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Prevalence and *in vitro* Antifungal Susceptibility Pattern of *Candida* species in a Tertiary Care Hospital, Rawalpindi, Pakistan

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Abstract.- *Candida* is an opportunistic pathogen commonly found in hospital settings. During recent years increase in the non albican species of *Candida* and their resistance against treatment of choice has been observed. The aim of this research was to study the prevalence and antifungal susceptibility pattern of *Candida* species from different hospital units. A total of 303 samples were included in this study. *Candida* species, initially identified by colony morphology, were further characterized by germ tube test, morphology on cornmeal as well as Chromagar and sugar assimilation profiles. Antifungal susceptibility pattern was checked by disc diffusion method (CLSI M44-A2). Five species of *Candida i.e., C. albicans, C. tropicalis, C. glabrata, C. parapsilosis* and *C. krusei* were isolated. *Candida albicans* was dominant (55.5%) over non albican species (45.5%). Candidiasis was more common in age group of 21-40 years and most of the isolates were reported from urine. Drug resistance was more common in non-albican species of *Candida.* Overall sensitivity rate of amphotericin B, fluconazole and caspofungin was 100%, 87.4% and 97.6%, respectively. The varied susceptibility of different *Candida* species proposes that proper identification and antifungal susceptibility testing must be done for efficacious treatment. Amphotericin still remains the drug of choice for candidiasis.

Key words: Candidiasis, non albicans *Candida*, chromagar, amphotericin B, fluconazole, caspofungin.

INTRODUCTION

It is becoming progressively more understandable that during the last few decades, development in modern health facilities has resulted in an increase in the incidence of fungal infections especially in hospitalized patients, globally (Jain *et al*., 2010). These infections prolong hospital stays and increase the financial burden on the patients (Schelenz, 2008). Almost 70% of all fungal infections in the hospital environment are due to yeast of genus *Candida* (Azie *et al*., 2012). Candidiasis (infections caused by *Candida*) has become a serious health problem owing to its high rate of morbidity and mortality (Almeida *et al*., 2013). *Candida* species colonize the mucosal surfaces *i.e*., mouth, urethra and gastrointestinal lining of healthy individuals (Jain *et al*., 2010). These commensals become pathogens because of any change in the host defense system (Colombo and Guimaraes, 2003). A number of risk factors such as invasive procedures, total parenteral nutrition, chemotherapy, and hospital environment are the major causes for the development of candidiasis in hospitalized patients (Schelenz, 2008). *Candida* species can cause infection exogenously from the hospital environment or hands of health care workers or endogenously *i.e.*, proliferation of microflora (Colombo and Guimaraes, 2003).

There are approximately 200 hundred species of *Candida* among which *C. albicans, glabrata, tropicalis, stellatoidea, parapsilosis, catemilata, ciferri, guilliermondii, haemulonii, kefyr* and *krusei* are most common in clinical infections (Pam *et al*., 2012). Mostly candidiasis is caused by five common species, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* (Pfaller *et al*., 2007). Common drugs used against candidiasis are amphotericin B and fluconazole belonging to polyenes and azoles groups of antifungal agents, respectively (Ashley *et al*., 2006). *Candida* species show different antifungal susceptibility pattern against the same drug. Some non-albicans species of *Candida, i.e., C. krusei* and *C. glabrata* show complete resistance and reduced susceptibility to fluconazole, respectively (Chander, 2009). This emphasizes that the correct identification and antifungal susceptibility of *Candida* species is

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essential for the selection of appropriate antifungal agent for successful therapy (Jain *et al*., 2010; Almeida *et al*., 2013).

In Pakistan candidiasis is studied in neonate's population (Farooqi *et al*., 2013; Arif *et al*., 2011) Extensive literature survey shows no study is conducted on the prevalence of *Candida* sp in a tertiary care center including all wards and sample specimen. The aim of the present research was to determine the prevalence and antifungal susceptibility of various *Candida* species in Holy Family Hospital, Rawalpindi, Pakistan.

MATERIALS AND METHODS

The current study was carried out from January 2010 to December 2012, by the joint collaboration of Department of Microbiology, Quaid-e-Azam University, Islamabad and Department of Pathology, Holy Family Hospital, Rawalpindi, Pakistan. Holy Family Hospital is a tertiary care hospital with facility 850 bed strength.

Sampling

A total of 303 *Candida* isolates were collected from different wards of the hospital. All clinical specimens were collected aseptically under strict sample collection standards for microbiological specimens.

