Original Article

"PHENOTYPIC EVALUATION OF ANTIFUNGAL SUSCEPTIBILITY PATTERN OF *Candida albicans* ISOLATED FROM DIFFERENT CLINICAL SAMPLES BY KIRBY-BAUER DISC DIFFUSION AND BROTH MICRODILUTION METHOD TO FLUCONAZOLE AND AMPHOTERICIN B"

Prema Nandini[1*], R.Sujatha[2]

[1*]Ph.D Scholar, Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India.
Email: pushpa.choclate@gmail.com
[2]Professor and Head of Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India.
Email: drsujatha152@gmail.com
*Corresponding Author: Prema Nandini

DOI: 10.47750/pnr.2023.14.02.337

Abstract

Introduction: Candidiasis is a major fungal infection where *Candida albicans* is the major cause of infections in humans. *Candida albicans* is an opportunistic pathogen under immune-compromised conditions and despite anti-fungal therapies, it has become lethal. Increase in the antimicrobial resistance in *C. albicans* is a matter of concern as it is in the human microbiome.

Aim and Objective: To determine the antifungal susceptibility pattern of *Candida albicans* isolated from different clinical samples against Fluconazole and Amphotericin B by Kirby Bauer disc diffusion method and Broth microdilution method.

Materials and Methods: This was a cross sectional study carried out in the Department of Microbiology at Rama Medical College Hospital and Research Centre Mandhana, Kanpur for a period of 1 year i.e, April 2018 to April 2019. A total of 70 isolates of Candida species from different clincal specimens like blood, BAL, urine, Pus, Et secreation and vaginal secreation were evaluated for its susceptibility against Fluconazole and Amphotericin B using Kirby Bauer disc diffusion method and Broth micro dilution method according to the CLSI guidelines 2018.

Results: Out of 70 isolates of Candida species 29 (41.4%) isolates were confirmed to be *C.albicans.* The ratio of Males 18 (62%) was more as compared to that of the Females 11(37.9%) with the maximum age of 31-40 being affected the most followed by 41-50 and least in the age group above 61 years of age. The number of isolates was maximum in the urine sample. A total of 12 (41.3%) samples of Candida were sensitive and 17 (58.6%) samples were resistant to Amphotericin B & 27(93%) samples of Candida were sensitive and 2(6.8%) samples were resistant to Fluconazole by Kirby bauer disc diffusion method. 28(96.5%) samples of Candida were sensitive and 1(3.4%) sample was resistant to Amphotericin B & 25 (86.2%) samples of Candida were sensitive and 4(13.7%) samples were resistant to Fluconazole by broth microdilution method.

Conclusion: The Antifungal susceptibility testing by broth microdilution method revealed that fluconazole was exceedingly resistant against *Candida albicans* (13.7%) and increasingly susceptible to Amphotericin B (96.5%). Antifungal susceptibility testing may possibly be used to calculate the clinical response, to forecast malfunction in management. Therefore, Proper

administration of antifungal drugs should be prioritized only with susceptibility result testing.

Keywords: *Candida albicans,* Antifungal resistance, Microdilution method, Disc diffusion method.

INTRODUCTION

Candida albicans is a yeast fungus that is normally present on the skin and mucous membranes such as oral cavity, vagina, and rectum. It can travel through the blood stream and cause infection in any part of the body. *C. albicans* is the most vital opportunistic pathogen where it normally resides in oral, conjunctival, gastrointestinal and genitourinary tracts. Moreover, infection caused by *Candida* species is called as candidiasis [1].

Antifungal resistance is a major concern in clinical practice and becoming a major problem. Intensive and long term use of antifungal drugs lead to decline in susceptibility and resistance patterns of *Candida* species [2]. Recently, resistance to common antifungals has been reported in different *Candida* species [3]. It should also be noted that *Candida* species differ in their antifungal susceptibility and virulence factors. The Antifungal susceptibility testing is an important tool in the management of *Candida* infection because it promotes accurate administration of antifungal agents, and as an aid in drug development as well as a means of tracking the development of antifungal resistance in epidemiological studies [4].

