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ORIGINAL ARTICLE

IDENTIFICATION OF *CANDIDA* SPECIES FROM CLINICAL SAMPLES AND THEIR ANTIFUNGAL SUSCEPTIBILITY PATTERNS

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ABSTRACT: OBJECTIVE AND BACKGROUND: The incidence of *Candida* infections has increased dramatically over the past few decades due to increase in the number of population susceptible to fungal infections. With multiple antifungal agents that are available and recovery of clinical isolates that exhibit inherent or developed resistance to commonly used antifungal agents, it has become imperative to do susceptibility testing routinely. The study was done to determine the predisposing factors, species incidence and susceptibility pattern of *Candida* isolates to commonly used antifungal agents. **METHODS:** A total of 108 *Candida* species were recovered from symptomatic clinical cases. *Candida* isolates were speciated by germ tube test, chlamydospore formation on corn meal agar and color produced on chromogenic media. Antifungal susceptibility test was done by disk diffusion method for nystatin, fluconazole, itraconazole, voriconazole and amphotericin-B. **RESULTS:** *Candida albicans* is the most frequently isolated species. However, non-*albicans Candida* species, taken as a group has predominated in clinical samples. Chromogenic agar medium showed good correlation in species identification in comparison with conventional germ tube test and chlamydospore formation on corn meal agar. *C. albicans (41), C. tropicalis (33), C. krusei (30)* and *C. glabrata* (04) were isolated. *Candida* species showed 95.4% susceptibility to amphotericin-B, 77.8% to voriconazole, 69.4% to nystatin, 64.1% to fluconazole and 63.9% to itraconazole. **CONCLUSION**: Increasing incidence of non-*albicans* species infection. Chromogenic medium can be used for species identification. Increasing resistance of *Candida* species to commonly used antifungal agents.

KEYWORDS: Chrom agar, Antifungal susceptibility test, *Candida* speciation.

INTRODUCTION: The genus *Candida* encompasses more than 163 species, only a few of which cause disease in humans.[1] *Candida* species are ubiquitous in nature, found on inanimate objects, in foods and on animals.[2] The *Candida* species are normal commensals of humans and are commonly found on skin and throughout the entire gastrointestinal tract. There is relatively high incidence of carriage on the skin of health care workers.[3] The overall carriage rate in healthy individuals has been estimated to reach 80%.[3] The most common isolated *Candida* species from the gastrointestinal tract of humans is *Candida albicans*, followed by C. *tropicalis* and C. *parapsilosis*.[4]

Candida is a small, thin walled, ovoid yeast like organism that measures 4-6μm in diameter and reproduces by budding. Organisms of this genus occur in three forms in tissues: blastospores, pseudohyphae and hyphae. *Candida* grows readily on simple medium; lysis centrifugation enhances its recovery from blood. Species are identified by biochemical testing or on special agar. Currently, automated devices are available.[2] The vast majority of *Candida* infections are of endogenous origin, human-to-human transmission is possible. Examples are thrush of newborn, which may be acquired from the maternal vagina, balanitis in the uncircumcised man, which may be acquired through contact with partner having *Candida* vaginitis. *Candida* infection can also be hospital acquired.[3]

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Candida species that are part of the normal flora can invade tissues and cause life threatening diseases in patients whose cell mediated immunity is decreased by disease or iatrogenic interventions.[4] With the widespread use of antibiotics, epidemic of HIV infection and increased number of patients with diabetes mellitus, previously undocumented manifestations of *Candida* infections have occurred, and the incidence of practically all forms of *Candida* infections has risen abruptly. *Candida* species are the seventh most common pathogen to cause nosocomial infections during the last four decades.[1] Now, *Candida* species constitute third to fourth most common cause of nosocomial blood stream infection.[1] *Candida* species is the fifth most common nosocomial urinary pathogen in India.[5]

With the introduction of antifungal agents, the causes of *Candida* infection shifted from an almost complete dominance of *C*. *albicans* to involvement of other species. Non-*albicans* species now account for approximately half of all cases of candidemia and hematogenously disseminated candidiasis. Recognition of this change is clinically important, since various species differ in susceptibility to antifungal agents.[2]

The study was undertaken to identify the prevalence of *C*. *albicans* and non-*albicans Candida* infection from clinical cases, to determine the susceptibility of the *Candida* species to commonly used antifungal agents in the region and to analyze the predisposing conditions for candidiasis.

