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Simultaneous Exposure to *Angiostrongylus vasorum* and Vector-Borne Pathogens in Dogs from Italy

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Abstract: Several drivers have recently fostered the expansion of *Angiostrongylus vasorum* throughout Europe, where Vector-Borne Pathogens (VBPs) are also spreading. However, the level of simultaneous risk of infection is still unknown in canine populations. This study evaluated the simultaneous exposure to *A. vasorum* and major canine VBPs in dogs of Italy. Sera of 294 dogs were subjected to two ELISAs, detecting *A. vasorum* circulating antigens and antibodies against the parasite, and to the following assays: (i) SNAP®4DX (IDEXX Laboratories Inc.) detecting *Dirofilaria immitis* antigens, and antibodies vs. *Borrelia burgdorferi*, *Anaplasma* spp. and *Ehrlichia* spp. and (ii) IFAT for the detection of antibodies vs. *Leishmania infantum*, *Babesia canis* and *Rickettsia conorii*. Twenty-two (7.5%, CI: 4.8–11.1%) and six (2%, CI: 0.7–4.4%) dogs scored positive for circulating *A. vasorum* antibodies and antigens, respectively. Seventeen dogs (5.8%, CI: 3.4–9.1%) were positive for *A. vasorum* antibodies + at least one VBP, three (1%, CI: 0.2–3%) for *A. vasorum* antigen + at least one VBP, while one dog (0.3%, CI: 0.01–1.88%) was positive for *A. vasorum* antigen + *A. vasorum* antibodies + *B. canis* antibodies. These results show that dogs living in different regions of Italy are at risk of simultaneous infections with both *A. vasorum* and VBPs. Despite the same scenario being likely in other countries of Europe, the current knowledge is scant. Therefore, further studies are warranted to amplify current epizootiological information and to understand whether control programs should be improved.

Keywords: *Angiostrongylus vasorum*; antigen; antibodies; vector-borne diseases

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1. Introduction

Over the last decade, nematodes of the genus *Angiostrongylus* have changed their distribution in Europe, with the spread of *Angiostrongylus vasorum* in canids [1,2], and new or unexpected findings of other species like *Angiostrongylus cantonensis*, *Angiostrongylus chabaudi* and *Angiostrongylus daskalovi* in different animal hosts [3–5]. Among them, *A. vasorum* is now regarded as a primary parasite of dogs throughout European regions, e.g., Iberian Peninsula, Mediterranean Basin, and northern, central, and eastern Countries [6–9].

Vector-Borne Diseases (VBDs) are also expanding in many territories, where they are a threat to animal and human health [10–14]. Dogs are frequently at risk of VBDs caused by non-parasitic (e.g., bacteria, viruses) and parasitic (e.g., protozoa) Vector-Borne pathogens (VBPs) transmitted by ticks and flying insects [10,12,15,16]. In Europe, the most important and distributed tick-borne bacteria are *Ehrlichia canis*, *Anaplasma platys*, *Anaplasma phagocitophylum*, *Rickettsia conorii* and *Borrelia* spp. [17–20]. The nematode

Dirofilaria immitis transmitted by mosquitoes, and the protozoans *Leishmania infantum* and *Babesia* spp. transmitted by sandflies and ticks, respectively, are the preeminent canine vector-borne parasites [21–23]. Many of these pathogens may also infect other animals, e.g., cats, and zoonotic VBPs shared by dogs and cats increase chances of interspecific transmission and of human infections [16,24–29].

Extrinsic and intrinsic drivers, e.g., global warming, travelling of pets with their owners, relocation and growth of human and animal population, and anthropization of wildlife habitats, may increase the distribution of pathogens transmitted by invertebrates and/or with an indirect biological cycle in both enzootic and free areas [15,29–32]. For instance, climate change has an impact on the spatial–temporal distribution of gastropod-borne nematodes and VBPs via its influence on the life cycle, survival, and reproduction rates of invertebrates and transmitted pathogens [33–35]. Increasing temperatures, changes in precipitations, greater climate variability, extreme weather events may favor the emergence of VBPs and of gastropod-borne nematodes in different ways. For instance, temperate areas becoming warmer are more suitable for vectors. This favors the introduction and establishment of new pathogens and vectors in previously free areas [33].

Wild animals serve as a source of infection for invertebrates and populations of domestic animals, especially if conurbation favors bridging infections [2,35,36].

