Original Research Article

Antifungal susceptibility of *Candida* by disc diffusion method of isolates from clinical cases of vulvovaginitis of a tertiary care hospital in Mumbai

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Abstract

Background: Vulvovaginal Candidiasis (VVC) is a grave public health problem affecting young and middle-aged females especially during their reproductive life. Though *Candida albicans* is the commonest species involved,recurrent episodes of VVC are caused by non-albicans *Candida* (NAC) spp. The NAC spp also show resistance to commonly used antifungal drugs leading to recurrent infections, complications and treatment failure. In addition, widespread use and over the counter availability of antifungals leads to resistance.Therefore, antifungal susceptibility testing of *Candida* isolates is mandatory for selection of an appropriate and accurate antifungal therapeutic agent**Objectives:** To perform in vitro antifungal susceptibility testing by disc diffusion method.**Materials and Methods:** A total of 150 vaginal Candida isolates were used to perform Antifungal susceptibility test by disc diffusion method. Mueller Hinton Agar with 2% Glucose and 0.5 µg/mL Methylene Blue Dye medium and six antifungal drugs Amphotericin B, Fluconazole, Itraconazole, Ketoconazole, Clotrimazole and Nystatin were used. Results were calculated using CLSI M 44-A2 (S3) for fluconazole and standard reference articles for other drugs.**Results and Conclusion:** Susceptibility of *Candida albicans* was 100% to Amphotericin B, Nystatin and 97.2% to Ketoconazole. NAC spp were less susceptible to Fluconazole and Clotrimazole as compared to *Candida albicans*. Resistance was found even to Amphotericin B and Nystatin among NAC spp. Accurate and reliable antifungal susceptibility testing is necessary to help the clinicians in better patient management and preventing the emerging antifungal resistance.

Keywords: Disc diffusion, non-albicans *Candida*, antifungals, vulvovaginitis

Introduction

Vulvovaginal candidiasis (VVC) is the commonest manifestation of genitourinary candidiasis in womenof reproductive age group.[1] Although *Candida* infections are common in seriously ill or otherwise immunocompromised patients, due to its ability to survive in various mucocutaneous sites, it can cause infections in otherwise healthy individuals as well. *Candida* spp. can be isolated from genital tract in upto 25% of asymptomatic healthy women of child bearing age.[2] Nearly 70-75% of women over 25 years of age reported to have atleast 1 episode of VVC during lifetime, the recurrence rate is 40–50% and 5-8% have recurrent vulvovaginal candidiasis (RVVC) which is characterized by 4 or more episodes of the disease over a period of 1 year.[3]*Candida albicans*is the common pathogen in 80-90% of cases of VVC.[4]However, in recent years, along with an increase in the incidence of candidiasis, there has been a shift from *Candida albicans* to non *albicans* spp.[5]Most commonly isolated NAC species include *Candida glabrata*, *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis*.[6]*Candida albicans* and *non-albicans* species present with same clinical picture but differ from each other with regard to epidemiology, virulence characteristics and antifungal susceptibility. This may be due to various factors like severe immunocompromised status of the host, exposure to broad spectrum antibacterial agents and widespread use of antifungals as well as over the counter availability of antifungals. Azoles are the treatment of choice for VVC. However, resistance has been reported especially in NAC species. The study of these pathogens has become necessary due to the increasing drug resistance. Also, an increase in the number of new and broad spectrum antifungal drugs has made it difficult to choose the proper antifungal treatment for candidiasis. [7]Therefore, antifungal susceptibility testing of *Candida* isolates is mandatory for selection of an appropriate and accurate antifungal therapeutic agent. Also, it helps in preventing chronic VVC by right drug administration. Antifungalsusceptibility testing may alter the clinical response,predict treatment failure, and assist in empirical selection of antifungals.Thus, the main objective of this study was to perform in vitro antifungal susceptibility testing by disc diffusion method using six antifungal drugs.

