# THE VAGINAL MICROFLORA AND CANINE HERPESVIRUS 1 ANTIBODY TITERS THROUGHOUT THE ESTROUS CYCLE OF BREEDING BITCHES

*De vaginale flora and antistoffentiters voor het caniene herpesvirus tijdens de oestrische cyclus van teven*

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## ABSTRACT

**The objective of this study was to make a qualitative and quantitative assessment of the vaginal microflora during the estrous cycle of 34 bitches from kennels with and without fertility disorders, in order to study whether any particular bacterial species is isolated more often in the kennels of low fertility status in general or during a specific stage of the estrous cycle. Furthermore, a serological follow-up of CHV1 antibody titers was performed to determine whether seroconversion, as a result of (re)infection or reactivation, occurs during a particular stage of the estrous cycle and, if so, how it affects fertility.**

**The number of bacteria isolated was significantly influenced by the stage of the estrous cycle. Bacterial counts were higher during pro-estrus and estrus. In the individual bitch the flora changed during the estrous cycle but when all samples were compared, no specific bacterial species could be associated with a given stage of the estrous cycle. No significant differences were found between bitches from kennels with and without reproductive disorders. In all but one bitch, the serum-neutralizing (SN) antibody titers to CHV1 did not change during the estrous cycle. In the one bitch that seroconverted, no effect on the fertility status was found. It was concluded that no correlation between the aerobic vaginal bacterial flora and/or CHV1 and fertility could be demonstrated.**

## SAMENVATTING

Het doel van deze studie was de aerobe vaginale flora van 34 teven afkomstig van kennels met en zonder fertiliteitsproblemen kwalitatief en kwantitatief op te volgen tijdens de oestrische cyclus, om na te gaan of er een bepaalde bacterie vaker geïsoleerd kon worden in kennels met fertiliteitsproblemen in het algemeen of tijdens een bepaald stadium van de cyclus. Verder werden van deze teven antistoffentiters voor CHV1 bepaald om na te gaan of er seroconversie optrad tijdens een bepaald cyclusstadium en zo ja, wat de invloed op de fertiliteit zou zijn.

Het cyclusstadium had een significante invloed op het aantal bacteriën. Tijdens pro-oestrus en oestrus werd een groter aantal kiemen geïsoleerd. Bij de individuele teef traden tijdens het verloop van de cyclus veranderingen op in de vaginale flora maar geen enkele kiem kon geassocieerd worden met een bepaald cyclusstadium. Verder werden er geen significante verschillen gevonden tussen de vaginale flora van teven op probleembedrijven en teven op niet-probleem bedrijven. Antistoffentiters voor CHV1 bleven constant tijdens het verloop van de cyclus, met uitzondering van 1 teef. De seroconversie die bij deze teef optrad, had geen invloed op de fertiliteit. Als besluit kan gesteld worden dat er geen verband tussen de aerobe vaginale flora en/of CHV1 en fertiliteit kon worden aangetoond.

## INTRODUCTION

Fertility problems are a frequent complaint in dog breeding. One major cause of infertility is mating at an inappropriate time. Breeders are often convinced, however, that an infectious agent is involved in reproductive failure. This has resulted in indiscriminate treatment of bitches with antibiotics before breeding (Freshman, 1991).The only bacterium so far known to be a specific cause of fertility problems in the bitch is *Brucella canis*, though it has not yet been demonstrated in Belgium. Many other bacterial species have been implicated as a cause of fertility problems. Early studies on the relationship between fertility and β hemolytic streptococci concluded that they were the etiological cause of infertility (Hare and Fry, 1938; Minett and Ellis, 1940). Later studies showed that bacterial species cultured from the vagina of infertile bitches do not differ from those found in healthy bitches (Allen and Dagnall, 1982). Streptococci, staphylococci, *Escherichia coli* and *Pasteurella multocida* are isolated most frequently from the vaginas of bitches with reproductive disorders, but researchers have failed to establish a causal link between these bacterial species and infertility (Björstrom, 1993). Some investigators claim that treatment is indicated when large numbers of these bacteria are found in pure culture (Okkens *et al.*, 1992) Other investigators have strong arguments against the treatment of healthy breeding bitches, since treatment with certain antibiotics may remove the normal flora and allow colonization of the vagina by *Mycoplasma* spp. and *Escherichia coli* (Ström and Linde-Forsberg, 1993). Besides bacterial infections, herpesvirus infection is also associated with fertility problems in dogs (Poste and King, 1971). In Belgium, Van Gucht *et al.* (2001) found a higher seroprevalence in bitches with fertility problems, whereas Ronsse *et al.* (2004) failed to establish an association between infertility and CHV1 antibody titers.