In this scenario, many European regions present a suitable environment for the circulation and spread of diseases transmitted by invertebrates and overlapping factors spur the emergence of canine angiostrongylosis and VBDs [32,34,37–39]. Accordingly, Italy is a major epizootiological hub for angiostrongylosis and VBPs, which are now enzootic throughout the Country in both domestic and wild animals, and dogs may suffer from one or more of these diseases at the same time [25,39–43].

Angiostrongylus vasorum has been found in Italian territories where it was considered unexpected (e.g., Northern regions) and it may occur simultaneously with *D. immitis* even where the monthly use of macrocyclic lactones would be able to prevent both diseases [42–46]. A recent study has proven that canine populations living in different regions of Italy are exposed to the most important VBPs despite the use of preventative treatments [39]. These findings indicate that continuous surveillance on local epizootiological scenarios is necessary for a constant refinement of control programs. Accordingly, the present survey investigated the simultaneous exposure of privately owned dogs living in different regions of Italy to *A. vasorum* and major VBPs.

2. Results

2.1. Angiostrongylus vasorum

Of the 294 dogs examined, 27 dogs (9.2%, 95% Confidence Interval, CI: 6.1–13.1%) were exposed to *A. vasorum*, i.e., Ab or Ag of *A. vasorum* were detected in 22 (7.5%, CI: 4.8–11.1%) and 6 (2%, CI: 0.7–4.4%) dogs, respectively, while 1 dog was positive for both Ab and Ag. Four (1.4%, CI: 0.3–3.4%) and 2 (0.7%, CI: 0.08–2.4%) dogs were positive for *A. vasorum* Ab or Ag only, respectively, while the other 21 were simultaneously positive also for other VBPs (Table 1—see Section 2.2.). Seventeen (5.8%; CI: 3.4–9.1%) positive dogs were female, while 10 (3.4%, CI: 1.6–6.1%) were male. Four dogs (1.4%, CI: 0.3–3.4%) positive to *A. vasorum* Ab lived mostly indoors, while all the other 23 dogs (7.8%. CI: 5–11.5%) lived permanently outdoors. The median age of dogs that seroreacted for *A. vasorum* was 36 months.

Table 1. Results of serological examinations (SNAP 4DX rapid test; Immunofluorescence Antibody Test, IFAT; ELISA): number/total (n/tot) and percentage (%) of dogs positive for different pathogens in Italy [§].

	SNAP 4DX				IFAT			ELISA *Av*					
Site	***An*** **n/tot (%)**	***Eh*** **n/tot (%)**	***Bb*** **n/tot (%)**	***Di*** **n/tot (%)**	***Li*** **n/tot (%)**	***Rc*** **n/tot (%)**	***Bc*** **n/tot (%)**	**Mixed VBP [a] n/tot (%)**	**Total VBP [b] n/tot (%)**	**Ag n/tot (%)**	**Ab n/tot (%)**	**Total *Av* [d] n/tot (%)**	**Mixed VBP + *Av* [c] n/tot (%)**
A	8/67 (11.9) -	- -	4/67 (6)	2/6 (3)	1/6 (1.6)	41/67 (61.2)	- -	10/67 (14.9)	45/67 (67.2)	- -	5/67 (7.5)	5/67 (7.5)	4/67 (6)
B	9/82 (11) -	- -	- -	1/82 (1.2)	3/82 (3.7)	39/82 (47.6)	18/82 (22)	18/82 (22)	48/82 (58.5)	- -	8/82 (9.8)	8/82 (9.8)	8/82 (9.8)
C	5/145 (3.4) -	- -	- -	2/145 (1.4)	14/145 (9.7)	54/145 (37.2)	7/145 (4.8)	12/145 (8.3)	69/145 (47.6)	6/145 (4.1)	9/145 (6.2)	14/145 (9.7)	9/145 (6.2)
Total	22/294 (7.5)	1/294 (0.3)	4/294 (1.4)	5/294 (1.7)	18/294 (6.1)	136/294 (46.3)	25/294 (8.5)	40/294 (13.6)	162/294 (55.1)	6/294 (2)	22/294 (7.5)	27/294 (9.2)	21/294 (7.1)

