Global Scientific JOURNALS

GSJ: Volume 9, Issue 12, December 2021, Online: ISSN 2320-9186

www.globalscientificjournal.com

PHENOTYPIC CHARACTERIZATION AND ANTIFUNGAL RESISTANCE PROFILES OF *Candida species* OBTAINED FROM CLINICAL SPECIMENS IN OKITIPUPA, ONDO STATE, NIGERIA.

AUTHORS:

1. NAME: ADETUWO OLAGUNJU JOHNSON

EMAIL: ADETUWOOLAGUNJU@GMAIL.COM

INSTITUTION: ADEKUNLE AJASIN UNIVERSITY AKUNGBA-AKOKO, NIGERIA

2. NAME: OGUNDANA FAITH NAOMI

EMAIL: FAITHNAOMI12@GMAIL.COM

INSTITUTION: ADEKUNLE AJASIN UNIVERSITY AKUNGBA-AKOKO, NIGERIA.

ABSTRACT

The aim of the study: This research work was carried out in order to identify *Candida* species Isolated from clinical Specimens obtained from hospitals in Okitipupa and its environs, and also to determine their antifungal resistance profiles.

Methods: The methods used for phenotypic analysis were germ-tube and chlamydospore production, culture in CHROMAgar Candida medium, sugar assimilation test, growth at 45^0C and cultured in Tween 80 agar. A total of seventy-four (74) *Candida* species isolated were characterized. Antifungal susceptibility test to Amphotericin-b, Ketoconazole, Clotrimazole and Fluconazole was performed using Mueller-Hilton agar disk diffusion procedures.

Results: The isolates were obtained from vagina swabs 24.3%, urine 14.9%, respiratory exudates 24.3%, skin and nails scrapping 10.8% blood 14.9% ear discharge 5.4% and plastics devices 5.4%. Out of the 74 isolates, 28 were *Candida albicans*, 9 were *Candida glabrata*, 5 were *Candida krusei* and 32 were *Candida tropicalis*. The isolates were fairly sensitive to the antifungal drugs used in this study. All the isolates showed 100% sensitivity to amphotericin-b, While the sensitivity to ketoconazole and Clotrimazole range between 75-90%. Candida *glabrata* and *Candida krusei* showed strong resistance to fluconazole. From this study, *Candida albicans* and *Candida tropicalis* were the most prevalent Candida species isolates obtained from clinical specimens, hence they were most common caused of candidiasis from this study.

Recommendation: The results obtained from this study have proven that phenotypic tests might be highly effective quicker and cheaper, in identifying *Candida* species from clinical samples, it also shown that there are some evidence of emerging resistance of Candida species to common use antifungal drugs. For the effective management of candidiasis infection there is need to identify all Candida species, as some have intrinsic resistance to commonly used antifungal drugs. There is also need to constantly carry out in vitro antifungal sensitivity test in order to establish an emerging resistance of candida species.

Key Words: *Candida,* species, Hospital, Susceptibility, Phenotypic, Antifungal, Identification**.**

INTRODUCTION

Candida is an important causes of human infections known as candidiasis Candidiasis mrange from mild infection such as onychomycosis to potentially fatal systemic candidiasis. The Candidal has been reported to be responsible for causative agents of bloodstream infections, and ranks fourth in the United States and seventh in Europe (Chakrabarti and Shivafrakash, 2008; ECMM, 2010; Fleming et al., 2002).

