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DATA ON CANINE HEARTWORM (*DIROFILARIA IMMITIS*) INFECTION AND OTHER VECTOR-BORNE PATHOGENS IN DOGS IN BUCHAREST AREA, ROMANIA

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Abstract

Heartworm disease is a serious cardiovascular and potentially fatal condition characterized by the presence of nematode Dirofilaria immitis in different developmental stages, found both in peripheral circulation, represented by microfilariae, and pulmonary artery and the right heart, represented by adult parasites. Diagnosis and identification of Dirofilaria species is complex involving antigen detection and microfilarial recognition. Therefore it is important for both animals and humans, improvement of rapid and efficient diagnostic protocols being a really powerful objective for epidemiological study progress.The purpose of the present study was to determine the exposure to D. immitis infection and other arthropod-borne pathogens of dogs living in Bucharest' adjacent area. For this we used modified Knott's technique and a point-of-care immunochromatographic test SNAP®4Dx® Plus. The modified Knott test is a concentration test that relies on lysing red blood cells and fixation of microfilariae for morphological examination, while, SNAP®4Dx® Plus represents an in-clinic diagnostic test that simultaneously screens dogs for 4 vector-borne diseases, including - Dirofilaria immitis antigen, and Ehrlichia canis, Ehrlichia ewingii, Anaplasma phagocytophilum, Anaplasma platys and Borrelia burgdorferi antibodies. A total of 175 dogs from Bucharest's adjacent area were included in the study. Of them, 21.14% were positive for D. immitis antigens. However, the modified Knott technique revealed a total of 16.57% samples positive for microfilariae of which 10.28% were D.immitis mf, 4.57%, D.repens mf. and 1.71% Acanthocheilonema mf. Additionally, 3.42% of dogs were positive for Anaplasma spp. antibodies, 1.14% for Ehrlichia spp, and 0.57% respectively, for B. burgdoferi.

In conclusion D.immitis infection in dogs from the greatest area of Bucharest is threatening high and therefore treatment and prophylaxis are needed to decrease the risks of disease since apparently healthy dogs harboring parasite serve as a reservoir of infection for other animals.

Key words*: Heartworm disease, vector-borne pathogens, Knott test, SNAP®4Dx® Plus test, dogs*

INTRODUCTION

Dogs are competent reservoir hosts of several zoonotic agents and can serve as a readily available source of nutrition for many blood feeding arthropods (Otranto et.al., 2009a). The explosion of canine population and their increasingly close relationship with humans in both urban and rural areas pose new concerns for human public health (Genchi et.al., 2011).

Canine vector-borne diseases (CVBDs) represent an important group of illnesses affecting dogs around the world. These diseases are caused by diverse range of pathogens transmitted by different arthropod vectors-ticks and insects (fleas, mosquitoes, phlebotomine sandflies). In addition to their veterinary importance, some of the CVBDs are of major zoonotic concern, with dogs potentially serving as reservoirs and sentinels for human infections (Otranto et. al., 2009b).

Dirofilaria immitis, a filarial nematode transmitted by mosquitoes has the highest prevalence, being endemic, in southern and central European countries, especially in those with mediteranean climate as Italy, Spain, Portugal, France and Greece, but its spread has impended in central and northern European countries, as Switzerland, Germany, Netherlands, United Kingdom, Croatia, Russia, Hungary, including in Romania (Morchón et al., 2012).

Mosquitoes (*Culicidae*) represent vectors and intermediate host for *Dirofilaria* spp., fleas and lice, being vectors for *A. reconditum* and

ticks for *A. dracunculoides* (Magnis et al., 2013).

For diagnosis of heartworm infection, in dogs, can be used blood tests that detect circulating microfilariae or adult antigens, but further diagnostic procedures are usually required to determine the severity of disease and treatment options (Knight, 1995). Moreover diagnosis and identification of *Dirofilaria* species is complex, involving antigen detection and microfilarial recognition (Roth et al., 1993; Genchi, 2005).

