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# *Mycoplasma haemocanis* Infection— A Kennel Disease?

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***Mycoplasma haemocanis*** **(formerly *Haemobartonella canis*) is a red blood cell parasite that causes disease mainly in immunosuppressed and splenectomized dogs. Clinical outbreak of the disease resulted in failure of a large experimental project. We aimed to identify whether *M. haemocanis* has increased prevalence in kennel-raised dogs. In a prospective study, we compared the prevalence of *M. haemocanis* in whole blood (anti-coagulated by use of EDTA) collected from pet dogs (University of Illinois, Urbana Champaign, Ill.; n = 60) with that in blood from dogs raised in three distinct kennels in western Europe (WE; n = 23), eastern Europe (EE; n = 20), and North America (NA; n = 20). Screening included antibody testing and microscopy of blood smears. The presence of *M. haemocanis* was identified using a polymerase chain reaction (PCR) assay for specific DNA of the organism. None of the pet dogs (0%) was test positive for *M. haemocanis* DNA. *Mycoplasma haemocanis* was found in dogs tested at all of the kennels. Infection rate in the three kennels was 30, 35, and 87%, respectively (all $P < 0.001$ versus control, $\chi^2$-test). Latent infection with *M. haemocanis* was not a single observation in kennel-raised dogs. Prevalence may be higher than that in a pet dog population. The potential exists for these latent infections to adversely affect or confound research results.**

*Mycoplasma haemocanis* and *M. haemofelis* (formerly known as *Haemobartonella canis* and *H. felis*, respectively) are the cause of an infectious disease in dogs and cats, respectively. These hemotropic mycoplasmas are small, gram-negative bacteria that cannot be grown in culture. The parasites attach to the surface of the red blood cell, and have the potential to cause severe alterations of the cell's shape, resulting in anemia (5, 22). It is known that an outbreak of clinically overt disease following infection with *M. haemocanis* is promoted by preexistent immunosuppression, parvovirus infection, lymphosarcoma, or microbial infection, but most importantly for research purposes, by splenectomy (6, 10, 11, 15, 23). Our group recently reported an outbreak of clinical infections with *M. haemocanis* in splenectomized beagle dogs used in our research laboratory (12). We had to abandon a large research project due to the fact that *M. haemocanis* could not be successfully eradicated in our laboratory animals. It is noteworthy that, in 1975, Pryor and Bradbury reported *M. haemocanis* in dogs at two separate research centers in the United States (19).

Infection with *M. haemocanis* is known to veterinary clinicians and is the subject of various case reports (2, 6, 9, 13, 23). Its manifestations include episodes of severe anemia and bleeding disorders in the dog, but may also follow a clinically inapparent courses of disease (5, 6, 13, 19). *Mycoplasma haemocanis* has world-wide distribution (5, 19, 21, 23); however, to the authors' knowledge, it has not been recognized as a kennel-related disease. It is believed that the organism is transmitted among dogs by blood-feeding arthropods or by direct inoculation (21), and there also is the possibility of transplacental infection (14). The fact that preexistent immunosuppression is required for clinical manifestation (11, 23) of disease in the dog increases the likelihood that an infection in kennel-raised dogs may go undetected.

Since kennel-raised dogs are supplied to research laboratories, our concern is that animals with unrecognized *M. haemocanis* infection may have affected research results in the past and will do so in the future. For the sake of maintaining quality of experimental research and avoiding use of compromised laboratory animals, our aim was to determine whether infection with *M. haemocanis* is a kennel-related issue.

The purpose of the prospective study reported here was to elucidate whether our previously reported case involving the clinical outbreak of mycoplasmosis in research dogs was a single observation, or whether infection with *M. haemocanis* is underestimated in kennel-raised dogs. We tested the null hypothesis, that we would not observe increased prevalence of infection with *M. haemocanis* in purpose-bred, kennel-raised beagle and mixed breed dogs compared with that in a pet dog population attended to a veterinary clinic.

## Materials and Methods

The study was approved by the Animal Protection Commission of the Bavarian Government and the institutional board for the care of animal subjects of the University of Illinois, College of Veterinary Medicine, Urbana Champaign, Illinois.

