

The prevalence of *Mycoplasma canis* in the vaginas of breeding bitches

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Abstract

Introduction: How bacterial infections of the reproductive tract cause infertility and the correlation between the health status of female dogs and the presence of *Mycoplasma canis* (*M. canis*) in the vagina are still unclear. The aim of this study was to determine the *M. canis* population in the vagina of breeding bitches and to correlate this microbial population with some fertility outcomes. **Material and Methods:** A total of 275 breeding bitches were included in the study. Vaginal samples were collected for microbiological and PCR testing. **Results:** *Mycoplasma canis* was identified in 34.91% of the samples. One-third of bitches from the problem-free group and 41.18% from the group with problems were positive. In general, there were no significant differences in the prevalence of *M. canis* between the groups (P-value > 0.05). *Mycoplasma canis* occurs in both mated and unmated bitches and was found in a large number of kennels (67%). There was a correlation between *M. canis* in the kennel and the incidence of single puppy deaths and low litter sizes. There was also some correlation between the presence of *M. canis* in the vagina with at least two other bacterial strains and reproductive disorders. **Conclusion:** Our results indicate that *M. canis* is part of the normal vaginal flora of breeding bitches, although a role for this bacterium in causing some reproductive disorders remains to be disproved.

Keywords: dog, microbiome, bacteria, *Mycoplasma canis*, vaginal flora.

Introduction

Research on the relationship between the health status of female dogs and the bacterial flora of their genital tracts has been conducted for years. However, the results obtained by different authors are contradictory and do not always pertain to breeders' dogs (15, 18, 22). In canine breeding, genitourinary tract infections and problems such as infertility, abortion, foetal resorptions and neonatal mortality are common complications that may have a bacterial source (10, 16, 25, 27).

The physiological bacterial flora in the genital tract of healthy bitches is thought to consist of a variety of microorganisms, mainly opportunistic pathogens (7, 13, 16, 17, 35). This bacterial flora varies with age, breeding status, oestrous cycle phase and season (3, 10, 12, 14, 16, 21, 29). The number of contaminants or normal flora routinely cultured from the caudal vagina can vary from

0.7 isolates per bitch in one report (26) to 2.4 in another (35). The physiological microflora consists of β -haemolytic *Streptococcus* spp. (e.g. *S. canis*), *Staphylococcus* spp. (mainly *S. intermedius* and *S. aureus*), *E. coli*, *Enterococcus faecalis*, *Pasteurella multocida*, *Bacillus* spp., *Corynebacterium* spp. and *Proteus* spp. (3, 16, 17, 35). Less frequent occurrences of *Klebsiella pneumoniae*, *Actinomyces* spp. and *Neisseria* spp. are also observed (6, 10, 15, 17). Some authors even suggest that the presence of *Lactobacillus* spp., *Ureaplasma* spp. and *Mycoplasma* spp. is debatable (10, 16, 17, 20, 22).

With a cell size of 300–800 nm, *Mycoplasma* spp. are the smallest bacteria isolated from the canine reproductive tract. Their role in the infertility in bitches has not been fully understood (4, 5, 13). They are found in the mucous membranes of the respiratory tract and the urogenital tract of many animal species besides dogs (5). They are opportunistic microbes and part of the natural

bacterial flora. Many authors confirmed that the development of the disease process caused by *Mycoplasma* spp. is largely determined by the individual immune response (9, 22), although the results of experimental infections suggest the real pathogenicity of these microorganisms (13). One previous report on the effect of *Mycoplasma* spp. infection on canine reproduction estimated that this group of bacteria is present on the mucosa of the reproductive tract in as many as 88% of bitches, and that this percentage is the same among fertile and infertile individuals (7). In several animal species (swine, cattle and small ruminants) peripheral involvement of *Mycoplasma* spp. in fertility disorders has been observed (11). There is also much debate about the negative effects of this bacterium on health in male dogs, as mycoplasma microorganisms have been isolated from male dogs with fertility disorders, in which they impaired semen quality (8). In addition to *M. canis*, the species routinely recovered from the vaginal tract of bitches include, *M. maculosum*, *M. cynos*, *M. molare*, *M. edwardii* and *M. spumans* (30). It is still unclear which factors have a direct influence on susceptibility to mycoplasma infections. In canine reproduction, questions about the possible virulence factors that are necessary to establish infections, the host immune responses that they trigger and their precise role in long-term disease pathogenesis still remain unanswered. For the same reasons, the prevalence of mycoplasma infections in dogs is underestimated and difficult to assess. Infections are often subclinical, *i.e.* do not cause visible health problems with pronounced clinical signs. Therefore, positive mycoplasma test results may not necessarily indicate ongoing disease processes. Furthermore, many are the researcher opinions that only the presence of *M. canis*, and not of other species of *Mycoplasma* spp. should be considered to trigger abortion, stillbirth or birth of weak puppies, neonatal and embryonic death (25, 33) or exacerbate urinary tract infections (19, 22). On the other hand, in some reports *M. canis* was isolated from the vagina of healthy bitches with good reproductive parameters; these results are partly contradictory (9, 16, 22).

