

# Trends in Antifungal Susceptibility among Clinical Isolates of *Candida* spp. Resistant to Antifungals and Natural Products

Sapna Rai<sup>1\*</sup>, Ashish Saraf<sup>2</sup>, Sanchali Padhe<sup>1</sup> and Kavita Sharma<sup>3</sup>

<sup>1</sup>Dept. of Microbiology, M. G. M. M., Jabalpur, M.P.

<sup>2</sup>Faculty of Life Science, MATS University, Raipur, C.G.

<sup>3</sup>Head Botany Arts & Commerce Girls College Raipur C.G

Article Info	Abstract
<b>Article History</b> Received : 29-01-2011 Revised : 18-03-2011 Accepted : 19-03-2011	A collection of 260 <i>Candida</i> strains isolates recovered from 198 patients from 13 different medical centers in Jabalpur over a period of 5 years. These were tested for resistance to various antifungals according to the guidelines of NCCLS documents M 38-P by various methods. The isolates which were found to be resistant to synthetic antifungals were selected and subjected to antifungal testing against natural herbs and spices. Amongst all the spices and natural herbs tested garlic was found to be the most effective against <i>Candida</i> strains.
<b>*Corresponding Author</b> Tel : +91-9300128897 Fax : +91-7714078998 Email:ashish.saraf22@gmail.com	
©ScholarJournals, SSR	<b>Key Words:</b> Clinical isolates, <i>Candida</i> sp., Antifungal agent

## Introduction

Antimicrobial resistance surveillance serves many purposes. The most common of which is detection and tracking of resistance trends and emerging new threats<sup>11,13,14,16</sup>. Clinically this is important for treatment recommendations and as a means to assess the prevalent pathogens causing serious infections. The isolates collected in this program can be used to assess the activities of new antimicrobial agents and to aid in the development and validation of new susceptibility methods<sup>11,16,10</sup>.

Over the past few decades the incidence of fungal infections has increased. The treatment of choice for infected patients remains amphotericin B, itraconazole, voriconazole etc. the appropriate susceptibility is decided by various in vitro susceptibility methods<sup>2,12,15,20,23,24</sup>. The studies to date that have documented the efficacies of agar based methods for the testing of susceptibilities to fluconazole or voriconazole have generally included adequate number of *Candida albicans* species but few *Candida glabrata* isolates. From susceptibility testing one can determine the resistivity of the clinical isolate to various antifungals by determining the MIC range against a sufficiently large number of isolates.

Apart from the synthetic antifungal antibiotic the naturally occurring herbal products and spices are known to have powerful antifungal properties. Research has proved their antifungal activities. Herbs like goldenseal, myrrh, walnut, licorice, lemongrass and spices like turmeric, cinnamon, clove, ajwain and medicinal plants like neem, tulsi, eucalyptus etc. have a great antifungal profile. Even the combination of these works in synergy and gives better results than a single herb<sup>1,19,22</sup>.

## Materials and Methods

**Organism:** A total of 260 clinical isolates of *Candida species* obtained from 13 medical centers of Jabalpur were tested. The collection included 175 isolates of *C. albicans*; 43 isolates of

*C. tropicalis*; 12 isolates of *C. krusei*; 17 isolates of *C. parapsilosis*; 5 isolates of *C. glabrata*; 8 isolates of *C. guilliermondii*. Isolates were identified using conventional methods, growth in CHROM Agar media and using Atlas of fungi. They were stored as water suspension until used. Prior to testing, each isolate was passaged at least twice on SDA with chloramphenicol to ensure purity and viability.

**Antifungal agents:** Voriconazole (Pfizer), fluconazole (Pfizer), itraconazole (Jansen), flucytosine (Sigma), amphotericin B (Hi media). Serial two fold dilutions were prepared exactly as outlined in NCCLS document M 27A (17). Final dilutions were prepared in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165M morpholine propane sulphonic acid (MOPS) buffer (Sigma). The dilutions were kept in Eppendorf's tubes until used and stored at -4°C in Quick freezer.

