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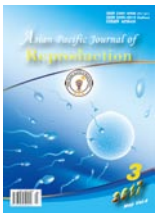

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Detection of bacterial biofilm in uterine of repeat breeder dairy cows

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ABSTRACT

Objective: To determine the possibility of presence of bacterial biofilm in the uterus of repeat breeder cows and to evaluate the effect of mucolytic agent in cleanup of uterus from biofilm. **Methods:** Twenty repeat breeder cows were selected from a large commercial dairy farm near Shiraz, Fars province, southern Iran. Uterine secretion samples were collected before and after uterine lavage with dimethyl sulfoxide (DMSO) 10% solution and periodic acid Schiff (PAS) staining was used to detect bacterial biofilm in uterine samples. After sampling, all cows were treated with two doses of PGF2 and intrauterine infusion of Cefquinome sulphate. Artificial insemination (AI) was performed after that. **Results:** Bacterial biofilms were found in 12 out of 20 animals (60%) in the first sampling with sterile saline lavage (before DMSO) and in 7 cows (35%) after DMSO lavage. Fourteen cows (70%) became pregnant after AI. This evidence showed the presence of bacterial biofilm in the uterus of dairy cows for the first time. Although non-significant, decrease in biofilm detection after DMSO lavage may suggest the potential ability of mucolytic agent for cleaning the uterus from bacterial biofilm. Also, high pregnancy rate after antibiotic treatment in the present study might be attributed to improved effect of antibiotic following lavage of uterine by DMSO. **Conclusions:** These findings should be investigated in future researches with more sample size.

1. Introduction

A major source of economic waste in dairy herds is Repeat breeder syndrome (RB). Failure of cows to conceive after 3 or more inseminations with fertile semen is classified as repeat breeder without any anatomic or infectious abnormality[1-4]. The RB syndrome remains a major problem in dairy farms. According to one report, the incidence of RB was 14% in 9 commercial herds[2].

There is clear evidence for chronic uterine damage in cows results after uterine infection[5]. Levine did not place great importance upon chronic uterine infection as a cause of failure to conceive in repeat breeding syndrome[1]. Seventy seven percent of infertile cows had endometritis[6], histological evidence of endometritis was found in fifty percent of the uterus obtained from an abattoir, yet only 12.5% showed gross lesions[7]. Hence, it is concluded that subclinical endometritis is a major contributor to the repeat breeder syndrome of bovine subfertility[5]. The RB syndrome remains a major problem in dairy farms. According to one report, the incidence of RB was 14% in 9 commercial herds[2]. In Australia, it has been suggested that at least 25% of cows will exhibit RBS[8]. Endometritis in mare can also be influenced by the offending pathogen and the immunological response to it. Different bacteria have shown different virulent factors and different modes of evading the immune response[9]. An abnormal uterine environment may cause endometritis and repeat breeding syndrome, therefore, the intrauterine environment improvement for embryo survival is the basis of different therapeutic protocols such as intrauterine

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administration of antibiotics and antiseptics which are commonly used to treat postpartum endometritis in cattle[10]. But these methods were not effective for treatment of subclinical endometritis[11,12]. To decrease RB syndrome in herds, the incidence of periparturient disease should be managed to reduce and minimize the depth and duration of nutrient deficiency in recently calved cows[13].

Some of the bacterial genus is involved in biofilm production, an adhesive matrix that supports growth and maintenance of bacterial micro-colonies. Resistance to antibiotics and both cellular and humeral immune defenses is inherent in the biofilms. Therefore, persistent or chronic infections exist even after prolonged antibiotic treatment[14,15].

Some bacteria or fungi form focal plaques of mare uterus, while other pathogens do not produce intra-uterine fluid, the 'hallmark' of endometritis. The establishment and chronicity of infection are formed by uterine response to a pathogen[16]. Biofilms formed from bacteria were resistant to common veterinary antibiotics[17]. Biofilm formation may be an important cause of chronic endometrial infection in the mare[18].

The authors have observed the opaque liquid or some particle in lavage fluid in lavage (by normal saline) of repeat breeder cows with clear discharge at estrus phase. This observation lead to a question about the nature of the particles and the possibility of the presence of bacterial biofilm was raised.

