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A Study on Virulence Determinants of Clinical Isolates of Candida Species and Their Correlation with Antifungal Resistance in a Tertiary Care Hospital in Eastern India.

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ABSTRACT

Background: Candida Species are normal flora of skin and mucosa of gastrointestinal tract (GIT) and genital region. Usually they invade host tissue and cause diseases in patients with impaired immunity. OBJECTIVE: The aim of this study is to determine virulence factors expressed by various clinical isolates of different Candida species and to correlate with their antifungal resistance pattern. **Methods:** A total of 120 Candida isolates obtained from different clinical samples and speciated by standard microbiological laboratory protocol. Haemolysin, phospholipase and esterase activities were detected by growth characteristics on SDA with blood, egg yolk agar medium and Tween- 80 opacity test medium respectively. Adherence was seen microscopically over buccal epithelial cell and biofilm formation was detected visually with 2% Safranin staining. Antifungal susceptibility was detected by disk diffusion method using Mueller Hinton Agar supplemented with glucose and methylene blue (As per CLSI guideline). **Results:** Out of 120 isolates, 51(42.50%) were C.albicans and 69 (57.5%) were Non albicans Candida (NAC) including C.tropicalis 32(26.67%), C. guilliermondii 21(17.50%), C. parapsilosis 12(10%) and C. glabrata 04(3.33%). Haemolysin, phospholipase and esterase activities were detected in 110 (91.67%), 95 (79.17%) and 82 (68.33%) respectively. Adherence seen in 98 (81.67%) and in 59 (49.17%) cases biofilm had formed. The antifungal susceptibility patterns revealed that 81 (67.5%) were susceptible to Fluconazole and 72 (60%) were susceptible to Itraconazole, whereas 100% were susceptible to Voriconazole and Amphotericin B. **Conclusion:** Detection of virulence factor will help in better understanding of the changing behavioural patterns of Candida isolates and open doorways to better management and assessing prognosis of the patients.

Keywords: Candida, Antifungal resistance.

INTRODUCTION

Candida species are normal flora of skin and mucosa of gastrointestinal tract (GIT) and genital region. They invade host tissue and capable of initiating infections in both immunocompetent and immunocompromised host, but the incidence of infections are more in immunocompromised individuals; hence, is rightly called the "disease of diseased".[1] Although Candida albicans is the most prevalent species involved in muco-cuteneous and disseminated infections, the incidence of candidiasis due to non albicans Candida (NAC) is increasing.[2] The proportion of infections due to non-albicans Candida (NAC) species is persistently rising and so is the need to identify clinical isolates of Candida up to species level.[3] Several factors like severe immunosuppression or illness, prematurity, use of broad spectrum antibiotics and empirical use of antimycotic drugs are reported to be associated with the change.[4] The transition of Candida spp. from commensal to potent pathogen is facilitated by a number of virulence factors such as adherence to host tissues and medical devices, biofilm formation, and secretion of extracellular hydrolytic enzymes.[2] Knowledge of these virulence factors will be an important tool to understand pathogenesis of candidiasis and in addition will help explore new antifungal drug targets for improved therapeutic regimens.[5] Therefore the present study was taken up with an aim to study the virulence factors and antifungal susceptibility profile of Candida species isolated from various clinical specimens.

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MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, R. G. Kar Medical College and

Hospital Kolkata. The study period was from January 2017 to June 2018. A total of 120 clinical isolates of Candida species from various clinical specimens were studied. The clinical samples included blood, urine, pus, high vaginal swab (HVS), nail clipping, surgical site infection and central line received from various intensive care units, wards and outpatient Department.

