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Direct endoscopic lavage and biopsy sampling and evaluation of uterine microflora in various stages of the canine estrous cycle

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Abstract

The microbial population of the uterus fluctuates during the estrous cycle. Microflora of uterus may affect the establishment and maintenance of pregnancy in bitches. The endoscopic samples obtained from the vagina and uterus of 20 female adult mixed-breed dogs. The uterine lavage samples were prepared for cytology, bacterial (aerobic and anaerobic) and fungal cultures. Uterine tissue samples were evaluated for the presence of *E. coli* by the polymerase chain reaction. The pure growth of bacteria was observed in seven plates out of the nineteen cultured samples (36.84%) and five Gram-negative and two Gram-positive bacteria were detected. The highest number of isolated bacteria was related to the samples of the diestrus and anestrus stages of the estrous cycle, while the lowest number of bacteria was observed in the samples of the estrous stage. Moreover, *Citrobacter spp.* was the most frequent group of isolated bacteria. The neutrophils were detected in the cytology of uterine samples. The fungi growth was observed in three uterine samples. *Cladosporium* and *Penicillium* isolated from the samples were related to the estrus stage, and yeast was grown in diestrus samples. The 16srRNA gene existed in all of the estrous uterine samples in which the bacterial culture was negative. However, the presence of this gene was proven in two samples (33.30%) of negative bacterial culture samples from the diestrus and anestrus stages. In conclusion, the normal bitches' uteri were infected with various bacteria in estrus, diestrus and anestrus stages of the estrous cycle, and it could coincide with the fungi infection.

Introduction

In spite of the culture and antibiotic susceptibility tests, the response to treatment may not be appropriate. Some researchers mentioned infection with viral or fungal agents and antibiotic resistance as the reasons for this issue.[1] In the culture of samples taken from the cranial portion of the vagina and uterus, bacteria are grown in proestrus and estrus samples and rarely observed in samples of other stages. Likely, the reason is the low number of bacteria in the samples taken in the diestrus and anestrus phases. In spite of inflammation of the genital tract, the results of bacterial culture were negative in some studies.[2] The researchers used different methods to prepare the vagina and uterine samples, detection of bacteria and comparison of bacterial flora of subfertile with healthy dogs.[3] Most of the samples have been taken from the uterus of bitches after ovariohysterectomy.[4] In recent years, direct and less invasive sampling techniques have been considered in subfertile bitches as part of a treatment program.[5] One of these methods is using a rigid endoscope to obtain a sample from the anterior part of the vagina or uterus. In this method, there would be a possibility of having a sterile and controlled collection of uterine lavage samples and biopsies in bitches at the same time.[3]

The aim of the present study was lavage and biopsy sampling with a rigid endoscope from the bitches' uteri at different stages of the estrous cycle. In addition to bacterial and fungal cultures, polymerase chain reaction (PCR) was used for those samples without bacterial growth to determine the presence of *E. coli*, as it is the most common bacteria reported in the reproductive system of a bitch.

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Materials and Methods

Animals and sampling. The experiment was approved by the Ethics Committee of Shiraz University, Shiraz, Iran (IACUC No: 4687/63).

Twenty female mixed breed adult bitches (1-2 years, 15.00 - 20.00 kg body weight) were selected randomly from a shelter of stray dogs that were going to a population control program. The antiparasitic drugs were administered and dogs were maintained at least for two weeks. Samples were taken from the vagina and uterus under physical (preferably) and chemical restraint (in some dogs). Vaginal cytology samples were obtained using wet cotton swabs from the cranial part of the vagina. The samples were fixed with methanol after being rolled and smeared on the glass slides. Slides were immersed in Gisma stain for 20 min and evaluated for detection of a stage of the estrous cycle. Uterine lavage samples and biopsies were obtained by a rigid endoscope.[6] The sterile rigid endoscope was covered with sterile disposable plastic film to prevent direct contact of an endoscope with a vaginal wall. An endoscope was inserted in the direction of the dorsal vaginal fold. When external os of the cervix was visible, sterile NG tube 8 Fr Foley catheters (Supamed, Tehran, Iran) with its wire stylet was entered into the cervix and uterus through the pathway of the endoscope. To harvest at least 1.00 mL of lavage sample, 10.00 mL of sterile normal saline injected into the uterus. Then the samples were poured into the microtubes. The biopsy forceps were entered into the uterus and punched the uteri tissue sample and placed in microtubes containing saline. The lavage and biopsy samples were transferred quickly to a laboratory for bacterial and fungal cultures and, and PCR.

Microbiological and mycological examination. The uterine lavage samples were centrifuged at 750 *g* for 10 min. Then, the sediments were inoculated into 5.00% sheep blood (Himedia, Mumbai, India) and MacConkey agar culture media (Himedia), and the plates were incubated at 37.00 ˚C for 24 to 48 hr. When discrete colonies were grown, they were observed under light microscopy and the results were recorded. To identify the aerobic and facultative anaerobic bacteria, standard biochemical tests were performed as well.[7] The same samples were inoculated onto Sabouraud's dextrose agar (SDA; Himedia) and were incubated at 25.00 ˚C for 2 to 3 weeks. The cultures were examined daily for any mycotic growth within the incubation period. Likewise, the fungal colonies were examined and their colony morphology or characteristics, such as texture, pigment, and rate of growth on media were recorded. Morphology of the fungi was identified by examining a small aliquot of the growth in lactophenol blue under a dry objective microscope. Finally, fungal isolates were identified at the genus level and used a duplicate culture for each sample.

