Evaluation of the biofilm formation capacity of *Pasteurella multocida* strains isolated from cases of fowl cholera and swine lungs and its relationship with pathogenicity[1]

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Pasteurella multocida is a Gram-negative bacillus that causes economic losses due to the development of respiratory diseases in several animal species. Among the mechanisms of virulence, the formation of biofilms is an important factor for bacterial survival in hostile environments. Studies of biofilm formation by *P. multocida* are needed because *P. multocida* is an important pathogen involved in respiratory infections. However, in contrast to other microorganisms, few studies of biofilm formation have examined *P. multocida*. Studies comparing the pathogenicity of microbial strains as a function of their biofilm production capacity are also rare. Consequently, the aim of this study was to evaluate the biofilm formation capacity of 94 *P. multocida* strains isolated from cases of fowl cholera and from swine lungs on polystyrene plates. The associations of the biofilm formation capacity with the pathogenicity index (PI) *in vivo* and with the presence of four genes (screened by PCR) of the *tad locus* (*tadB*, *tadD*, *tadE* and *tadG*), described as adhesion markers, were also determined. Strains from both animal origins were able to form biofilms. However, most of the specimens (52.13%) were classified as weak producers, and more than 40% of the strains of *P. multocida* (40.42%) did not produce biofilms. There was no significant difference ($p>0.05$) in the degree of biofilm production between the two sources of isolation. Of the analyzed strains, 56.52% contained all four genes (*tadB*, *tadD*, *tadE* and *tadG*). The PI arithmetic mean of the strains classified as non-biofilm producers was significantly different ($p<0.05$) from the PI of moderate-producer strains. The PI of specimens classified as weak biofilm producers also differed significantly ($p<0.05$) from that of the moderate-producer strains. The results indicate that even though the *P. multocida* strains isolated from cases of fowl cholera and swine lungs formed biofilms on polystyrene surfaces, adhesion was usually weak. The genes *tadB*, *tadD*, *tadE* and *tadG* were not significantly associated ($p>0.05$) with the production of biofilms and with the origin of a given strain. Finally, low virulence strains may suggest a higher biofilm formation capacity on polystyrene plates.

INDEX TERMS: *Pasteurella multocida*, *tad* locus, biofilm, pathogenicity.

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RESUMO.- [**Avaliação da capacidade de formação de biofilme por cepas de *Pasteurella multocida* isoladas de casos de cólera aviária e de pulmões de suínos e sua relação com a patogenicidade.**] *Pasteurella multocida* é um bacilo Gram negativo que ocasiona perdas econômicas, geralmente associadas a doenças respiratórias em diversas

espécies animais. Entre os mecanismos de virulência existentes, a formação de biofilmes demonstra ser um importante fator para a proteção e para a sobrevivência bacteriana em ambientes hostis. Estudos relacionados à formação de biofilmes por *P. multocida* são necessários, uma vez que este é um importante patógeno envolvido em infecções respiratórias. Entretanto, ainda são poucos os estudos desenvolvidos nesta área, quando comparados com aqueles envolvendo outros microrganismos. Também são os raros os estudos que comparam a patogenicidade das cepas com a sua capacidade de produção de biofilme. Neste contexto, o objetivo deste estudo foi avaliar a capacidade de formação de biofilme em placas de poliestireno de 94 cepas de *P. multocida* isoladas de casos de cólera aviária e de pulmões de suínos, associando-se com o índice de patogenicidade (IP) *in vivo* e com a presença de quatro genes do *locus tad* (*tadB*, *tadD*, *tadE* e *tadG*), descritos como marcadores de adesão e pesquisados através de PCR. As cepas de ambas as origens foram capazes de formar biofilme. Contudo, a maioria dos exemplares (52,12%) foi classificada como fracamente produtora e mais de 40% das cepas de *P. multocida* (40,42%) não produziram biofilme. Não foi observada diferença estatística (p>0,05) quanto ao grau de produção de biofilme entre as duas origens de isolamento. 56,52% das cepas analisadas apresentaram os quatro genes (*tadB*, *tadD*, *tadE* e *tadG*) concomitantemente. O IP médio das cepas classificadas como não produtoras de biofilme apresentou diferença estatística (p<0,05) em relação ao IP das cepas moderadamente produtoras. Os exemplares classificados como fracamente produtores de biofilme diferiram significativamente (p<0,05) do grupo de cepas moderadamente produtoras. Os resultados obtidos indicaram que, apesar de as cepas de *P. multocida* isoladas de casos de cólera aviária e do pulmão de suínos apresentarem capacidade de formar biofilme em superfícies de poliestireno, a adesão ocorreu geralmente de forma fraca. Os genes *tadB*, *tadD*, *tadE* e *tadG*, pertencentes ao locus *tad*, não apresentaram associação significativa com a produção de biofilme e nem com a origem de isolamento da cepa. Por fim, observou-se que as cepas de menor patogenicidade apresentaram uma maior capacidade de formação de biofilme em placas de poliestireno.

