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IN VITRO SENSITIVITY OF PATHOGENIC YEASTS TO FLUCONAZOLE

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ABSTRACT
The sensitivity of pathogenic yeasts, *Candida* (yeast like fungi) and *Cryptococcus species* to fluconazole were determined by broth micro dilution, broth macro dilution and agar dilution methods. Mean MICs showed good agreement between broth micro dilution and macro dilution methods. Agar dilution tests showed two fold higher MICs range. For *C. albicans* the MIC range observed was 1.25-80µg/ml by microdilution, 2.5-160µg/ml by macro dilution method while 2.5-160 µg/ml by agar dilution method. *C. albicans, C. tropicalis* and *C. glabrata* were less sensitive than other *Candida species* such as *C. gullermondi, C. krusei. C. parapsilosis, C. stellatoidea, C. pseudotropicalis* and *Cryptococcus neoformans.*

KEY WORDS: Fluconazole, Pathogenic Yeast, Sensitivity Testing.

INTRODUCTION

Fungi include a large group of eukaryotic organisms which depend for their nutrients as parasites or saprophytes. Yeasts are predominantly unicellular and uninucleate with round oval or elongate cells. Most of the yeasts are propagated by a process of budding with a daughter cell on blastospore, while very few species reproduce by fission (Glyn *et al*., 1985).Candidiasis is the worldwide infection caused by pathogenic yeast. The incidence of pathogenic yeasts was studied in many parts of the world. *Candida albicans* is the predominant species causing oral candidiasis, vaginitis and systemic candidiasis. *Cryptococcus neoformans* is the causative agent of cryptococcal meningitis (Rippon 1982. NG *et al*., 1998). It is reported that the fungal infection was present in 39% of diabetes mellitus patients during Nov.1991- July 1993. *Candida albicans* was the predominant organism (51.8%). Other *Candida species* causing fungaemia was *Candida glabrata* (15.6%) (Kauffman *et al*., 2000). The prevalence of mycotic vaginits caused by non albicans strains such as *T. glabrata (C.glabrata), C. parapsilosis* and *C. tropicalis* was 9.9% in 1988 and 17.2% in 1995. HIV -seropositive patients with recurrent vulovovaginal candidiasis constituted clinician referred patients (Spinillo, 1997).

Imidazole antimycotics constitute a group of antifungal drugs. Benzimidazole was the first antimycotic imidazole tested (1944) for its inhibitory activity towards pathogenic fungi by Woolley (Kerridge, 1986). At present the most important imidazoles like Clotrimazole, Miconazole, Econazole, Ticonazole, Isoconazole, Ketoconazole, Fluconazole, Voriconazole, Eberconazole, and Ravuconazole etc. are formulated for topical and oral purpose in the therapeutic management of yeast infections. Its mode of action is by inhibition of C-14-demethylation of trimethylsterols. The drugs interact with the haem ion of cytochrome P 450 (Kerridge, 1986). Fluconazole is a bistriazole antifungal structurally related to imidazole-derivative antifungal. It is used in deep fungal infections and superficial fungal infections. It is having marked activity against Candida species and offered a safe effective and convenient therapy, (Sobel and Brooker 1995). Due to the increased level of risk populations, the occurrence of fungal infections has significantly raised in past two decades. The morbidity and mortality in seriously immunocompromised patients was due to the infection of Candida species and was most commonly isolated fungal pathogens. In the nosocomial infections *Candida albicans* was the most commonly found and prevalent species in hospitalized individuals, so the management and treatment of fungal infection was critical. (Yang *et al*., 2012). There were four major classes of antifungal drugs for treatment and these include azoles, echinocandins, polyenes, and 5-flucytosine. Due to less side effects and low cost, fluconazole has become one of the best prescribed drugs.

It has following chemical structure.

