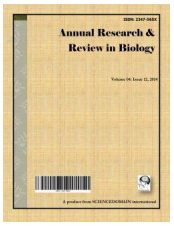

Annual Research & Review in Biology

24(3): 1-8, 2018; Article no.ARRB.39525
ISSN: 2347-565X, NLM ID: 101632869

Viability Kinetic Profile, Morphological Structure, and Physicochemical Characterization of *Candida albicans* Biofilm on Latex Silicone Surfaces

Derick Erl P. Sumalapao[1,2,3*], Esperanza C. Cabrera[2], Mary Jane C. Flores[2,3], Divina M. Amalin[2,3], Nelson R. Villarante[4], Marohren T. Altura[1] and Nina G. Gloriani[1]

[1]Department of Medical Microbiology, College of Public Health, University of the Philippines Manila, Manila, Philippines.
[2]Department of Biology, College of Science, De La Salle University, Manila, Philippines.
[3]Biological Control Research Unit, Center for Natural Sciences and Environmental Research, De La Salle University, Manila, Philippines.
[4]Department of Physical Sciences and Mathematics, College of Arts and Sciences, University of the Philippines Manila, Manila, Philippines.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/39525
Editor(s):
(1) Layla Omran Elmajdoub, Department of Zoology, Faculty of Science, Misurata University, Misurata, Libya.
(2) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Jun Sawai, Kanagawa Institute of Technology, Japan.
(2) S. Thenmozhi, Periyar University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/23306

Original Research Article

Received 20th January 2018
Accepted 18th February 2018
Published 23rd February 2018

ABSTRACT

Biofilm formed by *Candida albicans* on latex silicone surfaces was characterized by instrumental techniques such as fluorescence microscopy, scanning electron microscopy, and Fourier transform infrared spectroscopy. The growth and viability of *C. albicans* on the biofilm formed were described using different kinetic rate equations. *C. albicans* biofilm has a complex and heterogenous structure with hyphal elements and yeast cells entrenched within a polysaccharide matrix. Spectroscopic studies revealed specific stretching frequencies of O-H, C-O, and C=O which can be attributed to

**Corresponding author: E-mail: derick.sumalapao@dlsu.edu.ph;*

the presence of some functionalities in the biofilm formed by *C. albicans*. Viability of *C. albicans* behaved in accordance with the first-order kinetic equation on the first 48 h, then shifted to a second-order kinetic equation until the 72 h, and had a doubling time of 70 h. Information on model biofilms with emphasis on growth rates and morphogenesis, structural organization, and physicochemical characteristics can possibly explain resistance to some antifungal treatments and subsequent synthesis of newer generation drugs for fungal biofilm-related infections.

Keywords: Biofilm; Candida albicans; scanning electron microscopy; fluorescence microscopy; Fourier transform infrared spectroscopy; kinetic equations; latex silicone polymer.

1. INTRODUCTION

Adherence of microbial organisms to a surface results in the production of extracellular polymers [1] providing a structural matrix which favors biofilm formation [2]. Some of the early documented researches involving biofilms were in dairy processing lines [3] and ancient architectural structures [4]. These biofilms have a heterogenous structure with diverse genetics [1], and are involved in the pathogenesis of diseases [5] and persistence of infections [6]. When compared with freely suspended organisms, microbes involved in biofilm formation were relatively refractory to pharmacologic interventions [2,7,8]. There were extensive studies and even literature reviews done involving bacterial biofilms [9-11], but with less attention on medically relevant fungal biofilms.

Candidiasis has been increasing over the years and is associated with medical implant devices [12-17] including catheters, artificial joints, central nervous system shunts, dental implants, ocular lenses, vascular bypass grafts, and heart valves. Comprehensive investigation on bacterial biofilm formation involving these medical devices had been done [13], but less emphasis on fungal biofilm formation.

