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Microbiology

Species Identification of Candida Isolates in Various Clinical Specimens with Their Anti-fungal Susceptibility Patterns

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ABSTRACT

The Candida species are ubiquitous yeasts which are found on many plants and are a part of the normal flora of the alimentary tract of mammals and the mucocutaneous membranes of humans. Those that are a 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 intervention. The accurate species identification of Candida is important for the treatment, as not all species respond to the same treatment and also because of the problem of anti-fungal resistance.

The aims of the study were to isolate and identify the Candida species from clinical cases of candidiasis and to determine their anti-fungal susceptibility patterns and the predisposing conditions for candidiasis.

A total of 100 Candida isolates from various clinical specimens were identified by speciation on a chromogenic medium, HiChrome Candida differential agar. Susceptibility testing was carried out on 50 Candida isolates by using Amphotericin B, Fluconazole, Itraconazole, Voriconazole and Fluocytosine.

Candida albicans was the commonest species which was isolated (47%), followed by *Candida tropicalis* (30%). The Candida isolates were more susceptible to Amphotericin B (92%) and Fluocytosine (88%). Diabetes mellitus appeared to be the commonest predisposing factor for the Candida infections, followed by indiscriminate drug usage.

An increase in the predisposing conditions in recent years has resulted in an increasing incidence of Candida infections. Therefore, the species level identification of the Candida isolates, along with their anti-fungal susceptibility patterns can greatly influence the treatment options for the clinician and may have an impact on the patient care also.

Key Words: *Candida albicans*, non-albicans Candida, Fluconazole, Amphotericin B, Itraconazole, Fluocytosine, Voriconazole

KEY MESSAGE

- Candida spp., though they are a part of the normal flora, can cause infections in immunocompromised patients. In recent years, HIV infections, diabetes mellitus, multiple drug use and biomedical device usage have been identified as the commonly encountered risk factors for the Candida infections. The emergence of drug-resistant candidiasis is posing a serious threat to the patient care. Amphotericin B is effective against the non-albicans species of Candida. The species identification of the Candida isolates, along with the identification of their anti-fungal susceptibility patterns can influence the treatment options for the clinician and have a beneficial impact on the patient care.

INTRODUCTION

The Candida species are ubiquitous yeasts which are found on many plants and are a part of normal flora of the alimentary tract of mammals and the mucocutaneous membranes of humans [1]. The overall carriage rate in healthy individuals has been estimated to reach 80%. The most commonly isolated Candida species from the gastrointestinal tract of humans is *Candida albicans*, followed by *Candida tropicalis* and *Candida parapsilosis* [2]. *Candida glabrata* is most often isolated from the mouth. *Candida spp*. that are a 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 intervention [3-5]. In recent years, HIV infection has been identified as one of the most important predisposing conditions for candidiasis [6].

Candida albicans and related species which are pathogenic for humans, become resistant to the anti-fungal agents, in particular to the azole compounds, by the expression of the efflux pumps that reduce drug accumulation, the alteration of the structure or concentration of the anti-fungal target proteins and by the alteration of the membrane sterol composition. The clinical consequences of the anti-fungal resistance can be seen as the treatment failure in the patients and as the change in the prevalence of the Candida species which causes the infection [7-8].

Accurate species identification is important for the treatment of the Candida infections, as the non-albicans species of Candida continue to be increasingly documented and as not all the species respond to the same treatment [4]. The increase in the predisposing conditions in recent years has resulted in a

concurrent increase in the number of patients who suffer from candidiasis.

Hence, this study was undertaken to speciate Candida from the clinical cases of candidiasis, to determine the susceptibility of the Candida species to Fluconazole, Itraconazole, Fluocytosine, Voriconazole and Amphotericin B, and to analyze the predisposing conditions for candidiasis.

MATERIALS AND METHODS

A total of 100 Candida isolates from various clinical specimens (high vaginal swabs, blood, urine, exudates, biomedical devices, skin scrapings and nail clippings) were taken up for the study. A detailed clinical history was taken with regards to the age of the patient, sex, underlying disease/ conditions, immunodeficiencies, the Human immunodeficiency virus (HIV) status, diabetes mellitus, pregnancy, malnutrition, any ongoing treatment, burns, cancer and the type of candidiasis.

