Int.J.Curr.Microbiol.App.Sci (2017) *6*(12): 3593-3605

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 12 (2017) pp. 3593-3605**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.612.416

Study of Virulence Markers and Antifungal Susceptibility by Vitek-2 in Various Candida Species Isolated from Cases of Vulvovaginal Candidiasis

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Keywords

Candida species, Virulence markers, Antifungal susceptibility testing.

Article Info

Accepted:
26 October 2017
Available Online:
10 December 2017

A B S T R A C T

Vulvovaginal candidiasis (VVC) is the most common cause of vaginitis accounting for 17 to 39% of symptomatic women. Both *Candida albicans* and non *albicans Candida* species are involved in VVC. Amongst various virulence factors proposed for Candida, Biofilm formation, Proteinase and Extracellular Phospholipases are usually implicated in its pathogenicity. Concern is also rising with respect to antifungal resistance in both *Candida albicans* and non *albicans Candida.* Present study is carried out to study virulence function and antifungal susceptibility by Vitek 2C in isolates recovered from women in reproductive age group with signs and symptoms of VVC. Three vaginal swabs from 168 women of reproductive age with vulvovaginal candidiasis were collected. Direct microscopy and Gram's stained smear were examined for presence of budding yeast cells and pseudohyphae followed by isolation and identification of *Candida* species by conventional methods. The virulence factors studied were phospholipase, biofilm formation and proteinase. Antifungal susceptibility testing was carried out by VITEK 2C (Biomerieux). Vaginal swab of 55 (32.7%) subjects showed pure growth of Candida species. The most common species isolated was *C. albicans* (34.6%) followed by *C. tropicalis* (23.6%) and *C. glabrata* (21.8%). The production of virulence factor phospholipase was shown by most strains (83.6%) followed by biofilm formation (74.5%) and proteinase production (70.9%). MIC by VITEK-2 showed highest resistance to fluconazole (16.4%), followed by that to voriconazole (10.9%) and amphotericin B (5.4%). The study duly emphasizes the need for the determination of virulence factors and antifungal susceptibility testing for effective and prompt determination of not only the pathogenic state of candida species but also proper management of the VVC cases.

Introduction

VVC is defined as a condition with signs and symptoms of inflammation in the presence of Candida species and in absence of other etiology.[1] Women having VVC present with spectrum of manifestations ranging from asymptomatic colonization to severe acute symptomatic infection. VVC accounts for 17-39% of all cases of vaginitis among symptomatic women. Recent studies suggest that there is an increasing incidence of isolation of non albicans species from cases of VVC besides *C.albicans*.[2]

The transition of candida sp. from commensal to potential pathogen is determined by various host predisposing factors and virulence

attributes of infecting species such as adherence to host tissue and medical devices, biofilm formation and secretion of extracellular enzymes like phospholipases and proteinases[3]. Identifying these virulence factors in infecting pathogens and understanding their effects on human host is a major challenge.[4]

Concern is also rising about the emergence of antifungal resistance in Candida. Although amphotericin B and flucytosine continue to be more effective, resistance to azole group of antifungal agents, which are safe and effective, is of concern. Most *non albicans Candida* species have higher minimum inhibitory concentrations to the azole antifungal agents and infections they cause are often difficult to treat.[3] Any information on distribution and etiology of *Candida* is always very useful for epidemiological purpose. In view of the above, the present study has been undertaken with the following objectives.

The present study was undertaken with the following aim and objectives.

To demonstrate the following virulence markers in the candida species isolated:

-Phospholipase production
-In vitro biofilm / slime layer formation
-Proteinase production

To determine the Minimum Inhibitory Concentration (MIC) of *Candida* species using Vitek-2C system (Biomerieux).

Materials and Methods

Three high vaginal swabs were collected from168 women of reproductive age group attending Obstetrics and Gynecology (OBGY) department of tertiary care hospital of central India between January 01, 2016 and June 30, 2017 for the present study. The study was approved by institutional ethics committee. Patients fulfilling the defining criteria of vaginitis i.e. those with vaginal discharge, irritation, itching with or without pain in reproductive age group were included in the study.

