RESEARCH ARTICLE

In vitro antifungal activity of ketoconazole against clinical isolates of *Candida* and *Cryptococcus* spp.

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ABSTRACT

The incidence of fungal infections has been rising over the past few decades contributing to morbidity and mortality. Antifungal activity of ketoconazole was evaluated against clinical isolates of *Candida abicans*, *C. guilliermondii*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, *C. stellatoidea*, *C. tropicalis*, *C. pseudotropicalis* and *Cryptococcus neoformans* by broth macrodilution, broth microdilution and agar dilution methods. Ketoconazole showed strong antifungal activities against isolated strains of *Candida* and *Cryptococcus.* Ketoconazole effectively inhibited *C. albicans* in the MIC range of 1.25-80 µg/ml by broth micro dilution, 2.5-160 µg/ml by broth macro dilution and 2.5-160 µg/ml by agar dilution method. *Candida* spp were more resistant to ketoconazole than *Cryptococcus neoformans*, as *C. neoformans* was inhibited at lower MIC range of 1.25-40 µg/ml. Agar dilution method showed higher MIC values as compared to broth dilution. The results suggest promising antifungal properties of ketoconazole.

Key words: Antifungal activity, Ketoconazole, Susceptibility testing

INTRODUCTION

Candida spp. are common normal flora found on mucosal surfaces. In the presence of mucosal barrier breakdown or immunosuppression, these organisms become significant pathogens that can lead to increased morbidity and mortality. *Candida* species are major human fungal pathogens that cause both mucosal and deep tissue infections (Sardi et al. 2013). *Candida* species belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal tract and vagina, and are responsible for various clinical manifestations from mucocutaneous overgrowth to bloodstream infections. These yeasts are commensal in healthy humans and may cause systemic infection in immunocompromised situations.

Ketoconazole is an antifungal imidazole with broad spectrum activity against a variety of yeasts, dermatophytes and dimorphic fungi. It is

given orally as well as topically. Ketoconazole activity remains stable or increase in media enriched with serum proteins (Van-cutsem et al. 1986) and it affect the ergosterol systhesis in fungi by inhibiting the C-14 α-sterol demethylation step, leading to accumulation of methylated sterols which disrupt fungal membrane structure (Kerridge 1986).

Antifungal susceptibility testing is an important tool for physicians and includes macrobroth, microbroth, or disk methods. The Clinical and Laboratory Standards Institute (CLSI), formerly the NCCLS (National Committee on Clinical Laboratory Standards) provide standard methods for antifungal susceptibility testing (NCCLS, 1992; CLSI, 2008). In view of this, the susceptibility of pathogenic yeasts to ketoconazole was studied by various susceptibility test methods and was compared.

MATERIALS AND METHODS

Drug solution:

The antifungal drug ketoconazole used for determination of Minimum Inhibitory Concentration (MIC) of pathogenic yeasts was obtained from Ethnor Limited, Mumbai, India. A stock solution of ketoconazole 1600 µg/ml was prepared with 70% v/v ethanol by dissolving the required amount in 70% ethanol and vigorously shaken for about 30 min and then allowed to stand till complete dissolved. The stock solutions were then stored in refrigerator until use.

Cultures for sensitivity testing:

A set of 50 well characterized clinical isolates of pathogenic yeasts including *Candida abicans* (20 isolates), *C. guilliermondii* (04 isolates), *C. krusei* (04 isolates), *C. glabrata* (03 isolates), *C. parapsilosis* (05 isolates), *C. stellatoidea* (02 isolates), *C. tropicalis* (04 isolates), *C. pseudotropicalis* (03 isolates) and *Cryptococcus neoformans* (05 isolates) were used. Each isolate was originated from a different patient with clinical manifestations and was maintained on Sabouraud Dextrose agar.

Media for sensitivity testing:

RPMI-1640 medium was used for broth macro and microdilution. For agar dilution method, Sabouraud Dextrose Agar (SDA) was used. All media and chemicals were purchased from M/s. Hi Media Laboratories, Mumbai.

Inoculum preparation :

The yeast isolates were maintained on SDA slants. 24 to 48 hrs old cultures were used for inoculum preparation. A loopful of culture was inculated in 2 ml sterile saline and vortexed for 15 seconds. Yeast inoculum was then adjusted to 1 x 10^6 CFU/ml by adding sufficient sterile saline using NCCLS method (NCCLS M27-A2, 2002).