An: *Anaplasma* spp.; *Eh*: *Ehrlichia* spp.; *Bb*: *Borrelia burgdorferi*; *Di*: *Dirofilaria immitis*; *Li*: *Leishmania infantum*; *Rc*: *Rickettsia conorii*; *Bc*: *Babesia canis*; *Av*: *Angiostrongylus vasorum*; *Ag*: Antigen; Ab: Antibodies; [a] Dogs that tested positive for two or more pathogens investigated in this study; [b] Dogs positive for at least one VBP; [c] Dogs positive to at least one VBP and to *Angiostrongylus vasorum* (*Ag* or *Ab*) simultaneously; [d] Dogs positive for *Angiostrongylus vasorum* antigens or antibodies; [§] Sites of North-Eastern (i.e., Site A: Veneto and Friuli-Venezia Giulia), Central-Western (i.e., Site B: Giglio Island, Tuscany and Latium) and Central-Eastern (i.e., Site C: Umbria, Abruzzo, Marche) macroareas of Italy.

2.2. Vector-Borne Pathogens

Regardless of the positivity to *A. vasorum*, 162 (55.1%, CI: 49.2–60.8%) dogs were seropositive for at least one VBP, while 40 (13.6%, CI: 9.9–18.1%) scored positive to two or more VBPs (Table 1). Ab vs. *Rickettsia conorii* were found in 136 dogs (46.3%, CI: 40.5–52.1%), while 25 (8.5%, CI: 5.6-12.3%) and 18 (6.1%, CI: 3.7–9.5%) dogs seroreacted for *Babesia canis* and *L. infantum,* respectively. Twenty-two (7.5%, CI: 4.8–11.1%) dogs were seropositive for *Anaplasma* spp., while 4 (1.4%, CI: 0.3–3.4%) and 1 (0.3%, CI: 0.01–1.88%) dogs had antibodies against *B. burgdorferi* and *Ehrlichia* spp. *D. immitis* antigens were detected in 5 (1.7%, CI: 0.6–3.9%) dogs. Positivity rates for VBPs for each macro-area are detailed in Table 1. Of the dogs positive to at least one VBP, 86 (29.3%, CI: 24.1–34.8%) dogs were female, while 81 (27.6%, CI: 22.5–33.0%) were male: 117 (39.8%, CI: 34.2–45.6%) lived outdoors, while 50 (17.0%, CI: 12.9–21.8%) lived indoors. The median age of dogs that tested positive for one or more VBPs was 54 months.

2.3. Simultaneous Exposure

Twenty-one (7.1%, CI: 4.5–10.7%) dogs tested simultaneously positive to both *A. vasorum* (Ag or Ab) and at least one VBP. Of them, 17 (5.8%, CI: 3.4–9.1%) were positive for *A. vasorum* Ab + at least one VBP, 3 (1%, CI: 0.2–3%) were positive for *A. vasorum* Ag + at least one VBP, while 1 dog (0.3%, CI: 0.01–1.88%) was positive for both *A. vasorum* Ag and Ab, and for *B. canis* Ab.

Detailed information on the different combinations of seropositivity to the tested pathogens are reported in Tables 1 and 2, respectively.

Table 2. Number (n) and percentage (%) of dogs seropositive for *Angiostrongylus vasorum* antigens (Ag) and/or antibodies (Ab) and combined exposure to VBPs in the present study.

Simultaneous Exposure	n (%)
Angiostrongylus vasorum Ab + *Rickettsia conorii*	9 (3.0)
Angiostrongylus vasorum Ab + *Leishmania infantum*	1 (0.3)
Angiostrongylus vasorum Ab + *Anaplasma* spp. + *Rickettsia conorii*	3 (1.0)
Angiostrongylus vasorum Ab + *Rickettsia conorii* + *Babesia canis*	2 (0.7)
Angiostrongylus vasorum Ab + *Anaplasma* spp. + *Borrelia burgdorferi*	1 (0.3)
Angiostrongylus vasorum Ab + *Leishmania infantum* + *Rickettsia conorii* + *Babesia canis*	1 (0.3)
Angiostrongylus vasorum Ag + *Babesia canis*	2 (0.7)
Angiostrongylus vasorum Ag + *Rickettsia conorii*	1 (0.3)
Angiostrongylus vasorum Ab + Ag + *Babesia canis*	1 (0.3)

3. Discussion

The present results show that dogs living in Italy can be simultaneously exposed to *A. vasorum* and several VBPs, with different positivity rates according to the investigated geographic areas.

