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Vector-borne pathogens in clinically healthy military working dogs in eastern Austria

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ABSTRACT

Military working dogs have an increased risk of acquiring an infection with vector-borne pathogens due to kennel housing and regular exposure to wildlife and vectors. To evaluate the level of infections in clinically healthy dogs of the Austrian Armed Forces, 94 individuals of the Military Working Dog Training Centre (MWDTC) Kaisersteinbruch/eastern Austria were examined in August 2016, February 2019 and August 2019. A modified Knott test was used to determine the presence of microfilariae, PCR for DNA detection of filarioid nematodes (incl. *Dirofilaria*), *Leishmania* spp., piroplasms, *Borrelia* spp., *Bartonella* spp. and Anaplasmataceae, and serological examination for antibodies against *Borrelia burgdoferi* s. l. and *Leishmania infantum* in all dogs. Two dogs were positive for *Dirofilaria repens* in the Knott test, and one of them also by PCR. Six clinically healthy dogs (4.2%) were positive for *Babesia canis* (PCR). In serology, 10 (10.6%) of the dogs were positive for specific antibodies against *Borrelia burgdoferi* s. l. The results suggest that the current measures against arthropod vector exposure and the pathogens they can transmit are not fully sufficient for these dogs. Further investigations of the tick and mosquito fauna in this area will shed more light on the risk of exposure for both the dogs and the staff of the MWDTC.

1. Introduction

Vector-borne diseases are of great relevance to both human and animal health in tropical, but also in temperate climates, including central Europe. Filarioid helminths of veterinary and/or medical relevance are endemic in Mediterranean and eastern European regions, but an increasing number of occurrences of these pathogens are also reported from central Europe [1–3]. Tick-borne diseases of dogs, such as babesiosis and anaplasmosis, are endemic to Austria [4]. The incidence and frequency of vector-borne pathogens are directly related to the presence of vectors and the possibility of an encounter between the vector and a potential host [5]. Exposed persons and animals that spend much time outdoors in vector habitats could therefore have an increased risk of contracting arthropod vectors and consequently of acquiring infections with vector-borne organisms. Military working dogs spend a larger part of their lives outdoors and also train and work in areas that pet dogs rarely visit due to restricted access for non-military persons and animals. Therefore, their exposure to vectors in such a "small wilderness" must be considered higher than that for dogs in (peri-)urban areas. Similar to hunting dogs, they also reflect the exposure of wildlife to vectors and the pathogens they harbour. As recent studies show, working dogs that move a lot in the wild together with their trainers can also be suitable sentinels for human zoonotic vector-borne diseases as well [6].

The present study was conducted (a) to evaluate the presence of vector-borne infections in military dogs in eastern Austria and (b) to add knowledge on the ecological networks of vector-borne diseases and potential pathogen spill-overs to domestic animals and humans in the

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particular ecological setting of dogs in areas with restricted human activities. The dog population of the present study is located in an area in eastern Austria known to be endemic for various tick and mosquito species. To evaluate the presence of vector-borne pathogens in the dog population studied, blood samples from healthy military working dogs were examined for the presence of *Leishmania infantum, Babesia* spp., *Hepatozoon canis, Dirofilaria* spp., *Borrelia* spp., *Bartonella* spp. and Anaplasmataceae.

2. Materials and methods

2.1. Animals and blood samples

In total, 94 clinically healthy animals at the Military Working Dog Training Centre in Kaisersteinbruch (MWDTC), Burgenland (eastern Austria), were included in this study. The most common breeds (64.9%) were Rottweilers, followed by Labrador Retrievers (9.6%), Malinois (8.5%) and German Shepherds (5.3%). One dog each was a Tervueren, a Chihuahua and a Prager Rattler, for the rest (8.5%) the breed was unknown. More than half of the dogs (55.3%) were born at the MWDTC within the centre's own breeding program, and according to the centre's documentation system, 23.4% of the tested dogs were imported from other countries in Europe (Table 1). The gender distribution was balanced (51.1% males and 48.9% females). The majority of the dogs was between 6 months and 3 years old at the time of sampling (Fig. 1). None of the dogs had a history of travel (after acquisition) or mission to other countries.

The military working dogs were kept in kennels and treated monthly against gastrointestinal helminths (combination therapy with febantel, praziquantel and pyrantel; dose according to body weight). They were vaccinated against canine distemper virus, kennel cough (adenovirus type 2 and parainfluenza), hepatitis contagiosa canis, parvovirosis, leptospirosis and rabies.

