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Original Contribution

COMPARISON OF A RAPID ENZYME-LINKED IMMUNOSORBANT ASSAY TEST WITH AN INDIRECT IMMUNOFLUORESCENT ANTIBODY TEST IN DIAGNOSING *EHRLICHIA* AND *ANAPLASMA* INFECTIONS IN DOGS

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ABSTRACT
PURPOSE: The objective of this study is to compare the diagnostic value of commercial enzyme-linked immunosorbent assay (ELISA) with indirect immunofluorescent antibody test (IFA) in detecting immunoglobulin-G (IgG) antibodies to *Ehrlichia canis* and *Anaplasma phagocytophilum*. METHODS: Seventy-four serum samples, obtained from dogs believed to be naturally infected with *E. canis* or *A. phagocytophilum*, were analyzed. RESULTS: By ELISA, 48 (64.9%) samples were found positive for IgG to *E. canis*, 10 (13.5%) to *A. phagocytophilum*, 12 (16.2%) to both *E. canis* and *A. phagocytophilum*, and in 4 (5.4%) samples no presence of antibodies was detected. The number of serologically positive dogs for IgG was 44 (59.5%) to *E. canis*, 10 (13.5%) to *A. phagocytophilum*, 16 (21.6%) to both *E. canis* and *A. phagocytophilum*, and 4 (5.4%) were determined negative by means of IFA. In most samples the antibody titer did not exceed 1:80 but in 5 it reached a level of 1:320, and in other 4 of even above 1:640. CONCLUSIONS: This study shows that IFA assay is more sensitive than commercial ELISA rapid test when serum antibody titers are low.

Key words: *Ehrlichia canis*, *Anaplasma phagocytophilum*, ELISA rapid test kit, IFA, Vector-borne disease.

INTRODUCTION

Over the last few years there has been a growing tendency in numbers of home pet animals. The trend is particularly marked in pet dogs. They share our homes, sleep in our beds, and eat from our dishes. Taking into account the increasing interaction between animals and their owners, and the likelihood of pets becoming a source of infectious agents that could potentially harm humans, it is necessary to monitor animals' health condition (1), and above all to regularly screen for diseases characterized by long incubation periods or by predominance of subclinical infections.

Since late 1980s, vector-borne diseases caused by rickettsias in dogs have increasingly become a focus of research workers' interest. Such widespread diseases result from infections with *E. canis* and *A. phagocytophilum*. These infectious diseases are caused by bonded intracellular pathogens of the *Anaplasmataceae* family.

Dogs can be infected with these pathogens by ticks. The latter inoculate pathogens into recipients while continuously feeding on their blood. Target cells for further pathogen propagation are leucocytes and platelets. Diseases have been given names based on the type of infected cells. *E. canis* attacks monocytes, while *A. phagocytophilum* infects granulocytes (neutrophils, eosinophils). These diseases were respectively named canine monocytic ehrlichiosis and canine granulocytic anaplasmosis. Both infections have zoonotic potential.

Ticks of the *Rhipicephalus* genus are major transmitters of the above, and *R. sanguineus* is considered to be the specific vector of *E. canis* (2). The latter is also found in *R. turanicus*, and in the nymphs of *Dermacentor marginatus*. In

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experimental studies, *E. canis* has been proven in *Dermacentor variabilis* as well, even though it is not the common vector of this causative agent (3). The vector of *A. phagocytophilum* in dogs is *Ixodes ricinus*. It is widespread in almost all countries in Europe (4-8), Bulgaria included.

Veterinary clinics diagnose vector-borne diseases based on clinical signs, hematological changes, blood smear microscopy and serological tests. Typical clinical signs include fever, weight loss, vomiting, urinary-catarrhal rhinitis, lethargy, depression, limping, hemorrhage, lesions.

The hematological changes characteristic of ehrlichiosis and anaplasmosis are: thrombocytopenia, anemia, and leucopenia, and they are accordingly used as screening parameters in diagnostics of the diseases in question (9, 10). These paraclinical changes are, however, only indicative of nature, and further call for more specific diagnostic approaches when combined with other clinical markers. In blood cells (monocytes or granulocytes), the pathogens that cause ehrlichiosis and anaplasmosis form cell clusters called morules. They are easier to detect in buffy coat smears than in whole blood smears (11).