TERMOS DE INDEXAÇÃO: *Pasteurella multocida*, *locus tad*, biofilme, patogenicidade

INTRODUCTION

Pasteurella multocida is a Gram-negative bacillus that causes economic losses associated with numerous respiratory diseases in poultry farms (Moraes et al. 2014) and, in some cases, the processing of animal products (Ribeiro et al. 2012). *P. multocida* causes fowl cholera (FC) as well as progressive atrophic rhinitis, pneumonia and serositis in swine, hemorrhagic septicemia in cattle and pasteurellosis in rabbits (Boyce et al. 2010, Ferreira et al. 2012, Wilkie et al. 2012). Sporadic cases of infections in humans are usually associated with scratches and bites caused by dogs and cats (Hunt Gerardo et al. 2001, Kawashima et al. 2010). These infections can range from chronic to fatal sepsis, as is generally observed in the cases of FC and hemorrhagic septicemia (Wilkie et al. 2012).

Among various virulence mechanisms, the formation of biofilms is an important factor for bacterial survival and protection in hostile environments, such as in tissue or on an inert surface, with exposure to light, desiccation, osmotic pressure and to pH and temperature variations (Costerton et al. 1995, 1999, Sutherland 1997). A biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric extracellular matrix that is attached to a biotic or abiotic surface (Costerton et al. 1995, Donlan & Costerton 2002, Hall-Stoodley & Stoodley 2009). Infections involving sessile microorganisms are often chronic and difficult to treat (Costerton et al. 1999). The microbe's ability to escape the host immune system under these conditions and to resist high antimicrobial concentrations facilitates the persistence of the microorganism (Lemon et al. 2008, Silva et al. 2014).

The National Institutes of Health (NIH) estimates that 75% of infections in humans result from the formation and persistence of biofilms (Richards & Melander 2009). Considering the extensive role of biofilms in human infection, it is probable that biofilms are also related to a variety of infections in animals (Clutterbuck et al. 2007, Freeman et al. 2009). The major bacteria with known potential for biofilm formation include representatives of the genera *Pseudomonas*, *Vibrio*, *Salmonella* and *E. coli* (Freeman et al. 2009, Rajagopal et al. 2013). Recent studies suggest that *P. multocida* can also form biofilms, including in swine lungs (Ross 2006, Rajagopal et al. 2013, Moraes et al. 2014). However, such studies are rare compared with those involving other organisms. In addition, biofilm formation is usually not compared to the pathogenicity of a given isolate.

The main structures associated with virulence that have been identified in *P. multocida* strains are the capsule and lipopolysaccharide (LPS). However, other factors are known to be important virulence markers in *P. multocida* (Wilkie et al. 2012). These factors include genes encoding outer membrane proteins (*ompH*, *oma87*), genes related to iron metabolism (*hgbA*, *hgbB*, *exBD-tonB*) and those encoding fimbriae and adhesins (*ptfA, pfhA, tad*) (Ewers et al. 2006, Corney et al. 2007, Hatfaludi et al. 2010). The genes of the *tad locus* have been described as bacterial adhesion markers. Homologous regions of the biosynthesis locus play an important role in biofilm formation, colonization and pathogenesis of other *Pasteurellaceae* members and some Gram-negative bacteria (Tomich et al. 2007, Moraes et al. 2014).

Because *P. multocida* is an important pathogen involved in respiratory infections, studies of biofilm formation are necessary. Biofilms can be an important virulence factor for bacterial survival in the host (Rajagopal et al. 2013). Consequently, the aim of this study was to evaluate the biofilm formation capacity of *P. multocida* isolates *in vitro* and the relationship of biofilm formation capacity with pathogenicity, as well to detect genes of the *tad locus* in these strains.

