Int.J.Curr.Microbiol.App.Sci (2023) *12*(02): 253-262

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 12 Number 2 (2023)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2023.1202.024

Antifungal Susceptibility Pattern of Candida Species Isolated From Urine Samples in a Tertiary Care Hospital in Salem

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Keywords

IDSA- Infectious diseases society of America ,Immunodeficiency, fungal infection, broad spectrum antibiotics, *Candida albicans*

Article Info

Received:
05 January 2023
Accepted:
06 February 2023
Available Online:
10 February 2023

A B S T R A C T

Use of broad spectrum antibiotics, immunodeficiencies and advances in medical practice have all contributed to increased fungal infection over the two decades and Candida has emerged as a major cause of human disease, furthermore there is an increase in nonalbicans candida species in many countries. In this study, an attempt was made to speciate, characterize, and determine the antifungal susceptibility pattern of Candida isolated from urinary tract infections (UTIs). The main aim and objectives of this study to isolate and identify Candida from urine samples in a tertiary care hospital; To determine the in vitro efficacy of antifungal agents of Candida species. And to detect susceptibility of Candida species to antifungal drugs isolated from urine samples. Also to determine antifungal sensitivity pattern by disc diffusion method as per CLSI guidelines. This study was conducted in GMKMCH, Salem between July 2022 and November 2022 among patients of age group more than 18 yrs admitted having candiduria. Patient who was on antifungal therapy within past 6 months were not included in the study. Urine samples sent from patients admitted in GMKMCH with candiduria are cultured in SDA. Antifungal susceptibility test performed by disc diffusion method using Muller hinton agar with 2% glucose and 0.005% methylene blue as per CLSI M22 latest guidelines.22 Results interpreted by using standard method as per CLSI M 22 guidelines. Determination of Antifungal sensitivity pattern of Candida species isolated. Out of the 8 *C.tropicalis* isolates all the 8 were sensitive to Amphotericin B and Voriconazole, 5 were sensitive to Fluconazole and Ketaconazole and 6 were sensitive to Itraconazole. In *C.albicans* all the 9 were sensitive for Amphotericin B and voriconazole and 6 were sensitive to Fluconazole, Ketaconazole and Itraconazole. 100% of the isolates of *C.parapsilosis* were sensitive to Amphotericin B and Voriconazole, and 50% of them were sensitive to Fluconazole, Ketaconazole, 75% of them were sensitive to Itraconazole. In *C.glabrata* species 100% sensitivity for Amphotericin B,Voriconazole and Itraconazole was observed. *C.krusei* was 100% sensitivity for Amphotericin B and Voriconazole, 66% were sensitive to Fluconazole, 33% of them were sensitive for ketaconazole. *C.tropicalis* was the most common Non albicans candida isolated. 37% of isolates showed resistance to Fluconazole. Resistance to Fluconazole in clinical isolates of *C.tropicalis* has increased. According to this study, there was an increase in the incidence and antifungal resistance of Non albicans Candida species, specifically *C. krusei*, *C. glabrata* and *C. tropicalis*. Due to the advent of non-albicans Candida species causing UTIs that are intrinsically resistant to certain antifungal agents like azoles and increasing incidence of antifungal resistance, it is essential to monitor the antifungal susceptibility profile of Candida species causing candiduria.

