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Original Article

Candida species isolation, identification from Diabetics and their Antifungal Susceptibility Patterns

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ABSTRACT

Background and Objectives: The *Candida* infection has increased over the past two decades among diabetes patients. The specific *Candida* species isolated from diabetes patient and its antifungal susceptibility patterns are important factor which helps for establishment of empirical treatment protocol, however very limited institutional data are available on *Candida* infection from Nepali diabetes patients.

Material and Methods: In the current cross sectional research, different clinical samples from Nepali diabetes patients were selectively taken. The *Candida* species were isolated, identified and using Corn meal agar, HiChrom agar, sugar assimilation and fermentation tests and performed antifungal susceptibility test of *Candida* species isolated from different diabetes patients by disc diffusion method as per CLSI guide line given in document (M44-A).

Results: There were 321 *Candida* spp. isolated and identified with different Hi-Chrom media and sugar assimilation and fermentation tests. The highest number of *Candida* spp. were obtained from urine sample 201(37.6%) and least isolates were from nail infection 5(0.9%). Out of 321 *Candida* isolates, *C. albicans* was 88(27.4%) and non *albicans Candida* group were 233(72.6%). *Candida tropicalis* 122(38.0%) and *C. albicans* 88(27.4%) were the most common *Candida* spp. causing infections in patients. *Candida albicans* and non *albicans Candida* sp. which was 2nd highest isolates showed (100%) sensitive to amphotericin B and nyastatin. Whereas, resistance pattern was highest seen with fluconazole, ketoconazole and itraconazole, which was 13.6%, 22.7% and 21.6% respectively. *C. spherica* 8 (2.8%) showed (100%) sensitive to all the antifungal agents. Unidentified *Candida* species 11(3.4%) also exhibited similar resistance patterns to azole drug but they all were 100% sensitive to amphetericin B and nyastatin drugs.

Conclusions: Our findings show that non *albicans Candida* is the emerging fungal infection among diabetes patients followed by *C. albicans*. The rate of resistance is also increasing to azole group of antifungal drugs especially in *Candida* species isolates from Nepali diabetes patients.

Key word: Antifungal susceptibility test, *Candida* species, Diabetes patients, Hi-Chrom Agar

INTRODUCTION

Candida species were previously regarded as culture contaminants, which is a major causative agent of Candidaemia [1]. The

frequent occurrence of *Candida* infections in patients with diabetes mellitus has been recognized for many years [2]. The predisposing effects of diabetes have been confirmed by animal experiments, in which an induced diabetes state was associated with increased susceptibility to infection by pathogenic *Candida* species was found [3]. Different studies had shown that the prevalence of yeasts was greater among diabetes than healthy normal individuals [4]. On the other hand, non-diabetic individual also may experience Candidiasis when the immune system is temporally depressed by different factors like stress, alcohol as well as HIV with Hepatitis B patients [5]. It is also evident that Candidiasis affect some people on antibiotics since, antibiotics kills the bacteria, it provide an opportunity for *Candida* spp. to replace bacteria [6]. Nowadays, some *Candida* strains have become resistant to azole group of drug, especially in diabetics patients [7]. Drug resistance is becoming a major source of concern in diabetic patients. Therefore, a periodic surveillance of the antifungal susceptibility pattern of prevailing *Candida* spp. has become an important issue.

The present study thus aimed at determining novel characteristics in the incidence, distribution and antifungal susceptibility of *Candida* spp. isolated from diabetic patients from 1st January 2016 to 30th December 2018. The studies enable us to assess the change in antifungal sensitivity patterns of the isolated *Candida* spp.

MATERIALS AND METHODS

The cross sectional study was carried out between 1st January 2016 to 30th December 2018 in the Department of Microbiology at College of Medical Sciences Bharatpur, Nepal. The *Candida* species which were isolated from different samples like blood, urine, sputum, wound swab, oral swab and vaginal swab from clinically diagnosed diabetes patients (according to WHO) [8] attending CMS-TH, located at central part of Nepal were taken. An ethical approval was obtained from the institutional review board for performing the study.