In the past *Candida albicans* was the predominant species in most of the countries, causing up to two-thirds of all cases of invasive candidiasis (Pfaller and Diekema, 2007; Berman and Sudbery, 2002). However, other species of *Candida* have gained more attention nowadays due to rapid development of resistance to antifungal drugs. To address this there is need for proper speciation of *Candida* and also to carry-out antifungal sensitivity test for the new generation of commonly used antifungal drugs. Candida species are asexual yeast of the phylum Ascomycota, sub-phylum *Saccharomycota* and the class saccharomyces. They are genetically diploid with the presence of eight chromosomes, many species of Candidal are harmless commensals or endosymbiosis of host including humans. But other species can cause diseases (candidiasis) when they are moved from their micro niche in the body of their host or when their host is immune compromised or immunosuppressed. The genus *Candida* includes: C. *albicans*, C. *ascalaphidarum* C. *argentae*, C.*atlantica*, C.*dosseyi*, C.*dubliniesis*, C.*glabrata*, C. *kefer*, C. *krusei*, C. *lusitaniae*, C. *parapsilosis*, C. *tropicalis*, C. *theae* and several other species (Messer et al., 2003; Fleming et al., 2002). Although Candida *albicans* is by far the most common species causing infections in human (Abi-Said et al., 2008; Beck-Sague et al., 1993) the emergence of non-*albicans* *Candida* species as significant pathogens has however been well recognized during the past years (Fleming *et al.,* 2002). Numerous records have documented the increase of non-*albicans* species among hospitalized and immunosuppressed patients. The increased reporting may be

caused by advancement in laboratory diagnostic, yet the emergence of these opportunistic pathogens were favoured by the change in host susceptibility due to the growing number of immune compromised individuals in the population as a result of HIV pandemic and the use of long-term immunosuppressive therapy in cancer and organ transplant patients. *Candida* species are closely related but unique from each other with respect to epidemiology, virulence characteristics and antifungal sensitivity. All *Candida* species have been shown to cause a similar spectrum of disease ranging from vaginitis, mouth thrust, to systemic disease. The differences in disease severity and sensitivity to different antifungal agents have been reported (Fleck et al., 2008). However, other species of *Candida* including *Candida glabrata* have gained more attention nowadays due to rapid development of resistance to antifungal drugs (Mokaddas *et al.*,2007). Amphotericin-b, a Polyene fungicidal agent, has been the standard treatment for *Candidal* infections for decades, but the toxicity of its convectional form and the costs of its lipid forms limit its use (Beggs, 2012).

Therefore *Candida* spp. characterization is important for successful management of candidiasis. Distinction between species facilitates the understanding of the epidemiology of *Candida* species particularly regarding the reservoir and mode of transmission of effective measures to prevent and control transmission of resistant pathogens. These is also need to constantly carry-out in-vitro antifungal sensitivity test in order to establish any emerging resistance and determine antifungal drugs of choice.

MATERIALS AND METHODS

Sample collection:

A total of 250 different clinical samples were collected from patients being treated in hospitals in and around Okitipupa. The sample collected include 100 from respiratory tract (bronchial wash , tracheal secretion) and saliva, 50 from blood, 30 from urine, 20 from vagina swab, 10 from middle ear discharge, 10 from plastics devices, 30 from skin and nail scrapings.

Sample processing:

Samples were processed by standard microbiological methods. Skin and nail were cut into small fragments and boiled with 10% KOH for 20 minutes. Sputum and saliva were stored at 4°C prior to their used, urine samples were centrifuged sediment for culture and supernatant fluid used for sensitive test. Blood samples were diluted with heparin 1:1000 to prevent clotting. Vaginal swab samples were subjected to direct Gram stained smear examination as well as culture on SDA

Potato dextrose agar (PDA)

Potato dextrose agar was the media used in isolation *Candida* species.The PDA powder was dissolved in 1litre of boiled distilled water,sterilized by autoclaving at 121°C and 115mmHg for 15mins.Allow to cool to 45°C and mixed with diluted samples. Then aseptically poured into Petri-dishes allowed to set incubated at room temperature for 24 hours

Antibiotics mixture

The antibiotics used were a mixture of penicillin powder and streptomycin powder (the mixture was used to inhibit the growth of Gram +ve and –ve bacteria in agar medium). The mixture was prepared by dissolving 5g of streptomycin into 100ml of sterilized water and 5g of penicillin into 10ml of sterilized water. 20ml from the streptomycin mixture was pipette into the mixed penicillin and then shaken vigorously. 1ml each of the antibiotics of the antibiotics mixture was poured into each petri dish for culturing before pouring the PDA, and then swirled for even distribution.

SPECIES IDENTIFICATION

All the *Candida* isolates were subjected to germ tube test using normal human serum. Isolates were identified up to the species level on the basis of morphology on corn meal agar, growth on CHROMAgar *Candida* medium, growth at 45^0C on PDA and sugar assimilation test.

