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INVESTIGATION OF ARTHROPODS AND THEIR ASSOCIATED PROTOZOAL AND BACTERIAL PATHOGENS FROM APPARENTLY HEALTHY DOGS AND CATS IN FOUR SELECTED DISTRICTS OF SOUTHERN ETHIOPIA

PhD Dissertation

By
Hana Tadesse Seifu

Addis Ababa University, College of Veterinary Medicine and Agriculture

Department of Pathology and Parasitology

PhD program in Veterinary Parasitology

June, 2023

Bishoftu, Ethiopia

INVESTIGATION OF ARTHROPODS AND THEIR ASSOCIATED PROTOZOAL AND BACTERIAL PATHOGENS FROM APPARENTLY HEALTHY DOGS AND CATS IN FOUR SELECTED DISTRICTS OF SOUTHERN ETHIOPIA

A dissertation submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Parasitology

By

Hana Tadesse Seifu

Advisors

Professor Bersissa Kumsa (DVM, MSc, PhD, Professor)-College of Veterinary Medicine and Agriculture, Addis Ababa University

Professor Rudi Cassini (DVM, MSc, PhD, Associate professor)-University of Padova

June, 2023

Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture Department of Veterinary Pathology and Parasitology

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Title: **Investigation of Arthropods and Their Associated Protozoal and Bacterial Pathogens from Apparently Healthy Dogs and Cats in Four Selected Districts of Southern Ethiopia**

Submitted by:	Hana Tadesse Seifu	_____	_____
	Name of PhD Student	Signature	Date

Approved for submittion to dissertation assessment committee

Major Advisor:

Professor Bersissa Kumsa	_____	_____
Name	Signature	Date
Co-Advisor		
Professor Rudi Cassini	_____	_____
Name	Signature	Date
Department chairperson	_____	_____
Professor Getachew Terefe	_____	_____
Name	Signature	Date

Addis Ababa University College of Veterinary Medicine and Agriculture Department of VeterinaryPathology and Parasitology

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We, the members of the Examining Board of the final PhD open defense have read and evaluated the dissertation prepared by Hana Tadesse titled: Investigation of Arthropod-Borne Protozoal and Bacterial Pathogens in Dogs and Cats in Four Selected Districts of Southern Ethiopia, and recommend that as a partial fulfilment of the dissertation requirement for the degree of Philosophy in Veterinary Parasitology.

Chairman (title and name)	Signature	Date
Internal Examiner (title and name)	Signature	Date
External Examiner (title and name)	Signature	Date

I hereby certify that I have read the revised version of this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation advisor (title and name)	Signature	Date
Department chair (title and name)	Signature	Date

ABSTRACT

Dogs and cats are known to host several vector-borne diseases with zoonotic potential; however, little information is available on the epidemiology of these diseases in Ethiopia. The present study aims to investigate the diversity of arthropods and molecular identity of protozoal and bacterial vector-borne diseases in dogs and cats in selected districts of Gamo zone. Cross-sectional study design was used to determine the prevalence of arthropods and vector-borne diseases. Real-time and/or end-point PCR were used to identify vector-borne diseases. A total of 297 dogs and 110 cats were included for ectoparasites and blood samples. Questionnaires were applied for 290 dog and cat owners to obtain information concerning owner's knowledge about arthropods and vector-borne diseases. The overall prevalence of ticks, fleas and lice in dogs was 36.7%, 69.7% and 4.7%, respectively. Ectoparasite prevalence on dogs was as follows: fleas (*Ctenocephalides felis* (*C. felis*) (69.4%), *Echidnophaga gallinacea* (*E. gallinacea*) (1.3%), *Ctenocephalides canis* (*C. canis*) (1.0%), and *Pulex irritans* (*P. irritans*) (0.3%)), ticks (*Amblyomma variegatum* (*A. variegatum*) (22.9%), *Rhipicephalus sanguineus* (*R. sanguineus*) (14.1%), *Haemaphysalis leachi (H. leachi*) (8.8%), *Rhipicephalus praetextatus (R. praetextatus*) (4.0%), and *Rhipicephalus pulchellus* (*Rh. pulchellus*) (3.4%)), lice (*Heterodoxus spiniger* (*H. spiniger*) (4.0%) and *Trichodectes canis* (*T. canis*) (0.7%)) were identified. Blood samples were collected from a total of 273 dogs and then analyzed by real-time and/or end-point PCR for VBDs. The results of the study showed that *Hepatozoon canis (H. canis*) was the most prevalent pathogen (53.8%), followed by *Anaplasma phagocytophilum* (*A. phagocythophilum*) (7.0%), *Babesia canis rossi* (*B. canis rossi*) (3.3%), *Ehrlichia canis* (*E. canis*) (2.6%) and *Anaplasma platys* (*A. platys*) (2.2%). Furthermore, five samples tested positive for *Borrelia* spp., identified as *B. afzelii* (n = 3) and *B. burgdorferi* (n = 2), and two samples for *Rickettsia* spp., identified as *R. conorii* (n = 1) and *R. monacensis* (n = 1). On cats the prevalence of ticks was 2.7% and fleas was 21.8%. Fleas (*C. felis* (15.5%), and *E. gallinacea* (7.3%)) tick (*H. leachi* (2.7%)) was identified from cats. In cats, among *Hepatozoon* spp., *H. felis* (n=24; 21.8%) is the more widespread pathogen followed by *H. ingwe* (n=7; 6.4%), *H. lluiperdije* (n=7; 6.4%) and *H. canis* (n=1; 0.9%). *Babesia leo* (n=1; 0.9%) was identified from one cat. Eight cats were positive for bacterial pathogens, specifically *Bartonella* spp., (n=2; 1.8%) and *Rickettsia* spp., (n=6; 5.5%). All cats were negative for *Cytauxzoon* spp., *Anaplasma* spp., *Ehrlichia* spp., and *Borrelia* spp. Abundance of *C. felis*, in dogs was significantly higher ($p<0.001$), while *E. gallinacea* was

significantly more abundant in cats (p=0.002). Ectoparasite occurrence on dogs varied with different factors. A high prevalence of tick infestation was recorded in urban areas (p=0.020) and in lowlands (p<0.001). Nevertheless, the prevalence of *A. variegatum* in rural area and midland agroecology was significantly higher (p<0.001). *Rhipicephalus Sanguineus* prevalence was higher in urban areas (<0.001) and in dogs which lives in indoor environment (p=0.003). *H. leachi* prevalence was significantly higher in midland (p<0.001) and on adult dogs (p=0.001). Fleas are more prevalent in rural (p=0.029) areas compared to urban areas and females was slightly higher infested by fleas than male (p=0.047) dogs. *C. felis* was more prevalent in female (p=0.038) dogs than males. Around 88.3% owners in the study area had no knowledge about arthropods and VBDs. Majority of the owners (64.8%) never visited veterinary clinics. The findings of *A. phagocythophilum* and different species of the genera *Borrelia* and *Rickettsia* with zoonotic potential from dogs was unexpected and alarming, and calls for further investigation on the roles of dogs and on the tick species acting as vector in this specific context. Other pathogens (*H. canis, B. canis rossi, A. platys, and E. canis*) are already known to have an important impact on the dogs' health but have minor zoonotic potential as they were rarely or never reported in humans. Dogs from rural areas were found to be at higher risk for different pathogens, probably due to the presence of other wild canids in the same environment. Moreover, most of the pathogens reported in the studied cat population were identified for the first time in the country. In conclusion, the findings of the present study contribute to a better knowledge of dog and cat ectoparasites and on the epidemiology of associated vector-borne pathogens, in most cases relevant to human and animal health. Therefore, the data presented in this study are important for building new knowledge and this give rise to awareness creation and strengthening veterinary services for companion animals.

KeyWords: *Flea, Tick, Lice, vector-borne diseases, Dogs, Cats, Gamo zone, Ethiopia*

STATEMENT OF AUTHOR

This thesis is solely prepared for the accomplishment of the PhD degree where all sources of material used in this thesis have been duly acknowledged. As partial fulfillment of the PhD degree, this dissertation is submitted to Addis Ababa University, College of Veterinary Medicine and Agriculture, and is deposited in the college library to be made available to borrowers under the rules of the Library. In this thesis, there is no part, which has been submitted to obtain a degree, diploma, or certificate in my name in any institution. It is possible to have a brief excerpt from this thesis without a different agreement, provided that a truthful acknowledgment of the source is made. Requests for permission for a lengthy quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

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College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia

Date of Submission: _____

BIOGRAPHIC SKETCH

Hana Tadesse was born on 23 June, 1987 G.C in Jimma town. Her school life started at Mendera elementary school, and then continued to Jimma Acadamic and Vocational Training Institute where she successfully completed Ethiopian Higher Education Entrance Examination (EHEEE). Then in 2006 G.C she got the chance to join Jimma University, Collage of Agriculture and Veterinary Medicine, Jimma. She completed and graduated for the attainment of Degree of Doctor of Veterinary Medicine (DVM) in June 09, 2010 and continued her master's degree in Veterinary Epidemiology at the same university; graduated in November 28, 2013. Her working experience as Livestock Health Researcher started in the Southern Agricultural Research Institute, Arba Minch Agricultural Research Centre in June 2014 where she worked for 4 years. Then she started her PhD at Addis Ababa University in 2019 academic year.

DEDICATION

This dissertation manuscript is dedicated to all my family members.

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Drafted

4. Investigation on vector-borne pathogens circulating in cat populations and associated ectoparasites in selected areas of Ethiopia

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LIST OF ABBREVIATIONS

ABDs	Arthropod-borne diseases
ATBF	African tick bite fever
CNS	Central nervous system
CSD	Cat scratch disease
DIC	Disseminated intravascular coagulation
FTA	Flinders Technology Associates
HGE	Human granulocytic ehrlichiosis
ITS1, ITS2	First and second internal transcribed spacers
MSF	Mediterranean spotted fever
PCR	Polymerase chain reaction
PI	Post inoculation
RMSF	Rocky Mountain spotted fever
SFG	Spoted fever group
VBDs	Vector-borne diseases
HGA	Human granulocytic anaplasmosis
CGA	Canine granulocytic anaplasmosis
ACA	Acrodermatitis Chronica Atrophicans

1. INTRODUCTION

1.1.Background

Dogs (*Canis familiaris*) and cats (*Felis catus*) are the most popular companion animals in many parts of the world including Ethiopia (Feuerstein and Terkel, 2008; Gebremedhin et al., 2020; Endo et al., 2020). The global dog and cat population is estimated to be one-tenth of the human population (Smith et al., 2019). This impressivepopulation size and the ubiquitous spread of dogs and cats across human societies make dogs and cats an essential consideration for every nation considering control of pet borne zoonosis (Vallentine and AbdulRahman, 2009; Nguyen et al., 2020). The size of the overall pet population is related to the human population and increases as the human population increases. Dogs' and cats' ownership is affected by social and political instability and poverty (Macpherson et al., 2000). Domestic dogs and cats are often regarded as faithful friends and intimate companions of humans, and enjoy life together with humans (Chen et al., 2012). This human-animal bond can provide substantial positive benefits concerning emotional development and socialization.

The increase in dog and cat populations and their co-habitation with humans in urban and rural environments poses new threats to human health (Colwell et al., 2011; Otranto, 2015). Furthermore, this leads to widespread environmental contamination with ectoparasites and infectious agents; that might represent a significant source of infection for humans. Thus, dogs and cats might act as proxy species for the emergence of disease in the human population. They are among the main reservoirs, carriers, and transmitters of many zoonotic infections caused by bacteria and protozoa (Macpherson et al., 2000), ectoparasite infestations (Alasaad et al., 2011) and vector-borne pathogens (Kumsa et al., 2015b). Both dogs and cats, if not properly treated against ectoparasites, are most likely exposed to pathogens transmitted by them and can become competent reservoirs of several pathogens (Otranto et al., 2017).

In Ethiopia, much of the emphasis is on food animals and well-organized and documented data on dog and cat populations is unavailable. However, some reports indicate that Ethiopia has a pet

dog population of 5 million. In Addis Ababa alone dog population is estimated to be 250,000 to 350,000 (UAECP, 2011). About 50% of these dogs may be household dogs and the rest are free-roaming and may or may not be owned (MoA, 2000). No data is available on the size of the cat population. The health, nutrition and welfare aspects of dogs and cats are often inferior and their ownership is a tenuous connection with no tradition of lifelong care and responsibility. A study on demography and determinants of dog and cat ownership in three towns of West Shoa zone, Oromia Region revealed that about 75.3% of the owned dogs were considered guard dogs for protection of household property, while 73.2% were for love and affection and 33.0% were for companionship. Likewise, most owned cats were used for the protection of property from mice (83.7%), and companionship (43.9%) (Gebremedhin et al., 2020).

In tropical countries including Ethiopia, arthropods are very common and widely distributed due to the presence of very favourable climate and poor general hygiene and sanitation, lack of prevention and control (Vanderburg et al., 2014; Kumsa et al., 2014a). Arthropods act as vectors and reservoirs for several agents of emerging and re-emerging infectious diseases of medical and veterinary importance (Reif and Macaluso, 2009). Investigations indicate worldwide escalating problems from arthropods and VBDs caused by pathogens they transmit (De la Fuente and Estrada-Pena, 2012). Despite the large number of dog and cat populations in Ethiopia, only few researchs were conducted so far on zoonotic vector-borne pathogens. Consequent to uncontrolled populations of dogs and cats living near the increasing densities of human populations, effective control of canine and feline originated zoonotic disease is an extremely challenging task.

VBDs in dogs and cats have a major impact on animal health and welfare and, in many cases, also on human health. There is an urgent need to establish effective surveillance systems for most VBDs across various countries in order to facilitate a detailed risk analysis, which should include evaluation of potential spread to new areas and the possible introduction of new exotic species or disease agents. This requires clear and exhaustive knowledge on the distribution of VBDs in different areas, understanding of the diagnostic limitations pertaining to VBDs (Otranto and Wall, 2008).

In Ethiopia arthropods such as ticks, fleas and lice are common and widely distributed throughout all regions. Human contact with arthropods is favoured by several factors like 84% of the populations are involved in agriculture and thus farmers and their family members interact during their daily life activities with ectoparasite-infested dogs, cats, other domestic animals, wild animals and vegetation which is the natural habitats of arthropods (Kumsa et al., 2014b).

1.2.Problem statement

Ectoparasites are arthropods that live externally on their hosts. Globally, the estimated numbers of terrestrial arthropod species are ~7 million (Stork, 2018). Most parasitic arthropods have evolved and adapted to specific hosts. Their association with companion animals is known to cause damaging effects such as life-threatening anaemia, allergic dermatitis, pruritic and non-pruritic skin disorders (Omonijo and Sowemimo, 2017). However, the more relevant role of ectoparasites is related to the transmission of diseases with veterinary and public health significance. Fleas and ticks are the most common ectoparasites of dogs, and both vector a wide array of vector-borne pathogens that cause diseases such as borreliosis, bartonellosis, ehrlichiosis, rickettsiosis (Heylen et al., 2021).

Some ectoparasites can act as vectors of different pathogens such as *Hepatozoon* spp., *Babesia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Borrelia* spp., *Bartonella* spp., and *Rickettsia* spp. (Alcaíno et al., 2002; Nuchjangreed and Somprasong, 2007; Heukelbach et al., 2012). Vector-borne diseases (VBDs) and their vectors are present throughout Africa. They have been well studied in livestock of sub-Saharan Africa, but poorly investigated in companion animals (Heylen et al., 2021).

Arthropods which feed on dogs and cats cause direct damage and disease in their hosts through the transfer of saliva, injection of neurotoxins, skin injuries, allergic reactions and blood loss; indirectly, they may be vectors of bacteria, viruses, protozoa and helminths (Wall and Pitts, 2005). Studies have confirmed that arthropods such as ticks, fleas and lice are common

ectoparasites of domestic dogs and cats in many tropical countries (Abdulkareem et al., 2018; Tadesse et al., 2019; Siagian and Siregar, 2021). However, to date; the role of companion animals in the epidemiology of vector-borne pathogens (VBPs) in sub-Saharan Africa has not been addressed appropriately. In recent decades the socio-economic value of pets has increased in this area (Otranto and Wall, 2008; Heylen et al., 2021). Besides, human-mediated environmental alterations (i.e., expansion of farmland, urbanization, deforestation and establishments of settlements in natural ecosystems) give rise to increased risk of exposure to VBDs and new opportunities for novel transmission cycles (Heylen et al., 2021).

VBDs have a major impact on animal and human health, and are responsible for about 17% of all cases of human infectious disease worldwide (WHO, 2004). Many of these diseasesare expected to increase in prevalence as a result of factors linked to habitat modifications, the introduction of exotic vectors and climate change (van der Weijden et al., 2007).

In veterinary medicine VBDs have been considered important largely because of the losses they cause to livestock production, which may amount to millions of dollars per year (Bram et al., 2002). However, diseases transmitted by arthropods to dogs and cats have attracted increasing interest from the general public and the scientific community (Shaw and Day, 2005), not only because of their pathogenicity to the infected animals, but because many ABDs are zoonotic and impact on public health, particularly in developing countries (Irwin and Jefferies, 2004).

Increased disease transmission may be exacerbated by high abundance of companion animals and their close association with humans because they represent an easily available food source for arthropods and serve as reservoirs for pathogens. Indeed, over the last decades, the pet population worldwide has increased in both developed and developing countries; numerically dogs are equal about 10% of the human population (WHO, 2003). Furthermore, rapid movements of people and transportation of goods (van der Weidjenet al., 2007) have resulted in the introduction and establishment of several invasive vector species not previously present in

some areas, (Scholte and Schaffner, 2007) including novel pathogens (Duprey et al.,2006). Arthropod borne infectious diseases of the dog and cat reported in different parts of Africa (Moonga et al., 2019; Oliveira et al., 2018; Mtshali et al., 2017; Proboste et al., 2015; Heylen et al., 2021).

Like many other developing countries in Ethiopia arthropod borne infectious diseases of the dogs and cats are not well studied and no attention is given to the prevention and control programs. In Ethiopia a study was conducted by Kumsa et al. (2014a) in central Oromia to detect *Rickettsia felis* and *Bartonella henselae* in fleas from dogs and cats, but there are no molecular studies conducted directly using the blood of dogs and cats. Furthermore, some studies were conducted on the identification of arthropods from dogs and cats from other parts of the country (Tesfaye and Chanie, 2011; Kumsa and Mekonnen, 2011; Tadesse et al., 2019; Kumsa et al., 2019). To our knowledge, so far in Gamo zone there is no published and unpublished specific study on Arthropods and VBDs of dogs and cats.

1.3.Research Questions

Based on the stated problems this study was expected to address the following main research questions:

- Does the community have awareness about VBDs of dogs and cats in the selected districts?
- Do arthropods and VBDS of dogs and cats circulate in the selected districts?

1.4.Research Hypotheses

- There is no prevalence of vector-borne pathogens in dogs and cats in the study area.
- There is less knowledge about VBDs of dogs and cats among owners.

1.5.Objectives

1.5.1. General objective

- To investigate the diversity of arthropods and molecular identity of vector-borne pathogens in dogs and cats in selected districts of Gamo zone.

1.5.2. Specific objectives

- To investigate the molecular prevalence of protozoal and bacterial vector-borne pathogens in dogs and cats in the study districts.
- To identify the species diversity of ectoparasites from dogs and cats that are responsible for transmission of zoonotic pathogens in the study area.
- To determine the risk factors of ectoparasite infestation and associated pathogens in dogs and cats of the study areas.

2. LITERATURE REVIEW

2.1.Fleas, Flea-borne pathogens of dogs and cats

Fleas (order Siphonaptera or Aphaniptera) are small, wingless, obligate, blood-feeding insects and holometabolous. The order is relatively small, with about 2,500 described species of which approximately 90% occur on mammals and only 10% on birds within the family Pulicidae (Durden et al., 2005; Durden and Hinckle, 2009). The genus *Ctenocephalides* includes 13 species, identified according to morphological criteria based on the shape and structure of their genitalia and the presence and distribution of spinae, setae, and ctenidia on the body (Linardi and Santos, 2012). On cats and dogs, *C. felis* and *C. canis* are the two species of major importance worldwide. However, in most geographical areas, even on dogs, *C. felis* predominates. Adult fleas are highly modified for an ectoparasitic life and are structurally very different from most other insects. The other flea species found on dogs and cats was *Echidnophaga gallinacea* and *Pulex irritans. Echidnophaga gallinacea*is called sticktight flea because of its elongated shape and is frequently found on birds and only occasionally on dogs and cats in warm areas of the world (Iannino et al., 2017). *Pulex irritans* is globally distributed (Traversa, 2013) and mistakenly called the human flea, but it infests a wide variety of mammals, including dogs (Gracia et al., 2013), cats (Millan et al., 2007), and rats (He et al., 1997). It has been observed that *P. irritans* prefers to remain on dogs despite close contact with human beings. Although, this species does appear to transfer more readily among dogs and humans than *C. felis*, *C. canis* or *E. gallinacea* (Iannino et al., 2017).

