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Phenotypic Characterization of Clinical Isolates of *Candida* Species in Eastern Part of Nepal.

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ABSTRACT

Invasive candidiasis has emerged as a significant cause of morbidity and mortality. Its occurrence is mainly associated with the use of potent antibacterial and various immunosuppressive and cytotoxic drugs. To isolate and identify the *Candida* species from various clinical samples and characterize it phenotypically. A total of 1,180 clinical samples were received for fungal culture at department of microbiology, BP Koirala Institute of Health Sciences during April 2012 to March 2013. Specimens consisted of urine, sputum, high vaginal swab, nail, skin and blood. *Candida* species were characterize using various phenotypic methods such as Gram's stain, culture, Germ tube test, chlamydospore production, carbohydrate fermentation and assimilation, CHROMagar, and thermotolerance. Out of 1,180 clinical samples thirty six samples yielded yeast like growth. In addition, 18 yeasts isolates that grew in the bacterial culture media were also included. Out of 54 yeasts isolates, two were identified as *Rhodotorula* species & two as *Cryptococcus* species. Remaining 50 isolates were *Candida* species. Further characterization revealed *Candida albicans* 27 (54%), followed by *C. tropicalis 9* (18%), *C. krusei 6* (12%), *C. glabrata 5* (10%) and *C.parapsilosis 3* (6%). Among the various clinical samples, urine culture yielded the highest number of the *Candida* isolates (22), followed by sputum (14), high Vaginal swab (6), nail (4), skin (3) and blood (1). *C.albicans* 27 (54%) was the most predominant species from various clinical samples with non albicans species being 23 (46%).

Keywords: *Candida* species, phenotypic characterization, CHROMagar

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INTRODUCTION

Over the past three decades, invasive candidiasis has emerged as a significant cause of morbidity and mortality (1-2). Its occurrence is mainly associated with the use of potent antibacterial and various immunosuppressive and cytotoxic drugs (3).

Diversity and spectrum of *Candida* species of clinical significance is wide. Although *Candida albicans* predominated as the most frequently isolated yeast in the clinical laboratories in the past, recent studies have documented the rising trend of non - *albicans Candida* species (4). Identification and speciation of *Candida* species is essential as variation occurs among them in terms of virulence and susceptibility to antifungal drugs (4-5).

Various methods have been used to characterize clinical isolates of *Candida*. Although molecular techniques are highly sensitive and specific, they are not cost effective for routine use in resource constrained setup (6).Several phenotypic methods are available, however no single test has been found to be effective in establishing the identification of *Candida* upto species level. Therefore, a combination of the tests appears useful for speciation purpose.

Correct identification of *Candida* species is crucial, as it presents prognostic and therapeutical significance, allowing an early and appropriate antifungal therapy (7).

Objectives

Since knowledge about the spectrum of *Candida* species prevalent in our set up is limited, the present study undertaken at Department of Microbiology, BP Koirala Institute of Health Sciences, Dharan has made an attempt to establish the identification and characterization of *Candida* species from various clinical specimens by using a number of phenotypic methods.

MATERIALS AND METHODS

Present study was carried out in the Department of Microbiology, BPKIHS, Dharan, Nepal. Clinical specimens submitted to the mycology section during April 2012 to March 2013 were analyzed. In addition, yeasts that grew in the culture media used for the isolation of bacterial pathogens were also included. Specimens consisted of urine, sputum, high vaginal swab, nail, skin and blood.

Direct microscopy

Gram's staining was done for urine, sputum and high vaginal swab and Potassium Hydroxide Mount was performed for nail and skin samples (8-9).

Culture

Sputum, high vaginal swab and blood samples were cultured on blood agar and MacConkey agar. Urine samples were inoculated on cysteine lactose electrolyte deficient (CLED) agar and skin and nail samples were inoculated on Sabouraud's Dextrose Agar (SDA) and also on SDA agar containing chloramphenicol, cycloheximide and gentamicin (10).

Germ tube test

A small inoculum of the test yeast cells from a pure culture were suspended in 0.5 ml human serum. The suspensions were incubated at 37 °C for three hours after which a drop of the incubated serum were placed on a microscope slide and covered with a cover slip. The wet mounts were examined for presence of germ tubes using the 40 X objective. A yeast cell having about half the width and 3 to 4 times the length of the mother cell and not have the constriction at the neck of the parent cell was considered as true germ tube. More than 50% of the cells producing such germ tube per high power field were interpreted as true germ tube producer *Candida* species (11).