The various clinical specimens were collected and processed as per the standard microbiological procedures. The Candida isolates which were obtained were further speciated by the germ tube test, chlamydospore formation on corn meal agar and inoculation on chromogenic medium. The chromogenic medium, HiMedia CHROM agar®, has chromogenic substances which helps in the rapid identification of the Candida species, based on the reactions between the specific enzymes of the different species and the chromogenic substances. As per the colour code which is provided with the chromogenic media, *C. albicans* produces blue-green colonies, *C. tropicalis* produces dark blue- blue grey colonies, *C. glabrata* produces white to cream coloured colonies and *C. krusei* produces pale pink to purple, rough colonies.

Anti-fungal susceptibility testing [9, 10] was done for fifty isolates of Candida by using ATB Fungus 3® of Biomérieux. The ATB Fungus 3 strip enables the determination of the susceptibility of the Candida isolates to the antifungal agents in a semi-solid medium under conditions which are similar to the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) recommendations. The anti-fungals which were tested, included Fluconazole, Voriconazole, Itraconazole,Fluocytosine and Amphotericin B. The inoculated strips were used in duplicate (c and C) were read visually after incubation at 37°C for 24 hours. For each antifungal agent, the reading of the strips was started with the lowest concentration and the growth score was recorded for each of the wells and compared with the control wells as follows:

No reduction in growth	4
Slight reduction in growth	3
Distinct reduction in growth	2
Very weak growth	1
No growth	0

For Amphotericin B, the minimum inhibitory concentration (MIC) of the Candida species corresponded to its lowest concentration, thus enabling complete growth inhibition.

For Fluconazole, Itraconazole and Voriconazole, as the possibility of a trailing growth existed, the MIC corresponded to the lowest concentration of the anti-fungal agent, with which a score of 2, 1 or 0 was obtained.

For Fluocytosine, a growth was looked for and was quantified in both the wells and tested for two concentrations; the interpretation of the growth for the anti-fungals is as mentioned in [Table/Fig-1].

The results which were obtained, gave an MIC and classified the strain as sensitive, intermediate or resistant.

The anti-fungal breakpoints which were used were as recommended by the CLSI guidelines in mg/L [see Table/Fig-2].

RESULTS

A total of 100 clinical isolates of Candida from various clinical specimens were processed during the study period. A total of 38 isolates were obtained from high vaginal swabs [Table/Fig-3], followed by blood (16), urine (12), sputum (11) and others (23).

Candida albicans was the commonest species which was isolated (47%), followed by *C. tropicalis* (30%), *C. krusei* (14%) and *C. glabrata* (9%).

A higher incidence of *C. albicans* was found in the high vaginal swabs (HVS) and urine. A high incidence of *C. tropicalis* was found in the high vaginal swabs and sputum and a high incidence of *C. glabrata* was found in the high vaginal swabs, whereas *C. krusei* was found more in blood [Table/Fig-4].

Candidiasis was most common in the age group of greater than 18 years up to 45 years (54%) [Table/Fig-5], followed by the age group of greater than 60 years (22%).

The rate of isolation of the Candida species was more in females than in males [Table/Fig-6].

Growth Score		Results		Interpretation
c	C	c	C	
0/1/2	0/1/2	–	–	Sensitive (S)
3/4	3/4	+	–	Intermediate (I)
3/4	3/4	+	+	Resistant (R)

[Table/Fig-1]: Growth interpretation for antifungal drugs

Anti-fungal	Interpretation		
	Sensitive	Intermediate	Resistant
Fluocytosine	≤ 4	8–16	≥ 32
Amphotericin B	ND	ND	ND
Fluconazole	≤ 8	16–32	≥ 64
Itraconazole	≤ 0.125	0.25–0.5	≥ 1
Voriconazole	≤ 1	2	≥ 4

[Table/Fig-2]: Anti-fungal breakpoints recommended by CLSI
≤ - lesser than or equal to
≥ - greater than or equal to
ND - not formally defined by CLSI
For Amphotericin B, a MIC of > 2mg/L suggests resistance
Intermediate means susceptible dose- dependent (SDD)

Clinical specimens	Number
High vaginal swab (HVS)	38
Blood	16
Urine	12
Sputum	11
Bronchoalveolar lavage (BAL)	03
Endotracheal aspirate	06
Catheter tips	05
Pus/wound swabs	07
Ascitic fluid	02
Total	100

[Table/Fig-3]: Distribution of clinical specimens in the study

An analysis of the risk/predisposing factors in patients from whom the Candida species were isolated, showed that 32% had underlying diabetes mellitus, that 22% were on multiple antibiotics and that 12% had a history of catheter usage [Table/Fig-7].

