Vector-borne pathogens of zoonotic concern in hunting dogs of southern Italy

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Abstract

Dogs are commonly exposed to vector-borne pathogens (VBPs), yet few data are available on hunting dogs, which are often at high risk of infection due to their involvement in field activities. To investigate the occurrence of VBPs and evaluate the relative performance of different diagnostic tools, blood and serum samples were collected from hunting dogs (n = 1,433) in rural areas of southern Italy. All samples were tested by Knott's technique for filarioids, serologically (SNAP® 4Dx® Plus) for *Anaplasma* spp., *Borrelia burgdorferi* sensu lato, *Dirofilaria immitis* and *Ehrlichia*

spp. and molecularly (qPCR) for all except *B. burgdorferi* of the above pathogens plus *Babesia* spp. and *Leishmania infantum*. Logistic regression was run to evaluate the statistical associations between the risk of VBP infection and independent variables (such as geographic area of provenience, age class and sex) and K-Cohen formula for assessing the concordance among diagnostic tests. Overall, out of 321 dogs (22.4%) positive to at least one VBP, 28 (1.9%) were infected by filarial species at the Knott's technique. In particular, *Acanthocheilonema reconditum* was the most prevalent (1.6%), followed by *D. immitis* (0.2%) and *Dirofilaria repens* (0.1%). One hundred forty (9.8%) and 231 (16.1%) dogs scored positive to VBPs by serological and molecular methods, respectively. The most prevalent pathogens detected were *Ehrlichia* spp. (7.3%) with SNAP® 4Dx® Plus, and *A. reconditum* (7.7%) by qPCR. Statistics revealed a significant association ($p < 0.001$) between *A. reconditum* infestation and both *Ehrlichia* spp. seropositivity and geographical origin of dogs. An agreement of 99.9%, 94.0% and 95.7% for Knott - SNAP® 4Dx® Plus, Knott - qPCR and SNAP® 4Dx® Plus - qPCR for *D. immitis* was found, respectively. Data demonstrate a high prevalence of VBPs in hunting dogs, indicating that this group of animals is largely exposed to several arthropod vector species and suggesting the transmission risk of pathogens to humans in rural areas of southern Italy. A multi-diagnostic approach and a deeper cooperation among healthcare and stakeholders are required to prevent VBP infections to animals and humans.

Keywords: filarioids, hunting dog, Italy, vector-borne pathogens, zoonosis.

1. Introduction

Vector-borne diseases (VBDs) are caused by a wide range of infectious and parasitic agents transmitted by blood-feeding arthropods, such as ticks, fleas, lice, mosquitoes and phlebotomine sand flies (Otranto et al., 2009a). Some of the above VBDs (e.g., anaplasmosis, borreliosis, heartworm disease, leishmaniosis and subcutaneous dirofilariosis) are relevant for animal welfare,

as well as for their zoonotic potential (Maia et al., 2015). Moreover, the epidemiological scenario of VBDs is constantly evolving due to several social and environmental drivers (Otranto et al., 2017), such as changes in global temperature and ecosystems, increased mobility of animals and humans and chemoresistance towards insecticides and acaricides (Miró et al., 2013). All these factors may influence the spread of arthropods and vector-borne pathogens (VBPs) (Hofmann et al., 2019), eventually complicating the control of VBDs (Baneth et al., 2012; Dantas-Torres and Otranto, 2016). Although many studies are available on the occurrence of VBPs in companion dogs, fewer reports are accessible on working dogs (e.g., hunting dogs) which live in close contact with humans and wildlife (Otranto et al., 2015). However, the employment of dogs in hunting activities has a long history (Orr et al., 2019), being even supposed as one of the reasons for their initial domestication as pets (Olsen, 1985; Koler-Matznick, 2002), between 33,000 and 15,000 years ago (Orr et al., 2019). Hunting dogs spend a large part of their life in sylvan environments (Veneziano et al., 2018; Sonnberger et al., 2021), where arthropod vectors thrive, exposing themselves to a plethora of VBPs more than companion dogs (Miró et al., 2015; Veneziano et al., 2018; Sgroi et al., 2021a). For instance, a recent review on different domestic animals in Europe reported the highest prevalence (i.e., 12.2%) of zoonotic tick-borne pathogens (TBPs) in hunting dogs from Latvia (Springer et al., 2020), indicating this category of animals as a sentinel for the circulation of VBPs in pets and humans (Meyers et al., 2021; Sonnberger et al., 2021). Again, a recent citizen science survey in hunting areas of southern Italy revealed that ticks commonly infesting wild boars (i.e., *Dermacentor marginatus* - Sgroi et al., 2021b) were also prevalent on dogs (47.4%) and hunters (8.4%) which shared the same environments (Sgroi et al., 2021c). This would suggest that, if not properly treated with ectoparasiticides, hunting dogs may also act as reservoirs of several tick species and TBPs for animals and humans (especially hunters) in rural areas (Hornok et al., 2013; Dantas-Torres and Otranto, 2016; Toepp et al., 2018; Mahachi et al., 2020; Mendoza-Roldan et al., 2021a; Sgroi et al., 2021c). Accordingly, the simultaneous detection of zoonotic VBPs in canine populations of the Mediterranean basin (e.g., *Anaplasma* spp., *Dirofilaria* spp., *Ehrlichia* spp., and