**Natural products:** Turmeric, cinnamon, clove, ginger, garlic, neem, tulsi were used for the present study. The natural products were dried, powdered and then alcoholic extracts were prepared. The discs of natural products were prepared by soaking the discs in alcoholic extracts for a period of 24 hours and then dried in air. Two fold dilutions were prepared from powdered form in RPMI 1640 medium for broth dilution method.

**Susceptibility testing:** Reference antifungal Susceptibility testing of *Candida species* was performed by disc diffusion method as described by Barry et al (5) and BMD method as described in NCCLS documents (17). MIC's were determined with RPMI 1640 agar. An inoculum suspension adjusted to the turbidity of a 0.5 McFarland standard (~10<sup>6</sup> cells/ml), and incubation at 35°C for 48 hours. MIC interpretive criteria were those published by Rex et al and the NCCLS document M 27 A and were as follows: susceptible, MIC ≤8µg/ml; susceptible-

dose dependent, MIC = 16 to 32µg/ml; resistant, MIC ≥ 64µg/ml. The interpretive criteria for disc test were those published by Barry *et al.*<sup>5</sup> and the NCCLS document M 27 A (17): susceptible, zone diameter of ≥ 19mm; susceptible-dose dependent, zone diameter of 15 – 18 mm; resistant, zone diameter of ≤ 14mm.

**Quality control:** QC was performed for BMD & disc diffusion method in accordance with NCCLS document M 27 A<sup>17</sup> by using *C. albicans* 3809 as reference culture.

**Analysis of results:** The diameters of the zone of inhibition for the test antifungals were measured in mm by disc diffusion

method and their respective BMD MIC's were also studied. The errors were identified as susceptibility by one method and resistivity by other method.

**Results and Discussion**

The species distribution of *Candida* isolates in present investigation is summarized in Table 1. These isolates were obtained during the course of *Candida* surveillance studies and represent a total of 260 clinical isolates. Most of the species of *Candida* isolated in present study have been previously reported to cause serious infections in humans<sup>19,20,21, 22</sup>.

Table 1: Species distribution of *Candida* isolates

Species isolated	No. of isolates	% of isolates
<i>Candida albicans</i>	175	67.32
<i>Candida tropicalis</i>	43	16.54
<i>Candida krusei</i>	12	4.61
<i>Candida parapsilosis</i>	17	6.54
<i>Candida glabrata</i>	5	1.92
<i>Candida guilliermondii</i>	8	3.07
Total	260	100

Several species such as *C. krusei*, *C. guilliermondii*, and *C. parapsilosis* have been reported to express resistance to antifungal agents. Thus, it is evident that these non albicans species of *Candida* may be considered as opportunistic pathogens and that these may be responsible in posing

resistance problems for the currently used antifungal agents. From the table it is clear that the most frequently encountered *Candida* species is *C. albicans* followed by *C. tropicalis*, *C. parapsilosis*, *C. krusei* etc. The antifungal susceptibilities of the isolated *Candida* species are summarized in Table 2.

Table 2: In vitro susceptibility of *Candida* species against various antifungals

Antifungals	AMP B			5 FC			FLU			VORI			ITRA		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Susceptibility	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Disc diffusion	203	41	16	240	09	11	135	65	60	198	32	30	146	88	26
Broth dilution	196	53	11	168	75	17	112	98	50	206	28	26	185	69	06

AMP B –Amphotericin B, 5FC- 5 Fluocytosine, FLU-Fluconazole, VORI-Voriconazole, ITRA-Itraconazole.  
S-Sensitive; I-Intermediate; R-Resistant.

Table 3: In vitro susceptibility of *Candida* species against alcoholic extract of various natural herbs and spices measured as zone of inhibition in cms

S. No	Clinical fungal isolate	No. of isolate	neem	tulsi	ginger	Garlic	onion	Aloe vera
1	<i>Candida albicans</i>	16	1.4	0.4	1.0	1.6	1.8	0.6
2	<i>Candida tropicalis</i>	12	1.2	-	-	1.8	1.3	0.5
3	<i>Candida krusei</i>	05	1.4	0.4	1.2	1.5	1.2	-
4	<i>Candida parapsilosis</i>	04	1.2	-	1.0	1.6	1.3	0.5
5	<i>Candida glabrata</i>	07	1.2	-	1.0	1.6	1.4	0.4
6	<i>Candida guilliermondii</i>	03	1.0	-	1.2	1.4	-	0.6

