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ORIGINAL PAPER

Seroprevalence and risk factors associated with *Ehrlichia canis*, *Anaplasma* spp., *Borrelia burgdorferi* sensu lato, and *D. immitis* in hunting dogs from southern Italy

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Abstract Canine vector-borne diseases (CVBDs) are caused by a range of pathogens transmitted to dogs by arthropods. The present study investigates *Ehrlichia canis*, *Anaplasma* spp., *Borrelia burgdorferi* sensu lato, and *Dirofilaria immitis* seroprevalences in hunting dogs from southern Italy. Dogs (no. 1335) were tested using a commercial in-clinic enzyme-linked immunosorbent assay kit. Odds ratios (ORs) were calculated by logistic regression analysis to identify risk factors. Overall, 138/1335 dogs (10.3%) were seroreactive to at least one CVBD pathogen. *E. canis*, *Anaplasma* spp., *B. burgdorferi* s.l., and *D. immitis* seroprevalences were 7.6, 4.4, 0.3, and 0.2%, respectively. *E. canis* and *Anaplasma* spp. co-exposures were found in 30 dogs (2.2%), compared with *Anaplasma* spp. and *B. burgdorferi* s.l. co-exposures in 2 dogs (0.1%). Adult age was a risk factor for *E. canis* (OR 2.35) seroreactivity whereas hunting fur-bearing animals for *E. canis* (OR 4.75) and *Anaplasma* spp. (OR 1.87), respectively. The historical presence of tick infestation was identified as a risk factor for positivity to *E. canis* (OR 2.08) and *Anaplasma* spp. (OR 2.15). Finally, larger dog pack size was significantly associated with *E. canis* (OR 1.85) and *Anaplasma* spp. (OR 2.42) exposures. The results of the present survey indicated that hunting dog populations are at relative risk of CVBDs in southern Italy. Further studies are needed to evaluate the role of hunting dogs in the epidemiology of vector-borne organisms due to sharing common environments with wild, sympatric animal populations.

Keywords *Ehrlichia canis* · *Anaplasma* spp. · *Borrelia burgdorferi* · *Dirofilaria immitis* · Hunting dogs · Italy

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Introduction

Canine vector-borne diseases (CVBDs) are caused by a range of pathogens transmitted to dogs by arthropods including ticks and insects, many of which pose a zoonotic risk for human infection, with dogs potentially serving as reservoirs.

Canine monocityc ehrlichiosis (CME) caused by *Ehrlichia canis*, a Gram-negative obligate intracellular bacterium with a tropism for mononuclear leukocytes, is a widespread tick-borne infection, transmitted by *Rhipicephalus sanguineus*, the most common tick species found in the Mediterranean basin (Sainz et al. 2015). Human infection with *E. canis* has been reported in Latin America (Venezuela and Costa Rica) (Perez et al. 2006; Bouza-Mora et al. 2017). In dogs, ehrlichiosis can vary in severity from minimally symptomatic to fatal illness in chronic stages (Cardoso et al. 2012). Clinical presentation in dogs is typically characterized by fever, depression, myalgia, anorexia, lymphadenomegaly, anemia, and thrombocytopenia (Mircean et al. 2012).

Anaplasma phagocythophilum (formerly known as *E. phagocytophila* or *E. equi*) is an obligate intracellular Gram-negative bacterium that has tropism for neutrophilic granulocytes and is recognized as the causative agent of granulocytic anaplasmosis in dogs, cats, horses, sheep, and

humans. In Europe, *Ixodes ricinus* is the only known vector for *A. phagocythophilum* (Sainz et al. 2015). Although there is an overlap of the clinical features with *E. canis* infection, many *A. phagocytophilum*-infected dogs have a subclinical and self-limiting disease, as suggested by the high number of healthy seropositive dogs in *I. ricinus* endemic regions (Kohn et al. 2011).

A. platys (formerly known as *E. platys*), the cause of canine infectious cyclic thrombocytopenia (CICT), is an obligate intracellular Gram-negative bacterium that infects platelets and megakaryocytes (Latrofa et al. 2016). Clinical signs associated with CICT include lethargy, fever, anorexia, and bleeding disorders. *A. platys* may be transmitted by *R. sanguineus*, but vector competence has not conclusively demonstrated for this tick species (Ramos et al. 2014). In dogs, *A. platys* is found in co-infection with other vector-borne agents. Human infection with *A. platys* has been reported from the USA and Venezuela (Arraga-Alvarado et al. 2014; Breitschwerdt et al. 2014).

Borrelia burgdorferi sensu lato spirochetes infect a wide range of mammals, including dogs and humans. *I. ricinus* is the most important vector in Europe (Hovius 2013). Borreliosis, commonly referred as Lyme disease, is the most common tick-borne human infection in Europe and North America (Little et al. 2010, Rizzoli et al. 2011). In dogs, *B. burgdorferi* most often causes mild and non-specific clinical signs, such as fever, anorexia, lethargy, and lymphadenomegaly (Hovius 2013). More severe clinical manifestations, such as arthritis with lameness, neurologic disorders, and glomerulonephritis occur occasionally in dogs, although the specific role of *B. burgdorferi* in the development of renal failure remains unclear (Greene et al. 2012).