Media, reagents and materials

The culture media Sabouraud's dextrose agar (SDA), MacConkey agar and Cysteine Lactose Electrolytes Deficient (CLED) agar, Mueller Hinton and Chromagar were supplied by Oxoid Chemicals, Basingstoke, UK. Cornmeal agar was purchased from Becton, Dickinson and Co, Sparks, MD, USA. API 20C AUX were supplied by bioMerieux™ (France). All the other chemicals and reagents were commercial products of highest grade available.

Culturing

The specimens were inoculated on SDA, MacConkey's and CLED agar, and were incubated at 37^{o}C for 48 hrs. The cultures having colony morphology of dry, white to creamy color, pin point, opaque and fluffy growth on all above mentioned media were identified as *Candida* species (Chander, 2009; Fisher and Cook, 1998).

Identification

Simple gram staining was done for screening of yeast like cells and to confirm the purity of the culture. The isolates suspected as *Candida* on the basis of colony morphology on above mentioned media were examined in 10 % KOH. Speciation of *Candida* isolates was done by the germ tube test, morphology on corn meal agar and Chromagar. API 20 C AUX test results along with hypha formation on corn meal agar were noted. Final identities of the isolates were achieved by entering the 7-digit numerical profile into the API database Version 4.0 (Ng *et al*., 2000).

Antifungal susceptibility testing

Disc diffusion method was performed to check the antifungal susceptibility trend of *Candida* species by following the approved guidelines of CLSI M44-A2 (CLSI 2008). Antifungal discs of amphotericin (10µg), fluconazole (25µg) and caspofungin (5µg) were purchased from LiofilChem (Italy). The Mueller Hinton agar (Oxoid, UK) supplemented with glucose (2%) and methylene blue (0.5µg/ml) was used for antifungal susceptibility testing as recommended by CLSI M-44A. The inoculated plates were incubated at 35^{o}C for 48 hours. Zone of inhibition diameters measured after 48 hours, were interpreted using approved CLSI guidelines (2008) and the isolates were classified into resistant, intermediate and sensitive.

Statistical analysis

The data was analyzed by SPSS VER.20. p value was calculated for statistical significance of results. p value < 0.05 was considered as significant.

RESULTS

A total of 303 *Candida* isolates were collected from the Burn unit (BU), Medical Unit (MU), Medical Intensive Care unit (MICU), Gynecology (GYN), Pediatric (PD) and Surgical Unit (SU). The clinical *Candida* strains were isolated from urine, high vaginal swabs (HVS), sputum, pus swabs, catheter tips, body fluids and stool samples. These isolates were identified by conventional phenotypic methods.

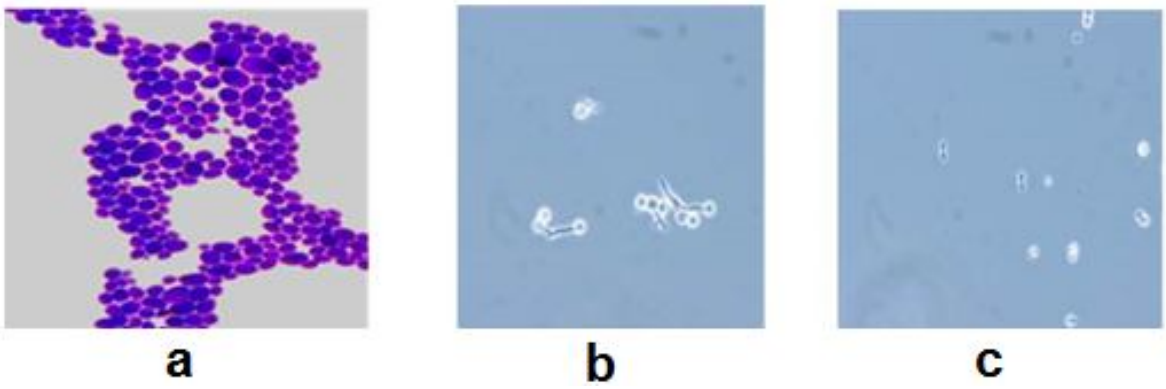

Fig. 1. Microscopy of *Candida* species (a) Gram's staining (b) Germ tube positive showing lateral tubes from *Candida* cells (c) Germ tube negative showing budding yeast cells.

