

## VECTOR-BORNE PATHOGENS-TESTING IN A ROMANIAN CANINE BLOOD BANK

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### Abstract:

**INTRODUCTION:** Canine blood banking in veterinary medicine is an expanding market. Once the demand for blood products increased all over the world, canine blood banks have focused attention on the risk of spreading diseases through blood transfused products. The need to preserve a healthy donor-pool, free of blood-borne infectious diseases, mainly in endemic areas, led to the implementation of appropriate protocols for screening canine blood donors using specific tests.

**OBJECTIVES:** The aim of this study was to evaluate the presence of *Anaplasma phagocytophilum*/*Anaplasma platys*, *Ehrlichia canis*/*Ehrlichia ewingii*, *Dirofilaria immitis* and *Borrelia burgdorferi* using the enzyme immunoassay technology (EIA) among the donors of a Romanian canine blood bank, from January 2015 to December 2016.

**METHODS:** Blood samples from 575 donors were collected and 1253 tests were performed with SNAP 4DX Plus® (IDEXX Laboratories, Fremont, CA) to reveal the presence of *D. immitis* antigens and the antibodies toward *A. phagocytophilum* and/or *A. platys*, *E. canis* and/or *E. ewingii* and *B. burgdorferi*.

**RESULTS:** The results of this holistic approach show that all blood samples provided negative results for *B. burgdorferi* and *E. canis*/*E. ewingii* (0/1253), while 0.87% (11/1253) samples provided positive results for *A. phagocytophilum*/*A. platys* and 6.94% (87/1253) for *D. immitis*.

**CONCLUSION:** The next studied topic would be to compare the results provided by the EIA technology with results of real time PCR and qPCR regarding these vector-borne pathogens.

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**Keywords:** blood transfusion; canine blood bank; vector-borne disease.

### Introduction

Over time, the approach of blood banking (BB) and transfusional medicine has evolved from an empirically and rare procedure, to a modern and currently available treatment (Greenwalt, 1997; Yagi & Bean, 2016). This trend has been closely connected to the evolution of blood bank equipment and diagnostic methods, making blood transfusions (BT) much safer (Wardrop, 2016). However, several risks are still present, related either to the donor, or to the recipient dogs (Yagi & Bean, 2016). In order to keep the donors and recipients safe, it is mandatory to fulfil a rigorous protocol for the donor selection. In this respect, Yagi & Bean (2016) recommended the criteria listed below for the selection of donors: age, weight, physical examination, behaviour, history of previously involvement in BT and other medication, preventive medication, haematological and biochemical blood analyses, blood type, infectious diseases screening, and the owner's attitude. Considering the above mentioned criteria, all blood donors should be tested for various blood-borne pathogens which can potentially cause diseases in the recipient (Wardrop, 2016).

Once canine BT became a routine procedure, there was a need to study the potential for transmission of infectious and parasitic diseases (Freeman, 1994; Owens, 2001; Stegeman, 2003; Reine, 2004; Wardrop, 2016). Canine blood banks need to implement programs of pre-emptive identification and screening of healthy blood donors (Wardrop et al., 2016), and, in this respect, it is mandatory to maintain an up-to-dated registry of donors. The design of an appropriate time table for donors' screening is difficult; some authors propose a full screening test on the first presentation and then re-testing at three-month interval. For all subjects agreed in the blood donation programs, Reine (2004) proposed monthly ectoparasite prophylaxis, to limit exposure to vectors of concerned disease and the

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correlation of the infectious diseases diagnostic tests used by the blood bank with the recent travel history of the donors. Blood-borne pathogens which can potentially cause diseases in the recipient could have geographic restrictions or breed predilection (Wardrop, 2016), and screening programs should also consider these factors. A physical exam is also important to be performed prior to each donation, to detect any subtle sign of infectious disease (Reine, 2004).