In Italy, *E. canis, A. phagocytophilum*, and *A. platys* infections have been reported in dogs in previous surveys (Antognoni et al. 2014; Pennisi et al. 2012; de Caprariis et al. 2011; Otranto et al. 2010; Trotta et al. 2009; Ebani et al. 2008; Torina et al. 2008; Torina et al. 2007; Corrain et al. 2007; de la Fuente et al. 2006; Solano-Gallego et al. 2006; Torina and Caracappa 2006). Due to use of different CVBD diagnostic techniques, varying geographical regions studied, and different selection criteria for inclusion of dogs in various studies, it is difficult to compare historical prevalence data generated throughout Italy. In a recent survey, *E. canis* was detected also in wild canids, as red foxes (*Vulpes vulpes*) and gray wolves (*Canis lupus*) (Santoro et al. 2016). Dogs in Italy are infrequently exposed to *B. burgdorferi* (Giudice et al. 2003; Mannelli et al. 1999).

Dirofilaria immitis, the cause of canine heartworm disease (CHD), is a filarial nematode that lives as adult in the right ventricle of the heart, extending into the pulmonary arteries. CHD is associated with exercise intolerance, dyspnoea, cough, and right-sided congestive heart failure (McCall et al. 2008). In Italy, mosquitoes of genera *Culex*, *Aedes*, and *Anopheles* transmit *D. immitis* (Otranto and Dantas -Torres 2010). Although there is a risk of zoonotic transmission, human heartworm infection is uncommon. In Europe, CHD occurs in Mediterranean basin countries, with the largest endemic area located along the Po River Valley in northern Italy (Otranto et al. 2013). Recent epidemiological data suggests a geographic expansion for *D. immitis* transmission throughout southern Italy and the surrounding islands (Del Prete et al. 2015; Pipia et al. 2014; Otranto et al. 2009).

Due to closer contact with wooded and rural areas, cohabitation in outdoor kennels and potentially less consistent use of acaricide products, hunting dogs are more likely to be exposed to CVBDs compared to other dog populations (e.g., household dogs) (Kordick et al. 1999; Solano-Gallego et al. 2006); however, few comparative studies from the same region have been published (Ebani et al. 2014). In Italy, serological and molecular data for *E. canis* and *A. phagocythophilum* were reported in a small number of hunting dogs from Central Italy by Ebani et al. (2013, 2015). The aim of the present study was to determine the CVBD seroprevalences in a large number of hunting dogs from southern Italy and to assess exposure risk factors.

Materials and methods

Study area

Avellino (40°54′55″N–14°47′22″E) and Salerno (40°41′00″N–14°47′00″E) provinces belong to the Campania region in southern Italy. The territory of the two provinces is contiguous and that of Salerno overlooks the Tyrrhenian Sea. It has a typical Mediterranean temperate climate along the coast that becomes progressively continental in the inland and mountainous areas. The study area has surface of 4527.81 square km, including the hunting district—Ambito Territoriale Caccia—of Avellino (ATC AV) and one of the two hunting districts of Salerno (ATC SA 1).

Study animals and sample size

This study included 1335 hunting dogs from 114 municipalities. The study was conducted as a component of the hunting dog's health assistance program of University of Naples Federico II, which was supported by the Italian management committees of the respective hunting districts (ATCs).

Blood samples were collected in 37 private veterinary hospitals located in the study area between March and October 2015. Animal sampling was performed by different veterinary operators (DP, BN, MS, LP, VV) during a routine health check.

The study was approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II (number of approval 0039904; date of approval 20 October 2014),

and a written consent was obtained from the owners of the hunting dogs.

After an overnight fast, 5 ml of blood withdrawn from the cephalic vein was collected into K3-EDTA anticoagulant tubes and immediately tested using in-clinic CVBD ELISA assay defined below.

The sample size was calculated using the formula proposed by Thrustfield (1995) for a theoretically "infinite" population inserting the following data: expected prevalence of 2.1% for *E. canis* based on the results of a similar large-scale study in canine population from Romania using the same in-clinic enzyme-linked immunosorbent assay (ELISA) (Mircean et al. 2012); confidence interval (99%) and desired absolute precision (1%).

A questionnaire was submitted to each owner to obtain information about dog's size (small, medium, large), type of coat (short hair or long hair), age (< 3 years, ≥ 3–8 years, ≥ 8 years), gender, cohabitation with other dogs (pack size), type of housing (indoor or outdoor), contact with other pet or farm animals (dogs, cats, horses, and ruminants), hunted animal species (game birds, wild boars, foxes, and hares), hunting periods in foreign countries, history of tick infestation, and general ectoparasite control practices.

