Candiduria—Study of Virulence Factors and Its Antifungal Susceptibility Pattern in Tertiary Care Hospital

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Abstract

Background The increased incidence of candiduria in hospitalized patients is due to the use of indwelling devices, long-term antibiotics, parenteral nutrition, and immunocompromised status of the patient. In this study, an attempt was made to speciate, characterize, and determine the antifungal susceptibility pattern of *Candida* isolated from urinary tract infections (UTIs).

Materials and Methods A total of 70 *Candida* isolates were obtained from urine samples. The isolated *Candida* species were studied for the production of virulence factors like phospholipase, protease activities, hemolysin, and biofilm production. Antifungal susceptibility testing of the isolated yeasts was done using Mueller-Hinton agar supplemented with 0.5 mg/mL methylene blue by E-test method for amphotericin B, fluconazole, caspofungin, and voriconazole.

Results Out of 70 isolates, *Candida tropicalis* was the most frequently isolated species (65.7%), followed by *Candida albicans* (14.3%), *Candida glabrata* (7.1%), *Candida krusei* (5.7%), *Candida parapsilosis* (4.3%), and *Candida dubliniensis* (2.9%). A total of 37.1% were biofilm producers, 62.9% showed proteinase activity, 38.6% were phospholipase positive, and 58.6% isolates showed hemolytic activity. Antifungal susceptibility profile of *Candida* species showed 38.6, 25.7, 15.7, and 12.9% resistance to amphotericin B, fluconazole, caspofungin, and voriconazole, respectively.

Conclusion A rising trend in isolation of non-albicans *Candida* from urinary isolates was noticed, which was statistically significant when comparing catheterized and noncatheterized urinary isolates from our study. However, there was no statistically significant difference when different virulence factor expressions were compared among *Candida* spp. isolated from catheterized and noncatheterized urinary samples. 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.

Keywords
- virulence
- biofilm
- proteinase
- phospholipase
- hemolysin

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Introduction

Candiduria, by definition, is the presence of yeast cells in urine. There is a rising trend of candiduria in hospitalized patients, due to the increased use of indwelling devices, parenteral nutrition, broad spectrum antibiotics, and chemotherapeutic agents.[1] Majority of patients diagnosed with candiduria do not manifest any symptoms and it is indeed very difficult to differentiate between bacteriuria and candiduria in symptomatic patients. In intensive care unit patients, presence or absence of symptoms should not be neglected, as candiduria are considered as one of the marker for invasive candidiasis. In addition, the expression of various virulence factors like proteinase, phospholipase, hemolysin and biofilm formation contributes to the pathogenicity of *Candida* spp. Identification of *Candida* species is very crucial for the administration of antifungals due to the intrinsic resistance of non-albicans *Candida* to azoles. In this study, an attempt was made to speciate, to study the virulence factors, and to determine the antifungal susceptibility pattern of *Candida* isolated from urinary tract infections (UTIs).

Materials and Methods

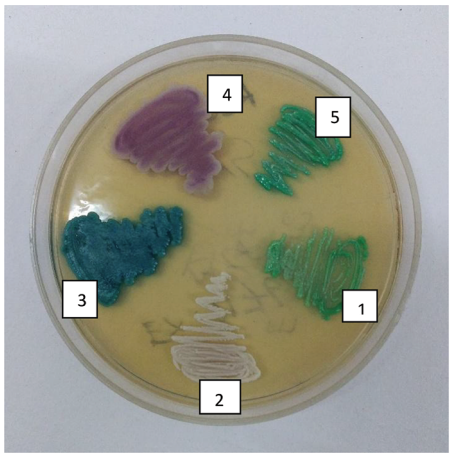
This prospective analytical study for a period of 1 year was conducted from a single center in a tertiary care hospital in South India. A total of 70 *Candida* spp. were studied from urine samples either mid stream urine or catheterized from OPD, wards, or ICU patients, which were sent to the microbiology laboratory for fungal culture and sensitivity. The inclusion criteria for the study were that the patients were above 18 years of age, *Candida* isolated as a pure growth with a significant colony count of ≥ 10^4 colony forming units (CFUs)/mL of urine, isolates where direct microscopic examination showed concomitant pyuria, and isolates recovered from the second sample of the same patient. Absence of pyuria or mixed growth was excluded from our study. This study was done after approval by Institutional Ethical committee.

