Biofilm Formation, Proteinase and Phospholipase Activities of *Candida parapsilosis* Isolated from Environment

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Received: July 24, 2019; Accepted: August 29, 2019

Abstract

Candida parapsilosis, although, is a human commensal of epithelial and mucosal tissues. This species is also frequently isolated from environments. *C. parapsilosis* is notable for the ability to form biofilms on medical devices. Therefore, it is frequently associated with nosocomial outbreaks. In addition, ability of *C. parapsilosis* producing secrete proteinase and phospholipase can causes more severe infection. The present study aimed to determine biofilm formation using crystal violet staining and hydrolytic enzyme production using spot assay on relevant agar medium of *C. parapsilosis* isolated from environment. In total, 8 of 30 yeast isolates (26.66 %) obtained from environment were *C. parapsilosis*. Biofilm formation of the isolated *C. parapsilosis* was variable. All of the isolates revealed the higher biofilm formation at room temperature (30 °C) compared to 37 °C. All the isolates showed moderate to high activity of proteinase but no activity of phospholipase. In contrast to biofilm formation, proteinase production of the isolates was increased at 37 °C. In conclusion, temperature is one important factor affecting biofilm formation and proteinase production. The formation of the biofilm is possibly beneficial for the isolates to live in the hostile environment. However, production of proteinase may play important role for *C. parapsilosis* to cause infection in human.

Keywords: *Candida parapsilosis*; biofilm formation; proteinase; phospholipase

1. Introduction

Candida parapsilosis is a ubiquitous yeast found in environment such as soil, water, air and plant. *C. parapsilosis* can be isolated from dwelling environments such as dishwasher, washing machine and tap water (Döğen *et al.*, 2017) and also be isolated from hospital environments such as beside table, doorknob, water taps and medical trolleys (Storti *et al.*, 2012; Sabino *et al.*, 2015). Medical importance of *C. parapsilosis* is that this species is currently recognized as the second or third most frequently isolated *Candida* species causing bloodstream infection (Pfaller *et al.*, 2011). In

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DOI: 10.14456/tjst.2020.11

addition, *C. parapsilosis* is frequently reported to associate with outbreak infections. Hands particular in areas surrounding the nails of health care workers are considered as an important source which facilitates the spreading (Reiss *et al.*, 2008; Hernández-Castro *et al.*, 2010). *C. parapsilosis* particularly infects premature infants and critically ill patients in intensive care units (Sarvikivi *et al.*, 2005; Tan *et al.*, 2010). The ability to grow in parenteral nutrition with high glucose concentration, to form biofilms on catheters or other medical devices and to consequently resist antifungal agents (de Toro *et al.*, 2011; Melo *et al.*, 2011) might contribute to successful infections by *C. parapsilosis.*

Biofilms, by definition, are communities of microorganisms that are surface associated or attached to one another embedded within a protective extracellular matrix (Costerton *et al.*, 1995; Finkel and Mitchell, 2011). The advantages of microorganism growing under biofilm in human is that to be protected from host immune response and to provide phenotypic resistance against antimicrobial agents. Furthermore, biofilms can serve as a reservoir for persistent infections (Donlan, 2001; d'Enfert, 2006; Bruzual *et al.*, 2007). Biofilm formation, therefore, has been considered as a potential virulence factor in a number of *Candida* species (Hasan *et al.*, 2009).

We have concerned that *C. parapsilosis* can be frequently isolated from contact surfaces. We, therefore, aimed in this study to isolate *C. parapsilosis* from critical areas of the hospital including pediatric ward, female medical ward and male medical ward. Isolation was additionally isolated from frequently contact surfaces such as handle on NGV bus, door knob and handrail. In addition, the isolated *C. parapsilosis* were tested for biofilm formation and production of proteinase and phospholipase at temperature relevant to environment (30 °C) and human body (37 °C). We showed that all *C. parapsilosis* isolates were able to form biofilm. All isolates showed moderate to high activity of proteinase but no activity of phospholipase. In addition, the temperature is one factor affecting biofilm formation and proteinase production.