About 982, diabetic patients suspected for fungal infections were examined for the presence of culture positive *Candida* spp. selectively taken for the research. Isolation and identification of *Candida* spp. were done from clinically diagnosed cases of diabetic patients clinical samples were cultured on Sabouraud's Dextrose agar (SDA) containing 0.5% chloramphenicol, PH 6.5 and incubated at 37^{o}C for 24 hours [9].

All moist creamy white colonies were picked up and used for preliminary identification on HiChrom for *Candida* species (HiMedia Lab. Ltd. Mumbai, India). All the plates were incubated at 37^{o}C for 24 hours. Different *Candida* species were identified on the basis of different colour on HiChrom agar. HiChrom agar gives *C. albicans*- blue green, *C. tropicalis*- dark blue with pink halo at the center, *C. krusei*- pink, large, rough spreading, *C. parapsillosis*- pale gremy and *C. dubliniensis*- dark green colonies [10]. Gram stain, Germ tube formation assay was done by inoculating and incubating *Candida* sp. at 37^{o}C in horse serum, and observed for germ tube after 2 hours, under 40X objective [11].

Carbohydrate fermentation test was done on medium containing 2% of sugars like dextrose, lactose, sucrose, glactose, trehalose and maltose in a medium containing yeast extract, peptone and bromothymol blue indicator. Acid and gas were noted and accordingly species identification was done. For sugar assimilation test, disc with 4%

respective sugar were prepared and placed on a *Candida* species inoculated on yeast nitrogen base agar and incubated aerobically at 37°C for 24 hours. Presence of growth around the disc indicated assimilation of that particular sugar which was also noted. Morphotyping of *Candida* spp. were performed using cornmeal agar inoculated by Dalmau plate method, incubated at 30°C for 2-5 days and observed microscopically for the presence of pseudohyphae, chlamydospores and blastospore formation [12].

Antifugal susceplibility test was performed for all the isolates of *Candida* spp. using disc diffusion method as per CLSI M44-A protocol. In this method, Muller Hinton agar supplemented with 2% Glucose and 0.5µg/ml methylene blue dye (GMB) medium were used. The commercially available antifungal disc were used and after inoculation and incubation of test organism at 37°C for 48 hrs, zone of inhibition were measured and recorded as per HiMedia comparison chart. The HiMedia antifungal discs used were Amphotericin-B (20µg), Clotrimazole (10µg), Fluconazole (10µg), Ketoconazole (10µg), Itraconazole (10µg) and Nystatin 100 units [13]. The tests were performed and interpreted according to the manufacturer instructions. *Candida albicans* ATCC 10231, *C. krusei* ATCC 14243 and *C. tropicalis* ATCC 201380 was used as quality control strain which were obtained from PGI Chandigarh, India.

Statistical analysis was done using SPSS version-18. Comparisons of species distribution and Antifungal susceptibility test were determined by the chi-square test for categorical variables and the Wilcoxon rank sum test for ordinal variables. A p-value <0.05 indicated statistical significance between any compared mean groups.

RESULTS

A total of 584 clinically confirmed diabetic patients volunteered to participate after signing consent for the present research. The patients belong to a wide age group from 13 years to 90 years with a mean age of 33 years. Among total 584 diabetic patient, 49 patient samples were excluded who were misdiagnosed, consent not willing to sign, complicated cases and ICU patients were excluded from the present research therefore, 535 cases only included in the present study.

Out of 535 diabetic patients 280 (52.3%) belong to the 16-30 years of age group. Males were predominant, with male and female ratio was 1.2:1. In our research, 86. 30% patients were from wards and remaining patients (13.7%) were from OPDs. The most common system involved was GIT (27.5%) followed by renal system (20.8%), respiratory system (19.3%) and CNS (11.2%). Diabetic coma was seen in 5 cases, while fever, multi-organ involvement and superficial infection were also seen in different diabetic patients.