**Origin of blood samples.** Sixty-three blood samples originated from dogs at three kennels in France, Hungary, and North

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**Table 1.** Specifications of animals in the various groups

| Group | Sex | Body wt (kg)* | Source | Breed |
|---|---|---|---|---|
| Control pets | Random† | 7-40 | Random source | Variable |
| EE | Random | 11-20 | Purpose-bred | Mixed breed |
| WE | Male | 16-20 | Purpose-bred | Beagles |
| NA | Female | 13-16 | Purpose-bred | Mixed breed |

*Body weight is given as range.
†Random sex equals a sex distribution of approximately 50% male and 50% female.
EE = Eastern European kennel; WE = western European kennel; NA = North American kennel; body wt = body weight.

America. Before the animals were purchased for research purposes, blood was submitted for laboratory screening. On the basis of the judgment of the kennel's veterinary clinician, the animals were healthy on standard clinical investigation. Blood collected in EDTA-containing tubes was cooled, and was submitted via courier transportation. On arrival at our institution, the blood samples were aliquoted for further analysis. Parallel to the acquisition of blood samples from kennel-raised dogs, blood samples from 60 pet dogs were taken independently over a one-month period. The pet dogs were consecutively admitted to the College of Veterinary Medicine, Urbana Champaign, because of various conditions. There was no prospective randomization for breed, sex, or body weight (for specifications of dogs, see Table 1).

**Detection of cross-reactive antibodies.** As an indirect sign of previous contact with *M. haemocanis* in the screened kennel-raised dogs, blood testing for cross-reactive antibodies was performed. Plasma samples were obtained from whole blood by use of centrifugation (4,000 ×*g*, 10 min) and were submitted to a specialized veterinary parasitologic laboratory (ALOMED Laboratories, Radolfzell-Boehringen, Germany) for detection of anti-*M. haemocanis* antibodies. Since *M. haemocanis* (*H. canis*) cross-reacts with *Rickettsia conori*, the sera were subjected to an indirect fluorescent antibody test, in which *R. conori* was used as antigen (*Rickettsia conori* -Spot IF, bioMerieux, Lyon, France). Fluorescein isothiocyanate (FITC)-labeled goat anti-dog IgM (μ) antibody and FITC-labeled goat anti-dog IgG (γ) antibody (KPL, Gaithersburg, Md.) were used to detect the presence of anti *M. haeomcanis* IgM and IgG antibodies in the canine sera using fluorescence microscopy. Antibody titer of the samples was measured in a dilution series using phosphate-buffered saline (PBS). Starting at a dilution of 1:40, the various dilutions of serum, as well as the FITC-conjugate, were incubated over a period of 1 h. At the end of the incubation period, the object slides were washed with PBS twice (5 min each time). A serum sample positive for *M. haemocanis* cross-reactive antibodies (IgM, 1:160) served as positive control, whereas PBS served as negative control. Positive and negative controls were assayed along with each object slide.

**Light microscopy, hemogram.** Blood smears were made in duplicate from whole blood samples and were dried and fixed, then underwent staining by use of the classic Giemsa method. For further analysis, parallel stains were forwarded to a veterinary laboratory specialist (ALOMED Laboratories); the others were tested at our laboratory. Morphologic/microscopic investigation by use of light microscopy was performed by two distinct blinded investigators. Positive and negative controls were not included. Hematologic analysis was performed by use of a Coulter counter (Model T40, Coulter Electronics, Krefeld, Germany). A single clinical pathologist at the University of Illinois performed all blood smear evaluations of samples from pet dogs, whereas the automated hematologic analysis was performed using a Cell-Dyne 3500 (Abbott Laboratories, Chicago, Ill.).

**Table 2.** Conditions for polymerase chain reaction (PCR) analysis

| Step of reaction | Temperature (°C) | Time (sec) | Cycles (n) |
|---|---|---|---|
| Predenaturation | 94 | 300 | 1 |
| Denaturation | 94 | 45 | |
| Annealing | 62 | 45 | 35 |
| Extension | 72 | 60 | |
| Extension | 72 | 600 | 1 |

**Table 3.** Overview of results obtained with the various methods

| Method | PCR | | Antibodies | | Smears | |
|---|---|---|---|---|---|---|
| Group | + | - | + | - | + | - |
| Control pets | 0 | 60 | ND | ND | ND | ND |
| EE | 6 | 14‡ | 15 | 4 | 10 | 2 |
| WE | 20 | 3‡ | 19 | 1 | 11 | 12 |
| NA | 7 | 13‡ | 16 | 4 | 16 | 4 |

‡$P < 0.01$ versus control group, $\chi^2$ test, corrected for multiple testing.
+ = indicative for *M. haemocanis* with the refering method.
- = not indicative for *M. haemocanis* with the refering method.
ND = not done.
*See* Table 1 for key.