Vector-borne pathogen-testing, as recommended by the Consensus Statements of the American College of Veterinary Internal Medicine (ACVIM) for canine blood donors, include 23 agents but with irregular geographic distribution and risk factors (Wardrop, 2016). Also, European studies revealed erratic geographic distribution of several vector-borne pathogens, like *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia canis*, *Dirofilaria immitis* and *Borrelia burgdorferi*, whose prevalence rates are higher in countries of southern Europe (Rizzoli et al., 2011; Vieira et al., 2014; Sainz et al., 2015; Ciuca et al., 2016).

In dogs, *A. phagocytophilum* and *A. platys* infections is causing granulocytic anaplasmosis and infectious cyclic thrombocytopenia; both can lead to chronic and persistent subclinical infections (Egenvall, 1998, 2000; Green, 2012). Also, *E. canis* and *E. ewingii* are vector-borne pathogens which can cause chronic and persistent infections (Starkey, 2015). Despite *D. immitis* does not meet the criteria of blood-borne pathogens which can potentially cause diseases in the recipient, because transfusion of microfilaria from an infected donor cannot cause heartworm disease in the recipient, microfilaria infected blood transfused to a recipient can potentially interfere with diagnostic testing can be a source of infection for the mosquito vectors and can carry *Wolbachia* spp. (Dingman, 2010). As a consequence, dogs infected with *D. Immitis* are not considered eligible as “healthy” donors, and thus, collection of blood from them is not suitable (Wardrop, 2016). *B. burgdorferi* is the etiological agent of Lyme disease, a tick-borne disease which may be expressed clinically asymptotically or chronically (Fritz, 2003). Although, the risk of acquiring Lyme disease from a transfused unit of packed red blood cells or platelets is negligible (Gerber, 1994; Ginzburg, 2013; Wardrop, 2016), the risk of infections should be considered as long the presence of *B. Burgdorferi* may be present in the blood of donors (Straubinger, 2000; Wardrop, 2016). All these state that healthy canine blood donors should not be screened for *B. burgdorferi*: even more, if a multi-test screening, designed to detect more pathogens, is providing a positive result for *B. burgdorferi*, that animal should not be excluded from the donor pool (Zhi, 2002).

## Material and Methods

### Animals and blood samples

The present study included 575 dogs (Table 1) identified as potential blood donors between January 2015 and December 2016 in a Romanian canine blood bank (Hemopet Blood Bank, Bucharest, Romania), of which 82 dogs, already tested in 2015, were re-evaluated at each blood donation realized in 2016.

	Dogs	Tested in 2015	Tested in 2016	Tested in 2015 and 2016	Total
	Number	270	223	82	575
	%	47	39	14	100

Source: Author

	Dogs	Tested in 2015	Tested in 2016	Total
	Number	647	606	1253
	%	52	48	100

Source: Author

Only 14% of all dogs donated blood in both years of study and have been tested in both years, while 86% were tested in 2015 or in 2016 (Table 1).

Over 24 months, 1253 blood samples were taken to perform serological tests (Table 2) by using a point-of-care ELISA test: SNAP 4DX Plus (IDEXX Laboratories, Fremont, CA). All dogs were tested at the time of each blood donation.

***Dogs testing for *Dirofilaria immitis*, *Anaplasma phagocytophilum*/*Anaplasma platys*, *Ehrlichia canis*/*Ehrlichia ewingii* and *Borrelia burgdorferi****

The dogs were tested for heartworm disease (*D. immitis*) and tick-transmitted pathogens (*A. phagocytophilum*/*A. platys*, *E. canis* / *E. ewingii* and *B. burgdorferi*) using SNAP 4DX Plus test (IDEXX Laboratories, Fremont, CA) according to the manufacturers' instructions.

*D. immitis* infection is detected with SNAP 4DX Plus using a monoclonal antibody which binds to the target circulating antigens of *D. immitis* and a labelled polyclonal antibody against soluble antigens of *D. immitis* adult worms (Stillman, 2014).