The 535 diabetic patient samples which included were urine from 201 cases (37.6%), sputum from 111 cases (20.7%), high vaginal swab (HVS) from 72 cases (13.5%), urinary catheter 42 cases (7.8%) and rest of all were from blood, aspirates and superficial infection 109 (20.4%). The most common risk factor found was prolonged antibiotic therapy in diabetes mellitus cases (Table-1).

There were 321 *Candida* spp. isolated and identified with different HiChrom media and sugar assimilation and fermentation tests as standard Microbiology protocol. The highest number of *Candida* spp. were obtained from urine sample 201(37.6%) and least isolates were from nail infection 5(0.9%). *Candida tropicalis* (38.0%) and *C. albicans* (27.4%)

were the most common *Candida* spp. causing infections in diabetes patients; followed by *C. glabrata* (9.9%), *C. parapsillosis* (8.5%), *C. krusei* (5.9%), *C. keyfer* (4.4%) unidentified *Candida* species (3.4%) and *C. spherica* (2.5%) were yeast like fungi causing fungal infections (Table-2).

Table 1: Profile of different samples collected from Diabetes patients

Samples	Number	Percentage
Blood	39	7.3%
Urine	201	37.6%
Sputum	111	20.7%
Trachial aspirate	08	1.5%
High vaginal swab	72	13.5%
Endotrachial tube	23	4.3%
Urinary catheter	42	7.8%
Abdominal drains	23	4.3%
Wound swab	11	2.1%
Nail infections	05	0.9%
Total	**535**	**100%**

Table-2: Profile for *Candida* species isolated from different diabetic patient clinical samples

***Candida* species**	**Number**	**Percentage**
Ca. albicans	88	27.4%
Ca. tropicalis	122	38.0%
Ca. glabrata	32	9.9 %
Ca. parapsillosis	27	8.5%
Ca. krusei	19	5.9 %
Ca. keyfer	14	4.4 %
Ca. spherical	8	2.5 %
Candida spp.	11	3.4 %
Total	**321**	**100** %

Out of 321 *Candida* isolates, *C. albicans* was 88 (27.4%) and non albicans *Candida* group were 233(72.6%). The present research revealed that non *albicans Candida* causing diabetes patients infections were more commonly by *C. tropicalis* 122(38.0%) followed by *C. glabrata* 32 (10.0%), *C. parapsillosis* 27(8.4%), *C. krusei* 19(5.9), *C.*

Table-3: Antifungal susceptibility pattern of various *Candida* species performed by disc diffusion method

***Candida* species**		**Amphotericin-B**		**Clotrimazole**		**Fluconazole**		**Ketoconazole**		**Itraconazole**		**Nystatin**	
		S	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**
***C. albicans* (n= 88)**	**No.**	88	-	77	11	74	14	68	20	69	19	88	-
	%	100	-	87.5	12.5	84.1	15.9	77.3	22.7	78.4	21.6	100	-
***C. tropicalis* (n= 122)**	**No.**	122	-	78	44	68	54	98	24	88	34	122	-
	%	100	-	63.9	36.1	55.7	44.3	80.3	19.6	72.2	27.8	100	-
***C. glabrata* (n= 32)**	**No.**	32	-	11	21	14	18	26	06	18	14	32	-
	%	100		34.3	65.6	43.7	56.2	81.2	18.8	56.3	43.7	100	
***C. parapsillosis* (n= 27)**	**No.**	27	-	25	2	19	8	22	5	23	4	27	-
	%	100	-	92.5	7.5	70.3	29.8	81.5	18.5	85.2	14.8	100	-
***C. krusei* (n= 19)**	**No.**	19	-	4	15	15	4	8	11	6	13	19	-
	%	100	-	21.1	78.9	78.9	21.1	42.1	57.9	31.5	68.5	100	-
***C. keyfer* (n= 11)**	**No.**	11	3	12	2	9	5	10	4	7	7	10	4
	%	78.6	21.4	85.7	14.3	64.2	35.7	71.4	28.5	50.5	50.5	71.4	28.5
***C. sherica* (n= 8)**	**No.**	8	-	8	-	8	-	8	-	8	-	8	-
	%	100	-	100	-	100	-	100	-	100	-	100	-
***Candida* spp (n= 11)**	**No.**	11	-	9	2	7	4	10	1	9	2	11	-
	%	100	-	81.8	18.2	63.6	36.3	90.9	9.1	81.8	18.2	100	-
Total (n= 321)	**No.**	**318**	**03**	**224**	**97**	**214**	**107**	**250**	**71**	**228**	**93**	**317**	**4**
	%	**99.1**	**0.9**	**69.8**	**30.2**	**66.7**	**33.4**	**77.9**	**22.1**	**71.0**	**28.9**	**98.8**	**1.2**

