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A STUDY OF ANTIFUNGAL SUSCEPTIBILITY TESTING AMONG NON ALBICANS CANDIDA IN PATIENTS OF VULVO VAGINAL CANDIDIASIS AT TERTIARY CARE CENTER

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ABSTRACT **Background &objectives:** Candida species are emerging as a significant pathogen certain species of Candida like Candida krusei are inherently resistant to azoles. In vitro susceptibility testing is essential for guiding therapy. The present study aims todetermine the antifungal susceptibility pattern ofNon albicansCandida isolates by disc diffusion and micro broth dilution method. **Methods:**This was a prospective study, conducted among 200 patients complaining of Vulvovaginal dischargeSpeciation was done as per standard microbiological methods. Non albicans Candida species were identified. Antifungal resistance was determined by disc diffusion method for fluconazole, Voriconazole and by microbrothdilution for fluconazole. Results: A total of 200 samples were collected from patients complaining of vaginal discharge . Out of them 69 were identified as Candida species , 31[44%] were C.albicans and 38 [56%] were non albicans Candida among them C.glabrata 22 (57%) , C.tropicalis 12 (31%), and 4 (10%) C.krusei. **Interpretation and conclusion:** In the present study, all the isolates of C. krusei 4 [10%] tested showed resistance to fluconazole by both disc diffusion and microbroth dilution methods . All isolates of C.glabrata 22 [57%] and C. tropicalis 12[31%] , tested were sensitive to fluconazole by both disc diffusion and microbroth dilution . For voriconazole there was no resistance among all isolates tested by disc diffusion method. It is essential to perform susceptibility testing for all the Candida isolates for providing crucial information about the resistance pattern and help in choosing the appropriate antifungal drug for therapy. Disc diffusion method which is easy to perform can be utilized for day to day practice.

KEYWORDS : Broth micro dilution, NonalbicansCandida, Disc diffusion

INTRODUCTION

Recurrent vulvovaginalcandidosis (RVVC) is defined as the occurrence at least four episodes of VVC in a one-year-period [1].Recurrent infection as seen in RVVC, unlike persistent infection, is characterized by the presence of intervals when the patient is free of symptoms [2]. Recurrent infections have a mutual relationship with NAC species infection where the occurrence of one predisposes to the other. Recurrent infections commonly lead to increased frequency of NAC species colonization, and also, the persistence of NAC species due to their resistance to antifungal agents might result in recurrent infections. This relationship is more common with C.glabrata [3].

Candida species have become a significant nosocomial pathogen with raise in drug resistant isolates. Species level identification is essential as certain species like Candida krusei are inherently resistant to azole drugs [4].

Antifungal drug therapies are selected empirically, but in vitro susceptibility testing are essential for guiding or altering the therapy. Fluconazole is the common azole antifungal drug that is being prescribed to immunocompromised individuals either as prophylaxis or for therapy [5].

Owing to the rise of drug resistant isolates the study of Antifungal drug resistance remains an area of significant importance [6]. The drug susceptibility testing methods must be precise, cost effective and easily reproducible. In the present study , two antifungal susceptibility methods, disc diffusion method and microbroth dilution method was performed to detect the resistance pattern of non albicans candida species isolated from vulvovaginal discharge collected from the patients of Vulvovaginal candidiasis .

MATERIALS AND METHODS:

This prospective study was done in the Department of Microbiology, Osmania General Hospital ,Telangana , India . A total of 69 Candida spp isolated from vulvovaginal discharge samples . All the 69 isolates were subcultured onto Sabouraud dextrose agar and incubated at 37 C for 24 to 48 hours and the growth of creamy white colonies were subjected to gram staining and further speciation was done as per standard Microbiological techniques (germ tube test, colour of the colonies in Chrom agar, morphology in corn meal agar) [7]. 38 isolates were identified as non albicans candida Further antifungal susceptibility testing was performed by disc diffusion method and broth micro dilution method .

