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Canine Cystitis - Biofilm Formation by Bacterial Isolates

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ABSTRACT

***Background*:** Biofilms have been reported as important virulent markers associated with drug resistance in urinary tract infections (UTIs) in humans and dogs. However, in veterinary medicine, researches involving biofilm formation, treatments and preventions have been limited; yet, it is still possible to find few studies demonstrating biofilm-forming bacteria associated with different comorbidities such as otitis, wound infections, UTIs, and endometritis. These studies generally select dogs with chronic and recurrent infections, which could be an important factor in antibiotic resistance. We aimed to evaluate biofilms in sporadic cystitis regarding prevalence and drug resistance.

***Materials, Methods & Results*:** Urine samples were collected by cystocentesis from 36 client-owned dogs under clinical and laboratory suspicion of non-recurrent urinary bladder infection (cystitis). Urine was aseptically plated onto blood agar, MacConkey, and CLED, followed by incubation for 24 to 48 h. Definitive identification of a potential pathogen was made by subculture collected from an isolated colony to obtain a pure culture. The gram staining method and specific biochemical tests (phenol red fermentation, lysine, phenylalanine, citrate, sulfide-indole-motility, and urease) were used to distinguish and classify the bacteria. After identification, the bacteria were tested for antimicrobial susceptibility by a standard disk diffusion method, using the following antimicrobials: amoxicillin with clavulanic acid, ampicillin, ceftriaxone, ciprofloxacin, clindamycin, cefazolin, cephalothin, erythromycin, gentamicin, norfloxacin, and sulfamethoxazole-trimethoprim. The biofilm-forming ability was determined based on a culture in Congo red agar (CRA), where biofilm producer strains formed black colonies with a dry crystalline surface, while non-biofilm producer strains formed red colonies with a smooth surface. A crystal violet dye assay was used to confirm the CRA results. Of the 36 urine samples collected from dogs with suspected cystitis, a total of 37 isolates were obtained, from mixed or pure cultures. The most prevalent bacteria were *Escherichia coli* (11/37), followed by *Staphylococcus* spp. (8/37), *Proteus* spp. (7/37), and *Enterococcus* spp. (5/37). Other less prevalent bacteria were *Klebsiella* spp., *Streptococcus* spp., and *Enterobacter* spp. As for biofilm-forming ability, 67.6% (25/37) of the bacterial isolates had biofilm formation in CRA and 54.05% (20/37) on the microplates containing crystal violet dye. There was no statistical difference in antimicrobial susceptibility between biofilm producer and non-biofilm producer bacteria.

***Discussion*:** We found a high proportion (> 54%) of *in vitro* biofilm-forming ability by different bacteria, which may indicate that biofilms may also be formed *in vivo*, in simple cystitis. Antimicrobial resistance was not noticed in bacteria capable of forming a biofilm; however, in a future study it is important to evaluate bacterial resistance *in vivo*, considering the possibility of having a different response than *in vitro*. In addition, the problem of the presence of a biofilm *in vivo* is that it can nullify the antimicrobial efficacy of therapeutic agents even with *in vitro* susceptibility. Besides the possibility of slow or incomplete diffusion of antibiotics through the extracellular matrix of the biofilm, aspects like hydration level, pCO_2, pO_2, pH, pyrimidine, and divalent cation concentration that negatively influence antimicrobial activity *in vitro* can also cause undesirable effects at the profound layers of the biofilm. In conclusion, all of the genera of bacteria isolated from dog's sporadic cystitis in this study were able to form a biofilm *in vitro*. The pathogenicity and antibiotic resistance of bacteria appears unrelated to biofilm formation *in vitro*.

Keywords: sessile bacteria, urine, simple cystitis, antibiotic resistance.

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INTRODUCTION

Biofilms have been described as important virulent markers attributed to the development of urinary tract infections (UTIs) in humans and dogs [6,8,18]. This complex group of microorganisms surrounded by an extracellular polymeric matrix facilitates adherence to the abiotic or biotic surfaces and microbial aggregation, it also makes the organism more virulent and is often associated with drug resistance [8,12].