Since it has been shown that the vaginal flora is influenced by the stage of the estrous cycle (Björstrom and Linde-Forsberg, 1992), the purpose of this study was to make a qualitative and quantitative assessment of the vaginal microflora during the estrous cycle of bitches from kennels with and without fertility disorders, in order to study whether any particular bacterial species is isolated more often on the kennels of low fertility status either in general or during a specific stage of the estrous cycle. Furthermore, little is known about how the estrous cycle affects CHV1 reactivation and subsequent seroconversion in dogs. Clinical signs upon CHV1 reactivation have been observed during heat and around parturition (Poste and King, 1971; Anvik, 1991), but no experimental data exist to confirm this. Therefore, a serological follow-up of CHV1 antibody titers was performed to determine whether seroconversion, as a result of (re)infection or reactivation, occurs during a particular stage of the estrous cycle and, if so, how this would affect fertility.

## MATERIALS AND METHODS

### Kennels

Four kennels were selected for this study. Selection was based on previously determined antibodies for CHV1 and the existence of a history of fertility problems. Low fertility was defined as a low conception rate. Before the onset of the study, two kennels (A and B) were experiencing fertility problems and problems of neonatal death (Table 1). In these kennels, all dogs investigated a year before the onset of this study were seropositive for CHV1 (16/16 in kennel A, 3/3 in kennel B). They were further described as having a "low" fertility status. The dogs in the other two kennels (C and D) had no fertility problems before the onset of this study and only 42% (5/12 in kennel C) and 75 % (3/4 in kennel D) of the dogs investigated were seropositive for CHV1. Their fertility status was described as "high". An overview is given in Table 1.

### Animals

Thirty-four breeding bitches were selected for this study. Because in most cases sampling started in anestrus, mainly bitches that were expected to come in season within 2-3 months were selected. For each bitch a full history was taken. This concerned the general condition, cycle and details about fertility. All bitches were followed during one estrous cycle. From each bitch, samples were taken at each of the four stages of the estrous cycle. In some bitches not all four stages of the estrous cycle were covered, due to the fact that samples could only be taken between September 2001 and March 2002 and because the owners sometimes did not notice in time that their bitch was in season. In 32 out of 34 bitches (94%), serum samples of at least three stages of the estrous cycle could be analyzed. In 16 out of 34 bitches (47%), all four stages were covered.

**Table 1. Characteristics of the investigated kennels.**

| Fertility status | Kennel | Kennel size | Breed | Kennel history (2000-2001) | | Present study (2001-2002) | | Time of mating (method) | Problems |
|---|---|---|---|---|---|---|---|---|---|
| | | | | N of dogs examined | N of dogs seropositive (%) | N of dogs examined | N of dogs seropositive (%) | | |
| Low | A | 114 | Mixed | 16 | 16 (100%) | 14 | 12 (86%) | Estrous behavior<br>Male* | Neonatal death<br>Low pregnancy rates |
| | B | 12 | Bernese mountain dog | 3 | 3 (100%) | 4 | 4 (100%) | Estrous behavior<br>Progesterone | Neonatal death<br>Low pregnancy rates |
| High | C | ± 100 | Mixed | 12 | 5 (42%) | 14 | 4 (29%) | Estrous behavior<br>Male* | None |
| | D | 15 | Bernese mountain dog<br>Japanese spaniel | 4 | 3 (75%) | 2 | 1 (50%) | Estrous behavior<br>Male* | None |