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Materials and methods

This study was carried out for a period of one and half years (May 2017 to October 2018) in the Microbiology department of TNMC and BYL Nair Ch. Hospital, Mumbai. A total of 150 vaginal *Candida* isolates from samples of vaginal discharge received in the microbiology laboratory during this period were included in the study.All the isolates received were processed by standard mycological tests.[8]After speciation by standard mycological tests, [9] Antifungal susceptibility testing was performed on the isolates by

disc diffusion method using CLSI (Clinical And Laboratory Standards Institute) document M 44-A2 (S3), titled Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts [10]The medium used for disc diffusion was Mueller-Hinton Agar with2% Glucose and 0.5 µg/mL Methylene Blue Dye. Antifungal discs used for testing were Amphotericin B (20µg), Fluconazole (25 µg), Itraconazole (10 µg), Ketoconazole (50 µg), Clotrimazole (10 µg) and Nystatin (100units/disc).Inoculum was prepared by using colonies of a 24-hour-old culture of *Candida* species and suspended in sterile saline. The turbidity of suspension was adjusted by a 0.5 McFarland standard. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension and the dried surface of a sterile Mueller-Hinton agar + 2% glucose and 0.5µg/ml methylene blue dye agar plate was inoculated by evenly streaking the swab over the entire agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.Antimicrobial discs were dispensed onto the surface of the inoculated agar plate. The distance was 24 mm from centre to centre. The plates were then incubated in the incubator at 37˚C for 24 hours. The plates were read after 24 hrs and the antifungal susceptibility of the isolates was interpreted as Susceptible (S), Susceptible- Dose Dependent (SDD) and Resistant (R). [10,11]*C.albicans*ATCC 90028 and *C.parapsilosis* ATCC 22019 were used as quality control.Zone diameter for Fluconazole was measured and compared with CLSI M 44-A2 (S3) [10] whereas the zone diameter for other azoles, Amphotericin B, Nystatin and Clotrimazole were interpreted with reference to standard articles.

Results

During the study period, a total of 150 *Candida* isolates from samples of vaginal discharge were isolated. Antifungal susceptibility was performed on three predominant species isolated, *Candidaalbicans*74(49.3%), *Candida glabrata*44(29.3%) and *Candida tropicalis*32(21.4%). Disc diffusion method of AFST was used for all the *Candida* isolates (n=150). Fluconazole AFST was done according to CLSI M 44A2 (S3) whereas the zone diameter for other azoles, Amphotericin B and Nystatin wereinterpreted with reference to standard articles.In our study,the susceptibility of *Candida albicans* was 100% to Amphotericin B, Nystatin and 97.2% to Ketoconazole. Susceptibility toFluconazole and Itraconazole was 86.5% and 60.8% respectively asshown in Figure 1.As depicted in Figure 2, *Candida glabrata* showed 100% susceptibility to Amphotericin B and 93.2% to both Nystatin and Ketoconazole. However, it was less susceptible to Fluconazole (59.1%) and Clotrimazole (56.8%). Least susceptibility was seen to Itraconazole (45.5%). *Candida tropicalis* showed 93.8% susceptibility to Amphotericin B, Nystatin and 84.4% to Ketoconazole. It showed almost same susceptibility to Fluconazole (68.8%) and Itraconazole (62.5%). Susceptibility to Clotrimazole was 50%, as represented in Figure 3.To sumarise, in Figure 4, overall high susceptibility of non albicans *Candida* (NAC) to Amphotericin B (97.4%) is promising. Increased susceptibility was also seen to Nystatin(93.4%) and Ketoconazole (89.5 %). Susceptibility to Fluconazole was 63.2% whereas almost equal susceptibility was seen to Itraconazole (52.6%) and Clotrimazole (53.9%).Overall, NAC spp were less susceptible to Fluconazole and Clotrimazole as compared to *Candida albicans* as shown in Table 1. Also, resistance was found even to Amphotericin B and Nystatin among NAC spp.