Alternatively number of yeast cells in suspension was counted by haemocytometer (Improved Naubauer Chamber). This stock inoculum was stored in refrigerator and used for sensitivity testing within two days of preparation. The stock inoculum was further confirmed by plating on SDA plates to determine the viable count. A working inoculum suspension was made by diluting the stock suspension with RPMI-1640 broth medium to get 1 x 10^2 CFU/ml.

Broth macrodilution and microdilution sensitivity testing:

The broth macrodilution test was performed in round bottom sterile glass tubes (12 x75mm) while broth microdilution test was performed by using sterile, disposable, multiwell microdilution plates (96 U-shaped wells) as per NCCLS reference method, (NCCLS M27-A2, 2002).

Agar dilution method :

The method described by Chakrabarti et al. (1995) was used with some modifications. Serial two fold dilutions of antifungal drugs were prepared in sterile distilled water in 10 sterile test tubes.

The tubes containing 9 ml sterile SDA medium were prepared and cooled to approximately 50^0c. One ml of diluted drug was mixed with 9 ml of liquid, molten, SDA. The contents were mixed and immediately transferred to sterile 10 cm petriplates and allowed to solidify. The plates were inoculated with 10 µl of inoculum (1x10^2 CFU/ml) using sterile micropipettes and incubated at 35^0c for 48 ± 2 hrs.

MIC reading :

Each test was performed in triplicate and the lowest concentration of the drug that inhibited the growth after 48 hrs without shaking was taken as minimal inhibitory concentration (MIC)

RESULTS AND DISCUSSION

The results of antifungal activity of ketoconazole to pathogenic yeasts by broth micro dilution, broth macro dilution and agar dilution method are presented in Table 1 and 2. The MIC range for *Candida* species observed was 0.08-20.0 µg/ml by broth micro dilution method, 0.15-20.0 µg/ml by broth macro dilution method while the range was 0.62-40.0 µg/ml by agar dilution method. *Cryptococcus neoformans* on the other hand were highly sensitive as indicated by MIC as low as 0.08 µg/ml by broth microdilution, 0.15 µg/ml by broth macrodilution and 0.62 µg/ml by agar dilution. MIC_{90} values were two to four fold greater than MIC_{50} for all the tested isolates. Out of 20 *Candida abicans* isolates only 15% (03) isolates demonstrated sensitivity at 0.62 µg/ml ketoconazole by broth microdilution. *Candida* species also exhibited high degree of resistance to 0.31 µg/ml ketoconazole with few exceptions. Among *Cryptococcus neoformans,* 100% isolates were inhibited at 0.31 µg/ml by broth microdilution, 2.5 µg/ml by broth macrodilution and 5.0 µg/ml by agar dilution.

Table 1. Antifungal activity as MICs (µg/ml) of ketoconazole against pathogenic yeasts by different sensitivity test methods.

Organism (No. of isolates tested)	Susceptibility test method	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Mean MIC (µg/ml)	MIC range (µg/ml)
C. albicans (20)	Broth microdilution	2.50	10.00	4.40	0.62-20.00
	Broth macrodilution	2.50	10.00	5.51	0.31-20.00
	Agar dilution	5.00	20.00	10.15	0.62-40.00
C. guilliermondii (4)	Broth microdilution	1.25	5.00	2.20	0.08-5.00
	Broth macrodilution	2.5	5.00	3.16	0.15-5.00
	Agar dilution	5.0	20.00	9.37	2.5-20.00
C. krusei (4)	Broth microdilution	1.25	2.5	2.34	0.62-5.00
	Broth macrodilution	1.25	2.5	2.34	0.62-5.00
	Agar dilution	2.5	5.0	5.00	2.5-10.00
C. glabrata (3)	Broth microdilution	5.00	20.00	10.0	5-20.00
	Broth macrodilution	10.00	20.00	11.66	5-20.00
	Agar dilution	20.00	40.00	23.33	10-40.00
C. parapsilosis (5)	Broth microdilution	2.5	10.00	4.06	0.31-10.00
	Broth macrodilution	5.0	20.00	8.5	2.5-20.00
	Agar dilution	10.0	40.00	13.5	2.5-40.00
C. stellatoidea (2)	Broth microdilution	1.25	2.5	1.87	1.25-2.5
	Broth macrodilution	0.62	1.25	0.93	0.62-1.25
	Agar dilution	5.0	10.00	7.5	5.00-10.00
C. tropicalis (4)	Broth microdilution	5.00	5.00	4.37	2.5-5.00
	Broth macrodilution	5.00	20.00	10.00	5.0-20.00
	Agar dilution	5.00	20.00	9.37	2.5-20.00
C. pseudotropicalis (3)	Broth microdilution	0.62	2.5	2.28	0.31-2.5
	Broth macrodilution	1.25	2.5	1.45	0.62-2.5
	Agar dilution	2.5	5.0	3.33	2.5-5.0
Cr. neoformans (5)	Broth microdilution	0.15	0.31	0.15	0.08-0.31
	Broth macrodilution	0.31	2.5	1.49	0.15-2.5
	Agar dilution	1.25	5.0	2.62	0.62-5.0

Table 2 : Number of pathogenic yeast isolates sensitive to ketoconazole concentrations by different sensitivity test methods.