The pupose of this study was to determine the possibility of presence of bacterial biofilm in the uterus of repeat breeder cows and to evaluate the effect of mucolytic agent in cleanup of uterus from biofilm. Fertility of repeat breeder cows after treatment with antibiotic following uterine lavage was also investigated. The authors hypothesized that there is bacterial biofilm in the uterus of some repeat breeder dairy cows which inhibit the action of antibiotics. Also, the authors suggested that using a mucolytic agent could clean the biofilm at least partly and consequently improve the effect of antibiotics and recover the fertility of treated animals.

2. Materials and methods

2.1. Animals

This study is a quasi-experimental study in that no independent control group was present. The animals were selected from a commercial large dairy farm near Shiraz, Fars province, southern Iran (29° 58′ 34″ N, 52° 40′ 45″ E). The cows were housed in free stall barns with sand bedding for cows. Cows calved throughout the year. The annual average milk yields was 11 000 kg per cow. The ration that was used for cows' nutrition was a combination of corn silage, alfalfa hay, and concentrates (containing corn meal, soybean meal, vitamins and minerals). The eligibility criteria were: cows with parturition 1-4 that were cycling and showed estrus, had no significant detectable pathologic defect associated with the reproductive tract, had returned to heat after ⩾3 services, and had clean discharge during estrus with body condition score (BCS) between 2.75-3.50 (scale 1-5).

2.2. Clinical examination

The cow's vulva was washed, disinfected and cleaned by using dry paper towel and then a clean, lubricated, gloved hand was inserted via the vulva and the mucus contents withdrawn manually for testing. The vaginal examination was done for confirming the clear mucus[19].

2.3. Uterine samples collection and bacterial culture

This study was carried out in 20 repeat breeder cows during January to March of 2015. Uterine secretion samples were were collected twice[20]. The first time 200 mL of sterile saline solution was injected into the uterus and agitated gently. The solution flowed out very gently from the uterus through the catheter. The second time 200 mL of dimethyl sulfoxide (DMSO, FWI, TULSA, USA) 10% solution was injected into the uterus and it flowed out through the catheter.

Uterine sample for biofilm evaluation of 20 repeat breeder cows was collected from first (sterile saline solution) and second uterine lavage (DMSO 10% solution) as described before. The volume of sample fluid ranged from 10 to 15 mL. Samples were maintained on ice prior to laboratory processing. After sampling, all cows were treated with two doses of $PGF_{2\alpha}$ (14 d interval) and intrauterine infusion of 25 mL syringe containing 900 mg Cefquinome sulphate (Cefquinome, Afarin Daroo, Iran). Artificial insemination was performed for all cows after antibiotic treatment. The parity of animals and milk production as well as reproductive parameters including number of services, days open and occurrence of pregnancy were recorded after treatment of these cows.

The samples were cultured on sheep blood agar and MacConkey agar (Merck, Germany), and incubated at 37 ℃ for 48 h. The same culture on sheep blood agar (Merck, Germany) was incubated anaerobically for up to 7 d. Standard biochemical tests were used for the isolation and identification of the isolates as described by Quinn.

The recovered fluid after culture was centrifuged at 250 g for 5 min. The resultant sediment suspension was used to prepare smears to be stained with Diff-Quik for cytologic examination[21].

2.4. Biofilm detection by periodic acid Schiff (PAS) staining

PAS staining was used for detection of bacterial biofilm in all samples directly. Briefly, uterine smears were treated for 5 min with Periodic acid solution (Sigma-Aldrich, USA), rinsed in distilled water, then treated for 15 min with Schiff reagent (Sigma-Aldrich, USA), subsequently washed in lukewarm tap water for 5 min and counterstained in Harris haematoxylin for 1 min.

2.5. Statistical analysis

Statistical analysis was performed using SPSS software (version 16). Continuous data were presented as mean, standard deviation (SD), median, minimum (min) and maximum (max); and categorical variables were displayed as number and percent. For comparison of continuous variables between pregnant and non-pregnant groups, two independent samples *t*-test was used. Comparison of PAS results before and after DMSO treatment was performed by McNemar test. For investigation of association between PAS results, bacterial culture and pregnancy with each other and with cytological examination results, Spearman's rank correlation coefficient was used. In all analyses, *P*-values less than 0.05 were considered as significant.

