Antifungal Susceptibility and Biofilm Formation of *Candida albicans* Isolated from Different Clinical Specimens

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ABSTRACT

Objective: Increasing antifungal resistance and biofilm formation among *Candida* species is an intimidating public health concern, especially at the hospital settings. In light of this, the current study was designed to assess the biofilm-forming ability of clinically isolated *Candida albicans* and determine their antifungal susceptibility against both the planktonic and sessile forms.

Methods: A total of 58 *Candida* isolates from different clinical samples received at the Microbiology laboratory of KIST Medical College and Teaching Hospital, Lalitpur, Nepal in between April to October 2018 were included in the study. Isolation and identification of *C. albicans* was done following standard microbiological procedures that comprised of microscopic observations along with germ tube formation and biochemical tests. Besides qualitative investigation of biofilm by tube method, it was also investigated quantitatively by crystal violet staining method and metabolic activity of the biofilm was assayed by tetrazolium (XTT) salt reduction method. Antifungal susceptibility pattern against common antifungal drugs was determined as planktonic and sessile Minimum Inhibitory Concentrations (MICs) by broth micro-dilution method.

Results: Out of 58 *Candida* recovered from the total samples, 21(36.2%) were identified as *C. albicans.* The vaginal swabs showed a higher prevalence (57.14%, 4/7) of *C. albicans* whereas none were recovered from the wound swabs. Qualitative study of biofilm formation showed that 4 (19.1%) *Candida albicans* were strong biofilm producers, 11 (52.3%) isolates were moderate and 6 (28.6%) produced weak or none biofilms, whereas a majority (85.7%) of the isolates gave biofilm positive test in microtiter plate assay. The metabolic activity of the biofilm revealed that the average absorbance following the metabolic reduction of tetrazolium salt was 0.577. Interestingly, both the methods used for assessing biofilm productions correlated well ($r=0.569$, $p=0.007$). Most of the isolates were susceptible to Fluconazole (80.9%) at MIC 0.12 µg/mL, Amphotericin B (76.19 %) at MIC 0.25 µg/mL and Clotrimazole (80.9%) at MIC 0.25 µg/mL. In addition, sessile forms of *C. albicans* was found to have 2 to 8 fold increases in MIC compared to the planktonic cells.

Conclusion: High prevalence of *C. albicans* in vaginal swabs may implicate that the women are more prone to vaginosis. The sessile forms are more resistant to antifungal agents and proper administration of antifungal targeting the biofilms should be prioritized only with susceptibility result interpretations.

Key words: *Candida albicans*, biofilm, antifungal resistance, minimum inhibitory concentration

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INTRODUCTION

Candidiasis is a fungal infection which is caused by the fungi of genus *Candida*, predominantly by *Candida albicans. C. albicans* is one of the major fungal pathogens causing invasive fungal infections (Horn et al 2012) and occupy top ranking among the infectious agents. *C. albicans* can invade wide range of anatomical sites including muco-cutaneous deep tissues and organs and superficial sites such as skin, nail and mucosa; oral cavity and vagina being the most frequently encountered (Ramage et al 2001a). *C. albicans* imposes a greater public health challenge because of its high mortality rates and increasing costs of care and duration of hospitalization and this is why *C. albicans* is considered to be of higher medical and economic importance (Almirante et al 2005; Lai et al 2012; Sardi et.al 2013).

Three among four women during their lifetime are affected from candidiasis especially during their child bearing age (Bongomin et al 2017). About 6% of Nepalese women suffer from recurrent vulvovaginal candidiasis where 'recurrent' refers to four episodes per year (Khwakhali and Denning 2015). Moreover, *C. albicans* and also minority of other *Candida* species were the second highest colonization to infections and the overall highest crude mortality along with being the third leading cause of catheter-related infections (Crump and Cillingon 2000; Ramage et al 2001a). The alteration of *Candida* from commensal of an anatomical sites to an opportunistic pathogen is mediated by host's weakened immune system and various virulence factors such as adherence, biofilm formation and hydrolytic enzymes that comes into play at suitable host environment for pathogen (Sardi et al 2013).