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Temperature tolerance

The isolates were cultured into SDA and incubated at 45 °C in ambient air for 72 hours after which they were observed for any growth. The SDA tube showing growth was identified as *Candida albicans* (9).

Chlamydospore production

The slide culture technique was adopted that involved stabbing the Corn Meal Agar with a 48 hour old yeast colony and covered with sterile cover slip. Then the plates were incubated at 20-25°C for 2-6 days. The growth was subjected to staining with lactophenol cotton blue and examined for chlamydospore production microscopically under high power objective. Those isolates that showed round, thick walled, blue coloured structure at the terminal ends of hyphae were considered as true chlamydospore producers (12).

CHROMagar

It was used for identification of different *Candida* species and detects any mixed colonies. The method is based on the differential release of chromogenic breakdown products from various substrates following differential exoenzyme activity. CHROMagar was purchased as powdered media and the plates were prepared according to the manufacturers' instructions. Using an inoculating needle, a single colony from a pure culture was seeded into CHROMagar media and incubated at 35 °C for 48 hours after which color changes was noted (13-14).

Sugar assimilation test

The assessment of the ability of yeast to utilize carbohydrates is based on the use of carbohydrate-free yeast nitrogen base agar and observing for the presence of growth around carbohydrate impregnated filter paper disks after incubation at 30°C for 96 hours. Carbohydrates used were glucose, lactose, trehalose, sucrose and raffinose (8-9).

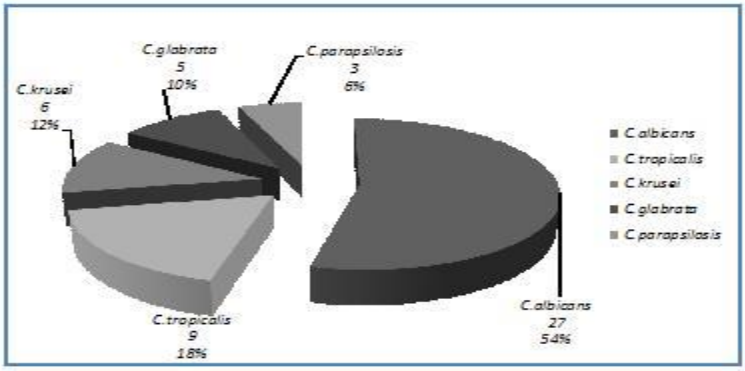
Sugar fermentation test

Four sugars i.e. glucose, lactose, sucrose and maltose at 2% concentration were used for differentiation of *candida* species on the basis of their fermentation (8-9).

RESULTS

A total of 1,180 clinical samples were received for fungal culture at department of microbiology, BP Koirala Institute of Health Sciences over a period of one year. Thirty six samples yielded yeast like growth. In addition, 18 yeasts isolates that grew in the bacterial culture media were also included. Out of 54 yeasts isolates, two were identified as *Rhodotorula* species & two as *Cryptococcus* species. Remaining 50 isolates were *Candida* species.

Figure 1 . Different species of *Candida* isolates (n=50)



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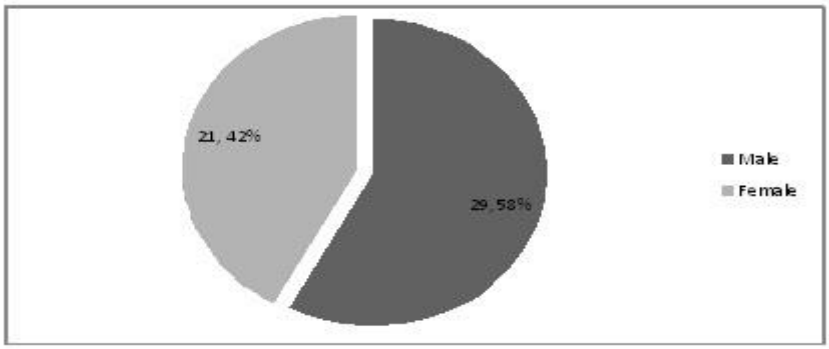
Further characterization revealed that *Candida albicans* 27 (54%) was the most predominant, followed by *C. tropicalis 9* (18%), *C. krusei 6* (12%), *C. glabrata 5* (10%) and *C.parapsilosis 3* (6%) in the descending order of frequency as shown in figure 1.