The clinical isolates resistant to antifungal were selected and subjected to the in vitro sensitivity against the natural products .The sensitivity of the strains was measured as zone of inhibition in cms. The sensitivity of the clinical isolates to natural extracts is expressed in table 3.The mean value of the number of isolates for each strain was studied. From the table it is clear that the resistant strains of clinically isolated *Candida spp.* are sensitive to spices and herbs used as a supplement in diet. The most effective amongst all the spices and herbs is garlic (*Allium sativum*), followed by onion(*Allium cepa*), neem

(*Azadirachta indica*), ginger (*Zingiber officinale*) and *Aloe vera*. Amongst all the *Candida* strains tested the neem extract was found to be effective against *C. albicans* and *C. krusei* followed by *C. tropicalis*, *C. parapsilosis* and *C. glabrata*. Tulsi (*Ocimum sanctum*) extract was found to be least effective. The antifungal activities of some spices and herbs have been reported by Anupam *et al.* 2005. The results of the present investigation are according to those obtained by Anupam *et al.* It is clearly illustrated from the table that the common spices used in our daily life and the extracts of natural herbs effects

the fungal growth adversely and therefore might be considered to control fungal diseases. The bioactive compounds in these plant extracts must be analyzed, purified and then can be used as drug for controlling fungal pathogens without the development of drug resistance in pathogens and also without any side effects.

#### Acknowledgement:

Authors are thankful to University Grants Commission for financial assistance.