Leishmania infantum) is a common finding, yet causing clinical and diagnostic challenges (De Tommasi et al., 2013; Kostopoulou et al., 2020). Although a number of diagnostic tools is available for the detection of the above pathogens in dogs, several limitations of these tests should be considered. For example, for TBPs such as *Babesia vogeli* or *Ehrlichia canis*, PCR is more useful than serology within the acute phase of infection, but less sensitive when animals are chronically infected, since the microorganism load may be below the threshold for DNA amplification (Otranto et al., 2010). Furthermore, SNAP® 4Dx® Plus test (IDEXX Laboratories, Inc., Westbrook, Maine, USA) is one of the most used and reliable techniques for a rapid point-of-care (POC) diagnosis of *Dirofilaria immitis* infestation and tick-borne infections in veterinary clinics, as well as in field studies, compared to the Knott's test and microtiter plate ELISA (Panarese et al., 2020). Based on the picture above, this study aimed to investigate the circulation of VBPs, including those of zoonotic concern, in hunting dogs from rural areas of southern Italy, evaluating the relative performance of different diagnostic tools.

2. Materials and Methods

2.1 Ethical approval

The protocol was approved by the Ethical Committee of the Department of Veterinary Medicine and Animal Productions of the University of Naples Federico II (protocol number: 0039904), in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2 Study area

The study was performed in different administrative provinces (i.e., Avellino, Napoli and Salerno) of the Campania region (southern Italy) (Figure 1), including a total surface of 123.417 km^2 with a typical Mediterranean temperate climate and progressively continental features in inland and mountainous landscapes.

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106 *2.3 Sampling*

107 Between April 2014 and September 2017, 57 private veterinary clinics and 215 private dog owners
108 were involved in the collection of blood and serum samples from hunting dogs (n = 1,433). During
109 clinical examination, signaling information including age class (< 2, 2 - 7, > 7 years old), sex and
110 coat length (short, medium, long) of each dog was recorded. For all dog owners, a questionnaire
111 survey was completed reporting number of dogs employed during hunting activity ("pack size", 0, 1
112 - 10, > 10), hunting typology (wild mammals, wild birds), administrative province and number of
113 ectoparasiticide treatments administered per year (0, 1, 2 - 6, > 6). All samples were delivered to the
114 Department of Veterinary and Animal Productions (University of Naples Federico II, Italy) for
115 morphological and serological examinations and to the IDEXX Laboratories, Inc. (Westbrook,
116 Maine, USA) for molecular analyses.

