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Research Article

In-Vitro Evaluation of Biofilm and Hemolysis activity of *Candida albicans* Isolated from Oral Cavity

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Abstract

Candida albicans is a member of the healthy human microflora, colonizing several niches in the body and can cause opportunistic infection under host debilitated and immunocompromised condition. The present study aimed to investigate the in-vitro hemolytic activity of *C. albicans* isolated from oral cavity and screen biofilm through three different methods. During the study, 200 oral rinse samples from general human population were analyzed in microbiology laboratory of Central Campus of Technology, Tribhuvan University, Hattisar, Dharan. Nepal. *Candida albicans* were isolated and identified by conventional microbiological procedures. The hemolytic activity was evaluated through two different Sabouraud dextrose broth media (SDB) containing 7% defibrinated human blood, one supplemented with 3% glucose (SDBwG) and the other without glucose (SDBwoG). The biofilm formation was screened through congo red agar, tube method and tissue culture plate method. In this present study, 42 (21%) isolates of *Candida albicans* were isolated from 200 oral rinse samples. Isolated *Candida albicans* exhibited mean hemolysis activity of 28.66% on human blood SDB without glucose and 43.55% on human blood SDB with 3% glucose. Tissue culture plate method was considered sensitive, specific and accurate method for quantitative screening of biofilm in comparison to tube method and congo red agar method. This research concluded that *Candida albicans* exhibited greater hemolytic activity in human blood with glucose (SDBwG) than in human blood without glucose (SDBwoG). This finding explains that an increased blood glucose concentration may contribute to increased hemolysis activity of *Candida albicans* that could play pathogenic role for inducing infection like oral candidiasis in debilitated host like diabetic patients. Tissue culture plate method can accurately screen biofilms than tube and congo red agar method.

Keywords: *Candida albicans*; Biofilm; Hemolysis; Tissue culture plate method; Nepal

Introduction

Candida albicans is the commensal fungal pathogen which can cause opportunistic infection when host becomes debilitated and immunocompromised (Spampinato and Leonardi, 2013). Most of the Candida infections affect the skin and mucosal membrane of the host (López-Martinez, 2010). *Candida* species can cause vaginitis, oral candidiasis, cutaneous candidiasis, candidemia, and systemic infections (Wachtler *et al.*, 2012). Changes in the host microbiota, changes in the host immune response and variations in the local environment enable proliferation of *C. albicans* to cause infection (Nobile and Jhonson, 2015).

Common risk factors for Candida infection are recent antibiotic use, pregnancy, diabetes mellitus, oral-contraceptives and inadequate therapy (Grigoriou *et al.*, 2006). Virulence factors, such as adherence, extracellular hydrolase production, hemolysis, phenotypic switching,

and filamentation may all influence the pathogenesis of *Candida* species (Wan *et al.*, 2015). Hemolytic capacity is an important virulence factor that allows fungi of the genus *Candida* to acquire iron from host tissues, which then is used by the fungus for metabolism, growth and host invasion during infection (Almeida *et al.*, 2009). A complement-mediated hemolysis induced by *C. albicans* was first reported by Manns *et al.*, (1994) and Luo *et al.*, (2001). The ability of *C. albicans* to utilize hemoglobin as an iron source was first described by Moors *et al.*, (1992).

In the oral cavity, extracellular iron is bound mainly to lactoferrin, a protein present in saliva, while intracellular iron is stored as ferritin. Although this element is bound to proteins and/or is present in the cytoplasm of cells, oral infections with *C. albicans* are frequent, suggesting that this yeast is able to take up different forms of iron from the oral cavity (Almeida *et al.*, 2008). Biofilm formation is one of the major virulence characteristic of *Candida albicans* which are resistant to antifungal therapeutics, the host immune system, and other environmental factors, creating clinical challenges (Gulati and Nobile, 2016). Therefore, the present study aimed to investigate the in-vitro hemolytic activity of *Candida albicans* isolated from oral cavity and screen biofilm formation through three different biofilm screening methods.