**Identification of specific *M. haemocanis* DNA.** The microorganism was identified by use of a polymerase chain reaction (PCR) technique that yields evidence for the presence of hemotropic mycoplasmas in dogs (5) and cats (3, 16), using a specific representative gene sequence that is largely congruent with minor variations for both subspecies (4). After standard proteinase-K treatment, the DNA was extracted from 2 ml of whole blood (NucleoSpin Blood Quick Pure No. 74056950, Mackerey-Nagel, Dueren, Germany) according to the manufacturer's protocol. The PCR assay was performed using sequenced specific primers for *M. haemocanis* (AF 407208) that were based on a BLAST genomic database search (1) at the National Center for Biotechnological Information, National Library of Medicine (http://www.ncbi.nlm.nih.gov). Cycling took place by use of a RoboCycler (Gradient 40, Stratagene, La Jolla, Calif.). The PCR product was a segment of 677 bp of the genome. As negative controls for the PCR assay, as well as for contamination, we omitted DNA in the samples and added everything else previously described. As a positive control, we used sequenced DNA from a dog that had previously tested positive for *M. haemocanis* at three independent laboratories. For PCR conditions, see Table 2.

**Data analysis, statistics.** Data were divided into 4 groups: n = 20 (EE), n = 23 (WE), n = 20 (NA), and n = 60 (IL, control pets). After the diagnostic procedures had been completed, the relationship of positive to negative PCR results at the 3 kennels EE, WE, and NA (observed distribution pattern) were tested against pet controls (IL, expected distribution pattern) by use of a $\chi^2$-test, or subsidiary by use of a Fisher's exact test (Sigmastat 2.0, Jandel Scientific, San Rafael, Calif.). Hematocrit, platelet count, and white blood cell count were tested using a Kruskall-Wallis analysis of variance on ranks, followed by a rank sum test.

In all tests, the α-error threshold was set to 1% and was corrected for repeated testing according to the Bonferrroni-Holm method ($P < 0.01$ divided by n; n = number of sequential test). Values obtained from hemograms are depicted as box plots.

## Results

Almost a third of the pet dogs examined at the University of Illinois were anemic (hematocrit < 35%). However, none of the blood samples from these 60 control animals were positive for *M. haemocanis* DNA (Table 3).