*Male and female were put together regularly during estrus to observe the behavior of the male as well as the behavior of the bitch

## Sampling

Blood and vaginal samples were taken at the four stages of the estrous cycle ( anestrus, pro-estrus, estrus and metestrus). From each bitch two vaginal swabs were obtained from the cranial vagina through a vaginal speculum. Before the speculum was inserted, it was disinfected with 70% alcohol and dried with a sterile tissue. One swab was placed in Stuart Transport Medium for bacteriological examination, the other one was taken for vaginal cytology. For cytological interpretation, the air-dried smears were stained with Diff-Quick and evaluated under the microscope. Blood samples were collected for the determination of CHV1 seroneutralizing (SN) antibody titers and for the examination of plasma progesterone concentrations.

## Staging the estrous cycle

Three methods were used for staging the estrous cycle: clinical signs, vaginal cytology and blood progesterone levels determined by means of radioimmunoassay (Henry *et al.*, 1987) (Table 2).

## Bacteriological examination

Primary isolation was done by directly streaking each swab onto 3 different media: Colombia agar containing 5% sheep blood (Gibco BRL, Paisly, U.K.), Colombia agar containing 5% sheep blood, colistin and nalidixic acid (CNA) (Oxoid, Basingstoke, UK) and MacConkey agar (Oxoid). Incubation was carried out at 37° C for a period of 24 h under aerobic conditions. Isolates were identified by their colonial morphology and biochemical characteristics according to Quinn *et al.* (1994). For *Streptococcus canis,* an additional agglutination test (Streptococcal Grouping Kit, Oxoid) was performed. A quantitative culture score was obtained by assessing the amount of growth of each colony type on the agar plate. No visible bacterial growth was scored as 0, between 1 and 10 colonies was scored as 1, 11-100 colonies was scored as 2, 101-300 colonies was scored as 3 and more than 300 colonies was scored as 4. Isolation of more than 5 morphologically distinct bacterial colonies was defined as a 'mixed culture'. For mixed cultures, the identification of bacterial species and culture score was not performed.

## Serology - serum neutralization test

All serum samples were first heated at 56° C for 30 min to inactivate the endogenous complement. Twofold serum dilutions up to 1/64 were performed in 96-well tissue culture microplates at 50 µl/well. Subsequently, 10 000 $TCID_{50}$ (tissue culture infectious dose with 50% end point) of the CHV1 93H61 strain diluted in 50 µl medium was added. The serum

**Table 2. Staging of the estrous cycle for sampling.**

| | Clinical signs | Vaginal cytology | Progesterone |
|---|---|---|---|
| **Anestrus** | No vulvar swelling<br>No sanguineous discharge | Basal and intermediate epithelial cells | <0.5 ng/ml |
| **Pro-estrus** | Vulvar swelling<br>Sanguineous discharge<br>Sexually attractive | Gradually more cornified cells<br>Some neutrophils | 0.5-2 ng/ml |
| **Estrus** | Acceptance of the male | >80% cornification<br>No neutrophils | 2-25 ng/ml |
| **Metestrus** | Rejection of the male | Large intermediate cells<br>Many neutrophils | >20-25 ng/ml |

**Table 3. Vaginal microflora isolated throughout the estrous cycle in kennels with high and low fertility status.**

| Fertility status | Total N of samples | N cultured positive | Mixed cultures | N of samples positive for*. | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Streptococci | *S. canis* | *E. coli* | Staphylococci | Enterococci | *Pasteurella* spp. |
| High | 50 | 49 | 12[a] | 20[a] | 8[a] | 12[a] | 5[a] | 7[a] | 3[a] |
| Low | 56 | 51 | 4[b] | 37[a] | 20[a] | 6[b] | 9[a] | 7[a] | 2[a] |

