Non-*albicans Candida* species: Emergence of neglected pathogens among population of Karachi

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Abstract: *Candida albicans* was considered as the principal cause of opportunistic candidiasis but nowadays, neglected non-*albicans Candida* (NAC) species are evolving as more virulent and drug resistant strains. This research was intended to assess pervasiveness of candidiasis mainly caused by NAC species in Karachi city. A total of 562 clinical isolates of *Candida* spp. collected during the period of one year were identified by microscopic as well as morphological (germ tube formation, characteristics on CHROM agar and Corn meal agar) and Biochemical (sugar assimilation and fermentation) characteristics. Doubtful species were further identified by using Remel RapID ™ yeast plus kit. The results were statistically analyzed by SPSS 16.0 version software. Isolated strains of candida revealed slight predominance of *C. albicans* (54.5%) over non- *albicans Candida* species (45.5%). Among NAC species, C. *tropicalis* and *C. glabrata* were isolated as the predominant species. These clinical species were procured mainly from urine samples of females (73.7%) of age group 20-30 years. No significant correlations exist between *Candida* species and their months of isolation as well as their isolation from different districts of Karachi. Emergence of NAC species may predict an upcoming threat in health care facilities and hence, require prompt management and accurate identification to suggest empirical antifungal therapy.

Keywords: *Candida albicans*, non-*albicans Candida*, opportunistic infections

INTRODUCTION

The greatest risk to human life is not only due to known pathogens but it is more because of unknown and underestimated pathogens. *Candida* species are the yeast fungi which are a part of normal flora of skin and mucous membrane. Being opportunistic organisms, they are one of the examples of those underestimated enemies which are a serious threat for human health nowadays. Candidiasis, infections caused by these versatile fungi, has been recognized as one of the most common fungal infection, whose clinical manifestations may range from relatively harmless superficial infections to serious systemic life-threatening infections having mortality rate as high as 40% (Hofs *et al*., 2016). This infection accounts for most of the invasive fungal infections worldwide while in USA it is considered as the fourth leading cause of nosocomial bloodstream infections (Healey *et al*., 2016). This situation is more alarming in countries of South Asia where the prevalence of candidiasis is almost 20-30 times greater than that in the western countries (Singh and Chakrabarti, 2017). Due to increasing use of broad-spectrum antibiotics and implanted medical devices, nearly all types of manifestations have been increasing and regarding as healthcare-related infections particularly among immune compromised individuals (Uppuluri *et al*., 2017).

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The genus *Candida* includes nearly 200 species while only 20 species are considered as clinically important (Hofs *et al.,* 2016). Among them, *Candida albicans* is the most repeatedly isolated specie responsible for causing different manifestations of candidiasis (Chauhan *et al*., 2019). But during last few years, there has been an alteration in the distribution of these infections and now more cases of candidiasis due to non-*albicans Candida* have been reported. This changing pattern is more pronounced in South Asian countries where 70-90% cases of candidiasis have been caused by NAC in recent years (Chakrabrti *et al.,* 2015). This epidemiological trend of prevalence of *Candida* species may also vary from country to country (Saunte *et al*., 2017). The spectrum of non- *albicans Candida* species also varies in different geographical locations. As observed in western countries, only *C. albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis and Candida krusei* were reported as causative agent of candidiasis. However, in south Asian countries there are more newly emerging species of *Candida* which accounts for candidiasis. As observed in a study conducted in India regarding candidemia, thirty-one species of *Candida* were procured from the clinical samples including above mentioned five species (Singh and Chakrabarti, 2017).

The main problem raised with the emergence of these NAC species was associated with their response to

antimycotic agents used to treat systemic infections. Most of these NAC species revealed reduced susceptibilities to these drugs. This high resistance towards antifungals was not only responsible for the aggravation of the course of disease, but also disseminated these drug resistant isolates among the community (Khadka *et al*., 2017). In the developed countries, there are many surveillance programs which keep a close check on emergence of such new infectious species but in developing countries like Pakistan such programs are lacking. Even in most of diagnostic laboratories, only *C. albicans* is identified among all *Candida* species while other species even isolated from severe systemic candidiasis cases have been reported as non-*albicans Candida* without any further identification (Ramos *et al*., 2018).

