High-throughput microfluidic real-time PCR for the simultaneous detection of selected vector-borne pathogens in dogs in Bosnia and Herzegovina

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Abstract
A scarcity of information on the occurrence of zoonotic vector-borne pathogens (VBPs), alongside a lack of human and animal health authorities’ awareness of pre-existing data, augment the risk of VBP infection for local people and limit our ability to establish control programs. This holds especially true in low-middle income countries such as Bosnia and Herzegovina (BiH). This dearth of information on zoonotic VBPs is bolstered by the inability of previously used diagnostic tests, including conventional molecular diagnostic methods, to detect the full spectrum of relevant pathogens. Considering this, we set out to apply a microfluidic qPCR assay capable of detecting 43 bacterial and protozoan pathogens from blood to accrue critical baseline data for VBPs occurrence in BiH. A total of 408 dogs were tested of which half were infected with at least one VBP of zoonotic or veterinary importance. Leishmania infantum was found in 18% of dogs, reaching a prevalence as high as 38% in urbanized areas of Sarajevo. These data highlight substantially higher levels of L. infantum prevalence when compared to that previously reported using conventional methods using the same samples. Additionally, this high-throughput microfluidic qPCR assay was able to detect pathogens rarely or never reported in canines in BiH, including Anaplasma phagocytophilum (3%), Anaplasma platys (0.2%), haemotropic Mycoplasma (1%) and Hepatozoon canis (26%). Our report of the endemity of important zoonotic pathogens and those of clinical significance to dogs emphasizes the need for urgent implementation of surveillance and control for VBPs in BiH, targeting both animal and human infections within the country.

KEYWORDS
Anaplasma, Bosnia and Herzegovina, companion animals, Leishmania, microfluidic real-time PCR, vector-borne zoonoses
1 | INTRODUCTION

Canine vector-borne pathogens (VBPs) encompass a broad diversity of disease-causing organisms, that include viruses, bacteria, protozoa, and helminths predominantly unified by their ability to be transmitted via blood-feeding arthropods, such as ticks, fleas, sandflies, and mosquitoes. Some VBPs generate severe disease solely in dogs, whilst others use canines as natural reservoir hosts of zoonotic agents thereby posing a risk to cohabiting humans as well (Otranto et al., 2017). To date, there has been very little information exploring the diversity and prevalence of canine VBPs in Bosnia and Herzegovina (BiH), with the few studies conducted thus far focusing on a limited number of key pathogen species, typically those of zoonotic significance (Ćoralić et al., 2018; Omeragić et al., 2018; Colella et al., 2019; Omeragić et al., 2021; Maksimović et al., 2022). These limited published studies have recorded high prevalence of canine VBP infection, exacerbated by a growing population of stray dogs since the end of the Bosnian war, that facilitates transmission and maintenance of VBPs in BiH (Ćoralić et al., 2018; Katica et al., 2019; Katica et al., 2020; Omeragić et al., 2021).

Prior investigations into canine VBPs in BiH have identified numerous zoonotic pathogens, in particular, a high prevalence of Leishmania spp. in dogs, alongside sporadic case reports of human infections (Alić et al., 2019; Colella et al., 2019; Obwaller et al., 2018; Vaselek, 2021). Human leishmaniasis are among the most important VBPs globally, implicated in approximately 0.2–0.4 million human cases of visceral leishmaniasis (VL) and 0.7–1.2 million cases of cutaneous leishmaniasis (CL) annually (Alvar et al., 2012). A proportion of these cases are caused by Leishmania infantum, a pathogen that utilizes canine as reservoir hosts (Alvar et al., 2012; Dantas-Torres, 2007; Okwor & Uzonna, 2016).

The tick species Ixodes ricinus and Dermacentor reticulatus are the most common ectoparasites infesting dogs in BiH (Krčmar et al., 2014; Omeragić, 2011). As a consequence, there have been reports of the bacterial VBP Borrelia burgdorferi sensu lato, which includes the etiological agents of Lyme disease that can be harboured by canids (Arapović et al., 2014; Dautović-Krkić et al., 2008), as well as the highly pathogenic, but not zoonotic, Babesia canis (Ćoralić et al., 2018). Additionally, the mosquito-transmitted filarial worms Dirofilaria immitis and Dirofilaria repens have been reported in Bosnian dogs (Omeragić et al., 2018; Omeragić et al., 2021), with evidence of infection in humans caused by the latter zoonotic nematode (Omeragić et al., 2021; Zvorničanin et al., 2020).