In contrast to lice or ticks, the flea body is laterally compressed. Adults are wingless and usually between 1 mm and 6 mm in length, with females being larger than males. The head is sessile on the prothorax and the body is covered with backwardly directed setae and, in many cases, with combs (also known as ctenidia). The thorax bears three pairs of legs, the third of which is particularly well developed for jumping. The mouthparts are modified for piercing, with a salivary canal for injecting saliva into the wound and a food canal along which blood is drawn. Both sexes are blood feeders (Day, 2016).

Fleas can cause discomfort to pets and their owners, skin diseases caused by fleabites, like allergic dermatitis, and anemia (Guaguère and Beugnet, 2006). Blood-feeding adults cause the anemia. Fleas feed by piercing the skin of the host and inserting the tip of the labrum-epipharynx to extract capillary blood. Flea feeding is more frequent at higher temperatures as a result of accelerated physiological activity and increased rate of water loss. Females of *C. felis felis* consume up to 13.6 µl of blood per day, which is equivalent to 15 times their body weight and they are able to reach a mean weight of 0.95 mg (Coutinho and Linardi, 2007).

Fleas infest man and animals and are the most frequent external parasites of companion animals worldwide. Some species are known to be vectors of zoonotic pathogens. Dogs and cats may play an important role either as reservoir of some of the pathogens or as transport vehicles for infected fleas between their natural reservoirs and human beings, thus playing a crucial step in the transmission cycle of flea-borne diseases (Iannino et al., 2017).

Fleas are competent vectors for numerous microbial pathogens of medical and veterinary importance and pathogen transmission is enhanced by their promiscuous feeding habits (Beugnet and Marié, 2009; Shaw et al., 2004). Plague and Murine typhus where historically known. But, with regard to cat and dog fleas, cat scratch disease (bartonellosis) and flea spotted fever (rickettsiosis) are well known. *Ctenocephalides felis* most probably originated from Africa and is more adapted to warm climates than cold. It is found in both rural and urban areas and on cats living indoors or outdoors (Franc et al., 1998; Alcaino et al., 2002; Gracia et al., 2008). Most species of flea are host-preferential rather than host-specific and will try to feed on any available animal. For example, *C. felis* has been found on over 50 different host species. Other factors that contribute to the potential of *C. felis* as a vector include transovarial transmission of some pathogens (*Rickettsia* species) and the transmission of pathogens such as *B. henselae* through adult flea faeces. Cat fleas also act as intermediate hosts for the common tapeworm of dogs and cats, *Dipylidium caninum*, and for the subcutaneous filarioid *Acanthocheilonema reconditum*, infesting dogs worldwide. Both helminths may occasionally be found in humans, particularly young children who, when playing with pets may inadvertently ingest infected fleas (Day, 2016).

2.1.1. *Rickettsia felis*

Flea-borne spotted fever (*Rickettsia felis*) is an obligate intracellular Gram-negative bacterium belonging to the spotted fever group (SFG), which may cause spotted fever rickettsiosis in humans. It was first described as a human pathogen in the early 1990s and has since been reported in patients worldwide. *Ctenocephalides felis* is currently the only known biological vector of *R. felis* (Reif et al., 2009). However, molecular evidence of *R. felis* in other species of fleas as well as in ticks suggests a variety of arthropod hosts. It has been demonstrated that this flea is able to maintain a stable infected progeny through transovarial transmission. Domestic dogs and cats are considered mammalian reservoir hosts for *R. felis*. Cat fleas may play an essential role in the transmission cycles resulting in potential risk of human exposure due to their indiscriminate feeding behavior. Additionally, *R. felis* can be circulating in cat fleas via transstadial and transovarial transmission consequently fleas acting as reservoirs and vectors. Therefore, understanding the host-vector relationship and epidemiology of *R. felis* is vital for pathogen surveillance and effective control measures in case of outbreaks as these animals share habitat with humans (Moonga et al., 2019). In human clinical signs of the illness are similar to those of murine typhus and other febrile illnesses such as dengue (Iannino et al., 2017). *Rickettsia felis* is now identified throughout the world and considered a common cause of fever in Africa (Brown and Macaluso, 2016).

Rickettsia felis is emerging worldwide as a flea-borne SFG human pathogen; it is detected frequently in *C.felis*, less often in other flea species (Brouqui et al., 2007). *C felis* is the vector and the recognised reservoir of *R felis*, which is vertically transmitted to successive generations of fleas (Wedincamp and Foil, 2002). Studies in parts of Europe have shown that *Ctenocephalides* infection rates range from 2.8% in Albania (Silaghi et al., 2012) to 54.2% in Andalucia, Spain (Márquez et al., 2006). In rare cases, *R.felis* DNA can be amplified also from the skin or gingiva of cats with negative blood PCR results (Lappin and Hawley, 2009).

2.1.2. *Bartonella* species

Bartonellae are emerging vector-borne pathogens that appear to be distributed in mammal's worldwide and cause multisystemic disease in dogs, cats, and humans. The *Bartonella* genus is best known for *Bartonella henselae*, the causative agent of cat scratch disease. Members of the genus *Bartonella* are generally transmitted by arthropod vectors. There are more than 40 named species within the genus, at least 17 of which are associated with human and/or animal disease (Pennisi et al., 2013; Taber et al., 2022). High prevalence was observed in areas where conditions are most favorable for arthropod vectors. Feline *B. henselae* infection was first reported in 1992 and canine *B. vinsonii* subsp. *berkhoffii* infection in 1994. Many *Bartonella* species appear to be well-adapted to extended survival in mammalian reservoir hosts. Prolonged bacteremia in clinically normal reservoir hosts is considered common. Cats appear to be the primary mammalian reservoir for the important zoonotic species, *B. henselae*, and also for *B. clarridgeiae* and possibly *B. koehlerae* (Guptill, 2010).

Infections by these bacteria in humans and animals can cause various clinical symptoms ranging from a mild, flu-like illness, to more severe manifestations, such as endocarditis, myocarditis, arthritis, hepatitis, and arthralgia. Numerous mammalian species, including domestic animals such as dogs, cats, as well as humans, serve as reservoir hosts for various *Bartonella* species. The vectors play a central role in the transmission of these bacteria and pets and their ectoparasites can pose a serious risk of zoonoses. (Chomelet al., 2006; Iannino et al., 2018).

Actually 30 species belong to the genus Bartonella and at least 13 species or subspecies are zoonotic (Cicuttin et al., 2014). Cat scratch disease (CSD) is the most common human infection caused by Bartonella species. Flea faeces are the main source of infection for *B.henselae* and can be inoculated by contaminated cat claws to other cats or accidentally to humans (Gil et al., 2013).

2.2.Ticks, Tick-borne pathogens of dogs and cats

Ticks are ectoparasites of mammals, birds and reptiles specializing as obligate blood sucking permanent ectoparasitic arthropods. One or more of the approximately 840 known species of ticks are found in most terrestrial regions of the earth. Two major families of ticks are ixodidae (hard bodies ticks) and argasidae (soft bodies ticks), with ixodidae being the largest and most important family (Ogbu et al., 2018). Ticks are relatively large, obligate blood-feeding ectoparasites, closely related to mites. They form a relatively small order of only about 800 species in the subclass Acari. The order can be broadly divided into 'hard' and 'soft' ticks, based on the possession of a dorsal scutum in the Ixodidae, which is absent in the Argasidae (the 'soft' ticks). Within the Ixodidae, species of the genera *Rhipicephalus* are of particular importance as vectors of disease for dogs and cats, since they may transmit a range of bacterial and protozoan pathogens. Hard ticks are usually relatively large and long-lived. During this time, they feed periodically, taking large blood meals, with long intervals off the host between each meal. Since a large proportion of the life cycle of most hard tick species occurs off the host, the habitat in which they live is of particular importance. It must include sufficient host numbers to sustain the tick population and have a high humidity to allow the ticks to maintain their water balance. Although many ixodid ticks are not host-specific, they are not indiscriminate in the hosts they parasitize. A few show an extremely wide host range, but most occur on a limited range of hosts, which they parasitize with varying intensities (Kettle, 1995; Day, 2016).

Ixodid ticks are relatively large (2–20 mm in length). The mouthparts are composed of a pair of four segmented palps (simple sensory organs), which aid host location. Between the palps lies a pair of heavily sclerotized, segmented appendages called chelicerae, housed in cheliceral sheaths. At the end of each chelicera are a number of tooth-like digits. The chelicerae are capable of moving back and forth and the tooth-like digits are used to cut and pierce the skin of the host animal during feeding. Below the chelicerae is the median hypostome, which emerges from the base of the palps (the basis capituli) and extends anteriorly and ventrally. The hypostome does not move, but is armed with rows of backwardly directed, ventral teeth. The hypostome is thrust into the hole cut by the chelicerae and the teeth are used to attach the tick securely to its host. As

the hypostome is inserted, the palps are spread flat onto the surface of the host's skin (Sonenshine and Roe, 2014; Day, 2016).

Ticks and TBDs have great economic and medical importance worldwide and they affect both animal and human health by sucking blood and also transmitting protozoan, bacterial, rickettsial, spirochetal and viral agents. Ticks can live in all regions of the world except Polar Regions. *Hepatozoonosis, Babesiosis, Anaplasmosis* and *Ehrlichiosis* are important tick-borne diseases of dogs and they influence a great variety of other domestic and wild animals (Chomel, 2011). More pathogens have been associated with ticks than any other bloodsucking arthropod but mosquitoes. Companion animals have always suffered from tick infestations. As veterinary medicine advances, signs and symptoms of disease that may have been missed before are now being detected. In addition, given the current trends of pet ownership and indeed treating companion animals as one of the family, there is more interest in the possible effects of tick infestation. (Berrada and Telford, 2009). Almost all the ticks of importance as vectors of disease in cats and dogs are three-host species, and it is this movement between different types of vertebrate hosts, and the fact that they are not strictly host-specific in their feeding preferences, that make ticks such important disease vectors. Wild animals are particularly important as reservoirs of pathogens through a wild animal/ tick/domestic animal cycle of contact. Several other factors contribute to the vectorial capacity of ticks. These include: secure attachment to their host; lengthy feeding periods allowing large numbers of pathogens to be ingested and transmitted; high rates of reproduction; and the transmission of pathogens between tick life cycle stages (transstadial transmission) and between generations via the egg (transovarial transmission). Infection of a host with tick-transmitted pathogens may be aided by salivary anticoagulants and other active compounds that modulate host cutaneous immunity and inflammation, while at the same time enhancing vasodilation in order to bring more blood to the feeding site. The salivary fluid is the principal avenue for disease transmission in the hard ticks (Hoogstraal and Aeschlimann, 1982; Day, 2016).

Table 1. Tick-transmitted pathogens of dogs

Disease agent	**Geographical distribution**	**Tick vectors**	**References**
Babesiosis			
B.canis canis	Tropical/semitropical worldwide	*Rhipicephalus sanguineus*, *Dermacentor reticulatus*, *Dermacentor marginatus*	Adachi et al. (1992); Kordick et al. (1999); Birkenheuer et al. (1999); Zahler et al. (2000); Irwin and Hutchinson (1991); Welzl et al. (2000); Morita et al. (1996); Taboada et al. (1992); Schetters et al. (1997)
B. canis vogeli	Tropical/semitropical worldwide	*Rhipicephalus sanguineus*	
B. canis rossi	Southern Africa	*Haemaphysalis leachi*	
B. gibsoni	Africa, Asia, USA, southern Europe, Middle East	*Haemaphysalis bispinosa*, *Rhipicephalus sanguineus*	
Hepatozoonosis			
H. canis	Southern Europe, Middle East, Far East, Africa	*Rhipicephalus sanguineus*, *Amblyomma maculatum*, *Haemaphysalis longicornis*	Mathew et al. (1998); Macintireet al. (1997); Baneth et al. (1996); Kakoma et al. (1994); Hegarty et al. (1997); Gauntet al. (1996); Harrus et al. (1998); Harrus et al. (1999); Harrus et al. (1997)
H. americanum	Southern USA	*Amblyomma americanum*	
Ehrlichia canis **genogroup**			
E. canis	Southern USA, southern Europe, Africa, Middle East, Eastern Asia	*Rhipicephalus sanguineus*	Gothe (1999); Grindem et al. (1999); Breitschwerdt et al. (1998); Kordick (1999); Schouls (1999); Kakoma (1994); Hegarty et al. (1997); Gaunt et al. (1996); Harrus et al. (1998); Harrus et al. (1999); Harrus et al. (1997)
E. chaffeensis	USA	*Amblyomma americanum*, *Dermacentor variabilis*	
E. ewingii	USA	Unconfirmed	

***E. phagocytophila* genogroup**			
E. phagocytophila	Northwestern Europe		
E. equi	USA	Ixodes species[a]	Greig et al. (1996); Johansson et al. (1995); Egenvall et al. (2000)
Human granulocytic ehrlichiosis agent	Midwestern and northeastern USA, northwestern Europe		
E. platys	USA, southern Europe, Middle East	Unconfirmed	
***Borreliosis* (Lyme disease)**			Levy and Magnarelli (1992); Straubinger et al. (1997a); Straubinger et al. (1997b); Schouls (1999); Fillipuzzi-Jenny et al. (1993); Junttila et al. (1999); Lorvich et al. (1994); Azuma et al. (1994); Ma et al. (1996); Chang et al. (1995)
B. burgdorferi sensu lato genogroups:			
B. burgdorferi sensu stricto	North America, Europe, Middle East		
B. garinii	Europe, Asia	Ixodes species[a]	
B. afzelii	Europe, Asia		
B. japonica	Japan		
Spotted fever group			Mumcuoglu et al. (1993); Grindem et al. (1999); Kordick et al. (1999); Drancourt and Raoult (1994); Weiser and Greene (1989)
R.conorii (Boutonneuse fever)	Southern Europe, Middle East, Africa	*Rhipicephalus sanguineus*	
R.rickettsii (Rocky Mountain spotted fever)	USA	*Dermacentor andersoni*, *Dermacentor variabilis*	

[a]Species dependent on geographical location, unconfirmed for *B. japonica*.

Source: Shaw et al., 2001.

2.2.1. Bacterial pathogens transmitted by ticks

2.2.1.1.*Ehrlichia* species

Canine ehrlichiosis is caused by tick-transmitted intracellular bacteria of the genus *Ehrlichia*, which, in dogs, have been identified parasitizing monocytes, granulocytes and platelets. Three genogroups of ehrlichiae have now been identified by 16S rRNA30 phylogenetic analysis. Genogroup III includes *E. canis*, which is responsible for widespread disease in tropical and temperate areas of the world. The geographical distribution of *E. canis* has expanded with the distribution of *R. sanguineus*. Other genogroup III species, *E. chaffeensis* and *E. ewingii*, have been identified by PCR in naturally infected dogs, with or without ehrlichiosis symptoms (Breitschwerdt et al., 1998; Kordick et al., 1999). These species have more restricted geographical distributions but a potentially wider range of tick vectors than *E. canis*. A subspecies of *E. risticii* (Genogroup I) has also been reported to cause an atypical syndrome of monocytic ehrlichiosis in dogs in the USA (Kakoma I et al., 1994).

Disease manifestations caused by members of the *E. canis* genogroup (genogroup III) infecting dogs can be indistinguishable (Breitschwerdt et al., 1998), and there can be strain variation in pathogenicity (Hegarty et al., 1997). At present, information on the pathogenesis of experimental monocytic ehrlichiosis relates primarily to *E. canis*. Immunological destruction of platelets occurs in acute disease and anti-platelet antibodies have been found in naturally occurring and experimental cases. Autoantibodies decrease platelet life-span, and interfere with platelet membrane glycoproteins, causing inhibition of aggregation. Other factors such as splenic sequestration and the production of a cytokine, platelet migration-inhibition factor, are also involved in the pathogenesis of thrombocytopenia. Hyperviscosity owing to hyperproteinaemia adds further to platelet dysfunction and can result in ocular and central nervous system (CNS) abnormalities. Subclinical persistent infection owing to splenic sequestration of organisms is common (Harrus et al., 1998). Severe life threatening chronic ehrlichiosis can develop following persistent infection and can be associated with irreversible bone marrow destruction. Factors that

predispose to myelofibrosis are not understood. *Ehrlichiosis* is more severe in certain breeds (e.g., German shepherd) and in younger animals. However, coinfection, immune status and strain variation could all play a role (Harrus et al., 1999). Genogroup II *Ehrlichiae* of pathogenic significance includes the *Ehrlichia phagocytophila* group and *Ehrlichia platys*. Strains (currently species) within the *E. phagocytophila* genogroup (*Ehrlichia equi*, human granulocytic ehrlichiosis agent and *E. phagocytophila*) are transmitted by Ixodes spp., ticks, and are the major causative agents of canine *ehrlichiosis* in the northern and western USA (Greig et al., 1996) and in northern and central Europe (Johansson et al., 1995). Infection with species of the *E. phagocytophila* group is generally associated with less severe clinical signs than *ehrlichiosis* caused by *E. canis*. Persistent sub-clinical infection has recently been identified with the granulocytic *Ehrlichia* species in dogs in Sweden (Egenvall et al., 2000). However, the severe chronic disease seen with *E. canis* in susceptible dogs has not been reported. As granulocytic *ehrlichiosis* is transmitted by Ixodes spp., ticks, coinfection with *Borrelia* in dogs is probable, as reported in humans. Canine cyclic thrombocytopenia, caused by *E. platys*, was first reported in the USA and appears to be a problem in several southern European countries, Israel, Taiwan and Venezuela (Harrus et al., 1997). Although it is assumed to be transmitted by *R. sanguineus*, its natural mode of transmission is uncertain. Pathogenicity is generally low but *E. platys* infection might play a role in coinfection with other arthropod-borne diseases (ABDs) (Shaw et al., 2001).

Ehrlichiosis is also known as canine *rickettsiosis*, canine hemorrhagic fever, canine typhus, tracker dog disease, and tropical canine pancytopenia (spelling) is a tick-borne disease of dogs usually caused by the organism, *E. canis*. Humans can become infected by *E. canis* and other species after tick exposure (Ogbu et al., 2018). *Ehrlichia* is a *Rickettsia* of the *Anaplasmataceae* family, Gram-negative intracytoplasmic bacteria that invade and multiply within leukocytes and platelets in the peripheral blood of various species of domestic and wild mammals. It causes *Ehrlichiosis* (Borjesson, 2000), a world-wide distributed zoonosis (Perez et al., 2005), concentrated in tropical and subtropical regions due to the geographical distribution of its vector tick Ixodidae, *R. sanguineus* which can rarely infect humans (Dantas-Torres et al., 2006). In Brazil, *R. sanguineus* is the main vector for *E. canis* in urban areas, although in rural areas human infection seems to be related to the genus *Amblyomma* (Labruna and Pereira, 2001).

Several species of *Ehrlichia* infect dogs such as *A. platys*, *E. equi*, *E. ewingii*, *E. risticii*, *E. chaffeensis*, *E. sennetsu* and *E. canis*. The latter being the main species that infects dogs producing several clinical symptoms Stiles (2000), fever, anorexia, vomiting, loss of weight, enlargement of the liver, spleen and lymph nodes, epistaxis, hemorrhage and thrombocytopenia (Moreira et al., 2003).

Diagnosis is achieved most commonly by serologic testing of the blood for the presence of antibodies against the *Ehrlichia* organism. Many veterinarians routinely test for the disease, especially in enzootic area. During the acute phase of infection, the test can be falsely negative because the body will not have had time to make antibodies to the infection. As such, the test should be repeated. PCR test can be performed during this stage to detect genetic material of the bacteria. The PCR test is more likely to yield a negative result during the subclinical and chronic disease phases. In addition, blood test may show abnormalities in the numbers of red blood cells, white blood cells, and most commonly platelets, if the disease is present. Uncommonly, a diagnosis can be made by microscopical examination of blood smear for the presence of the *Ehrlichia* morulae, which sometimes can be seen as intracytoplasmic inclusion bodies within a white blood cell (Dumler et al., 2007).

Serologic evidence of previous studies around the world indicated that *E. canis* is present among dogs throughout all continents with a prevalence ranging from 30 to 80% in some countries of Africa (Azzag et al., 2015; Inokuma et al., 2006; Kelly et al., 2004; Ndip et al., 2005; Davoust et al., 2006), while in some Asian countries it was 0.2 to 30% (Inokuma et al., 1999; Stich et al., 2008). In Europe, a prevalence ranging from 2 to 50% have been found. A study realized in the USA detected most often *Ehrlichia* antibodies in dogs in the Southeast, with 1.3% of samples testing positive, whereas other regions showed lower numbers ranging from 0.3 to 0.6% (Bowman et al., 2009). Depending on technique used, different results were found in Oklahoma, where the prevalence of *E. canis* was 10.8% by serology, and 3.1% by the polymerase chain reaction (PCR) (Murphy et al., 1998). Among dogs belonging to the U.S.A military forces, seropositivity to *E. canis* ranged from 8% in cold zones (above 45^{o} latitude) to 24% in temperate places (between 40 and 45^{o} latitude); a 13% prevalence was found in tropical zones (below 40^{o}

latitude). Several studies on *E. canis* prevalence have been realized in Brazil. In Grenade, 43.8% of dogs tested were positive for *E. canis* (Yabsley et al., 2008) while in Mexico, studies performed in the southern area found 44% of seropositive dogs to *E. canis* with ELISA testing (Rodriguez-Vivaz et al., 2005).