A biofilm is an organized community that is regulated by molecular mechanism of regulation of gene expression in response to fluctuations in cell density that is the exchange of chemical signals among cells in a process known as quorum sensing (Yu et al 2012). The formation of the biofilm *in vivo* mediates the important work in pathogenesis. Moreover, development of biofilm is also related to the developmentof the resistance towards the antifungal agents and contributes to the increasing disease incidence which is supported by the fact that sessile cells within the biofilm are less susceptible to antifungal agents than the planktonic cells (Silva et al 2017). The treatment of biofilm-based infection remains an escalating clinical problem because of lack in biofilm specific drugs for *C. albicans* (Nobile and Johnson 2015).

Antibiotic susceptibility testing is one of the ways to determine the resistance of the organism towards the antimicrobial agents and determination of minimum inhibitory concentration is the best choice to understand the actual degree of susceptibility or resistance towards the antimicrobial agents. Some species of the *Candida* like *C. glabrata* and *C. krusei* are intrinsically resistant to Fluconazole (Izquierdo et al 2015). It has also been reported that these Fluconazole resistant *C. albicans* strains appear to be cross-resistant to other azoles (Richardson and Warnock2012). Similarly, rare but reported case of Amphotericin B resistance of *Candida* has been attributed to the alterations in the cell membrane, including reduced amounts of ergosterol, and were isolated following prolonged treatment (Richardson and Warnock 2012). With the increasing number of clinical isolates' resistance towards the commonly used antifungal agents, more specifically, by the production of biofilm in case of *C. albicans*, there is a growing need for antifungal susceptibility testing of the biofilm-producers which can contribute towards the pool for therapeutic approaches. In this regard, this study aims at determining the hospital-based prevalence of *C. albicans,* assess their biofilm formation and standard (planktonic) and sessile susceptibility against some commonly used antifungals.

METHODS

This was a qualitative, laboratory-based cross-sectional study carried out from April to October 2018 in KIST Medical College and Teaching Hospital, Lalitpur. A total of 58 *Candida* isolates were collected from different clinical samples (sputum, high vaginal swabs, associated catheter devices and wound swabs) from the patients visiting both inpatient and outpatient departments. Clinical samples were grown on three different media: Sabouraud Dextrose agar (SDA) supplemented with 0.05 mg/L chloramphenicol, Cystein Lactose Electrolyte Deficient (CLED), and Hichrom agar for 24-48 hours at 35-37°C. All the media used were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Identification of isolates was done based on colony characteristics, microscopic observation, germ tube formation test, and biochemical tests (Shrestha et al 2020). A yeast suspension was prepared from the pure culture of yeast as follows: First, a small amount of stock culture was inoculated on Sabouraud Dextrose Agar (SDA) containing Chloramphenicol by using a sterile loop and incubated at 37°C for 24–48 h. The yeast was then harvested and suspended in RPMI-1640 medium at turbidity equal to

optical density (OD) of 0.5 McFarland. The final suspension was adjusted to contain 1×10^7 yeast cells/mL (Subramanya et al 2017).

Qualitative biofilm measurement was done by inoculating the fresh yeast culture in nutrient broth at 37° C for 48 hours. After 48 hours, the liquid was pipetted out, washed with 2 mL phosphate buffer solution (PBS) and again pipetted out slowly to avoid disruption of the biofilm. It was then air-dried, stained with 1% crystal violet (CV) for 25 mins and washed with distilled water solubilized with 2 ml of 30% glacial acetic acid resulting in visible biofilm. Quantitative biofilm measurement was performed by crystal violet staining method (staining for biomass) in 96 well microtiter plates (Inci et al 2010) and metabolic activity of the biofilm was further determined by a well-established enzymatic method using XTT (2, 3-bis (2-methoxy-4-nitro-5-sulffophenyl)-2H-tetrazolium-5-carboxanilide) as previously described (Pierce et al 2010).