The urine samples obtained were immediately processed in the microbiology laboratory by semiquantitative method as per standard procedures. For fungal culture, samples were inoculated on to Sabouraud dextrose agar (SDA) and incubated at 25°C. Identification of the growth on SDA plates was done by colony morphology, Gram-staining, and standard biochemical reactions.[2] Chromogenic Agar (HiMedia) was used to identify *Candida* species (►**Fig. 1**). Speciation of *Candida* isolates was done by testing for germ tube formation, growth on corn meal agar, sugar assimilation, and sugar fermentation tests.[3]

Candida isolates were studied for the production of virulence factors like phospholipase, protease activity, hemolysin, and biofilm production by following methods:

Phospholipase Activity Detection

After the inoculum showed growth on egg yolk agar, detection of *Candidal* phospholipase activity was done measuring the zone of precipitation. The organism was considered to be positive for phospholipase activity, if they produce a zone of precipitation surrounding the colony. The ratio of the diameter of the colony to the total diameter of the precipitation zone (including the colony) is measured as the value of phospholipase activity (Pz). If the measured value of Pz value is less than one of 1 (Pz < 1), it indicates positive phospholipase activity and Pz value equal to 1 means no phospholipase activity **(►Fig. 2)**. *Candida albicans*—reference strains (ATCC 10231 and ATCC 24433) were the positive controls.[4-6]



1-Candida albicans 2- Candida parapsilosis 3- Candida tropicalis 4- Candida krusei 5-Candida dubliensis

Fig. 1 Growth of *Candida* spp. on Hicrome Agar.

Proteinase Activity Determination

Measurement of *Candidal* extracellular proteinase activity was done as per the technique described by Staib,[7] using bovine serum albumin. Degradation of the protein was determined when an opaque zone was seen around the colony that was unstainable with amido black.

Calculation of proteinase activity (Prz) was done by measuring the ratio of the colony to the diameter of the proteolytic unstained zone. A Prz value corresponding to one signified no activity, and value less than one meant positive proteinase activity. *C. albicans* reference strains (ATCC 10231 and ATCC 10261) were the positive controls.

Hemolysin Activity Determination

Evaluation of hemolysin activity was done by a blood plate assay as explained by Manns et al.[8] The ratio of the diameter of the colony to that of the translucent zone of hemolysis (in mm) around the colony was determined as the hemolytic index (Hz value). *C. albicans* (ATCC 90028) was used as the positive control (see ►**Fig. 3**).[4,8]

Detection of Biofilm Production

Biofilm formation was detected by method proposed by Branchini and Pfaller.[9] A loopful of colony from SDA plate was inoculated into 10 mL of Sabouraud liquid broth with 8% glucose supplemented and kept for incubation at 37°C for 48 hours. Following this, the broth was aspirated from the tube and the walls were stained using Safranin. Biofilm formation was graded as negative (0+) or 1+, 2+, and 3+

corresponding with weak, moderate, and strong positivity (►**Fig. 4**).

Antifungal Susceptibility Testing

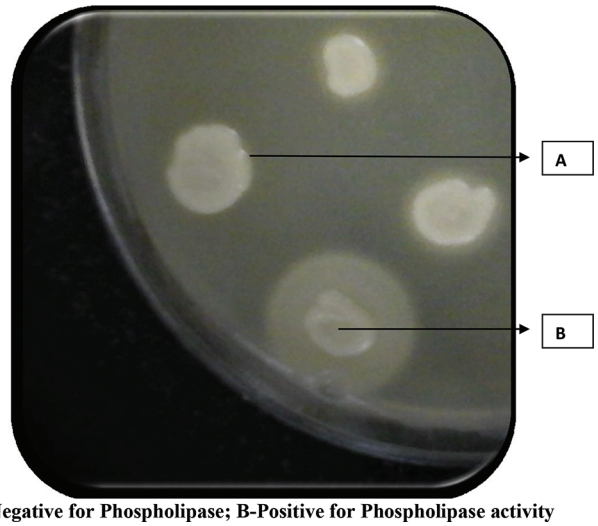
Antifungal susceptibility testing of the isolated yeasts was performed on Mueller-Hinton agar supplemented with 0.5 mg/mL methylene blue by E-test method (Hi-Media) for—amphotericin-B, fluconazole, caspofungin, and voriconazole. Interpretation of susceptibility was done using the breakpoint criteria defined by the CLSI document M44.[10] The MIC ranges used in this study for various antifungal drugs were—amphotericin B (0.002–32 µg/mL), fluconazole (0.016–256 µg/mL), voriconazole (0.002–32 µg/mL), and caspofungin (0.002–32 µg/mL) (►**Fig. 5**).

