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Vector-borne diseases in red foxes from Carinthia

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Wien, den 10.07.2023 Charlena Poonyth

Table of contents:

List of abbreviations .....

1. Introduction 1

2. Causative agents 3

2.1. Bacterial pathogens 3

2.1.1. *Mycoplasma* spp. 3

2.1.2. Anaplasmataceae 5

2.1.3. *Bartonella* spp. 7

2.1.4. *Rickettsia* spp. 9

2.2. Helminths 11

2.2.1. *Dirofilaria* spp. 11

2.3. Protozoal pathogens 13

2.3.1. Piroplasmidae 13

2.3.2. *Hepatozoon* spp. 15

3. Vectors 18

3.1. Arthropods 18

3.2. Fleas 18

3.3. Mosquitoes 19

4. Material and Methods 21

4.1. Sample collection 21

4.2. Location 21

4.3. Extraction of sample material 23

4.4. Molecular analysis 23

4.4.1. DNA Extraction 23

4.4.2. PCR 24

4.4.3. Principles of the PCR 24

4.4.4. Procedure 24

4.4.5. Gel electrophoresis 27

5. Results 36

6. Discussion 41

7. Summary 45

8. List of tables and figures 47

9. Bibliography 48

10. References 64

11. Acknowledgements..65

List of abbreviations

AGES: Agentur für Gesundheit und Ernährungssicherheit

bp: base pair

BSC: biosafety cabinet

CSD: cat scratch disease

DEBONEL: Dermacentor-borne necrosis erythema and lymphadenopathy

d.p.i.: days post-infection

°C: degrees Celsius

FIV: feline immunodeficiency virus

HGA: human granulocytic anaplasmosis

IAFGP: *Ixodes scapularis* tick antifreeze glycoprotein

IFAT: indirect fluorescent antibody test

IST: intergenic transcribed spacer

MIF: microimmunofluorescence

min: minute(s)

ml: millilitre

MST: multispacer typing

µl: microlitre

rpm: rounds per minute

s: second(s)

S: Svedberg unit

SFG: spotted fever group

s.l.: sensu lato

sp./spp: species (singular/plural)

TBE: Tris-Borat-EDTA-Buffer

TBF: tick-borne fever

TIBOLA: tick-borne lymphadenopathy

V: Volt

1. Introduction

The province of Carinthia is the most southern province of Austria, bordering the Eastern Alps. It nurtures Austria's second most densely forested province with a forest area of 592,000 hectares (1). Human activities such as hunting, and game management have contributed to the progressive use of forest land and instilled migration of game species towards new areas. A prerequisite to the transmission of vector-borne diseases is the presence of a vector, a host, and a favourable environment for the pathogen. Climatic conditions being of utmost importance to the survival and spread of the latter. The aftermath brought about by global warming and its undeniable anthropogenic nature has been confirmed through ongoing research and raised concern in the light of alien species being introduced to Austria (2). This gears towards the emergence of previously unknown vector-borne diseases and the permanent establishment of invasive species within new areas of Central Europe.

With the rise of vector-borne diseases within the sylvatic population and at the same time an overlapping of habitats between wildlife and domestic animals, public and animal health are subjects at risk. Further contributors to the spread of vector-borne pathogens include travelling, trade, socioeconomic factors, and the lack of surveillance programmes. In Austria, the ovitrap monitoring programme has re-assessed the geographical distribution of the plethora of mosquito species across the country and established the presence of the Japanese bush mosquito, *Ae. japonicus*, with a particular concentration in the south (3). These surveillance programmes enable the recognition of patterns, acting as supporting data, when assessing the prevalence of certain vector-borne pathogens in a given area and the incidental occurrence of their corresponding vectors.

The aim of this study was to test for the presence of seven (hemophagocytic) vector-borne pathogens in red foxes (*Vulpes vulpes*) from Carinthia, Austria. The red fox belongs to the order Carnivora and family Canidae, regrouping Austria's most abundant wild canid population (4, 5). They are known to be monogamous, free-ranging species influenced by seasonal change (4, 6). Red foxes harbour a range of zoonotic agents, thus acting as reservoirs for many of these vector-borne pathogens. The occurrence of *Mycoplasma* spp., *Dirofilaria* spp., Anaplasmataceae, *Bartonella* spp., *Rickettsia* spp., Piroplasmida, and *Hepatozoon* spp. was evaluated during the course of this study, representing an adjunct to the current published data on the red fox population from other parts of Austria (7–9). To this date, there have been no

published studies on the occurrence of vector-borne pathogens in Carinthia specifically. The sample size used for this study is larger in comparison to several previous studies carried out in Austria (7), enhancing the accuracy of the results and providing more representative results.

2. Causative agents

The selected causative agents have been classified according to their phylogeny. This chapter will provide a detailed analysis regarding their classification, methods of diagnosis, mode of transmission, prevalence and the symptoms induced by an infection.

2.1. Bacterial pathogens

2.1.1. *Mycoplasma* spp.

Mycoplasmas are bacteria of the family Mycoplasmataceae, that belong to the order of Mycoplasmatales. They are known as ubiquitous, intracellular gram-negative bacteria characterised by the absence of a cell wall. Within the order Mycoplasmatales exist haemoplasmas (haemotropic mycoplasmas), which currently regroup the formerly known genera of *Haemobartonella* and *Eperythrozoon*, whose target cells are erythrocytes. *Mycoplasma haemocanis* (Mhc) and *Candidatus* Mycoplasma haemominutum (CMhm) have been identified in dogs, while *Mycoplasma haemofelis* (Mhf), CMhm, and *Candidatus* Mycoplasma turicensis (CMt) were observed in cats. The classification into *Candidatus* is carried out with the species, where there is a lack of complete characterisation based on their bacterial culture (10).

Their morphology is referred to as pleomorphic, as they range from rod-like, coccoid to ring-shaped structures that can be found either solitary or in chains on red blood cells. Currently, PCR has been recognised as the golden standard for the diagnosis and differentiation of haemoplasma species in dogs and cats (11–16). They affect a wide variety of mammalian species, typically associated with infectious causative agents for anaemia. Their parasitism involves attaching to the walls of the host's red blood cells and potentially penetrating the cell.

The primary suspected mode of transmission of haemoplasma involves blood transfusions (infected blood), haematophagous ectoparasites and vertical transmission through the placenta or through direct contact, which entails fighting and biting. This was supported by studies demonstrating the presence of haemoplasma DNA in saliva, on the gingiva, and on claw beds of infected cats (12) or in the case of dogs, an increased number of infections was recorded in fighting dogs in Japan, in comparison to other breeds (17). While in Europe, feline haemoplasmas are expected all over the continent, canine haemoplasmas have solely been

identified in dogs originating from Mediterranean countries (18, 19, 14, 20). In dogs, the transmission via the dog tick *Rhipicephalus sanguineus* has been confirmed, with the prevalence for Mhc in Europe having been reported at 0.9 % in Switzerland, 0.5–14.3 % in Spain, 5.8 % in Italy, and 5.8 % in France, suggesting a correlation between the detection of canine haemoplasma in endemic regions with *R. sanguineus* (21, 14, 19, 22). Additionally, an interspecies transmission between rodents and cats might also play a role in the spread of feline haemoplasmas (12). The potential mechanical transmission via other vectors, in particular mites, has been discussed in dogs that presented with mange and tested positive for canine haemoplasma (19). The role of fleas in the transmission of haemoplasmas is not to be neglected, as these have proven to contribute significantly to the infections in wild mammals in Patagonia, Argentina (23).

There seems to be a higher risk of infection in male, adult dogs supporting the theory behind direct transmission and a potential influence of older age prolonging the risk of exposure to the pathogen (20, 24). Another study found more young dogs to be positive for canine haemoplasma infections and hypothesised that this could be due to the association between their increased activity and thus, leading to a higher exposure rate to tick vectors (19). However, gender as a risk factor in the transmission of canine haemoplasma was contradicted by previous studies (21, 14). In cats, the risk factor analysis stated that older age, male gender, and FIV positivity were predisposing factors for infections with feline haemoplasmas (22).

Infected animals mostly present themselves as asymptomatic carriers with chronic infections, but some severe cases have been recorded. The most common clinical findings included haemolytic anaemia, lethargy, anorexia, and fever, accompanied by splenomegaly and less commonly, icterus. The anaemia arises due to an immune-mediated haemolysis, that classifies as a regenerative anaemia. Nevertheless, co-pathogens such as mange may impact on the condition of the animals, alongside an immunocompromised state, which influences the fatality of the disease (19). Whilst in cats, there has been a strong link between the simultaneous co-infection of FIV and feline haemoplasmas, this did not imply an enhanced pathogenicity but instead suggested a similar mode of transmission (25). To this date, very little is known about the zoonotic potential of haemoplasmas regarding the possibility of humans contracting it, too. One report mentions the infection of a veterinarian with *Mycoplasma haematoparvum,* though providing no substantial evidence of humans being suitable hosts for the latter (26).

2.1.2. Anaplasmataceae

Anaplasmataceae also known as Ehrlichiaceae is a family, that regroups nine genera: *Aegyptianella, Anaplasma, Candidatus* Cryptoplasma*, Ehrlichia, Lyticum, Candidatus* Neoehrlichia*, Neorickettsia, Paranaplasma* and *Wolbachia* (27). They belong to the order of Rickettsiales, which encompasses obligate intracellular gram-negative bacteria. *Anaplasma phagocytophilum* is a zoonotic agent responsible for quite an array of tick-borne diseases in mammals, for instance, human granulocytic anaplasmosis (HGA) in humans, canine and equine granulocytic anaplasmosis in dogs and horses, tick-borne fever (TBF) in small ruminants and in cattle.

The bacteria possess two morphotypes, a larger reticulate (RC) form, and a smaller dense-core (DC) form, that encapsulates condensed protoplasm (28, 29). Anaplasmataceae distinguish themselves from other members of the Rickettsiaceae family, by replicating via intracytoplasmic vacuoles (morulae) within the cytoplasm of their host cells (28, 29). The detection of the morulae is one of the methods of detection used to test for the presence of *A. phagocytophilum*, alongside serological tests and PCR (30, 31). Using the Romanowsky staining with Diff-Quick enables the detection of the latter, in which case purple-stained mulberry-like clumps referred to as morulae become visible (28). Cholesterol is essential for the survival and infection of *A. phagocytophilum*, which is ensured by an upregulation of host cell cholesterol levels upon infection. A study provides detailed information on how cholesterol is transported to the intracellular inclusion body containing the bacteria and later on, built into its cell membrane (32). Moreover, the bacteria reveal an interesting “regulatory hijacking” strategy, which is deemed at escaping the defensive mechanisms of the host’s immune system. This is carried forward by subverting the neutrophilic antimicrobial defences, downregulating the generation of reactive oxygen species, inhibiting host cell apoptosis, and subverting autophagy of host cells such that a remodelling of the host cell’s cytoplasm makes more room and nutrients available to the bacteria (28).

Arthropods play a critical role in upholding the existence of *A. phagocytophilum*. Similar to mammalian hosts, the bacteria also undertake a modulation of signalling pathways in ticks, mainly of the actin phosphorylation (33). Another interesting aspect of its life cycle involves its seemingly facultative symbiosis with ticks, which is demonstrated through the acquired resistance towards cold climates in infected ticks. *Ixodes scapularis* ticks, that were infected

with *A. phagocytophilum* had upregulated their antifreeze glycoprotein (IAFGP) (34). Whilst ticks are not dependent on *A. phagocytophilum*, the contrary is very much true for the survival of the latter, which requires ticks as a host. This symbiosis entails an advantageous condition for infected ticks, that become able to overcome colder temperatures, thus increasing their survival rate in the environment. It is to be noted that ticks do not appear to suffer from the infection, rather they benefit from it (28).

In mammals, *A. phagocytophilum* replicates within neutrophilic and eosinophilic granulocytes and endothelial cells. Whereas in ticks the replication takes place in the salivary glands and midgut cells (28). Their persistence in nature relies on the continuous interspecies contact between vertebrates and ticks. Mammals are infected through the bite of an infected tick, typically a hard tick belonging to the genus *Ixodes*. In Europe, *I. ricinus* is mainly responsible for the transmission of this pathogen (30). The prevalence in ticks in Europe is substantially low with 1.5 % in Switzerland (35), 2.69 % in Slovakia (36), and a mean prevalence in Eastern European regions endemic for *I. persulcatus* tick from 1.7 to 16.7 % (37). Currently, the transovarial transmission has been ruled out, indicating that only nymphs and adult ticks are considered vectors. Adult ticks have been estimated to possess twice the chance of contracting an infection, as they take one more blood meal than nymphs (37).

In Austria, a high rate of infection with *A. phagocytophilum* in wild ruminants, primarily in roe deer was documented, reinforcing the idea of roe deer being a natural reservoir (38). There seems to be a positive correlation between the high prevalence of *A. phagocytophilum* and the presence of a high density of roe deer in a given area (39–42). The pathogen does infect other deer species too, such as red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and sika deer (*Cervus nippon*), which are present in Europe. Aside from wild ruminants, rodents represent the second largest group of carriers of *A. phagocytophilum*. However, in Europe, rodents seem to bear a different strain of *A. phagocytophilum* in comparison to the one affecting wild ungulates, humans, and ticks (37). This suggests that the role of rodents as potential carriers of this zoonotic agent, can be viewed as negligible. Wild boars have also been evaluated for the presence of *A. phagocytophilum* and were found to be potent carriers with a prevalence of infection at 16.7 % in Slovakia (43). Less data is available when it comes to the occurrence of *A. phagocytophilum* in red foxes in Europe. Generally, the prevalence of infection in red foxes was 2.7 % in Poland (44), 4 % in the Czech Republic (45), and 16 % in Italy (46). Although not representative, because of its small sample size, the prevalence of *A. phagocytophilum* in red foxes in Germany was 8.2 % (47).

Domestic animals suffering from an infection with *A. phagocytophilum* typically present themselves with fever, leukopenia, and thrombocytopenia. Additional symptoms may include anorexia, depression, distal limb oedema, lameness, and ataxia (28, 48, 49). The number of cases of human granulocytic anaplasmosis (HGA) in Europe has been rising over the past decades, with 22 cases recorded by 2006 from Germany, the Netherlands, Spain, Sweden, Norway, Croatia, Poland, Italy, Austria, and France (50, 51). Most of these had been reported from July to August, which can be explained by the seasonal activities carried out in tick habitats, that coincide with the peak activity of the latter around this time of the year (50). Nonetheless, these statistics do not compare with the recorded cases in the United States, where the prevalence of HGA is higher. An imported case of HGA in Austria has been reported, suggesting that humans themselves contribute to the dissemination of the bacteria across continents through international travel (52). While *A. phagocytophilum* is viewed as a zoonotic agent, given the relatively low occurrence in red foxes in Central Europe and the diversity of strains, it remains unclear whether red foxes carry strains with high zoonotic potential and thus represent a reservoir.

2.1.3. *Bartonella* spp.

Bartonella regroups a genus of bacteria of the family Bartonellaceae (53). *Bartonella* are classified as intracellular gram-negative alpha-proteobacteria, that are fastidious, aerobic bacilli (54). They represent an arthropod-borne zoonotic agent, that infects domestic mammals, wildlife, and humans. They have evolved with an array of adaptive immune evasive mechanisms, thus inducing a pertaining silent infection in their mammalian hosts (55). Presently, there are approximately 40 recorded species/subspecies of *Bartonella*, of which 17 are suspected to be of zoonotic relevance for humans (56, 57, 55, 54).

Bartonella is described as pleomorphic rods, which are ideally made visible with Gimenez stain, though this is not specific to the latter (58). Other diagnostic methods include isolation and culture and serology including serologic assays, that make use of enzyme-linked immunosorbent assay (ELISA) (59, 54). Molecular diagnosis using PCR has facilitated the diagnosis of *Bartonella*-associated diseases in humans and hence resolved the uncertainty around the aetiology of diseases such as cat scratch disease (CSD) in humans (60). Interestingly, the genome size shows a direct proportionality with host specificity. *Bartonella*

species, that are associated with infection in rodents were found to have larger genomes, while the *B. bacilliformis* species infecting humans showed the smallest genome (61, 54).

One of the prominent features of *Bartonella* includes their bacterial type IV secretion systems, which have enabled them to adapt to their respective mammalian hosts. Additionally, they possess critical bacterial adhesins ensuring an efficient translocation of type IV secretion effector proteins (62). *Bartonella* is transmitted by vectors, such as ticks, fleas, biting flies (63), and mosquitoes (64). Feline bartonellae have been the focus of many studies revealing, that fleas seem to be a key factor in the transmission of *B. henselae* (65). In cats, *Ctenocephalides felis*, the cat flea, is mainly responsible for transmitting this bacterium. Direct transmission of infected fleas, leading to flea bites and ingestion of the latter or their faeces have been discussed in relation to the mode of transmission (66) among cats. Little is known about the primary infection niche, but it has been hypothesized that endothelial cells might play an important role due to the presence of a marked tropism for these cells. Once they enter the bloodstream of their mammalian hosts, the bacteria usually require a period of about five days before inducing bacteriaemia (67). Their common approach involves selecting mature erythrocytes, adhering to them, followed by an invasion aimed at intracellular replication. The replication is carried out in a membrane-bound vacuole until a certain density level is attained, after which a lag phase is reached. This static phase can be withheld for the remaining lifespan of the erythrocyte (68).

Red foxes seem to be carriers of *B. rochalimae*, which is considered pathogenic for humans and macaques (69). It is not very clear yet, how this strain is contracted from animal to human, but previous studies indicate that aside from fleas and ticks, lice should be considered too (70). Infected humans present clinical signs such as splenomegaly, fever and anaemia (69). Aside from wild canids, *B. rochalimae* can also infect dogs, though it is believed that these are accidental hosts. *B. rochalimae* has been demonstrated to be responsible for infective endocarditis in dogs (71). There statistics published about the prevalence of *B. rochalimae* in red foxes in Europe are scarce, published studies are mostly restricted to the Czech Republic (45), Germany (47), Italy (46), Poland (72), Romania (73) and France (74). A prevalence of 0.2 % was recorded in a study from western Austria (5). One study mentions a recorded prevalence of 1.6 % in (northern) Spain (75), hence leaving room for more elucidation on this topic.

2.1.4. *Rickettsia* spp.

Within the previously mentioned order of Rickettsiales exists a family known as Rickettsiaceae. This family is further divided into the genera termed *Rickettsia* and *Orientia*. These are obligate intracellular gram-negative bacteria, of which many are considered to be in a symbiotic relationship with their specific arthropod hosts (76). They belong to one of the oldest known zoonotic agents.

Most referred to as an alphaproteobacterial subgroup, Rickettsiaceae have been of great interest in gathering more evidence to prove the endosymbiotic theory. The origin of the mitochondria as an organelle in eukaryotic organisms and the shared similarities between this organelle and members of the family of Rickettsiaceae (as well as the *Rickettsia*-like endosymbionts, RLE) led to the assumption, that mitochondria underwent a polyphyletic evolution. One of the assumptions states, the dependence of Rickettsiaceae upon a eukaryotic host organism as being essential for the import of certain proteins, for which a set of transferred genes comes along (77). Further assumptions are testifying for a mutualistic relationship between these intracellular prokaryotic organisms and a pro-eukaryote at the time.