Antifungal susceptibility testing (AST) was performed and interpreted for all the *C. albicans* isolates by modified Kirby-Bauer disk diffusion method. Antifungal discs of Fluconazole (10 µg), Ketoconazole (10 µg), Clotrimazole (10 µg), and Amphotericin B (10 µg) were used for susceptibility testing on Muller Hinton Agar (MHA) supplemented with 2% glucose and 5 µg/mL methylene blue. Results of AST were interpreted following Clinical and Laboratory Standards Institute guidelines (CLSI, 2009) and susceptibility criteria were determined as per the recommendation guidelines provided by the company (HiMedia, Mumbai, India). Planktonic MICs and Sessile MICs (SMICs) were determined against the drugs-Amphotericin B, Clotrimazole and Fluconazole using broth micro-dilution methods (Ramage et al 2001b) following guidelines outlined in the Clinical and Laboratory Standards Institute (CLSI) documents (CLSI 2012).

RESULTS

Out of 58 candida species, 21 (36.2%) were reported as *Candida albicans* whereas 37 (63.8%) belonged to non-albicans *Candida* species (NCAC) (Figure 1).

The majority of *Candida* isolates were recovered from urine 29 (50%) followed by sputum 15 (25.9%), high vaginal swab (HVS) 7 (12.1%), catheter tip 6 (10.3%) and wound swab 1 (1.7%). Majority of *Candida* species (n=29) were isolated from urine among which 9 (31.0%) were *Candida albicans*. The prevalence of *Candida albicans* was seen higher in high vaginal swabs (57.1%) followed by sputum (46.7%) and urine (31.0%). Only 1 isolate out of 6 from catheter tip culture (16.66%) was *C. albicans*; whereas the only one Candida recovered from wound swab was non-albicans *Candida* species (Table 1).

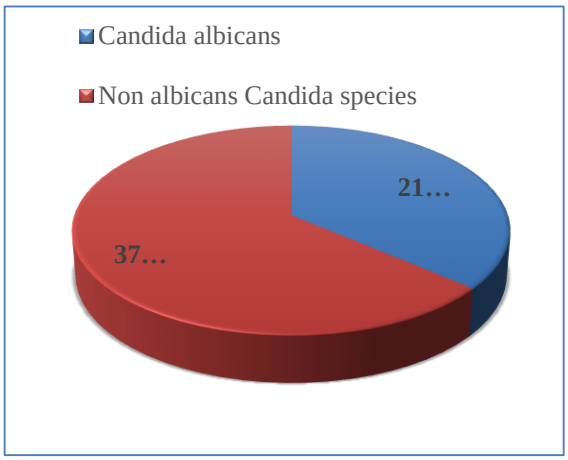

Figure 1: Distribution of *Candida* isolates

Qualitative study of biofilm formation showed that 4 (19.1%) *Candida albicans* were strong biofilm producers. 11 (52.3%) *Candida albicans* isolates were moderate and 6 (28.6%) of total *C. albicans* produced weak or none biofilms. A majority (85.7%, 18/21) of *C. albicans* isolates gave biofilm positive test in microtiter plate method as specified by crystal violet staining. The OD was intensely positive in 5 (23.8%) isolates, while moderate and mild biofilm formation was shown by 6 and 7 *C. albicans* isolates respectively. Three OD values were observed below the cut-off values representing negative or no biofilm formation. The optical densities of all the isolates are given in figure 2. The optical density value for each isolate represents the average of 2 replicate values. The mean OD value was 0.0549 with standard deviation of 0.319. The maximum reading was 0.112 corresponding to the strongest biofilm forming isolates while lowest was 0.011.

The metabolic activity of the biofilm revealed that the average absorbance reading following the metabolic reduction of tetrazolium salt was 0.577. The maximum optical density value corresponding to the highest metabolic activity in biofilm cells was 1.127; while the lowest OD reading was 0.090. All the value represents the metabolic activity of the biofilm cell (Figure 3).