MATERIALS AND METHODS

***Pasteurella multocida* samples.** For this study, we selected 94 strains isolated from clinical cases of FC (n=55) and from swine lungs of healthy animals (n=39) at slaughter. All strains were stored in total sheep blood at a temperature of -80°C. Reactivation

and preliminary tests for the confirmation of the purity of *Pasteurella multocida* samples were performed as described by Glisson et al. (2008) and Furian et al. (2013).

The strains were reactivated in brain heart infusion (*Brain Heart Infusion* - BHI - Oxoid; Basingstoke, Hampshire, UK) broth and incubated at 37°C for 24 hours. After this period, the strains were plated on blood agar (Oxoid) supplemented with 5% defibrinated sheep's blood and on MacConkey agar (Oxoid), a culture medium in which *P. multocida* growth does not occur. The strains were then incubated again at 37°C for 24 hours. Subsequently, the morphology of the colonies present on blood agar was evaluated. Giemsa staining was used to observe the bipolar characteristics of the bacterial cells. Finally, evaluation of catalase and oxidase, in addition to biochemical tests using the Bactray III system (Laborclin; Pinhais, Paraná, Brazil), were performed. One colony was selected for DNA extraction using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). A PCR protocol for species--specific amplification of a 460-bp fragment of the *kmt* gene was performed, as described by Townsend et al (1998). Reference strains of *P. multocida* (ATCC 15742, ATCC 12945) were selected as positive controls.

Evaluation of biofilm formation capacity at 37°C. The methodology used was adapted from the studies of Stepanovic et al. (2000), Silva et al. (2014) and Borges (2015).

Initially, the strains were plated on TSA agar without glucose (Trypticase Soy Agar, Merck, Darmstadt, Germany) and incubated at 37°C for 24 hours. Subsequently, a colony of each sample was inoculated in TSB broth without glucose (Trypticase Soy Broth - TSB - Difco; Detroit, USA) and incubated at 37°C for 24 hours. After this period, the culture was diluted in TSB broth to achieve a concentration corresponding to 1 McFarland scale unit (Probac; São Paulo, Brazil), which indicates a concentration of approximately $3x10^8$ CFU/mL. Then, 200µL of the bacterial suspension from each sample was inoculated in triplicate in polystyrene 96-well flat-bottomed plates (Kasvi, Curitiba, PR, Brazil), including the strains selected as positive controls, and the plates were incubated at 37°C for 24 hours. One strain of *Salmonella enteritidis* and one strain of *Escherichia coli* in our lab previously classified as strong biofilm producers were selected as positive controls. The negative control was TSB broth without glucose, which was added to three wells of each plate.

After incubation, the bacterial suspensions were removed, and the plates were washed three times with 250 µL of sterile 0.9% sodium chloride solution to remove planktonic cells. Thereafter, 200µL of methanol (Nuclear; Diadema, SP, Brazil) was added to fix the adherent bacterial cells. The contents of the wells were removed after 15 minutes, and the plates were dried at room temperature. A 200-µL aliquot of 2% Hucker crystal violet solution was added to stain the fixed cells. After 5 minutes, the plates were washed in water and dried again at room temperature. To perform the reading, 200µL of 33% glacial acetic acid (Nuclear) was added, and the absorbance was measured 1 hour later by an ELISA reader at 550nm (Biotek; Winooski, USA). The absorbance value of each strain (DO_a) was obtained by calculating the arithmetic mean of the triplicate wells. The cutoff point was defined as three deviations above the absorbance mean of the negative control (DO_c). To determine biofilm formation capacity, the following classification was used: non-adherent ($DO_a \leq DO_c$), weakly adherent ($DO_c < DO_a \leq 2xDO_c$), moderately adherent ($2xDO_c < DO_a \leq 4xDO_c$) and strongly adherent ($4xDO_c < Do_a$).

Strain pathogenicity index (PI). The pathogenicity indices (PIs) of the 94 strains were associated with biofilm formation capacity at 37°C. The PIs were previously established by Pilatti et al. (2016). In this preliminary study, the strains were classified into three groups of pathogenicity (high, intermediate, low) on a scale from 0 to 10, calculated from the experimental inoculation of one--day-old chicks. The high-pathogenicity group comprised strains with PIs ranging from 8 to 10, the intermediate-pathogenicity group had PIs between 4 and 7, and the low-pathogenicity group included strains with indices from 0 to 3 (Pilatti et al. 2016).

