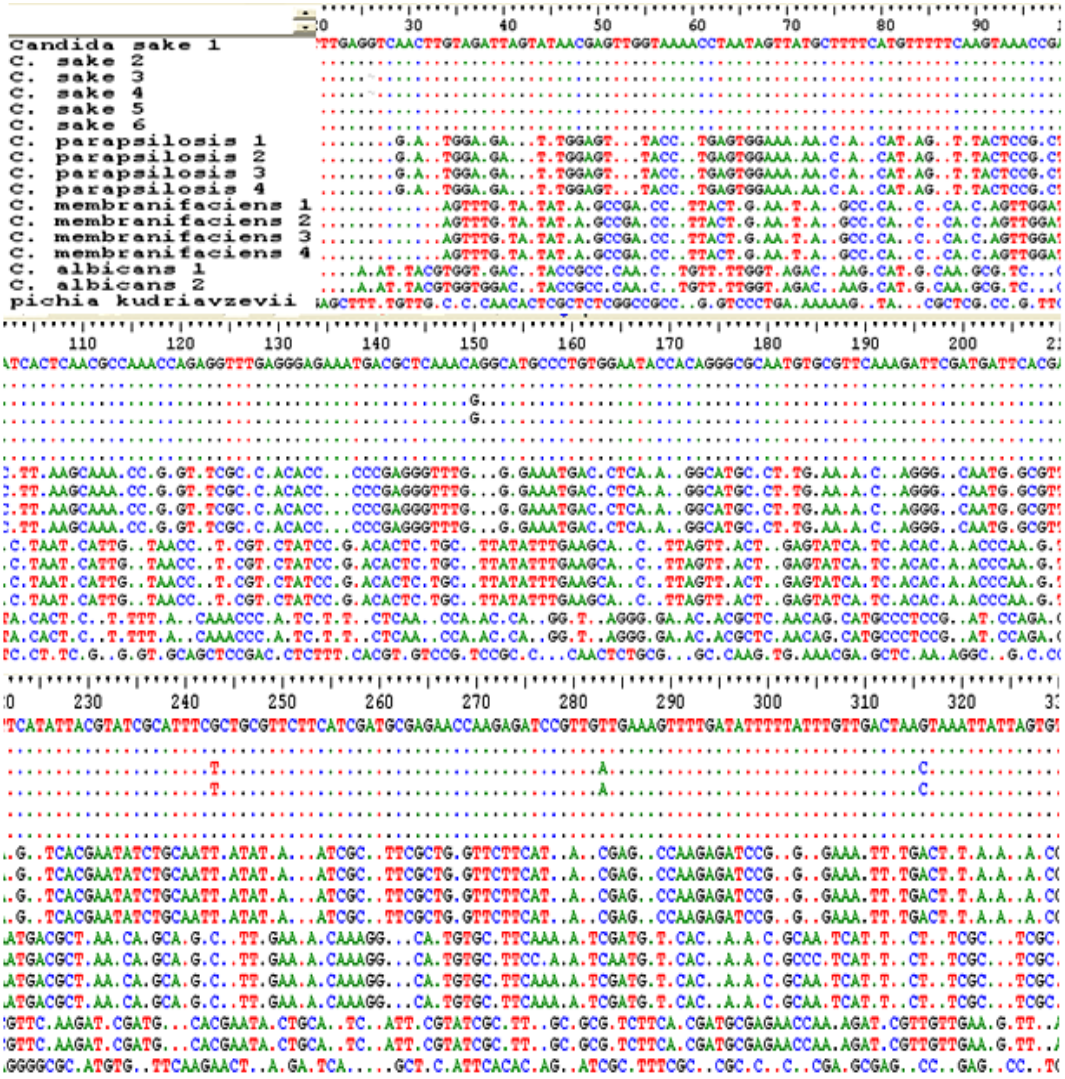

Figure (2): The Sequence Multiple-alignment of sequence analysis of ITS2 region amplified by ITS5/ITS4.