Detection of *A. platys* antibodies is based on the cross-reactivity with the p44 protein of *A. phagocytophilum* (Zhi, 2002). For *E. ewingii* previously exposure of dogs, p28 outer membrane protein is used, an antigen proved to bind highly specific *E. ewingii* antibodies (Gusa, 2001; Zhang, 2008; O'Connor, 2010; Stillman, 2014). Presence of *E. canis* antibodies in serum samples is based on the detection of the main proteins p30 and p30-1 (Ohashi, 1998; Chandrashekar, 2010; Stillman, 2014), while for *B. burgdorferi* antibodies detection is targeting the VlsE protein-derived C6 peptide (Chandrashekar et al., 2010; Stillman, 2014).

Briefly, all blood samples, reagents and SNAP tests were kept for 30 min at room temperature (18-22°C) before being used. Four drops of enzyme-labelled conjugate are mixed with three drops of whole blood in a tube and added to the sample well of the SNAP device. The sample-conjugate mixture flows through the matrix, interacts with the test and control spots deposited on the matrix, and reaches the activation circle in approximately 30-60 seconds. The device is then activated (by depressing or "snapping" the activation), which results in the release of wash buffer and substrate solution from the reagent tank of the device.

Positive results are visualized by the formation of coloured reaction products; the assay is complete in 6-10 minutes depending on the test. The development of the colour in the positive control indicates that the assay reagents are properly working (Wild, 2013).

**Results and Discussion**

Between January and December 2015, 352 dogs were selected as potential donors, but only 302 were included in the blood donor registry, while 50 were excluded due to the positive results of the performed immunological tests (46 dogs with *Dirofilaria immitis* Ag, and 4 dogs with *A. phagocytophilum*/*A. platys* Ab) (Table 3).

Throughout 2016, the Blood Bank team selected 223 new dogs as potential donors, and only 188 were included in the blood donor register, while 35 dogs were excluded due to the positive results of the performed immunological tests (33 dogs with *Dirofilaria immitis* Ag, and 2 dogs with *A. phagocytophilum*/*A. platys* Ab).

Interestingly, of the 302 donors registered in 2015, only 27.15% (n=82) were maintained as donors during the year 2016, while 72.85% were not found on the donors' group, even if their status in the donor registry was maintained (Table 1).

During the year 2016, only 82 blood donors from the previous year were included in Donors Registry and have donated blood, and five were removed from the blood bank due to the positive results of the immunological tests performed in 2016 (2 dogs with *Dirofilaria immitis* Ag, and 3 dogs with *A. phagocytophilum*/*A. platys* Ab) (Table 4).

Despite several Romanian reports concerning the circulation of *Ehrlichia canis* and *B. burgdorferi* in dogs (Kiss et al, 2011; Mircean et al, 2012; Morar et al, 2015; Enache & Coprean, 2015) and in tick-vectors (Vladimirescu et al, 2016), in our study non *Ehrlichia* and *Borrelia* species were detected in the samples from the evaluated dogs in 2015 and 2016. Our data supports the negative results of Andersson et al. (2017) in Romanians canine blood samples collected during 2013 and 2014, in Snagov (Iflov County), a village located in the same region of the dogs tested in this study.

**Table 3: Blood samples tested between January 2015 and December 2016 in Hemopet Blood Bank**

Vector-borne pathogen	2015			2016			2015-2015		
	Negative	Positive	Total	Negative	Positive	Total	Negative	Positive	Total
<i>Dirofilaria immitis</i> antigen	597	50	647	569	37	606	1166	87	1253
<i>A. phagocytophilum</i> / <i>A. platys</i> antibodies	640	7		602	4		1242	11	
<i>E. ewingii</i> / <i>E. canis</i> antibodies	647	0		606	0		1253	0	
<i>B. burgdorferi</i> antibodies	647	0		606	0		1253	0	

Source: Authors

**Table 4: History of blood harvests and immunological tests performed in the blood donors included in the Donor Registry which later became positive**

