B-4923
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RESEARCH ARTICLE Indian Journal of Animal Research

Detection of Biofilm Formation by *Escherichia coli* and *Staphylococcus aureus* Associated with Canine Pyometra

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ABSTRACT

Background: Antibiotic resistance is one of the major problems encountered in the therapy of canine pyometra. The ability of bacteria to form biofilm is implicated as one of the factors responsible for this. *Escherichia coli* and *Staphylococcus aureus* are the predominant bacteria associated with pyometra in canines and are known for their biofilm formation. Keeping in this view, a preliminary study was conducted to detect the biofilm forming strains of *E. coli* and *S. aureus,* if any, associated with canine pyometra.

Methods: A total of 25 samples were collected, which included uterine discharges from cases of closed pyometra and anterior vaginal swabs from open pyometra. The isolates of *E. coli* and *S. aureus* were identified based on the cultural, morphological and biochemical characteristics. These isolates were subjected to antibiotic sensitivity test employing disc diffusion method. For biofilm detection, the isolates were screened by Congo red agar method, tube method and tissue culture plate method.

Result: From 25 samples, two *Streptococcus* spp., thirteen *Staphylococcus* spp., *seven E.coli*, five *Klebsiella* spp. and two *Pseudomonas* spp. were isolated. All the isolates were found to be multi-drug resistant on antibiogram. The tissue culture plate and Congo red agar method was found more sensitive to detect the biofilm formation by *S. aureus* and *E.coli* isolates, respectively. The biofilm forming strains showed higher degree of antibiotic resistance in comparison with non-formers, indicating it as one of the major reasons for failure of antibiotic therapy in canine pyometra.

Key words: Antibiotic resistance, Biofilm detection, Canine pyometra.

INTRODUCTION

Pyometra is the most common reproductive emergency in veterinary medicine and is a serious and life threatening condition seen in middle-aged and old bitches. The most common infectious cause of pyometra is of bacterial origin and the predominant bacterium associated is *Escherichia coli*. The other organisms implicated include *Staphylococcus aureus*, *Streptococcus* spp., *Pseudomonas* spp. and *Proteus* spp. (Rautela and Katiyar, 2019). The medical management of pyometra is possible, but the current treatment protocols are costly, time consuming and include risk. In some cases, even after successful medical management, re-occurrences were noticed on subsequent cycle (10%-40%) and within 2 years (77%) (Fieni *et al*., 2014). One possible explanation for this may be the ability of microorganisms to form biofilm *in vivo*.

Biofilm is a thick layer consisting of extra cellular polymeric substances. It protects the microbes from harsh external conditions such as nutritional deprivation, pH changes, disinfectants *etc* and also helps to evade host immune system and the treatments directed against the infection. Studies on pyometra showed that *E. coli* and *S. aureus* isolates associated with pyometra could produce biofilm that might contribute to antibiotic resistance and failure of therapy (Fiamengo *et al*., 2020). Biofilm forming organisms will adhere on infected areas and form a matrix, which inhibit the penetration of antimicrobial agents (Rose and Poppens, 2009). Today biofilm formation by pathogenic bacteria is emerging as a great barrier in antibiotic therapy.

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How to cite this article: Amrutha, M.S., Sankar, S., Mani, B.K., Harshan, H.M., Vidya, P. and Paulson, S.T. (2022). Detection of Biofilm Formation by *Escherichia coli* and *Staphylococcus aureus* Associated with Canine Pyometra. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4923.

Submitted: 26-04-2022 **Accepted:** 12-10-2022 **Online:** 14-11-2022

Very few literatures are available on the biofilm forming ability of *E. coli* and *S. aureus* associated with pyometra in companion animals like dogs. The detection of biofilm forming organisms, if present, in canine pyometra would be of great help in modifying the existing therapeutic protocols and also spur the development of new therapeutic approaches aiming at increasing treatment efficacy and minimizing treatment length and side-effects. In addition, the pathogens with potential for biofilm formation could be transferred to humans via close contact with their companion animals and may contribute to the problem of antibiotic resistance.

MATERIALS AND METHODS

A total of 25 samples were collected from bitches, which included anterior vaginal swab in case of open pyometra and uterine discharges from closed pyometra (during ovario hysterectomy) during the year 2021. Samples were collected from Kerala Veterinary and Animal Sciences University Veterinary Hospitals. For isolation, Brain Heart Infusion Agar (BHIA), MacConkey Agar (MAC), Eosin Methylene Blue Agar (EMB) and Mannitol Salt Agar (MSA) were used. The isolates were identified based on the cultural, morphological and biochemical characteristics (Quinn *et al*., 1994). Mueller-Hinton agar (MHA) was used for antibiotic susceptibility testing employing Kirby-Bauer disc diffusion method (CLSI, 2018). The following antibiotic discs with known concentration in microgram (mcg) or international unit (IU) per disc were used (Table 1).

