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Phenotypic and molecular characterization of *Candida* species in urine samples from renal failure patients

Mohammed S. Alhussaini[1,*], Noha F. El-Tahtawi[2], AhmadM. Moharram[3]

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Saudi Arabia
[2]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Al-Dawadmi, Shaqra University, Saudi Arabia
[3]Department of Botany and Microbiology, Faculty of Science,Assuit University,Egypt

Email address:
malhussaini@su.edu.sa (M. S. Alhussaini)

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Abstract: The prevalence of Candida infections of the urinary tract (candiduria)has increased significantly over the past few years due to various predisposing factors especially in hospitalized patients. The predisposing factors frequently associated with candiduria are urinary tract instrumentation, prior antibiotic use, prolonged hospital stay, extremes of age, diabetes mellitus, female sex and use of immunosuppressive therapy. The purpose of this study was to apply different diagnostic techniques for characterization of *Candida* species in urine samples from renal failure patients using urinary catheters. Results showed that 20 % of renal failure patients were suffering from candiduria. Females were more affected than males (64% versus 36% of culture positive cases). Diabetes mellitus and catheterization were the obvious risk factors. Different *Candida* isolates showed variations in their phenotypic (macroscopic and microscopic) characteristics when cultured on CHROMagar Candida, corn meal agar and bovine serum. *Candida albicans* was the most common species being isolated from 54% of positive cases. *C.krusei* (Teleomorph: *Pichia kudriavzevii*), *C. glabrata* and *C.tropicalis* were respectively identified in 24%, 16% and 6% of samples. Positive germ tube test (in serum) and production of chlamydospores (on corn meal agar) were only confined to *C. albicans*. Sequencing of rRNA gene (18S covering ITS1 and ITS2) confirmed the identification of the four *Candida* species. In vitro antifungal sensitivity test (disc diffusion method) revealed that all *Candida* strains were inhibited by Amphotericin-B and Nystatin. Other compounds as Clotrimazole, Itraconazole, Ketoconazole, Tioconazole, Fluconazole and Sertaconazole were effective against 50% - 67% of *Candida* strains. All isolates of *C. glabrata* were resistant to Clotrimazole and Sertaconazole.It is highly recommended to follow standard hygienic precautions to avoid cross contamination by *Candida* during hemodialysis. Complications following candiduria can be avoided by early diagnosis and proper treatment with effective antifungal agents.

Keywords: Candiduria, Renal Failure Patients, Chromagar Candida, RNA Gene Sequencing

1.Introduction

Candiduria or presence of *Candida* species in the urine is rarely encountered in otherwise healthy people with structurally normal urinary tract (Bukhary,2008,Kauffman, 2005 and Schonebeck and Ansehn, 1972). It is, however, of common occurrence in hospitalized patients. *Candida* spp. account for almost 10-15%of nosocomial urinary tract infections (UTIs) (Kauffman,2005, Lundstrom and Sobel, 2001 and Kauffman *et al*., 2000).In the United States, Shay and Miller (2004) estimated that the incidenceof candiduria was 25,000 cases per year. Moreover, approximately one-third of hospitalized patients with urine cultures yielding *Candida* were in the Intensive care unit (ICU) where bladder catheter use was high. However, as early as 1986 Platt and colleagues reported that 26.5% of all UTIs with bladder catheter usage were due to *Candida* species. This observation was later substantiated by others who found that 90% of *Candida* UTIs in a large tertiary care centre in the United States were related to bladder catheters (Berrouane *et al*., 1999). On a national scale, surveillance studies have indicated that 25% of all UTIs in ICUs are caused by *Candida* species (Banerjee *et al*., 1999 and Jarvis *et al*.,1999) and the length of stay in such units influences the incidence significantly.

Candiduria is a frequently documented condition in ICU, but it remains a common dilemma, faced by clinicians,

whether determining if a patient is suffering from a fungal infection or if the fungal presence is only due to normal colonization. In hospitalized patients, the urinary tract (UT) is one of the most propitious anatomical sites for the development of infections, once it is normally colonized by such microorganisms (Schaberg *et al*., 1991; Guler *et al*., 2006). However, the lack of a proper and safer protocol to characterize candiduria as a UT infection isusually a serious problem (Kauffman, 2005). On the other hand, candiduria has been considered an early marker of disseminated fungal infection in critically ill patients.

The genus *Candida* includes several species implicatedin human pathology such as *C. albicans*, *C. tropicalis,C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. kefyr*, *C. guilliermondii* and *C. dubliniensis*(Sulivan *et*al. , 1995).*Candida albicans* is by far the most common cause of mucosal yeast infection, being the sole species recovered from up to 70% of HIV infected individuals and up to 90% of cases of Candida vaginitis (Coleman *et al*., 1993; Sobel, 2007). Other *Candida* species can be recovered alone or coisolated with *C. albicans* from sites of mucosal infection (Coleman *et al*.,1995).

