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Original article

MOLECULAR DETECTION OF *EHRLICHIA CANIS* AND *ANAPLASMA PHAGOCYTOPHILUM* IN BLOOD SAMPLES FROM DOGS IN BULGARIA

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Summary

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The aim of the study was to develop a molecular diagnosis protocol of monocytic ehrlichiosis and granulocytic anaplasmosis in dogs by conventional polymerase chain reaction (PCR) and to compare the results from PCR and a rapid serological test. One hundred and six blood samples from dogs were tested by the rapid serological test SNAP 4Dx Plus (IDEXX Laboratories, Westbrook, ME) and by conventional PCR. Sixty-two of them (58.49%) were positive for antibodies to *Ehrlichia canis/Ehrlichia ewingii* and 14 (13.21%) for antibodies to *Anaplasma phagocytophilum/Anaplasma platys*. In 24 (22.64%) blood samples, antibodies against both pathogens were simultaneously detected. Six (5.66%) samples were seronegative. Fourty-six of the 106 tested samples (43.4%) were positive for a 345 bp segment of the ribosomal gene of family *Anaplasmataceae.* In 28 of them the presence of a 444 bp fragment of the *ankA* gene of *A. phagocytophilum* was detected, and in 26: a 409 bp fragment of the gene of *E. canis*. Nine samples were simultaneously positive for genetic sequences of *E. canis* and *A. phagocytophilum*. The target DNA fragments specific for the two studied pathogens were not detected in one of the *Anaplasmataceae*-positive samples. In the remaining 60 cases (56.6%), the presence of a 345 bp segment of the ribosomal gene was not detected. In the present study, the DNA of *E. canis* and of *A. phagocytophilum* was detected for the first time in Bulgarian dogs by the conventional PCR.

Key words: *16S rRNA*, *Anaplasmataceae*, *ankA*, antibodies, canine blood

INTRODUCTION

Over the past few decades, a large number of rickettsial pathogens have been discovered in dogs and established later as human pathogens (Nicholson *et al.*, 2010; Parola *et al.*, 2013). Some of these pathogens can pose a serious threat to animal health and represent a diagnostic challenge for veterinarians due to the broad

spectrum of clinical manifestations, long incubation periods and the frequent occurrence of co-infections (ESCCAP, 2023). In Europe, the most important diseases of this group are monocytic ehrlichiosis, with its main etiological agent *E. canis*, and granulocytic anaplasmosis caused by *A. phagocytophilum*. Rickettsiae bind to glycoproteins on the surface of the target cells – monocytes or neutrophils, and enter cells by endocytosis, multiplying in membrane-bound vacuoles as microcolonies called morulae (Carrade *et al.*, 2009).

The diagnosis of monocytic ehrlichiosis and granulocytic anaplasmosis is based on anamnestic data, clinical signs, epidemiological data and, above all, on laboratory tests. A number of serological and molecular genetic methods have been developed for laboratory diagnosis and in recent years, a great advantage is given to diagnosis of these diseases by polymerase chain reaction (PCR) based analyses (Khatat *et al.*, 2021; Diniz & de Aguiar, 2022).

A significant disadvantage of rapid serological tests is their inability to detect acute infections. Positive results cannot be directly related to an active infection, but only determine whether the patient has been exposed to the pathogen. Early in the course of the disease, there is often a "lag" between the development of clinical signs and the appearance of detectable, circulating antibodies. In the case of ehrlichiosis or anaplasmosis however, blood PCR will determine if the pathogen is present and whether the disease is active (Beall *et al.*, 2022; Waner 2022; Aziz *et al.*, 2023).

