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ORIGINAL ARTICLE

Identification and pattern of antifungal susceptibility of *Candida* species isolated from cases of vaginitis in a tertiary care hospital in India

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ABSTRACT

Background and Objectives: Vulvovaginal candidiasis (VVC) is one of the most frequent reasons for gynecological consultations. *Candida albicans* is responsible in the majority of cases. Lately, VVC caused by non-albicans *Candida* spp. (NAC), which are resistant to routinely used antifungals, is on the rise. This study was designed to determine the prevalence of *Candida* in patients suffering from vaginitis and to assess the predisposing factors along with identification of *Candida* species and evaluation of their susceptibility profile.

Materials and Methods: High vaginal swabs were collected from 225 women. Sample processing consisted of Gram stain and culture onto Sabouraud's dextrose agar and HiChrom *Candida* Differential agar. Isolates were identified and speciated using VITEK2 Compact System. Susceptibility testing was done using VITEK2 AST-Y S08 cards and disc diffusion.

Results: *Candida* spp. were isolated from 94 (41.8%) of the cases. *C. albicans* was the predominant species (71.6%) followed by other NAC spp. (28.4%). Pregnancy and diabetes were the most frequently implicated risk factors (67.1% and 44.4%). High resistance was observed in NAC spp. as opposed to *C. albicans* to all antifungal agents tested.

Conclusion: Empirical therapy with routinely used antifungals can be initiated for *C. albicans*. In the case of NAC spp., identification should be followed by susceptibility testing.

Keywords: Candidiasis; Vaginitis; Resistance; Antifungals; Reproductive health

INTRODUCTION

The inflammation of the vulvovaginal area with the established presence of *Candida* spp. by culture and in the absence of other infectious agents is referred to as vulvovaginal candidiasis (VVC). The patients present with symptoms of vulval pruritus, erythema, curd-like discharge, and a grey-white pseudo membrane (1). VVC accounts for a majority of gynecology consultations on an outpatient basis and affects more than two-thirds of women at least once during their lifetime (2, 3). The disease, though rarely lethal, is responsible for a high magnitude of morbidity around the world (4). Although *Candida albicans* (*C. albicans*) is isolated from a majority of the cases, lately, increased isolation of non-albicans *Candida* (NAC) spp. which have reduced susceptibility to routinely used antifungals has complicated

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the situation (5). This increase in resistant species can be attributed to the indiscriminate use of over-the-counter anti-fungal agents, leading to severe financial implications due to treatment failure in such cases (6).

The rise in the frequency of VVC caused by NAC spp. with an increase in resistance to routinely used antifungals has led to protocols being developed for prompt identification and in-vitro antifungal susceptibility testing. Given the lack of data in this region regarding VVC, this study was undertaken to identify the *Candida* species causing VVC and study their antifungal susceptibility profile.

MATERIALS AND METHODS

This was a cross-sectional, hospital-based study conducted in the Department of Microbiology, in collaboration with the Department of Gynecology and Obstetrics of an apex tertiary care hospital for a period of 18 months (November 2017 to May 2019). Before starting the study, approval was sought from the Institutional Ethics Committee.

Patient and samples. Women over 18 years of age who presented with self-reported symptoms of vaginal infection and were labelled as vaginitis on examination by the attending gynecologist were included in the study. Patients already on antifungals, who refused to participate and those already recruited in the study were excluded. Simultaneously, samples from 100 women attending the gynecology clinic for unrelated causes, who mandated a vaginal examination were taken and comprised the control group for the study. Informed consent was taken at the time of inclusion in the study from the cases and the controls along with a standard proforma used to document socio-demographic and clinical information.

Specimen collection. Upon admission, the attending gynecologist performed a detailed clinical examination and recorded the signs of vaginal infection. A sterile vaginal speculum was inserted to examine and appreciate the state of the cervix. Two high vaginal swabs (HVS) were collected aseptically, labelled and transported. A total of 225 patients were enrolled in the study.