ANTIFUNGAL SUSCEPTIBILITY TEST

Morphological examination on Corn meal agar (CMA)

Pseudohyphae and chlamydospore production by Candida species was observed by streaking the isolates on Corn meal agar plates supplemented with tween 80 (1%). Each plate was divided into 4 quadrants. Using a sterile needle, the yeast colony was lightly touched in each quadrant and streaked. Cover glass slip was flamed sterilized and placed over control part of streak after it had cooled. The plates were incubated at 25 for 4 days. The cultures were examined for various morphological features such as pseudohyphae, blastospores or chlamydospores.

Germ tube test

The germ tube test was used for presumptive identification of *Candida albicans*. It is a quick method where the production of germ tube is observed within two hours. For this test a fresh growth from a pure culture was obtained. A very light suspension of the test isolates in 0.5ml

of sterile serum was prepared. And incubated at 37 for exactly two hours. One drop from incubated serum was transferred on a slid with cover slip. Observed under microscope for the production of germ tubes. Germ tube represents the initiation of hyphal growth arising from yeast cell. Candida albicans strain ATCC 90028 was used as control for germ tube test. Each test was considered positive only 30% of the cells produced the germ tubes.

Cultured into Potato Dextrose Agar (PDA)

All samples were cultured onto Potatoe Dextrose Agar (SDA) (HiMedia, Mumbai, India) plates supplemented with 0.05% (W/V) chloramphenicol. Cultures were incubated at 37oC for 24-48 hours after which the growing fungi were purified and kept in slants for further used.

Cultured into CHROMagar Candida

The isolates were cultured into CHROMagar. The manufacturer instructions were followed: 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^{0}C, with gentle stirring, then poured into sterile Petri dishes and allowed to solidify. Separate colonies from all *Candida* isolates on PDA were subcultured onto CHROMagar Candida and incubated at 37^{0}C for 48 hr. Presumptive identification was done based on colony colour of the growing *Candida* strains. **Growth at 45^{0}C**

The isolates were incubated at 45^{0}C in PDA and Emmons medium (2% SDA) for 10 days .The ability of any of the isolates to grow at this temperature will be useful in phenotypic identification of *Candida albicans.*

Microscopic examination:

All the respiratory specimens and exudates were examined in 10% KOH. In addition, the smear were Gram stained to look for Gram positive budding yeast cells and pseudo-hyphae.

SUGAR ASSIMILATION TEST PROCEDURE:

Agar plates were inoculated with the test *Candida* isolates, then sterile paper disc containing various sugars are placed on the culture plates and then incubated at room temperature for 24 hours. Growth adjacent to any of the disc is a positive test for sugar assimilation.

ANTIFUNGAL SUSCEPTIBILITY TEST

The antifungal susceptibility of *Candida* species isolated from clinical samples was determined by disc diffusion method on glucose methylene blue Muller Hinton agar.

Antifungal Susceptibility Test of Yeasting method was used as explained by CLSI guidelines M44-A (2010).

ANTIFUNGAL AGENTS USED

Antifungal agents used were Amphotericin-B (100µg), Fluconazole (25µg), Ketoconazole (50µg) and Clotrimazole (50µg). Glucose methylene blue Mueller Hinton agar (GM-MH) was prepared by addition of 2% glucose and 0.5µg of methylene blue to Mueller Hinton agar. The inoculum was prepared by picking four distinct colonies of approximately 1mm from 24 hours old cultures grown on Sabouraud's dextrose agar (SDA). Colonies were suspended in 5ml of sterile 0.85% saline. This suspension was vortexed to adjust the turbidity yielding $1\times10^6$to 5×10^6 cells/ml and streaked on the entire surface of GM-MH agar the antifungal discs were placed 24mm apart from each other. The plates were then incubated at 37 for 24hours the plates were read after 48hours.zone diameter were interpreted as per the approved CLSI M44-A guidelines. The quality control test was performed by using *C.parapsilosis (ATCC22019) C.krusei (ATCC6258)* and *C. albicans (ATCC90028*).