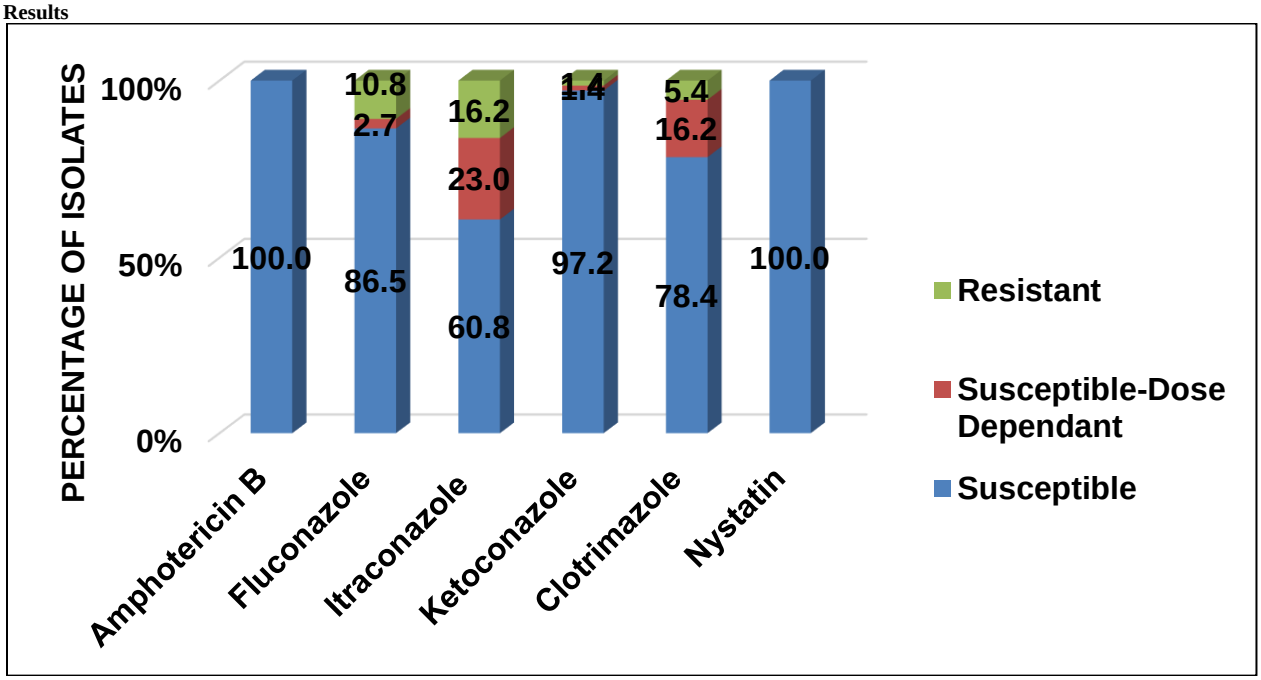

Fig 1:Antifungal susceptibility of *Candida albicans*

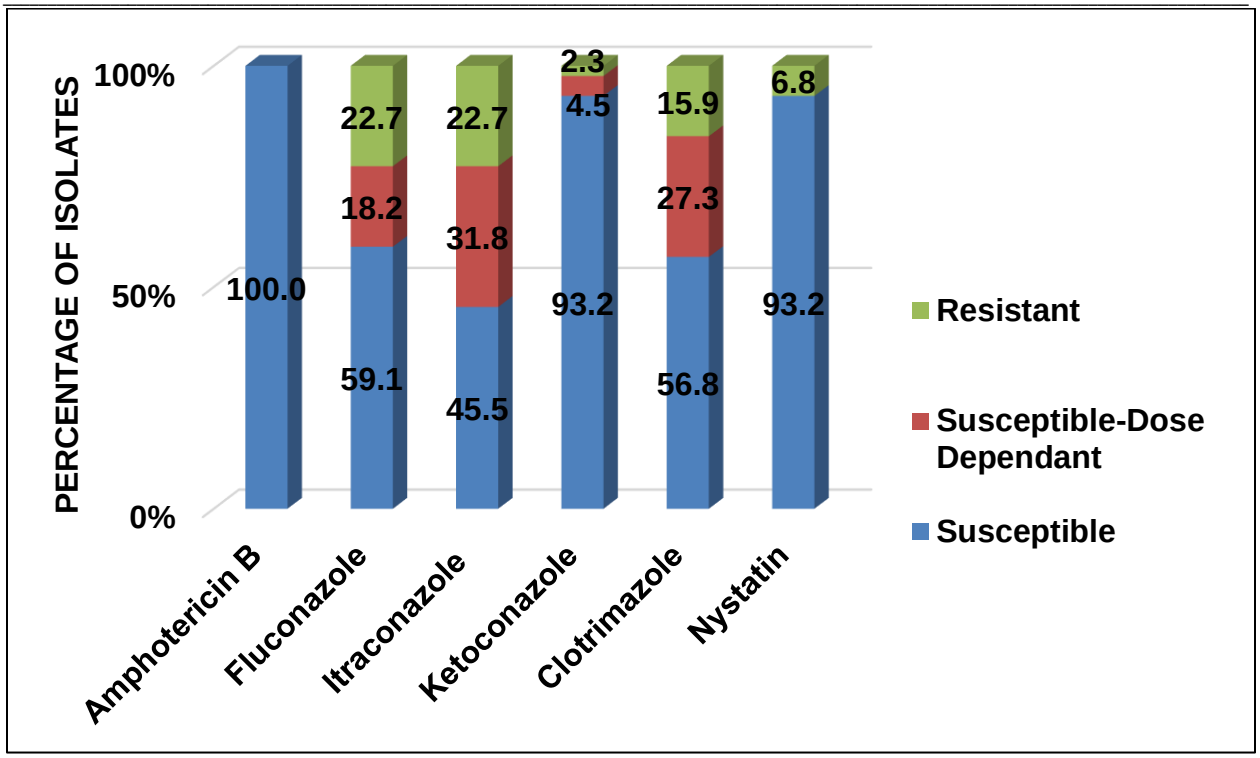

Fig 2:Antibiotic susceptibility of Candida glabrata

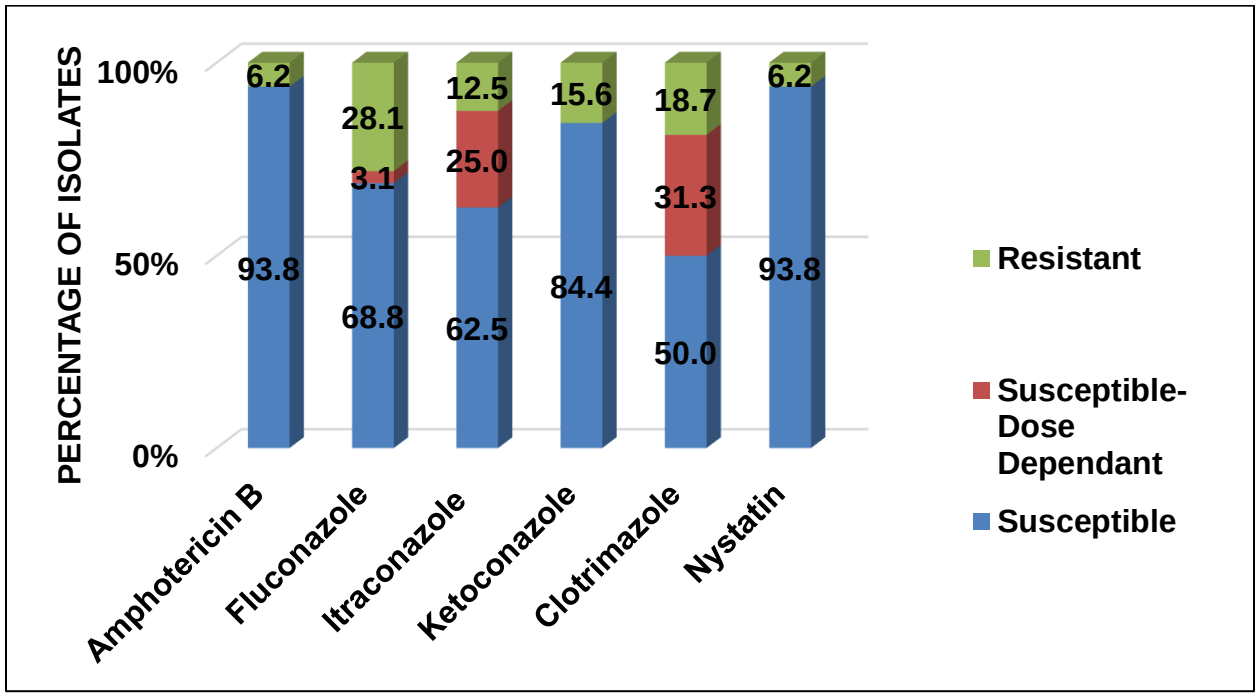

