Parasites & Vectors

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Vector-borne pathogens in dogs from the Republic of Kosovo

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Abstract

Background Canine vector-borne pathogens (CVBP) are transmitted by arthropod vectors such as ticks, fleas, mosquitoes, and phlebotomine sand flies and are of global veterinary and medical importance. Dogs are important reservoir hosts, which may develop potentially life-threatening clinical signs. The Balkan area harbors diverse vector fauna and associated CVBPs, and data, particularly from the Republic of Kosovo, are scarce. Considering the high number of stray and privately owned dogs primarily kept outside, living in close contact with dogs might promote spillover of zoonotic pathogens to human populations. To combat these diseases, a One Health approach is required. Therefore, our study molecularly analyzed samples of dogs for CVBP.

Methods Blood samples of 276 dogs originating from all seven districts of Kosovo collected from 2021 to 2022 were screened using polymerase chain reaction (PCR) and sequencing for a substantial set of pathogens, including *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Ehrlichia* spp., Filarioidea, *Hepatozoon* spp., *Mycoplasma* spp., *Rickettsia* spp., and *Trypanosoma* spp. Prevalence rates were statistically assessed on the basis of various factors such as sex, breed, age, and district.

Results In total, 150 (54.3%) dogs tested positive for at least one pathogen, comprising eight species of five genera. The most prevalent pathogens detected were *Candidatus* Mycoplasma haematoparvum (55; 19.9%), *Hepatozoon canis* (52; 18.8%), and *Mycoplasma haemocanis* (49; 17.8%). We also detected double (32; 11.6%) and triple (5; 1.8%) infections, with the latter involving combinations of *Mycoplasma* spp., *Dirofilaria repens*, *Dirofilaria immitis*, *H. canis*, or *Babesia vulpes*. In addition, prevalence rates were calculated and mapped by district. Of all included factors, significant prevalence differences were found for purebred/mixed breed dogs as well as between age groups.

Conclusions This study provides the first comprehensive polymerase chain reaction (PCR)-based screening and detection of vector-borne pathogens in dogs from Kosovo and highlights the circulation of pathogens with high veterinary importance and zoonotic potential.

Keywords Dogs, PCR, Kosovo, Vector-borne pathogens

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Background

Canine vector-borne pathogens (CVBP) comprise a wide range of globally distributed pathogens, including viruses, bacteria, protozoan parasites, and helminths that are transmitted by arthropod vectors such as ticks, fleas, mosquitoes, and phlebotomine sand flies [1]. Dogs can act as reservoir hosts and many CVBPs might develop after long incubation periods without pathognomonic clinical signs, which makes diagnosis challenging. Some also potentially cause life-threatening complications [2]. Combating these diseases requires a One Health approach as many of these pathogens have zoonotic potential, and living in close contact with dogs might promote spillover to human populations [3].

Among many others, the tick-borne pathogens *Anaplasma* spp., *Ehrlichia* spp., and *Rickettsia* spp., the mosquito-borne nematodes *Dirofilaria immitis* and *Dirofilaria repens*, as well as the sand fly-borne protozoan parasites *Leishmania* spp. have been reported to infect both humans and dogs [4–6]. In recent decades, globalization (e.g., commercial transportation, tourism, and dog travel), along with climate and environmental changes, has promoted the growth of vector populations, the shift in CVBD distribution across Europe, and their introduction to previously nonendemic areas [7].

The Balkan area harbors a diverse vector fauna; however, data on CVBD are often scant and based on heterogeneous detection methods (e.g., serology, polymerase chain reaction (PCR)-based) or completely missing for some countries [8–10]. In the Republic of Kosovo, a landlocked country in the center of the Balkans bordered by Montenegro, Serbia, Albania, and North Macedonia, data on vector-borne diseases are scarce, particularly for dogs. Particularly, the high number of stray dogs and their exports to other European countries urge for detailed understanding of CVBDs in the country. Few recent studies indicate the endemicity of important vector species and the circulation of associated diseases. The most abundant tick species, *Ixodes ricinus*, was found infected with *Anaplasma phagocytophilum*, *Babesia microti*, *Borrelia* spp., and *Rickettsia* spp. [11, 12]. In addition, antibodies against *Anaplasma*, *Borrelia*, and *Ehrlichia* in dogs have been reported by Sinani et al. in 2020 [13]. Noteworthy, data on the brown dog tick (*Rhipicephalus sanguineus* sensu lato) and its importance as a vector of CVBPs are currently lacking from Kosovo. The mosquito fauna has been investigated to comprise 13 species of six genera, and *Aedes albopictus*, the Asian tiger mosquito, was detected in 2020 in the southern part of the country for the first time [14, 15]. *Dirofilaria immitis* seropositive dogs, which had prevalence rates up to 28.6%, were observed in six of the seven districts of Kosovo [13]. Recent studies have highlighted a diverse sand fly fauna comprising nine species, with the first detection of *Leishmania infantum* in vector species and canine leishmaniasis seroprevalence in six of seven districts [16–18].

Considering the high number of stray dogs and many privately owned dogs that are primarily kept outside, further elucidation of the circulation of CVBDs, particularly those of zoonotic concern, is crucial to combat their transmission and spread. Therefore, this study aimed to analyze blood samples of dogs that have previously been subject to a canine leishmaniasis seroprevalence study [18] by targeting the detection of selected vector-borne pathogens with PCR and sequencing.