The general management against ectoparasites consisted of spot-on application of permethrin (Exspot®, MSD, Vienna, Austria) until 2017, and after that, of oral application of fluralaner (Bravecto®; MSD, Vienna, Austria) every three months. Dogs deployed in military missions abroad received an additional selamectin treatment as a spot-on before leaving (Stronghold®, Zoetis, Vienna, Austria).

Apart from episodic military missions across Austria, when off duty, the enrolled dogs were kennelled in their handler's residence, or in indoor or outdoor kennels until they were assigned to a military dog handler at the MWDTC, and spent their complete life in the MWDTC. Five of the tested dogs were privately owned by the employees at the MWDTC, but also spent much time at the MWDTC.

Blood (EDTA and serum) samples were collected in August 2016, February 2019 and August 2019 from the *Vena saphena lateralis* or the *Vena cephalica*, and transferred to the University of Veterinary Medicine for further analysis. The dogs were clinically examined before sampling, and the handlers were interviewed for the medical history of the dogs. Being a dynamic population, not all dogs could be sampled at specified days, because they were not always available at the centre at the time of blood collection; some had left and new animals had joined or were born. In the MWDTC's own Rottweiler breeding unit, the annual numbers of births varied; usually one or two litters were borne each year. A single sample was obtained from 56.4% of the individuals, two samples were collected from 34.0% and three samples from 9.6% of the dogs.

In total, 144 blood samples from the 94 dogs were available for PCR testing. *Borrelia burgdorferi* s. l.-specific serology and *Leishmania* spp.-specific serology were performed once for all dogs or for 88 of the dogs (all except the youngest puppies born in 2019), respectively.

Ethical approval for this study was obtained from the Ethics Committee of the University of Veterinary Medicine Vienna.

Table 1
Origin of the included dogs ($n = 94$).

Origin	Number of dogs [%]
Austria – military centre	52 [55.3]
Austria - elsewhere	20 [21.3]
Germany	4 [4.3]
Hungary	2 [2.1]
Netherlands	2 [2.1]
Slovenia	2 [2.1]
France	1 [1.1]
Croatia	1 [1.1]
Czech Republic	1 [1.1]
Greece	1 [1.1]
Unkown	8 [8.5]

2.2. Modified Knott test

For the detection of *Dirofilaria* spp. microfilariae, a modified Knott test [7]. was performed with all blood sample ($n = 144$).

2.3. Serology

In total 94 blood sera of the military working dogs were tested once for *Borrelia burgdorferi* s. l. -specific antibodies by using a kinetic ELISA (KELA) [8] and a commercially available line immunoblot assay (LIA, Virotech, Rüsselsheim, Germany) [9], detecting IgG antibodies. Samples were also tested with an in-house indirect immunofluorescence antibody test (IFAT) for the detection of *Leishmania infantum*-specific antibodies (IgG). For this, serial serum dilutions (1,20 to 1360) were prepared with PBS. Subsequently, antigen-coated slides (prepared from L. *infantum* grown in vitro in RPMI 1640 supplemented with HEPES, sodium bicarbonate, 10% FCS and penicillin/streptomycin at 28 °C under 5% CO_2) were transferred to a humidity chamber and each field was covered with 25 µl of diluted serum. The slides were then incubated at 37 °C for 30 min, washed twice in PBS (2 × 7 min) and incubated for another 30 min at 37 °C with secondary antibody (FITC-conjugated anti-dog-IgG, The Binding Site, Birmingham, UK), diluted 1:30 and mixed with a drop of Evans Blue solution to reduce background fluorescence. The slides were then covered with PBS-glycerine and a cover slip, and examined by fluorescence microscopy at 500× magnification under immersion oil. Positive and negative control sera were prepared in parallel.

2.4. Molecular analyses

DNA was extracted from whole blood and peripheral blood mononuclear cells (PBMCs) using the erythrocyte lysis buffer of the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. PCRs were conducted in an Eppendorf Mastercycler® Pro, Eppendorf, Hamburg, Germany to screen for DNA of filarioid helminths, *Leishmania* spp., Anaplasmataceae, *Bartonella* spp., *Borrelia burgdorferi* s.l. and piroplasms (Table 2).

PCR products were separated by electrophoresis on 2% agarose gels stained with Midori Green Advance (Nippon Genetics Europe, Düren, Germany). Finally, purified PCR products were sequenced by LGC Genomics GmbH (Berlin, Germany), and sequences were compared for similarity using the Basic Local Alignment Search Tool (BLAST) in GenBank® (http://www.ncbi.nlm.nih.gov/BLAST).

3. Results

All dogs appeared clinically healthy during clinical examinations and blood collection. Individual dogs had histories of performance-orthopaedic issues. However, clinical signs were limited to individuals and did not extend over longer time periods. There were no reports of reduced performance, loss of body mass, circulation problems or other abnormalities reported by the trainers.