Organism (No. of isolates)	Method	No. of isolates inhibited at stated concentration (μg/ml)									
		0.08	0.15	0.31	0.62	1.25	2.5	5.0	10.0	20.0	40.0
C. albicans (20)	Broth Micro	--	--	--	3	8	12	16	19	20	20
	Broth Macro	--	--	1	3	6	10	15	18	20	20
	Agar Dilution	--	--	--	1	1	6	12	16	18	20
C. guilliermondii (4)	Broth Micro	1	1	1	1	2	3	4	4	4	4
	Broth Macro	--	1	1	1	1	2	4	4	4	4
	Agar Dilution	--	--	--	--	--	1	2	3	4	4
C. krusei (4)	Broth Micro	--	--	--	1	2	3	4	4	4	4
	Broth Macro	--	--	--	1	2	3	4	4	4	4
	Agar Dilution	--	--	--	--	--	2	3	4	4	4
C. glabrata (3)	Broth Micro	--	--	--	--	--	--	2	2	3	3
	Broth Macro	--	--	--	--	--	--	1	2	3	3
	Agar Dilution	--	--	--	--	--	--	--	1	2	3
C. parapsilosis (5)	Broth Micro	--	--	1	1	1	3	4	5	5	5
	Broth Macro	--	--	--	--	--	1	3	4	5	5
	Agar Dilution	--	--	--	--	--	1	2	4	4	5
C. stellatoidea (2)	Broth Micro	--	--	--	--	1	2	2	2	2	2
	Broth Macro	--	--	--	1	2	2	2	2	2	2
	Agar Dilution	--	--	--	--	--	--	1	2	2	2
C. tropicalis (4)	Broth Micro	--	--	--	--	--	1	4	4	4	4
	Broth Macro	--	--	--	--	--	--	2	2	4	4
	Agar Dilution	--	--	--	--	--	1	2	3	4	4
C. pseudotropicalis (3)	Broth Micro	--	--	1	2	2	3	3	3	3	3
	Broth Macro	--	--	--	1	2	3	3	3	3	3
	Agar Dilution	--	--	--	--	--	2	3	3	3	3
Cr. neoformans (5)	Broth Micro	2	4	5	5	5	5	5	5	5	5
	Broth Macro	--	1	3	3	4	5	5	5	5	5
	Agar Dilution	--	--	--	1	3	3	5	5	5	5

In the past few decades the incidence of fungal infections has increased dramatically with a special focus on *Candida* species that are the most widespread and threatening fungal pathogens today, and are responsible for the majority of invasive and non-invasive fungal infections. Ketoconazole is an antifungal imidazole compound that has a significant activity against a broad range of superficial and systemic infections caused by pathogenic yeasts, dermatophytes, and filamentous fungi, including *C. albicans* (Beena et al. 2016).

The results showed that broth microdilution and broth macrodilution has good co-relation while MICs by agar dilution were found to be two-fold greater than broth dilution methods. The high activity of ketoconazole was displayed against *Cr. neoformans, C. pseudotropicalis,* and *C. stellatoidea* by broth dilution methods. These results by broth microdilution and broth macrodilution are in agreement with Hacek et al. (1995). Colombo et al. (1995) reported MIC range for *C. albicans* ≤ 0.03->32 μg/ml, for *Cr. neoformans* ≤ 0.03-0.5 μg/ml, and for *C. glabrata* 0.125-8 μg/ml. While Torres-Rodriguez et al. (1999) reported (Microbroth) highest MIC of 1 μg/ml for *C. albicans* and other species except 2 μg/ml for *C. krusei* and 4 μg/ml for *C. glabrata*.