In cats, *E. canis* has been detected by PCR in blood samples (Ayllón et al., 2012; Hegarty et al., 2015; Breitschwerdt et al., 2002; Oliveira et al., 2009; Braga et al., 2012; Braga et al., 2013; Braga et al., 2014; Maia et al., 2014; André et al., 2015). Less frequently, *E. chaffeensis* and *E. ewingii* have also been detected (Hegarty et al., 2015; Braga et al., 2012). Cats seropositive for *E. canis* have been reported in Europe (6.0–18.0%) (Ebani et al., 2014; Persichetti et al., 2016; Solano-Gallego et al., 2006; Ayllón et al., 2012; Aguirre et al., 2004; Ortuño et al., 2005), Brazil (5.5%) (Braga et al., 2012) and the USA (0.7%) (Hegarty et al., 2015).

2.2.1.2.*Anaplasma* species

Granulocytic *anaplasmosis* is a tick-borne disease caused by *Anaplasma phagocytophilum,* a rickettsial pathogen, causing granulocytic blood infections in humans and animals (Alleman et al., 2008). The clinical appearance of infection in dogs is defined in different ways: granulocytic ehrlichiosis Bexfield et al. (2005), most commonly as anaplasmosis or granulocytic anaplasmosis. *Anaplasma phagocytophilum* is a Gram negative, obligate intracellular pleiomorphic agent. Up to 2001, it belonged to the genus *Ehrlichia phagocytophila*. This genus included *E. phagocytophila* (causative agent of the tick-borne fever in cattle, goats and sheep), *Ehrlichia equi* (causing granulocytic ehrlichiosis in horses) and an unnamed agent of human granulocytic ehrlichiosis (HGE) (Lillienook et al., 1998). On the basis of sequential analysis of 16S rRNA and groESl operons, these three aetiological agents were united in one species and renamed to *A. phagocytophilum*. Target cells for *A. phagocytophilum* are neutrophil leukocytes and sometimes eosinophils (Pusterla et al., 1999). The earliest time when anaplasmae could be seen is 4 to 18 days after the infection as elementary bodies of 0 to 6 μm or morules of 4 to 6 μm

size in the cytoplasm of blood neutrophils. Microscopically, morules could be registered for a short period of time, usually for 4 to 8 days (Egenvall et al., 1998).

Granulocytic anaplasmosis in dogs is sporadically detected in different European countries: Sweden (Egenval et al., 1996, 1998); Norway (Ekerstad et al., 1996); Switzerland (Pusterla et al., 1997); Italy (Gravino et al., 1997); Austria (Kirtz., 2000); Great Britain (Bexfield., 2005); Slovenia (Tozon et al., 2003); Germany (Jensen et al., 2007); and Czech Republic (Melter et al., 2007). In the USA, the disease (neutrophilic ehrlichiosis) was detected for the first time in 1971 in a German shepherd dog from Arkansas (Ewing et al., 1997). In 1998 it was also studied in 6 dogs from North Carolina and Virginia (Goldman et al., 1998). The latest data showed a remarkable wide prevalence, it was found in all states except for Mississippi and Nebraska (Alleman, 2008). *A. phagocytophilum* was evidenced for the first time in Australia in 2001, whereas the first incidence in Canada was in 2005 (Lester et al., 2005). The commonest vector of *A. phagocytophilum* in Europe is the tick *I. ricinus*. In 1995 it was identified as a disease vector for the first time in Sweden (Johansson et al., 1995), and then in Switzerland (Pusterla et al., 1999). The vectors in America are the ticks *I. scapularis* and *I. pasificus* (Davoust and Marie, 2007). *A. phagocytophilum* is characterized with a trans-stage transmission. An infection with *A. phagocytophilum* could occur through blood transfusion (Ewing et al., 1997). There is no sex-related predisposition in dogs with regard to granulocytic anaplasmosis. Pets are not a real source of infection for humans, but are sentinel animals. Such are also cats, among which the disease is also encountered. The seroprevalence in cats varies from 4.3% to 38% (Billeter et al., 2007).

The detection of *A. phagocytophilum* morules in granulocytes is a sufficient prerequisite to identify the disease (Pusterla et al. 1997). This is evidenced in 5% to 37% infected granulocytes in dogs with clinical signs of *A. phagocytophilum* infection. For a more reliable diagnosis however, the performance of additional analyses is advised (Sirigireddy and Ganta, 2005), such as indirect immunofluorescence, PCR and isolation. Until now, all isolated strains have been cultivated on the human cell line HL-60 (Goodman et al., 1996). Since 2006, the ELISA

SNAP 4Dx test kit appeared on the market (IDEXX Laboratories, Westbrook, Maine, USA), that has a high sensitivity (99.4%) and specificity (100%). It detects IgM and IgG antibodies against *A. phagocytophilum* (Ogbu et al., 2018).

Antibody prevalence to *A. phagocytophilum* in cats has been reported as 4.5–33.3% in Italy (Ebani et al., 2014; Persichetti et al., 2016), 2.0–8.0% in Spain (Solano-Gallego et al., 2006; Ayllón et al., 2012), 16.2% in Germany (Hamel et al., 2012),22.1% in Sweden (Elfving et al., 2015), and 1.8–38.0% in the USA (Magnarelli et al., 2005; Billeter et al., 2007; Hegarty et al., 2015). *Anaplasma phagocytophilum* DNA was recently amplified in 0.4% of blood samples from cats admitted to veterinary clinics in southern Germany (Bergmann et al., 2015). *Anaplasma platys* replicates in mature platelets and is the agent of thrombocytotropic anaplasmosis, a disease well documented worldwide in dogs. It has occasionally been detected in cats by PCR analysis (Hegarty et al., 2015; Lima et al., 2010; Qurollo et al., 2014). *Anaplasma bovis* has also been found by PCR, in feline blood samples from Japan (Sasaki et al. 2012). A novel, unclassified *A. platys*-like strain from cats was characterised in Sardinia (Italy). This strain, despite its tropism for platelets, is closely related to others identified in ruminants (Zobba et al., 2015). Anaplasma, *Ehrlichia* and *Rickettsia* species infections have been generally less studied in cats than in dogs. (Allison and Little, 2013).

Table 2. Members of the Ehrlichia, Anaplasma and Rickettsia genera detected in cats in various countries

	Countries in which infection has been reported*
***Ehrlichia* genus**	
E. canis	Canada, USA, Brazil, Portugal
E. chaffeensis	USA, Brazil
E. ewingii	USA
Ehrlichia species	Italy, USA, Kenya, France
***Anaplasma* genus**	
A. phagocytophilum	USA, Sweden, Finland, Poland, Switzerland, Germany, Italy, Spain
A. platys	USA, Brazil
A. platys-like	Italy
A. bovis	Japan
***Rickettsia* genus**	
R. rickettsii	USA
R. conorii	Spain
R. massiliae	Spain
Rickettsia species	Italy

*Note that this list may not be exhaustive. It is possible that feline Rickettsial infections are occurring in countries from which reports have not, to date, been published; particularly in areas where the competent tick vectors (see text) are abundant

Source: (Pennisi et al., 2017)

2.2.1.3.*Borrelia* species

Canine *borreliosis* is caused by a spirochete, *B. burgdorferi* sensu lato, which is transmitted by ticks of the genus Ixodes. At least four genospecies, with geographical distributions primarily in the northern hemisphere, cause disease in humans (Filipuzzi-Jenny et al., 1993). In northern Europe, the distribution of *borreliosis* is expanding and infected *I. ricinus* ticks are now commonly found in urban areas (Talleklint and Jaenson, 1998; Junttila et al., 1999). Much of the published *borreliosis* research relates to the Genogroup I species, *B. burgdorferi* sensu stricto, which is the primary isolate in humans and dogs in the USA. There is considerable genetic heterogeneity between North American and European isolates of *B. burgdorferi* (Lovrich et al., 1994). In Europe, human *borreliosis* is also caused by *Borrelia garinii* and *Borrelia afzelii* and in Japan, by *Borrelia japonica*, a separate genospecies. The degree to which *B. garinii*, *B. afzelii* and *B. japonica* contribute to canine infection and disease is currently unclear. However, infection with this *Borrelia* spp., could explain the disparity in pathogenicity and clinical syndromes described (Azuma et al., 1994). Although a high proportion of dogs are seropositive in endemic areas, relatively few develop clinical signs (Levy and Magnarelli, 1992). Several mechanisms have been incriminated in causing joint damage. The production of the inflammatory mediator nitric oxide is upregulated, as is interleukin 8, a cytokine that recruits neutrophils into infected synovial membranes (Straubinger et al., 1997a). Neurological abnormalities occur in some cases of *borreliosis*, most particularly in Japan (Azuma et al., 1994). Immunological cross-reactions to bacterial and self-antigens are important in human neuro *borreliosis*, and antibodies to flagellin, one of the most immunogenic *Borrelia* antigens, have been shown to cross-react with neuroaxonal proteins. In canine *borreliosis*, cutaneous and cardiac diseases are rare. Persistent infection with *Borrelia* even after antibiotic therapy is reportedly common in dogs (Straubinger et al., 1997b). The organism is sequestered in the skin, connective tissue, joints and CNS. Reactivation of infection with recrudescence of disease can occur in immunocompromised individuals or in coinfection (Shaw et al., 2001).

2.2.1.4.Spotted fever group *Rickettsia*

Rickettsia conorii, the agent of boutonneuse fever in humans in southern Europe, the Middle East and southern Africa, is reported to infect dogs (Mumcuoglu et al., 1993), but clinical signs of disease have not been reported. Mediterranean spotted fever (MSF) is caused by *R. conorii* and is transmitted by *R. sanguineus* in Europe, Africa, and Asia. The disease's many other names (Marseilles fever, Kenya tick typhus, Astrakhan spotted fever, Israeli tick typhus, and Indian tick typhus) attests to its broad geographic distribution. Experimentally, dogs have been demonstrated to be a competent mammalian reservoir capable of transmitting *R. conorii* to uninfected ticks. Cases occur in the summer months when ticks are most active. MSF has been reported to occur in those traveling to endemic areas (Blanton, 2019).

Amplification of rickettsial DNA by polymerase chain reaction (PCR) has been applied to the detection of a number of rickettsial species from peripheral blood, plasma, tissues (fresh frozen or paraffin-embedded), and from swabs collected from the ulcerated base of eschars. A variety of genes have been targeted (citrate synthase, outer membrane protein A, outer membrane protein B, and the 17-kDa lipoprotein gene), but no gene target appears more effective than another (Paris and Dumler, 2016; Portillo et al., 2017). Conventional, nested, and quantitative real-time PCR assays have been used. When rickettsial DNA is amplified using conventional or nested PCR techniques, sequencing can offer a species-specific diagnosis. When amplification is performed using certain primer sets, restriction fragment length polymorphism analysis can help to tidentify the etiologic agent (Peniche-Lara et al., 2013). Real-time PCR has improved analytical sensitivity over conventional and nested PCR assays it can detect fewer than 10 copies of genomic DNA per reaction (Paris and Dumler, 2016). Furthermore, with the use of species-specific probes, real-time PCR assays can diagnose to the species level without sequencing (Denison et al., 2014). Despite the ability of PCR techniques to detect very small quantities of DNA, the very limited number of circulating *rickettsiae* hinders the clinical sensitivity of PCR when used on whole blood or serum. A review analyzed data available in the literature and reports a median sensitivity of 18% (Paris and Dumler, 2016). In those with RMSF, PCR is more often positive and with higher copy numbers in those with fatal outcomes (Kato et al.,

2016). PCR is much more useful when applied to rash or eschar biopsy specimens, where it has a sensitivity of 48 to 92%. Swabbing the ulcerated base of an eschar with a saline dipped sterile cotton swab is an effective method to detect the nucleic acids of *Rickettsia* (Morand et al., 2018). Since PCR requires the expense of a thermocycler, reagents, and technical expertise, more inexpensive and easier to use nucleic amplification tests are desirable. The loop-mediated isothermal amplification assay is such a method and offers the ability to detect fewer than 100 copes of DNA per reaction (Hanaoka et al., 2017; Dittrich et al., 2014). Recombinase polymerase amplification assays are also inexpensive with field applicability for use in resource-limited regions (Chao et al., 2015).

2.2.2. Protozoan pathogens transmitted by ticks

2.2.2.1.*Babesia* specis

Babesiosis caused by different *Babesia* species is a disease with a worldwide distribution characterized by erythrocyte destruction causing mild to severe systemic clinical manifestations (Boozer and Macintire, 2003). Historically, *Babesia* infection in dogs was identified based on the morphologic appearance of the parasite in the erythrocyte. All large forms of *Babesia* (2.5-5.0 m) were designated *Babesia canis*, whereas all small forms (1.0-2.5 m) were considered as *Babesia gibsoni* (Boozer and Macintire, 2003). However, the development of molecular methods based on the characterization of the nuclear small subunit-ribosomal RNA gene (18S rDNA), the first and second internal transcribed spacers (ITS1, ITS2) loci as well as the intervening 5.8S coding region of the rRNA gene and hsp 70 (Yamasaki et al., 2007) and cytochrome B (Criado et al., 2006) genes has demonstrated that more piroplasmid species infect dogs. These include the small piroplasms *Babesia conradae* (Kjemtrup and Conrad, 2006; Kjemtrup et al., 2006), *Babesia* microti-like piroplasm (Zahler et al., 2000) which is also referred to as *Theileira annae* (Camacho-Garcia, 2006; Zahler et al., 2000) or “Spanish dog isolate” (Yeagley et al., 2009), Theileria spp., (Matjila et al., 2008c) and the yet unnamed large form *Babesia* spp., (Birkenheuer et al., 2004b) in addition to those initially described (Carret et al., 1999; Matjila et al., 2004; Zahler et al., 1998). *Babesia rossi, B. canis* and *B.vogeli* previously considered as subspecies of

B. canis are identical morphologically but demonstrate tremendous variations in geographic distribution, vector specificity, genetic characteristics, and the clinical signs which they induce in dogs, and are therefore currently considered separate species (Caccio et al., 2002; Carret et al., 1999; Irwin, 2009; Zahler et al., 1998). *Babesia microti*-like infections have been reported in foxes in North America (Birkenheuer et al., 2010) and in Spain (Gimenez et al., 2009). In addition, molecular detection of *B. rossi* infection was found in African wild dogs in South Africa (Matjila et al., 2008a). The geographical distribution of the causative agent and thus the occurrence of *babesiosis* are largely dependent on the habitat of relevant vector tick species. Knowledge of the prevalence and clinicopathological conditions caused by *Babesia* species infecting dogs and wild canids around the world is of epidemiologic and medical interest (Solano-Gallego and Baneth, 2011).

Babesiosis in domestic cats is a rarer clinical infection in comparison with its canine counterpart. Clinical domestic feline *babesiosis* has mostly been reported from South Africa where infection is mainly due to *Babesia felis*, a small *Babesia* that causes anemia and icterus (Penzhorn et al., 2004; Schoeman et al., 2001). *Babesia felis* also infects African wild felids including lions, cheetahs and servals (Bosman et al., 2007). Other reports of domestic feline *babesiosis* have mostly been sporadic. *Babesia cati* was reported from a cat in India (Mudaliar et al., 1950) and a few cases of infection in domestic cats by unnamed *Babesia* parasites were reported in France, Germany, Thailand and Zimbabwe (Bourdeau, 1996; Jittapalapong and Jansawan, 1993; Moik and Gothe, 1997; Stewart et al., 1980). A large form *Babesia*, *B. canis presentii*, was described in cats from Israel (Baneth et al., 2004). Interestingly, the presence of *Babesia* species typical to dogs in domestic cats is detected sporadically by molecular techniques often without compelling evidence of clinical infection. Molecular evidence for infection by *B. canis* in cats was provided in a study from Spain and Portugal in which a partial DNA sequence from the small subunit RNA gene identified as belonging to *B. canis* was amplified from three cats and the *B. microti*-like piroplasmid from two cats (Criado-Fornelio et al., 2003b). In addition, *B. vogeli* has been identified by blood smear examination and PCR in stray cats from metropolitan Bangkok, Thailand (Simking et al., 2010). Another small piroplasm infecting felines is *Cytauxzoon felis* which infects the bobcat (*Lynx rufus*) and domestic cats. Itis related to Theileria and *Babesia* and

is endemic in the United States (Holman and Snowden, 2009; Meinkoth and Kocan, 2005). Molecular recognition of *Cytauxzoon*-like parasites has been reported in domestic cats (Criado-Fornelio et al., 2004) and in Iberian Lynx (Millan et al., 2007, 2009) in Spain and in a domestic cat in France (Criado-Fornelio et al., 2009).

Babesia rossi has to date been restricted to Africa and *B. canis* has mostly been reported from Europe, whereas *B. vogeli* and *B. gibsoni* have wide distributions in both the old and new world continents. Molecular studies on canine *Babesia* infection in Europe have demonstrated *B. canis* infection in Croatia, Poland (Caccio et al., 2002), Hungary (Foldvari et al., 2005), Russia (Rar et al., 2005), Switzerland (Porchet et al., 2007) and Germany (Zahler et al., 1998). Infection with both *B. canis* and *B. vogeli* has been reported in Slovenia (Duh et al., 2004), France (Caccio et al., 2002), Spain (Criado-Fornelio et al., 2007), Portugual (Cardoso et al., 2008) and Albania (Hamel et al., 2009). In Italy, *B. canis* is mainly described in the north and less frequently in central Italy while *B. vogeli* is predominantly found in central and southern Italy (Solano-Gallego et al., 2008). Additionally, canine *B. vogeli* infections have been reported in Turkey (Gulanber et al., 2006) and recently, the first case of autochthonous *babesiosis* caused by *B. canis* in a dog was reported from Norway (Oines et al., 2010). An outbreak of autochthonous canine *babesiosis* caused by *B. canis* was also reported in the Netherlands (Matjila et al., 2005). *Babesia microti*-like piroplasm infections were reported in north-western Spain (Camacho et al., 2001) and have since been detected also in Croatia (Beck et al., 2009) and the United States (Yeagley et al., 2009). Occasional clinical cases of *B. gibsoni* were reported in Spain (Criado-Fornelio et al., 2003a), Germany (Hartelt et al., 2007) and Italy (Trotta et al., 2009).

Table 3. Distribution, vectors, and cytological characteristics of selected *Babesia* spp., that infect dogs (modified from Solano-Gallego, 2008)

Species	**Geographic distribution**	**Proven or putative vector (s)**	**Size (m)**	**Cytological appearance**	**References**
B. rossi	South Africa, Nigeria, Sudan	*Haemophysalis elliptica* (formerly *Haemaphysalis leachi*)	2 × 5	Usually paired	Matjila et al. (2008b); Oyamada et al. (2005); Sasaki et al. (2007)
B. canis	Europe	Dermacentor spp., *Rhipicephalus sanguineus*	2 × 5	Usually paired	Bourdoiseau (2006); Cassini et al. (2009); Iori et al. (2010)
B. vogeli	Africa, Asia, Europe, North/Central/South America, Australia	*Rhipicephalus sanguineus*	2.5 × 4.5	Single or paired	Criado-Fornelio et al. (2007); Jefferies et al. (2003); M'Ghirbi and Bouattour (2008); Oyamada et al. (2005); Passos et al. (2005); Sasaki et al. (2007)
Unnamed large form *Babesia*	Eastern USA	Unknown	2x6	Ameboid, paired piriform	Birkenheuer et al. (2004b); Sikorski et al. (2010)
B. gibsoni	Southeast Asia, United States, South America, Australia, Europe	*Haemaphysalis longicornis*, *Haemaphysalis bispinosa*? *Rhipicephalus sanguineus*?	1 × 3	Usually singular	Birkenheuer et al. (2005); Hartelt et al. (2007); Jefferies et al. (2007b); Miyama et al. (2005); Trapp et al. (2006)
B. conradae	USA (California)	*Rhipicephalus sanguineus*?	0.3-3	Ring, tetrade, ameboid	Kjemtrup and Conrad (2006)
B. microti-like (*Theileria annae*)	Spain (Galicia, Burgos), Croatia, North America[a]	*Ixodes hexagonus*?, *Ixodes ricinus*?, *Rhipicephalus sanguineus*?	1 × 2.5	Usually singular	Beck et al. (2009); Camacho et al. (2003); Gimenez et al. (2009); Lledó et al. (2010); Iori et al. (2010); Yeagley et al. (2009)

[a] The B. microti-like piroplasm has been described in dogs in Galicia, Croatia, and the USA and in foxes in Burgos (Spain) and North America (USA and Canada).