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118 *2.4 Morphological, serological and molecular procedures*

119 All blood samples (n = 1,433) were analyzed on the day of collection by the modified Knott's
120 technique to detect microfilariae (mfs), which were counted and measured under 400x
121 magnification via digital system (i.e., Leica, DM 200, Germany) (Lindsey, 1965; Balbo and
122 Panichi, 1968). In addition, serum samples were analyzed by using a rapid POC device (i.e.,
123 SNAP® 4Dx® Plus - IDEXX Laboratories, Inc., Westbrook, ME, USA) to identify exposure of dogs
124 to TBPs (i.e., *Anaplasma* spp., *Ehrlichia* spp., *Borrelia burgdorferi* sensu lato) and *D. immitis*.
125 Then, in order to molecularly detect the above pathogens (with the exception of *B. burgdorferi*) plus
126 *Babesia* spp. and *L. infantum*, real-time PCR was performed by a reference veterinary diagnostic
127 laboratory using a comprehensive panel for VBPs (Tick/Vector Comprehensive RealPCR™ Panel
128 Canine, IDEXX Laboratories, Inc.). Briefly, total nucleic acid was extracted from whole blood
129 using a commercial kit (Life Technologies, Valencia, CA) according to manufacturer's instructions.
130 Real-time PCR reactions were performed on a LightCycler LC480 instrument (Roche Diagnostics)

to amplify target gene/sequences (Genbank) from the following pathogens: *Anaplasma phagocytophilum* (*msp2* - DQ519570), *Anaplasma platys* (*groEL* - AY848753), *Babesia canis* (*hsp70* - AB248735), *Babesia gibsoni* (*hsp70* - AB248731), *B. vogeli* (*hsp70* - EF527401), *E. canis* (*p27* - AF403710), *Ehrlichia ewingii* (*p27* - AY428950), *Ehrlichia chaffeensis* (*p27* - AF403711), *L. infantum* (*Gp63* - Y08156), *Acanthocheilonema reconditum* (ITS-2- AF217801), *D. immitis* (18S rRNA - AB973231) and *Dirofilaria repens* (*COI* - AJ271614). The commercial real-time PCR also included positive and negative controls for each assay, quality controls for sample extraction efficiency and a control for monitoring environmental contamination.

2.5 Statistical Analysis

The K-Cohen formula (K) was run to establish the percentage agreement among diagnostic tests employed, with value of 0 - 20%, 21 - 40%, 41 - 60%, 61 - 80% and 81 - 100% considered as poor, fair, moderate, strong and high, respectively (Maggi et al., 2014). Exact binomial 95% confidence intervals (CIs) were established for proportions found in the present work, using the Epitools - Epidemiological Calculators software (Sergeant, 2018). A regressive logistic model analysis was performed using the *A. reconditum* positivity status as a dependent variable, since it was the most prevalent pathogen in this study. Whereas independent variables of dogs (i.e., age class, sex, coat length, pack size, hunting typology, administrative province, number of ectoparasiticide treatments administered per year and co-infection with different pathogens) were included in the multivariate model as potential predictors of *A. reconditum* infestation. Dog breed was not considered as an independent variable, since all animals belonged to hunting breeds. The distribution of dogs enrolled and those positive to VBPs, according to the different administrative provinces of the study area, was determined *via* ArcGIS (version 10.3; ESRI, Redlands, California, USA).

3. Results

Out of 1,433 hunting dogs, 321 (i.e., 22.4%, 95% CI: 20.3 - 24.6) tested positive for VBPs by using at least one diagnostic tool. Details on the geographical distribution of dogs enrolled and those positive to VBPs, according to the different provinces of the study area, are shown in Figure 1.