This research study was aimed not only to assess the emergence of *Candida* infections in Karachi City but it mainly focused on exploration of non-albicans *Candida* species responsible for these manifestations. These NAC are usually not identified up to specie level in most of the diagnostic laboratories of country which not only complicates the process of disease but also fails treatment measures as most of these NAC are resilient to many antimycotic agents.

MATERIALS AND METHODS

Collection of clinical isolates of candida

Five hundred and sixty two isolates of *Candida* spp. were obtained by convenient sampling from Diagnostic laboratory of Dow University of Health Science (DUHS) during one year (October 2016-September 2017). The details regarding source of isolation, and demography were also recorded from patient's medical record.

Identification of candida isolates up to specie level

The isolates of *Candida* were conventionally identified by using following standard diagnostic criteria (Larone, 1995; Mahmoudabadi *et al*., 2015).

Microscopic examination

The clinical isolates were examined under the microscope by using Gram staining techniques or wet mount technique.

Primary culture

For identification, the clinical isolates of *Candida* were streaked on Sabouraud's Dextrose agar (Oxide, UK) for 24 hours at 37°C. The growth characteristics of appeared colonies were recorded.

Germ tube test

This test is used widely to differentiate between *C. albicans* and *C. dubliniensis* from Non-*albicans Candida* species. The isolated colony from fresh culture of *Candida* was inoculated in sterile human serum (0.5ml) and incubated for 2 hours at 37°C. One drop of serum after incubation was transferred on glass slide and observed for the formation of germ tube under high power (40X) microscope. At least 05 germ tubes in the entire wet preparation were considered as positive result. *C. albicans* ATCC 14053 was used as positive control while *C. glabrata* ATCC 9258 was used as negative control.

Characteristics on Chromogenic agar

For the differentiation up to specie level, the *Candida* isolates were further streaked on Brilliance *Candida* Agar (Oxoid, CM1007) and incubated at 37°C for 48 hours. The different *Candida* species produce different colored colonies on this medium such as C. *albicans* and *C. dubliniensis* produce light green colored colonies while *C. tropicalis* produces dark metallic blue colonies. In the same way, *C. krusei* produces dry irregular pink colored colonies however, other species such as *C. kefyr, C. parapsilosis, C. glabrata, C. lusitaniae* exhibit yellow, beige and brown colored colonies.

Characteristics on corn meal agar with tween 80

The formation of chlamydospores and blastospores by *Candida* spp. and their specific arrangement was observed by streaking fresh culture of *Candida* on corn meal agar (Oxoid CM0103) with tween 80 by using Dalmau's plate method and incubated at 25°C for 3 to 5 days. The plates were observed directly under 40X lens of microscope.

Physiological/Biochemical characterization

The *Candida* species were further analyzed for their physiological and biochemical characteristics.

Carbohydrates fermentation test

The ability of carbohydrate fermentation was detected by inoculating colonies of *Candida* in yeast nitrogen base broth. After incubation, the heavy inoculum (0.1ml) was inoculated in carbohydrates fermentation broth containing inverted Durham tube and 2% sterilized sugar for seven days. The production of acid and gas was observed and recorded (Goel *et al.,* 2018)

Carbohydrate assimilation test

The ability to assimilate carbohydrate was investigated by preparing yeast inoculum (2ml) in yeast nitrogen base (YNB) which was then mixed well with 18ml of melted agar and poured in sterilized petri plates. After solidification of media, filter paper discs of 6mm diameter soaked in 10% sugar solution were aseptically placed on media and incubated for at least three days at 37°C. Presence of yeast growth around the disc indicated assimilation of that particular carbohydrate (Goel *et al.*, 2018).