STATISTICAL ANALYSES

Data were statistically described in terms of frequencies and percentages. The chi-square test was used to evaluate the differences in prevalence between *C. albicans* and non-albicans *Candida* species

RESULTS

Among phenotypic features, colony morphology of each isolate was studied using potatoe dextrose agar and CHROMAagar Candida media..

Table 1: Morphology of Candida species on PDA media

Candida species	Colonies colour/ appearance
C. albicans	White to cream, smooth and soft
C. glabrata	White to cream
C. krusei	White to cream

C. tropicalis	White to cream

On PDA medium colonies of Candida albicans were white to cream coloured smooth and soft, whereas Candida tropicalis, Candida glabrata and Candida krusei produced white to cream coloured colonies.

Table 2. Identification of *Candida* species by culture in CHROMAgar medium after 48 hours.

Species	Colonies colour/ appearance
C. albicans	Bluish-green
C. tropicalis	Metallic blue
C. glabata	White to creamy
C. krusei	Pink colonies with pale, flat and speading with fringe

A total 38% isolates showed bluish-green colonies in CHROMAgar medium, indicating C. *albicans,* C. tropicalis (42%), C. glabrata (12%) and C. krusei (7%) gave metallic blue ,pink to lilac and pink with pale flat colonies respectively.

Table 3. Identification of *Candida* species by chlamydospore production test (CH)

Species	Chlamydospore Production
C. albican	+ve
C. tropicalis	-ve
C. glabrata	-ve
C. krusei	-ve

+ve= means it Produced chlamydospore; -ive = it didn't Produced chlamydospore

Production of pseudohyphae, chlamydospore and blastospores was observed on corn meal agar supplemented with Tween 80. *C. albicans* on CMA showed dimorphic morphology that is yeast as well as pseudohyphae with terminal chlamydospores, *Candida tropicalis* exhibited pine forest arrangement of pseudohyphae, *Candida glabrata* and *Candida k*rusei yeast only.

Table 4. Identification of Candida species by germ-tube test(GT)

Candida species	**Germ-tube Test**
Candida albicans	**+ve**
Candida glabrata	**-ve**
Candida krusei	**-ive**
Candida tropicalis	**-ve**

Candida albicans formed germ tube while other Candida species didn't form germ tube

Table 5. Identification of Candida species by growth at 45 test.

***Candida* species**	**Growth at 45^0C**
C. albicans	**+ve**
C. *tropicalis*	**-ve**
C. krusei	**-ve**
C. glabrata	**-ve**

+ve means it can grow at 45 -ve means it cannot grow at 45^0C

Only C. albicans growed at 45^0Cwhile other Non-*Candida albicans* species didn't grow at 45^0C

Table 6: S**ugar Assimilation test for Candida isolates**

Species	Glu	Mal	Suc	Lac	Mel	Raf	Cel	Tre	Xyl
C.albicans	+ve	+ve	+ve	+ive	+ive	+ve	-ve	+ve	+ve
C.tropicalis	+ve	+ve	+ve	+ive	+ive	-ve	+ve	+ve	+ve
C. glabrata	+ve	+ve	+ive	+ve	+ive	-ve	-ve	+ve	-ve
C. krusei	+ve	+ve	+ive	+ve	+ve	-ve	-ve	-ve	-ve

Glu= Glucose, Mal= maltose, Suc= Sucrose, Lac= lactose, Mel=melibiose, Raf=Raffinose Cel= cellobiose, Tre = Trehalose, Xyl=xylose

Regarding the pattern of sugar assimilation, all the isolates, except C. krusei assimilated glucose, trehalose and maltose.The lactose, raffinose and melibose were not assimilated by all the isolates. C. tropicalis was only isolates that assimilated cellobiose.

Table 7: Distribution of *Candida* isolates in various clinical samples

Candida isolates	Respiratory exudates(A)	Blood(B)	Urine(C)	Vagina swab(D)	Middle ear discharge(E)	Plastic devices(F)	Skin and nails scrapings(G)
C.albicans	6(33.3)	3(27.3)	6(54.5)	7(38.9)	1(25.0)	2(50.0)	3(37.5)
C.tropicalis	7(38.9)	4(36.4)	4(36.4)	10(55.6)	2(50.0)	1(25.0)	4(50.0)
C.glabrata	3(16.7)	2(18.2)	1(9.1)	1(5.6)	0(0)	1(25.0)	1(12.5)
C.krusei	2(11.1)	2(18.2)	0(0)	0(0)	1(25.0)	0(0)	0(0)

TOTAL A=18 B=11 C=11 D=18 E=4 F=4 G=8

Figures in the brackets Indicate % distribution of *Candida* isolates in each clinical sample.

