Isolation and Identification of *Candida spp*. In immunocomparomised patients and Detection some virulence factors.

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Abstract

One hundred thirty patients undergoing chemotherapy for different types of cancer attending Tikrit Teaching Hospital from January 2013 to Augusto 2013 were enrolled in this study. Their age were range between "11-83 years old". 68 blood samples and 62 sputum samples were collected in this study.

Candida spp. Infection was 14(20.5%) isolates from patients blood and 5(8.1%) isolates from patients sputum, were infections more in blood *C.albicans* 8(57.8%) , *C.glabrata* 3(21.4%) *C.krusei* 2(14.3%) and *C.parasillosis* while isolates from sputum were 3(60%) of *C.albicans* and *C.glabrata* 2(40%). The isolated were identified according morphological , Cultural and biochemical characteristic.

Fungal isolates in present study produced various virulence factors hemolysin and protease production were produced in *C.albicans* 8(72.8), 11(100%) respectively, while *C.glabrata* hemolysin production were 4(80%) and (40%) of protease production , while *C.parasillosis* isolate were produced protease and hemolysin. C.krusei were produce only hemolysin 1(50%).

Introduction

Medical mycology education crisis after 1970s, the incidence increased of fungal diseases in immunocompromised hosts stimulated awareness and growth in medical mycology [(1)].

More than 200 species that have been described, *Candida* is ubiquitous. Some species are normal habitants of the human microbiological flora of the skin, as well as the gastrointestinal, genitourinary, and even respiratory tracts [(2,3)] but only 10% are known to cause infections in humans [(4,5)] Five most common species (as *Candida albicans, C.glabrata, C.parapsilosis, C.tropicalis* and *C.krusei*) cause 95–97% of human candidemias [(4,6)]. The remaining 3–5% of *Candida* blood stream infections are caused by 12–14 different species, and although these are considered rare causes of candidemias, several have occurred in nosocomial clusters and exhibit innate or acquired resistance to one or more established antifungal agents[(2)]. *Candida* infections of the latter category are also referred to as candidemia and usually confined to severely immunocompromised persons , such as cancer, transplant and AIDS patients [(7)]. , this yeast exhibits a number of different morphological forms under different environmental conditions; such forms include budding yeast cells (blastospores, blastoconidia), pseudohyphae (elongated cells which appear as filamentous cell chains), true hyphae, and clamydospores [(9)]. *Candida species* are possess a number of virulents factors (8) such as phospholipase , hemolysis and protease. That helps fungi in nutrient uptake , tissue invasion, adherence and dissemination [(10)]. Hemolysin in red blood cells has also suggested to provide a survival strategy for fungi during opportunistic infections. In Candida , the secretion of hemolysin coupled with iron uptake facilitates hyphal invasion during disseminated candidiasis [(11)].

Proteases are a group of enzymes, whose catalytic function is to hydrolysed peptide bonds of proteins and break them [(12)]. Secretion of proteinases by pathogen is mandatory in order to degrade the tissue barriers and obtain nutrition at the infection site [(13)].

Extracellular proteases may play a role in adherence and survival of the pathogen on mucosal surface [(14)] , invasion of host tissues [(15,16)] and digestion of immunoglobulins[(16,17)]. there are a number of publications investigating protease production in *Candida* spp. isolated from different sources [(18)].

Several researchers have reported that production of Secreted aspartyl proteinases is also correlated with hyphal formation, adherence, and phenotypic switching [(19,20)].

this study aimed to :

- To isolation and identification of candida species infection from immunocompromised patients. By using conventional methods (BHI agar/broth , colony morphology, germ tube test).
- To screening the ability of candida species isolates to produce , protease, hemolysin as a virulence factors.

Materials & methods:

Samples Collection:

Sputum and blood samples were collected from 130 patients undergoing chemotherapy their age varried "11-83 years old", for different types of cancer attending Tikrit Teaching Hospital from January 2013 to Augusto 2013.

Blood samples were cultured on BHI agar/broth for isolation media according Glenn& John (1975)[(21)] and Sputum samples were cultured on (SDA).

1. Identification Of Candida Isolates:

- Germ Tube Test was determined according to Binesh & Kalyani (2011)[(22)].
- Chlamydospore Formation all *Candida* isolates were tested for the production of chlamydospores in corn meal agar (CMA) with 1% Tween 80. (2005)[(23)].

- Sugar Fermentation Test : a set of sugars consists of Glucose, Maltose, Sucrose,Trehalose, Galactose, D-xylose and Lactose, which were used for identification and differentiation between *Candida* spp., the test was done by inoculating tubes containing fermentation media and 2% sugar with part of the colony, shaking gently then incubated at 28-30C° for3 days. The positive result was recorded by changing the color of phenol red to yellow and production of CO2 gas bubbles in Durham tube [25].
- Carbohydrate Assimilation Test: the test depends on the ability of different species of yeasts to grow in various sugar solutions (glucose, lactose, trehalos, raffinose, Maltose , Melibiose, Galactose, D-xylose and sucrose).