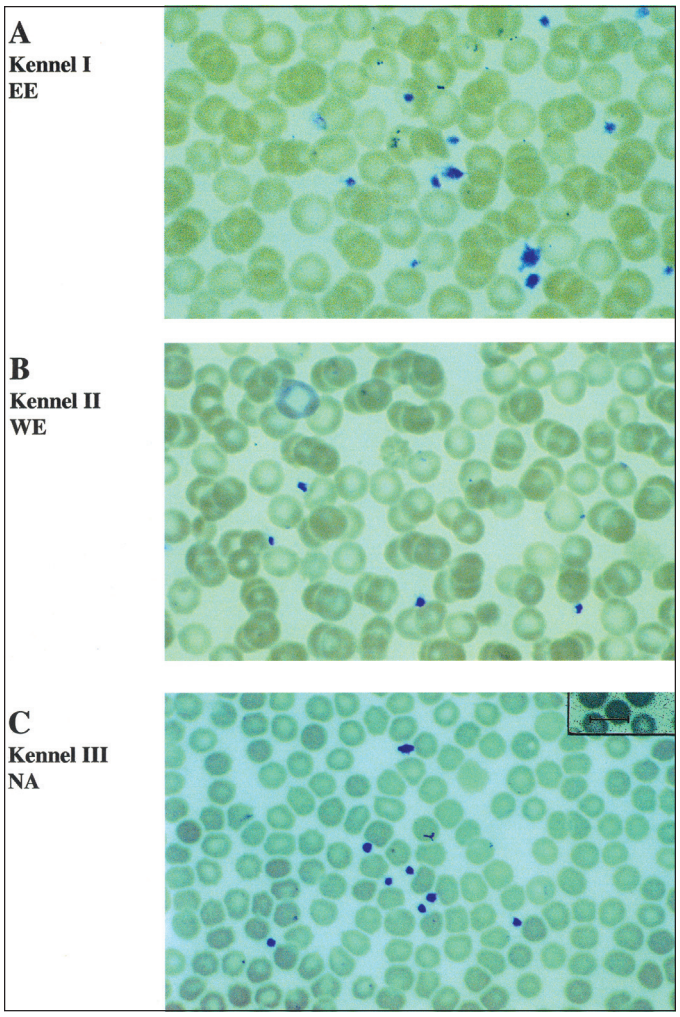


**Figure 1.** Blood smears from dogs at the eastern European (panel A, EE), western European (panel B, WE), and North American (panel C, NA) kennels at time of blood sample collection. Dogs were deemed healthy during investigation by the institutional veterinary physician. Small basophilic inclusion bodies are seen on panels A, B, and C.

All animals were classified by the institutional veterinary clinicians of the kennels as clinically healthy at the time of blood sample collection. However, *M. haemocanis* was presumptively identified by use of light microscopy of blood smears from several dogs at each of the 3 kennels (Fig. 1, Table 3). In kennel EE, 6 of 20 (30%; $P < 0.001$ versus control), in kennel NA, 7 of 20 (35%, $P < 0.001$ versus control), and in kennel WE, 20 of 23 (87%, $P < 0.001$ versus control) dogs were positive for *M. haemocanis* DNA on the basis of results of PCR analysis (Fig. 2). In dogs originating from kennels, testing for cross-reactive antibodies revealed high titer in a higher percentage of animals (79%, EE; 80%, NA; and 96%, WE) than the percentage of animals that were positive for specific DNA. The presence of *M. haemocanis* was regularly suspected by two independent investigators (Table 3).

**Hemograms.** Hematocrit in dogs from the WE and NA kennels and platelet count in dogs from the EE kennel were higher than values in control pets. White blood cell counts were high in dogs from all kennels compared with pet controls (Fig. 3, all $P < 0.01$). Erythrocyte indices (data not shown) were devoid of pathologic findings such as hyper- (hypo-) chromasia or volemia.

