Revista da Sociedade Brasileira de Medicina Tropical
Journal of the Brazilian Society of Tropical Medicine
Vol.:52:e-20180182: 2019
doi: 10.1590/0037-8682-0182-2018

Short Communication

Biofilm formation by blood isolates of *Candida parapsilosis* sensu stricto in the presence of a hyperglycidic solution at comparable concentrations of total parenteral nutrition

***Thalita Caroline Herek*[1], *Vinícius Ribeiro Menegazzo*[2], *Mayara Baptistucci Ogaki*[1], *Hugo Felix Perini*[1], *Luciana Furlaneto Maia*[3] and *Marcia Cristina Furlaneto*[1]**

[1]. Departamento de Microbiologia, Universidade Estadual de Londrina, Londrina, PR, Brasil.
[2]. Departamento de Ortopedia e Traumatologia, Universidade Estadual de Londrina, Londrina, PR, Brasil.
[3]. Departamento de Tecnologia em Alimentos, Universidade Tecnológica Federal do Paraná, PR, Brasil.

Abstract

Introduction: Administration of total *parenteral nutrition* (TPN) via catheters increases the risk for candidemia from *Candida parapsilosis*. **Methods:** *C. parapsilosis* sensu stricto blood isolates were evaluated for ability total biomass biofilm formation and morphogenesis in presence of glucose at TPN equivalent concentrations. **Results:** Biofilms were increased at high glucose concentrations (25-30%) compared to the control medium. Significant increase in filamentous forms was observed in cultures with 30% glucose. **Conclusions:** Biofilm formation by *C. parapsilosis* sensu stricto in hyperglycidic medium may contribute to its pathogenic potential for fungemia related to TPN catheters.

Keywords: *Candida parapsilosis*. Biofilm. Glucose. TPN.

Parenteral nutrition via oral or enteral routes for a predefined time is widely used in patients unable to obtain adequate nutrition. Solutions containing glucose, proteins, fat, and micronutrients are transferred to the patient through a central venous catheter[1].

Being an invasive technique that connects the external environment directly to the bloodstream and providing nutrients for microorganism growth, parenteral nutrition can render the patient susceptible to infection by pathogens. In particular, total parenteral nutrition (TPN) is a risk factor associated with the development of invasive *Candida* species infections[2].

Concerning the species *Candida parapsilosis*, administration of parenteral nutrition has been shown to strongly influence yeast growth, with high susceptibility to blood infection development[3]. *C. parapsilosis* is a human commensal microorganism and its ability to adhere to prosthetic materials as well as its potential for biofilm formation in high glucose concentrations solutions makes it an important opportunistic pathogen in cases of candidemia associated with parenteral overfeeding and use of intravascular devices[4].

C. parapsilosis has emerged as one of the most common *Candida* species in candidemia surveys worldwide. A multicenter study in Brazil found that *C. parapsilosis* is responsible for 24.1% of candidemia episodes[5]. In a study carried out at the University Hospital of State University of Londrina (Av. Robert Koch, 60 - Operária, Londrina - PR) our group showed that *C. parapsilosis* was the third most common species isolated from candidemia, causing 22% of the episodes[6].

Biofilm formation is an important virulence factor of *C. parapsilosis.* In fact, biofilm-forming *C. parapsilosis* isolates have been associated with higher mortality rates compared with biofilm-deficient isolates[4]. Thus, evaluation of the role of high-glucose concentrations, at comparable concentrations of TPN, in biofilm formation by *C. parapsilosis* is highly relevant and can extend our knowledge regarding the pathogenicity of this species.

For this study, thirteen isolates of *C. parapsilosis* sensu stricto obtained from blood cultures were evaluated (44.10;

Corresponding author: Dra. Marcia Cristina Furlaneto. ORCID: 0000-0002-1188-1320
e-mail: furlaneto@uel.br
Received 9 May 2018
Accepted 4 July 2018

65.10; 262.10; 275.10; 357.10; 390.10; 471.10; 551.10; 117.11; 159.95; 185.11; 230.11; 450.11). The isolates were obtained from patients at the University Hospital of State University of Londrina-PR, in the period of 2010 and 2011[6] and were stored at -20°C in Sabouraud medium supplemented with 20% glycerol.