In this study, the viability kinetic profile, morphological structure, and physicochemical characteristics of biofilm formed by *Candida albicans* on latex silicone surfaces were described using standard plating method, scanning electron microscopy, fluorescence microscopy, and Fourier transform infrared spectroscopy. Given the highly complex nature of biofilm formation including specific extracellular material production, information obtained from this study will further our understanding of the biology of *C. albicans* biofilm with emphasis on biofilm growth rates, structural organization, and physicochemical characterization as these can possibly explain resistance of some antifungal treatments and subsequent synthesis of newer generation drugs designed for fungal biofilm-related infections.

2. MATERIALS AND METHODS

2.1 Organism, Culture Conditions, and Inoculum

Candida albicans isolate, obtained from a clinical specimen of a hospitalized patient, was kindly provided by the Department of Medical Microbiology, College of Public Health, University of the Philippines Manila. The identity of *C. albicans* was established using api20C-AUX system, germ tube formation, and urea and nitrate assimilation tests. In this study, *C. albicans* was grown and subcultured at 37°C on Sabouraud dextrose agar (SDA) for 24 h. For the inoculum preparation, three colonies were placed in 5 ml sterile distilled water warmed to 37°C [18] with the final inoculum adjusted to 5.61 (± 0.06) $\log_{10}$ CFU/ml using serial dilution with viable fungal counts (5.54 to 5.66 $\log_{10}$ CFU/ml) confirmed on SDA plates.

2.2 Biofilm Formation

A modified method [19,20] for growing biofilms was employed. Discs with surface area of 0.5 cm^2 (0.5 cm by 1.0 cm) from latex silicone urinary catheter material (Fr18, Surgitech+®, Fujian Bestway Medical Polymer Corp) were individually placed in glass culture plates (Pyrex®), subsequently autoclaved and oven-dried.

For the biofilm formation, 100 µl of the standardized cell suspensions of the final inoculum were applied to the surface of each disc, supplemented with 1 ml 50 mM glucose, and then incubated for up to 72 h at 37°C. Discs without cells, in the control setup, were incubated in medium containing 1 ml 50 mM glucose. Both the biofilm formation and control experiments were performed in triplicates.

2.3 Biofilm Characterization

The biofilm formed were described based on morphological structure, physicochemical characteristics, and viability profile of *C. albicans* on latex silicone surfaces.

2.3.1 Fluorescence microscopy

Assessment on the morphological structure of the biofilm formed using fluorescence microscopy was performed by the Molecular Science Unit Laboratory, De La Salle University, Manila, Philippines. After removal from the medium, the latex silicone discs were subsequently gently washed with 5 ml 0.15 M phosphate buffer solution to remove nonbiofilm cells [19,21]. The latex silicone discs with biofilms were stained for 1 min with 2 ml Sypro® Ruby (Invitrogen, USA), transferred to microscope slides, and subsequently examined under a fluorescence microscope (Applied Spectral Imaging, Yokneam, Israel) which fluoresced in the UV range (λ_{max}= 450 nm).

2.3.2 Scanning Electron Microscopy

After removal from the medium, the latex silicone discs were subsequently gently washed with 5 ml 0.15 M phosphate buffer solution to remove nonbiofilm cells [19,21]. The samples were sent to the Chemistry Instrumentation Room II, De La Salle University, Manila, Philippines for freeze-drying. Dried biofilm specimens were subsequently sent to Surface Morphology Laboratory, De La Salle University, Manila, Philippines for scanning electron microscopy analysis. The general surface morphology of the biofilm and the changes in the porosity of the surfaces were detected.

2.3.3 Fourier transform infrared (FTIR) spectroscopy

Dried biofilm specimens were subsequently analyzed for spectral imaging in the Chemistry Instrumentation Room II, De La Salle University, Manila, Philippines. The FTIR spectra were recorded at room temperature in the range of 4000–500 cm^{-1} on a ThermoScientific Nicolet 6700 FT-IR spectrometer using a KBr pellet sampling method.

2.3.4 Viability Kinetic Profile

Viable population of *C. albicans* in the biofilm formed on catheter surfaces was monitored serially (0, 4, 8, 12, 24, 36, 48, 60, and 72 h). After removal from the medium, the latex silicone discs were subsequently gently washed with 5 ml 0.15 M phosphate buffer solution to remove nonbiofilm cells [19,21]. Using a sterile scalpel, biofilm organisms were scraped from the discs to remove any remaining cells. For viable fungal colony counts, the samples were plated on SDA and incubated for 24 h at 37°C. Each test was done in triplicate with results expressed as means CFU/ml.