Antifungal susceptibility testing by disc diffusion method

Antifungal Susceptibility Testing for Candidaisolates was done by Disc diffusion method, as per CLSI Guidelines on Antifungal Susceptibility testing in M-44A 2 document.

Disc diffusion method:

Glucose methylene blue (GMB) stock solution was prepared

GMB stock solution was prepared and autoclaved for 15 minutes at 121 °C. The solution was stored at room temperature and were not refrigerated as this may cause precipitation.

Mueller-Hinton agar with 2% glucose and 0.5 µg/ml methylene blue

Mueller-Hinton agar was prepared according to the manufacturer's instructions.

GMB solution with methylene blue was poured into the freshly prepared and cooled Mueller Hinton agar medium and then they were poured into plastic, flat-bottomed petri dishes to a depth of approximately 4 mm. Then agar medium was allowed to cool to room temperature and stored at refrigerator temperature (2 to 8 °C) .The agar medium should have a pH between 7.2 and 7.4 at room temperature.

Inoculum preparation:

Inoculum was prepared by picking five distinct colonies from a 24-hour-old culture of Candida species and the colonies were suspended in 5 ml of sterile normal saline. The turbidity was adjusted equivalent to 0.5 McFarland standards (1x 106to 5 x 106cells/ml) resulting in semi-confluent growth.

Inoculation of the agar plate:

A sterile cotton swab was dipped into the suspension and rotated several times. The excess fluid was removed from the swab by pressing firmly against the inside wall above the fluid level. Then a lawn culture was made on the moisture to be absorbed, and then the antifungal disc were dispensed onto the plate.

Application of disks to inoculated plates:

Antifungal discs were dispensed onto the surface of an inoculated agar plate by means of a sterile forceps and pressed down. The discs were evenly distributed on the plate with a distance of 24 mm from centre to centre of the discs.

Incubation:

Plates were inverted and incubated at 37°C within 15 minutes after placing the discs. The zone is read after 20-24 hours of incubation. If no visible growth with particular strains, the plate is re-incubated for 48

hours and then read. Zone of inhibition was measured at the point where there was prominent reduction in growth.

TABLE – 1 : Zone interpretation chart for Disc diffusion method

SOURCE DOCUMENT CLSI M44-A2			
DRUGS	SENSITIVE	SDD / I R	RESISTANT
Fluconazole	≥19	15-18	≤14
Voriconazole	≥17	14-16	≤13

Broth microdilution:

- Although the antifungal broth macrodilution test was the first method proposed by the CLSI, this test is cumbersome for use in clinical laboratory. The broth microdilution has become the most widely used technique. Now a days most of the laboratories use broth microdilution method, as it is more practical, less cumbersome with more consistent MIC results.
- The inter-laboratory agreement of the microdilution MICs has also been found to be much better than macrodilution method.
- It is easy to perform, trays may be sealed in plastic bags,& stored frozen at -70°C for up to 6months without deterioration of drug potency.

CLSI recommends RPMI 1640 medium with L-glutamine, without sodium bicarbonate and buffered with morpholine propane sulfonic acid at 0.165M as the test medium .

PREPARATION OF STOCK SOLUTION
DRUG WEIGHT CALCULATION:

- W(mg) = V(ml) X C (µg/ml) / p (µg/mg)
- W=weight of the drug powder required
- V=volume of stock solution to be made
- C=concentration of stock solution
- P=potency of antifungal power

For water insoluble drugs:

- Stock solutions are to be prepared at 100 times the highest concentration to be tested. The measured amount of antifungal powder is dissolved in 3ml DMSO in a test tube and labelled as 'stock solution'.
- For water soluble drugs:
- Stock solutions are to be prepared at 10 times the highest concentration to be tested. The measured amount of antifungal powder is mixed first in 1ml distilled water in a test tube, then add 7ml of RPMI 1640 media to it and label it as 'T1'.