PCR detection of *E. canis* DNA can be achieved as early as 4–10 days after experimental infection (Iqbal *et al.*, 1994). Several procedures have been developed based on the detection of various target genes (e.g. *16S rRNA, p28, p30, dsb, VirB9*) but the *16S rRNA* (Inokuma *et al.*, 2003; Vinasco *et al.*, 2007; Nazari *et al.*, 2013), and p30-based PCR assays (Stich, 2002) are most commonly used. PCR allows for the earlier detection of *A. phagocytophilum* compared to microscopic examination. In experimentally infected dogs, PCR results of whole blood samples were positive 6–8 days before morulae appeared (Egenvall *et al.*, 1998). In adittion, the DNA of the pathogen can be identified after infection longer than morulae are detected by use of microscopy (Shaw *et al.*, 2001). Several conventional PCR assays have been developed to detect *A. phagocytophilum* DNA in peripheral blood, skin, bone marrow or spleen. Target genes in the majority of assays used the *16S rRNA* gene or the outer surface protein gene *msp2* (*p44*). PCRs based on the latter gene are usually specific for *A. phagocytophilum*. Some authors use molecular assays targeting genes such as *msp4*, *groEL*, *rrs*, *epank1* or *ankA* of *A. phagocytophilum* (Walls *et al.*, 2000; Massung & Slater 2003; Santos *et al.*, 2004; de la Fuente *et al.*, 2008).

The aim of the study was to develop a protocol for the molecular diagnosis by conventional polymerase chain reaction (PCR) of monocytic ehrlichiosis and granulocytic anaplasmosis in dogs and to compare the performance of the PCR to that of a rapid serological test.

MATERIALS AND METHODS

Animals and samples

One hundred and six dogs, patients of the Small animal clinic at the University Veterinary Hospital of Trakia University (97 privately owned pets and 9 dogs housed in the Municipal Shelter for Stray Animals, Stara Zagora), were included in this study. All investigations were performed with

the consent of the owners. The animals exibited depression, fever, anorexia, weight loss, easy fatigability, spontaneous haemorrhages, anaemia, and enlarged lymph nodes, i.e. clinical signs characteristic of monocytic ehrlichiosis or granulocytic anaplasmosis. Blood samples from all dogs were obtained of the *vena cephalica antebrachii externa* using vacuum containers with EDTA as anticoagulant. Haematological, biochemical and serological studies, as well as downstream molecular genetic investigations were made. The established clinical signs and haematological changes were the only basis for the inclusion of animals in the study. All selected 106 dogs were serologically tested with SNAP®4Dx Plus (IDEXX Laboratories, Westbrook, ME): a rapid diagnostic kit for the simultaneous detection of antibodies to *A. phagocytophilum/A. platys, E. canis/E. ewingii, B. burgdorferi* and *Dirofilaria immitis* antigen, intended for use in veterinary clinics. Its sensitivity is 93.4% for *E. canis* and 94.1% for *Anaplasma* spp. The specificity is 96.8% for *E. canis* and 98.4% for *Anaplasma* spp.

DNA extraction

The High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) was used to isolate DNA from 200 µL whole blood following the manufacturer's instructions. The extracted DNA was stored at –20 ºC until the PCR assays were performed. A spectrophotometric assay was used to determine the purity and concentration of the extracted DNA samples.

PCR amplification

Standard screening conventional PCR was performed on all 106 samples, using genus-specific primers: forward EHR 16SD – F-5`-GGTACCYACAGAAGAAGTCC-3` and reverse EHR 16SR – R-5`-TAG CACTCATCGTTTACAGC-3` (Parola *et al.*, 2000). These primers amplify the 345 bp fragment of the *16S rRNA* gene from the DNA of rickettsiae of the family *Anaplasmataceae* including *Ehrlichia canis, E. chaffensis, E. muris*, *Anaplasma marginale, A. equi, A. phagocytophilum, A. platys, A. centrale, Wolbachia pipientis, Neoricketsia senetsu, N. risticii, N. helminthoeca* (Inokuma *et al.*, 2001). A second PCR was performed on samples positive from the screening PCR using *A. phagocytophilum* species-specific primers: forward LA1 – (F) 5`-GAGAGAT GCTTATGGTAAGAC-3`, and reverse LA6 - (R) 5`- CGTTCAGCCATCAT TGTGAC- 3' (Caturegli *et al.*, 2000; Walls *et al.*, 2000) that amplify approximately a 444 bp fragment of the *ankA* gene. A third PCR was also performed on positive samples from the first round PCR using primers specific for *E. canis* species and complementary to a 409 bp fragment of the *16S rRNA* gene: forward CANIS – (F) 5`-CAATTATTTATAGCCTCTGGC TATAGGA-3` and reverse GA1UR – (R) 5`- GAGTTTGCCGGGACTTCTTCT- 3` (Inokuma *et al.*, 2003).