Although further research is required as a lot is still unclear regarding the precise classification of *Rickettsia*, they are divided into four phylogenetic groups at present (78):

1. Spotted fever group (SFG)
2. Typhus group
3. *Rickettsia bellii* group
4. *Rickettsia canadensis* group

Rickettsiae target microvascular endothelial cells inducing endothelial dysfunction. They take refuge in the host cell's cytosol, where they modulate the transport mechanisms to their advantage. The typical entryway of the pathogen into its host's bloodstream involves inoculation through a feeding tick or mite. Macrophages and/or dendritic cells represent their next target (79). Although vertical and horizontal transmission are described, there is a limited understanding of the relevance of the vector-host interaction for the survival of rickettsiae (80).

The main focus was placed on the SFG, which has zoonotic potential and was found responsible for severe diseases in humans and animals (81). The indirect microimmunofluorescence (MIF), which was developed in 1978 for serologic typing, long

remained as a reference method for the detection of rickettsiae (82). With the discovery of molecular diagnostic methods, such as multiple-gene sequencing, a more precise classification of the genus *Rickettsia* was made possible. Previously implemented guidelines were reviewed and altered to ensure a distinct recognition of new *Rickettsia* species (83). The most accurate method for the distinction between two species was narrowed down to genotyping bacterial strains in their most variable regions, which correspond to the variable intergenic spacers. This technique is referred to as multispacer typing (MST) and was deemed to be most useful in tracking back rickettsial isolates from one specific source with individual passage history. It has been most advantageous in increasing the reproducibility rate, simplifying the interpretation and incorporation of collected data for future use (84).

Rickettsia typhi and *R. slovaca* were diagnosed in red foxes from Spain using immunofluorescence assays (85). Another study used molecular techniques and identified *R. massiliae, R. aeschlimannii* and *R. slovaca* in red foxes from Catalonia (Spain) (86). *Rickettsia slovaca* is the most prevalent species in Western and Central Europe (43). A recent study established the presence of *R. massiliae* and *R. felis* in arthropods, that fed on red foxes in France (87). *R. massiliae* has mainly been recorded in ticks belonging to the *Rhipicephalus* group in Mediterranean countries (88–93) and Switzerland (94).

Hard ticks of the family Ixodidae act as main vectors and reservoirs for SFG rickettsiae. *Dermacentor reticulatus* has been shown to expand its geographical distribution, thus invading new areas of Northern and Central Europe (95). Other vectors include fleas, human body lice (96, 97), and mosquitoes (98, 99). Tick-borne rickettsioses are more common in Europe. Infections with *R. slovaca* result in tick-borne lymphadenopathies or *Dermacentor*-borne necrosis erythema and lymphadenopathy (TIBOLA/DEBONEL) (100, 101). They are characterised by an eschar around the tick bite site, typically on the scalp. However, compared to other rickettsial infections in humans, the one caused by *R. slovaca* rarely is accompanied by fever (102, 103). Recent outbreaks of rickettsiosis in humans across Europe were also associated with *R. felis*. These were responsible for flea-borne spotted fever rickettsiosis in humans following flea bites, after which a rash eventually became visible.

2.2. Helminths

2.2.1. *Dirofilaria* spp.

Dirofilaria refers to a filarioid nematode belonging to the family Onchocercidae and to the order Rhabditida. Currently, 27 species have been identified, among which *D. repens* and *D. immitis* are the most well-known zoonotic agents responsible for human dirofilariasis (78, 104). Other less frequently mentioned species, that still bear a zoonotic potential include *D. tenuis, D. ursi, D. striata,* and *D. subdermata. Dirofilaria immitis* primarily infects dogs causing heartworm disease, but can also infect wild canids such as coyotes, jackals, wolves, and foxes. *Dirofilaria repens* on the other hand is little to non-pathogenic for dogs and mostly responsible for a subcutaneous infection. In humans, it may lead to larva migrans syndrome, often found in the ocular region and less frequently in other organs, such as the lungs (105–108).

Mosquitoes of the genera *Aedes, Culex, Anopheles,* and *Mansonia* play a significant role as intermediate hosts (109–111). *Dirofilaria immitis* and *D. repens* are highly synanthropic when it comes to their vertebrate host. Female mosquitoes comprise the blood-feeding arthropod vectors, as male mosquitoes do not take blood meals (112). The first larval stage (L1) starts with microfilariae, that are ingested by female mosquitoes, when taking a blood meal on an infected vertebrate host. The microfilariae make their way to the Malpighian tubules situated in the mosquito's digestive tract, where they evolve to the second larval stage (L2). It takes about 11–13 d.p.i. for them to undergo maturation from the first to the third larval stage (L3). However, the third developmental phase is influenced by the surrounding temperature, which should be ≥ 26 °C (113). L3 larvae then migrate to the stylet of the mosquito, where they remain until inoculation of a vertebrate host occurs, following the upcoming blood meal. The larvae are transmitted via the haemolymph through the bite wound (109). Their first site of penetration involves connective tissues, after which they move towards the muscles and molt into their fourth larval stage (L4). The fifth larval (L5) development phase occurs within muscular tissues in the thoracic/abdominal cavities, where they are recognised as immature adults. These are capable of migrating to pulmonary arteries, which is the site of final maturation to become sexually mature worms and allow copulation to take place (113).

Studies have found a symbiotic relationship between *Dirofilaria* species and the bacterium *Wolbachia.* They evaluated the role of this particular interaction in larval development within vertebrate hosts (114). Further studies showed the presence of *Wolbachia* in somatic gonads

and the intestinal wall, indicating the complexity of that symbiosis (115). Commonly implemented diagnostic methods for the detection of *Dirofilaria* in dogs include antibody-testing, Knott's test or any other tests aiding the concentration of microfilaria for further microscopical study. Additionally, histochemical staining can be used to determine phosphatase activity. Lastly, PCR represents a molecular method of diagnosis.

In *D. immitis* non-indigenous areas, imported domestic dogs from endemic countries create a reservoir, upon which the parasite gets introduced into the local mosquito community (116). The first reported case in a human in Austria goes back to 1978 (117). Humans represent accidental hosts and are of no benefit to the parasite, as they are unable to parasitise within this host. The infective larvae either die or fail to pursue their larval development into mature adult nematodes (117). In Europe, the greatest area deemed endemic for *D. immitis* is situated in the Po River Valley (Italy), where its prevalence surpassed 50 % (118) and when assessed in dogs not receiving prophylactic treatment, it reached up to 80 % (119). Austria's current status as potential pre-endemic country might be challenged in the years to come, given the previous doubling of recorded cases in a short period of time (120). The fact that Austria has not yet been labelled as endemic, must be due to the lack of canine reservoirs because of the differences in dog keeping and hence a delayed introduction to the local mosquito community (121, 122).

While *D. immitis* requires a mild, tropical climate, *D. repens* was already known as endemic to the Old World, which includes Italy, France, Spain, Portugal, and Germany, but not considered endemic in Austria. Recent studies have shown that mosquitoes in eastern Austria (Burgenland) were already possible vectors for *D. repens* (121). Whilst *D. repens* has been detected in dogs in Austria, the majority of which had a travelling history to endemic countries. Some had an unknown travelling history and the few reported cases, where there was no travelling abroad involved prior to the infection, are assumably autochthonous cases. The suspicion around the autochthonous cases is further reinforced as these were detected in regions close to the border to Slovakia and Hungary, which are both at present known to be endemic. Red foxes in eastern and western Austria have been screened for the presence of filarioid helminths (5), however to this date no positive results have been published. Hence, the main reservoirs remain domestic animals (dogs), of which those originating from or having travelled to an endemic country are the most likely to introduce *Dirofilaria* spp. Nevertheless, climate change is contributing to more favourable conditions, especially for *D. immiti*s. Consequently, the introduction of new invasive mosquito species capable of serving as vectors

to *Dirofilaria* spp. in Austria, raises concern about the country's (pre-endemic) status (121, 111).

2.3. Protozoal pathogens

2.3.1. Piroplasmidae

Piroplasmidae refers to an order, that regroups the genera *Babesia, Theileria* and *Cytauxzoon*. They are recognised as protists/protozoans belonging to the phylum of Apicomplexa, that generally include arthropod-borne intra-erythrocytic parasites. Five evolutionary lineages have been discovered in the order Piroplasmida (123). Their lifecycle comprises a phase, in which they undergo merogony (asexual reproduction) within mammalian erythrocytes, whilst the gamogony (sexual reproduction) occurs in ticks. They share a taxonomic proximity with *Plasmodium* spp. as both are classified as apicomplexan parasites (124–126, 123). As such, piroplasms also vary in morphological constitution during their lifecycle. This involves sporozoites, trophozoites and merozoites. The latter are characterised by their pear-shaped appearance within the erythrocyte in some *Babesia* species (127).

Theileria spp. and *Babesia* spp. differ from each other in their initial approach. *Babesia* spp. do not require intra-leukocytic schizogony before invading the host's erythrocytes (128). *Theileria* spp. have shown to first invade monocytes and lymphocytes, in which they proceed with a process known as schizogony. This involves the asexual multiplication of the internalised sporozoites, generating merozoites. Targeted leukocytes may also undergo neoplastic transformation following schizogony, which results in an uncontrolled proliferation (128). However, in comparison to *Babesia* spp., *Theileria* sporozoites are non-motile (129, 130). Following internalisation, the sporozoites evolve into multinucleate schizonts (131, 130). The process of schizogony induces structural changes within the schizonts and is terminated with the release of single-nucleated merozoites into the host's bloodstream. Merozoites pursue erythrocytes, in which merogony then takes place (132).

The multiplication of these merozoites, in the case of *Babesia* spp. sporozoites occur within the host's erythrocytes upon internalisation. Merogony includes the next evolutionary step, which involves the trophozoites and their asexual division, setting more merozoites free (133–136). This process leads to the destruction of the invaded erythrocyte and consequently to the bursting of the latter, thus releasing merozoites into the bloodstream. These carry on by targeting healthy, undamaged erythrocytes. It is to be noted, that merogony is considered an

asynchronous process. Hence, trophozoites and merozoites can be observed in the host's bloodstream at the same time (137). The process of sexual multiplication (gamogony) leads to the surge of gametocytes within the mammalian erythrocytes.

Their next target involves an invertebrate host, namely a tick. Typically, ticks take a blood meal on an infected mammal and hence, the gametocytes enter the vector. The maturation and differentiation of gametocytes occur in the lumen of the tick's gut (138–141). Consequently, the metamorphosis of the latter brings about gametes, commonly termed "Strahlenkörper" or spiky-rayed stages (142). The fertilisation of the gametes results in zygotes, that target the tick's epithelial cells to carry on with meiosis. This gives rise to kinetes, which will be moving to the salivary glands of the tick for sporogony. There is a distinction between the kinetes of *Theileria* spp. and *Babesia* spp. While *Theileria* spp. have primary kinetes, that readily proceed with the invasion of the salivary gland, *Babesia* spp. require further asexual multiplication in diverse tissues, generating secondary kinetes, that are then able to invade the salivary glands of the tick. Subsequently, the final stage involves sporogony, whereby sporonts are being formed. Their development results in creating a sporoblast, that awaits the vector's attachment to the next mammalian host. Once attached, it is reported that the sporozoites mature within 48 h (143–147) and are released into the tick's saliva (128).

The presence of *Babesia* spp. in animals can be tested by Giemsa-staining thin blood smears, which can be viewed under a microscope. Another method uses the indirect fluorescent antibody test (IFAT). Currently, the molecular approach using PCR is considered the gold standard for detection as it has a higher sensitivity and specificity (148, 149). Canine babesiosis is often responsible for severe symptoms in dogs, accompanied by pale mucous membranes, hyperthermia, haemoglobinuria, anaemia, thrombocytopenia, lethargy and anorexia (150, 151, 148). In foxes, no clinical cases have been reported yet, but carcasses of foxes, that tested positive for *B. vulpes*, exhibited splenomegaly and kidney enlargement (152).

In Europe, ticks, mainly *D. reticulatus, R. sanguineus* s.l. and *I. ricinus*, are vectors for canine babesiosis. Although, the occurrence of *Babesia canis* is scarce in red foxes (153). Red foxes are mostly affected by *B. vulpes,* also referred to as *T. annae* or *Babesia microti*-like (154–157). The classification as *T. annae* is deemed obsolete, as there is still no proof to this date of schizogony or transovarial transmission in ticks (158, 148). There is a steady increase of reported cases across Europe, with 72.2 % in (NW) Spain (159), 50 % of foxes in Austria (7), 46.4 % in Germany (160), 20 % in Hungary (161), 5.2 % in Croatia (162). *Ixodes hexagonus*

is suspected to be the main vector of this protozoan in countries with a mild, Atlantic climate (163). This hypothesis was further reinforced by studies carried out in Spain, that found *I. hexagonus* dense regions to coincide with a high prevalence *B. microti*-like infections (164, 159). This does not cancel out the possibility for other arthropod vectors in Europe, as there were reports of *B. microti*-like infections from countries, that are not endemic to *I. hexagonus* (165). Molecular screening for *Babesia cf. microti* in Bosnia and Herzegovina proved the presence of piroplasmid DNA in *D. reticulatus* (166).

A closely related species, *B. microti,* carries the highest zoonotic potential, resulting in three different outcomes. Some manifest an illness accompanied by mild to moderate viral-like symptoms, others present with an acute, severe course of disease, that can end up in death or chronic relapses and lastly asymptomatic infections (167). The first case of human infection in Austria was recorded in 2003. This study established other potential zoonotic *Babesia* species besides *B. divergens*, in this case with proximity to *B. microti* (168).

2.3.2. *Hepatozoon* spp.

Hepatozoon represents a genus of protozoal parasite, a member of the Hepatozoidae family and associated with the apicomplexan phylum. *Hepatozoon* spp. are characterised as arthropod-borne parasitic agents, that rely on an invertebrate host for their sexual development (sporogony). An interesting feature of this protozoan is its unique mode of transmission, which involves the ingestion of an infected definitive host (tick) (169, 170). Approximately 50 species of *Hepatozoon* are currently known to infect mammals (171). Most attention is brought to *H. canis* and *H. americanum*, when it comes to their sympatric host population, which involves dogs and their wild counterparts (172). *Hepatozoon canis* figures as one of the most frequently diagnosed blood parasite in red foxes in Europe (173), although it is believed to be non-endemic to countries situated in the middle latitude in Europe (174).

The life cycle of *Hepatozoon* spp. is unique, as it requires the oral uptake of an infected invertebrate host, containing the oocytes. In this case, the intermediate host is the canid, whilst the invertebrate hosts are haematophagous vectors such as ticks, mites, sand flies, tsetse flies, mosquitoes, fleas and lice among others. Upon ingestion of an infected tick carrying oocysts, sporozoites enter the dog's intestine and pursue the invasion of the gut wall (155). Their target cells involve mononuclear cells, ideally monocytes and macrophages, which ensure the

transfer of the latter to lymphoid organs (175). The liver, kidneys and lungs might be affected as well. Within these tissues occurs the maturation of secondary meronts through the process merogony. The meronts develop either into macromerozoites or micromerozoites. Micromerozoites are responsible for the invasion of neutrophilic granulocytes and monocytes, whilst macromerozoites are believed to generate secondary meronts (170).

Rhipicephalus sanguineus has been described as the main arthropod-vector for *H. canis* in Europe (169, 170). There are records of other tick species being infected with *H. canis*, among which *I. ricinus* in Italy (176). The definitive invertebrate host contracts the disease, following a blood meal on the infected intermediate host (155). This induces an uptake of parasitised white blood cells, which are transported to the tick's intestine, where they set male and female gamonts free. Gametogony, which involves the sexual reproduction of these protozoal parasites, occurs in the tick. This stage ends with the production of a zygote, which divides to give rise to oocysts, that reside within the tick's body. The oocysts of *H. canis* contain up to hundreds of membrane-bound sporocysts, which in turn confine sporozoites. Sporozoites are the infectious agents, that enable canids to contract canine hepatozoonosis (127).

Within ticks, *H. canis* can be transmitted vertically, larvae can pass it on to nymphs and consequently to adult ticks. Nymphs and adult ticks can both contain the infectious sporozoites (177). Another mode of transmission, that might be of greater importance for the spread of hepatozoonosis among red foxes, is intrauterine transmission (178). Studies demonstrated no difference between the prevalence of the disease in adult and juvenile foxes. Hence, the assumption is that foxes must have been infected via the placenta (179). The possibility for foxes or other canids to contract the disease by feeding on another conspecific, as is the case for *H. americanum* still remains to be elucidated for *H. canis* (180).

Hepatozoon spp. can be identified using microscopy and stained blood smears for the detection of intracellular gamonts, histopathology, serology or using molecular diagnostic methods, such as PCR (155). Some studies used IFAT to detect antibodies produced against *H. canis* in Israel (181, 182), Japan (183) and Turkey (184). Molecular diagnostic methods bear the highest sensitivity, when it comes to testing for *H. canis* (184, 185). A positive correlation between the high prevalence of *H. canis* and the natural habitat *of R. sanguineus* has been observed (186). This theory is further reinforced by reports of autochthonous infections occurring in Mediterranean countries, which are endemic to *R. sanguineus* (184, 187). A prevalence of 14.0 % within the dog population in southern Italy was recorded in a

study (188) and 72.7 % in Malta (189). In red foxes, the prevalence detected was at 22.2 % in Hungary (190). The prevalence in Austria varied across the country, with one study stating a prevalence of 58.3 % (7) in north-eastern Austria and another reporting a prevalence of 18.5 % in blood samples and 29.8 % in spleen samples in western Austria. (5). However, there have been reports from northern countries proving the presence of *H. canis* within their fox population, where *R. sanguineus* is known to be non-endemic (191, 192). Thus, it can be assumed that other tick species might be the cause for the infection of foxes (193, 194) in these areas, as well as free-roaming canids coming from neighbouring countries endemic to *H. canis*, that act as reservoirs (174, 195).

In dogs, the disease manifests itself with splenomegaly and hepatomegaly (155). The dogs generally suffer from chronic, subclinical infections in previously healthy dogs, but can result in extreme lethargy accompanied by cachexia and anaemia in more severe cases (182, 196, 184, 197–199). The situation among red foxes is similar, however, co-infections with other diseases such as *B. vulpes* might contribute to the pathological findings (152). The first recorded case of *H. canis* in Austria was in a golden jackal (174). This led to the assumption that free-roaming wild canids, such as red foxes and golden jackals, act as reservoirs and could be the cause for the introduction of *H. canis* in non-endemic areas (191). To this date, there are no records about the zoonotic potential of *Hepatozoon* spp. in regard to humans (200).

3. Vectors

In this study we focus on vector-borne diseases, which inherently implies a vector's importance in maintaining such diseases within a population. An overview of the most common vectors and their carrier role for the seven specific vector-borne agents chosen is provided in Figure 1 below.