TABLE - 2 :PREPARATION OF ANTIFUNGAL SOLUTION FOR WATER SOLUBLE DRUGS

Antifungal Solution (Water Soluble Drug) (Double Strength)					
Step	Concentration (µg/ml)	Source	Volume (ml) + Medium (ml)	Intermediate concentration (µg/ml)	Final Concentration at 1:10 (µg/ml)
1	5120	Stock	1+7	640	64
2	640	Step 1	1+1	320	32
3	640	Step 1	1+3	160	16
4	160	Step 3	1+1	80	8
5	160	Step 3	0.5+1.5	40	4
6	160	Step 3	0.5+3.5	20	2
7	20	Step 6	1+1	10	1
8	20	Step 6	0.5+1.5	5	0.5
9	20	Step 6	0.5+3.5	2.5	0.25
10	2.5	Step 9	1+1	1.25	0.125

MIC TESTING:Yeast inocula standardized spectrophotometrically and diluted in RPMI medium to obtain a final concentration of 0.5 to 2.5 * 103 CFU/ml .In microbroth dilution, 100µl of each antifungal agent at a concentration two times the targeted final concentration were dispensed in the wells of flat bottom 96 well micro titre plates. A constant volume (100 µl) of inoculum was added to each microdilution well containing 100 µl of serial dilution of the antifungal agents to reach final concentration and incubated at 35ºC for 48 hrs for Candida.

- Mark the 96 well 'U' shaped microtitre plate vertically with test strains from A to H and horizontally from T1 to T12.
- Add 100 µl of each prepared drug dilution vertically in all wells in one column for each dilution.
- The same is repeated for all dilutions from T1 to T10.
- Now add 100 µl of prepared inoculum in the wells marked T1 to T10, in one row for one fungal strain.
- Column T1 will contain the highest concentration,& column T10 will contain the lowest concentration of drug.
- To T11, add only inoculum without drug. It serves as growth control.
- To T12, add only RPMI medium without drug. It serves as media control.
- Now the microtitre plate is covered with a lid & incubated @ 35°C for 48hrs. Readings are taken at 24hrs & again at 48hrs.
- Results are read visually with reading mirror.

READING AFST RESULT

Each microdilution well is then given a numerical score as follows:

- 0 – optically clear
- 1 – slightly hazy
- 2 – prominent decrease in turbidity
- 3 – slight reduction in turbidity
- 4 – no reduction of turbidity

Reading in each well should be compared with that of growth control well

Candida species [Yeast] quality control

- Candida albicans ATCC – 90028
- Candida parapsilosis ATCC – 90018 were used

Drug dilution range

1) Fluconazole 64 to 0.12 µg/ml

MIC of azoles were defined as the lowest concentration that inhibit growth by 50% in microbroth& 80% in macrobroth dilution

TABLE -3 DRUG DILUTION RANGE

Drug tested	Susceptible	Dose Dependent	Resistant
Fluconazole	≤ 8µg/ml	16-32 µg/ml	≥64µg/ml

RESULTS:

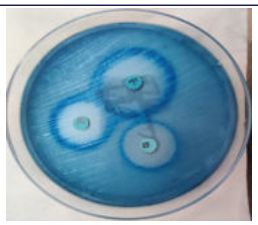
A total of 200 samples were collected from patients complaining of vaginal discharge . Out of them 69 were identified as Candida species , 31[44%] were C.albicans and 38 [56%] were non albicans Candida among them C.glabrata 22 (57%) , C.tropicalis 12 (31%), and 4 (10%) C.krusei. In the present study, all the isolates of C. krusei 4 [10%] tested showed resistance to fluconazole by both disc diffusion and microbroth dilution methods .All isolates of C.glabrata 22 [57%] and C. tropicalis 12[31%] , tested were sensitive to fluconazole by both disc diffusion and microbroth dilution . For voriconazole there was no resistance among all isolates tested by disc diffusion method.