Statistical Analysis

All data were entered into MS Excel 2010 and analyzed. Percentages were calculated for categorical variables.

Results

In this study, 70 adult patients were diagnosed with candiduria, 43 (61.4%) of them were female and 27 (38.6%) were male. Age group analysis showed that 12 (17.1%) patients were between 18 and 30 years, 10 (14.3%) were between 31 and 45 years, 16 (22.9%) were between 46 and 60 years, 26 (37.1%) were between 61 and 75 years, and 6 (8.6%) were older than 76 years (►**Table 1**). Out of 70 isolates, 12 (17.1%) were from outpatients and 58 (82.8%) were isolated from inpatients. Isolated *Candida* spp. from catheterized and noncatheterized urine samples were found to be 30 (42.9%) and 40 (57.1%), respectively (►**Table 2**). Among 30 isolates from catheterized urine samples, 10 (33.3%) and 20 (66.7%) were found to be *C. albicans* and non-albicans *Candida,* respectively. Among 40 *Candida* isolates from catheterized urine samples, only 2 (5%) were identified as *C. albicans*, the remaining 38 (95%) were identified as non-albicans *Candida*. The predominant species isolated in our study was *Candida tropicalis* representing 65.7% of isolates, followed by *C. albicans* (14.3%), *Candida glabrata* (7.1%), *Candida krusei* (5.7%), *Candida parapsilosis* (4.3%), and *Candida dubliniensis* (2.9%) (►**Table 3**). Positivity values of hemolysin, proteinase, phospholipase, and biofilm in isolates from catheterized sample were 56.7, 70.0, 43.3, and 40.0%, respectively, and from noncatheterized samples were around 60, 57.5, 35, and 35%, respectively (►**Table 4**). Antifungal susceptibility profile of *Candida* species showed 38.6% were resistant to amphotericin B, 27.1% were resistant to fluconazole, 15.7% resistant to caspofungin, and 14.3% resistant to voriconazole (►**Tables 5** and **6**).



A-Negative for Phospholipase; B-Positive for Phospholipase activity

Fig. 2 Phospholipase production in Egg yolk agar.

Discussion

Candiduria is rarely seen as a community-acquired infection in a structurally normal urinary tract and in healthy people. It has been increasingly reported as an important subgroup of nosocomial UTIs (10–15%) and almost all are caused by *Candida* spp.[11] In a recent case-controlled study, it has been shown that the risk of developing candiduria was increased 12-fold after urinary catheterization, sixfold after the use of broad spectrum antibiotics and urinary tract abnormalities, fourfold following abdominal surgeries, twofold in the presence of diabetes mellitus, and onefold on corticosteroid administration.[12]

In our study, majority of patients identified with candiduria were in the age group of 61 to 75 years (37.1%), followed by 46 to 60 age group (22.9%). Studies have shown high prevalence rate of UTIs in elderly people, which is concordant with our study.[13] However, there are studies showing higher prevalence rates in patients belonging to 51 to 60 years of age group.[14]

Frequency of distribution of candiduria in females (43, 61.4%) was higher in our study when compared with males (27, 38.6%). Several similar studies have shown female predominance in the incidence of candiduria with prevalence rates of 61.2, 66.2, and 70.2% which are in agreement with our study.[15-17]

In our study, non-albicans *Candida* spp. emerged as the predominant pathogen contributing to 82.9% of fungal UTI. Of which, *C. tropicalis* was most frequently isolated (65.7%), while *C. albicans* were identified in only 17.1% of the cases. The result of the present study is in agreement with other studies, where increased prevalence of non-albicans *Candida* causing UTI was reported.[18-20] Overall, there has been a rising trend of non-albicans *Candida* spp. causing candiduria, the most prevalent of which was *C. tropicalis*.[18]