The polyenes, azoles, echinocandins, nucleoside analogs, and allylamines are used with varying efficacy depending on the type and site of infection and the sensitivity of the *Candida* species. Azole antifungal drugs are the mainstay of management of infections with *Candida* species. The most commonly prescribed antifungal used for most *C. albicans* infections is fluconazole, a member of the azole class of antifungals [5]. There is an extensive use of fluconazole for chemoprophylaxis and treatment of fungal infections due to their favorable oral bioavailability and safety. Moreover the environmental stress with exposure to antifungal drugs can mediate resistance. With the increased incidence of Candida infections, there has also been development of resistance to antifungal agents specially the azole group [3]. The indiscriminate, inadequate use of antifungal drugs, especially azole group have contributed for increase in emergence of resistance strains of Candida.

Thus, the role of in-vitro laboratory tests in selection of antifungal therapy has become crucial. Hence, this study was undertaken to identify the *Candida* isolates from various clinical samples up to species level and to find out their antifungal susceptibility by Kirby-Bauer disc Diffusion and Broth Microdilution Method to Fluconazole and Amphotericin B.

MATERIAL AND METHODS

This was a cross sectional study carried out in the Department of Microbiology at Rama Medical College Hospital and Research Centre Mandhana Kanpur for a period of 1 year i.e, April 2018 to April 2019. The Ethical clearance was obtained from the Ethical Committee of RMCH&RC, Mandhana, Kanpur. The Demographic details and clinical history along with the relevant clinical investigations was recorded after the informed consent. Candida isolates from all clinical specimen in pure culture were included in our study whereas, repeat isolates from same clinical specimen of same patient and isolation of Candida species from mix culture were excluded from the study.

All the clinical samples were subjected to culture on 5% Blood agar, and MacConkey agar. Gram staining of all the positive cultures was performed, and those showing yeast like budding cells were sub-cultured on SDA and HiChrome agar for species identification. Germ tube test was performed to differentiate Candida albicans and NACA. Further identification was done by Chrom agar, sugar assimilation tests using commercially prepared sugar discs sucrose, maltose, dextrose, trehalose, lactose and dulcitol from HiMedia and studying micro morphology on corn meal agar.

A total of 70 isolates of Candida species from different clincal specimens like blood, BAL, Urine, Pus, Et secreation and Vaginal secreation were included in our study.

Antifungal sensitivity of Candida isolates was done by Kirby-Bauer disc diffusion method. Mueller Hinton agar supplemented with 0.2% glucose and 0.5µg/ml methylene blue dye medium (MH-GMB) was used for this purpose against azole group Fluconazole 25ug and Amphotericin B 25ug procured from Hi-media Laboratories Pvt Ltd India. The broth micro dilution method was done to determine the minimum inhibitory concentrations (MICs) according to the CLSI guidelines 2018 [6].

RESULTS

A total of 70 isolates of Candida species was included in the present study out of which 29 (41.4%) isolates were confirmed to be *C.albicans.*

Table No. 1: The Type of Candida species isolates

Type of Fungal isolates	**Number of Isolates**	**Percentage**
C. albicans	29	41.4 %
C.tropicalis	20	28.5 %
C.glabrata	17	24.2 %
C. krusie	4	5.7 %

Table No. 2: Genderwise distribution of the *Candida albicans*

Gender	**Total no. of Cases studies (N=29)**	**Percentage**
Male	18	62%
Female	11	37.9%

The ratio of Males 18 (62%) was more as compared to that of the Females 11(37.9%) [Table No. 2] with the maximum age of 31-40 being affected the most followed by 41-50 and least in the age group above 61 years of age [Table No. 3].

Table No. 3: Age wise distribution of *Candida albicans* patients from the study

S. No.	Age (in years)	No. of Cases	Percentage
1.	0- 10	-	-
2.	11-20	3	10.3 %
3.	21-30	4	13.7 %
4.	31-40	10	34.4 %
5.	41-50	9	31 %
6.	51-60	2	6.8 %
7.	≥61	1	3.4 %

Table No. 4: Type of Sample Isolated from *Candida albicans*

Type of Sample	**Number of Isolates**
BAL	1
Urine	15
Pus	5
Et secretion	3
Vaginal secretion	4
blood	1

The maximum number of isolates was found in the urine sample followed by the pus and least in the BAL and the blood sample [Table No. 4].