Identification of Candida species

All the isolates of Candida species were identified by standard laboratory techniques based on colour of the growth on Chrom- agar (HIMEDIA, Mumbai, India), germ tube formation, chlamydospores and blastospores production on corn meal agar. Biochemical tests were performed via sugar fermentation and assimilation methods.[6]

Antifungal susceptibility test

Antifungal susceptibility test was performed via disc diffusion method using Mueller Hinton Agar supplemented with glucose and methylene blue. The used discs included Amphotericin B (10 µg), Fluconazole (25 µg), Itraconazole (10 µg), and Voriconazole (1 µg). The inhibition zone was measured and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[7] The control C. albicans ATCC 10231, C. glabrata ATCC 15126, and C. tropicalis ATCC 10610 strains were also used in this study.

Determination of virulence factors of Candida species

Preparation of yeast suspension:

Yeast suspension was prepared to evaluate different virulence factors. Small amount of a colony of a 24 – 48 hours culture of the organism was suspended in sterile Phosphate buffer solution (pH 7.2). The turbidity was matched to 0.5 McFarland standards.[8]

Examine for Phospholipase activity:

For determination of phospholipase activity Egg yolk agar media was used [5, 8, 9]. 13.0 g Sabouraud's Dextrose agar, 11.7 g NaCl, 0.11 g CaCl2 were dissolved in 184 ml of distilled water and sterilized by standard autoclave. Egg yolks were mixed and centrifuged at 500 g for 10 minutes at room temperature. 20 ml of the supernatant was added to the sterilized media for final preparation.[8] 10 µl of yeast suspension was inoculated over the agar surface. The plates were incubated at 370C for 5 days. Presence of enzyme activity was assessed by formation of precipitation zone around the yeast colonies.[8,9] [Figure 1]

The phospholipase index (Pz) was defined as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone. A Pz value of 1 denoted no phospholipase activity; Pz < 1 indicated phospholipase production by the isolate. The lower the Pz value, the higher the phospholipase activity.[1]

Figure 1: Phospholipase activity over egg yolk agar

Examination for Haemolytic Activity:

Sabouraud Dextrose agar containing 7% blood and 3% glucose was used to determine haemolytic activity. The pH of the media was adjusted to 5.6 ± 0.2. 10 µl of yeast suspension was inoculated over the agar surface and the media was incubated for 2 days at 370C. Presence of haemolytic activity was assessed by the formation of a transparent /semitransparent zone around the inoculation site.[5,9] [Figure 2]

The presence of a zone of haemolysis around the colony indicated haemolysin production. Haemolytic activity (Hz) was calculated in terms of the ratio of diameter of the colony to that of the translucent zone of haemolysis (in mm).[5]

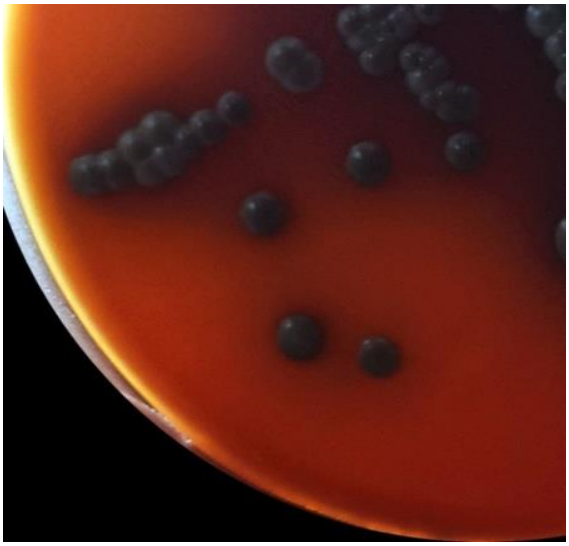

Figure 2: Haemolysin activity by Candida isolates

Adherence assay:

The adherence assay described by Kimura et al,[10] was used with minor modification in the present study. Buccal epithelial cells (BEC) from healthy donor were collected by the help of sterile cover

slips and washed thrice and suspended in sterile Phosphate Buffered Solution (pH 7.2). 0.5 McFarland standard matched suspension of BEC and yeast suspension were mixed and incubated at 370C for 45 minutes with gentle shaking. The suspension was centrifuged at 3000 rpm for 5 minutes and the sediment was repeatedly washed in PBS. A smear was prepared from the sediment and stained by Gram's Method. Adhered yeast cells over the epithelial cells were examined under microscope.[10] [Figure 3]