3.1. Arthropods

The most common vector for the pathogens mentioned above are ticks. The most prevalent ticks in Europe belong to the order of Ixodida and are members of the family of Ixodidae. Approximately 16 tick species are known to feed on foxes, although displaying regional differences across Europe (193). Over the past two decades, a lot of data has been collected about the distribution of ticks over Europe. *Rhipicephalus sanguineus* was found to move further north from the Mediterranean countries, where it is endemic. Moreover, the geographical distribution of *D. reticularis* has extended over most of Europe, though it used to be scarce in cold continental climates (50). *Ixodes ricinus* has been mentioned as the most common tick in Europe. Nonetheless, it used to be rare at high altitudes and in cold regions such as northern Sweden. Currently, it has been reported at higher altitudes than before and in colder regions, where it used to be non-existent (201). In Germany, the most frequent tick species collected from red foxes were *I. ricinus*, *I. hexagonus, I. canisuga* and *I. kaiseri* (202–207). The fox tick, *I. canisuga*, was mainly found on red foxes in a study carried out in Hungary, although its prevalence in Austria was reported to be significantly lower than in Germany or Hungary. *Haemaphysalis concinna* was also reported, however with a noticeable decrease in prevalence towards western Austria (208).

3.2. Fleas

Chaetopsylla globiceps also known as the fox flea, is most commonly found on red foxes in the wild (208). A recent study in Turkey identified four commonly found flea species on red foxes: *Pulex irritans*, *C. globiceps*, *Ctenocephalides canis* and *Ctenophalides felis* (209). Aside from their main involvement in the spread of *Bartonella spp.*, little is known about the role they play in the spread of other vector-borne diseases. In Hungary the human flea, *Pulex irritans,* was found to be more common on red foxes than the fox flea, thus suggesting the aftermath

of urbanisation and closer contact with humans and domestic animals as key factors for this shift. Another flea species that can be found on red foxes is the hedgehog flea, *Archaeopsylla erinacei,* which is believed to be due to the predator nature of the red fox towards hedgehogs (208).

3.3. Mosquitoes

There is little information about mosquito species acting as vectors in the transmission of *Dirofilaria* spp. to red foxes. A study from Portugal identified seven species as carriers *of D. immitis*: *Culex theileri*, *Culex pipiens* f. *pipiens* and f. *molestus*, *Anopheles maculipennis* s.l. and *Anopheles atroparvus*, *Aedes caspius*, and *Aedes detritus* s.l. However, *D. repens* was not detected in any of the mosquitoes collected (210). In Italy, xeno-monitoring was carried out on *Cx. pipiens*, *Ae. caspius*, *Ae. vexans* and *Cx. modestus.* Overall, 2.2 % tested positive for *D. immitis* and 0.21 % tested positive for *D. repens*. The highest prevalence being in *Ae. caspius*, followed by *Ae. vexans*. *Dirofilaria repens* was only found in *Cx. pipiens* (211). Another study from Corsica Island (France) showed similar results with *Cx. pipiens* s.l. and *Ae. caspius* testing positive for both *D. immitis* and *D. repens*. However, an additional species has tested positive for *D. immitis*, *Ae. albopictus* (212). This raises concern, as this mosquito also referred to as “tiger mosquito”, has been invading more and more countries in Europe, threatening to become endemic, and *Aedes albopictus* was demonstrated to be a natural vector of *D. immitis* in Italy (213).

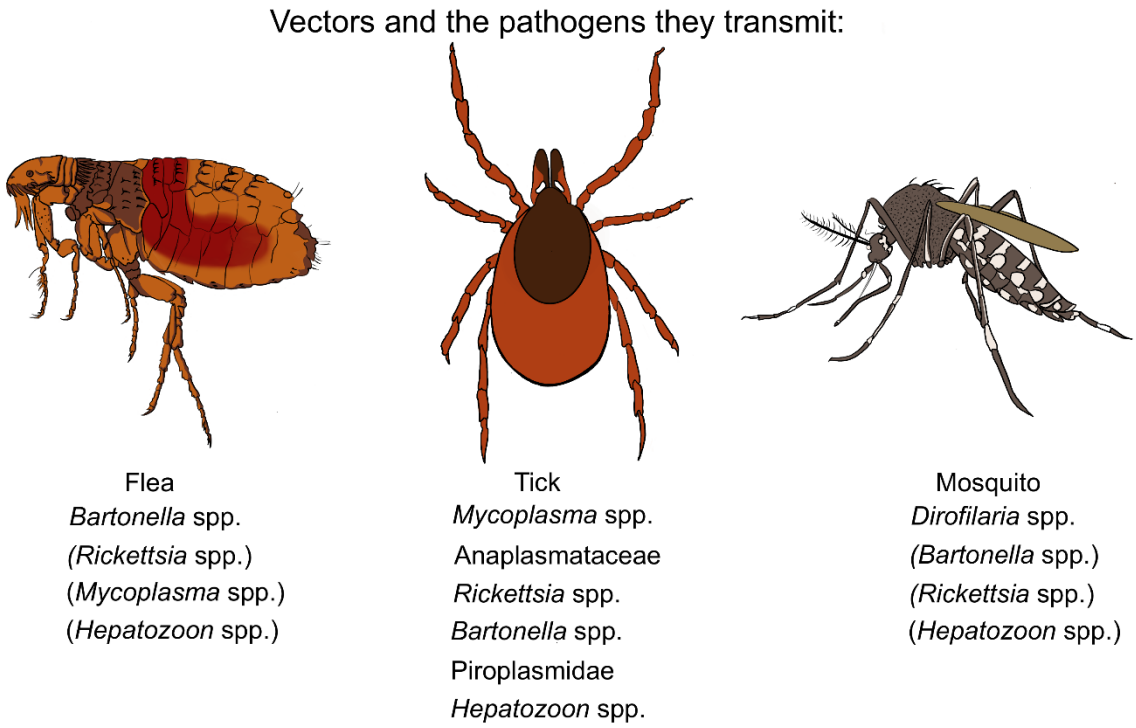


Figure 1: Arthropods as vectors of pathogens.

4. Material and Methods

4.1. Sample collection

The carcases of a total of 243 red foxes (*Vulpes vulpes*) were collected in collaboration with local hunters in Carinthia, Austria. Red foxes were shot during their yearly hunting season, which runs from December to February. For this study, we were provided with material from carcasses, that had been shot around December of 2021 until February of 2022. The data for the hunting location of the animals was provided by the hunters. Once shot, the animal carcases were delivered to the AGES (Agentur für Gesundheit und Ernährungssicherheit) situated in Innsbruck, where their respective data such as age, sex (Table 1) of each animal and body condition score were recorded at necropsy.

Table 1: Data regarding age and sex of red fox carcasses collected.

	Juvenile	*Adult*	*Unknown (age)*	*Sum (∑):*
Male (♂)	64	52	2	118
Female (♀)	75	45	4	124
Unknown (sex)	1	0	-	1
Sum (∑):	**140**	**97**	**6**	**∑ =243**

4.2. Location

The red foxes were shot in different locations throughout Carinthia, a federal state located in the southernmost part of Austria and makes about 9.536 km^2.

On this map, the different hunting locations are displayed. The outlier is from the location Velden (GJ Velden), which is part of the rural district Villach-Land (see Figure 2). The GPS Location of the hunting areas was inputted into Google Maps and with the help of pins, marked

down for a clearer visual representation. Figure 3 tabulates the districts, in which carcasses were collected by the hunters.

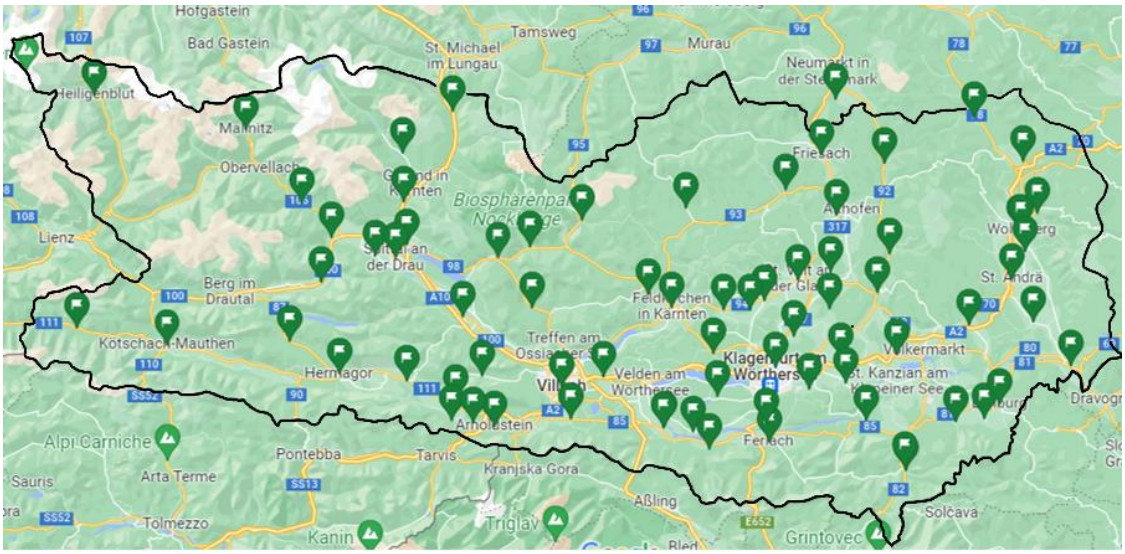


Figure 2: Map of Carinthia showing the locations at which samples have been collected (Google Maps).

Table 2: Total of carcasses collected per district.

Federal district	Sum of samples collected
St. Veit a.d. Glan	33
Klagenfurt	18
Klagenfurt-Land	36
Spittal an der Drau	39
Villach	10
Villach-Land	36
Völkermarkt	17
Wolfsberg	28
Feldkirchen	8
Hermagor	16
Unknown	2
Total (∑)	243

4.3. Extraction of sample material

The small intestines of each fox were removed and screened for *Echinococcus multilocularis* (fox tapeworm) and later placed in a freezer at -80° C for two weeks to eliminate any risk of infection. The spleen of each animal was extracted by cutting it with a pair of scissors and placed in stool collection tubes. These were then also placed in a freezer at -80° C for two weeks. Blood was collected using a 1 ml syringe. The blood was drawn from the thoracal cavity, mostly in the circumference of the heart and placed in 1.5 ml tubes individually.

4.4. Molecular analysis

4.4.1. DNA Extraction

The deoxyribonucleic acid (DNA) was individually extracted from the animal blood and tissue cells. Using this approach, the AGES was tasked with the collection of the foxes' spleen and blood. Thereupon, the materials were sent to Dr. Kniha from the Institute of Specific Prophylaxis and Tropical Medicine (Medical University of Vienna). The DNA was extracted using the QIAGEN Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol for the purification of total DNA from Animal or Blood Cells (DNeasy 96 Protocol). Their kit supplies the ready-to-use endopeptidase (Proteinase K) along with its storage buffer. For this experiment, the protocol required buffer ATL for spleen samples in the first step (no necessary for blood). The DNA was extracted by cutting off two to three pieces (approximately 10 mg) of splenic material. This was followed by an enzymatic clearing of the lysate, in which 100 µl of blood were pipetted onto 20 µl of Proteinase K and filled up with 120 µl of PBS, to make up a total volume of 220 µl. In order to identify the position of each sample, the use of a 96-well plate register was recommended (*as per the manufacturer's instructions*). Subsequently, 200 µl of the buffer (Buffer AL) was added to each well. It is important to ensure that the Buffer AL is free of ethanol.

The process did not involve an automated DNA-Isolation, instead, micro-spin columns were used for the binding of the DNA. The samples were incubated in the Eppendorf Thermo Mixer C and centrifuged using the Centrifuge Sigma 1-16.

4.4.2. PCR

The polymerase chain reaction (PCR) describes a method used to amplify a specific DNA segment, synthesising millions of copies for further research in the laboratory.
The PCR and sequence analysis were completed at the Institute of Parasitology of the Veterinary Medicine University of Vienna.

4.4.3. Principles of the PCR

The PCR is aimed at rapidly providing a very large number of copies of a targeted DNA region. The amplification is carried out using a DNA template, a DNA polymerase (Taq polymerase), two DNA primers, deoxynucleoside triphosphates (dNTPs), a buffer solution and bivalent cations (magnesium).

4.4.4. Procedure

The process involves an automated heating and cooling pattern using a thermocycler.

In this experiment, three different thermal cyclers were used: Mastercycler® nexus (Eppendorf), Mastercycler EP gradient S (Eppendorf) and the Biometra Tone 96 (Analytik Jena). This experiment focused on seven vector-borne pathogens, namely: *Mycoplasma* spp., filarioid helminths, Anaplasmataceae (*Ehrlichia canis*), Piroplasmida, *Rickettsia* spp., *Bartonella* spp. and *Hepatozoon* spp. These were tested in three sets, i.e., blood samples B1–33, B35–131, B133–236 and spleen samples MD1–83, MD84–165 and MD166–243. The equipment and reagents used for this experiment are listed below (214).

Equipment:

- Centrifuge
- Thermocycler
- Microwave

- UV transilluminator
- Voltage source (electrophoresis unit)
- Gel casting tray
- Well combs
- Micropipettes (10 µl, 10–100 µl and 100–1000 µl)
- PCR tubes
- Eppendorf tubes (1000 µl and 1500 µl)
- Racks

Reagents:

- PCR primers
- DNA (DNA-template PCR amplicon)
- dNTP Mix
- Go Taq (DNA) Polymerase
- Water
- Agarose (powder)
- Buffer (Green)
- DNA loading dye
- DNA ladder
- DNA gel stain (Midori green Advance®)

The primers used in this experiment are listed in Table 3 below with their sequences:

Table 3: List of primers for the respective causative agents

Target organism	**Primer name**	**Primer sequence**	**Amplicon size**
Mycoplasma spp.	HBT-F (Forward Primer)	5’ – ATA CGG CCC ATA TTC CTA CG – 3′	600 bp
	HBT-R (Reverse Primer)	5’ – TGC TCC ACC ACT TGT TCA – 3′	

Filarioid helminths	COlint_F (Forward Primer)	5’ – TGA TTG GTG GTT TTG GTA A – 3’	668 bp
	Colint_R (Reverse Primer)	5’ – ATA AGT ACG AGT ATC AAT ATC – 3‘	
Piroplasmida (Nest 1)	BTH-1F (Forward Primer)	5’ – CCT GAG AAA CGG CTA CCA CAT CT – 3’	700 bp
	BTH-1R (Reverse Primer)	5’ – TTG CGA CCA TAC TCC CCC CA – 3’	
Piroplasmida(BTH_G _Nest 2)	GF2	5’ – GTC TTG TAA TTG GAA TGA TGG – 3’	561 bp
	GR2	5’ – CCA AAG ACT TTG ATT TCT CTC – 3’	
Anaplasmataceae	EHR16SD_for (Forward Primer)	5’ – GGT ACC YAC AGA AGA AGT CC – 3’	345 bp
	EHR16SR_rev (Reverse Primer)	5’ – TAG CAC TCA TCG TTT ACA GC – 3’	
Bartonella spp.	bartgd_for (Forward Primer)	5’ – GAT GAT GAT CCC AAG CCT TC – 3’	179 bp
	B1623_rev (Reverse Primer)	‘5 – AAC CAA CTG AGC TAC AAG CC – 3’	
Rickettsia spp.	Ricketts_ITS_for (Forward Primer)	5’ – GAT AGG TCG GGT GTG GAA G – 3’	350 -500 bp
	Ricketts_ITS_rev (Reverse Primer)	5’ – TCG GGA TGG GAT CGT GTG – 3’	
Hepatozoon spp.	H14Hepa18SFw (Forward Primer)	5’ – GAA ATA ACA ATA CAA GGC AGT TAA AAT GCT – 3’	620 bp
	H14Hepa18SRv (Reverse Primer)	5’ – GTG CTG AAG GAG TCG TTT ATA AAG A – 3’	

Preparing the master mix:

The preparation of the master mix was formulated such that each new set of samples had a freshly prepared master mix. The ratios were according to the university's protocols and adjusted to the number of samples in one set.

The reagents were placed under a biosafety cabinet (BSCs) to thaw. In the meantime, the rack was filled with the required number of PCR tubes and Eppendorf tubes, which were then labelled accordingly. The first step involved transferring all the reagents to make up the master mix (for this set of samples) into an Eppendorf tube. Starting with the largest volumes, i.e. water and moving towards the smallest required volume of reagent (in this case dNTP mix). This precise order was also deemed suitable to prevent any further damage to the polymerase, as the Go Taq Polymerase is not supposed to be kept at room temperature. The master mix was briefly centrifuged, to prevent droplets from adhering to the sides of the tube and settle the tube's content. Using a micropipette with a 10–100 µl (yellow) capacity, 20 µl of the master mix was transferred into each PCR tube. Once every PCR tube was visually verified for its content, i.e. (20 µl of) master mix, the lids were gently pressed onto the tubes.

4.4.5. Gel electrophoresis

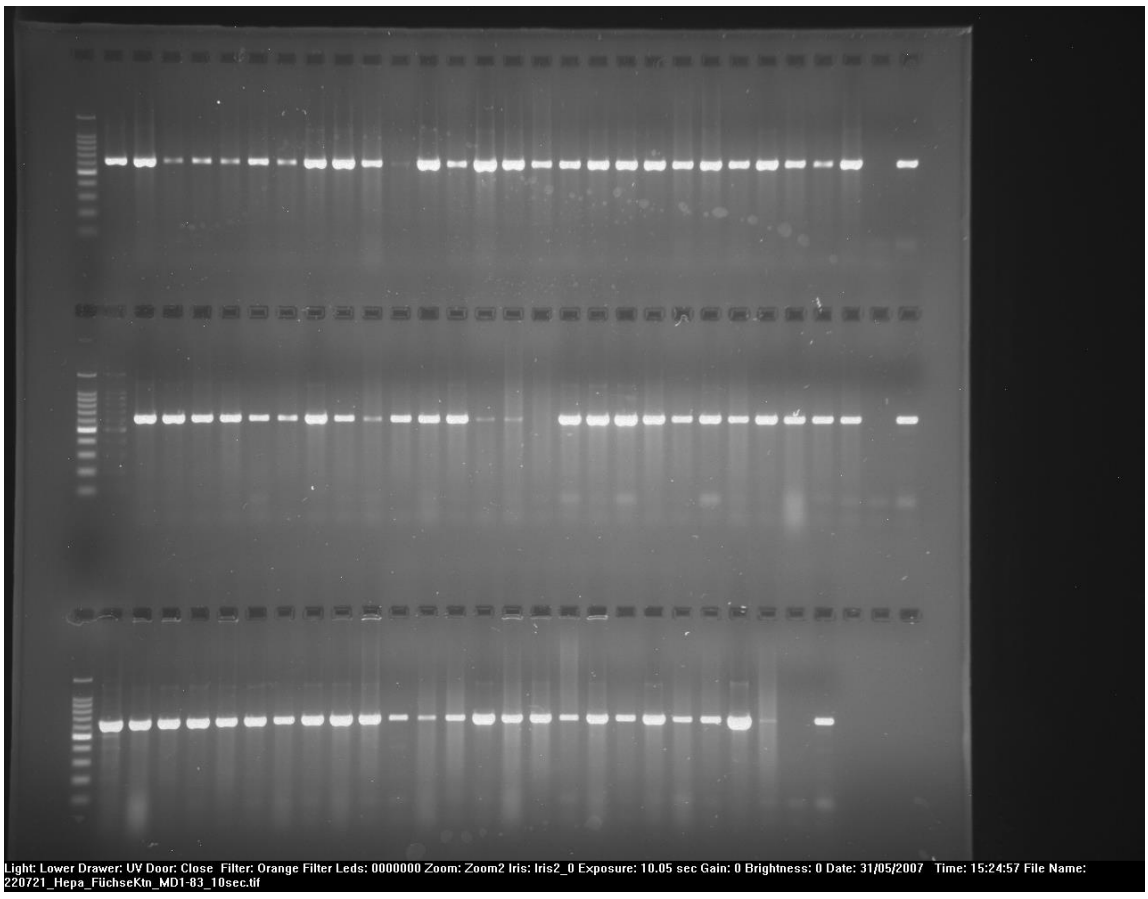
Gel electrophoresis refers to a laboratory technique, that involves an electric current, used to separate biological molecules such as proteins, DNA, RNA and other macromolecules according to their charge and size. Since all DNA fragments have an identical amount of charge per mass, the separation focusses only on the size. DNA is a nucleic acid composed of nucleotides, deoxyribose and a phosphate group. Due to the presence of this phosphate group, the DNA fragments are negatively charged, hence they will migrate towards the positive electrode. By staining the gel with a DNA-binding dye, the DNA fragments can be made visible as bands. The gel is used as a medium through which the DNA shall migrate. It is placed in a gel box, which has a positive and negative electrode positioned at both ends. A buffer solution is added to the box to facilitate the conduction of current.