DNA extraction and detection of *tad* locus genes. An aliquot of 1 mL of BHI broth inoculated overnight with each strain was used for DNA extraction using the commercial extraction kit NucleoSpin (Macherey Nagel; Düren, Germany). The extracted DNA was stored at -2 °C until analysis by PCR to detect the four genes of the tad locus (*tadB*, *tadD*, *tadE* e *tadG*). The oligonucleotides used were obtained from previous studies (Table 1)"

To amplify the *tadD* gene, we used the PCR protocol described by Furian et al. (2016). The amplification of the *tadB*, *tadE* and *tadG* genes was adapted from the protocol described by Moraes et al. (2014). The reaction mix was composed of 2. µL of 10X buffer, 0. µL of dNTPs (1 mM – Ludwig Biotec; Alvorada, Rio Grande do Sul, Brazil), 0. µL of primers (2 pmol – Invitrogen; Carlsbad, CA, USs), 1.5 U of GoTaq® Hot Start Polymerase (Promega; Madison, Wisconsin, USs), 1.2 µL of $MgCl_2$ (2 mM - Promega) and 17.5 µL of ultra-pure water.

The amplification reactions were performed in a Swift MaxPro thermocycler (Esco Technologies; Singapore) under the following conditions: initial denaturation at 9 °C for 5 minutes followed by 30 cycles of denaturation at 9 °C (30 seconds), annealing at 5 °C (30 seconds) and extension at 7 °C (60 seconds), and a final extension step at 7 °C (10 minutes). Electrophoresis of the amplified products was performed in agarose gels stained with 1.5% ethidium bromide. The gels were photo-documented (Alpha Innotech; San Leandro, California, USs) and then interpreted. The standard strains *P. multocida* ATCC 12945 and *Avibacterium gallinarum* ATCC 13360 were selected as positive and negative controls, respectively.

Statistical analysis. Descriptive statistical analysis was used to determine the absolute and relative frequency of virulence genes as well as the grouping of the samples according their biofilm formation capacity. The non-parametric chi-square ($\chi 2$) and Fisher's exact tests were used to analyze the distribution of the strains classified in each biofilm group. The nonparametric Kruskal-Wallis and Mann-Whitney tests were used to compare the means of the pathogenicity indices of strains with their biofilm formation capacity. The Statistical Package for Social Sciences (SPSS) was used for statistical analysis, adopting as a reference a significance level of 5% and a confidence level of 95%.

RESULTS

The *Pasteurell. multocida* strains isolated from both sources formed biofilms on polystyrene plates, as shown in Table 2. However, most of the specimens (52.13%) were classified as weakly adherent, and more than 40% of the strains (40.42%) did not produce biofilms. Although only swine

Table 1. Sequences of *tad* gene oligonucleotides and the respective amplicon sizes

Gene	Primers sequence (5'- 3')	Amplicon	Reference
tadB	TTCGCCTAATTGTCCCGTTA		
	TGGAAGTTAGGGCAATACCG	150 bp	Moraes et al. (2014)
tadD	TCTACCCATTCTCAGCAAGGC		
	ATCATTTCGGGCATTCACC	416 bp	Tang et al. (2009)
tadE	TGGATTCGTCCCAAGAGAAC		
	ATCTCTCCTACGGGGAGTCG	195 bp	Moraes et al. (2014)
tadG	AACTTGCCCAATTGTTCTCG		
	CCTTCTGGTTGGACTTCTGC	224 bp	Moraes et al. (2014)

strains were classified as moderate producers (Table 2), there was no significant difference (p>0.05) in the degree of biofilm production between the two isolate sources".

All PCR protocols were specific for the detection of *tad locus* genes. Of the 94 strains of *P. multocida*, 56.52% possessed all four genes analyzed (*tadB*, *tadD*, *tadE* and *tadG*).

Analysis of the frequencies of the genes according to the origin of isolation (Table 3) revealed that more than 80% of the avian and swine strains were positive for *tadB* and 100% were positive for *tadE* and *tadG*, regardless of source. In contrast, the *tadD* gene was detected in 50.91% of avian strains and 82.05% of the strains isolated from swine".