Biofilm formation was analyzed using cultures in microtiter plates with modifications[7]. Strains were subcultured in Sabouraud dextrose agar (4% glucose, 1% peptone, 0.25% yeast extract; 2% agar) for 48 h at 37°C. A fraction of cells was then subcultured in Sabouraud broth (4% glucose, 1% peptone, 0.25% yeast extract), supplemented with different concentrations of glucose (5, 15, 25, or 30%) for 18 h at 37°C under agitation (120 rpm).

After this step, the cellular density was adjusted to 1 x 10^8 cells/ml in Sabouraud broth at the tested glucose concentrations. Next, 200 µl of this suspension wastransferred to polystyrene microtiter, flat-bottomed plates, followed by incubation for 24 h at 37°C, under static conditions. Then, 100 µl of supernatant was removed and the same volume of fresh Sabouraud medium was added to the wells; plates were incubated for 48 h under the same conditions. Following incubation, non-adherent cells were removed by washing the biofilms twice with sterile ultrapure water. Biofilm-forming ability was assessed through total biomass quantification by crystal violet (CV) staining[7]. Thus, after washing, biofilms were fixed with 200 µl methanol for 15 min. The microtiter plates were allowed to dry at 28°C, 200 µl of crystal violet solution (1% v/v) was added to each well and plates were incubated at 28°C for 5 minutes. The wells were gently washed with sterile, ultrapure water, and 200 µl of acetic acid (33% v/v) was added to dissolve the stain. Absorbance of the solution was measured using a microplate reader (Bio-Tek L 808) at 570 nm. Experiments were performed in triplicates three biological replicates and the geometric mean of biofilm production for each isolate was determined. Isolates were divided into terciles according to biofilm production (OD_{570nm} values) to establish cut-offs. This division provided cut-offs to classify isolates as low, intermediate, and high biofilm-forming as described previously[8].

To analyze the presence of filamentous forms (pseudohyphae) among biofilm cells, six isolates (65.10, 471.10, 450.11, 262.10, 275.10, and 357.10) of *C. parapsilosis* sensu stricto were randomly chosen. To this end, after biofilm formation, the microplate wells were washed with PBS to remove non-adherent cells. Then, 200 µl of the same buffer was added and the adherent cells were removed mechanically using a sterile micropipette tip. A 10-µl aliquot of each solution was applied to a hemocytometer chamber and direct count of 1000 cells was performed to estimate the number of blastoconidia and pseudohyphae present in the biofilms. Data were expressed in terms of percentage of filamentous forms in relation to the total number of cells. For the analysis of biofilm formation and morphogenesis in different culture media, ANOVA and Tukey's test were used.

All isolates formed biofilms in Sauboraud medium (control culture) and in Sauboraud medium supplemented with glucose at concentrations equivalent to that of TPN (**Table 1**). The highest median values of total biofilm biomass were observed in cultures supplemented with 25% and 30% glucose (data not shown). Absorbance values related to total biomass allowed classification of isolates as low biofilm-forming ($OD_{570nm} < 1.593$) and high biofilm-forming ($OD_{570nm} > 2.654$). Isolates with OD_{570nm} between the above values were classified as intermediate biofilm-forming (**Table 1**).

Accordingly, in the control culture (Sauboraud, 4% glucose), 23% (n = 3), 54% (n = 7), and 23% (n = 3) of the isolates were

TABLE 1: Biofilm formation by blood isolates of *Candida parapsilosis* sensu stricto.