2. Materials and Methods

2.1 Yeast isolation

This study was conducted at Thammasat University, Pathum Thani province, Thailand. Isolation of *C. parapsilosis* was carried out in critical areas of hospital including pediatric ward, female and male medical ward. Isolation was additionally isolated from frequently contact surfaces such as handle on NGV bus, door knob and handrail. A total of 203 swab samples were monthly random collected during October 2017 - March 2018. These included 87 samples from general contact surfaces (bus handles, door knobs and handrails) and 116 samples from critical contact surfaces at the patient ward as mentioned above (hospital bed). The collection was performed in the morning before routine cleaning.

The swab samples were suspended in 5 ml of Sabouraud dextrose broth (SDB) with 0.005 % chloramphenicol and 0.5 % propionic

acid and further incubated at room temperature (30 °C). After 6 days of incubation, the turbid broth culture without contaminated mold was further cultured on Sabouraud dextrose agar (SDA) with 0.005 % chloramphenicol and 0.5 % propionic acid. Single colony was re-streaked at least 3 times on the same agar medium. The obtained yeast isolates were sent to identify based on sequencing analysis of ITS region (Macrogen, Korea).

2.2 Culture condition

For assessment of biofilm formation, an overnight culture of isolated yeast was prepared in 5 mL of yeast nitrogen base (YNB) medium containing 50 mM glucose and incubated at 30 °C with shaking at 120 rpm. Cells were harvested and washed twice with sterile 0.85 % NaCl. After resuspension of pellet in YNB medium with 50 mM glucose, cells were adjusted to 0.5 McFarland (1-5 × 10^6 CFU/mL) standard.

For assessment of proteinase and phospholipase production, yeast cells were grown overnight in YPD medium (1 % yeast extract, 2 % peptone and 2 % dextrose) at 30 °C with shaking at 120 rpm. Cells were harvested and washed twice with 0.85 % NaCl. Yeast cells were resuspended and adjusted to 0.5 McFarland standard with sterile saline.

Temperature of each following experiment in this study was at 30 °C (natural environment) and 37°C (clinically relevant environment).

2.3 Biofilm formation assay

Briefly, biofilm was allowed to form on polystyrene 96-well plate (Corning®) using YNB medium containing 50 mM glucose as growth medium. Each well was added with 200 μL of adjusted yeast cell suspension to 0.5 McFarland. Non-adherent yeast cells were removed after incubation at 30 and 37 °C for 90 mins by washing twice with 250 μL of 0.85 % NaCl. The well was added with 200 μL of fresh medium and further incubated at 30 and 37 °C for 48 h for the biofilm development. Crystal violet (CV) staining was used to quantify the amount of biofilm. Briefly, the 48 h biofilm was gently washed twice with 250 μL of 0.85 % NaCl. After air dried for 30 min, biofilm in the well was stained with 250 μL of 0.1 % CV solution for 15 min. Afterward, CV solution was removed and the well was gently washed twice with 250 μL of sterile distilled water. The well then was destained with 250 μL of 95 % ethanol for 45 min. Two-hundred μL of supernatant was transferred to a new 96-well microtiter plate. The amount of CV was spectrophotometrically determined at 595 nm. The optical density of each sample was subtracted from that of the blank. The assay was carried out at least two independent experiments and with three technical replicates. *C. parapsilosis* ATCC 22019 was included as negative control.

2.4 Hydrolytic enzyme assay

Assessment of the ability to produce secreted proteinase by a spot assay was performed as previously described (Pannanusorn *et al.*, 2013). Briefly, 5 μL of adjusted yeast cells was spotted on Yeast Carbon Base (YCB) agar

containing 0.2 % bovine serum albumin (BSA). The plate was incubated at 30 and 37 °C for 5 days. Each isolate was tested with three technical replicates. Afterward, the plate was stained with 0.1 % Amido black solution (0.1 % Amido black, 1 % acetic acid and 40 % methanol). After 15 min, staining solution was discarded and the plate was rinsed with excess volume of distilled water. The plate was then destained overnight with 50 % methanol.