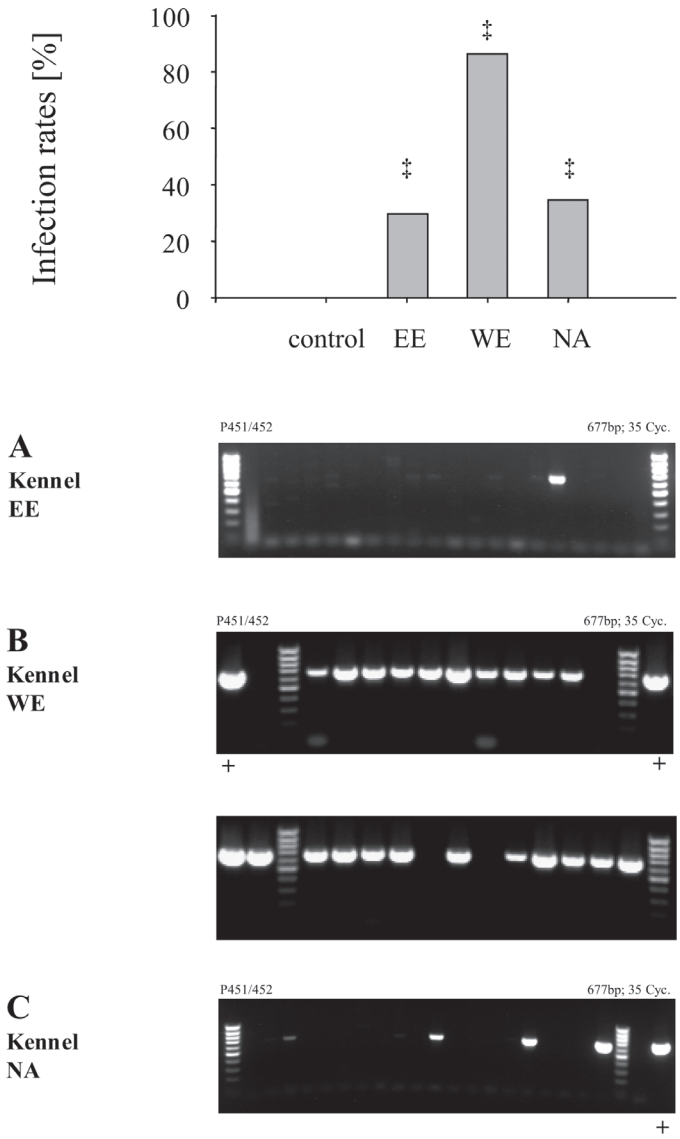


**Figure 2.** The bars in the upper panel depict the percentage infection rates obtained in the various groups of dogs. Significant differences between the groups as tested by use of a $\chi^2$-test are indicated by a double dagger. The panels A, B, and C depict agarose gels of DNA extracts (proteinase K) derived from blood samples from dogs at the eastern European (panel A, EE), western European (panel B, WE), and North American (panel C, NA) kennels. Highly positive results are indicated by white horizontal bars. Results that could only be visualized by use of prolonged light exposure were subjected to densitometric analysis, and were compared with negative controls.

## Discussion

The main result of our study is that, in contrast to what we had hypothesized, there was a significantly higher frequency of *M. haemocanis* infection in the kennel dogs than in control pets, in which *M. haemocanis* was absolutely absent. Thus, we concluded that occult infection with *M. haemocanis*, as recently reported by our group, is not a single observation.

Results of diagnostic procedures provide conclusive evidence that most kennel dogs that were tested had previously been exposed to *M. haemocanis* (79 to 96%), and that a considerable number of dogs from the three kennels had specific DNA in the blood at the time of investigation (30 to 87%). The rates of blood