Fifty isolates of Candida were subjected to anti-fungal susceptibility testing [Table/Fig-8]. Sixty percent of the Candida isolates were found to be sensitive to Fluocytosine and Voriconazole, whereas 56% of the isolates were sensitive to Amphotericin B. Itraconazole (48%) and Fluconazole (32%) were found to be less sensitive against Candida. The highest resistance (32%) was seen against Fluconazole.

S. No.	Specimen	Total No.	C. albicans	C. tropicalis	C. krusei	C. glabrata
1.	HVS	38	19	12	03	04
2.	Blood	16	05	05	04	02
3.	Urine	12	09	01	01	01
4.	Sputum	11	03	07	01	–
5.	BAL	03	01	01	01	–
6.	Endotracheal aspirates	06	01	02	02	01
7.	Wound swabs	07	04	02	–	01
8.	Catheter tips	05	04	–	01	–
9.	Ascitic fluids	02	01	–	01	–
Total		100	47	30	14	09

[Table/Fig-4]: Distribution of Candida species isolated from various clinical specimens

S. No.	Age of patient	C. albicans	C. tropicalis	C. krusei	C. glabrata
1	≤ 1 year	02	01	–	–
2	> 1 yr – ≤ 18 yrs	02	02	01	–
3	> 18 yrs – ≤ 45 yrs	28	16	04	06
4	> 45 yrs> – ≤ 60 yrs	07	05	04	01
5	> 60 years	08	06	05	03

[Table/Fig-5]: Age distribution of patients from whom Candida species were isolated

Gender	C. albicans	C. tropicalis	C. krusei	C. glabrata	Total
Male	14	10	07	05	36
Female	33	20	07	04	64

[Table/Fig-6]: Gender-wise distribution of patients with Candida isolates

Predisposing factors	Number of patients
Diabetes mellitus	32
History of drug intake & secondary to the disease	22
On catheters	12
Sepsis	10
Use of Intrauterine devices	10
Pregnancy	08
HIV positive	06

[Table/Fig-7]: Distribution of predisposing factors in patients with Candida isolates

DISCUSSION

Candidiasis is a primary or secondary infection which involves a member of the genus, Candida. The clinical manifestations of the disease are extremely varied, ranging from acute, subacute and chronic to an episodic involvement. It may be localized to the mouth, lungs or the gastrointestinal tract, or may become systemic as in septicaemia, endocarditis and meningitis.

A total of 100 Candida isolates from various clinical specimens were included in our study, of which high vaginal swabs showed the highest number of isolates (38%), followed by blood (16%) and urine (12%). Studies which were done earlier by Pfaller et al, have reported Candida species as the seventh most common nosocomial pathogen hospital wide and as that which caused 25% of all the urinary tract infections. These findings were confirmed in the most recent study which was conducted by them for the SENTRY Antimicrobial Surveillance Programme.

C. albicans was the most frequently isolated species. Many other species of Candida are also being reported in the current literature [11, 12]. In this study as well, the most frequently isolated species was *C. albicans,* accounting for 47% of the infections, followed by *C. tropicalis* (30%), *C. krusei* (14%) and *C. glabrata* (9%) respectively.

Comparative studies on different Candida species which were isolated in their studies by different researchers showed that the isolation of *C. albicans* was the highest in each of them, except in Chakrabarti's study [13], which showed that the isolation of *C. tropicalis* was the highest (42%) and that the isolation of *C. albicans* was 25%. Dastidher (72.8%), Gupta D (64%) and Mokaddas et al (39.5%) found *C. albicans* to be the commonest isolate [14].