The PCR amplification in the first and second rounds was set up within a 20 µL reaction mixture containing 10 µL ready to use buffer mix (KAPA2G Robust Hot Start ReadyMix with dye 2×; Roche Diagnostics, Mannheim, Germany) with 5U Taq DNA polymerase, 1.5 mM $MgCl_2$ and 1 mM dNTPs, 10 pmol of each primer, 2 µL of DNA template and sterile destilled water to a final volume of 20 µL. The reaction mixture in the third round assays were similar, except for the concentration of $MgCl_2$ (5 mM).

A positive and a negative control were used in each PCR assay. The negative

control contained all the PCR mix components except for DNA template (NTC – non-template control). Positive DNA samples kindly provided by Prof. Handan Cetinkaya from the Department of Parasitology at the Faculty of Veterinary Medicine of Istanbul University, Turkey were used as a positive control. Amplification was carried out in a Aeris™ PCR Thermal Cycler (Esco Micro Pte Ltd, Singapore) at appropriate thermal cycling parameters.

The amplification conditions were as follows: first PCR with primers EHR 16SD/ EHR 16SR – initial denaturation for 3 min at 95 ºC, followed by 35 cycles of 30 s at 94 ºC, 35 s at 55 ºC, 35 s at 72 ºC, and a final extension for 7 min at 72 ºC; second PCR with primers LA1/ LA6 – initial denaturation for 5 min at 95 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 44.2 ºC (the annealing temperature was optimised using gradient PCR), 90 s at 72 ºC, and a final extension for 7 min at 72 ºC; third PCR with primers CANIS/ GA1UR – initial denaturation for 5 min at 95 ºC, followed by 40 cycles of 30 s at 94 ºC, 30 s at 63 ºC, 90 s at 72 ºC, and a final extension for 5 min at 72 ºC.

Amplification products were visualised by 1.5% agarose gel electrophoresis, after staining with ethidium bromide (0.5 mg/mL) and exposure to ultraviolet light. A molecular marker was used in each electrophoresis for amplification of fragment sizes ranging from 100 bp to 1000 bp (DirectLoad™ PCR 100 bp Low Ladder, Sigma-Aldrich Chemie GmbH, Germany).

Statistical analysis

The determination of 95% confidence limits was performed with the help of statistical software GraphPad InStat v. 3.00 (GraphPad Software Inc., La Jolla, CA). The percentage agreement and association between the results obtained with the serological technique and the molecular test was assessed using the Cohen's Kappa index. Percentages of 0–20%, 21– 40%, 41–60%, 61–80% and 81–100% were interpreted as poor, fair, moderate, strong and high percentages, respectively. Thus, values of the Cohen's Kappa index of 0 indicated no agreement; 0–0.2: slight agreement; 0.2–0.4: fair agreement; 0.4–0.6: moderate agreement; 0.6–0.8: substantial agreement; 0.8–1.0: near perfect agreement and 1: perfect agreement.

RESULTS

Sixty-two (58.49%) of all 106 tested samples were positive for anti-*Ehrlichia* IgG antibodies (Table 1), 14 (13.21%) were

Table 1. Results of PCR and serological tests for A. *phagocytophilum* and *E. canis* in 106 dog blood samples

	PCR n (%)	Confidence level (95%)	Serology n (%)	Confidence level (95%)
Positive for *A. phagocytophilum* only	19 (17.92%)	11.15÷26.57	14 (13.21%)	7.41÷21.17
Positive for *E. canis* only	17 (16.04%)	9.63÷24.43	62 (58.49%)	48.51÷67.98
Positive for both *E. canis* and *A. phagocytophilum*	9 (8.49%)	3.96÷15.51	24 (22.64%)	15.08÷31.97
Negative for both pathogens	61 (57.55%)	47.57÷67.09	6 (5.66%)	2.11÷11.91

Note: One sample was positive for *Anaplasmataceae,* but negative for *E. canis* or *A. phagocytophilum* DNA.

