Ciência Rural, Santa Maria, v.49:02, e20180574, 2019 http://dx.doi.org/10.1590/0103-8478cr20180574
ISSNe 1678-4596
MICROBIOLOGY

Biofilm formation by *Prototheca zopfii* isolated from clinical and subclinical bovine mastitis in distinct growth conditions under different dyes

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ABSTRACT: *Prototheca spp. have been reported as an emergent environmental mastitis pathogen in several countries. Biofilm formation is a significant factor associated with different degrees of virulence developed by many microorganisms, including Prototheca spp. The present study aimed to compare two growth conditions and two staining dyes to determine which combination was more appropriate to evaluate qualitatively and quantitatively the production of biofilm by P. zopfii. Biofilm formation was evaluated in polystyrene microplates under static and dynamic growth conditions and staining with crystal violet or cotton blue dye. All P. zopfii isolates from cows with mastitis were classified as biofilm-producers in all growth conditions and staining. The cotton blue dye proved to be more appropriate method to classify the intensity of P. zopfii biofilm production.*
Key words: *Prototheca zopfii*, dairy cattle; bovine mastitis, intramammary infections, microplate biofilm assay, virulence factor.

Formação de biofilme por *Prototheca zopfii* isolados de mastite bovina clínica e subclínica em distintas condições de crescimento sob diferentes corantes

RESUMO: *Prototheca spp. tem sido relatado como um patógeno ambiental causador de mastite bovina em vários países. A formação de biofilme é um fator associado a diferentes graus de virulência desenvolvidos por muitos microrganismos, incluindo Prototheca zopfii. O presente estudo teve como objetivo comparar duas condições de crescimento e dois corantes para determinar a combinação mais adequada para avaliar qualitativa e quantitativamente a produção de biofilme por P. zopfii. A formação de biofilme foi avaliada em microplacas de poliestireno sob condições estáticas e dinâmicas de crescimento e coloração com cristal violeta ou azul de algodão. Todos os isolados de P. zopfii de vacas com mastite foram caracterizados como produtores de biofilme, independentemente das condições de crescimento e coloração. O corante azul de algodão demonstrou ser o método mais adequado para classificar a intensidade de produção de biofilme de P. zopfii.*
Palavras-chave: *Prototheca zopfii, bovino de leite, mastite bovina, infecções intramamárias, ensaio de biofilme em microplaca, fator de virulência.*

INTRODUCION

Prototheca spp. are unicellular achlorophyllous algae that reproduce asexually (SUDMAN & KAPLAN, 1973), and are widely distributed in the environment, especially in the presence of water and organic matter (PORE et al., 1983). These microorganisms have also been isolated from diverse animal species, particularly from dairy cattle. The infections of mammary gland of cows by *Prototheca* spp., especially *Prototheca zopfii*, normally result in reduced milk production, early culling, as well as public health concerns (COSTA et al., 1997; VARGAS et al., 1998; SALERNO et al., 2010). *P. zopfii* has been reported to be a causative agent of bovine mastitis across Europe (ROESLER et al., 2006), as well as in Japan (ONOZAKI et al., 2013) and in Brazil (SALERNO et al., 2010). In Brazilian dairy cattle, protothecosis has been described as an important cause of environmental mastitis, occasioning great damage to the mammary gland and resulting in animal discard (SALERNO et al., 2010).

Received 07.20.18 Approved 01.02.19 Returned by the author 02.08.19
CR-2018-0574.R2

Mastitis diagnosis caused by *P. zopfii* may be performed through morphology and phenotypic methods (PORE, 1985; VARGAS et al., 1998; ROESLER e*t* al. 2006); although, molecular methods are necessary for genotyping (AOUAY et al., 2008). There is no effective therapeutic protocol for treatment of *P. zopfii* induced bovine mastitis due to its intrinsically high resistance to conventional antimicrobials, antifungals and antiseptic compounds (LASS-FLÖRL & MAYR, 2007). Although, the algae's poor response to conventional therapy has long been known (COSTA et al., 2004), only recently it has been demonstrated that the biofilm formed by *P. zopfii* is associated with microorganism persistence in the mammary gland, and consequently to antimicrobial resistance (MORANDI et al., 2016).