Carbohydrate assimilation medium was poured in Petri dishes and inoculated with *Candida* spp., then wells were made by cork borer in the inoculated plates, each well were filled with 2% sugar and incubated plates at 30C° for 2-4 days [25].

2. Haemolysin Activity test: Determination of haemolysin activity was evaluated with a blood plate assay[26] .
3. Protease Production test: The protease production was determined according to Aoki [27] using a test medium consisting of SDA plates containing bovine serum albumin (BSA).

Results and discussion:

Out of 130 samples collected form patients undergoing chemotherapy 19(14.6%) fungal isolates were found .

Results of blood culture showed *candida* isolates were detected in 14(20.5%) blood culture while 5(8.1%) *candida* isolates were found sputum samples. No growth were detected in 54(79.5%) of blood culture and 57(91.9%) of sputum.

Table (1): candida spp. In blood and sputum specimens .

Culture results	Blood		Sputum		Total	
	No	%	No	%	No	%
Growth	14	20.5	5	8.1	19	14.6
No growth	54	79.5	31	91.9	111	85.4
Total	68	100	62	100	130	100

According to blood culture results the most frequently isolated fungal was *C.albicans* 8(57.2%) followed by *C.glabrata* 2(14.3%), *C.krusei* 3(21.4%), then *C.parasillosis* 1(7.1%) as illustrated in table (2).

Table (2): Number and percentage of identified fungal isolates from blood culture samples.

Fungal isolates	Number of isolates	%
C. albicans	8	57.2
C. krusei	2	14.3
C. glabrata	3	21.4
C. parasillosis	1	7.1
Total	14	100

The present study revealed that the rate of Candid isolates by used blood culture method in immunocompromised patients (as shown in table (2). there are many studies which have been conducted on fungal isolates from bloodstream by used blood culture method[34] but in iraq less study for comparing this results to other study[28].

Only one study in Iraq which have been conducted on candidemia was study by AL-Asehagi, (2012)[28] in Salah al din who found that *C. albicans* were isolated from (17.6%) blood, While that reported by Chen et al (2006)[29] in Australia, who isolated (32.1%) Theoklis *et al*. (2005)[30] and Diekema *et al*. (2002)[31] who found that *candida spp*. were isolated from (32%) in USA, which were agree with our results. And were *C.albicans* (37.9%), were *C.glabrata* (13.8%) and were *C.parapsilosis* (11.2%)in study conducted by Hsiue et al(2009)[32] in Taiwan. while in study reported by Lynn et al (2002)[33] studied 50 blood samples collected from immunocompromised patients in Texas and the results showed that (76%) of samples were candida spp of which *c.albicans* and *C.glabrata* (24%) for each of them, were *C.glabrata* (18%) were *C.krusei*. Kovacicova et al (2001)[34] at in the Slovak Republic was isolated (52.9%) were *C.albicans*, were (32.1%) were non *C.albicans* of prospective was completed on 140 patients with fungaemia.

these different results may be due to the difference of technique for blood culture as the sample size to the volume of media and daily inverting inoculated broth with blood sample. In addition, the method used in our study by BHI broth/agar as show in Fig(1).

Fig 1: Blood culture in BHI agar/broth media.

For sputum samples the results showed that C.albicans were most isolated 3(60%), followed by *C.glabrata* 2(40%), as illustrated in table (3).

Table (3): Fungal isolates from sputum culture samples.

Fungal isolates	**Number of isolates**	**%**
C.albicans	**3**	**60**
C. glabrata	**2**	**40**
Total	**5**	**100**

our results for sputum culture showed the *C.albicans* were more (60%).

There were many studies which have been conducted on opportunistic fungi in immunocompromesid patients. One of these studies was by Yongabi *et al*.(2009)[36] isolated 12 isolates of *C.albicans* from 98 sputum. which were agreement with our results ,and Jaffer (1998)[37] in Babylon province reported that *C. albicans* (69.2%) was the most common isolate in a study of pulmonary fungal infection, less frequency are *C. tropicalis* (19.2%), *C. kefyr* (7.6%) and *C. krusei* (3%). In other study were 26 (27%) of *C.albicans* and non albicans spp. were 29(30%) [38].

The reason for these variations in all studies may be due to sample size, environment factors, nutrition requirements and virulence factors of this fungi [39], It is not easy to determine the pathogenic role of fungal isolates from the respiratory tract, to differentiate between infections, colonization and contamination [40].