Limited and contradictory knowledge exists regarding the qualitative composition of the natural vaginal microflora of breeding bitches, and knowledge specific to *M. canis* is matchingly deficient. The objective of this study was to assess the population of *M. canis* in the vagina of fertile, problem-free breeding bitches and compare it with a group experiencing reproductive problems. Additionally, we aimed to establish a correlation between the presence of this microbe in the vaginal environment and fertility outcomes, including pregnancy development, uterine infection, resorption, abortion and neonatal mortality. This study aimed to draw conclusions that are applicable to the entire population of breeding bitches by obtaining results from a large group of individuals. The use of a significant sample size increases the statistical significance of the findings. To the best of our knowledge, no similar study has been conducted before.

Material and Methods

Sample collection. Client-owned breeding bitches presented for routine gynaecological examination to the Clinic of the Department of Animal Reproduction, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Poland, were included in this study. The owners were informed of the purpose of the study. Two vaginal swabs for microbiology and PCR testing were collected from each of 275 anoestrous bitches of 35 different breeds, aged from 1 to 8 years, kept in 82 kennels. The first swab was for microbiological culture on an appropriate growth medium and the second one was for PCR testing. A sterile cotton swab without culture medium was used (Invasive sterile EUROTUBO Collection swab, Deltalab, Rubí, Spain). A sterile Hannover-type vaginal speculum for bitches (Eickemeyer, Tuttlingen, Germany) of 150 mm in length and 5, 10 or 15 mm diameter was used to collect the samples, with the size adjusted to the bitch. The microbiological samples were promptly delivered to the laboratory, most often within 8–12 h of collection. The study included bitches randomly selected from a group of clinical patients with no systemic or organ diseases, except for symptoms of vaginitis. Animals undergoing any pharmacological treatment were excluded. Allocation to a clinical group (healthy or with fertility disorders) was based on clinical examination, medical history, and information provided by the owners in a questionnaire which they filled out. The points explored in the questionnaire written for this research were presented in a clear and concise manner to minimise confusion or misinterpretation by the respondents and the investigative questions sought purely specific information. The analysis of questionnaire data was an important part of the assumptions made about the study groups and the later interpretation of the results. A total of 198 bitches were classified as without reproductive problems, while 68 were classified as having problems such as infertility, abortion, foetal resorptions and neonatal mortality, reported by the owners during last 12 months. The study analysed the percentage of *M. canis*-positive individuals in the kennels with different reproductive problems and the problems' relationship to mating (sexual contact with male dogs). Additionally, the correlation between *M. canis* and the rest of the aerobic microbiome in the vaginas of the examined dogs was evaluated.

Identification of vaginal bacterial populations.

The methodology for the isolation of aerobic bacteria has been previously described by Jagódka *et al.* (16). For pre-incubation, vaginal swabs were incubated in non-selective tryptic soy broth (Oxoid, Basingstoke, UK) at 37°C for 24 h under aerobic conditions. Samples were then transferred to Columbia agar supplemented with 5% defibrinated sheep blood, MacConkey and Chapman agars and Edwards medium using a calibrated inoculation loop. All agars and the medium were supplied by Oxoid. Bacteria were cultured at 37°C for

48 h under aerobic conditions. The grown isolates were subjected to microbiological analysis, which included evaluation of bacterial colony morphology, Gram staining, selected biochemical tests (tests for catalase, coagulase and oxidase; API 20E and API 20NE tests (bioMérieux, Lyon, France)); Christie–Atkins–Munch–Peterson reaction test; and selected latex tests (PathoDextra Strep grouping kit (Remel Europe, Dartford, UK) and Staphylect Plus (Oxoid)).