MATERIALS AND METHODS: The study was conducted in A. J. Institute of Medical Sciences, Mangaluru, Karnataka between 1st February 2013 and 31 May 2013. Permission from institution Ethics Committee was taken. Various samples that were sent to Department of Microbiology for routine culture and identification were analyzed. A detailed history was taken with regards to age, sex, and underlying clinical condition, use of antibiotics, diabetes and HIV status. Isolation of *Candida* species was determined to be non-pathogenic/pathogenic based on clinical and standard microbiological criteria. Isolates found to be non-significant clinically were excluded. A total of 108 *Candida* species were isolated from exudates, urine, sputum, blood, high vaginal swabs and biomedical devices from symptomatic clinical cases.

The clinical specimens were collected and processed as per standard microbiological procedures. The *Candida* isolates were further speciated by germ tube test, chlamydospore formation on corn meal agar, urease test and inoculation onto chromogenic medium. The chromogenic medium used is HiMedia CHROM agar®. Species identification was done based on the color code which was provided with the media (Figure 1).

Antifungal susceptibility testing was done for all the 108 isolates by using modified Kirby-Bauer technique as per CLSI guidelines. All the isolates were sub cultured onto Sabouraud dextrose agar (SDA) to ensure purity and viability. Incubation temperature was maintained at $35^0C \pm 2^0C$. Inoculum is prepared by picking five distinct colonies from a 24hr old culture of *Candida* species. Colonies were suspended in 5ml of sterile 0.85% saline solution. The suspension was vortexed to get uniform turbidity and adjusted visually to 0.5 McFarland standard.[6]

Within 15 minutes, suspension was inoculated onto Antimycotic sensitivity media obtained from HiMedia Lab®. Antimicrobial disks are placed evenly so that they are not closer than 24mm from centre to centre.[6] Antifungal disks were obtained from Hi Media Lab®. Following antifungal agents were used: Nystatin 100units, fluconazole 10µg, itraconazole 10µg, voriconazole 1µg and amphotericin B 100units. The plates were placed in an incubator at 37^0C for 20-24hr (Figure 2).

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After the completion of incubation period, zone of inhibition was measured to the nearest whole millimeter at the point at which there was a prominent reduction in growth. Pinpoint micro colonies at the zone edge or large colony within a zone were ignored.[6] Incubation was extended to 48hr whenever insufficient growth was observed after 24hr of incubation.[6] Zone diameter was interpreted as susceptible, intermediate and resistant as per the instruction manual of the manufacturer. *Candida albicans* ATCC 90028 was used as a standard.[6]

RESULTS: Of the 108 isolates that were isolated, 32 were from sputum samples, 29 from urine, 15 from high vaginal swabs, 10 from pus swabs, 10 from biomedical devices, 8 from blood and 4 from throat swab (Table 1 and chart 1). *Candida albicans* was isolated from 41 samples, *Candida tropicalis* from 33, *Candida krusei* from 30 samples and *Candida glabrata* from 4 samples. (Table 1 and chart 2) None of the patients were reactive to HIV antibodies by laboratory reports. Thirty two patients were on antibiotics and 32 were both on antibiotics and steroid medications. Four patients were only on steroid medication. Pregnant women constituted 14 cases and 8 were on anti-diabetic medications. Of the 14 pregnant women 5 were on insulin for gestational diabetes (Table 2 and 3).

It was noted from the study that overall, *Candida* species was sensitive to amphotericin-B in 95.4% cases, to voriconazole in 77.8%, to nystatin in 69.4%, fluconazole in 64.1% and itraconazole in 63.9%. *Candida albicans* isolates were 100% susceptible to amphotericin-B, 78% to voriconazole, 76% to itraconazole, 63% to fluconazole and 61% to nystatin. *Candida tropicalis* was 94% susceptible to amphotericin-B, 79% voriconazole, 76% susceptible to nystatin & itraconazole and 70% to fluconazole. *Candida krusei* was 93% susceptible to amphotericin-B, 80% to voriconazole, 70% to nystatin and 33% to itraconazole. *Candida glabrata* was 100% susceptible to amphotericin-B, 50% susceptible to nystatin & voriconaozole and 25% fluconazole & itraconazole. (Table 4)

DISCUSSION: Candidiasis is primary or secondary infection which involves members of the genus Candida. The manifestations may vary from acute, sub-acute to chronic and episodic involvement. Infection may be localized or systemic.[4]