High similarity of multiple alignment partial sequence of ITS and ITS2 regions amplified by ITS5/ITS4 and ITS3/ITS4 primer pairs respectively. Seventeen and 16 isolates of *Candida* spp. (6n and 5n for *C.sake*,2n for *C.albicans*,4n for both *C.parapsilosis* and *C.membrenifaciens* and one isolate for *Pichiakudriavzevii* for to regions respectively. All isolates of *C.sake* showed highly similarity with leading sequence with low variation in some sequences sites.These variations may be Single Nucleotide Polymorphism(SNP) or bases substitution ,more variation was observed at the end of sequences of *C.sake*(Figures2,3).Other species showed intra or inter- species variations, sometimes similarity in all species may due to the uniformity of belonging to same *Candida*genus.

The phenotypic identification assays showed fluctuation results compare with molecular identification assays were showed reliable coincidence results, many isolates of *Candida* spp. were identified as *C.kefyr* based on their reaction color on CHROMagar while identified as *C.membranifaciens* based on whole ITS region and ITS2 in rDNA region, and also with some isolates of *C.albicans*, and *C.albicans* Ssp.*dubliniesis* (Table 1).

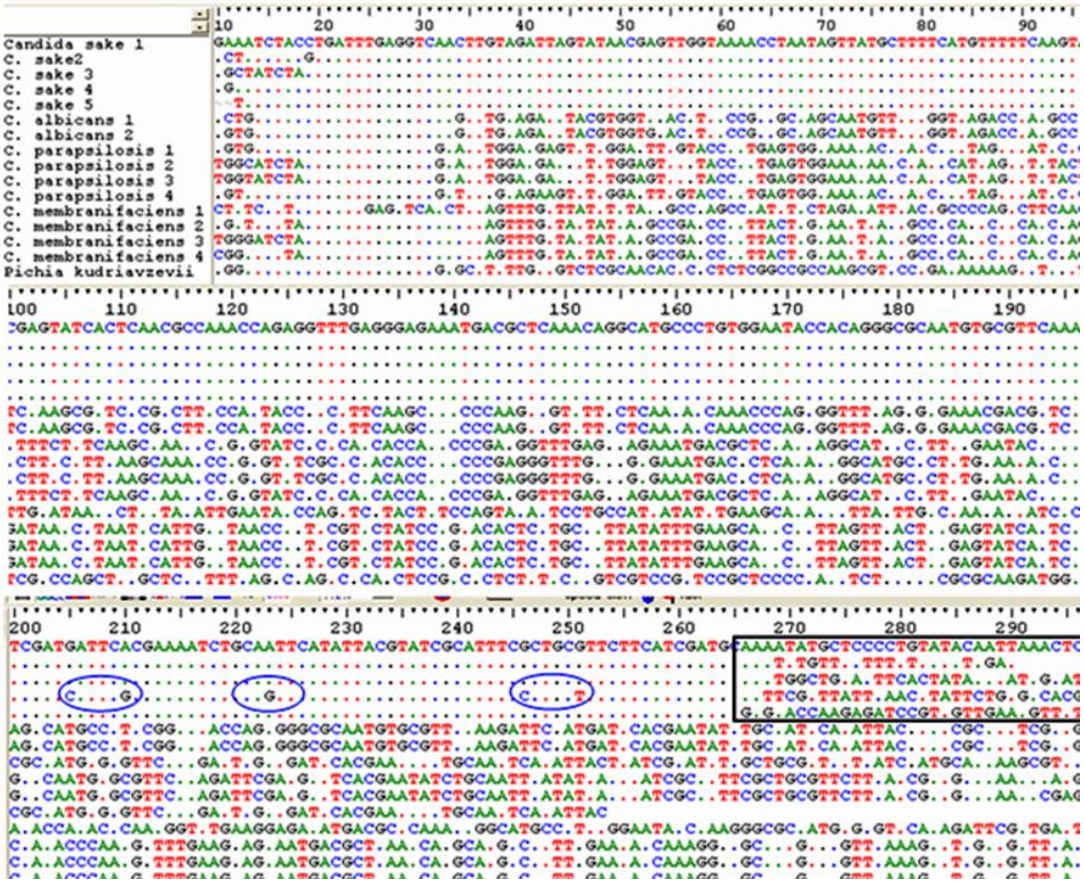

Figure (3): Multiple-alignment of sequence analysis of ITS2 region amplified by ITS3/ITS4.

Table (1): Comparisonamong of phenotypic identification and molecular identification based on sequence analysis data.