In this experiment, the agarose gel was prepared using 1.8 g of agarose, 100 ml of 1 x Tris-Borat-EDTA buffer (TBE) to which 35 ml of distilled water were added. This solution was heated up using a microwave to bring it to a boil. It was left to cool down under a BSC for about

10–20 minutes and manually checked for its temperature. Afterwards, the gel was stained using the DNA-binding dye, Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Germany). 30-well combs were placed into the gel to create pockets. Once the gel had hardened and the well combs could be extracted, the gel was wrapped into plastic foil and placed into the refrigerator for later use.

Prior to loading the gel, the gel box was filled up with 1 x TBE-Buffer, so that the gel was fully immersed in it. Each well was loaded with 5 µl of PCR product. 10 µl of a 100 bp DNA Ladder (Promega, USA) was used as a standard reference. For each experiment, one negative and 1-2 positive controls were loaded at the very end to validate the screening results. The gel box was turned at 120 V and ran for a total of 40-45 minutes. After the gel has run, it was visualised under UV light using the UV transilluminator from DNR Bio-Imaging Systems. The positive control band was used as a reference to determine, which of these samples had tested positive for the causative agent of the specific vector-borne disease (see Figure 3). Positive results were sent to (LGC Genomics, Berlin, Germany) for the sequence analysis.

Figure 3: Gel capture of spleen samples 1-83 for *Hepatozoon* spp.



***Mycoplasma* spp.**

The following PCR protocol (215) was used to screen for *Mycoplasma* spp. within the 16S rRNA gene (see Table 4). The 16S rRNA is a gene that is present in all bacteria, enabling a distinct differentiation between prokaryotic and eukaryotic species. Its length is approximately 1500 bp with nine variable regions among conversed regions. Furthermore, sequencing the 16S rRNA gene enables the classification of different strains at species and subspecies levels.

Table 4: Master mix protocol for *Mycoplasma* spp.

Master mix Protocol for Mycoplasma (blood samples)		
Reagents (stock solutions)	**Volume for 1 PCR reaction (1 Eppendorf tube)/µl (µl)**	**Volume required for 10 PCR reactions/µl (µl)**
DNA	5.00	50.00
HBT-F (forward primer)	1.00	10.00
HBT-R (reverse primer)	1.00	10.00
5x Buffer (Green)	5.00	50.00
dNTP mix	0.20	2.00
Go Taq Polymerase	0.125	1.25
Water	12.675	126.75
Total	25.00	250.00

The temperature profile for this PCR was set at 94 °C (initial denaturation) for 2 min, followed by 40 cycles of 95 °C, 60 °C and 72 °C each for 1 min and a final extension at 72 °C for 7 min.

Filarioid helminths

Dirofilaria is a genus belonging to the Onchocercidae family. These are filarioid nematodes, of which *Dirofilaria immitis* (dog heartworm) and *Dirofilaria repens* are the most well-known. Different species of mosquitoes serve as intermediate hosts for this autochthonous infection.

The screening for *Dirofilaria* was carried out using the following protocol (see Table 5).

Table 5: Master mix protocol for *Dirofilaria* spp.

Master mix Protocol for Dirofilaria (blood samples)		
Reagents (stock solutions)	**Volume for 1 PCR reaction/µl (µl)**	**Volume required for 10 PCR reactions/µl (µl)**
DNA	5.00	50.00
COlint_F (forward primer)	2.00	20.00
COlint_R (reverse primer)	2.00	20.00
5x Buffer (Green)	5.00	50.00
dNTP mix	0.20	2.00
Go Taq Polymerase	0.125	1.25
Water	10.675	106.75
Total	25.00	250.00

The temperature profile, that was selected for this experiment started off with an initial denaturation at 94 °C for 2 min, followed by 8 cycles of 94 °C and 51 °C for 45 s and 72 °C for 1.5 min, that had the temperature reduced by 0,5 °C for each cycle. This was followed by 25 cycles of 94 °C and 45 °C for 45 s and 72 °C for 1.5 min. The final extension was set at 72 °C for 7 min.

Piroplasmida

Piroplasmida regroup an order of parasites of the phylum Apicomplexa. In this experiment, the BTH-1F and BTH-1R primer aim to amplify *Babesia* spp., *Theileria* spp. and *Hepatozoon canis* DNA. Compared to the prokaryotic 16S rRNA gene, that had been targeted in the previous protocols, this protocol targets the eukaryotic 18S rRNA gene (216). It is used for the identification of fungi and lower eukaryotic species, as well as subspecies (217).

Furthermore, this study makes use of a nested PCR, which involves two consecutive PCRs with two different sets of primers (see Tables 6 and 7). This allows the amplification of genes in low abundance within the DNA sample and is done to reduce the occurrence of unexpected products, due to primers binding to incorrect regions of the DNA. The first PCR run generates the amplification of a larger fragment of rRNA using the first set of primers. The second run targets the product from the first run. However, this time making use of another set of primers that bind a smaller region of the amplicon, thus reducing the yield of unwanted products (217, 218).

Table 6: Master mix protocol for Piroplasmida (Nest 1)

Master mix Protocol for Piroplasmida (blood samples)		
Reagents (stock solutions)	**Volume for 1 PCR reaction/µl (µl)**	**Volume required for 10 PCR reactions/µl (µl)**
DNA	5.00	50.00
BTH-1F (forward primer)	0.25	2.50
BTH-1R (reverse primer)	0.25	2.50
5x Buffer (Green)	5.00	50.00
dNTP mix	0.20	2.00
Go Taq Polymerase	0.125	1.25
Water	14.175	141.75
Total	25.00	250.00

The temperature profile selected for the first set of PCRs for Piroplasmida started off with an initial denaturation at 94 °C for 2 min, followed by 40 cycles at 95 °C for 30 s, 68 °C and 72 °C for 1 min. The final extension was set at 72 °C for 10 min.

Table 7: Master mix protocol for Piroplasmida (Nest 2)

Master mix Protocol for Piroplasmida (blood samples)		
Reagents (stock solutions)	**Volume for 1 PCR reaction/µl (µl)**	**Volume required for 10 PCR reactions/µl (µl)**
Nest 1 product	5.00	50.00
GF2 (forward primer)	0.50	5.00
GR2 (reverse primer)	0.50	5.00
5x Buffer (Green)	5.00	50.00
dNTP mix	0.20	2.00
Go Taq Polymerase	0.125	1.25
Water	13.675	136.75
Total	25.00	250.00

Two different master mixes and temperature profiles were required for the nested PCR. The final product of the second PCR run was then screened using gel electrophoresis.

The second PCR run was performed using a temperature profile, that started off with an initial denaturation at 94 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 60 °C and 72 °C for 1 min and a final extension of 72 °C for 10 min.

Anaplasmataceae

This protocol (219) enables the detection of both *Anaplasma* spp. and *Ehrlichia* spp DNA. (see Table 8). The primers used targeted the prokaryotic 16S gene.

Table 8: Master mix protocol for Anaplasmataceae

Master mix Protocol for Anaplasmataceae (blood samples)		
Reagents (stock solutions)	**Volume for 1 PCR reaction/µl (µl)**	**Volume required for 10 PCR reactions/µl (µl)**
DNA	5.00	50.00
EHR16SD-for (forward primer)	1.00	10.00
EHR16SR-rev (reverse primer)	1.00	10.00
5x Buffer (Green)	5.00	50.00
dNTP mix	0.20	2.00
$MgCl_2$ (25 mM)	1.50	15.00
Go Taq Polymerase	0.125	1.25
Water	11.175	111.75
Total	25.00	250.00

In the case of Anaplasmataceae, the selected temperature profile started off with an initial denaturation at 95 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 54 °C and 72 °C for 30 s and a final extension at 72 °C for 5 min.

***Bartonella* spp.**

For the detection of *Bartonella* spp., the protocol used is shown below in Table 9 (220).

Table 9: Master mix protocol for *Bartonella* spp.

Master mix Protocol for Bartonella spp. (blood samples)		
Reagents (stock solutions)	**Volume for 1 PCR reaction/µl (µl)**	**Volume required for 10 PCR reactions/µl (µl)**
DNA	5.00	50.00
bartgd_for (forward primer)	1.00	10.00
B1623_rev (reverse primer)	1.00	10.00
5x Buffer (Green)	5.00	50.00
dNTP mix	0.20	2.00
Go Taq Polymerase	0.125	1.25
Water	12.675	126.75
Total	25.00	250.00

The temperature profile selected for *Bartonella* spp. started off with an initial denaturation at 95 °C for 10 min, followed by 30 cycles at 95 °C for 15 min, 60 °C for 1 min and 72 °C for 20 min. The final extension was set at 72 °C for 5 min.

***Rickettsia* spp.**

Using the protocol below (221), the DNA was screened for Rickettsia spp. (see Table 10). ITS stands for internal transcribed spacer and refers to the spacer DNA found between the ribosomal RNA and rRNA genes in the chromosome. It is useful when determining the relationship between species belonging to the same genus or that are closely related.

Table 10: Master mix protocol for *Rickettsia* spp.

Master mix Protocol for Rickettsia spp. (blood samples)		
Reagents (stock solutions)	**Volume for 1 PCR reaction/µl (µl)**	**Volume required for 25 PCR reactions/µl (µl)**
DNA	5.00	50.00
Ricketts_ITS_for (forward primer)	1.00	10.00
Ricketts_ITS_rev (reverse primer)	1.00	10.00
5x Buffer (Green)	5.00	50.00
dNTP mix	0.20	2.00
Go Taq Polymerase	0.125	1.25
Water	12.675	126.75
Total	25.00	250.00

The temperature profile selected for Rickettsia spp. started off with an initial denaturation at 96 °C for 4 min, followed by 35 cycles at 94 °C and 52 °C for 1 min and 72 °C for 2 min. The final extension was set at 72 °C for 3 min.

***Hepatozoon* spp.**

For the screening of *Hepatozoon* spp. an established protocol was used, as shown in Table 11 below (166).

Table 11: Master mix protocol for *Hepatozoon* spp.

Master mix Protocol for Hepatozoon spp. (blood samples)		
Reagents (stock solutions)	**Volume for 1 PCR reaction/µl (µl)**	**Volume required for 10 PCR reactions/µl (µl)**
DNA	5.00	50.00
H14Hepa18SFw (forward primer)	2.00	20.00
H14Hepa18SRv (reverse primer)	2.00	20.00
5x Buffer (Green)	5.00	50.00
dNTP mix	0.20	2.00
Go Taq Polymerase	0.125	1.25
Water	14.675	146.25
Total	25.00	250.00

The temperature profile selected for *Hepatozoon* spp. started off with an initial denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C, 58 °C and 72 °C for 1 min each. The final extension was set at 72 °C for 7 min.

The tables (Table 1–15) below represent the master mix ratios used for the spleen samples. It is to be noted, that the same temperature profiles were used for the PCR of spleen-derived DNA, as for the PCR of blood-derived DNA.

For the spleen samples, the same protocols were implemented, and volumes adjusted accordingly.

5. Results

In this study, more spleen samples were available than blood samples. Out of 243 blood samples, blood samples from 59 juvenile animals, 47 adult animals and four of unknown age were not available for analysis. Spleen samples from 14 animals were not available, three of which were adults and eleven juveniles. It is to be noted, that a total of 140 juvenile animals and 97 adult animals were sampled for blood and splenic material respectively. Six animals were not identifiable regarding their age and one animal in regards of its sex.

A total of 133 blood samples and 229 spleen samples were tested for the presence of seven groups of vector-borne pathogens. The results coincide with the previously published data from other regions in Austria (7, 5). *Babesia* spp. and *H. canis* being the most common protozoal vector-borne agents in Carinthia's red fox population. Figures 4 and 5 give a representative overview of the number of blood and spleen samples that tested positive for specific causative agents in this study. Figure 6 enables the comparison between the affected animals according to age, sex, and causative agent.

In this study, *Hepatozoon canis* was identified as the vector-borne pathogen with the highest prevalence: 81.2 % (108/133) in blood samples and 98.7 % (226/229) in spleen samples have been recorded. The confidence interval for the recorded cases of *Hepatozoon* spp. in red foxes, for this experiment, was calculated and tabulated in Table 20 below.

Table 12: Confidence Interval for *Hepatozoon* spp. in blood and spleen samples

95 % Confidence Interval for *Hepatozoon* spp.			
Blood samples		**Spleen samples**	
Lower limit	**Upper limit**	**Lower limit**	**Upper limit**
73.7 %	86.9 %	96.2 %	99.6 %

Babesia spp. were the second most prevalent vector-borne pathogens, with a recorded prevalence of 74.4 % (99/133) in blood samples and 51.1 % (117/229) in spleen samples. One spleen sample from an adult, male fox from St. Veit a.d. Glan (Table 21) was suspected to be positive for *B. microti*. *Mycoplasma* spp. were the third most prevalent vector-borne pathogens recorded in red foxes from Carinthia. A prevalence of 34.6 % (46/133) in blood samples and 6.7 % (20/229) in spleen samples was recorded. The sequence analysis revealed the

presence of *Mycoplasma haemocanis* isolates (CMT001), uncultured *Mycoplasma* sp. clone 98B, uncultured *Mycoplasma* sp. clone PP158, and uncultured *Mycoplasma* sp. clone SK1F1 within the blood and spleen samples collected in this experiment.

Co-infections with the three most prevalent pathogens recorded in the respective blood and spleen samples have been tabulated in Table 14. The most common co-infection was shown in spleen samples, where 98 samples tested positive for both Piroplasmidae and *Hepatozoon* spp. 48 blood samples tested positive for the Piroplasmidae and *Hepatozoon* spp. Whilst 40 blood samples tested positive for *Mycoplasma* spp., Piroplasmidae and *Hepatozoon* spp.

Surprisingly, one spleen sample from a juvenile, male fox shot in Villach-Land (Table 13) tested positive for *A. phagocytophilum*. However, the corresponding blood sample did not test positive for *A. phagocytophilum*. Hence, the recorded prevalence of the latter was at 0.4 %, which is negligible when compared with the prevalence in neighbouring countries (45, 46).

Figure 4: Sum of blood samples testing positive for different vector-borne pathogens.

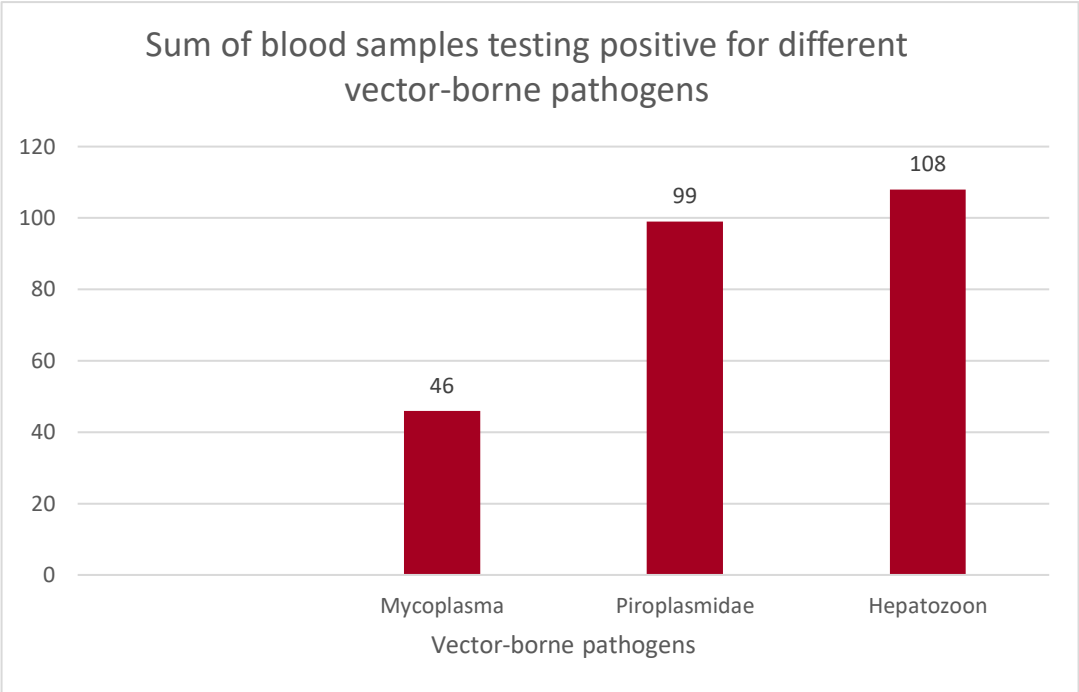


Figure 5: Sum of spleen samples testing positive for different vector-borne pathogens.