Table 1: Distribution of *Candida* species in different clinical specimens

Sample	*C. albicans* n (%)	NCAC n (%)	Total n (%)
Urine	9 (31.0%)	20 (68.1%)	29 (50.0)
Sputum	7 (46.7%)	8 (53.3%)	15 (25.9)
HVS	4 (57.1%)	3 (42.9%)	7 (12.1)
Catheter tip	1 (16.7%)	5 (83.3%)	6 (10.3)
Wound swab	0	1 (100%)	1 (1.7)
Total	21 (36.21%)	37 (63.79%)	58 (100)

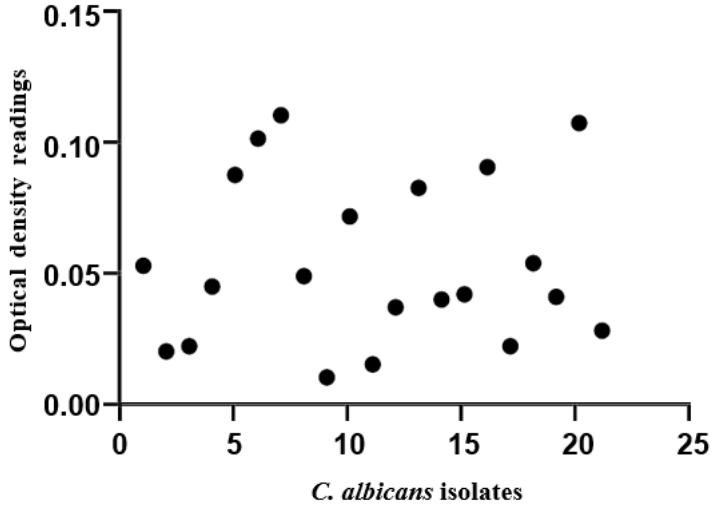

Figure 2: Optical density of biofilm production by crystal violet method by *C. albicans*

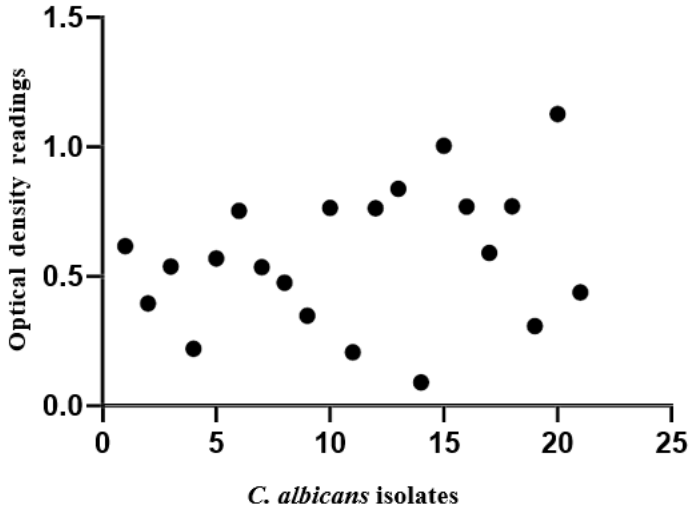

Figure 3: Optical density of biofilm formation by XTT metabolic reduction by *C. albicans*

Spearman correlation was used to assess the consistency of the two semi-quantitative methods of biofilm production used in this study. Interestingly these two methods correlated well with correlation coefficient (r) 0.569 and p=0.007. However, the maximum and minimum reading belonged to different isolates in two methods. For CV maximum reading represented isolate no.7 while isolate no. 20 exhibited maximum OD value in XTT assay. Similarly, the lowest colorimetric value among these two methods corresponds to two different isolates (Figure 4).

Most of the *C. albicans* were fairly susceptible to Amphotericin B, Fluconazole and Clotrimazole with 76.2%, 71.4% and 66.7% susceptibility respectively. Highest level of resistance was observed with Ketoconazole (52.4%) followed by Amphotericin B (23.8%) (Table 2).