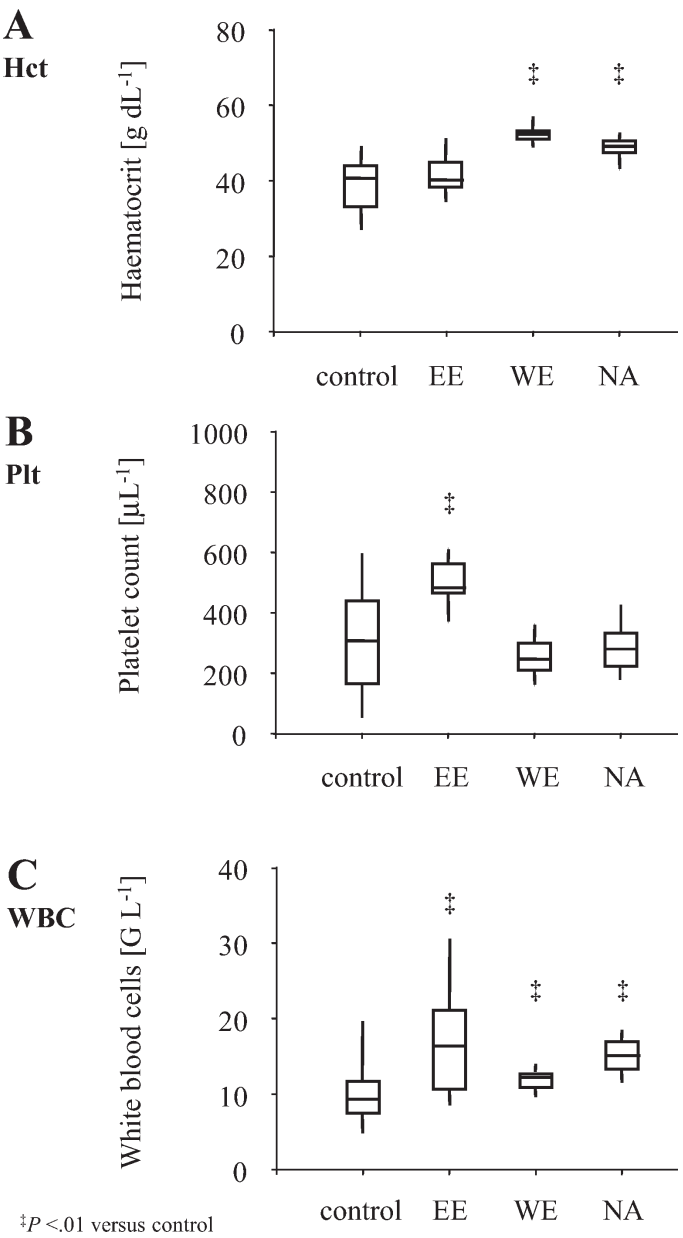


**Figure 3.** Red blood cell (A), white blood cell (B), and platelet (C) count data obtained from the healthy pet group (control, IL, n = 60), and the eastern European (EE, n = 20), western European (WE, n = 23), and North American (NA, n = 20) kennel groups at the time of blood sample collection. Significant differences between the groups as tested by use of Kruskall-Wallis analysis of variance on ranks, followed by a rank sum test are indicated by a double dagger. The *P*-values are adjusted for repeated testing.

smears classified test positive for *M. haemocanis* and the numbers of serum samples with significant titer of cross-reactive antibodies were seemingly inconsistent with results of DNA analysis. Since PCR analysis is considered the gold standard, the differences may be explained by problems with morphologic/microscopic diagnosis of blood smears. A reduced percentage of test positive blood smears compared with PCR results may be due to prolonged storage between blood sample collection and analysis, where *M. haemocanis* may have disintegrated from the red blood cell membrane and thus was not present on the blood smear during microscopy. On the other hand, a higher rate of positive findings in blood smears compared with PCR findings may indicate false-positive results (i.e., type-β error). Clerical errors with basophilic stippling, Howell-Jolly bodies, or stained artifacts are common errors. Moreover, we observed a higher number of dogs from EE and NA kennels with significant titer of cross-reactive antibodies than of dogs with positive PCR results. We further observed a higher number of dogs from WE and EE kennels with significant antibody titer than of dogs with positive blood smears. This can be explained by the fact that, at time of blood withdrawal, some of the dogs had previous contact with the parasite and subsequent antibody synthesis might have taken place, while at the same time, parasitemia was absent. So far, it is not known where *M. haemocanis* hides during absence of parasitemia. Perhaps the positive antibody titer only represents previous exposure and the dog has cleared the parasite. There was a significantly higher rate of infection of dogs at the kennels compared with that in the control pet dogs.

Although a control population was included in this prospective trial, the control population of pet dogs was not prospectively randomized or matched in terms of age, sex, or breed to the kennel-raised dogs. The study design also would have been stronger if control dogs were provided from geographic locations corresponding to that of the kennels. The kennel-raised dogs were chosen at random for the purpose of sale. The systematic survey of the dogs studied was initiated exclusively in those that were classified as healthy by the referring institutional clinicians of the various kennels. Due to the fact that blood samples were submitted to our institution via courier, differences in transportation time as well as differences in cooling quality might have affected hemograms and blood smears in an unpredictable manner. These factors may have contributed to the differences observed in the hemograms. However, final diagnosis of whether infection was present was confirmed by use of three methods: detection of cross-reactive antibodies, morphologic/microscopic assessment, and isolation and identification of DNA. To verify our findings, blood smears were examined by two separate investigators. The control group from which we report absence of *M. haemocanis* is rather small (n = 60) and therefore calls for a larger prospective study. However, despite the small sample sizes, statistical analysis revealed satisfying *P*-values (< 0.001), with negligible β-errors (0.92 to 1.00) in all instances.

*Mycoplasma haemocanis* formerly belonged to the family of *Anaplasmataceae*, order *Rickettsiales*. It is classified today as *Mollicute*, genus *Mycoplasma*. The parasite was reported first from Germany in 1928 (13). Today, it exists in the Mediterranean, North and South America, Asia, and Africa (5). *Mycoplasma haemocanis* is typically located in deeply indented folds of the erythrocyte membrane (5, 22). It mostly appears in coccoid form (0.2 to 0.4 µm). It also appears in rings or doughnut forms, rods (0.2 to 0.4 µm) (22), and streptococcal-like chains or bows (20, 22, 25). The organism can be visualized by use of stains such as Giemsa, Wright's and toluidine and methylene blue.