Serological assay

Serological analyses were performed using an in-clinic assay test system (SNAP® 4Dx® Plus, IDEXX Laboratories Inc., Westbrook, ME, USA) based on enzyme immunoassay technique and following the manufacturer's instructions for use. The device consists of a coated membrane matrix with five spots in the reaction area (result window). Three spots are impregnated respectively with peptide antigens from p30 and p30–1 outer membrane immunodominant proteins of *E. canis*, a peptide from the immunodominant major outer surface protein (p44/MSP2) of *A. phagocytophilum* and the synthetic C6 peptide derived from IR6 region within the membrane protein of *B. burgdorferi*. Regarding the European *Borrelia* species, Krupka et al. (2009) in mice experimentally infected by *B. burgdorferi* and *B. garinii* have detected antibodies against C6 peptide. *D. immitis* analyte is derived from polyclonal antibodies specific for a carbohydrate antigen of the adult female nematodes. The fifth spot serves as a positive control. According to Stillman et al. (2014), sensitivity and specificity of the in-clinic ELISA were for detection of antibodies against *A. phagocytophilum* (93.2 and 99.2%, respectively), *A. platys* (89.2 and 99.2%, respectively), *B. burgdorferi* (96.7 and 98.8%, respectively), *E. canis* (97.8 and 92.3%, respectively), and *E. ewingii* (96.5 and 93.9%, respectively). Sensitivity of the assay for detection of *D. immitis* was 98.9%, with 99.3% specificity.

The SNAP® 4Dx® Plus test uses a peptide from a major outer surface protein (p28) of *E. ewingii* on the *Ehrlichia* portion of the test. In addition to the SNAP® 4Dx® Plus, a Knott's test was performed on *D. immitis* seropositive dogs. Furthermore, the seropositive samples were qualitatively tested for antibodies to *Leishmania infantum* using the SNAP® *Leishmania* in order to rule out a possible coinfection with this protozoan. The locations of seropositive dogs were mapped using a Geographical Information System (ARC GIS 10.1, ESRI Corporation, USA).

Clinical examination and complete blood cell count

For each SNAP® 4Dx® Plus seropositive dog, a complete clinical examination was performed by participating veterinarians (DP, BN, NDA, FDP, VV). A complete blood cell count was done using a semi-automatic cell counter (HM5, Abaxis, USA). The body condition score (BCS) was assessed using a nine-point system (Laflamme 1997).

Statistical analysis

The analysis was performed by coauthor LA with dedicated software (Prism® 6.0, GraphPad Software Inc., La Jolla, CA, USA). Data are reported as absolute number, percentage of the total and the relative 95% confidence interval (95% CI). A two-tailed chi-square test was applied to evaluate the effect of each risk factor, and the odds ratio (OR) and the relative 95% CI were calculated for the significant variable (Petrie and Watson 2013). Contingency tables were used to identify whether the distribution of clinical signs or hematological abnormalities varied among the different CVBDs or among dogs that were exposed to more than one CVBD. Significance was set at $P < 0.05$.

Results

Overall, the average age of this hunting dog population was 3.6 ± 2.4 years (ranging from 3 months to 14 years). There were 749 males and 586 females, including the following breeds: 493 Italian Segugio, 393 English Setter, 107 Epagneul Breton, 61 mixed-breeds, 46 Pointer, 43 Beagle, 33 Ariegeois, 32 Griffon Bleu de Gascogne, 21 Italian Bracco, 20 Kurzhaar, and 86 of other 14 hunting breeds. All of dogs lived in rural environments and the pack size varied from 2 to more than 13 dogs. The majority of dogs (1203/1335; 90.1%) were in contact with pets or farm animals. Regarding management practices, all the owners reported administration of ectoparasiticides to their dogs.

Overall, 138/1335 dogs (10.3%; CI 8.7–12.1%) were seropositive to one CVBD pathogen.

The seroprevalences for the pathogens were *E. canis* 7.6% (102/1335; CI 6.3–9.2%), *Anaplasma* spp. 4.4% (59/1335; CI 3.4–5.7%), *B. burgdorferi* s.l. 0.3% (4/1335; CI 0.1–0.8%), and *D. immitis* 0.2% (3/1335; CI 0.05–0.6%). Co-infection

with *E. canis* and *Anaplasma* spp. was found in 30 dogs (2.2%; CI 1.5–3.2%), while co-infection with *Anaplasma* spp. and *B. burgdorferi* s.l. in only 2 animals (0.1%; CI 0.02–0.5%). The distribution of the ELISA positive dogs for the pathogens in the study area is shown in Fig. 1.

L. infantum antibodies were detected in 7 (5.1%; CI 2.5–11.1%) out of 138 CVBD seropositive dogs. Three *Leishmania* seropositive dogs had antibodies against *E. canis*, two against *Anaplasma* spp., and two against both pathogens.

Seroprevalence analyses in relation to the detailed characteristics of the hunting dog population hypothesized as potential risk factors associated with the exposure to any of the different CVBD pathogens are summarized in Table 1. Due to the very low prevalence in the study population, *B. burgdorferi* s.l. and *D. immitis* positivities were not included in the risk factor statistical analysis.