No.	Breed	Gender	Age (years)	Location	Habitat	Occupation	Freedom of movement	Exposure to vectors	Immunological testing	
									Date	Result*
1.	Malinois	Male	4	Crevedia, Dambovita	Rural	Guard	Yes	Yes	Jan 06, 2015	-
									Mar 06, 2015	-
									Apr 06, 2015	D+
2.	Malinois	Male	4	Unknown	Unknown	Guard	Yes	Yes	Jan 11, 2015	-
									Mar 10, 2015	D+
3.	Golden retriever	Female	4	Bucharest	Urban	Companion	Yes	Yes	Feb 18, 2016	-
									Mar 17, 2016	D+
4.	Golden retriever	Male	2	Bucharest	Urban	Companion	Yes	Yes	Jan 15, 2015	-
									Feb 18, 2015	D+
5.	Crossbred	Male	4	Braila	Rural	Stray	No	Unknown	Aug 18, 2015	-
									Sept 18, 2015	D+
6.	German shepherd dog	Male	3	Crevedia, Dambovita	Rural	Companion	Yes	Yes	Aug 27, 2015	-
									Oct 07, 2015	-
									Nov 09, 2015	-
									Dec 30, 2015	-
									Feb 03, 2016	D+
7.	German shepherd dog	Female	2	Crevedia, Dambovita	Rural	Companion	Yes	Yes	Oct 07, 2015	-
									Nov 09, 2015	-
									Dec 30, 2015	-
									Mar 10, 2016	-
									Apr 21, 2016	-
May 27, 2016	D+									
8.	German shepherd dog	Female	2	Crevedia, Dambovita	Rural	Companion	Yes	Yes	Feb 03, 2016	-
									Mar 10, 2016	-
9.	Boxer	Female	3	Unknown	Unknown	Companion	Yes	Yes	Jan 08, 2015	-
									Feb 15, 2015	A+
10.	Romanian mioritic shepherd dog	Female	10	Campulung, Arges	Rural	Shepherd	Yes	Yes	Jan 17, 2015	-
									Jul 15, 2015	-
									Oct 31, 2015	A+
11.	Romanian mioritic shepherd dog	Female	2	Campulung, Arges	Rural	Shepherd	Yes	Yes	Nov 03, 2015	-
									Dec 17, 2015	-
									Mar 15, 2016	-
									May 08, 2016	-
									Jul 06, 2016	A+
12.	Romanian mioritic shepherd dog	Female	2	Campulung, Arges	Rural	Shepherd	Yes	Yes	Nov 03, 2015	-
									Dec 17, 2015	-
									Jan 28, 2016	-
									Mar 15, 2016	-
									May 08, 2016	A+
13.	American staffordshire terrier	Female	4	Tartasesti, Dambovita	Rural	Companion	Yes	Yes	Aug 26, 2015	-
									Apr 21, 2016	-
									May 27, 2016	A+

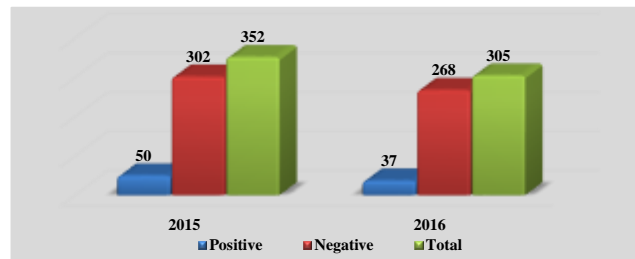
\* "-": negative result; "D+": positive result for *Dirofilaria immitis* Ag; "A+": positive result for *A. phagocytophilum*/*A. platys* Ab

Source: Authors

***Dirofilaria immitis* positive dogs study**

Positive tests results for *D. immitis* were recorded in 87/1253 samples (Table 3) collected from 575 dogs (Table 1) over a period of 24 months (2015-2016). In 2015, there were recorded 50 positive results in 352 dogs selected as potential donors, while in 2016 there were 37 positive results in 305 dogs (223 new dogs were selected as potential donors and 82 dogs were maintained as donors of the previous year) (Figure 1).