On detecting candiduria, treating physicians have to decide whether it is a result of UTI (upper or lower) or bladder colonization, or urine sample contamination. Contamination can be differentiated from colonization or infection by obtaining another urine sample to verify candiduria.[1]

A study on the predominance of *Candida* species isolated, reported *C. albicans* being seen in more than 51% cases, followed by *C. glabrata* and *C. tropicalis*.[13] Pathogenicity of *Candida* is enhanced by several virulence determinants like host surface adherence, secretion of hydrolytic enzymes, and formation of pseudohyphae.[14]

Table 1 Age and sex distribution pattern of *Candida* spp. from urine samples (n = 70)

Age	Male	Female	No %
15–30	1	11	12 (17.1%)
31–45	6	4	10 (14.3%)
46–60	6	10	16 (22.9%)
61–75	12	14	26 (37.1%)
> 76	2	4	6 (8.6%)
Total	27 (38.6%)	43 (61.4%)	70 (100%)

Table 2 Distribution of cases among urinary isolates of *Candida*

IP/OP	Catheterized	Noncatheterized	No (%)
OP	0	12	12 (17.1%)
IP	30	28	58 (82.8%)
Total	30 (42.9%)	40 (57.1%)	70 (100%)

Table 3 Percentage of distribution of urinary isolates of *Candida* species

Species	Catheterized (30)		Noncatheterized (40)		Total		*p*-Value
	No	%	No	%	No	%	
Candida albicans	10	33.3	2	5	12	17.1	The *p*-value is 0.001854. Significant at $p < 0.05$.
Non-albicans *Candida*	20	66.7	38	95	58	82.9	
• *Candida tropicalis*	15	50.0	31	77.5	46	65.7	
• *Candida glabrata*	1	3.3	3	7.5	4	5.7	
• *Candida krusei*	0	0.0	3	7.5	3	4.3	
• *Candida parapsilosis*	2	6.7	1	2.5	3	4.3	
• *Candida dubliniensis*	2	6.7	0	0	2	2.9	
Total	30	100.0	40	100	70	100	

Table 4 Detection of virulence among *Candida* isolated from catheterized and noncatheterized urine samples

Virulence factors	Catheterized (30)		Noncatheterized (40)		Total		*p*-Value	
	Positive	%	Positive	%	No	%	*p*-Value	Significance
Hemolysin	17	56.7	24	60	41	58.6	0.779344	Not significant at $p < 0.05$.
Proteinase	21	70.0	23	57.5	44	62.9	0 0.137233	
Phospholipase	13	43.3	14	35	27	38.6	0 0.47843	
Biofilm	12	40.0	14	35	26	37.1	0 0.668326	

In the current study, a rising trend in isolation of non-albicans *Candida* from urinary isolates was noticed, which was statistically significant when comparing catheterized and noncatheterized urinary isolates in our study ($p > 0.05$ using Fisher's exact test).[20,21]

Also, owing to the problem of intrinsic resistance of some non-albicans *Candida* species to azole group of antifungal agents, empirical treatment of candiduria with azole group of antifungal agents would not be effective. This further validates the importance of speciation of *Candida*.

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.[21]

In our study, proteinase activity was demonstrated in 62.9% of the isolates, 58.6% showed hemolysin positivity, phospholipase activity in 38.6% isolates, and 37.1% were biofilm producers. Study by Alenzi,[22] on the virulence factor of urinary isolates of *Candida* showed similar percentage

Table 5 Antifungal susceptibility profiles of Candida species isolated from urine (n = 70)