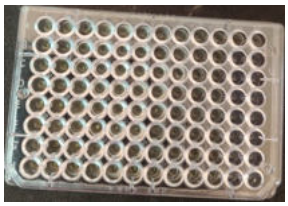
TABLE -4 ANTIFUNGAL RESISTANCE PATTERNS OF NON CANDIDA ISOLATES

	Susceptibility pattern of Candida species(CLSI)						
	Disc Diffusion Method				Microbroth dilution		
Drugs tested	Voriconazole (zone diameter)		Fluconazole (zone diameter)		Fluconazole		
Name and no. of isolates tested	>17mm	<12mm	>17mm	<13mm	MIC ranges		
	Sensitive	Resistant	Sensitive	Resistant		Sensitive	Resistant
C.glabrata (22)[57%]	22	0	22	0	<16µg/ml;S>64µg/ml;R	22	0
C.tropicalis (12)[31%]	12	0	12	0	<2µg/ml; S>8µg/ml ;R	12	0
C. Krusei (4)[10%]	4	0	0	4	<2µg/ml; S>8µg/ml ;R	0	4

OBSERVATIONS

FIGURE -1
FIGURE -2





Disc diffusion method Muller Hinton agar Microbroth dilution by Microtitre plate method with Methylene blue and glucose

DISCUSSION:

The overall prevalence of non albicans species in our study was (56%) which was more than that of C .albicans (44%).

Most common non albicans species isolated in our study have been C.glabrata (57%), C. tropicalis (31%), C. krusei (10%) . Antifungal susceptibility testing was carried out for all 38 non albicans Candida isolates by both Disc Diffusion and microbroth dilution method.

In ourstudy, all the isolates of C. krusei 4 [10%] tested showed resistance to fluconazole by both disc diffusion and microbroth dilution.

There was no resistance for fluconazole among C.glabrata 22 [57%] and C. tropicalis 12[31%] , by both disc diffusion and microbroth dilution .

For voriconazole there was no resistance among all isolates tested by disc diffusion .

We speculate this increasing detection of non-albicans species are probably related to the widespread and inappropriate use of antimycotic treatments (self medication, topical use, long-term treatments and repeated candidial episodes).

Hence, the reliable and rapid identification method of Candida species is a fundamental goal of microbiology laboratories.

In vitro antifungal susceptibility testing is becoming important because of the emergence of new non albicans species and the increased inherent and acquired resistance to azoles .

Agar-based antifungal susceptibility testing is easy to perform and inexpensive for routine laboratories. CLSI M44-A disc diffusion testing with glucose methylene blue Muller Hinton agar is a very convenient method for antifungal susceptibility testing.

Study done by DeepaBabain [8] et al reported resistance for fluconazole by C.glabrata[48%],C.krusei [100%],C.tropicalis [25%].Study done by jayachandran[9] et al reported resistance for fluconazole by C. glabrata [60%],C.krusei [100%] , C .tropicalis [26%].Study done by bitew et al [10]reported resistance for fluconazole by C.glabrata [0%],C.krusei [100%],C.tropicalis [0%].Study done by venugopal et al [11]reported resistance for fluconazole by C. glabrata [0%],and C.tropicalis [0%].

TABLE- 5 : Percentage Of Fluconazole Resistance in different studies

Author	Year	Place	Candida glabrata	Candida krusei	Candida tropicalis
Deepababin et al	2013	Kerala	48%	100%	25%
			25	19/19	32
Jayachandran AL et al	2018	Tamilnadu,india	60%,	100%	26%
			6/10	11/11	8/30
Bitew et al	2018	Ethiopia	0%	100%	0%
			0/3	15/15	0/2
Venugopal et al	2020	Saudi Arabia	0%		0%
			0/1		0/10
Present study	2022	India	0%	100%	0%
			0/22	4/4	0/12

Conclusion :

- In routine practice drug of choice for VVC is fluconazole as C.krusei is intrinsically resistant to fluconazole, resulting in treatment failure.
- Definitive laboratory procedures are of paramount importance to identify and to speciate candida isolates to ensure appropriate and effective use of antifungals.

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