C. albicans has been the yeast most commonly isolated from urine, accounting for 50%–70% of isolates in various studies. *C. glabrata* and *C. tropicalis* are the next most common species found in cultures of urine. *Candida parapsilosis*, a common cause of candidemia in both adults and neonates, is uncommonly isolated from urine ofadults (Kauffman *et al*.,2000 ; Ayeni *et al.,*1999 and Stofer *et al*.,1994).*C. parapsilosis* is found more often in urine from neonates and is usually associated with systemic infection in this population (Wainer *et al*.,1997).In Brazil Vidigal *et al* (2011) reported a candiduria case in a 64-year-old male patient from intensive care unit (ICU) who developed candiduria due to *C.tropicalis*, which complicated to fatal candidemia despite antifungal treatment.

Researches in many laboratories is carried out to develop new drugs or drug delivery systems, but the development of approaches that allows quick and accurate identification of disease-causing yeasts is also necessary, especially because the incidence of human disease caused by the less common *Candida* species has increased. Thus, the identification of *Candida* species is very important in the diagnostic laboratory, because such identification shows prognostic and therapeutical significance,allowing the early and correct antifungal therapy (Godoy *et al*., 2001, Milan and Zaror, 2004).

The purpose of this study was to identify different *Candida* spp. isolated from renal failure patients by phenotypic and genotypic methods and to evaluate their sensitivity or resistance to currently used antifungal therapeutic agents.

2. Material and Methods

2.1. Collection of Urine Samples

Urine samples were collected from 250 patients visiting the University Hospitals in Cairo, Assiut and Sohag Governorates, Egypt during July and August 2012. All patients were complaining of renal failure (150males and 100females) and frequently use urine catheters for more than 10 days.

2.2. Phenotypic Identification of CandidaIsolates

2.2.1. A- Culturing on Sabouraud's Dextrose Agar (SDA)

All samples were cultured onto Sabouraud's Dextrose Agar (SDA) (HiMedia, Mumbai, India) plates supplemented with 0.05% (W/V) chloramphenicol(Bhavan *et al*., 2010).Cultures were incubated at 37ºC for 24-48 hours after which the growing fungi were purified and kept in slants for further phenotypic and molecular studies.

2.2.2. B- Culturing on CHROMagar Candida

Chromogenic media contain chromogenic substrates which react with enzymes secreted by the target microorganisms to yield colonies of varying colours (Pfaller *et al*., 1996).CHROMagar Candida Differential agar (CHROMagar Company, Paris, France) is a selective and differential medium, which facilitates rapid isolation and presumptive identification of some yeasts from mixed cultures.The medium contained (g/L): agar 15; peptone 10.2; chromogenic mix 22; chloramphenicol 0.5; pH: 6.1. According to the manufacturer 47.7 grams of the powdered medium were slowly dispersed in 1 liter of sterile distilled water and brought to a boil by repeated heating until complete fusion of agar grains. The medium was cooled in a water bath to 45-50°C, with gentle stirring, then poured into sterile Petri dishes and allowed to solidify. Separate colonies from all *Candida* isolates on SDA were subcultured onto CHROMagar Candida and incubated at 37ºC for 48 hr. Presumptive identification was done based on colony colour of the growing *Candida* strains.

2.2.3. C- Germ-Tube Test

Small inoculum of suspected Candida cultures were inoculated into 1 ml of human serum (Sigma-Aldrich, Germany) in a small tube and incubated at 37 ºC for 2 hours. After incubation, a loop-full of culture was placed on a glass slide, overlaid with a cover-slip and examined microscopically for the presence or absence of germ-tubes. Formation of germ tubes was seen as long tube like projections extending from the yeast cells with no constriction or septa at the point of attachment to the yeast cells. The germ tube is indicative of *C. albicans* and *C. dubliniensis* (Bhavan *et al.,* 2010).

2.2.4. D- Culturing on Corn Meal Tween 80 Agar (CMA)

As recommended by Koehler *et al*. (1999) and Ellis *et al.* (2007), chlamydospore formation by certain Candida species (*C. albicans* and *C. dubleniensis*) is a encouraged by culturing on CMA. This test is negative with other Candida species.

All yeast isolates were subcultured on SDA and and in glycerol water (15%V/V) and kept under low temperature

for further molecular and in vitro antifungal sensitivity test. Isolates were also given a code number assigned to the Assiut University Mycological Centre (AUMC).

2.2.5. Genotypic Identification of Yeast Isolates

Fungi were individually grown on Sabouraud's Dextrose agar and incubated at 37° C for 2 days. A small amount of fungal growth was scraped and suspended in 100µl of distilled water and boiled at 100° C for 15 minutes and stored at -70° C. Cultures were sent to SolGent Company (Daejeon, South Korea) for rRNA gene sequencing. Fungal DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene (also referred to as rDNA) was amplified using the polymerase chain reaction (PCR) technique in which two universal fungal primers ITS1 (forward) and ITS4 (reverse) were incorporated in the reaction mixture. Primers used for gene amplification have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC -3'). The rRNA gene is composed of the following regions: small subunit (SSU) which is the 18S region, the ITS1, the 5.8S, the ITS2 and the large subunit (LSU)which is the 28S region as shown in Fig 1. The PCR reaction mixture was prepared using Solgent EF-Taq as follows: 10X EF-Taq buffer 2.5 µl, 10 mM dNTP (T) 0.5 µl, primer (Forward-10 picomol) 1.0 µl, primer (Reverse -10 picomol) 1.0 µl, EF-Taq polymerase (2.5U) 0.25µl, DNA template 1.0 µl, Distilled Water (to 25 µl). Then the amplification was carried out in a thermal cycler under the following conditions: one round of denaturation at 95°C for 15 sec followed by 30 cycles of denaturation at 95°C for 20 sec, annealing at 50°C for 40 sec and extension at 72°C for 1 min, with a final extension step at 72 C for 5 min.