Adult age was a risk factor for *E. canis* exposure. Hunting fur-bearing animals (boars, hares, foxes), large dog pack size (> 10 animals), and a history of tick infestation(s) were significantly associated with *E. canis* and *Anaplasma* spp. exposures. Table 2 shows the significant risk factors for *E. canis* and *Anaplasma* spp., while *B. burgdorferi* s.l. and *D. immitis* risk factors were not calculated.

A clinical examination was performed on 124/138 seropositive dogs (89.9%). The clinical features observed in dogs infected by *E. canis*, *Anaplasma* spp., and *B. burgdorferi* s.l. are summarized in Fig. 2. Lymphadenomegaly (41.1%; CI 38.5–55.8%), elevated rectal temperature (45.6%; CI 37.1–54.3%), and splenomegaly (42.0%; CI 33.7–50.7%), as determined by abdominal palpation, represented the most frequent clinical findings among these hunting dogs. Clinical abnormalities were not found in three dogs that were *D. immitis* antigen positive, all of which were confirmed by identification of circulating microfilariae using Knott's test (concentration ranged from 44 to 60 microfilariae/ml of blood).

Statistically, there were no differences in the frequency of clinical findings among dogs seroreactive to *E. canis*, *Anaplasma* spp., or *B. burgdorferi* s.l.; to *E. canis*, *Anaplasma* spp., and their respective co-infections; and between *Anaplasma* spp., *B. burgdorferi* s.l., and their respective co-infections.

Complete blood cell count (CBC) results for 120/138 (86.9%) CVBD seroreactors are depicted in Fig. 3. A normal CBC was detected in 61.8% (CI 49.2–73.3%) of dogs with *E. canis*, in 46.1% (CI 26.6–66.6%) with *Anaplasma* spp., in 50.0% (CI 1.3–98.7%) with *B. burgdorferi* s.l., in 36.0% (CI 18.0–57.5%) co-infected by *E. canis* and *Anaplasma* spp., and in all dogs with *D. immitis* and co-infected by *Anaplasma* spp. and *B. burgdorferi* s.l..

Thrombocytopenia was found in 38.2% (CI 26.7–50.8%) of dogs with *E. canis*, in 34.6% (CI 17.2–55.7%) with *Anaplasma* spp., in none of the dogs with *B. burgdorferi* s.l. or with

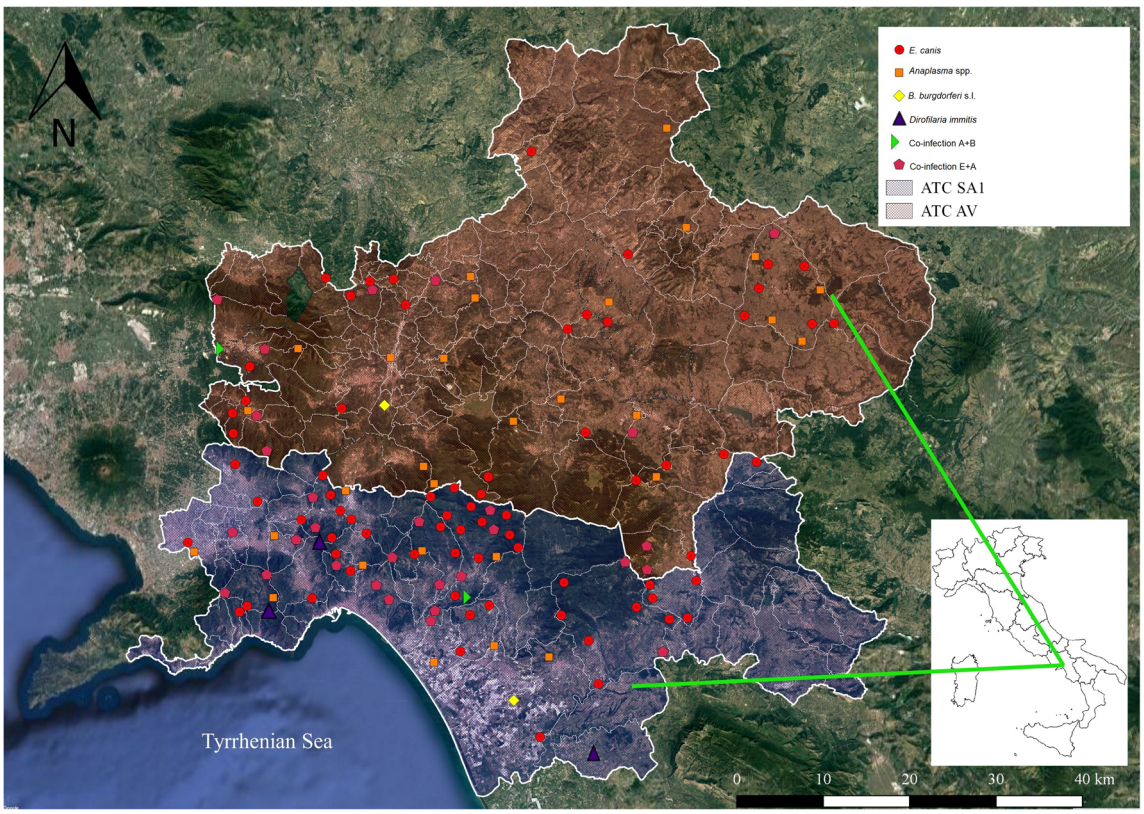

Fig. 1 Map showing the distribution of the hunting dogs seropositive for the pathogen agents in the study area