Methods

Study area

The present study was conducted in the Republic of Kosovo, a landlocked country in the center of the Balkan Peninsula in South-Eastern Europe. It is located between latitudes 41° and 43° N and longitudes 20° and 22° E. The study area has a continental climate with Mediterranean and Alpine influences. Kosovo is divided into seven districts according to the law of Kosovo, namely Pristina (01), Mitrovica (02), Peja (03), Prizren (04), Ferizaj (05), Gjilan (06), and Gjakova (07). In the countryside, agricultural activities include farming various animals (cattle, sheep, goats, poultry, and pigeons). Many dogs (stray, kept in private households, or shepherd dogs) are widely present.

Dog samples and sample size

For this study, available blood samples taken in the frame of a *Leishmania* seroprevalence study in Kosovo [18] were used, which originated from dogs in private households, stray dogs (kept in shelters), and shepherd dogs. All samples were collected following the basic ethical principles and were marked by the name of the dog or chip number, location, age, breed, sex, and health status (random pathology, e.g., dermatitis, arthritis, tumor, and vasculitis).

DNA extraction and PCR-based pathogen detection

Before DNA isolation, samples were vortexed, 200 µL blood was placed in a new tube, and 200 µL phosphate-buffered saline (PBS) was added. Thereafter, 200 µL of AL buffer was added, vortexed, and incubated at 56 °C at 550 rpm for 10 min. Then, DNA isolation was performed using the Dneasy® Blood and Tissue Kit 250 (Qiagen, Hilden, Germany) following the manufacturer's protocol with final elution in 100 µL. The DNA was stored at −20 °C until further use.

All DNA samples were tested by PCR for the presence of DNA of the following pathogens: *Anaplasma* spp., *Babesia* spp., *Bartonella* spp., *Ehrlichia* spp.,

Filarioidea, *Hepatozoon* spp., *Mycoplasma* spp., *Rickettsia* spp., and *Trypanosoma* spp. In addition, samples showing positive results for filarioid helminths were analyzed with species-specific PCRs for *D. immitis* and *D. repens* for inclusion of potential mixed infections. Primers and PCR protocols are presented in Table 1.

All PCRs were performed with either a GoTaq® DNA Polymerase Master Mix (Promega, Walldorf, Germany) or a 2×EmeraldAmp® GT PCR Master Mix (Takara Bio Europe AB, Göteborg, Sweden) in a final volume of 25 µL with either an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) or Biometra® Cycler (Analytik Jena, Jena, Germany). Bands were analyzed, cut out

Table 1 PCR-based protocols for the detection of various pathogens used in this study

Organism target (length)	Primer 5′-3′	Protocol	References
Anaplasmataceae 16S rRNA (345 bp)	EHR16SD-for: GGTACCYACAGAAGAAGTCC EHR16SR-rev: TAGCACTCATCGTTTACAGC	95 °C/2 min; 35 cycles: 94 °C/1 min, 54 °C/30 s, 72 °C/30 s; 72 °C 5 min	[113]
Babesia[a] 18S rRNA (700 bp)	BTH-1F: CCTGAGAAACGGCTACCACATCT BTH-1R: TTGCGACCATACTCCCCCCA	94 °C/2 min; 40 cycles: 95 °C/30 s, 68 °C/1 min, 72 °C/1 min; 72 °C 10 min	[114]
18S rRNA (561 bp)	GF2: GYYTTGTAATTGGAATGATGG GR2: CCAAAGACTTTGATTTCTCTC	94 °C/2 min; 40 cycles: 95 °C/30 s, 60 °C/1 min, 72 °C/1 min; 72 °C 10 min	[115]
Bartonella 16S–23S rRNA (179 bp)	bartgd_for: GATGATGATCCCAAGCCTTC B1623_rev: AACCAACTGAGCTACAAGCC	95 °C/10 min; 30 cycles: 95 °C/15 s, 60 °C/1 min, 72 °C/20 s; 72 °C 5 min	[37]
Filarioidea[b] *coxI* (668 bp)	COlint_F: TGATTGGTGGTTTTGGTAA COlint_R: ATAAGTACGAGTATCAATATC	94 °C/2 min; 8 cycles with 0.5 °C reduction/step: 94 °C/45 s, 51 °C/45 s, 72 °C/1.5 min; 25 cycles: 94 °C/45 s, 45 °C/45 s, 72 °C/1.5 min; 72 °C 7 min	[116]
Hepatozoon 18S rRNA (620 bp)	H14Hepa18SFw: GAAATAACAATACAAGGCAGTTAAAATGCT H14Hepa18SRv: GTGCTGAAGGAGTCGTTTATAAAGA	95 °C/2 min; 35 cycles: 95 °C/1 min, 58 °C/1 min, 72 °C/1 min; 72 °C 7 min	[37]
Mycoplasma 16S rRNA (600 bp)	HBT-F: ATACGGCCCATATTCCTACG HBT-R: TGCTCCACCACTTGTTCA	94 °C/2 min; 40 cycles: 95 °C/1 min, 60 °C/1 min, 72 °C/1 min; 72 °C 7 min	[117]
Rickettsia 23S/5S rRNA (350–550 bp)	ITS_F: GATAGGTCGGGTGTGGAAG IST_R: TCGGGATGGGATCGTGTG	96 °C/4 min; 35 cycles: 94 °C/1 min, 52 °C/1 min, 72 °C/2 min; 72 °C 3 min	[118]
Trypanosomatidae[a] 18S rRNA (~1320 bp)	Tryp_18S_F1: GTGGACTGCCATGGCGTTGA Tryp_18S_R1: CAGCTTGGATCTCGTCCGTTGA	96 °C/5 min; 35 cycles: 94 °C/1 min, 56 °C/1 min, 72 °C/1 min; 72 °C 5 min	[119]
18S rRNA (~960 bp)	Tryp_18S_F2: CGATGAGGCAGCGAAAAGAAATAGAG Tryp_18S_R2: GACTGTAACCTCAAAGCTTTCGCG	96 °C/5 min; 35 cycles: 94 °C/1 min, 56 °C/1 min, 72 °C/1 min; 72 °C 5 min	
Dirofilaria immitis[c] COI (203 bp)	DI COI-F1: AGTGTAGAGGGTCAGCCTGAGTTA DI COI-R1: ACAGGCACTGACAATACCAAT	94 °C/2 min; 32 cycles: 94 °C/30 s, 58 °C/30 s, 72 °C/30 s; 72 °C 7 min	[120]
Dirofilaria repens COI (209 bp)	DR COI-F1: AGTGTTGATGGTCAACCTGAATTA DR COI-R1: GCCAAAACAGGAACAGATAAAACT	94 °C/2 min; 32 cycles: 94 °C/30 s, 58 °C/30 s, 72 °C/30 s; 72 °C 7 min	[120]