The Fluconazole MIC value of different *C. albicans* isolates were in the range 0.06 to >64 µg/mL after 24 hours incubation at 37ºC. Most of *C. albicans* isolates (80.9%) were fairly susceptible (≤8µg/mL) to Fluconazole, 4.8% were susceptible dose dependent while 14.3% were resistant (≥64 µg/mL). The Amphotericin B MIC value of different *C. albicans* isolates were in the range 0.12 to ≥16 µg/mL after 24 hours incubation at 37ºC. Majority of *C. albicans* weresusceptible to Amphotericin B. 76.2 % of *C. albicans* had an MIC of ≤1 µg/mL while the rest 23.8% had MIC greater than 1µg/mL. Moreover, the Clotrimazole MIC value of different *C. albicans* isolates were in the range of 0.12 to ≥16µg/mL after 24 hours incubation at 37ºC. Most of the *C. albicans* isolates (76.2%) were fairly susceptible to Clotrimazole, whereas 21.8% isolates had MIC greater than 1µg/mL (Table 3).

Majority of *C. albicans* biofilm (71.5%) were resistant to Fluconazole with sessile minimum inhibitory concentration greater than 1024 µg/mL. Biofilms of 6 (28.5%) isolates were inhibited by Fluconazole; however, the minimum inhibitory concentration at 50% inhibition was 2 to 4 folds higher than the maximum concentration used for the planktonic susceptibility testing. Amphotericin B showed a variable minimum inhibitory concentration on the biofilm between different isolates. As indicated by SMIC50, 61.9% of *Candida* isolates biofilms were fairly susceptible to Amphotericin B and remaining 39.1% were comparatively resistant with SMIC50 1µg/mL and above. In addition, Clotrimazole was relatively less effective towards *C. albicans* biofilms. Only 3 individual biofilms of *C. albicans* had SMIC50 and SMIC80 below 1µg/mL. A total of 6 (28.6%) *C. albicans* biofilms were totally resistant to Clotrimazole with SMIC50 and SMIC80 greater than the maximum used concentration (16µg/mL). Two to eight fold increase in minimum inhibitory concentration was observed between planktonic and sessile cells (Table 4).

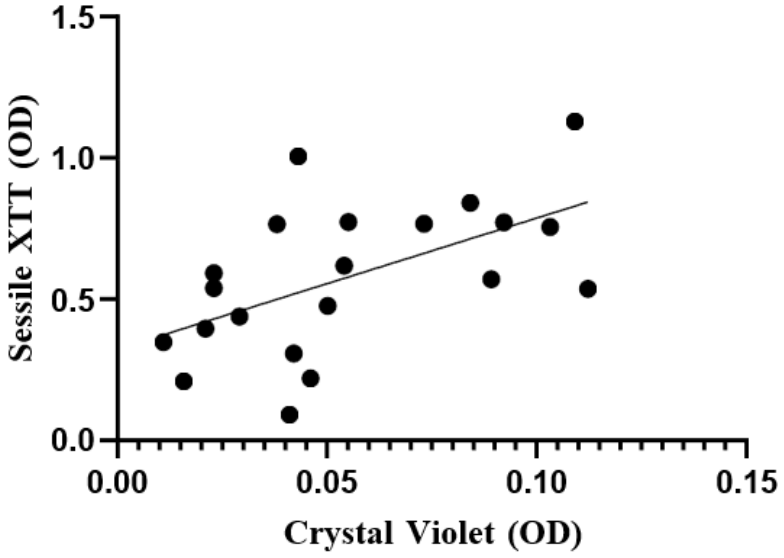

Figure 4: Correlation of methods of biofilm formation (Crystal violet correlated well withsessile metabolic activity).

Table 2: Antifungal susceptibility test of *C. albicans* against commonly used antifungalagents by disk-diffusion method

Antifungal drugs	Antifungal susceptibility pattern (n=21)		
	Resistant, n (%)	SDD, n (%)	Susceptible, n (%)
Amphotericin B	5 (23.8%)	0	16 (76.2%)
Clotrimazole	2 (9.5%)	5 (23.8%)	14 (66.7%)
Fluconazole	4 (19.1%)	2 (9.5%)	15 (71.4%)
Ketoconazole	11 (52.4%)	2 (9.5%)	8 (38.1%)

Table 3: Response of *C. albicans* isolates to different concentrations of antifungal drugs