Introduction

Use of broad spectrum Antibiotics, Immunodeficiencies and advances in medical practice have all contributed to increased fungal infection over the two decades and Candida has emerged as a major cause of human disease, furthermore there is an increase in nonalbicans candida species in many countries (Subramanian Pramodhini *et al.*,). Candiduria is defined as the presence of yeast cells in urine (Kauffman *et al.*, 2011). It is increasing nowadays due to increased use of indwelling catheters, broad spectrum antibiotics (Sullivan *et al.*, 1996). Majority of the patients are asymptomatic and in symptomatic patients it is difficult to differentiate between bacteriuria and candiduria. Candiduria is considered as one of the marker of invasive candidiasis. *Candida albicans* and related species become resistant to antifungals. The emerging pathogens are resistant to conventional antifungal therapy. Moreover clinicians are usually most interested in quickly knowing the resistance of candida species to antifungals especially fluconazole. The importance of early and accurate identification of Candida species along with susceptibility testing for timely initiation of appropriate therapy cannot be overstated. In this study, an attempt was made to speciate, characterize, and determine the antifungal susceptibility pattern of Candida isolated from urinary tract infections (UTIs). Infectious diseases society of America (IDSA) guidelines 2016 states that – candidiasis is a serious, life threatening infection that needs to be treated early, aggressively and appropriately. (Pffaler and Diekema, 2007) There are various antifungals used in the treatment of candidiasis.

These consist of fluconazole, voriconazole, caspofungin, amphotericin B, and lipid formulations of amphotericin B. Fluconazole is most commonly used in the treatment of candidemia. Resistance to antifungal drugs is increasing in recent years (De Gregoria and Lee, 1982). The resistance of *C.albicans, C.tropicalis* and *C.glabrata* to fluconazole is increased compared to other drugs, due to indiscriminate use of fluconazole for long periods (Segal and Elan, 1998). *C.krusei* is intrinsically resistant to fluconazole. IDSA also states that, more than 90 % of invasive candidiasis is caused by *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis,* and *C. krusei*, and these have unique virulence potential, antifungal susceptibility and epidemiology (Peter G. Pappas and Carol A. Kauffman, 2016). All these factors has led to increase in the mortality and morbidity rates in patients with fungal infections warranting rapid identification and antifungal susceptibility testing at the earliest.

The main aim and objectives of this study to isolate and identify Candida from urine samples in a tertiary care hospital; To speciate and evaluate their distribution with the age, clinical diagnosis and comorbid conditions. To determine the in vitro efficacy of antifungal agents of Candida species. And to detect susceptibility of Candida species to antifungal drugs isolated from urine samples. Also to determine antifungal sensitivity pattern by disc diffusion method as per CLSI guidelines.

Materials and Methods

Type of study- Descriptive study.

Place of study- GMKMCH, Salem

Period of study – July 2022 to November 2022.

Study population- patients of age group more than 18 years and both sexes admitted in GMKMC with candiduria.

Study sample – urine sample.

Inclusion criteria-Patients of age group more than 18 years with Candiduria admitted in GMKMC.

Exclusion criteria-Patient on antifungal therapy within last 6 months duration

Urine samples sent from patients admitted in

GMKMCH with candiduria are cultured in SDA. Identification of candida species done by gram staining, germ tube test, hichrome candida agar and other standard methods. Antifungal susceptibility test performed by disc diffusion method using Muller hinton agar with 2% glucose and 0.005% methylene blue as per CLSI M22 latest guidelines.(Michael Klevay *et al.,* 2005) Results interpreted by using standard method as per CLSI M 22 guidelines. Determination of Antifungal sensitivity pattern of Candida species isolated

Collection and processing of urine sample

Urine

After washing the hands with soap and water, the female patients were instructed to clean the area around the urethral opening with soap and water, after cleaning, the area was dried by using a sterile gauze pad, and then the mid stream urine was collected with the labia held apart. In case of male patients, the same procedure was followed after retracting the foreskin. Clean catch midstream urine was collected in a sterile, dry, leak proof, transparent screw capped container. The urine was processed within 2 hrs. Catheter Collection: The area was disinfected before proper collection of samples. Urine samples were aspirated using a sterile syringe and needle (gauge no.28), through the soft rubber connecter between catheter and collecting tubing. The macroscopic appearance of the urine was noted. Then the urine is examined for the pus cells, budding yeast cells and pseudohyphae by wet mount preparation. If budding yeast cells are present in wetmount then the urine samples are streaked in Sabouraud dextrose agar SDA slants and incubated at 37◦c for 24 hours[3]. From the colonies obtained in SDA Gram staining performed to identify gram positive budding yeast cells. Then germ tube test is performed by adding 5 to 6 colonies to 0.5 ml of freshly prepared serum and incubated at 37◦c for 2 to 4 hours to differentiate candida species into candida albicans and noncandida albicans by germ tube formation. Isolates producing germ tubes were presumptively identified as *C.albicans* or *C.dubliniensis*. (Wasington Winn *et al.*,; Mackie and Mccartney) Colonies inoculated in SDA were kept at 45°C in a waterbath. *C.albicans* were able to grow at this temperature while *C. dubliniensis* did not grow. CHROM agar is a selective media for the isolation and identification of different species of Candida.. Isolates were plated directly from SDA to HI-Chrom agar and incubated at 30°C for 48 hours.