Identification

The *Candida* strains demonstrated budding in 10% KOH and stained purple on Gram's staining (Fig. 1a). Germ tube test results showed that all *C. albicans* were germ tube positive (Fig. 1b). On Chromagar, the strains of *C. albicans* and *C. tropicalis* formed green and blue colored colonies, respectively (Fig. 2). *C. krusei* produced shades of pink color after 48-72 h of incubation and the colonies were dry, flat with white edges. *C. glabrata* formed brown small colonies while *C. parapsilosis* colonies were of white natural color. On corn meal agar germ tube positive isolates formed chlamydospores (Fig. 3), while *C. tropicalis*, *C. krusei* and *C. parapsilosis* formed pseudohyphae. Strains of *C. glabrata* did not produce any hyphae or pseudohyphae. The API tests identified the *Candida* species on the basis of fermentation and utilization of different sugars. The affirmative test by API yielded five species of *Candida* in total *i.e.*, *C. albicans, C. tropicalis, C. glabrata, C. krusei* and *C. parapsilosis*. Overall chromagar identified 92% (n=279) of strains correctly while API 20 C AUX identified all strains correctly with more than 90 % discrimination.

Distribution of Candida *spp. according to patient's gender and age*

Nosocomial candidiasis was more frequent in female patients (56%) as compared to male patients (44%) as shown in Table I. The majority of the patients (42%) fall in the age group of 21-40 years (Fig. 4).

Distribution of Candida species in different hospital wards and clinical specimens

Most of the *Candida* spp. were isolated from the general Medical Unit (MU) 52%, Medical Intensive Care Unit 18%, Gynecology ward 17.5%, Surgical ward 5%, Pediatric ward 4.5% and Burn Unit 3% (Fig. 5). *Candida* spp. were mostly isolated from urine samples 38.6%, HVS 18.3%, sputum 13.5%, catheter tips 12.5%, pus 9.2%, wounds 5.2%, body fluids 2% (n=6) and stool samples 0.7% (Table I).

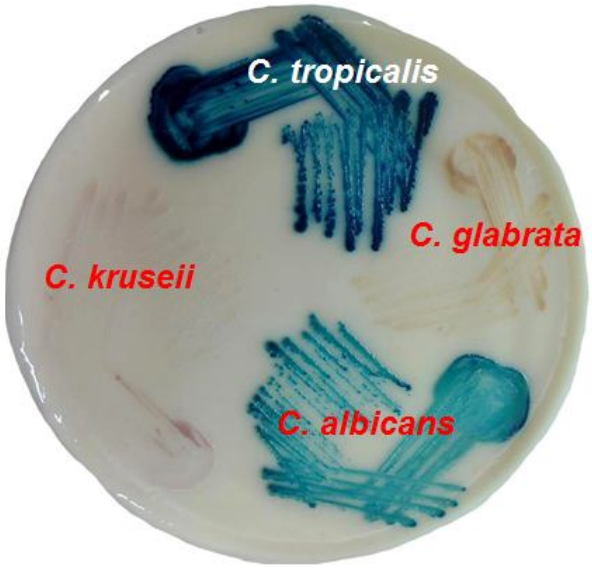

Fig. 2. Growth of different *Candida* species on Chromagar plate.

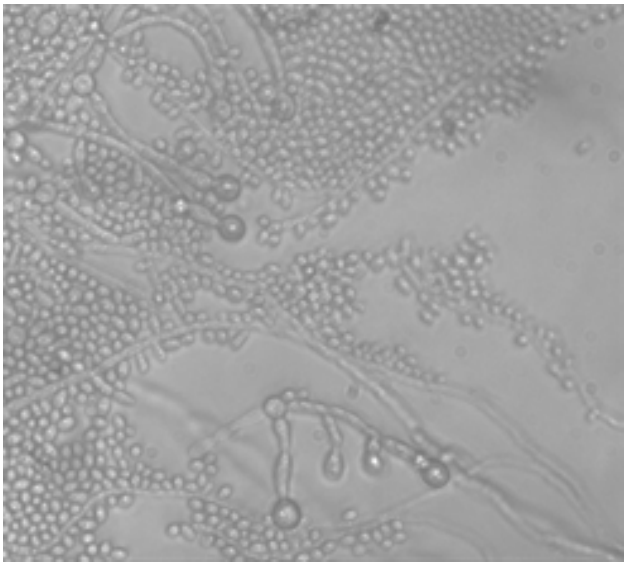

Fig. 3. Chlamydospores production by *C. albicans* on corn meal agar plate.