Babesia rossi is considered to cause the most severe disease manifestations, among the large babesial species that infect dogs and the disease is most prevalent in summer (Jacobson, 2006; Reyers et al., 1998). *Babesia* infection in cats is associated with anorexia, lethargy, anemia and icterus. Information on the clinical manifestations of domestic feline *babesiosis* is limited mostly to publications on *B. felis* infection in South Africa (Ayoob et al., 2010b; Penzhorn et al., 2004; Schoeman et al., 2001). In a study on *B. felis* that included 56 cats (Schoeman et al., 2001), 80% were less than 3 years old and there was no specific breed or gender predilection. Most cats were anorectic and lethargic. Macrocytic hypochromic regenerative anemia was present in the majority of infected cats. Hyperbilirubinemia was present in 86% of the cats and alanine aminotransferase activity was elevated in 89%. Thirty two percent of the cats were concurrently infected with feline leukemia virus (FeLV) and 14% with feline immunodeficiency virus (FIV). *Babesia canis presentii* infection in a cat from Israel co-infected with FIV and Candidatus *Mycoplasma haemominutum* was accompanied by fever, icterus, moderate anemia and thrombocytopenia which resolved following anti-babesial therapy (Baneth et al., 2004).

The polymerase chain reaction (PCR) is a sensitive and specific diagnostic technique which is frequently employed for the diagnosis of *babesiosis*. It is particularly useful for detection of infection in dogs with a low parasitaemia levels and for speciation of parasites. A large number of PCR assays and protocols using a variety of gene targets have been described. A semi-nested PCR able to detect and discriminate DNA from *B. canis*, *B. rossi*, *B. vogeli* and *B. gibsoni* has been described (Birkenheuer et al., 2003a). In addition, a reverse line blotting (RLB) technique in which PCR products are hybridized to a membrane containing specific probes for the several babesial species and possibly also for other pathogens has been developed for simultaneous detection and speciation of piroplasms and co-infections. The RLB confirmed the presence of *B. vogeli* in addition to *B. rossi* in dogs from South Africa (Matjila et al., 2004). PCR-restriction fragment length polymorphism is also used to separate between canine *Babesia* species (Carret et al., 1999; Jefferies et al., 2007a; Solano-Gallego et al., 2008). High-resolution melting curve quantitative fluorescence resonance energy transfer PCR has been developed to discriminate between species based on melting curves analysis (Wang et al., 2010).

Prevention of *babesiosis* relies mostly on topical and environmental acaricidal treatments aimed at reducing the exposure to vector ticks and pathogen transmission to the dog or cat. Collars, spot on formulations and sprays are the most popular and effective means of controlling tick infestations on individual animals and a variety of products which include permethrin, amitraz, fipronil, imidacloprid and other chemicals for protection of individual animals are available from commercial companies (Berrada and Telford, 2009; Brianti et al., 2010; Last et al., 2007; Otranto et al., 2010). These topical ectoparasiticides either repel ticks and prevent attachment or kill ticks within 24-48 h after application. Decrease of tick burdens in the environment can be achieved using conventional and slow release acaricidal formulations applied by spray or powder. Biologic control by means of organisms pathogenic specifically to ticks may be used in the future as environmental control measures (Fernandes and Bittencourt, 2008). As *Babesia* species are transmitted by blood product transfusions, it is highly recommended to screen canine blood donors for *Babesia* infection on a regular basis (Wardrop et al., 2005). Non-vectorial transmission of babesiae by blood transfusions and by dog-to-dog fighting is preventable and should be of special concern as it can be responsible for the incursion of *babesiosis* into previously non-endemic areas. Vaccines against *B. canis* are commercially available in some countries in Europe. One vaccine contains culture derived soluble parasite antigens from a homologous *B. canis* stock and a second bivalent vaccine contains culture derived soluble antigens of heterologous origins with a European *B. canis* stock and a South African *B. rossi* addition (Moreau et al., 1989; Schetters, 2005; Schetterset al., 2009b). Both vaccines induce partial protection against disease caused by *B. canis* manifested by decreased severity of clinical signs, parasitaemia or duration of clinical disease induced by infection challenge. While the former vaccine conferred protection only against strains of *B. canis*, the latter was also partially protective against *B. rossi*. Studies to evaluate vaccination against *B. gibsoni* have been reported using several types of vaccination techniques including recombinant antigen and DNA vaccines (Fukumoto et al., 2005b, 2007, 2009).

Human *babesiosis* caused by several *Babesia* species is an important emerging tick-borne zoonotic disease (Gray et al., 2010). A fatal *Babesia divergens* infection reported in 1956 was the first confirmed case of human *babesiosis* (Skrabalo and Deanovic, 1957). *Babesiosis* has since

then regarded as an important and potentially life-threatening zoonotic infection of humans (Homer et al., 2000).

The spectrum of *Babesia* pathogens that infect dogs and cats is gradually being elucidated with the aid of new molecular techniques and meticulous clinical investigation. Species of *Babesia* that cannot be distinguished morphologically cause diverse diseases and are transmitted by different vector ticks. Non-vector transmission by blood transfusion and directly from dog to dog is of special concern and could be responsible for the spread of infection to areas that were previously non-endemic. Accurate detection and species recognition are important for the selection of the correct therapy, predicting the course of disease and for following the epidemiologic trends related to infection by different species globally (Solano-Gallego and Baneth, 2011).

Canine *babesiosis* is caused by the intraerythrocytic protozoan parasite *Babesia canis*, and increasingly by *Babesia gibsoni*, which is extending its range in the USA Birkenheuer et al. (1999) and Europe. Molecular studies have identified novel *Babesia* spp., which infects dogs (Zahler et al., 2000). Although the pathogenicity of *B. gibsoni* is uniformly high, pathogenicity varies among strains of *Babesia canis*: *Babesia canis rossi*, the prevalent strain in South Africa, causes severe clinical disease; *B. canis canis* in Europe is moderately pathogenic; and *Babesia canis vogeli* infection causes relatively mild disease worldwide (Irwin and Hutchinson, 1991). The relative importance of tick species in the transmission of canine *babesiosis* varies with geographical location. The clinico-pathogenesis of *babesiosis* caused by *B. canis canis* and *B. gibsoni* involves progressive haemolytic anaemia. By contrast, the more severe disease caused by *B. canis rossi* can involve hypoxic, hypotensive shock with disseminated intravascular coagulation (DIC), systemic inflammatory response syndrome and multiple organ dysfunction syndromes. The severity of disease also varies with the species of vector, and the age, breed and immune status of the dog (Irwin and Hutchinson, 1991). Erythrocyte-bound auto antibodies are involved in the haemolytic form of the disease (Adachi et al., 1992). There can be erythrocyte autoagglutination and many dogs with *babesiosis* give a positive Coombs' test.

Methaemoglobinaemia and methaemoglobinuria occur secondary to oxidative damage in parasitized red blood cells (Morita et al., 1996)). As a consequence of the methaemoglobinaemia, there is enhanced damage by anti-erythrocyte antibodies and erythrophagocytosis. Persistent infection with *B. canis* or *B. gibsoni* is common in endemic areas (Taboada et al., 1992). Although these animals appear healthy unless subjected to stress, they provide a reservoir of infection for susceptible animals and have suboptimal athletic performance (Taboada et al., 1992).

The distribution of canine *Babesia* parasites is worldwide and is dependent on the presence of the specific tick vector responsible for their transmission, such that the *Babesia canis vogeli* which is transmitted by *R. sanguineus* is seen in North Africa, South America, Southern and Eastern Africa, and European countries (Duh et al., 2004; Matjila et al., 2008a,b; Oyamada et al., 2005; Eiras et al., 2008; M'ghirbi and Bouattour, 2008); *Babesia canis rossi* transmitted by *Haemaphysalis elliptica* (Apanaskevich et al., 2007) is seen in South Africa and Sudan (Oyamada et al., 2005; Matjila et al., 2008b), whereas *Babesia canis canis* transmitted by *D. reticulatus* is distributed mainly in Europe (Caccio et al., 2002; Duh et al., 2004; Solano-Gallego et al., 2008). *Dermacentor reticulatus* is an exophilic and ditropic tick found mainly in forests, but adapted to suburban habitats and is the major vector species of canine *babesiosis* in France (Bourdoiseau, 2006). The small *Babesia* (*B. gibsoni*, *B. conradae* and *Babesia* (Theileria) *annae*) which has three genetically distinct entities are found mainly in Asia (Matjila et al., 2008a, b). *Babesia conradae* has been shown to be closely related to piroplasm isolates from wildlife and humans and distributed around the California area of USA while *B.* (Theileria) *annae* is similar to *Babesia microti* and *Theileria equi* and occurs mainly in Spain. The vector for this emergent canine infection has not been described, although *Ixodes hexagonus* is suspected based on their presence upon dogs in North-West of Spain and the relative absence of other ticks (Dixit et al., 2010).

2.2.2.2.*Hepatozoon* specis

Canine and feline *hepatozoonosis* is caused by protozoon parasites from the genus *Hepatozoon*. There are more than 340 species in this genus, and so far, 2 have been found to infect dogs, *Hepatozoon canis* and *Hepatozoon americanum*, and 3 infect domestic cats, *Hepatozoon felis*, *Hepatozoon silvestris*, and *Hepatozoon canis* (Baneth, 2011; Giannelli et al., 2017). The life cycle of *Hepatozoon* spp., involves transmission by hematophageous arthropod vectors, ixodid ticks in the case of the *Hepatozoon* spp., which infect dogs. The arthropod vector harbors the oocyst stage of the parasite and is consumed by the vertebrate host when transmission occurs (Baneth, 2011). Different *Hepatozoon* spp., affect a variety of target organs in carnivores and elicit subclinical to severe life-threatening infections with considerable diversity between the infecting species (Modry´ et al., 2017).

Canine *hepatozoonosis* is a tick-transmitted, protozoan disease caused by species of the intraleukocytic parasite *Hepatozoon*. Unlike most other tick-borne diseases, *Hepatozoon* is transmitted by ingestion of an infected tick, rather than tick bites. *Hepatozoon canis* commonly infects dogs in Africa, southern Europe, the Middle East and Asia, reflecting the geographical distribution of its major vector, *R. sanguineus*. However, unique clinical features suggest that a separate species (*Hepatozoon americanum*) transmitted by the tick *Amblyomma maculatum* causes disease in dogs in the southern USA and that this disease is spreading (Mathew et al., 1998; Macintire et al., 1997). The clinical spectrum of *H. canis* infection ranges from subclinical to severe life-threatening disease (Macintire et al., 1997; Baneth et al., 1996)). *Hepatozoon canis* is commonly associated with coinfection with other diseases, in particular ehrlichiosis and leishmaniosis in endemic areas, and clinical presentations are variable (Shaw et al., 2001).

Hepatozoonosis is a tick-borne disease of wild and domestic carnivores caused by protozoan parasite, *Hepatozoon canis* that is transmitted by ticks, usually the brown dog tick, *R. sanguineus*. The mode of transmission is unusual; the tick picks up the organism from an infected host while biting the animal. An uninfected host gets the disease by eating the tick, not from being bitten by the tick. Because of the long prepatent period of the parasite, this disease is not developed soon after tick bites of particular tick season, but there are all possibilities of occurrence of the disease all year round. The life cycle of the apicomplexan protozoon *Hepatozoon canis* in its natural hosts *Rhipicephalus sanguineus* (tick) and *Canis familiaris*

(domestic dog) was studied in an experimental infection. Tick nymphs were fed on a naturally infected dog, or they were infected by percutaneous injection of blood. Dogs were inoculated by ingestion of adult ticks containing mature oocysts. Gamonts were in syzygy 24 hr. after percutaneous injection of ticks. Early oocysts were detected 96 hr. after nymph repletion, and mature oocysts in adult ticks were infective to dogs 40 days post molt. Merogony was detected in dog bone marrow from 13 days post inoculation (PI) and included meronts containing 20-30 micromerozoites, and a second type with 2-4 macromerozoites. Monozoic cysts were observed in the spleen in conjunction with merogony. Gamontogony with infection of leukocytes by micromerozoites occurred from 26 days post inoculation (PI), and gamontparasitemia, which completed the life cycle, was detected 28 days PI. The length of the life cycle from nymphal attachment to parasitemia in dogs was 81 days. Increased body temperatures were evident from 16 to 27 days PI and paralleled the time of intensive bone marrow merogony. Skeletal pain and recumbence were manifested in dogs (Baneth et al., 2007).

The *H. canis* among dogs is widespread. Its distribution corresponds to that of the vector tick *R. sanguineus* in continents/countries such as Africa, Europe and Asia, including the Middle East, the pacific and Indian Ocean islands (Gevery, 1993; Baneth, 2006). In Japan Direct microscopy using Giemsa-stained blood smears revealed *H. canis* gametocytes in the peripheral blood of 23.6% of dogs. Based on PCR, 42.9% dogs were positive using the common primer and 41.3% were positive using the specific primer. The investigation indicated that all screened areas, except for Sado Island and Atsumi Peninsula, were infected. Yaku Island had the highest infection rate (84.6% in males and 100.0% in females), while Ishigaki Island showed the lowest infection rates (8.3% in males and 17.7% in females) (El-Dakhly et al., 2013). Epidemiological survey revealed that in different regions of Pakistan higher prevalence of *H. canis* is observed in Islamabad 22.4% compared to Lahore 5.8% and Multan 8%. Using PCR, 11.9% of dogs were found positive for the presence of *H. canis* DNA (Qamar et al., 2017). In Israel the prevalence of *H. canis* is 33.1% and 1% of the dogs had detectable parasites in their blood smears (Baneth et al., 1996). In Aegean coast of Turkey, the prevalence of *Hepatozoon* spp., infection was 10.6% by blood smear parasitology and 25.8% by PCR. IFAT revealed that 36.8% were positive for antibodies reactive with *Hepatozoon* spp. The PCR products of 18S rRNA gene of *Hepatozoon*

spp., isolated from six infected dogs, one isolate originating from each of the six different locations, were sequenced. The results of sequence analysis indicate that they are closely related to Indian and Japanese isolates of *H. canis* (Karagenc et al., 2006)

The initial studies on the seroprevalence of *H. canis* in dogs showed high to low percentage of infection in different countries such as 36% in Portugal, 17.6% in Nigeria, 2.5% in India, 2.3% in Israel and 2.1% in Thailand (Gevrey, 1993). The distribution is closely related to that of the definitive tick host *R. sanguineus* (Baneth et al., 2001). The host range in carnivores is not yet elucidated, except for the domestic dog (Ogbu et al., 2018).

The microscopic detection of *H. canis* gamonts in blood smears stained according to Romanovski-Giemsa, Pappenheim or with Hemacolor is the commonest diagnostic approach to this infection. The protozoa concentration is directly related to the severity of the illness. Gamonts are oval shape with dimensions of 812/3-6 μm and are detected in the cytoplasm of neutrophils and rarely in that of monocytes. Schizonts could be observed in histological or touch impression preparations from lymph nodes, spleen, and bone marrow. Schizonts are round or oval, with a diameter of about 30 μm and contain 2 or 4 macromerozoites or over 20 micromerozoites. Histologically, microschizonts with the so-called “wheel spoke” shape could be observed (Baneth, 2006). For sero-diagnostic purposes, indirect immunofluorescent antibody test (IFAT) and ELISA are applied. Previously prepared antigen of *H. canis* gamonts is used. These assays are performed mainly in epidemiological studies (Baneth et al., 2002).

Infection with multiple tick-transmitted pathogens, or with multiple genotypes of the same pathogenic species, can occur in an individual animal following heavy exposure to ticks (Breitschwerdt et al., 1998; Kordick et al., 1999). The same tick species can be a vector for several pathogens and coinfection of individual ticks can occur (Schouls et al., 1999). Infection with tick-borne pathogens can also be complicated by other arthropod-borne diseases that share the tick biohabitat, such as leishmaniosis. In dogs, coinfection with combinations of *Ehrlichia*, *Bartonella*, *Babesia*, *Hepatozoon*, *Leishmania* and *Rickettsia* species occurs in endemic areas. The role of such coinfection is being clarified by PCR (Kordick et al., 1999). Coinfection could

partially explain variations in clinical presentation, pathogenicity and response to therapy (Shaw et al., 2001).

2.3.Lice (Phthiraptera), louse-borne pathogens of dogs and cats

Lice are small (1-2 mm), dorsoventrally flattened, wingless insects. They are host-specific, obligate parasites and spend their entire life cycle (about three weeks) on the host. Lice are small insects that live in close contact with the skin and hair of their hosts. They may cause direct injury to the skin and act also as vectors or intermediate hosts of pathogens. According to their feeding habits they are classified as sucking lice or chewing lice. The species found on companion animals include, *Trichodectes canis* and *Heterodoxus spiniger* (both chewing lice). Cats have only one chewing lice (*Felicola subrostratus*). (Bowman et al., 2002; Bowman, 2014). *Trichodectes canis* are distributed worldwide. *H. spiniger* is found mainly in warm tropical or subtropical regions (Dantas-Torres and Figueredo, 2007). The chewing louse *T. canis* (family *Mallophaga*) and *H. spiniger* (family *Boopidae*), are found in dogs in the tropics. Transmission is usually by direct contact between dogs although immature lice can survive for up to three or four days off-host, long enough to be transmitted indirectly via brushes, combs and bedding. Neglected animals in overcrowded, dirty conditions are particularly susceptible (Craig, 2011). In dogs *Trichodectes* feeds on hair, skin debris, scale and inflammatory exudate. It can act as intermediate host for the tape worm, *Dipylidium caninum*. Lice are typically found at the base of hairs, and adult lice have a claw at the end of each foot enabling them to stay attached. Eggs (nits) are laid by females at the base of hairs and attached firmly to the hairs by cement. They are about 1 mm long, whitish and operculate, and hatch out within about six days to produce nymphs which then moult three times before becoming adults. Each female lays about 300 eggs and can live up to eight weeks (Craig, 2011). Lice develop through an incomplete metamorphosis. All of them spend their entire life on the host and display a high order of host specificity. The eggs or nits are glued by the female to the hair shaft. The duration of the life cycle varies according to species (Bowman, 2014).

Usually, the owners do not notice lice infestations on their pets and they are found only at a more thorough examination of the fur. In dogs, pruritus is the main clinical sign. A rough, dry, matted coat can be observed as well as erythema, scaling, crusting, and hair loss (specifically around ears, neck, shoulders, groin, and rectal regions). Restless behaviour is sometimes noticed by the owner. Anaemia is possible, especially in young or immunesuppressed animals, caused by blood loss due to heavy infestations, and less frequently *T. canis* or *H. spiniger* infestations. Lice are most commonly found around the head and back, accumulating under mats of hair, and around the pinnae. Pruritus with poor coat and alopecia are classic features although severity of pruritus is variable. A pinnal-pedal scratch reflex is sometimes seen. Papules, scale, crusting, excoriations and secondary infections may be severe. Dogs with lice often have a poor, dirty, matted coat although this can be associated with neglect as much as the lice themselves. Affected dogs may also smell quite bad and are often irritable. Some animals, however, are asymptomatic carriers. Heavy infestations of sucking lice, especially in young animals, may produce anaemia (Craig, 2011).

In dogs Diagnosis is suggested from history and clinical signs but confirmation requires finding the lice. Lice are visible to the naked eye but biting lice move very quickly and may be difficult to see. A magnifying glass may be helpful. Skin scrapings, tape strips and hair plucks can be used to demonstrate both lice and nits. Skin biopsies are unnecessary, histopathology revealing varying degrees of superficial perivascular dermatitis. The differential diagnosis includes allergic skin diseases, sarcoptic mange, cheyletiellosis and harvest mite infestation (Craig, 2011).

The prognosis is usually good with appropriate treatment. Mats should be clipped and the patient and all in-contact dogs treated with an appropriate insecticide. Fortunately, lice are relatively easy to eradicate although products may need to be re-applied in line with manufacturer's instructions. Many of today's commercially available insecticidal products are effective and licensed for treating biting lice in dogs (Craig, 2011). These products are considered effective against, but unlicensed for, sucking lice. Brushes, combs and bedding should be cleaned thoroughly and the premises sprayed where appropriate with a suitable environmental insecticide (Craig, 2011). Advice should be given to owners regarding importance of general hygiene and

environmental cleanliness.The source of infestation should be investigated with a view to preventing re-contamination (Craig, 2011).

