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Antifungal Resistant in Non-*albicans Candida* Species are Emerging as a Threat To Antenatal Women with Vulvovaginal Candidiasis

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Vulvovaginal candidiasis (VVC) is caused by *Candida* species. It has been associated with impact on economic cost. Currently, Non-*albicans Candida* species are more resistant to azoles and get converted from harmless to pathogenic state due to several virulence factors. Monitoring of the antifungal susceptibility pattern is important to know the resistant pattern of *Candida* species. Thus the objective of this research was to the identification of *Candida* in species level and to evaluate the antifungal resistance pattern in *Candida* species isolated from the vaginal discharge of antenatal women with vulvovaginal candidiasis. This prospective study was done in SRM MCH & RC, Chennai, India, from March 2017 to December 2018. An aggregate of 342 vaginal swabs were gathered from antenatal women of symptomatic and asymptomatic VVC. Antifungal susceptibility test was done by the disk diffusion method as per the CLSI guidelines. A total of 112 *Candida* species were isolated from 342 high vaginal swabs. Out of 112 *Candida* isolates, 65 (58%) were Non-*albicans Candida* (NAC) and 47 (42%) were *C. albicans*. In this study, 103/112(91.6%) of *Candida* isolates hadthe highest sensitivity to voriconazole and 26/112(23.2%) of *Candida* isolates had the highest resistance to miconazole. NAC species are emerging as potential threats to cause infection and posing a therapeutic challenge. Early empirical antifungal therapy and further research to improve diagnostic, prevention and therapeutic strategies are necessary to reduce the considerable morbidity and mortality.

Keywords: *Candida,* Resistant, Fluconazole, Non-*albicans Candida*, Vulvovaginal Candidiasis, Antifungal, Antenatal women.

In antenatal women, Vulvo-Vaginal Candidiasis (VVC) is one of the commonest fungal infections caused by *Candida* species[1,2]. The prevalence rate of VVC is more than 40% worldwide and 5-10% antenatal women suffered recurrent VVC[3]. The usual presentations are persistent curdy white vaginal discharge with itching, bad odour, irritation, pain in the lower abdomen and induration of vulva[4]. During pregnancy, VVC has been related to adverse outcomes such as low birth weight, preterm birth, miscarriage and premature rupture of the membrane[5,6]. The etiological agents of VVC are *Candida albicans* and Non-*albicansCandida* (NAC). Among NAC,*C. glabrata* is the second most common *Candida* species isolates from

VVC. Other species are *C. tropicalis, C. krusei, C.parapsilosis, C. kefyr, C. rugosa, C. dubliniensis* and *C. guiliermondi*[7, 8]. There is increased scientific and epidemiological interest on NAC species as their prevalence is at increasing trend all around the world. In complicated VVC, NAC species are commonly found than *C. albicans*[9].Pathogenic mechanisms of NAC aren't understood clearly as those of *C. albicans* where progressively broad research has been done.Prolonged antifungal treatment, diabetes mellitus, more established age and early antifungal uses are situations that lead to arise in the prevalence rate of NAC species[2]. *C. albicans* and NAC species possess several virulence factors like extracellular production of hydrolytic enzymes, hyphae formation, phenotype switching and cell adhesion[10]. A significant characteristic of NAC in that they are intrinsically resistant to the first line azole drugs, resulting in treatment failure[11]. In India, very limited data are available on NAC causing vaginal infection in antenatal women and its antifungal susceptibility. Incidence of antifungal resistance to *Candida* specieshas been on a growing trend over the past decade. Azoles are the drug of choice for VVC; yet, resistance has been reportable increased in NAC species[12, 13]. Causes of azoles drugs resistance in *Candida* species is due to continued treatment and repeated use of antifungal for recurrent candidiasis[14]. Several studies have described the mechanisms of azoles resistance, such as elevated expression of gene encoding lanosterol demethylase (*ERG11*), a point mutation in the *ERG11*, the gene coding the multidrug efflux pumps, *CaMDR1*, *CDR1* & *CDR2*[8]. It has become essential to have a close check of antifungal susceptibility and resistance mechanisms in an environment of rapidly changing resistant pattern. There are many studies, which have assessed the method of susceptibility patterns for *Candida*. "Clinical and Laboratory Standards Institute (CLSI)" has been described as the standard procedure for antifungal susceptibility test of fungus. Macro tube dilution method is time-consuming and more laborious in most of the clinical laboratories[15, 16]. Micro broth dilution method has been found to be acceptable[16]. Though micro broth dilution tests can commonly be read after 24 hours and 48 hours[15, 17]the epsilometer test (E- test) is an exclusive method, within 24 hours of incubation can give trustworthy results with minimum labour[16]. A simple disk diffusion test has some significant benefits for practical reasons in a clinical laboratory[18-21].Therefore, the aim of this study was to identify the *Candida* species level and investigate antifungal susceptibility patterns in *Candida* species from vaginal discharge in order to determine any emerging resistance and to develop standard treatment guidelines for VVC of antenatal women, particularly in settings where the diagnosis depends on clinical overview or restricted research facility testing.