Materials and Methods

Study Design

This study was a cross sectional lab-based study conducted in Dharan sub-metropolitan city from June 2018 to January 2019 after receiving ethical approval from Nepal Health Research Council, Kathmandu, Nepal. During the study, 200 oral rinse samples from general human population were analyzed in microbiology laboratory of Central Campus of Technology, Tribhuvan University, Hattisar, Dharan. Informed consent was obtained from the participants before carrying out the study.

Sample Collection

The participants were provided 10 mL of sterile saline and asked for 1-minute oral rinse and then the oral rinse sample was inoculated in a broader capped sterile container. The oral rinse samples were transported to microbiology laboratory maintained in ice box. The oral rinse samples were labeled with participant's identification number and processed within 2 hours of collection.

Isolation and Identification

Isolation of Candida from the oral cavity was performed as described by Samaranayake *et al.*, (1986). About 50µl of oral rinse sample was inoculated in Sabouraud dextrose agar (HiMedia, India) with chloramphenicol (0.05gm/l) and incubated at 37°C for 3-4 days. *Candida albicans* were identified through colony characteristics, simple staining, germ tube and Chlamydospore formation as described by Beheshti *et al.*, (1975). For germ tube test the pure isolated colony of *Candida albicans* was dispensed in 0.5mL of serum and incubated at 37°C for 2 hours. After incubation an aliquot was taken in a clean slide and was observed under oil immersion for the formation of germ tube. In addition, Chlamydospore formation test was performed in which the pure isolated and suspected colony was placed in corn meal agar (HiMedia, India) with coverslip over the inoculum and incubated at 25°C for 3-4 days. Culture was taken on a clean slide and observed under microscope for formation of Chlamydospore. Candida that could form germ tube in serum and Chlamydospore in corn meal agar was identified as *Candida albicans*.

Hemolysis Activity

The hemolysis activity was evaluated as described by Malcok *et al.* (2009) through two different Sabouraud dextrose broth media (SDB) (HiMedia, India) containing 7% defibrinated human blood, one supplemented with 3% glucose (SDBwG) and the other without glucose (SDBwoG). McFarland 2 turbidity standard yeast suspension was prepared in Sabouraud dextrose broth (HiMedia, India). About 10µL of this suspension was inoculated into 2mL of SDBwG and SDBwoG media separately. Then the tubes were incubated at 37°C in 5% CO_2 for 48 hours. By the end of incubation, tubes were centrifuged at 1800 rpm at 4°C for 10 minutes in order to separate non-hemolyzed erythrocytes. The released hemoglobin in the supernatants was then quantified by spectroscopic analysis using the Cyanmethemoglobin. For quantification about 10 µl of supernatants were transferred to sterile polyethylene tubes, and 5 mL of Drabkin's reagent (KCN, $K_3Fe(CN)_6$, $NaHCO_3$, KH_2PO_4, TritonX-100) were added. The absorbance of the mixture was measured at 540 nm. Standard hemostat suspension was prepared in order to compare the hemoglobin content of this standard with those of the test samples. About 100 µL of defibrinated human blood was mixed with 1430 µl cold water (final dilution of blood is 7%), and incubated at 4°C for 30 minutes in order to lyse the erythrocytes. The hemoglobin content of this standard was then determined by same procedure. The degree of the hemolysis (percentage value) was calculated according to the formula below: (Absorbance of test tubes / absorbance of standard hemostat) × 100.

Biofilm Assay

Congo red Agar Method (CRA):

The *Candida albicans* culture was streaked on surface of Congo Red Agar (HiMedia, India) additionally supplemented with 1% glucose and incubated at 37ºC for 24-48 hours (Freeman *et al.*, 1989). Black- or purple-colored colonies with dry crystalline consistency was interpreted as positive biofilm producing strains. Red colored colonies were interpreted as negative for biofilm production. This experiment was repeated for three times.