3. Results

PAS staining revealed the presence of bacterial biofilm in uterus samples as red complex which is indicated in Figure 1. Overall, 15 out of 20 cows (75%) were positive for bacterial biofilm in PAS staining. The bacterial biofilms were found in 12 out of 20 animals (60%) in the first sampling with sterile saline lavage. After the injection of DMSO 10% solution, just 7 cows (35%) were positive for bacterial biofilms (Figure 2). No significant difference for PAS results was detected before and after DMSO lavage (P=0.22).

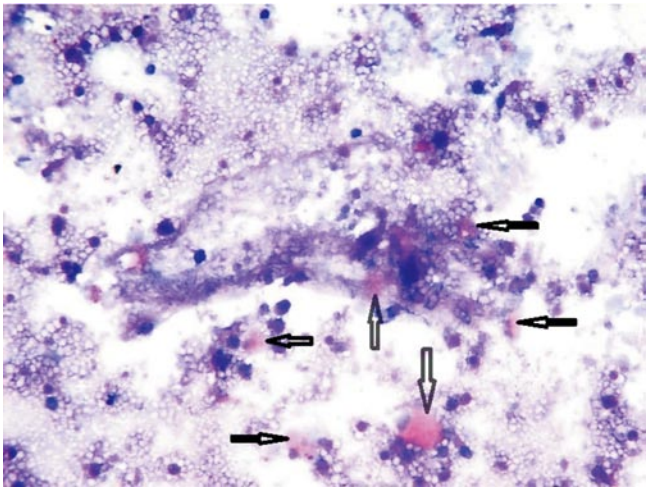

Figure 1. Periodic acid-Schiff stain (×100) of uterine samples. Red complex revealed bacterial biofilm (Shown by arrows).

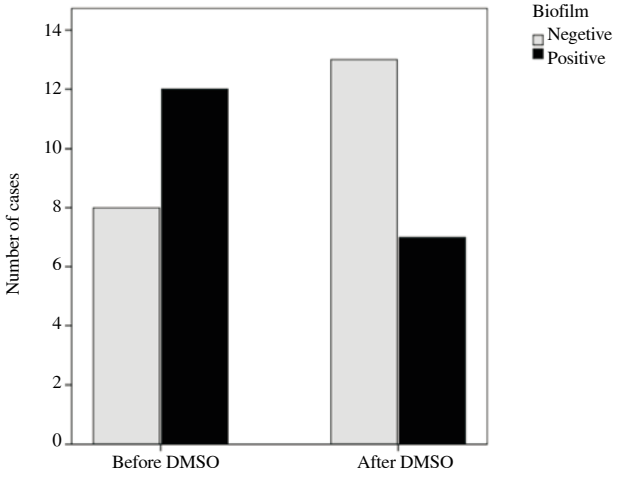

Figure 2. Comparison of detection of bacterial biofilm using PAS staining before and after uterine lavage by MMSO in 20 repeat breeding cows.

Bacterial growth in culture was observed in eight animals. All of these cows were positive in PAS staining (r_s=0.47, P=0.036).

From 20 repeat breeder animals, 14 became pregnant after treatment. Parity, milk production, number of insemination and days open did not show significant difference between pregnant and non-pregnant cows (Table 1). Eleven cows from PAS positive cows (73%) in comparison with three from PAS negative group (60%) became pregnant after treatment (P=0.60). The corresponding measures for pregnancy status in culture positive and culture negative cows were 6 and 8 cows, respectively (P=0.71).

Table 1

Descriptive statistics for 20 selected repeat breeder animals based on their final pregnancy status.

Parameter	Pregnant (n=14)		Non-pregnant (n=6)		
	Mean	SD	Mean	SD	P-value
Parity	2.4	0.9	1.8	1.1	0.24
Number of inseminations	5.6	1.2	5.6	1.0	0.96
Last recorded milk	36.5	9.2	29.4	12.1	0.17
Days open	280.4	75.6	300.0	81.7	0.61

Cytological results for all cows are shown in Table 2. The number of monocytes and eosinophils were zero in all. No significant correlations for cytological parameters with presence of bacterial biofilm, bacterial growth in culture and pregnancy status were observed (P>0.05 in all cases). However, Spearman's rank correlation coefficient showed slightly significant negative association of polymorphonuclear leukocytes with pregnancy in study group (r_s=-0.48, P=0.054).