Of the animals sampled, 28 (i.e., 1.9%, 95% CI: 1.4 - 2.8) were positive for filarial species, being *A. reconditum* the most prevalent (*n* = 23, 1.6%), followed by *D. immitis* (*n* = 3, 0.2%) and *D. repens* (*n* = 2, 0.1%), with one co-infested dog (0.07%, *A. reconditum* - *D. repens*). The microfilaremia average was 33 mfs/ml/positive dog (minimum 1 - maximum 120 mfs). Most of the positive animals (*n* = 16, 57.1%) showed microfilaremia ranging from 11 to 50 mfs/ml, whereas 7 (25%), 3 (10.7%) and 2 (7.1%) dogs displayed values from 1 to 10, 51 to 100 and > 100, respectively. The average length of the mfs was of 263.3 µm (min. 250.1 - max. 271.2 µm), 302.5 µm (min. 281.5 - max. 306.7 µm) and 359.7 µm (min. 346.2 - max. 376.2) for *A. reconditum*, *D. immitis* and *D. repens*, respectively. Overall, 140 (i.e., 9.8%, 95% CI: 8.3-11.4) and 231 (i.e., 16.1%, 95% CI: 14.3 - 18.1) dogs scored positive for at least one VBP by serological and molecular methods, respectively. The most prevalent pathogens detected were *Ehrlichia* spp. (*n* = 104, 7.3%) with SNAP® 4Dx® Plus, and *A. reconditum* (*n* = 110, 7.7%) by qPCR. Most of the co-infections were by *Anaplasma* spp. - *Ehrlichia* spp. (*n* = 28, 1.9%) serologically and by *A. reconditum* - *E. canis* (*n* = 6, 0.4%) molecularly. Details on serological and molecular results are listed in Table 1, according to different pathogens diagnosed, including co-infection cases. Statistical analyses reported a significant association ($p < 0.001$) between *A. reconditum* infestation and both *Ehrlichia* spp. seropositivity and geographical origin of dogs (Table 2). A high agreement among diagnostic tools employed was found for *D. immitis* positivity, being of 99.9%, 94.0% and 95.7% for Knott - SNAP® 4Dx® Plus, Knott - qPCR and SNAP® 4Dx® Plus - qPCR, respectively. The questionnaire survey revealed that 978 dogs (i.e., 68.2%) had been infested at least one time by ticks (with number of ticks reported ranging from 1 to > 20) during the hunting activities and 30 animals (i.e., 2.1%) had never received any ectoparasiticide treatment in their life. All dogs were apparently healthy, showing no symptoms or clinical signs ascribable to VBPs.

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183 *4. Discussion*

184 This survey indicates a broad involvement of hunting dogs in the maintenance of arthropod vectors
185 and VBPs, some of which are of zoonotic concern. The overall prevalence of VBPs herein found
186 (i.e., 22.4%) is in accordance with large scale surveys carried out in Spain (i.e., 22.1%, n = 4,643 -
187 Montoya-Alonso et al., 2020) and Greece (i.e., 25.6%, n = 1,154 - Kostopoulou et al., 2020),
188 confirming the endemicity of these infections in canine population of the Mediterranean basin
189 (Mendoza-Roldan et al., 2021b). In fact, a multi-center investigation on 345 dogs from 17 endemic
190 countries (13 of which belonging to the Mediterranean area) reports a prevalence of 35% for at least
191 one VBP, with values up to 54% in Spain (Schäfer et al., 2019).

192 Among filarial species herein detected, the higher occurrence of *A. reconditum* (flea-borne
193 nematode), compared to *D. immitis* and *D. repens* (both mosquito-borne nematodes), indicates a
194 more likely exposure of hunting dogs to fleas than Culicidae (Dantas-Torres and Otranto, 2013;
195 Otranto et al., 2013; Gizzarelli et al., 2019). The high seroprevalence of *Ehrlichia* spp. (7.3%) and
196 *Anaplasma* spp. (4.1%), combined with co-infections by these TBPs (1.9%), suggests that hunting
197 dogs were infested by *Rhipicephalus sanguineus* sensu lato and *Ixodes ricinus* ticks, which are
198 vectors of these pathogens (Sgroi et al., 2021c) perpetuating throughout the year in the examined
199 areas (Lorusso et al., 2010). In addition, the simultaneous exposure of dogs to flea and tick
200 populations is furtherly suggested by the high molecular prevalence of *A. reconditum* (7.7%), *E.*
201 *canis* (2.7%) and *A. platys* (4.5%), as well as by the statistical association ($p < 0.001$) between *A.*
202 *reconditum* infestation and *Ehrlichia* spp. seropositivity herein found. The higher molecular
203 proportion of *B. vogeli* (1.4%) than *B. canis* (0.1%) in hunting dogs is in accordance with the
204 distribution of ticks acting as vectors of these piroplasmids in Italy. In fact, *B. vogeli* is mainly
205 reported from central and southern Italy, where *R. sanguineus* s.l. is the predominant tick species on
206 dogs (Solano-Gallego et al., 2008), whereas *B. canis* is more widespread in northern regions,
207 according to the occurrence of *Dermacentor reticulatus* (Olivieri et al., 2016). The low molecular