Despite of the great concern worldwide on vector-borne diseases generally (Knols et al., 2007) and on CVBDs particularly, little is known about the occurrence and prevalence of vector-borne pathogens in dogs in different areas of Romania.

Therefore, the present study was conducted to investigate and determine the exposure to *D. immitis* infection and other arthropod-borne pathogens of dogs living in Bucharest's adjacent area.

MATERIALS AND METHODS

A total of 175 dogs, were enrroled in the present study. The dogs were older than 1 year, without previous chemoprophylaxis or treatment by microfilaricide products and living in Bucharest's adjacent area.

All dogs were screened using a modified Knott's technique (Bowman, 2003) for microfilariae detection and by a point-of-care immunochromatographic test *SNAP®4Dx® Plus*, for qualitative detection of antigens and antibodies (IDEXX Laboratories, 2008) of different arthropod-borne pathogens (Figure 1).

SNAP®4Dx®Plus represents an in-clinic ELISA test, for qualitative detection of *Ehrlichia canis* and *Ehrlichia ewingii*, *Borrelia burgdorferi*, *Anaplasma platys* and *Anaplasma phagocytophilum* antibodies, as well as simultaneously detection of *Dirofilaria immitis* antigens in serum, plasma or whole blood from the dog, with a specificity of 98-99% (IDEXX Laboratories, Westbrook, ME).

Enzyme-linked immonosorbent assays are designed to detect heartworm adult antigens, which are considered highly specific, as cross reactivity with other canine parasites (i.e. *D. repens, Dipetalonema* spp.) does not occur (Venco et al., 2001).

These tests allow detection of adult heartworm antigens produced only by female worms and may provide information about worm burden (McCall et al.,1992; Knight, 1995).

The sensitivity is actually very high, but false negative results may occur in prepatent period or very light infections or when only male worms are present (Knight, 1995).

We obtained 4 ml of blood collected in EDTA tubes from all dogs, selection including a wider area and more varied places in town and its surroundings. Thus, 67 were street dogs, 22 were German Shepherd from a military unit and the remaining 86 were mixed-breed companion dogs, owned by native people, brought to the Faculty of Veterinary Medicine-Bucharest for various investigations. Blood samples and tests were kept at room temperature for 30 minutes prior to testing. We used both, whole blood and serum obtained by centrifugation. The use of serum gives an accuracy of 99.2%, a confidence of 100% and a specificity of 95% according to the manufacturers (IDEXX Laboratories, 2008).

There have been distributed 3 drops of whole blood/ serum sample to be analyzed with the pipette contained in the test kit in a 2 ml Eppendorf tube, adding above 4 drops of conjugate and mixing 2-3 times.

The SNAP was placed horizontally, then the mixture was added in the orifice for the sample content. The sample will arrive in the reading window before reaching the activation circle (30-60 seconds).The complex antigen (Ag)/ conjugate or antibody (AB) /conjugate binds to the labeled antigen or antibody. Once the activation circle has began to change color, the activator was pressed firmly to align the SNAP device body. The sample will flow back over the array. Bidirectional flow provides a second opportunity to bind to the antibody. Washing solution cleans matrix debris that could interfere with the results. Colorless substrate solution reacts with the enzyme conjugate. Each enzyme converts multiple substrate molecules from colorless to blue, amplifying

the signal. This reaction forms blue spots in the window reading device color indicating a positive result.

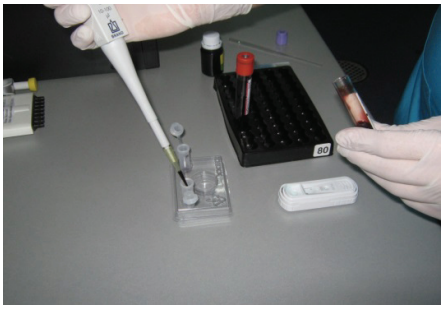

Figure 1. Comparative testing of blood samples by *SNAP®4Dx® Plus* and modified Knott test

Results were issued after 8 minutes, using SNAP shot Dx Analyzer device that records and interprets the response colorimetric enzyme activity on the surface of SNAP test. During the procedure, the analyzer records digital images of each SNAP device using an algorithm to calculate the specific test results using own bar code, thus minimizing subjective interpretation.