Other canine VBPs that are prevalent in this country include the Oriental eyeworm Thelazia callipaeda, a zoonotic nematode parasite implicated in occupational human infections in Eastern Europe (Colella et al., 2016; Hodžić et al., 2014; Paradžik et al., 2016), and the aposymbiont pathogen Hepatozoon canis (Hodžić et al., 2015). However, there have been no reports to date of bacterial VBPs infecting Bosnian canines, likely due to a dearth of research given that evidence of such pathogens has been found in neighboring countries (Huber et al., 2017; Laušević et al., 2019). In addition, rickettsial pathogens have been molecularly detected in Bosnian ticks, including the zoonotic bacterial species Anaplasma phagocytophilum (Hodžić et al., 2017), the causative agent of potentially lethal human granulocytic anaplasmosis.

This dearth of information on zoonotic VBPs in BiH is caused by both the limited studies so far conducted and the inability of previously used diagnostic tests to detect the full spectrum of relevant pathogens. For example, conventional molecular methods such as endpoint PCR and real-time PCR (qPCR) comprise some of the most sensitive and specific diagnostic methods available for the detection of VBPs, however, they can typically only detect a limited number of pathogens simultaneously and may require large volumes of template DNA per reaction (Gondard et al., 2018; Huggins et al., 2021; Michelet et al., 2014). More recently, advances in microfluidic technology have allowed for the development of microfluidic qPCR methods that employ microchips printed in chamber arrays as large as a 96 × 96, allowing for the simultaneous running of up to 9216 singleplex nanoliter quantity reactions (Gondard et al., 2020). The large number of reactions possible at any one time permits testing for numerous pathogens from multiple samples concurrently, reducing the amount of time, labour, and reagents used when compared to conventional qPCR, whilst also being able to easily identify coinfections. For example, microfluidic qPCR has been used to detect water-borne and tick-transmitted pathogens, permitting testing for as many as 37 organisms at the same time in various studies that have used it to unearth zoonoses as well as rare VBPs of veterinary importance (Gondard et al., 2020, 2018; Michelet et al., 2014; Sprong et al., 2019).

Given the significant paucity of research exploring VBPs and the potential zoonotic risk posed by some of them, we set out to use a microfluidic qPCR to test for 43 bacterial and protozoan pathogens and accrue baseline data for their occurrence in BiH. This is particularly timely given that economic and societal upheaval in BiH following the Bosnian war have had wide-reaching impacts and may have potentially made the country vulnerable to an increase in neglected diseases such as those caused by VBPs.

2 | MATERIALS AND METHODS

2.1 | Sample collection and DNA extraction

In 2018, blood samples were collected from 408 domestic dogs kept under different living conditions (free-roaming [n = 127], client-owned [n = 134], sheltered [n = 142] and not reported [n = 4]) from different urban localities of BiH, namely Sarajevo (n = 138), Gračanica (n = 63), Zenica (n = 44), Goražde (n = 40), Bihać (n = 40), Mostar (n = 35), Livno (n = 14), Gornji Vakuf-Uskoplje (n = 12), Ođak (n = 11) and Tuzla (n = 10) (Figure 1). Two millilitres of whole blood were collected per dog by a veterinarian via cephalic or jugular puncture into an anti-coagulation ethylenediaminetetraacetic acid (EDTA) tube and stored at −20°C until further analysis. Genomic DNA was extracted from these samples using the GenUP DNA Kit (Biotechnribbit, Germany), following the manufacturer’s recommendations.
2.2 DNA pre-amplification

All DNA samples were subjected to pre-amplification to enrich DNA content compared to host DNA using the PerfeCTa PreAmp SuperMix (Quanta Biosciences, Beverly, USA), following the manufacturer’s guidelines. All the primers were pooled (except positive control Escherichia coli culture EDL 933 strain), with a final and equal concentration of 200 nM each. The pre-amplification reaction was performed in a final volume of 5 µl containing 1 µl Perfecta Preamp 5x, 1.25 µl pooled primers mix, 1.5 µl distilled water and 1.25 µl DNA. PCR cycling conditions were one cycle at 95°C for 2 min followed by 14 cycles at 95°C for 10 s and 60°C for 3 min. At the end of the cycling program, the reactions were diluted at 1:10 and stored at −20°C until further use.