Fig No. 1: *Candida albicans* on Sabouraudextrose agar

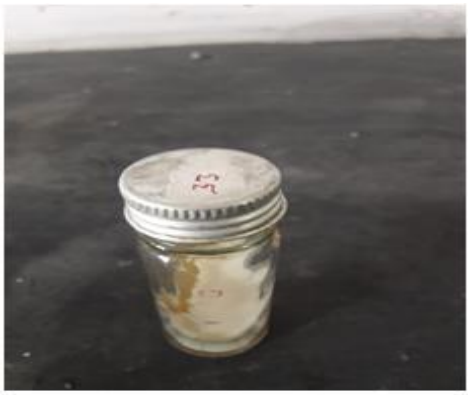

Fig No. 2: *Candida albicans* on germ tube formation

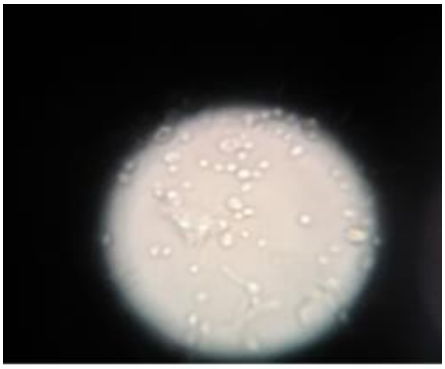

Fig No. 3: *Candida albicans* on Hichromagar

Out of 70 isolates a total of 29 isolates of *Candida albicans* were isolated. A total of 12 (41.3%) samples of Candida were sensitive and 17 (58.6%) samples were resistant to Amphotericin B & 27(93%) samples of Candida were sensitive and 2(6.8%) samples were resistant to Fluconazole by Kirby bauer disc diffusion method.

28(96.5%) samples of Candida were sensitive and 1(3.4%) sample was resistant to Amphotericin B & 25 (86.2%) samples of Candida were sensitive and 4(13.7%) samples were resistant to Fluconazole by broth microdilution method.

Graph No. 1: Graphical Representation of *Candida albicans*

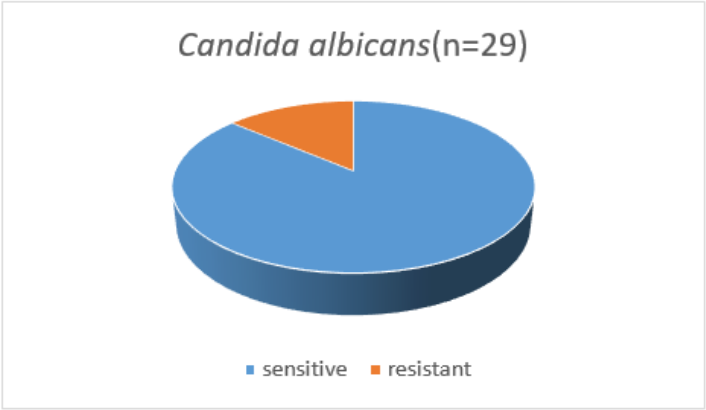

Fig. No.4: Sensitivity pattern by Kirby-Bauer disc diffusion method

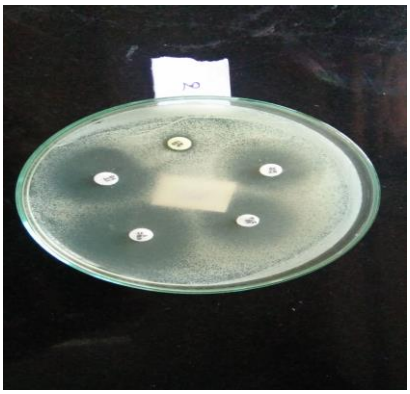

Fig No. 5 (a)

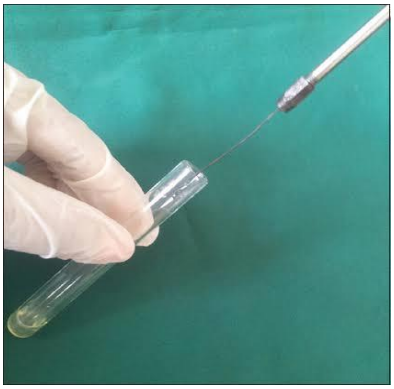

Fig No. 5 (b)