Studies over the years have shown that there is a considerable increase in the non-albicans Candida isolates. Our study showed that non-albicans Candida were isolated at a higher rate (53%) than *C. albicans* (47%), which was in agreement with the findings of the studies by Mokaddas et al, who also showed the non-albicans Candida incidence (60.5%) to be higher than that of *C. albicans* (39.5%) [14]. A study by Chakrabati A also showed non-albicans Candida to have a higher incidence (75%) than *C. albicans* (25%). These findings seem to suggest that non-albicans Candida are emerging as important pathogens.

The speciation of Candida is important to provide a database for a given area of study. The choice of antifungals is also dependent on the species of Candida. The azoles being effective against

Species	No.	FC			Amp B			FCA			ITR			VCR		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
C. albicans	18	14	04	0	12	06	0	06	08	04	10	06	02	12	04	02
C. tropicalis	16	10	04	02	10	04	02	06	06	04	08	06	02	10	04	02
C. krusei	10	04	04	02	02	06	02	02	02	06	04	04	02	06	02	02
C. glabrata	06	02	02	02	04	02	0	02	02	02	02	0	04	02	02	02
Total	50	30	14	06	28	18	04	16	18	16	24	16	10	30	12	08

[Table/Fig-8]: Antifungal susceptibility testing patterns
FC- Fluocytosine, Amp B- Amphotericin B, FCA- Fluconazole, ITR- Itraconazole, VCR- Voriconazole, S- Sensitive, I- Intermediate, R- Resistant

C. albicans and *C. tropicalis*, are found to be ineffective against *C. krusei* and *C. glabrata*. Other studies which have reported on the epidemiology of candidaemia, have stressed the importance of the speciation of Candida, as it provides accurate information on the disease incidences and the trends in most of the infections[11, 12].

Frazer, Victoria J and co-workers reported the importance of the association of candidaemia which was caused by various species of Candida with the disease outcome. Studies which were conducted by them, showed that in patients with sustained candidaemia, the mortality which was associated with the non-albicans Candida species was higher, with a statistical significance. It is important however, to clearly differentiate between Candida which occur as contaminants and those which occur as pathogens [11].

Though candidiasis can occur at all ages, studies by Dalal PJ and Kelkar SS at Mumbai showed the highest incidence of candidiasis to be in the age group of 21-40 years [15]. These findings were in concurrence with those of our study, where the age group of >18 years up to <45 years was that which had the highest incidence of candidiasis.

In our study, the incidence of candidiasis was higher in females than in males. In a similar study by Kandhari KC et al, the incidence was found to be higher in females (61.2%) than in males (38.8%). This could be due to the higher number of samples which were collected from female patients.

We have studied the association of the risk factors in all the 100 patients from whom the Candida species were isolated. As can be seen in [Table/Fig-7], diabetes mellitus was the most frequently associated risk factor. Experimental evidence in vitro shows that a glucose concentration of 150mg/100ml increases the growth of Candida [16]. This may probably hold true in the human body, that an increase in the concentration of glucose in the tissues, blood and urine promotes the growth of Candida. A comparison of the incidence of diabetes among the cases of candidiasis is shown in [Table/Fig-9]. Kandhari KC et al found 11 cases of diabetes in a total of 54 cases of candidiasis, with an incidence of 20.4%. Shroff PS studied 150 patients with cutaneous candidiasis and found 22 patients to be diabetic. The findings of the present study correlated well with those of other studies, as in our study, 32 out of the 100 cases of candidiasis showed an incidence of 32%. A history of multiple drug usage was the second most frequently associated risk factor (22%). The drugs which were incriminated were mainly corticosteroids, antibiotics and contraceptive pills [17, 18]. According to Rippon [19], there is some effect of the antibiotics on the host tissue, which predisposes it to invasion by the organism, and the antibiotics itself may stimulate the growth of Candida. The most important effect of antibiotics is the elimination and alteration of the bacterial flora that holds the population of Candida in check. Winner and Hurley, in 1964, have found considerable evidence (clinical and experimental) in support of the enhancement of the development of candidiasis by systemic antibiotics and corticosteroids. They demonstrated a 21-30% increase of the Candida infections when a patient was treated with antibiotics.