On basis of the phenotypic and biochemical tests 74 Candida strains including 28 *Candida albicans*, 32 *Candida tropicalis*, 12 *Candida glabrata* and 7 *Candida krusei* these were isolates from various clnical samples.

Table 8: Antifungal sensitivity profiles of *Candida* species isolated from clinical samples

Candida isolate	Number of isolate	Amphotericin-B		Ketoconazole		Fluconazole		Clotrimazole	
		Se	Re	Se	Re	Se	Re	Se	Re
Candida albicans	28	28(100)	0(0)	26(93)	2(7)	27(96.4)	1(3.4)	27(96.4)	1(3.6)
Candida glabrata	9	9(100)	0(0)	3(43)	6(57)	2(29)	7(71)	5(55.6)	4(44.4)
Candida krusei	5	5(100)	0(0)	4(10)	1(20)	2(40)	3(60)	2(60)	3(60)
Candida tropicalis	32	32(100)	0(0)	22(69)	10(31)	20(63)	12(37)	22(69)	10(31)
TOTAL		74	74(100)	0(0)		55(74.3)	19(25.7)	51(68.9)	23(31.1)
56(75.6)	18(24.4)								

Se=sensitivity; Re=resistance; figures in brackets indicate % of sensitivity and resistance

All the 74 isolates were sensitive to amphotericin-B (100% sensitivity). While 55 isolates were sensitive to ketoconazole (74.3% sensitivity).Also 51 isolates were sensitive to fluconazole (68.9% sensitivity) and 56 isolates were sensitive to clotrimazole (75.7% sensitivity).

Discussion

The phenotypic method for identification of *Candida* species is simple and inexpensive while compared with molecular method, but it imposes some limitations; which are time consuming and more often unable to discriminate C. albicans and C. dubliniensis. However combinations of two or more phenotypic methods can be useful for the presumptive identification of these

species but they would require a pure culture and five or longer days for differentiation between C. albicans and C. dubliniensis isolates. It was observed in this study that CHROMAgar *Candida* medium is a useful medium for isolation of *Candida* species, the same observation was reported by other researchers (Pfaller et al., 2010; Anne-Marie, 2009). Among the *C.tropicalis* isolates all the 32 showed blue colour (100% excellent specificity). All the 28 isolates*C.albicans* showed light green colour (100% excellent specificity).

And all the 5 isolates *Candida krusei* showed pale pink colour (100% excellent specificity) but among the 9 isolates of *Candida glabrata* 6 showed cream white colour (66.7% specificity) and the remaining three were confirmed by carbohydrate assimilation test, since *Candida glabrata ca*nnot assimilate sucrose. The pattern of carbohydrate assimilation is considered a reliable test and is generally used for correct identification of Candida species. The result obtained confirmed the identification of: *Candida* albicans, C. tropicalis, Candida glabrata and C. krusei. *Candida tropicalis* was the predominant pathogen (43.0%) followed by *Candida albicans* (38.0%). In this study the isolation of *Candida* species from the respiratory exudates is common (24.3%). This occurs as a result of dissemination through oropharyngeal or gastric contents as observed by Gari and Kindo in their study (2012). The high presence of candida species in the blood samples (14.9%) is an indication of bloodstream infection caused by various *Candida* species. Which are significant cause of morbidity and mortality in hospitalized patients as reported by various researchers in many countries worldwide (ECMM, 2010; Beck-Sague et al.,1993; Bedini et al., 2006). Candidaemia is associated with many Invasive medical procedures such as endoscopy, intravascular catheters aggressive cancer incisions, intrauterine device, and underlying diseases like diabetes. Candida *tropicalis* was the most common *Candida* species isolated from blood samples (36.4%) in this study, therefore may be the most commonly caused bood infection, C. albicans was the next predominant species in blood samples analysed (27.3%), follow by *C. glabrata* (18.2%) and *Candida krusei* (18.2%). Also out of 30 urine samples analysis, the presence of *Candida* species was discovered in five samples. *Candida albicans* was predominantly (54.5%) followed by *Candida tropicalis* (36.4%). Chakrabarti (2005) observed that the presence of indwelling catheter favours the development of candiduria in 38% of pediatrics group and 43% of adult patients as they observed in their study. Therefore, candidiasis of the urinary tract are strongly associated with the presence of urinary catheter. The high prevalence of Candida species in vagina swab (24.3%) with *Candida tropicalis* predominatly (55.5%) perhaps indicated that vulvo-vaginal candidiasis (vaginitis) is a common condition in women, and might be related with the