No.	Phenotypic identification based on CHROMagar	identification based on (ITS5/ITS4) &(IS3/ITS4)	identification based on sequence		Final identification
			ITS sequencing	ITS2 sequencing	
1	C.parapsilosis	C.parapsilosis	C.parapsilosis	C.parapsilosis	C.parapsilosis
2	C.parapsilosis	C.parapsilosis	C.parapsilosis	C.parapsilosis	C.parapsilosis
3	C.parapsilosis	C.parapsilosis	C.parapsilosis	C.parapsilosis	C.parapsilosis
4	C. kefyr	C. kefyr	C.parapsilosis	C.parapsilosis	C.parapsilosis
5	C. kefyr	C. kefyr	C.membranifaciens	C.membranifaciens	C.membranifaciens
6	C. kefyr	C. kefyr	C.membranifaciens	C.membranifaciens	C.membranifaciens
7	C. kefyr	C. kefyr	C.membranifaciens	C.membranifaciens	C.membranifaciens
8	C. kefyr	C. kefyr	C.membranifaciens	C.membranifaciens	C.membranifaciens
9	C. krusie	C. krusie	P. kudriavzevii	P. kudriavzevii	P. kudriavzevii
10	C. albicans	C. albicans	C.sake	C.sake	C.sake
11	C. albicans	C. albicans	C.sake	C.sake	C.sake
12	C. sake	C. albicans	C.sake	C.sake	C.sake
13	C. dubliniesis	C. albicans	C.sake	C.sake	C.sake
14	C. dubliniesis	C. albicans	C.sake	C.sake	C.sake
15	C. albicans	C. albicans	C.sake	C.sake	C.sake
16	C. dubliniesis	C. albicans	C.albicans	C.albicans	C.albicans
17	C. albicans	C. albicans	C.albicans	C.albicans	C.albicans

3.3.. Phylogeny tree:

The phylogenetic tree(UPGM) for16 *Candida*spp was constructed based on sequences of ITS region. The isolates of *Candida*spp were isolated from cancer patients showed closed related intra-isolates groups as in Figure (4), results 5 clusters of *Candida* spp. the observation show *C.sake* in cluster 1, *C.parapsilosis* in cluster2, *C.albicans* in cluster 3, *C.membranifaciens* in cluster 4 and *Pichiakudriavzevii* in the cluster 5.

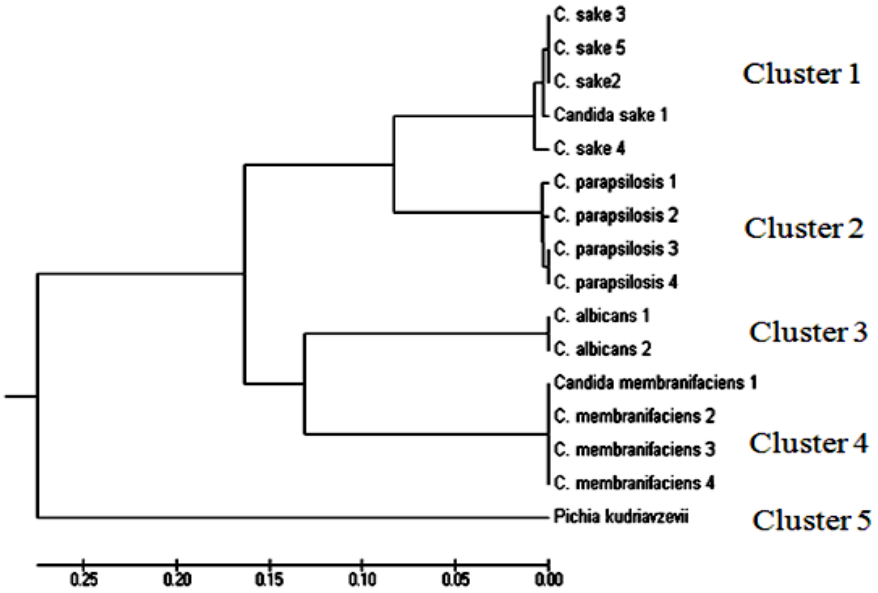

Figure (4): Phylogeny tree (UPGM) based on sequence of 16 *Candida*sppisolates.