The ability of bacteria to produce a biofilm is often associated with drug resistance [15,19,20]. Nevertheless, this can be contradicted by studies that show that not all bacteria able to form a biofilm necessarily demonstrate multidrug resistance [1,4].

In veterinary medicine, researches involving biofilm formation, treatments and preventions have been limited; however, it is still possible to find few studies demonstrating biofilm-forming bacteria associated with different comorbidities such as otitis, wound infections, UTIs, and endometritis [5,6,14,17]. These studies generally select dogs with chronic and recurrent infections, which could be an important factor in antibiotic resistance. We aimed to evaluate biofilms in sporadic cystitis regarding prevalence and drug resistance.

MATERIALS AND METHODS

Animals and samples

Urine samples were collected by cystocentesis from 36 client-owned dogs of different breeds, sex, and age treated at the Small Animal Clinic of the Veterinary Hospital at the Federal University of Uberlândia (HV-UFU), in Uberlândia, MG, Brazil. The dogs were under clinical (dysuria, hematuria, and anorexia were common clinical signs noted) and laboratory suspicion, based on urinalysis, of non-recurrent urinary bladder infection (cystitis).

Bacterial frequency, biofilm-forming ability, and antimicrobial susceptibility tests of the urine samples were analyzed at the Infectious Disease Laboratory of UFU.

Urine culture

According to the chosen literature, bacteria were isolated and identified [13]. Urine was aseptically plated onto blood agar[1], MacConkey[1], and cystine lactose eletrolyte deficient (CLED)[1], followed by incubation for 24 to 48 h. Definitive identification of a potential pathogen was made by subculture collected from an isolated colony to obtain a pure culture. The gram staining method and specific biochemical tests (phenol red fermentation, lysine, phenylalanine, citrate, sulfide-indole-motility, and urease) were used to distinguish and classify the bacteria.

Antimicrobial susceptibility test

After identification, the bacteria were tested for antimicrobial susceptibility using the Kirby-Bauer disk diffusion method following the Clinical and Laboratory Standards Institute guidelines [3]. Tests were performed using the following antimicrobials (all Sensifar-Vet®)[2]: amoxicillin with clavulanic acid (AMC), ampicillin (AMP), ceftriaxone (CRO), ciprofloxacin (CIP), clindamycin (CLI), cefazolin (CFZ), cephalothin (CFL), erythromycin (ERY), gentamicin (GEN), norfloxacin (NOR), and sulfamethoxazole-trimethoprim (SUT).

It should be noted that cephalothin and cefazolin were used only for gram-negative bacteria, and clindamycin and erythromycin only for gram-positive bacteria.

Congo red agar and crystal violet assay to assess biofilm-forming capability

The biofilm-forming ability was determined based on a Congo red agar (CRA)[3], inoculating and incubating the agar plates at 37ºC for 24 h. A crystal violet dye assay was used to confirm the CRA results.

The ability to produce biofilm *in vitro* by those bacteria that formed black colonies in CRA was confirmed by a 96-well microplate[1] test using crystal violet dye[4].

Bacteria were cultured individually for 12 h in brain heart infusion (BHI) broth[1] at 37ºC. The cell suspension was then inoculated onto sterile 96-well polystyrene microplates, diluted at 1:200 in tryptic soy broth (TSB) containing 0.25% glucose[5], and incubated for 24 h at 37ºC under agitation and renewal of the medium after 12 h [2].

After 24 h, the wells were washed 3 times with 200 µL of sterile phosphate-buffered saline (PBS, pH 7.4), immediately followed by the addition of 200 µL methanol for fixation, after which the plate was oven dried at 37ºC. Then, 200 µL of 1% crystal violet dye

was added for 5 min. The plates were then washed with distilled water and, after drying, 200 μL of 33% acetic acid[6] was added and the 96-well microplates were evaluated for absorbance by determining the optical density of each well at a wavelength of 492 nm (Multiskan GO)[7].