Processing of samples. One swab was used for the preparation of a Gram smear which was used to document the presence of yeast cells as depicted in Fig. 1. The second swab was inoculated onto freshly prepared Sabouraud's dextrose agar (SDA) with chloramphenicol (HiMedia Laboratories Pvt. Ltd, India) and HiCrome Candida Differential Agar (HCDA) (HiMedia Laboratories Pvt. Ltd, India). SDA tubes were examined for growth after 24-48 hours of incubation. *Candida* colonies appear opaque white to creamy on SDA. HCDA is a chromogenic differential media recommended for the rapid isolation and characterization of *Candida* species from mixed cultures as illustrated in Fig. 2. The plates were examined for growth and color after 24-48 hours of incubation. Further processing was done using the growth on HCDA.

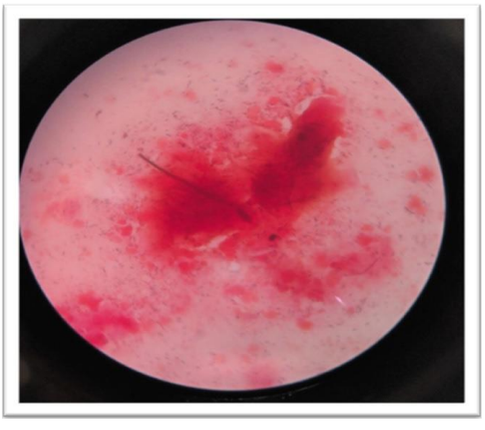

Fig. 1. Photomicrograph of a Gram smear of HVS showing *Candida* cells with pseudohyphae and epithelial cells (1000x).

*(A – *C. krusei*, B – *C. glabrata*, C – *C. parapsilosis*, D – *C. albicans*, E – *C. kefyr*)

Fig. 2. *Candida* colonies on HiCrome Candida Differential Agar showing various pigmentations.

Candida species were identified using the VITEK2 Compact system (bioMérieux) (software version 8.01). Briefly, a standardized inoculum suspension (1.8 to 2.0 McFarland) was placed into a sterile polystyrene test tube along with a VITEK2 cassette for each isolate. The loaded cassettes were placed inside the VITEK2 instrument. The card consists of colorimetric reagents that are incubated and interpreted automatically. The reagent cards have 64 wells that contain an individual test substrate which measures various metabolic activities. An optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel. The time of incubation varied from 9.1 to 27.1 hours (7).

Antifungal sensitivity was done by VITEK2 AST YS08 card which gave susceptibility to fluconazole (FLC), voriconazole (VRC), amphotericin B (AP), caspofungin (CAS), micafungin (MYC), and flucytosine (FLU) and disc diffusion method for itraconazole (ITR), ketoconazole (KET), and clotrimazole (CLO) (HiMedia Laboratories Pvt. Ltd, India). The disc diffusion method was done as per the CLSI guidelines (8). Mueller Hinton Agar with 2% glucose and methylene blue was inoculated with a 0.5 McFarland suspension of the growth for performing disc diffusion. A lawn culture was made on the agar with discs containing antifungal agents (CLO-10mcg, KET-10mcg, and ITR-10mcg) applied to the surface of the inoculated agar and pressed to ensure optimal contact. Plates were inverted and incubation was done at 37°C. After incubating for 24 hours, the plates were examined and the zones of inhibition surrounding the discs were measured and compared with established zone size ranges as depicted in Table 1 to determine the sensitivity.

Table 1. The interpretative criteria as per the manufacturer's guidelines (HiMedia)

Antifungal Agent (HiMedia)	Disk Content	Zone Diameter, Nearest Whole (mm)*			
		S	S-DD	R	NS
Ketoconazole	10 µg	>30	23-29	<22	-
Itraconazole	10 µg	>15	10-14	<9	-
Clotrimazole	10 µg	>20	12-19	<11	-

*Susceptible (S), susceptible-dose dependent (S-DD), resistant (R), and non-susceptible (NS) interpretive categories are defined in Section 8.2 of CLSI document M44-A2 (8).

Statistical analysis. All the categorical variables have been shown in terms of frequency and percentage. Sensitivity and predictive values were calculated using standard statistical tests (Chi-square test). Results have been discussed on a 5% level of significance. *p*-value <0.05 was considered significant while $p < 0.01$ was considered highly significant. The analysis was done with the help of statistical software, SPSS version 22 (9).