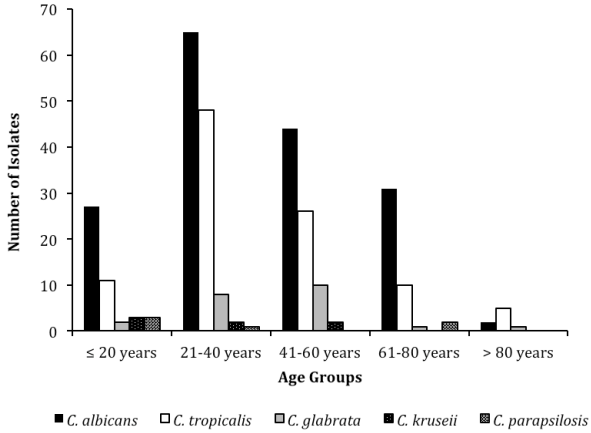

Fig. 4. Prevalence of *Candida* species in different age groups.

Species-wise distribution of Candida

The results of this study showed that *C. albicans* was the most abundant species (n=168, 55.5%) isolated from clinical samples. While among the non-albicans species, *C. tropicalis* (n=99, 32.6%) was the most frequently reported followed by *C. glabrata* (7.6%), *C. krusei* (2.3%) and *C. parapsilosis* (2%). *C. glabrata* was not found in wounds, body fluids and stool samples, whereas *C. parapsilosis* was only isolated from urine samples and catheter tips samples (Table I).

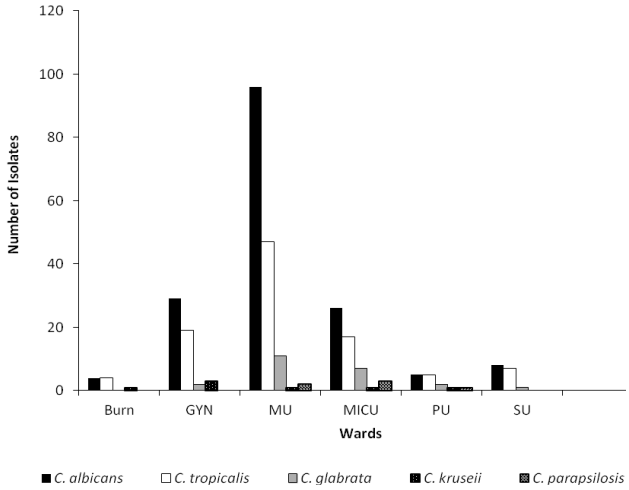

Fig. 5. Distribution of *Candida* species in different wards. (GYN, Gynecology; MU, Medical unit; MICU, Medical intensive care unit; PU, Pediatric care unit; SU, Surgical unit).

Antifungal susceptibility testing

All the species of *Candida* were sensitive to amphotericin B as shown in Table II. Resistance rate of fluconazole was 1.2%, 8.1%, 17.3% and 100% for *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei*, respectively. Resistance to fluconazole was not seen in *C. parapsilosis* as shown in Table II. Resistance to caspofungin was very low. Resistance to caspofungin was only observed in *C. tropicalis* (2.1%) and *C. glabrata* (22%). Overall the sensitivity rate of amphotericin, fluconazole and caspofungin was 100%, 87.4% and 97.6%, respectively (Table II). *C. tropicalis* and *C. glabrata* exhibited higher resistance to fluconazole as compared to *C. albicans*, though *C. tropicalis* was more resistant towards fluconazole than caspofungin (*p* value 0.06). *C. glabrata* showed significantly higher resistance (*p* value <0.00005) to caspofungin as compared to all other isolated species. Resistance to fluconazole was significantly higher ($p < 0.001$) in non albicans *Candida* (NAC) than *C. albicans*. Similarly caspofungin also exhibited significant resistance ($p < 0.0253$) towards NAC as compared with *C. albicans* (Table III).

DISCUSSION

Fungal infections have always been a problem for hospitalized patients but during the last

Table I.- Distribution of different *Candida* species in different specimens.