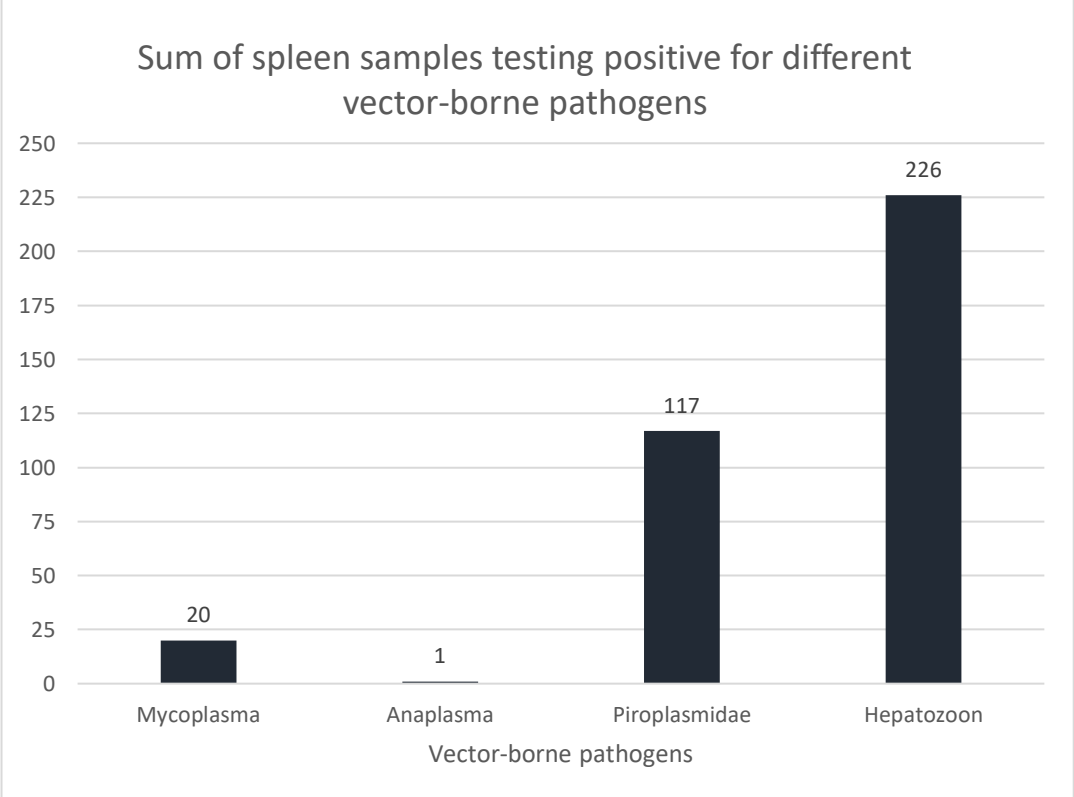
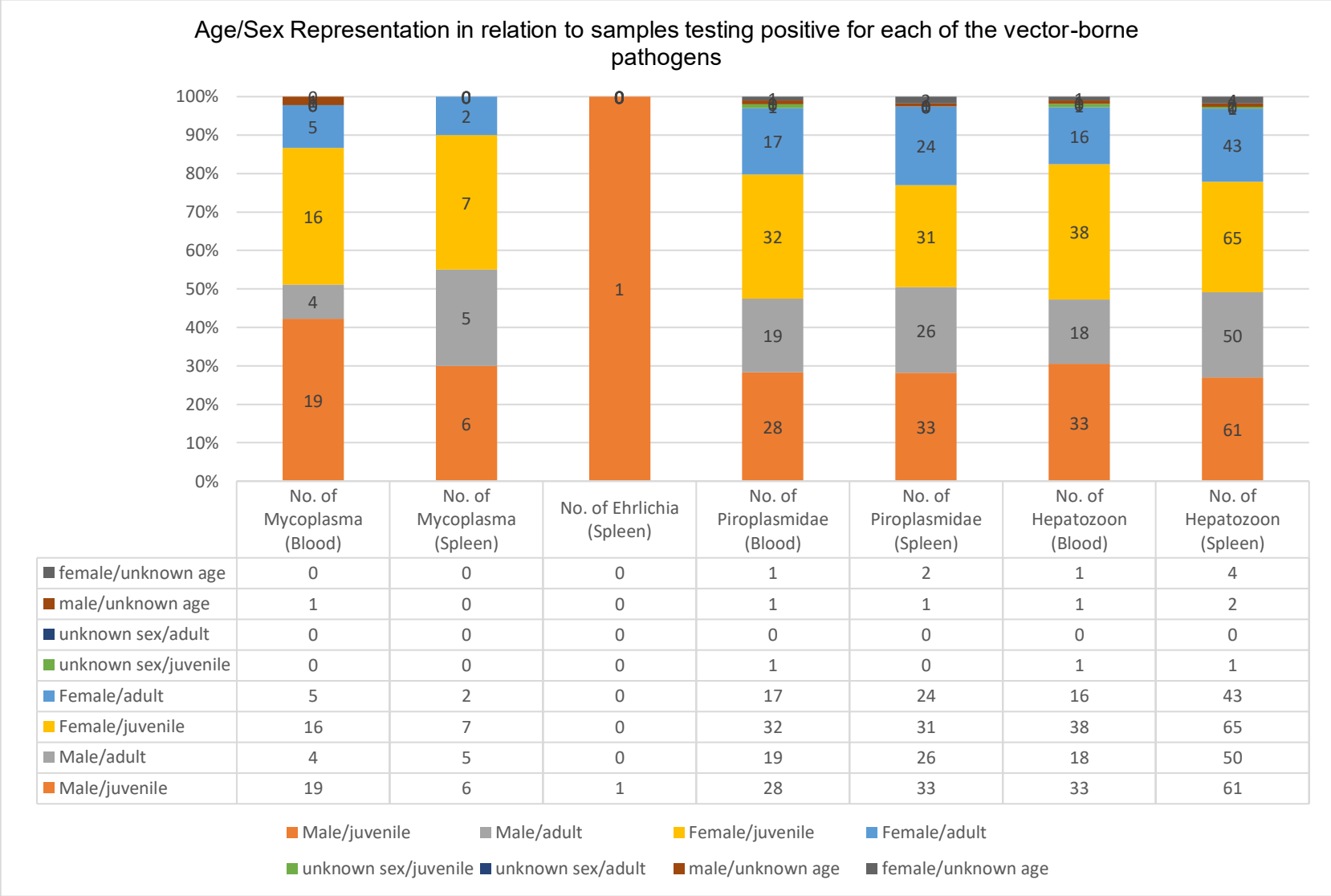


Figure 6: Distribution of affected animals according to pathogen, age and sex



	No. of Mycoplasma (Blood)	No. of Mycoplasma (Spleen)	No. of Ehrlichia (Spleen)	No. of Piroplasmidae (Blood)	No. of Piroplasmidae (Spleen)	No. of Hepatozoon (Blood)	No. of Hepatozoon (Spleen)
female/unknown age	0	0	0	1	2	1	4
male/unknown age	1	0	0	1	1	1	2
unknown sex/adult	0	0	0	0	0	0	0
unknown sex/juvenile	0	0	0	1	0	1	1
Female/adult	5	2	0	17	24	16	43
Female/juvenile	16	7	0	32	31	38	65
Male/adult	4	5	0	19	26	18	50
Male/juvenile	19	6	1	28	33	33	61

Table 13: Vector-borne pathogens recorded per district in Carinthia.

Vector-borne pathogens recorded per district in Carinthia								
District	***Mycoplasma* spp.**		**Anaplasmataceae**		**Piroplasmidae**		***Hepatozoon* spp.**	
	Blood	Spleen	Blood	Spleen	Blood	Spleen	Blood	Spleen
Feldkirchen	1	0	0	0	0	6	0	8
Hermagor	1	2	0	0	9	6	9	15
Klagenfurt	1	4	0	0	7	5	9	15
Klagenfurt-Land	2	7	0	0	19	23	20	32
Spittal an der Drau	4	3	0	0	15	17	16	35
St. Veit a. d. Glan	2	5	0	0	12	14	10	31
Villach	0	2	0	0	3	3	2	10
Villach-Land	5	12	0	1	16	18	19	34
Völkermarkt	1	1	0	0	2	13	5	17
Wolfsberg	3	9	0	0	14	11	16	27
Unknown	0	1	0	0	2	1	2	2
Sum =	20	46	0	1	99	117	108	226

Table 14: Co-infections within red foxes from Carinthia.

Co-infections with two or more vector-borne pathogens				
Samples	***Mycoplasma* spp., Piroplasmidae and *Hepatozoon* spp. positive**	***Mycoplasma* spp. and Piroplasmidae positive**	***Mycoplasma* spp. and *Hepatozoon* spp. positive**	**Piroplasmidae and *Hepatozoon* spp. positive**
Blood	40	3	3	48
Spleen	13	0	6	98

6. Discussion

Although a fair number of blood samples from juvenile animals were missing, the prevalence of most of the causative agents detected for this study was highest in samples derived from juvenile animals. The blood samples that tested positive for *Mycoplasma* spp. were collected from 35 juvenile red foxes, of which 19 were male and 16 were female and nine animals were adults, excluding one animal of unknown age. The spleen samples, that tested positive for the *Mycoplasma* spp. were collected from 13 juvenile animals and seven adult animals. When assessing the possibility of gender or age being a risk factor, more juvenile animals were found to be positive for *Mycoplasma* spp. and predominantly male animals. This could be related to their higher activity levels and consequently higher exposure rates to vectors, as hypothesised in another study with dogs (19). Male gender as a risk factor was shown in a study involving hunting dogs from southern Italy (24) and another study from Chile with free-ranging dogs and wild foxes (20). This leads to the assumption, that male animals are roaming around in search for their vixen during this period of the year, as mating takes place in winter. Thus, increasing their chances of coming across the vectors when covering long distances and possibly moving across different climatic regions (222). It is yet unclear, which ectoparasites are mainly responsible for the infection with *Mycoplasma* spp. in red foxes. In dogs, *Rhipicephalus sanguineus* is suspected to be the main vector, as it would correlate with the higher prevalence of *Mycoplasma* spp. infections in Mediterranean countries, where it is endemic (18, 19, 14, 20). Although feline haemoplasmas have been mentioned to be occurring all over Europe, this experiment suggests, that foxes might be more prone to infections with canine haemoplasmas (18, 19, 14, 20). This could either be due to red foxes being unsuitable hosts for feline haemoplasmas or the lack of a common vector, ensuring the interspecies transmission of feline haemoplasmas. Another study carried out in Patagonia, Argentina demonstrated that fleas were effective carriers of haemoplasma, but could not establish a link between positively tested animals and their respective ectoparasites (23). As this study only focuses on the detection of vector-borne diseases within the red fox population, there is uncertainty regarding their ectoparasitic load. No recording of their ectoparasitic load has been undertaken.

Positive samples were sent for sequencing to LGC Genomics, Berlin, Germany. In most cases, *Mycoplasma haemocanis* isolates were detected with some uncultured *Mycoplasma* sp. clones. The isolate *Mycoplasma haemocanis* CMT001 was identical to the strain detected in blood samples from feral dogs (*Canis lupus familiaris*) originating from Mexico (223) and in a dog

from South Korea with a history of transfusion-associated infection (224). An identical sequence was also identified in South American grey foxes (*Lycalopex griseus*) and Andean foxes (*L. culpaeus*) from Patagonia, Argentina (23). In the case of uncultured *Mycoplasma* sp. clone PP158, an identical strain has been confirmed in a study carried out on dogs in Australia (225). The uncultured *Mycoplasma* sp. clone 98B detected in this experiment was confirmed to be identical to the strain identified in a study involving red foxes from Slovakia (226).There are no comparative studies for the occurrence of *Mycoplasma* spp. in red foxes from Austria, enticing further research in this field.

In the case of Piroplasmida, blood samples of 61 juvenile animals and 36 adult animals tested positive, excluding two animals of unknown age. Sixty-four juvenile animals and 50 adult animals tested positive with their respective spleen samples, but three animals remained unidentifiable regarding their age. Blood samples were more susceptible to the test, in comparison with spleen samples, considering the ratio of blood to spleen samples available for this experiment. While 99 blood samples and 117 spleen samples tested positive for *Babesia* spp., only 47 animals tested double positive. A recent study states that blood samples are more suitable for the identification of *Babesia* cf. *microti* (= *B. vulpes*) (5). Ticks are known to be the main vectors of *Babesia* spp. in red foxes, especially the hedgehog tick, *I. hexagonus* (163). This can be explained by the predatory nature of the fox towards the latter. Hence, the transmission of this arthropod vector must be due to the sympatric co-occurrence of red foxes and hedgehogs. Hedgehogs are a protected species, which restricts the information available about their status as carriers of diseases. Nonetheless, they are known to be heavily infested with ticks and fleas, which in turn supports this hypothesis (8). Other possible vector ticks include *I. ricinus* and *I. canisuga* (160). The prevalence of *Babesia* spp. in red foxes has previously been reported at 50.7 % in blood samples from western Austria (5) and 50 % in north-eastern Austria (7). Little is known about the eventuality of *Babesia vulpes* being zoonotic and its potential carriers.

Anaplasma phagocytophilum was detected in a spleen sample of a male juvenile animal originating from Villach-Land. The occurrence of *A. phagocytophilum* in Europe remains relatively low, even though the main tick vector is *I. ricinus*, which is the most frequent tick occurring across Europe. The strain of *A. phagocytophilum* possessing the highest prevalence in Austria is found in roe deer followed by rodents (38). The causative agent can be classified into host-specific strains, thus bearing a low zoonotic potential. The present study considers this result to be negligible in the transmission of *A. phagocytophilum* to other species. There is

no conclusive evidence on the possibility of humans contracting this bacterial vector-borne pathogen from red foxes, but the possibility of this occurring should not be eliminated.

Hepatozoon spp. could be identified in the blood of 72 juvenile animals and 34 adult animals, excluding two animals of unknown age. 127 juvenile animals and 93 adult animals, excluding six animals of unknown age all tested positive with their respective spleen samples. Nearly all animals tested positive for *Hepatozoon* spp. when testing their corresponding splenic tissue. This could be due to the process of merogony involving lymphoid organs and thus leading to a higher parasitic load in the spleen (179). Hence, splenic material is considered more suitable for the detection of *Hepatozoon* spp. (5). No risk factor could be observed in this study, especially when considering age and sex. The main tick vector of this apicomplexan parasite is *R. sanguineus*, which is not endemic in Austria. Interestingly, this does not correlate with the high prevalence of this vector-borne pathogen in Austria; 58.3 % (7), thus suggesting that other tick species might be acting as vectors. Another speculation would be that vertical transmission plays a bigger role in maintaining the pathogen within the red fox population. This might explain the timely occurrence within juvenile as well as adult animals. Furthermore, given the high occurrence within the red fox population, but a seemingly low prevalence or absence within domestic dogs, this might lead to the assumption, that red foxes are better-suited hosts than domestic dogs (227). Free-roaming wild canids such as the grey wolf or golden jackal have been mentioned to contribute to the spread of vector-borne diseases across countries, especially when coming from *R. sanguineus*-endemic regions, where the chances of contracting *H. canis* are higher (191). It is still unclear if this vector-borne pathogen might pose a threat to domestic dogs (8).

All the collected samples tested negative for filarioid helminths (incl. *Dirofilaria* spp.), *Rickettsia* pp. and *Bartonella* spp. This is in accordance with previous findings in Austria (121), suggesting that dogs, possibly from overseas (or that have travelled overseas) remain the primary reservoir for *Dirofilaria* spp. in Austria. Nonetheless, awareness of the expanding topographic distribution of the following mosquito genera, *Aedes, Culex and Anopheles* within Austria and its neighbouring countries, is not to be disregarded. In the case of *Rickettsia* spp., another study carried out in western Austria identified *Candidatus* Neoehrlichia sp. with a prevalence of 0.4 % (5). It is remarkable, that although neighbouring countries such as Slovakia and Switzerland have detected *R. massiliae* and *R. slovaca* within their respective red fox and tick populations, they appear to be absent in red foxes in Austria (43, 94). Although, there is little knowledge about the pathogenicity of the vectors found in Austria. Most studies

focussed on collecting samples from the host (red fox) directly, rather than sampling the vectors (ticks). Hence, further research is necessary to determine the presence of this pathogen within the local tick population and thus conclude, whether there might be an imminent risk of infection for red foxes. *Bartonella rochalimae* was detected in Austria with a prevalence of 0.2 % (5), however, in this study *Bartonella* spp. was not identified. There is little data available about the occurrence of this bacterium in red fox populations across Europe, mostly restricted to the Czech Republic (45), Germany (47), Italy (46), Poland (72), Romania (73), Spain (75) and France (74). Generally, the prevalence was low and negligible, when evaluating the role of red foxes as carriers of this zoonotic agent. Currently, there is no published data on the local flea population of red foxes or their vector-bearing role in Austria, which could lead to more conclusive sequence data.

Overall, this study was carried out on animals, that had been shot during the winter season. Austria typically experiences a humid snow climate, which is not ideal for many tick species, as well as mosquito species. Hence, leading to the assumption that certain vector-borne pathogens might have displayed a lower prevalence in the red fox population around this time of the year. Additionally, other carriers of arthropod vectors and subsequently vector-borne pathogens, such as the hedgehog, undergo hibernation during this time of the year. This in turn might reduce the interspecies encounters and spread of vector-borne diseases around this period of the year.

7. Summary

A better understanding of the occurrence of vector-borne diseases within the red fox population in Carinthia was gained through this experiment. Hunting being a common leisure activity within this state of Austria, there is an imminent sympatric situation between humans, domestic animals (hunting dogs), and wildlife. Red foxes have been described to partake with a major role in Austrian wildlife, representing the most common wild canid species. Their role as disease carrier is of utmost interest for the assessment of the possible transmission and emergence of vector-borne diseases within their territory. The primary aim of this study was to perform a molecular screening for seven chosen vector-borne pathogens, of which some were already known to occur frequently within the red fox population in other states of Austria, as well as neighbouring countries. The focus was placed on the following vector-borne pathogens: *Mycoplasma* spp., filarioid helminths., Anaplasmataceae, *Rickettsia* spp., *Bartonella* spp., Piroplasmida, and *Hepatozoon* spp.

Six of the mentioned vector-borne pathogens from this experiment have been identified as zoonotic agents, possibly putting human health at risk. This alone highlights the importance of monitoring the occurrence of the latter within the red fox population, in order to raise awareness and prevent possible outbreaks. A key factor in limiting the spread of vector-borne diseases lies in effective monitoring and setting up a good public health system. The data collected in this study enables to draw conclusions based on the current occurrence of vector-borne diseases in Carinthia and backs up preventive measures, that have already been set in place, such as tick prophylaxis in domestic animals and humans. Furthermore, this study emphasises the life cycle, mode of transmission and possible symptoms following an infection with the mentioned vector-borne pathogens. Since this study revolves around pathogens, that require an intermediate host or reservoir to be able to infect other species, the respective vectors and their prevalence within Austria have been underlined.

Throughout this study, comparisons were drawn between other states in Austria and neighbouring countries with the aid of published studies. This also included similar studies from other continents, that pointed at interesting differences in the identity of the vectors and prevalence of vector-borne diseases within their red fox populations. The findings of this study align with other studies carried out in Austria and correlate with the situation in Central Europe. Furthermore, the results of this study apprise of the change in the distribution of vectors and

hence the emergence of vector-borne diseases within new areas of Austria. This could be linked to global warming, increased travelling, and the trade of animals.

The red fox carcasses were obtained through the collaboration with local hunters from Carinthia, that sent them to the AGES in Innsbruck, who undertook the recording of age, sex and body condition. The DNA extraction was performed at the Institute of Specific Prophylaxis and Tropical Medicine. Finally, the PCR extraction was accomplished at the Institute of Parasitology at the Veterinary Medicine University of Vienna.