Based on the relative frequencies of the four genes studied, the relationship between the presence of these genes and bacterial adhesion was established. Of the avian strains positive for the four genes screened, 61.53% were classified as biofilm producers, including a strong producer strain. Among the strains isolated from swine, 57.68% of the strains that were positive for the four genes were classified as biofilm producers, including three classified as moderate producers. However, the absence of individual *tadB* and *tadD* genes was not significantly associated (p>0.05) with the variation in the biofilm formation capacity of the *P. multocida* strains.

Following the evaluation of biofilm formation and grouping of the samples, the analyzed strains were compared to their respective PIs (Table 4), which were previously obtained by an *in vivo* study (Pilatti et al. 2016). The mean PI of the *P. multocida* strains classified as non-biofilm producers differed significantly (p<0.05) from the mean PI of moderate biofilm producer strains (Fig 1). Similarly, the PIs of specimens classified as weak biofilm producers differed significantly from PIs of the moderate producer strains. However, there was no significant difference (p>0.05) between the PIs of the strains that did not produce biofilms and the PIs of weak biofilm producers (Fig 1).

DISCUSSION

Several pathogenic bacteria present in animals produce biofilms (Costerton et al. 1999). However, studies of the production of these structures by *Pasteurell multocida* strains are rare (Olson et al. 2002, Romanò et al. 2013).

Table 2. Classification of the 94 strains of *Pasteurella multocida* isolated from poultry and swine according to the degree of biofilm production at 37°C

Biofilm production classification	Avian (n=55)	Swine (n=39)
non-biofilm producers	25 (45.45%)	13 (33.33%)
weak biofilm producers	29 (52.73%)	20 (51.28%)
moderate biofilm producers	0 (0.00%)	6 (15.39%)
strong biofilm producers	1 (1.82%)	0 (0.00%)
all biofilm producers	30 (54.54%)	26 (66.67%)

Table 3. Absolute and relative frequency (%) of the four virulence genes (*tadB, tadD, tadE and tadG*) according to the origin of the *Pasteurella multocida* strain

Origin	Virulence genes			
	tadB	*tadD*	*tadE*	*tadG*
Avian (n=55)	46 (83.64)	28 (50.91)	55 (100%)	55 (100%)
Swine (n=39)	34 (87,18)	32 (82.05)	39 (100%)	39 (100%)

Table 4. Classification of the samples according to their biofilm formation, pathogenicity index and pathogenicity group