The diameter of colony (A) and diameter of clear zone around the colony (B) indicated proteolytic activity were measured. Ratio of A to B represented to the precipitation zone (Pz) value. Degree of proteolytic activity was classified as no activity (Pz = 1), low activity ($0.64 \leq Pz < 1$) and high activity ($Pz < 0.64$) (Sanitá *et al.*, 2014). *C. parapsilosis* ATCC 22019 was included as positive control.

The ability of yeast cells to produce phospholipase was assessed using a spot assay as previously described. Sabouaraud dextrose agar containing 1 M NaCl, 5 mM $CaCl_2$ and 8 % egg yolk (Oxoid®) (Ge *et al.*, 2011) was spotted by 5 μL of adjusted yeast cells. The plate was incubated at 30 and 37 °C for 5 days. The assay was carried out with three technical replicates. Opaque zone around the colony indicated to phospholipase activity. The Pz value and degree of activity was as same as described above. *C. albicans* ATCC 10231 was included as positive control.

2.5 Statistical analysis

SPSS was used for statistical analysis. p-value < 0.05 indicated significance. The capacity of biofilm formation, proteinase and phospholipase production between 30 and 37 °C were compared using paired sample T-test.

3. Results

3.1 Isolation of *C. parapsilosis* from environment

A total of 203 swab samples were collected from environment during October 2017 - March 2018. The emphasis was made on clinical wards frequently reported for *C. parapsilosis* infection and common surfaces with frequent contact. In the preliminary experiments using 0.35 % propionic acid, isolation of yeast was limited due to overgrowth of mold. However, contamination of mold was successfully eliminated after increasing concentration of propionic acid to 0.5 % in the later experiments.

Of the 203 samples (116 were collected from hospital bed and 87 were from common contact surfaces), only 30 samples (14.77 %) were successfully isolated as yeast. Based on ITS sequencing, *Candida* spp. were mostly found in the environment. *C. parapsilosis* was the most frequently isolated among *Candida* spp. (n = 8, 26.66 %), followed by *C. tropicalis* (n = 6, 20 %), *C. albicans* (n = 4, 13.33 %) and *C. grabrata* (n = 3, 10 %). We found that most isolates of *C. parapsilosis* were from bus handle (n = 4). Pediatric ward was the only place of hospital environment where *C. parapsilosis* were obtained (n = 2) (Table 1). All isolates of *C. parapsilosis* used in this study were shown in Table 2.

Table 1 Isolation of yeast from hospital environments and common contacted surfaces.

Yeast	Source of isolation (number of samples)						
	Pediatric ward (62)	Female medical ward (29)	Male medical ward (25)	Handrail (41)	Bus handle (6)	Door knob (9)	Total
C. parapsilosis	2			1	4	1	8
C. tropicalis	3	2	1				6
C. albicans		3				1	4
C. glabrata	2	1					3
C. dubliniensis		1					1
C. intermedia				1			1
C. guilliermondii	1						1
C. famata		1					1
Rhodotorula mucilaginosa	1			1			2
Hanseniaspora opuntiae	1						1
Yeast	1	1					2
Total	11	9	1	3	4	2	30

Table 2 Laboratory ID of the isolated *C. parapsilosis.*

Isolate number	Laboratory ID	Source
1	CP-ENV 1	Door knob
2	CP-ENV 3	Bus handle
3	CP-ENV 4	Bus handle
4	CP-ENV 5	Bus handle
5	CP-ENV 6	Bus handle
6	CP-ENV 21	Pediatric ward
7	CP-ENV 23	Pediatric ward
8	CP-ENV 28	Handrail

3.2 Biofilm formation of the isolated *C. parapsilosis*

We further characterized biofilm formation of the isolated *C. parapsilosis* using crystal violet staining to quantify an amount of biofilm. To mimic environmental condition and clinical condition, the formation of biofilm was tested at 30 and 37 °C, respectively. Biofilms of all *C. parapsilosis* at the tested temperatures were variable. However, biofilms of all isolates were stable as the same tendency of formation was revealed at both 30 and 37 °C. Biofilm of all *C. parapsilosis* at 30 °C was significantly higher than biofilm at 37 °C ($p < 0.05$) (Figure 1).