A detailed clinical examination was done and documented. Two vaginal swabs were subjected to KOH wet mount microscopy and Gram's stain for presence of budding yeast and pseudohyphae. Subsequently, third swab was inoculated on SDA for yeast isolation. Growth of *Candida* was confirmed by colony morphology, Gram's stain and speciated by its characteristics on CHROME agar (Hi-media Mumbai), sugar assimilation and fermentation tests.[4]

The virulence factors studied were biofilm formation, and production of phospholipase, and proteinase.

Detection of virulence markers

Phospholipase detection[5]

All isolates were screened for their extracellular phospholipase activity by the following procedure: Egg yolk medium consisting of 13.0 g Sabouraud's dextrose agar (SDA), 11.7g NaCl, 0.111 g $CaCl_2$, and 10% sterile egg yolk was used. The egg yolk was centrifuged at 5000g for 30 min at room temperature, and 20 ml of the supernatant was added to the sterilized medium. Extracellular phospholipase activity was detected by inoculating 10µl aliquots of the yeast suspension (approximately 10^8 yeast cells /ml) into the wells punched onto the surface of the egg yolk medium. The diameter of the precipitation zone around the well was measured after incubation at 37°C for 48 h. The phospholipase index (Pz) was defined as the ratio of the diameter of the growth to the

total diameter of the growth plus the precipitation zone. Pz ≥1 indicated no phospholipase activity. Pz<1 indicated positive phospholipase activity. *C. albicans* ATCC 10231 was used as positive control.

Proteinase detection[5]

Candidaproteinase was detected by modified Staib method. Proteinase activity was measured in terms of Bovine Serum Albumin (BSA) degradation. Bovine serum albumin medium (dextrose 2%, KH_2 PO_4 0.1%, $MgSO_4$ 0.05%, agar 2% was mixed after cooling to 50°C with 1% bovine serum albumin solution) was used. Proteinase activity was detected by inoculating 10 µl aliquots of the yeast suspension (approximately 10^8 yeast cells /ml) into the wells punched onto the surface of the medium. The plates were incubated at 37°C for 2 days. After incubation, the plates were fixed with 20% trichloroacetic acid and stained with 1.25% amidoblack. Decolourisation was performed with 15% acetic acid. Opaqueness of the agar, corresponding to a zone of proteolysis around the wells that were not stained with amidoblack indicated degradation of the protein. The diameter of unstained zones around the well was considered as a measure of proteinase production. The proteinase index (Pz) was measured in terms of the ratio of the diameter of the growth of the unstained zone. Pz ≥1 – no proteinase activity detected in the strain while Pz<1 – positive for proteinase production. *C. albicans* ATCC 10231 was used as positive control.

In vitro biofilm formation/slime production[6]

Biofilm production was determined by visual methods. Colonies from the surface of SDA plate were inoculated into a polystyrene tube (Falcon conical tube with screw cap) containing 10 ml of Sabouraud-dextrose broth (SDB) supplemented with glucose (final concentration 8%). After incubation at 35°C for 48 h, the broth in the tubes was gently aspirated. The tubes were washed with distilled water twice and then stained with 2% safranin for 10 min. They were then examined for the presence of an adherent layer. Biofilm production was scored as negative (−), weak (+), moderate (++) or strong (+++). The biofilm producer Staphylococcus *epidermidis* ATCC 35984 was used as a positive control.

Antifungal susceptibility testing

MIC determination by VITEK 2[7]

Minimum Inhibitory Concentration (MIC) is the lowest drug concentration that prevents visible microorganism growth after overnight incubation.AST-YS01 card of Vitek 2 system was used for susceptibility testing of amphotericin B (AmB), fluconazole (FLC) and voriconazole (VRC). MIC of the antifungals was determined as described in the company's manual.