Serological tests such as indirect immunofluorescence and ELISA are also applied to reach an accurate diagnosis. In recent years, the PCR method of molecular biology has also found a wide range of application.

Indirect immunofluorescence for determination of antibodies to these diseases is considered to be the "gold standard" in serological diagnostics (12). Several rapid commercial assays have been developed to detect immunoglobulin-G (IgG) and immunoglobulin-M (IgM) antibodies to *E. canis* and to *A. phagocytophilum*, that make use of whole blood or patient serum to reach quick diagnosis in clinical cases. Such assays include Immunocomb (Biogal, Israel), which uses as an antigen the whole cell of rickettsias, and Snap 4 Dx Test (IDEXX Laboratories Inc., USA), which uses two *E. canis*-specific proteins (p30 and p30-1), and p44 for *A. phagocytophilum*.

The aim of the presenting study was to compare the commercial ELISA rapid test with the immunofluorescence method in the serological diagnosis of the diseases in question.

MATERIALS AND METHODS

The study was conducted for the period between April 2018 and January 2019 in the laboratories of the Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria.

SAMPLE

Seventy-four blood samples were collected from dogs with clinical signs and hematological changes characteristic of vector-borne rickettsiosis. Venous blood from dogs was obtained by means of a Vacutainer tube, with EDTA for collected plasma. Samples were stored at -20°C until tested.

ELISA

Serum samples were tested for IgG-specific antibodies to *A. phagocytophilum/A. platys*, *E. canis/ewingii* and *B. burgdorferi*, and for the specific antigen of *D. immitis* using a commercial ELISA assay kit (SNAP® 4Dx®; IDEXX Laboratories, Inc. U.S.A.). The SNAP® Test's peptide-based technology allows for determination of only highly specific antibodies for *Anaplasma spp.*, Lyme disease, and *Ehrlichia spp.* which, in turn, helps to eliminate false positives. In the commercial ELISA assay kit, the specific IgM and IgG antibodies were used to test the specific antigen of *D. immitis*. The synthetic peptide from the major surface protein (p44/MSP2) was utilized to detect the specific antibodies of *A. phagocytophilum.* The P30 and P30-1 outer membrane proteins of *E. canis* were used to demonstrate the specific antibodies of *E. canis*, and the C6 peptide of *B. burgdorferi* was utilized for testing for its specific antibodies. The ELISA test was performed following the manufacturers' instructions. Two drops of test serum were mixed with horse-radish-peroxidase-conjugated proteins. Antigen-antibody complexes were bound to a membrane filter and visualized using a substrate. Any color development in the sample spots indicated the presence in the sera of heartworm antigen, *A. phagocytophilum* antibody, *B. burgdorferi* antibody, or *E. canis* antibody.

IFA

Sera were analyzed by an indirect immunofluorescence antibody test using IFA assay kits "Mega FLUO® *Ehrlichia canis* (Megacor Diagnostik GmbH) and "Mega FLUO® *Anaplasma phagocytophilum* (Megacor Diagnostik GmbH) for detection of anti-*Ehrlichia canis* IgG antibodies and anti-*Anaplasma phagocytophilum* IgG antibodies. Dogs' sera were diluted in PBS (pH 7.2-7.4) and dropped onto the slide wells to allow an antigen-antibody reaction at 37°C in the case of a positive sample. By subsequent washing with PBS, non-bound unspecific serum proteins

were washed off. Evaluation of the sera was done afterwards by utilizing a fluorescence microscope (filter system for FITC) with 400x magnification. Serial two-fold dilutions of the positive test samples were prepared in PBS to determine the endpoint titer (the highest dilution of serum demonstrating immunofluorescent-positive cells).

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).