RESULTS

A total of 225 patients who were clinically diagnosed with vaginitis and 100 controls were included in this study. The controls were selected by simple random sampling. Among the study population, a total of 153 (68%) patients attended the outpatient department whereas 72 (32%) were sampled from the inpatient department. The control group was taken from those patients attending the outpatient department. The age distribution of the study population is depicted in Fig. 3. The risk factors and symptoms are depicted in Tables 2 and 3.

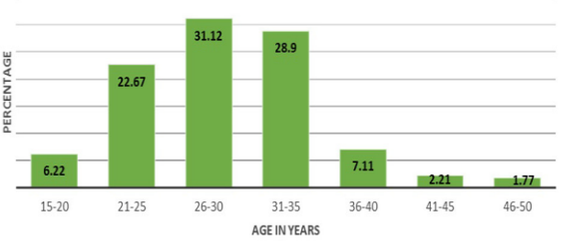

Fig. 3. Age-wise distribution of cases

Of 225 cases, 94 (41.8%) were positive for *Candida* spp. on culture while only 16 (16%) of the controls were positive for *Candida* spp. on culture as depicted in Table 4. Of the 94 cases that had a positive culture on the primary selective media, only 57 (60%) had a positive Gram smear. A total of 102 *Candida* isolates were recovered from 94 patients. *C. albicans* was the most common species both in the controls (100%) as well as the cases (71.6%). The remaining 28.4% of the cases were due to the NAC spp. and consisted of 4 different species. *C. glabrata* (n=16, 15.7%) was the most commonly isolated NAC spp. followed by *C. kefyr* (n=7, 6.9%), *C. krusei* (n=5,4.9%), and *C. parapsilosis* (n=1,1%) as illustrated in Fig. 4.

In contrast to the five different spp. causing candidi-

Table 2. Risk factors present in the studied population

Risk factors	Total	Positive for VVC		*p*-value
		N	%	
Married	159	78	49	0.001*
Pregnant	151	74	49	0.002*
Diabetes	100	65	65	0.000*
Antibiotic Usage	39	20	51.2	0.213
Menopause	6	2	33.3	0.574
OCP	3	2	66.6	0.573
Immunosuppressive state	1	1	100	0.418

OCP-Oral contraceptive pills
*Statistically significant

Table 3. Clinical presentation of the studied population

Clinical features	Total	Positive for VVC		*p*-value
		N	%	
Pruritus+Erythema+Discharge	20	19	95	0.000*
Pruritus + Discharge	43	42	97.6	0.000*
Discharge + Erythema	44	38	86.3	0.000*
Pruritus + Erythema	46	21	45.6	0.616
Erythema	102	42	41.1	0.893
Discharge	107	82	76.6	0.000*
Pruritus	129	52	40.3	0.682

*Statistically significant

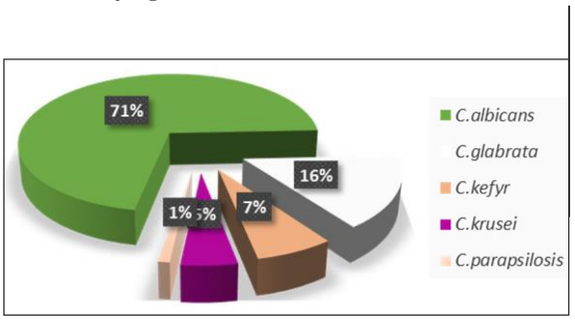

Fig. 4. *Candida* spp. isolated from cases of VVC

Table 4. Isolation of *Candida* spp. amongst cases and controls

Subjects	Total	Positive for *Candida* spp.	*p*-value
Cases	225	94 (41.8%)	0.0028*
Controls	100	16 (16%)	

asis in the cases, only one *Candida* spp. was found in the controls (*C. albicans*). Mixed infection with two species of *Candida* was seen in 8 (8.5%) cases while the remaining patients (n=86, 91.5%) had only one *Candida* spp. isolated.

Antifungal susceptibility was done for all isolates recovered during the study period. In general, higher rates of resistance for all antifungals were observed in NAC spp. as opposed to *C. albicans* as depicted in Table 5.