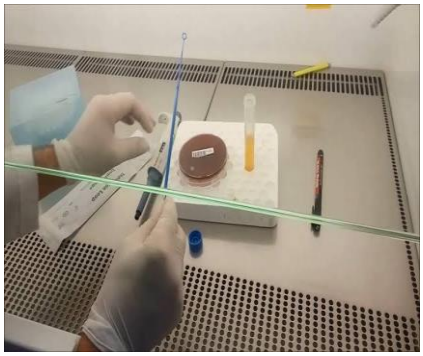

Fig No. 5 (C): The Antifungal sensitivity pattern by broth microdilution method

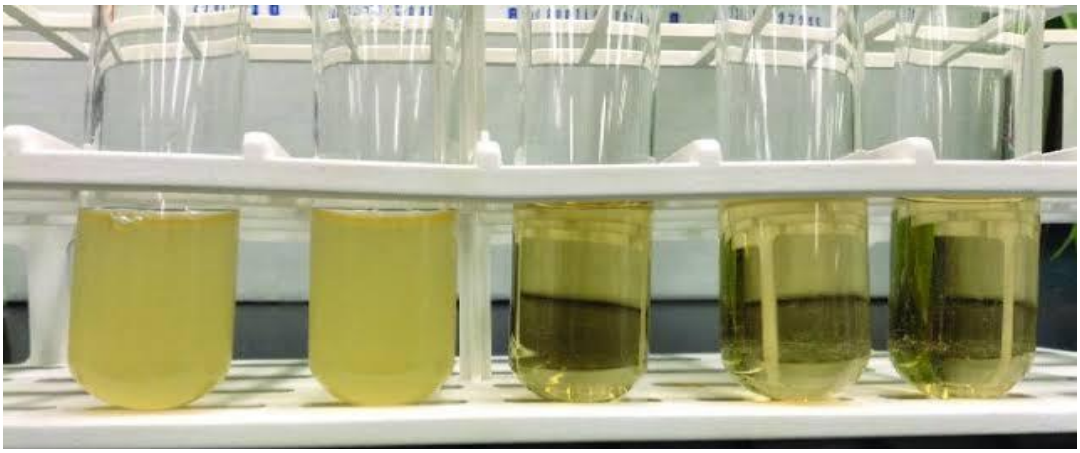

Table No. 5: Antifungal Sensitivity pattern of *Candida albicans* against fluconazole by Kirby Bauer disc diffusion method according to the CLSI guidelines

Antifungal-Fluconazole	**Number of isolates N=29**	**Percentage of isolates**
Sensitive	27	93 %
Resistant	2	6.8 %

A total of 27(93%) samples of Candida were sensitive and 2(6.8%) samples were resistant to Fluconazole by Kirby bauer disc diffusion method [Table No. 5].

Table No. 6: Antifungal Sensitivity pattern of *Candida albicans* by CLSI broth Microdilution method

Antifungal-Fluconazole	**Number of isolates N= 29**	**Percentage of isolates**
Sensitive	25	86.2%
Resistant	4	13.7%

In the Table No. 6 it was illustrated that out of 29 isolates of *C.albicans* tested for susceptibility pattern by CLSI broth microdilution method 25 isolates(86.3%) were sensitive and 4 isolates(13.7%)were resistant to Fluconazole showing Mic>/=64ug/ml.

Table No. 7: Antifungal Sensitivity pattern of *Candida albicans* against Amphotericin B by Kirby Bauer disc diffusion method

Antifungal-AMP B	**Number of isolates N= 29**	**Percentage of isolates**
Sensitive	12	41.3 %
Resistant	17	58.6 %

A total of 12 (41.3%) samples of Candida were sensitive and 17 (58.6%) samples were resistant to Amphotericin B [Table No. 7]

Table No. 8: Antifungal Sensitivity pattern of *Candida albicans* against Amphotericin B by CLSI broth microdilution method

Antifungal-AMP B	**Number of isolates N= 29**	**Percentage of isolates**
Sensitive	28	96.5 %
Resistant	1	3.4 %

In the Table No. 8 it was illustrated that 96.5% were sensitive whereas, 3.4% were resistant against Amphotericin B by broth microdilution method.