S. No.	Workers' study	Total no. of cases	Diabetic cases	Percentage
1.	Kandhari KC et al	54	11	20.4
2.	Shroff PS et al	150	22	14.66
3.	Present study	100	32	32

[Table/Fig-9]: Comparison of incidence of diabetes mellitus in various studies

Since the advent of the contraceptive pill, there has been a controversy about the predisposing role of oral contraceptives in candidial vulvovaginitis. Bourg suggested that progestational steroids, by causing changes which were similar to those which were found in pregnancy, might be responsible for an increased incidence of candidiasis among the patients who took oral contraceptive pills [20]. Jackson and Spain found that the chances of a patient having vaginal candidiasis were significantly less among those which used a combination regimen. These hormones probably act by promoting changes which are similar to those which are seen in the luteal phase of the menstrual cycle and in early pregnancy. In the present study, four patients were on oral contraceptives and ten patients who had intrauterine devices had vulvovaginitis, out of the 38 cases which were studied. It has been established that the prevalence of genital candidiasis increases in pregnancy. The high hormone level leads to a proportional increase in the glycogen content of the vagina, thus producing a favourable environment for the growth of Candida. Undoubtedly, the reduced glucose tolerance and the increased incidence of glycosuria render some patients more susceptible to candidiasis [16].

In the present study, eight pregnant women had candidiasis. The occurrence of candidial vulvovaginitis is also important from another viewpoint. There is a real risk of transmission of the infection to the newborn infant. In a study by Woodruff et al, it was found that maternal infection resulted in a 35 times greater chance of the child developing oral thrush than a baby which was born of a non-infected mother. The other significant risk factors were the presence of urinary and indwelling catheters (in 12%), sepsis (in 10%) and HIV infection in about 7%. Studies have shown that patients with underlying risk factors such as those which are mentioned in [Table/Fig-7], are at an increased risk of developing blood stream infections which are caused by Candida [21]. In our study, the second most common specimen was blood. During the past decade, there has been increasing appreciation of the significance of candidaemia. Dismissal of the isolation of the Candida species from a single blood culture as a skin contaminant has been questioned as a potentially hazardous interpretation [22]. The dogma that two blood cultures are required to confirm the significance of the first blood culture is being questioned. This could lead to a delay in administering potentially life saving therapy. It is said that all blood cultures that yield Candida species must be considered to be significant until they have been proved otherwise.

ANTIFUNGAL TESTING ASPECTS

The in vitro susceptibility testing of antifungal agents is becoming increasingly important because of the introduction of new antifungal agents and the recovery of clinical isolates that exhibit inherent or developed resistance to Amphotericin B, Fluocytosine, the azole group of drugs or Nystatin during chemotherapy. In the present study, antifungal susceptibility testing was done for 50 Candida isolates by using ATB Fungus 3 of Biomérieux. The *C. albicans* isolates were 100% susceptible to Amphotericin B and Fluocytosine and showed 22% resistance to Fluconazole. The *Candida tropicalis* isolates were 87.5% susceptible to Fluocytosine, Amphotericin B, Itraconazole and Voriconazole and showed 25% resistance to Fluconazole. The *Candida krusei* isolates were 80% susceptible to Amphotericin B, Itraconazole and Voriconazole, and showed 60% resistance to Fluconazole and 40% resistance to Fluocytosine. The *C. glabrata* isolates were 100% susceptible to Amphotericin B and showed 66.66% resistance to Itraconazole and 33.33%

resistance to Fluconazole. The findings of the present study correlate with those of Yonghao Xu et al's study in which *C. krusei* showed 40% resistance to Fluconazole and *C. glabrata* showed 9.3% resistance and 100% resistance to Fluocytosine respectively [23]. The findings of the present study also correlated with those of a study by Jin-Sol Lee et al, in which *C. glabrata* showed 38% resistance to Itraconazole and C. krusei showed 100% resistance to Fluconazole [24]. *C. krusei* and *C. glabrata* showed high resistance to Fluconazole and Itraconazole respectively, probably due to their innate resistance to these drugs.

To conclude, an increase in the predisposing conditions in recent years has resulted in an increasing incidence of Candida infections. Therefore, the species level identification of the Candida isolates along with their antifungal susceptibility patterns can greatly influence the treatment options for the clinician and may have an impact on the patient care.

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