The results of bioinformative pairwise sequence alignment data for two each of *Candida* species were showed distinct of each species based on the data of the score and the Query cover percent as in Figures (5-8) .The pairwise sequence alignment data of two *C,albicans* isolates showed high score 558 and 91% of query cover, this means belonging for same species ,while the same bioinformative between the others species showed them as a distinct species for example: The pairwise sequence alignment data of two , *C.membrenifaciens*and *C.albicans* showed high score 159 and 37% of query cover, this means belonging distinct species(Figure 5)

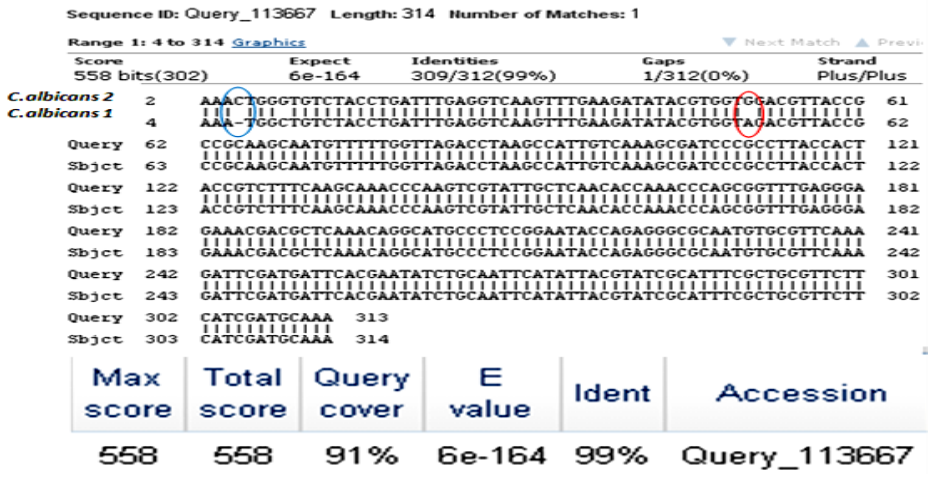

Figure (5): The pairwise sequence alignment data of two , *C.albicans* and *C.albicans* showed high score 558 and 91% of query cover, this means belonging to the same species.red cycles mismatches ,blue cycles gaps .

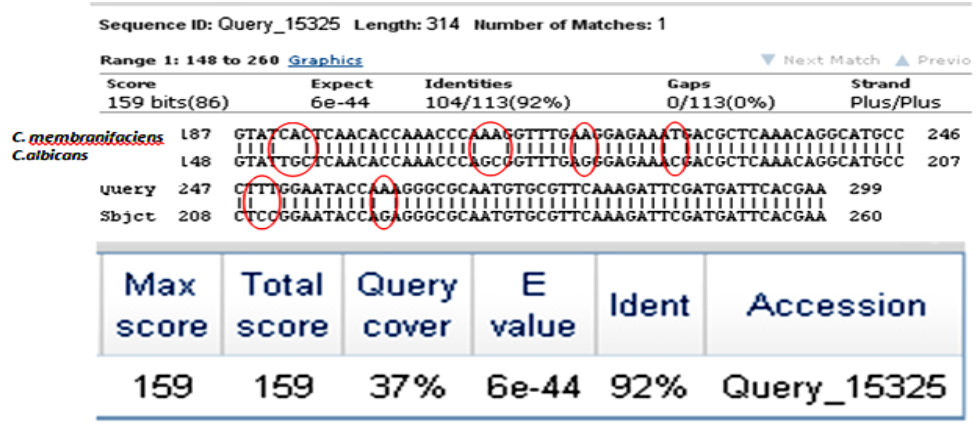

Figure (6):The pairwise sequence alignment data of two , *C.membrenifaciens*and *C.albicans* showed high score 159 and 37% of query cover, this means belonging distinct species.