* with the exception of the mixed cultures (cultures containing more than 5 species)
[a-b] values with different superscripts within the same column are statistically significant ($P < 0.05$)

**Table 4 . Influence of the estrous cycle on the vaginal microflora.**

| Fertility status | Stage of the estrous cycle | Total N of samples | N. cultured positive | Mixed cultures | % of samples positive for* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Streptococci | *S. canis* | *E. coli* | Staphylococci | Enterococci | *Pasteurella* spp. |
| High | Anestrus | 11 | 10 | 1 | 33 | 0 | 0 | 22 | 11 | 11 |
| | Pro- estrus | 10 | 10 | 5 | 80 | 20 | 60 | 0 | 40 | 0 |
| | Estrus | 14 | 14 | 2 | 58 | 25 | 42 | 25 | 25 | 17 |
| | Metestrus | 15 | 15 | 4 | 55 | 36 | 36 | 0 | 9 | 0 |
| Low | Anestrus | 13 | 12 | 0 | 75 | 42 | 8 | 25 | 0 | 0 |
| | Pro-estrus | 12 | 12 | 0 | 75 | 33 | 8 | 17 | 25 | 17 |
| | Estrus | 13 | 13 | 1 | 83 | 50 | 25 | 17 | 25 | 0 |
| | Metestrus | 18 | 14 | 3 | 82 | 45 | 9 | 18 | 9 | 0 |

* with the exception of the mixed cultures (cultures containing more than 5 species)

mixture was incubated at 37° C for 23 h. Afterwards, 25 µl of diluted guinea-pig complement was added to each well. After incubation at 37° C for 1 h, each virus-serum mixture was added to a MDCK cell monolayer. Microplates were then further incubated in a $CO_2$ incubator at 37° C and microscopic examination for the presence of characteristic CHV1 cytopathic effect (CPE) was performed over a period of 5 days after incubation. Minimal essential medium (MEM) enriched with 5% foetal calf serum (FCS) (Gibco BRL, Paisly, UK), 100 U/ml penicillin (Continental Pharma, Brussels), 0.1 mg/ml streptomycin (BUFA Belgium), 0.1 mg/ml kanamycin (Kela Veterinaria, Sint-Niklaas, Belgium) and 0.3 mg/ml glutamine (BDH Laboratory supplies, Dorset, UK) was used for dilution of sera, virus and cell suspensions. The SN titer was defined as the reciprocal of the highest serum dilution inhibiting CPE in 100% of all cultures (Ronsse *et al.*, 2002). The same SN test was used for the determination of CHV antibodies in all bitches at all sampling times, including those determined prior to this study.

### Statistical analyses

The bacteriological results of the vaginal flora were evaluated using univariate logistic regression (SPSS, 11.0). For each bacterial species, an analysis was performed in which the frequency of the absence (0) or presence (1) of a bacterial species was compared between kennels of high and low fertility status and between the different stages of the estrous cycle. Since bacterial counts were not recorded on a normally distributed scale, these results were analyzed by an ordinal logistic regression (SPSS, 11.0) using bacterial growth categories (1-4). Also the frequency of the absence (0) or presence (1) of CHV1 antibodies was compared between kennels of high and low fertility status using univariate logistic regression (SPSS, 11.0).