Biofilms are communities of microorganisms attached to a surface and surrounded by an extra cellular matrix (O'TOOLE et al., 2000). These slime-embedded communities become highly resistant to both antimicrobials and host defenses (FUX et al, 2005). Although, the formation of biofilms by *P. zopfii* had been reported, there is no agreement between the current characterization protocols (GONÇALVES et al., 2015; KWIECINSKI, 2015; MORANDI et al., 2016). Therefore, given the relevance of this issue, the aim was to compare two growth conditions and two staining dyes to determine which combination was more appropriate to evaluate qualitatively and quantitatively the production of biofilm by *P. zopfii*.

MATERIALS AND METHODS

Thirty-two strains of *P. zopfii* isolated from cows with clinical (n=27) and subclinical (n=5) mastitis were used in this study. Clinical mastitis was characterized by abnormalities at visual observation and udder palpation, including udder swelling, hardness of the affected quarter, pain, watery milk, and reduced milk yield. Subclinical mastitis was considered when the visible inflammatory changes were absent in the milk or udder. All *Prototheca* spp.- positive milk samples were obtained from five medium-scale dairy farms, with 100 to 380 lactating cows, each producing 12-20 liters of milk daily and milked mechanically twice a day. All dairy farms were located in four Brazilian States from the South (Rio Grande do Sul and Paraná), Southeast (São Paulo) and Northeast (Pernambuco) regions. Milk samples were plated on defibrinated sheep blood agar (5%) and Sabouraud dextrose agar and incubated aerobically at 37°C for 72h. Yeast-like colonies were submitted for identification by phenotypic tests and carbohydrate fermentation (trehalose, fructose, galactose and glycerol) (PORE, 1985; VARGAS et al., 1998; ZAROR et al., 2011).

Biofilm assays were performed as previously described by RODRIGUES et al. (2010) with few modifications. All isolates were grown in tryptone soya broth (TSB) with 1% glucose added, and incubated at 37°C for 48h. Next, an inoculum was prepared for all isolates and standardized at approximately 10^8 $CFUmL^{-1}$ which corresponded to optical density (OD) of 0.24±0.02 by spectrophotometric observation at 600nm (OD600). Subsequently, 20µl of each *P. zopfii* suspension was added to 96-well polystyrene microplates containing 180µl/well TSB added 1% glucose. The microplates were incubated at 37°C for 24h under static or dynamic growth conditions (100 revolutions per minute, rpm). After incubation, the content in the wells was carefully aspirated, and each well was washed twice with 0.01M sterile saline phosphate buffer (PBS; pH 7.4) to remove all non-adherent cells. After drying of the plates at room temperature, the adherent cells were stained for 5min. with 100µl of 0.25% crystal violet (Synth®, Cat. No. 42535/42555) or 0.05% cotton blue (Newprov®, Cat. No. 771). Lastly, the content of the wells was aspirated, washed and dried as previously described. After, 200µl of a 60:40 alcohol/acetone solution was added to solubilize the stained biofilm. Biofilm was quantified by spectrophotometric observation at 550nm. To ensure quality control, the ATCC 25923 *Staphylococcus aureus* was used as positive control for biofilm formation (MARQUES et al., 2007), while the medium alone served as negative control. All tests were performed in triplicate. The arithmetic mean of the triplicates was calculated and the strains showing absorbance values greater than the negative control were considered positive for biofilm formation. Strains were classified regarding the biofilm-forming ability using the results of arithmetic mean, where OD_{ct} refers to the negative control and OD_{is} refers to the strain analyzed. *P. zopfii* isolates were classified as weak ($OD_{ct}<OD_{is}<2.OD_{ct}$), moderate ($2.OD_{ct}<OD_{is}<4.OD_{ct}$) or strong ($4.OD_{ct}<OD_{is}$) biofilm-producers (STEPANOVIĆ et al., 2003). Results of static and dynamic growth conditions, as well as the staining assays, were compared using the OD values averages of each triplicate by statistical methods. The homogeneity of variances among groups was assessed using the Levene's test and the comparisons among those groups were carried out using one-way ANOVA and Tukey's test (Statistica 7.0 Software). The minimum significance level was set at $P\leq0.05$.