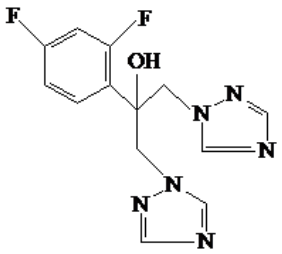


Figure 1. Structure of Fluconazole

The methods commonly used for the antifungal sensitivity testing are broth microdilution, broth macrodilution, agar dilution and disc diffusion (Pfaller, 1992). National Committee for clinical Laboratory Standards (NCCLS).Villanova, Pa has proposed standard reference broth macrodilution method (NCCLS standards M27-A,1997) which was revised subsequently in 2002 (NCCLS,2002) with the inclusion of broth microdilution method for antifungal sensitivity testing of yeasts. In the present study, sensitivity of various *Candida species* and *Cryptococcus neoformans* to flucoazole was studied by various sensitivity test methods and were compared.

MATERIALS AND METHODS:

Fluconazole was obtained from Sarabhai, Pharmaceuticals Vadodara, India. A stock solution (1600 µg/ml) was prepared by dissolving fluconazole in 70%v/v ethanol. The stock solution was then stored in refrigerator until use.

Culture and Inoculums:

A total of well characterized clinical isolates of pathogenic yeasts were selected including *C. albicans* (20 isolates), C. *gulliermondii* (04 isolates), *C. krusei* (04 isolates), *C. glabrata* (03 isolates), *C. parapsilosis* (05 isolates), *C. stellatoidea* (02 isolates), *C. tropicalis* (04), *C. pseudotropicalis* (03) and *Cryptococcus neoformans* (05) were selected for sensitivity testing. Each isolate was originated from different patient with clinical manifestations and was maintained on Sabouraud Dextrose Agar. An inoculums of approximately 1x102 (CFU/ml) was prepared and used for sensitivity testing by broth micro dilution, broth macro dilution and agar dilution method.

This test was performed in round bottom sterile glass tubes (12 x75mm) using NCCLS reference method, NCCLS M27- A2 (2002). A working drug solution of 160 g/ml of fluconazole was prepared. The broth micro dilution test was performed by using sterile disposable, multiwell micro dilution plates (96 U-shaped wells). The procedure described by NCCLS standards (2002) was followed. Agar dilution test was performed in petriplates containing fluconazole incorporated in Sabouraud Dextrose Agar and inoculating agar surface with 10 µl of inoculums (approx. 1x 102 CFU/ml) as spot. The final drug concentrations in the medium were 160-0.3 g/ml from plate 1 through plate 10. Plate 11 and 12 with drug free solid medium were used as sterility and growth control respectively.

RESULTS AND DISCUSSION

In vitro sensitivity of pathogenic yeasts to fluconazole by broth micro dilution, broth macro dilution and agar dilution method is shown in Table 1 & 2. The MIC range for *C.albicans*, observed was 1.25-80 µg/ml by broth micro dilution method. 2.5-160 µg/ml by broth macro dilution method while the range was 2.5-160 µg/ml by agar dilution method. The fluconazole MIC range observed was 1.25-160 µg/ml by all the three antifungal susceptibility methods for *Candida species* while MIC range was 1.25-40 µg/ml for *Cryptococcus neoformans*.

The activity of fluconazole against pathogenic *Candida species* and *Cr. neoformans* was comparatively more by broth micro dilution than broth macro dilution and agar dilution. The agreement between broth macro dilution and agar dilution was good in some cases while agreement between broth macro dilution and broth micro dilution was good in case of *C. stellatoidea, C. pseudo tropicalis,* and *C. Glabrata*. The activity of fluconazole against pathogenic yeasts i. e. *Candida species* other than *Candida albicans* observed is 1.25-20 µg/ml for *C. gulliermondii*, 2.5-20 µg/ml for *C. krusei*, 10-80 µg/ml for *C. glabrata*, 5-80 µg/ml for *C.parapsilosis*, 2.5-5 µg/ml for *C.stellatoidea*, 5-20 µg/ml for *C. tropicalis*, 0.62-5 µg/ml for *C. pseudotropicalis* and 1.25-40 µg/ml for *Cr. neoformans*.