3.3 Proteinase production of isolated *C. parapsilosis*

Clear zone around the colony after staining with amido black dye indicated for proteinase production. Conversely to biofilm formation, proteinase production of all *C.*

parapsilosis isolates was increased at 37 °C compared to 30 °C. However, significant difference of Pz value was observed in only 3 isolates (CP-ENV 1, CP-ENV 5 and CP-ENV 6) (Table 3).

3.4 Phospholipase production of *C. parapsilosis*

Opaque zone around the colony growing on egg yolk agar medium indicated phospholipase production. After incubation at temperature 30 and 37 °C, opaque zone was not revealed in any tested isolates of *C. parapsilosis*. This indicated no production of phospholipase by *C. parapsilosis*. Reference strains of *C. albicans* ATCC 90028 and ATCC 10231 were included in this study as a negative and positive control, respectively. Phospholipase was not observed in the negative control ATCC 90028 at both 30 and 37 °C. The positive control ATCC 10231 showed a high production of phospholipase (Pz = 0.45) and temperature tested had no effect on the production by this strain.

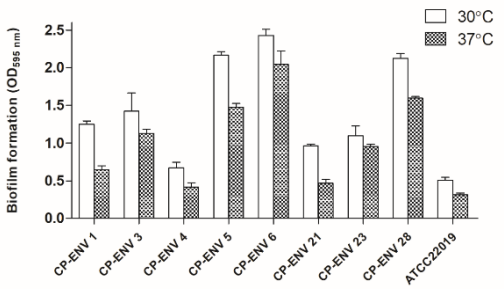

Figure 1 Biofilm formation on 96-well plate of eight isolates of *C. parapsilosis* at 30 and 37 °C.

Table 3 Proteinase production of eight isolates of *C. parapsilosis* at 30 and 37 °C.

ID	30 °C		37 °C		p-value
	Pz	Interpretation	Pz	Interpretation	
CP-ENV 1	0.47	High	0.23	High	0.0052 *
CP-ENV 3	0.70	Moderate	0.65	Moderate	0.6195
CP-ENV 4	0.68	Moderate	0.64	Moderate	0.4778
CP-ENV 5	0.72	Moderate	0.24	High	< 0.0001*
CP-ENV 6	0.68	Moderate	0.36	High	0.0052*
CP-ENV 21	0.73	Moderate	0.46	High	0.1338
CP-ENV 23	0.88	Moderate	0.61	High	0.0512
CP-ENV 28	0.68	Moderate	0.66	Moderate	0.9353

4. Discussion

Our study revealed that *Candida* spp. are the most often isolated yeast in the environment. The result is in consistent to several studies which reported that *C. parapsilosis* is the first leading species isolated from different environment (Cordeiro *et al.*, 2010; Storti et al., 2012; Döğen et al., 2017). This is probably due to the persistent of *C. parapsilosis* on dry surface is longer than other species (Welsh *et al.*, 2017; Bougnoux *et al.*, 2018). Our result also showed that the important pathogenic yeasts such as *C.*

albicans, *C. glabrata* and *C. tropicalis* are commonly found in the hospital environment but not on the contacted surfaces in common area. Our result certainly emphasized to the basic hand hygiene and surface disinfection are essentially needed for preventing the transmission of the pathogenic yeasts. Interestingly, isolation of *C. parapsilosis* in domestic environment such as kitchen surfaces, kitchen sink, kitchen drain, dishwasher and washing machine and tap water have been reported (Zalar *et al.*, 2011; Babič *et al.*, 2015; Novak Babič *et al.*, 2016; Zupančič *et al.*, 2016; Döğen et al., 2017). Likewise the hospital environment, the most prevalence species of *Candida* spp. is *C. parapsilosis* (Storti *et al*., 2012). This obviously reveals the persistence of *C. parapsilosis* in diverse habitat. Important virulence factors, however, facilitating survival of *C. parapsilosis* in such a hostile environment is still not clear.