Uninoculated tryptic soy broth (TSB)[1] with glucose was used as a negative control. Each strain was tested in triplicate and the test repeated twice. The mean was determined by averaging the proportion of each isolate individually. Strains with absorbance readings higher than 0.1 were considered to be biofilm formers.

Statistics

Descriptive statistics, as well as frequency distribution variables, were used for each group. Drug resistance variables were compared between biofilm and non-biofilm bacteria using the Mann-Whitney test for non-parametric variables. *P*-values of < 0.05 were considered significant (GraphPad Prism. Version 6.0 for Windows)[8].

RESULTS

Of the 36 urine samples collected from dogs with suspected cystitis, a total of 37 isolates were obtained, from mixed or pure cultures. The most prevalent bacteria were *Escherichia coli* (11/37), corresponding to 29.8% of the total. *Staphylococcus* spp. (8/37) were identified in 21.6% of the isolates, *Proteus* spp. (7/37) in 18.9%, and *Enterococcus* spp. (5/37) in 13.5% of the isolates. Other less prevalent bacteria were *Klebsiella* spp., *Streptococcus* spp., and *Enterobacter* spp.

The biofilm producer strains formed black colonies with a dry crystalline surface, while the non-biofilm producer strains formed red colonies with a smooth surface [7] (Figure 1).

As for biofilm-forming ability, 67.6% (25/37) of the 37 bacterial isolates had biofilm formation in CRA and 54.05% (20/37) on the microplates containing crystal violet dye, as shown in Table 1.

The antimicrobial susceptibility was analyzed, as shown in Table 2. No statistically significant differences were found between the population of biofilm producer and non-biofilm producer bacteria.

Table 1. Distribution of bacterial isolates from urine samples collected from dogs with cystitis and evaluation of biofilm formation on Congo Red Agar and Microplate test with violet crystal dye.

Bacteria	Total	Biofilm-forming ability	
		Congo red agar	Microplates containing crystal violet dye
		n	n
Escherichia coli	11	7	5
Staphylococcus spp.	8	7	5
Proteus spp.	7	1	1
Enterococcus spp.	5	5	4
Klebsiella spp.	3	2	2
Streptococcus spp.	2	2	2
Enterobacter spp.	1	1	1
Total of isolates	37	25	20

Table 2. Total of biofilm producer and non-biofilm producer of each bacteria and profile of antimicrobial resistance of each antibiotic used in biofilm former and non-biofilm former bacteria isolated from urine collected from dogs with cystitis.

