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

Bijay Kumar Shrestha1*, Jenish Shakya1

1Department of Microbiology, Central Campus of Technology, Tribhuvan University, Hattisar, Dharan, Nepal

**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-Martínez, 2010). *Candida* species can cause vaginitis, oral candidiasis, cutaneous candidiasis, candidemia, and systemic infections (Wachtler et al., 2012). Changes in the host microflora, changes in the host immune response and variations in the local environment enable proliferation of *C. albicans* to cause infection (Nobile and Johnson, 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, host microbiota, changes in the host immune response and variations in the local environment enable proliferation of *C. albicans* to cause infection (Nobile and Johnson, 2015).
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 characteristics 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 Samaranyake 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. Congo Red Agar (HiMedia, India) was added. Then the tubes were incubated at 37°C for 24 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 spectrophotometric analysis using the Cyanemthemoglobin. For quantification about 10 µl of supernatants were transferred to sterile polyethylene tubes, and 5 µL of Drabkin’s reagent (KCN, K,Fe(CN),, NaHCO,, KHPO,, 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 37°C 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 were 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 37°C for 24 hours for biofilm formation. The unbound cell was discarded and washed 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 (ODx) 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 (ODnc). The following classification was used for the determination of biofilm formation: no biofilm production (ODx≤ODnc), weak biofilm production (ODnc<ODx≤2.ODnc), moderate biofilm production (2.ODnc<ODx≤4.ODnc) and strong biofilm production (4.ODnc<ODx) 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        |

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 (Lamichhanee 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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**References**

Almeida R S, Wilson D and Hube B (2009) *Candida albicans* iron acquisition within the host. FEMS yeast research 9(7): 1000-1012. DOI: 10.1111/j.1567-1364.2009.00570.x

Almeida RS, Brunke S, Albrecht A, Thewes S, Laue M, Edwards JE, Filler SG and Hube B (2008) The hyphal-associated
adhesin and invasin Als3 of Candida albicans mediates iron acquisition from host ferritin. *PLoS Pathog* **4**(11): e1000217. DOI: 10.1371/journal.ppat.1000217

Beheshti F, AG Smith and GW Krause (1975) Germ tube and chlamydospore formation by *Candida albicans* on a new medium. *Journal of clinical microbiology* **2**(4): 345-348

Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM and Beachey EH (1985) Adherence of coagulase-negative Staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* **22**(6): 996-1006.

Dorocka-Bobkowska B, Zozulinska-Ziolkiewicz D, Wierusz-Wysocka B, Hedzekel W, Szumala-Kakol A and Budtz-Jorgensen E (2010) Candida-associated denture stomatitis in type 2 diabetes mellitus. Diabetes research and clinical practice **90**(1): 81-86. DOI: 10.1016/j.diabres.2010.06.015

Freeman DJ, Falkiner FR, Keane CT (1989) New method for detecting slime production by coagulase negative staphylococci. *Journal of clinical pathology* **42**(8): 872-874. DOI: http://dx.doi.org/10.1136/jcp.42.8.872

Grigoriou O, Baka S, Makrakis E, Hassiakos D, Kapparos G, Kouskouni E (2006) Prevalence of clinical vaginal candidiasis in a university hospital and possible risk factor. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **126**(1): 121-125. DOI: 10.1016/j.ejogrb.2005.09.015

Gulati M and Nobile CJ (2016) *Candida albicans* biofilms: development, regulation, and molecular mechanisms. *Microbes and infection* **18**(5): 310–321. DOI: 10.1016/j.micinf.2016.01.002

Hassan A, Usman J, Kaleem F, Omai M, Khalid A and Iqbal M (2011) Evaluation of different detection methods of biofilm formation in the clinical isolates. *Brazilian journal of infectious Diseases* **15**(4): 305-311. DOI: http://dx.doi.org/10.1590/S1413-86702011000400002

Khatri S, Sumana MN, Mahale RP and Kishore A (2015) Analysing three different screening methods for biofilm formation in clinical isolates of *Candida*. *Journal of Evolution of Medical and Dental Sciences* **4**(83): 14515-14524. DOI: 10.14260/jemsds/2015/2065