keyfer 14(4.4%) *C. spherica* 8(2.5%) and unidentified *Candida* species 11(3.4) as shown in Table-3.

Candida albicans and non *albicans Candida* sp. which was 2nd highest isolates showed (100%) sensitive to amphotericin B and nystatin. Whereas, resistance pattern was highest seen with fluconazole, ketoconazole and itraconazole, which was 13.6%, 22.7% and 21.6% respectively. *C. tropicalis* 122 (38.0%) showed resistance to azole group of drugs and 36.1% were also resistance to clotrimazole. *C. glabrata* 32 (10.0%) the third highest isolated species showed, 21(65.6%) were resistance to clotrimazole, 16(50.0%) to fluconazole, 14(43.7%) to itraconazole and 6 (18.8%) to ketoconazole. *Candida keyfer*, *C. krusei* and *C. parapsillosis* 27(8.4%) also showed similar type of azole drug resistance pattern. *C. spherica* 8(2.8%) showed (100%) sensitive to all the antifungal agents. Unidentified *Candida* species 11(3.4%) also exhibited similar resistance patterns to azole drug but they all were 100% sensitive to amphetericin B and nystatin drugs as shown in Figure-1.

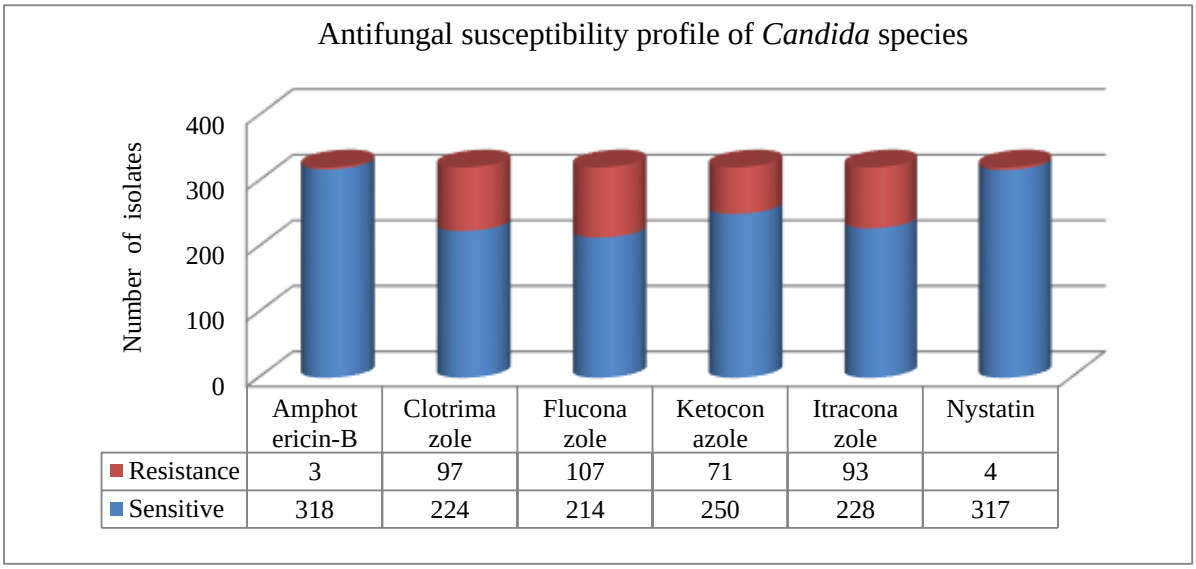

Figure-1: Shows the susceptibility pattern of *Candida* species isolated from diabetes patients

DISCUSSION

Over the past few decades, there has been a significant increase in the number of reports of systemic and mucocutaneous yeast infections with *Candida* species other than *C. albicans* [14]. The emergence of more fungal infections among diabetic patients requires advance type of medical management, needs long time hospital stay and also increases economic burden to the diabetic patients. The potential clinical importance of species and its identification has been recognized, as *Candida* species differ in the expression of virulence factors and antifungal susceptibility patterns [15].

Current and rapid identification of various *Candida* isolated is definitely warranted not only for proper patient management as various species respond differently to various antifungal agents but also to prevent emergence of drug resistance. *Candida* species is yeast like fungal cell, which is a part of normal microbial normal flora of human

body. *Candida* spp. are known as opportunistic fungal pathogens, as it causes infection when person is immune-compromised, immune-suppressed or diabetic. In diabetic patients *Candida* causes diseases like oral, vaginal thrush, intestinal Candidiasis and also onychomycosis [16]. Modern medical procedure of various kinds attributed to the risk for developing Candidiasis among diabetic patients. Therefore, early isolation, identification and antifungal susceptibility testing is necessary for decreasing patient mortality rate.