Results and Discussion

Among the 168 subjects included in the study, vaginal swab of 55 (32.7%) subjects showed pure growth of *Candida* species. The most common species isolated was *C. albicans* (34.6%) followed by *C. tropicalis* (23.6%) and *C. glabrata* (21.8%) (Table 1). All the 55 isolates recovered showed the presence of one or more of the three virulence factors (phospholipase, biofilm formation and proteinase production) studied. The production of virulence factor phospholipase was shown by most strains (83.6%) followed by biofilm formation (74.5%) and proteinase production (70.9%). Among *C. albicans*, most strains produced phospholipase (94.7%), followed by biofilm formation (89.5%) and proteinase production (84.2%). Among the

NAC isolates, just as *C. albicans*, most produced phospholipase (77.8%), followed by biofilm production (66.7%) and proteinase production (63.9%). Among the biofilm formers *C. albicans* (41.5%) showed the maximum positivity, followed by *C. glabrata* (22.0%), *C. tropicalis* (19.5%) and other species. Among proteinase producers again *C. albicans* (41.0%) showed maximum positivity, but it was followed by *C. tropicalis* (17.9%), *C. glabrata* (17.9%), and other species. Among phospholipase producers, though again *C. albicans* (39.1%) showed maximum positivity, it was followed by *C. glabrata* (23.9%), *C. tropicalis* (21.7%) and other species (Table 2).

As per MIC results determined by Vitek 2C (Biomerieux) highest resistance was seen to fluconazole (16.4%), followed by that to voriconazole (10.9%) and amphotericin B (5.4%). Higher resistance seen to fluconazole as compared to others was statistically not significant (*p-value*: 0.56).In case of *C.albicans*, resistance to amphotericin B, fluconazole and voriconazole was 10.5%, 31.6% and 21.1% respectively. The only isolate of *C. krusei* showed resistance to fluconazole only (100%), Resistance in *C. parapsilosis* was to the tune of 50% to all the three antifungals tested. MIC of *C. tropicalis* strains suggested resistance to fluconazole (7.7%) and voriconazole (7.7%) (Table 3).

As shown in Table 4, 18.2% of our isolates were resistant. Among these, 40% were resistant to single drug and 60% were multi drug resistant (40% to the two azoles and 20% to all the three antifungals). With respect to individual species, 36.8 % of *C. albicans*, 100% *C. krusei*, 50% of *C. parapsilosis* and 7.7% of *C. tropicalis* showed resistance to one or more antifungal tested. Among the resistant *C. albicans* strains, 42.9% showed resistance to one of the three antifungals, 42.9% to two antifungals (fluconazole and voriconazole) while 14.2% showed resistance to all the three antifungals tested (amphotericin B, fluconazole, voriconazole). In case of *C. parapsilosis* 50% strains were resistant, and 1 strain showed resistance to all the three antifungals tested. The only isolate of *C. krusei* recovered in the present study was resistant to a single drug, fluconazole (100%). Of the 13 isolates of *C. tropicalis*, 1 (7.7%) isolate was resistant and this was resistant to both the azoles tested.

In the present study *C. albicans* was the commonest species isolated (11.3% among the study subjects and 34.6% among isolates). Earlier report from Egypt (86.6%) [8], Kuwait (73.9%) [9] Yemen (65.9%)[10] and Saudi Arabia (59%)[11] have also reported higher rate of isolation of *C. albicans* in cases of VVC. Worldwide, rates of the isolation of *C. albicans* in cases of VVC ranges between 47% and 89% in studies from Nicaragua[12], Australia[13,14], Turkey[15], Iran[16], Nigeria[17,18] and India[19].

In our study the rate of isolation of *NAC* was higher (21.4%) among study subjects and 65.4% among the isolates), than that of *C. albicans* (11.3% among the study subjects and 34.6% among isolates). Higher isolation of *NAC* over *C. albicans* has also been reported by Kikani *et al.*,[426] (55.6% vs. 44.4%), Deepa Babin *et al.*,[19] (64.5% vs. 35.5%) and Namrata *et al.*,[20](53% vs. 47%).

However, there have been reports of higher isolation of the commonest species, *C. albicans* over *NAC* from Tehran[21] (65.1% vs. 34.9%), Sudan[22] (92 % vs. 8%), Egypt[23] (60.3% vs. 39.7%), Turkey[24] (59.9% vs 40.1%) and India[25] (66% vs. 34%).