Along with Snap tests, we used a concentration method called modified Knott test which relies on lysing red blood cells and fixation of microfilariae for morphological examination.

For each sample obtained, we mixed 1 ml of whole blood collected in EDTA tube, with 9 ml of 2% formalin, then centrifuged 5-8 minutes at 1500 rpm. The supernatant was discarded from the centrifuge tube and the sediment was mixed with equal parts of the dye, methylene blue 1: 1000.We spread the colored sediment on a slide, put a coverslip, and examined microscopically (10X, 20X and 40X objective).

Examination of whole sediment allows finding the number of microfilariae in a milliliter of blood. We prefered this method because formalin fixed microfilariae in extension allows their measurement.

RESULTS AND DISCUSSION

Overall, out of 175 dogs, investigated by *SNAP®4Dx® Plus*, 37 (21.14% [$CI_{99\%}$=27.6-46.4]) was positive for *D. immitis* antigens.

However, the modified Knott technique revealed a total of 29 samples of microfilariae (16.57%), of which 18 were *D.immitis* mf., 8 *D.repens* mf. and 3 *Acanthocheilonema* mf. (Table 1).

Microfilariae of *D.immitis* measured between 290 to 330 µm in length and 5 to 7 µm in diameter, with a straight tail and a spindle-shaped cephalic extremity (Figure 2).

D. repens microfilariae measured between 350 and 385 µm in length and 7 to 8 µm in diameter, with a curved tail and rounded cephalic extremity (Figure3). *Acanthocheilonema* species differentiation were not possible by this method (Figure 4).

However, given the variety of canine filariae, the detection of microfilariae alone does not give an accurate diagnosis, because although filaria species can be identified by an evaluation of cephalic and caudal morphologies, these features are often difficult to differentiate (Simón et al., 2012).

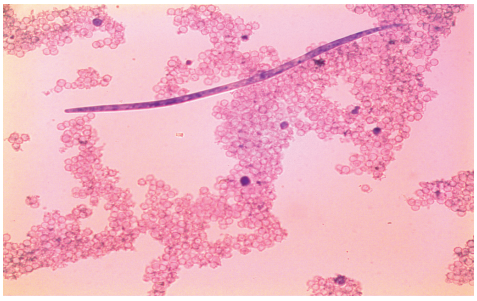

Figure 2. *Dirofilaria immitis* - microfilariae by modified Knott test, O.B x 20, (original NIKON microscope)

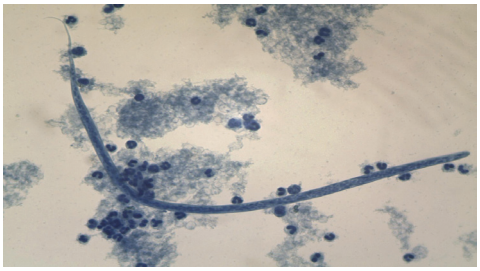

Figure 3. *Dirofilaria repens* - microfilariae by modified Knott test, O.B x 20, (original OPTIKA microscope)

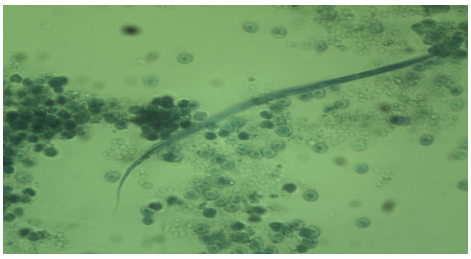

Figure 4. *Dipetalonema* spp. microfilariae by modified Knott test, O.B x 20, (original OPTIKA microscope)

Occult infections occur during prepatent period (larval stage) or unisexual infections when worms become sterile after microfilaricidal treatment, or when the body produces antibodies that cause destruction host's microfilariae. (Rawlings et al., 1982).
In this study, a case of occult infection has been reported (positive with *SNAP®4Dx® Plus*, but negative for microfilariae.)
Additionally, 6 dogs (3.42% [CI99%=1.38-10.62]) were positive for *Anaplasma* spp. antibodies, 2 dogs(1.14% [CI99%=-0.73-4.73]) were positive for *Ehrlichia* spp, and one dog (0.57% [CI99%= -0.94-2.94]) respectively, for *B. burgdoferi* (Figure 5). No mixed infection were recorded.