Sample	Host	Biofilm production classification	Pathogenicity index (PI)*	Groups of Pathogenicity*
1	Chicken	Non-biofilm producer	3.27	Low
2	Chicken	Non-biofilm producer	6.93	Intermediate
3	Chicken	Non-biofilm producer	5.07	Intermediate
4	Chicken	Non-biofilm producer	4.8	Intermediate
5	Chicken	Non-biofilm producer	6.43	Intermediate
6	Chicken	Non-biofilm producer	5.46	Intermediate
7	Chicken	Non-biofilm producer	4.88	Intermediate
8	Chicken	Non-biofilm producer	6.56	Intermediate
9	Chicken	Non-biofilm producer	10	High
10	Chicken	Non-biofilm producer	10	High
11	Chicken	Non-biofilm producer	10	High
12	Chicken	Non-biofilm producer	10	High
13	Chicken	Non-biofilm producer	10	High
14	Chicken	Non-biofilm producer	8.51	High
15	Chicken	Non-biofilm producer	10	High
16	Chicken	Non-biofilm producer	10	High
17	Chicken	Non-biofilm producer	8.69	High
18	Chicken	Non-biofilm producer	10	High
19	Chicken	Non-biofilm producer	10	High
20	Chicken	Non-biofilm producer	10	High
21	Chicken	Non-biofilm producer	8.6	High
22	Chicken	Non-biofilm producer	9.18	High
23	Chicken	Non-biofilm producer	8.93	High
24	Chicken	Non-biofilm producer	10	High
25	Chicken	Non-biofilm producer	7.75	High
26	Chicken	Weak biofilm producer	2.29	Low
27	Chicken	Weak biofilm producer	3.31	Low
28	Chicken	Weak biofilm producer	2.17	Low
29	Chicken	Weak biofilm producer	1.33	Low
30	Chicken	Weak biofilm producer	4.43	Intermediate
31	Chicken	Weak biofilm producer	7.44	Intermediate
32	Chicken	Weak biofilm producer	3.95	Intermediate
33	Chicken	Weak biofilm producer	5.63	Intermediate
34	Chicken	Weak biofilm producer	4.69	Intermediate
35	Chicken	Weak biofilm producer	5.17	Intermediate
36	Chicken	Weak biofilm producer	4.39	Intermediate
37	Chicken	Weak biofilm producer	4.56	Intermediate
38	Chicken	Weak biofilm producer	7.36	Intermediate
39	Chicken	Weak biofilm producer	7.9	High
40	Chicken	Weak biofilm producer	7.8	High
41	Chicken	Weak biofilm producer	7.57	High
42	Chicken	Weak biofilm producer	8.26	High
43	Chicken	Weak biofilm producer	9.13	High
44	Chicken	Weak biofilm producer	10	High
45	Chicken	Weak biofilm producer	7.82	High
46	Chicken	Weak biofilm producer	10	High
47	Chicken	Weak biofilm producer	7.93	High
48	Chicken	Weak biofilm producer	10	High
49	Chicken	Weak biofilm producer	8.06	High
50	Chicken	Weak biofilm producer	8.87	High
51	Chicken	Weak biofilm producer	8.44	High
52	Chicken	Weak biofilm producer	7.69	High
53	Chicken	Weak biofilm producer	8.13	High
54	Chicken	Weak biofilm producer	10	High
55	Chicken	Strong biofilm producer	2.99	Low
56	Swine	Non-biofilm producer	0.33	Low
57	Swine	Non-biofilm producer	2.42	Low
58	Swine	Non-biofilm producer	2.25	Low
59	Swine	Non-biofilm producer	5.81	Intermediate
60	Swine	Non-biofilm producer	4.93	Intermediate
61	Swine	Non-biofilm producer	5.44	Intermediate
62	Swine	Non-biofilm producer	5.24	Intermediate
63	Swine	Non-biofilm producer	3.79	Intermediate
64	Swine	Non-biofilm producer	4.73	Intermediate
65	Swine	Non-biofilm producer	3.5	Intermediate
66	Swine	Non-biofilm producer	3.98	Intermediate

(Cont.) Table 4. Classification of the samples according to their biofilm formation, pathogenicity index and pathogenicity group

Sample	Host	Biofilm production classification	Pathogenicity index (PI)*	Groups of Pathogenicity*
67	Swine	Non-biofilm producer	3.6	Intermediate
68	Swine	Non-biofilm producer	6.11	Intermediate
69	Swine	Weak biofilm producer	3.12	Low
70	Swine	Weak biofilm producer	2.1	Low
71	Swine	Weak biofilm producer	2	Low
72	Swine	Weak biofilm producer	1.17	Low
73	Swine	Weak biofilm producer	1.75	Low
74	Swine	Weak biofilm producer	2.6	Low
75	Swine	Weak biofilm producer	1.33	Low
76	Swine	Weak biofilm producer	1.33	Low
77	Swine	Weak biofilm producer	1.67	Low
78	Swine	Weak biofilm producer	6.77	Intermediate
79	Swine	Weak biofilm producer	4,89	Intermediate
80	Swine	Weak biofilm producer	7,5	Intermediate
81	Swine	Weak biofilm producer	6,31	Intermediate
82	Swine	Weak biofilm producer	6,65	Intermediate
83	Swine	Weak biofilm producer	6,42	Intermediate
84	Swine	Weak biofilm producer	7,2	Intermediate
85	Swine	Weak biofilm producer	7,25	Intermediate
86	Swine	Weak biofilm producer	10	High
87	Swine	Weak biofilm producer	10	High
88	Swine	Weak biofilm producer	8,27	High
89	Swine	Moderate biofilm producer	1,25	Low
90	Swine	Moderate biofilm producer	1,33	Low
91	Swine	Moderate biofilm producer	1,42	Low
92	Swine	Moderate biofilm producer	2,5	Low
93	Swine	Moderate biofilm producer	1,58	Low
94	Swine	Moderate biofilm producer	5,26	Intermediate

*Pillatti et al. (2016)