Isolate	Total Biomass (median values ± SD)					Categorization[3]				
	SAB.[1]	Supplementation[2]				SAB.[1]	Supplementation[2]			
		5%	15%	25%	30%		5%	15%	25%	30%
44.10	2.703 ± 0.140	1.600 ± 0.243	1.549 ± 0.300	3.043 ± 0.042	4.628 ± 0.205	+	+ / -	-	+	+
65.10	2.080 ± 0.328	1.576 ± 0.150	3.226 ± 2.063	4.548 ± 0.137	4.704 ± 0.281	+ / -	-	+	+	+
471.10	3.415 ± 0.267	3.086 ± 1.290	2.659 ± 0.874	2.977 ± 0.370	4.570 ± 0.241	+	+	+	+	+
551.10	1.638 ± 0.225	1.708 ± 0.027	2.417 ± 1.147	2.339 ± 0.294	2.477 ± 0.712	+ / -	+ / -	+ / -	+ / -	+ / -
122.11	2.567 ± 0.008	3.097 ± 1.088	1.492 ± 0.054	2.602 ± 0.0615	3.120 ± 0.411	+ / -	+	-	+ / -	+
230.11	2.634 ± 0.528	2.693 ± 0.936	2.920 ± 0.311	4.923 ± 0.033	3.321 ± 0.314	+ / -	+	+	+	+
450.11	1.736 ± 0.076	1.756 ± 0.161	1.647 ± 0.097	3.474 ± 0.027	3.489 ± 0.721	+ / -	+ / -	+ / -	+	+
262.10	1.881 ± 0.044	1.637 ± 0.061	2.144 ± 0.799	2.587 ± 0.015	3.753 ± 0.234	+ / -	+ / -	+ / -	+ / -	+
275.10	1.387 ± 0.296	1.469 ± 0.124	2.006 ± 0.920	3.142 ± 1.231	3.043 ± 0.238	-	-	+ / -	+	+
357.10	4.665 ± 0.054	4.517 ± 0.002	4.547 ± 0.142	5.067 ± 0.078	6.282 ± 0.009	+	+	+	+	+
390.10	1.528 ± 0.081	1.167 ± 0.274	1.761 ± 0.801	2.789 ± 0.775	2.328 ± 0.086	-	-	+ / -	+	+ / -
117.11	1.719 ± 0.232	1.424 ± 0.035	2.466 ± 0.051	2.854 ± 0.651	3.269 ± 0.201	+ / -	-	+ / -	+	+
185.11	1.353 ± 0.208	1.433 ± 0.198	1.713 ± 0.406	2.478 ± 0.103	2.153 ± 0.542	-	-	+ / -	+ / -	+ / -

[1] Sabouraud medium; [2] Percentage of glucose supplementation to Sabouraud medium; [3] (+) high biofilm-forming, (+ / -) intermediate biofilm-forming, (-) low biofilm-forming.