Identification of *Mycoplasma canis* by PCR.

The study used a PCR to confirm quickly whether *M. canis* was present in the material tested. The reactions were performed using the HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions in a Nexus gradient thermocycler (Eppendorf, Hamburg, Germany). All assays were conducted with positive and negative controls. The conditions for the reaction, primer information and visualisation were previously described by Jagódka *et al.* (16). The primer sequences and reaction conditions were as follows: 5'CACCGCCCGTCACACCA (forward) and 5'CTGTCGGGGTTATCTCGAC3' (reverse), 247 base-pair amplicon size and 51°C annealing temperature (5).

Statistical analyses. Statistical inference methods were used to analyse the results (23, 31). To characterise the community structure, clustering was imposed using statistical series to determine the absolute and relative percentages of the analysed characteristics in the studied groups. The normality of the distribution was assessed using the Shapiro–Wilk test, while the equality of variance in different samples was gauged using the Levene test. Statistical analyses were conducted to demonstrate significant differences in the presence of *M. canis* among the tested groups. The fixed factors were the type of bacterial strains. In order to determine the significance of the relationship between the study groups with respect to the frequency of *M. canis*, a contingency analysis was performed using the chi-squared test of independence, also determining Yule's ϕ contingency coefficient and Spearman's rank correlation coefficient. The assumption of normality or equality of variance was not met; therefore, the non-parametric Kruskal–Wallis test was used instead of analysis of variance. In cases where significant differences between averages were found, multiple comparisons between average ranks were conducted. The level of significance for all statistical tests was set at P -value < 0.05. The statistical calculations were performed using MS Excel 2019 software (Microsoft, Redmond, WA, USA) and Statistica, v. 13.3.0 (TIBCO Software, Palo Alto, CA, USA).

Results

Mycoplasma canis was a very common vaginal bacterium and was isolated from more than one third of the examined dogs (96 out of 275; 34.91%) (Table 1).

It was identified in 66 out of 198 (33.33%) dogs from the problem-free group and in 28 out of 68 (41.18%) from the one with problems (Figs 1 and 2). In general, no difference was found between the prevalence of *M. canis* strains ($H = 4.98$, P -value = 0.1732) in the group of bitches with reproductive problems and this prevalence in the group of healthy dogs.

Mycoplasma canis was identified in 56 out of 169 bitches (33.14%) which had naturally mated once or more (Fig. 3). However, 40 out of 106 bitches (37.74%) which had never naturally mated were also *M. canis* positive (Fig. 4). There was no significant effect of the presence of *M. canis* on mating efficiency ($X^2 = 0.717$, P -value = 0.699, $RSpr = 0.06$, P -value = 0.424).

Table 1. Prevalence of aerobic bacteria in population of breeding bitches

	Number of bitches	Percentage of bitches
<i>Streptococcus</i> spp.	107	38.91
<i>Staphylococcus</i> spp.	106	38.55
<i>M. canis</i>	96	34.91
<i>E. coli</i>	88	31.99
<i>Bacillus</i> spp.	45	16.36
<i>Proteus</i> spp.	32	11.64
<i>Pseudomonas</i> spp.	11	4.00
<i>Klebsiella</i> spp.	7	2.55
<i>Citrobacter</i> spp.	1	0.36

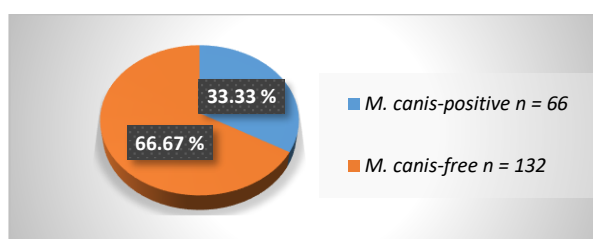


Fig. 1. The prevalence of *Mycoplasma canis* in the problem-free (healthy) group of breeding bitches (n = 198)