Candida species	MIC interpretative value			Observation		
	S	SDD or I	*R*	*S*%	SDD or I (%)	*R* (%)
Candida tropicalis (46)						
Amphotericin B	≤ 1		≥ 2	31		15
Fluconazole	≤ 2	4	≥ 8	30	4	12
Voriconazole	≤ 0.12	0.25–0.5	≥ 1	41	2	3
Caspofungin	≤ 0.25	0.5	≥ 1	38	2	6
Candida albicans (10)						
Amphotericin B	≤ 1		≥ 2	8		2
Fluconazole	≤ 2	4	≥ 8	7	3	0
Voriconazole	≤ 0.12	0.25–0.5	≥ 1	8	2	0
Caspofungin	≤ 0.25	0.5	≥ 1	9		1
Candida glabrata (5)						
Amphotericin B	≤ 1		≥ 2	2		3
Fluconazole	≤ 8	16–32	≥ 64	2	2	1
Voriconazole	0	0	0	–		–
Caspofungin	≤ 0.12	0.25–0.5	≥ 1	2	1	2
Candida krusei (4)						
Amphotericin B	≤ 1		≥ 2	1		3
Fluconazole	0	0	0	–		–
Voriconazole	≤ 0.5	1	≥ 2	0		4
Caspofungin	≤ 0.25	0.5	≥ 1	2	1	1
Candida parapsilosis (3)						
Amphotericin B	≤ 1		≥ 2	1		2
Fluconazole	≤ 2	4	≥ 8	2		1
Voriconazole	≤ 0.12	0.25–0.5	≥ 1	2	1	0
Caspofungin	≤ 2	4	≥ 8	2		1
Candida dubliniensis (2)						
Amphotericin B	≤ 1		≥ 2	0		2
Fluconazole	≤ 2	4	≥ 8	1		1
Voriconazole	≤ 0.12	0.25–0.5	≥ 1	1		1
Caspofungin	≤ 0.25	0.5	≥ 1	1	1	0

Abbreviations: MIC, minimum inhibitory concentration; SDD, susceptible-dose-dependent.

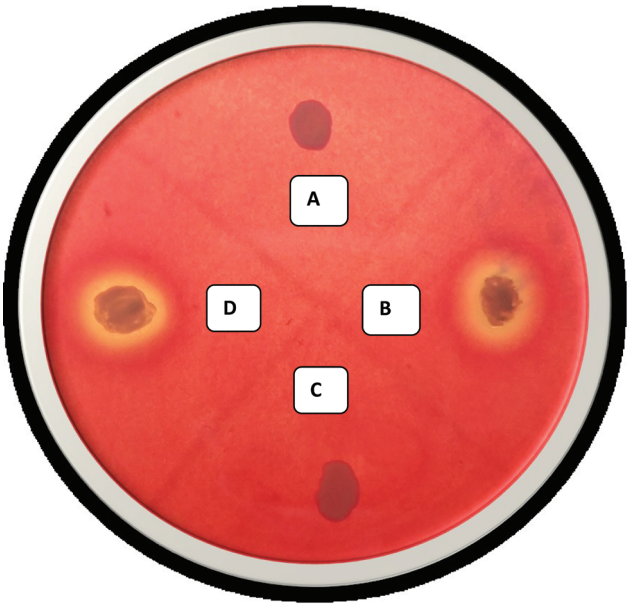
of expression of virulence factors for biofilm and phospholipase activity, except for proteinase activity, which was on higher rate in our study. Proteinases degrade host epithelial and mucosal barrier proteins including keratin, collagen, and mucin. These help *Candida* in resisting the cellular and humoral components of the host immunity by degrading antibodies, complement, and cytokines.[23]

In a different study by Sachin et al,[24] phospholipase, proteinase, and hemolysin activity was seen in 60.9, 59.1, and 51.8% of *Candida* isolates, respectively. A similar study by Udayalaxmi et al,[25] among urinary isolates of *Candida* detected that 50% of their isolates were biofilm producers, 97.6% were hemolysin positive, and 37.1% showed phospholipase activity. The difference in the virulence factor expression among clinical isolates of *Candida* depends on various factors such as the infecting *Candida* species, geographical region, infection site type, and stage of infection as well as host immunity. A comparative study of different virulence factors expression among *Candida* spp. isolated from catheterized and noncatheterized urinary samples showed no statistically relevant difference in the production of virulence factors such as hemolysin, phospholipase, proteinase, and biofilm producers. Thus virulence factor expression is influenced by various other contributing factors such as infecting species, geographical origin, type, site and stage of infection, underlying risk factors, comorbidities, and host immune response.