DISCUSSION

Despite having a worldwide distribution, the frequency of VVC is greatly affected by local and regional socioeconomic factors (10). As such, updated epidemiological data from different areas is an important tool for health service planning. The present study was carried out to find out the epidemiology of VVC in the region, and identify the various *Candida* spp. involved along with antifungal susceptibility testing of the isolates.

The prevalence of *Candida* spp. in the patients who were recruited in the study was 41.8% (n=94) whereas, amongst the controls, it was 16% (n=16) (*p*-value < 0.01). As the controls were recruited based on the absence of symptoms of vaginitis, it can be safely assumed that the isolation of *Candida* in this subset suggested colonization rather than true infection. Studies done elsewhere have reported different rates of infection (6, 11-13). Multiple factors like geographical location, socioeconomic status of patients, underlying factors and methods of testing can affect the prevalence rates between studies.

Patient age ranged from 18-50 years (median=29 years). The majority of the patients were in the age group of 26-30 years (31.1%) followed by 31-35 years (28.9%) and 21-25 years (22.6%). Similar results were reported elsewhere (6, 11-13). The high incidence of VVC in this age group has been attributed to lower levels of protective cervical antibodies, increased sexual activity, and the influence of reproductive hormones (14).

Amongst the patients enrolled, 159 (70.6%) were married with 151 (67.1%) being pregnant. Amongst the pregnant females, the highest percentage of the patients was in the third trimester (n=117, 77.5%), followed by the second trimester (n=20, 13.2%) and

Table 5. Antifungal susceptibility profile of *Candida* spp. recovered from the study population

Organism n (%)	Fluconazole*			Voriconazole*			Amphotericin B*		
	S	SDD	R	S	SDD	R	S	SDD	R
C. albicans	72 (98.6)	0	1 (1.4)	73 (100)	0	0	73 (100)	0	0
NAC	11 (37.9)	13 (44.8)	5 (17.2)	29 (100)	0	0	29 (100)	0	0
All Isolates	83 (81.4)	13 (12.7)	6 (5.9)	102 (100)	0	0	102 (100)	0	0
	Caspofungin*			Micafungin*			Flucytosine*		
	S	SDD	R	S	SDD	R	S	SDD	R
C. albicans	73 (100)	0	0	72 (98.6)	0	1 (1.4)	73 (100)	0	0
NAC	13 (44.8)	15 (51.7)	1 (3.4)	27 (93.1)	0	2 (6.9)	24 (82.8)	0	5 (17.2)
All Isolates	86 (84.3)	15 (14.7)	1 (1)	99 (97.1)	0	3 (2.9)	97 (95.1)	0	5 (4.9)
	Itraconazole#			Ketoconazole#			Clotrimazole#		
	S	SDD	R	S	SDD	R	S	SDD	R
C. albicans	66 (90.4)	5 (6.8)	2 (2.7)	20 (27.4)	30 (41.1)	23 (31.5)	58 (79.5)	10 (13.7)	5 (6.8)
NAC	24 (82.8)	4 (13.8)	1 (3.4)	6 (20.7)	10 (34.5)	13 (44.8)	17 (58.6)	7 (24.1)	5 (17.2)
All Isolates	90 (88.2)	9 (8.8)	3 (2.9)	26 (25.5)	40 (39.2)	36 (35.3)	75 (73.5)	17 (16.7)	10 (9.8)

* AST done by VITEK2 #AST done by Disc Diffusion

first trimester (n=14, 9.2%) Similar findings were observed in other studies where the incidence of VVC increases with gestational age and is highest in the third trimester (15, 16).

The most prominent risk factor for VVC in the study was found to be marriage (n=159, 70.6%) followed by pregnancy (n=151, 67.1%) and diabetes (n=100, 44.4%) with a significant association (*p*-value < 0.01). Several patients had multiple risk factors. Our results were in agreement with other studies (17, 18). It has been well documented that *Candida* spp. require a carbon source for growth and pregnancy provides the ideal environment due to a high level of reproductive hormones which induce an increase in glycogen content in the vaginal epithelial cells (10). Some studies have also reported that estrogen has a direct effect on the growth of *Candida* and its adherence to the vaginal epithelium (19).