RESULTS

From the total of blood samples tested by means of ELISA with SNAP® 4Dx®; IDEXX, 48 (64.9%) were positive for IgG antibodies to *Ehrlichia*, 10 (13.5%) were positive for IgG antibodies to *Anaplasma*, in 12 (16.2%) blood samples antibodies were found simultaneously to both pathogenic causative agents, i.e. *Ehrlichia* and *Anaplasma*, and in 4 (5.4%) serum samples no antibodies were detected **(Table 1).**

The same blood samples were also tested employing an indirect immunofluorescent antibody test. By IFA, the presence of antibodies to *E. canis* was determined in 44 (59.5%) of the samples **(Figure 1),** 10 (13.5%) of the samples revealed the presence of antibodies to *A. phagocytophilum*, in 16 (21.6%) antibodies to both *E. canis* and *A. phagocytophilum* pathogens were found, and in 4 (5.4%) of the serum samples no antibodies were found to the above infectious agents **(Figure 2) (Table 1).**

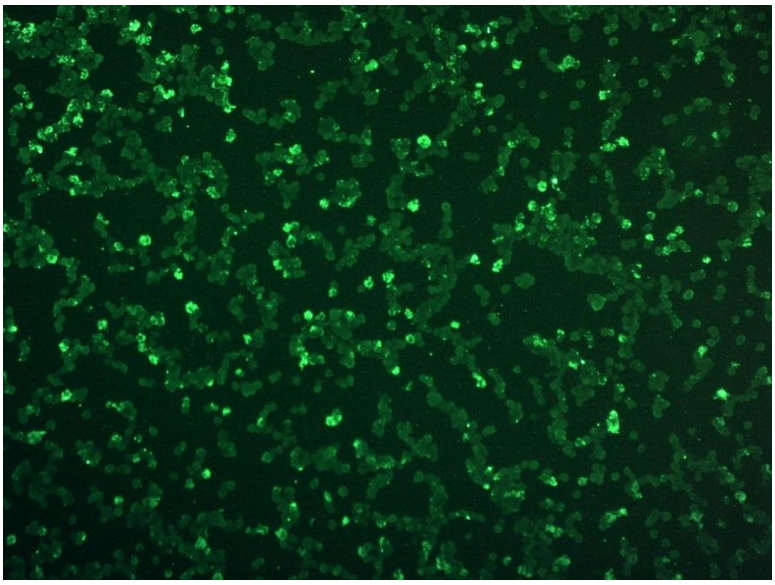

Figure 1. Positive sample for *E.canis* antibodies

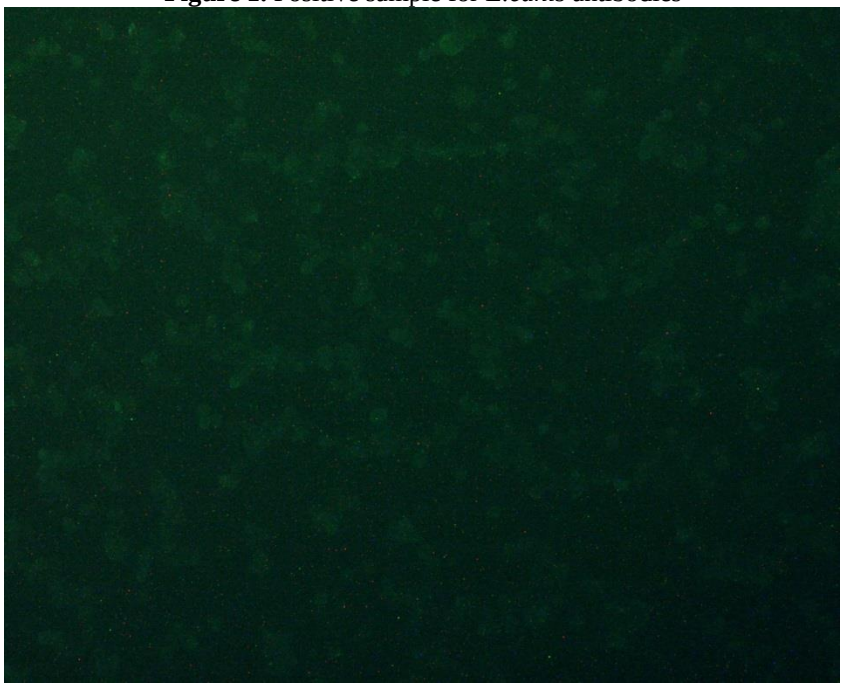

Figure 2. Negative sample

Four of the samples, which were only positive for antibodies to *E. canis* in the ELISA SNAP® 4DX® test, in IFA showed the presence of antibodies to *A. Phagocytophilum,* as well **(Table 1).**

Table 1. *ELISA and indirect IFA test results for detection of antibodies to Ehrlichia and Anaplasma in serum samples*