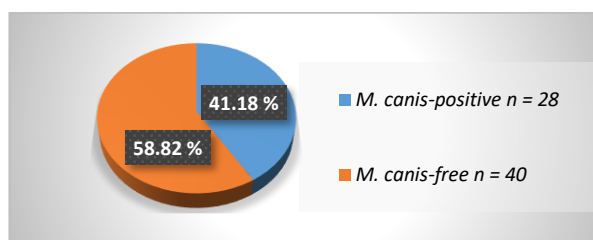


Fig. 2. The prevalence of *Mycoplasma canis* in the group of breeding bitches with fertility disorders (n = 68)

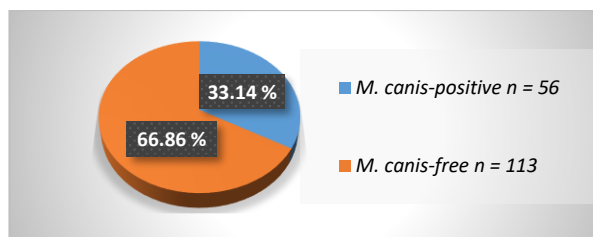


Fig. 3. The prevalence of *M. canis* in the group of bitches which had naturally mated at least once (n = 169)

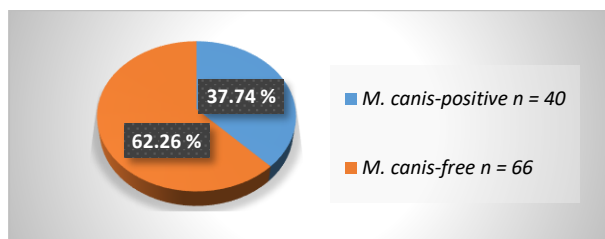


Fig. 4. The prevalence of *Mycoplasma canis* in the group of bitches which had never naturally mated (n = 106)

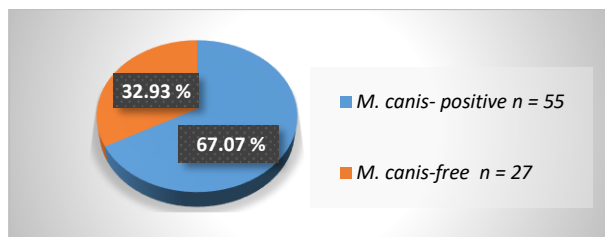


Fig. 5. The prevalence of *Mycoplasma canis* in the dog kennels of breeding bitches (n = 82)

Mycoplasma canis was identified in 55 out of 82 kennels (67.07%) (Fig. 5). *Mycoplasma canis*-positive dogs were found in 77.78% of the kennels with single puppy deaths (P-value < 0.05). Out of the 29 kennels

with low litter sizes, 20 of them (68.97%, P-value < 0.05) were *M. canis* positive. The bacterium was confirmed in 14 out of 24 kennels with resorptions (58.33%) and in half of the kennels with puppy deaths >50% of a litter (Table 2).

Besides *M. canis* (96/275), other common bacterial isolates from the vaginal tract of all dogs were *Streptococcus* spp. (107/275), *Staphylococcus* spp. (106/275) and *Escherichia coli* (88/275). Less common strains were *Bacillus* spp., *Proteus* spp., *Pseudomonas* spp., *Klebsiella* spp. and *Citrobacter* spp. (Table 1). In no case was the incidence of *M. canis* found as the only bacterium, and it was always recorded as co-occurring with different aerobic bacteria. Statistically significant differences were found between *M. canis*-positive bitches with one additional bacterial strain when comparing the group of healthy bitches with those with fertility disorders (H = 95.0, P-value = 0.001). In the majority of cases in problem-free bitches (41.67%), *M. canis* co-occurred with a single species (Fig. 6.). In the group of bitches with problems, *M. canis* was found most often co-occurring with two other aerobic bacteria (15.63% of cases) (RSpr = 0.97, P-value < 0,001, X2 = 270.6, P-value < 0.001, ϕ = 0.71) (Fig. 6).

