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Biofilm Production of *Leptospira* spp. Strains

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ABSTRACT

***Background*:** Leptospirosis is a zoonosis that affects many species of mammals and occurs endemically in Brazil. The biofilm matrix provides structure and protection to the biofilm cells working as a physical barrier to antibiotic agents, which are attached or consumed by the matrix components. However, this attribute varies according to the matrix, antimicrobial agent and biofilm age. Leptospira may change morphologically according to environmental conditions, including cell aggregation and biofilm formation. Leptospira can colonize the ducts of kidney from hosts for a long time, forming a biofilm, which is believed to be an important factor for their maintenance in animals and in the environment. Thus, the objective of this research was to determine the biofilm formation capacity of four strains of *Leptospira interrogans*.

***Materials, Methods & Results*:** The strains were typified by WHO/FAO/OIE and National Collaborating Center for Reference and Research on Leptospirosis (Kit Biomedical Research, Amsterdam, Netherlands). *Leptospira interrogans* strains, two isolated from cattle and two isolated from dogs were biofilms tested for adhesion on polystyrene plates, extracellular matrix composition and confocal microscopy. In the plating adhesion test, the suspension was inoculated into 96-well sterile polystyrene microplates with flat bottom at a ratio of 1:200 in EMJH medium, followed by 24 h incubation at 28°C, with medium renewal after 12 h. After this period the wells were washed three times with sterile PBS and following incubation; the plates were dried in the oven at 60°C for 30 min and added 200 µL of 1% violet crystal for five min. Subsequently, the plates were washed with distilled water, after complete removal, 200 µL of acetic acid 33% was added and the readings were performed at 570 nm in the ELISA reader. The proteins and polysaccharides were quantified in a scraped pooled sample diluted in 0.85% sterile saline solution to achieve an optimal amount for testing used reagents of the BCA kit. The polysaccharide content was determined by adding into a tube, an aliquot of 0.5 mL from the pooled sample, 0.5 mL of phenol and then immediately 2.5 mL of sulfuric acid. The solution was homogenized and left to react for 15 min at room temperature. The reading was performed at 490 nm in ELISA reader. The strains were compared regarding polysaccharides and protein matrices using analysis of variance (ANOVA) and Tukey test. At confocal microscopy the strains were incubated with the tested polypropylene material for 24 h. The materials were washed with sterile phosphate buffer and stained with propidium iodide. The reading was performed using a Laser Scanning Confocal Microscope (Zeiss 710) with laser excitation (488 nm) and 580-680 nm emission filters for propidium iodide (red marking). All strains displayed strong adherence on microplate and the amount of polysaccharides in biofilm was not statistically different among the studied strains, but the amount of protein was significantly different in strain 4 ($P > 0.5$). The confocal microscopy showed the adherence of the *Leptospira* spp. strains to polypropylene material after washing.

***Discussion*:** Biofilm production plays an important role in the maintenance of a chronic infection by *Leptospira interrogans* with renal colonization. The exopolysaccharide (EPS) has various functions, such as checking insolubility in water; giving the three-dimensional conformation of the biofilm; protecting cells from physical (mechanical action, irradiation and temperature variations), chemical.

Keywords: biofilm formation, extracellular matrix, *Leptospira interrogans*.

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INTRODUCTION

Leptospirosis is a zoonotic disease with world wide distribution and endemic in tropical countries. Leptospiras can survive for several days in the environment, under ideal pH, temperature and humidity conditions. Infected animals may carry bacteria in kidneys, and shed it in the urine, transmitting to other animals, humans and contaminating the environment

Biofilm formation is characterized by the expression of an extracellular matrix, which has structural and physiological functions. The biofilm matrix provides structure and protection to the biofilm cells [14,21] working as a physical barrier to antibiotic agents, which are attached or consumed by the matrix components. However, this attribute varies according to the matrix, antimicrobial agent and biofilm age [13].

Although biofilm formation may differ even for a single strain grown under different environmental conditions, the structure and composition of the extracellular matrix follows common principles. Typically, a biofilm consists of amyloid, adhesive fimbriae, surface protein, exopolysaccharide, and extracellular DNA [8,21].

Biofilms have been shown to be a major cause of cross-contamination of food products and disease transmission [16,18]. Leptospira can change its morphology according to environmental conditions, including cell aggregation [18] and biofilm formation [17]. Biofilm formation by *Leptospira* sp. can play an important role in their ability to survive in different environmental habitats including their host [17].

However, there are few studies on biofilm formation by leptospires, in spite of their high pathogenicity. This study aimed to evaluate the biofilm-forming ability of *Leptospira* spp. strains isolated from infected dogs and cattle.

