



PERFORMANCE OF LABORATORY ELISA AND RAPID ELISA TESTS FOR *EHRlichia* spp. AND *ANAPLASMA* spp. ANTIBODY DETECTION IN DOGS

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Summary

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The aim of the study was to compare the performance of two diagnostic approaches for the detection of antibodies against *Ehrlichia canis* (*E. canis*) and *Anaplasma phagocytophilum* (*A. phagocytophilum*). Two types of tests were used. Anti-*E. canis* ELISA Dog (IgG) and Anti-*A. phagocytophilum* ELISA Dog (IgG) are ELISA kits for the detection of relevant antibodies in laboratory conditions, and SNAP[®] 4Dx Plus is a pet-side ELISA-based serological screening test for simultaneous detection of antibodies against *A. phagocytophilum/A. platys*, *E. canis/E. ewingii*, *B. burgdorferi* and *Dirofilaria immitis* antigens. A total of 61 blood samples obtained from dogs with clinical signs and haematological changes suspect for granulocytic anaplasmosis or monocytic ehrlichiosis were analysed. Antibodies against *E. canis* were found out in 29 (47.54%) and *A. phagocytophilum* in 7 (11.48%) of the samples tested by laboratory ELISA. When using the SNAP test, the results were 35 (57.38%) and 11 (18.03%), respectively. Using the laboratory ELISA kit, 18 samples (29.50%) were positive for antibodies against both pathogens vs 9 (14.75%) samples tested by SNAP. The comparison of the two tests showed a greater agreement of the results in the detection of antibodies against *Ehrlichia* spp. (52 samples) than against *Anaplasma* spp. (44 samples). This difference was attributed to possible cross-reactions.

Key words: *Anaplasma phagocytophilum*, *Ehrlichia canis*, ELISA test kit, SNAP[®] 4Dx Plus Test

INTRODUCTION

During the last years, the dog population in Bulgaria has markedly increased. The trend includes both higher number of pets and of stray dogs inhabiting urban and peri-urban areas. This implies a necessity

for evaluation of risk from exposure to vector-borne diseases, including monocytic ehrlichiosis and granulocytic anaplasmosis. These tick-borne infections with cosmopolitan spread are caused by *E.*

canis and *A. phagocytophilum* – obligate Gram-negative intracellular bacteria from the families *Ehrlichia* and *Anaplasmataceae* of the Rickettsiales order. In Bulgaria, monocytic ehrlichiosis was detected in 2003 (Tsachev, 2006a), whereas granulocytic anaplasmosis – in 2008 (Tsachev *et al.*, 2008). The diagnosis of these infections is a challenge due to the variety of clinical manifestations, frequent cases with subclinical course or disease chronicity. Infection with *Ehrlichia* or *Anaplasma* has to be suspected when dogs live in or have travelled to endemic regions, have been bitten by ticks, exhibit the typical clinical signs or specific haematological and blood biochemical alterations (Harrus & Waner, 2011; Khatat *et al.*, 2021).

During the years, several in-clinic screening tests for detection of *A. phagocytophilum* and *E. canis* seroprevalence have appeared in response to the need for rapid diagnostics. They are based on enzyme-linked immunosorbent assay (ELISA) or immunochromatographic techniques. The SNAP test (SNAP[®] 4Dx Plus, IDEXX Laboratories, Westbrook, ME) is one of the most commonly used. This is a rapid ELISA diagnostic test for simultaneous detection of antibodies against the major immunodominant p30 and p30-1 *E. canis* proteins, the p28 protein of the outer surface of *E. ewingii*, the major surface protein p44/MSP2 of *A. phagocytophilum/A. platys*. Also, it permits detection of antibodies against *B. burgdorferi* and the *Dirofilaria immitis* antigen. The test is designed for use in veterinary clinics. Its sensitivity is 97.1% for detection of *E. canis*, and 90.3% for *Anaplasma* spp. The reported specificity for *E. canis* is 95.3%, whereas for *Anaplasma* spp. – 94.3% (O'Connor, 2015).