Tube method:

A qualitative assessment of biofilm formation was done as described by Christensen *et al.* (1985). The 10 mL Tryptic Soy Broth (TSB) (HiMedia, India) supplemented additionally with 1% glucose was inoculated with a loop full of *Candida albicans* from overnight culture plates and incubated for 24 hours at 37°C. The tubes was decanted and washed with Phosphate Buffer Saline (HiMedia, India) (pH-7.2) and dried. Then the tubes were stained by 0.1% crystal violet (HiMedia, India). Stain was removed by deionized water. Tubes were then dried in inverted position for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not considered biofilm. This experiment was repeated for three times.

Tissue Culture Plate method:

The quantification of biofilm was performed according to Christensen *et al.*, (1985). In this method, 5mL of overnight culture of *Candida albicans* was prepared in Sabouraud dextrose broth. Then, 100 µl of diluted culture was inoculated in a sterile 96-well polystyrene tissue culture plate well containing tryptic soy broth (HiMedia, India) additionally supplemented with 1% glucose. The plate was incubated at 37ºC for 24 hours for biofilm formation. The unbound cell was discarded and washes several times with sterile Phosphate Buffer Saline (HiMedia, India) (pH-7.2). About 125µl of 0.1% crystal-violet solution was added and left for 10-15 minutes incubation. The plate was washed and left inverted for dry at 60°C for 30 minutes to fix the biofilms. The quantitative determination was performed by solubilizing the biofilm by adding 125µl of 30% acetic acid (HiMedia, India) to each well and incubated the plate for 15 minutes at room temperature and later it was transferred to another tissue culture plates for reading the absorbance at 570nm by ELISA plate reader (Loncare LR-620 microplate reader, Medical Technology Co., Ltd.). Interpretation was made on optical density (OD) of test wells. The experiment was performed in triplicates. The optical density (OD_s) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (OD_{nc}). The following classification was used for the determination of biofilm formation: no biofilm production ($OD_s \leq OD_{nc}$), weak biofilm production ($OD_{nc} < OD_s \leq 2.OD_{nc}$), moderate biofilm production ($2.OD_{nc} < OD_s \leq 4.OD_{nc}$) and strong biofilm production ($4.OD_{nc} < OD_s$) as described by Stepanovic *et al.*, (2007).

Quality Control

Strain of *Candida albicans* ATCC 24433 was used as a positive control for the study. In this study, quality and accuracy of all test was maintained by following standard procedures of collection, isolation and identification. For quality control, media and reagents were prepared, stored and utilized as recommended by the manufacturing company.

Data Analysis

The data was documented in MS-EXCEL 2010 and was analyzed using statistical Package for Social Sciences (SPSS) version 16.0. The p value of equal or less than 0.05 at 95% confidence interval was used for statistical significance.

Results and Discussion

Hemolysis Percentage by Candida albicans in Two Different Liquid Media

In this study, total of 42 (21%) *Candida albicans* were isolated from 200 oral rinse samples. From 63 positive samples of Candida identified, 42 (66.66%) were identified as *Candida albicans*. The isolated *Candida albicans* exhibited mean hemolysis degree of 28.66% on human blood SDB without glucose and 43.55% on human blood SDB with 3% glucose (**Table 1**).

Comparative Study of Biofilm Assays

The comparative analysis of biofilm formation of isolated *Candida albicans* was analyzed by three methods; tissue culture plate method, tube method and congo red agar method (**Table 2).**

Sensitivity, Specificity and Accuracy of Biofilm Screening Methods

The tissue culture plate method was found to be most accurate and standard method for screening biofilm as compared to tube method and congo red agar method. The parameters like sensitivity, specificity, and negative predictive value, positive predictive value and accuracy were calculated (**Table 3**).

Table 1: Mean hemolysis percentage by *Candida albicans* in two different liquid media.