[a] Nested PCR, [b]touchdown PCR, [c]*Dirofilaria* discrimination for mixed infections

from the gel, and purified, as described elsewhere [16]. The samples were sent to Microsynth (Microsynth Austria GmbH, Vienna, Austria) and LGC Genomics (LGC Genomics GmbH, Berlin, Germany) for Sanger sequencing. The sequences were uploaded in the National Center for Biotechnology Information (NCBI) sequence database (accession numbers in the results section) and compared with reference sequences using the Basic Local Alignment Search Tool (BLAST) in NCBI GenBank.

Statistical analysis and mapping of prevalences

Data were prepared with Microsoft Excel for Mac and analyzed with RStudio for Mac [19]. Categorical data were analyzed using Fisher's exact test, using overall prevalence as a predictor variable. Odds ratios (OR) with exact 95% confidence intervals (CI) were estimated. A two-sided P-value < 0.05 was considered statistically significant. Prevalence was mapped with QGIS [20] using first-level administrative divisions of Kosovo (year 2015) taken from https://earthworks.stanford.edu/catalog/stanford-zh532mm5047.

Results

Detected pathogens

Overall, samples of 276 dogs were analyzed, comprising 138 females and 138 males from all seven districts collected in 1 year between summer 2021 and spring 2022. The mean age was 3.8 years (standard deviation (SD): 2.7 years), with the youngest dog being 4 months old and the oldest 16 years old. In total, 107 (38.8%) were purebred and 169 (61.2%) were mixed breeds. Of all dogs, 238 (86.2%) were classified as healthy and 38 (13.8%) as disrupted (random pathology unrelated to canine leishmaniasis such as dermatitis, arthritis, tumor, or vasculitis). Of the samples, 50 originated from Pristina district (01), 40 from Mitrovica (02), 35 from Peja (03), 38 from Prizren (04), 35 from Ferizaj (05), 40 from Gjilan (06), and 38 from Gjakova (07).

DNA of at least one pathogen was detected in 150 (54.3%; 95% CI 48.3–60.3) dogs, comprising eight pathogens of five genera (Table 2). Altogether, 55 (19.9%; 95% CI 15.5–25.2) DNA samples were positive for *Candidatus* Mycoplasma haematoparvum, 52 (18.8%; 95% CI 14.5–24.1) for *Hepatozoon canis*, 49 (17.8%; 95% CI 13.5–22.9) for *Mycoplasma haemocanis*, 15 (5.4%; 95% CI 3.2–9.0) for *Dirofilaria immitis*, 11 (4.0%; 95% CI 2.1–7.2) for *D. repens*, 4 (1.5%; 95% CI 0.5–3.9) for *Babesia vulpes*, 3 (1.1%; 95% CI 0.3–3.4) for *B. gibsoni*, and 1 (0.4%; 95% CI 0–2.3) for *Anaplasma phagocytophilum*. No *Bartonella* spp., *Ehrlichia* spp., *Rickettsia* spp., and *Trypanosoma* spp. DNA was detected.

Significant differences in overall prevalence were only found for the parameters of breed and age group (Table 3). The prevalence was significantly higher in purebred compared with mixed-breed dogs (OR = 1.7, P = 0.04), and significantly higher prevalence rates were found in the age groups 4–6 years (OR = 2.6, P = 0.03), 6–8 years (OR = 4.1, P = 0.02), and over 8 years (OR = 2.8, P = 0.05) compared with the youngest dogs from the 0–2 years age group (Table 3). No significant differences were observed between sexes or health status.