## RESULTS

### Bacteriology

#### *Vaginal bacterial flora in relation to the fertility status*

A total of 106 samples were taken from 34 bitches in four kennels at the four stages of the estrous cycle. The fertility status of the kennel had no significant effect on the number of bacteria isolated (P=0.17). The organisms isolated most often were streptococci (63.3% of the samples), *Escherichia coli* (20%), staphylococci (15.6%), enterococci (15.1%) and *Pasteurella* spp. (5.6%). Table 3 shows the vaginal bacterial flora in relation to the fertility status. In the two kennels with low fertility status, streptococci, including *Streptococcus canis,* tended to be isolated more often than in kennels with high fertility status, though this difference was not statistically significant (P=0.096). Moreover, the isolation rate of *S. canis* was not significantly different between the kennels with a high fertility status and those with a low fertility status (P=0.082). In one of the two kennels with a low fertility status, all the bitches were negative for *S. canis*. *E. coli* was isolated significantly more often in the two kennels with a high fertility status (P<0.05). Mixed cultures were significantly more present in kennels with high fertility (P<0.05).

#### *Relationship between the vaginal microflora and the stage of the estrous cycle*

All of the vaginal samples in pro-estrus and estrus were bacteriologically positive. In anestrus, 92% of the samples were positive and in metestrus 88% of the samples were positive. A larger number of bacteria were found during pro-estrus and estrus. Indeed, the stage of the estrous cycle had a significant influence on the number of colonies which were present (P<0.01). Negative cultures were only observed during anestrus and metestrus. Mixed cultures were found during all stages of the estrous cycle. In the individual bitch, the flora changed during the estrous cycle. Although changes occurred in the flora of the individual bitch, no specific bacterial species could be associated with a certain stage of the estrous cycle (Table 4).

### Serology

At the start of the study, 21 out of 34 bitches (62%) were seropositive (Table 1). Five of these bitches belonged to the kennels with a high fertility status, and 16 belonged to the kennels with a low fertility status. Eighteen bitches remained seropositive for CHV1 throughout the entire estrous cycle with no changes in seroneutralization titers. Two bitches were initially slightly positive (SN titer of 6 and 12) but became seronegative during the course of the estrous cycle. One bitch seroconverted from 3 in anestrus into 96 and 192 in estrus and metestrus. Fertilization of this bitch during the estrous cycle under investigation was successful. In kennels of low fertility status,

significantly more bitches were seropositive for CHV1 ($P<0.01$).

## DISCUSSION

Little is known about the temporal dynamics of the canine vaginal flora and canine herpesvirus 1. In the present study, no specific bacterial species could be associated with a given stage of the estrous cycle, although changes did occur in the vaginal flora of the individual bitch. These results are in agreement with the findings of Olson and Mather (1978), though they are in contrast with the results of Björstrom and Linde-Forsberg (1992). The latter investigators found a correlation between certain bacterial species and a specific stage of the estrous cycle. In our study, no association between the canine vaginal flora and the fertility status could be made. The first possible explanation as to why no association was found could be the limited amount of data. But there are several other studies in which the bacterial species cultured from the vaginas of infertile bitches did not differ from those found in healthy bitches (Allan and Dagnall, 1982; Baba *et al.*, 1983; Bjüstrom and Linde-Forsberg, 1992; Bjüstrom, 1993; Watts *et al.*, 1996), and in most of these studies more bitches were sampled than in ours (Allen and Dagnall (1982), 143; Baba et al. (1983), 82; Björstrom and Linde-Forsberg (1992), 59; Björstrom (1993), 203 and Watts et al. (1996), 32). However, there was no chronological follow-up of the bitches during the estrous cycle, with the exception of the study of Björstrom and Linde Forsberg (1992) and the study of Watts (1996). This last study only concerned healthy bitches, while we monitored both healthy bitches and bitches with problems. Our study also differs in the fact that we performed both a qualitative and a quantitative assessment of the vaginal flora of the four stages of the estrous cycle, while in the study of Björstrom and Linde-Forsberg (1992) only a qualitative assessment was performed.