Fig 3:Antibiotic susceptibility of Candida tropicalis

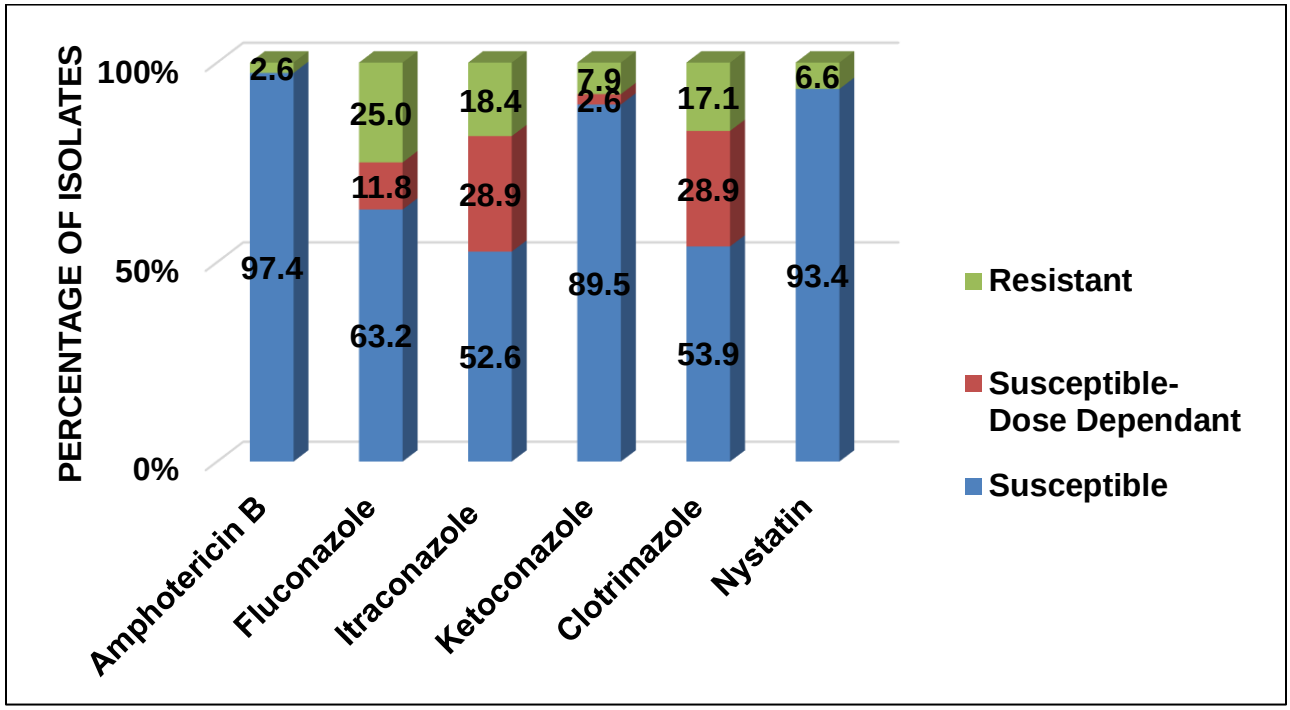

Fig 4:Antibiotic susceptibility of total non albicans Candida

Table 1 : Comparison of Antifungal susceptibility pattern of *Candida albicans* and NAC

Antifungal drugs	*Candida albicans*	Non-albicans*Candida*
Amphotericin B	100%	97.4%
Fluconazole	89.2%	75%
Itraconazole	83.7%	81.6%
Ketoconazole	98.6%	92.1%
Clotrimazole	94.6%	82.9%
Nystatin	100%	93.4%