Escherichia coli (Biofilm: 5; Non-biofilm: 6)						
Antibiogram	Resistant		Intermediate		Susceptible	
	Biofilm	Non-biofilm	Biofilm	Non-biofilm	Biofilm	Non-biofilm
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
AMC	0 (0.0)	2 (33.3)	3 (60.0)	0 (0.0)	2 (40.0)	4 (66.7)
AMP	3 (60.0)	2 (33.3)	0 (0.0)	0 (0.0)	2 (40.0)	4 (66.7)
CFL	3 (60.0)	5 (83.3)	0 (0.0)	1 (16.7)	2 (40.0)	0 (0.0)
CFZ	1 (20.0)	4 (66.7)	0 (0.0)	0 (0.0)	4 (80.0)	2 (33.3)
CRO	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	5 (100.0)	4 (66.7)
CIP	2 (40.0)	2 (33.3)	0 (0.0)	2 (33.3)	3 (60.0)	2 (33.3)
GEN	0 (0.0)	1 (16.7)	1 (20.0)	0 (0.0)	4 (80.0)	5 (83.3)
NOR	2 (40.0)	1 (16.7)	0 (0.0)	0 (0.0)	3 (60.0)	5 (83.3)
SUT	2 (40.0)	3 (50.0)	1 (20.0)	0 (0.0)	2 (40.0)	3 (50.0)
Staphylococcus spp. (Biofilm: 5; Non-biofilm: 3)						
Antibiogram	Resistant		Intermediate		Susceptible	
	Biofilm	Non-biofilm	Biofilm	Non-biofilm	Biofilm	Non-biofilm
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
AMC	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (100.0)	3 (100.0)
AMP	5 (100.0)	3 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CRO	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (80.0)	3 (100.0)
CIP	1 (20.0)	1 (33.3)	0 (0.0)	1 (33.3)	4 (80.0)	1 (33.3)
CLI	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (80.0)	3 (100.0)
ERY	1 (20.0)	0 (0.0)	1 (20.0)	0 (0.0)	3 (60.0)	3 (100.0)
GEN	2 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (60.0)	3 (100.0)
NOR	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	5 (100.0)	2 (66.7)
SUT	4 (80.0)	2 (66.7)	0 (0.0)	0 (0.0)	1 (20.0)	1 (33.3)
Proteus spp. (Biofilm: 1; Non-biofilm: 6)						
Antibiogram	Resistant		Intermediate		Susceptible	
	Biofilm	Non-biofilm	Biofilm	Non-biofilm	Biofilm	Non-biofilm
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
AMC	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (100.0)	5 (83.3)
AMP	1 (100.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	5 (83.3)
CFL	0 (0.0)	1 (16.7)	0 (0.0)	1 (16.7)	1 (100.0)	4 (66.7)
CFZ	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	1 (100.0)	5 (83.3)
CRO	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	6 (100.0)
CIP	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	6 (100.0)
GEN	1 (100.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	5 (83.3)
NOR	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	6 (100.0)
SUT	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (100.0)
Enterococcus spp. (Biofilm: 4; Non-biofilm: 1)						
Antibiogram	Resistant		Intermediate		Susceptible	
	Biofilm	Non-biofilm	Biofilm	Non-biofilm	Biofilm	Non-biofilm
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
AMC	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (100.0)	1 (100.0)
AMP	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (100.0)
CRO	3 (75.0)	0 (0.0)	1 (25.0)	1 (100.0)	0 (0.0)	0 (0.0)
CIP	1 (25.0)	1 (100.0)	1 (25.0)	0 (0.0)	2 (50.0)	0 (0.0)
CLI	4 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ERY	2 (50.0)	0 (0.0)	2 (50.0)	1 (100.0)	0 (0.0)	0 (0.0)
GEN	1 (25.0)	0 (0.0)	2 (50.0)	1 (100.0)	1 (25.0)	0 (0.0)
NOR	2 (50.0)	1 (100.0)	1 (25.0)	0 (0.0)	1 (25.0)	0 (0.0)
SUT	4 (100.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