Co-infections

Of all positive samples, 113 (40.9%) were single, 32 (11.6%) were double, and 5 (1.8%) were triple infections. All pathogens except *A. phagocytophilum* were associated with at least one double infection. Generally, co-infections with two pathogens were highest, including *H.*

Table 2 Detected pathogens by sex, health status, and breed

Pathogen	Sex		Health status		Breed	
	Female	Male	Normal	Disrupted	Purebred	Mixed
A. phagocytophilum	1 (0.7%)	–	1 (0.4%)	–	1 (0.9%)	–
Babesia	1 (0.7%)	6 (4.4%)	5 (2.1%)	2 (5.3%)	7 (6.5%)	–
B. gibsoni	1 (0.7%)	2 (1.5%)	1 (0.4%)[a]	2 (5.3%)[a]	3 (2.8%)	–
B. vulpes	–	4 (2.9%)	4 (1.7%)	–	4 (3.7%)	–
Dirofilaria	13 (9.4%)	7 (5.1%)	17 (7.1%)	3 (7.9%)	9 (8.4%)	11 (6.5%)
D. immitis	9 (6.5%)	6 (4.4%)	12 (5.0%)	3 (7.9%)	7 (6.5%)	8 (4.7%)
D. repens	9 (6.5%)	2 (1.5%)	10 (4.2%)	1 (2.6%)	6 (5.6%)	5 (3.0%)
Hepatozoon canis	24 (17.4%)	28 (20.3%)	45 (18.9%)	7 (18.4%)	15 (14.1%)	37 (21.9%)
Mycoplasma	45 (32.6%)	60 (43.5%)	90 (37.8%)	15 (39.5%)	51 (47.7%)[a]	54 (32.0%)[a]
M. haemocanis	24 (17.4%)	25 (18.1%)	40 (16.8%)	9 (23.7%)	18 (16.8%)	31 (18.3%)
Candidatus M. haematoparvum	21 (15.2%)	34 (24.6%)	50 (21.0%)	5 (13.1%)	32 (29.9%)[a]	23 (13.6%)[a]

[a] Significant difference (P < 0.05)

Table 3 Overall prevalence associated with different factors

Parameter	Factor	Sample (*n*)	Positive (%)	OR (95% CI)	*P*-value
Sex	Female	138	70 (50.7%)	Reference	–
	Male	138	80 (58.0%)	1.3 (0.8–2.2)	0.28
Health Status	Disrupted	128	238 (53.8%)	Reference	–
	Normal	22	38 (57.9%)	1.2 (0.6–2.5)	0.73
Breed	Mixed	83	169 (49.1%)	Reference	–
	Purebred	67	107 (62.6%)	1.7 (1.0–2.9)[a]	0.04
Age	0–2 years	42	17 (40.5%)	Reference	–
	2–3 years	31	63 (49.2%)	1.4 (0.6–3.4)	0.43
	3–4 years	58	27 (46.6%)	1.3 (0.5–3.1)	0.68
	4–6 years	61	39 (63.9%)	2.6 (1.1–6.3)[a]	0.03
	6–8 years	23	17 (73.9%)	4.1 (1.2–15.4)[a]	0.02
	> 8 years	29	19 (65.5%)	2.8 (0.9–8.4)[a]	0.05
District	Pristina 01	50	24 (48.0%)	Reference	–
	Mitrovica 02	40	20 (50.0%)	1.1 (0.4–2.7)	1
	Peja 03	35	20 (57.1%)	1.4 (0.6–3.8)	0.51
	Prizren 04	38	26 (68.4%)	2.3 (0.9–6.3)	0.08
	Ferizaj 05	35	21 (60.0%)	1.6 (0.6–4.3)	0.38
	Gjilan 06	40	17 (42.5%)	0.8 (0.3–2.0)	0.67
	Gjakova 07	38	22 (57.9%)	1.5 (0.6–3.8)	0.4

[a] Significant difference ($P < 0.05$)

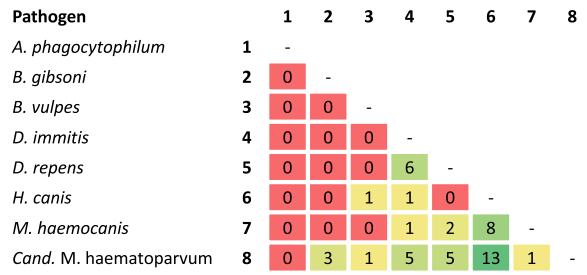

Fig. 1 Number of detected double infections

canis and *Candidatus* M. haematoparvum (13/32, 40.6%), followed by *H. canis* and *M. haemocanis* (8/32, 25.0%) and *D. immitis* and *D. repens* (6/32, 18.8%) (Fig. 1).

No significant differences in overall double infections associated with sex, health status, or breed were detected, neither between age groups nor districts.

Co-infections with three pathogens were observed in five dogs, all involving *Mycoplasma*, four *Dirofilaria*, two *H. canis*, and one *B. vulpes* (Supplementary Table 1). No significant differences between triple infections and risk factors were detected; however, a much higher but not significant triple infection rate was observed in purebred compared with mixed breeds (OR = 6.5, $P = 0.08$).

Prevalence by district

Overall prevalence rates were highest in Prizren (68.4%) and lowest in Gjilan (42.5%) (Fig. 2). In dogs originating from Gjakova, seven of eight pathogens were detected, followed by Pristina, Peja, and Prizren with six pathogens, and Mitrovica, Ferizaj, and Gilan with five pathogens (Table 3). *Dirofilaria immits*, *H. canis*, *M. haemocanis*, and *Candidatus* M. haematoparvum were detected in all seven districts, whereas *Anaplasma phagocytophilum* was only detected in a dog from Gjakova 07.