#### References

- [1] Anupam, N., Dev, K., Gajanan, B. Zore, S., Mohan K. (2005). Potentials of plant oil as inhibitors of *Candida albicans*. *FEMS Yeast Res.* 5: 867-873.
- [2] Arendrup, M., Lundgren, B., Jensen, I. M., Hensen, B. S. and Frimondt Mofler, N. (2001). Comparison of E-test and a tablet diffusion test with the NCCLS broth microdilution method for fluconazole and amphotericin B susceptibility testing of *Candida* isolates. *J. Antimicrob. Chemother.* 47: 521-526.
- [3] Barcheisi, F., Tortorano, A.M., Di Francesco, L.F., Cogliati, M., Scaliso, G. and Viviani, M. A. (1999). In vitro activity of five antifungal agents against uncommon clinical isolates of *Candida* spp. *J. Antimicrob. Chemother.* 43: 295-299.
- [4] Barry, A. L., Pfaller, M.A., Rennie, R. P., Fuchs, P. C. and Brown, S.D. (2002). Precision and accuracy of fluconazole susceptibility testing by broth microdilution, Etest and disc diffusion methods. *Antimicrob. Agents Chemother.* 46: 1781-1784.
- [5] Barry, A.L. and Brown, S.D (1996). In vitro studies of two triazole antifungal agents (voriconazole[UK-109,496] and fluconazole) against *Candida* spp. *Antimicrob. Agents Chemother.* 40: 1948-1949.
- [6] Dick, J.D., Rosengard, R.R., Merz, W.G., Stuart, R.K., Hutchins, G.M. and Saval, R. (1985). Fatal disseminated candidiasis due to Amphotericin B resistant *Candida guilliermondii*. *Am. Inter. Med.* 102: 67-68.
- [7] Espinell Ingroff, A., Boyle, K. and Sheehan, D.J. (2001). In vitro antifungal activities of voriconazole and reference agents as determined by NCCLS methods: review of the literature. *Mycopathologia.* 150:101-115.
- [8] Espinell Ingroff, A. (1998). In vitro activity of the new triazole voriconazole[UK-109,496] against filamentous and dimorphic fungi and common and emerging yeast pathogens. *J. Clin. Microbiol.* 36: 198-202.
- [9] Goldman, M., Pottage, J.C. and Weaver, D.C. (1993). *Candida krusei* fungemia. *Medicine (Baltimore)* 72:143-150.
- [10] Hunter, P.A. and Reeves, D. S. (2002). The current status of surveillance of resistance to antimicrobial agents. Report on a meeting. *J. Antimicrob. Chemother.* 49: 17-23.
- [11] Jones, R. N., and the MYSTIC Advisory Board. (2000). Detection of emerging resistance patterns within longitudinal surveillance systems: data sensitivity and microbial susceptibility. *J. Antimicrob. Chemother.* 46(Topic T2): 1-8.
- [12] Kronvall, G., and I. Karlson. 2001. Fluconazole and voriconazole multi disc testing of *Candida* species for disc calibration and MIC estimation. *J. Clin. Microbiol.* 39: 1422-1428.
- [13] Lewis, D. (2002). Antimicrobial resistance surveillance: methods will depend on objectives. *J. Antimicrob. Chemother.* 49:3-5.
- [14] Masterton, R.G. (2000). Surveillance studies: how can they help the management of infection? *J. Antimicrob. Chemother.* 46(Topic T2): 53-58.
- [15] Morace, G., Amato, G., Bistoni, F., Fadda, G., Marone, P., Montagna, M.T., Oliveri, S., Polonelli, L., Rigoli, R., Mancuso, I., Face, S.La, Masucci, L., Romano, L. Napoli, C., Tato, D., Buscema, M.G., Belli, C. M.C., Picirillo, M.M., Coni, S., Covan, S., Fanti, F., Cavana, C., Alo, F.D. and Pitzurra, L. (2002). Multicenter comparative evaluation of six commercial systems and the National Committee for Clinical Laboratory Standards M27-A broth microdilution method for fluconazole susceptibility testing of *Candida* species. *J. Clin. Microbiol.* 40: 2953-2958.
- [16] Morris, A. K. and Masterton, R. G. (2002). Antibiotic resistance surveillance action for international studies. *J. Antimicrob. Chemother.* 49:7-10.
- [17] National Committee for Clinical Laboratory Standards. (1997). Reference methods for broth dilution antifungal susceptibility testing of yeasts. Approved Standards M 27-A. National Committee for Clinical Standards, Wayne. Pa.
- [18] Nguyen, M.H., Peacock, J.E., Morris, Jr., A.J., Tanner, D.C., Nguyen, M.L., Snyderman, D.R., Wagener, M.M., Rinaldi, M.G. and Yu, V.L. (1996). The changing face of candidemia: emergence of non *Candida albicans* species and antifungal resistance. *Am. J. Med.* 100:617-623.
- [19] Peter, S. (1997). Antimicrobial effect of spices and herbs. Hospitality Institute of Technology and Management; St. Paul, Minnesota.
- [20] Pfaller, M.A., Diekema, D.J., Messer, S. A., Boyken, L. and Hollis, R. J. (2001) The ARTEMIS Global Antifungal susceptibility program participants group. Activity of Fluconazole and voriconazole DETERMINED by broth microdilution, Etest and disc diffusion methods AGAINST 1586 recent clinical species: report from the Global Antifungal susceptibility program, 2001. *J. Clin. Microbiol.* 40: 852-856.
- [21] Pfaller, M.A., Messer, S.A., Hollis, R.J., Jones, R.N., Doein, G.V., Brandt, M.E. and Hajjaj, R. A. (1999). Trends in species distribution and susceptibility to fluconazole among blood stream isolates of *Candida* species in the United States. *Diagn. Microbiol. Infect. Dis.* 33: 217-222.
- [22] Rubin, Z. A. and Somani, J. (2004). New options for the treatment of invasive fungal infections. Proceedings of seminar in Oncology 31 April 2004. 91-98.
- [23] Simor, A.E., Goswell, G., Louie, L. and Lee, M. (1997). Antifungal susceptibility testing of yeast isolates from blood cultures by microbroth dilution and E test. *Eur. J. Clin. Microbiol. Infect. Dis.* 16:693-697.
- [24] Warnock, D.W., Johnson, E. M. Rodgers, T. R. (1998). Multicentre evaluation of the E test method for antifungal susceptibility testing of *Candida* spp. and *Cryptococcus neoformans*. *J. Antimicrob. Chemother.* 42:321-331.