2.4 Statistical and Numerical Analyses

All experiment setups were performed in triplicates in monitoring the viability kinetic profile and in describing the morphological structures of the biofilm formation. Quantitative measures were subjected to analysis of variance and Bonferroni test. Nonlinear curves were generated to describe the relationship between fungal population count and time. Several kinetic equations [22,23] were employed in elucidating the rate mechanisms behind biofilm formation (Table 1). All statistical and numerical analyses were performed using Microsoft Excel© and STATA© V12.0 software at 5% level of significance.

Table 1. Nonlinear and linearized forms of the biofilm growth models

Kinetic model	Nonlinear model	Linearized form
Zero-order	$\frac{dP_t}{dt} = k_o$	$P_t = k_0 t + P_o$
First-order	$\frac{dP_t}{dt} = k_1 P_t$	$\log P_t = \log P_o + \frac{k_1}{2.303} t$
Second-order	$\frac{dP_t}{dt} = k_2 {P_t}^2$	$\frac{-1}{P_t} = k_2 t + \left(\frac{-1}{P_0}\right)$

P_t (CFU/ml): population at time t (h); k_o (CFU/ml/h): zero-order rate constant;
k_1 (CFU/ml/h): first-order rate constant; k_2 (ml/CFU/h): second-order rate constant

3. RESULTS AND DISCUSSION

Viable counts of *C. albicans* in the biofilm formed on the surface of latex silicone urinary catheter when monitored over the 72-h period exhibited an increasing nonlinear trend over time (Fig. 1). The viable population counts did not significantly differ over the first 36-h monitoring period (P>0.05). There is a significant increase in the viable counts identified starting on the 36 h up to the 72 h, and these significantly differed from the baseline value (P<0.05). However, these viability counts on 36 h to 72 h did not significantly differ (P>0.05).

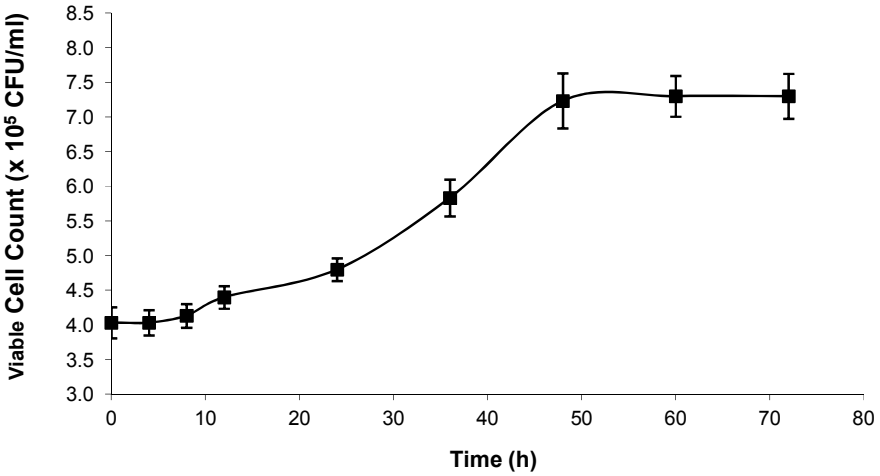

Fig. 1. Viability kinetic profile of *C. albicans* grown on latex silicone discs with plate counts reflecting measurements (means ± standard error) of *C. albicans* population in the biofilms

Kinetic profile on the viability of *C. albicans* on the biofilm showed that when population *P* was plotted against time *t*, the relationship reflects a zero-order rate mechanism as justified by the value of the coefficient of determination close to unity (Table 2). However, examination of the linear plots of log *P* against *t* describing the first-order kinetic process revealed better parameter estimates, while the linear plots of *-1/P* against *t* yielded parameter estimates for the second-order kinetic model. Regardless of the kinetic equation describing the viability of *C. albicans* in the biofilm, it appeared that the doubling time of the organism is approximately 70 h.