2.3 High-throughput microfluidic real-time PCR

The BioMark real-time PCR system (Fluidigm, USA) was used for high-throughput microfluidic real-time PCR amplification using 48.48 dynamic arrays (Fluidigm, USA). These chips dispensed 48 samples and 48 assays into individual wells, followed by on-chip real-time PCR reactions in individual chambers and thermal cycling, resulting in 2,304 individual reactions as reported in Michelet et al., 2014. Targeted microorganisms were Borrelia spp., Borrelia burgdorferi sensu stricto, Borrelia garinii, Borrelia afzelii, Borrelia valaisiana, Borrelia lusitaniae, Borrelia spielmanii, Borrelia bissettii, Borrelia miyamotoi, Anaplasma spp., Anaplasma marginale, Anaplasma platys, Anaplasma phagocytophilum, Anaplasma ovis, Anaplasma centrale, Anaplasma bovis, Ehrlichia spp., Ehrlichia canis, Neoehrlichia mikurensis, Rickettsia spp., Rickettsia conorii, Rickettsia slovaca, Rickettsia massiliae, Rickettsia helvetica, Rickettsia aeschlimannii, Rickettsia felis, Bartonella spp., Bartonella henselae, Francisella spp., Coxiella spp., Apicomplexa, Babesia microti, Babesia vogeli, Babesia ovis, Babesia bovis, Babesia caballi, Babesia venatorum, Babesia divergens, Mycoplasma spp., Theileria spp., Hepatozoon spp., Leishmania spp. and Leishmania infantum. Targeted genes and primers/probes set for the pathogens above listed are reported in Gondard et al., 2020; Michelet et al., 2014; Sprong et al., 2019.

Briefly, amplifications were performed using 6-carboxyfluorescein (FAM)- and black hole quencher (BHQ1)-labelled TaqMan probes with PerfeCTa qPCR ToughMix, Low ROX (QuantaBio) following a previously described protocol (Michelet et al., 2014). Two kinds of controls per chip were used for experimental validation: a negative water control to exclude contamination and internal control, to check for the presence of qPCR reaction inhibitors comprising Escherichia coli strain.
EDL933 DNA and relevant primers and probe to detect this (Gondard et al., 2020). Data were acquired on the BioMark Real-Time PCR System and analyzed using the Fluidigm Real-time PCR Analysis Software to obtain crossing point (CP) values. Each sample was run in duplicate and considered positive only when both reactions displayed a CP value. Samples with a CP value > 30 were considered negatives (cut-off value).

To ascertain the species of pathogens for which only targets at genus level were detectable via microfluidic real-time PCR (i.e., haemotropic Mycoplasma spp., Babesia spp., and Hepatozoon spp.), selected positive samples for the above-mentioned genera were also tested via conventional and nested PCRs using primers listed in Table 1. Amplicons were sequenced in both directions by LGC Genomics, Germany, using Sanger sequencing and nucleotide sequences were assembled for 204 dogs (50%; CI: 45–55%) were found positive for at least one VBP across all field sites investigated (Figure 2, Table 2). The estimated prevalence of VBP infection in dogs with at least one infection ranged from 8% (CI: 1–35%) in Gornji Vakuf-Uskoplje to 57% (CI: 45–69%) and 63% (CI: 47–76%) in Gračanica and Bihać, respectively (Figure 2). The most frequently identified target was Apicomplexa in 141 dogs (35%; CI: 30–39%), followed by Babesia/T. theileri (22–31%) and L. infantum in 74 dogs (18%; CI: 15–22%) (Table 2). Additionally, the zoonotic bacterial VBP A. phagocytophilum was detected in 11 canines (3%; CI: 2–5%), with peaks of 8% in Bihać and Gornji Vakuf-Uskoplje (Table 2).