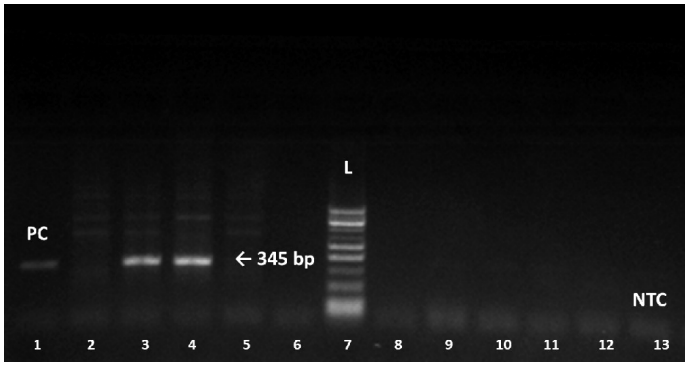

Fig. 1. Amplification of the *16S rRNA* gene with EHR 16SD/ EHR 16SR primers, with approximate size 345 bp. Lane 1: positive control (PC); lanes 2, 5, 6, 8–12: negative samples; lanes 3–4: positive samples; lane 7: 100 bp DNA ladder (L), lane 13: negative control (NTC).

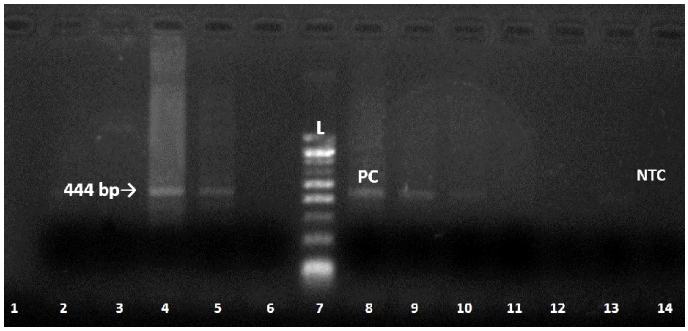

Fig. 2. Amplification of the ankA gene with LA1/ LA6 primers, with approximate size 444 bp. lanes 2, 3, 6, 11–13: negative samples; lanes 4, 5, 9, 10: positive samples, lane 7 = 100 bp DNA ladder (L); lane 8: positive control (PC); lane 14: negative control (NTC).

positive for anti-*Anaplasma* IgG antibodies, 24 (22.604%) were simultaneously positive for antibodies against both causative pathogenic agents. In 6 (5.66%) samples no antibodies were detected by SNAP®4Dx Plus.

All 106 DNA samples isolated from venous blood were tested by PCR analysis for presence of genetic sequences specific for the *16S rRNA* gene of rickettsiae from the *Anaplasmataceae* family (Fig. 1). Of the examined samples, 46 (43.4 %) were positive for a 345 bp fragment of the *Anaplasmataceae* ribosomal gene. In the remaining 60 cases (56.6%) the presence of this genetic fragment was not detected.

PCR amplification with the genus-specific primers demonstrated that out of the 46 samples positive for *Anaplasmataceae*, 28 showed the presence of a 444 bp fragment of the *ankA* gene of *A. phagocytophilum* (Fig. 2). Also, in 26 blood samples, the 409 bp fragment of the *16S rRNA* gene of *E. canis* was confirmed (Fig. 3). Nine of the samples were simultaneously positive for genetic sequences of *E. canis* and *A. phagocytophilum*. In one patient, the target nucleotide fragments, specific for the genes of the two studied pathogens, were not detected.