Discussion

Vulvovaginal Candidiasis is a major health concern as affecting women of reproductive age group both physically and psychologically. If not treated appropriately and on time, it may lead to recurrence and also further complications.In the management of this condition, Clotrimazole and Fluconazole are the two most commonly used antifungal drugs by the clinicians.[12]However, an increasing resistance to these drugs is observed owing to the widespread and inappropriate use of antimycotic treatments. Other factors like self medication, topical use, long term treatments and recurrent candidal episodes further contribute to the resistance.[13]This is superadded by the emergence of non-albicans *Candida* spp further leading to recurrent episodes of vaginal Candidiasis. Hence, antifungal susceptibility of *Candida* spp before commencing empirical antifungal therapy enables correct management and thereby prevents emergence of resistance and occurrence of recurrent cases. All the *Candida albicans* in our study were susceptible to Amphotericin B and Nystatin. This correlates with the study of Gandhi et al[14] that reports 100% susceptibility of *Candida albicans* to both Amphotericin B and Nystatin. Similarly, Muthusamy et al[15] reported 100% susceptibility of *Candida albicans* to Amphotericin B. However, Lakshmi et al[16] reported 80% susceptibility of *Candida albicans* to Amphotericin B.Among the azoles, *Candida albicans* exhibited highest susceptibility of 97.2% to Ketoconazole in our study. Similar findings were reported by Lakshmi et al[16], where *Candida albicans* showed highest susceptibility to Ketoconazole (89%). In our study, susceptibility to Fluconazole was 86.5% whereas Gandhi et al[14] reported 78% susceptibility to Fluconazole. Susceptibility to Clotrimazole in our study was 78.4% which was similar as compared to 79% reported by Gandhi et al.[14] Least susceptibility of *Candida albicans* was shown for Itraconazole (60.8%) in our study. This correlated with the findings of Gandhi et al [14] and Lakshmi et al[16] that showed 49.38% and 56% susceptibility to Itraconazole, which is lowest compared to other azoles. Thus, susceptibility of *Candida albicans* to Ketoconazole is higher as compared to other azoles. In our study, all the *Candida glabrata* isolates were susceptible to Amphotericin B. This correlated with the findings of Gandhi et al[14] which also showed 100% susceptibility of *Candida glabrata* to Amphotericin B. But, Babin et al[17] reported 80% susceptibility to Amphotericin B. Also, susceptibility to Nystatin in our study was 93.2% however Gandhi et al[14] reported it as 100%.Among the azoles, in our study, *Candida glabrata* showed 93.2% susceptibility against Ketoconazole and 59.1% against Fluconazole. Similar results were reported by Gandhi et al[14] which showed 90% susceptibility of *Candida glabrata* to Ketoconazole and 79% susceptibility to Fluconazole. However, Babin et al[17] reported 32% susceptibility to Fluconazole. Susceptibility to Itraconazole was low in both present study and