	ELISA SNAP® 4Dx® IDEXX		Indirect immunofluorescence	
	Numbers	Percent (CL*)	Numbers	Percent (CL*)
Ehrlichia	48	64.9 (52.7÷74.3)	44	59.5 (45.7÷70.3)
Anaplasma	10	13.5 (6.7÷22.1)	10	13.5 (6.7÷22.1)
Ehrlichia and *Anaplasma*	12	16.2 (8.7÷25.3)	16	21.6 (13.0÷31.6)
Antibody negative samples	4	5.4 (1.4÷11.6)	4	5.4 (1.4÷11.6)
Total	74		74	

* CL - confidence limit

All 74 samples were tested by means of IFA to determine the range of *E. canis* and of *A. phagocytophilum* antibody titers. The results are presented in Table 2. Sixty samples with a titer of at least 1:40 to *E. canis* were determined. In most samples, the antibody titer did not exceed 1:80, in 3 its level was 1:160, in 5 it reached 1:320, and in another 4 it reached a level of above 1:640. Fourteen samples were found negative.

We determined antibodies to *A. phagocytophilum* in 26 samples. Then again, in 18 of the samples the antibody titer levels were 1:80. Only one sample showed levels of 1:160 and 1:320. The percentage of samples with a titer of 1:640 and above was higher, i.e. 8.1% (6 animals) **(Table 2).**

Table 2. *IFA Titer Ranges*

	IFA titers					
	Up to 1:40 n (%) (CL*)	Up to 1:80 n (%) (CL*)	Up to 1:160 n (%) (CL*)	Up to 1:320 n (%) (CL*)	1:640 & higher n (%) (CL*)	Negative n (%) (CL*)
Ehrlichia canis (n=74)	60 (81.1) (71.5÷89.1)	48 (64.9) (53.7÷75.2)	3 (4.1) (0.81÷9.7)	5 (6.7) (2.1÷13.4)	4 (5.4) (1.4÷11.6)	14 (18.9) (10.9÷28.5)
Anaplasma phagocytophilum (n=74)	26 (35.2) (24.8÷46.3)	18 (24.3) (15.3÷34.6)	1 (1.4) (0÷5.2)	1 (1.4) (0÷5.2)	6 (8.1) (3.0÷15.3)	48 (64.8) (53.6÷77.3)

* CL - confidence limit

DISCUSSION

In Bulgaria, the infection with *E. canis* in dogs was first encountered in 2006, and with *A. phagocytophilum* in 2008 (13, 14), respectively. In the years to follow, numerous studies were submitted that gave evidence to the wide spread of these pathogens in Bulgaria (15, 16). Since then, the diagnostic in the small animal clinics has been relying mostly on rapid ELISA tests or on immunochromatographic assays that aim to quickly detect antibodies to these agents. Specificity is the most important measure that determines the ability of a given test to confirm clinically suspected cases. Sensitivity, on the other hand, allows to correctly determine illness in animals with low antibody titers. Several studies have shown that ELISA assays are characterized by lower specificity and sensitivity compared to IFA (17, 18), especially in cases of low antibody titer.

In our study, by use of ELISA (SNAP® 4Dx®) we determined antibodies to *Ehrlichia spp.* in 81.1% of the samples, to *Anaplasma spp.* in 29.5%, and to both antigens in 16.2%. Results indicate that the tested animals were predominantly infected with *Ehrlichia* pathogens. In that respect, our results differ from those presented by *Pantchev et al.*, (16). Their team performed tests on 167 dog samples (where dogs exhibited no indication of vector-transferable rickettsiosis) and determined 77 samples positive for *A.*

phagocytophilum, and 35 positive for *E. canis*. At the same time, the results they obtained for *E. canis* and *A. phagocytophilum* co-infections (in 30 samples) were comparable with the 16.2% we determined as to the presence in the tested samples of antibodies to both pathogens.