Exposure to *A. vasorum* was herein found in all the studied macro-areas. Thus far, this metastrongyloid has been rarely recorded outside Central and Southern Italy, in particular seldom in Northern-Western territories [41]. Nevertheless, a relatively high proportion of dogs of the present study tested seropositive in Site A. This and other recent findings [42] indicate that *A. vasorum* has currently expanded its distribution range and confirm that also dogs living in North-Eastern regions of Italy are at risk of canine angiostrongylosis. A past study investigated the occurrence of *A. vasorum* in dogs from a small region (Liguria) of North-Western Italy, showing ~1 and ~3% positivity to Ag and AB, respectively [47]. The present data indicate that *A. vasorum* can occur in Northern Italy more frequently than previously recorded, although the nematode remains more distributed in Central Italy rather than in the North of the country [41]. This difference is likely due to the chemoprevention for *D. immitis* using macrocyclic lactones (i.e., milbemycin oxime and moxidectin) that are active also against *A. vasorum*, routinely performed in Northern Italy [44]. No positive dogs were detected in Giglio Island of Site B, most probably attributable to the absence of foxes, the natural reservoirs of *A. vasorum*, that are not present in minor Italian islands [48]. However, a future introduction of the parasite in such spots, e.g., via dogs travelling with their owners in touristic areas, is not unlikely, as metastrongyloids can perpetuate their lifecycle in absence of their natural wild reservoirs [49].

As in previous studies, the number of dogs with detectable *A. vasorum* Ab was here higher if compared to the number of dogs showing circulating Ag [8,47,50]. Many reasons can account for this discrepancy, e.g., the inclusion of dogs (i) sampled during prepatency when Ag is not detectable yet, (ii) after a natural clearance of the infection (iii) after antiparasitic treatment, based on longer persistence of Ab [51,52]. Moreover, individual variations in the production of Ab against *A. vasorum* (i.e., the production of antibodies may vary individually and some dogs can even remain antibody negative) may further reduce the number of detected positive dogs [53,54].

Dirofilaria immitis was here found in all study sites and most of the positive dogs lived in central regions, which are now regarded as stably enzootic. Seropositivity to *L. infantum* was detected in dogs living on the island of Giglio and in Umbria/Abruzzo/Marche, but not in Friuli Venezia Giulia region (i.e., Site B, the easternmost study site). The lack of positive dogs may not reflect a true absence in this latter region, as competent vectors are already established in this area [55]. The presented results and recent analysis [56] ultimately prove that, to date, all Italian regions should be considered potentially enzootic for *D. immitis* and *L. infantum* and corroborate the changing epizootiological scenario of dog dirofilariosis in Italy, following an expansion from Northern areas southwards [56,57]. Hence, worthy of note is the establishment of hyperendemic foci of dirofilariosis in the extreme South of Italy, and vice versa for leishmaniosis [58–61].

The seropositivity of the here studied dog populations to tick borne pathogens fits with past and most recent surveys carried out in Italy and confirm that dogs are exposed to a multitude of pathogens throughout the country [17,39,62,63] (Table 3). The level of exposure to given pathogens in each geographic area is in accordance with the biology of their vectors. For instance, *R. conorii* and *Anaplasma* spp. are transmitted by ticks (e.g., *Rhipicephalus sanguineus* and *Ixodes ricinus*) which are widespread and active throughout the year [64–66]. Conversely, the main vector of *B. canis* (*Dermacentor* spp.) is little distributed in northern territories and limited to a short season [65,67]. On the other hand, the positivity values in central and southern areas could be due to possible cross-reactions with other *Babesia* species, e.g., *Babesia vogeli* or others, transmitted by widely spread ticks like *R. sanguineus* [63,68,69]. Analogously, the occurrence of *B. burgdorferi* in northern Italy is explained by the wide distribution of its major vector *I. ricinus* in northern areas, with forested environments and suitable climate [65].

Table 3. Positivity (%) of *Angiostrongylus vasorum* and Vector-Borne Pathogens in dogs of different European countries between 2006 and 2021, detected using different diagnostic techniques. *Av*: *Angiostrongylus vasorum* (Baermann test, serological detection of antigens (Ag) or antibodies (Ab)). *Li*: *Leishmania infantum* (serological detection of antibodies, PCR); *Di*: *Dirofilaria immitis* (serological detection of antigens, Knott's test, PCR); *Eh*: *Ehrlichia* spp. (serological detection of antibodies, PCR); *Anaplasma* spp. (serological detection of antibodies, PCR); *Bo*: *Borrelia* spp. (serological detection of antibodies, PCR); *Rc*: *Rickettsia conorii* (serological detection of antibodies, PCR); *Ba*: *Babesia canis* (serological detection of antibodies, PCR). Only countries for which information on both *A. vasorum* and VBPs is available are shown in the table.