Pathogen typing

Anaplasma phagocytophilum was detected in one female dog only. The obtained sequence was identical to bacteria described in dogs from South Korea (MK239931) [21] and grey wolves (*Canis lupus*) from Germany (MN790646) [22].

DNA of *B. vulpes* (PP462098) was detected in 2.9% of the blood samples, which were identical to parasites documented in dogs from Russia (MT509981) [23]; Kyrgyzstan (OR116236) [24]; Spain [25, 26]; France [27]; and a confiscated pit bull terrier in the USA named *Babesia* sp. "Spanish dog" (EU583387) [28]. Moreover, it was examined in *Dermacentor reticulatus* in Austria [29] and raccoon dogs (*Nyctereutes procyonoides*) in Austria [30] and South Korea (OM510442). It was mainly detected in red foxes (*Vulpes vulpes*) in Austria [31, 32], Italy (KY486299), Spain (KT223483), Turkey [33], China (MW192450), the UK [34], the Czech Republic [35], Slovakia (KY175167), Croatia [36], and Bosnia and Herzegovina (KP216411) [37]. *Babesia gibsoni* (PP462099) was confirmed in three dogs, and sequences were identical to parasites described in dogs from Italy (MT752610) [38], the USA (DQ184507), Saint Kitts and Nevis (JX112784) [39], India (MN134517), Sri Lanka (OQ396762), China (CP141527), Taiwan (FJ769386), Japan (LC012808), and Myanmar (LC602469). An identical haplotype was reported from a male Boxer in Austria with travel history to Serbia, showing a co-infection with *B. canis* and *B. gibsoni* [40].

Four different haplotypes of *H. canis* (PQ836159–PQ836162) were observed. In 25 (9%) of the dogs,

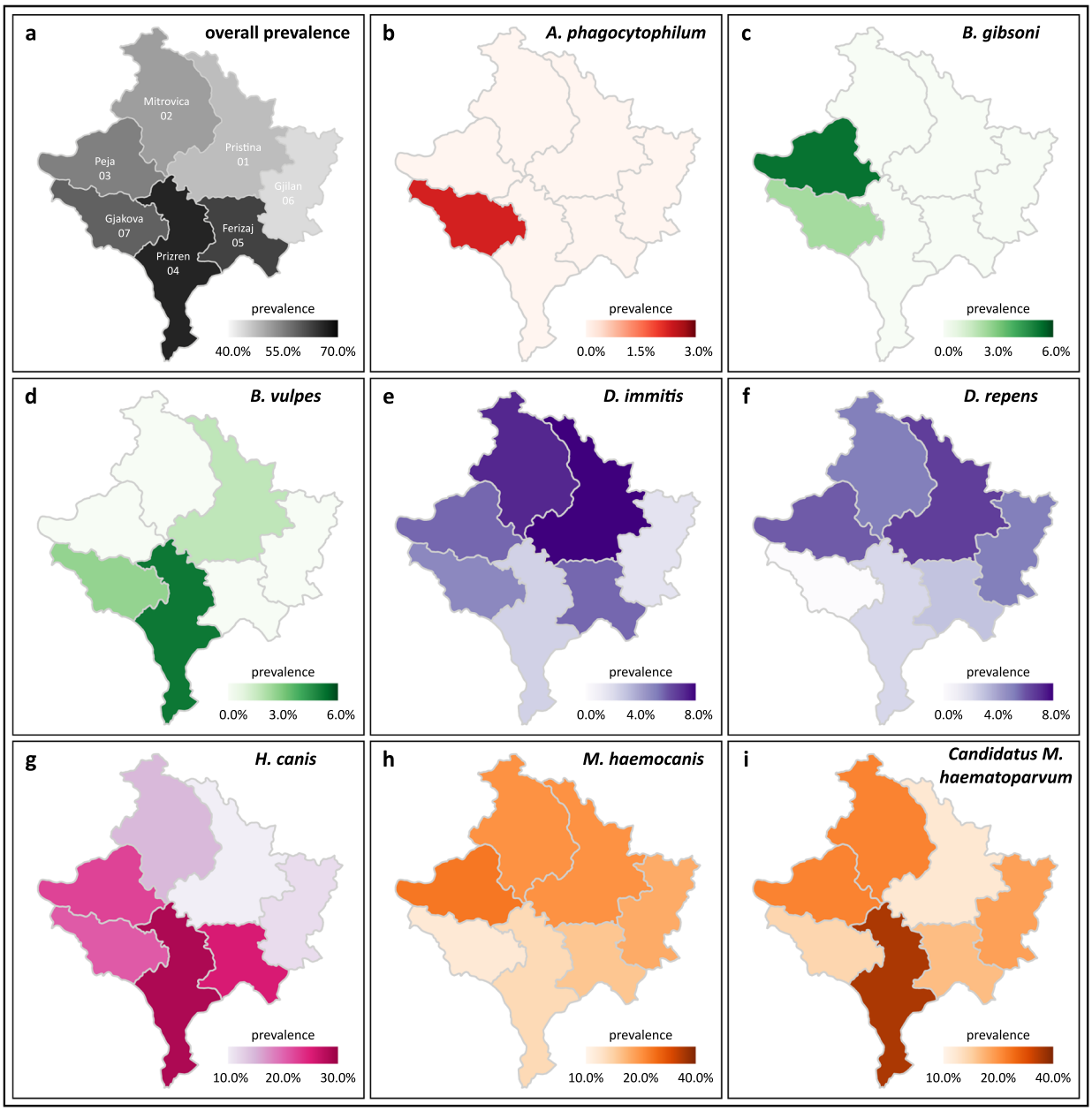

Fig. 2 Prevalence of detected pathogens by district. Overall prevalence (**a**), *A. phagocytophilum* (**b**), *Babesia gibsoni* (**c**), *Babesia vulpes* (**d**), *Dirofilaria immitis* (**e**), *Dirofilaria repens* (**f**), *Hepatozoon canis* (**g**), *Mycoplasma haemocanis* (**h**), and *Candidatus* Mycoplasma haematoparvum (**i**)