The various species of Candida were identified by their colony color, size, texture, and presence of color diffusion into the surrounding agar presumptively in 48hrs *C. albicans* ATCC 90028 was used as the control strain.: Colours produced by various Candida species *Candida albicans*- Green, *C. dubliniensis* -dark green, *C.tropicalis* -dark blue, *C.krusei*- Dry pink, *C.parapsilosis*- white to pale pink, *C.glabrata* -white to pink. The findings were recorded and tabulated.

Sugar fermentation

Liquid medium containing peptone, 2% sugar and indicator was poured in a test tube (~5ml) and Durham's tube was placed into each tube. Heavy inoculums of yeast colonies were suspended into each tube and incubated at 25°C for 1 week (Nur Yapar) tubes were examined at 48- 72 hours interval for acid (pink color) and gas (in durham's) production. *C.albicans* ATCC 90028 was used as the control strain. Sugar assimilation (Auxonographic techniques): 18ml quantities of agar and distilled water was dispensed in screw capped tubes. Autoclaved at 121°C for 20 minutes and stored at 4°c. A heavy inoculums yeast suspension was prepared from 24 hours old culture in 2ml Yeast Nitrogen Broth. The prepared suspension was poured into 18 ml of molten agar cooled at 45°C, and then poured into a 90mm Petri plate. The Petri plate was set at room temperature until the agar surface hardens. Sugar discs were placed in a circle with sterile forceps, such that they were at least 30mm was present between centers of each disc and was incubated at 37°C 3-4 days. Presence of growth around each disc indicates assimilation (Larone, 2011): Fermentation of different sugars by different species. *C.albicans* ATCC 90028 was used as the control strain.

Antifungal susceptibility testing

Antifungal Susceptibility Testing for Candida isolates was done by Disc diffusion method, as per CLSI Guidelines on Antifungal Susceptibility testing in M-51A document. Mueller-Hinton agar with 2% glucose and 0.5 µg/ml methylene blue. Inoculum was prepared by picking five distinct colonies from a 24-hour-old culture of Candida species and the colonies were suspended in 5 ml of sterile normal saline.

The turbidity was adjusted equivalent to 0.5 McFarland standards (1x 10^6 to 5 x 10^6 cells/ml) resulting in semi-confluent growth. A lawn culture was made on the dried surface (Wickerham and Bruton, 1948) of the agar by streaking the cotton swab according to the standard three directional method. The plate was left open for 3 – 5 minutes, allowing excess moisture to be absorbed, and then the antifungal disc were dispensed onto the plate. Plates were inverted and incubated at 35°C ±2°C within 15 minutes after placing the discs. The zone is read after 20-24 hours of incubation. If no visible growth with particular strains, the plate is re-incubated for 48 hours and then read. Zone of inhibition was measured at the point where there was prominent reduction in growth.

Results and Discussion

In this study 47urine samples obtained from various wards had yeast cells and pseudohyphae on wet mount examination. Repeat urine sample was collected from these patients to rule out mere colonisation, Only when the repeat sample showed yeast cells and pseudohypae on wet mount examination was processed further. Out of the 47 samples for which repeat sample is collected 26 samples had shown yeast cells on wet mount.