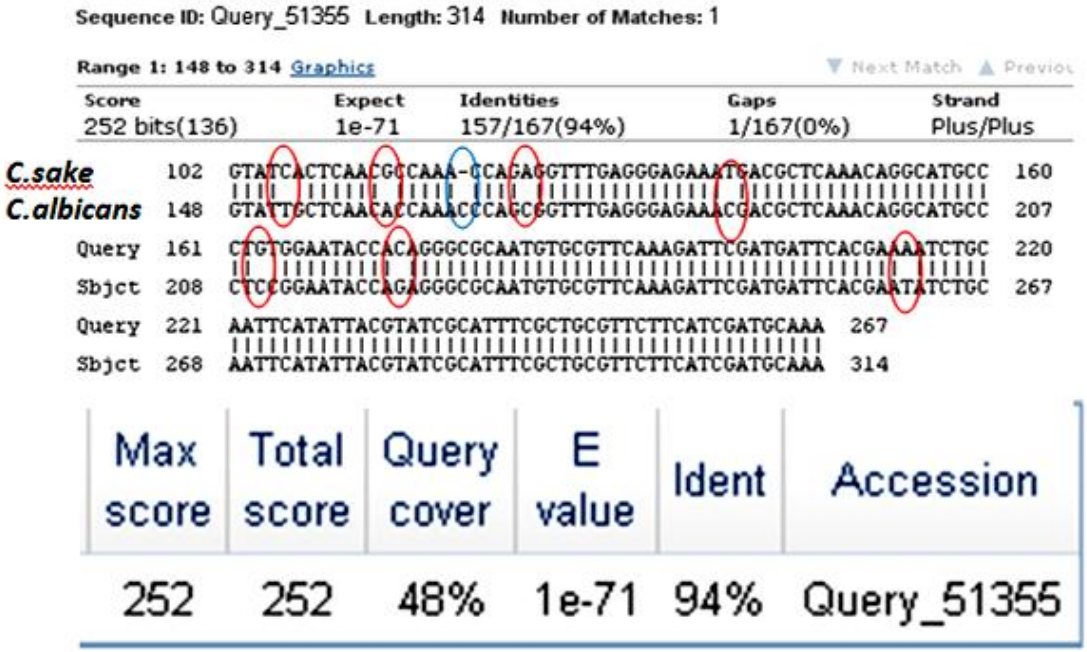

Figure (7): The pairwise sequence alignment data of two , *C.sake* with *C.albicans* showed high score 252 and 48% of query cover, this means belonging distinct species.red cycles mismatches ,blue cycles gaps .

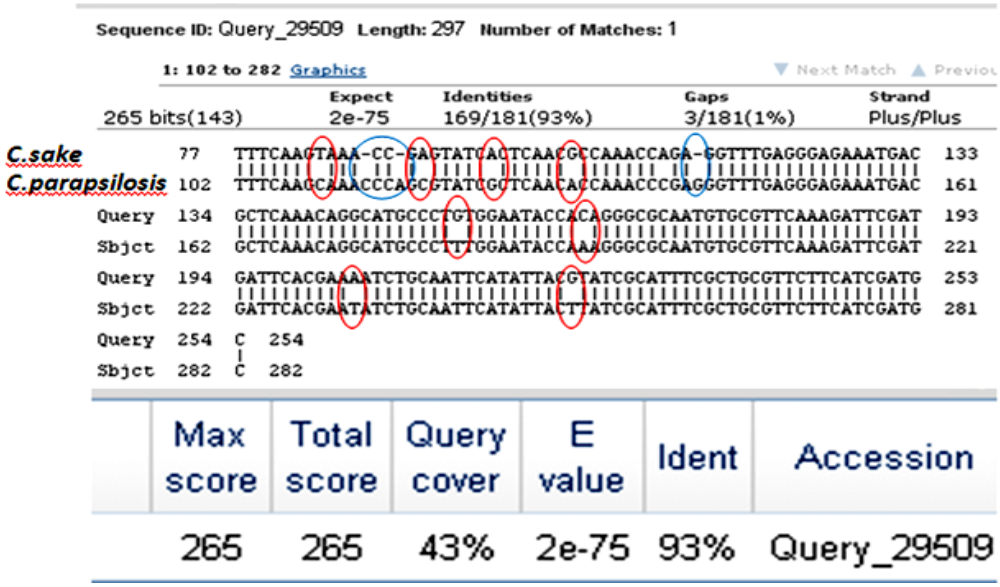

Figure (8): The pairwise sequence alignment data of two , C.sake with C.parapsilosis showed high score 265 and 43% of query cover, this means belonging distinct species . red cycles mismatches ,blue cycles gaps .