In the present study, *C. tropicalis* (23.6 %) was the second most commonest isolate after *C. albicans*. Deepa Babin *et al.*,[19] (29.4%) and Namrata Kalia *et al.*,[20] (24.1%) from

India have also reported similar findings. Studies have reported the rates of *C. tropicalis* isolation in cases VVC ranging between 4% and 26.4%.[9,12,19]

However, *C. glabrata*has been reported to be the second most common isolate in cases of VVC from Saudi Arabia[11] (31%), Turkey[15] (34.5%), Australia[14] (20%), Egypt[8] (12.7%) and India[19] (11%). In the present study *C. glabrata* (21.8%) was the third commonest isolate after *C. albicans* and *C. tropicalis.* Other species isolated in the present study were *C. dubliniensis* (7.3%), *C. lusitaniae* (5.5%), *C. parapsilosis* (3.6%), *C. guilliermondii* (1.8%) and *C. krusei* (1.8%). Rates of isolation of *C. dubliniensi s*have been reported to range between 0.17% to 29.5%, while that of *C. krusei* from 3% to 15.7%.[11,12,15,19]

Cultural, ethnic and epidemiological differences may influence the isolation rate of different yeast from vulvovaginitis samples. However, it goes beyond doubt that there is need to speciate *Candida*, be it for epidemiological purpose or from point of view of ensuring institution of the correct antifungal.

Virulence markers

All pathogenic microorganisms have developed mechanisms that allow successful colonization or infection of the host. As a result, most pathogens, including *Candida* species, have developed an effective battery of putative virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease, and overcome host defenses. The virulence factors expressed or required by *Candida* species, and in particular *C. albicans*, to cause infections may well vary depending on the type of infection (i.e. mucosal or systemic), the site and stage of infection, and the nature of the host response. It seems apparent that a panel of virulence attributes is involved in the infective process, but no single factor accounts for *Candida* virulence and not all expressed virulence attributes may be necessary for a particular stage of infection. In present study all the 55 isolates recovered showed the presence of one or more of the three virulence factors (biofilm formation, proteinase and phospholipase production) studied. The presence of each of these virulence factors was seen more among *C. albicans* as compared to *non albicans Candida* (Table 2).

It has been reported in several studies that aspartic proteinases are produced at higher rates by *C. albicans*, as compared to *NAC*. In our study too, production of proteinase was seen more among *C. albicans* (84.2%) as compared to *NAC* (63.9%). Koga-ito *et al.,*[26] showed higher proteinase production among the *C. albicans* strains isolated from oral candidiasis patients when compared to those isolated from control individuals. Costa *et al.,*[27]found that 88.1% of *Candida albicans*and 69.8% of *non-albicans Candida* isolates produced proteinase. Vinitha *et al.,* [5]detected proteinase activity in 74.5% of Candida species isolated from the blood samples. The proteinase-producing capacity of *Candida nonalbicans* (50.4%) was less than that of *C. albicans* (67.3%). Somansu Basu *et al.,*[28] also reported that *C. albicans* from various clinical sources, exhibited strong proteinase (66.6%). Vivek *et al.,*[29] reported proteinase activity in 61.4% of their isolates, and observed maximum proteinase activity in *C. albicans* isolates followed by that in *C. glabrata*, *C. krusei* and *C. tropicalis*. They are of the opinion that the secreted proteinases are responsible for the adhesion and tissue invasion.

Table.1 Candida species isolated by conventional techniques

Candida species	Total n [%]
C. albicans	19 [34.6]
C. tropicalis	13 [23.6]
C. glabrata	12 [21.8]
C. dublinensis	04 [7.3]
C. lusitaniae	03 [5.5]
C. parapsilosis	02 [3.6]
C. guilliermondi	01 [1.8]
C. krusei	01 [1.8]
Total	**55**