Germ tube test done for these 26 samples. Out of the 26 isolates tested for germ tube production 9 were germ tube positive and 17did not produce germ tubes.

Age wise distribution

13case were from the age group of more than 60 and 9 were from age group of 40 to 60 years and 4 cases were from 18 to 40 years of age.

Association of comorbid condition

17 cases were with comorbid conditions like diabetes, CVA, autoimmune diseases, cancer etc

All the 26 Candida isolates were checked for growth on CHROM agar and the colour changes were noted. The speciation was made according to the colour noticed and sugar fermentation and assimilation test and speciation is given in Table

All the candida isolates were sensitive to Amphotericin B and Voriconazole. Sensitivity to Fluconazole is lowest in *C.krusei* and almost 70% in other species. Very low sensitivity to ketaconazole is noted among all candida species. Overall sensitivity to Itraconazole is around 80% among all species of candida.40% of *C. krusei* is resistant to fluconazole and 80% to Ketaconazole.

Over the last two decades, fungal infections have increased at an alarming rate. Candidiasis has emerged as a very important opportunistic infection. In the last 20 years with the surge of non albicans Candida species, Clinical importance of species level identification is important as they differ in expression of virulence factors and antifungal susceptibility.

The present study was undertaken to speciate the Candida isolated from urine samples, to detect their antifungal susceptibility pattern. The study also concentrated on the changes observed in species distribution, the shift towards non albicans Candida species. in our hospital. The reported incidence of fungal infections associated with Non albicans Candida species is increasing in recent times. This is related largely to a dramatic increase in the number of individuals with deficient cellular immunity, particularly those infected with HIV and individuals

receiving immunosuppressive treatments, both in organ transplantation and in anticancer therapy. (Sullivan *et al.*, 1996)

Out of the 26 isolates tested for germ tube production 9 were germ tube positive and 17 did not produce germ tubes. In the study conducted by Deorukhar *et al.*, (2014) in Maharashtra, India a total of 523 Candida spp. were isolated from various clinical specimens, Non albicans Candida species had a higher prevalence compared to *C.albicans*. In our study, the highest incidence of candidiasis was found to be in persons more than 60 years.

Table.1 Zone Size Interpretive chart For Antifungal Susceptibility Test

Drugs	Sensitive	Intermediate	Resistant
Fluconazole	≥19	15-18	≤14
Voriconazole	≥17	14-16	≤13
Ketoconazole	≥28	27-21	≤ 20
Itraconazole	≥15	10-14	≤ 9
Amphotericin - B	≥19	15-18	≤14

Table.2

Colour -chrom agar	Species	No
Blue purple	*C.tropicalis*	8
Light green	*C.albicans*	9
Mauve	*C.parapsilosis*	4
Purple	*C.glabrata*	2
Pink	*C.krusei*	3

Table.3 Species Identification by Sugar Fermentation

Species	Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose
C.tropicalis	8	8	8	-	8	8
C.albicans	9	9	-	-	9	9
C.parapsilosis	4	-	-	-	-	-
C.glabrata	2	-	-	-	-	2
C.krusei	3	-	-	-	-	-

Table.4 Species Identification by Sugar Assimilation

Candida species	Glucose	Maltose	Sucrose	Lactose	Galactose	Xylose	Trehalose
C.tropicalis	8	8	8		8	8	8
C.albicans	9	9	9		9	9	9
C.parapsilosis	4	4	4		4	4	4
C.glabrata	2	2	2				2
C.krusei	3	3	3				

Table.5 Antifungal Susceptibility pattern of Candida species

Name of the Candida Species	Amphotericin B sensitive	Voriconazole	Fluconazole	Ketaconazole	Itraconazole
C. tropicalis	8	8	5	5	6
C.albicans	9	9	6	6	6
C. parapsilosis	4	4	2	2	3
C. glabarata	2	2	1	1	2
C.krusei	3	3	2	1	-