Coinfections by two pathogens were diagnosed in 36 dogs (9%; CI: 6–12%), with the most common being H. canis with L. infantum (n = 204 dogs; 4%; CI: 2–6%), followed by A. phagocytophilum with H. canis (n = 7 dogs; 2%; CI: 1–4%) and Apicomplexa with L. infantum (n = 7 dogs; 2%; CI: 1–4%) (Table 3).

For the samples found positive by microfluidic real-time PCR that could not be characterized to species level for example samples found positive to Apicomplexa, Hepatozoon and haemotropic Mycoplasma we used cPCR and Sanger sequencing to gain species-level resolution. This allowed us to detect Babesia canis, H. canis, Mycoplasma haemotroparum and Mycoplasma haemocanis with all sequences showing 100% identity with reference sequences. These sequences have been deposited in GenBank under accession numbers MK107800-MK107818).

No model could be fitted with predictor variables for positivity to at least one VBP. However, we found evidence of an association between owned [OR 2.26 (95% CI: 1.17–4.5), p = 0.01] and stray [OR 2.23 (95% CI: 1.15–4.45), p = 0.02] dogs compared to sheltered animals on the odds of being infected by L. infantum.

4 DISCUSSION

The deployment of a high-throughput microfluidic qPCR method has elucidated a spectrum ofVBPs that Bosnian canines are infected with, including those that are zoonotic, with at least half of all dogs sampled infected with one VBP. In addition, this research has unearthed the presence of VBPs (e.g., A. phagocytophilum, A. platys, haemotropic...
mycoplasmas and *H. canis*) rarely or never reported in Bosnian dogs, therefore expanding the known geographical distributions of these pathogens.

With the aid of this highly sensitive technique, we were able to detect the zoonotic VBP *L. infantum* in 18% of dogs sampled across BiH, with a particularly high prevalence around urbanized areas of BiH, such as Sarajevo (38%) and Mostar (23%). These data highlight substantially higher levels of *L. infantum* prevalence when compared to that previously reported (Colella et al., 2019). Nonetheless, this study found similar foci for *L. infantum* infection to the data presented here, with prevalence highest in Mostar (16.7%), followed by Sarajevo (4.9%) (Colella et al., 2019).

*Leishmania infantum* is a causative agent of VL and CL in humans with prior research having an estimated annual incidence of 2–3 cases per 100,000 in BiH (Alvar et al., 2012), alongside occasional case reports (Gvozdenović & Miladinović, 1959; Obwaller et al., 2018). Our identification of *L. infantum* in dogs in BiH is made even more pertinent given that when national institutes and ministries in the country were surveyed regarding the presence of endemic animal leishmaniosis, this disease was not known to exist, despite recent research demonstrating it to be endemic (Berriatua et al., 2021; Colella et al., 2019). Given that autochthonous cases of animal leishmaniasis are notifiable in BiH, it would appear that contemporary data on *L. infantum* prevalence may not be effectively communicated to local ministries and veterinarians, resulting in cases of animal leishmaniosis potentially being undiagnosed and unreported (Berriatua et al., 2021). Substantial efforts should be made to keep local veterinarians and clinicians abreast of such findings regarding the prevalence of *L. infantum* in BiH due to the substantial risk this pathogen poses to humans and animals in the country (Colella et al., 2019). Additionally, our research identified *L. infantum* in concomitant infections, for example with *H. canis* in 4% of dogs, a finding that has not previously been made in BiH and may have pathological implications.

The identification of dogs infected with *A. phagocytophilum* is a key finding given that dogs act as sentinel hosts for this important zoonotic pathogen, the aetiology of granulocytic anaplasmosis, which is a potentially lethal disease in humans (Carrade et al., 2009; Ganta, 2021). *Anaplasma phagocytophilum* infects canines as accidental hosts in which the disease is usually self-limiting, exhibited through clinical signs such as temporary lethargy, fever, inappetence, and lameness (Carrade et al., 2009). This pathogen has been only recently reported from dogs in BiH (Maksimović et al., 2022), although molecularly identified in Bosnian...
TABLE 2 Number of positive dogs and pathogen prevalence with 95% confidence intervals found for vector-borne pathogen infections in 408 dogs from ten different field sites in Bosnia and Herzegovina, as detected by microfluidic qPCR