2. Identification of *Candida spp*. Isolates:

Candida was identified depending on the morphological features on culture medium, germ tube formation, pseudohypha formation and chlamydospores according ([41, 42] showed table (4).

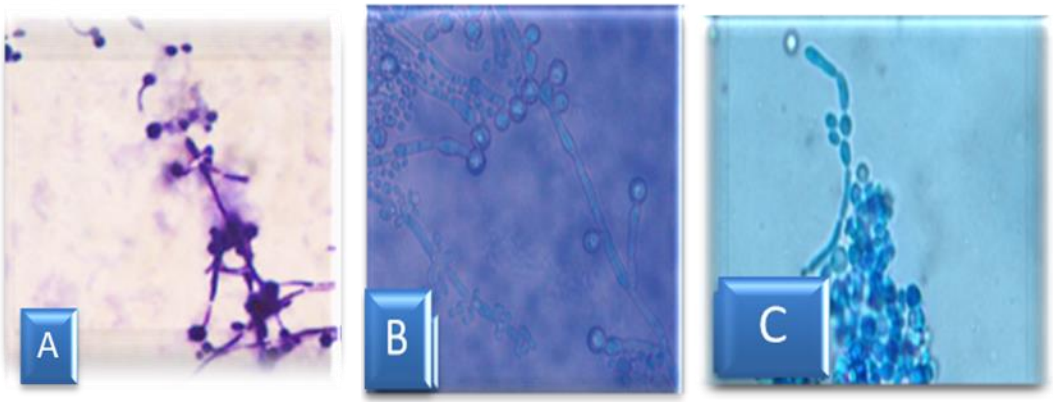
Table (4): Morphology feature and some tests used for identification of *Candida* Spp.

Candida spp.	***C.albicans***	***C.krusei***	***C.glabrata***	***C.parasillosis***
Color colony	white to cream	white to cream	white to cream	white to cream
Germ tube	+	_	_	_
Chlamy-dospore	+	_	_	_
Pseudo-hyphae	+	+	_	+

(+) positive , (_): negative.

The *C.albicans* isolates was showed positive result and the formation of germ tubes was seen as long tube like projections extending from the yeast cells (Fig 2A), there was no constriction at the point of attachment to the yeast cells. These tubular extensions represent an early stage in the formation of true hyphae. The germ tubes were formed within two hours of incubation and this is a unique diagnosis characteristic of *C. albicans* differentiates it from other fungi. This results agreement with Mohammed (2008)[43] , when they mentioned that "All *C. albicans* strains were germ tube test (GTT) positive when tested directly from the colony, and all non-*albicans* species were GTT negative when tested directly from the colony".

C.albicans produced Chlamydospore than other species. They were spherical, thick-wall, and usually produced on suppurating cells that occur along pseudohyphae or at the tip of hyphae (Fig 2B). Approximately 90% of Isolates produced chlamydospore when inoculated by Dalmau technique or streak technique on cornmeal–Tween 80 agar . Pseudohyphae and hyphae with clusters of blastospores are also produce on this agar fig (2C) . *C.parasillosis, C.krusei* and *C.glabrata* dosents produce chlamydospores [44].



Fig(2): A: Germ Tube of C.albicans stained by gram stains (40X) B:Chlamydospores of C. albicans Cultured on CMA-tween 80 at 30℃ staning by gram stain B: Microscopic morphology of *C. albicans* in mycelial phase with blastoconidia budding from the pseudohyphae staining by LPCB stain (40x).

The biochemical tests were performed according to the tests described by Ellis *etal*.(2007)[45].

The isolates were identified by used urease test , sugar assimilation and sugar fermentation. The results of this tests as in table (5).

Table (5): Biochemical tests used for identification of *Candida* spp.

Candida spp	*C.albica-ns*	*C.krusei*	*C.glabrata*	*C.parasillosis*
Urease	_	_	_	_
Fermentation tests				
Glucose	+	+	+	+
Maltose	+	_	+	_
Sucrose	_	_	_	_
Trehalose	V	_	V	_
Galactose	V	_	_	+
D-xylose	+	_	_	+
Lactose	_	_	_	_
Assimilation tests				
Melibiose	_	_	_	+
Raffinose	_	_	_	_
Glucose	+	+	+	+
Maltose	+	_	_	+
Sucrose	V	_	_	+
Trehalose	+	_	V	+
Galactose	+	_	_	+
D-xylose	+	_	_	+
Lactose	_	_	_	_

(+) positive , (_): negative , (V): variable .