Fig.1

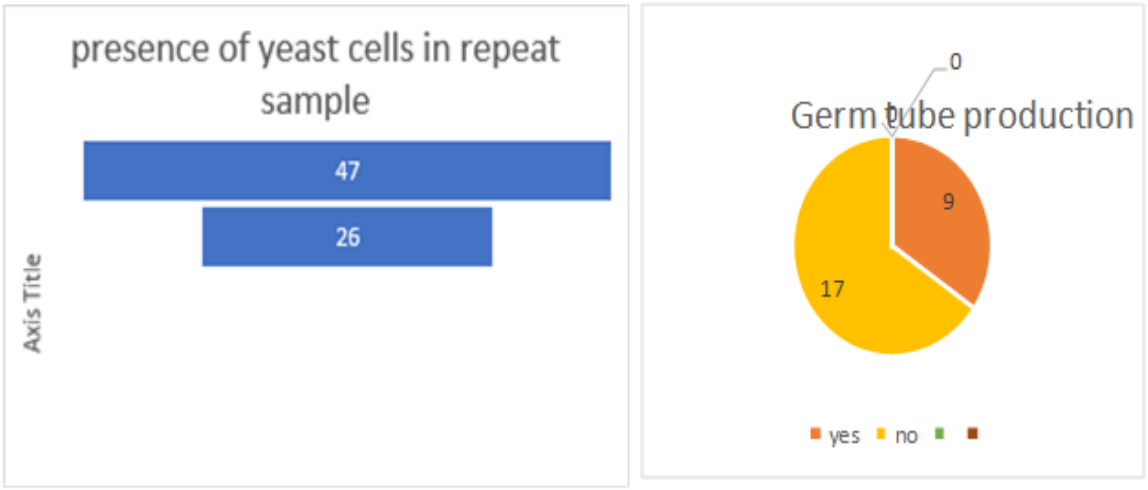


Fig.2

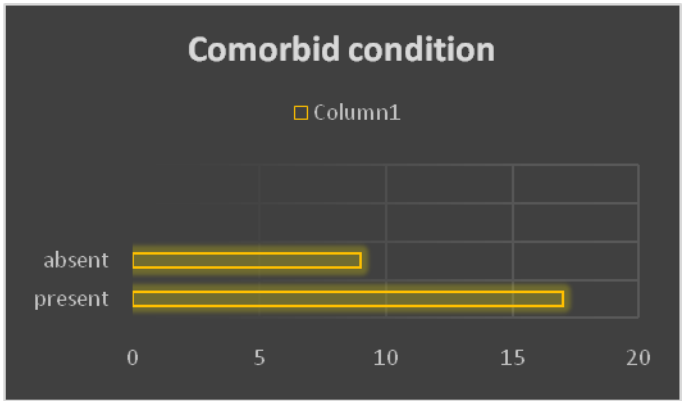


Fig.3

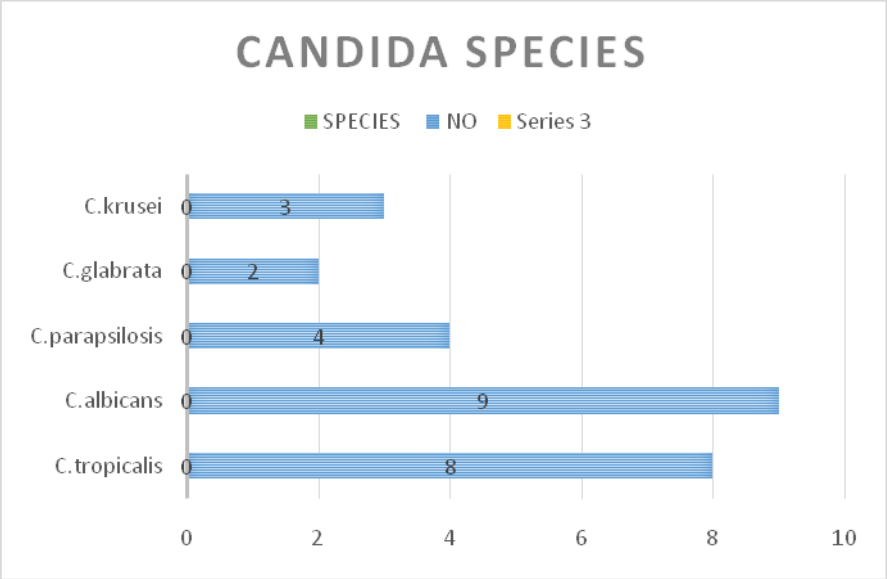