Biofilm for ming potential of *S. aureus* and *E. coli* isolates were determined qualitatively by Congo red agar method and tube method and quantitatively assessed by tissue culture plate method.

Congored agar method

The method described by Freeman *et al.* (1989) was followed. Black colonies with a dry crystalline consistency indicated biofilm production.

Tube method

The method proposed by Christensen *et al.* (1985) was used. The scoring for tube method was done according to the result of control strains. Biofilm formed was scored as 1- weak/ none, 2-moderate and 3- high/strong.

Tissue culture plate method

This quantitative method was described by Christensen *et al*. (1995). Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA auto reader at wavelength 570 nm. The isolate showing average OD value less than 0.120 was considered as non-biofilm producer, the average OD value between 0.12 and 0.24 was considered as moderate biofilm producers, while the value more than 0.24 was considered as strong biofilm producers.

RESULTS AND DISCUSSION

All samples were cultured on to BHIA, MacConkey, EMB and MSA. Based on the colony characters, Gram's staining and biochemical characteristics, 15 isolates were Gram positive cocci (2 *Streptococcus* and 13 *Staphylococcus*) and14 were Gram negative bacilli (7*E. coli*, 5 *Klebsiella* spp. and 2 *Pseudomonas* spp.). Out of 25 samples, eight *S. aureus* and seven *E. coli* isolates were obtained based on the cultural, morphological and biochemical characteristics. In our study, *S. aureus* (32%) was the most prevalent organism, followed by *E.coli* (28%). Similar observations were made by Khan *et al*. (2007), who isolated 66.66% Gram positive organisms and 33.33% Gram negative organisms from pyometra cases. Among these, the predominant organisms isolated were *Staphylococcus* spp. followed by *E. coli*. Singathia *et al.* (2013) reported similar observations. As per Niyas *et al.* (2020), in majority of cases, *S. aureus* and *E. coli* were the causative agents associated with pyometra.

Table 1: Antibiotic concentration present in the discs used for antibiogram.

Antibiotics	Concentration/Disc
Amoxycillin-clavulanicacid	30 mcg
Cefotaxime	30 mcg
Ceftriaxone	30 mcg
Ceftriaxone-tazobactam	30 mcg
Co-trimoxazole	30 mcg
Ciprofloxacin	30 mcg
Enrofloxacin	10 mcg
Gentamicin	30 mcg
Metronidazole	5 mcg
Tetracycline	30 mcg

The isolates were subjected to antibiogram using the common antibiotics employed for the therapy of pyometra. Fifty per cent of *S. aureus* isolates showed sensitivity to tetracycline, followed by enrofloxacin (37.5%) and co-trimoxazole (25%), but 100 per cent resistance to amoxy-clav, ceftriaxone, ceftriaxone-tazobactam, ciprofloxacin, gentamicin and metronidazole. All the isolates were found to be multidrug resistant, showing resistance to at least two classes of antibiotics. Similar results were documented by Mustapha *et al*. (2020), who isolated *E. coli*, *S. aureus*, *Pseudomaonas* spp. and *Streptococcus* spp. from uterus of the dogs with open cervix-pyometra. Both Gram positive and Gram negative isolates were more sensitive to enrofloxacin. Maity *et al*. (2009) isolated *S. aureus* from cases of pyometra followed by *Proteus* spp., *E. coli* and *Klebsiella* spp. and the isolates were found sensitive to gentamicin, enrofloxacin, ciprofloxacin, ceftriaxone, chloramphenicol and oxytetracycline. Lee *et al*. (2000) isolated *E. coli*, *Serratia marcescens*, *S. aureus* and *Salmonella* from pyometra cases. Isolates were more susceptibile to enrofloxacin, followed by norfloxacin, nalidixic acid, chloramphenicol, trimethoprim-sulfamethazole, tetracycline and gentamicin.