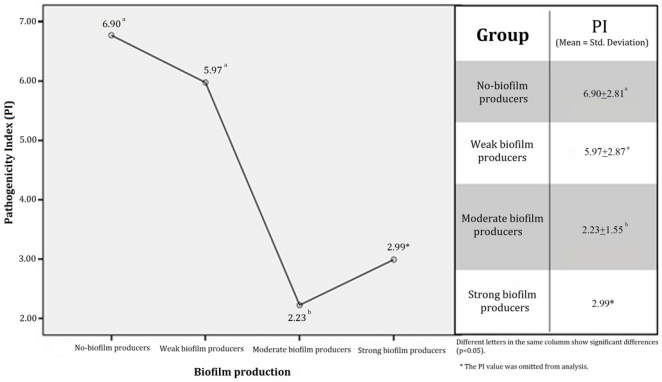

Fig.1. Distribution of *Pasteurella multocida* strains of avian and swine origin according to their biofilm formation and to their pathogenicity index (PI) calculated from the experimental inoculation of chicks.

Biofilm-forming bacteria adhere to practically all surfaces *in vitro* under suitable conditions. Among these conditions, the restriction of nutrients, such as TSB broth without glucose (used in the present study), is an important condition for the formation of biofilms (Christensen et al. 1982, Dewanti & Wong 1995). Furthermore, Olson et al. (2002) determined that TSB broth supplementation with 2% of fetal bovine serum and incubation in 10% CO_2 are necessary conditions for *P. multocida* biofilm formation. Such conditions should be evaluated in future studies for comparison with the results obtained here.

Several substrates, both hydrophobic and hydrophilic, are used to stimulate the fixation of bacteria *in vitro* (Rajagopal et al. 2013). The polystyrene selected in this study is an inert material often used to assess biofilm formation capacity in bacterial species (Silva et al. 2014, Borges 2015, Narayanan et al. 2016, Salimena et al. 2016). A recent study by Rajagopal et al. (2013) also reported the adhering ability of *P. multocida* to an inert surface formed by bentonite clay.

The potential of *P. multocida* strains to form biofilms was observed in this study by growing the organism under nutrient restriction and on polystyrene plates. Regardless of origin, the strains analyzed exhibited low levels of adhesion without strong biofilm production, as described for other bacteria, such as *Salmonella spp*. (Marquezini 2015) and *Pseudomonas aeruginosa* (Hassett et al. 2009). However, *P. multocida* did exhibit the ability to form biofilms. Thus, research on the adhesion capacity of microorganisms is essential because *P. multocida* is an important respiratory pathogen and the production of these structures could contribute to the increased virulence of the organism (Rajagopal et al. 2013).

It is also important to note that there are different ways to evaluate the production of biofilms, including the congo red agar method, bioluminescent assay, air-liquid interface, microscopy examination, among others (Freeman et al. 1989, Donlan et al. 2001, Hassan et al. 2011, Cabarkapa et al. 2015, Karched et al. 2015). A clear picture of attachment cannot be obtained without considering the effects of the substratum, conditioning films forming on the substratum, hydrodynamics of the aqueous medium, characteristics of the medium and various properties of the cell surface (Donlan 2002). Thus, the use of different quantitative or qualitative assessment methods may present different results, especially with *P. multocida*, and should be compared in the future.

The degree of biofilm formation was compared with the PI obtained for the same strain, which can be considered a measure of the *in vivo* pathogenicity of the isolate (Pilatti et al. 2016). Pathogenicity is related to the characteristics of a microorganism that are involved in the capacity to cause infection, whereas virulence determines the level of disease caused by a specific pathogenic microorganism (Kubatzky 2012). However, the low biofilm formation capacity of the pathogenic *P. multocida* strains examined in this study suggests that virulence factors are predominant in the process of infection. For example, the main *P. multocida* surface components, lipopolysaccharide and capsule, play important roles in the resistance to phagocytosis and the inhibition of complement and the activity of antimicrobial peptides (Wilkie et al. 2012). In addition, other virulence factors are involved in the survival of the microorganism in the infected host, and pathogenic bacteria generally have multiple adhesins to distinct cell types (Harper et al. 2006, Kline et al. 2009, Wilson & Ho 2013).