MATERIAL AND METHODS

This research was done in the Department of Microbiology and Obstetrics & Gynaecology

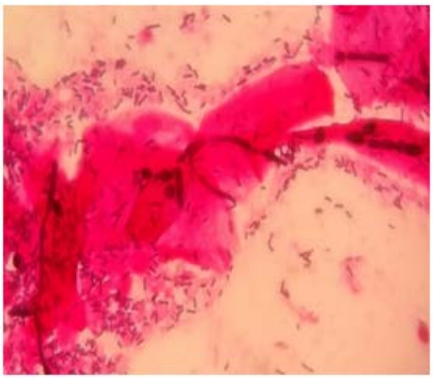

Fig. 1. Direct microscopy of Gram-stained showing Gram-positive budding yeast cell with pseudohyphae, Gram-positive bacilli and epithelial cell (100X)

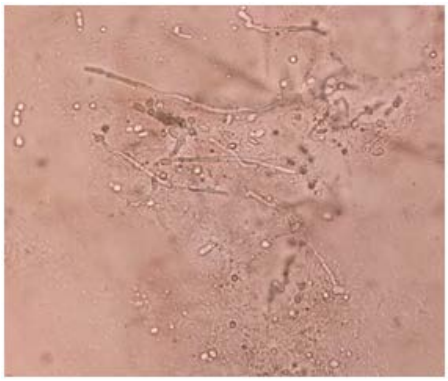

Fig. 2. KOH mount showing budding yeast cell with pseudohyphae (40X)

(OG) at SRM MCH and RC, Kattankulathur, Tamil Nadu, India, from March 2017 to December 2018 after getting the approval from the "Institutional Ethical Committee (IEC NO-1090/IEC/2017)". Informed consent of the patients was taken before including them into the study. Inclusion criteria for cases of VVC were all women attending the obstetric clinic with or without symptoms of curdy white discharge, itching, odour, pain, irritation, and swelling. Women with clinically diagnosed VVC on antifungal treatment were excluded from the study.

Collection of specimen and processing

Totally 342 vaginal swabs were collected from antenatal women of symptomatic and asymptomatic VVC. Samples were processed for direct microscopy by 10 % potassium hydroxide (KOH 10%) mount (Figure 2)and Gram stain(Figure 1 & 4). The culture was performed on "Sabouraud's Dextrose Agar (SDA)"(Figure 3)with gentamicin and incubated at 37°C as well as at 25°C for 24-48 hours.

***Candida* species identification by the conventional method**

Germ tube test (Figure 5) was done to differentiate *Candida albicans* from NAC[22]. Subsequently, *Candida species* were identified by culture on Chrome agar (Himedia, India) according to manufactures instructions(Figure 6). Cornmeal agar with tween 80 (Dalmau plate technique) performed as described in the previous study for chlamydospores and blastopores formation[23](Figure 7). Sugar fermentation(Figure 8) and assimilation tests were done to identify