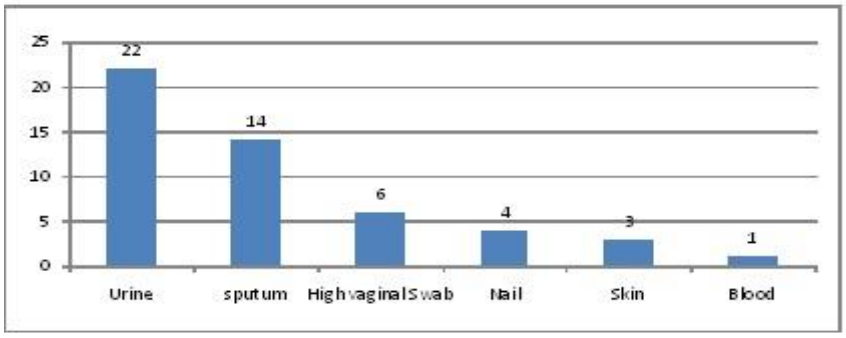
Most of the isolates were obtained from the specimens from male patients were 29(58%), details of this distribution is depicted in figure 2.

Figure 2. Sex distribution of studied patients



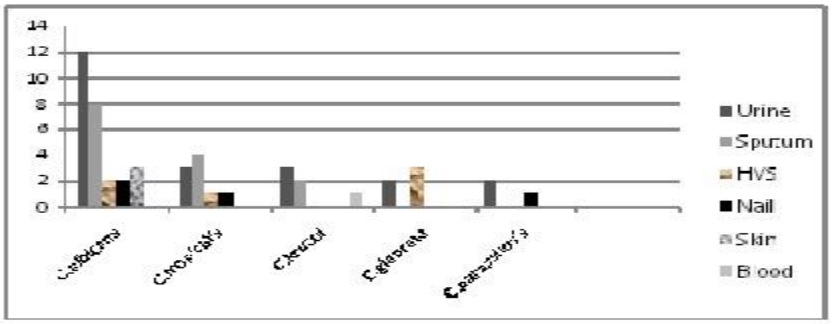
Among the various clinical samples, urine culture yielded the highest number of the *Candida* isolates (22), followed by sputum (14), high Vaginal swab (6), nail (4), skin (3) and blood (1) as demonstrated in figure 3.

Figure 3. Distribution of clinical specimens that yielded the growth of *Candida* species(n=50)



C. albicans was the most common species isolated from the different clinical samples, urine being the most frequent specimen. Among the other species *C. tropicalis* and *C.glabrata* were isolated at higher frequency from sputum and high vaginal swab respectively. Details of the *candida* species with respect to the specimen is shown in figure 4.

Figure 4. Distribution of *Candida* species isolated from various clinical specimens (n=50)



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Candida infection was most commonly found in the age group of 21-40 years (46%) followed by the age group 41-60 years (38%) as shown in table 1.

Table 1. Age wise distribution of patients with *Candida* isolates.

Age groups	Various Candida species					
	C.albicans	*C.tropicalis*	*C.krusei*	*C.glabrata*	*C.parapsilosis*	Total
5-20 yrs	2	1	0	0	0	3
21-40 yrs	9	5	4	3	2	23
41-60 yrs	12	2	2	2	1	19
>60 yrs	4	1	0	0	0	5
Total	27(54%)	9(18%)	6(12%)	5(10%)	3(6%)	50

Table 2. Distribution of *Candida* isolates among various samples originated in different wards (n=50)

Origin	Samples	Various Candida species					Total
		C.albicans	*C.tropicalis*	*C.krusei*	*C.glabrata*	*C.parapsilosis*	
Medicine	Urine	6	-	2	2	2	12
	Sputum	2	2	-	-	-	4
	Blood	-	-	1	-	-	1
General OPD	Urine	3	3	1	-	-	7
	Sputum	1	1	-	-	-	2
Intensive care unit	Sputum	5	1	2	-	-	8
Dermatology	Nail	2	1	-	-	1	4
	Skin	3	-	-	-	-	3
Gynecology	High vaginal swab	2	1	-	3	-	6
Surgery	Urine	3	-	-	-	-	3
Total		27	9	6	5	3	50

Table 3. Growth and colonial characteristics of *Candida* species (n=50)