A decade ago, the only readily available method to obtain the diagnosis of *M. haemocanis* was direct identification in blood smears. As indicated by our present data, *M. haemocanis* may be absent in the blood (11) even when the dog has an infection. Further, correct microscopic diagnosis may be hampered by stained artifacts, basophilic stippling, or Howell-Jolly bodies. Although it has not been established, serologic testing for cross-reactive an-

tibodies might be helpful. However, final diagnosis is accomplished by DNA isolation from whole blood by use of the PCR technique (3, 5, 16). Of note, PCR analysis requires presence of parasitemia in the blood. The diagnosis of *M. haemocanis* infection in dogs is currently problematic, since the disease may be clinically inapparent, especially in absence of splenectomy, immunosuppression, or coexistent disease.

In principle, the disease in dogs may exist in two forms. The acute form, with pronounced anemia and including anorexia, lethargy, weight loss, inappetence, and fever, mainly becomes manifested in immunosuppressed or splenectomized individuals (2, 5, 6, 11, 20, 25). Coinfection with *Babesia canis* or *Ehrlichia* spp. may be present (25). A chronic or latent form of infection has been reported in non-splenectomized dogs in which definitive clinical signs of disease are not apparent. In this form of infection, the parasites are found only periodically and in low numbers in the blood. This is the most likely form of infection in the kennel-raised dogs reported here. Profound alterations of the hemogram, including red and white blood cells and platelets, have been reported (2, 6, 9-11). These changes include risk of profound anemia, co-infection, and bleeding episodes (6). Clinical disease may develop quickly, within 2 weeks after inoculation (19, 25), or later after 2 months (5, 6, 19), with mild or incomplete signs of disease. The usual therapy is oxytetracycline (20 to 40 mg/kg of body weight/d) or doxycycline (5 to 10 mg/kg/d) (11). Systemic antimicrobial therapy does not, however, fully eliminate *M. haemocanis* from the blood (11, 12). The possible outcomes of *M. haemocanis* infection in the dog include recovery, persistence of the parasite, and late relapses of the infection (6). The course of the disease is largely incalculable.

The occurrence of *M. haemocanis* in the kennel or laboratory setting could be mitigated by controlling the possibility of transmission by blood-feeding arthropods, especially the dog tick, *Rhipicephalus sanguineus* (21). It is also transmitted by oral or direct penetrating blood inoculation (21). However, the likelihood of transplacental transmission (14) would perpetuate an infection, once established, in kennel-raised dogs. Whether this form of infection is important in the transmission of *M. haemocanis* needs to be investigated.

The exact damage done by the mycoplasmal parasite to the red blood cell is unknown. These cells might simply be destroyed by the adhering microorgansisms (22). Probably exerted by type-II autoimmune reactions, hemolysis, accompanied by Coombs-positive anemia (7) and thrombocytic purpura, may occur (6). *Mycoplasma haemocanis* severely alters shape and deformability of affected red blood cells (5, 22). It is obvious that this may interfere with microvascular perfusion and oxygen unloading at the tissue level. Research dogs are often splenectomized prior to commencing an experimental study, since the spleen as blood reservoir is contractile and expels a considerable amount of red blood cells, resulting in variable hematocrit and red blood cell count (8, 17, 18, 24). However, splenectomy has been documented to facilitate overt disease in the infected dog (6, 11, 15, 23). Thus, when using splenectomized dogs as experimental models, development of acute hemoplasmosis as a possible complication must be considered. Nonetheless, acute and latent infections in dogs with *M. haemocanis* may lead to misinterpretation of subsequent experimental results. At present, research in splenectomized dogs cannot be recommended, and the scientific validity of any research performed in dogs must be questioned.

In conclusion, we report the results of a prospective study in dogs from various kennels in Europe and North America. According to the commercial breeders, none of the blood samples originated from a clinically ill animal. Nevertheless, *M. haemocanis* was present in dogs at all of the kennels tested. Infection of dogs with *M. haemocanis* might be an underestimated problem, especially in kennel-bred dogs, which are subjected to biomedical research. Further studies in this field, in particular screening methods to establish an effective surveillance allowing exclusion of infected animals, are urgently needed.

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