Fig. 3. On SDA *Candida* has grown as smooth, creamy white pasty colonies

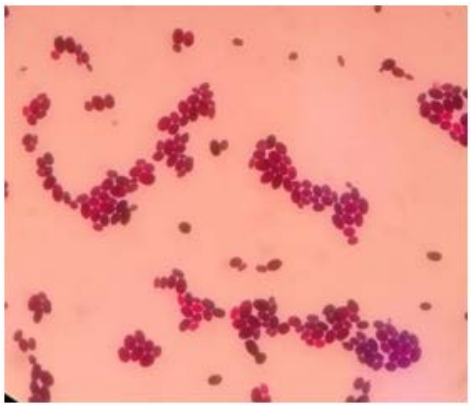

Fig. 4. Gram stained from a culture showing Gram-positive budding yeast cell (100X)

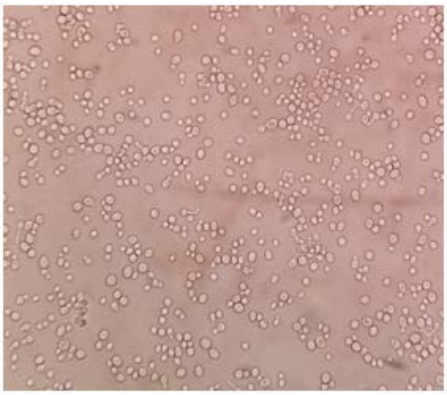

Fig. 5. Germ tube formation by *Candida albicans* (40X)

Fig. 6. On chrome agar, *C. albicans* produced light green and *C. krusei* produced a pink colony

Candida species as proposed by Giri *et al*[24].

Inoculum preparation and susceptibility testing by disk diffusion method

As per the "CLSI" (M44-A) procedures, the inoculum was set up by picking five particular colonies from a 24 hr old culture of *Candida* species. Colonies were suspended in 5ml of sterile 0.9% normal saline[25]. The suspension was vortexed to get uniform turbidity and adjusted visually to 0.5 McFarland standards. After 15 minutes, the suspension was inoculated onto "Mueller Hinton Glucose Agar (MHGA)" with 2% glucose and 0.5 µg/ml of methylene blue were used for susceptibility testing (Figure 9 & 10). Fowling antifungal disks were used: fluconazole (10 µg), ketoconazole (30 µg), clotrimazole (10 µg), nystatin(100 U), voriconazole (1 µg), miconazole (30 µg) and amphotericin-B (20 µg). Antifungal disks are placed evenly so that they are not closer than 24 mm from the centre to centre. The plates were placed in an incubator at 37°C for 24-48hr. *C.albicans* ATCC90028, *Candida tropicalis* ATCC 750, *Candida krusei* ATCC6258 and *C.Parapsilosis* ATCC22019 were used as a control. All the antifungal disks, control strains and culture media were parched from Hi-Media, Mumbai, India. The zones of inhibitions were as resistant (R), susceptible dose-dependent (SDD) and susceptible (S) as per the CLSI guidelines for fluconazole and voriconazole, while for other antifungal agents, interpretive breakpoints were referred from published paper[13, 25-27](Table 1).

Statistical analysis

Data were entered into an excel sheet and analysed by using EpiTools epidemiological calculators online software. Z test has been used to find the difference in proportions and a 5% level of significance has been used.

RESULTS

Totally 112 *Candida* species were isolated from 342 high vaginal swabs. Out of 112 *Candida* isolates, 65/112(58%) were Non-*albicans Candida* (NAC) and 47/112(42%) were *C. albicans*. Among NAC,23/112(20%) were *C.glabrata*, followed by 21/112(19%) *C.tropicalis*, 11/112(10%) *C. parapsilosis* and 10/112(9%) were *C.krusei* as shown in figure 11.