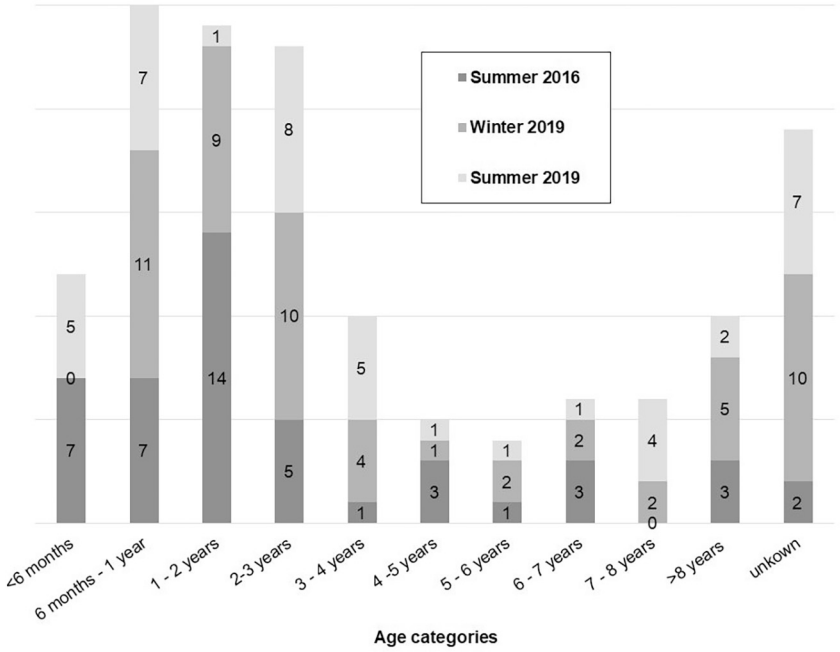

Fig. 1. Age of the dogs at the time of blood sampling.

Table 2
PCR protocols for molecular analyses.

Organism	Locus	Primer sequences	Amplification protocol	Product size [bp]	Reference
Leishmania	SSU rRNA	LEI_70L_for: 5′-CGCAACCTCGGTTCGGTGTG-3′ LE_70R_rev: 5′-CGCGGTGCTGGACACAGGGTA-3′	94 °C 2 min 40×: 94 °C 1 min, 65 °C 90 s, 72 °C 90 s 72 °C 1 0 min	345 bp	SPANAKOS et al., 2002
Anaplasmataceae	16S rRNA	EHR16SD_for: 5′-GGTACCYACAGAAGAAGTCC-3′ EHR16SR_rev: 5′-TAGCACATCATCGTTTACAGC-3	95 °C 2 min 35×: 94 °C 1 min, 54 °C 30 s, 72 °C 30 s 72 °C 5 min	345 bp	PAROLA et al., 2000
Filarioid nematodes	COI	H14FilaCOIRw: 5′-GCCTATTTTGATTGGTGGTTTTGG-3 H14FilaCOIRv: 5′-AGCAATAATCATAGTAGCAGCACTAA-3′	95 °C 2 min 35×: 95 °C 1 min, 53 °C 1 min, 72 °C 1 min 72 °C 5 min	724 bp	HODŽIĆ et al., 2015
Piroplasms	18S rRNA	Nest 1: BTH—1F: 5′-CCTGAGAAACGGCTACCACATCT-3′ BTH-1R: 5′-TTGCGACCATACTCCCCCCA-3′ Nest 2: G-2_for: 5′-GTCTTGTAATTGGAATGATGG-3′ G-2_rev: 5′-CCAAAGACTTTGATTTCTCTC-3′	Nest 1: 94 °C 2 min 40×: 95 °C 30 s, 68 °C 1 min, 72 °C 1 min 72 °C 10 min Nest 2: 94 °C 2 min 40×: 95 °C 30 s, 60 °C 1 min, 72 °C 1 min 72 °C 10 min	Nest 1: 700 bp est 2: 561 bp	ZINTL et al., 2011
Borrelia	16S rRNA	Borr_allg_for: 5′-ACGCTGGCAGTGCGTCTTAA-3′ Borr_allg_rev: 5′-CTGATATCAACAGATTCCACCC-3′	94 °C 5 min 40×: 94 °C 1.5 min, 63 °C 2 min, 72 °C 2 min 72 °C 10 min	674 bp	LIEBISCH et al., 1998
Bartonella	gltA	BhCS.871p: 5′-GGGGACCAGCTCATGGTGG-3′ BhCS.1137n: 5′-AATGCAAAAAGAACAGTAAACA-3′	94 °C 5 min 40×: 94 °C 1 min, 54 °C 1 min, 72 °C 1 min 72 °C 10 min	379 bp	NORMAN et al., 1995