In cats, scratching is the main sign, associated with a rough, dry coat, crusting, or scaling. Predilection sites include face, back and pinna causing non-specific skin lesions characterized by scaling, papules, and crusts. The degree of pruritus is variable and damage to the skin from scratching may result in alopecia and crusts, inflammatory excoriation. Louse infestations in cats are infrequently diagnosed. Heavy infestations occur typically only in animals' incapable of grooming such as very young animals or diseased cats with underlying severe conditions (e.g., feline leukaemia virus and feline immunodeficiency virus). The species of louse found in cats is the biting louse; *F. subrostratus*. As well as being a cause of severe pruritus, *Felicola* may be associated with miliary dermatitis. Cats may sometimes be asymptomatic carriers of *Felicola*. Diagnosis is confirmed by demonstration of lice or nits (Craig, 2011). Some insecticidal products are specifically licensed for use against *F. subrostratus* in cats. Advantix is toxic to cats and should always be avoided in this species. Cats should not be allowed to come into contact with animals treated with Advantix until the application site is dry. Cats should not be allowed to groom the application site on a dog treated with Advantix (Craig, 2011).

Trichodectes canis are yellow-coloured and run around very quickly. The head is much wider than the thorax and they measure approximately 2 mm in length. They are usually found on the head (especially the ears), the back and the tail. *Linognathus setosus* have pincer-like tarsal claws for clinging to the hairs of their hosts. The thorax is wider than the head. They measure 1.5 to 2.5 mm in length, and are grey to dusk red. These lice have sedentary habits and move slowly.They are found on the head, the eyelids and on the ventral part of the neck and chest. *Heterodoxus spiniger* has a subtriangular head. The thorax is longer than wide, while the head is wider than long. They measure 2.5 mm and are typically found anywhere on the host, moving around rapidly. *Felicola subrostratus* is characterized by the triangular shape of the anterior portion of the head which is wider than the thorax, and measures 1.2 to 1.5 mm. They are found on the head, the back, the ear pinna and rarely inside the auditory canal (Durden, 2018). Treatment is effective only against active stages on hosts (nymphs and adults) whereas eggs are not affected.

Topical or systemic treatments are available. (Kohler-Aanesen et al., 2017). Prophylactic use of imidacloprid, fipronil, moxidectin or selamectin monthly is effective in preventing louse infestations, but avoidance of infested animals is also recommended. Lice are highly host specific so cat or dog lice do not parasitize humans. Lice are vectors of *D. caninum* for dogs and cats and *Acanthocheilonema reconditum* for dogs.

2.4.Molecular diagnostic tests

The genome of a pathogen contains all the information required to produce the proteins and RNA molecules that it requires to successfully propagate itself. Genes that are essential for life often have DNA sequences that are highly similar amongst apparently unrelated organisms. The subtle changes in DNA sequences that occur due to random mutation, and which offer a selective advantage or are selectively neutral over geological time frames, differentiate one pathogen from another and these differences can be exploited to allow differentiation of these organisms. The recent expansion in the number and range of pathogens that have had their genomes sequenced, and the amount of sequence data generated from clinical isolates, have provided new avenues for the identification of previously difficult-to-detect organisms. Deoxyribonucleic acid (DNA) provides an excellent template on which to base a diagnostic assay, due to its stability and unique structure. A number of commercially available technologies exist for reliable and reproducible extraction of highly pure nucleic acids from all classes of pathogens in a variety of different sample types, including tissues and blood. This extracted DNA can then be used in any of a number of assays that utilize the nucleotide basepairing characteristics of DNA to generate a diagnostic signal (Day, 2016).

2.4.1. Polymerase chain reaction

Polymerase chain reaction (PCR) is a method that involves the amplification of target DNA sequences by repeated cycles of synthetic oligonucleotide primer-driven DNA synthesis. The key to the process is the use of a thermostable DNA polymerase (such as that derived from the hot

spring bacterium *Thermus aquaticus*), which is optimally active at elevated temperatures (75–80°C) and maintains its activity when heated to the temperatures required to melt double-stranded DNA (e.g. 95°C). Therefore, newly synthesized double-stranded DNA can be dissociated, by heating, to act as templates for subsequent rounds of primer binding and DNA synthesis while maintaining polymerase enzyme activity (Eddlestone et al., 2007; Day, 2016).

As the technology involved in determining the nucleotide sequences of pathogen genes has developed, a vast array of sequence information has been placed in databases such as GenBank. This information can be used to determine areas of genes that are conserved between species, genera and families of pathogenic organisms, or areas that are specific to individual strains. In parallel with this explosion of information, the commercial synthesis of oligonucleotide probes and the generation of economically priced, rapid DNA extraction kits mean that DNA-based diagnostic methods have become widely available. In many cases, where microbial culture is impossible, slow or undesirable because of biohazard considerations, PCR is becoming the method of choice in the diagnostic laboratory because of its sensitivity, selectivity and speed (Day, 2016).

A number of PCR methodologies are available, but the two most commonly used are conventional (end-point) and real-time (quantitative) PCR (qPCR). The primary difference between these methodologies is in the strategy used to measure the accumulation of the products from the amplification reaction. Conventional PCR measures the products at the end of the thermocycling protocol, while real-time PCR measures the accumulation during the thermocycling protocol using a fluorescent dye or fluorogenic-dye labelled probe (Day, 2016).

A number of different test formats are available for qPCR, but the common factor is that as PCR product is produced, the amount of fluorescence increases proportionately. Fluorescence is monitored throughout the assay and these data are converted into quantitative results reflecting the amount of pathogen in the sample (rather than the qualitative results given by conventional PCR). Therefore, this method has great advantages in assessing pathogen ‘load’ and responses to

treatment. A number of different thermocycling platforms have been developed, many with the ability to monitor fluorescence at a number of different wavelengths and thus multiple fluorescent dyes within a single sample. This gives rise to the potential to develop 'multiplex' assays where a number of different organisms, gene targets or reaction controls can be probed simultaneously. In multiplex systems, labelled probes with different fluorescent dyes are used with distinct, non-overlapping signal spectra. At each cycle during the assay, the machine assesses the fluorescence produced by the binding of each probe independently. This has the potential to increase the speed and reduce the costs of testing for multiple pathogens in a single sample. However, these multiplex assays can be technically difficult and costly to develop and require careful optimization to ensure that the individual reactions work efficiently within the multiplex, such that there is no reduction in assay sensitivity, particularly as infection with multiple agents is possible. A more common application of the multiplex potential of qPCR is the incorporation of an internal control reaction, which ensures the successful extraction and amplification of the DNA from a sample to reduce the chance of false-negative results due to experimental error or PCR inhibitors within the sample. The major problem with all PCR-based methodologies is their extreme sensitivity. This makes contamination a major concern, and laboratory design should consider the need to keep reagent preparation, DNA extraction from samples, thermal cycling and agarose electrophoresis analysis (where conventional PCR is used) physically separate to minimize the generation of false-positive results (Eddlestone et al., 2007; Day, 2016).

2.5.Status of vector-borne diseases of dogs and cats in Ethiopia

Rickettsia felis has been reported in *C. felis* and *P. irritans* specimens collected from human dwellings in the southwestern regions of Ethiopia, *R. felis* was detected in 21% of *C. felis*, 11% *P. irritans* and 6% *C. canis* collected from dogs and cats in Ethiopia (Mediannikov et al., 2012; Kumsa et al., 2014a) and 11% prevalence of antibodies against *Bartonella* spp., in cats from Addis Ababa and *Bartonella* spp., related to *B. elizabethae* in small mammals in northern Ethiopia (Meheretu et al., 2013) were detected. *Rickettsia typhi* has been reported in 62% of *Rattus rattus* collected from buildings in Addis Ababa, Ethiopia (Azad and Beard, 1998).

Previous studies on *rickettsia* in Ethiopian ixodid ticks have documented the presence of *rickettsia* spp., in *Amblyomma variegatum*, *Amblyomma cohaerens* and *Rhipicephalus* spp., in central Ethiopia (Philip et al., 1966) and *Amblyomma* spp., (*Amblyomma gemma*, *A. Varigatum* and *Am. cohaerens*) in the central and eastern regions of Ethiopia (Burgdorfer et al., 1973). *Rickettsia aeschlimannii*, the agent of SFG rickettsiosis, had been detected in *Hyalomma marginatum rufipes* and *R. africae*, the agent of African tick bite fever (ATBF), has been detected in *Amblyomma lepidum* and *A. Variegatum* ticks from eastern Ethiopia (Mura et al., 2008), and also *R. africae* had been detected in pools of *Amblyomma* and *Rhipicephalus* ticks (Pader et al., 2012).

Rickettsia africae has been molecularly confirmed in a 62-year-old French man returning after 2 months stay in western part of Ethiopia (Stephany et al., 2009). A case of relapsing fever *borreliosis* with cutaneous eschar and radiculopathy in a 77-year-old French woman traveler returning from Ethiopia was reported to be transmitted by hard ticks using molecular tools (Socolovschi et al., 2012). Spoted fever group *Rickettsia* DNA was detected in dried thin blood smears prepared to test malaria in children with febrile illnesses at Soddo Christian Hospital in Wolaitta Soddo, Ethiopia (Aarsland et al., 2012).

A new *Borrelia* species distant from both relapsing fever group and Lyme *borreliae* was detected in 7.3% of the *Amblyomma cohaerens* collected from cattle in southwest parts of Ethiopia (Mediannikov et al., 2013). Similarly, new *Borrelia* species has been detected in 8/119 (6.7%) *Amblyomma cohaerens*, 1/42 (2.4%) *Amblyomma gemma*, 3/53(5.7%) *Ammblyomma variegatum*, 5/22(22.7%) *Amblyomma* larvae and 3/60(5%) *Amblyomma* nymphs (Kumsa et al., 2015b) that cluster at intermediate position in between the recurrent fever and the Lyme disease *borreliae* group using 16S genus-specific qPCR.

A study conducted by Kumsa et al. (2012), revealed the presence of *Rickettsia* species DNA in lice from Ethiopia. *Heterodoxus spiniger* on dogs were identified. Molecular identification of lice using an 18S rRNA gene analysis confirms the identified lice species by morphological methods and detected. Hordofa and Adugna, (2017) detected *Babesia canis* (9.9%) and *Babesia gibsoni* (6%) from dogs in and around jimma town.

3. MATERIALS AND METHODS

3.1.Description of the Study area

Gamo Zone is located in Southern Nations, Nationalities, and People's Regional state between 5°55′N and 6°20′N latitude and between 37° 10′E and 37°40′E longitude. Elevation ranges between 600 and 4,207 m above sea level, and it covers an area of 6,735 km2. The annual average temperature ranges from 15°C to 28°C, whereas the mean annual rainfall ranges from 200 mm to 2,000 mm. The rainfall pattern can be characterized as a bimodal minor rainy season (September–November) and the major rainy season (March–May). The main rainy season accounts for 70–90% of the total annual rainfall. Air temperature largely depends on the altitude, it decreases with increasing altitude. Most of the natural vegetation consists of woodland and savannas. In the highlands, afro montane forests are found (Shalishe et al., 2011). Sampling was carried out between November 2020 and January 2021 in four districts of Gamo zone in Southern Ethiopia: Arba Minch town, Chencha town, Arba Minch zuria and Gerese. These districts were selected by the Gamo zone administration, being the ones with higher dog and cat populations, and households to be visited for sample collection were porposively identified. Arba Minch zuria district elevation ranges from a warm semiarid midland (1200masl) around eastern part to a cool and humid highland (3000masl) in north western part near Chencha town. Arba Minch town has a warm semiarid climate which is lowland lies at an altitude of 1285masl. Gerese district lies in an altitude of 2400masl which has also cool and humid highland characteristics. Chencha town is located at an altitude of 2732masl. The territory of the Zone is divided into three agroecology zones, named in local language Dega (high land), Woinadega (mid land) and Kolla (low land), on the basis of their climatic/geographic aspects. All agroecological Classification was done based on Ethiopian Ministry of Agriculture guidelines (MoA, 1998).

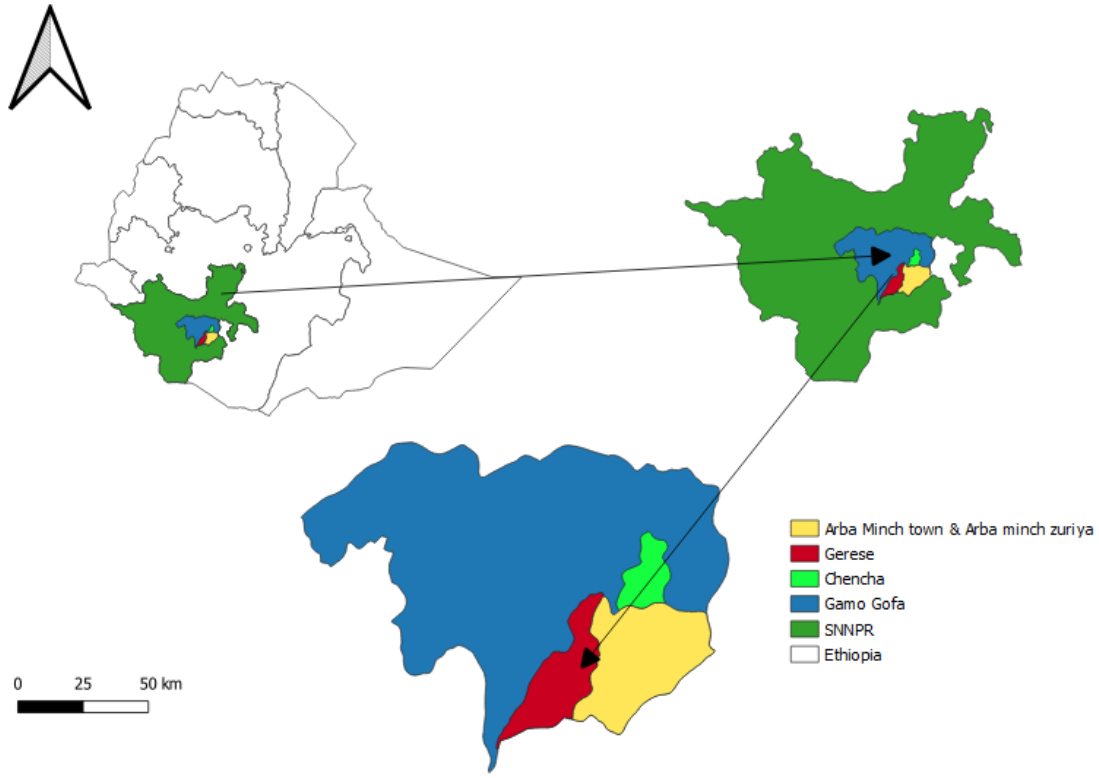


Figure1. Map of the study districts (Created by QGIS 3.28.4). The map shows the location of the districts in Ethiopia

3.2.Study population

All the study dogs and cats were included irrespective of their sex, age and life style. The sex (female, male) and age (young, adult) were recorded for each study animal. Age was estimated by dental formulary and owner's information. The animals were categorized into two age groups, young (≤ 1 year) and adult (> 1 year) according to Kumsa and Mekonnen (2011) and Kumsa et al. (2019). In this study, only owned dogs and cats were included for the investigation of different kinds of vector-borne pathogens and infestation. A total of 297 dogs and 110 cats were identified for ectoparasites and blood sample collection. Information on sex, age (i.e., young, adult), district of provenance, location (i.e., urban, rural), lifestyle (i.e., outdoor, indoor) and agroecology (i.e., highland, midland, lowland) was recorded for each dog and cat. Most of the

owned dogs are usually left to free roaming during the day in the proximity of their home. Stray dogs and cats were not included in the sample, since their safe containment and management is extremely difficult in this context. Only asymptomatic dogs and cats were included in the study. Overall, 407 pet animals (297 dogs and 110 cats) from 21 Peasant Assocaitation (PA) (the lowest administrative unit), were included from Arba Minch town (n=8 Kebeles), Arba Minch zuria district (n=6 Kebeles), Chencha town (n=4 Kebeles) and Gerese district (n=3 Kebeles). The details of the sampling effort are reported in Table 6

3.3.Study design and Sample size determination

A cross-sectional study design was used to assess the presence of ectoparasites and the molecular prevalence of vector-borne pathogens of owned dogs and cats through house-to-house survey in purposively selected households, and based on owners' willingness to participate. A structured questionnaire was conducted within each household to collect information on the pet animal owner, on his/her general knowledge about VBDs and previous specific health problem related to VBDs or reasons for taking the pet animal to the local health post. Dogs and/or cats present in the house were visited and ectoparasites and blood samples were collected as described below. Due to the practical and logistic defeculties to collect blood samples from dogs and cats in the study area, for dogs and cats a minimum sample size of 276 and 97 animals was defined respectively at the begning of the study, in order to estimate the prevalence of concerned pathogens with a 6% accepted error in dogs and 10% in cats (95% confidence level), given an expected prevalence of 50% and an infinite population. Sample size was determined based on Thrusfield, (2008) considering the factors related to the study area and animal species.

3.4.Flea, lice and tick collection and identification

The skins of all dogs and cats were palpated and visually inspected thoroughly for the presence of ticks, lice, and fleas by going through all parts of the body for 10-15min. All ticks were removed carefully to ensure that the mouth-parts remained intact by using forceps. For the

collection of lice and fleas, each animal was combed using a standard fine metal flea comb (12 teeth per 1 cm) for 10–15 min (Marchiondo et al., 2007). After combing, the flea comb was held over a white plastic tray and fleas and lice collected with forceps from the tray. All the collected ticks, lice, and fleas from each dog and cat were moved into labeled specimen bottles containing 70% ethanol and subsequently transferred to the laboratory. Specimens from all animals were counted, recorded and identified thoroughly in the laboratory of Addis Ababa University, College of Veterinary Medicine and Agriculture, Parasitology Laboratory. Each ectoparasite was identified at genus or species level and their sex was determined under a microscope according to the descriptions of Hoogstraal (1956), Okello-onen et al. (1999) and Walker et al. (2000; 2013) for ticks, and Wall and Shearer (1997) and Taylor et al. (2007) for lice and fleas. Adult ticks were identified at species level whereas larvae and nymphs were identified only at genus level (Hoogstraal, 1956; Walker et al., 2000; 2013).

3.5.Blood sample Collection

Whole blood samples were collected from the cephalic vein of the selected dogs and cats and applied (100 μL) to classic Flinders Technology Associates (FTA™) Nucleic Acid Collection Cards (Whatman®, Maidstone, UK), then air dried, coded and stored in sealed FTA pouches with a silica gel desiccant (Sigma Aldrich, Co., Life Sciences, St. Louis, MO, USA) until analysis. FTA cards with blood samples were transported to the Laboratory of Parasitology of the University of Padova, Legnaro (PD), Italy, as per authorization of the Ethiopian Biodiversity Institute and Italian Ministry of Health (Prot. N. 0002711, 22 April 2021) (Appendix 5).

3.6.DNA extraction

DNA was extracted from dried blood spots found in the FTA cards that were punched (diameter of punches: 6 mm) and one punch was put into an Eppendorf tube labeled with the same number reported on the FTA cards. The samples were processed using NucleoSpin™ Tissue extraction

kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The purified DNA was stored in the freezer at −20 °C until further molecular analysis.

3.7.PCR assays

The detection of bacterial pathogens was performed using either end-point or real-time PCR assays. End-point PCR reactions were performed using a BiometraTAdvanced thermocycler (Analytic Jena, Gottingen, Germany), Phire Hot Start II PCR Master Mix (Thermo Scientific, Vilnius, Lithuania) and 0.3 μM of each primer. The presence of *Anaplasma phagocythophilum*, *Anaplasma platys*, *Ehrlichia canis* (only in dogs) and *Borrelia burgorderferi* sensu latu was checked with primers targeting a portion of the gene encoding the groEL heat shock protein (Alberti et al., 2005; Otranto et al., 2010; Lee et al., 2003), while *Rickettsia* sp. DNA was detected using the primer pair described by Regnery et al. (1991) and Choi (2005) which targets a portion of the citrate synthase gene (gltA). Primer sequences and expected product length have been summarized in Table 4. Amplification products were visualized by electrophoresis on 2% agarose gel stained with SybrSafe DNA Stain (Invitrogen, Thermo Scientific, Carlsbad, CA, USA). The real-time PCR assay were used in cat samples for the detection of *Bartonella* spp. *Bartonella* spp., DNA was targeted to the *ssrA* gene (Diaz et al., 2012) and was performed on a Light Cycler® 96 (Roche Diagnostic GmbH, Mannheim, Germany) real-time PCR instrument using QuantiNova Probe RT-PCR kit (Qiagen, Hilden, Germany), 0.8μM of each primer and 0.25μM of probe. Positive and negative controls have been included in each run of all assays.