Table No. 9: Antifungal Sensitivity pattern of *Candida albicans* against Amphotericin B by CLSI broth microdilution method

Antifungal	**Kirby bauer disc diffusion Method**		**Broth microdilution method**	
	Sensitive	**Resistant**	**Sensitive**	**Resistant**
Fluconazole	27(93%)	2(6.8%)	25(86.2%)	4(13.7%)
Amphotericin b	12(41.3%)	17(58.6%)	28(96.5%)	1(3.4%)

Table No. 10: Sample wise resistance pattern of *C.albicans* against Fluconazole and Amphotericin B by CLSI Kirby bauer disc diffusion method

Type of Sample	**Fluconazole**	**Amphotericin B**
BAL	0	0
Urine	1	13
Pus	0	2
Et secretion	0	1
Vaginal secretion	1	1
blood	0	0

Table No. 11: Sample wise resistance pattern of *C.albicans* against Fluconazole and Amphotericin B by CLSI broth microdilution method

Type of Sample	**Fluconazole**	**Amphotericin B**
BAL	0	0
Urine	3	1
Pus	0	0
Et secretion	0	0
Vaginal secretion	1	0
blood	0	0

In the present study it was observed that by Kirby bauer disc diffusion method 2(6.8%) were resistant to Fluconazole whereas, 4(13.7%) showed resistance to Fluconazole by Broth microdilution method. It was also observed that 17 (58.6%) showed resistance against Amphotericin B by Kirby bauer disc diffusion method and 1 (3.4%) was found to be resistant by Broth microdilution method.

In the study it was also observed that the maximum number of sample observed resistant was found in the urine sample.

DISCUSSION

Candidiasis is a major fungal infection, and *Candida albicans* is the major cause of infections in humans [3]. It is also an important part of the normal microbial flora in the oral cavity, gastrointestinal tract, and vagina in healthy humans. Mediate adhesion, biofilm formation, invasion into host cells, yeast-to-hypha transition (phenotypic switching), secretion of hydrolases, contact sensing, and thigmotropism are the pathogenic potentials of *C. albicans* [7].

A total of 70 isolates of Candida species was included in the present study out of which 29 (41.4%) isolates were confirmed to be *C.albicans.* This study was in support with the study performed by L. Sherry et al.,[8] where the rate of *Candida albicans* was found to be maximum. In the present study it was observed that the ratio of Males 18 (62%) was more as compared to that of the Females 11(37.9%) with the maximum age of 31-40 being affected the most followed by 41-50 and least in the age group above 61 years of age. There were other studies which were parallel to our study where the male was more common. R A Kashid et al.,[9] reported the isolation of Candida species was higher in males (55.10%) with male to female ratio of 1:0.81. In another study by Amar CS et al., more Candida isolates from male and the male female ratio was reported as 0.66:1[10]. The study by B S G Sailaja et al.,[11] was similar to our study where the maximum age of 31-40 being affected the most but in contrast with the study by Arasi et al.,. which reported that more Candida strains in age group >60 years [12]. The maximum number of isolates was found in the urine sample. These finding were similar to the study by Alvarez-Lerma et al.,[13]. and CA Kauffmann et al., [14] Sankarankutty Jay and Vipparti Harita [15] reported that more strains were isolated from Urine.

In the present study a total of 12 (41.3%) samples of Candida were sensitive and 17 (58.6%) samples were resistant to Amphotericin B & 27(93%) samples of Candida were sensitive and 2(6.8%) samples were resistant to Fluconazole by Kirby bauer disc diffusion method. 28(96.5%) samples of Candida were sensitive and 1(3.4%) sample was resistant to Amphotericin B & 25 (86.2%) samples of Candida were sensitive and 4(13.7%) samples were resistant to Fluconazole by broth microdilution method, which was incompatible with the study conducted by kamal Uddin Zaidi.et.al., [16] which showed 56.5% resistance and 43 % sensitivity to Fluconazole and which was in comparison with the studies conducted by Lulu Zhang.et.al., [17] which showed 10.6% resistance and 89.2% sensitivity to fluconazole and study conducted by shirshaklamsalet.al.,[18]which showed 80.9%susceptibility and 9.1% resistance to fluconazole.