Out of all 38 seropositive *A. phagocytophilum* samples, 27 were found to be

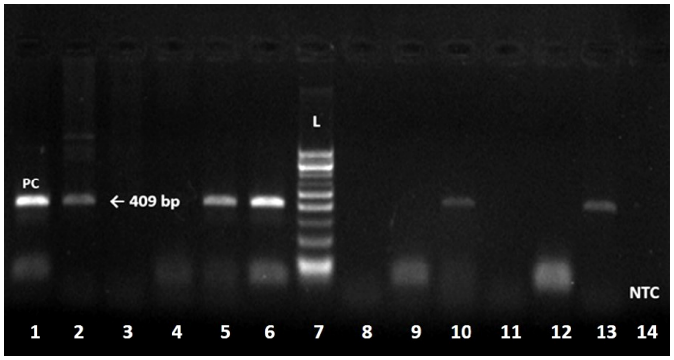

Fig. 3. Amplification of the *16S rRNA* gene with CANIS/ GA1UR primers, with approximate size 409 bp. Lane 1: positive control (PC); lanes 2, 5, 6, 10, 13: positive samples; lanes 3, 4, 8, 9, 11, 12: negative samples; lane 7: 100 bp DNA ladder (L); lane 14: negative control (NTC).

Table 2. Associations between the serological and PCR test for *A. phagocytophilum* and *E. canis* in 106 dog blood samples

A. phagocytophilum		PCR		Statistical analysis
		Pos	Neg	% of agreement: 88.7
SNAP® 4Dx®	Pos	27	11	Cohen's κ 0.74
	Neg	1	67	Substantial agreement
E. canis		PCR		Statistical analysis
		Pos	Neg	% of agreement: 39.62
SNAP® 4Dx®	Pos	24	62	Cohen's κ: 0.08
	Neg	2	18	Slight agreement

Pos=positive; Neg= negative.

PCR positive and the remaining 11 were PCR-negative. In addition, a sample negative for antibodies against *A. phagocytophilum* was found to contain DNA of this rickettsia. Correlation analysis showed 88.7% agreement and Cohen's κ of 0.74, which was interpreted as substantial agreement (Table 2). Significantly different results were established with regard to *E. canis*. Out of the 86 seropositive samples, 24 were PCR-positive and the remaining 54 were negative. Also, two seronegative samples demonstrated presence of ehrlichial DNA. The correlation analysis showed an agreement of 39.62% and Cohen's κ of 0.08, accepted as slight agreement (Table 2).

DISCUSSION

The current study presents molecular genetic evidence for the presence of active *A. phagocytophilum* and *E. canis* infections in dogs with clinical signs of the above diseases. Although these pathogens have been indirectly confirmed in previous serological studies of clinically sick and healthy dogs (Tsachev, 2006; Tsachev *et al.*, 2008; Pantchev *et al.*, 2015; Manev, 2020; Arnaudov, 2021), their direct detection by molecular diagnostic methods has never been performed in Bulgaria. Thus, the present study is the first to record active circulation of *A. phagocytophilum* and *E. canis* in dogs exhibiting clinical signs of disease.

Our results from a species-specific *16S rRNA* sequence identification for *E. canis* in dogs by PCR-analysis showed prevalence about two times lower than that reported by Petrov (2018) in the Republic of North Macedonia, a neighbouring country of Bulgaria. The author found the presence of *16S rRNA* sequence for *E. canis* in 45% of the examined dogs. In this research as well as in ours, animals with clinical, haematological and serological evidence of monocytic ehrlichiosis were also included. The differences in the results between our studies and data of Petrov can be explained by the country-specific frequency of tick invasion, measures taken by owners to protect dogs against ectoparasites, treatment with tetracyclines in previously diagnosed infections with some of the pathogens, or study group selection. According to Bartsch & Greene (1996), doxycycline therapy results in the elimination of *E. canis* from the blood. Anti-*Ehrlichial* IgG antibodies are known to persist for several months to years after doxycycline therapy and microbiological recovery (Moroff *et al.*, 2014). Another possible explanation is the longer survival of the bacteria in splenic macrophages than in blood monocytes, i.e. "hiding" of *Ehrlichia* in tissue macrophages in chronically infected animals, as determined by Harrus *et al.* (1998; 2004). Although the dogs included in our study had clinical signs compatible with monocytic ehrlichiosis, it is possible that some of them had a chronic but subclinical and self-limiting infection.