Secondly when fertility is discussed, it is likely that the uterine flora is of more significance than the vaginal flora. Several studies have shown, however, that the uterine flora is similar to that of the vagina and the cervix (Baba *et al.*, 1983; Watts *et al.*, 1996), which is probably due to the fact that in dogs the cervix is open during estrus under the influence of estrogens (Verstegen *et al.*, 2001) and the ascension of lower genital tract microorganisms into the uterus can frequently occur. However, they are likely to be eliminated by the normal uterine defense mechanisms, such as neutrophils and local immunity (Wiesenfeld *et al.*, 2002). This is in agreement with the findings of Watts *et al.* (1996), who rarely found bacteria in the uterus during metestrus and anestrus, though of course their study only concerned healthy bitches. It may be possible that under certain circumstances (hormonal imbalance, large numbers of bacteria) the bacteria are not always eliminated. Then they may act either directly on the endometrium or indirectly through the release of inflammatory mediators that cause morphological changes leading to reduced implantation rates. The fact that the bacteria act in this way as facultative pathogens may explain why *S. canis* is also isolated from perfectly fertile bitches.

Sex hormones may possibly play a role in infertility, but little is known about the influence of sex hormones on the canine vaginal and uterine flora. In other species it is known that sex hormones have an influence on the number of bacteria isolated (Larsen *et al.*, 1976; Larsen *et al.*, 1977). This is in agreement with the present results, since we found a higher number of bacteria during pro-estrus and estrus. Baba *et al.* (1983) also found higher counts at estrus, though their study did not cover an entire breeding cycle. Furthermore, in several mammalian species, high progesterone levels are associated with increased susceptibility to infection (Dhaliwell *et al.*, 2001). In pyometra, it is hypothesized that not only bacteria but also increased progesterone concentrations are involved in the pathogenesis (Noakes *et al.*, 2001). Several authors believe that the presence of large numbers of bacteria is important (Okkens *et al.*, 1992; van Duijkeren, 1992). This could not be confirmed in our study since the number of bacteria isolated was not affected by the fertility status of the kennel.

Another explanation for not finding an association between the presence of bacteria and fertility is that other bacteria than the ones investigated in this study, such as *Mycoplasma* spp. and anaerobic bacteria, are involved in fertility problems. *Mycoplasma* spp. is often isolated from the vagina of healthy bitches and is reported to be associated with fertility problems in bitches when concentration levels exceed $10^6$ CFU/ml (Mimouni, 1996). Since it is still unclear whether bacteria are involved in infertility, recommendations for treatment are premature and further research is mandatory, especially at the level of uterine colonization and uterine defense mechanisms in fertile and infertile bitches.

The estrous cycle was clearly influencing the vaginal microflora, but had no apparent effect on

canine herpesvirus 1. All seronegative dogs remained seronegative. Some of the 20 seropositive bitches remained seropositive and some became seronegative. Only one bitch seroconverted during estrus. Since this bitch already had a positive CHV1 antibody titer, it was likely that reactivation or reinfection had occurred. In spite of the seroconversion during estrus, the bitch became pregnant upon mating. Therefore, an association between CHV1 and infertility could not be made in this study. This is in contrast with the study done by Poste and King (1971), who observed a decrease in pregnancy rates in bitches with genital lesions caused by CHV1. Van Gucht *et al.* (2001) also found a correlation between the circulation of CHV1 in kennels and fertility problems.

Another observation showed that in two bitches the SN titers dropped below detection limits during the course of the estrous cycle. If in these bitches reactivation were to occur at the moment of parturition, the mother would infect her puppies. Then neonatal death could occur because the puppies would not be protected by colostral antibodies. This scenario is not inconceivable because parturition represents a stress situation for the bitch and in these situations virus reactivation and excretion can take place (Ronsse *et al.*, 2004). Therefore, the possibility of vaccinating such bitches should be considered.

In conclusion, CHV1 antibody titers were not affected by the estrous cycle, since seroconversion was only demonstrated in one bitch. Furthermore, no association between the aerobic vaginal bacterial flora and/or CHV1 and fertility could be demonstrated.

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