Totally 112 *Candida* isolates were tested for antifungal susceptibility testing by disk diffusion method. Among 112 *Candida* isolates, 65(58%) were Non-*albicans Candida* (NAC) and 47(42%) were *C. albicans*. In this study *Candida* species had shown highest sensitivity to voriconazole 101/112(90.1%) followed by amphotericin B 100/112(89.2%), nystatin 92/112(82.1%), fluconazole 83/102(81.3%), clotrimazole 85/112(75.8%), ketoconazole 79/112(70.5%) and miconazole 75/112(66.9%) as shown in table 2. In the present study *Candida* species had shown highest resistance to miconazole 26/112(23.2%) followed by ketoconazole 25/112(22.3%), clotrimazole 19/112(17%), fluconazole 16/102(15.6%), nystatin 16/112(14.2%), amphotericin B 12/112(10.8%) and voriconazole 9/112(8.1%). *Candida albicans* had shown the highest sensitivity to amphotericin B 43/47(91.4%) and voriconazole 40/47(85.1%) butthe highest resistant to ketoconazole 10/47(22%). *C.glabrata* had shown the highest sensitivity to amphotericin B21/23(93.3%) while 7/23 (30%) resistance to Miconazole. *C. tropicalis* had shown a maximum of 20/21(95.2%) sensitivity against Voriconazole and 6/21(28.5%) resistance to Miconazole. *C. parapsilosis* had shown 11/11(100 %) sensitivity against voriconazole and 3/11(27%)

Table 1. Interpretive break point of different antifungal agents

Drugs with concentration (µg)	Susceptible (mm)	Susceptible dose-dependent (mm)	Resistant (mm)
Fluconazole (10 µg)	≥ 19	15-18	≤ 14
Ketoconazole (30 µg)	≥ 28	21-27	≤ 20
Clotrimazole (10 µg)	≥ 20	12-19	≤ 11
Miconazole (30 µg)	≥ 20	12-19	≤ 11
Voriconazole (1 µg)	≥ 17	14-16	≤ 13
Amphotericin-B (20 µg)	≥ 15	10-14	<10
Nystatin(100 U)	≥ 15	10-14	No zone

µg=micrograms, mm=millimetre.

resistance to miconazole. *C.krusei* had shown 9/10(90 %) sensitivity to voriconazole and highest resistance 5/10(50%) against Clotrimazole (Table 2).

Comparisons between *C. albicans* and NAC species with resistance antifungal agents as shown in table 3. There was a statistically significant difference ($p<0.05$) between *C. albicans* and NAC species in terms of resistance against amphotericin B and voriconazole.

DISCUSSION

In antenatal women, VVC is one of the commonest fungal infections. The causative agents of VVC are *C. albicans* and NACspecies such as *C. glabrata* and *C. tropicalis* appear to be increasing. *C.glabrata* is the 2nd commonest agent in vaginal infections[28-30]. There are various effective antifungal drugs that are used for treating *Candida* vulvovaginitis, Understanding of the antifungal susceptibility patterns is key in guiding proper therapy and selection of antifungal drugs for vulvovaginal candidiasis.

Antifungal resistance in NAC is increasing day by day due to reiteration and long duration of antifungal therapy resulting in treatment failures and the emergence of azole resistance in *Candida.* Antifungal susceptibility testing may offer good treatment outcome by monitoring the drugs resistance and antifungal efficiency[31]. Several studies have been described the mechanisms of azoles resistance such as elevated expression of gene encoding lanosterol demethylase (*ERG11*), alteration in the *ERG11*, the gene coding the multidrug efflux pumps, *CaMDR1*, *CDR1* and 2 [32].

Candida identification at the species level is crucial, in the view of emerging different *Candida* species and the distinctive antifungal susceptibility profiles[33-35]. In the present study, *C. albicans* were 42% but the prevalence of NAC species were 58%. An earlier study was done by El-Sayed *et al*.[36] has reported a higher prevalence rate of *C. albicans* in VVC as 86%, thoughthe prevalence