8. List of tables and figures

Table 1: Data regarding age and sex of red fox carcasses collected. 21
Table 2: Total of carcasses collected per district. 22
Table 3: List of primers for the respective causative agents 25
Table 4: Master mix protocol for Mycoplasma spp. 29
Table 5: Master mix protocol for Dirofilaria spp. 30
Table 6: Master mix protocol for Piroplasmida (Nest 1) 31
Table 7: Master mix protocol for Piroplasmida (Nest 2) 31
Table 8: Master mix protocol for Anaplasmataceae 32
Table 9: Master mix protocol for Bartonella spp. 33
Table 10: Master mix protocol for Rickettsia spp. 34
Table 11: Master mix protocol for Hepatozoon spp. 35
Table 12: Confidence Interval for Hepatozoon spp. in blood and spleen samples 36
Table 13: Vector-borne pathogens recorded per district in Carinthia. 40
Table 14: Co-infections within red foxes from Carinthia. 40

Figure 1: Vectors and their transmissible pathogens 20
Figure 2: Map of Carinthia showing the locations at which samples have been collected (Google Maps). 22
Figure 3: Gel capture of spleen samples 1-83 for Hepatozoon spp. 28
Figure 4: Sum of blood samples testing positive for different vector-borne pathogens. 38
Figure 5: Sum of spleen samples testing positive for different vector-borne pathogens. 38
Figure 6: Distribution of affected animals according to pathogen, age and sex 39

9. Bibliography

1. Carinthia. Bio-economic principle - Carinthia; 2023 [cited 2023 Jun 27]. Available from: URL: https://carinthia.com/en/business-technology/bio-economic-principle/.

2. Republic of Austria, Federal Ministry of Agriculture, Forestry, Environment and Water Management. The Austrian Forest Programme [cited 2023 Jun 27]. Available from: URL: https://info.bml.gv.at/dam/jcr:e8e49622-bff5-4ccf-b948-ee6cecfd0902/Waldprogramm_20englisch.pdf.

3. AGES. Ovitrap monitoring of alien gnat species in Austria; 2023 [cited 2023 Jun 27]. Available from: URL: https://www.ages.at/en/research/project-highlights/ovitrap-monitoring-of-alien-gnat-species-in-austria.

4. Zecchin B, Nardi M de, Nouvellet P, Vernesi C, Babbucci M, Crestanello B et al. Genetic and spatial characterization of the red fox (Vulpes vulpes) population in the area stretching between the Eastern and Dinaric Alps and its relationship with rabies and canine distemper dynamics. PLoS One 2019; 14(3):e0213515.

5. Hodžić A, Mrowietz N, Cézanne R, Bruckschwaiger P, Punz S, Habler VE et al. Occurrence and diversity of arthropod-transmitted pathogens in red foxes (Vulpes vulpes) in western Austria, and possible vertical (transplacental) transmission of Hepatozoon canis. Parasitology 2018; 145(3):335–44.

6. Medkour H, Laidoudi Y, Marié J-L, Fenollar F, Davoust B, Mediannikov O. MOLECULAR INVESTIGATION OF VECTOR-BORNE PATHOGENS IN RED FOXES (VULPES VULPES) FROM SOUTHERN FRANCE. J Wildl Dis 2020; 56(4):837–50.

7. Duscher GG, Fuehrer H-P, Kübber-Heiss A. Fox on the run--molecular surveillance of fox blood and tissue for the occurrence of tick-borne pathogens in Austria. Parasites Vectors 2014; 7:521.

8. Duscher GG, Leschnik M, Fuehrer H-P, Joachim A. Wildlife reservoirs for vector-borne canine, feline and zoonotic infections in Austria. Int J Parasitol Parasites Wildl 2015; 4(1):88–96.

9. Cambridge Core. Occurrence and diversity of arthropod-transmitted pathogens in red foxes (Vulpes vulpes) in western Austria, and possible vertical (transplacental) transmission of Hepatozoon canis | Parasitology | Cambridge Core; 2023 [cited 2023 May 21]. Available from: URL: https://www.cambridge.org/core/journals/parasitology/article/occurrence-and-diversity-of-arthropodtransmitted-pathogens-in-red-foxes-vulpes-vulpes-in-western-austria-and-possible-vertical-transplacental-transmission-of-hepatozoon-canis/492CA92B43D9562F094A6F7B0BB092AC.

10. Foley.janet. Hemotropic Mycoplasma Infections in Animals - Circulatory System - MSD Veterinary Manual; 2023 [cited 2023 Jun 27]. Available from: URL: https://www.msdvetmanual.com/circulatory-system/blood-parasites/hemotropic-mycoplasma-infections-in-animals.

11. Tasker S, Helps CR, Day MJ, Harbour DA, Shaw SE, Harrus S et al. Phylogenetic analysis of hemoplasma species: an international study. J Clin Microbiol 2003; 41(8):3877–80.

12. Willi B, Boretti FS, Meli ML, Bernasconi MV, Casati S, Hegglin D et al. Real-time PCR investigation of potential vectors, reservoirs, and shedding patterns of feline hemotropic mycoplasmas. Appl Environ Microbiol 2007; 73(12):3798–802.

13. Peters IR, Helps CR, Willi B, Hofmann-Lehmann R, Tasker S. The prevalence of three species of feline haemoplasmas in samples submitted to a diagnostics service as determined by three novel real-time duplex PCR assays. Vet Microbiol 2008; 126(1-3):142–50.

14. Wengi N, Willi B, Boretti FS, Cattori V, Riond B, Meli ML et al. Real-time PCR-based prevalence study, infection follow-up and molecular characterization of canine hemotropic mycoplasmas. Vet Microbiol 2008; 126(1-3):132–41.

15. Willi B, Meli ML, Lüthy R, Honegger H, Wengi N, Hoelzle LE et al. Development and application of a universal Hemoplasma screening assay based on the SYBR green PCR principle. J Clin Microbiol 2009; 47(12):4049–54.

16. Barker EN, Tasker S, Day MJ, Warman SM, Woolley K, Birtles R et al. Development and use of real-time PCR to detect and quantify Mycoplasma haemocanis and "Candidatus Mycoplasma haematoparvum" in dogs. Vet Microbiol 2010; 140(1-2):167–70.

17. Sasaki M, Ohta K, Matsuu A, Hirata H, Ikadai H, Oyamada T. A molecular survey of Mycoplasma haemocanis in dogs and foxes in Aomori Prefecture, Japan. jpr 2008; 18(2):57–60. Available from: URL: https://www.jstage.jst.go.jp/article/jprotozoolres/18/2/18_57/_article/-char/ja/.

18. Willi B, Novacco M, Meli M, Wolf-Jäckel G, Boretti F, Wengi N et al. Haemotropic mycoplasmas of cats and dogs: transmission, diagnosis, prevalence and importance in Europe. Schweiz Arch Tierheilkd 2010; 152(5):237–44.

19. Novacco M, Meli ML, Gentilini F, Marsilio F, Ceci C, Pennisi MG et al. Prevalence and geographical distribution of canine hemotropic mycoplasma infections in Mediterranean countries and analysis of risk factors for infection. Vet Microbiol 2010; 142(3-4):276–84.

20. Di Cataldo S, Cevidanes A, Ulloa-Contreras C, Sacristán I, Peñaloza-Madrid D, Vianna J et al. Widespread Infection with Hemotropic Mycoplasmas in Free-Ranging Dogs and Wild Foxes Across Six Bioclimatic Regions of Chile. Microorganisms 2021; 9(5).

21. Kenny MJ, Shaw SE, Beugnet F, Tasker S. Demonstration of two distinct hemotropic mycoplasmas in French dogs. J Clin Microbiol 2004; 42(11):5397–9.

22. Ravagnan S, Carli E, Piseddu E, Da Rold G, Porcellato E, Zanardello C et al. Prevalence and molecular characterization of canine and feline hemotropic mycoplasmas (hemoplasmas) in northern Italy. Parasit Vectors 2017; 10(1):132.

23. Millán J, Travaini A, Cevidanes A, Sacristán I, Rodríguez A. Assessing the natural circulation of canine vector-borne pathogens in foxes, ticks and fleas in protected areas of Argentine Patagonia with negligible dog participation. Int J Parasitol Parasites Wildl 2019; 8:63–70.

24. Cortese L, Beall M, Buono F, Buch J, Pacifico L, Neola B et al. Distribution and risk factors of canine haemotropic mycoplasmas in hunting dogs from southern Italy. Vet Microbiol 2020; 251:108910.

25. Bergmann M, Englert T, Stuetzer B, Hawley JR, Lappin MR, Hartmann K. Risk factors of different hemoplasma species infections in cats. BMC Vet Res 2017; 13(1):52.

26. Maggi RG, Mascarelli PE, Havenga LN, Naidoo V, Breitschwerdt EB. Co-infection with Anaplasma platys, Bartonella henselae and Candidatus Mycoplasma haematoparvum in a veterinarian. Parasit Vectors 2013; 6:103.

27. Hördt A, López MG, Meier-Kolthoff JP, Schleuning M, Weinhold L-M, Tindall BJ et al. Analysis of 1,000+ Type-Strain Genomes Substantially Improves Taxonomic Classification of Alphaproteobacteria. Front Microbiol 2020; 11:468.

28. Rikihisa Y. Mechanisms of obligatory intracellular infection with Anaplasma phagocytophilum. Clin Microbiol Rev 2011; 24(3):469–89.

29. Lin M, Kikuchi T, Brewer HM, Norbeck AD, Rikihisa Y. Global proteomic analysis of two tick-borne emerging zoonotic agents: anaplasma phagocytophilum and ehrlichia chaffeensis. Front Microbiol 2011; 2:24.

30. Woldehiwet Z. The natural history of Anaplasma phagocytophilum. Vet Parasitol 2010; 167(2-4):108–22.

31. Dzięgiel B, Adaszek Ł, Winiarczyk S. Wild animals as reservoirs of Anaplasma phagocytophilum for humans. Przegl Epidemiol 2016; 70(3):428–35.

32. Xiong Q, Lin M, Rikihisa Y. Cholesterol-dependent anaplasma phagocytophilum exploits the low-density lipoprotein uptake pathway. PLoS Pathog 2009; 5(3):e1000329.

33. Sultana H, Neelakanta G, Kantor FS, Malawista SE, Fish D, Montgomery RR et al. Anaplasma phagocytophilum induces actin phosphorylation to selectively regulate gene transcription in Ixodes scapularis ticks. J Exp Med 2010; 207(8):1727–43.

34. Neelakanta G, Sultana H, Fish D, Anderson JF, Fikrig E. Anaplasma phagocytophilum induces Ixodes scapularis ticks to express an antifreeze glycoprotein gene that enhances their survival in the cold. J Clin Invest 2010; 120(9):3179–90.

35. Lommano E, Bertaiola L, Dupasquier C, Gern L. Infections and coinfections of questing Ixodes ricinus ticks by emerging zoonotic pathogens in Western Switzerland. Appl Environ Microbiol 2012; 78(13):4606–12.

36. Pangrácová L, Derdáková M, Pekárik L, Hviščová I, Víchová B, Stanko M et al. Ixodes ricinus abundance and its infection with the tick-borne pathogens in urban and suburban areas of Eastern Slovakia. Parasit Vectors 2013; 6(1):238.

37. Stuen S, Granquist EG, Silaghi C. Anaplasma phagocytophilum--a widespread multi-host pathogen with highly adaptive strategies. Front Cell Infect Microbiol 2013; 3:31.

38. Polin H, Hufnagl P, Haunschmid R, Gruber F, Ladurner G. Molecular evidence of Anaplasma phagocytophilum in Ixodes ricinus ticks and wild animals in Austria. J Clin Microbiol 2004; 42(5):2285–6.

39. Jensen PM, Hansen H, Frandsen F. Spatial risk assessment for Lyme borreliosis in Denmark. Scand J Infect Dis 2000; 32(5):545–50.

40. Carpi G, Cagnacci F, Neteler M, Rizzoli A. Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area. Epidemiol Infect 2008; 136(10):1416–24.

41. Rizzoli A, Hauffe HC, Tagliapietra V, Neteler M, Rosà R. Forest structure and roe deer abundance predict tick-borne encephalitis risk in Italy. PLoS One 2009; 4(2):e4336.

42. Rosef O, Paulauskas A, Radzijevskaja J. Prevalence of Borrelia burgdorferi sensu lato and Anaplasma phagocytophilum in questing Ixodes ricinus ticks in relation to the density of wild cervids. Acta Vet Scand 2009; 51(1):47.

43. Víchová B, Majláthová V, Nováková M, Stanko M, Hviščová I, Pangrácová L et al. Anaplasma infections in ticks and reservoir host from Slovakia. Infect Genet Evol 2014; 22:265–72.

44. Anaplasma phagocytophilum infection of red foxes [Vulpes vulpes]; 2009. Available from: URL: https://bibliotekanauki.pl/articles/50073.pdf.

45. Hulínská D, Langrová K, Pejcoch M, Pavlásek I. Detection of Anaplasma phagocytophilum in animals by real-time polymerase chain reaction. APMIS 2004; 112(4-5):239–47.

46. Ebani VV, Verin R, Fratini F, Poli A, Cerri D. Molecular survey of Anaplasma phagocytophilum and Ehrlichia canis in red foxes (Vulpes vulpes) from central Italy. J Wildl Dis 2011; 47(3):699–703.

47. Härtwig V, Loewenich FD von, Schulze C, Straubinger RK, Daugschies A, Dyachenko V. Detection of Anaplasma phagocytophilum in red foxes (Vulpes vulpes) and raccoon dogs (Nyctereutes procyonoides) from Brandenburg, Germany. Ticks Tick Borne Dis 2014; 5(3):277–80.

48. Ben Said M, Belkahia H, Héni MM, Bouattour A, Ghorbel A, Gharbi M et al. Seroprevalence of Anaplasma phagocytophilum in well maintained horses from northern Tunisia. Trop Biomed 2014; 31(3):432–40.

49. Heikkilä HM, Bondarenko A, Mihalkov A, Pfister K, Spillmann T. Anaplasma phagocytophilum infection in a domestic cat in Finland: Case report. Acta Vet Scand 2010; 52(1):62.

50. Beugnet F, Marié J-L. Emerging arthropod-borne diseases of companion animals in Europe. Vet Parasitol 2009; 163(4):298–305.

51. Kowalski J, Hopfenmüller W, Fingerle V, Malberg H, Eisenblätter M, Wagner J et al. Seroprevalence of human granulocytic anaplasmosis in Berlin/Brandenburg, Germany: an 8-year survey. Clin Microbiol Infect 2006; 12(9):924–7.

52. Markowicz M, Schötta A-M, Wijnveld M, Stanek G. Human granulocytic anaplasmosis acquired in Connecticut, USA, diagnosed in Vienna, Austria, 2015. Diagn Microbiol Infect Dis 2016; 84(4):347–9.

53. registry-migration.gbif.org. Prokaryotic Nomenclature Up-to-Date (PNU); 2023.

54. Okaro U, Addisu A, Casanas B, Anderson B. Bartonella Species, an Emerging Cause of Blood-Culture-Negative Endocarditis. Clin Microbiol Rev 2017; 30(3):709–46.

55. Harms A, Dehio C. Intruders below the radar: molecular pathogenesis of Bartonella spp. Clin Microbiol Rev 2012; 25(1):42–78.

56. Breitschwerdt EB. Bartonellosis: one health perspectives for an emerging infectious disease. ILAR J 2014; 55(1):46–58.

57. Chomel BB, Kasten RW. Bartonellosis, an increasingly recognized zoonosis. J Appl Microbiol 2010; 109(3):743–50.

58. Molecular medical microbiology; 2015. Available from: URL: https://scholar.google.com/citations?user=dmyvu-eaaaaj&hl=de&oi=sra.

59. Barka NE, Hadfield T, Patnaik M, Schwartzman WA, Peter JB. EIA for detection of Rochalimaea henselae-reactive IgG, IgM, and IgA antibodies in patients with suspected cat-scratch disease. J Infect Dis 1993; 167(6):1503–4.

60. Anderson B, Kelly C, Threlkel R, Edwards K. Detection of Rochalimaea henselae in cat-scratch disease skin test antigens. J Infect Dis 1993; 168(4):1034–6.

61. Berglund EC, Frank AC, Calteau A, Vinnere Pettersson O, Granberg F, Eriksson A-S et al. Run-off replication of host-adaptability genes is associated with gene transfer agents in the genome of mouse-infecting Bartonella grahamii. PLoS Genet 2009; 5(7):e1000546.

62. Chomel BB, Boulouis H-J, Breitschwerdt EB, Kasten RW, Vayssier-Taussat M, Birtles RJ et al. Ecological fitness and strategies of adaptation of Bartonella species to their hosts and vectors. Vet Res 2009; 40(2):29.

63. Halos L, Jamal T, Maillard R, Girard B, Guillot J, Chomel B et al. Role of Hippoboscidae flies as potential vectors of Bartonella spp. infecting wild and domestic ruminants. Appl Environ Microbiol 2004; 70(10):6302–5.

64. Rudolf I, Blažejová H, Mendel J, Straková P, Šebesta O, Rettich F et al. Bartonella species in medically important mosquitoes, Central Europe. Parasitol Res 2020; 119(8):2713–7.

65. Sréter-Lancz Z, Tornyai K, Széll Z, Sréter T, Márialigeti K. Bartonella infections in fleas (Siphonaptera: Pulicidae) and lack of bartonellae in ticks (Acari: Ixodidae) from Hungary. Folia Parasitol (Praha) 2006; 53(4):313–6.

66. Foil L, Andress E, Freeland RL, Roy AF, Rutledge R, Triche PC et al. Experimental infection of domestic cats with Bartonella henselae by inoculation of Ctenocephalides felis (Siphonaptera: Pulicidae) feces. J Med Entomol 1998; 35(5):625–8.

67. Dehio C. Bartonella-host-cell interactions and vascular tumour formation. Nat Rev Microbiol 2005; 3(8):621–31.

68. Schülein R, Seubert A, Gille C, Lanz C, Hansmann Y, Piémont Y et al. Invasion and persistent intracellular colonization of erythrocytes. A unique parasitic strategy of the emerging pathogen Bartonella. J Exp Med 2001; 193(9):1077–86.

69. Eremeeva ME, Gerns HL, Lydy SL, Goo JS, Ryan ET, Mathew SS et al. Bacteremia, fever, and splenomegaly caused by a newly recognized bartonella species. N Engl J Med 2007; 356(23):2381–7.

70. Parola P, Shpynov S, Montoya M, Lopez M, Houpikian P, Zeaiter Z et al. First molecular evidence of new Bartonella spp. in fleas and a tick from Peru. Am J Trop Med Hyg 2002; 67(2):135–6.

71. Henn JB, Gabriel MW, Kasten RW, Brown RN, Koehler JE, MacDonald KA et al. Infective endocarditis in a dog and the phylogenetic relationship of the associated "Bartonella rochalimae" strain with isolates from dogs, gray foxes, and a human. J Clin Microbiol 2009; 47(3):787–90.

72. Karbowiak G, Vichova B, Majlathova V, Hapunik J, Petko B. Anaplasma phagocytophilum infection of red foxes (Vulpes vulpes). Ann Agric Environ Med 2009; 16(2):299–300.

73. Dumitrache MO, Matei IA, Ionică AM, Kalmár Z, D'Amico G, Sikó-Barabási S et al. Molecular detection of Anaplasma phagocytophilum and Borrelia burgdorferi sensu lato genospecies in red foxes (Vulpes vulpes) from Romania. Parasites Vectors 2015; 8:514.