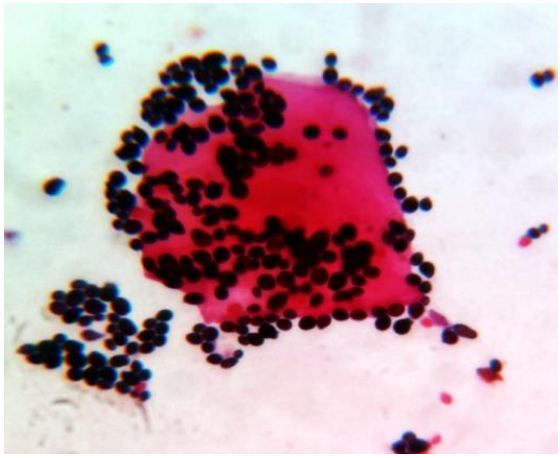

Figure 3: Adherence assay by Candida species (400 X)

Examination for Esterase activity:

Tween 80 opacity test media was used for detection of esterase activity. 1% peptone, 0.5% NaCl, 0.01% CaCl2 and 1.5% agar were dissolved in distilled water and sterilized by standard autoclave. 0.5% Tween 80 was added to the media after cooling it to 500C. The final pH was adjusted to 6.8. 10 µl of yeast suspension was inoculated and media was incubated at 370C for 10 days. Esterase activity was assessed by presence of a halo pervious to light around the yeast colonies. [Figure 4].[8,11]

Esterase activity (Ez) was calculated in terms of the ratio of diameter of the colony to that of the zone of opacity around the colony (in mm).

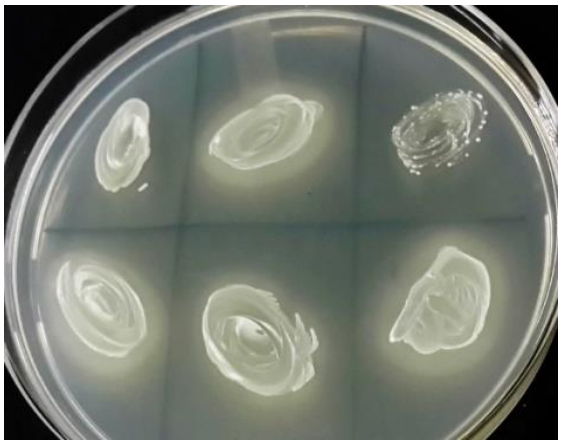

Figure 4: Esterase activity over Tween 80 opacity media

Determination of biofilm production:

Biofilm production was determined visually by the method described by Yigit et al.[12] with mild modification. Colonies from surface of SDA plate were inoculated into each well of polystyrene micro titre plate tube containing 10 µl of Sabouraud-dextrose broth (SDB) supplemented with glucose (Final concentration 8%). After incubation at 350C for 48 hours, the broths in the wells were gently aspirated. The wells were washed with distilled water twice and then stained with 2% Safranin for 10 mins. They were then examined for the presence of an adherent layer. Biofilm production was scored as negative (-), weak (+), or strong (++). The biofilm producer Staphylococcus epidermidis ATCC 35984 was used as a positive control [Figure 5].[1,5]