Table 2. Antifungal susceptibility pattern of various *Candida species*

Antifungal agents		*C. albicans* n=47(%)	Non-*albicans Candida* (NAC) *C.glabrata* n=23(%)	*C. tropicalis* n=21(%)	*C. parapsilosis* n=11(%)	*C. krusei* n=10(%)	Total n=112(%)
FCZ	Susceptible	39(83)	19(82)	16(76)	9(81)	NA*	83/102(81.3)
	SDD	-	1(4)	-	2(19)	NA*	3/102(2.9)
	Resistant	8(17)	3(14)	5(24)	-	NA*	16/102(15.6)
VCZ	Susceptible	40(85.1)	21(91.3)	20(95.2)	11(100)	9(90)	101(90.1)
	SDD	2(4.2)	-	-	-	-	2(1.7)
	Resistant	5(10.6)	2(8.7)	1(4.7)	-	1(10)	9(8.1)
KCZ	Susceptible	35(74)	15(65)	15(71)	8(72)	6(60)	79(70.5)
	SDD	2(4)	2(8.6)	2(10)	1(9)	1(10)	8(7.1)
	Resistant	10(22)	6(26)	4(19)	2(19)	3(30)	25(22.3)
CLOT	Susceptible	40(85)	16(69)	17(80)	9(81)	3(30)	85(75.8)
	SDD	1(2)	3(13)	1(4.7)	1(9.5)	2(20)	8(7.1)
	Resistant	6(13)	4(17)	3(14)	1(9.5)	5(50)	19(17)
MCZ	Susceptible	36(76)	14(61)	12(57)	7(63)	6(60)	75(66.9)
	SDD	4(4.5)	2(8.6)	3(14.2)	1(9.5)	1(10)	11(9.8)
	Resistant	7(14.8)	7(30)	6(28.5)	3(27)	3(30)	26(23.2)
AMP	Susceptible	43(91.4)	21(93.3)	18(85.7)	10(90.9)	8(80)	100(89.2)
	SDD	-	-	-	-	-	-
	Resistant	4(8.6)	2(8.7)	3(14.2)	1(9.1)	2(20)	12(10.8)
NS	Susceptible	39(82.9)	20(86.9)	16(76.1)	9(81.8)	8(80)	92(82.1)
	SDD	2(4.2)	-	1(4.7)	-	1(10)	4(3.5)
	Resistant	6(12.7)	3(13)	4(19)	2(18.1)	1(10)	16(14.2)

SDD= Susceptible Dose Dependent, NA*= *C.krusei* intrinsic resistance to fluconazole. FCZ=Fluconazole (10µg), KCZ=Ketoconazole (30µg), CLOT=Clotrimazole (10µg), NS=Nystatin (100U), VCZ=Voriconazole (1µg), MCZ=Miconazole (30µg) and AMP=Amphotericin-B (20µg).

rates of 59%, 65% and 73% were reported by Al-Hedaithy *et al*.,[37]Al-mamari *et al*.,[38] Al-fouzan *et al*.,[39] respectively. Worldwide the prevalence of *C. albicans* in VVC falls somewhere in the range of 47% and 89% in various investigations[40-46].

In this study, there was an increasing rate of NAC species. In VVC *C. albicans* has been observed as the main disease-causing agent. Nevertheless, in complicated VVC, NAC species are commonly found than *C. albicans*[9].That might be because of the increased prevalence of drugs resistance, prolong antifungal treatment, diabetes mellitus, more established age, earlier antifungal uses and poor hygienic conditions that lead to increase in the prevalence of infection by NAC[2]. However, there is increased scientific and epidemiological interest in NAC species as their prevalence is rising all around the world. In the present study, 20% of *C. glabrata* which was the second commonest speciesfollowed by 19% of *C. tropicalis,* 10% of *C. parapsilosis* and 9% of *C. krusei*. Results of the present study are concordant to the study done by the various researchers[37,42,43]. Prior investigations have revealed rates of *C. tropicalis* in VVC extended from 5% to 26%[36, 40, and 46] whereas rates of *C. Krusei* ranged from 5% to 15%[37, 40, 43 and 46] and 0.6% for *C. parapsilosis*[37].

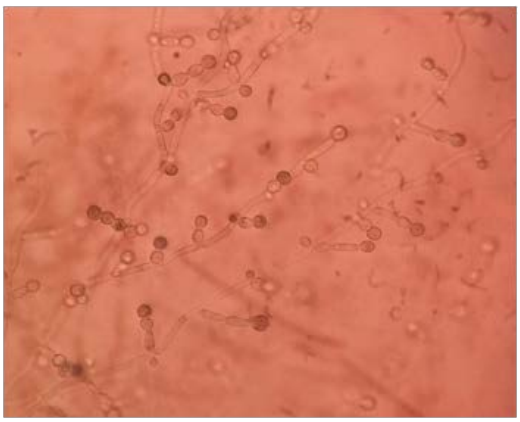

Fig. 7. Chlamydospore formation by *C. albicans* on corn meal agar (10X)