The different age groups of patient were included in this present study. Mostly *Candida* infections were seen in age group >20 to < 50 years of diabetes patients which corroborates the result with other researcher [17]. They showed *Candida* infection was almost uniformly distributed in all age group of immunocompromised patients.

In the current investigation, HiChrom agar along with other essential biochemical test was used for *Candida* spp. identification. Most of the researcher found HiChrom is better than SDA, which was in agreement with the findings of the study done by Chakraborti *et al*. [18]. The results were quite superior to conventional SDA in terms of suppressing the bacterial growth. It was quite easy to identify species on the basis of biochemical and colour on HiChrom agar colonies. Few fungal species did not grow on SDA therefore they were classified as unidentified *Candida* species.

In diabetes patients, the most common type of *Candida* species causing infections were seen in urinary tract infection, respiratory tract infection and blood stream infections [19]. In the present research, culture isolates were mostly obtained from of urine samples (37.6%) fallowed by sputum (20.7%) in addition to a varying percentage from other samples like high vaginal swab (13.5%), urinary catheter (7.8%), blood (7.3%), abdominal drain (4.3%), wound swab (2.1%) and nail infection (0.9%). Different studies reported the predominance of *C. albicans* as a leading cause of invasive Candidiasis. However, several research around the world have witnessed a change in the epidemiology of *Candida* infections in diabetic patients, characterized by a progressive shift from a predominance of *C. albicans* towards a predominance of non *albicans Candida* (NAC) remained 2nd highest isolated species from diabetic Nepali patients. Similar types of report were also found from different researchers around the world like USA, Europe and South America (Brazil). Which shows it's predominance and it is slowly giving way to increase in NAC species such as *C. tropical* [20]. This may be an explanation for the increase fungal infections, it is totally depends on immunity of diabetes patients.