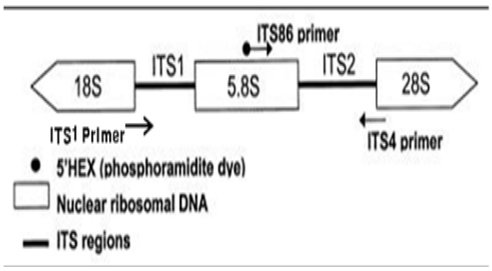

Fig.1. *Schematic representation of the fungal ribosomal gene containing the primer target areas used in the amplification of the 18S region (partial sequencing), ITS1, 5.8S, ITS2 (complete sequencing) and 28S (partial sequencing).*

The PCR products were then purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing. The purified PCR products were reconfirmed (using size marker) by electrophoreses on 1% agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers. Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

2.2.6. Antifungal Susceptibility Test

The disc diffusion test was performed according to the procedure described in the Clinical and Laboratory Standard Institute (CLSI, 2004). Cell suspensions of individual Candida strains were prepared in 2 ml of sterile 0.85% saline solution. The turbidity was adjusted to yield 0.5 McFarland standard (approximately $5x10^3$ cells/ml. Six kinds of antifungal agents obtained from HiMedia Company in India were tested. The interpretative breakpoints of these antifungal agents were done according to Ellis (2011a) as shown in table 1.

Table 1. *Interpretative breakpoints of antifungal agents.*

Antifungal agents (Abbreviation)	Concentration /disc	Zone of activity in mm		
		Sensitive	Intermediate	Resistant
Amphotericin-B (AM-B)	100U	≥15	10 - 14	<10
Nystatin (NYS)	100U	≥15	10 - 14	<10
Clotrimazole (CLO)	10ug	≥20	12 - 19	≤11
Fluconazole (FLU)	10ug	≥19	15 - 18	≤14
Itraconazole (ITR)	10ug	≥23	14 - 22	≤13
Ketoconazole (KET)	10ug	≥28	21 - 27	≤20
Sertaconazole (SER)	10ug	≥23	14 - 22	≤13
Tioconazole (TIO)	10ug	≥23	14 - 22	≤13

3. Results and Discussion

3.1. Prevalence of Candiduria in Relation to Sex and Age of Patients

Of the 250 renal failure patients studied, 150 were males and 100 were females. Candiduria was confirmed in only 50 patients (20% of total cases) by positive culturing on Sabouraud's agar medium. Among these cases, 32 (64%) were females and the remaining 18 (36%) were males (Tables2 &3). The age of Candida infected persons was ranging from 27 to 80 years. These results are nearly similar to those reported by Ang *et al* .(1993) who found that the incidence of candiduria was 6.5- 20% among hospitalized patients. Pfaller *et al*. (1991) reported that the frequency was 22.1% and is strongly associated with the presence of a urinary catheter. Slightly higher frequencies (25% and 28.7%) have been reported by Badawi *et al*.(2004) and Pakshir *et al*. (2004) respectively.

The higher prevalence of candiduria in females than in males confirms the work of Passos *et al*.(2005) who found that the percent of candiduria in females was 61.6% compared to 38.4% in males. More recently, Rashwan *et*

al.(2010) observed candiduria in 34.4% females versus 14.9% in males. A plausible explanation of this phenomenon is the presence of Candida in the genital tract of women complaining of vaginal candidiasis. This also confirms the work of Febre *et al.* (1999) who found five of eight patients with positive vaginal secretions and later showed the presence of the same yeast species in their urine.

Table 2. *Epidemiology of candiduria, characteristics of yeasts cultured onSDA, CMA and CA Candida media and identification after phenotyping and (*rDNA sequencing).*