Additionally, laboratory ELISA tests were developed, available for use by veterinary practitioners like Anti-*E. canis* ELISA Dog (IgG) and Anti-*A. phagocytophilum* ELISA Dog (IgG), manufactured by EUROIMMUN Medizinische Labordiagnostika AG, Luebeck, Germany. The tests provide semi quantitative *in vitro* evaluation for presence of IgG antibodies against *E. canis* or *A. phagocytophilum* in blood serum or plasma. According to the manufacturer, the specificity is 92% and 96% for *E. canis* and *A. phagocytophilum* respectively whereas reported specificity values are 100% (*E. canis*) and 97% (*A. phagocytophilum*).

The aim of the study was to compare the performance of in-clinic and laboratory ELISA tests for detection of antibodies against *E. canis* and *A. phagocytophilum* in dogs with clinical signs and haematological changes specific for monocytic ehrlichiosis or granulocytic anaplasmosis.

MATERIALS AND METHODS

The tests were performed from September 2019 to June 2020 in the immunodiagnostic laboratory to the Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria.

Samples

Blood samples from 61 dogs with clinical signs and haematological changes specific for monocytic ehrlichiosis or granulocytic anaplasmosis were analysed. Of them, 52 samples were collected from pets (29 males and 23 females) whereas 9 samples (5 males and 4 females) were from stray dogs. All dogs have been recently infected with ticks. Blood was collected from v.

cephalica in vacutainers with potassium EDTA. The tubes were carefully mixed to prevent thrombocyte aggregation. Blood plasma was separated by centrifugation and stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

Tests

Plasma samples were tested for antibodies against *Ehrlichia* spp. and *Anaplasma* spp. using Anti-*E. canis* ELISA Dog (IgG), Anti-*A. phagocytophilum* ELISA Dog (IgG) (EUROIMMUN Medizinische Labordiagnostika AG, Luebeck, Germany) and SNAP[®] 4Dx Plus test (IDEXX Laboratories, Westbrook, ME).

The commercial EUROIMMUN kits use specific recombinant antigen, hence their high specificity and sensitivity. The kit contains microplates with wells coated with recombinant purified antigen. In the first stage of the test, 100 μL of the calibrator, positive control, negative control and diluted samples were incubated in individual wells at $37\text{ }^{\circ}\text{C}$ for 30 minutes. After three washings with buffer, 100 μL enzyme conjugate (peroxidase-labelled anti-dog IgG) was added to each well to develop the colour reaction. Plates were incubated at $37\text{ }^{\circ}\text{C}$ for 30 minutes followed by another triple washing. The third step was addition of 100 μL chromogen/substrate solution in each well. The incubation was at room temperature ($18\text{-}20\text{ }^{\circ}\text{C}$) for 15 minutes. The last step before spectrophotometry was the addition of 100 μL stop solution to microplate wells. The measurement of absorption was done at a detection wavelength of 450 nm and reference wavelength 620 nm within 30 minutes after stop solution addition.

A semi-automated ELISA system (ELISA Reader LEDETECT 96, Labexim Produkt, Biomed Dr. Weisser GmbH) and ELISA Washer were used.

The results were interpreted semi quantitatively on the basis of extinction coefficient using the formula: Extinction coefficient = Control or sample extinction/calibrator extinction. Samples with extinction coefficient <0.8 were determined as negative, those with extinction coefficient >0.8 and 1.1: as borderline and those with with extinction coefficient >1.1 : as positive.

The same plasma samples were tested for IgG antibodies against *E. canis* and *A. phagocytophilum* with SNAP[®] 4Dx Plus as well following manufacturer's instructions. Enzyme-labelled conjugate was mixed with patient plasma in a tube and added to the sample well of the SNAP device. The mix conjugated with the sample flowed through the matrix interacting with test and control spots to reach the activation circle after 30-60 s. Afterwards, the device was activated, washing buffer and substrate solution were released from reagent reservoirs of the device. Positive results were visualised by forming colour reaction products. Test results were read within 8 minutes after device activation. The presence of positive control spot indicated that test reagents were available and active. The appearance of test spots was interpreted as positive results for antibodies against *Ehrlichia* or *Anaplasma*.