The results showed greater efficacy of broth macro dilution and agar dilution over broth micro dilution sensitivity testing. There is good co-relation observed between broth macro dilution and agar dilution in case of isolates of *C. albicans, C. krusei, C. glabrata, C. parapsilosis, C. stellatoidea and C. tropicalis*. The high activity of fluconazole was displayed against *C. stellatoidea, C. pseudotropicalis,* while *C. albicans* showed less sensitivity to fluconazole. Fluconazole MIC <10 g/ml for pathogenic yeasts reported by Hacek et al., (1995). *C. albicans, C. parapsilosis, C. tropicalis, Cr. neoformans, C. glabrata and C. guilliermondii* has shown MIC < 10 g/ml from July 1993 to June 1994 study.37% resistant *Candida* strains by agar dilution method were reported by Chakrabarti *et al*., (1995). In resistance mechanism Candida species required > 64 g/ml fluconazole concentration reported by White et al., (2002). However 50% inhibition range of 0.05 - > 100 g/ml fluconazole for *C. albicans* while for *C. glabrata* 6.3-> 100 µg/ml for *C. tropicalis* 0.1 - > 100 g/ml and *for C. parapsilosis* 0.2-1.6 g/ml was reported by Odds *et al*,. (1995). The interpretative criterion for susceptibility of fluconazole was proposed by NCCLS 2002 and Rex *et al*., (1997).Hong Nguyen and Yu (1998) reported the *Cr. neoformans* isolates with MIC of 8 g/ml as fluconazole susceptible, 16-32 g/ml as susceptible dose dependent and 64 g/ml as fluconazole resistant using broth macro dilution method. Pfaller *et al*., (2002) also

considered MIC value for classifying clinical isolates of *Candida* species as fluconazole susceptible (MIC 8 g/ml), susceptible dose dependent (MIC 16-32 g/ml) and resistant MIC 64 g/ml using broth micro dilution method.

Table 1: Number of pathogenic yeast isolates sensitive to Fluconazole concentrations by Different Sensitivity Test Method

Sr. No.	Organism (No. of isolates)	Method	No. of isolates inhibited at stated concentration (g/ml)									
			0.31	0.62	1.25	2.5	5	10	20	40	80	160
1	*C. albicans* (20)	Broth Microdilution	--	--	3	6	10	14	17	19	20	20
		Broth Macrodilution	--	--	--	5	9	11	15	17	20	20
		Agar Dilution	--	--	--	1	7	10	13	15	18	20
2	*C. guilliermondii* (4)	Broth Microdilution	--	--	1	3	4	4	4	4	4	4
		Broth Macrodilution	--	--	1	3	4	4	4	4	4	4
		Agar Dilution	--	--	--	--	1	2	4	4	4	4
3	*C. krusei* (4)	Broth Microdilution	--	--	--	2	2	3	4	4	4	4
		Broth Macrodilution	--	--	--	--	2	3	4	4	4	4
		Agar Dilution	--	--	--	--	2	3	4	4	4	4
4	*C. glabrata* (3)	Broth Microdilution	--	--	--	--	--	1	2	3	3	3
		Broth Macrodilution	--	--	--	--	--	--	2	3	3	3
		Agar Dilution	--	--	--	--	--	--	1	2	3	3
5	*C. parapsilosis* (5)	Broth Microdilution	--	--	--	--	2	4	4	5	5	5
		Broth Macrodilution	--	--	--	--	--	1	4	4	5	5
		Agar Dilution	--	--	--	--	--	--	2	4	5	5
6	*C. stellatoidea* (2)	Broth Microdilution	--	--	--	1	2	2	2	2	2	2
		Broth Macrodilution	--	--	--	--	2	2	2	2	2	2
		Agar Dilution	--	--	--	--	2	2	2	2	2	2
7	*C. tropicalis* (4)	Broth Microdilution	--	--	--	--	2	4	4	4	4	4
		Broth Macrodilution	--	--	--	--	1	2	4	4	4	4
		Agar Dilution	--	--	--	--	2	3	4	4	4	4
8	*C. pseudotropicalis* (3)	Broth Microdilution	--	1	2	3	3	3	3	3	3	3
		Broth Macrodilution	--	--	1	3	3	3	3	3	3	3
		Agar Dilution	--	--	--	1	3	3	3	3	3	3
9	*Cr. neoformans* (5)	Broth Microdilution	--	--	1	2	2	5	5	5	5	5
		Broth Macrodilution	--	--	--	1	4	5	5	5	5	5
		Agar Dilution	--	--	--	--	1	3	4	5	5	5