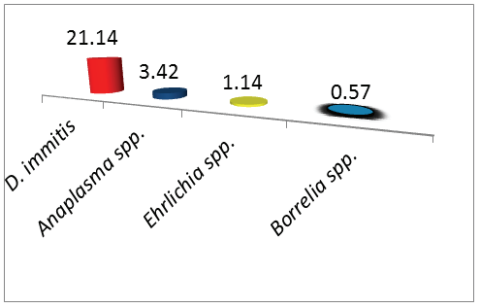

Figure 5. Seroprevalence of *D.immitis, Anaplasma* spp. *Ehrlichia* spp and *Borrelia* spp. using *SNAP®4Dx® Plus*

Table 1. Prevalence of arthropode-borne pathogens (*Dirofilaria* spp. *Anaplasma* spp., *Ehrlichia* spp, and *Borrelia burgdoferi* sensu lato), stratified by category of dogs and detection methods

Dog category	No.of tested	Detection methods				
		Microscopy (Modified Knott test)	Ag ELISA (SNAP 4DX Plus)			
		Nos. Positive microfilariae spp. (%)	Nos. Positive *D.immitis* (%)	Nos. Positive *Anaplasma* spp (%)	Nos positive *Ehrlichia* spp (%)	Nos. Positive *Borrelia* spp (%)
Stray dogs	67	14 (20.89%)	19 (28.35%)	4 (5.97%)	-	-
Police dogs	22	4 (18.18%)	3 (13.63%)	-	-	-
Pet dogs	86	11 (12.79%)	15 (17.44%)	2 (2.32%)	2 (2.32%)	1 (1.16%)
Total	175	29 (16.56%)	37 (21.14%)	6 (3.42%)	2 (1.14%)	1 (0.57%)

These diagnostic approaches are a powerful outfit for epidemiological studies and should allow the assessment and screening of large area, considering that they have a good sensitivity and do not require invazive techniques.

Modified Knott test is more sensitive than direct smear test, because, it concentrates microfilaria, so they are less likely to be missed during microscopic examination. Nevertheless, antigen tests are very specific, but they are not always sensitive, and modified Konott test is a method that requires

time and experience, thus molecular techniques are the next step certifying the truthfulness samples.

Present finding supports similar studies of other authors, in Romania, as Mircean et al. (2012) reported seroprevalence values as follows:*D.immitis* (3.3%), *A.phagocytophilum* (5.5%), and E. *canis* (2.1%). Also, Ionita et.al. 2012, acquired the following results *D. immitis*,18.68%,*A. phagocytophilum*, 16.00%, *E. canis*, 4.00%.

Another survey conducted in 2014 by Ciucă et al. which refers to Romania's northern counties, of region Moldova, certify a seroprevalence of 2.10% of *D.immitis,* considering that most of this area has a temperate continental climate with eastern influences aridity and Baltic scandinavian influences in the north, having a cool and moist character but, reveals an expanding in the northeast areas of the country, where the climate conditions support transmission of heartworm.

Increasingly prevalence in almost all our results is due to superiority assessment tests, including new etiologic pathogens, but also by particular ecological conditions (climate, biotopes) associated with the distribution and abundance of arthropods in the studied area.

CONCLUSIONS

These findings show that people need to be informed about the risk of zoonotic potential of dogs that harbour parasites.

In particular, *D.immitis* infection in dogs from the greatest area of Bucharest is threatening high and therefore treatment and prophylaxis are needed to decrease the risks of disease since apparently healthy dogs harboring parasite serve as a reservoir of infection for other animals.

Therefore, the findings are expected to serve as a reference for future investigations and control actions in order to protect dogs and limit the risk of transmission of vector-borne agents to humans.

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