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 of agreement between the two tests was calculated by the formula:

$$\text{Percentage agreement} = \frac{\text{Agreements}}{\text{Agreements} + \text{Disagreements}} \times 100$$

RESULTS

Out of 61 canine blood samples tested with ELISA EUROIMMUN kits, antibodies against *E. canis* were present in 47 or 77.05% of samples (29 samples positive for *E. canis* only and 18 positive for both *E. canis* and *A. phagocytophilum*). Another 25 samples or 40.98% were positive for antibodies against *A. phagocytophilum* (7 samples positive for *A. phagocytophilum* only and 18 positive for both *E. canis* and *A. phagocytophilum*). Seven samples (11.48%) had no antibodies against these infectious agents (Table 1).

With SNAP® 4Dx, 44 samples or 72.13% were positive for antibodies against *Ehrlichia* spp (35 samples positive for *E. canis* only and 9 positive for both *E. canis* and *A. phagocytophilum*), 20 samples or 32.79% had antibodies against

Anaplasma spp. (11 samples positive for *A. phagocytophilum* only and 9 positive for both *E. canis* and *A. phagocytophilum*) and six plasma samples (9.84%) were negative (Table 1).

The comparative analysis of results from both tests showed agreement with respect to anti-*E. canis* antibodies in 52 samples with percentage agreement of 85.25%. Disagreement was recorded for 9 samples (14.75%): in 6 cases (9.84%) SNAP® 4Dx Plus was negative and anti-*E. canis* ELISA Dog (IgG) – positive. In the rest 3 samples (4.91%) the result was the opposite (Table 2).

Data for presence of anti-*A. phagocytophilum* antibodies were identical in 44 samples with 72.13% percentage agreement. In the other 17 tested samples (27.87%) the tests gave different results: in 11 cases (18.03%) SNAP® 4Dx Plus

Table 1. Results from EUROIMMUN ELISA and SNAP® 4Dx® IDEXX testing for IgG against *E. canis* and *A. phagocytophilum*

	EUROIMMUN ELISA Kit		ELISA SNAP® 4Dx® IDEXX	
	Number	Percent (95% CL)	Number	Percent (95% CL)
Positive only for anti- <i>Ehrlichia</i> antibodies	29	47.54% (45.43÷59.49)	35	57.38% (50.35÷64.41)
Positive only for anti- <i>Anaplasma</i> antibodies	7	11.48 % (6.09÷20.14)	11	18.03% (11.01÷25.06)
Positive for both anti- <i>Ehrlichia</i> and anti- <i>Anaplasma</i> antibodies	18	29.50% (17.56÷37.62)	9	14.75% (7.72÷21.78)
Negative samples	7	11.48% (2.81÷16.87)	6	9.84% (2.81÷16.87)

CL – confidence limits

Table 2. Comparison of results from SNAP® 4Dx® IDEXX and EUROIMMUN ELISA testing of 61 samples for antibodies against *E. canis* and *A. phagocytophilum*

<i>E. canis</i>		EUROIMUN ELISA Kit		<i>A. phagocytophilum</i>		EUROIMUN ELISA Kit	
		Pos	Neg			Pos	Neg
SNAP® 4Dx®	Pos	41	3	SNAP® 4Dx®	Pos	14	6
	Neg	6	11	SNAP® 4Dx®	Neg	11	30

was negative and anti-*A. phagocytophilum* ELISA Dog (IgG) was positive (Table 2).

For three samples (4.92%) there was a disagreement between test results for both pathogens.

DISCUSSION

This study attempted to compare the results from different ELISA (in-clinic and laboratory) tests for detection of antibodies against *A. phagocytophilum* and *E. canis* in blood plasma of spontaneously infected dogs.

According to results, the seroprevalence of *Ehrlichia* spp was higher – 72.13% with SNAP® 4Dx Plus and 77.04% with Anti-*E. canis* ELISA Dog (IgG) (EUROIMMUN). The respective data for *Anaplasma* spp. were 32.79% for the rapid test and 40.98% for Anti-*A. phagocytophilum* ELISA Dog (IgG) (EUROIMMUN). These results are similar to reported seroprevalence of *E. canis* in North Bulgaria (37.5%; Tsachev *et al.*, 2006b) and South Bulgaria (30%; Tsachev *et al.*, 2006a) and seroprevalence data of Arnaudov (2021) – 30.4% antibodies against *E. canis* and 26.1% against *A. phagocytophilum*. Our results were however different from those presented by Pantchev *et al.* (2015), Borisov *et al.* (2017) and Manev (2020), who reported predominance of IgG against *Anaplasma* spp. The latter three studies were conducted in animals without clinical or haematological signs of vector-borne rickettsiosis that may explain the found lower seroprevalence and the greater number of positive samples for antibodies against *A. phagocytophilum*. The differences in testing results of suspectly infected and clinically healthy dogs may be attributed to the high seroprevalence with relatively low number of clinically ill dogs, specific for