Lamichhane RS, Boaz K, Natarajan S and Shrestha M (2015) Assessment of Candidal carriage in patients with Type II Diabetes Mellitus. *Journal of Pathology of Nepal* **5**(9): 733-738. DOI: 10.3126/jpn.v5i9.13784

Lopez-Martinez R (2010) Candidiosis, a new challenge. *Clinics in Dermatology* **28**(2): 178–184. DOI: 10.1016/j.clindermatol.2009.12.014

Luo G, Samaranayake LP and Yau JYY (2001) Candida species exhibit differential in vitro hemolytic activities. *J Clin Microbiol* **39**(8): 2971-4. DOI: 10.1128/JCM.39.8.2971-2974.2001

Malcok HK, Aktas E, Ayyildiz A, Yigit N and Yazgi H (2009) Hemolytic activities of the Candida species in liquid medium. *The Eurasian Journal of Medicine* **41**(2): 95-98. PMID: 25610076; PMCID: PMC4299835.

Manandhar S, Singh A, Varma A, Pandey S and Shrivastava N (2018) Evaluation of methods to detect in vitro biofilm formation by staphylococcal clinical isolates. *BMC research notes*. **11**(1):714. DOI: 10.1186/s13104-018-3820-9

Manns MJ, Mosser MD and Buckley RH (1994) Production of a hemolytic factor by *Candida albicans*. *Infect Immun* **62**(11): 5154–6.

Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T and Rattan A (2006) Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. *Indian J Med Microbiol* **24**(1): 25-9. DOI: 10.4103/0255-0857.19890

Moors MA, Stull TL, Blank KJ, Buckley HR and Mosser DM (1992) A role for complement receptor-like molecules in iron acquisition by *Candida albicans*. *J Exp Med* **175**(6): 1643-51. DOI: 10.1084/jem.175.6.1643

Nobile CJ and Johnson AD (2015) *Candida albicans* Biofilms and Human Disease. *Annu Rev Microbiol* **69**: 71-92. DOI: 10.1146/annurev-micro-091014-104330

Obradovic RR, LG Kecic, AN Pejicic, MS Petrovic, ND Zivkovic and DM zivkovic (2011) Diabetes mellitus and oral candidiasis. Acta Stomatologica Naissi **27**(63): 1025-1034. DOI: 10.5937/asn1163025O

Samaranayake LP, MacFarlane TW, Lamey P-J and Ferguson MM (1986) A comparison of oral rinse and imprint sampling techniques for the detection of yeast, coliform and *Staphylococcus aureus* carriage in the oral cavity. *Journal of Oral Pathology* **15**(7): 386–388. DOI: 10.1111/j.1600-0714.1986.tb00646.x

Singh A, Verma R, Murari A and Agrawal A (2014) Oral candidiasis: An overview. *Journal of oral and maxillofacial pathology: JOMFP* **18**(Suppl 1), S81–S85. 10.4103/0973-029X.141325

Spampinato C and Leonardi D (2013) Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. *BioMed Research International* **1–13**. DOI: 10.1155/2013/204237

Stepanovic S, D Vukovic V, Hola GD, Bonaventura S, Djukic I, Cirkovic and F. Ruzicka (2007) Quantification of biofilm formation in clinical isolates of *Candida albicans*. *BMC Microbiol* **7**: 51-5. DOI: 10.1186/1471-2180-7-5

Wächtler B, Cititulo F, Jablonowski N, Forster S, Dalle F, Schaller M, Wilson D and Hube B (2012) *Candida albicans*-epithelial interactions: dissecting the roles of active penetration, induced endocytosis and host factors on the infection process. *PLoS one* **7**(5): e36952.

Wan L, Luo G, Lu H, Xuan D, Cao H, Zhang J (2015) Changes in the hemolytic activity of Candida species by common electrotyes. *BMC Microbiol* **15**(1): 171. DOI: 10.1186/s12866-015-0504-7