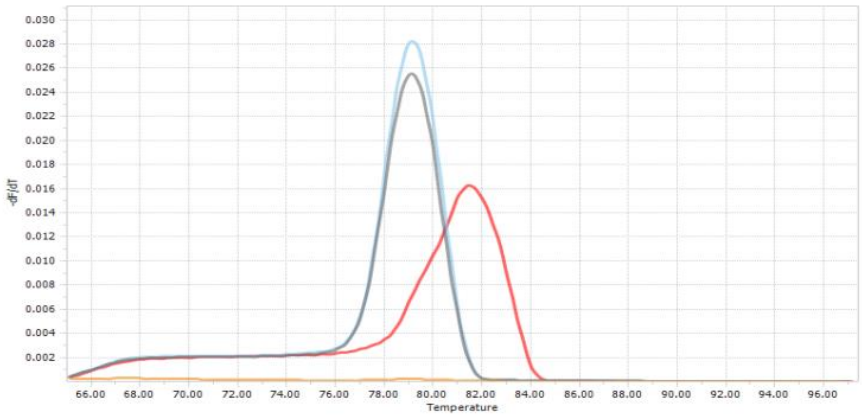
For the detection of protozoal pathogens (*Babesia* spp., and *Hepatozoon* spp.), DNA extracts were initially analyzed by real-time PCR using the QuantiNova SYBR® Green PCR Kit (QIAGEN Group, Hilden, Germany) with primers previously described by Tabar et al. (2008) targeting the 18S-rRNA gene spanning the V4 region (Table 4). The assay was performed in the Roche LightCycler®96 thermocycler (La Roche Ltd, Basel, Switzerland) with the following amplification cycle: incubation at 95 °C for 2 min, followed by 45 cycles of amplification steps at 95 °C for 5 s and 60 °C for 10 s, concluding at 95 °C for 10 s, 65 °C for 1 min and 97 °C for

1s. The melting curve analysis was performed by continuously monitoring the fluorescence while decreasing the temperature from 95 °C to 65 °C. The melting temperature of the more common canine and feline piroplasms of the genera *Hepatozoon*, *Cytauxzoon* and Babesia (respectively, *H. canis* and *B. canis*) were identified and compared with those of other relevant piroplasms, potentially present in other domestic or wild hosts (Grillini et al., 2022). The identification of target pathogens in dogs and cats were achieved through the melting temperature analysis (figure 2 and 3). In particular, the melting temperature for *H. canis* (79/79.5 °C) was determined using an isolate of *H. canis* as positive control obtained through preliminary analysis of 20 samples of the present study. The melting temperature for *B. canis* (81.5 °C) was determined using as positive control a *B. canis canis* field sample provided by the Istituto Zooprofilattico Sperimentale delleVenezie (Legnaro, Italy). The melting temperature for *Hepatozoon* feline species (i.e., *H. felis*, *H. ingwe*, *H. luiperdije*) (78/78.5 °C) was determined both through preliminary analysis on samples of the present study and those provided by the Parasitology laboratory at the department of Animal Medicine, Production and Health, University of Padua (Legnaro, Italy). The melting temperature for *Cytauxzoon europaeus* (81 °C) was obtained using a field sample provided by the Parasitology laboratories at the department of Animal Medicine, Production and Health, University of Padua (Legnaro, Italy).

Positive (i.e., DNA of sequenced field samples) and negative (no DNA added) controls were added in each PCR reaction and all samples were tested in duplicate. Selected samples that resulted positive at real-time PCR analysis for *H. canis* and *B. canis* and all cat positive samples were submitted to end-point PCR targeting a 373 bp fragment of the 18S-rRNA gene using the same primers as before (Table 4). End-point PCR was performed using Invitrogen Taq DNA polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA), following the manufacturer's instructions.

Table 4. Primer sets for the PCR amplification of different pathogens

Pathogen	**Primers**	**Expected amplicon length**	**Ref.**
Anaplasma phagocytophilum *Anaplasma platys*	F (Ephpl-569F) ATGGTATGCAGTTTGATCGC R (Ephpl-1193R) TCTACTCTGTCTTTGCGTTC	624bp	Alberti et al 2005
Ehrlichia canis	F (Ecan-163S) AAATGTAGTTGTAACGGGTGAACAG R (Ecan-573AS) AGATAATACCTCACGCTTCATAGACA	410bp	Otranto et al. 2010
Borrelia burgdorferi s.l	F (GF) TACGATTTCTTATGTTGAGGG R (GR) CATTGCTTTTCGTCTATCACC	310bp	Lee et al 2003
Rickettsia spp.	F (Rp877p) GGGGGCCTGCTCACGGCGG R (Rp1258n) ATTGCAAAAAGTACAGTGAACA	381bp	Regnery et al. 1991
	F (Rp896p) GGCTAATGAAGCAGTGATAA R (Rp1233n) GCGACGGTATACCCATAGC	338 bp	Choi et al. 2005
Bartonella spp.(real-time PCR)	F (ssrA-F) GCTATGGTAATAAATGGACAATGAAATAA R (ssrA-R) GCTTCTGTTGCCAGGTG Probe FAM-ACCCCGCTT AAACCTGCGACG-BHQ1	301 bp	Diaz et al. (2012)
Piroplasms (real-time PCR)	F CCAGCAGCCGCGGTAATTC R CTTTCGCAGTAGTTYGTCTTTAACAAATCT	373 bp	Tabar et al. (2008)
Piroplasms (end-point PCR)	F CCAGCAGCCGCGGTAATTC R CTTTCGCAGTAGTTYGTCTTTAACAAATCT	373 bp	Tabar et al. (2008)



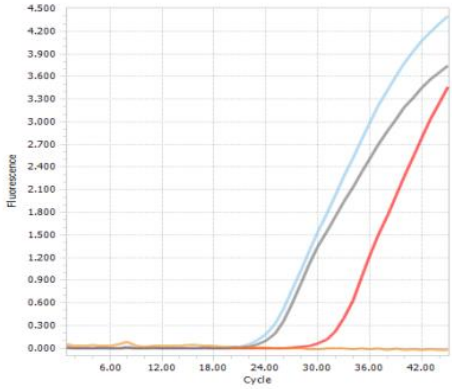


Figure 2. Specific temperature of melting (Tm) and cycle threshold (Ct) for *Hepatozoon canis* (light blue and purple lines), *Babesia canis* canis (red line) and negative control (orange line) in dogs

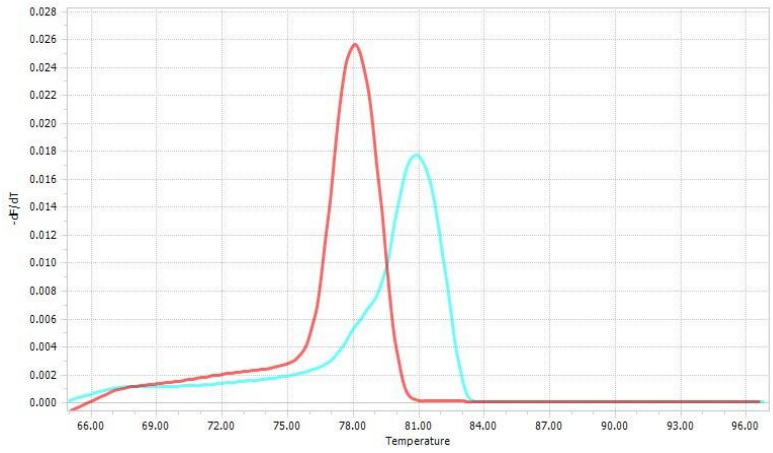


Figure 3. Melting temperature (Tm) for *Hepatozoon felis* (red line) and *Cytauxzoon europaeus* (light blue line) in cats

Amplified PCR products from positive samples were purified using EXOSAP-it® (ExoSAP-IT™ PCR Product Cleanup, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Bidirectional Sanger sequencing of all purified products was carried out at Macrogen Spain (Madrid, Spain) or at StarSEQ® GmbH facilities (Mainz, Germany), using the same primers of original PCR. The nucleotide sequences were assembled and edited using ChromasPro v.2.1.8 (Technelysium Pty Ltd., Brisbane, Australia) and compared to those deposited in GenBank® using BLAST (https://blast.ncbi.nlm.nih.gov/Blast) (accessed on 25 January 2023).

3.8.Ethical Clearance

The study protocol was approved by the Animal Ethics Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture (Agreement No.VM/ERC/01/13/021). All necessary permits were obtained including permission of administration and agricultural office of the Districts and verbal consent from each animal owner.

3.9.Statistical analysis

Descriptive statistics (i.e., prevalence and mean abundance values) was used to summarize and display in tables the level of infestation for each group of ectoparasites (ticks, lice and fleas) for both dog and cat species. Pearson Chi-square (X^2) test, or Fisher's exact test when appropriate, were applied to assess significant differences between dog and cat infestation rates for the different parasite groups and species. Similarly, within each of the two host species, the same approach was used to assess differences in groups identified by age group, sex, life style, urban/rural areas and agroecology. Concerning dogs, age class include young (up to 12 months) and adult (olderthan one year), whereas life style was categorized in indoor (if the dog had access to indoor also partially) and outdoor (if dog was always outdoor). Likewise, cats were categorized as previously described in young (up to 6months) and adult (older than 6months) (Thomas et al., 2016), and the life style differentiated in indoor (if the cat was always indoor)

and outdoor (if the cat had access to outdoor also partially). The urban or rural environment and the agroecology zone were based on information provided by local administrations. Differences in the infestation burden between dog and cats were investigated only for relevant groups or species of parasites (i.e. for the ones with higher abundance values), by means of a non-parametric approach (Mann Whitney U-Test).

The prevalence values of the different vector-borne pathogens and their 95% confidence intervals (95% CI) were calculated using the Clopper–Pearson exact method for each pathogen identified. Sex (i.e., male, female), age (i.e., young: 0–24 months, adult: ≥25 months), location (i.e., urban, rural), lifestyle (i.e., only outdoor, mixed indoor–outdoor), agroecology (i.e., highland, midland, lowland) and presence of ticks were used as variables to calculate prevalence for all pathogen species sufficiently prevalent and summarized descriptively. The Pearson Chi-square test, or the Fisher's exact test when more appropriate, was used to compare proportions for the more prevalent identified pathogens, and, when more than one factor significantly associated with a specific parasite resulted, a multivariable logistic regression model was used to evaluate the association between the potential risk factors and that species. The model fitness was assessed by the Hosmer–Lemeshow goodness-of-fit test. A probability p-value < 0.05 was regarded as statistically significant. For some vector-borne pathogens, statistical analysis was not conducted considering the very low number of positive animals. Data analysis was performed with IBM SPSS Statistics for Windows, version 28 (IBM Corp., Armonk, N.Y., USA).

4. RESULTS

4.1.Owner's characterstics

Overall, 290 dog/cat owners were included in this study and interviewed, specifically 143 from Arba Minch town, 85 from Arba Minch Zuria district, 49 from Gerese district and 13 from Chencha town (Table 5). Two-hundred fifty-six (88.3%) dog/cat owners had no knowledge about arthropods and VBDs of dogs and cats. Only one-third of the interviewed persons had ever visited a veterinary clinic and most of the dog owners went to veterinary clinic for rabies vaccination (n=96, 33.1%). Most of the owners only kept dogs (n=190, 65.5%). The other owners have both dogs and cats (n=100, 34.5%). The highest number of dogs and cats in a single household was six and two respectively.

Table 5. Results of the questionnaire survey on general characteristicsof pet owners (n=290)

Variables	**Category**	**N of owners (%)**
Age	7-25	46(15.9)
	26-55	197(67.9)
	>55	47(16.2)
Gender	Male	231(79.7)
	Female	59(20.3)
Education	Illiterate	82(28.3)
	Elementary	92(31.7)
	High school	53(18.3)
	College	62(21.4)
	Theology	1(0.3)
Knowledge about VBDs	Yes	34(11.7)
	No	256(88.3)
Person infected in the household	No	256(88.3)
	Leishmania (cutaneous)	1(0.3)
	Relapsing fever	1(0.3)
	Typhus	31(10.7)
	Typhus and *Leishmania*	1(0.3)
Purpose of having dog/cat	Companion and guard	2(0.7)
	Companion and rat protection	100 (34.5)
	Guard	188(64.8)
Pet origin	Breeding in the house	69(23.8)
	Neighbor	176(60.7)
	Other places	45(15.5)
Reason for Vet. clinic visit	No visit	188(64.8)
	Ectoparasite infestation	2(0.7)
	Infection and stomach upset	2(0.7)
	Loss of appetite and weight loss	2(0.7)
	Rabies vaccine	96(33.1)

4.2.General information on animal's characterstics

Overall, 407 pet animals (297 dogs and 110 cats) from 21 Peasant Assocaitation (PA) (the lowest administrative unit), were included Table 6.

Table 6. Number of pet animals sampled according to District, sex, age class and provenance area

District	**Cat**				**Dog**				**Total**
	Female		Male		Female		Male		
	Adult	Young	Adult	Young	Adult	Young	Adult	Young	
Arba Minch town									
Lowland (urban)	23	7	7	3	33	9	71	41	194 (47.7%)
Arba Minch Zuria									122 (30.0%)
Lowland (rural)	7	1	2	1	17	6	16	8	58 (14.0%)
Midland (rural)	6	2	9	4	12	5	20	6	64 (15.7%)
Chencha town									
Highland (urban)	4	0	2	0	4	1	4	3	18 (4.4%)
Gerese									73 (17.9%)
Highland (rural)	1	0	1	0	2	4	3	4	15 (3.7%)
Highland (urban)	14	6	7	3	9	2	8	9	58 (14.3%)
Sub-total	55	16	28	11	77	27	122	71	
Total	**110**				**297**				**407**

4.3.Ectoparasites identified in dogs and cats

Generally, eight genera of ectoparasites were identified comprising five tick species (*Rhipicephalus sanguineus, Rhipicephalus pulchellus, Rhipicephalus praetextatus, Amblyomma variegatum* and *Haemaphysalis leachi*), four flea species (*Ctenocephalides felis, Ctenocephalides canis, Echidnophaga gallinacea* and *Pulex irritans*) and two lice species (*Heterodoxus spiniger* and *Trichodectes canis*).

Overall, the prevalence for tick species in dogs was 36.7% compared to 2.7% in cats, with a significant difference. Similarly, the prevalence for fleas was much higher ($p < 0.001$) in dogs (69.7%) compared to cats (21.8%), although in dogs the prevalence for lice was 4.7% and lice was totally absent in cats (Table 7). Considering the prevalence of the different species, *R. Sanguineus* and *A.variegatum* were the more prevalent among dogs, whereas only the tick *H.leachi* was found in few cats. *Heterodoxus spiniger* was the most prevalent lice species in dogs. The only species most prevalent in cats compared to dogs was the flea *E. gallinacea*.

Table 7. Comparison of prevalence values of different ectoparasites between dogs and cats

Parasite species	**Dogs (n=297)**		**Cats (n=110)**		**p-value**
	Pos	**Prev (%)**	**Pos**	**Prev (%)**	
Ticks (overall)	109	36.7	3	2.7	<0.001
R. sanguineus	42	14.1	0	0.0	<0.001
R. pulchellus	10	3.4	0	0.0	>0.05
A. variegatum	68	22.9	0	0.0	<0.001
Rh. praetextatus	12	4.0	0	0.0	0.042
H. leachi	26	8.8	3	2.7	0.048
Fleas (overall)	207	69.7	24	21.8	<0.001
P. irritans	1	0.3	0	0.0	>0.05
C. felis	206	69.4	17	15.5	<0.001
C. canis	3	1.0	0	0.0	>0.05
E. gallinacea	4	1.3	8	7.3	0.004
Lice (overall)	14	4.7	0	0.0	0.014
H. spiniger	12	4.0	0	0.0	0.042
T. canis	2	0.7	0	0.0	>0.05

The mean abundance values (Table 8) were generally low (i.e., less than one ectoparasite/host) for all species, a part from the flea *C. felis* in dogs, whose mean number was 7.0 fleas/host. In most cases only adult ticks were found, a part from *R. sanguineus*, whose larval stages (nymphs and larvae) were collected with similar frequency than adults, and from *A. variegatum*, which was infesting dogs mainly during the nymphal stage. The higher presence of the flea *E. gallinacea* in cats was confirmed also by the significantly higher abundance ($p=0.002$) in this host species, compared to dogs, where the hen flea was only sporadically encountered.

Table 8. Number of collected specimens of the different species of ectoparasites (according to the development stage) and significant difference in the mean abundance between dogs and cats

Parasite species			**Dogs (n=297)**		**Cats (n=110)**		p-value (Mann-Whitney U-Test)
			N collected specimens	Mean abundance	N collected specimens	Mean abundance	
Ticks	*R. sanguineus*	**Total**	**216**	**0,7**	**0**		
		male	70		0		
		female	57		0		
		nymph	76		0		
		larvae	13		0		
	R. pulchellus	**Total**	**16**	**0,1**	**0**		
		male	9		0		
		female	7		0		
	A. variegatum	**Total**	**146**	**0,5**	**0**		
		male	4		0		
		female	0		0		
		nymph	136		0		
		larvae	6		0		
	Rh. praetextatus	**Total**	**17**	**0,1**	**0**		
		male	9		0		
		female	8		0		
	H. leachi	**Total**	**60**	**0,2**	**3**	**0,0**	
		male	17		0		
		female	43		3		
Fleas	*P. irritans*	**Total**	**1**	**0,0**	**0**		
		male	1		0		
	C. felis	**Total**	**2076**	**7,0**	**29**	**0,3**	**p<0.001**
		male	541		7		
		female	1535		22		
	C. canis	**Total**	**29**	**0,1**	**0**		
		male	22		0		
		female	7		0		
	E. gallinacea	**Total**	**31**	**0,1**	**78**	**0,7**	**p=0.002**
		males	6		11		
		females	25		67		
Lice	*H. spiniger*	**Total**	**41**	**0,1**	**0**		
		males	24		0		
		females	17		0		
	T. canis	**Total**	**7**	**0,0**	**0**		
		males	3		0		
		females	4		0		

4.4.Risk factors for ectoparasite presence

Differences among subgroups of dogs identified by risk factors and statistical significance are reported in Table 9 and Table 10

Table 9. Overall prevalence (P) of tick, flea and lice for the subgroups of dogs (n=297) and statistical differences (p-value in bold when significant)

			Tick			**Flea**			**Lice**		
Factor	**Variable**	**Tested**	**Positive**	**P (%)**	**p-value**	**Positive**	**P (%)**	**p-value**	**Positive**	**P (%)**	**p-value**
Urbanisation	Urban	194	62	32.0	**0.020**	127	65.5	**0.029**	10	5.2	>0.05
	Rural	103	47	45.6		80	77.7		4	3.9	
Agroecology	Lowland	201	79	39.3	**<0.001**	134	66.7	>0.05	9	4.5	>0.05
	Midland	43	27	62.8		31	72.1		2	4.7	
	Highland	53	3	5.7		42	79.2		3	5.7	
Ageclass	Young	150	48	32.0	>0.05	104	69.3	>0.05	7	32.0	>0.05
	Adult	147	61	41.5		103	70.1		7	41.5	
Sex	Female	104	39	37.5	>0.05	80	76.9	**0.047**	3	37.5	>0.05
	Male	193	70	36.3		127	65.8		11	36.3	
Life style	Indoor	15	11	73.3	**0.003**	9	60.0	>0.05	1	6.7	>0.05
	Outdoor	282	98	34.8		198	70.2		13	4.6	

Table 10. Prevalence (P) of the main ectoparasites for the subgroups of dogs (n=297) and statistical differences (p-value in bold when significant)

			Ticks									**Fleas**			**Lice**		
			R. sanguineus			***A. variegatum***			***H. leachi***			***C. felis***			**Lice**		
Factor	**Variable**	**Tested**	**+ve**	**P (%)**	**p-value**	**+ve**	**P (%)**	**p-value**	**+ve**	**P (%)**	**p-value**	**+ve**	**P (%)**	**p-value**	**+ve**	**P (%)**	**p-value**
Urbanisation	Urban	194	42	21.6	**<0.001**	23	11.9	**<0.001**	14	7.2	>0.05	126	64.9	**0.024**	10	5.2	>0.05
	Rural	103	0	0		45	43.7		12	11.7		80	77.7		4	3.9	
Agroecology	Lowland	201	42	20.9	**<0.001**	41	20.4	**<0.001**	13	6.5	**<0.001**	133	66.2	>0.05	9	4.5	>0.05
	Midland	43	0	0		25	58.1		12	27.9		31	72.1		2	4.7	
	Highland	53	0	0		2	3.8		1	1.9		42	79.2		3	5.7	
Ageclass	Young	150	16	10.7	>0.05	32	21.3	>0.05	5	3.3	**0.001**	103	68.7	>0.05	7	32.0	>0.05
	Adult	147	26	17.7		36	24.5		21	14.3		103	70.1		7	41.5	
Sex	Female	104	14	13.5	>0.05	25	24.0	>0.05	10	9.6	>0.05	80	76.9	**0.038**	3	37.5	>0.05
	Male	193	28	14.5		43	22.3		16	8.3		126	65.3		11	36.3	
Life style	Indoor	15	6	40.0	**0.003**	5	33.3	>0.05	3	20.0	>0.05	9	60.0	>0.05	1	6.7	>0.05
	Outdoor	282	36	12.8		63	22.3		23	8.2		197	69.9		13	4.6	

The ticks in dogs showed an opposite trend related to urbanization; *A. variegatum* was most abundant in dogs from rural areas (43.7% vs. 11.9%), whereas *R. sanguineus* and *R. pulchellus* were present only in urban areas. As well, fleas prevalence appears higher in rural areas compared to urban areas (p=0.029), and this is mainly due to *C. felis*, the predominant species of fleas. Based on agroecology, significantly lower prevalence of ticks was identified from dogs living in highland (5.7%). Male and female dogs were also similarly attractive for all ectoparasites, with the only exception of the flea *C. felis* that seems to prefer female hosts (p=0.038). Finally, *R. Sanguineus* was more prevalent in dogs which lived indoor environment (40.0%).