granulocytic anaplasmosis (Foley *et al.*, 2001; Kohn *et al.*, 2008; Bowman *et al.*, 2009).

The high percentage of agreement (85.25%) from comparative analysis in this study demonstrated similar specificity of both ELISA tests. In 6 out of 9 samples with different result, SNAP® 4Dx Plus result was negative while the Anti-*E. canis* ELISA Dog (IgG) result was positive. In these samples, the extinction coefficient determined by EUROIMMUN ELISA was between 1.2 and 1.4, presuming a relatively low antibody titre. Thus, these results of ours showed the probably higher sensitivity of laboratory ELISA test. Comparable data were reported by Harrus *et al.* (2002) from comparison of indirect rMAP2 ELISA, dot-ELISA (Immunocomb® Biogal, Israel) and Snap® 3Dx. A strong positive correlation was found out among results of the three tests and the standard (immunofluorescence analysis). According to the authors, 16 out of 17 disagreements corresponded to lower titres ($\leq 1:320$). This supported the hypothesis that sensitivity was lower in the low antibody titre range, yet high in titres higher than 1:320. In a similar study, Bélanger *et al.* (2002) also observed a slightly higher sensitivity of indirect ELISA vs the SNAP test, again in presence of low anti-*E. canis* antibody titres. The conclusions of both research teams supported the thesis that quantitative ELISA tests allowed identifying acutely infected animals through detection of seroconversion, as clinically ill dogs develop high titres within several weeks after the infection (Harrus *et al.*, 1998). This opportunity for confirmation of time course in antibody titres presents several advantages to laboratory ELISA compared to SNAP® 4Dx Plus. The main issue is adding quantitative information to dis-

crimination between positive and negative results. The rapid in-clinic tests are however less expensive, are easy to perform and provide a result within 10–15 minutes, which makes them useful in small animal clinic setting. Furthermore, the physician may decide to perform treatment or other diagnostic tests while the patient is still in the clinic.

The comparison of results for anti-*A. phagocytophilum* antibodies showed a lower agreement (72.13%). They matched in 44 out of 61 tested samples. Eleven out of the 17 samples with different results were negative in SNAP® 4Dx Plus and positive in Anti-*A. phagocytophilum* ELISA Dog (IgG) tests. Also, 10 out of 11 samples were at the same time positive for Anti-*E. canis* antibodies in both tests. The extinction coefficient for *E. canis* in these samples was much higher compared to that for *A. phagocytophilum*. This allowed assuming that perhaps, the cause was cross-reactivity of Anti-*A. phagocytophilum* ELISA Dog (IgG) test. This assumption is further supported by the observed very high extinction coefficients for anti-*E. canis* antibodies – over 3.0. A similar conclusion was made also by Solano-Gallego *et al.*, (2006) assuming cross-reactivity between *E. canis* and *A. phagocytophilum*, when titres of antibodies against one of pathogens were very high. Unlike us, the researchers performed the testing via indirect immunofluorescence assay (IFA) and SNAP® 3Dx.

The six samples positive in SNAP® 4Dx Plus but negative in Anti-*A. phagocytophilum* ELISA Dog (IgG) formed a heterogeneous group with regard to Anti-*E. canis* antibody results, suggesting the possible involvement of antibodies against *A. platys*, which are detected by the rapid ELISA test (Sainz *et al.*, 2015).

CONCLUSION

The comparison of the two tests showed a greater agreement of the results for detection of antibodies against *Ehrlichia* spp. (85.25%) than against *Anaplasma* spp. (72.13%).

The results from this study necessitate more investigations through comparison to a reference serological technique such as immunofluorescence analysis and molecular genetic methods (PCR).

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