Representative microscopic images of the biofilm formed on latex silicone surfaces at magnifications of x10 and x20 were obtained (Fig. 2). The thickness of the mature biofilm appears varied over the different areas in the surface of the catheter. Individual yeast cells appeared discernible with the less dense biofilm matrix. Moreover, imaging of the biofilm on the latex silicone surface of a urinary catheter by scanning electron microscopy reflects a heterogenous structure, a network of extracellular matrix strands, characterized by the presence of hyphal forms and yeast cells (Fig. 3). These *C. albicans* hyphal elements were implicated in fungal infections [24-26].

The Fourier transform infrared spectroscopic profile of the biofilm formed by *C. albicans* can be observed from 800-1100 cm^{-1} infrared regions (Fig. 4). The prominent peaks identified in the spectral images at 1655 cm^{-1} and 1038-1081 cm^{-1} are due to C=O and C-O stretching, respectively which can be attributed to the presence of β-glucan and mannan moieties. Moreover, the broad –OH stretch of the sugar was distinctively observed at 3396 cm^{-1}. These peaks clearly indicate the development of biofilm generated from *C. albicans*.

Table 2. Parameter estimates and error analysis of the biofilm growth models

Kinetic model	Parameter estimates	
Zero-order	k_0	0.0549
	P_o	3.8412
	R^2	0.9402
	SSE	1.0424
	p	1.55e-5
First-order	k_1	0.0099
	P_o	3.9486
	R^2	0.9435
	SSE	0.0060
	p	1.27e-5
Second-order	k_2	0.0018
	P_o	4.0140
	R^2	0.9404
	SSE	0.0012
	p	1.54e-5

k_o (CFU/ml/h): zero-order rate constant; k_1 (CFU/ml/h): first-order rate constant; k_2 (ml/CFU/h): second-order rate constant; R^2: coefficient of determination; SSE: sum of squares of the error; p: p-value

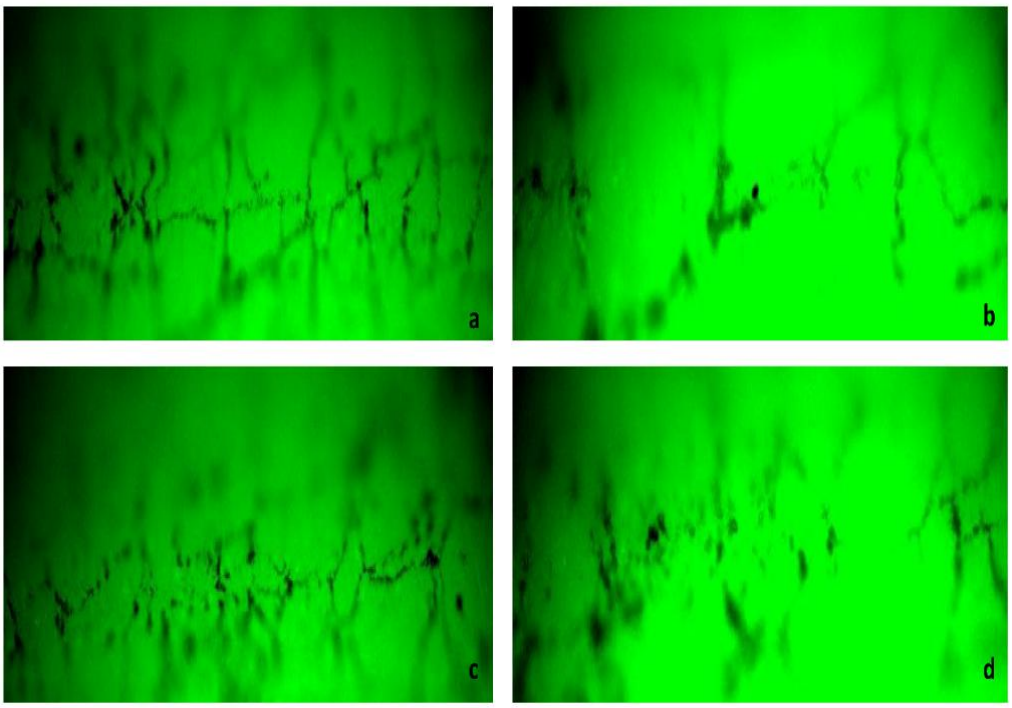

Fig. 2. Fluorescence microscopic images of *C. albicans* biofilm grown on latex silicone discs stained 72 h of development. Magnification: x10 (a,c), x20 (b,d)