| Field Site  | Vector-Borne Pathogens | A. platys | A. phagocytophilum | Apicomplexa | B. vogeli | Hepatozoon spp. | L. infantum | Haemotropic Mycoplasma | At least 1 VBP |
|------------|------------------------|----------|------------------|----------|----------|-----------------|-----------|---------------------|----------------|
| Sarajevo (n = 138) |            | 0(0%;0–3%) | 2(1%;0–5%) | 36(26%;19–34%) | 2(1%;0–5%) | 17(12%;8–19%) | 52(38%;30–46%) | 1(0%;0–4%) | 75(54%;46–62%) |
| Gražanica (n = 63) |            | 0(0%;0–6%) | 3(5%;2–13%) | 30(48%;36–60%) | 0(0%;0–6%) | 18(29%;19–41%) | 5(8%;3–17%) | 2(3%;0–11%) | 36(57%;45–69%) |
| Tuzla (n = 10) |            | 0(0%;0–28%) | 0(0%;0–28%) | 3(30%;11–60%) | 0(0%;0–28%) | 2(20%;6–51%) | 0(0%;0–28%) | 0(0%;0–28%) | 3(30%;11–60%) |
| Mostar (n = 35) |            | 1(3%;1–15%) | 0(0%;0–10%) | 9(26%;14–42%) | 0(0%;0–10%) | 10(29%;16–45%) | 8(23%;12–39%) | 0(0%;0–10%) | 17(49%;33–64%) |
| Livno (n = 14) |            | 0(0%;0–22%) | 0(0%;0–22%) | 5(35%;16–61%) | 0(0%;0–22%) | 4(29%;12–55%) | 0(0%;0–22%) | 1(7%;1–31%) | 6(43%;21–67%) |
| Goražde (n = 40) |            | 0(0%;0–9%) | 2(5%;1–17%) | 14(35%;22–50%) | 0(0%;0–9%) | 17(43%;29–58%) | 2(5%;1–17%) | 1(3%;0–13%) | 19(47%;33–63%) |
| Odžak (n = 12) |            | 0(0%;0–24%) | 0(0%;0–24%) | 3(25%;9–53%) | 0(0%;0–24%) | 1(8%;1–35%) | 0(0%;0–24%) | 0(0%;0–24%) | 3(25%;9–53%) |
| G. Vakuf (n = 12) |            | 0(0%;0–24%) | 1(8%;1–35%) | 0(0%;0–24%) | 0(0%;0–24%) | 0(0%;0–24%) | 0(0%;0–24%) | 0(0%;0–24%) | 1(8%;1–35%) |
| Zenica (n = 44) |            | 0(0%;0–8%) | 0(0%;0–8%) | 16(36%;24–51%) | 0(0%;0–8%) | 14(32%;20–47%) | 5(11%;5–24%) | 0(0%;0–8%) | 19(43%;30–58%) |
| Bihać (n = 40) |            | 0(0%;0–9%) | 3(8%;3–20%) | 25(63%;47–76%) | 1(3%;0–13%) | 24(60%;45–74%) | 2(5%;1–17%) | 1(3%;0–13%) | 25(63%;47–76%) |
| All Sites (n = 408) |            | 1(0.2%;0–1%) | 11(3%;2–5%) | 141(35%;30–39%) | 3(0.7%;0–2%) | 107(26%;22–31%) | 74(18%;15–22%) | 6(1%;0.6–3%) | 204(50%;45–55%) |