In another research of 400 blood samples from dogs in the European part of Turkey, another neighbouring country of Bulgaria, Cetinkaya *et al.*, (2016) found that out of 109 seropositive samples, 24 were PCR positive for *E. canis*, 16 for *A. phagocytophilum* and 24 for *A. platys*. Co-infection with more than one pathogen was detected in 12 samples. The researchers reported 22% correlation between positive serological and PCR results for *E. canis*, 15% for *A. phagocytophilum*, and 22% for *A. platys*. These data are very similar to our results regarding the etiological agent of monocytic ehrlichiosis, a disease in which a chronic phase and a long-lasting hosting are both observed. At the same time, they differ significantly for the other rickettsia which is probably due to selection of the samples. Cetinkaya *et al.* (2016) tested 100 dogs, each coming from shelters in the cities of Istanbul, Edirne, Tekirdağ and Kırklareli, while we investigated animals with evidence of a vector-borne disease at the time of sampling.

In an investigation in hunting dogs, sheltered or recently captured street dogs, Filipović *et al.*, (2018) found a seroprevalence of 28.8% for *Anaplasma* spp. but a negative PCR result for *A. phagocytophilum*. The authors assume that dogs in Serbia, bordering Bulgaria to the east, became infected with *A. phagocytophilum*, but got rid of the pathogen as a result of a developed protective immune response, evidenced by the presence of antibodies in the dogs' serum. Our study included animals with evidence of acute infection, so it is logical that the percentage of PCR positives was also high, while Filipović *et al.* (2018) have studied animals at risk of being infected but without clinical and haematological evidence of the disease. Essentially, their study is a serological screening followed by a PCR assay, unlike ours which was a diagnostic one. Probably that is why their results in the part for PCR detection differed significantly from ours. However, regarding the seroprevalence, the results of both studies were very similar.

In another investigation for vector-borne diseases (VBDs) of 1,433 blood samples from hunting dogs in Southern Italy, Sgroi *et al.*, (2022) found 29 seropositive and 59 PCR positive samples for *Anaplasma* spp. An interesting fact is that the authors detected 58 PCR positive samploes for *A. platys* and only 1 for *A. phagocytophilum*, but did not propose a hypothesis for this result. In the same study on *Ehrlichia* spp. 76 seropositive and 32 PCR positive samples, respectively were detected. In contrast to our study, which included animals with clinical and haematological changes characteristic of monocytic ehrlichiosis or granulocytic anaplasmosis, Sgroi *et al.* (2022) studied only hunting dogs without selecting the animals by disease symptoms. However, similar to the results of our study, the authors also found a high percentage of agreement between serological (SNAP® 4Dx Plus) and PCR tests for *Anaplasma* spp. and low percentage of agreement for *Ehrlichia* spp.

Our results differ from those presented by René-Martellet *et al.* (2015) in a Mediterranean study, that tested by PCR 366 dog samples from 78 clinics in Spain, Portugal and Italy. All the dogs selected by the veterinarians and included in the study were sick and met at least three criteria compatible with monocytic ehrlichiosis. The study reported seroprevalence for *E. canis* of 26% and positive PCR results of 11%. Antibodies to *Anaplasma* spp. were present in 9% of the samples. In subsequent PCR testing, however, no sample was proven positive for *A. phagocytophilum*. Differences in results are likely due to sample selection. In addition, René-Martellet *et al.* (2015) suggested the existence of 'hotspots' of infections. They assumed that the risk of morbidity in southern Italy was higher than in other areas, including southern Spain and Portugal. Our tests were on animals with anamnestic, clinical and haematological data suggestive of a vector-borne rickettsial disease from only one area and this probably has affected the results. Furthermore, 100 out of the 106 dogs tested by us were seropositive for at least one of the pathogens sought, while in the study by René-Martellet *et al.* (2015) for antibodies to *Anaplasma* spp. and *E. canis* a total of 123 (35%) of the included animals tested positive. The comparison of the ratios between seropositive and PCR positive results with regard to *E. canis* showed that very similar results: 35/92 or 38% in the study by René-Martellet et. al. (2015) and 31/106 (29%) in the present study.