prevalence of *L. infantum* (0.3%) in hunting dogs, in an area endemic for canine leishmaniosis (CanL) (Piantedosi et al., 2016; Mendoza-Roldan et al., 2020), is probably related to the poor sensitivity of PCR on blood samples for the diagnosis of this infection (Otranto et al., 2009b; Iatta et al., 2021).

The present study also highlights the limitations in the diagnosis of VBPs through a single diagnostic approach, supporting the use of multiple tools for the detection of these pathogens. In fact, although the high agreement among the tests employed (i.e., K value, 99.9%, 94.0% and 95.7% for Knott - SNAP® 4Dx® Plus, Knott - qPCR and SNAP® 4Dx® Plus - qPCR, respectively), differences in the prevalence of pathogens were recorded. For instance, a higher prevalence of filarioids has been found molecularly (7.9%) than by Knott test (1.9%), whereas a lower proportion of *Ehrlichia* spp. infection was diagnosed by qPCR (2.7%) compared to serology (7.3%). Consequently, these results confirm that the combination of different diagnostic methods is recommended to increase the probability of finding positive animals (Otranto et al., 2010), as previously demonstrated for CanL (Otranto et al., 2009b), especially in hunting dogs which are likely exposed to multiple VBPs (Sgroi et al., 2021c). The absence of clinical signs in hunting dogs suggests the subclinical nature of several VBP infections (Montoya-Alonso et al., 2020), which represent a further hindrance in the diagnosis of VBDs. Despite the low percentage of animals never treated with ectoparasiticides (2.1%), the proportion of those infested by ticks (68.2%) is indicative for the presence of these arthropods in hunting environments, as well as a scarce treatment compliance of owners. In accordance, a survey from northern-central Italy reveals that up to 63.2% of owners treat their dogs only when already infested by ectoparasites (Colombo et al., 2021). Therefore, the use of ectoparasiticides in hunting dogs should be carefully performed toward reducing the likelihood of pathogens circulation, minimizing the risk of infection to other animals and humans. Indeed, the occurrence of several zoonotic filarial, bacterial and protozoan agents herein detected (i.e., *D. immitis*, *D. repens*, *A. phagocytophilum*, *E. canis*, *L. infantum*) confirms the

public health concern for hunters, with up to 8.4% of them infested by ticks in a previous survey from the same study area (Sgroi et al., 2021c).

5. Conclusions

Hunting dogs are broadly exposed to arthropod vectors and may represent a risk of VBP transmission to humans in rural areas of southern Italy. A multi-diagnostic approach for a prompt diagnosis of VBP infections in dogs and a deeper cooperation among healthcare and stakeholders are needed to improve the quality of management strategies against VBPs, in order to guarantee animal and human welfare in a one health perspective.

Author contributions

Giovanni Sgroi: Conceptualization, Writing-original draft. **Francesco Buono**: Formal analysis, Investigation. **Roberta Iatta**: Supervision, Writing-review & editing. **Melissa Beall**: Methodology, Formal analysis. **Ramaswamy Chandrashekar**: Supervision. **Jesse Buch**: Supervision. **Diego Piantedosi**: Methodology, Investigation. **Vincenzo Veneziano**: Project administration. **Domenico Otranto**: Supervision, Writing-review & editing.

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463 **Figure legend**

464 **Figure 1.** Map showing the number of hunting dogs enrolled and the overall prevalence of VBP
465 infections, along with the odds ratio values, according to the three provinces of the study area.
466 Abbreviations: Avellino (AV), Napoli (NA) and Salerno (SA).

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473 **Table 1**

474 Number and percentage of hunting dogs tested positive to different vector-borne pathogens on the
475 total number examined (*n* = 1,433), according to serological and molecular tools employed.