74. Henn JB, Chomel BB, Boulouis HJ, Kasten RW, Murray WJ, Bar-Gal GK et al. Bartonella rochalimae in raccoons, coyotes, and red foxes. Emerg Infect Dis 2009; 15(12):1984–7.

75. Gerrikagoitia X, Gil H, García-Esteban C, Anda P, Juste RA, Barral M. Presence of Bartonella species in wild carnivores of northern Spain. Appl Environ Microbiol 2012; 78(3):885–8.

76. Labruna MB, Walker DH. Rickettsia felis and changing paradigms about pathogenic rickettsiae. Emerg Infect Dis 2014; 20(10):1768–9.

77. Emelyanov VV. Rickettsiaceae, rickettsia-like endosymbionts, and the origin of mitochondria. Biosci Rep 2001; 21(1):1–17.

78. Merhej V, Raoult D. Rickettsial evolution in the light of comparative genomics. Biol Rev Camb Philos Soc 2011; 86(2):379–405.

79. Walker DH, Hudnall SD, Szaniawski WK, Feng HM. Monoclonal antibody-based immunohistochemical diagnosis of rickettsialpox: the macrophage is the principal target. Mod Pathol 1999; 12(5):529–33.

80. Helminiak L, Mishra S, Kim HK. Pathogenicity and virulence of Rickettsia. Virulence 2022; 13(1):1752–71.

81. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev 2005; 18(4):719–56.

82. Raoult D, Roux V. Rickettsioses as paradigms of new or emerging infectious diseases. Clin Microbiol Rev 1997; 10(4):694–719.

83. Fournier P-E, Dumler JS, Greub G, Zhang J, Wu Y, Raoult D. Gene sequence-based criteria for identification of new rickettsia isolates and description of Rickettsia heilongjiangensis sp. nov. J Clin Microbiol 2003; 41(12):5456–65.

84. Fournier P-E, Zhu Y, Ogata H, Raoult D. Use of highly variable intergenic spacer sequences for multispacer typing of Rickettsia conorii strains. J Clin Microbiol 2004; 42(12):5757–66.

85. Lledó L, Serrano JL, Isabel Gegúndez M, Giménez-Pardo C, Saz JV. Antibodies to Rickettsia spp. and Borrelia burgdorferi in Spanish Wild Red Foxes (Vulpes vulpes). J Wildl Dis 2016; 52(1):122–5.

86. Ortuño A, Sanfeliu I, Nogueras MM, Pons I, López-Claessens S, Castellà J et al. Detection of Rickettsia massiliae/Bar29 and Rickettsia conorii in red foxes (Vulpes vulpes) and their Rhipicephalus sanguineus complex ticks. Ticks Tick Borne Dis 2018; 9(3):629–31.

87. Marié J-L, Davoust B, Socolovschi C, Mediannikov O, Roqueplo C, Beaucournu J-C et al. Rickettsiae in arthropods collected from red foxes (Vulpes vulpes) in France. Comp Immunol Microbiol Infect Dis 2012; 35(1):59–62.

88. Beati L, Raoult D. Rickettsia massiliae sp. nov., a new spotted fever group Rickettsia. Int J Syst Bacteriol 1993; 43(4):839–40.

89. Matsumoto K, Ogawa M, Brouqui P, Raoult D, Parola P. Transmission of Rickettsia massiliae in the tick, Rhipicephalus turanicus. Med Vet Entomol 2005; 19(3):263–70.

90. Fernández-Soto P, Pérez-Sánchez R, Alamo-Sanz R, Encinas-Grandes A. Spotted fever group rickettsiae in ticks feeding on humans in northwestern Spain: is Rickettsia conorii vanishing? Ann N Y Acad Sci 2006; 1078:331–3.

91. Bacellar F, Regnery RL, Núncio MS, Filipe AR. Genotypic evaluation of rickettsial isolates recovered from various species of ticks in Portugal. Epidemiol. Infect. 1995; 114(1):169–78.

92. Willi B, Boretti FS, Cattori V, Tasker S, Meli ML, Reusch C et al. Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with hemolytic anemia in Switzerland. J Clin Microbiol 2005; 43(6):2581–5.

93. Mura A, Masala G, Tola S, Satta G, Fois F, Piras P et al. First direct detection of rickettsial pathogens and a new rickettsia, 'Candidatus Rickettsia barbariae', in ticks from Sardinia, Italy. Clinical Microbiology and Infection 2008; 14(11):1028–33.

94. Bernasconi MV, Casati S, Péter O, Piffaretti J-C. Rhipicephalus ticks infected with Rickettsia and Coxiella in Southern Switzerland (Canton Ticino). Infection, Genetics and Evolution 2002; 2(2):111–20. Available from: URL: https://www.sciencedirect.com/science/article/pii/s1567134802000928.

95. Földvári G, Široký P, Szekeres S, Majoros G, Sprong H. Dermacentor reticulatus: a vector on the rise. Parasit Vectors 2016; 9(1):314.

96. Angelakis E, Bechah Y, Raoult D. The History of Epidemic Typhus. Microbiol Spectr 2016; 4(4).

97. Houhamdi L, Fournier P-E, Fang R, Lepidi H, Raoult D. An experimental model of human body louse infection with Rickettsia prowazekii. J Infect Dis 2002; 186(11):1639–46.

98. Socolovschi C, Pages F, Ndiath MO, Ratmanov P, Raoult D. Rickettsia species in African Anopheles mosquitoes. PLoS One 2012; 7(10):e48254.

99. Barua S, Hoque MM, Kelly PJ, Poudel A, Adekanmbi F, Kalalah A et al. First report of Rickettsia felis in mosquitoes, USA. Emerg Microbes Infect 2020; 9(1):1008–10.

100. Parola P, Rovery C, Rolain JM, Brouqui P, Davoust B, Raoult D. Rickettsia slovaca and R. raoultii in tick-borne Rickettsioses. Emerg Infect Dis 2009; 15(7):1105–8.

101. Portillo A, Santibáñez S, García-Álvarez L, Palomar AM, Oteo JA. Rickettsioses in Europe. Microbes Infect 2015; 17(11-12):834–8.

102. Oteo JA, Ibarra V, Blanco JR, Martínez de Artola V, Márquez FJ, Portillo A et al. Dermacentor-borne necrosis erythema and lymphadenopathy: clinical and epidemiological features of a new tick-borne disease. Clin Microbiol Infect 2004; 10(4):327–31.

103. Silva-Pinto A, Santos MdL, Sarmento A. Tick-borne lymphadenopathy, an emerging disease. Ticks Tick Borne Dis 2014; 5(6):656–9.

104. Michalski ML, Bain O, Fischer K, Fischer PU, Kumar S, Foster JM. Identification and phylogenetic analysis of Dirofilaria ursi (Nematoda: Filarioidea) from Wisconsin black bears (Ursus americanus) and its Wolbachia endosymbiont. J Parasitol 2010; 96(2):412–9.

105. Human dirofilariasis due to Dirofilaria (Nochtiella) repens: an update of world literature from 1995 to 2000; 2000. Available from: URL: https://www.esda.vet/media/attachments/2021/08/19/europe1.pdf#page=67.

106. Otranto D, Eberhard ML. Zoonotic helminths affecting the human eye. Parasit Vectors 2011; 4:41.

107. Sałamatin RV, Pavlikovska TM, Sagach OS, Nikolayenko SM, Kornyushin VV, Kharchenko VO et al. Human dirofilariasis due to Dirofilaria repens in Ukraine, an emergent zoonosis: epidemiological report of 1465 cases. Acta Parasitol 2013; 58(4):592–8.

108. Di Cesare A, Otranto D, Di Giulio E, Simonato G, Latrofa MS, La Torre F et al. Microfilarial periodicity of Dirofilaria repens in naturally infested dogs. Parasitol Res 2013; 112(12):4273–9.

109. Simón F, Siles-Lucas M, Morchón R, González-Miguel J, Mellado I, Carretón E et al. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin Microbiol Rev 2012; 25(3):507–44.

110. Capelli G, Genchi C, Baneth G, Bourdeau P, Brianti E, Cardoso L et al. Recent advances on Dirofilaria repens in dogs and humans in Europe. Parasit Vectors 2018; 11(1):663.

111. Genchi C, Kramer L. Subcutaneous dirofilariosis (Dirofilaria repens): an infection spreading throughout the old world. Parasit Vectors 2017; 10(Suppl 2):517.

112. Heartworm infection in humans and animals; 2001.

113. Dirofilaria immitis and D. repens in dog and cat and human infections; 2007. Available from: URL: https://www.pagepress.org/journals/index.php/mappe/article/download/7154/6816.

114. McGarry HF, Egerton GL, Taylor MJ. Population dynamics of Wolbachia bacterial endosymbionts in Brugia malayi. Mol Biochem Parasitol 2004; 135(1):57–67.

115. Ferri E, Bain O, Barbuto M, Martin C, Lo N, Uni S et al. New insights into the evolution of Wolbachia infections in filarial nematodes inferred from a large range of screened species. PLoS One 2011; 6(6):e20843.

116. Brown HE, Harrington LC, Kaufman PE, McKay T, Bowman DD, Nelson CT et al. Key factors influencing canine heartworm, Dirofilaria immitis, in the United States. Parasit Vectors 2012; 5:245.

117. Riebenbauer K, Weber PB, Walochnik J, Karlhofer F, Winkler S, Dorfer S et al. Human dirofilariosis in Austria: the past, the present, the future. Parasit Vectors 2021; 14(1):227.

118. Heartworm infection in humans and animals; 2001.

119. Morchón R, Carretón E, González-Miguel J, Mellado-Hernández I. Heartworm Disease (Dirofilaria immitis) and Their Vectors in Europe - New Distribution Trends. Front Physiol 2012; 3:196.

120. Sonnberger K, Duscher GG, Fuehrer H-P, Leschnik M. Current trends in canine dirofilariosis in Austria-do we face a pre-endemic status? Parasitol Res 2020; 119(3):1001–9.

121. Fuehrer H-P, Auer H, Leschnik M, Silbermayr K, Duscher G, Joachim A. Dirofilaria in Humans, Dogs, and Vectors in Austria (1978-2014)-From Imported Pathogens to the Endemicity of Dirofilaria repens. PLoS Negl Trop Dis 2016; 10(5):e0004547.

122. Kulmer L-M, Unterköfler MS, Fuehrer H-P, Janovska V, Pagac M, Svoboda M et al. First Autochthonous Infection of a Cat with Dirofilaria immitis in Austria. Pathogens 2021; 10(9).

123. Schreeg ME, Marr HS, Tarigo JL, Cohn LA, Bird DM, Scholl EH et al. Mitochondrial Genome Sequences and Structures Aid in the Resolution of Piroplasmida phylogeny. PLoS One 2016; 11(11):e0165702.

124. Burki F, Inagaki Y, Bråte J, Archibald JM, Keeling PJ, Cavalier-Smith T et al. Large-scale phylogenomic analyses reveal that two enigmatic protist lineages, telonemia and centroheliozoa, are related to photosynthetic chromalveolates. Genome Biol Evol 2009; 1:231–8.

125. Janouskovec J, Horák A, Oborník M, Lukes J, Keeling PJ. A common red algal origin of the apicomplexan, dinoflagellate, and heterokont plastids. Proc Natl Acad Sci U S A 2010; 107(24):10949–54.

126. Arisue N, Hashimoto T. Phylogeny and evolution of apicoplasts and apicomplexan parasites. Parasitol Int 2015; 64(3):254–9.

127. Protozoa. In: Parasiticide Screening, Volume 1. Elsevier; 2019. p. 379–540.

128. Jalovecka M, Hajdusek O, Sojka D, Kopacek P, Malandrin L. The Complexity of Piroplasms Life Cycles. Front Cell Infect Microbiol 2018; 8:248.

129. Shaw MK. Theileria parva: sporozoite entry into bovine lymphocytes is not dependent on the parasite cytoskeleton. Exp Parasitol 1999; 92(1):24–31.

130. Shaw MK. Cell invasion by Theileria sporozoites. Trends Parasitol 2003; 19(1):2–6.

131. Shaw MK. The same but different: the biology of Theileria sporozoite entry into bovine cells. Int J Parasitol 1997; 27(5):457–74.

132. Shaw MK, Tilney LG. How individual cells develop from a syncytium: merogony in Theileria parva (Apicomplexa). J Cell Sci 1992; 101 (Pt 1):109–23.

133. Rudzinska MA, Trager W. Formation of merozoites in intraerythrocytic Babesia microti: an ultrastructural study. Can J Zool 1977; 55(6):928–38.

134. Fawcett DW, Conrad PA, Grootenhuis JG, Morzaria SP. Ultrastructure of the intra-erythrocytic stage of Theileria species from cattle and waterbuck. Tissue Cell 1987; 19(5):643–55.

135. Montero E, Rodriguez M, Oksov Y, Lobo CA. Babesia divergens apical membrane antigen 1 and its interaction with the human red blood cell. Infect Immun 2009; 77(11):4783–93.

136. Lobo CA, Rodriguez M, Cursino-Santos JR. Babesia and red cell invasion. Curr Opin Hematol 2012; 19(3):170–5.

137. Jalovecka M, Bonsergent C, Hajdusek O, Kopacek P, Malandrin L. Stimulation and quantification of Babesia divergens gametocytogenesis. Parasites Vectors 2016; 9(1):439.

138. Rudzinska MA, Spielman A, Riek RF, Lewengrub SJ, Piesman J. Intraerythrocytic 'gametocytes' of Babesia microti and their maturation in ticks. Can J Zool 1979; 57(2):424–34.

139. Bishop R, Musoke A, Morzaria S, Gardner M, Nene V. Theileria: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. Parasitology 2004; 129 Suppl:S271-83.

140. Becker CAM, Malandrin L, Depoix D, Larcher T, David PH, Chauvin A et al. Identification of three CCp genes in Babesia divergens: novel markers for sexual stages parasites. Mol Biochem Parasitol 2010; 174(1):36–43.

141. Becker CAM, Malandrin L, Larcher T, Chauvin A, Bischoff E, Bonnet SI. Validation of BdCCp2 as a marker for Babesia divergens sexual stages in ticks. Exp Parasitol 2013; 133(1):51–6.

142. Mehlhorn H, Schein E. The piroplasms: "A long story in short" or "Robert Koch has seen it". Eur J Protistol 1993; 29(3):279–93.

143. Mehlhorn H, Schein E, Warnecke M. Electron-microscopic studies on Theileria ovis Rodhain, 1916: development of kinetics in the gut of the vector tick, Rhipicephalus evertsi evertsi Neumann, 1897, and their transformation within cells of the salivary glands. J Protozool 1979; 26(3):377–85.

144. Karakashian SJ, Rudzinska MA, Spielman A, Lewengrub S, Piesman J, Shoukrey N. Ultrastructural studies on sporogony of Babesia microti in salivary gland cells of the tick Ixodes dammini. Cell Tissue Res 1983; 231(2):275–87.

145. Takahashi K, Kawai S, Yaehata K, Kawamoto S, Hagiwara K, Kurosawa T et al. Sporogony of Theileria sergenti in the salivary glands of the tick vector Haemaphysalis longicornis. Parasitol Res 1993; 79(1):1–7.

146. Mackenstedt U, Gauer M, Fuchs P, Zapf F, Schein E, Mehlhorn H. DNA measurements reveal differences in the life cycles of Babesia bigemina and B. canis, two typical members of the genus Babesia. Parasitol Res 1995; 81(7):595–604.

147. Guimarães AM, Lima JD, Ribeiro MF. Sporogony and experimental transmission of Babesia equi by Boophilus microplus. Parasitol Res 1998; 84(4):323–7.

148. Miró G, Checa R, Paparini A, Ortega N, González-Fraga JL, Gofton A et al. Theileria annae (syn. Babesia microti-like) infection in dogs in NW Spain detected using direct and indirect diagnostic techniques: clinical report of 75 cases. Parasites Vectors 2015; 8(1):217. Available from: URL: https://link.springer.com/article/10.1186/s13071-015-0825-2.

149. Fukumoto S, Xuan X, Shigeno S, Kimbita E, Igarashi I, Nagasawa H et al. Development of a polymerase chain reaction method for diagnosing Babesia gibsoni infection in dogs. J Vet Med Sci 2001; 63(9):977–81. Available from: URL: https://www.jstage.jst.go.jp/article/jvms/63/9/63_9_977/_article/-char/ja/.

150. LOBETTI R. G. Canine babesiosis. Comp. Cont. Educ. Pract. Vet. 1998; 20:418–30. Available from: URL: https://cir.nii.ac.jp/crid/1571135650666450432.

151. Simões PB, Cardoso L, Araújo M, Yisaschar-Mekuzas Y, Baneth G. Babesiosis due to the canine Babesia microti-like small piroplasm in dogs-first report from Portugal and possible vertical transmission. Parasites Vectors 2011; 4:50.

152. Mierzejewska EJ, Dwużnik D, Koczwarska J, Stańczak Ł, Opalińska P, Krokowska-Paluszak M et al. The red fox (Vulpes vulpes), a possible reservoir of Babesia vulpes, B. canis and Hepatozoon canis and its association with the tick Dermacentor reticulatus occurrence. Ticks Tick Borne Dis 2021; 12(1):101551. Available from: URL: https://www.sciencedirect.com/science/article/pii/s1877959x20304209.

153. Cardoso L, Cortes HCE, Reis A, Rodrigues P, Simões M, Lopes AP et al. Prevalence of Babesia microti-like infection in red foxes (Vulpes vulpes) from Portugal. Vet Parasitol 2013; 196(1-2):90–5. Available from: URL: https://www.sciencedirect.com/science/article/pii/s0304401713000125.

154. Alvarado-Rybak M, Solano-Gallego L, Millán J. A review of piroplasmid infections in wild carnivores worldwide: importance for domestic animal health and wildlife conservation. Parasit Vectors 2016; 9(1):538.

155. Baneth G. Perspectives on canine and feline hepatozoonosis. Vet Parasitol 2011; 181(1):3–11. Available from: URL: https://www.sciencedirect.com/science/article/pii/s0304401711002767.

156. Baneth G, Florin-Christensen M, Cardoso L, Schnittger L. Reclassification of Theileria annae as Babesia vulpes sp. nov. Parasit Vectors 2015; 8:207.

157. Baneth G, Cardoso L, Brilhante-Simões P, Schnittger L. Establishment of Babesia vulpes n. sp. (Apicomplexa: Babesiidae), a piroplasmid species pathogenic for domestic dogs. Parasites Vectors 2019; 12(1):129. Available from: URL: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-019-3385-z.

158. Baneth G, Florin-Christensen M, Cardoso L, Schnittger L. Reclassification of Theileria annae as Babesia vulpes sp. nov. Parasites Vectors 2015; 8(1):207. Available from: URL: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-015-0830-5.

159. Checa R, López-Beceiro AM, Montoya A, Barrera JP, Ortega N, Gálvez R et al. Babesia microti-like piroplasm (syn. Babesia vulpes) infection in red foxes (Vulpes vulpes) in NW Spain (Galicia) and its relationship with Ixodes hexagonus. Vet Parasitol 2018; 252:22–8.