4.3.1 Screening of protease production fungi isolates.

In this study, the extracellular protease production of *Candida* spp. , was detected in 8(72.8%) of tested *C.albicans* , 5(40%)of *C.glabrata* and 1(100%) *C.parasilliosis*, while *C.krusei* were unable to protease production showed table (6).

Table (6): Protease Production by fungal isolates.

Fungal isolates	Number isolates	Protease	
		No	%
C.albicans	**11**	**8**	**72.8**
C.glabrata	**5**	**2**	**40**
C.krusei	**2**	**0**	**0**
C.parasillosis	**1**	**1**	**100**

Result of present study agree with where De Bernardis [46] reported high *in vitro* protease activity in all *C. parapsilosis* strains isolated in patients with also Kanatrcioglu and Yucel [12] reported *in vitro* protease production in a majority of *C. albicans*, *C. kefyr*, *C. lipolytica*, *C. parapsilosis* and *C. tropicalis* clinical isolates, while *C. glabrata*, *C. guillermondii*, *C. krusei*, *C. lusitaniae* and *C. rugosa* were unable to protease positive. While Yamamoto (1992)[47] discovered that the majority of *C. tropicalis* and *C. parapsilosis* isolates had proteolytic activity while none of the tested *C.glabrata* strains secreted the enzyme.

Fig (4): Protease activity A: *C.albicans*. B: *C.glaabrat*

3. Screening of hemolysis production by fungi isolates:

our results indicated that 11(100%) of *C.albicans* , 4(80%) of *C.glabrarta* , 1(50%) of *C.krusei* and (100%) of *C.parasillosis* isolates were able hemolysin production showed table (7), These findings agree with Ramesh *et al.*[49] who compared the hemolytic activity of 50 *Candida* strains isolated from patients with HIV and 10 *Candida* strains isolated from immunocompetent patients. All strains produced hemolysis, but haemolytic activity was significantly higher for *C.albicans* strains isolated from HIV patients when compared to those isolated from immunocompetent patients. And Rossoni *et al.*[50] reported that *C.albicance* (100%) produced hemolysins, *C.glabrarta and C.krusei* each of all, (40%) of *C.parasillosis* isolates were hemolysin.

Table (7): hemolysin Production some by some fungal isolates.

Fungal isolates	Number isolates	Hemolysis	
		No	**%**
C.albicans	**11**	**11**	**100**
C.glabrata	**5**	**4**	**80**
C.krusei	**2**	**1**	**50**
C.parasillosis	**1**	**1**	**100**

Luo *et al.* (2001)[51] observed that species of *Candida* are capable of producing one or more types of hemolysins *in vitro* and that species differ in the production of these activities.

These differences are possible to be due to the environmental conditions, the source of isolate and the detection method that was used in the detected of the enzyme .

Fig (5): hemolysin activity of *C.albicans* isolate fraction of hemolysis in sheep blood agar at 37 ◦C.

In conclusion, according to results of this study *C.albicans* are the most common specie of blood among the total *Candida* infections, and most *candida* species isolates have ability to produce significant amount protease, hemolysin.

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عزل وتشخيص انواع المبيضات لدى المرضى المثبطين مناعيا والتحري عن بعض عوامل الضراوة

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الملخص

تم جمع 130 عينة دم وقشع من المرضى الخاضعين للعلاج الكيمياوي الراقدين والمراجعين لمستشفى تكريت التعليمي من كلا الجنسين من تتراوح اعمارهم (11–83) سنة ، تم جمع 68 عينة دم و62 عينه قشع ضمن هذه الدراسة.

بلغت نسبة الإصابة بالفطريات (20.5%)14 عزله من مجرى الدم و (8.1%)5 عزلة من القشع ، كانت اعلى نسبة اصابات من مجرى الدم هي *Candida albicans* بنسبة (57.8%)8 وكانت *C.glabrata* بنسبه (21.4%)3 و *C.krusei* بنسبة (14.3%)2 و *C.parasillosis* بنسبة (7.1%)1 بينما اكثر العزلات من القشع كانت لل *C.albicans* بنسبة 3(60%) و كانت *C.glabrata* بنسبه (40%)2 حي تم تشخيص العزلات وفقا لخواصها الشكلية والمز رعية و الكيموحيوية .

تم في هذا البحث التحري عوامل الضراوة متنوعة للمبيضات المعزولة مثل قدرتها على انتاج hemolysin ، و protease كانت الانتاج في *C.albicans* بنسبة 8(72.8%) و 11(100%) على التوالي ، بينما *C.glabrata* كانت منتجه لل hemolysin في بنسبة 4(80%) و انتاجها protease كانت بنسبة 2(40%) ، بينما عزلة *C.parasillosis* كانت منتجة لل protease و hemolysin بينما *C.krusei* كانت منتجة فقط لل hemolysin بنسبة (50%)2.