[ ] - Figures in parenthesis are percentages from vertical total

Table.2 Candida species-wise distribution of virulence factors

Candida spp	**Biofilm formation n[%]**	**Proteinase production n[%]**	**Phospholipase production n[%]**
C. albicans (n=19)	17 [41.5]	16[41.0]	18[39.1]
C. glabrata (n=12)	09[22.0]	07[17.9]	11[23.9]
C. tropicalis (n=13)	08[19.5]	07[17.9]	10[21.7]
C. dubliniensis (n=04)	03[7.3]	04[10.3]	04[8.7]
C. parapsilosis (n=02)	02[4.9]	00[0.0]	01[2.2]
C. lusitaniae (n=03)	01[2.4]	03[7.7]	01[2.2]
C. krusei (n=01)	01[2.4]	01[2.6]	00[0.0]
C. guilliermondii (n=01)	00[0.0]	01[2.6]	01[2.2]
Total	41	39	46

[ ]: Figures in parenthesis are percentages from vertical total

Table.3 MIC (µg/ml) results in different *Candida* species by Vitek 2

	Amphotericin-B			Fluconazole			Voriconazole		
Candida species (n)	**S ≤1**	**I 2**	**R ≥4**	**S ≤2 */ ≤8****	**I 4*/ 16-32****	**R ≥8 */ ≥64 ****	**S ≤0.125*/ ≤0.5 **/ 1 *****	**I 0.25-0.5 */ 1 **/ 2 *****	**R ≥1 */ ≥2**/ ≥4*****
C. albicans (19)	15 (79.0)	2 (10.5)	2 **(10.5)**	11 (57.9)	2 (10.5)	6 **(31.6)**	15 (79.0)	0 (0.0)	4 **(21.1)**
C. dubliniensis (4)	4 (100)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)
C. glabrata (12)	12 (100)	0 (0.0)	0 (0.0)	11 (91.6)	1 (8.4)	0 (0.0)	12 (100.0)	0 (0.0)	0 (0.0)
C. guilliermondii (1)	1 (100)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
C. krusei (1)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	1 **(100.0)**	1 (100.0)	0 (0.0)	0 (0.0)
C. lusitaniae (3)	3 (100)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)
C. parapsilosis (2)	1 (50.0)	0 (0.0)	**1 (50.0)**	1 (50.0)	0 (0.0)	**1 (50.0)**	1 (50.0)	0 (0.0)	**1 (50.0)**
C. tropicalis (13)	13 (100)	0 (0.0)	0 (0.0)	11 (84.6)	1 (7.7)	**1 (7.7)**	12 (92.3)	0 (0.0)	**1 (7.7)**
Total (55)	49 (89.0)	3 (5.5)	3 **(5.5)**	42 (76.4)	4 (7.2)	9 **(16.4)**	49 (89.1)	0 (0.0)	6 **(10.9)**

S - Sensitive, I - Intermediate, R - Resistant
Interpretive criteria of Fluconazole / Voriconazole for:
**-C. albicans / C. parapsilosis, **C. krusei, ***other Candida spp.*

Table.4 Multidrug resistance among resistant isolates

	No. resistant (%)	**R to single drug**	**Multidrug resistance pattern**	
Candida species (n)			**F+V**	**A+F+V**
C. albicans (n=19)	7 (36.8)	3 [42.9]	3 [42.9]	1 [14.2]
C. krusei (n=1)	1 (100)	1 [100]	0	0
C. parapsilosis (n=2)	1 (50.0)	0	0	1 [100.0]
C. tropicali s(n=13)	1 (7.7)	0	1 [100]	0
Total (n=55)	10 (18.2)	4 (7.3) [40.0]	4 (7.3) [40.0]	2 (3.6) [20.0]

Note: None of the strains showed resistance to A+F and A+V
R – Resistant, A-Amphotericin B, F-Fluconazole, V-Voriconazole
() Figures in parenthesis are percentages from 'n'
[] Figures in parenthesis are from numbers mentioned in column "No. resistant" of respective species

Among the *NAC* isolates in the present study maximum proteinase positivity was seen in *C. tropicalis* (17.9%), *C. glabarata* (17.9%) and other species. Suzane Katy *et al.,*[30] reported proteinase activity in 33.3 % *C. tropicalis* and in 20% *C. glabrata*, followed by that in 15.3% *C. parapsilopsis*, recovered from vaginal secretions. Our results are similar to this study especially with respect to *C. glabrata*. However, none of our *C. parapsilosis* isolates produced proteinase. Candido *et al.,*[31] have opined that *NAC* species do not secrete proteinase.