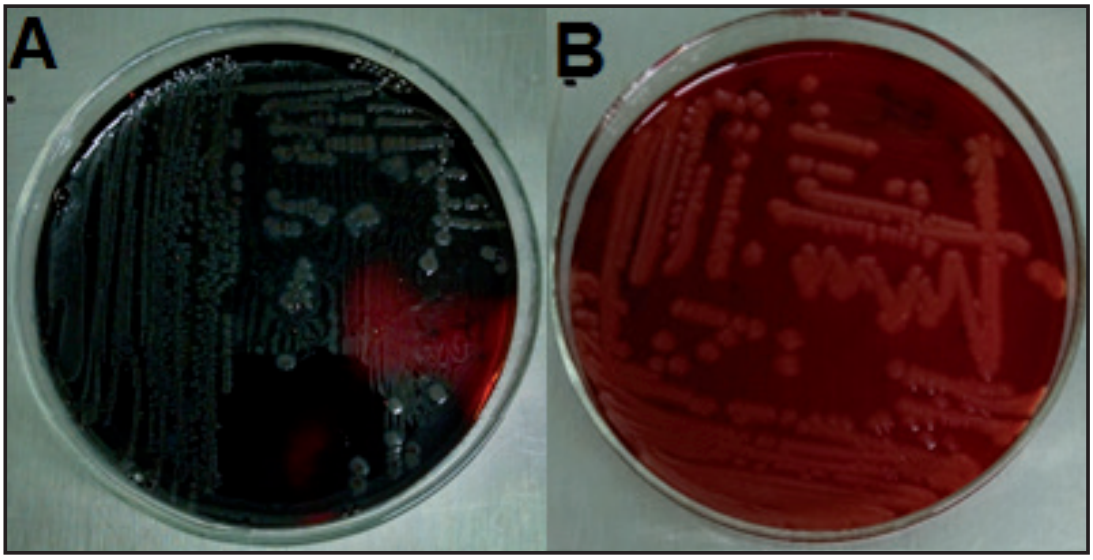

Figure 1. A- *Escherichia coli* isolated from canine urine, biofilm producer, forming black colonies with a dry crystalline surface on Congo Red Agar. B- *Proteus mirabilis* isolated from canine urine, non-biofilm producer, forming red colonies with smooth surface on Congo Red Agar.

DISCUSSION

Most studies in humans have shown that biofilm producing bacteria account for up to 80% of all infections, with the urinary system being one of the most affected in which biofilms are able to cause serious issues [16] and are often associated with drug resistance [8,12]. Our results showed that biofilms could not be associated with drug resistance and have important prevalence even in sporadic cystitis.

In this study, we identified similar bacteria to other research [10] that analyzed recurrent cystitis, where the most prevalent bacteria were *Escherichia coli*, followed by *Proteus* spp., *Staphylococcus* spp., *Enterococcus* spp., and others.

Regarding the assays to assess biofilm-forming capability, CRA assays was chosen due to their rapid technique that demonstrates the capacity of the strain to form a biofilm by screening of matrix production in bacteria through the connection of Congo red with polysaccharides, producing a colorful complex [8]. The crystal violet dye binds to negative charges and quantifies the total biofilm biomass by the attraction to the extra polymeric substances and the bacteria [11]. Although there is no standardized method to identify biofilm formation, this has been used in many studies [8] and could be used routinely.

We found a high proportion (> 54%) of *in vitro* biofilm-forming ability, which may indicate that biofilms may also be formed *in vivo*, in simple cystitis. Antimicrobial resistance was not noticed in bacteria capable of forming a biofilm; however, in a future study it is important to evaluate bacterial resistance *in vivo*, considering the possibility of having a different response than *in vitro*.

Other research [9] corroborates our findings, where the authors noticed that non-biofilm-forming *E. coli*, isolated from canine urine, had a higher tendency to multidrug resistance (MDR) in comparison to biofilm-forming *E. coli*. Other studies showed no relation between drug resistance and biofilm formation; like where the authors isolated different bacteria from blood, urine, and respiratory samples from hospitals and concluded that MDR isolates do not demonstrate a trend to being greater biofilm producers than non-MDR isolates [1]. Another study [4], analyzed antibiotic susceptibility and biofilm production in 135 clinical isolates from 87 patients and verified that a comparable level of biofilm production was found with both multidrug-resistant organisms (MDRO) and not-MDRO, with no significant differences between groups.

However, the problem of the presence of biofilms *in vivo* is that they can nullify the antimicrobial efficacy of therapeutic agents even with *in vitro* suscep-

tibility. Besides the possibility of slow or incomplete diffusion of antibiotics through the extracellular matrix of the biofilm, aspects like hydration level, pCO_2, pO_2, pH, pyrimidine, and divalent cation concentration that negatively influence antimicrobial activity *in vitro* can also cause undesirable effects at the profound layers of the biofilm [12].

CONCLUSIONS

Escherichia coli, *Staphylococcus* spp., *Proteus* spp., *Enterococcus* spp., *Klebsiella* spp., *Streptococcus* spp., and *Enterobacter* spp. were the main bacteria isolated from the urine of dogs with sporadic cystitis and all of the genera of bacteria isolated in this study were able to form biofilms *in vitro*. The results show that it is possible to have biofilm formation *in vitro* by different bacteria from the urinary tract of dogs with sporadic cystitis. The pathogenicity and antibiotic resistance of bacteria seem unrelated to biofilm formation *in vitro*.

MANUFACTURERS

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