Table 1 Seroprevalence (%) and confidence interval (95%) of *E. canis*, *Anaplasma* spp., *B burgdorferi* s.l and *D. immitis* in hunting dogs in southern Italy

Factor	No. of dogs tested	*Ehrlichia canis*			*Anaplasma* spp.			*Borrelia burgdorferi* s.l.			*Dirofilaria immitis*		
		Positive	%	CI 95%	Positive	%	CI 95%	Positive	%	CI 95%	Positive	%	CI 95%
Size													
Small	29	3	10.3	2.2–27.3	2	6.9	0.9–22.8	0	0.0	0.0–11.9	0	0.0	0.0–11.9
Medium	1008	75	7.4	5.9–9.2	43	4.3	3.1–5.7	4	0.4	0.1–1.0	3	0.3	0.1–0.9
Large	298	24	8.0	5.2–11.7	14	4.7	2.6–7.7	0	0.0	0.0–1.2	0	0.0	0.0–1.2
Coat													
Short haired	584	50	8.6	6.4–11.1	32	5.5	3.8–7.6	0	0.0	0.0–0.6	0	0.0	0.0–0.6
Long haired	751	52	6.9	5.2–9.0	27	3.6	2.4–5.2	4	0.5	0.1–1.4	3	0.4	0.1–1.2
Age													
< 3	524	23	4.4	2.8–6.5	17	3.2	1.9–5.1	0	0.0	0.0–0.7	1	0.2	0.0–1.1
≥ 3–8	751	72	*9.6**	7.6–11.9	40	5.3	3.8–7.2	4	0.5	0.1–0.4	2	0.3	0.0–1.0
≥ 8	60	7	*11.7**	4.8–22.6	2	3.3	0.4–11.5	0	0.0	0.0–6.0	0	0.0	0.0–6.0
Gender													
Male	749	59	7.9	6.0–10.0	29	3.9	2.6–5.5	2	0.3	0.0–1.0	1	0.1	0.0–0.7
Female	586	43	7.3	5.4–9.8	30	5.1	3.5–7.2	2	0.3	0.0–1.2	2	0.3	0.0–1.2
Size pack													
≤ 10	1135	78	6.9	5.5–8.5	42	3.7	2.7–5.0	4	0.4	0.1–1.1	1	0.1	0.0–0.5
> 10	200	24	*12.0**	7.8–17.3	17	*8.5**	5.0–13.3	0	0	0.0–0.9	2	1.5	0.2–5.4
Indoor overnight													
Yes	1287	100	7.8	6.4–9.4	59	4.6	3.5–5.9	4	0.3	0.1–0.8	3	0.2	0.0–0.7
No	48	2	4.2	0.5–14.2	0	0.0	0.0–7.4	0	0.0	0.0–7.4	0	0.0	0.0–7.4
Contact with other pet or farm animals													
Yes	1203	94	7.8	6.4–9.5	52	4.2	3.2–5.6	3	0.2	0.0–0.7	1	0.1	0.0–0.5
No	132	8	6.1	2.6–11.6	7	5.3	2.1–10.6	1	0.8	0.0–4.1	2	1.5	0.2–5.4
Hunted animal species													
Game birds	618	17	2.7	1.6–4.4	19	3.1	1.9–4.8	0	0.0	0.0–0.5	0	0.0	0.0–0.5
Fur-bearing animals	717	85	*11.8**	9.6–14.4	40	*5.6**	4.0–7.5	4	0.6	0.2–1.6	3	0.5	0.1–1.4
Hunting in foreign countries													
Yes	43	0	0.0	0.0–8.2	0	0.0	0.0–8.2	0	0.0	0.0–8.2	0	0.0	0.0–8.2
No	1292	102	7.9	6.5–9.5	59	4.6	3.5–5.8	4	0.3	0.1–0.8	3	0.2	0.0–0.7
Tick history infestation													
Yes	780	75	*9.6**	7.6–11.9	44	*5.6**	4.1–7.5	1	0.1	0.0–0.7	1	0.1	0.0–0.7
No	555	27	4.9	3.2–7.0	15	2.7	1.5–4.4	3	0.5	0.1–1.6	2	0.4	0.0–1.3
TOTAL	1335	102	7.6	6.3–9.2	59	4.4	3.4–5.7	4	0.3	0.1–0.8	3	0.2	0.0–0.6

B. burgdorferi s.l. and *D. immitis* positivities were not included in the statistical analysis because of their very low prevalence in the study population