A variety of factors are known to predispose to superficial and deep seated candidiasis. These include prolonged antibiotic therapy, invasive therapeutic procedures, radiotherapy, AIDS pandemic, recipients of solid organ transplant and those on immunosuppressive therapy.[7] All these factors act either by altering the balance of normal microbial flora of the body or by lowering the host resistance.[1] Of the 108 isolates that are recovered from clinical cases, 32 patients were on antibiotics, 32 were both on antibiotics and steroid medications in which 22 were on inhaled steroids and 10 were on systemic steroids. Four patients were only on steroid medication and no antibiotics. Pregnant women constituted 14 cases and uncontrolled diabetes were 8 in number. None of the cases were found to be reactive to HIV antibodies from laboratory reports.

The results of chromogenic medium exactly paralleled that of conventional method. Chromogenic medium was found to be superior to SDA and antimycotic sensitivity media in terms of suppressing the bacterial growth. Use of chromogenic medium allows laboratories to identify and speciate *Candida* rapidly and at a lower cost.[8] The speciation of *Candida* is important, to know the incidence of the infection and since antifungal susceptibility tests are not routinely done in most laboratories, speciation itself helps clinician in choosing correct antifungal agent in many clinical cases i.e. azoles are effective against C. *albicans* and *C. tropicalis*, but found to be ineffective against C. *krusei* and *C. glabrata*.[9]

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Of the 108 *Candida* isolates recovered from various clinical samples, sputum samples had the highest isolation of *Candida* followed by urine, high vaginal swab, exudates, blood and throat swabs. *Candida albicans* was the most frequently isolated species. Non-*albicans Candida* predominated in all infections and has emerged as important pathogens.

The incidence of fungal infections has increased dramatically over the past few decades due to the increase in the number of population susceptible to *Candida* infections.[10] With multiple antifungal agents that are available and recovery of clinical isolates that exhibit inherent or developed resistance to commonly used antifungal agents it has become imperative to do susceptibility testing routinely so that clinician can use appropriate antifungal agent to reduce the morbidity and mortality.[1,11]

Standardized methods for antifungal susceptibility testing have been available for many years. The CLSI standardized broth micro-dilution method remains a reference method for antifungal susceptibility testing. Clinically relevant interpretative breakpoints are available and quality control strains are validated.[12] Prior studies have shown that agar based susceptibility testing (E-test and disk diffusion) are more practical as compared to broth micro-dilution due to their simplicity and reproducibility. Requirement of additional equipment (spectrophotometer) is also eliminated.[13]

With the disk diffusion method the susceptibility pattern that is obtained correlates with similar studies done in the region by others using ATB Fungus 3 of Biomerieux and disk diffusion method where they found *C. albicans* isolates were 100% susceptible to amphotericin-B, *C. tropicalis* showed 25% resistance to fluconazole.[4,8]

Some of the limitation of the study is that, antifungal susceptibility test was done on antimycotic sensitivity media obtained commercially from HiMedia® and susceptibility zone size is interpreted based on manufacturer's guidelines. There are no CLSI guidelines for susceptibility testing of antifungal agents other than fluconazole and voriconazole by disk diffusion method. Correlation between disk diffusion for sensitivity is good with broth dilution, but for resistance the correlation differs.[14]

To conclude, an increase in the predisposing conditions in recent years has resulted in an increasing incidence of *Candida* infections. Non-*albicans Candida* species are increasingly becoming more common cause of infection. With multiple antifungal agents and their varying susceptibility patterns it has now become a necessity to perform antifungal susceptibility testing routinely. Disk diffusion method is simple and user friendly but continued refinement of technique is needed to provide clinicians a more meaningful report for a better therapeutic outcome.