RESULTS AND DISCUSSION

All *P. zopfii* isolates were classified as biofilm-producers in all growth conditions and staining methods analyzed. Absorbance values of *P. zopfii* stained with crystal violet and cotton blue grown under static or dynamic conditions are presented in Table 1. All isolates stained with crystal violet were classified as strong biofilm producers under static or dynamic growth conditions (Table 2). However, the microorganisms stained with cotton blue displayed different degrees of biofilm formation. Under static condition, 68.75% (22/32) were classified as moderate, 21.9% (7/32) as strong and 9.37% (3/32) as weak biofilm-producers (Table 2). *P. zopfii* isolates stained with cotton blue under dynamic conditions presented the same proportion of strong biofilm producers, while moderate isolates dropped to 53.1% (17/32), and the weak isolates proportion increased to 25.0% (8/32) (Table 2). However, statistical analyses did not reveal significant differences (P>0.05) with crystal violet and cotton blue grown under static or dynamic conditions.

Concerning the virulence factors and pathogenic aspects of this microorganism there is scarce information. Since the biofilm-formation

Table 1 - Absorbance values of *Prototheca zopfii* stained with crystal violet and cotton blue and grown under static and dynamic conditions.

Isolate	----------Crystal violet----------		----------Cotton blue----------	
	Static	Dynamic	Static	Dynamic
2	3.25±0.09	3.32±0.05	0.17±0.09	0.17±0.16
4	3.16±0.12	3.28±0.04	0.18±0.20	0.48±0.13
5	3.41±0.04	1.96±0.80	0.28±0.01	0.09±0.06
6	2.28±0.08	2.95±0.64	0.17±0.03	0.07±0.07
7	2.97±0.18	3.05±0.18	0.11±0.04	0.02±0.02
8	3.27±0.03	3.30±0.03	0.14±0.05	0.24±0.06
9	2.49±0.68	3.05±0.37	0.10±0.001	0.02±0.01
10	3.24±0.09	3.34±0.03	0.12±0.04	0.03±0.02
11	2.94±0.35	2.78±0.45	0.26±0.07	0.13±0.06
12	1.87±0.17	2.68±0.63	0.07±0.03	0.19±0.02
13	2.68±0.52	2.83±0.40	0.05±0.02	0.13±0.02
14	2.43±0.75	3.08±0.09	0.09±0.01	0.16±0.003
15	2.91±0.07	2.98±0.39	0.09±0.01	0.18±0.007
16	2.66±0.21	1.90±0.27	0.15±0.03	0.03±0.006
17	3.20±0.07	2.57±0.37	0.26±0.06	0.10±0.03
18	2.78±0.24	3.34±0.15	0.13±0.03	0.19±0.05
19	3.20±0.16	3.34±0.08	0.09±0.03	0.26±0.07
20	2.37±0.69	2.69±0.43	0.19±0.04	0.36±0.16
21	3.34±0.15	3.31±0.02	0.033±0.09	0.11±0.003
166AD	2.91±0.36	4.34±1.44	0.15±0.06	0.19±0.09
172AD	3.37±0.02	3.15±0.18	0.25±0.12	0.07±0.03
172AE	2.54±0.60	2.98±0.34	0.12±0.01	0.20±0.03
192AE	1.92±0.15	1.64±0.13	0.10±0.02	0.05±0.02
267AD	1.66±0.92	1.96±0.42	0.14±0.07	0.13±0.05
267PE	2.47±0.21	2.73±0.34	0.11±0.04	0.10±0.02
287AD	1.74±0.34	2.96±0.29	0.022±0.01	0.016±0.01
287PE	3.31±0.03	1.96±1.00	0.18±0.06	0.12±0.03
288AD	3.00±0.28	3.37±0.05	0.32±0.18	1.00±0.05
289AD	1.85±0.40	3.19±0.28	0.19±0.04	0.23±0.10
304PD	3.35±0.04	3.34±0.05	0.22±0.02	0.15±0.05
308AD	3.22±0.03	1.00±0.24	0.14±0.05	0.17±0.12
SB119/12	3.31±0.10	2.89±0.41	0.17±0.02	0.12±0.04

Note: the controls (media only) absorbance values were subtracted from *P. zopfii* absorbance values. The results represent the mean±standard deviation (SD) for each triplicate.