Table 2. The association between the presence of *Mycoplasma canis* in the kennel and the incidence of selected reproductive disorders

Reproduction disorders	Number of kennels	<i>M. canis</i> positive	% of kennels
Small litters	29	20	68.97*
Resorption	24	14	58.33
Puppy deaths > 50% of litter	12	6	50.00
Single puppy deaths	18	14	77.78*

* – statistically significant

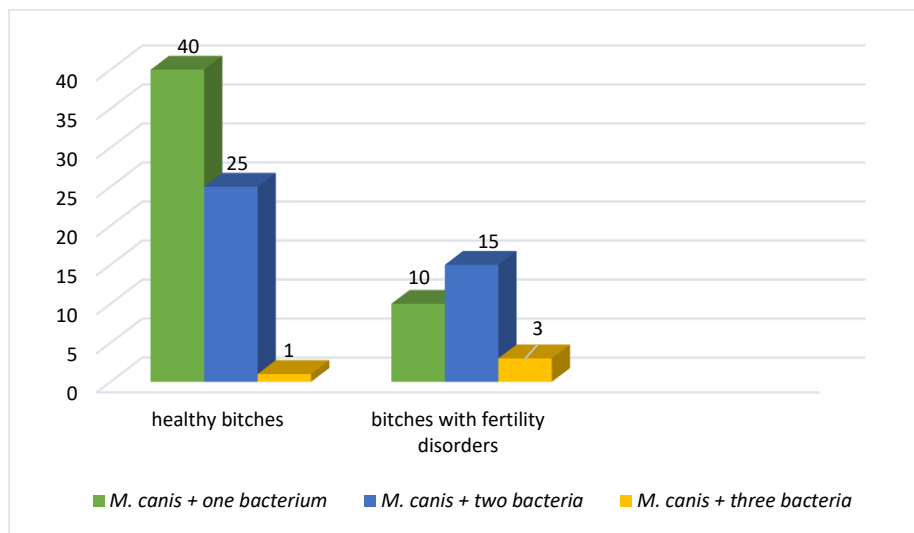


Fig. 6. The correlation between *Mycoplasma canis*-positive bitches and the number of additional bacterial strains

Discussion

While many bacteria reside in the canine vaginal canal, only brucellosis is a true venereally transmitted bacterial disease in canines. Other bacterial populations

already exist in the reproductive tract of dogs, and normal immune defence mechanisms quickly clear any infection that occurs during breeding (25). It cannot be unequivocally confirmed that the presence of *M. canis* in the genital tract of a bitch will result in reproductive

disorders. However, a detailed history and any clinical signs should be confronted with laboratory results whether an increase in the number of these bacteria on the vaginal mucosa could have a negative effect on fertility. In our study, we confirmed some association between the presence of *M. canis* in the vagina of breeding bitches and a decrease in selected reproductive parameters, but in our opinion this bacterial species should be considered as saprophytic and only conditionally pathogenic. The finding we obtained in this research, highlights the importance of new verification the status of *M. canis* in the bacterial flora of breeding bitches and its clinical approach.

In comparison to the cohort sizes of previous research in this area, the sample size in the current study was large. This study aimed to determine the prevalence of *M. canis* in the vagina of healthy breeding bitches and compare it with the prevalence in bitches characterised by selected reproductive problems important from the clinicians' and breeders' point of view. The research's first significant finding was the confirmation that *M. canis* is a prevalent pathogen in the genital tract of breeding bitches (34.91%). The second was that the prevalence of *M. canis* was similar in the both compared groups of dogs. This particular result was somewhat consistent with most of the conclusions of previous research on *Mycoplasma* spp., where the prevalence in female dogs ranged 23–75% (18, 20, 22), although those authors' conclusions about the pathogenicity of this bacterium remain to be substantiated. Therefore, based on our results, its agency in causing reproductive disorders in breeding bitches is therefore still uncertain.

The effect of natural mating on the frequency of *M. canis* in the study population was analysed for the first time. The insignificant differences in the frequency of this bacterium between bitches that had not mated (37.74%) and bitches that had (33.14%) confirmed the suspicion that *M. canis* is a saprophytic bacterium that resides in the canine vagina and is not transmitted necessarily by mating. *Mycoplasma canis* occurred in both mated and unmated bitches, so sexual transmission of this bacterium may be questionable. *Mycoplasma canis* was previously thought to be most often transmitted by direct sexual contact, which makes interesting this finding's support for non-sexual acquisition of this bacterium. Furthermore, in both groups of dogs in our research, no association of *M. canis*-positivity with mating efficiency was observed. This finding, along with others we obtained in our research, highlights the importance of new verification. Individuals just being introduced into breeding may be considered a particularly interesting experimental group.