Identification by using rapid identification kit system

Remel RapID ™ yeast plus system kits, a qualitative micro conventional method, was used for the final identification of *Candida* species which had some doubts

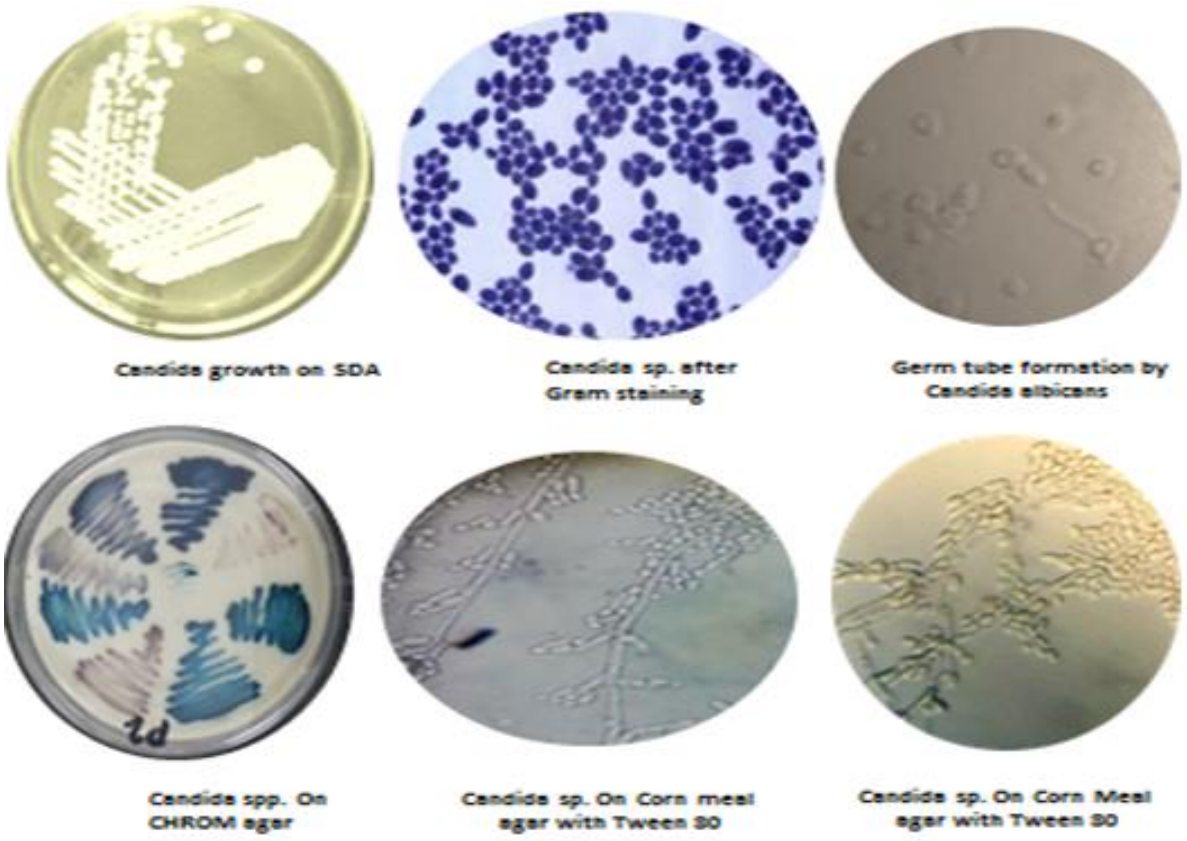


Fig. 1: Identification of Candida and Non albicans Candida species by different methods

in identification by above conventional methods. The freshly grown culture of *Candida* spp., mixed with sterile saline was added in the panel of kit system as per manufacturer's protocol and incubated for 4 hours at 30°C. Later, regents A and B (provide with kit) were added according to protocols and the appeared results were correlated with Remel RapID^{TM} yeast plus system computation data base to identify *Candida* species (Noni *et al.*, 2019).