Escherichia coli were sensitive to tetracycline (71%) followed by enrofloxacin (57%) and co-trimoxazole (37.5%) respectively and showed resistance against ceftriaxone-tazobactam (85.7%) followed by amoxy-clav, cefotaxime, ceftriaxone, ciprofloxacin and metronidazole (100%). Here also, multidrug resistance could be observed in all the isolates. Similar observations are reported by Siqueira *et al*. (2009), where *E.coli* was isolated from cases of UTI, pyometra and feces of dogs and the isolates showed sensitivity to norfloxacin, ciprofloxacin and enrofloxacin. Multidrug resistance could be observed in 13.5 percent of isolates. Hagman and Greko (2005) isolated *E. coli* from most cases of pyometra and reported that the isolates showed low resistance to enrofloxacin (4%), tetracycline (4%), ampicillin (10%), gentamicin (0%), streptomycin (5%), sulfamethoxazole (8%) and trimethoprim (2%). Similarly, in

the present study, both *S. aureus* and *E. coli* isolates were found sensitive to enrofloxacin and tetracycline. Approximately similar observations reported by Serafini *et al*. (2020). In their studies, both Gram positive and Gram negative isolates were sensitive to enrofloxacin (100%), followed by cephalexin (30%) and resistant to pencillin (90%) followed by ampicillin (80%).

The isolates were then subjected to different biofilm detection methods which included Congo red agar method, tube method and tissue culture plate method. Similar methods were used by Atshan and Shamsudin (2011) and Vasanthi *et al*. (2014).

Congo red agar method is considered as a qualitative method for biofilm detection and in the present study, out of eight *S. aureus*, two isolates (25%) produced strong biofilm, two (25%) showed moderate biofilm formation and weak or non-biofilm producers were four (50%). This was similar to the observation made by Mathur *et al*. (2006), who documented that out of eight *Staphylococcus* isolates, three showed (5.26%) positive biofilm formation. In a study, Sohail *et al*. (2018) observed that 50% of *S. aureus* were found to be weak, 27% were moderate and 23%were strong biofilm producers in Congo red agar method. Present study showed that sensitivity of Congo red agar method in detecting biofilm producers was very low for *S. aureus* isolates. Similar observations were made by Bose *et al*. (2009). Nasr *et al.* (2012) reported that even though Congo red agar method was found to be the easier method for biofilm detection, it was not suitable for biofilm detection in *S aureus* isolates.In case of *E. coli*, all the isolates revealed strong biofilm production with Congo red method. Nachammai *et al*. (2016) reported that 70% *E. coli* isolated from pyometra cases showed positive results in Congo red agar method and he documented that results of Congo red agar method did not correlate well with other two methods. Similar results were observed by Dadawala *et al*. (2010), where out of 14 *E. coli* isolates, 12 were detected as biofilm producers with Congo red method. Dhanalakshmi *et al*. (2018), in their studies documented that Congo red method and tube method showed more sensitivity compared to tissue culture plate method.

In tube method, two (25%) out of eight *S. aureus* produced strong biofilm, four isolates (50%) were found to be moderate biofilm producers and two (25%) were weak or non-biofilm producers. The findings were similar to the observation of Hassan *et al* (2011), who reported that 19% of isolates were strong biofilm producers. Sharvari and Chithra (2012) could identify 20.5 per cent strong biofilm producers. These findings were inaccordance with our results. Out of seven *E. coli* isolates, one isolate (14.2%) produced strong biofilm, two (28.5%) were identified as moderate biofilm producers and four (57.14%) were noticed as weak or non-biofilm producers. The results were similar to the findings of Ponnuswamy *et al*. (2012), who analysed *in vitro* biofilm formation of ~~e~~uropathogenic *E. coli* isolates and reported that 17% isolates were strong biofilm producers and 23.6% were weak producers. In the present study, tube method could detect only few number of biofim forming *S. aureus* and *E.coli*. The main reason for the differences in the results in various studies with respect to tube method might be the errors arising during visual interpretation. In addition, it was difficult to differentiate between strong and moderate biofilm producers by visual examination that interfered with the results. Similar observations were reported by Christensen *et al.* (1982).

Tissue culture plate method is considered as the quantitative method for biofilm detection. In present study, four *S. aureus* isolates (50%) were strong biofilm producers, one was (12.5%) moderate producer and three (37.5%) were non- biofilm producers (Table 2). Similar observations were made by Gad *et al*. (2009), who identified 56.6 per cent as strong biofilm producers. In our study, tissue culture plate method showed high degree of sensitivity compared to other two methods. In a study by Mohamed *et al* (2016), 78% of biofilm formation by S. *aureus* was detected by tissue culture plate method. Nimbalkar and Bose (2014), detected 59% biofilm producers by tissue culture plate method and considered it as the gold standard method for biofilm detection. Present study agreed with the findings. In case of *E. coli*, no strong biofilm producers could be detected with this method (Table 3). The reason behind this could be interpreted in two ways. Considering tissue culture plate method as the gold standard, only 25 samples were included in the study and from that, we got only seven *E. coli* isolates, which might be non-biofilm forming strains. The other explanation is that, for detection of biofilm forming *E. coli* strains, tissue culture plate method is least sensitive in

Table 2: Optical density values in tissue culture plate method for biofilm detection of *S. aureus* isolates at 570 nm.