The species identification of *Candida* is important to provide a database for given area of study. In India, *C. tropicalis* showed most common cause of *Candida* infection in immunocompetant individual. In the current research, *Candida albicans* causing infection were (27.4%) followed by NAC like *C. tropicalis* (38.0%) and *C. grabrata* (9.9%). Findings of our study were quite similar to the Indian subcontinent, which showed 40-50% of *Candida* infections were due to *C. tropicalis* as most emerging dominant species causing infection among diabetes patients [21]. In old literature comparative studies had done on different *Candida* species which were isolated in their studies by different researchers showed that *C. albicans* were in highest number [22]. Studies over a year have shown that there is considerable increase NAC infection in diabetes patients [23], although this could not be clearly explained,

this could be attributed to mode of exposure, type of *Candida* species emerging in that particular area, personal hygiene and immunity of the person.

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 acquired resistance to amphotericin B, flucytosine and the azole group of drugs used for chemotherapy. The choice of antifungal agents is also dependent on the *Candida* species. In the present investigation, antifungal susceptibility testing was done for 321 *Candida* species by Kirby Bauer disc diffusion method. The *C. albicans* and non *albicans Candida* group of isolates were 99.9% sensitive to amphotericin- B and nyastatin and only 0.1% NAC isolates showed resistance to these drugs.

The results of our study are in accordance with the result of other studies in this respect. As regards the antifungal sensitivity of *Candida* species study by Badiee *et al*. [24] 31 of C*. albicans* strains were sensitive 100% to amphotericin-B and flucytosine, and few isolates were also resistance to nystatin at the rate of (1.0%). Birhan *et al* and Casalinuovo *et al*. [25-26] investigated the antifungal sensitivity of *Candida* isolated from patients found that 12.5% *C. albicans* were resistance to fluconazole and 5.3% to clotrimazole. 14.3% *C. parapsillosis* was resistance to fluconazole, 33.3% *C. tropicalis* was resistance to ketoconazole (Figure-1). In this research, it was noted that fluconazole 33.4% was mostly showing resistance followed by clotrimazole 30.2%, itraconazole 28.9% and ketoconazole 22.1%. Amphotericin B 0.9% and nystatin 1.2% only showed resistance in *Candida* species isolated from diabetes patients. It showed these are the two drugs which we can limit the use and can be saved for future use for the treatment in the complicated Candidiasis cases.

Some of the NAC species have been increasing their virulence factors noticed among diabetes patients. Findings in our study have confirmed and corroborated the earlier reports of increasing resistance to fluconazole among isolates of *C. tropicalis* from Asia pacific region. *C. parapsillosis*, *C. krusei*, *C. keyfer* and *C. spherica* have shown a predilection. Most of the diabetes patients were reported to have antibiotics and few also said to have azole groups of antifungals for three days. These may not be effective way of treatment that's why patients might have persistent *Candida* infection for long time. Hypothetical treatment, over the counter medication, antibiotic treatment in fungal cases and shorter antifungal treatment contributes to re-emergence of similar infections and also it promotes antifungal drug resistance. Therefore, result shows that increasing azole group of antifungal drug resistance pattern in *Candida* spp. against antifungal agents needs continuous species identification and antifungal sensitive test before treatment.

CONCLUSION

In the present research we found that non *albicans Candida* species (NAC) was the predominant species responsible for various fungal infections among diabetes patients. Pre disposing condition like diabetes has high incidence of non *albicans Candida* infection which is intrinsically resistant to commonly used antifungal agents. HiChrom agar is the fast growing, inexpensive and rapid identification culture media for *Candida* species. *C. tropicalis* was the increasing incidence of major NAC responsible for fungal infection in diabetes patients. Antifungal

susceptibility test result exhibits drugs like amphotericin B and nystatin are the highly sensitive drug whereas, azoles and clotrimazole are showing resistance to commonly used antifungal agents for both *C. albicans* and NAC. Therefore, early isolation, identification and antifungal susceptibility test is essential for clinician to treat patient and also it also reduces the chances for drug resistance by restricting empirical use of antifungal agents.

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