In the present study both *Candida* and *Cryptococcus species* were considered as fluconazole susceptible (MIC 8 g/ml), susceptible dose dependent (MIC 16-32 g/ml) and resistant (MIC 64 g/ml). Among *C. albicans* 50%, 45% and 35% isolates were found to be susceptible by broth micro dilution, broth macro dilution and agar dilution method respectively. On the other hand 5%, 15% and 25% isolates were found to be resistant by broth micro dilution, broth macro dilution and agar dilution method respectively. Among *C. guilliermondii*, 100% isolates were susceptible by broth macro and micro dilution but only 25% isolates were susceptible and remaining 75% isolates were susceptible dose dependent and none of the isolate was found to be resistant to fluconazole by agar dilution method.

For *C. krusei*, 50% isolates were sensitive and 50% isolates were susceptible dose dependent by all sensitivity testing methods. Among *C. glabrata* only one isolate (33%) was found to be resistant by agar dilution method while 20% isolates *of C. parapsilosis* were resistant by broth macro dilution and agar dilution methods. 100% isolates of *C. stellatoidea* and *C. pseudotropicalis* were sensitive by all the sensitivity test methods. No resistance was reported in *C. tropicalis* and *Cr. neoformans* by different test methods. Among *Cr. neoformans*, 100% isolates were sensitive by broth micro dilution, 80% isolates were sensitive by broth macro dilution and only 20% isolates were found to be sensitive by agar dilution method. Fungtome *et al*. (1998) reported MIC range of 0.25-16 g/ml for *Candida albicans*, 8 - > 64 g/ml for *C. krusei*, MIC 0.13 – > 64 g/ml for *C. tropicalis*, and 1 - > 64 for *C. glabrata*. For *C. parapsilosis* and *C. krusei* the MIC range of fluconazole was 0.06 – 4 g/ml as reported by Barry *et al*. (2000). *C. krusei* is naturally resistant to fluconazole (Goa *et al*. 1995, Klastersky, 1995 and Boschman *et al*., 1998). Colombo *et al*. (1995) reported observations by comparing NCCLS reference method with E test. Fluconazole MIC range for *C. albicans* and *other*

Candida species was 0.125 - > 32 g/ml. The present study results co-relates with Hong Nguyen *et al*. (1998). MIC by agar dilution and by broth macrodilution method were greater for *C. parapsilosis* than reported by Barry *et al*. (2000). Our results for *C. albicans* also co-relates with Chakrabarti *et al*. (1995) and White *et al*. (2002). *C. krusei* in our study required maximum 20 g/ml of fluconazole concentration. The MIC range was 2.5-20 g/ml by broth microdilution, broth macrodilution and agar dilution method.

Table 2: MIC range, MIC50 MIC70 and MIC90 and mean MIC of pathogenic yeasts for Fluconazole by different Sensitivity Test Methods.