Clinical breakpoints for ketoconazole have not been proposed but they are close to the break points for itraconazole (White et al. 2002). The break points proposed were MIC ≤ 0.125 μg/ml susceptible, MIC

0.25 to 0.5 µg/ml susceptible dose dependent and MIC ≥ 1 µg/ml resistant. Odds et al. (1995) reported MIC_{50} range by broth microdilution method as ≤ 0.025 > 25 µg/ml for *C. albicans,* while for *C. glarata* ≤ 0.25 – 13 µg/ml, for *C. tropicalis* ≤0.025 – 6.3, for *C. parapsilosis* ≤ 0.025 – 0.1, for *C. krusei 0.05-1.6* µg/ml and for *C. guilliermondii* 0.05-0.2 µg/ml. *C. parapsilosis* and *C. krusei* exhibited higher MIC values as compared to previous reports of Barry et al. (2000) and Odds et al. (1995). This may be due to the development of resistance in these species.

Sensitivity patterns of *C. albicans* showed agreement with Odds et al. (1995) and Colombo et al. (1995). However the poor agreement was seen with the previous reports of Torres – Rodriguez et al. (1999) for all *Candida* species. The results also showed higher MIC range than reported by Torres-Rodriguez et al. (1999) and Barry et al. (2000). MICs for *C. glabrata* slightly co-relates with Odds et al. (1995), while for *C. tropicalis* (by broth microdilution method), MIC range was 2.5 – 5 µg/ml which was slightly less than the reports of Odds et al. (1995).

In summary the results of this study clearly imply that ketoconazole possess strong anticandidal and anticryptococcal activity thereby suggesting its use in the therapeutic management of human infections caused due to *Candida* species and *Cryptococcus neoformans.*

Conflicts of interest: The authors stated that no conflicts of interest.

REFERENCES

Barry AL, Pfaller MA, Brown SD, Espinel-Ingroff A, Ghannoum MA, Knapp C, Rennie RP, Rex JH and Rinaldi MG (2000) Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. *J. Clin. Microbiol*., 38: 3457 – 3459.

Beena Shino, Faizal C, Peedikayil, Jaiprakash SR, Gufran AB, Kottayi S, and Jose D (2016) Comparison of antimicrobial activity of chlorhexidine, coconut oil, probiotics, and ketoconazole on *Candida albicans* isolated in children with early childhood caries: an *in vitro* study. *Scientifica*. Article ID 7061587, 5 pages.

Chakrabarty A, Ghosh A, Kanta A, and Kumar P (1995) *In vitro* antifungal susceptibility of *Candida*. *Ind. J. Med. Res.,* 102: 13-19.

CLSI (2008) Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition; CLSI document M27-A3. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.

Colombo AL, Barchiesi F, McGough DA, and Rinaldi MG (1995) Comparison of E test and National Committee for Clinical Laboratory Standards broth macrodilution method for azole antifungal susceptibility testing. *J. Clin. Microbiol*. 33: 535-540.

Hacek DM, Noskin GA, Trakes K and Peterson LR (1995) Initial use of broth microdilution method suitable for *in vitro* testing and fungal isolates in a clinical microbiology laboratory. *J. Clin. Microbiol*. 33 : 1884 – 1889.

Kerridge D (1986) Mode of action of clinically important antifungal drugs. *Ad. Microb. Physiol*. 27: 1-72.

National Committee for Clinical Laboratory Standards (1992) Reference method for broth dilution antifungal susceptibility testing of yeasts. Proposed standard M-27 P National Committee for Clinical Laboratory Standards, Villanova Pa.

National Committee for Clinical Laboratory Standards (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved standard. 2nd ed. NCCLS document $M27-A_2$, NCCLS, Wayne, Pennsylvania.

Odds FC, Vranckx L, and Woestenborghs F (1995) Antifungal susceptibility testing of yeasts: Evaluation of technical variables for test automation. *Antimicrob. Agents Chemother*. 39: 2051-2060.

Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM and Mendes Giannini MJS, (2013) Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med Microbiol*., 62, 10–24.

Torres-Rodriguez JM, Mendez R, Lopez-Jodra, Morera Y, Espasa M, Jimenez T and Lagunas C (1999) *In vitro* susceptibilities of clinical yeast isolates to the new antifungal eberconazole compared with their susceptibilities to clotrimazole and ketoconazole. *Antimicrob. Agents Chemother.* 43: 1258-1259.

Van CJ, Van GF and Janssen PAJ (1986) The *in vitro* evaluation of azoles. In: *In vitro* and *in vivo* evaluation of antifungal agents. *Iwata K. and Vanden Bossche H. (Ed.). Elsevier Sci Pub.* 51-64..

White TC, Scott H, Francis D (2002) Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrob. Agents Chemother.* 46 (6): 1704 – 1713.