a haplotype (PQ855756) was found that was identical to dog samples from India (PP859411), Zambia (LC331054), Australia (MG062866) and from Eurasian golden jackals (*Canis aureus*) in Romania (KX712129; [41]) and *Ixodes holocyclus* in Australia (MG758124; [42]). Haplotype 2 (PQ836160) could be confirmed in four dogs and was identical to *H. canis* described in dogs from Cuba (MN393911), Uruguay (OR814220), and Malawi (LC169075; [43]); an Iberian wolf (*Canis lupus signatus*; PP574309) from Spain; red foxes (*V. vulpes*) in Austria (KM115969; [31]); Pampas foxes (*Lycalopex gymnocercus*) in Brazil; *C. aureus* from Austria (Mitkova et al. 2017); and *Haemaphysalis longicornis* in China and Japan (MT107096, LC169075; [44]). The third haplotype (PQ836161) was observed in two animals and was identical to pathogens in dogs in the Czech Republic (KU893127; [45]); red fox samples from Austria (KM115984, [31]) and the Czech

Republic (ON128264); and raccoon (*Procyon lotor*) in the Czech Republic (OQ816791; [35]). A single sample (PQ836162) varied in one bp from the most abundant haplotype (PQ836159).

Three haplotypes of *D. repens* were documented in this study (PP552045–PP552047). Haplotype 1 (PP552045) was with one bp difference identical to findings in dogs in Italy (MT345575) [46], Slovenia (OP494254) [47], Austria (MW590257) [48], the Czech Republic (MW675691) [49], and Finland (KY828979); humans in the Czech Republic (KR998257, MW017212) [50], Slovenia (OP494269) [47], Croatia (KX265049) [51], and Finland (KY828978) [52]; and *Anopheles plumbeus* in Austria (MF695085) [53]. Haplotype 2 was identical to findings in dogs in Italy (KX265048) [51]; a human in Croatia (MT847642) [54]; and *An. daciae* in Germany (KF692102) [55]. The third haplotype was identical to findings in a dog in the Czech Republic (MW675692) [49] and humans in Italy (KT899073) [56] and Spain (MH780816) [57].

Only one haplotype (PP552044) of *D. immitis* with global distribution was documented in this study. It was identical to isolates collected in dogs from Slovenia (OP494255) [47], Hungary (KM452920) [58], Italy (AM749229, FN391553) [59], Iran (KR870344, MZ266350) [60], Bangladesh (KC107805) [61], Myanmar (ON259772) [62], Thailand (MT027229) [63], China (EU159111) [64], and Chile (OP811228) [65]. Moreover, the partial mt *COI* sequence was identical to *D. immitis* examined in grey wolf in Italy (DQ358815) [66]; golden jackal (*Canis aureus*) in Iran (MZ266360) [67]; coyote (*Canis latrans*) in the USA (ON062409) [68]; *Culex pipiens* s.l. in Spain (LC107816) [69] and Hungary (KM452924) [58]; *Cx. quinquefasciatus in* Myanmar (OL721654) [70]; domestic cat in the USA (OQ359099) [71]; and humans in Iran (MH920260) and Thailand (MW577348) [72].

Mycoplasma haemocanis (PQ846586) isolates were identical to samples collected in dogs in Bosnia and Herzegovina (MK107818, MK107817, MK107816), Turkey (KX641903), and also Asia and South America. Moreover, the same haplotype was documented in a red fox in Slovakia (KX752055) and cats in Brazil (KM275246, KM275242). Two haplotypes of *Candidatus* Mycoplasma haematoparvum (PQ846588, PQ846589) were documented. The haplotype (PQ846588) was identical to samples collected in dogs from Bosnia and Herzegovina (MK107815, MK107814), Italy (MH094850), Switzerland (EF416569), Romania (KY433884), and also Cuba (MZ221181), Iran (KU886262, KC762746), Iraq (PP903626), and Thailand (KT359592). Moreover, this haplotype was documented in a human with extensive animal contact (KF366443; [73]). In addition, a single sample (PQ846589) differed in one bp.

Discussion

This study comprehensively reports, to the best of our knowledge, the first PCR-based screening and detection of vector-borne pathogens in dogs from Kosovo. Altogether, the DNA of eight pathogens of five genera was successfully amplified and sequenced. The observed overall prevalence of 54.3% highlights the circulation of various vector-borne pathogens in dogs in all districts of the country.

Of all evaluated factors, we found significantly higher infection rates in purebred dogs compared with mixed-breed dogs. Generally, genetic disorders or cancer predispositions can cluster in inbred dog populations [74, 75]. For infectious diseases, breed predispositions to disease are controversially discussed, for some infectious agents, e.g., *Leishmania infantum*, significantly higher seroprevalence rates have been found in breeds such as the Doberman Pinscher or Boxer breeds [76], while Ibizan hounds have been observed to be more resistant to *Leishmania* infections [77]. Particularly, the tick-borne pathogens *A. phagocytophilum* and *Babesia* spp. were only found in purebred dogs in our study. In contrast, Facile et al. [78] detected significantly higher infection rates and double infections of tick-borne pathogens in mixed-breed dogs than in purebreds.