*$P < 0.05$ between groups

B. burgdorferi s.l. and *Anaplasma* spp. co-infection. Anemia was documented in 22.1% (CI 12.9–33.8%) of dogs with *E. canis*, in 26.9% (CI 11.6–47.8%) with *Anaplasma* spp., in 50.0% (CI 1.3–98.7%) with *B. burgdorferi* s.l., and in 32.0% (CI 14.9–53.5%) with *E. canis* and *Anaplasma* spp. co-infection. Leukopenia was found in 5.9% (CI 1.6–14.4%) of dogs with *E. canis*, in 3.8% (CI 0.1–19.6%) with *Anaplasma* spp., and in 12.0% (CI 2.4–31.2%) of dogs co-infected by *E. canis* and *Anaplasma* spp. None of the dogs with single infection due to *B. burgdorferi* s.l. were leukopenic. The contingency tables applied to the distribution of the above-mentioned hematological abnormalities did not highlight any difference among the different types of CVBD infections.

Discussion and conclusion

This study documents that hunting dogs in southern Italy are exposed to five organisms that cause CVBDs. Our results are

Table 2 Significant risk factors associated with *E. canis* and *Anaplasma* spp. seropositivity

Pathogen agent	Variable	O.R.	Lower confidence limit 95%	Upper confidence limit 95%	*P* value (Pearson)
Ehrlichia canis	Age	2.35	1.482	3.872	$P < 0.01$
	Pack size	1.85	1.118	2.957	$P < 0.05$
	Hunted animal species	4.75	2.864	8.365	$P < 0.01$
	Tick history infestation	2.08	1.321	3.276	$P < 0.01$
Anaplasma spp.	Pack size	2.42	1.315	4.265	$P < 0.01$
	Hunted animal species	1.87	1.089	3.340	$P < 0.05$
	Tick history infestation	2.15	1.185	3.908	$P < 0.05$

consistent with a recent study that found an increasing gradient of CME incidence risk from northern towards southern areas, particularly in Italy (Renè-Martellet et al. 2015). In addition to *E. canis*, the *Ehrlichia* peptides in the in-clinic rapid ELISA assay used in this study detects *E. chaffeensis*, *E. ewingii*, and *E. muris* antibodies (Stillman et al. 2014). In a study from North America that used *Ehrlichia* species-specific peptides, *E. ewingii* was the most seroprevalent *Ehrlichia* spp. infecting dogs (Qurollo et al. 2014). To date, tick transmission of *E. chaffeensis*, *E. ewingii*, and *E. muris* has not been reported in European dogs; for this reason, the *Ehrlichia* spp. seropositivity found in this study was attributed exclusively to *E. canis*. The *E. canis* seroprevalence (7.6%) in hunting dogs living in Campania region is similar to the value reported by Ebani et al. (2014) in a dog population living in a rural environment in Central Italy (no. 721; 7.12%). No seroprevalence data are available for comparative purposes on the general dog population of Campania region. In Apulia and Sicily regions of southern Italy, higher *E. canis* seroprevalences were reported in stray dogs co-housed in kennels (from 14.9 to 46.0%) that were infested with large numbers of *R. sanguineus* ticks, and these findings may be explained by irregular and partially effective metaphylactic treatment schemes against ectoparasites (Otranto et al. 2008; Pennisi et al. 2012).

The overall *Anaplasma* spp. seroprevalence in the present study (4.4%) was lower than the *A. phagocytophilum* seroprevalence, determined by an indirect fluorescent antibody test (IFAT) with a low cut-off value (1:40), reported in hunting dogs from central Italy (no. 215; 14.8%) (Ebani et al. 2013). Differences in the serological methods used in these two studies most likely account for the substantial seroprevalence differences among the two dog populations. When testing for exposure to *A. phagocytophilum*, the SNAP® 4Dx® Plus test was calibrated by the manufacturer to be positive at an IFAT titer of approximately 1:100 or greater (O'Connor 2015).

It is important to underline that *A. platys* infection in dogs has been previously described in southern Italy (de Caprariis

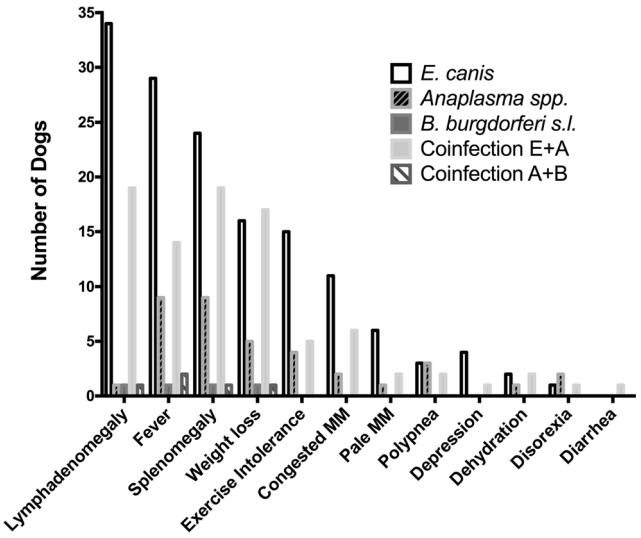

Fig. 2 Clinical features in the seropositive dogs. Co-infection E + A: co-infection with *E. canis* and *Anaplasma* spp.. Co-infection A + B: co-infection with *Anaplasma* spp. and *B. burdorferi* s.l. Congested MM: congested mucous membranes. Pale MM: pale mucous membranes