160. Najm N-A, Meyer-Kayser E, Hoffmann L, Herb I, Fensterer V, Pfister K et al. A molecular survey of Babesia spp. and Theileria spp. in red foxes (Vulpes vulpes) and their ticks from Thuringia, Germany. Ticks Tick Borne Dis 2014; 5(4):386–91. Available from: URL: https://www.sciencedirect.com/science/article/pii/s1877959x14000399.

161. Farkas R, Takács N, Hornyák Á, Nachum-Biala Y, Hornok S, Baneth G. First report on Babesia cf. microti infection of red foxes (Vulpes vulpes) from Hungary. Parasites Vectors 2015; 8(1):55. Available from: URL: https://link.springer.com/article/10.1186/s13071-015-0660-5.

162. Dezdek D, Vojta L, Curković S, Lipej Z, Mihaljević Z, Cvetnić Z et al. Molecular detection of Theileria annae and Hepatozoon canis in foxes (Vulpes vulpes) in Croatia. Vet Parasitol 2010; 172(3-4):333–6. Available from: URL: https://www.sciencedirect.com/science/article/pii/s0304401710003304.

163. Sándor AD, D'Amico G, Gherman CM, Dumitrache MO, Domșa C, Mihalca AD. Mesocarnivores and macroparasites: altitude and land use predict the ticks occurring on red foxes (Vulpes vulpes). Parasites Vectors 2017; 10(1):173. Available from: URL: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-017-2113-9.

164. BARANDIKA JF, ESPÍ A, OPORTO B, Del CERRO A, BARRAL M, POVEDANO I et al. Occurrence and genetic diversity of piroplasms and other apicomplexa in wild carnivores. Parasitology Open 2016; 2.

165. Beck R, Vojta L, Mrljak V, Marinculić A, Beck A, Zivicnjak T et al. Diversity of Babesia and Theileria species in symptomatic and asymptomatic dogs in Croatia. Int J Parasitol 2009; 39(7):843–8. Available from: URL: https://www.sciencedirect.com/science/article/pii/s0020751909000277.

166. Hodžić A, Alić A, Fuehrer H-P, Harl J, Wille-Piazzai W, Duscher GG. A molecular survey of vector-borne pathogens in red foxes (Vulpes vulpes) from Bosnia and Herzegovina. Parasites Vectors 2015; 8:88.

167. Vannier E, Gewurz BE, Krause PJ. Human babesiosis. Infect Dis Clin North Am 2008; 22(3):469-88, viii-ix.

168. Herwaldt BL, Cacciò S, Gherlinzoni F, Aspöck H, Slemenda SB, Piccaluga P et al. Molecular characterization of a non-Babesia divergens organism causing zoonotic babesiosis in Europe. Emerg Infect Dis 2003; 9(8):942–8.

169. BANETH GA, SAMISH M, ALEKSEEV E, AROCH I, SHKAP V. Transmission of Hepatozoon canis to Dogs by Naturally-Fed or Percutaneously-Injected Rhipicephalus sanguineus Ticks. Journal of Parasitology 2001; 87(3):606–11.

170. Baneth G, SAMISH M, SHKAP V. Life cycle of Hepatozoon canis (Apicomplexa: Adeleorina: Hepatozoidae) in the tick Rhipicephalus sanguineus and domestic dog (Canis familiaris). Journal of Parasitology 2007; 93(2):283–99.

171. Harvey JW. Chapter 5 - Evaluation of Leukocytic Disorders. In: Harvey JW, editor. Veterinary Hematology. Saint Louis: W.B. Saunders; 2012. p. 122–76 Available from: URL: https://www.sciencedirect.com/science/article/pii/B9781437701739000051.

172. Vincent-Johnson N, Baneth G, Allen KE. 99 - Hepatozoonosis. In: Sykes JE, editor. Infectious diseases of the dog and cat. Fifth edition. London: Elsevier; opyright 2023. p. 1230–47 Available from: URL: https://www.sciencedirect.com/science/article/pii/B9780323509343000999.

173. Lesiczka PM, Rudenko N, Golovchenko M, Juránková J, Daněk O, Modrý D et al. Red fox (Vulpes vulpes) play an important role in the propagation of tick-borne pathogens. Ticks Tick Borne Dis 2023; 14(1):102076.

174. Duscher GG, Kübber-Heiss A, Richter B, Suchentrunk F. A golden jackal (Canis aureus) from Austria bearing Hepatozoon canis--import due to immigration into a non-endemic area? Ticks Tick Borne Dis 2013; 4(1-2):133–7.

175. Canine Parasites and Parasitic Diseases. Elsevier; 2019.

176. Gabrielli S, Kumlien S, Calderini P, Brozzi A, Iori A, Cancrini G. The first report of Hepatozoon canis identified in Vulpes vulpes and ticks from Italy. Vector Borne Zoonotic Dis 2010; 10(9):855–9.

177. Giannelli A, Ramos RAN, Di Paola G, Mencke N, Dantas-Torres F, Baneth G et al. Transstadial transmission of Hepatozoon canis from larvae to nymphs of Rhipicephalus sanguineus. Vet Parasitol 2013; 196(1-2):1–5.

178. Murata T, Inoue M, Tateyama S, Taura Y, Nakama S. Vertical transmission of Hepatozoon canis in dogs. J Vet Med Sci 1993; 55(5):867–8. Available from: URL: https://www.jstage.jst.go.jp/article/jvms1991/55/5/55_5_867/_article/-char/ja/.

179. Cardoso L, Cortes HCE, Eyal O, Reis A, Lopes AP, Vila-Viçosa MJ et al. Molecular and histopathological detection of Hepatozoon canis in red foxes (Vulpes vulpes) from Portugal. Parasites Vectors 2014; 7:113.

180. Johnson EM, Panciera RJ, Allen KE, Sheets ME, Beal JD, Ewing SA et al. Alternate pathway of infection with Hepatozoon americanum and the epidemiologic importance of predation. J Vet Intern Med 2009; 23(6):1315–8.

181. Circulating Antibodies to Hepatozoon Canis Demonstrated by Immunofluorescence; 1994. Available from: URL: https://journals.sagepub.com/doi/pdf/10.1177/104063879400600127.

182. Baneth G, Shkap V, Presentey BZ, Pipano E. Hepatozoon canis: the prevalence of antibodies and gametocytes in dogs in Israel. Veterinary Research Communications 1996; 20(1):41–6. Available from: URL: https://link.springer.com/article/10.1007/bf00346576.

183. Inokuma H, Ohno K, Yamamoto S. Serosurvey of Ehrlichia canis and Hepatozoon canis infection in dogs in Yamaguchi Prefecture, Japan. J Vet Med Sci 1999; 61(10):1153–5. Available from: URL: https://www.jstage.jst.go.jp/article/jvms/61/10/61_10_1153/_article/-char/ja/.

184. Karagenc TI, Pasa S, Kirli G, Hosgor M, Bilgic HB, Ozon YH et al. A parasitological, molecular and serological survey of Hepatozoon canis infection in dogs around the Aegean coast of Turkey. Vet Parasitol 2006; 135(2):113–9.

185. Aktas M, Özübek S, Altay K, Balkaya İ, Utuk AE, Kırbas A et al. A molecular and parasitological survey of Hepatozoon canis in domestic dogs in Turkey. Vet Parasitol 2015; 209(3-4):264–7.

186. Zur Verbreitung der Braunen Hundezecke Rhipicephalus sanguineus (Latreille 1806) in Österreich und deren Bekämpfungsmöglichkeiten; 1986. Available from: URL: https://www.researchgate.net/profile/heinrich-prosl/publication/267303408_zur_verbreitung_der_braunen_hundezecke_rhipicephalus_sanguineus_latreille_1806_in_osterreich_und_deren_bekampfungsmoglichkeiten/links/5470c6ce0cf24af340c37ebd/zur-verbreitung-der-braunen-hundezecke-rhipicephalus-sanguineus-latreille-1806-in-oesterreich-und-deren-bekaempfungsmoeglichkeiten.pdf.

187. Vojta L, Mrljak V, Curković S, Zivicnjak T, Marinculić A, Beck R. Molecular epizootiology of canine hepatozoonosis in Croatia. Int J Parasitol 2009; 39(10):1129–36. Available from: URL: https://www.sciencedirect.com/science/article/pii/s0020751909001015.

188. Pacifico L, Braff J, Buono F, Beall M, Neola B, Buch J et al. Hepatozoon canis in hunting dogs from Southern Italy: distribution and risk factors. Parasitol Res 2020; 119(9):3023–31.

189. Licari E, Takács N, Solymosi N, Farkas R. First detection of tick-borne pathogens of dogs from Malta. Ticks Tick Borne Dis 2017; 8(3):396–9.

190. Tolnai Z, Sréter-Lancz Z, Sréter T. Spatial distribution of Anaplasma phagocytophilum and Hepatozoon canis in red foxes (Vulpes vulpes) in Hungary. Ticks Tick Borne Dis 2015; 6(5):645–8.

191. Majláthová V, Hurníková Z, Majláth I, Petko B. Hepatozoon canis infection in Slovakia: imported or autochthonous? Vector Borne Zoonotic Dis 2007; 7(2):199–202.

192. Najm N-A, Meyer-Kayser E, Hoffmann L, Pfister K, Silaghi C. Hepatozoon canis in German red foxes (Vulpes vulpes) and their ticks: molecular characterization and the phylogenetic relationship to other Hepatozoon spp. Parasitol Res 2014; 113(7):2679–85.

193. D'Amico G, Juránková J, Tăbăran FA, Frgelecová L, Forejtek P, Matei IA et al. Occurrence of ticks in the subcutaneous tissue of red foxes, Vulpes vulpes in Czech Republic and Romania. Ticks Tick Borne Dis 2017; 8(2):309–12.

194. Mitková B, Hrazdilová K, Steinbauer V, D'Amico G, Mihalca AD, Modrý D. Autochthonous Hepatozoon infection in hunting dogs and foxes from the Czech Republic. Parasitol Res 2016; 115(11):4167–71.

195. Vojta L, Mrljak V, Curković S, Zivicnjak T, Marinculić A, Beck R. Molecular epizootiology of canine hepatozoonosis in Croatia. Int J Parasitol 2009; 39(10):1129–36. Available from: URL: https://www.sciencedirect.com/science/article/pii/s0020751909001015.

196. Baneth G, Weigler B. Retrospective case-control study of hepatozoonosis in dogs in Israel. J Vet Intern Med 1997; 11(6):365–70.

197. Marchetti V, Lubas G, Baneth G, Modenato M, Mancianti F. Hepatozoonosis in a dog with skeletal involvement and meningoencephalomyelitis. Veterinary Clinical Pathology 2009; 38(1):121–5.

198. Mundim AV, Morais IA de, Tavares M, Cury MC, Mundim MJS. Clinical and hematological signs associated with dogs naturally infected by Hepatozoon sp. and with other hematozoa: a retrospective study in Uberlândia, Minas Gerais, Brazil. Vet Parasitol 2008; 153(1-2):3–8. Available from: URL: https://www.sciencedirect.com/science/article/pii/s0304401708000393.

199. Sakuma M, Nakahara Y, Suzuki H, Uchimura M, Sekiya Z, Setoguchi A et al. A case report: a dog with acute onset of Hepatozoon canis infection. J Vet Med Sci 2009; 71(6):835–8. Available from: URL: https://www.jstage.jst.go.jp/article/jvms/71/6/71_6_835/_article/-char/ja/.

200. Krista Williams, BSc, DVM, CCRP, Catherine Barnette, DVM. Hepatozoonosis in Dogs | VCA Animal Hospitals; 2023 [cited 2023 Jun 27]. Available from: URL: https://vcahospitals.com/know-your-pet/hepatozoonosis-in-dogs.

201. Gray JS, Dautel H, Estrada-Peña A, Kahl O, Lindgren E. Effects of climate change on ticks and tick-borne diseases in europe. Interdiscip Perspect Infect Dis 2009; 2009:593232.

202. Cornely M, Schultz U. Zur Zeckenfauna Ostdeutschlands. Angew Parasitol 1992; 33(3):173–83.

203. Prevalence of Borrelia (B.) burgdorferi sensu lato in red foxes (Vulpes vulpes) in eastern Brandenburg; 2000.

204. Liebisch A, Walter G. Untersuchungen von Zecken bei Haus- und Wildtieren in Deutschland: Zum Vorkommen und zur Biologie der Igelzecke (Ixodes hexagonus) und der Fuchszecke (Ixodes canisuga). Dtsch Tierarztl Wochenschr 1986; 93(9):447–50.

205. Zur Parasitenfauna des Rotfuchses in Berlin (West); 1991.

206. Meyer-Kayser E, Hoffmann L, Silaghi C, Pfister K, Mahling M, Passos LMF. Dynamics of tick infestations in foxes in Thuringia, Germany. Ticks Tick Borne Dis 2012; 3(4):232–9.

207. Hornok S, Sándor AD, Beck R, Farkas R, Beati L, Kontschán J et al. Contributions to the phylogeny of Ixodes (Pholeoixodes) canisuga, I. (Ph.) kaiseri, I. (Ph.) hexagonus and a simple pictorial key for the identification of their females. Parasites Vectors 2017; 10(1):545. Available from: URL: https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-017-2424-x.

208. Sréter T, Széll Z, Varga I. Ectoparasite infestations of red foxes (Vulpes vulpes) in Hungary. Vet Parasitol 2003; 115(4):349–54. Available from: URL: https://www.sciencedirect.com/science/article/pii/s0304401703002164.

209. Aydın MF, Balkaya I, Aktaş M, Dumanlı N. Erzurum İlinde Üç Kırmızı Tilkide (Vulpes vulpes) Kene (Ixodoidea) ve Pire (Siphonaptera) Türleri. Turkiye Parazitol Derg 2011; 35(2):110–3.

210. Ferreira CAC, Pinho Mixão V de, Novo MTLM, Calado MMP, Gonçalves LAP, Belo SMD et al. First molecular identification of mosquito vectors of Dirofilaria immitis in continental Portugal. Parasites Vectors 2015; 8:139.

211. Latrofa MS, Montarsi F, Ciocchetta S, Annoscia G, Dantas-Torres F, Ravagnan S et al. Molecular xenomonitoring of Dirofilaria immitis and Dirofilaria repens in mosquitoes from north-eastern Italy by real-time PCR coupled with melting curve analysis. Parasites Vectors 2012; 5:76.

212. Younes L, Barré-Cardi H, Bedjaoui S, Ayhan N, Varloud M, Mediannikov O et al. Dirofilaria immitis and Dirofilaria repens in mosquitoes from Corsica Island, France. Parasites Vectors 2021; 14(1):427.

213. Cancrini G, Di Frangipane Regalbono A, Ricci I, Tessarin C, Gabrielli S, Pietrobelli M. Aedes albopictus is a natural vector of Dirofilaria immitis in Italy. Vet Parasitol 2003; 118(3-4):195–202.

214. Jenny Molloy, Stephane Fadanka, Nadine Mowoh, Cordellia Cordellia Fulai. Preparing 1x PCR Master Mix; 2022.

215. Criado-Fornelio A, Martinez-Marcos A, Buling-Saraña A, Barba-Carretero JC. Presence of Mycoplasma haemofelis, Mycoplasma haemominutum and piroplasmids in cats from southern Europe: a molecular study. Vet Microbiol 2003; 93(4):307–17.

216. Mitreva M. The Microbiome in Infectious Diseases. In: Infectious Diseases. Elsevier; 2017. 68-74.e2.

217. Pelt-Verkuil E, Belkum A, Hays JP. Principles and Technical Aspects of PCR Amplification. Dordrecht: Springer Science + Business Media B.V; 2008.

218. Thompson A, Bench S, Carter B, Zehr J. Coupling FACS and genomic methods for the characterization of uncultivated symbionts. Methods Enzymol 2013; 531:45–60.

219. Parola P, Roux V, Camicas JL, Baradji I, Brouqui P, Raoult D. Detection of ehrlichiae in African ticks by polymerase chain reaction. Trans R Soc Trop Med Hyg 2000; 94(6):707–8.

220. Norman AF, Regnery R, Jameson P, Greene C, Krause DC. Differentiation of Bartonella-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J Clin Microbiol 1995; 33(7):1797–803.

221. Vitorino L, Zé-Zé L, Sousa A, Bacellar F, Tenreiro R. rRNA intergenic spacer regions for phylogenetic analysis of Rickettsia species. Ann N Y Acad Sci 2003; 990:726–33.

222. Red Fox Breeding - Mating & Monogamy | Wildlife Online; 2023 [cited 2023 Jun 27]. Available from: URL: https://www.wildlifeonline.me.uk/animals/article/red-fox-breeding-mating-monogamy.

223. Arenas P, Gil-Alarcón G, Sánchez-Montes S, Soto-Trujillo MP, Fernández-Figueroa E, Rangel-Escareño C. Molecular detection of Bartonella, Ehrlichia and Mycoplasma in feral dogs of El Pedregal de San Angel Ecological Reserve in Mexico City. Rev Bras Parasitol Vet 2019; 28(4):728–34.

224. Kim J, Lee D, Yoon E, Bae H, Chun D, Kang J-G et al. Clinical Case of a Transfusion-Associated Canine Mycoplasma haemocanis Infection in the Republic of Korea: A Case Report. Korean J Parasitol 2020; 58(5):565–9.

225. Barker EN, Langton DA, Helps CR, Brown G, Malik R, Shaw SE et al. Haemoparasites of free-roaming dogs associated with several remote Aboriginal communities in Australia. BMC Vet Res 2012; 8(1):55. Available from: URL: https://bmcvetres.biomedcentral.com/articles/10.1186/1746-6148-8-55.

226. Koneval M, Miterpáková M, Hurníková Z, Blaňarová L, Víchová B. Neglected intravascular pathogens, Babesia vulpes and haemotropic Mycoplasma spp. in European red fox (Vulpes vulpes) population. Vet Parasitol 2017; 243:176–82. Available from: URL: https://www.sciencedirect.com/science/article/pii/S0304401717302972.

227. Doris Fasching. Zecken und von Zecken übertragene Krankheitserreger im Umfeld von Militärhunden in Kaisersteinbruch; 2020. Available from: URL: https://phaidra.vetmeduni.ac.at/view/o:265.

10. References

Amt der Kärtner Landesregierung, State office (Landesamtdirektion), accessed 27 June 2023

https://carinthia.com/en/business-technology/bio-economic-principle/

Republic of Austria, Federal Ministry of Agriculture, Forestry, Environment and Water Management (BMLFUW), 2007. The Austrian Forest Programme, accessed 27 June 2023

untitled (bml.gv.at)

Agentur für Gesundheit und Ernährungssicherheit, accessed 27 June 2023

https://www.ages.at/en/research/project-highlights/ovitrap-monitoring-of-alien-gnat-species-in-austria

MSD Manual Veterinary Manual, accessed on 27 June 2023

https://www.msdvetmanual.com/circulatory-system/blood-parasites/hemotropic-mycoplasma-infections-in-animals

Krista Williams, Catherine Barnette, Hepatozoonosis in Dogs, VCA animal hospitals, accessed on 27 June 2023

Hepatozoonosis in Dogs | VCA Animal Hospitals (vcahospitals.com)

Red fox breeding – Mating and Monogamy accessed on 27 June 2023

https://www.wildlifeonline.me.uk/animals/article/red-fox-breeding-mating-monogamy

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