RESULTS

Collection and Identification of Candida isolates

To assess the emergence pattern of *Candida* species in cases of candidiasis in Karachi City, five hundred and sixty two isolates of *Candida* species of clinical origin were procured and identified up to specie level by standard conventional methods. After complete identification, it was revealed that among 562 isolates, 54.5% (306) isolates were of *Candida albicans* while 45.5% (256) were non-albicans *Candida* species (NAC). These NAC comprised *C.glabrata* (16.7%), *C.tropicalis* (16.5%), *C. rugosa* (3.8%) and *C.krusei* (3.9%), *C. parapsilosis* (1.4%), *C. guilliermondii* (1.4%), *C. kefyr* (0.9%), *C. zeylanoides* (0.5%), *C. apicola* (0.2%) and *C. lipolytica* (0.2%) (table 1), fig. 1.

Gender wise distribution of Candida species

The highest numbers of *Candida* isolates were obtained from females i.e. 414/562 (73.7%) as compared to males 148/562 (26.3%). Among females, *C.albicans* was isolated as the predominant specie (54.3%) while NAC spp.were isolated with slighter rate (45.7%). Among the NAC species, *C. glabrata* was isolated as predominant spp. In male patients, almost similar distribution of *Candida* species was observed however, *C. tropicalis* was isolated as the predominant NAC specie (table 1).

Age wise distribution of Candida species

The *Candida* isolates were obtained from nine age groups of patients. Among these groups, the overall incidence of *Candida* spp. was higher in age group 20-30 years which was followed by age group 60-70 years. Interestingly, lesser *Candida* isolates were obtained from extreme age groups i.e. 0-10 and >80 (fig. 3).

Month wise distribution of Candida species

The *Candida* isolates were collected throughout the year i.e. from October 2016 to September 2017. The results obtained describe no significant variation in the number of isolations during different months. The statistical analysis also demonstrated a weak correlation (0.523<1) between different months of isolation of *C. albicans* and NAC species (table 2).

Table 1: Distribution of *Candida* species isolated from different genders

Candida species	Isolated from Females		Isolated from Males		Total No. of isolates	
	No.	%[a]	No.	%[a]	No.	%[b]
C. albicans	225	54.3	81	54.7	306	54.5
C. tropicalis	61	14.7	32	21.6	93	16.5
C. glabrata	71	17.2	22	14.9	94	16.7
C. parapsilosis	5	1.3	3	2.0	8	1.4
C. krusei	18	4.1	4	2.7	22	3.9
C. guilliermondii	7	1.7	1	0.7	8	1.4
C.kefyr	4	0.7	1	0.7	5	0.9
C. rugosa	18	5.1	3	2.0	21	3.8
C. zeylanoides	2	0.5	1	0.7	3	0.5
C. apicola	1	0.2	0	0	1	0.2
C. lipolytica	1	0.2	0	0	1	0.2
Total no	414	100	148	100	562	100

*[a] Percentages calculated from total number of *Candida* isolates from that particular gender.
*[b] Percentages calculated from total number of *Candida* isolates.

Table 2: Distribution *Candida* species isolated in different months of a year

Months	*Candida albicans*	Non-*albicans Candida* species
October	25 (8.17%)	15 (5.86%)
November	26 (8.50%)	20 (7.81%)
December	22 (7.19%)	10 (3.91%)
January	31 (10.13%)	36 (14.06%)
February	22 (7.19%)	25 (9.77%)
March	29 (9.48%)	20 (7.81%)
April	25 (8.17%)	23 (8.98%)
May	25 (8.17%)	24 (9.38%)
June	24 (7.87%)	25 (9.77%)
July	25 (8.17%)	21 (8.20%)
August	29 (9.48%)	20 (7.81%)
September	23 (7.52%)	17 (6.64%)
Total no of Cases	306 (100%)	256 (100%)

* Co- relation value 0.523 <1.

Table 3: Distribution of major *Candida* species among seven districts of Karachi

District of Karachi	Clinically isolated *Candida* species					
	Candida albicans	%	non-*albicans Candida* species	%	Total	%
Karachi Central	103	33.66	81	31.64	184	32.74
Karachi East	86	28.10	61	23.83	147	26.16
Karachi West	35	11.44	26	10.16	61	10.85
Karachi South	42	13.73	46	17.97	88	15.66
Korangi	25	8.17	22	8.59	47	8.36
Malir	15	4.90	20	7.81	35	6.23
Total	306	100.00	256	100.00	562	100.00

* Statistical analysis by Chi-Square Test revealed p value 0.43>0.05.