4.5.Prevalence of vector-borne pathogens in dogs and cats

Overall, 383 (273 from dogs and 110 from cats) blood samples were analyzed by end-point/real time PCR. For dogs, most of the sampled animals (n = 141; 51.6%) came from Arba Minch town, followed by Arba Minch Zuria district (n = 85; 31.1%), Gerese district (n = 36; 13.2%) and Chencha town (n = 11; 4%). Dogs lived in urban areas (64.5%), more than in rural areas (35.5%). Most of the dogs were male (65.9%), whereas only 34.1% were females. According to age classes, they were similarly distributed between young (51.3%) and adult (48.7%), ranging from 2 to 240 months. Similarly, for cats most of the samples Collected from Arba Minch town (n=40; 36.4%), Arba Minch zuria district (n=32; 29.1%), Gerese district (n=32; 29.1%) and Chencha town (n=6; 5.4%).

4.5.1. Molecular Analysis

Out of 273 analyzed dog blood samples, 53.8% (147/273; 95%CI: 48–60%) showed positive results for *H. canis*, followed by 9.2% of the samples showing positive for *A. phagocytophilum*/*A. platys* (25/273; 95%CI: 6–13%), 3.3% for *B. canis rossi* (9/273; 95%CI: 2–6%), 2.6% for *E. canis* (7/273; 95%CI: 1–5%), 1.8% for *B. burgdorferi* s.l. (5/273; 95%CI: 1–4%) and 0.7% for *Rickettsia* sp. (2/273; 95%CI: 0–3%). Out of 147 animals positive for *H. canis*, 17 were also infected with at least one bacterial pathogen, while three out of the seven dogs positive for *E. canis* were coinfected with another bacterial species (i.e., one each with *A. phagocytophilum*, *A. platys* and *B. burgdorferi* s.l.), and finally one animal was coinfected with *B. burgdorferi* s.l. and *A. platys*.

All samples positive for bacterial pathogens at PCR assays were purified, sequenced and analyzed with Basic Local Alignment Search Tool (BLAST). Nineteen out of the 25 samples positive at the *A. phagocytophilum*/*A. platys* screening assay (Genbank: from ID OQ319089 to ID OQ319113) showed the highest identity percentage (99.81–100%) with analogous sequences of *A. phagocytophilum* strains, while six were more similar (99.66–100%) to *A. platys*. All nucleotide sequences obtained from the seven samples positive at PCR for *E. canis* DNA detection (Genbank: from ID OQ319077 to ID OQ319083) proved to be similar or identical (99.05–100%) to the *E. canis* groEL sequences published in the GenBank database. Three (Genbank: from ID OQ319086 to ID OQ319088) of the five samples positive for *B. burgdorferi* s.l. exhibited a nucleotide sequence with 99.63–100% similarity to those of *B. afzelii*, while the other two (Genbank: from ID OQ319084 to ID OQ319085) were identical to *B. burgdorferi* analogous sequences. Finally, out of the two samples positive for *Rickettsia* sp. during the first screening PCR, one (Genbank: from ID OQ319075) was identical to gltA sequences of *R. conorii* and the other (Genbank: from ID OQ319076) to *R. monacensis*.

Out of the 147 samples positive at real-time PCR for *Hepatozoon*, 88 were randomly selected and submitted to end-point PCR analysis, and all of them yielded positive results. Among them, 40 samples (Genbank: from ID OQ300438 to ID OQ300477) were Sanger sequenced and the BLAST analysis revealed 100% identity with sequences already present in Genbank® and identified as *H. canis*. The nine samples positive for *B. canis* at real-time PCR were also submitted to end-point PCR, but only six samples resulted positive, while three gave a negative outcome.

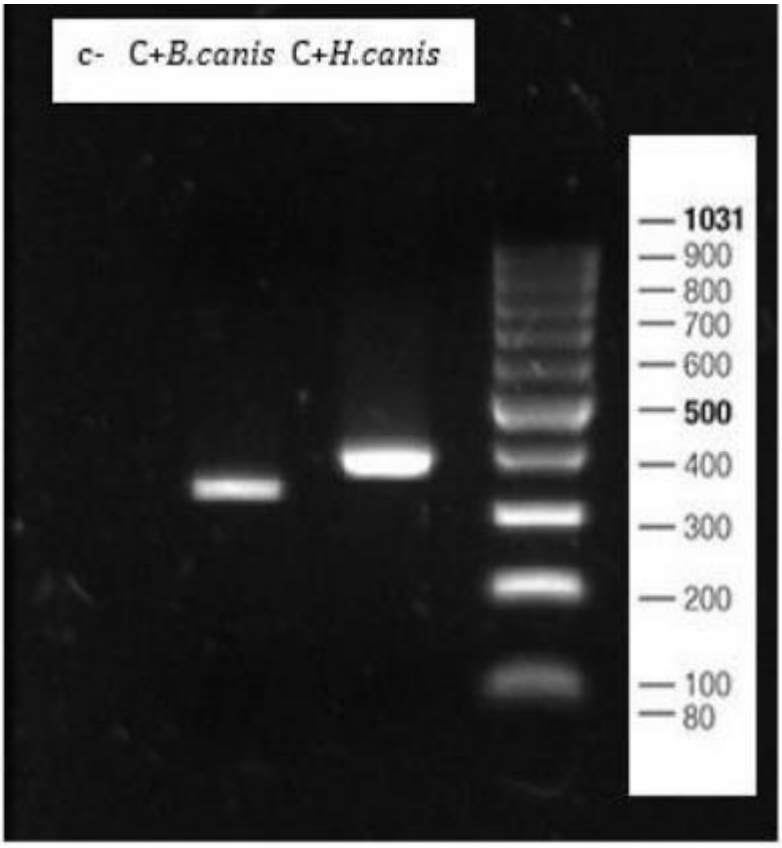


Figure 4. Electrophoretic gel depicting negative control, *Hepatozoon canis* and *Babesia canis*

The sequences of the six samples (Genbank: from ID OQ300497 to ID OQ300502) showed 99.26% identity with *B. canis rossi* isolate, reported in black-backed jackals in South Africa (Penzhorn et al., 2017). Considering that all results obtained at end-point PCR and sequencing were consistent with the identification of *H. canis* for the samples positive at real-time PCR with a melting temperature of 79/79.5 °C, the positive results obtained by the sole realtime PCR for the remaining samples (n = 59) were assumed as concordant to this identification.

In cats, *Hepatozoon* spp., is confirmed as the most spread vector-borne pathogen like in dogs. Thirty-eight (34.6%) cats were positive to *H. felis*-complex with percentage of identity from 99.27% to 100% with the deposited sequences referrable to this complex (i.e. *Hepatozoon felis, Hepatozoon ingwe, Hepatozoon lluiperdije*). *Hepatozoon canis* (n=1; 0.9%) and *Babesia leo* (n=1; 0.9%) were identified only in one cat each, showing 100% percentage of identity with deposited sequences. Moreover, eight cats were positive for bacterial pathogens and specifically 2 for *Bartonella* spp., and 6 for *Rickettsia* spp. The sequence analysis is still on-going, and will be based on the use of other target genes for *Rickettsia* spp., in consideration of the molecular complexity of this genus. All cats were negative for *Cytauxzoon* spp., *Anaplasma* spp., and *Borrelia* spp.

4.5.2. Factors Influencing Pathogens' Distribution

The differences in the proportion of positive animals to the different pathogens according to the considered risk factors are shown in Table 10. *Rickettsia* and *Borrelia* species were not displayed in this table, neither were their results analyzed through statistical analysis, in consideration of the very few positive samples. The prevalence of the bacterial pathogens was similar in all subgroups for all factors investigated, apart from one factor for *A. phagocythophilum*, which was indeed more prevalent in the lowland agroecology ($p = 0.032$). On the contrary, protozoal pathogens (*H. canis* and *B. canis rossi*) showed significant differences for more than one factor. The prevalence of *H. canis* was higher in rural areas ($p < 0.001$) and in tick-infested dogs ($p =$

0.030). Concerning agroecology, dogs living in highland were significantly less infected by *H. canis* ($p = 0.022$), while the opposite was found for *B. canis rossi*, which showed the highest prevalence in the highland dog population ($p = 0.001$). Finally, younger dogs were more affected by *B. canis rossi* ($p = 0.003$).

Table 11. Distribution of positivity in the investigated dog population according to different factors

			H. canis		*A. phagocythophilum*		*B. canis rossi*		*E. canis*		*A. platys*	
Factor	**Variable**	**Tested**	**P**	**p-Value**	**P**	**p-Value**	**P**	**p-Value**	**P**	**p-Value**	**P**	**p-Value**
Sex	Female	92	53.3	>0.1	4.3	>0.1	6.5	=0.065	3.3	>0.1	2.2	>0.1
	Male	181	54.1		8.3		1.7		2.2		2.2	
Age	Young	140	51.4	>0.1	5.7	>0.1	**6.4**	**=0.003**	2.9	>0.1	2.1	>0.1
	Adult	133	56.4		8.3		**0.0**		2.3		2.3	
Life-style	Mixed	14	50.0	>0.1	7.1	>0.1	0.0	>0.1	7.1	>0.1	0.0	>0.1
	Outdoor	259	54.1		6.9		3.5		2.3		2.3	
Agroecology	Lowland	186	**56.5**	**=0.022**	**9.7**	**=0.033**	**0.5**	**=0.001**	3.8	>0.1	2.7	>0.1
	Midland	40	**62.5**		**0.0**		**7.5**		0.0		0.0	
	Highland	47	**36.2**		**2.1**		**10.6**		0.0		2.1	
Location	Urban	176	**45.5**	**<0.001**	8.5	>0.1	1.7	=0.072	4.0	=0.053	3.4	=0.066
	Rural	97	**69.1**		4.1		6.2		0.0		0.0	
Tick infestation	Neg	172	**48.8**	**=0.030**	7.0	>0.1	3.5	>0.1	2.9	>0.1	2.3	>0.1
	Pos	101	**62.4**		6.9		3.0		2.0		2.0	
Total		273	53.8		7.0		3.3		2.6		2.2	

P: prevalence. Significant differences ($p < 0.05$) at statistical analysis are highlighted by bold characters.

The multivariable logistic regression model developed for *H. canis* (Table 11) confirmed the strong protective effect of living in an urban context and in highland.

Table 12. Results of the logistic regression model for H. canis (Hosmer–Lemeshow test: p = 0.880)

Factor	Variable	Odds Ratio	95% CI per Odds Ratio		p-Value
			Lower	Upper	
Location	rural		reference		
	urban	0.283	0.144	0.555	<0.001
Agroecology	lowland		reference		
	midland	0.478	0.195	1.171	>0.050
	highland	0.404	0.203	0.806	0.010

The multivariable logistic regression model developed for *B. canis rossi* (Table 12) identified agroecology as the most influential factor on the presence of the parasite, with midland and highland at higher risk compared to lowland.

Table 13. Results of the Multiple regression model for B. canis rossi (Hosmer–Lemeshow test: p = 0.903)

Factor	Variable	Odds Ratio	95% CI per Odds Ratio		p-Value
			Lower	Upper	
Agroecology	lowland		reference		
	midland	19.207	1.801	204.900	0.014
	highland	17.935	1.961	164.063	0.011

Concerning cats, the statistical analysis were conducted only for *Hepatozoon felis*-complex, and only one of the considered factors showed to influence significantly the prevalence of this pathogen. The prevalence values were increasingly higher (p<0.001) along with the altitude, with highland (56.8%) showing the highest value, followed by midland (38.1%) and by lowland (17.6%).

5. DISCUSSION

Canine and feline arthropods and their associated VBDs are a major threat for their natural vertebrate hosts worldwide, and some of them have also an important zoonotic potential (e.g., *Anaplasma* spp., *Rickettsia* spp., *Borrelia* spp., and *Bartonella henselae*). However, scattered information on the diffusion of these ectoparasites and pathogens is available for most of the African countries. In Ethiopia, few data is available on the prevalence of feline and canine ectoparasites and VBPs (Kumsa and Mekonnen, 2011; Kumsa et al., 2019; Heylen et al., 2021; Madder et al., 2022). The present study investigated the epidemiological role played by dogs and cats concerning the different species of ectoparasites and VBDs in four districts of southern Ethiopia. Consequently, this study may provide useful information on the arthropods and their assocated VBDs from dogs and cats.

5.1.Ectoparasite distribution

Overall, eight genera of ectoparasites were identified, including five species of ticks (*R. sanguineus, R. pulchellus, R. praetextatus, A. variegatum* and *H. leachi*), four flea species (*C. felis, C. canis, E. gallinacea* and *P. irritans*) and two lice species (*H. spiniger* and *T. canis*). In general, the studied populations showed a quite high richness in ectoparasite and VBP species, and dogs confirmed their higher susceptibility, as generally known (Morelli et al., 2021). On the contrary, investigated cats were mostly affected by fleas, whereas ticks and lice seem to infest this host only sporadically. In fact, ectoparasites and VBP were more prevalent and abundant in dogs than in cats, similarly to what already found in the other two studies conducted in both dog and cat populations living in sympatry in other parts of Ethiopia (Kumsa and Mekonnen, 2011; Kumsa et al., 2019). In particular, in the present study the prevalence for tick species was significantly higher in dogs (36.7%) compared to cats (2.7%), and similarly the prevalence for fleas was much higher in dogs (69.7%) than in cats (21.8%). Finally, low prevalence values of lice (4.7%) were recorded in dogs, whereas these ectoparasites were totally absent in cats.

Concerning ticks, the overall burden in the dog population of investigated areas was lower than what reported in other developing countries, where management conditions may be similar. Studies conducted on the prevalence of ticks in dogs in India (Abd Rani et al., 2011), Nigeria (Ekanem et al., 2010), and Pakistan (Jafri and Rabbani, 1999) revealed infestation ranging from 45% to 55%, and by far higher prevalence values were recorded in some areas of Nigeria, exceeding the 90% (Agbolade et al., 2008). On the contrary, overall tick prevalence was higher of the one found in dog populations of European countries, where they aregenerally below the 20% (Cassini et al., 2009; Smith et al., 2011).This difference is probably due to the wide use of preventive measures (i.e., collars and spot-on devices) in owned dogs.

Considering the different species of ticks, *A. variegatum* and *R. sanguineus* were the more prevalent tick species among dogs in the area, as already observed in another study in Nigeria (Elelu et al., 2022). However, the separate counts for adults and larval stages performed allowed to demonstrate that nearly all *A. variegatum* tick specimens were larvae or nymphs (142/146, 97.3%), while adults were predominant (127/216, 58.7%) in *R. sanguineus*. Besides, *A. variegatum* was the most abundant tick in dogs from rural areas (43.7%), whereas *R. sanguineus* and *Rh. pulchellus* were only present in urban areas, in agreement with other authors that have found higher prevalence of *R. sanguineus* in urban areas compared to the rural ones (Shimada et al., 2003; Neves et al., 2004). Authors findings suggest that dogs act as a preferred host for larval stages of *A. Variegatum*.This happens mostly in rural areas, where probably other animal species that usually harbor the adult stages of this tick species (e.g., domestic and wild herbivores) are more abundant and live in sympatry with dogs. On the other side, the two tick species known to be strongly associated with the dog, i.e., the brown dog tick (*R. sanguineus*) and the African dog tick (*H. leachi*), were indeed commonly found on dogs, although not with a high abundance (on average, less than 1 specimen per host). The other two species of the genus *Rhipicephalus* were sporadically found and only in dogs, suggesting a marginal epidemiological role of both dogs and cats in these species' life cycle. *Haemaphysalis leachi* was the only tick species able to infest also cats, which usually resulted very resistant to ticks infection, probably due to a mix of natural resistance, local management and specific behavior.

Fleas were found as the most prevalent and abundant group both in dogs and cats, and specifically *C. felis* was the only ectoparasite species exceeding the 50% prevalence. Other three studies conducted on dogs in Ethiopia, respectively in Jimma town (Tadesse et al., 2019), in Gondor town (Tesfaye and Chanie, 2011) and in Hawassa town (Kumsa and Mekonnen, 2011), reported that the most prevalent ectoparasite identified was *C. felis*, followed by *C. canis*. The above findings confirmed that in Ethiopia, *C.felis* is the predominant flea species found on both dogs and cats, replacing *C. canis* on domestic dogs, generally regarded as the predominant species in many countries worldwide. This might reflect the wider range of environments suitable for the survival, development and reproduction of C. *felis* as compared to all the other flea species.

In this study *C. felis* was commonly found also in cats, with lower prevalence and burden than in dogs. This finding is in agreement with most of the studies on cat ectoparasites, although values may vary greatly according to geographical areas and inclusion criteria. A study conducted in free roaming domestic cats in central USA reveled 72% cats with flea infestation. As well as, a shelter survey that evidenced flea infestation in 73% of cats (Thomas et al., 2016), reporting amuch higher prevalence than in this study. In a different one, fleas were identified in 15% of the examined cats (Beugnet et al., 2014), that is very similar to the value reported in here. Moreover, in one of the very few studies conducted in Ethiopia on cats, the prevalence for *C. felis* was higher than in this study (Kumsa et al., 2019). On the contrary *E. gallinacea* was the only ectoparasite more abundant in cats, compared to dogs. The higher abundance of *E. gallinacea* on cats than on dogs in the present study may reflect the greater association and exposure of cats to chickens or wild birds than dogs, as has been suggested earlier by others (Kumsa et al., 2019). It has been accepted that *E. gallinacea* is a species frequently found on birds, and occasionally reported on dogs and cats, due to transient infestations through contact with infested birds. *Pulex irritans* was found in dogs, but on a sporadic base, while *C. canis* was absent in cats. The higher prevalence and abundance of *P. irritans o*n dogs than on cats may reflect the closer association of dogs to humans than cats as has been suggested before (Kumsa et al., 2019).

As regards lice, this ectoparasite group was observed only in dogs with low prevalence, and specifically two species *H. spiniger* (4.0%) and *T. canis* (0.7%) were recorded. The obtained results are consistent with the known limited diffusion and importance of lice in dogs and cats. However, other studies conducted in different countries and socio-economic settings, found different results. In particular, low prevalence of lice infestation was observed in urban cats in Brazil (Mendes-de-Almeida et al., 2011) and in a nationwide survey in Italy (Genchi et al., 2021). On the contrary, Opeyemi et al. (2019) reported high prevalence of *H. spiniger* in an area of Nigeria. Taking into account thiscomparison, the authors can conclude that lice infestation has a limited importance in the area.

Concerning risk factors, fleas and some tick species showed higher prevalence in dogs from rural areas compared to the one in urban areas (p=0.029). In sub-Sharan Africa higher prevalence of ectoparasites in dogs was found in rural areas of Nigeria, Ghana, Tanzania and Kenya compared to urban areas with the exception of Uganda and Namibia which has higher prevalence in urban areas (Heylen et al., 2021). In general, rural area may be considered at major risk for interspecies transmission, considering a higher presence of domestic animals.This aspect can increment mostly the presence of ectoparasite species involving also other hosts in their life cycle, such as the case of *A. variegatum* in this study. Both *A. variegatum* and *H. leachi* were significantly more prevalent in midland, whereas *R. sanguineus* was found only in the lowland. No significant differences were found among agroecology areas for fleas and lice. Adult and young dogs were similarly infested by all types of ectoparasites, a part from *H. leachi* that was predominantly found in older animals.

Based on agroecology, significantly lower prevalence of ticks was identified from dogs which live in highland. No significant differences were found for fleas and lice, suggesting that colder climate can mainly affect ectoparasite with a prolonged environmental phase, such as ticks. Moreover, in other study dogs from lowlands showed a higher percentage of parasitism and a greater biodiversity of parasites than dogs from highlands (Bermúdez and Miranda 2011).

Finally, sex and age classes seemed to have low influence in ectoparasite burden. In this study adult and young dogs were similarly infested by all types of ectoparasites, a part from *H. leachi* which is mostly found in older animals. The literature reports contrasting indication on this aspect, with similar finding observed by Agu et al. (2020) in Nigeria. At the same time, a study conducted in Iran revealed that the prevalence of ectoparasites was higher in adult dogs compared to the young ones (Mosallanejad et al., 2012), while higher incidence among dogs younger than1 year old was reported India (Raut et al., 2006). In the study here in, male and female dogs were equally infested, with the exception of *C. felis* that prefers female dogs (p=0.038). This result is agreed with Agu et al. (2020) which reported the same result in Nigeria. In contrast, in other studies the prevalence was higher in male dogs (Mosallanejad et al., 2012; Jamshidi et al., 2012), or in female (James-Rugu and Jidayi, 2004; Dantas-Torres et al., 2009; Arong et al., 2011; Ul-Hasan et al., 2012).