Country	*Angiostrongylus vasorum*			*Leishmania infantum*	*Dirofilaria immitis*	*Ehrlichia* spp.	*Anaplasma* spp.	*Borrelia burgdorferi*	*Rickettsia conorii*	*Babesia canis*	References
	Baermann	*Ag*	*Ab*								
Bulgaria		0–0.6	0	0	10.5–16.2	1.34–21	13.4–46.1	0.7–2.4		16.2	[70–72]
Czech Republic	0.4	4.8	3.6				3.4 *	6.5–10.3			[73–75]
Denmark	1.1–3.5										[76]
Finland		CR				0.3	5.3	2.9			[77,78]
France		1.8	3.5		0.2	0.3	2.7	1.1			[79,80]
Germany	0.4–7.4	0.5	2.2	5–23.5	1–1.4	0.1–15.1	1.5–43.2	4.5	0.8	0.1–11.5	[51,81–87]
Greece	1.1	1.6–2.0	1.4–3.0	6.5–25.2		1.5–12.5	1–6.2	0–0.1	11 *–46.5	0.5	[18,88–92]
Hungary		3.1	4.09	0–2.6 *	1.3 *–2.6	0.2	1.3 *–15.4	0.4		11.5–50 *	[93–95]
Ireland	0.5										[96]
Italy	0.4–12.6	0.8–0.9	2.3–3.8	2.5–54.1	8.9	0.4–46	0.5 *–38	0.3–1.7	40.5–72	10.3–70	[39–41,47,63,97–106]
Poland		1.3	1.79			0 *–1.5	8–12.3	3.8–11			[107–109]
Portugal	2.0	0–2.6	1.9–5	18.2–60.4 *	9.4				0.5 *–38.5		[6,37,38,110–114]
Serbia	CR			10.6		0–0.9	0–28.8	0 *–1.8		2.7 *–52.3	[115–117]
Slovakia	CR	3.1	4.4				11.7	2.8			[50,118–120]
Spain		0.8		5.3–30	0–6.25	0.9–16.7	1.3–19	0–6.3	42–56.4		[121–131]

* PCR; CR: Case Report.

Worthy of note is the positivity to *R. conorii*, as dogs are reliable epidemiological sentinels for monitoring potential risk of human exposure to spotted fever group rickettsioses. Regardless of the potential of cross-reactions with other *Rickettsia* species, *R. conorii* is the most frequent affecting dogs in the Mediterranean basin, and the present data indicate a high exposure to infected tick arthropods, which act as vectors of these pathogens [63].

Knowledge on the simultaneous risk for angiostrongylosis and VBDs in dog populations of Europe is very poor, as studies have thus far been conducted only in a few countries, e.g., Portugal [37] and Bulgaria [70,71]. In the latter studies, cases of co-exposure /co-infection by *A. vasorum* and VBPs have not been found [37]. In Italy, a similar study was carried out a few years ago in kenneled dogs of central Italy, though limited to *L. infantum*, *A. vasorum* and *Dirofilaria* spp. (and other endoparasites) [106]. Unfortunately, a comparison with the number of dogs co-infected by VBPs and *A. vasorum* is not possible, as this information is not shown in ref. [106]. Further differences in the diagnostic methods and/or protocols, e.g., the use of Baermann test for *A. vasorum* vs. antigen detection by ELISA or rapid assay [52], of higher screening dilution for *L. infantum* and of a different commercial kit for *D. immitis* antigen detection, impair any comparison with the present results.

This study has confirmed once again the applicability of serological methods in large-numbers epizootiological studies and investigations on pet metastrongyloid angiostrongylids [47,50,51,132–135]. The usefulness of serology should be considered in planning future studies relying on standardized methods towards comparable results from different regions and settings.

Many European countries are enzootic for *A. vasorum* and VBPs (Table 3). Hence, the simultaneous exposure to these pathogens is a realistic threat even where this has not been verified by purposed studies, especially if one considers that these pathogens are spreading in both enzootic and previously free regions [35,36,136,137].