Case No.	Age by years	Sex	Diabetes mellitus	Growth On SDA agar	Color on CA	Chlamydo-spores on CMA	Identification based on rDNA sequencing
1	67	Male	+	Budding cells & Hyphae	Pink	-	*Candida krusei* (Teleomorph: *Pichiakudriavzevii*)
2	75	Male	-	Budding cells & Hyphae	Blue	-	*C. tropicalis**
3	53	Female	-	Budding cells & Hyphae	Pink	-	*C. krusei** (Teleomorph: *Pichia kudriavzevii*)
4	35	Female	-	Budding cells & Hyphae	Green	+	*C. albicans**
5	49	Male	+	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)
6	54	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
7	44	Male	-	Budding cells	Green	+	*C. albicans*
8	45	Female	-	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)
9	55	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
10	57	Male	-	Budding cells	Pink	-	*C. glabrata**
11	70	Female	+	Budding cells	Green	+	*C. albicans*
12	68	Female	-	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)
13	70	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
14	65	Female	-	Budding cells	Pink	-	*C. glabrata**
15	45	Male	-	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)
16	75	Male	-	Budding cells & Hyphae	Green	+	*C. albicans*
17	60	Female	+	Budding yeast	Pink	-	*C. glabrata**
18	37	Female	-	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)
19	38	Male	-	Budding cells & Hyphae	Green	+	*C. albicans*

Case No.	Age by years	Sex	Diabetes mellitus	Growth On SDA agar	Color on CA	Chlamydo-spores on CMA	Identification based on rDNA sequencing
20	72	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
21	44	Female	+	Budding cells	Green	+	*C. albicans*
22	78	Male	+	Budding cells	Pink	-	*C. glabrata*
23	68	Female	-	Budding cells & Hyphae	Blue	-	*C. tropicalis*
24	27	Male	-	Budding cells	Green	+	*C. albicans*
25	80	Female	-	Budding cells	Green	+	*C. albicans*
26	42	Male	-	Budding cells & Hyphae	Blue	-	*C. tropicalis*
27	30	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
28	47	Female	-	Budding cells	Green	+	*C. albicans*
29	33	Female	+	Budding cells & Hyphae	Green	+	*C. albicans*
30	43	Male	-	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)
31	39	Female	-	Budding cells	Pink	-	*C. glabrata**
32	40	Male	-	Budding cells	Green	+	*C. albicans*
33	37	Male	-	Budding cells & Hyphae	Green	+	*C. albicans*
34	36	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
35	51	Male	+	Budding cells	Green	+	*C. albicans*
36	31	Female	-	Budding cells	Green	+	*C. albicans*
37	28	Male	-	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)
38	53	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
39	35	Female	-	Budding cells	Green	+	*C. albicans*
40	31	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
41	34	Male	+	Budding cells & Hyphae	Blue	-	*C. tropicalis*

Case No.	Age by years	Sex	Diabetes mellitus	Growth On SDA agar	Color on CA	Chlamydo-spores on CMA	Identification based on rDNA sequencing
42	50	Female	-	Budding cells	Pink	-	*C. glabrata**
43	54	Female	-	Budding cells	Pink	-	*C. glabrata*
44	43	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
45	34	Female	-	Budding cells & Hyphae	Green	+	*C. albicans*
46	37	Male	+	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)
47	55	Female	-	Budding cells	Green	+	*C. albicans*
48	75	Female	-	Budding cells	Pink	-	*C. glabrata**
59	77	Female	-	Budding cells	Green	+	*C. albicans*
50	32	Female	-	Budding cells & Hyphae	Pink	-	*C. krusei* (Tel: *Pichiakudriavzevii*)

3.2. Risk Factors for Candiduria

All patients investigated in this study were urinary catheterized (Table 3).According to Klotz and Smith (1990) yeasts are able to adhere to the catheter, allowing colonization in this device. Passos *et al*.(2005) showed that in92.6% cases of candiduria the patients had a urinary catheter. Similar results have also been given by Kobayashi *et al*. (2004) who verified that 84.4% of the patients with candiduria had a catheter. Álvarez-Lerma *et al*. (2003) mentioned that although infection by yeasts in patients with indwelling urinary catheter has not been well defined till now, use of invasive procedures has been reported as an important factor for development of Candida infection.

Table 3.*Summary of cases of candiduria and germ tube test of Candida strains.*

Criteria	Number (%)
Gender	Total = 50
Males	18 (36%)
Females	32 (64%)
Cathetarization	50(100%)
Diabetes mellitus	10 (20%)
Males	6 (12%)
Females	4 (8%)
Germ tube test	Total = 50
Positive	27 (54%) *Candida albicans* strains
Negative	23 (46%) Non-*Candida albicans* strains

Results of the present study (Tables 2 & 3) showed also that 10 (20%) of *Candida* infected patients were diabetic with males outnumbering females (6 versus 4 cases). Previous reports showed higher prevalence of candiduria (36.7%) in diabetes mellitus patients (Behiry *et al*.,2010). During their study of risk factors for nosocomial candiduria, Guler *et al*. (2006) showed that candiduria was increased by 12-fold after urinary catheterization, 6-fold each after use of broad spectrum antibiotics and urinary tract abnormalities, 4-fold following abdominal surgery, 2-fold in the presence of diabetis mellitus and one-fold in association with corticosteroid administration.