Microfilariae were detected by the Knott test in two samples, both from 2016, resulting in a relative prevalence of 1.4%. These were identified morphologically as *Dirofilaria repens*. One of the positive dogs was a male Rottweiler born in Hungary and the other one a Malinois from Austria. Only the sample from the latter dog could be confirmed as *D. repens* by sequencing after PCR. *Babesia canis* DNA was detected in six samples (4.2%) from 2016 (Table 3). None of these positive dogs had had any missions abroad. Except for one Malinois from Hungary, the *B. canis*-positive dogs (three Rottweilers, one Tervueren and a further two Malinois) were from Austria, and the three Rottweilers were born in the MWDTC (Table 3). The Malinois, which was positive for *D. repens,* was also positive for *B. canis*. Of the *B. canis* DNA sequences, five samples were 100% and one 99% similar to isolates found in *Dermacentor reticulatus* and in foxes in Austria (KY693669.1, KY447296.1) [10]. The PCR for other pathogens (Anaplasmataceae and *Bartonella* spp.) were all negative.

None of the animals was positive for *Leishmania* spp.-specific antibodies in serology, but ten individuals (10.6%) were classified as

Table 3
Dogs positive for *Babesia canis and Dirofilaria repens* by PCR and amplicon sequencing. MWDTC: Military Working Dog Training Centre.

Breed	Age at sampling [years]	Origin	Pathogens detected	Accession nos.
Rottweiler	1	MWDTC	*Babesia canis*	MW588421
Tervueren	3	Austria	*Babesia canis*	MW588421
Malinois	6	Hungary	*Babesia canis*	MW588420
Malinois	4	Austria	*Babesia canis/ Dirofilaria repens*	MW588421/ MW590257
Rottweiler	11	MWDTC	*Babesia canis*	MW588421
Rottweiler	0.5	MWDTC	*Babesia canis*	MW588421

positive for *Borrelia burgdorferi* s. l.-specific antibodies either in the KELA or in the line immunoassay (Table 4). Again, the Malinois, which was already positive for *D. repens* and *B. canis*, was also positive for *Borrelia burgdorferi* s. l.-specific antibodies.

4. Discussion

4.1. Dirofilaria repens

In the modified Knott test microfilariae of *D. repens* were detected in two out of 94 dogs. Only one of those blood samples produced a positive result in the PCR, and sequence analysis confirmed the morphological identification of *D. repens*. This filarioid species is known to be endemic in Austria and has been detected in dogs with and without a history of travel to other endemic countries [1,11], as well as in the *Anopheles maculipennis* complex, *An. algeriensis*, and *An. plumbeus* from eastern Austria [2,12]. Since one of the two positive dogs in the present study was born in Hungary, a known endemic country for *D. repens* [13], it could not be unequivocally determined whether this specific infection was of autochthonous origin; however, the infected Malinois was born and bred in Austria and had no known travel history; consequently, an autochthonous infection is assumed for this case. The local transmission of *D. repens* is associated with two conditions; the presence of competent mosquito vector species, which are present in Austria [2], and the presence of a minimum number of dogs with circulating microfilariae in the blood [14]. The MWDTC and adjacent area would fulfil both conditions. The dogs of the Centre are housed in kennels, spending most of their lives outdoors and exposed to mosquito vectors. No *D. immitis* antigen test was performed and thus an infection with macrofilariae of *D. immitis* only might have been missed.

4.2. Babesia canis

DNA of *Babesia canis* was detected in six of the 94 dogs. Five animals originated from Austria and one from Hungary. The main vector for this parasite, *Dermacentor reticulatus*, is known to be endemic in eastern Austria. The MWDTC is located in an area where *D. reticulatus* infections in dogs have been detected previously [15], and the presence of *B. canis* in *D. reticulatus* later confirmed endemicity for this pathogen [10]. Previous reports also indicated that *B. canis* is endemic in eastern Austria [16,17]. The prevalence of *B. canis* of 6.4% in the present study can be considered low to moderate compared to other European countries [18]. Different studies showed variable prevalences. Sled dogs also kept under outdoor conditions in Poland showed a much higher prevalence for *B. canis* with 25.3% positivity in 82 dogs tested by PCR over a 2-year period [19]. A study from 2013 showed a seroprevalence of 12.8% in 90 dogs in eastern Austria [17]. Military dogs in extensive husbandry in Portugal ($n = 100$) had a seroprevalence of 3% [20]. Seropositive dogs (19.8% of 197 tested dogs) without any clinical signs were found in a study in Romania [21].