In our study, antifungal susceptibility profile of *Candida* species showed 27 (38.6%), 19 (22.7%), 11 (15.7%), and

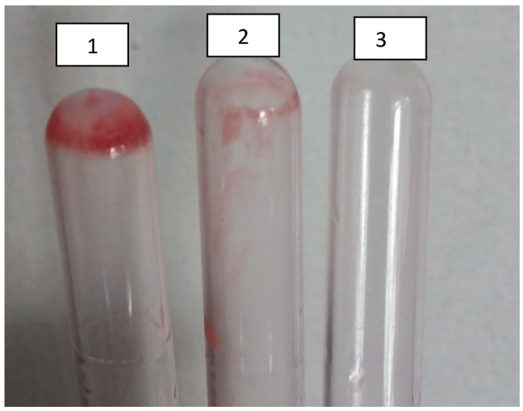
Table 6 Overall susceptibility pattern of *Candida* species isolated from urine (n = 70)

Candida species	Amphotericin B		Caspofungin		Voriconazole		Fluconazole	
	S	R	S	R	S	R	S	R
Candida tropicalis (46)	31	15	40	6	43	3	34	12
Candida albicans (10)	8	2	9	1	10	0	10	0
Candida glabrata (5)	2	3	3	2	3	2	4	1
Candida krusei (4)	1	3	3	1	0	4	0	4
Candida parapsilosis (3)	1	2	2	1	3	0	2	1
Candida dubliniensis (2)	0	2	2	0	1	1	1	1
Total (70)	43 (61.4%)	27 (38.6%)	59 (84.3%)	11 (15.7%)	60 (85.7%)	10 (14.3%)	51 (72.9%)	19 (27.1%)



A, C -No hemolysis; B, D -Positive for hemolysin

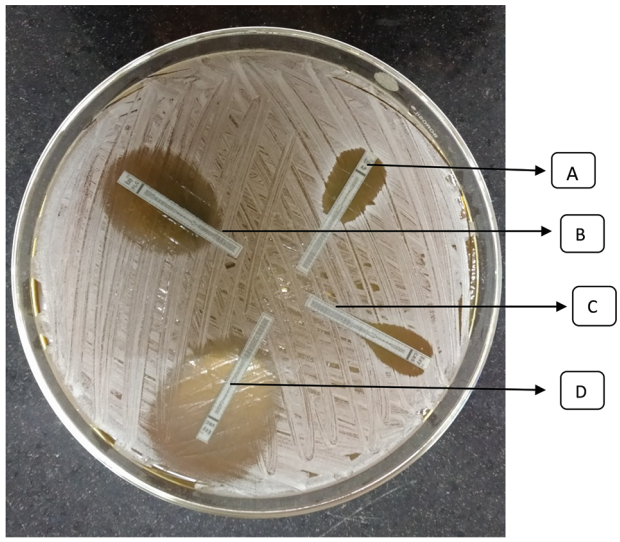
Fig. 3 Hemolysin production of *Candida* spp. in Blood agar.



1-Strong positive 2-Weak positive 3-Negative

Fig. 4 Biofilm formation by tube adherence method.

10 (12.3%) resistance to amphotericin B, fluconazole, caspofungin, and voriconazole, respectively. Similar percentage of resistance to fluconazole 22/90 (24.4%) and



A- Amphotericin B ; B-Fluconazole; C-Caspofungin ; D-Voriconazole

Fig. 5 Antifungal susceptibility testing by E-test method.

voriconazole 17/90 (18.88%) was observed in a study by Marak et al[14] Another study by Yenisehirli et al,[26] on antifungal susceptibility of *C. albicans*, observed 34% resistance to fluconazole and 14% resistance to voriconazole well comparable to our study. The resistance rates derived from our study for fluconazole and voriconazole are similar to that of earlier studies. The reduced susceptibility of *Candida* spp. to fluconazole and voriconazole is likely due to long-term and widespread use of antifungals among the study subjects.

Conclusion

A rising trend in isolation of non-albicans *Candida* from urinary isolates was noticed, which was statistically significant when comparing catheterized and noncatheterized urinary isolates from our study. However, there was no statistically significant difference when different virulence factors expressions were compared among *Candida* spp. isolated from catheterized and noncatheterized urinary samples. 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. 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.

Approval

This study was done after approval by Institutional Human Ethical Committee.

Conflict of Interest

None declared

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