Table 2 - Classification of *Prototheca zopfii* biofilm-forming ability according to the growth and staining conditions.

Isolate	----------Cotton blue dye----------			----------Crystal violet dye----------		
	Static	Dynamic	Classification S/D*	Static	Dynamic	Classification S/D
2	3.31	3.93	M/M	12.26	12.68	S/S
4	3.55	8.80	M/S	9.33	8.60	S/S
5	4.43	2.06	S/M	14.96	7.80	S/S
6	3.35	1.96	M/W	12.91	10.61	S/S
7	2.48	1.31	M/W	16.53	10.93	S/S
8	2.91	5.09	M/S	12.32	12.61	S/S
9	2.33	1.28	M/W	13.97	10.95	S/S
10	2.68	1.42	M/W	17.94	11.86	S/S
11	4.14	2.47	S/M	13.03	10.61	S/S
12	1.99	3.47	W/M	10.76	9.73	S/S
13	1.67	3.21	W/M	10.29	10.96	S/S
14	2.31	3.70	M/M	7.40	8.13	S/S
15	2.24	4.16	M/S	11.08	11.50	S/S
16	2.91	1.41	M/W	11.91	7.56	S/S
17	4.26	2.16	S/M	14.09	9.88	S/S
18	2.78	3.39	M/M	15.53	11.87	S/S
19	2.23	5.49	M/S	12.07	12.75	S/S
20	3.80	6.93	M/S	7.25	7.23	S/S
21	5.02	2.31	S/M	14.69	12.46	S/S
166 AD	3.05	4.27	M/S	11.07	16.28	S/S
172 AD	4.10	1.84	S/W	14.79	11.91	S/S
172 AE	2.80	4.26	M/S	7.69	7.91	S/S
192 AE	2.19	1.58	M/W	8.86	6.67	S/S
267 AD	3.07	3.11	M/M	5.38	5.54	S/S
267 PE	2.55	2.63	M/M	7.51	7.32	S/S
287 AD	1.29	1.20	W/W	10.08	10.64	S/S
287 PE	3.47	3.12	M/M	12.47	7.92	S/S
288 AD	5.31	2.26	S/M	16.69	11.99	S/S
289 AD	3.53	3.92	M/M	10.69	11.39	S/S
304 PD	4.09	3.57	S/M	12.61	12.77	S/S
308 AD	2.71	3.01	M/M	14.20	4.42	S/S
119/12	3.32	3.06	M/M	12.48	11.19	S/S

*Correspond to static (S) or dynamic (D) growth condition. M–moderate, S–strong, W–weak.
Note: the values were obtained by the division of arithmetic mean of the absorbance values of each isolate by the mean of the control (culture medium only).

has been reported as an important virulence factor of *Prototheca* spp., this study aimed to evaluate the biofilm formation by 32 *P. zopfii* isolates testing different methodologies employing two growth conditions, and two different dyes.

In the present study, all *P. zopfii* isolates were classified as biofilm producers. No significant difference in the biofilm-forming ability was detected with different staining methods (P>0.05), it was verified that the cotton blue dye was more appropriated to quantify biofilm formation by *P. zopfii*. According to the literature, crystal violet dye has been used mainly in biofilm assays with bacteria (O'TOOLE et al., 2000), while cotton blue dye has been employed in fungal and yeast biofilm studies (JAYASINGHEARACHCHI & SENEVIRATNE, 2006; SIQUEIRA et al., 2008). In this study, the characterization of *P. zopfii* as weak, moderate or strong biofilm-producer was possible using the absorbance results from 0.05% cotton blue dye only. Several studies have demonstrated that microorganisms, even those from the same species,

display variable biofilm-forming ability (O'TOOLE et al., 2000), as observed using cotton blue staining. There is a hypothesis about the ability of *P. zopfii* strains to form biofilm depending on their genotypes which claims that the strains belonging to the genotype 1 are weak to moderate whilst those of the genotype 2 are strong biofilm producers (MORANDI et al., 2016). However, without a standardized methodology this hypothesis cannot be tested appropriately.