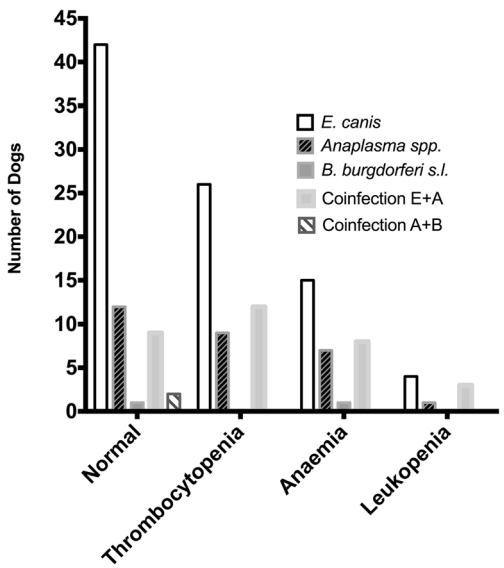

Fig. 3 Hematological findings in the seropositive dogs. Co-infection E + A: co-infection with *E. canis* and *Anaplasma* spp. Co-infection A + B: co-infection with *Anaplasma* spp. and *B. burdorferi* s.l. Reference ranges from Bush (1991): red blood cells (RBCs)—5.5–8.5 × 10^{12}/l; packed cell volume (PCV)—37–55%; hemoglobin (Hb)—12–18 g/dl; white blood cells (WBCs)—6–17 × 10^{9}/l; platelets (PLTs)—200–500 × 10^{9}/l

et al. 2011; de la Fuente et al. 2006; Sparagano et al. 2003). The *Anaplasma* peptide in the SNAP® 4Dx® Plus ELISA platform detects *A. platys* and *A. phagoctyophilum* antibodies. Therefore, we report our results as indicative of exposure to *Anaplasma* spp. in hunting dogs, but specific identification at species level would require organism visualization or PCR amplification of organism-specific DNA sequences.

There are limited *B. burgdorferi* s.l. seroprevalence data for the general dog population in Italy. Ebani et al. (2014) reported a seroprevalence of 1.47% in dogs living in central Italy, and Mannelli et al. (1999) did not find serological evidence of *B. burgdorferi* s.l. exposure in dogs on the Thyrrenian coast of central Italy. Our results also document a low *B. burgdorferi* seroprevalence (0.3%) in the study area, which is consistent with other serosurveys conducted in southern European countries, such as Portugal (0.2%) (Cardoso et al. 2012).

In the seropositive clinically sick dogs, there was an overlap of symptoms and hematological changes for the different investigated CVBDs, either as a single infection or as co-infections. Previously, de Caprariis et al. (2011) in a longitudinal study in young dogs naturally infected by vector-borne pathogens emphasized the clinical challenge associated with assigning a specific clinical sign or hematological abnormality to a particular CVBD. Due to substantial overlap in both clinical and hematological abnormalities, an etiological diagnosis requires species-specific serological assays or molecular assays that confirm the infecting species on the basis of organism-specific DNA sequences.

A substantial number of hunting dogs in this study (32/138; 23.2%) were exposed to more than one CVBD pathogen. Although it has been hypothesized that the presence of two, or more CVBDs, is responsible for alteration, and worsening, of clinical manifestations, typically associated with singular infections, the pathogenic consequences of vector-borne co-infections are minimally documented (De Tommasi et al. 2013). However, veterinarians should keep in mind that CVBDs co-infections may make diagnosis and treatment more difficult, as well as adversely affect the prognosis. Pennisi et al. (2012) in a serological survey in southern Italy (Stretto di Messina) found seropositivity to at least two tick-borne pathogens in 57% of examined dogs, suggesting the possibility that a single tick species may be a vector for multiple pathogens. In our study, two dogs were co-exposed to *Anaplasma* spp. and *B. burgdorferi* s.l., and this is not surprising because both organisms has the same vector (*I. ricinus*), and *A. phagocytophilum* DNA has been detected in ticks from central and northern Italy (Carpi et al. 2009; Veronesi et al. 2011). Dual infection with *A. phagocytophilum* and *B. burgdorferi* s.l. has been reported for *I. ricinus* (8.3% out of 303 examined adults ticks) in northern Poland (Stańczak et al. 2004). In Europe, CME is transmitted by *R. sanguineus* and in our study, 30 dogs were co-exposed to *E. canis* and *Anaplasma* spp.