Data on dominance of the seropositive for *Anaplasma spp.* were presented by our neighboring countries like Serbia (19, 20), Croatia (21), and Romania (22). Results, similar to ours, were reported in Italy (23) that revealed seroprevalence of 7.07% for *E. canis*, and 4.68% for *A. phagocytophilum*, i.e. a ratio of almost 2:1 in favor of *E. canis.* The differences in seroprevalence were probably correlated to the region of origin of the tested animals where the infection with *E. canis* must have been prevalent. Our data show that exposure of the animals, from which we took samples, was higher to *E. canis* than to *A. phagocytophilum.*

Our findings determined that only 4 samples tested negative for both antigens, in both ELISA and IFA assays. The reason, for the very low percentage of negative results, is that only animals with clinical symptoms and hematological data already suggestive of the presence of an infection with *Anaplasma* or *Ehrlichia* were tested. The negative result obtained by both assays testifies that these animals have not been infected.

The SNAP® 4Dx® ELISA test used in our study is of high sensitivity and specificity. The high coincidence rate between this test and IFA, which is considered the serological "gold standard" test for detection and titration of *E. canis* antibodies, (12) confirmed its qualities. We found a 100% coincidence rate when testing for *Ehrlichia* and 84.6% (22/26) when testing for *Anaplasma*. Similar data were presented by *Harrus et al.*, (18) in a comparative analysis of three ELISA assays with IFA. Their analysis determined a coincidence rate of 91% in the results obtained by SNAP 3Dx and by IFA for *E. canis*. In our study, all samples positive for *Ehrlichia* in ELISA were also positive in IFA. A similar correlation was also found by *O'Connor et al.*, (24) for samples with an antibody titer of above 1:160 determined by IFA. The same research workers reported that in samples determined positive, using IFA at titers lower than 19, only 6 were found positive by a commercial rapid ELISA test.

The results we obtained in researching the presence of antibodies to *Anaplasma* were different. Just 4 samples, that revealed antibodies to *Ehrlichia* by means of ELISA, showed a presence of antibodies to *Anaplasma* by means of IFA, with titers of 1:40. That might be due to cross- reactions (25, 26), or low titers, in which cases IFA appears to be the more sensitive (17). When selecting a method of serological testing, it should be taken into account that animals with acute infection develop higher titers within a few weeks of infection (27). IFA testing allows differentiating between acutely infected animals and animals with non-acute infections.

When repeating the test in 1 to 2 weeks, titers are expected to go significantly higher in acutely infected animals. And on the contrary, titers will either remain low in those with latent infection, or no antibodies will be detected in uninfected animals. Repeated serological testing helps solve the problem with sensitivity at lower titer samples. By using IFA, a progressive increase in antibody titers has been reported in cases of natural and experimental infections with *E. canis* in the course of the first 5 months after contracting the disease, which points to the continuous antigen stimulation. Titer levels remain high for a period of about a year and then drop (18). Having, both, its advantages and flaws, the IFA method is broadly applied to identify *E. canis* and *A. phagocytophilum* antibodies in cases of acute infections.

Infection with *E. canis* or *A. phagocytophilum* fosters the development of specific antibodies. In experimental infections, IgM and IgA antibodies appear within 4 to 7 days of infection, whereas IgG antibodies can be detected 15 days after infection with titers of 1:160 and 1:640 (28). The initial presence of IgG antibodies seems to depend on the infective dose that the dog has received (29). IgG antibodies have even been found in 3 days of infection (30). High antibody titers have been more frequently determined in dogs with active *A. phagocytophilum* infection rather than in uninfected dogs (31). The latter is in line with the high-level titer (over 1:320) of antibodies to *E. canis* that we detected in 9 dogs, and to *A. phagocytophilum* in 7 dogs. The results allow us to infer that these animals have had an active infection.

Furthermore, by using IFA, we detected antibody titers of 1:40 to *A. phagocytophilum* in 4 samples, which tested negative by utilizing ELISA. Those samples were probably taken from animals with a recent infection, where the antibody titer was too low to be detected by the SNAP® 4Dx® test.

CONCLUSION

The obtained results show that SNAP® 4Dx® ELISA assay kit might be useful in field diagnostics to determine the presence of antibodies. For research purposes, however, the application of IFA is crucial. The study also emphasizes the importance of serological testing during the acute stages of the disease. The interpretation of this information might prove beneficial to clinicians in helping them make an accurate diagnosis, which, in turn, might account for better treatment and prognosis. The comparison of quantitative data shows a positive and significant correlation between the IFA IgG titers and the results obtained by means of ELISA.

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