3.3. Phenotypic Characteristics of CandidaIsolates

Preliminary identification of the different *Candida* isolates was successfully done using conventional phenotypic methods which included growth on (SDA), germ tube test, formation of pseudohyphae, chlamydospore production and colony colour on chromogenic media (Table 2).

a.Growth on Sabauroud's dextrose agar (SDA):

All *Candida* isolates showed good growth on (SDA). Colonies were white to cream in colour, smooth, glabrous and yeast- like in appearance. Microscopic morphology showed spherical to subspherical budding yeast cells with several isolates producing pseudohyphae. Although simple and inexpensive, these criteria are not enough for identification for *Candida* species.

b. Growth on corn meal tween 80 agar (CMA):

All cultures of Candida grew well on this medium but only 27/50 (54%) were able to produce chlamydospores suggestive of *C. albicans* (see table 2).

c. Germ tube formation by *Candida* isolates:

In the present work germ-tube production was observed in 27/50 (54%) of *Candida* strains, which were identified

as *C. albicans* whereas the remaining strains 46% failed to produce germ-tubes, being identified as non-*Candida albicans* (Table 2 and Fig. 2). This ratio is markedly lower than that reported by Kangogo *et al*.(2011), who found 112/130 (86%) of *Candida spp*. produced germ-tubes and were identified as *Candida albicans* and 18/130 (13.9%) were identified as non-*Candida albicans*. The germ-tube production test has the advantage to be simple and efficient in the economical and fast identification of *C. albicans* (Fisher and Cook,1998 and Lacaz *et al.,*2002). Some authors evaluated sensitivity and specificity of the germ-tube test, finding results between 93 and 98.8%, and between 73.3 and 100%, respectively (Cambell *et al*.,1998; Conceicao *et al.*,2005 and Gatica *et al*.,2002).

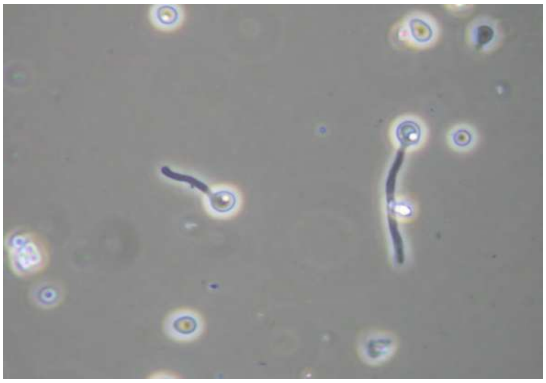

Figure 2.*Germ tube of C. albicans (magnification x 400) by phase contrast microscope.*

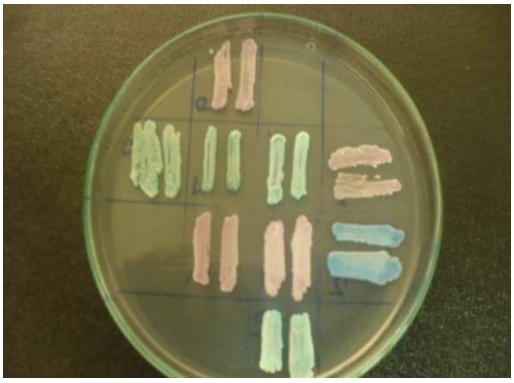

Figure 3. *Colony colors on CHROMagar Candida medium. Light green: C. albicans, Dark blue : C. tropicalis, pink: C. glabrata and C. krusei.*

d. Growth on Chromagar Candida (CA) medium:

Results of the present study (Table 3) revealed that (27/50) 54% of *Candida* isolates yielded several shades of green colonies on CA suggestive of *C. albicans*. Only (20/50) 40% of isolates developed a pink color suggestive of *C. krusei* or *C. glabrata*. Discrimination of these two species was possible on the basis that *C. krusei* can form both budding cells and pseudohyphae whereas *C. glabrata* is not able to produce peudohyphae (Ellis et al., 2007). Accordingly *C. krusei* and *C. glabrata* were represented by 24% and 16% of total Candida is in renal failure patients. The present data showed also that 6% of *Candida* isolates developed a distinctive dark blue color on CA typical of *C. tropicalis.* Chromogenic media have the advantage of rapid identification of *Candida* species, technically simple preparation (by boiling), rapid and cost effective compared to technically demanding time consuming and expensive conventional method (Vijaya *et al*., 2011).There are several reports confirming the high prevalence of *C. albicans* in urine samples (Weinberger *et al*., 2003). Among all fungi isolated from the urine, 40-65% was found to be *C. albicans* (Vincent *et al*., 1998). In their study performed with 751 patients with candiduria, Kauffman *et al*.(2000) found that *C. albicans* was present in 51.8% and *C. glabrata* in 15.6% of the patients. Weinberger *et al*. (2003) isolated *C. albicans* in 56.4%, *C. tropicalis* in19%, *C. glabrata* in 15.7%, *C. parapsilosis* is in 6.1% and *C. krusei* in1.8% of the cases. Although the prevalence of *C. albicans* is higher, the proportion of non- Candida fungi involved in urinary pathogenesis increases over the course of time (Vincent *et al.,* 1998). Orovcova *et al*. (1996) observed *C. albicans* in 72% of the cases and non- *Candida albicans* pathogens in 28%, in their series of 50 patients. More recently, Kangogo *et al*. (2011) recorded that out of the 150 isolates130/150 (86.7%) yielded several shades of green colonies and identified as *C. albicans*. Only 4/150 (2.7%) of isolates produced pink colonies on CA typical of *C.krusei* and also 2.7% of isolates developed a dark blue colour typical of *C. tropicalis*. In Egypt, Girgis *et al*. (2009) found that the average counts of *Candida* spp. isolated from urine samples were 21.14, 14.14, 15.14 and 8.35 CFU/ml for *C. albicans*, *C. glabrata, C. tropicalis* and *C. parapsilosis*, respectively during the sampling period. The minimum counts were 18, 11, 11 and 5 CFU/ml for *Candida albicans*, *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis*, respectively. While the maximum counts were 24, 17, 19 and 12 CFU/ml for these species respectively. The highest counts of isolated *Candida spp*. were *C. albicans* in urine samples, while the lowest counts were *C. parapsilosis* during the sampling months. In New York, USA, Jain *et al*. (2007) obtained 67 *Candida* isolates from urine samples from 55 patients. The species distribution was *C. albicans* (54%), *C. glabrata* (36%), and *C. tropicalis* (10%).