Otranto et al. (2010) found in dogs living in a shelter in Apulia region of southern Italy a high prevalence of *A. platys* and *Babesia vogeli* coinfections, supporting the suspicion that *R. sanguineus* ticks are likely vectors for both pathogens. *A. platys* DNA has been PCR amplified from *R. sanguineus* ticks in southern Italy, further supporting that this tick species a putative vector of *A. platys* (Ramos et al. 2014). On the basis of our findings, we speculate that hunting dogs in southern Italy may become co-infected with *A. platys* and *E. canis* by *R. sanguineus* tick bites.

Three dogs without a travel history to heartworm endemic areas were positive to *D. immitis*, confirming the appearance of autochthonous foci of this filarial infection in previously non-endemic areas, such as Campania and Apulia (Del Prete et al. 2015; Giangaspero et al. 2013). This is potentially a consequence of the environmental changes that have an impact on the vector's geographical distribution, density, and activity pattern (Genchi et al. 2011). A serological approach for detection of CHD in this specific subpopulation of dogs is very suited, because many hunters in southern Italy had the habit to extra-label use of macrocyclic lactones (mainly ivermectin) on their dogs giving rise to occult infections. Courtney and Zeng (2001) have reported that the sensitivity of rapid assay tests for *D. immitis* is dependent on the number of adult female worms. The infected dogs in our study had a low degree of microfilaremia, and thus it cannot be ruled out that the prevalence may be underestimated in a non-hyperendemic area, such as southern Italy.

In these hunting dogs, adult age was a risk factor for *E. canis* seroreactivity, probably due to a longer duration of exposure to *R. sanguineus*, the vector for this pathogen that induces long-lasting infections in some dogs, in accordance to what was previously observed (Costa et al. 2007). Hunting fur-bearing animals (hares, foxes, boars) was a risk factor for exposure to *E. canis*, *Anaplasma* spp., and *B. burgdorferi* s.l., respectively; these data can be explained by the closer contact with vegetation and wild mammals that are reservoirs for tick-borne pathogen agents, request by this type of hunting (Santoro et al. 2016; Westmoreland et al. 2016). Furthermore, *E. canis* and *Anaplasma* spp. infections were significantly more frequent in hunting dogs with a history of previous tick infestation. Finally, a large dog pack size was significantly associated with *E. canis* and *Anaplasma* spp. exposures, being probably related to the sharing of the arthropod vectors and could be correlated to the monotropic three-host life cycle of *R. sanguineus*.

Overall, the prevalence of the examined CVBDs was relatively low considering the likelihood of frequent environmental exposure to ticks and mosquitoes. In the interpretation of these data, it must be considered that all the hunters treated their dogs with ectoparasiticide molecules, as a result of being informed of the risks of pathogen transmission by ticks and other vectors. The mean number of treatments per year was

6.1 (range 1–12). Ectoparasiticide treatments were performed monthly in 27% of hunting dogs (Veneziano, personal communication). A more accurate assessment of the effect of preventive measures on the development of CVBDs is not obtainable, due to a number of variables regarding the ectoparasite treatment modalities recorded at questionnaire survey (variability of the treatment schemes during the year and the hunting season, empirical dosage calculation, molecules used, association of more molecules, route of administration, extra-label use of macrocyclic lactones) and was beyond the aim of the study design.

To our knowledge, this is the first large-scale serological survey on hunting dogs in southern Italy. Over the last few decades, changes in ecosystems due to urbanization occurred, resulting in thinning of the boundaries between domestic animals and wildlife. For example, some rural landscapes have become periurban areas, providing very attractive food sources for adaptable species (such as the red fox) (Mackenstedt et al. 2015). This new scenario has created a changing dynamic interaction between wildlife, domestic animals, and humans. Understanding these changes will be crucial for epidemiological studies and preventive strategies for CVBDs in hunting and pet dogs in this area. Some specific ecological variants, such as hunting practice, may facilitate parasite circulation from domestic canids to wildlife and vice versa (Otranto et al. 2015). Recently, many studies have investigated the role of wild animals as epidemiological reservoir for many parasites that may infect other susceptible species sharing the same habitats, including humans (Piantedosi et al. 2016; Tolnai et al. 2015; Duscher et al. 2015; Hodžić et al. 2015; Cardoso et al. 2013). Using PCR on tissue samples from red foxes in southern Italy, Santoro et al. (2016) amplified *E. canis* DNA from 55 (52%) out of 105 animals. Epidemiologically, monitoring the circulation of CVBDs in a region by testing serum samples from hunting dogs may represent a useful sentinel population, as well as a compromise between difficulties associated in testing wild animals. In this way, hunting dogs can act as sentinel animals for monitoring wildlife zoonotic infections, as recently demonstrated by Gómez-Morales et al. (2016) for *Trichinella* spp.

In conclusion, the results of the present survey indicate that the hunting dog population in southern Italy is at low risk for contracting CVBDs. Further studies are needed to evaluate the role of hunting dogs in monitoring the epidemiology of zoonotic vector-borne agents, as these dogs are exposed to wild animals and vectors residing in the same environments.

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Compliance with ethical standards The authors declare that the research comply with the current Italian laws.

Conflict interests The authors declare that they have no competing interests.

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