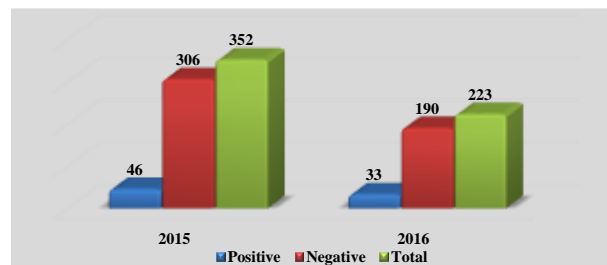
Figure 1: Positive canine blood donors to *Dirofilaria immitis* antigen revealed at the step of donor selection.



Source: Author

In the group of dogs tested before their first blood donation (Figure 2), the highest prevalence of the positive results was recorded in 2015: 58.22% (46/79). However, if it is related to dogs tested before their first blood donation yearly, the prevalence of positive results was higher in 2015 than in 2016: 14.79% (33/223) and 13.06% (46/352), respectively.

Figure 2: *Dirofilaria immitis* antigen positive results in dogs evaluated by Hemopet Blood Bank to be included in the Donor Registry (dogs tested before their first blood donation)

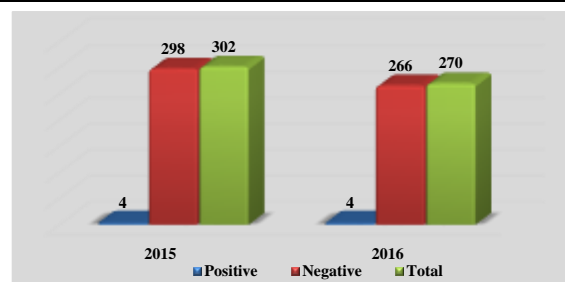


Source: Author

Only eight positive cases were recorded in the group of the dogs retested after their first donation (dogs included in the registry of donors who donated several times during 2015 and 2016): 1.32% (4/302) positive animals were recorded in 2015, and 1.48% (4/270) in 2016 (Figure 3).

The results of *D. immitis* antigen detection highlights the necessity of continuously testing for this pathogen in all blood donors and supports the previously epidemiological studies concerning the prevalence of *D. immitis* in Southern (Mircean et al, 2012) and central East (Ciucă et al, 2016) regions of country.

Figure 3: *Dirofilaria immitis* antigen positive results in donor dogs evaluated by Hemopet Blood Bank to be kept in the Donor Registry (dogs retested after their first donation)

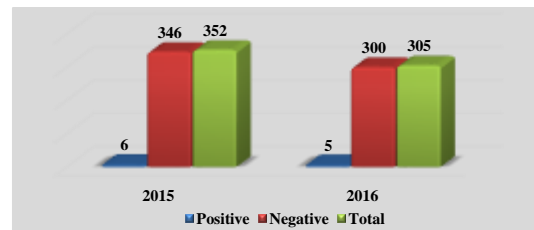


Source: Author

### ***A. phagocytophilum/A. platys* positive dogs study**

Seropositivity to *A. phagocytophilum/A. platys* was the second main cause of non-acceptance / rejection in the register of dog donors. Positive tests results were recorded in 11/1253 samples (Table 3) collected from 575 dogs (Table 1) over a period of 24 months (2015-2016). While 7 tests were positive in 2015 and in 2016 the number decreased to only 4 positive tests (Figure 4).