Fig.4

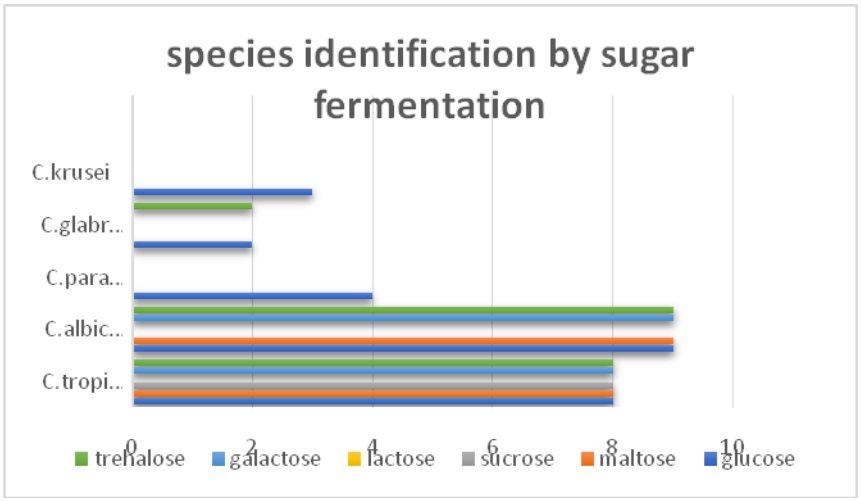


Fig.5

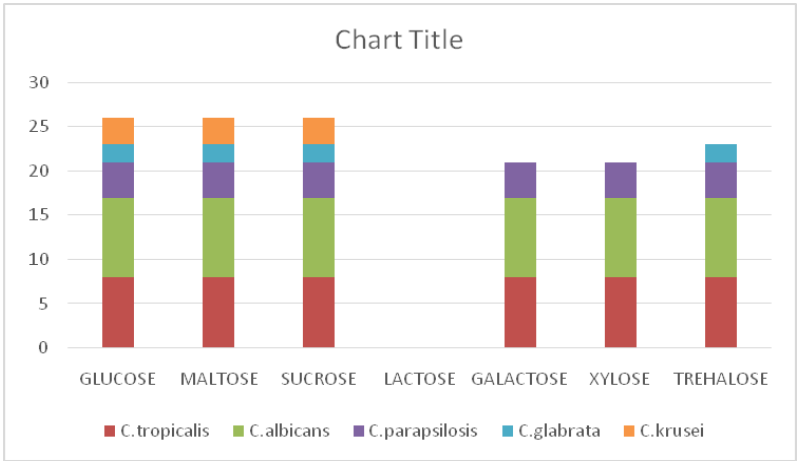
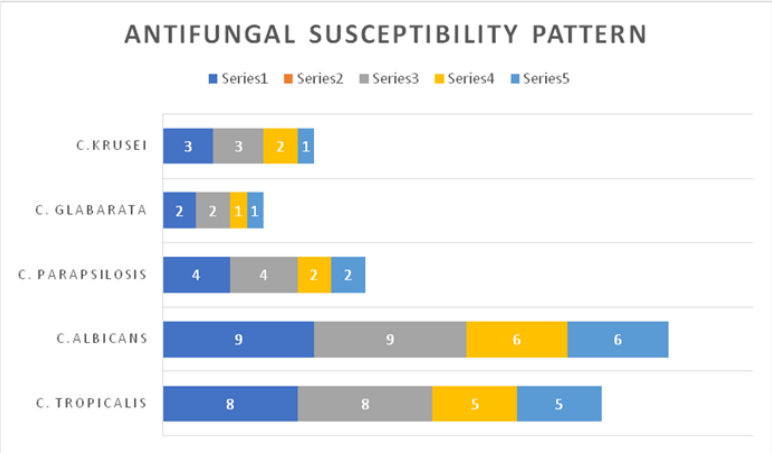