Species	Total number of isolates	Color on Chrome agar	Germ tube test		Chlamydospores on CMA	Growth at 45°C
			positive	negative		
C.albicans	27	Light green	25	2	+	+
C.tropicalis	9	Blue colour	-	9	-	-
C.krusei	6	Purple fuzzy colour	-	6	-	-
C.glabrata	5	Pink smooth colour	-	5	-	-
C.parapsilosis	3	Pale colour	-	3	-	-

Table 4. Results of sugar assimilation test (n=50)

Candida species	Glucose	Sucrose	Lactose	Trehalose	Raffinose
C. albicans (27)	+	+	+	+	-
C. tropicalis (9)	+	+	-	+	-
C. krusei (6)	+	-	-	-	-
C. glabrata (5)	+	-	-	+	-
C. parapsilosis (3)	+	+	-	+	-

Table 5. Results of sugar fermentation test (n=50)

Candida species	Glucose	Sucrose	Lactose	Maltose
C. albicans (27)	AG	-	-	AG
C. tropicalis (9)	AG	AG	-	AG
C. krusei (6)	AG	-	-	-
C. glabrata (5)	AG	-	-	-
C. parapsilosis (3)	AG	-	-	-

A=AcidProduction, G=GasProduction.

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DISCUSSION

In the recent times the number of serious opportunistic yeast infections has increased considerably, particularly due to the global rise in the population of immunocompromised patients. Over the past decade, there has been a significant increase in the number of reports of systemic and mucosal yeast infections with both *albicans* and non-*albicans Candida* species worldwide (15). Definite diagnosis with identification up to species level is useful as it helps in prompt institution of appropriate antifungal therapy.

In our study an attempt has been made to investigate fifty *Candida* isolates from various clinical samples in BPKIHS, Dharan. Isolates were recovered from blood, urine, sputum, high vaginal swab, nail and skin. The commonest species isolated in our study was *Candida albicans* (27) which accounts for 54% of the total isolates. The predominance (54%) of *C. albicans* in this study correlates to the studies reported in India (16). A study conducted by Kangogo *et al.,* in Kenya reported that out of 150 isolates, 130 (86.7%) were of *C. albicans*, whereas 13.3% were non albicans *Candida* (17). According to Zaini *et al.,* out of 313 *Candida* isolates, 199 (63.5%) were identified as *C. albicans* and 114 (36.5%) as non *albicans Candida* (18).

A study from South India and Karnataka subsequently reported the distribution of *C. albicans* and non *albicans Candida* species as 39.64%, 47% and 60.36%, 53% respectively in various clinical samples obtained from hospitalized patients (19, 22).

Predominance of non *albicans Candida* in the above studies could be explained by the fact that most of the samples were from hospitalized patients with predisposing factors like prolonged medication, pregnancy and urinary catherization (19, 20). These factors are known to compromise the defense mechanism of the individuals rendering them more vulnerable to infection by the various species of *Candida*.

Among the 23 (46%) non- albicans, *C. tropicalis* (18%) was the most common species isolated in our study. A study conducted by Basu *et al*., recovered *C. tropicalis* in a similar frequency followed by *C. krusei* and *C. glabrata* (21).

In the present study, *Candida albicans* were predominantly isolated from urine samples (44%) followed by sputum (28%), high vaginal swab (12%), nail (8%), skin (6%), and blood (2%). Our finding of higher number of isolation of *Candida* from urine is in agreement with many reports which have shown the increased incidence of *Candida* infection in the genitourinary tract in all areas of medical and surgical practice. Candidal colonization of the urinary tract is common in patients with diabetes, patients receiving broad-spectrum antibiotics or immunosuppressants, or those with long term urinary catheters (21, 23).

Candida is a normal inhabitant of the mouth and can be recovered from sputum in 20 to 55% of normal subjects (24). The role of *Candida* in pulmonary candidiasis and its diagnosis is still controversial. Isolation of *Candida* species from respiratory specimens is frequent in mechanically ventilated patients. Respiratory samples constituted, 14 (28%) of sources of *Candida* in our study. The isolated *Candida* species from the patients in our study are very much significant, as most of the patients (57.2%) were in intensive care unit with many underlying risk factors. The remaining patients (42.8%) from whom *Candida* were recovered from sputum samples were from outpatient setting and could not be followed up.

Candida species have been found to be one of the most common pathogens causing blood stream infections (9). Nevertheless in our study, only one *Candida* isolate was obtained from blood specimen. This could be due to the limited number of blood specimens included in this study.

Although candidiasis can occur at all ages, a report from Mumbai indicated its highest incidence in the age group of 21-40 years (25). These findings are in concurrence with those of ours, where we found highest number of *Candida* was obtained from the age group of 21-40 years (table-1).