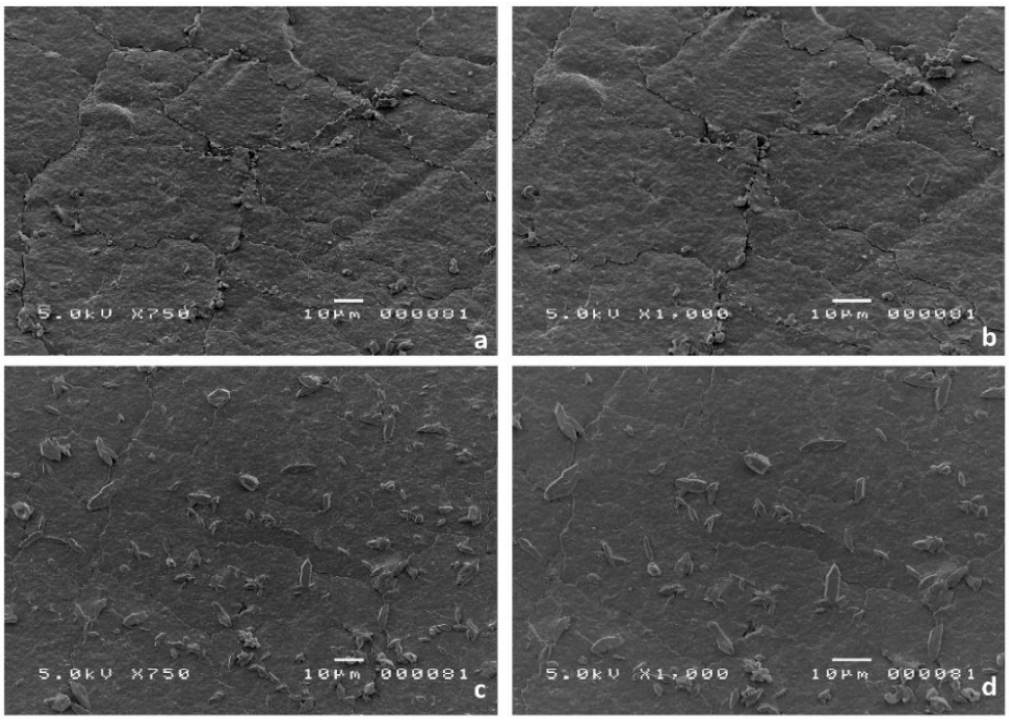

Fig. 3. Scanning electron micrographs of latex silicone surfaces (a,b) and *C. albicans* biofilm on latex silicone surfaces in medium containing 50 mM glucose after 72 h development (c,d)