Candida Isolates	CLSI breakpoints (µg/mL)							
	Amphotericin B			Fluconazole			Clotrimazole	
	S (≤ 4)	SDD (8–16)	R (≥16)	S (≤8)	SDD (16-32)	R (≥64)	S (≤1)	R (>1)
Candida albicans	16 (76.2%)	3 (14.3%)	2 (9.5%)	17 (81.0%)	1 (4.7%)	3 (14.3%)	16 (76.2%)	5 (21.8%)

Table 4: Sessile MICs of different *C. albicans* isolates against three different antifungals

C. albicans isolate	**Sample**	**Amphotericin B**		**Fluconazole**		**Clotrimazole**	
		SMIC50	SMIC80	SMIC50	SMIC80	SMIC50	SMIC80
1	Urine	1	2	512	1024	4	8
2		2	4	>1024	>1024	2	4
3		1	1	>1024	>1024	1	2
4		<0.12	<0.12	>1024	>1024	0.5	0.5
5		0.5	1	>1024	>1024	>16	>16
6		0.5	1	1024	>1024	2	4
7		>16	>16	>1024	>1024	2	4
8		0.5	1	128	256	2	4
9		2	4	32	64	0.12	0.25

10	Sputum	0.25	0.5	1024	1024	1	2
11		<0.12	<0.12	>1024	>1024	>16	>16
12		0.5	1	>1024	>1024	>16	>16
13		0.5	1	>1024	>1024	4	8
14		0.25	0.5	>1024	>1024	>16	>16
15		0.5	1	>1024	>1024	2	4
16		1	2	>1024	>1024	>16	>16
17	HVS	<0.12	<0.12	512	1024	1	2
18		0.5	1	>1024	>1024	0.25	0.5
19		0.5	1	256	512	2	4
20		1	2	>1024	>1024	2	4
21	Catheter tip	1	2	>1024	>1024	>16	>16

DISCUSSION

Candida species remain to be a predominant cause of fungal infection ranking higher among the yeasts. This study reported *C. albicans* as a significant fungal pathogen accounting for 36.2% among total yeasts isolates which was inconsistent with the previous studies (Kandel et al 2017; Khadka et al 2017; Subramanya et al 2017). This relative lower figure can be attributed to different factors such as demographics of the patients, their immune status and exposure to pathogen as well as site of infection (Gnat et al 2019). The changing epidemiology of candidiasis and an increasing trend of non-albicans *Candida* over the last three decades certainly must be taken into account and health personnel must be aware of the importance and implication of the non-albicans *Candida* while diagnosing and especially during the selection of the antifungal drugs although no significant differences have been found regarding their susceptibility (Sobel et al 2011; Chow et al 2008).

In this study majority of *Candida* species were isolated from urine of which 31.0% were *C. albicans*. On the other hand, the prevalence of *C. albicans* was seen higher in high vaginal swab as 57.1% of the *Candida* species were *Candida albicans*-an indication that the vaginal candidiasis by *C. albicans* was common. *Candida* species can be isolated from oral samples like sputum or throat swab in both commensal state and in cases of the oral candidiasis (Raju and Rajjapa 2011). In this study one fourth of the *Candida* species (25.9%) were isolated form sputum samples among which *Candida albicans* accounted for 46.7%. There exists a wide variation in the prevalence of *C. albicans* in sputum in different researches ranging from 24% to 70% (Khadka et al 2017, Jha et al 2006). This indicated that species are more likely to be isolated from sputum samples either it be as commensal or as pathogenic which highlights the importance of clinical symptoms represented as thrush or oral candidiasis. None of the isolates were obtained from blood and CSF probably because these are normally sterile

body fluids and candidemia and *Candida* meningitis is not commonly reported and carry grave prognosis (Reef and Mayer 1995).

The qualitative method of biofilm formation among *C. albicans* showed that 71.4% of *C. albicans* isolates were biofilm producers while remaining 28.6 % were either weak biofilm producers or non-biofilm producers. Similar study performed in India reported 61% biofilm producers among a total of 26 *C. albicans* isolates (Sida et al 2016). The biofilm-producing *C. albicans* isolates were further identified by microtiter plate method using crystal violet assay and reported that 85.7% of *C. albicans* isolates were biofilm producers. A comparable study done in Nepal documented 75% of *C. albicans* isolates were biofilm positive (Subramanya et al 2017). A high degree of biofilm forming ability represents the potential of the pathogen to cause the invasive disease (Hasan et al 2009).