The present study demonstrated male preponderance 29(58%), a finding similar to that of Kashid *et al.,* in which involvement was higher in males (55.10%) as compared to the females (44.8%) (26). Singh *et al.,* in a study conducted in Chandigarh, India concluded male sex being a risk factor for developing fungaemia (27). Detection and identification of microorganisms depends on the availability of easy to perform screening and cost- effective methods. The medium most widely used for the isolation of *Candida* and other yeast species

from clinical specimens is Sabouraud Dextrose Agar (SDA) (28). This is a general – purpose medium that supports the growth of most pathogenic fungi. However SDA is not a differential medium and colonies of different pathogenic yeast species grown on this agar cannot be easily distinguished from each other. In addition at times SDA may support the growth of bacteria. Therefore, a medium is required which can inhibit the growth of bacterial contaminant as well as detect the mixed fungal growth.

CHROMagar *Candida* fulfills all these requirements. It is a selective and differential medium which is widely used for the rapid identification and differentiation of *Candida* species from clinical specimens. It is superior to Sabouraud dextrose agar in terms of its ability to inhibit bacterial growth (29). This special medium contains chromogenic substrates which are acted upon by enzymes produced by the microorganism to yield microbial colonies with varying pigmentation, hence allowing the differentiation of species by color and morphology (30).

The observation made in our study is that CHROMagar *Candida* medium is a useful medium for isolation of *Candida* species. Among the *C. albicans* all the 27 isolates showed light green colour, 9 isolates of *C. tropicalis* showed blue colour, 6 isolates of *C.krusei* isolates showed purple fuzzy colonies and all the 5 isolates of *C. glabrata* showed cream to pink smooth colonies. However, limited number of *C.parapsilosis* (2 out of 3) isolated in our study could not be distinctly identified in this medium, thus resorted to carbohydrate assimilation and fermentation tests for speciation.

Horvath *et al.*, and Davise *et al*., in their study described that CHROMagar *Candida* can readily be used for primary isolation of yeast from clinical specimens. Use of this medium allows rapid identification of clinically important *Candida* species (30, 31).

Since *C. albicans* is the yeast species most often isolated from clinical material, most clinical laboratories approach yeast identification by applying simple rapid tests such as germ tube formation to distinguish *C. albicans* from other species.

However newly described yeast, *C. dubliniensis is* very similar to *C. albicans* in many characteristics like germ tube formation and chlamydospore production. It, in a way, increases the likelihood of misidentification of *C. dubliniensis* strains as *C. albicans* underscoring the importance of other rapid, simple and cost effective methods which allows identification of *C. dubliniensis* and its discrimination from *C. albicans* in a routine diagnostic set up. CHROMagar *Candida* is one of such methods in which identification of *Candida* including *C. dubliniensis* is possible by the formation of colored colonies distinct for each clinically significant *Candida* species (32, 33), no growth at 45°C and lack of ability to assimilate xylose (34). In our investigation, no *C. dubliniensis* was isolated, all the *C. albicans* isolates grew at both 37° C and 45° C and chlamydospores were not typical of *C. dubliniensis*.

C.albicans showed the terminal chlamydospores, *C.tropicalis* formed blastospores singly or in small groups, *C. krusei* showed pseudohyphae with cross-matchsticks or tree–like blastoconidia, *C.glabrata* formed the yeast only and *C.parapsilosis* formed the giant hyphae and blastospores at nodes on corn meal agar.

Out of 27 *C. albicans* species, 7.4 % were germ tube negative. This is in accordance with other reports that up to 5 % of *Candida albicans* are germ tube negative (35-36). All the *C. albicans* were positive for chlamydospores. They all showed distinct growth at 37°C and 45°C temperatures. Non *albicans Candida* did not form any chlamydospores, therefore carbohydrate fermentation and assimilation tests along with colour production on CHROMagar was used to establish their identity.

Although the present study revealed *C. albicans* as the predominant species among clinical candidal isolates in our set up, non *albicans Candida* appear to be emerging as significant yeast and warrant routine discrimination in clinical laboratories.

CONCLUSION

In the context of rising concern about the opportunistic infections globally, it becomes imperative for us to take timely steps for early recognition of the clinically significant yeast isolates prevalent in our society. In

addition, further study with inclusion of large number of samples, longer duration and clinico-epidemiological correlation is recommended in future.

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