Gender	*Candida* spp. in Different Specimens [n (%)]								
	Urine	**HVS**	**Sputum**	**Tip**	**Pus**	**Wound**	**Fluids**	**Stool**	**Total**
Male	69(23)	-	22(7.2)	14(4.6)	15(4.9)	9(2.9)	1(0.3)	2(0.7)	132(44)
Female	48(15.8)	55(18.3)	19(6.3)	24(7.9)	13(4.3)	7(2.3%)	2(0.7)	-	171(56)
Species									
C. albicans	55(18.1)	30(9.9)	31(10.3)	21 (6.9)	17(5.6)	7(2.3)	5(1.7)	2(0.7)	168(55.5)
C. tropicalis	39(12.9)	20(6.7)	9(2.9)	14(4.6)	8(2.6)	8(2.6)	1(0.3)	-	99(32.6)
C. glabrata	15(4.9)	2(0.7)	1(0.3)	2(0.7)	3(1%)	-	-	-	23(7.6)
C. krusei	3(1)	3(1)	-	-	-	1(0.3)	-	-	7(2.3)
C. parapsilosis	5(1.7)	-	-	1(0.3)	-	-	-	-	6(2)
Candida spp.	117(38.6)	55(18.3)	41(13.5)	38(12.5)	28(9.2)	16(5.2)	6(2)	2(0.7)	303(100)

Table II.- Results of antifungal susceptibility profile of *Candida* species.

Different species of *Candida*	Antifungal drugs [n (%)]						
	Amphotericin (10µg)		**Fluconazole (25µg)**			**Caspofungin (5µg)**	
	S >15 mm.	**R (NZ)**	**S ≥19mm**	**SDD 18-15mm**	**R ≤14mm**	**S ≥11mm**	**R ≤10mm**
Candida albicans	168 (100)	-	163 (97)	3 (1.8)	2 (1.2)	168 (100)	-
Candida tropicalis	99 (100)	-	80 (80.8)	11 (11.1)	8 (8.1)	97 (97.9)	2 (2.1)
Candida glabrata	23 (100)	-	16 (69.7)	3 (13)	4 (17.3)	18 (78)	5 (22)
Candida krusei	7 (100)	-	-	-	7 (100)	7 (100)	-
Candida parapsilosis	6 (100)	-	6 (100)	-	-	6 (100)	-
Total	303 (100)		265 (87.4)	17 (5.6)	21 (7)	296 (97.6)	7 (2.4)

S, sensitive; SDD, sensitive dose dependent; R, resistant; NZ, no zone.

Table III.- Comparison of antifungal susceptibility profile between *C. albicans* and non-albicans *Candida*.

Antifungal drugs	Fluconazole (25µg)		Caspofungin (5µg)	
	S (%) ≥19mm	**R (%) ≤14mm**	**S (%) ≥11mm**	**R (%) ≤10mm**
Candida albicans	98.8	1.2	100	0
Non-albicans *Candida*	86	14	95	5
p value	0.34	0.001	0.720	0.025

thirty years fungal infections due to *Candida* spp., especially those that are caused by non-albicans species, increased enormously (Colombo and Guimaraes, 2003). The proper and prompt diagnosis of this pathogenic yeast is important for its effective treatment (Nadeem *et al*., 2010). Gram staining and germ tube test were found easy and reliable techniques for the identification of *Candida* spp. Chromagar correctly identified more than 92% of *Candida* strains which is in consistence with the previous study done by Ozcan (Ozcan *et al*., 2010). API tests and cornmeal agar were found to be best conventional methods for the identification of all *Candida* species but however in our present settings of resources constrained laboratories it cannot be used routinely. Identification by chromagar is cost effective, rapid and easy method as API 20 C AUX test is laborious and expensive (US$20=2000PKR) and socio economic status of local patients of Pakistan makes it difficult to use this test on regular basis (Nadeem *et al*., 2010).

According to our results, the *Candida* infection rate was higher in females (56%) as compared to males (44%), which is in accordance with findings of Almeida *et al.* (2013) and Roy *et al.* (2013). Even though candidiasis can occur at any age, our study showed that the isolation of *Candida* was largely from the age group of 21-40 years. In

comparison some studies of the region, Patel *et al*. (2012) and Roy *et al*. (2013) also reported the highest incidence of *Candida* spp. infection in the same age group. In our study, we reported maximum number of *Candida* spp. from urine samples. *Candida* spp. are reported as seventh most common nosocomial pathogen in hospital settings causing 25% of all the urinary tract infections in some of previous studies (Roy *et al*., 2013; Patel *et al*., 2012; Pfaller *et al*., 1996). Almeida *et al*. (2013) had also reported most of *Candida* spp. from urine samples in his study (62%).