In this study, overall resistance was 26(23.2%) of miconazole followed by 25(22.3%) ketoconazole, 19(17%) clotrimazole, 16(15.6%) fluconazole, 16(14.2%) nystatin, 12(10.8%) amphotericin B and 9(8.1%) voriconazole. Resistance to miconazole, ketoconazole and clotrimazole is of great concern as these are the first line azoles used for the treatment of vulvovaginal candidiasis. Fluconazole is a contraindication to antenatal women but the resistance of fluconazole have a great concern as it is the most common azole used for the treatment of systemic candidiasis such as candidemia. In the present study, there was an increase in resistance of azoles except for voriconazole but other drugs such as amphotericin B and nystatin showed good efficacy against *Candida*. The present finding revealed 15.6% were fluconazole resistant by *Candida* species, similarly, studies from Egypt[37] and Taiwan[47] has reported the same rates of fluconazole resistance. Higher resistance rates were accounted for from Brazil[13] whereas no resistance from Kuwait[39]. In the present study, 39(83%) were fluconazole sensitivity to *C. albicans*, which is higher than the study reported by *Babinet al.*[46]. Highest fluconazole resistant seen in this study was *C. tropicalis* 5 (24%) which is lower than the study reported by Sachin *et al*.[30]. In addition to that, Dota *et al* reported increased resistance to

Table 3. Comparison between *C. albicans* and NAC species with resistant antifungal agents

Antifungal agents	Number of resistance *C. albicans*, n=47(%)	Number of resistance NAC, n=65(%)	P-value
Amphotericin B	4(8.6)	8(12.3)	0.0001*
Nystatin	6(12.7)	10(15.3)	0.697
Fluconazole	8(17)	8(12.8)	0.482
Voriconazole	5(10.6)	4(6.1)	0.0001*
Clotrimazole	6(13)	13(20)	0.33
Ketoconazole	10(22)	15(23)	0.9
Miconazole	7(14.8)	19(29.2)	0.0746

* Statistically significant ($p<0.05$)

fluconazole (32%) by Kirby Bauer method, while lower resistance was noticed by micro broth dilution method[13]. In this study, clotrimazole had shown 40(85%) sensitivity to *C. albicans* followed by 9(81%) of *C.parapsilosis* which is quite high in the study reported by *Ajitha et al.*[28]. Another study conducted by *Sachin et al*.,[30] reported that 50% clotrimazole resistance to *C. parapsilosis* and 20% by *C. albicans,* whereas in our finding 5(50%) clotrimazole resistance was seen in *C. krusei*.The results of the present study revealed 35(74%) were ketoconazole sensitive to *C.albicans* and 3(30%) of ketoconazole was resistance against *C. krusei* which was highest, while a study was done by *Sachin et al.,* reported that 25% ketoconazole was resistance by *Candida* species[30]. Among the azole drugs, voriconazole showed good efficacy against *Candida.* In the present finding, all the *C. parapsilosis* was 11(100%) sensitive to voriconazole whereas 5(10.6%) resistance by *C.albicans* but lower resistant rates was reported by *Baghdadi et al.*[48]. Nystatin was 20(86.9%) sensitive to *C. glabrata* and 6(12.7%) resistance against *C. albicans;* the finding of this study is concordant with the study done by *Sherinet al.*[19]. Amphotericin B was 21(93.3%) sensitive to *C.*

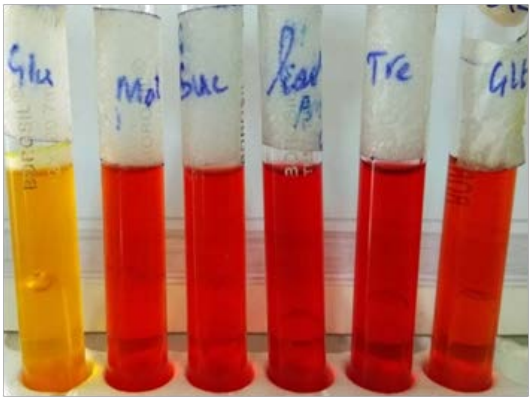

Fig. 8. Sugar fermentation test of *Candida* species

Fig. 9. Antifungal Susceptibility test of *Candida* isolates by disk diffusion methods