Biofilm formation may also function as a survival mechanism under adverse conditions in less virulent strains or with *P. multocida* involved in secondary infections. For example, *P. multocida* can colonize swine lungs in the form of biofilms, causing lesions that are generally difficult to tre-

at because of the blockade of antimicrobial action and the monocyte phagocytic system (Donlan & Costerton 2002, Moraes et al. 2014). Despite these results, the correlation of biofilm formation capacity with the pathogenicity of a given strain should be further evaluated in future studies with a larger number of strains classified as strong biofilm producers. This relationship was not possible to evaluate statistically in this study due to the presence of only a single strong biofilm producing strain.

In addition, the lesions evaluated through the experimental inoculation (Pilatti et al. 2016) involve mechanisms and virulence factors previously surveyed (Furian et al. 2016) that are not directly related to biofilm formation. For example, the widespread petechiae on serosal and epicardial surfaces of the inoculated animals were an indication of the consumptive coagulopathy common to endotoxemia (Boyce et al. 2010). Likewise, death after inoculation is probably associated with a massive bacteremia and endotoxic shock with the lysis and subsequent release of endotoxins that damage the host tissues (Harper et al. 2006). On the other hand, bacteria in biofilms can be viewed as biogenic habitat formers at a microscale (Flemming et al. 2016) and they are not directly related to these lesions. By generating a matrix, bacteria in biofilms create a physically distinct habitat that provides shelter, promotes the accumulation of nutrients and fundamentally alters both the physicochemical environment and interactions among the organisms therein (Flemming et al. 2016).

The genes of the *tad* locus belong to a group of putative adhesins (Hatfaludi et al. 2010). The Tad system, which is present in many bacterial genera, is the main subtype of the type III secretion system, and related genes encode proteins necessary for the formation of Flp fimbriae, which can be essential for biofilm formation (Tomich et al. 2007, Hatfaludi et al. 2010). Among the four genes selected for analysis, *tadE* and *tadG* were detected in 100% of FC and swine lung isolates. In contrast, the frequency of the *tadB* and *tadD* genes was variable depending on the source. Similarly, Moraes et al. (2014) detected the *tadA*, *tadB*, *tadC*, *tadE*, *tadF* and *tadG* genes in 100% of isolates from swine lungs, except for *tadD*, as reported in the current study.

However, the presence or the absence of the gene establishes a linear relationship among samples, which may not be true, since the expression and the interaction are not considered. Furthermore, several genetic factors participate in biofilm formation (Beloin & Ghigo 2005), which is influenced by environmental factors, such as pH, temperature and concentration of nutrients in the medium (Steenackers et al. 2012, Flemming et al 2016). Thus, these genetic factors, especially when they are involved in the early stages of biofilm formation, can be functionally replaced or overridden by others, depending on the media and growth conditions. Similarly, finding common bacterial biofilm gene-expression patterns through global expression analysis remains difficult (Beloin & Ghigo 2005).

The *tadD* gene encodes an adhesin with a non-specific adhesion pattern (May et al. 2001) and was previously investigated in genetic profile analysis studies of *P. multocida* strains isolated from swine, cattle and poultry (Tang et al. 2009, Katsuda et al. 2013, Moraes et al. 2014, Furian et al. 2016). These studies also highlight the presence of variations in the gene and its positive association with *P. multocida* serotype A (Tang et al. 2009) and negative association with serogroup D, as previously observed in a study by our group (Furian et al. 2016).

Although *tadD* is in a locus associated with the formation of biofilms, including the presence of biofilms in lung lesions in swine and cattle (Khamesipour et al. 2014, Moraes et al. 2014), the presence of *tadD* in individual strains in the current study was not significantly associated with biofilm formation capacity. This finding is probably explained by the presence of other genes of the same locus related to the formation of biofilms and mutations in the *tadD* gene that interfere with the adhesion potential of *P. multocida* (Fuller et al. 2000).

CONCLUSIONS

The evaluation of the biofilm formation capacity of *Pasteurella multocida* strains isolated from cases of FC and swine lung after slaughter demonstrated that the adhesion of these strains was usually weak, despite their ability to form biofilms on polystyrene surfaces.

The *tadB*, *tadD*, *tadE* and *tadG* genes of the *tad* locus were simultaneously detected in most of the analyzed strains, and a significant association was not observed ($p>0.05$) between the presence of these genes and the origin of the strain.

Similarly, there was no association ($p>0.05$) between the presence of the *tadB* or *tadD* genes and the biofilm formation capacity of the strains. Finally, low virulence strains may suggest a higher capacity for biofilm formation on polystyrene plates.

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