The most prominent presenting symptom among the study population was pruritus (n=129, 57.3%) followed by discharge (n=107, 47.5%) and erythema (n=102, 45.3%). Many patients presented with multiple symptoms. In the study, VVC was commonly associated with thick, white curd-like discharge and its association was significant (*p*-value <0.01).

Several identification systems have been developed and are commercially available. However, microscopy remains valuable as a quick and inexpensive method of identification, although it lacks sensitivity. Direct microscopic examinations of the Gram-stained smear of HVS revealed budding yeast cells only in 57 (25.3%) of cases and all of these cases yielded *Candida* spp. on culture. Among the culture-positive cases, the sensitivity of the Gram smear was 60%. Therefore, as previously reported, a negative smear result does not rule out the presence of disease. It reemphasizes the fact that culture is more sensitive than microscopy and should be preferred over microscopy as has been advocated by other authors (20).

A total of 102 *Candida* isolates were recovered from 94 patients. Mixed infection with two species of *Candida* was seen in 8 (3.57%) cases while the remaining 94 (96.5%) had a single spp. isolated. *C. albicans* was the most common species in cases (n=73, 71.6%) as well as controls (n=16, 100%). The remaining 29 (28.4%) cases were due to the NAC spp. and consisted of 4 different species.

Though *C. albicans* (71.6%) remained the predominant species isolated in the present study, NAC spp. were isolated in considerable proportions (28.4%). Furthermore, in accordance with some studies, *C. glabrata* (15.7%) was the commonest NAC spp. isolated (18, 21). With higher resistance levels in NAC spp. to the commonly prescribed azole-based treatments, the consequences for women affected by these strains might be incapacitating. Therefore, identification up to the species level should be routinely done for *Candida* infections to ensure targeted therapy. A striking observation in this study is that NAC spp., although low in number, still accounts for a consid-

erable proportion of the cases in VVC (n=29, 28.4%). The high isolation of NAC spp. in this hospital-based study may be because complicated, chronic, and unresponsive cases of VVC are usually referred to this center.

Antifungal susceptibility testing for VVC was rarely indicated in the past given the limited reports of VVC caused by resistant *C. albicans* strains, and low prevalence rates of NAC spp. (22). In the present study a substantial number of NAC spp. (28.4%) were isolated from cases of VVC. The antifungal susceptibility depicted in Table 5 shows the highest overall resistance to KET (35.3%), followed by CLO (9.8%) and FLC (5.9%). NAC spp. in the study showed higher resistance rates as compared to *C. albicans.* Similar results have been reported elsewhere (18, 21). Worth mentioning is that although all isolates of *C. albicans* in the study were sensitive to most of the antifungals, one isolate was found to be resistant to FLC.

An increase in NAC infections and the emergence of azole-resistant *C. albicans* in VVC cases was seen in this study. Resistance to commonly used antifungal agents among NAC spp. makes it imperative that species identification be carried out in routine laboratories for the initiation of appropriate antifungal therapy. Also, surveillance of susceptibility profiles ensures that the empiric treatment regimens are validated regularly.

CONCLUSION

Vulvovaginal candidiasis cannot be diagnosed by clinical criteria alone. Culture is valuable for identifying the species of *Candida* and performing antifungal susceptibility, especially in pregnant and/or diabetic patients who present with discharge. This helps in the avoidance of empirical therapy which can prevent the emergence of resistance to antifungal agents. *C. albicans* was the most commonly isolated species, the majority of the strains being sensitive to all the antifungals tested. Empirical therapy with routinely used antifungals can be initiated for this species without the need for drug susceptibility testing in our setting. In the case of NAC spp., identification should be followed by susceptibility testing as the majority of the NAC spp. were found to be resistant to commonly used antifungal agents. The emergence of resistant NAC spp. can lead to treatment failures leading to recurrent VVC.

The study could not find a statistically significant influence between the use of broad-spectrum antibiotics, oral contraceptive pills and immunosuppressive states with the incidence of VVC. The reason could be the smaller number of patients with these predisposing factors. Further studies need to be done to find out a correlation between them. Also, yeast could not be identified as the causative agent in the majority of the symptomatic women. This finding requires due consideration and indicates the need to look for other etiological agents causing vaginitis.

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