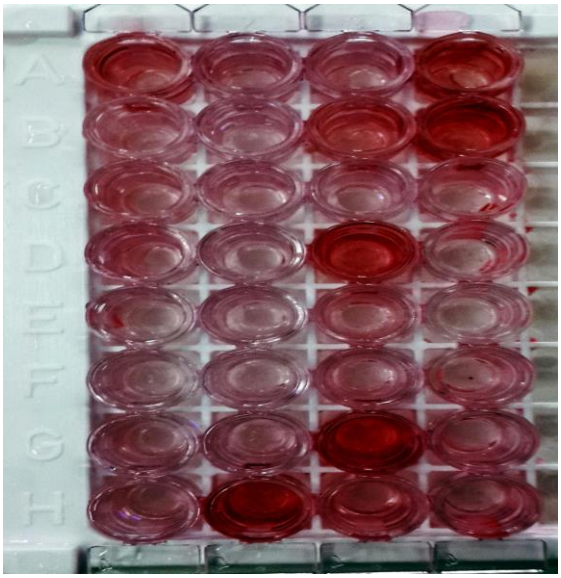

Figure 5: Biofilm formation by different Candida species

All culture media, reagents and chemicals were obtained from HI MEDIA private limited, Mumbai, India. All statistical analysis were done using Chi-square test and Fischer exact test. The software used for the statistical analysis was SPSS version 22.0 and Graph pad Prism 7.

RESULTS

During the study period a total of 120 Candida isolates were collected from different specimens for species identification, detection of virulence factors and antifungal susceptibility testing. Out of these 120 candida isolates, 51(42.50%) were C.albicans and 69 (57.5%) were Non albicans Candida (NAC) (Figure-6) including C.tropicalis 32(26.67%), C. guilliermondii 21(17.50%), C. parapsilosis 12(10%) and C. glabrata 04(3.33%). [Table 1]

Table 1: Distribution of Candida species identified from different clinical samples (n=120)

Type of Specimen	No (%)	C. albicans	C. tropicalis	C. guilliermondii	C. glabrata	C. parapsilosis
Urine	38 (31.67)	14 (11.67)	14 (11.67)	07 (5.83)	0	03 (2.5)
High vaginal swab	47(39.17)	21 (17.5)	10 (8.34)	08 (6.67)	0	08 (6.67)
BAL fluid	06 (5)	02 (1.67)	03 (2.5)	01 (0.83)	0	0
Blood	13 (10.83)	07 (5.83)	01 (0.83)	03 (2.5)	02 (1.67)	0
Pus	06 (5)	02 (1.67)	03 (2.5)	0	0	01 (0.83)
Nail clipping	03 (2.5)	02 (1.67)	01 (0.83)	0	0	0
Surgical site infection	03 (2.5)	03 (2.5)	0	0	0	0
Central venous tip	04 (3.33)	0	0	02 (1.67)	02 (1.67)	0
Total	120 (100)	51 (42.50)	32 (26.67)	21 (17.5)	04 (3.33)	12 (10)

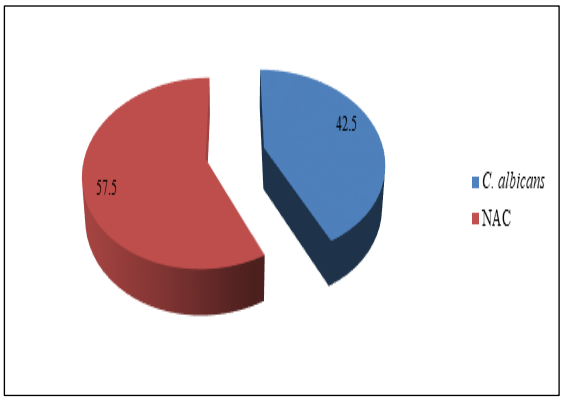

Figure 6: Distribution of isolated Candida species (n= 120)

Out of all 120 Candida isolates, 110 (91.67%) were haemolysin producer, 95 (79.17%) were phospholipase producer and 82 (68.33%) were esterase producer. Non albicans Candida (NAC) were found to be significantly more enzyme/toxin producer than Candida albicans. [Table 2].

Table 2: Haemolysin, Phospholipase and Esterase activity exhibited by Candida species (n=120).