Sr. No .	Organism (No. of Isolates)	Sensitivity Test Methods	MIC range	MIC50	MIC70	MIC90	Mean MIC
1	*C. albicans* (20)	Broth Microdilution	1.25-80	5	20	40	14.56
		Broth Macrodilution	2.5-160	10	20	80	22.62
		Agar Dilution	2.5-160	10	40	80	38.12
2	*C. guilliermondii* (4)	Broth Microdilution	1.25-5	2.5	2.5	5	2.81
		Broth Macrodilution	1.25-5	2.5	2.5	5	2.81
		Agar Dilution	5-20	10	20	20	13.75
3	*C. krusei* (4)	Broth Microdilution	2.5-20	2.5	10	20	8.75
		Broth Macrodilution	5-20	5	10	20	10
		Agar Dilution	5-20	5	10	20	10
4	*C. glabrata* (3)	Broth Microdilution	10-40	20	40	40	23.33
		Broth Macrodilution	20-40	20	40	40	26.66
		Agar Dilution	20-80	40	80	80	46.66
5	*C. parapsilosis* (5)	Broth Microdilution	5-40	5	10	40	14.0
		Broth Macrodilution	10-80	20	20	80	30.0
		Agar Dilution	20-80	20	40	80	40.0
6	*C. stellatoidea* (2)	Broth Microdilution	2.5-5	2.5	5	10	3.75
		Broth Macrodilution	5	5	5	5	5.0
		Agar Dilution	5	5	5	5	5.0
7	*C. tropicalis* (4)	Broth Microdilution	5-10	5	10	10	7.5
		Broth Macrodilution	5-20	10	20	20	13.75
		Agar Dilution	5-20	5	10	20	10.00
8	*C. pseudotropicalis* (3)	Broth Microdilution	0.62-2.5	1.25	2.5	2.5	1.45
		Broth Macrodilution	1.25-2.5	2.5	2.5	2.5	2.08
		Agar Dilution	2.5-5	5	5	5	4.16
9	*Cr. neoformans* (5)	Broth Microdilution	1.25-5	2.5	5	5	3.75
		Broth Macrodilution	5-10	5	5	10	5.5
		Agar Dilution	5-40	10	20	40	17.0

All the values are in g/ml

Fig. 2. A. Minimum inhibitory concentration (MIC) values for fluconazole against yeast isolates

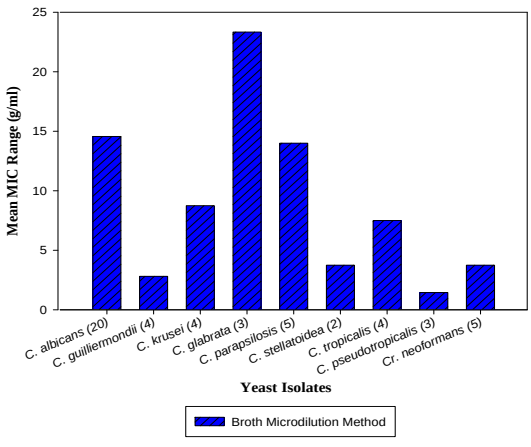


Fig. 2. B. Minimum inhibitory concentration (MIC) values for fluconazole against yeat isolates

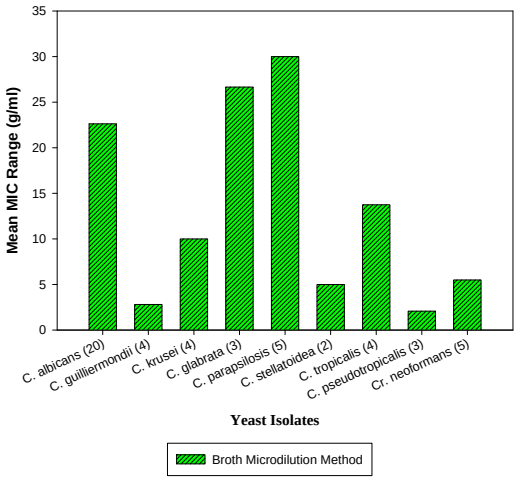
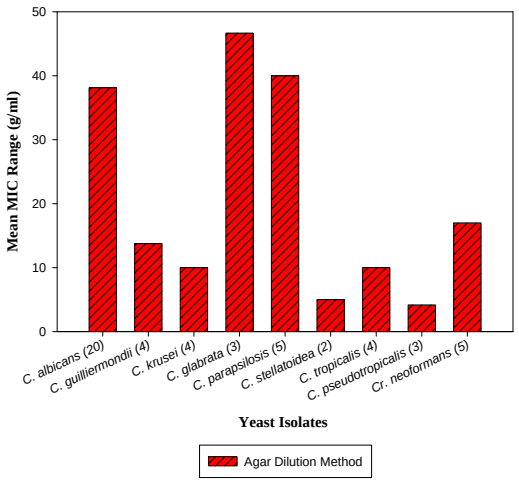


Fig. 2. C. Minimum inhibitory concentration (MIC) values for fluconazole against yeast isolates



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