Although Holtzman *et al.* (13) demonstrated that mycoplasma is a pathogen that causes purulent endometritis or endometrial cysts in some intranasally infected bitches, Janowski *et al.* (2008) reported in contrast that the frequency of positive tests for *Mycoplasma* spp. was high in healthy bitches, suggesting that the bacterium may be part of the

physiological flora of the vaginal mucosa (17). Suggestive of the same, in the study by Doig *et al.* (7) where 75 females were examined, the percentages of individuals with *Mycoplasma* spp. were comparable in fertile and infertile groups, and were high (>60%). Likewise Maksimović *et al.* (22) showed the presence of *Mycoplasma* spp. in swabs from 122 healthy females in 34% of owner-bred bitches and in 39% of stray bitches.

Our other important finding is the widespread presence of *M. canis* in dog kennels; a significant proportion of them were home to *M. canis*-positive individuals. This is another argument confirming the prevalence of this bacterium and proving its rather saprophytic nature in the vaginal environment in bitches.

Previous studies have isolated *M. canis* from male dogs with urogenital disease and infertility even after prolonged antibiotic therapy. It has also been cultured from the prostate, epididymis and the chronically inflamed bladder wall (19). Doig *et al.* (7) isolated several species of mycoplasma from the vagina and prepuce and suggested a possible association of ureaplasma with infertility in male dogs, but conclusive evidence is still lacking (4). Experimental infection with *M. canis* resulted in chronic urethritis and epididymitis in 50% of males tested, and in females, an enlarged uterus and endometritis were observed (28). However, further research is required to determine whether *M. canis* is associated with genital tract infections and infertility in female dogs (1, 19).

Unquestionably, our results confirm the opinion of Domrazek *et al.* (9) that *M. canis* is a part of the bacterial flora of the majority of breeding bitches, in which is a contention formed from the observation that 77.94% of the females tested in their study were positive. On the other hand, they showed that infected bitches were fertile and produced numerous healthy litters. Moreover, there was also no significant effect of *M. canis* on the incidence of abortions in the studied bitches, which is not in line with our conclusions. We confirmed a statistical correlation between the presence of *M. canis* in the kennel and the incidence of single puppy deaths and low litter sizes. There is no such correlation with regard to the bacterium's presence and occurrence of resorption in pregnant bitches and puppy deaths >50% of a litter. Our study also shows that there is a statistical correlation between the copresence of *M. canis* and at least two other bacterial species in the vaginal environment and the group allegiance of the individual bitch to the group with reproductive disorders.

With reference to the above publications and on the basis of our study conducted, the real impact of *M. canis* on the fertility of bitches should be considered and reappraised. We agree with other authors that the vaginal mucosa is not sterile and the presence of microbes on its surface does not always indicate an ongoing disease process (2, 3, 27, 32, 34). Furthermore, the presence of *M. canis* in the vaginas of both healthy and diseased breeding bitches is inconclusive as regards the bacterium's causation of fertility problems. This may be

due to differences between strains and their virulence (24). Future studies should focus on comparing the phenotypes and genotypes of isolated *M. canis* strains.

This study aimed to draw conclusions that are applicable to the entire population of breeding bitches by obtaining results from a large group of individuals. The use of a significant sample size has increased the statistical significance of the findings.

Conclusion

It cannot be unequivocally confirmed that the presence of *M. canis* in the genital tract of a bitch will result in reproductive disorders. Detailed patient histories and any clinical signs should be contrasted with laboratory results before it can be confidently predicted whether an increase in the number of these bacteria on the vaginal mucosa could have a negative effect on fertility. In our study, we confirmed some association between the presence of *M. canis* in the vagina of breeding bitches and a worsening of selected reproductive parameters, but in our opinion this bacterial species should be considered as saprophytic and only conditionally pathogenic. The finding we obtained in this research highlights the importance of new verification of the status of *M. canis* in the bacterial flora of breeding bitches and a reconsideration of the agency of this bacterium in fertility disorders by clinical practitioners.

Conflicts of Interests Statement: The authors declare there is no conflict of interests regarding the publication of this article.

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Animals Rights Statement: Ethical review and approval were not required for this study as the vaginal samples were collected during routine veterinary examinations of the animals. The animals were treated in accordance with the Code of Good Veterinary Practice. The owners of the dogs gave their informed consent for inclusion of their animals in the study.

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