No dog in the present study showed clinical signs indicative of babesiosis, despite parasitaemia. Clinically inapparent infections with *B. canis* have been reported previously [19,22]. In addition to the risk to the infected individual to develop a clinically apparent babesiosis (which can take a fatal course in untreated dogs; Köster et al., 2015), the potential of parasite transmission by clinically healthy dogs (either to the vector tick or to other dogs by blood transfusion) should not be neglected. Infected dogs are therefore a risk factor for the spread of *B. canis* in Austria and neighbouring countries.

4.3. Borrelia burgdorferi sensu lato

No dog was positive for *B. burgdorferi* s. l. when tested with PCR, but 10.6% were positive for specific antibodies against *B. burgdorferi* s.l., indicating previous tick exposure and infections. A determination of the seroprevalence of *B. burgdorferi* s. l. from 90 dogs from the same area in Austria using a different method showed positive results in 31.1% of the dogs [4]. In Germany, a prevalence of 6.9% for antibodies against *B. burgdorferi* s.l. in dogs was reported [23]. In Hungary, a country bordering eastern Austria, healthy dogs were examined and 0.4% were seropositive for *B. burgdorferi* s. l. [24]. Thus, the seroprevalence determined in the present study may be considered moderate compared to other studies.

The distribution of *B. burgdorferi* s. l. is strictly linked to the distribution of its main vector, *Ixodes ricinus* [25]. In the province of Styria, in south-eastern Austria, the prevalence of *B. burgdorferi* s. l. in *I. ricinus* ticks collected from 2002 to 2003 was 25.7% [26]. It can be assumed that military working dogs experience relatively high risks of contracting *Borrelia* spp.-infections due to the exposure to the tick vector *I. ricinus,* which is the most abundant tick species in eastern Austria, and is known for its wide pan-European distribution [15,27,28].

Studies from Germany show a similarly high prevalence of *B. burgdorfer* s. l. in dogs and humans [29,30]. These results imply that the military dog handlers may experience a similar infection risk (and infection prevalence) as their dogs. Additionally, another pathogenic *Borrelia* species, *B. miyamotoi*, has recently been reported from Austria, with one case of associated relapsing fever in a patient [31,32].

5. Conclusion and outlook

In the present study, dogs exposed to mosquitoes and ticks in their

Table 4
Dogs serologically positive for *Borrelia.*

Breed	Age at sampling [years]	Origin	KELA (units)	VlsE0Mix dog	OspA0Mix	DbpA0Mix	OspC0Mix	39 kDa (BmpA)	58 kDa	83 kDa
Rottweiler	6	MWDTC	347.7	3	0	0	0	2	2	3
Rottweiler	10	France	116.8	1	0	0	0.5	0	0.5	0
Rottweiler	3	MWDTC	309.7	2	0	0	0	0.5	1	3
Malinois	4	Austria	131.4	1	0	0	0	0.5	1	0
Rottweiler	1	MWDTC	117.8	2	0	0	1	0	0	0
Rottweiler	3	MWDTC	114.5	1	0	0	0	0	0	2
Malinois	unknown	Unknown	110.8	1	0	0	0	0.5	0	2
unkown	unkown	Unknown	122.6	1	0	0	0	0.5	0	2
Rottweiler	2	MWDTC	97.2	2	0	0	1	0	0.5	0
Rottweiler	1	MWDTC	175.7	2	0	0	0.5	0	0	0

home environment were tested for a range of vector-borne infections. Infections with the mosquito-borne filarioid *D. repens,* the tick-borne protozoan *B. canis* and the tick-borne spirochetes of the *B. burgdorferi* s. l.-complex were detected, indicating considerable exposure to these pathogens. It should also be mentioned that the parasite management of the MWDTC might reduce the risk of transmission of VBDs and thus the prevalence in untreated and unprotected populations might be higher.

The population in this study was a group of dogs that has extensive outdoor access in an area endemic for culicid species [12,33] and *D. reticulatus,* which is present almost all year round [15]. The dogs move mainly in a military area which is partially restricted for hikers and constitutes a "pristine" landscape, not affected by extensive human activities. Such military areas with restricted public activities are also known to harbour wild carnivores, such as wolves [34] which could constitute a canid reservoir for infectious agents of dogs [35]. This emphasises the need to incorporate the monitoring of such infections both in humans and dogs in the surveillance of vector-borne diseases relevant to public health. The presence of potentially competent vectors is currently being investigated.

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