Our identification of the haemotropic M. haemogregarina and M. haemocanis in Bosnian dogs, as well as A. platys, was also novel data that extend the known endemic geographical range of these VBPs. Although none of these pathogen species is associated with significant clinical signs in dogs, when identified in VBP infections, some can produce mild disease in canines, potentially causing severe anaemia, leukopenia, and thrombocytopenia (Coralić et al., 2018). M. haemoparvum and M. haemocanis are the aetiological agents of canine cyclic thrombocytopenia, a disease known to exacerbate canine anaemia and leukopenia in the context of VBP coinfections. Similarly, M. haemoparvum can also produce mild disease in canines, with cases of canine haemoglobinuria and thrombocytopenia associated with significant clinical signs in dogs when identified in VBP infections (Coralić et al., 2018). Although none of these pathogen species is associated with significant clinical signs in dogs, when identified in VBP infections, some can produce mild disease in canines, potentially causing severe anaemia, leukopenia, and thrombocytopenia (Coralić et al., 2018). M. haemoparvum and M. haemocanis are the aetiological agents of canine cyclic thrombocytopenia, a disease known to exacerbate canine anaemia and leukopenia in the context of VBP coinfections. Similarly, M. haemoparvum can also produce mild disease in canines, with cases of canine haemoglobinuria and thrombocytopenia associated with significant clinical signs in dogs when identified in VBP infections (Coralić et al., 2018).
TABLE 3  Number of positive dogs and pathogen prevalence with 95% confidence intervals found for vector-borne pathogen coinfections in 408 dogs from ten different field sites in Bosnia & Herzegovina, as detected by microfluidic qPCR

| Field Site | A. phagocytophilum & H. canis | Apicomplexa & L. infantum | B. vogelli & H. canis | Apicomplexa & Mycoplasma | H. canis & L. infantum | H. canis & Mycoplasma |
|------------|-------------------------------|---------------------------|----------------------|--------------------------|------------------------|------------------------|
| Sarajevo (n = 138) | 2 (1%; 0–5%) | 6 (4%; 2–9%) | 0 (0%; 0–3%) | 1 (0%; 0–4%) | 7 (5%; 2–10%) | 0 (0%; 0–3%) |
| Gračanica (n = 63) | 1 (2%; 0–8%) | 1 (2%; 0–8%) | 0 (0%; 0–6%) | 1 (2%; 0–8%) | 2 (3%; 0–11%) | 0 (0%; 0–6%) |
| Tuzla (n = 10) | 0 (0%; 0–28%) | 0 (0%; 0–28%) | 0 (0%; 0–28%) | 0 (0%; 0–28%) | 0 (0%; 0–28%) | 0 (0%; 0–28%) |
| Mostar (n = 35) | 0 (0%; 0–10%) | 0 (0%; 0–10%) | 0 (0%; 0–10%) | 0 (0%; 0–10%) | 2 (6%; 2–19%) | 0 (0%; 0–10%) |
| Livno (n = 14) | 0 (0%; 0–22%) | 0 (0%; 0–22%) | 0 (0%; 0–22%) | 1 (7%; 1–31%) | 0 (0%; 0–22%) | 0 (0%; 0–22%) |
| Goražđe (n = 40) | 1 (3%; 0–13%) | 0 (0%; 0–9%) | 0 (0%; 0–9%) | 0 (0%; 0–9%) | 1 (3%; 0–13%) | 1 (3%; 0–13%) |
| Odžak (n = 12) | 0 (0%; 0–24%) | 0 (0%; 0–24%) | 0 (0%; 0–24%) | 0 (0%; 0–24%) | 0 (0%; 0–24%) | 0 (0%; 0–24%) |
| G. Vakuf (n = 12) | 0 (0%; 0–24%) | 0 (0%; 0–24%) | 0 (0%; 0–24%) | 0 (0%; 0–24%) | 0 (0%; 0–24%) | 0 (0%; 0–24%) |
| Zenica (n = 44) | 0 (0%; 0–8%) | 0 (0%; 0–8%) | 0 (0%; 0–8%) | 0 (0%; 0–8%) | 2 (5%; 1–15%) | 0 (0%; 0–8%) |
| Bihać (n = 40) | 3 (8%; 3–20%) | 0 (0%; 0–9%) | 1 (3%; 0–13%) | 0 (0%; 0–9%) | 2 (5%; 1–17%) | 1 (3%; 0–13%) |
| All Sites (n = 408) | 7 (2%; 1–4%) | 7 (2%; 1–4%) | 1 (0.2%; 0–1%) | 3 (0.7%; 0–2%) | 16 (4%; 2–6%) | 2 (0.5%; 0–2%) |

missed (Gondard et al., 2020; Huggins et al., 2020). Moreover, our microfluidic qPCR’s pre-amplification step, which has previously been demonstrated to greatly improve the sensitivity of this assay, may have permitted enhanced detection capability of the VBPs herein detected (Gondard et al., 2020; Michelet et al., 2014). This improved sensitivity may provide us with more accurate estimated prevalence data, particularly given that these VBPs usually circulate at low parasite DNA concentrations or generate transient parasitaemia in dogs (de Caprariis et al., 2011; Oliva et al., 2006; Paradis et al., 2010). For example, our pre-amplification step in this study may explain the increased detection of L. infantum in approximately 15% more dogs than the percentage of positives elucidated before (Colella et al., 2019).