3.4. Molecular Characterization of Candida Isolates

Ten representative isolates of *C. albicans*, *C. tropicalis* (one isolate for each), *C. krusei* (5) and *C. glabrata* (3) were chosen for rRNA gene sequencing. After sequence alignment and establishment of the phylogenetic tree, identification of these isolates was confirmed especially when compared with the type strains accessed from the Gene bank (Fig.2).The phylogenetic tree showed four well defined clades clearly distinguishing the four different species of *Candida*. The first is the *C. tropicalis* clade which comprises strain assigned AUMC 9236 recovered in the present work from urine compared to other closely related strains from different sources in the world. The second clade comprised *C. albicans*(AUMC 9239) compared to the type strain of the same species (NRRL Y-12983T) and others. Clade 3 included *Pichia kudriavzevii* which is the teleo-

morphic (ascosporic) state of *C. krusei.*

Yeasts of the genus *Pichia* are widely distributed; they can be found in natural habitats, such as soil, freshwater, tree exudates, insects, plants and fruits, and also as contaminants in a variety of foods and beverages. Some *Pichia* species contribute desired effects in the early stages of wine fermentation, several types of brines, and different types of cheeses; while others have been described as human pathogens (Bakir *et al*., 2003; Otag *et al*., 2005).

The fourth clade in Fig. 2 is the *C. glabrata* which comprised the type strain of this species (ATCC 2001T= NRRL Y-65T).

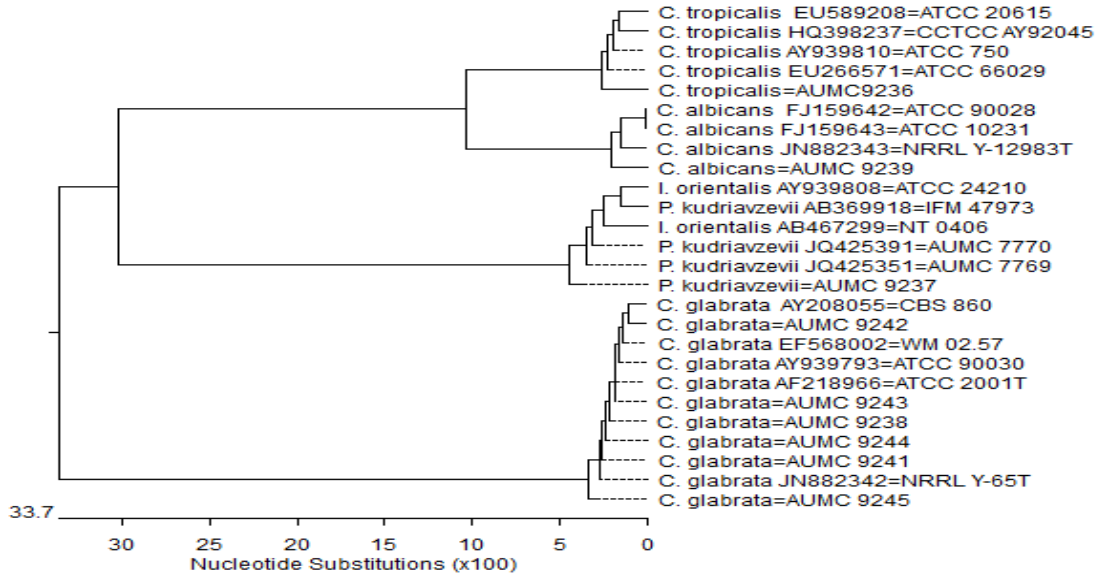

Fig 4. *Phylogenetic tree for fungal species isolated from urine sample of renal failure patientsin Egypt (given AUMC Numbers), C= Candida, I = Issatchenkia, and P= Pichia. Yeast species from Gene bank are given accession Numbers (e.g. EU589208, etc.)The scale indicates the number of nucleotide substitutions per site. Reference type strains (T) of corresponding fungi are involved in the tree (given CBS, ATCC, CCTCC, NRRL, IFM, NT, and WM numbers.*