As the scenario of the distribution of canine parasites and VBDs is in continuous evolution, further large-scale studies are warranted for more comprehensive knowledge on the epizootiological risks in European territories, and to understand if and how the routine use of broad-spectrum endo-ectoparasiticides needs a refinement based on local settings. The use of ectoparasiticides and/or repellents is the most effective strategy to minimize the risk of diseases transmitted by arthropod vectors, while dog angiostrongylosis and dirofilariosis can be prevented by the monthly administration of macrocyclic lactones like milbemycin oxime and moxidectin [138–141]. Although cases of LOE/ML-resistant strain of heartworm are known to occur in North America, this is not the case in Europe [142,143]. Several broad-spectrum formulations are available on the market to protect dogs and those containing an isoxazoline and either or milbemycin oxime or moxidectin may be used for the prevention of ticks, *D. immitis* and *A. vasorum* infections. Although isoxazolines do not have a repellent activity, their fast onset of action has the potential to reduce the risk of disease transmission of tick-borne pathogens that are not immediately transmitted to the host, such as *Babesia* or *Borrelia* [144]. Repellents (e.g., synthetic pyrethroids) have a fast onset of action on many insects and ticks, and they are also irritant or repulsive for many ectoparasites [144,145]. Their use is thus particularly important for the prevention of *L. infantum* [146,147] and of pathogens that are passed within a few hours or immediately after the tick's bite [148]. In fact, in recent years, the use of systemic isoxazolines to reduce the transmission of leishmaniosis or human VBDs has been evaluated [149–151]. Further studies in this field are still necessary and, to date, the combination of repellency and parasiticidal activity is still the best approach to prevent pathogen transmission by arthropods. Therefore, an adequate preventative measure should be put in place on a case-by-case basis considering the lifestyle of the dog, the geographical distribution of vectors and of the related transmitted diseases.

In conclusion, canine populations of Italy and in parts of other European countries are at factual risk of angiostrongylosis and VBDs at the same time. A standardization of the diagnostic techniques used in future epizootiological surveys is here advocated, along with

the establishment of international monitoring tools with real-time mapping of positive animals in determined areas, e.g., as available in the USA via the CAPC (capcvet.org).

4. Materials and Methods

Overall, 294 privately owned dogs from North-Eastern (Friuli Venezia-Giulia and Veneto = site A), Central-Western (Giglio Island of Tuscany, Latium = Site B) and Central-Eastern (Umbria, Abruzzo, Marche = Site C) Italy were enrolled in the study, i.e., 67, 82 and 145 in each site, respectively. All the dogs were apparently healthy at the clinical examination. Overall, 150 dogs were female, 144 were male and the median age was 48 months. Of the 294 dogs included, 187 lived permanently outdoors, while 107 lived mostly indoors.

Dogs were enrolled during routine medical checks by local veterinarians and all dog owners signed a written consent form before sampling. All sera samples were subjected to two ELISAs detecting *A. vasorum* circulating antigen (Ag) (Sensitivity 95.7%, Specificity 94.0%) and specific antibodies (Ab) (Sensitivity 81.0%, Specificity 98.8%) against the parasite [54,152]. The optical density (OD) threshold was determined for both Ag (A_{405} nm = 0.153) and Ab test (A_{405} nm = 0.266) using the mean OD value plus three standard deviations of 291 (Ag) and 244 (Ab) samples. All dogs were tested also for VBPs using (i) SNAP®4DX (IDEXX Laboratories, Inc., Westbrook, ME, USA) detecting *D. immitis* Ag (Sensitivity 99%, Specificity 99.3%) and Ab vs. *B. burgdorferi* (Sensitivity 94.1%, Specificity 96.2%), *Anaplasma* spp. (Sensitivity 90.3%, Specificity 94.3%) and *Ehrlichia* spp. (Sensitivity 97.1%, Specificity 95.3%) and (ii) IFAT for Ab vs. *L. infantum* (MegaFLUO Leish-Megacor Diagnostik GmbH) (Sensitivity 96.9%, Specificity 98.7%), *B. canis* (MegaFLUO BABESIA *canis*-Megacor Diagnostik GmbH) and *R. conorii* (MegaFLUO RICKETTSIA *conorii*-Megacor Diagnostik GmbH) using screening dilutions of 1:100, 1:160 and 1:64, respectively.

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