In addition, all age classes above 4 years compared with 0–2 years of age showed significantly higher infection rates, being highest in the age class of 6–8 years with a prevalence of 73.9%. This is in line with other studies, particularly in endemic regions, where age correlates with the time of exposure to vectors during a dog's life [79, 80].

We also observed higher but not significant prevalence rates in males (58%) compared with females (50.7%), which is in line with literature. Several studies on tick-borne pathogens have reported that sex is not a risk factor or a slight predisposition in male dogs, possibly related to their behavior, which exposes them to ticks more frequently [81–83].

We report the first detection of *A. phagocytophilum* by PCR in a dog from Kosovo. Only one dog originating from Gjakova was positive, resulting in a low overall prevalence of 0.4%, which is similar to a study from neighboring Albania reporting a prevalence among dogs of 1% [84]. While prevalences based on PCR and serology are generally not comparable, we would like to highlight that Sinani et al. [13] reported an *A. phagocytophilum* seroprevalence of 25% in dogs from Kosovo, which is comparable to other Balkan countries such as Croatia (4.5%) [85], Bosnia and Herzegovina (20.7%) [86], and Serbia (28.8%) [87] and confirms circulation. Discrepancies between serological and polymerase chain reaction (PCR) results are commonly reported [84, 86], as PCR is

generally the most sensitive diagnostic method, but often only detects recent infections. While antibody tests can still detect past exposure through the presence of antibodies, PCR may become negative over time [88].

Two *Babesia* species were detected, namely *B. vulpes* and *B. gibsoni*, which need further discussion. To date, only one study indicated the circulation of *Babesia* in dogs from Kosovo, namely *B. canis*, the main *Babesia* species infecting dogs [89]. *Babesia vulpes*, formerly known as *Theileria annae*, primarily infects red foxes (*V. vulpes*), which most certainly represent the reservoirs, as high asymptomatic infection rates are observed regularly [90]. On the contrary, sporadic infections have been reported from dogs, and the vector involved is yet unclear; different *Ixodes* species, as well as *R. sanguineus* sensu lato, have been suspected [91–93]. *Babesia gibsoni* is a parasite of dogs, and the principal vector is *R. sanguineus* sensu lato [94]. In dogs, prevalence rates of both species are regularly low, but both small *Babesia* species are known to cause acute and chronic clinical manifestations in dogs, such as fever, lethargy, anorexia, mild-to-severe thrombocytopenia, and mild-to-severe regenerative anemia due to hemolysis, among others [95]. Noteworthy, we detected a significantly higher infection rate in dogs with disrupted health status compared with healthy dogs. In addition to our data, *B. vulpes* and *B. gibsoni* have been molecularly detected in dogs from Croatia and Serbia [95], highlighting the co-circulation of both species in Balkan countries. Thus, species identification should be applied with diagnosis in dogs and detection in ticks to evaluate potential vector species.

The detection of *Dirofilaria* DNA in dogs from all seven districts of Kosovo underlines the wide spreading of the pathogen in this region. To date, only *D. immitis* seroprevalences have been reported from dogs from Kosovo. However, *D. repens* has been reported in other Balkan countries such as Croatia, Bosnia and Herzegovina, and Serbia [96, 97]. Dirofilariosis is a disease of great veterinary importance with high zoonotic potential. *Dirofilaria repens* infections often develop asymptomatically, but nonspecific dermal alterations have been reported, such as skin nodules, pruritus, thinning, itching, and asthenia [96]. On the contrary, *D. immitis* is usually located in the heart of carnivores and causes heartworm disease (HWD) [98]. In humans, *Dirofilaria* generally does not complete its life cycle. However, few cases reported the detection of adult *D. repens*-producing microfilariae [54]. Usually, *D. repens* causes subcutaneous nodules or ocular manifestations, whereas *D. immitis* can cause pulmonary nodules [98]. Several mosquito species of different genera (*Aedes*, *Anopheles*, and *Culex*) have been implicated in the transmission of both *Dirofilaria* species [99]. In Kosovo, potential vector species, such as *Aedes vexans*, *Anopheles maculipennis* s.l., or *Culiseta annulata*, can be found. Particularly, *A. maculipennis* s.l. is highly abundant in all seven districts of Kosovo. The recent detection of *Aedes albopictus*, another potent vector, in Kosovo, might be involved in future transmission cycles if it spreads [14, 15]. Considering the frequent export of dogs from Kosovo to other (Central) European countries, the spread of dirofilariosis to previously nonendemic countries is likely and should be monitored by improved screening and preventive measures.