Location wise distribution of Candida species obtained during the study

Candida isolates in this study were obtained from main Laboratory of DUHS having seven collection centers located in six different districts of Karachi City. It has been observed that highest number of *Candida* isolates were obtained from Karachi Central (32.74%) followed by Karachi East (26.16%) and Karachi South (10.85%) (table 3.1). Moreover, the results revealed almost parallel isolation of *C.albicans* and NAC species from all districts of Karachi which was also depicted by insignificant relationship between these species and their area of isolation when analyzed statistically (p=0.43>0.05) (table 3).

Table 4: Distribution of *Candida* species with respect to clinical specimen

Clinical Specimens	*C.albicans*	*C.tropicalis*	*C.glabrata*	*C.parapsilosis*	*C.krusei*	*C.guilliermondii*	*C.kefyr*	*C.rugosa*	*C.zeylanoides*	*C.apicola*	*C.lipolytica*	No of isolates
Urine	171 (53.9%)	61 (19.2%)	51 (16.1%)	5 (1.6%)	8 (2.5%)	4 (1.2%)	3 (0.9%)	12 (3.8%)	0 (0%)	1 (0.3%)	1 (0.3%)	317-56.4%
High Vaginal swab	67 (46.2%)	17 (11.7%)	38 (26.2%)	2 (1.4%)	9 (6.2%)	3 (2.1%)	1 (0.7%)	6 (4.1%)	2 (1.4%)	0 (0%)	0 (0%)	145-25.8%
Sterile fluids	7 (77.8%)	2 (22.2%)	0 (0.0%)	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	0 (0%)	9-1.6%
Pus	5 (55.5%)	2 (22.2%)	0 (0.0%)	0 (0%)	2 (22.2%)	0 (0%)	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	0 (0%)	9-1.6%
Blood	7 (50%)	1 (7.1%)	3 (21.4%)	1 (7.1%)	0 (0.0%)	0 (0%)	0 (0%)	2 (14.3%)	0 (0%)	0 (0%)	0 (0%)	14-2.4%
Oral Swab	6 (40%)	5 (33.3%)	1 (6.7%)	0 (0%)	1 (6.7%)	0 (0%)	0 (0%)	1 (6.7%)	1 (6.7%)	0 (0%)	0 (0%)	15-2.7%
Sputum	41 (93.1%)	3 (6.8%)	0 (0.0%)	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	0 (0%)	44-7.8%
medical devices	2 (33.3%)	2 (33.3%)	0 (0.0%)	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	2 (33.3%)	0 (0%)	0 (0%)	0 (0%)	6-1.1
Stool	0 (0%)	0 (0.0%)	0 (1.1%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	0 (0%)	2-(0.4%)
Ear swab	0 (0%)	0 (0.0%)	0 (0.0%)	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1-(0.2%)
Total	306 (54.4%)	93 (16.6%)	93 (16.6%)	8 (1.4%)	21 (3.7%)	8 (1.4%)	4 (0.7%)	24 (4.3%)	3(0.5%)	1 (0.2%)	1 (0.2%)	562

Clinical specimens and gender wise distribution of Candida isolates

Candida isolates were acquired from different clinical specimens obtained from both genders. In females the highest number of *Candida* isolates were obtained from urine (53.14%) followed by high vaginal swab (35.2%). However, in males, the *Candida* species were mainly isolated from urine (65.54%) followed by sputum (13.5%). Interestingly a significant relationship (P = 0.000<0.05) was found between gender and *Candida* isolates from different clinical specimens (fig. 4).