indiscriminately used of antimicrobial agents, low vaginal pH, diabetes mellitus, sexually transmitted infections and contraceptive drugs

The most effective antifungal agent used in this study is amphotericin B, all *Candida* isolates showed 100% sensitive to amphotericin-B. Amphotericin-B was the most active agent against *C.albicans* (MIC50, 1µg/ml). The activities of amphotericin-B against the non-*Candida albicans* were (MIC50, 4µg/ml). *Candida tropicalis*, *Candida glabrata and Candida krusei* were all showed strong resistance to fluconazole, ketoconazole and clotrimazole (<65.0% susceptibility). While *Candida albicans* was sensitive to all azoles (>93.0% susceptibility) the result agreed with observation of Mokaddas et al., (2007) and Mulu et al., (2013), they observed that *Candida* species with fluconazole resistance have been seen more in recent years. Resistance to fluconazole among non-Candida albicans (NAC) varies from 37.0% to 63.0%, Candida *tropicalis*, *Candida glabrata*, and *Candida krusei* showed strong resistance to fluconazole in this study. The maximum resistance was seen in *Candida tropicalis (63.0%). Other* researchers have also noted an increased in resistance to fluconazole in clinical isolates of *Candida tropicalis* (Beggs et al., 2006; Beggs et al., 2007; Ather and Winner, 2010; Lyon et al., 2010).

CONCLUSION

This research work proved that CHROMAgar *Candida* medium with other phenotypic methods is found to be useful in identification of *Candida* species from clinical samples. Also non-*Candida albicans* (NAC) were predominant isolates. This research showed that there are emerging non-*Candida albicans* as important opportunistic pathogens. Also all *Candida* isolates were fairly sensitive to Amphotericin-B but marked resistance towards Azoles. Rapid identification of *Candida* species directly from clinical samples may help to reduce the long staying in hospital and high overall costs that are associated with management of candidiasis. An increase in the resistance to antifungal agents by Non Candida albicans species has made antifungal sensitivity test essential for routine Medical Microbiology services.

REFERENCES

1. Abi-said, D., Anaissie, E. and Uzon, O. (2008). The epidemology of hematogenous candidiasis caused by different *Candida* species. *Clinical Infectious Diseases* **24**: 1122-1128.

2. Anne-Marie, F. (2009). Evolution of CHROMagar *Candida*, a new differential Isolation Medium for presumptive Identification of Clinical important*Candida* species. *Journal of Clinical Microbiology* **32**: 1923-1926.

3. Ather, M.A. and Winner, H.I. (2010). Development of resistance by *Candida* species to polyene antibotics in vitro. *Jounal of Medical Microbiology* **4**: 505-517.

4. Beck-Sague, C.M. and Jarvis, W.R. (1993). National nosocomial infections surveillance system. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. *Journal Infectious Diseases* **167**: 1247-1250.

5. Bedini, A., Venturelli, C., Mussini,C., Guaraldi, G., Codeluppi, M., Borghi, V., Rumpianesi, F., Barchiesi, F., Esposito, R. (2006). Epidemiology of candidaemia and antifungal susceptibility patterns in an Italian tertiary-care hospital. Clin Microbiol Infect. **2**(1):75-80.