Phospholipases act as a virulence factor by degrading cell membranes. In this study, the phospholipase activity was more associated with *C. albicans* (94.7%) as compared to *NAC* (77.8%) (Table 2). Costa *et al.,*[27] reported phospholipase production in 55.9% *C. albicans* isolates, and only in 37.7% *non-albicans Candida.* In another study conducted by Samaranayake *et al.,*[32] 80% isolates of *C. albicans* recovered from HIV patients were phospholipase-positive. In the study of Somansu Basu *et al.,*[29], 48.7% clinical isolates of *C. albicans* demonstrated phospholipase activity. Vinitha *et al.,*[5] reported that phospholipase activity was detected in 44.14% isolates, which included 46.93% of *C. albicans* and 42% *NAC.* Suzane Katy *et al.,*[30] demonstrated phospholipase activity in 8.9% isolates from vaginal secretions and maximum production was seen in *C. albicans* (42.8%) isolates. Vinita *et al.,*[5] in their study have shown that even though all the isolated strains were pathogenic, not all produced phospholipase as virulent factor, and have suggested that the virulence of *Candida* species is attributed not to a single factor but to a combination of several factors, like proteinase, phospholipase, biofilm production etc. Fule *et al.,*[33] reported phospholipase activity in 81.1% of all their vaginal isolates studied. They could not demonstrate any phospholipase activity in *NAC*. Mahmoudabadi *et al.,*[34], reported that all clinical isolates of *C. albicans* from VVC showed phospholipase activity while Basu *et al.,*[28], reported this enzyme activity in 66.6% of vaginal isolates.

In present study among the *NAC* species *C. glabarata* (23.9%) and *C. tropicalis* (21.7%) showed maximum phospholipase production. Suzane Katy *et al.,*[33] reported phospholipase activity in 6.6% *C. glabrata*, while none in *C. tropicalis* and *C. krusei*. Sachin *et al.,*[35] reported high phospholipase activity in *C. tropicalis* (57.2%).

Borst and Fluit[36] believe that phospholipase activity could be associated with strain characteristics, geographical region and infection type, which probably justifies the difference in prevalence of this activity, observed in different studies.

The formation of *Candida* biofilms carries important clinical repercussions because of the ability of cells within biofilms to withstand host immune defenses. *Candida* biofilms adversely impact the health of the patients with increasing frequency and severity and with soaring economic sequelae. However, Thamke *et al.,*[37] did not find any significant difference in ability of biofilm formation by *C. albicans* isolated from cases of VVC (40 %) and those from asymptomatic women (27.27%).

Different strains of *C. albicans* and different Candida species differ in their capacities to form biofilms[38]. In the present study, 41(74.5%) *Candida* isolates showed biofilm production, and biofilm formation by *C. albicans* (89.5%) was higher than *NAC* (66.7%). Yigit *et al.,*[6]reported higher positivity in *C. albicans (*88.2%) strains as compared to *NAC* (51.6%) as did Ilknur Dag *et al.,*[39](*C. albicans* vs *NAC*: 39.3% vs 37.79%). However, Tumbarello *et al.,*[40]

reported that biofilm production by *C. albicans* was significantly lower (22.6%) than that by all other *non-C. albicans* species (33.3%). Among biofilm producers, the *NAC* species producing biofilm in our study after *C. albicans* (41.5%) were *C. glabrata* (22%) and *C. tropicalis* (19.5%)., Tumbarello *et al.,*[40] reported biofilm formation in 30% of *C. tropicalis* and in 21% of *C. parapsilosis*.

Thorough search of literature revealed that there are few studies on simultaneous detection of different virulence factors. Our study revealed that of the 55 isolates that produced one or the other virulence factors, 44 (80%) produced two or more. In fact 27 (49.1%) produced all the three. Among combination, biofilm together with phospholipase was commonest (14.5%). Out of all, in *C. albicans* the commonest combined virulence factor activity seen was proteinase and phospholipase (16.7%). Among *NAC*, it was biofilm and phospholipase (87.5%).