DNA extraction. Each sample was homogenized in 1.00 mL distilled water and centrifuged (13,000 *g* for 30 sec). Likewise, the supernatant was removed and added with 1.50 mL distilled water and centrifuged at 13,000 *g* for 30 sec. This step was repeated to lavage the tissues. After that, 500 µL of lysis buffer (Tris-HCL; Cinnagen, Tehran, Iran), EDTA (Cinnagen), 0.20% Tween (Cinnagen) and proteinase K (Sinaclon, Tehran, Iran) with the final concentration of 1.00 mg was added and incubated overnight at 37.00 ˚C. The enzyme digested samples was mixed with 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) for 15 min. Then the mixture was centrifuged 10,000 *g* for 10 min. The DNA from the supernatant was purified by adding an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1), and centrifuged at 10,000 *g* for 1 min. Additionally, the DNA was precipitated from the aqueous phase by adding 2.50 volume of absolute ethanol and incubated at – 20.00 ˚C for 1 hr. Furthermore, the resultant DNA pellet was washed with 70.00% ethanol twice, dried, and resuspended in 50 µL distilled water. Finally, the extracted DNA was held in – 20.00 ˚C for further analysis.

Analysis of samples by PCR. The primers of 16srRNA-F (5'ATCAACCGAGATTCCCCCAGT-3') and 16sr RNA-R (5'-TCACTATCGGTCAGTCAGGAG -3') were selected for detection of *E. coli*. The PCR mixture contained 0.75 µL dNTPs (each at 0.20 mM; Sinaclon), 1.00 µL of each primer (20.00 pmol; Sinaclon), 0.75 µL 50.00 mM $MgCl_2$ (Sinaclon), 2.50 µL 10X PCR buffer (Sinaclon), 0.20 Taq DNA polymerase (5.00 U μL^{-1}; Sinaclon), and 5.00 µL of template DNA. Likewise, sterile distilled water was added to bring the final volume to 25.00 µL. The PCR conditions were as follows: 5 min at 95.00 ˚C, 35 cycles of 1 min at 95.00 ˚C, 50 sec at 55.00 ˚C, 1 min at 72.00 ˚C, and a final extension of 10 min at 72.00 ˚C.

Agarose gel electrophoresis. The PCR products were subjected to electrophoresis in 1.00% agarose gels containing ethidium bromide (Cinnagen) and visualized under UV light.

Results

In most studies, the uterine samples were collected from bitches undergoing ovariohysterectomy, so the number of their samples was greater than our study. We did not perform statistical analysis due to the small sample size and there was no control group. In this study, it was possible to take lavage and biopsy samples of the uterus from all bitches (n = 20). The restlessness of some female dogs was the most important reason for the prolongation of sampling. Therefore, sedative we used in some dogs. It was easier to pass the lavage tube and biopsy forceps in estrus dogs. In some cases where the vaginal wall was collapsed in the fornix and obscured the observation of the external os of the cervix, a little air was blown into vagina.

Table 1. Results of fungal and bacterial cultures of bitches' uterine lavage samples in different stages of the estrous cycle.

Stage of the estrous cycle (no.)	Bacteriology	Mycology	Uterus cytology
Estrous (6)	*Bacillus lentus* (n=1) *Staphylococcus saprophyticus* (n=1) *Proteus mirabilis* (n=1)	*Clodosporium* (n = 1) *Penicilium* (n = 1)	Neutrophils (negative)
Diestrus (9)	*E. coli* (n=1) *Citrobacter* (n=1)	Yeast (n = 2)	Neutrophils (negative)
Anestrus (4)	*Citrobacter* (n=1) *Klebsiela* (n=1)	Yeast (n = 1)	Neutrophils (negative)
Proestrus (0)	-	-	-

Samples from one of the dogs were missed during the preparation for bacterial and fungi cultures, and gene extraction. The growth of bacteria was monitored on the MacConkey and blood agar, and the bacteria were grown purely in seven plates out of the 19 cultured samples (36.84%). There were five Gram-negative and two Gram-positive bacteria. The most number of bacteria were isolated from the samples of diestrus and anestrus stages, and the least bacteria were grown in a culture of the estrus stage samples (Table 1). None of the randomly selected dogs was in the proestrus stage. Accordingly, we did not present any data in Table 1 for the proestrus stage samples. *Citrobacter* was the most isolated bacteria (Table 2). We did not observe neutrophils in all lavage samples of the uterus (Table 1). Five uterine samples were positive in fungi plate cultures. The identified fungi were *Cladosporium* and *Penicillium* in estrus and yeast was grown in diestrus and anestrus stages (Table 2).