It is worthy to mention that the genotypic identification based on rRNA gene sequencing showed close agreement with phenotypic identification for all *Candida* species studied in this investigation. However, molecular techniques have the advantages of relatively processing time and high sensitivity and specificity. The amplification feature of the PCR assay make it ideal for detecting low yeast levels from minimal volume of clinical samples. DNA based diagnosis tests have also the potential to decrease the time taken for the laboratory identification of pathogens that are growing slowly or difficult to culture (Khlif *et al*.,2007). Moreover, rapid identification of the fungal pathogens such as *Candida s*pecies may help to reduce the hospital stay and high overall costs associated with management of *Candida* infections and is also of great value in epidemiological studies.

3.5. Sensitivity of Candida Isolates to Antifungal Therapeutic Agents

The data presented in table 3 and Fig. 5 revealed that Nystatin was the most active drug against the majority of *Candia* strains tested (36/37 strains). Also high proportion of *Candida* strains was sensitive to Clotrimazole, Amphoterici-B and Tioconazole (28, 26, and 20 strains respectively). On the other hand the highest number of resistant Candida strains was observed with Sertaconazole (29/37), followed in a descending order by Itraconazole (21), Fluconazole (19), Tioconazole (15) and Clotrimazole (9). Resistance to Nystatin and Amphotericin-B was only exhibited by 1 and 2 strains respectively. All isolates of *C. glabrata* (9 tested isolates) were completely resistant to Fluconazole and Sertaconazole. Among the 7 isolates of *C. krusei* only two were susceptible to Fluconazole. These findings are nearly similar to those reported by Nawrat *et al*. (2000) who found Amphotericin-B effective against 100% of *Candida* isolates. The same authors noticed also that 72.1% of *Candida* strains were sensitive to Ketoconazole, 61.4% to Fluconazole and 47.1% to Itraconazole. The study of Kronvall and Karlsson (2001) showed that Most *Candida* strains were susceptible to Fluconazole with the exception of *C. glabrata* and *C. Krusei*. Working with 270 isolates of *Candida*, Ellis (2011b) showed that all isolates of *C. albicans* were susceptible to Fluconazole and Clotrimazole. Resitance to Fluconazole was manifested by 20% of *C. glabrata* isolates. As suggested by Bukhary (2008), Fluconazole is the drug of choice if the organism isolated is not *C. glabrata* or *C. krusei*. Oral fluconazole has a more delayed but more lasting effect on candiduria than Amphotericin-B bladder irrigation. Patients receiving Amphotericin-B bladder irrigation had higher rates of eradication two days after the beginning of therapy than those receiving oral Fluconazole but the cure rates were similar one month after the beginning of therapy. According to Sobel *et al*. (2000), treatment

with Fluconazole , 200mg/day, for 7 – 14 days or with intravenous Amphotericin-B deoxycholate at doses 0.3 - 1.0 mg/kg/day for 1 – 7 days has been successful based on moderate evidence from a randomised clinical trial.

In conclusion, more work is still needed to cover more hospitals and a broader spectrum of patients complaining of candiduria. Discovery of new antifungal agents effective against Candida strains resistant to the currently used antifungal is a must.

Table 4.*Sensitivity of Candida species to antifungalagents (Zone of activity in mm expressed as Sensitive (S), Intermediate (I) and Resistant (R).*