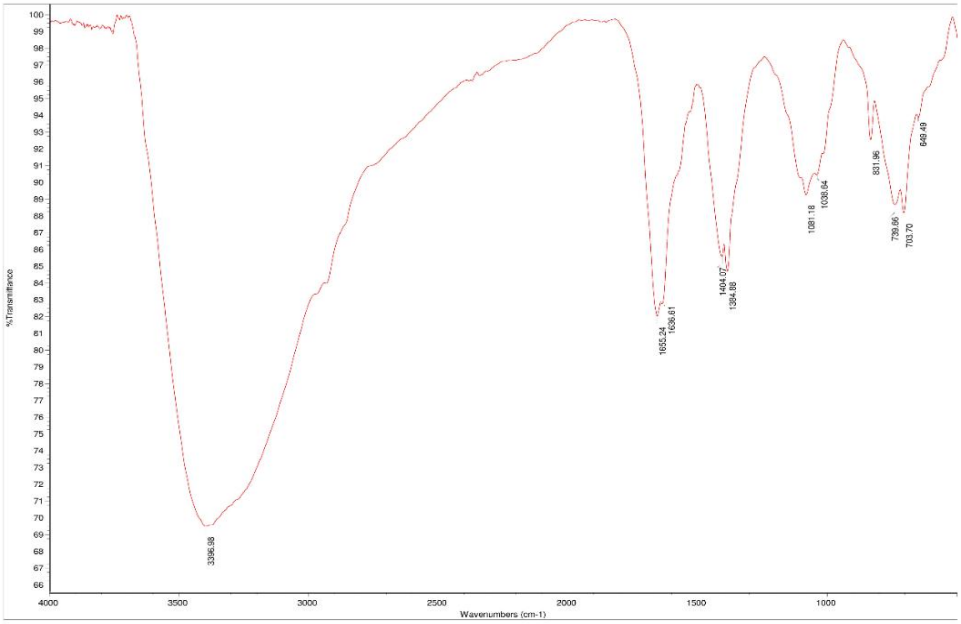

Fig. 4. Fourier transform infrared spectroscopic image of *C. albicans* biofilm formed on latex silicone discs in medium containing 50 mM glucose after 72 h development

In biofilm formation, many bacterial species were found to have and share distinct developmental phases such as extracellular matrix production comprised of cell wall-like polysaccharides (glucose and mannose residues) [9-11,27]. These extracellular polymeric compounds found in bacterial biofilms were identified to have physical interaction with antibiotics resulting to some form of resistance against these drugs [28,29].

Biofilm formation involving fungi is influenced by fungal species and morphogenesis [14,19,30-32], type of contact surface [15,19,30,32], and some environmental factors [33] such as oxygen availability [34] and pH [35]. In the present study, glucose was identified to promote *C. albicans* biofilm formation. Moreover, biofilm formation by *C. parapsilosis* is favored by high-glucose medium [19] suggesting possible device-related infections among patients receiving parenteral nutrition [14].

4. CONCLUSION

Given the complex nature of fungal biofilm formation, studies on pathogenic species isolated from medically and clinically relevant indwelling devices monitored under physiologic conditions can be further performed to unveil the true biology and nature of fungal biofilms. In the present study, information on model biofilms with focus on growth rates, structural organization, and physicochemical characteristics can possibly explain resistance of antifungal drugs and subsequent development of novel therapies for biofilm-based mycoses. Demonstration of common biofilm features will extend the findings of this study beyond fungi to polymicrobial infections, as new information and insights will influence several disciplines ranging from environmental microbiology to pharmaceutical drug designs intended for biofilm-associated infections.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Glenn G. Oyong for facilitating the fluorescence microscopic examination of the biofilm.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Jin Y, Yip HK, Samaranayake YH, Yau JY, Samaranayake LP. Biofilm-forming ability

of *Candida albicans* is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. J Clin Microbiol. 2003;41(7):2961–2967.