MATERIALS AND METHODS

Leptospira spp. *strains*

Leptospira interrogans strains (two isolated from cattle and two isolated from dogs) were provided by Laboratory of Bacterial Zoonosis from Universidade de São Paulo (USP), São Paulo, Brazil. Bacteria were maintained in Ellinghausen-McCullough (EMJH) and semi-solid FLETCHER media at 28°C. The strains were typified by WHO/FAO/OIE and National Collaborating Center for Reference and Research on Leptospirosis (Kit Biomedical Research)[1].

Seroreativity of strains was classified by microscopic agglutination test (MAT) with monoclonal antibodies [10]. The pulsed-field gel electrophoresis was performed as previously described [5].

Strains were identified as: strain nº1: M5/90 1990 *Leptospira interrogans* serovar Icterohaemorragiae/Copenhageni; strain nº2: L06 2001 *Leptospira interrogans* serovar Canicola in dogs; strain nº3: L014 2001 *Leptospira interrogans* serovar Canicola; and strain nº 4: L010 2001 *Leptospira interrogans* serovar Icterohaemorragiae/Copenhageni in cattle. All tests were performed in triplicate with three replications.

Plate adhesion test

The plating adhesion test was performed as previously described with few modifications [5]. The bacteria were cultured individually in EMJH medium supplemented with 10% rabbit serum for seven days. Then, the suspension was inoculated into 96 well sterile polystyrene microplates with flat bottom at a ratio of 1:200 in EMJH medium, followed by 24 h incubation at 28°C, with medium renewal after 12 h. The wells were washed three times with sterile PBS (10 mM, pH 7.4) and following incubation; the plates were dried in the oven at 60°C for 30 min. Then, 200 µL of 1% violet crystal was added for 5 min. Subsequently, the plates were washed with distilled water, after complete removal, 200 µL of acetic acid 33% was added and the readings were performed at 570 nm in the ELISA reader[2]. Non-inoculated wells with EMJH worked as control. Strains of biofilm producing bacteria had absorbance greater than 0.1. Each strain was tested in triplicate, three times. The intensity of the slime production was scored as follows: strong (greater than 0.3), moderate (> 0.2 and <0.3) and low (> 0.1 and <0.2) [6]

Quantification of matrix proteins

The proteins and polysaccharides were quantified in a scraped pooled sample diluted in 0.85% sterile saline solution to achieve an optimal amount for testing. A 12.5 µL aliquot of the pooled sample, diluted at the same ratio with sterile saline, was added to a 96-well microplate. Then, 200 µL of the mixed reagents of the BCA kit (Sigma)[3] was added, homogenized for 30 s and incubated for 30 min at room temperature. The readings were performed at 562 nm in ELISA reader[2], and a phosphate buffer as blank, as demonstrated with adaptations for *Leptospira* [1].

Quantification of polysaccharides - phenol-sulfuric acid method

The polysaccharide content was determined by adding into a tube, an aliquot of 0.5 mL from the pooled sample, 0.5 mL of phenol (50 g/L) and then immediately 2.5 mL of sulfuric acid (95-97% - Isofar). The solution was homogenized and left to react for 15 min at room temperature. The reading was performed at 490 nm in ELISA reader [2] using a phosphate buffer as blank [7]. The strains were compared regarding polysaccharides and protein matrices using analysis of variance (ANOVA) and Tukey test.

Confocal microscopy

The strains of *Leptospira interrogans* were incubated with the tested polypropylene material in 8-well sterile plate with flat bottom at 1: 200 in EMJH at 28°C for 24 h, with medium renewal after 12 h. In the Histology Laboratory from Universidade Federal de Uberlândia (UFU), the materials were washed with sterile phosphate buffer and stained with propidium iodide. The reading was performed using a Laser Scanning Confocal Microscope (Zeiss 710)[4] with laser excitation (488 nm) and 580-680nm emission filters for propidium iodide (red marking).

RESULTS

All strains were identified as biofilm producers by adhesion test in microplate and classified as strong, since biomass absorbance was greater than 0.3. Analysis of the biofilm matrix showed higher concentration of protein compared to polysaccharide, according to (Figure 1). The amount of polysaccharides in biofilm was not statistically different among the studied strains, but the amount of protein was significantly different in strain 4 ($P > 0.5$)

Shows the images of the confocal microscopy and the adherence of the *Leptospira* spp. strains to polypropylene material after washing (Figure 2).

DISCUSSION

All strains showed strong adherence to plates after one day of incubation. This result differs from a previous study in which two strains of *Leptospira* sp., one saprophytic and other pathogenic were tested in 12-well polypropylene plates, washed and stained with crystal violet, with readings 600 nm, and after 2 days of incubation a light adhesion was observed [17].