In this study XTT results were interpreted directly under the absorbance value as the ability to form the biofilms. The average OD value was 0.577 with highest and lowest value 1.127 and 0.090 respectively. The range of optical density value in similar study was observed in between 1.0 and 1.5 which indicated a higher biofilm forming capacity of the test isolates (Pierce et al 2010). Biofilm quantification as determined by crystal violet and XTT assay correlated well with each other. This correlation can be because of the increased number of living and dividing cells than metabolically inactive ECM as the biofilm in early phase was used for the study. The obvious difference in absorbance value can be attributed to difference in the enzymatic action, dye intensity and also because the experiment was carried in different days under different laboratory environments for two different methods. Based on the strength and weakness of both the tested methods along with the correlation date, it is reasonable to support the use of both methods for biofilm quantification and alongside it is safe to suggest that neither of the methods be used alone for the biofilm quantification.Together and in parallel these two methods are good indicators of biofilm production by clinical isolates.

Antifungal susceptibility profile revealed that 76.2% of *C. albicans* were susceptible to Amphotericin while the highest level of resistance was observed with Ketoconazole (52.4%). This finding is similar to the previous study done in Nepal (Khadka et al 2017). Most *C. albicans* (80.9%) were susceptible to Fluconazole with an MIC of ≤8 µg/mL while 14.29% were resistant (≥64 µg/mL). Similarly, majority of the *C. albicans* isolates were susceptible to Clotrimazole; only 9.525% of *C. albicans* isolates had MIC ≥16 µg/mL. The susceptibility pattern obtained in this study against azole antifungals are in agreement with a previous study where higher susceptibility rates were observed against azole antifungal drugs (Rathod et al 2012). However, some investigations reported higher resistance rate of Fluconazole and Clotrimazole and other azole drugs (El houssaini et al 2018; Zhang et al 2015). The development of resistance against azole antifungals can be due to the alteration of the lanosterol 14 α demethylase target enzyme because of either overexpression or mutation in the ERG11 gene encoding the enzyme (Henry et al 2000). Another reason can be because of the overexpression of efflux pumps mediated by the activation of expression of ATP binding cassette or major facilitator superfamily transporters (Paul et al 2014). In addition, Majority of the *C. albicans* isolates were susceptible to Amphotericin B with 76.19 % of *C. albicans* having an MIC of ≤1 µg/mL while the rest 23.81% had an MIC > 1 µg/mL. Amphotericin B is still widely used drug of choice for most fatal and disseminated fungal infection; however, its high cost makes it difficult to afford by majority of the patients especially in the developing countries.

Susceptibility of clinical *C. albicans* biofilms to Amphotericin B is often reported to be higher than azoles antifungal agents. Azole antifungal agents are often known to have decreased susceptibility against *Candida* biofilms (Tobudic et al 2012). Sessile MIC value for Fluconazole was found to be 2 to 4 times higher than the maximum concentration used for the plankton susceptibility testing. However, 8-fold increase in sessile MIC for Clotrimazole was observed. The finding in this study is in line with the literatures as higher MICs were seen in biofilm-associated cells as compared to planktonic cells (Pierce et al 2008; Shuford et al 2007). Obtaining the standard MIC results from clinical microbiology laboratory might be insufficient when it comes to initiate appropriate dosing level to completely eradicate an infection.

CONCLUSION

Candida albicans is still the most prevalent fungal pathogenic yeast but the prevalence rate was found to be less than the expected range. Higher proportion of non-albicans *Candida* was found among the clinical isolates. It was apparent form the study that species has higher prevalence in urine sample and vaginal candidiasis is common among Nepalese women caused by *C. albicans*. Majority of the *C. albicans* isolates were biofilm-producing drug resistant which remain as a challenge for therapeutic world with decreased susceptibility against common

antifungals. Proper administration of antifungal drugs should be prioritized only with susceptibility result testing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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