2. Donlan RM. Biofilms and device-associated infections. Emerg Infect Dis. 2001;7(2):277–281.
3. Austin JW, Bergeron G. Development of bacterial biofilms in dairy processing lines. J Dairy Res. 1995;62(3):509–519.
4. Ortega-Morales O, Guezennec J, Hernandez-Duque G, Gaylarde CC, Gaylarde PM. Phototrophic biofilms on ancient Mayan buildings in Yucatan, Mexico. Curr Microbiol. 2000;40(2):81–85.
5. Potera C. Forging a link between biofilms and disease. Science. 1999;283(5409): 1837-1839.
6. Costerton JW. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. Trends Microbiol. 2001;9(2):50–52.
7. Donlan RM. Role of biofilms in antimicrobial resistance. Asaio J. 2000; 46(6):S47–S52.
8. Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob Agents Chemother. 2001;45(9):2475–2479.
9. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic approaches to study of biofilms. Methods Enzymol. 1999;310:91–109.
10. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol. 2000;54:49–79.
11. Watnick P, Kolter R. Biofilm, city of microbes. J Bacteriol. 2000;182(10):2675–2679.
12. Jarvis WR. Epidemiology of nosocomial fungal infections, with emphasis on *Candida* species. Clin Infect Dis. 1995; 20(6):1526–1530.
13. Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. Crit Care Med. 1999;27(5):887–892.
14. Shin JH, Kee SJ, Shin MG, Kim SH, Shin DH, Lee SK, Suh SP, Ryang DW. Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: Comparison of bloodstream isolates with isolates from other sources. J Clin Microbiol. 2002;40(4):1244–1248.
15. Douglas LJ. *Candida* biofilms and their role in infection. Trends Microbiol. 2003; 11(1):30–36.
16. Taff HT, Nett JE, Andes DR, Comparative analysis of *Candida* biofilm quantitation assays. Med Mycol. 2012;50(2):214-218.
17. Mohamed SA, Al-Ahmadey ZZ. Biofilm formation and antifungal susceptibility of *Candida* isolates from various clinical specimens. British Microbiology Research Journal. 2013;3(4):590-601.
18. Andes D, Nett J, Oschel P, Albrecht R, Marchillo K, Pitula A. Development and characterization of an in vivo central venous catheter *Candida albicans* biofilm model. Infect Immun. 2004;72(10):6023-6031.
19. Hawser SP, Douglas LJ. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. Infect Immun. 1994;62(3):915–921.
20. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen *Candida albicans*: development, structure, and drug resistance. J Bacteriol. 2001; 183(18):5385–5394.
21. Nett JE, Brooks EG, Cabezas-Olcoz J, Sanchez H, Zarnowski R, Marchillo K, Andes DR. Rat indwelling urinary catheter model of *Candida albicans* biofilm infection. Infect Immun. 2014;82(12):4931-4940.
22. Sumalapao DEP. Physiologic kinetic profile of glycemic response in a single dose of clonidine. Natl J Physiol Pharm Pharmacol. 2017;7(7):701-706.
23. Sumalapao DEP, Mesina JART, Cabrera EC, Gloriani NG. Viability kinetics of *Lactobacillus casei* Shirota strain in a commercial fermented milk drink during refrigerated storage. Natl J Physiol Pharm Pharmacol. 2017;7(11):1242-1246.
24. Yaar L, Mevarech M, Koltin Y. A *Candida albicans* RAS-related gene (Ca*RSR1*) is involved in budding, cell morphogenesis and hypha development. Microbiology. 1997;143:3033–3044.
25. Gale CA, Bendel CM, McClellan M, Hauser M, Becker JM, Berman J, Hostetter MK. Linkage of adhesion, filamentous growth, and virulence in *Candida albicans* to a single gene, INT1. Science. 1998; 279(5355):1355–1358.

26. Yamada-Okabe T, Mio T, Ono N, Kashima Y, Matsui M, Arisawa M, Yamada-Okabe H. Roles of three histidine kinase genes in hyphal development and virulence of the pathogenic fungus *Candida albicans*. J Bacteriol. 1999;181(23):7243–7247.
27. Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev. 2000;64(4):847–867.
28. Hoyle BD, Costerton JW. Bacterial resistance to antibiotics: the role of biofilms. Prog Drug Res. 1991;37:91–105.
29. Gilbert P, Das J, Foley I. Biofilm susceptibility to antimicrobials. Adv Dent Res. 1997;11(1):160–167.
30. Baillie GS, Douglas LJ. Role of dimorphism in the development of *Candida albicans* biofilms. J Med Microbiol. 1999;48:671–679.
31. Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. Infect Immun. 2002;70(2):878–888.
32. Li X, Yan Z, Xu J. Quantitative variation of biofilms among strains in natural populations of *Candida albicans*. Microbiology. 2003;149:353–362.
33. Hawser SP, Baillie GS, Douglas LJ. Production of extracellular matrix by *Candida albicans* biofilms. J Med Microbiol. 1998;47(3):253–256.
34. Xu KD, Stewart PS, Xia F, Huang CT, McFeters GA. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl Environ Microbiol. 1998; 64(10):4035–4039.
35. Stoodley P, DeBeer D, Lappin-Scott HM. Influence of electric fields and pH on biofilm structure as related to the bioelectric effect. Antimicrob Agents Chemother. 1997;41:1876–1879.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/23306