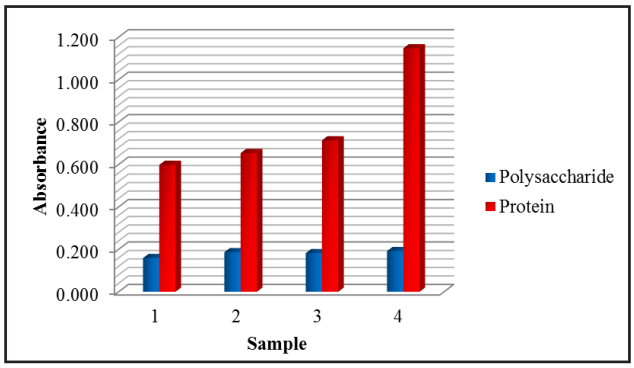

Figure 1. Biochemical composition of protein and polysaccharide extracellular matrix of *Leptospira interrogans* strains, Canicola and Icterohaemorrhagiae/ Copenhageni serovars tested by BCA Kit and phenol-sulfuric acid method.

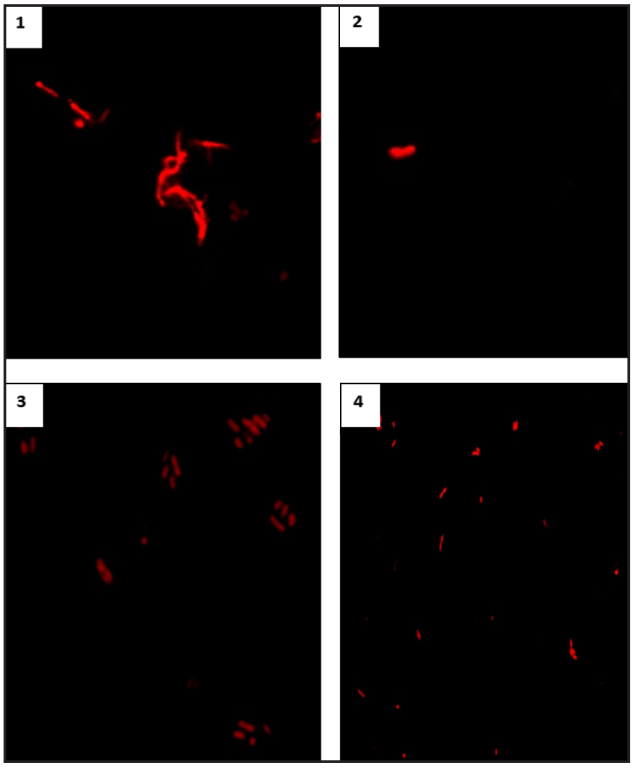

Figure 2. Images of the confocal microscopy method showing the biofilms of the *Leptospira interrogans* strains serovar Canicola and Icterohaemorragiae/Copenhageni on polypropylene material. The pictures are ordered in the sequence: Strains 1 and 2, and below strains 3 and 4.

A recent study tested 21 *Leptospira* spp. strains in 96 well polystyrene plates. The results showed adherence *in vitro* for 8 strains [11] and reported 600 nm reading after 48 h, similar to this study, with 570 nm readings after 24 h for four adherent strains.

The exopolysaccharide (EPS) has various functions, such as checking insolubility in water; giving the three-dimensional conformation of the biofilm; protecting cells from physical (mechanical action, irradiation and temperature variations), chemical (chemical agents used in industrial hygiene procedures) stress (competi-

tors and predators) biological; and the role in the nutrient input [4,9]. To this end, the EPS brings doubtless benefits to the survival of microorganisms despite being a large burden to the cell, due to the energy required for its synthesis [6,12] .Therefore, the characterization of the biofilm matrix is important for understanding the pathogenicity of strains.

The biofilm matrices had higher amount of protein than polysaccharide. In general, the protein biofilms have better mechanical properties than those based on polysaccharides [3]. Also, proteins are important to the biofilm maturation process since they interact with special polysaccharides, named polysaccharide intercellular adhesion (PIA) in cell-cell aggregation [15].

The leptospiral colonization in placental tissues of mice proved the cell aggregation ability of pathogenic *Leptospira* sp. *in vivo* [2]. Also, the biofilm can play an important role in maintaining a chronic infection by *Leptospira interrogans* with kidney colonization [17].

CONCLUSION

Strains of *Leptospira interrogans* serovar Canicola and Icterohamorrhagiae / Copenhageni from dogs and cattle formed biofilms and showed high adherence in the tested materials.

MANUFACTURERS

[1]Kit Biomedical Research. Amsterdam, Netherlands.
[2]Thermo Scientific Multiskan Co. Hudson, NH, USA.
[3]Sigma Chemical Co. St. Louis, MO, USA.
[4]Carl Zeiss AG Corporate. Oberkochen, Baden-Württemberg, Germany.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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