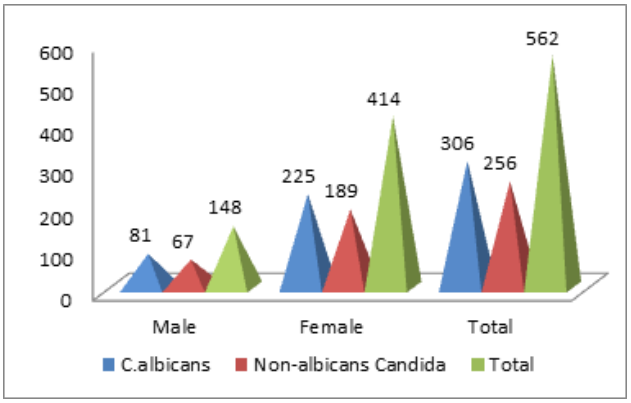


Fig. 2: Distribution of *Candida spp*. with respect to gender of patients

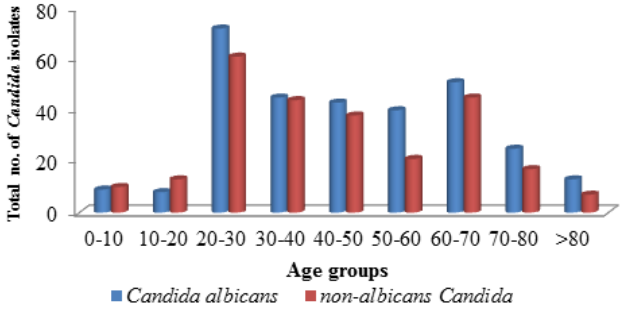
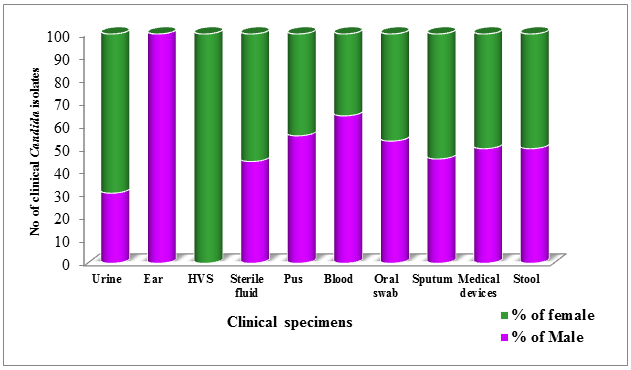


Fig. 3: Distribution of *Candida* species isolated from different age groups of patients

Clinical specimen wise distribution of Candida species

Different *Candida* species in this study were obtained from different clinical specimens but the highest no. of these species was obtained from urine (56.4%). The overall isolation pattern exhibited preponderance **of** *C. albicans* over NAC spp. from sputum (93%), sterile fluids (78%), pus (56%) and urine samples (54%). While, the NAC species were predominantly isolated from clinical specimens like ear swab (100%), oral swab (60%), stool 100%, medical devices 64%, HVS (54%) as compared to *C. albicans*. The specie wise isolation from clinical specimens revealed that *C. albicans* (53.9%), *C. tropicalis* (19.2%) and *C. glabrata* (16.1%) were isolated predominantly from urine samples while from high vaginal swab *C.albicans* (46.2%) was followed by *C. glabrata* (table 4)



*Statistical analysis by Chi-Square test revealed p value 0.000 < 0.05

Fig. 4: Distribution of clinical specimens with respect to gender of patients.

DISCUSSION

The unrevealed or underrated pathogens are sometimes more injurious than known pathogens and it was found true with etiology of candidiasis. *C. albicans* has been recognized as the major causative agent of all forms of candidiasis since beginning and the main focus of diagnostic laboratories are always to report this organism as pathogen only while other non-*albicans Candida* species are always considered as unimportant therefore remains unidentified up to specie level. But with time, the epidemiological pattern of candidiasis has been changing and in the recent years the species other than *C. albicans* are also evolving as more virulent and drug resistant species. This emergence of NAC species has been reported from all over the world (Tasneem *et al.,* 2017; Quindos *et al*., 2018).