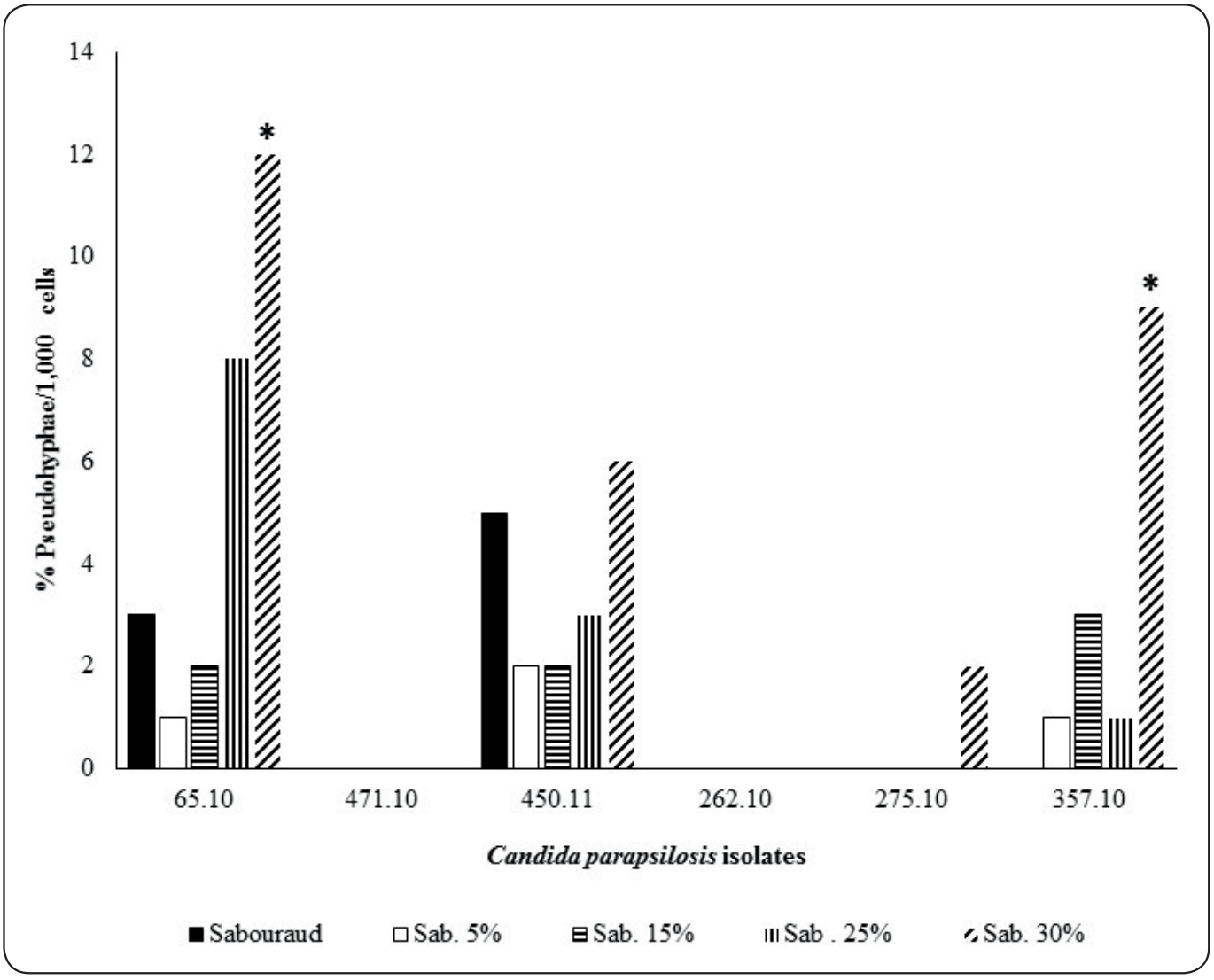

FIGURE 1: Percentage of pseudohyphae among biofilms cells of *C. parapsilosis* sensu stricto at different glucose concentrations. Statistically significant at *P < 0.05 for Sabouraud medium supplemented with 30% glucose *vs*. others culture media.

classified as high, intermediate, and low biofilm-forming, respectively. This categorization was altered with glucose supplementation. For instance, in a medium with 25% and 30% glucose, 69% (n = 9) and 77% (n = 10) of the isolates, respectively, were classified as high biofilm-forming. Under these culture conditions no isolate was categorized as low biofilm-forming, which was contrasting to the observations in the control culture and media supplemented with lower glucose concentrations (**Table 1**).

Occurrence of filamentous forms (pseudohyphae) among biofilm cells was variable among blood isolates of *C. parapsilosis* sensu stricto. As shown in **Figure 1**, morphogenesis among biofilm cells was absent in two isolates (471.10 and 262.10). Presence of glucose at concentrations equivalent to that of TPN affected differentiation into pseudohyphae among biofilm cells (**Figure 1**). In the presence of 30% glucose, the isolates 65.10 and 357.10 exhibited significantly higher percentages of pseudohyphae among biofilm cells compared to those in other media tested (**Figure 1**). Further, the isolate 275.10 showed capacity of differentiation in pseudohyphae during biofilm growth only in culture medium supplemented with 30% glucose, suggesting that high carbohydrate concentrations may induce polymorphisms in this isolate. Biofilms produced in polystyrene under high glucose concentrations also showed larger and more oval cells in relation to those cultivated in Sabouraud medium (data not shown).