6. Beegs, W.H., Andrews, F.A. and Sarosi, G.A. (2007). Combined action of amphotericin B and 5-Fluorocytosine on pathogenic yeast susceptibility to either drug alone. *Chemotherapy* **27**: 247-251.

7. Beggs, W.H. (2012). Comparison of Miconazole and Ketoconazole induced release of k^+ from*Candida* species. *Journal of Antimicrobial Chemotherapy* **11**: 381-383.

8. Beggs, W.H., Sarosi, A., and Walker, D. (2006). Synergistic action of Amphotericin B and Rifampin against Candida species. *Journal of Infectious Diseases* **133**: 206-209.

9. Chakrabarti, A., Chatterjee, S. and Shivafrakash, M.R (2008). Overview of opportunistic fungal infection in India. *Japan journal of Medical Mycology* **49**: 162 – 172.

10. Clinical and Laboratory Standard Institute (CLSI) (2004). Reference method for Antifungal Disk Diffusion susceptibility testing of yeasts; Approved Guidelines-second edition. CLSI document M44-A2. *Clinical and Laboratory Standard Institute*, Wayne, Pennsylvania 19087-1898 USA.

11. Deorukhhar, S. and Saini, S. (2014). Virulence markers and antifungal susceptibility profile of Candida glabrata: An emerging pathogen. *British Microbiology Research Journal***4**(1): 35 – 45.

12. Dick, J.D., Metz, W.G. and Saral, R. (2006). Incidence of polyene-resistant yeasts recovered from clinical specimens. *Antimicrobial Agents chemotherapy* **18**: 158-163.

13. European Confederation of Medical Mycology (ECMM) Survey of Candidemia in Italy (2010): Antifungal Susceptibility patterns of 261 non-*albicansCandida* isolates from blood. *Journal of Antimicrobial Chemotherapy* **52**: 679-682.

14. Fleck, R., Dietz, A. and Hof, H. (2008). Susceptibility of *Candida* Species to five antifungal agents in German University hospital assessed by the reference broth microdilution method and Etest. Journal of Microbial chemotherapy **57**: 769 – 771.

15. Fleming, R.V., Wash, T.J. and Anaissie, E.J. (2002). Emerging and less common fungal pathogens. *Infectious diseases Clinical North America* **16**(4): 915-933.

16. Gari, S. and Kindo, A.J. (2012). A review of Candida species causing blood stream infection. Indian Journal of Medical Microbiology **30(**3): 270-278.

17. Lyon, G.M., Karatela, S., Sunay, S. and Adel, Y. (2010). Antifungal susceptibility testing of *Candida* isolates from *Candida* surveillance study. *Journal of Clinical Microbiology* **48**: 1270-1275.

18. Messer, S.A., Jones. R.N. and Fritsche, T.R. (2006). International surveillance of *Candida* species and *Aspergillus* species: Report from the SENTRY Antimicrobial surveillance program (2003). *Journal of Clinical Microbiology* **44:** 1782 – 1787.

19. Mokaddas, E.M., Al-Swelh, N.A. and Khan, Z.U. (2007). Species distribution and antifungal susceptibility of *Candida* blood stream isolates in Kuwait: a 10 years study. *Journal of Medical Microbiology***. 56**(2): 225-259.

20. Mulu, A., Kassu, A., Anagaw, B., Mages, B., Gelaw, A., Alemayehu, M., Belyhun, Y., Biadglegne, F., Hurissa, Z., Moges, F. and Isognl, E. (2013). Frequent detection of "azole" resistant species among late presenting AIDS patients in North West Ethiopia. *BMC infectious Diseases* **13**(1): 82.

21. Pfaller, M.A. (2012). Nosocomial Candidiasis: Emerging species, Reservoir and modes of transmission. *Clinical Infectious Diseases* **36:** 589-594.

22. Pfaller, M.A. and Diekema, D.J. (2007). Epidemiology of Invasive Candidiasis: a persistent public health problem. Clinical Microbiology review **20**: 133-163.

23. Pfaller, M.A., Huston, A. and Coffman, S. (2010). Application of CHROMagar *Candida* for Screening of clinical specimens for *Candida albicans, Candida tropicalis, Candida krusei and Candida glabrata. Journal of clinical Microbiology* **21**: 58-61.