The static or dynamic growth conditions had no significant influence in the production of biofilms by *P. zopfii* (P>0.05); however, it was possible to verify that the growth conditions (static and dynamic) had impact on the biofilm formation, which was noticeable when cotton blue was used (Table 2). Nevertheless, besides the technical particularities, the biofilm formation is also modulated by several growth conditions and nutrient sources, as well as interactions with different microorganisms and environmental conditions (COSTERTON et al., 1995), making any comparison among microorganisms a challenge. Studies carried out on biofilm formation in isolates of *Prototheca* spp.; although restricted, present a large variability in their experimental design (Table 3) resulting from the lack of standardization.

In table 3 we summarized the current knowledge about biofilm formation by *Prototheca* spp. In 2015, the first study showing the biofilm formation by *P. zopfii* was reported (GONÇALVES et al., 2015). The authors evaluated 10 subclinical isolates under different incubation temperatures (25°C and 37°C) and suggested some influence of this parameter on biofilm formation of *Prototheca* sp. The authors classified the isolates as weak, moderate, and strong biofilm-producers, and reported six weak and four moderate biofilm-producers employing a 48 h of incubation at 37°C. Later, another study testing 46 *P. zopfii* isolates identified 90% as strong biofilm-producers after a 24 h incubation at 37°C and by using safranin dye (MORANDI et al., 2016). In the present study, employing a 24h incubation at 37°C, using crystal violet dye, as suggested by GONÇALVES et al. (2015), it was verified that 100% of the isolates were strong biofilm-producers. However, when cotton blue dye was used it was possible to classify the isolates as weak, moderate or strong biofilm-producers. Nevertheless, when in the static condition 90.65% of *P. zopfii* isolates were classified as moderate to strong and under dynamic condition there was a decrease in number of moderate and strong biofilm-forming isolates (75.00%) (Table 2).

The present research reinforces previous findings that indicate *P. zopfii* as a potent *in vitro* biofilm-producer, which may contribute to chronic bovine mammary mastitis, as well as the persistence of algae in milking machines or farm environments (OSUMI et al., 2008). Based on these results, it is possible to propose that the most adequate conditions for testing the formation and classification of biofilm by *P. zopfii* would be static growth condition, a 24-

Table 3 - Studies involving biofilm formation by *Prototheca* spp.

Study	Isolates/% positive	Tº	Mode of incubation	Dye/OD	Culture medium	Degree
Gonçalves et al. (2015)	10/100	37ºC/25ºC	48h/dynamic (156 rpm)	1% crystal violet/570nm	TSB+0.6% yeast extract	W, M or S
Farrag et al. (2015)	1/100	37ºC	48h/static*	1% crystal violet/570nm	YNB broth+0.9% glucose	-
Kwiecinski (2015)	4/100	37ºC	24h/static*	0.4% safranin/520nm	Minimal medium+10% human heparinized plasma or 10% skimmed milk	-
Morandi et al. (2016)	46/100	37ºC	24h/static	0.4% safranin/450nm	Sabouraud broth + 6% glucose	W, M or S
Present study	32/100	37ºC	24h/static and dynamic (100rpm)	0.25% crystal violet or 0.05% cotton blue/550nm	TSB+1% glucose	W, M or S

TSB: Tryptic Soy Broth; YNB: Yeast Nitrogen Base; *: We assumed the mode as static once it is normally used and because the authors have not described the incubation as dynamic.
Degrees: W: Weak; M: Moderate; S: Strong.

hour incubation at 37°C and staining with 0.05% cotton blue. Nevertheless, we suggested that additional studies are necessary to characterize the mechanisms involved in biofilm production by *P. zopfii* to prevent and control the infections caused by this important pathogen.

ACKNOWLEDGEMENTS

The authors acknowledge the support received from development agencies to search Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (finance code 001).

DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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