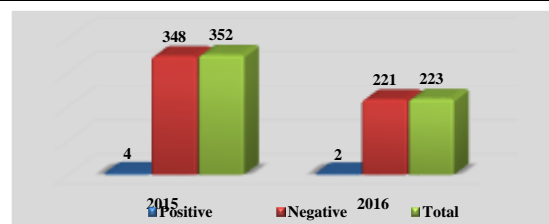
Figure 4: Prevalence of positive results (antibodies) to *A. phagocytophilum*/*A. platys* in candidate blood donors, before blood harvest procedure



Source: Author

Dogs tested before their first blood donation provided 1.13% (4/352) positive results in 2015, and 0.89% (2/223) in 2016 (Figure 5).

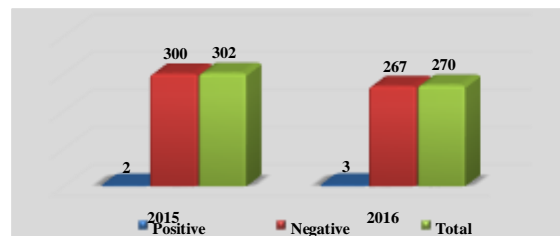
Figure 5: *A. phagocytophilum*/*A. platys* positive results (antibodies) in dogs evaluated by Hemopet Blood Bank to be included in the Donor Registry (dogs tested before their first blood donation)



Source: Author

In the group of dogs tested before their first blood donation, the positive results recorded in 2015 (n=4) were higher than in 2016 (n=2). In the group of the dogs retested after their first donation, there were five positive results: 0.66% (2/302) positive animals were recorded in 2015, and 1.11% (3/270) in 2016 (Figure 6).

Figure 6: *A. phagocytophilum*/*A. platys* positive results (antibodies) in donor dogs evaluated by Hemopet Blood Bank to be kept in the Donor Registry (dogs retested after their first donation)



Source: Author

Positive results obtained in all groups of donors and in both years of study recommend the necessity of *A. phagocytophilum*/*A. platys* continuous testing of donor pool, and this recommendation is also supported by epidemiologically studies performed in Romania that proved the risk of granulocytic anaplasmosis mainly in Central and Southern Romania, with a greater risk in the Southern lowland region (Matei et al, 2017), and clustered foci in southern regions and in the western part of the country (Mircean et al, 2012).

As it was stated by Kidd (2003), ideally all dogs should be screened for *D. immitis*, *A. phagocytophilum* / *A. platys*, *E. canis* / *E. ewingii* and *B. Burgdorferi*. Canine blood donors should be treated with ectoparasite prophylaxis to minimize exposure to potential vectors. The prophylaxis medicine should be chosen with deep caution, because diseases transmission typically takes at least 24 to 72 hours of tick blood feeding (Kidd, 2003).

### Conclusion

Canine blood donor screening for *Dirofilaria immitis* antigen revealed 14.20% (50/352) positive animals in 2015 and 12.13% (37/305) in 2016. The prevalence of infected dogs retested after their first donation (already included in the Donor Registry) was quite low relative to the number of blood

donors [1.32% (4/302) in 2015, and 1.48% (4/270) in 2016]. The prevalence of infected dogs tested before their first blood donation (not included in the Donor Registry) was quite high relative to the number of the selected potential blood donors [13.06% (46/352) in 2015, and 14.79% (33/223) in 2016]. The screening for *A. phagocytophilum*/*A. platys* antibodies revealed positive results: 1.70% (6/352) in 2015, and 1.63% (5/305) in 2016. Both groups of tested dogs (already included and not included in the Donor Registry) reveal low prevalence of infected dogs. Only 1.13% (4/352) in 2015, and 0.89% (2/223) in 2016 of dogs already included in the Donor Registry were *A. phagocytophilum* / *A. platys* positives (antibodies), and 0.66% (2/302) in 2015, and 1.11% (3/270) in 2016 of dogs not included in the Donor Registry were positives. None of the 575 dogs evaluated by the 1253 immunological tests, performed over 24 months (2015-2016), provided positive results for *E. ewingii* / *E. canis* antibodies and *B. burgdorferi* antibodies.

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