Sl. No.	Samples	No. of samples	*C. albicans*	*C. tropicalis*	*C. krusei*	*C. glabrata*
1	Sputum	32	15	10	06	01
2	Urine	29	10	12	06	01
3	High vaginal swab	15	09	03	02	01
4	Pus swab	10	03	02	05	0
5	Biomedical devices	10	01	06	03	0
6	Blood	08	02	0	05	01
7	Throat swab	04	01	0	03	0
	Total	**108**	**41**	**33**	**30**	**4**

Table 1: Distribution of *Candida* species in different samples

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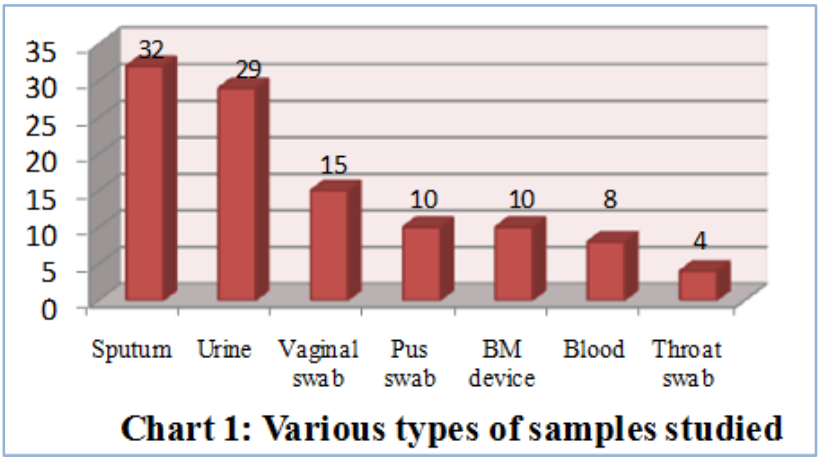

Chart 1: Various types of samples studied

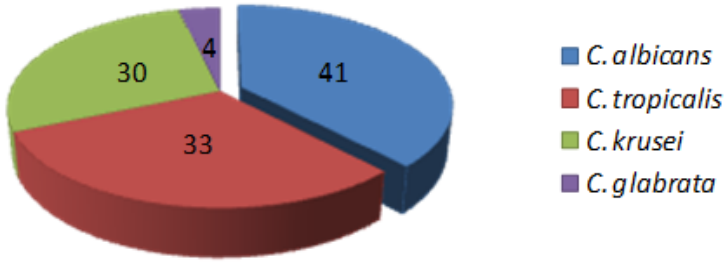

Chart 2: Various species of *Candida* isolated

Sl. No	Risk factors	No. of samples	*C. albicans*	*C. tropicalis*	*C. krusei*	*C. glabrata*
1	Male	47	14	16	16	01
2	Female	61	27	17	14	03

Table 2: Distribution of *Candida* species based on gender

Sl. No.	Risk factors	No. of samples	*C. albicans*	*C. tropicalis*	*C. krusei*	*C. glabrata*
1	No known predisposing factors	23	08	10	05	0
2	Antibiotics alone	32	13	10	08	01
3	Antibiotics and steroids	32	13	09	09	01
4	Steroids alone	04	01	02	01	00
5	Pregnancy*	14	06	02	04	02
6	Antidiabetic* medications	03	0	0	3	0

*Total number of patients who were on antidiabetic medications were eight and five were gestational diabetic on insulin

Table 3: Distribution of *candida* species based on risk factors

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Figure 1: *Candida* species grown On Chromogenic medium

Figure 2: Antifungal Susceptibility test

Species	Total	NS			FLC			IT			AP			VRC		
		S (%)	I	R	S (%)	I	R	S (%)	I	R	S (%)	I	R	S (%)	I	R
C. albicans	41	23 (61)	02	16	22 (63)	04	15	31 (76)	02	08	40 (100)	01	0	30 (78)	02	09
C. tropicalis	33	21 (76)	04	08	20 (70)	03	10	23 (76)	02	08	31 (94)	0	02	26 (79)	0	07
C. krusei	30	23 (70)	0	07	*			08 (33)	02	20	28 (93)	0	02	22 (80)	02	06
C. glabrata	04	02 (50)	0	02	01 (25)	0	03	01 (25)	0	03	04 (100)	0	0	02 (50)	0	02
Non-*albicans*	67	46 (75)	04	17	21 (36)	03	13	32 (54)	04	31	63 (94)	0	04	50 (78)	02	15
Total	108	69 (69.4)	06	33	43 (64.1)	07	28	63 (63.9)	06	39	103 (95.4)	01	04	80 (77.8)	04	24

NS-Nystatin, FLC-Fluconazole, IT-Itraconazole, AP-Amphotericin-B, VRC-Voriconazole, S-Susceptible, I-Intermediate, R-Resistant

* *C. krusei* is intrinsically resistant to fluconazole

Table 4: Antifungal Susceptibility Pattern

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