Virulence Factors	Total (%)	C. albicans (%)	NAC (%)	p value
Haemolysin	110 (91.67)	43 (35.84)	67 (55.83)	0.0177
Phospholipase	95 (79.17)	45 (37.5)	50 (41.67)	0.0420
Esterase	82 (68.33)	40 (33.33)	42 (35)	0.0485

98 (81.67%) Candida species were shown to be adherent to buccal squamous epithelium cell, out of which 44 (36.67%) were C. albicans and 54 (45%) were NAC. 27 (22.5%) C.albicans species were biofilm producer whereas 32 (26.67%) NAC were biofilm producer [Table 3].

Table 3: Adherence activity and biofilm formation shown by different Candida species (n=120).

	Adherence	Biofilm
C. albicans (%)	44 (36.67)	27 (22.5)
NAC (%)	54 (45)	32 (26.67)
Total (%)	98 (81.67)	59 (49.17)
p value	0.2621	0.4771

All isolates were susceptible to Voriconazole (1 µg) and Amphotericin B (10 µg). 32.5% Candida isolates were Fluconazole resistant in which 10% were Candida albicans and 22.5% were NAC, whereas 40% were Itraconazole resistant in which 16.67% were Candida albicans and 23.33% were NAC. [Figure 7 & Table 4].

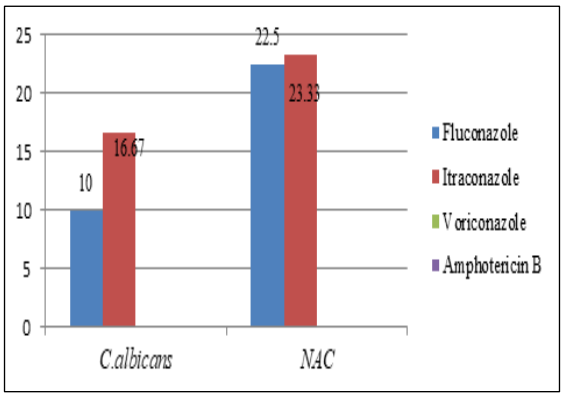

Figure 7: Distribution of different Candida species according to resistant pattern (n=120).

Table 4: Distribution of Candida species according to antifungal sensitivity testing (S = Susceptible, R = Resistant)

	Fluconazole (25 µg)		Itraconazole (10 µg)		Voriconazole (1 µg)		Amphotericin B (10 µg)	
	S	R	S	R	S	R	S	R
C. albicans (n=51)	39 (76.47)	12 (23.53)	31 (60.78)	20 (39.22)	51 (100)	0	51(100)	0
C. tropicalis (n=32)	24 (75)	8 (25)	22 (68.75)	10 (31.25)	32 (100)	0	32 (100)	0
C. guilliermondii (n=21)	11 (52.38)	10 (47.62)	11 (52.38)	10 (47.62)	21 (100)	0	21 (100)	0
C. glabrata (n=04)	1 (25)	3 (75)	01 (25)	03 (75)	4 (100)	0	4 (100)	0
C. parapsilosis (n= 12)	6 (50)	6 (50)	07 (58.33)	05 (41.67)	12 (100)	0	12 (100)	0
Total (n=120)	81 (67.5)	39 (32.5)	72 (60)	48 (40)	120 (100)		120 (100)	

Table 5: Distribution of Candida species in relation with virulence factor production and antifungal drug resistant pattern (n=120) (FLC Fluconazole, ITR Itraconazole).