Fig.6



These findings were in concordance with the study conducted by Holley *et al.*, (2009) in France among 137 patients admitted in ICU, where the median age of patients was 63 years. This might be due to the co-morbid conditions like diabetes mellitus, renal failure, neoplastic disease, use of chemotherapeutic agents etc, which is common in this age group.

C.tropicalis was the most common Non albicans candida isolated. 37%of isolates showed resistance to Fluconazole. Resistance to Fluconazole in clinical isolates of *C. tropicalis* has increased. Yang *et al.*, (2004) in Taiwan described a higher incidence of Fluconazole resistance in Candida tropicalis, Virulence factors expression by Candida species contribute to the pathogenesis by secreting various hydrolytic enzymes by facilitating its adherence to host tissue, cell membranes rupture, mucosal surfaces and blood vessels invasion, and evading immune system of host. Phospholipases hydrolyses the fatty acids from phospholipids of mammalian cell membranes, thereby destabilizing the membranes, which in turn facilitate the tissue invasion and dissemination of infections. To establish their persistence and survival in the host cells, hemolysin act upon by RBC lysis and acquire elemental iron from hemoglobin. Biofilm formation is considered as one of the most important crucial virulence factors by attaching to body sites and further proliferation. Thus, virulence factors help the organisms to evade host defense mechanisms and also to establish their pathogenicity (Schaller *et al.*, 2005).

A significant epidemiological shift to higher isolation of Non albicans Candida species was noticed. According to our study, there was an increase in the incidence and antifungal resistance of Non albicans Candida species, specifically *C. krusei*, *C. glabrata* and *C. tropicalis*. Due to this rise in non-albicans Candida species causing UTI that are intrinsically resistant to certain antifungal agents like azoles and increasing incidence of antifungal resistance, it is essential to monitor the antifungal susceptibility profile of Candida species causing candiduria.

The risk of developing candiduria was increased 12-fold after urinary catheterization, six fold after the use of broad spectrum antibiotics and urinary tract abnormalities, fourfold following abdominal surgeries, two fold in the presence of diabetes mellitus, and one fold on corticosteroid administration. The isolation and their resistance pattern make us to consider the need for surveillance system for fungal isolates. Isolation of Candida in urine is often ignored as a commensal or a contaminant and therapeutic intervention to patients with candiduria always needs to be individualized considering the underlying risk factors, extent of the disease and renal function, to arrest further dissemination of infections. At the same time mere

colonisation should not be treated because it results in increasing resistance to antifungal drugs. Due to the advent of non-albicans Candida species causing UTIs that are intrinsically resistant to certain antifungal agents like azoles and increasing incidence of antifungal resistance, it is essential to monitor the antifungal susceptibility profile of Candida species causing candiduria. More studies on the relevance of virulence factors of Candida are needed to further understand the pathogenesis of candidiasis and also to guide the exploration of new antifungal drug targets to ensure better outcomes for patients as mortality with candiduria can be high in debilitated patients and those in advanced age.

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How to cite this article:

Suganya, R G. Kavitha and Rajesh Sengodan. 2023. Antifungal Susceptibility Pattern of Candida Species Isolated From Urine Samples in a Tertiary Care Hospital in Salem. *Int.J.Curr.Microbiol.App.Sci.* 12(02): 253-262. **doi:** https://doi.org/10.20546/ijcmas.2023.1202.024