Microorganism	Human blood SDB without glucose (%)	Human blood SDB with 3% glucose (%)
Candida albicans	28.66	43.55

Table 2: Biofilm formation by *Candida albicans* by three methods

Biofilm formation	Tissue culture plate method	Tube method	Congo Red Agar method	p-value
High	2 (4.76%)	2 (4.76%)	1 (2.38%)	
Moderate	27 (64.28%)	16 (38.09%)	15 (35.71%)	P<0.05
Weak	9 (21.42%)	10 (23.80%)	11 (26.19%)	
None	4 (9.52%)	14 (33.33%)	15 (35.71%)	
Total isolates	42	42	42	

Table 3: Sensitivity, Specificity and accuracy of Biofilm Screening Methods

Biofilm Screening Method	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
Tube method	71.4	62.85	27.77	91.66	64.28
Congo Red Agar method	27.77	16.66	20	23	21.42

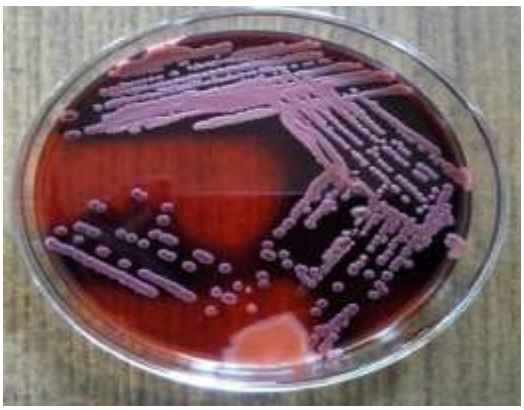

Fig. 1: Biofilm formation in Congo Red Agar plate

Fig. 2: Biofilm formation in Test tube.

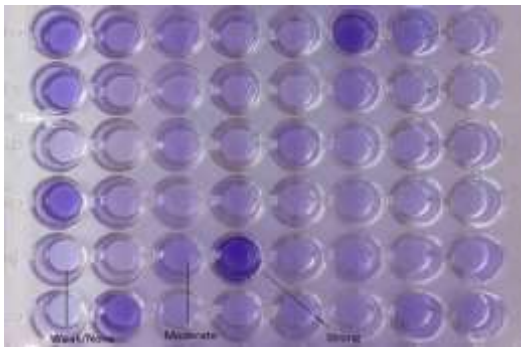

Fig. 3: Biofilm formation in Tissue Culture plate.

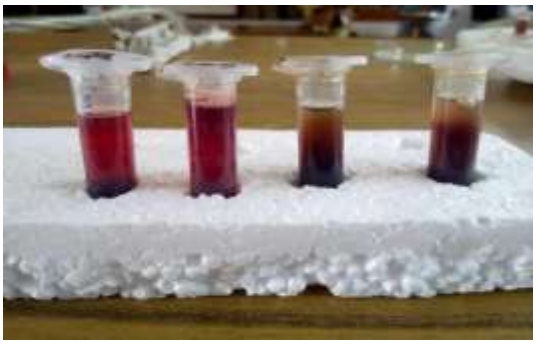

Fig. 4: Hemolysis activity in SDBwG (Right) and SDBwoG (left).

Candida albicans is known to be carried in oral cavity of 50% of world's population as a part of normal flora (Singh *et al.*, 2014). Many risk factors are associated with colonization by Candida. Poor oral hygiene, diabetic conditions, immunosuppressive therapy in cancer disease, diet habit has shown increasing prevalence of Candida species (Dorocka-Bobkowska, 2010).