Virulence factors		FLC (25 µg) (%)	p value	ITR (10 µg) (%)	p value
Haemolysin	Produced	36 (30)	0.8613	40 (33.33)	0.0140
	Not produced	03 (2.5)		8 (6.67)	
Phospholipase	Produced	26 (21.67)	0.0193	43 (35.83)	0.0234
	Not produced	13 (10.83)		5 (4.17)	
Esterase	Produced	20 (16.67)	0.0053	38 (31.67)	0.0373
	Not produced	19 (15.83)		10 (8.33)	
Adherence	Produced	28 (23.33)	0.0525	36 (30)	0.1233
	Not produced	11 (9.17)		12 (10)	
Biofilm	Produced	26 (21.67)	0.0078	35 (29.17)	0.0000
	Not produced	13 (10.83)		13 (10.83)	

DISCUSSION

Over the past few years, Candida has been recognized as one of the most important etiological agents of nosocomial infections.[13] The increased frequency of Candida infections to a certain extent coincides with advances in the field of medicine.[14] NAC spp. once dismissed or ignored as non-pathogenic, commensal, or contaminant have emerged as potential pathogens.[15]

In the present study, NAC species (57.5%) were more isolated than C.albicans (42.5%) from all clinical specimens, which was shown by other recent studies also.[4] A total of 120 Candida isolates were collected from different clinical specimens, of which high vaginal swab samples showed the highest number of isolates (39.17%), followed by urine (31.67%) and blood (10.83%) respectively. The incidence of C. albicans was 42.5% where as C. tropicalis was 26.67%. C. guilliermondii also showed an incidence of 17.5% and C.glabrata was 3.33%. Incidence of C. parapsilosis was 10%. Basu et al found an incidence of C. albicans of 45.8% where as C. tropicalis was 24.7%, C. guilliermondii was 3.5% and C.glabrata was 1.1%.[16]

The phospholipase activity which is thought to help the yeast in tissue invasion was found in 37.5% of C. albicans and 41.67% of non albicans candida. Phospholipase activity shown by NAC species was significantly more than that shown by Candida albicans (p value =0.0420) which was also shown by other studies [9] supporting more virulence and pathogenecity of NAC.

Out of 120 Candida isolates, 91.67% were haemolysin producer, out of which 35.84% were C. albicans and 55.83% were NAC. The hemolysin activity, shown by NAC was significantly more than that shown by Candida albicans species (p value = 0.0177). Ruchika Butola et al (2015),[9] showed that Candida albicans showed significantly more haemolytic activity. It may be due to the fact that being a tertiary hospital, more critical and unresponsive cases were transferred here, making a shift towards more virulent and pathogenic non albicans Candida infection.

Only 68.33% isolates showed esterase activity out of which 33.33% were C. albicans and 35% were NAC and esterase activity in NAC was significantly more than C. albicans (p= 0.0485). Esterase activity were found to be more in C. albicans in the study of Deepthi T et al,[8] and Aktas et al,[17] This finding also supports the more virulent and more pathogenic emerging non albicans Candida infection. The diversity in expression of various extracellular enzymes leads to exaggerated synergistic effect showing better adaptability of the fungi to its new found environment.[18]

Upon entering a mammalian host, with environment changes such as elevated temperature and acidic pH, Candida changes their nutritional requirement from saprophytic to parasitic and starts to manifest virulence factors. Adhesion is the first step to infection in a host which helps the pathogen to avoid being washed away by mucosal secretions. It is mediated by Adhesins and starts with nonspecific interactions such as Vander Waals, Brownian movement, and hydrophobic and ionic interactions, and later becomes more permanent with receptor-ligand interactions.[19] Majority of the isolates in our study (81.67%) expressed adherence to buccal squamous epithelium cells out of which 36.67% were C. albicans and 45% were NAC. The highest percentage adherence to BECs was also observed in isolates of non albicans Candida in other studies also.[20,21]

Candida spp. possesses ability to form biofilm on most, if not all, medical devices and lead to resistant health care associated infections.[2,22] Singhai et al.[23] reported Candida associated catheter related sepsis in 7.4% of patients with peripheral intravascular catheters. Biofilm formation is one of the most extensively investigated virulence factors of Candida species.[24] In the current study, NAC (26.67%) was the major biofilm producer than Candida albicans (22.5%), which is consistent with other studies also.[21]

Azoles exert direct effects on the fatty acids of cell membranes and inhibit ergosterol biosynthesis through their interactions with lanosterol demethylase converting lanosterol to ergosterol in fungal cell membranes,[25] leading to the depletion of ergosterol in the membrane Overall,[26,27] biofilm-forming Candida cells have the ability to develop resistance against azoles, especially Fluconazole and clotrimazole [28] which also supports the present study showing different virulence factors

(phospholipase, esterase or biofilm production) expressed by various Candida isolates have significantly greater impact on development of resistance to azoles (Fluconazole and Itraconazole).