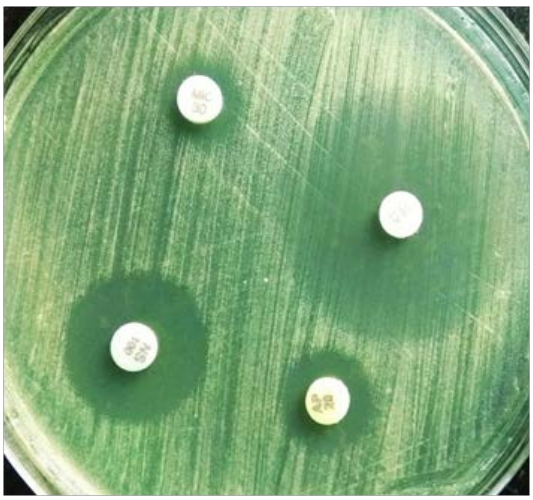

Fig. 10. Antifungal Susceptibility test of *Candida* isolates by disk diffusion methods

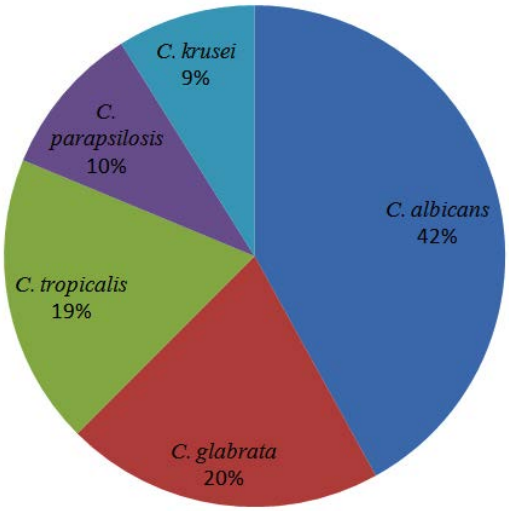

Fig. 11. Distribution of *Candida* species (*n=112*)

glabrata and 2(20%) resistance against *C.krusei* in this study which is higher than the rate reported by *Ajitha et al*.[28]. In the present study, overall fluconazole was 83(81.3%) sensitive, while the study done by *Kelen et at.,*[8] showed 35.5% and *Dharmik et al*.,[4] showed 97.2%.In this study, the overall Clotrimazole was 85(75.8%) sensitive and ketoconazole was 79(70.5%) which were almost near to the study done by *Dharmik et al*.,[4] as 80%. However, in this present study, as compared to *C. albicans*the majority of NAC had shown a high level of resistance towards antifungal drugs (Table 3). A huge alteration in the epidemiologic patterns of *Candida* and furthermore the development of resistance among already susceptible species because of increased uses of over-the-counter antifungal agents. The prior study reported that there is a higher MIC value by most NAC species; consequently, it is very hard to treat[9]. An enormous report discovered that among NAC of vaginal isolates, *C. glabrata*is emergence as more resistant to azoles as compared to isolates from bloodstream infection[11, 21]. In the present study, there was a statistically significant difference ($p<0.05$) between *C. albicans* and NAC species in terms of resistance against amphotericin B and voriconazole. Whereas, no statistically significant difference ($p>0.05$) between *C. albicans* and NAC species resistance against other antifungal agents as shown in table 3.

CONCLUSION

The study offers information about *Candida* species distribution and antifungal susceptibilityactivity of *Candida* isolated from antenatal women of VVC. In this study, there was a clear shift in the prevalence of infection by *Candida albicans*to those by NAC. NAC species are emerging as a potential threat for causing cause infection and posing a therapeutic challenge. Early empirical antifungal therapyand further research to improve diagnostic, prevention and therapeutic strategies are necessary to reduce the considerable mortality and morbidity. Appropriate selection of drugs for the treatment of *Candida* infections and antifungal susceptibility testing must be performed regularly.The study was directed on a low number of isolates and in a tertiary care hospital which is the limitation of the study.

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