Hepatozoon canis is an apicomplexan protozoan parasite infecting domestic dogs and wild canids worldwide, and the brown dog tick, *R. sanguineus* sensu lato, serves as a vector. Transmission to the host typically happens by ingestion of ticks containing oocysts of the parasite, but vertical transmission in foxes is suspected [100, 101]. In our study, we found a *H. canis* infection rate of around 19%, similarly to other surveys reporting PCR-based prevalences in dogs of 17% in Albania [102], of 18.2% in Serbia [103], and of 26% in Hungary [104]. The infection in dogs is often subclinical but can manifest as a severe life-threatening disease with fever, cachexia, lethargy, and anemia [105], with adverse effects resulting from co-infections with other bacteria or hemoparasites [106]. We did not observe a significant difference between infection rates of healthy and disrupted dogs. However, *H. canis* presence was associated with 22 co-infections (including 2 triple infections) involving *Babesia*, *Dirofilaria*, and *Mycoplasma*, which could result in more severe clinical cases.

Similar infection rates were also detected for the hemotropic bacteria *Candidatus* Mycoplasma haematoparvum (19.9%) and *Mycoplasma haemocanis* (17.8%). Those hemotropic mycoplasmas, or hemoplasmas, are commonly known to cause chronic, subclinical infection in immunocompetent dogs but can cause hemolytic anemia in splenectomized dogs. Lower prevalence rates have been reported in Albania (8.8% for *M. haemocanis*) [84] and Greece (4.2% for *Candidatus* M. haematoparvum and 5.6% for *M. haemocanis*) [107]. The natural mode of transmission of canine haemoplasmas is currently unknown; however, *R. sanguineus* sensu lato ticks have been hypothesized as vectors owing to the usually higher prevalence rates observed in *R. sanguineus* sensu lato endemic Mediterranean countries [108, 109]. In addition, other hematophagous insects such as fleas (Siphonaptera), sucking lice (Anoplura), or keds (Hippoboscidae) have been assumed to play a role as vectors [110]. Considering the potential involvement of *R. sanguineus* sensu lato in the transmission of *Babesia*, *Hepatozoon*, and hemotropic *Mycoplasma*, the currently unclear status of this tick species in Kosovo should give

rise to further studies assessing their presence in the country.

Noteworthy, the detection of DNA of vector-borne pathogens by PCR is not necessarily associated with symptoms and does not confirm active infections. In addition, we did not detect *Bartonella*, *Rickettsia*, or trypanosomatids (including *Leishmania*), probably owing to different reasons. *Bartonella* infections in dogs seem rare in Balkan countries and have been minorly addressed. For instance, Hamel et al. [102] did not detect *Bartonella* DNA in dogs from Albania, and only an infection rate of 0.7% was detected in dogs from the Czech Republic [111]. The circulation of *Rickettsia* in the Balkans seems to be evident and increasing. However, detection in dogs is underreported. In addition, the bacteria might only be detectable in blood shortly after infection, and, thus, serology or the PCR-based screening of other tissue might be more appropriate [112]. Similarly, the absence of *Leishmania* DNA in our samples can be explained. An overall canine leishmaniasis seroprevalence of 4.2% was observed among our samples in a previous study by Xhekaj et al. [18], and the endemicity of leishmaniasis is apparent in the country [16]. However, owing to the intracellular nature of the parasites, the circulation in the blood is higher shortly after infection. Generally, lymph nodes, spleens, or skin samples of infected animals should be favored, if available.

Conclusions

For the first time, we have molecularly assessed and proven the infection of dogs with various vector-borne pathogens in the Republic of Kosovo. Our study highlights the circulation of pathogens with high veterinary importance and zoonotic potential and urges for the development of disease control strategies. High numbers of stray and shelter dogs kept outside might promote the local transmission of CVBDs. Particularly, the implementation of routine monitoring of shelter dogs (which are mostly captured stray dogs) by serology combined with PCR-based diagnosis of recent infections could be a tool to monitor and counteract the establishment of new disease transmission hotspots. However, successful countermeasures include effective treatment, if available, which can be costly and might display a limiting factor.

Given the underreported nature of vector-borne diseases in Kosovo, our results should definitely be used to raise awareness among veterinarians and serve as baseline data for further regular studies.

Abbreviations

AL buffer	Lysis solution buffer
CVBD	Canine vector-borne disease
CVBP	Canine vector-borne pathogens
CI	Confidence intervals
OR	Odds ratio
PBS	Phosphate-buffered saline
rpm	Rounds per minute
PCR	Polymerase chain reaction
SD	Standard deviation
s.l.	Sensu lato

Supplementary Information

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Supplementary Material 1: Table 1. Triple infections by sex, health status, and breed.

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Author contributions

B.X.—data curation, formal analysis, methodology, and writing—review and editing. E.K.—formal analysis, methodology, writing—original draft, and writing—review and editing. L.W—methodology, and writing—review and editing. I.H.—methodology and writing—review and editing. B.E.—methodology and writing—review and editing. J.S.—supervision and writing—review and editing. A.C.—supervision and writing—review and editing. K.S.—project administration, supervision, and writing—review and editing. H.P.F.—data curation, formal analysis, methodology, writing—original draft, and writing—review and editing.

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Availability of data and materials

No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

Blood samples were tested as remnants of a previously published study on *Leishmania* seroprevalence [18], which was conducted in compliance with the regulations of the Department of Hygiene, Welfare, and Ethology of Animals, Faculty of Agriculture and Veterinary, University of Prishtina "Hasan Prishtina". Sampling was performed following the approval of the Faculty on 19 March 2021. Scientific research works that include investigation of vector-borne emerging diseases in dogs are performed to diagnose animal diseases and improve animal welfare. No suffering was caused during the sample collection.

Consent for publication

Informed consent was obtained from all owners of dogs involved in the study.

Competing interests

The authors declare no competing interests.

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