The susceptibility pattern obtained in the present study against azole antifungal fluconazole was also in agreement with a previous study by Rathod et.al.,[19] where higher susceptibility rates were observed against fluconazole. The development of resistance against azole antifungals can be due to alteration of the lanosterol 14 alpha demethylase target enzyme because of either overexpression or mutation in Erg11 gene encoding the enzymehenry et.al 2000 [20].

In the present study, we examined the antifungal susceptibility and resistance of antifungal agents of Fluconazole against *C. albicans* in disk diffusion and a micro-dilution method. The zone of inhibition of a different antifungal agent against *C. albicans* was observed at a concentration of 25µg/ml.. Our findings were not in accordance with the study conducted by Fadda et al.,2008 [21] where decreased susceptibility to azoles in *C. albicans* was observed.

In the current study *candida albicans* sensitivity to amphotericin B was observed to be 96%sensitivity which was in comparison with the study by P.Badiee et.al which showed 99.5% sensitivity to amphotericin B. resistance rates of *C. albicans* to amphotericin B were reported to be 2.6% [22] and 7% [23] in Shiraz and Mazandaran which is in comparison with the present study which showed 4% resistance to Amphotericin B.

Minimum inhibitory concentration was tested at the final concentrations ranging from 0.5µg/ml to 256µg/ml. The dilution that showed no growth indicate the concentration at which the fungal growth was inhibited and the lowest concentration showing no colour was recorded in terms of the MIC value.. Early detection of drug susceptibility to the organism was carried out for a successful treatment of any infectious disease [24]. The diagnosis and identification of Candida species along with its antimicrobial susceptibility pattern in patients is very important for maintaining the rational use of antifungals.

This study carries significance due to an increase in the antifungal resistance of *Candida* which is a lethal threat to immune compromised and hospital acquired infections. Intrinsic resistance to antifungal therapy had been reported [17,18]. It was also observed that the antifungal resistances are developed due to the treatment. It is very important to understand the mechanisms of drug resistance to improve the efficiency of treatment since *Candida* infections have high impact on immune compromised patients.

CONCLUSION

Antifungal susceptibility testing may possibly be used to calculate the clinical response, to forecast malfunction in management. Therefore, the diagnosis and identification of Candida species along with its antimicrobial susceptibility pattern in patients will help the clinician in selecting the appropriate antifungal agent and thus contribute to overall reduction in cost of treatment and duration of hospital stay.

REFERENCES

1. Aydin E, Karakas A, Savasci U, Akpak YK, Caymaz SO, Avci SAM, et al. Identification of Candida species isolated from clinical samples and investigating antifungal susceptibility in Turkey. Acta Med. 2014; 30:561.

2. Almeida AA, Mesquita CS, Svidzinski TI, Oliveira KM. Antifungal susceptibility and distribution of Candida spp isolates from the University Hospital in the municipality of Dourados, State of Mato Grosso do Sul, Brazil. Rev Soc Bras Med Trop. 2013; 46(3):335–9.

3. Yenisehirli G, Bulut N, Yenisehirli A, Bulut Y. In vitro susceptibilities of Candidaalbicans isolates to antifungal agents in Tokat, Turkey. Jundishapur J Microbiol. 2015; 8(9):e28057.

4. Badiee P, Alborzi A, Shakiba E, Farshad S, Japoni A. Susceptibility of Candida species isolated from immunocompromised patients to antifungal agents. East Mediterr Health J. 2011;17(5):425–30

5. Song JLHarry JB Eastman RTO liver BG et.al The Candida albicanslanosterol 14-alpha-demethylase (ERG11) gene promoter is maximally induced after prolonged growth with antifungal drugs. PMID 15047513 2009; 6: 56.

6. Clinical and Laboratory Standards Institute (CLSI).Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard, 3rd ed. CLSI document M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA. 2018.