Overall, the microfluidic qPCR method used in this study may be highly suited to large-scale epidemiological surveys of canine VBPs, particularly in countries with scarce prior data in conjunction with large free-roaming dog populations that may facilitate easy and unbridled VBP transmission (Katica et al., 2020; Omeragić et al., 2021). The large number of samples that can be screened concurrently for numerous VBP species, makes microfluidic qPCR techniques amenable to surveys that rely on a high-throughput diagnostic capability (Gondard et al., 2020; Michelet et al., 2014). Nonetheless, cross-validation might be needed for the characterization of some VBPs to a species level that is, through the use of conventional PCR and Sanger sequencing, which rules the microfluidic method less suited to diagnosis in a clinical setting, for which more specific and bespoke molecular or serological tests may be available.

Here our report of endemicity of the highly pathogenic and zoonotic pathogens L. infantum and A. phagocytophilum alongside a lack of awareness by the relevant animal and human health authorities regarding the occurrence of these pathogens in BiH, emphasizes the urgent need for allocation of resources to implement surveillance and control, targeting both animal and human infections within the country.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

ETHICS STATEMENT
The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to. The study was performed in accordance with the Veterinary law of Bosnia and Herzegovina (“OJ BiH”, no: 34/02), the Game law of Bosnia and Herzegovina (“OJ BiH”, no: 34/06), the Law on Prevention of Animal Health Protection in Bosnia and Herzegovina (“OJ BiH”, no: 15/18).

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Alić, A., Prašović, S., Čamo, D., Coralić, A., Preldžić, D., Duscher, G. G., & Hodžić, A. (2019). Fatal visceral leishmaniasis in a dog caused by Leishmania infantum in Bosnia and Herzegovina: A case report. Veterinary Parasitology: Regional Studies and Reports, 15, 100260. https://doi.org/10.1016/j.vsprsr.2018.100260

Alvar, J., Vélez, I. D., Bern, C., Herrera, M., Desjeux, P., Cano, J., Jannin, J., & de Boer, M. (2012). Leishmaniasis worldwide and global estimates of its incidence. Plos One, 7, 35671. https://doi.org/10.1371/journal.pone.0035671

Arapovic, J., Skocibusic, S., Grgic, S., & Nikolic, J. (2014). The first evidence of Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., & de Boer, M. (2012). Leishmaniasis worldwide and global estimates of its incidence. Plos One, 7, 35671. https://doi.org/10.1371/journal.pone.0035671

Brikčažić, M., Matijatko, V., Kučer, N., Foršek, J., Rafaj, R. B., Grden, D., Torti, M., Mayer, I., & Mrljak, V. (2010). Molecular evidence of natural infection with Babesia canis in dogs from Southern Slovenia. Veterinary Parasitology: Regional Studies and Reports, 15, 100260. https://doi.org/10.1016/j.vsprsr.2018.100260

Carrade, D. D., Foley, J. E., Borjesson, D. L., & Sykes, J. E. (2009). Canine Babesioses in Nonin-vestigated Areas of Serbia. Veterinary Parasitology, 159(9), 535–538. https://doi.org/10.1016/j.vetpar.2015.1797

Ganta, R. (2021). The canine host serving as a sentinel species for tick-borne diseases caused by Anaplasma, Ehrlichia and Borrelia pathogens impacting human health in the USA. (Master’s thesis). Kansas State University.