The present study showed that all C. albicans isolates are susceptible to Amphotericin B and Voriconazole which was also shown by Shivanand et al,[29] and Araj et al,[30] All other non albicans Candida isolates in the present study were also susceptible to Voriconazole and Amphotericin B. There are so many studies showing similar type of pictures all over India. V. Gandhi et al,[31] from Ahmadabad and Sachin C. Deorukhkar et al,[32] from Maharashtra showed also that percentage of resistance of Candida isolates to Amphotericin B was 3% and 4.63% respectively, whereas Jaswinder Kaur Oberoi et al,[33] from New Delhi showed a bit higher resistance pattern to Amphotericin B (10.4%). Deepthi T et al,[8] from Andhra Pradesh showed 100% susceptibity of Candida species to Voriconazole, which was similar to our study. Although Jaswinder Kaur Oberoi et al,[33] and V. Gandhi et al,[31] found higher resistance pattern to Voriconazole (11.4% and 23% respectively).

The most frequently used antifungal Fluconazole was reported resistant in maximum number of cases in various studies. The percentage of resistance to Fluconazole was reported in various studies ranges between 11.7% (Xess et al., (2007) New Delhi),[34] to 37.5% (Gupta et al., (2001) New Delhi),[35] Other studies showed various percentage of resistance, S. Giri et al., (2013) Tamil Nadu,[36] 30.8%, Kumar et al., (2005) Chennai,[37] 17.2%, Sachin C. Deorukhkar (2012), Maharastra,[32] 19.07%, Jaswinder Kaur Oberoi et al,[33] 21.2%, Kothari et al., (2009) New Delhi,[38] 36% and V. Gandhi et al,[31] 25%. In the present study also, we noticed the resistance pattern to Fluconazole was 32.5% whereas to Itraconazole it was 40% which was also consistent with V. Gandhi et al,[31] 36% and Jaswinder Kaur Oberoi et al,[33] 45.7%. On the contrary, Kothari et al,[38] found 76% candida isolates susceptible to Itraconazole.

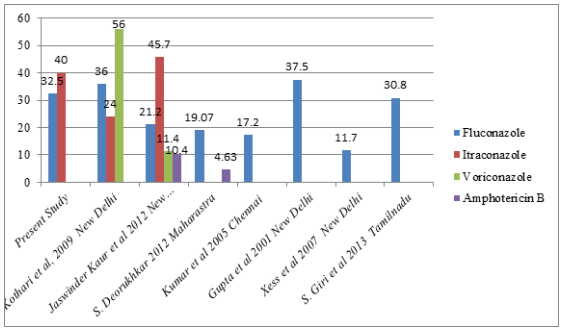

Figure 8: Comparison of antifungal resistance in all Candida species isolated from various clinical specimen.

In all studies including the present study, it was found that Non albicans candida isolates showing more resistance than C.albicans did, depicting more virulent and resistant, emerging threat of Non albicans candida infection to modern health care system.

CONCLUSION

Increased incidence of candidiasis along with antifungal resistance has become an important healthcare issue worldwide now a day. NAC species exhibit a great degree of variation not only in their pathogenecity but also in their antifungal susceptibility profile. The identification of virulence attributes specific for each species and their correlation with each other will aid in the understanding of the pathogenesis of infection [5]. The importance of early and accurate identification of infecting Candida species along with susceptibility testing for timely institution of appropriate therapy cannot be overstated [5].

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