Gandhi et al[14] study being 45.5% and 52.63% respectively. *Candida glabrata* showed 56.8% susceptibility to Clotrimazole in our study whereas it was 95% reported by Gandhi et al[14]. Thus, susceptibility of *Candida glabrata* to Amphotericin B, Nystatin and Ketoconazole was higher as compared to other drugs. An increasing resistance to Fluconazole and Clotrimazole is seen among *Candida glabrata*.Susceptibility of *Candida tropicalis* to Amphotericin B and Nystatin was 93.8% in our study whereas Gandhi et al[14] reported it as 83.4% and 100% respectively. However, 100% susceptibility to Amphotericin B was reported by Muthusamy et al.[15] This shows that resistance has started developing for Amphotericin B and Nystatin among *Candida tropicalis.*Among the azoles in our study, *Candida tropicalis* showed highest susceptibility of 84.4% for Ketoconazole, followed by 68.8% for Fluconazole. However Gandhi et al[14] reported more susceptibility to Fluconazole (83%) as compared to Ketoconazole (75%). Muthusamy et al[15] showed 100% susceptibility of *Candida tropicalis* to Fluconazole whereas 84% susceptibility was reported by Kandati et al[4]. Thus, there is a wide variation reported by various studies amongst susceptibility of *Candida tropicalis* to Fluconazole. Itraconazole susceptibility of *Candida tropicalis* was 62.5% in our study. Similar finding was reported by Gandhi et al[14] and Babin et al[17] susceptibility being 66.6% and 68.7% respectively. Susceptibility of *Candida tropicalis* to Clotrimazole was lowest (50%) in our study however 75% suceptibility was reported by Gandhi et al.[14] This indicates that even *Candida tropicalis* along with *Candida glabrata* is showing increasing resistance to Clotrimazole.Overall, our study highlights that among the non albicans *Candida,* susceptibility to Ketoconazole, Fluconazole, Clotrimazole and Itraconazole was 89.5%, 63.2%, 53.9% and 52.6% respectively. Thus among azoles, highest susceptibility was shown to Ketoconazole followed by Fluconazole in our study. This correlated with the findings of Lakshmi et al[16] that reported highest susceptibility of 76% against Ketoconazole. However, greater susceptibility was reported by Lakshmi et al[16] to Clotrimazole(69%) and Itraconazole (62%) compared to Fluconazole (58%). Thus, among non albicans *Candida* Ketoconazole is more susceptible as compared to other azoles. In our study, non albicans *Candida* showed 97.4% and 93.4% susceptibility to Amphotericin B and Nystatin whereas Lakshmi et al[16] reported 92% and 72% susceptibility against these drugs. These findings are alarming as increasing resistance is developing among non albicans*Candida* for higher drugs like Amphotericin B and Nystatin. This may be due to non judicious prescribing of antifungal drugs for longer duration thus making the resistance to these drugs alarming when used in case of chronic infections, complications and when lower drugs are resistant.Comparing the overall susceptibility of *Candida albicans* and non albicans *Candida*, in our study, susceptibility of *Candida albicans* and non albicans*Candida* to Amphotericin B was 100% and 97.4% respectively. However, Lakshmi et al[16] reported 80% and 92% susceptibility by *Candida albicans and* non-albicans*Candida* respectively. The susceptibility of both *Candida albicans* and non-albicans *Candida* to Nystatin was 100% and 93.4% respectively in our study whereas Lakshmi et al[16] reported a susceptibility of 76% and 72% to both *Candida albicans* and non albicans *Candida*. Thus, in our study, greater susceptibility was shown to higher drugs by both *Candida albicans* and non albicans *Candida*.Increased susceptibility to Clotrimazole was shown by *Candida albicans* (94.6%) as compared to non albicans *Candida* (82.9%) in our study. This correlated with the findings of Lakshmi et al[16] that showed 78% susceptibility by *Candida* albicans and 69% by non albicans *Candida*. *Candida albicans* was more susceptibile to Fluconazole (89.2%) compared to non albicans*Candida* (75%) in our study. This finding correlated with that reported by Lakshmi et al[16] where *Candida albicans* showed higher susceptibility to Fluconazole (60%) as compared to non albicans *Candida* (58%). Thus, we see that non albicans *Candida* show decreased susceptibility to Fluconazole as compared to *Candida albicans.*Susceptibility of *Candida albicans* and non albicans *Candida* towards Ketoconazole was 98.6% and 92.1% respectively which is higher compared to Fluconazole in our study. Similarly Lakshmi et al[16] reported higher susceptibility by *Candida albicans*(89%) and non albicans *Candida* (76%) towards Ketoconazole. These findings show that among the azoles greater susceptibility was shown by both *Candida albicans* and non albicans *Candida* to Ketoconazole compared to other azoles.Azoles are the most frequent class of antifungals used to treat *Candida* infections. Fluconazole is the preferred treatment for many *Candida* infections as it is less expensive, exhibits limited toxicity and is available for oral administration. Clotrimazole is the commonly used drug in vaginal pessary.[12] However, several *Candida* species exhibit resistance against azole antifungals.[18] This may be a cause of recurrent infections and treatment failure by non albicans *Candida.* Fluconazole is also used in the syndromic management of sexually transmitted infections (STI) in the Kit for vaginal discharge, where treatment is based on syndromes rather than specific diseases identified on testing. This may affect its use in the syndromic management of STI, thus emphasizing the need of doing speciation of *Candida* and thereby antifungal susceptibility to know the susceptibility of various *Candida* spp and to prevent the emergence of resistance.

Conclusion

Antifungal susceptibility testing of *Candida* species should be made mandatory before initiation of empirical therapy to avoid the overuse of antifungal drugs. Our study highlights that *Candida* isolates were more susceptible to Amphotericin B, Nystatin and Ketoconazole as compared to Clotrimazole and Fluconazole. Fluconazole is widely used as oral drug for treatment of vaginal discharge as it is less expensive and exhibits limited toxicity. It is also used in the Kit provided by the National STI/RTI prevention and control program for vaginal discharge. Also, Clotrimazole is commonly used in vaginal pessary. But in our study, susceptibility to Fluconazole and Clotrimazole was less.Thus, speciation and antifungal susceptibility will play a vital role in appropriateselection of antifungal agents for the treatment offungal infections prior to the initiation of therapy. This will also limit the emergence and spread of drug resistance preventing infections and complications of vaginal candidiasis.Also, STI clinics in tertiary care centres and peripheral hospitals should support an approach of targeted screening for the diagnosis of vaginal discharge in patients. Emphasis should also be given to providing education and reproductive healthcare to patients attending outpatient department.

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