This changing trend of candidiasis was also perceived in this study, where *C. albicans* (54.4%) was isolated as the predominant specie as compared to NAC species (45.6%) but this difference was not so marked and therefore isolation of these NAC species can be considered as more or less parallel to *C. albicans.* These findings are in accordance with other studies conducted in Pakistan where *C. albicans* was still the major causative agent of candidiasis but NAC species were also following these rates (Tasneem *et al.,* 2017; Jamil *et al.,* 2017). The study conducted in Turkey and Nepal also supported our findings and reported almost parallel isolation of both *Candida* species (Sharma et *al.,* 2016; Calgin and Cetinkol, 2018). The marked predominance of *C. albicans* over NAC species were reported only from some European countries while from most of other parts of world this pattern has been changed (Lamoth *et al.,* 2018).

In United States, the prevalence of NAC species has been exceeded more than 50% and *C. glabrata* has emerged as predominant pathogen followed by *C. parapsilosis* (Matsumoto *et al.*, 2014; Cleveland *et al*., 2015; Lamoth *et al.,* 2018). However, in Latin America and Africa this picture was little different and *C.albicans* was still the major pathogenic specie followed by *C. parapsilosis* (Doi *et al.,* 2016). Similarly, in Europe and Australia, *C.albicans* was still appeared as major causative agent but increased prevalence of *C.glabrata* has been reported from all over there (Chapman *et al.,* 2017; Trouve *et al.,* 2017). The shift towards non-*albicans Candida* species was more evident from Asia. The data from India showed that these NAC species (63.2%) were isolated as the predominant pathogen as compared to *C.albicans* (36.8%) and *C.tropicalis* was isolated as the major NAC specie (Deorukhkar *et al.,* 2014). Similar picture was also observed in a previous study where *C.tropicalis* superseded over *C.albicans* when isolated from tuberculosis patients (Naz and Perween, 2004; Bilal et al., 2018) China also presented the similar picture where NAC species superseded over *C.albicans* (Gong *et al.,* 2016). In Japan, the predomination of NAC species was also evident while *C.parapsilosis* was appeared as the most prevalent pathogen (Hirano, 2018). However, from Pakistan, the NAC species such as *C.tropicalis* (38%), *C.parapsilosis* (18%) and *C. glabrata* 16% superseded over *C.albicans* which was isolated from only 12% of adult population (Farooqui, 2013). These findings from Pakistan did not correlate with our findings which might be due to difference in site of isolation of these species.

In the present study, *C. tropicalis* and *C. glabrata* were isolated as predominant species after *C. albicans*. The isolation of *C. tropicalis* was in accordance with many studies and it was reported as major NAC specie from most of Asian countries but isolation of *C.glabrata* in high proportion was unusual because this specie was mostly reported as prominent pathogen from America and Europe (Lamoth *et al.,* 2018). This geographic discrepancy in prevalence of NAC species might be due to certain factors such as chronic illness, use of indwelling medical devices and demographic details of patients (Mohamed *et al.,* 2018).

The demographic information obtained during the study also revealed that the species of *Candida* were largely sequestered from female (73.7%) patients as compared to males (26.3%). This high prevalence might be due to their isolation from urine specimens as well as high vaginal swabs. In females, *Candida* species are found as normal commensal of urogenital areas and in certain circumstances these commensals become opportunistic pathogen and cause infections. Therefore, genitourinary candidiasis is one of the most frequent type of candidiasis occurs in females where it usually causes vulvovaginal candidiasis. Even in males these *Candida* species may cause severe genital infections such as balanoposthitis and balanitis. Moreover, candiduria is another prevalent infection caused by these species in both genders (Uppuluri *et al*., 2017). In the present study, a slightly

higher prevalence of *C.albicans* over other NAC species might be because of the fact that most of the isolates were obtained from urine and high vaginal swab and it was evident that among other species, *C.albicans* has more ability of strong adherence with vaginal and oral mucosa lining (Patel *et al*., 2012; Vieira de Meloa *et al*., 2019). This finding is in accordance with previous studies where urine specimens were the main source of isolation of *Candida* species (Khadka *et al.,* 2017, Wiebusch *et al.*, 2017).

CONCLUSION

The recent profile of Candida species in this study suggests an upcoming threat by newly arising non-*albicans Candida* species particularly *C. tropicalis* and *C. glabrata* which are already reported as drug resistant species. Therefore, there is a need to manage these non-*albicans Candida* species in population.

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