The results of the PCR test for the detection of *E. coli* gene revealed that the 16srRNA gene existed in 100% of the samples taken at the estrous stage which were negative in the bacterial culture. However, the 16srRNA gene existed only in two samples of diestrus and anestrus stages which were negative in the bacterial culture.

Table 2. Positive uterine samples in terms of bacterial (n = 7) and fungal (n = 5) cultures based on the type of bacteria and fungi.

Bacteria	Positive dogs in culture (%)
Citrobacter	2 (28.57%)
Escherichia coli	1(14.28%)
Klebsiela	1(14.28%)
Proteus mirabilis	1(14.28%)
Staphylococcus saprophyticus	1(14.28%)
Bacillus lentus	1(14.28%)
Yeast	3(60.00%)
Clodosporium	1(20.00%)
Penicilium	1(20.00%)

Discussion

The uteri of healthy bitches were not sterile in estrus, diestrus and anestrus stages of the estrous cycle. Researchers observed 26.43 - 68.00% bacterial growth among collected samples from canine uteri and noticed that the estrous cycle and cervix opening could influence the presence of bacteria in a healthy uterus.[4,8] The isolation of bacteria from the uterus of clinically normal bitches suggests that low levels may be indicative of normal flora or contaminants of the sampling and culture procedures. The low level of recovery of anaerobic bacteria was similar to previous studies.[4,8,9] In the present study, mycoplasma were not isolated from the uterine lavage samples in different stages of the estrous cycle as other researchers did.[8,9] This indicated that we used a careful sterile technique for sampling. In the present study, 75.00% of bacteria were isolated from diestrus and anestrus samples.

Other researchers reported the highest percentage of the bacterial growth (37.50%) in diestrus phase,[8] similar bacterial culture in all stages of the estrous cycle,[4] and the lowest bacterial growth in the metestrus stage.[2] In contrast, it has been reported that bacteria were not naturally found in the uterus.[10]

In the present research, *Citrobacter* (28.57%) was the most frequent bacterial growth. It was interesting that Gram-negative bacteria were grown from samples of diestrus stages while Gram-positive bacteria were grown in the estrus stage samples. The highest percentage of the bacterial growth was observed for *Staphylococcus* (57.60%) in uterine samples,[2] *E. coli* in vaginal culture, yeast (45.50%) and *E. Coli* (30.90%) in uterine samples[11] and *Bacillus* and *Proteus Mirabilis* species were in eighth and ninth orders of cultured samples, respectively.[3] The microbial flora of bitches vagina were affected by breed, estrous cycle, and season.[12]

Neutrophils were not observed in the uterus cytology samples. However, others stated that the presence and percentage of neutrophils were affected by the stage of the estrous cycle and pyometra.[13]

Different studies investigated the fungi flora in the reproductive tract of camels, rabbits, and dogs.[11,14,15] In the present study, we observed fungi growth in three samples of normal bitch uteri. However, Gunay *et al*. have found yeasts (45.50%) to be the most prevalent microorganisms in uterine samples of ovariectomized bitches. Others found yeasts in samples from anestrus (20.00%), proestrus (32.00%), estrus (28.00%), diestrus (12.00%), and pregnant (8.00%) dogs.[11] In a study on 30 rabbits, researchers separated seven fungi types from the uterus samples. It was included yeast (40.00%), Cladosporiaceae (23.00%), Alternaria (6.60%), Scopulariopsis (6.60%), and Bipolaris (3.00%), and they were found in all estrous cycles.[14]

In most studies, *E. coli* was the most frequently isolated bacteria from bitch's uteri samples, [3,11,16,17] however, in the present study, *Cytobacter* was the most frequently isolated bacteria. We performed PCR on non-grown bacteria samples to confirm that this bacterium was not grown following the sampling process. In the PCR test, all uterine samples from the estrous stage with negative bacterial culture were positive for the presence of 16srRNA. However, the presence of this gene was confirmed in two samples (33.30%) of diestrus and anestrus stages which were negative in bacterial culture. A study performed PCR on samples of dogs with pyometra and detected the strain of *E. coli*. Sancak and Dhaliwal have studied samples from 17 dogs infected with pyometra using PCR. *E. coli* strains isolated from the uterine and the fecal samples included ETEC, VTEC, EIEC, EPEC, and EAggEC. In the uterine samples, isolated *E. coli* strains were EPEC and VT1 (41.00%) and EIEC (5.00%). However, VT2, EHEC, ETEC, and EAggEC strains were not present.[18]

Bitches' uteri were not sterile in different stages of the estrous cycle, and bacterial and fungi flora were different in the various phases of the estrous cycle. Fungi were isolated from the uterine lavage samples in healthy dogs. In spite of the negative culture results in some uterine samples, there was *E. coli* gene, especially, in the estrus phase samples. It should be stated that the small number of samples and lack of control samples might have limited the conclusions from the results of the present study. However, we could set up direct sampling from bitch's uterus as a clinical non-invasive method.

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Conflict of interest

The authors declare that there is no conflict of interest.

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