A retrospective study on molecular and serological detection of *A. phagocytophilum* in dogs presented by Schäfer *et al.*, (2023) summarises data for dogs positive for *A. phagocytophilum* by direct pathogen detection (PCR) and by antibody detection (IFA/ELISA) in Germany for the period 2008–2020. The authors reported 4.9% samples positive by genetic tests and 23.3% seropositive samples. The results showed a significantly lower percentage of agreement between the tests than in our study regarding *A. phagocytophilum*. The probable reason is the sample selection, as our study included only animals with clinical signs and haematological data of VBDs, whereas Schäfer *et al.* (2023) presented no information on the health status of the dogs.

Although Bulgaria is a country with a temperate continental climate, our PCR results are very similar to a number of studies in tropical and subtropical regions. For example, Lara *et al.* (2020) found seroprevalence for *E. canis* of 60% and positive PCR results in 37% of the tested samples obtained from dogs inhabiting the

Caribbean island of St. Kitts. Mittal *et al.* (2017) found seroprevalence for *E. canis* of 19% and PCR positivity in 6% of 225 animals tested in India. Again, the correlation between a positive serological and PCR tests was about 30%, a result very similar to ours, however in this study, only clinically healthy animals were selected for the study because of the increased risk of tick infestation. Malik *et al.* (2018) reported 28% PCR positivity for *E. canis* among dogs in three areas of Pakistan.

The results of this study are similar to reports from warm and humid regions. Climate changes, associated with rise in temperatures worldwide are the main factors involved in the density and life cycles of vectors as well as their habitats. These changes pose a threat of area expansion on the part of different tick species, thus increasing the risk of spreading VBDS such as ehrlichioses and anaplasmoses in temperate countries (Fouque & Reeder, 2019). In support of the last statement is the retrospective study by Schäfer *et al.* (2023) analysing data from serological and PCR studies over a 13-year period in Germany. The authors reported an apparent increase in affected dogs in northeastern Germany as well as in general over time. According to the researchers, changes in climate, land use, wildlife reservoirs, and population density can affect the range and population size of many tick vectors such as *I. ricinus*, which may have influenced changes in detection of *A. phagocytophilum* in dogs in Germany over time (Schäfer *et al.*, 2023).

Our PCR results proved the presence of active infections with *E. canis* and *A. phagocytophilum*. Simultaneously, there was a disagreement between serological and PCR tests results for *E. canis*. Serological tests used in pet clinics give information about an encounter with the pathogen but not about the presence of an active infection. Often, there is a "lag" between the development of clinical signs and haematological changes at disease onset and detection of circulating antibodies, possible between post infection days 7 and 14. On the other hand, anti-*Ehrlichia* IgG antibodies persist for several months to years after treatment and elimination of the rickettsia from the immune system (Nair *et al.*, 2016). These shortcomings of serodiagnosis highlight the need for more careful interpretation of results when deciding whether or not to treat the patients (Sainz *et al.*, 2015). The use of PCR tests allows detecting *A. phagocytophilum* and *E. canis* DNA in the infected host, indicating the presence of active infection and the need for therapy. Therefore, in endemic areas, after receiving a positive serological test result, the samples should be tested by molecular diagnostic methods for early detection of acute infections caused by *A. phagocytophilum* and *E. canis*.

In conclusion, *E. canis* and *A. phagocytophilum* in dogs were detected by conventional PCR for the first time in Bulgaria. It enables the quick and timely recognition of animals with an active infection and is a prerequisite for subsequent adequate intervention from the part of veterinary specialists.

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