7. Mayer FL, Wilson D, Hube B. Candida albicans pathogenicity mechanisms. Virulence. 2013; 4(2):119–28.

8. L. Sherry, R. Kean, E. McKloud et al., "Biofilms formed by isolates from recurrent vulvovaginal candidiasis patients are heterogeneous and insensitive to fluconazole," Antimicrobial Agents and Chemotherapy. 2017; vol. 61, no. 9.

9. R A Kashid S Belawadi G Devi D Dadich Characterization and antifungal susceptibility testing for Candida species in a tertiary care hospital J Health Sci Res 20112217

10. C S Amar Ashish J V Hajare Y Belagali Study of prevalence and antifungal susceptibility of candida Int. J Pharm Bio Sci. 2013. 4236181

11. Sailaja B, Prasad P**.** A study on isolation of Candida species in various clinical samples in a tertiary health care unit. Indian Journal of Microbiology Research. 2019; 6: 258-260.

12. Chitralekha Saikumar A. Arasi Samyuktha Isolation, Identification and Speciation of Candida Species from Various Clinical Specimens in a Tertiary Care Hospital in Chennai Sch J App Med Sci201758F34608. Indian Journal of micro2biology Research.

13. F lvarez-Lerma J Nolla-Salas C Len M Palomar R Jord N Carrasco Candiduria in critically ill patients admitted to intensive care medical units Intensive Care Med 2003297106976.

14. C A Kauffman Candiduria Clin Infect Dis 2005413717Supplement 6

15. Sankarankutty Jay Vipparti Harita Candida Species Isolated from Various Clinical Samples and their Susceptibility Patterns to Antifungals. J Med Microbiol Infec Dis. 201311226.

16. Kamal Uddin Zaidi, AbiniMani, VijayThawani, ArtiMehra. Total Protein Profile and Drug Resistance in Candida albicansisolated from clinical samples. Mol Bio Int 2016; 2016:4982131.doi:10.1155/2016/4982131

17. Lulu Zhang, XiaodongShe, Daniel Merenstein et.al. Fluconazole Resistance patterns in Candida species that colonize women with HIV infection. Current Therapeutic research. 2014; 76(C):84-89.

18. Lamsal,S., Adhikari, S., Raghubhansi, B.R., Sapkota,S., et.al Antifungal Susceptibility and Biofilm formation of Casndida albicans isolated from different clinical specimens. Tribhuvan University Journal of Microbiology,8(1),53-62.https://doi.org/10.3126/tujm.v8.41195.

19. Rathod SD, KlausnerJD, KruppK, ReingoldAL, AdhivananP.Epidemiologic features ofv Vulvovaginal Candidiasis among Reproductive Age women in India. Infect Dis Obst Gynaecol. 2012;2012:859071.

20. Henry, K.W., Nickels, J.T., Edlind, T.D.(2000). Up regulation of Erg Genes in Candida Species by Azoles and other sterol Biosynthesis Inhibitors. Antimicrob. Agents chemother.44,2693-2700.doi:10.1128/AAC.44.10.2693-2700.2000.

21. Fadda ME, Podda GS, Pisano MB, Deplano M, Cosentino S. Prevalence of Candida species in different hospital wards and their susceptibility to antifungal agents: Results of a three year survey. J Prev Med Hyg 2008; 49(2):69-74.

22. Shokohi T, Bandalizadeh Z, Hedayati MT, Mayahi S. In vitro antifungal susceptibility of Candida species isolated from oropharyngeal lesions of patients with cancer to some antifungal agents. Jundishapur J Microbiol. 2011;4(Suppl 1):S19–26.

23. Badiee P, Alborzi A, Shakiba E, Ziyaeyan M, Rasuli M. Molecular identification and in-vitro susceptibility of Candida albicans and C dubliniensis isolated from immu-nocompromised patients. Iran Red Crescent Med J. 2009;2009(4):391–7

24. Nazir A, Masoodi T. Spectrum of Candida species isolated from neonates admitted in an Intensive Care Unit of teaching hospital of Kashmir, North India. Journal of Laboratory Physicians. 2018; 10 (3):255-259.