In the study of D'Eca Junior A *et al.,*[41], 29.3% of the isolates produced both phospholipases and proteinases. Kantarcioglu and Yucel *et al.,*[42] observed that 56 out of 60 strains of *C. albicans* and 2 out of 4 strains of *C. kefyr* tested produced both phospholipases and proteinases. A study conducted by Shimizu *et al.,*[43] investigated the ability of different *Candida* species to simultaneously produce hyaluronidase, chondroitin sulfatase, protease and phospholipase in order to evaluate whether they were related to *Candida* pathogenicity. They determined that with the exception of the *C. albicans* strains, none of the strains produced all four enzymes simultaneously.

Multidrug resistance (Table 4)

Among the 55 isolates studied, drug resistance was seen in 18.2% isolates (10 of 55 isolates) and among these 60% (40% to the two azoles and 20% to all the three antifungals studied) showed multidrug resistance. Various mechanisms that contribute to the development of MDR have been implicated in Candida as well as in other human fungal pathogens, and some of these include over expression of or mutations in the target enzyme of azoles, lanosterol 14 alpha-demethylase, and transcriptional activation of genes encoding drug efflux pump proteins belonging to ATP-binding cassette (ABC) as well as to major facilitator superfamilies (MFS) of transporters. The ABC transporters, CDR1, CDR2, and an MFS pump CaMDR1, play a key role in azole resistance as deduced from their high level of expression found in several azole-resistant clinical isolates[44].

Multidrug resistance across C. *albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* was found in 22% of the isolates studied by Razzaghi-Abyaneh *et al.,*[45]. In our study this was seen in *C. albicans*, *C. tropicalis* and *C. parapsilosis*.

Consideration of the interactions between azoles and amphotericin B (AmB) has become clinically significant in recent years. Prior exposure to azole especially fluconazole has resulted in pronounced increase in resistance to subsequent exposures to AmB[46]. Clinical isolates of *C. tropicalis* and *C. glabrata* have been reported to be resistance to both azoles and amphotericin B. This was seen in one isolate each of *C. albicans* and *C. parapsilosis* in our study. For *C. tropicalis*, 6.3% of the isolates in one study were found resistant to azoles[47]. In our study the only isolate of *C. tropicalis* found resistant was so to both azoles tested. Among Candida isolates, there is a strong positive correlation between fluconazole MICs and those of itraconazole, voriconazole, posaconazole, and ravuconazole, indicating considerable cross-resistance[48]. Thus, resistance to fluconazole

may serve as a surrogate marker in predicting resistance to the other extended-spectrum triazoles with *Candida* spp. An analysis of cross-resistance among fluconazole and the other triazoles demonstrated that isolates of *Candida* spp for which fluconazole MICs are ≥64 mcg/mL (resistant) also tend to be less susceptible (MICs>2 mcg/mL) to voriconazole also. In our study all our 6 multidrug resistant isolates were resistant to both fluconazole and voriconazole.

Antifungal drug resistance is particularly more serious when it develops not only against the administered drug, but also to other non-related chemical compounds. Hence, it is important to develop new methods of diagnosis, that are more rapid and efficient, and that allow not only unequivocal identification of which strain(s) are causing the infection and to which compounds they are resistant to, or at least prone to develop resistance to. An overall better understanding of the patient's clinical history and of the strain that is causing the infection is determinant to choose the most efficient therapeutic option.

The study duly emphasizes the need for the determination of virulence factors and antifungal susceptibility testing for effective and prompt determination of not only the pathogenic state of candida but also proper management of the VVC cases.

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How to cite this article:

Rafat Siddiqi, D.K. Mendiratta, Atul Rukadikar and Sandhya Gadre. 2017. Study of Virulence Markers and Antifungal Susceptibility by Vitek-2 in Various Candida Species Isolated from Cases of Vulvovaginal Candidiasis. *Int.J.Curr.Microbiol.App.Sci.* 6(12): 3593-3605.
doi: https://doi.org/10.20546/ijcmas.2017.612.416