Medically, *C. parapsilosis* is a second or third most common species of *Candida* causing candidiasis worldwide (Takakura *et al.*, 2004; Sandven *et al.*, 2006; Tortorano *et al.*, 2006; Odds *et al.*, 2007; Cisterna *et al.*, 2010; Marra *et al.*, 2011). In Thailand, although there are few studies of *Candida* spp. distribution in hospital setting, a similar epidemiological result was revealed (Boonyasiri *et al*., 2013; Faksri *et al*., 2014). This species is often related to outbreak infections (Dizbay *et al.*, 2008; Brillowska-Dabrowska *et al.*, 2009; da Silva Ruiz *et al.*, 2013; Wang *et al.*, 2016). Unlike other *Candida* spp., most cases of infection caused by *C. parapsilosis* is from exogenous source. There have been reported that contaminated hands of health care workers with *C. parapsilosis* is considered as an important source of infection (Trofa *et al.*, 2008). In addition, *C. parapsilosis* possess biofilm forming ability as virulence factor which facilitates the spreading (Pannanusorn *et al*., 2013) and influences clinical outcomes of patients (Soldini *et al.*, 2018). The isolated *C. parapsilosis* in our study showed a variable capability of biofilm formation. Environmental factors can influence the formation of biofilm in *in vitro* (Frade and Arthington-Skaggs, 2010; Estivill *et al.*, 2011; Pereira *et al.*, 2015). Although, *C. parapsilosis* can grow in temperature ranging from 10-37 °C (Döğen et al., 2017). Most studies of biofilm formation in *C. parapsilosis* were performed at 37 °C where is a human relevant temperature (Pannanusorn *et al.*, 2014; Ziccardi *et al.*, 2015; Soldini *et al*., 2018). Architecture of biofilm was well described under this condition. Diversity of biofilm was also revealed (Pannanusorn *et al*., 2014). In our experiment, we compared biofilm formed at temperature relevant to environment (30 °C) and human body (37 °C). All isolated of *C. parapsilosis* formed significantly higher amount of biofilm at 30 °C compared to biofilm formed at 37 °C. This may imply the significant of biofilm for *C. parapsilosis* to survive in the environment. However, further studies need to be performed to make a conclusive information.

Hydrolytic enzyme such as proteinase, lipase and phospholipase are virulence factors facilitating infection (Trofa et al., 2008; Chow *et*

al., 2012). Here, we investigated the production of proteinase and phospholipase in our isolates at 30 and 37 °C. Generally, the result of hydrolytic enzyme production comparing between two temperatures was conversed to biofilm production. All isolates tested in this study can produce proteinase. This is consistent to several studies which reported many isolates of *C. parapsilosis* with positive proteinase activity (Ziccardi *et al*., 2015; Shirkhani *et al.*, 2016). Additionally, the production of proteinase was increased at 37 °C compared to proteinase produced at 30 °C. This results emphasis the role of proteinase involving in human infection.

Phospholipase activity of all isolates in this study was not detected at both temperatures. Generally, prevalence of *C. parapsilosis* or other non- *albicans Candida* spp. producing phospholipase is relatively low compared to *C. albicans* (Marcos-Arias *et al.*, 2011; Shirkhani *et al*., 2016). However, there is still a discrepancy in prevalence of *C. parapsilosis* producing phospholipase. Our result is in agreement to the studies which report no phospholipase production in all clinical isolates (Ziccardi *et al*., 2015; Shirkhani *et al*., 2016). This contrasts to a report which presented a relatively high number of *C. parapsilosis* clinical isolates with positive phospholipase activity (Pharkjaksu *et al.*, 2018). Nevertheless, we noted that the number of isolate in our study is limited. A larger sample to be tested will obviously provide much more conclusive information.

5. Conclusion

C. parapsilosis is the most frequently isolated *Candida* spp. from environment. Temperature is one important factor affecting biofilm formation and proteinase production. The formation of the biofilm is beneficial for the isolates to live in the hostile environment. Production of proteinase is increased at temperature relevant to human body. This possibly implies the important role of proteinase for *C. parapsilosis* to cause infection in human. However, to make conclusive information, clinical isolates and environmental isolates should be compared in further experiments.

6. Acknowledgements

The authors gratefully acknowledge the financial support provided by Thammasat University Research Fund under the TU New Research Scholar, Contract No. 08/2559). We would like to express our great appreciation to Thammasat University Hospital for the permission to collect samples from hospital environment.

7. References

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