This study reported the prevalence of five species *i.e.*, *C. albicans, C. tropicalis, C glabrata, C. krusei* and *C. parapsilosis. C. albicans* was dominant over the non albicans species of *Candida* (54.5%). Results of our study are consistent with that of Ariff *et al*. (2011) reporting that *C. albicans* was the most prevalent among all the species, followed by *C. tropicalis* and *C. glabrata*. Resende *et al*. (2002) reported similar results about the *Candida* spp. distribution in hospitalized patients *i.e.*, with *Candida albicans* (51%) the most common species followed by *C. tropicalis* (33%), *C. parapsilosis* (8%), *C. glabrata* (5%), *C. krusei* (2%) and *C. guilliermondii* (1%). Non-albican *Candida* species were also isolated in a significant number (45.5%). An equal percentage of *C. albicans* and *C. tropicalis* were reported in patients from Pediatrics, Burn and Surgical Units. These findings suggest that prevalence of non-albicans species is increasing. The result shows that among non-albicans species, *C. tropicalis* was more frequent which is in accordance with the study of Agarwal *et al*. (2004). These were followed by *C. glabrata* among the non-*albicans* species. *C. krusei* and *C. parapsilosis* were the least reported *Candida* spp. These results are in agreement with the results of a recently study done in Taiwan who reported that the *C. albicans* (39%) is the most prevalent species, followed by *C. tropicalis* (24%), *C. glabrata* (23.8%), *C. parapsilosis* (8%) and *C. krusei* (1.7%) and other species (2.6%), respectively (Yang *et al*., 2013).

Our results of antifungal sensitivity showed that amphotericin was sensitive against all species of *Candida*. This is in consistence with a study in India (Bhaumik *et al*., 2012). Significantly higher resistance was observed in NAC as compared with *C. albicans* against antifungal drugs like other studies in the world (Almeida *et al*., 2013; Yang *et al*., 2008). Our results of antifungal sensitivity showed that *C. albicans* was highly susceptible to fluconazole with a resistance rate of only 1.2%. Lyon *et al*. (2010) also observed similar rate of resistance against fluconazole in *C. albicans*. We observed that resistance to fluconazole was high in non-albicans species of *Candida*. 8 out of 99 (8.1%) strains of *C. tropicalis* showed resistance against fluconazole. Yang *et al*. (2008) also observed an increased resistance to fluconazole in the species of *C. tropicalis* and similar to our results he found no resistance in *C. parapsilosis* strains. It was observed that *C. glabrata* showed high resistance to fluconazole (17.3%) whereas *C. krusei* strains showed complete resistance to it. Another study in Pakistan also reported complete resistance to fluconazole in species of *C. krusei* and high resistance in *C. glabrata* (Farooqi *et al*., 2013). The study showed that high resistance was common in NAC as compared to *C. albicans*.

Resistance to caspofungin was very rare in the isolated species of *Candida*. Strains of *C. albicans*, *C. parapsilosis* and *C. krusei* showed no resistance to caspofungin. This result is in agreement with the study of Pfaller *et al*. (2010). Strains of *C. tropicalis* were highly susceptible to caspofungin with only 2 strains showing resistance to caspofungin. A study in Malaysia also confirms resistance against caspofungin in *C. tropicalis* (Hamid *et al*., 2012). Resistance to caspofungin was also witnessed in some strains of *C. glabrata*. Increased resistance to caspofungin among species of *C. glabrata* has also been observed by SENTRY Antimicrobial Surveillance Program (2008–2009) (Pfaller *et al*., 2010).

CONCLUSIONS

It is concluded from our study, that Candidiasis is increasing with a fast rate among nosocomial infections, with high exposure to females and people in the age group of 21-40 years. The conventional method such as Chromagar is an easy technique, as it readily identifies the fluconazole resistant species. The most accurate conventional method for the identification of

Candida species was the sugar fermentation tests in combination with the hyphae formation on cornmeal agar, as it has the ability to identify the rarely isolated species. The prevalence of *C. albicans* was still higher than non-albican species from the hospitalized patients. But in some hospital units the non-albican species are more common. Resistance to antifungals is rare in *C. albicans*. Increasing resistance in non-albican species especially in *C. glabrata* makes it important than ever to properly identify and test the antifungal susceptibility for its proper treatment to effectively control its mortality rate.

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Conflict of interests

The authors declare that they have no conflict of interest.

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