Sample no.	OD values			Average OD
*Positive control	0.111809	0.108432	0.121351	0.113864
*Negative control	0.070222	0.069752	0.0725345	0.070836167
C14256	0.246412	0.246496	0.233982	0.242297
C16616	0.0701348	0.0731078	0.0772895	0.0735107
C13478	0.142466	0.114352	0.147083	0.1346336
C122	0.0759096	0.0690385	0.0781702	0.074372767
C419	0.242466	0.234352	0.247083	0.2413
C1698	0.25926	0.251804	0.24363	0.251566
C2130	0.0692645	0.0751242	0.0740897	0.072826133
C1375	0.23449	0.244747	0.243489	0.24090

Table 3: Optical Density values in tissue culture plate method for biofilm detection of *E. coli* isolates at 570 nm.

Sample no.	OD values			Average OD
*Positive control	0.111809	0.108432	0.121351	0.113864
*Negative control	0.070222	0.069752	0.0725345	0.070836
C11398	0.0703741	0.0716104	0.0830181	0.075000867
C12750	0.0632623	0.0705556	0.0702492	0.068022367
C14876	0.0709781	0.0659442	0.0730669	0.0699964
C863	0.095516	0.0879306	0.102299	0.095249067
C1896	0.0706531	0.0719134	0.0688073	0.070457933
C1205	0.0677826	0.0827794	0.0700847	0.0735489
C1375	0.0714981	0.0712266	0.0685976	0.0704407

*Biofilm forming and negative strains of *S.aureus* and *E.coli* maintained in the department of veterinary microbiology, college of veterinary and animal sciences, mannuthy is taken as positive and negative controls.

comparison with other two. Further studies employing more number of isolates are needed to confirm both the interpretations.

In the present study, biofilm producing *S. aureus* showed high degree of resistance compared to non-biofilm producers. Similar observations were made by Singh *et al.* (2017), who reported that biofilm positive Staphylococcal isolates showed high resistance to the commonly employed antibiotics. Umadevi and Sailaja (2014) made similar observations, where biofilm forming *S.aureus* isolates showed a high rate of antibiotic resistance. Kwon *et al.* (2008) also documented a high rate of biofilm formation in multidrug resistant *S. aureus*. As in the case of *S. aureus* isolates, high rate of antibiotic resistance were shown by biofilm producing *E. coli* isolates in comparison with non-biofilm producers. Similar conclusions were drawn by Golia *et al*. (2012), who studied the correlation between biofilm formation of uropathogenic *E. coli* and antibiotic resistance pattern and documented that biofilm producing isolates exhabited high degree of resistance towards broad spectrum antibiotics. Raya *et al* (2019) analysed *in vitro* biofilm formation and antimicrobial resistance of *E. coli* in diabetic and non-diabetic patients and found that resistance was higher in biofilm producing *E. coli* compared to non-biofilm isolates. Risal *et al*. (2018) reported similar conclusions. In short, four strong biofilm producing *S. aureus* showed resistance to 90% of the antibiotics employed and moderate biofilm producers showed approximately similar profile. In case of *E. coli*, one strong biofilm producer, showed resistance to all the antibiotics tested. Two moderate biofilm producers showed about 90% resistance to the antibiotics. Among the total 15 cases, ovariohysterectomy was recommended for five cases, with strong biofilm producers, which showed resistance to all the antibiotics tested and hence could not be treated with antibiotics.

CONCLUSION

Antibiotic resistance is a globally emerging thereat in the therapy of clinical infections worldwide, both in human and veterinary medicine. Pyometra is a medical emergency in dogs and there are multiple factors responsible for antibiotic resistance in the treatment. Among them, the role of biofilm forming bacterial organisms is not explored. The present study documented biofilm forming strains of *E. coli* and *S. aureus* as a significant factor responsible for antibiotic resistance in pyometra. This could pave the way for initiating appropriate intervention in the therapy of pyometra and advocating appropriate and judicious use of antibiotics.

ACKNOWLEDGEMENT

Authors acknowledge the Dean, College of Veterinary and Animal Sciences, Mannuthy for providing the necessary facilities needed for the study.

Conflict of interest: None.

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