Species of the genus *Candida* are the second-most common cause of medical device associated infections, and are related to high mortality rates, reaching 50% in patients in intensive care units[9]. Among non-*Candida albicans* species, *C. parapsilosis* is recognized as one of the main species responsible for candidemia in hospitalized patients. This is partly due to the ability of *C. parapsilosis* to form biofilms in solutions with high glucose concentrations[10] which is correlated with the formation

of strong and structured biofilms[11], in addition to its capacity for colonizing different medical devices[4].

TPN is an effective method of delivering nutrients to the blood stream to meet the patients' protein and energy requirements[1]. Carbohydrates are the main source of calories in almost all TPN formulations and are tightly connected to protein metabolism. Formulation of TPN regimens can be individualized to meet specific requirements or standardized to cover the nutritional needs of a larger patient population. The standard parenteral carbohydrate solution is glucose, which is used at variable concentrations according to established clinical guidelines. For instance, a standard parenteral nutrition formula for adults contains 10 - 30% glucose[12,13].

Supplementation of culture media used in the present study was performed to evaluate the response of *C. parapsilosis* sensu stricto blood isolates to the presence of glucose at concentrations equivalent to those found in catheters during TPN administration. Sabouraud culture medium was chosen to contribute to this equivalence, because this medium is rich in proteins and minerals. For *C. albicans*, high fat and high carbohydrate solutions are known to influence biofilm development[14]. The effect of this supplementation affects the growth, architecture, and morphology of cells present in the biofilm, resulting in an increased number of hyphae compared to those in biofilms formed in culture medium without supplementation[14].

The role of parenteral nutrition in blood infections caused by *Candida* sp. increases about 3.6 times when compared with that in patients not subjected to nutrition by this route[15]. The present study shows that high glucose concentrations stimulate biofilm formation and cell differentiation in blood isolates of *C. parapsilosis* sensu stricto. Regarding the potential of pseudohyphae differentiation, our data suggest a possible relationship between high glucose concentration and morphogenesis potential (**Figure 1**). These alterations may change the parasite-host relationship of these microorganisms or the patterns of recognition, adhesion, and subsequent biofilm formation. Biofilms formed by *C. parapsilosis* isolates exhibit little structured architecture and mostly composed of clustered blastoconidia[7]. When grown at high glucose concentrations, these biofilms, in addition to producing more biomass, also present cells in the form of pseudohyphae. In a study with oral isolates of *C. parapsilosis* the presence of glucose contributed to the development of a strong biofilm in terms of total biomass[11]. The morphogenesis capacity presented by *Candida* species has a fundamental role in good biofilm development. Moreover, these structures are directly related to the virulence of these yeasts; for instance, blastoconidia are related with the ability to spread into the bloodstream, and filamentous forms show the ability of invading tissues and other substrates[9].

Given the epidemiological relevance of *C. parapsilosis* sensu stricto and the importance of parenteral nutrition in patients who cannot obtain nutrients by other means, the present study indicates that glucose at concentrations equivalent to parenteral nutrition stimulates *in vitro* biofilm production as well as cellular polymorphism. The biological characteristics of microorganism, in addition to the relevance of medical device usage in the clinic and the patients' immune deficit subjected to parenteral nutrition renders utmost importance to the knowledge of these virulence characteristics, as these characteristics may potentially favor catheter colonization and development of candidemia.

Acknowledgements: Thalita Caroline Herek held a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Brazil - Finance Code 001. Marcia Cristina Furlaneto is grateful to *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) for the PQ fellowship.

Conflict of interest: The authors declare that there are no conflicts of interest.

Financial support: This work was supported by PROPPG/State University of Londrina-Brazil.

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