4. Discussion

Our results of isolation and identification of *C.sake* and *C.membrenifaciens* and other species in the blood samples of ICU patients were coincidence with recently studies[32].The final identification of 17 *Candida* spp.(Table 1) showed that molecular markers more accurate for identification based on[33-35]. The missing identification based on phenotypic markers being fluctuation,CHROMagar considered as preliminary identification test but not accurate for ever,this interpretation was reviewed in[16,18].

The variation of PCR product for both region showed coincidence. The identification of *Candida*spp was followed(Fujita *et al*.[36] andBellemain*et al*.[37]. These sequences variations may raised due to micro evolutionary trends in any species groups,*Imran and Ali*[18] mention to the same sequences variation in *C.glabrata* and *C.parapsilosis*.

Due to the fluctuation of the identification of *Candida*spp based on CHROMagar for example: some of isolates were identified as *C.albicans* based on green color on CHROMagar but identified as *C.sake* based on sequences analysis ,the same case of *C.albicans* Ssp.*dubliniesis* identified as *C.albicans* based on Sequencing methods (Table 1). These confusion in identification between *Candida* spp. Wassolved the confuse by pairwise sequence alignment between two each species, and the results of paiwise alignment showed genetic variation and microevolution degree for each alignment (Figures 5-8).

Our finding, with regards to the lacking previous data on Candidemia in Iraq,the finding *C.parapsilosis*,*C.sakeC.membrenifaciens*and *C.albicans* being the most frequent recovered species in this study is indicative of unique trait specific to the epidemiology of candidemia in ICU. Moreover, similar to our finding, many studies were reported the isolation most of the *Candida* spp. from neighboring countries from Sudia Arabia[23], Gulf region[28] and Iran[26] and other countries in Europe and USA[40].

Most of previous identification studies were based on small sample sizes ranged from 30-50 clinical isolates such as Vijayakumaret al.,[27] based on 39 consecutive clinical isolates patients from various ICUs .

Our study suffered from several limitations. First, because we used blood culture positivity as the criterion for diagnosing candidemia instead of direct PCR detection, this estimation could be less than the actual frequency of candidemia.Second, small samples number makes our results less accurate to estimate the actual prevalence of candidemia in Iraq. Finally, we faced difficulties in the following up our patient groups for along

period due to the critical patient's cases,such as death of the patients and built our explanation on the results of culture positive diagnosis.

On the other hand the misidentification of blood septicemia ,which may be due to the systematic candidiasis causes problems in ICU for patients, may be indistinguishable from bacterial septicemia, and these infection are severe difficult to diagnose may lead to severely disease from 1-year to elderly patients.The major risk factors for developing IC include prior antibiotic use, central venous catheterization, a recent major surgery, use of steroids, dialysis and immunosuppression[38].According to a recent survey, 50-80% of the critically ill patients who were admission to ICU had already been exposed to risk factors for IC, 5-15% had *Candida* colonization on admission and 5-30% actually had IC(Leroy et al.,2009)[39].

The results of this study were agreed with review of Yapar[40]that only 15 out of 150 Candida spp isolated from patients as infectious agents , it has been determined that in 95% of infections, involved *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis,* and *C. krusie*.The contribution of this study showed the foggy picture of candidemia in Iraq ,and emphasis the requirement for futures studies about candidemia and IC incidence compare with huge studies in development countries were performed .

Our conclusion,in spite ofthe ICU receiving multi-hundreds of patients in each year, undergo any one of multi-accidents types such as car accidents, wars accidents ,terrorist attacks and Cerebrovascular accidents(CVA) cases and the patients were exposed to different levels of injuring cases and contaminated with fungi and bacteria . Unfortunately the studies in Iraq gave low attention of identification species caused candidemia based and depending on simple conventional methods for identification compare with finding our study, due to these reasons the value of this study still low and the picture of IC is ambiguous because the absence of pervious interest studies in Iraq ,also the local epidemiological data on fungal infections in ICU staff hospitals are lacking . Our perspective of the incidence and the impact of IC in our ICUs should be further clarified in future investigations.

Acknowledgments

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