Table 2

Results of cytological examination in 20 selected repeat breeder animals.

Parameter (%)	Mean	SD	Median	Min	Max
Epithelial cells	70	36.5	89	0	97
Polymorphonuclear leukocytes	7	11.9	3	0	50
Lymphocytes	3	3.7	3	0	10

4. Discussion

The results of this study revealed the presence of bacteria biofilm in bovine uterus in repeat breeder cows for the first time. However, no clinical signs of endometritis were observed in cases affected by biofilm. The lavage of uterus is a mechanical method for removal of biofilm from uterus. This study showed that the use of DMSO solution for uterus lavage caused decrease in biofilm detection from 60% to 35% in repeat breeder cows. Decrease in biofilm detection after DMSO lavage may suggest the potential ability of mucolytic agent for cleaning the uterus from bacterial biofilm. This is in agreement with previous work which has shown that the uterine lavage plus $PGF_{2\alpha}$, without any antibiotic is effective in the treatment of repeat breeder cows[12]. Although the change in biofilm detection before and after DMSO treatment in the present study was not significant, this finding is interesting and merits more investigation with more significant sample size in future researches with two parallel groups randomized design.

As mentioned, DMSO as a mucolytic material might cause clearing of biofilm and bacterial lipopolysaccharides. Therefore the uterus condition was prepared for better response to the antibiotic infusion. Seventy percent pregnancy rate after antibiotic treatment in the present study might be attributed to improved effect of antibiotic following uterine lavage by DMSO in repeat breeder cows. Biofilms resist antibiotic treatment and contribute to bacterial persistence in chronic infections[22,23]. New antimicrobial drugs that inhibit bacterial virulence and biofilm formation are needed[24].

However, based on the present results, using uterine lavage before antibiotic treatment with a mucolytic agent such as DMSO may be considered an alternative opportunity until new drug discovery. This is consistent with previous report about synergistic antibiofilm activity for combinations of classical antimicrobial agents and other compounds such as the mucolytic agent N-acetylcysteine, ethanol, or the chelating agent EDTA on infected catheters[25].

There was significant positive association between presence of bacterial biofilm and bacterial growth in culture. All culture positive samples were PAS positive, too. Our results indicate that bacteria isolated from the uterus are capable of producing a biofilm. The results presented in this research agree with other published studies performed in horse in which the majority of bacterial isolates cause biofilm formation.

No significant association between cytological results and either bacterial biofilm or bacterial growth in culture and pregnancy were observed in this study. However, a tendency tosignificant negative association of polymorphonuclear leukocytes with pregnancy in study group was detected. Cervical neutrophil percentages showed no significant difference between cows conceived or not conceived with 1 artificial insemination[21]. This study was carried out in a large commercial dairy farm. Therefore the selection of positive and negative control group in this study was impossible and all cows were treated by one protocol. Pregnancy rate of seventy showed the successful procedure for treatment of repeat breeder cows.

The main drawback of this study is the lack of independent control group. This study was carried out in a large commercial dairy farm. All cows were treated by one protocol and selection of control group in this study was impossible due to setting limitations. Therefore, the results of the effect of DMSO on bacterial biofilm and fertility of dairy cows should be interpreted with caution.

In conclusion, this is the first report on detection of biofilm in uterus of dairy cows. The effect of DMSO on clearing the uterus from bacterial biofilm as well as improving the effect of antibiotic treatment on fertility of repeat breeder cows needs more investigation in future researches. The time of biofilm formation in uterus of dairy cattle is not clear. Further research is necessary to demonstrate the time of onset and various determinants of biofilm formation in dairy cows.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Authors contributions

Ahmadi was the general coordinator, designer of clinical part of this project. Derakhshandeh was the designer of microbiological part. Shirian and Daneshbod were done periodic acid Schiff (PAS) staining. Ansari-Lari analysed of data. Nazifi did the cytologic examination.

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