Vector-borne pathogens	**Serology no. (%)**	**95% CI***	**PCR no. (%)**	**95% CI***
Single infections				
Anaplasma spp.	29 (2.0)	1.4 - 2.9	59 (4.1)[a]	3.2 - 5.3
Borrelia burgdorferi sensu lato	1 (0.07)	0.01 - 0.4	-	-
Ehrlichia spp.	76 (5.3)	4.3 - 6.6	32 (2.2)[b]	1.6 - 3.1
Dirofilaria immitis	3 (0.2)	0.07 - 0.6	2 (0.1)	0.04 - 0.5
Acanthocheilonema reconditum	-	-	98 (6.8)	5.6 - 8.3
Dirofilaria repens	-	-	2 (0.1)	0.04 - 0.5
Babesia spp.	-	-	19 (1.3)[c]	0.8 - 2.1
Leishmania infantum	-	-	4 (0.2)	0.08 - 1.0
Sub-total	**109 (7.6%)**	**6.3 - 9.1**	**216 (15.1)**	**13.3 - 17.0**
Co-infections				
Anaplasma spp. - *Borrelia burgdorferi* sensu lato	1 (0.07)	0.01 - 0.4	-	-
Anaplasma spp. - *Ehrlichia* spp.	28 (1.9)	1.4 - 2.8	-	-
Anaplasma spp. - *Borrelia burgdorferi* sensu lato - *Dirofilaria immitis*	1 (0.07)	0.01 - 0.4	-	-
Borrelia burgdorferi sensu lato - *Dirofilaria immitis*	1 (0.07)	0.01 - 0.4	-	-
Acanthocheilonema reconditum - *Anaplasma platys*	-	-	5 (0.3)	0.1 - 0.8

Acanthocheilonema reconditum - Ehrlichia canis	-	-	6 (0.4)	0.2 - 0.9
Acanthocheilonema reconditum - Leishmania infantum	-	-	1 (0.07)	0.01 - 0.4
Anaplasma platys - Babesia vogeli	-	-	2 (0.1)	0.04 - 0.5
Ehrlichia canis - Babesia vogeli	-	-	1 (0.07)	0.01 - 0.4
Sub-total	**31 (2.2)**	**1.5 - 3.0**	**15 (1.0)**	**0.6 - 1.7**
Total	**140 (9.8)**	**8.3 - 11.4**	**231 (16.1)**	**14.3 - 18.1**

476 *Exact binomial 95% confidence intervals

477 [a]*Anaplasma platys* (*n* = 58) and *Anaplasma phagocytophilum* (*n* = 1)

478 [b]All *Ehrlichia canis*

479 [c]*Babesia vogeli* (*n* = 17) and *Babesia canis* (*n* = 2)

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484 **Table 2**

485 Multiple logistic regression analysis, based on the *Acanthocheilonema reconditum* positivity status

486 as a dependent variable, according to selected independent variables of hunting dogs enrolled.

Variables	**Primary category**	**Reference category**	**95% CI***	***p*†**	**ORs‡**
Age[a]			0.9 - 1.1	0.605	1.0
Sex	Male	Female	0.8 - 2.0	0.233	1.3
Coat length	Long	Short	0.8 - 2.4	0.190	1.4
	Medium	Short	0.1 - 2.0	0.190	0.5
Pack size[b]			0.9 - 1.0	0.853	1.0
Hunting typology	Wild mammals	Wild birds	0.8 - 2.4	0.260	1.4
Province	Naples	Avellino	1.2 - 6.1	0.0001	2.7
	Salerno	Avellino	2.4 - 8.3	0.0001	4.5
Ectoparasiticide treatments			0.9 - 1.0	0.530	1.0
***Ehrlichia* spp. seropositivity**	Positive	Negative	1.5 - 4.9	0.001	2.7

487 *Exact binomial 95% confidence intervals

488 †*p* values

489 ‡Odds ratios

490 [a]Age class (< 2; 2 - 7; > 7 years old)

491 [b]Number of dogs employed during hunting activity (0, 1 - 10, > 10)