Hodžić, A., Fuehrer, H. P., & Duscher, G. G. (2017). First molecular evidence of zoonotic bacteria in ticks in Bosnia and Herzegovina. Transboundary and Emerging Diseases, 64(4), 1313–1316. https://doi.org/10.1111/tbed.12473

Hodžić, A., Alić, A., Fuehrer, H. P., Harl, J., Wille-Pilzai, W., & Duscher, G. G. (2015). A molecular survey of vector-borne pathogens in red foxes (Vulpes vulpes) from Bosnia and Herzegovina. Parasites and Vectors, 8(1), 1–7. https://doi.org/10.1186/s13071-015-0692-x

Hodžić, A., Latrofa, M. S., Annoscia, G., Alić, A., Beck, R., Lia, R. P., Dantas-Torres, F., & Otranto, D. (2014). The spread of zoonotic Thelazia calliptera in the Balkan area. Parasites and Vectors, 7(1), 1–6. https://doi.org/10.1186/1756-3305-7-525

Huber, D., Reil, I., Duvnjak, S., Jurković, D., Lukačević, D., Pilat, M., Beck, A., Mihaljević, Z., Voja, L., Polkinghorne, A., & Beck, R. (2017). Molecular detection of Anaplasma platys, Anaplasma phagocytophilum and Wolbachia sp. but not Ehrlichia canis in Croatian dogs. Parasitology Research, 116(11), 3019–3026. https://doi.org/10.1007/s00436-017-5611-y

Huber, D., Reil, I., Duvnjak, S., Jurković, D., Lukačević, D., Pilat, M., Beck, A., Mihaljević, Z., Voja, L., Polkinghorne, A., & Beck, R. (2017). Molecular detection of Anaplasma platys, Anaplasma phagocytophilum and Wolbachia sp. but not Ehrlichia canis in Croatian dogs. Parasitology Research, 116(11), 3019–3026. https://doi.org/10.1007/s00436-017-5611-y

Huggins, L. G., Koehler, A. V., Ng-Nguyen, D., Wilcox, S., Schunack, B., & Traub, R. J. (2021). A novel metabarcoding diagnostic tool to explore protozoan haemoparasite diversity in mammals: A proof-of-concept study using canines from the tropics. Scientific Reports, 9(1), 12644. https://doi.org/10.1038/s41598-019-49118-9

Huggins, L. G., Koehler, A. V., Schunack, B., Inpankaew, T., & Traub, R. J. (2020). A host-specific blocking primer combined with optimal DNA
Michelet, L., Massetti, L., Schunack, B., Colella, V., & Traub, R. (2021). Novel high-throughput multiplex qPCRs for the detection of canine vector-borne pathogens in the Asia-Pacific. Microorganisms, 9(5), 1092. https://doi.org/10.3390/microorganisms9051092

Katica, M., Obradović, Z., Ahmed, N. H., Mehmedika-Suljić, E., Stanić, Ž., & Obradović, Z. (2019). Hard tick infestation of dogs in the Tuzla area (Bosnia and Herzegovina). Veterinarski Arhiv, 84(2), 177–182.

Laušević, D., Stanić, Ž., Nenadović, K., Đurić, J., Đurić, I., & Bacić, D. (2021). Seroprevalence of Rickettsia conorii, Ehrlichia canis and Coxiella burnetii in dogs from Montenegro. Acta Parasitologica, 66(4), 769–777. https://doi.org/10.2478/1s11666-019-00098-w

Katica, M., Obradovic, Z., Ahmed, N. H., Mehmedika-Suljić, E., Stanić, Ž., Mohamed, R. S. A., & Dervišević, E. (2020). Interdisciplinary aspects of possible negative effects of dogs on humans in Bosnia and Herzegovina. Medicinski Glasnik, 17, 1–6. https://doi.org/10.17392/1187-20

Krčmar, S., Ferizbegović, J., Lonić, E., & Kamberović, J. (2014). High-throughput multiplex qPCRs for the detection of canine vector-borne bacteria. Pathogens, 9(4), 258. https://doi.org/10.3390/paths9040258

Huggins, L., Massetti, L., Schunack, B., Colella, V., & Traub, R. (2021). Novel high-throughput multiplex qPCRs for the detection of canine vector-borne pathogens in the Asia-Pacific. Microorganisms, 9(5), 1092. https://doi.org/10.3390/microorganisms9051092

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