Antifungal agents / Strain No. and Name		AM-B (100U)	CLO (10µg)	FLU (10µg)	ITR (10µg)	KET (10µg)	NYS (100U)	SER (10µg)	TIO (10µg)
9206	*C. krusei*	16 S	22 S	23 S	15 I	00 R	23 S	7 R	00 R
9207	*C. albicans*	10 I	22 S	27 S	13 R	20 I	25 S	12 I	27 S
9208	*C. albicans*	22 S	24 S	34 S	17 I	23 I	28 S	00 R	00 R
9209	*C. krusei*	15 S	25 S	15 I	12 R	00 R	20 S	00 R	20 S
9210	*C. albicans*	10 I	7 R	00 R	00 R	00 R	24 S	00 R	10 R
9211	*C. glabrata*	18 S	00 R	00 R	16 I	25 I	28 S	00 R	00 R
9213	*C. krusei*	13 I	00 R	00 R	00 R	00 R	25 S	10 R	00 R
9214	*C. albicans*	11 I	27 S	27 S	15 I	22 I	25 S	00 R	23 S
9215	*C. glabrata*	16 S	00 S	00 R	00 R	00 R	35 S	11R	00 R
9217	*C. albicans*	25 S	00 S	28 S	00 R	22 I	28 S	11 R	00 R
9218	*C. glabrata*	17 S	00 S	00 R	00 R	00 R	32 S	00 R	00 R
9219	*C. krusei*	24 S	27 S	27 S	20 I	32 S	30 S	15 I	27 S
9220	*C. albicans*	24 S	24 S	23 S	10 R	17 R	38 S	00 R	18 I
9221	*C. albicans*	22 S	26 S	25 S	16 I	26 I	32 S	00 R	31 S
9222	*C. albicans*	8 R	00 R	00 R	00 R	00 R	19 S	00 R	00 R
9223	*C. glabrata*	13 I	21 S	00 R	11 R	22 I	25 S	00 R	10 R
9224	*C. tropicalis*	25 S	27 S	00 R	15 I	26 I	32 S	11 R	27 S
9225	*C. albicans*	22 S	24 S	00 R	17 I	00 R	26 S	12 I	30 S
9226	*C. albicans*	22 S	26 S	26 S	15 I	00 R	37 S	00 R	20 S
9227	*C. tropicalis*	21 S	26 S	00 R	16 I	00 R	37 S	7 R	25 S
9229	*C. albicans*	23 S	30 S	27 S	13 R	24 I	34 S	9 R	28 S
9230	*C. albicans*	18 S	23 S	26 S	12 R	20 R	23 S	10 R	26 S
9231	*C. krusei*	23 S	26 S	00 R	16 I	00 R	26 S	11 R	27 S
9232	*C. glabrata*	22 S	25 S	00 R	16 I	25 I	35 S	10 R	30 S
9233	*C. albicans*	23 S	28 S	00 R	15 I	00 R	35 S	7 R	25 S
9234	*C. albicans*	13 I	23 S	32 S	11 R	24 I	25 S	00 R	23 S
9235	*C. albicans*	20 S	29 S	00 R	15 I	25 I	25 S	17 I	28 S
9236	*C. tropicalis*	13 I	19 S	20 S	13 R	22 I	25 S	00 R	21 S
9237	*C. krusei*	10 I	25 S	12 R	11 R	13 R	25 S	16 I	13 I
9239	*C. albicans*	15 S	25 S	22 S	12 R	17 R	25 S	21 I	23 S
9241	*C. glabrata*	10 I	00 R	00 R	00 R	21 I	20 S	00 R	00 R
9242	*C. glabrata*	22 S	00 R	00 R	00 R	00 R	10 R	00 R	00 R
9244	*C. glabrata*	15 S	00 R	00 R	00 R	00 R	27 S	00 R	00 R
9245	*C. glabrata*	16 S	00 R	00 R	00 R	00 R	27 S	00 R	00 R
9246	*C. albicans*	20 S	21 S	30 S	13 R	00 R	25 S	00 R	27 S
9247	*C. krusei*	9 R	20 S	15 I	00 R	11 R	21 S	10 R	10 R
9248	*C. albicans*	17 S	00 R	00 R	14 I	25 I	22 S	21 I	35 S

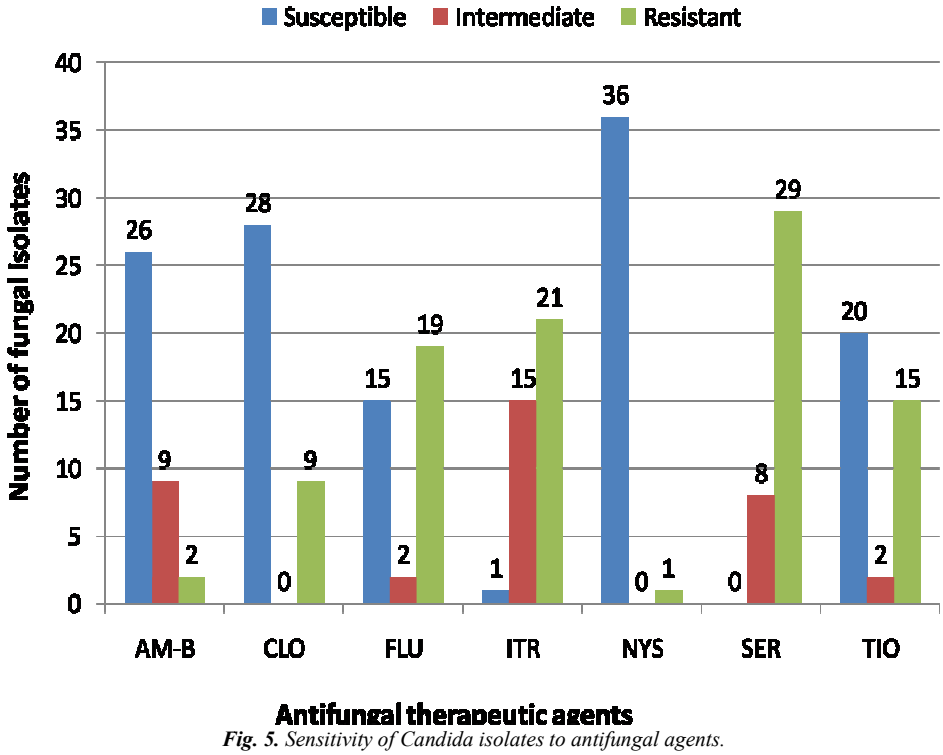

Fig. 5. *Sensitivity of Candida isolates to antifungal agents.*

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