In this present study, the mean hemolytic activity of *Candida albicans* in human blood with glucose (SDBwG)

was 43.55% and in human blood without glucose (SDBwoG) was 28.66%. This study reported that *Candida albicans* exhibited higher hemolytic activity in glucose enriched environment. Malcok *et al.,* (2009) reported that most species of Candida exhibited hemolytic activity in glucose enriched medium and hence suggested the parallel combination of diabetic condition with pathogenesis of *Candida albicans.* In-vitro comparative analysis of hemolytic degree of *Candida albicans* in presence and absence of glucose level strongly explains that, increased blood sugar level increases the hemolysis activity of *Candida albicans*. This study explains the pathogenic role of *Candida albicans* in glucose enriched condition which might correlate its pathogenicity in Diabetes mellitus inducing oral colonization and oral Candidiasis. Many researchers conducted throughout the world have reported cases of oral candidiasis in diabetic patients, known as oral thrush (Obradovic *et al.,* 2011). In one study, higher prevalence of Candida carriage was reported in oral cavity of diabetic patients when compared with non-diabetic population (Lamichhanae *et al.,* 2015). Pathogenic microorganisms are capable of acquiring iron for survival and establish infection in host that addresses its pathogenicity. Since there is little iron in human body, the most microorganisms derive iron from hemoglobin. So to destroy hemoglobin, they secrete enzyme like Hemolysin (Malcok *et al.,* 2009).

Biofilm of Candida is made up of layers of cells embedded in matrix of extracellular polymeric materials. It is the surface-attached microbial community that contributes virulence factor (Khatri *et al.,* 2015). In this study 1% glucose was additionally supplemented to growth media as additional sugar helps in biofilm formation (Manandhar *et al.,* 2018). In this study the strong, moderate, weak and none biofilm producers screened by Tissue culture plate method were 4.7%, 64.2%, 21.42% and 9.52% respectively. The strong, moderate, weak and none biofilm producers screened by tube method were 4.7%, 38%, 23.80% and 33.33% respectively. The strong, moderate, weak and none biofilm producers screened by congo red agar method were 2.3%, 35.71%, 26.19% and 35.71% respectively. Number of false positive and false negative were reported in the Tube and congo red agar methods. In addition, it was difficult to discriminate strong, moderate and weak biofilm producers in tube method and congo red agar method due to phenotypic variations.

Sensitivity and specificity of tube method was found to be 71.4% and 62.8% respectively with accuracy of 64.2%. For congo red agar method the sensitivity and specificity was found to be 27.7% and 16.6% with accuracy of 21.4%. The screening analysis was similar even in present study which supports different other similar findings performed before which strongly explains that tube method is least sensitive for screening biofilms (Mathur *et al.,* 2018). Hassan *et al.,* (2011) concluded tissue culture plate method as gold standard technique for screening biofilm as compared to tube method and congo red agar method. Even in this study, the tissue culture plate method was considered sensitive, specific and accurate method for quantitative screening of biofilm in comparison to tube method and congo red agar method.

Conclusion

This research concludes that *Candida albicans* exhibited greater hemolytic activity in human blood with glucose (SDBwG) than in human blood without glucose (SDBwoG) media. This finding suggests that an increased blood glucose concentration may contribute to increased hemolysis activity of *Candida albicans* that could play pathogenic role in inducing infection like oral candidiasis in debilitated host like diabetic patients. However, further studies need to be conducted in order to study host-parasite relationship and pathogenicity. *Candida albicans* can produce biofilm which is known to play significant role in pathogenicity and tissue culture plate method was found to be standard method for screening biofilm.

Ethical Approval

This study was carried out after receiving ethical approval from Nepal Health Research Council, Kathmandu, Nepal. Approval was also obtained from Department of Microbiology of Central Campus of Technology, Hattisar, Nepal. Informed consent was obtained from the participants before carrying out the research.

Authors' Contribution

Bijay Kumar Shrestha designed the concept, performed laboratory work, analyzed and interpreted data, critically revised the manuscript for intellectual contents and drafted the manuscript. Jenish Shakya participated in laboratory work, quality control setting, data analysis and manuscript drafting. Both the authors contributed for final approval of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

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