5.2.Vector borne pathogens in dog and cat

In this study, 273 and 110 dog and cat blood samples were studied using molecular methods with the aim of investigating the presence and distribution of bacterial and protozoal tick and flea-borne pathogens with zoonotic potential and determining the associated risk factors.

5.2.1. *Anaplasma phagocytophilum*

A relevant finding of the study was the relatively high number of dogs (n = 19) that tested positive for *A. phagocytophilum*, a zoonotic emerging pathogen that causes Human granulocytic anaplasmosis (HGA). This finding was unexpected and at the same time alarming. An increase in human cases of this disease has been reported worldwide in recent years (Bakken and Dumler, 2015; Matei et al., 2019; CDC 2002). *Anaplasma phagocytophilum* is a multi-host pathogen characterized by a huge genetic variability influencing the host affinity of the bacterium. The isolates from dogs and humans are characterized by a strong similarity (Huhn et al., 2014; Jahfari

et al., 2014), and consequently dogs have been proposed as a sentinel for human infection risk (Hornok et al., 2013). Moreover, because of their proximity to humans, dogs may represent an amplifier and a source of infected ticks in a highly inhabited environment (Hornok et al., 2013). However, the enzootic cycle of this infectious agent appears complex and it is not completely clarified (El HamianiKhatat et al., 2021).

In Africa few studies have been conducted to detect the DNA of *A. phagocythophilum* in dog blood. The prevalence estimation (6.6%) of the present study was higher than what was recorded in previous surveys, conducted in many African countries including Algeria (Azzag et al., 2015), Morocco (El HamianiKhatat et al., 2021), Ghana (Clarke et al., 2014), Angola (Sili et al., 2021), South Africa (Kolo et al., 2016) and other Sub-Saharan African countries (Heylen et al., 2021), that reported positive findings ranging from 0% to 2.1%. However, the biomolecular methods and primers used differed among these studies. Other zoonotic strains of Anaplasma, genetically similar to *A. phagocytophilum*, were also detected in dogs in South Africa (Inokuma et al., 2005; Kolo et al., 2020) and in Zambia (Vlahakis et al., 2018). Our observation is instead similar to those found in the dog populations of many European countries, such as Germany (5, 7%), Romania (5, 7% and 6.2%), Italy (6.0%) and Slovakia (8.0%). Similar values were reported in other countries worldwide, such as Turkey (6.0%), the United States (7.6%) and Brazil (7.1%). However, higher prevalence values were reported in both European and Asian countries: Hungary (11%), Poland (14%), China (13.2%, 10.9%, and 11.9%), Jordan (39.5%) and Iran (57.3%) (El HamianiKhatat et al., 2021). In dogs, *A. phagocytophilum* is known as the causative agent for Canine granulocytic anaplasmosis (CGA) which can evolve asymptomatically or with few symptoms followed by a rapid positive evolution, or sometimes with an acute symptomatology characterized by fever, joint pain, gastrointestinal disorder and lethargy (Carrade et al., 2009). The dogs under study were asymptomatic and appeared clinically healthy at the moment of blood collection (even if mild or previous symptoms may have gone unnoticed), and therefore the detected prevalence can be considered remarkable. Although *A. phaocytophilum* is primarily transmitted by ticks of the genus Ixodes in the USA (*I. scapulari, I. pacificus*), Europe (*I. ricinus*) and Asia (*I. persulacatus*), other tick species were also reported to play a role in its epidemiological cycle. In Africa some studies showed *A. phagocytophilum* in

Rhipicephalus sanguineus, *Haemophysalis elliptica* and *Hyalomma* spp. (Van Wyk et al., 2022; Mtshali et al., 2017; Hegab et al., 2022; Mghirbi et al., 2012). In the dog population under this study, ticks belonging to the genus *Rhiphicephalus* and *Haemophysalis* were found, with the species *R. sanguineus* and *H. leachi* (unpublished data) highly represented. It can hypothesize the involvement of these tick species in transmission through their bite, but further studies should be performed to better understand the role of these ectoparasites. The observation of 2.2% *A. platys* in dogs in the present study is also relevant from a public health perspective. This pathogen has occasionally been reported as a zoonotic agent (Arraga-Alvarado et al., 2014; Breitschwerdt et al., 2014; Maggi et al., 2013). In the study the prevalence of different kinds of VBDs in cats was lower than dogs, which is similar to the infestation patern of ectoparasites. In the study the DNA of *Anaplasma* spp., was not amplified from cats. In European cats, antibody prevalence for *A. phagocytophilum* was ranging from 0% to 33.3%, and in USA the prevalence was ranging from 4.3% to 37.6% (Schäfer and Kohn, 2020). In Angola the prevalence of *Anaplasma bovis* in cats was 1.0% which mainly affects cattle (Oliveira et al., 2018). Direct pathogen detection via PCR or within a blood smear was positive in 0–23.1% of cats in Europe, and in 0–6.9% of cats in the USA. The absence of *Anaplasma* spp., in our study in cats could be explained by varying climate, environment, tick populations and the difference of reservoir host populations from Europe and USA. Although, the study have been performed in an area not endemic to *Ixodes* species of ticks. In these study most of the cats living only indoors which leads to less likely to have vector contact. Moreover, the intensive grooming behavior of cats might lead to the removal of ticks before the transmission of pathogens. Furthermore, cats may show lower numbers of *A. phagocytophilum* in circulating neutrophilic granulocytes in comparison with dogs, potentially leading to false negative PCR results (Lappin et al., 2004; Schäfer and Kohn, 2020). In this study most of the cats studied were live in indoor environment and has no tick infestation.

5.2.2. *Anaplasma platys* and *Ehrlichia canis*

Unlike with *A. phagocytophilum*, dogs are considered the natural host for *A. platys*, the causative agent for canine cyclic thrombocytopenia (Nicholson et al., 2010). *Rhipicephalus sanguineus* is also implicated as the main competent vector of this bacterium, but DNA of *A. platys* has been detected in other tick species. *Anaplasma platys* is distributed worldwide and has been detected in several dog populations in African countries, such as Algeria (Azzag et al., 2015), Morocco (El HamianiKhatat et al., 2021), Egypt (Hegab et al., 2022; Selim et al., 2021), Cape Verde (Lauzi et al., 2016), Ivory Coast (Matei et al., 2016), Kenya (Matei et al., 2016), Uganda (Proboste et al., 2015), Angola (Cardoso et al., 2016), Zambia (Vlahakis et al., 2018) and South Africa (Kolo et al., 2020), with the percentage of positivity changing in relation to the geographical area and the type of animal population considered (Heylen et al., 2021). The results of the present study demonstrated the circulation of this pathogen in dogs in Ethiopia. It was found to have a higher prevalence in urban dogs than in rural animals, although not in a significant way. As suggested by Heylen et al. (2021), this could be due to the behavior of *R. sanguineus* ticks, which prefer man-made constructions to hide in cracks and crevices to oviposit or molt and consequently infest dogs near these structures.

The same consideration could be made for *Ehrlichia canis*, transmitted mainly by *R. sanguineus* and with the dog as the primary host. It causes canine monocytic ehrlichiosis (Sainz et al., 2015) in dogs, which is characterized by clinical, subclinical and chronic phases. Since the sampled animals were asymptomatic, the positive dogs were probably in the chronic phase of infection. Although the prevalence was lower than those obtained in other African studies (Kolo et al., 2016; Bessas et al., 2016; Kolo et al., 2020; Heylen et al., 2021; Hegab et al., 2022), the detection of this pathogen deserves attention not only because of the disease's consequences for dogs' health, but also because of the occasional reports of *E. canis* acting as a zoonotic agent (Perez et al., 1996; Dumler and Walker, 2020).

5.2.3. *Borrelia burgdorferi* s.l.

Blood samples also tested for other important zoonotic bacteria belonging to *Borrelia burgdorferi* s.l. complex (of the Lyme group *borreliae*). *Borrelia burgdorferi* is the causative agent of Lyme disease, which is the most prevalent tick-borne zoonoses in the United States and Europe (Marques et al., 2021), but which is poorly studied in Africa although infection has been observed in humans (Diouf et al., 2021; Hammouda et al., 1995). Dogs are occasionally infected with this pathogen, which can cause symptoms including fever, general malaise, polyarthritis, lameness and lymph node enlargement (Chomel, 2015). The dog, sharing the same environment as humans, is considered a good indicator of the risk for people to become infected. The circulation of *B. burgdorferi* has been ascertained in dogs in Egypt both directly by molecular methods and indirectly by serological testing, and has been found in *R. sanguineus* collected from an infected dog (Elhelw et al., 2021; Selim et al., 2021). On the other hand, this pathogen was not found in the ticks of dogs in South Africa (Mtshali et al., 2017). The genospecies *B. afzelii* and *B. burdorferi* detected in this study, along with *B. garinii*, are the main causative agents of Lyme *borreliosis* in Europe and the US and are linked to different symptoms in humans (Marques et al., 2021). To the best of the authors' knowledge, this is the first report of *B. afzelii* circulation in Africa. Unlike *B. burgdorferi*, which causes primarily arthritis, this genospecies is associated mainly with Acrodermatitis Chronica Atrophicans (ACA) in humans. Our data thus provide interesting information on the circulation of this zoonotic pathogen. However, further studies should be conducted to obtain an idea of the real spread of *borreliae* in different African environments so as to determine the species of hosts and vectors that play a role in its epidemiological cycle.

5.2.4. *Rickettsia* spp.

Two dogs tested positive for *R. conorii* and *R. monacensis*, bacterial species belonging to the SFG *Rickettsia*. *Rickettsia conorii*, which is the causative agent of the human Mediterranean Spotted Fever (MSF), distributed mostly in the area surrounding the Mediterranean Sea and in

Sub-Saharan Africa. MSF is the most important tick-borne disease occurring in North Africa (Parola et al., 2013). This pathogen is transmitted primarily by *R. sanguineus*, which is considered the reservoir. Infected dogs are usually asymptomatic, but febrile illness has been observed (Solano-Gallego et al., 2015). DNA of *Rickettsia* spp., was reported in *H. elliptica* and *R. sanguineus* ticks from dogs in South Africa (Van Wyk et al., 2022), in Ghana (Nimo-Paintsil et al., 2022) and in Algeria (Bessas et al., 2016), and in the blood of dogs in Nigeria (Kamani et al., 2013) and in Angola (Barradas et al., 2017). *Rickettsia monacensis* causes MSF-like illness and is an emerging human pathogen. It was detected in ticks from North Africa (Morocco, Tunisia, Algeria), but it was rarely reported from dogs. It was detected by molecular methods in one animal in Cape Verde (Lauzi et al., 2016) and in different species of ticks (i.e., *R. sanguineus*, *Ixodes boliviensis*, *I. ricinus*) collected from dogs living in different parts of the world, such as the United States (Lineberry et al., 2022), Costa Rica (Campos-Calderón et al., 2016) and Romania (Ionita et al., 2016). Our data confirmed the circulation of SFG rickettsiae in Ethiopian dogs, and, since the dog is usually considered a sentinel of rickettsial infections in humans, other studies would be recommended to understand the real spread of these pathogens, their epidemiology and the risk for people to contract infection.

In cats the *Rickettsiaceae* family includes the spotted fever group (SFG) and the typhus group agents (Allison and Little, 2013). *Rickettsiaceae* family was transmitted by tick and flea species in cats (Parola et al., 2005; Lappin, 2018). In Central Oromia, Ethiopia *Rickettsia felis* DNA was detected in 21% of fleas, primarily *C. felis* (Kumsa et al., 2014a). Similarly, in Zambia, *R. felis* DNA was detected from 3.7% of cat fleas infesting dogs, co-infected with *Rickettsia asembonensis* with higher prevalence than the Ethiopian study. Furthermore, 37.7% of cat flea samples tested positive for *R. asembonensis*, a member of spotted fever group *rickettsiae* of unknown pathogenicity (Moonga et al., 2019). On another study Candidatus *Rickettsia senegalensis* was identified from cat fleas in Senegal (Mediannikov et al., 2014). By using indirect immunofluorescence assays, from cats in Zimbabwe and South Africa *Rickettsia conorii* (Zimbabwe; 34% and South Africa; 19%) and *Rickettsia typhi* (Zimbabwe; 7% and South Africa; 10%) was detected. These results indicate that cats may become infected with members of the spotted fever and typhus groups of rickettsiae and those cats can, therefore, be used as indicators

of the presence of these organisms (Matthewman et al., 1997). In other parts of the world SFG *Rickettsiae* were detected in the blood of 17.01% cats and 1.28% fleas in Bangkok, Thailand (Phoosangwalthong et al., 2018), 0.4% in the blood of cats in Southern Germany (Bergmann et al., 2015), from endemic areas of brazil 65.7% shelter cats and 31.2% free-roaming cats were sero-positive for one *Rickettsia* species. Although, 18.8% of those cats are infested with different tick species (Mendes et al., 2019). In the United States, fleas collected from free-roaming domestic cats 16.5% were positive for the genus-specific 17-kDa protein antigen gene of *Rickettsia* (Brown et al., 2022). In another study from USA *R. felis* DNA was detected in the blood of domestic cats at 0.53% (Hoque et al., 2020). In central Italy the serological prevalence of *R. felis* was 17.89% and, 14.73% for *R. conorii*, (Ebani et al., 2021).

5.2.5. *Hepatozoon* spp.

In the surveyed dog population, a large percentage of samples (53.8%) tested positive for *H. canis*, indicating that this pathogen is commonly found in the Ethiopian territory. The sequences obtained from positive samples of the study area were found to be very similar to specimens isolated from the black-backed jackal (*Canis mesomelas*) in South Africa (Viljoen et al., 2021), from the grey wolf (*Canis lupus*) in Germany (Hodžić et al., 2020) and from the dog in India (Lakshmanan et al., 2018). The presence of this piroplasm in several African countries was already documented. For instance, in a study conducted in Sudan (Oyamada et al., 2005), 42.3% of dogs were positive for *H. canis* and in a subsequent study in Nigeria 20.3% of samples were positive for the same parasite (Sasaki et al., 2008). These studies confirmed the tendency of this parasite to circulate in African dog populations with medium or high prevalence values, as in our study area. Moreover, areas at lower altitudes (i.e., lowlands and midlands) and located in a rural context showed a significantly higher prevalence of *H. canis* (71.8%). This could be explained by the fact that these areas constitute a more suitable environment for the survival and development of different stages of tick species acting as vectors of *H. canis*.

Hepatozoon spp., is a protozoan parasite of peripheral blood neutrophils in cats. Similarly to what recorded for dogs, *Hepatozoon* spp., was confirmed as the most spread vector-borne pathogen also in cats in the study area. In the present study, 38 (34.55%) cats resulted positive to the so-colled *H. felis*-complex, with very high percentage of identity with deposited sequences referrable to *Hepatozoon felis*, *Hepatozoon ingwe* and *Hepatozoon lluiperdije*. Before 21st century, *Hepatozoon* species were usually classified based on host identity, clinical lesions/symptoms and morphological characteristics. The morphological characteristics to differentiate *Hepatozoon* species refer to gamont and nucleus dimensions, position of nucleus within the gamont, number an arrangement of vacuoles (Van As et al., 2013; Borges-Nojosaet al., 2017). Unfortunately, the reading of stained blood smear was not possible in the present study since the samples were collected through FTA card. Thanks to advanced molecular techniques, it is nowadays possible establish the phylogenetic relationships that occur between species and/or halotypes of the same species (Davies and Johnston, 2000; Perkins and Keller, 2001; Netherlands et al., 2018; Harris et al., 2019).The difficulty of finding unique sequence identities (e.g., a sample obtained a 99% sequence identity with both *H. felis* and *H. ingwe*) among deposited sequences in GenBank is one of the limitations. In fact, the pairwise difference is very low in the sequences and it is difficult to define them at the species level. As suggested by Van As et al. (2020) both morphology and the repercussion on host cells are meaningful features to take into account to identify *Hepatozoon* species. Considering the low pairwise difference and the impossibility of using morphological parameters, suggested to enclose all positive samples of the study under the name of *H. felis*-complex.

Domestic cat *hepatozoonsis* has been reported from several countries worldwide including: India, South Africa, Nigeria, USA, Brazil, Israel, Spain, France and Portugal (Baneth, 2011; Vilhena et al., 2013). Most studies have focused on reporting the detection of feline *hepatozoonosis* and there is little knowledge on its pathogenesis, transmission, and epidemiology. *Hepatozoon felis* appears to be the predominant species of *Hepatozoon* which infects cats worldwide; however, there is evidence that *H. canis* also infects domestic cats (Baneth et al., 2013). Surveys of *Hepatozoon* infection in domestic cats describe variable rates of infection in different areas. Studies using PCR detection from Spain have shown diverse

prevalence rates with 16 % in a cat colony from Barcelona (Ortuño et al., 2008), and 4 % in cats from the Barcelona area (Tabar et al., 2008). A comparative study carried out in Bangkok, Thailand, where both canine and feline *hepatozoonosis* are prevalent, has reported a high infection rate of 32 % in 300 cats by PCR. A positive association was found between the rates of infected dogs and cats in the same districts and 18S rDNA sequences from cats and dogs were closest to *H. canis* (Jittapalapong et al., 2006). A study from Brazil evaluated 200 blood samples from Sao Luis and found only one cat infected with a *Hepatozoon* spp., which clustered with *H. felis* on a phylogenetic analysis (de Bortoli et al., 2011). *Hepatozoon* spp., DNA was amplified by PCR from the blood of 55 of 152 (36 %) surveyed cats in Israel (Baneth et al., 2013). DNA sequencing determined that all of the positive cats were infected with *H. felis* and a significant association was found between infection and outdoor access. The prevalence in Italy was 5.1% and 16.5% (Giannelli et al., 2017; Grillini et al., 2021). The arthropod vectors of *H. felis* have not been described and its specific routes of transmission have not been fully elucidated yet. Its wide geographic distribution is likely to be due to transmission by some ubiquitous vector such as a common flea, mite or tick species, or to alternative routes of transmission such carnivorism of a yet unknown wildlife intermediate host. Evidence for the transplacental transmission of *H. felis* from infected queen to the fetus has been described and it could be an important route of parasite transmission (Baneth et al., 2013). *Hepatozoon* DNA sequences from domestic cats are closely related to those reported from wildlife felids and other carnivores from India, Korea, Japan, Tanzania, Brazil and Argentina, and thus *H. felis* may also be responsible for wild life carnivore infection (Baneth et al., 2013). Genetic diversity within partial 18S rRNA sequences from *Hepatozoon* protozoan parasites from domestic cats in South Africa was assessed and compared against published data to assess global biogeographic patterns (Harris et al., 2019). *Hepatozoon felis* genotype I was identified from 15% of cats from Maio Island, Cape Verde (Pereira et al., 2019). *Hepatozoon luiperdjie* infecting neutrophils and *Hepatozoon ingwe* infecting lymphocytes were detected in South African captive and wild leopards (*Panthera pardus*) at the prevalence rate of *Hepatozoon luiperdjie* in peripheral blood of (8/16; 50%) individual *P. pardus* sampled co-infected with *Hepatozoon ingwe* in (6/16; 38%), and was the sole species of *Hepatozoon* detected in (2/16; 13%) of *P. pardus*. Prevalence of 63% (5/8) in males and 38% (3/8) in females infected (van As et al 2020).

5.2.6. *Babesia* spp.

Concerning the *Babesia* spp., detected, in our study a small percentage (3.3%) of animals tested positive, and these samples were identified as *B. canis rossi*. This subspecies is particularly virulent for dogs and commonly reported in Sub-Saharan Africa (Penzhorn, 2020), where the black-backed jackal was identified as a reservoir of *B. canis rossi* due to the high prevalence of the parasite in this host (Penzhorn et al., 2018). Indeed, nearly a third of jackals (29.7%) were infected by *B. canis rossi* in South Africa. Black-backed jackals are generally distributed in Northeast Africa, from Somalia and Eastern Ethiopia southward to Tanzania, and in Southwest Africa, from Southwestern Angola and Zimbabwe to the Western Cape Province in South Africa (Penzhorn et al., 2018). It is therefore reasonable to suspect that black-backed jackals are also responsible for maintaining *B. canis rossi* infection in Ethiopia, and specifically in the study area, since they are found in the nearby Nech Sar National Park (Serekebirhan and Jacob, 2011). This hypothesis is supported by its significantly higher prevalence in rural areas, where dogs share the same environment as wild canids and have a major risk of coming in contact with ticks and their pathogens. It is, however, more difficult to interpret why the prevalence of *B. canis rossi* was significantly higher with altitude, an opposite trend compared to the other pathogens. Finally, its higher prevalence in young dogs was probably related to the dog's immunity, since acquired immunity to *B. rossi* increases with age, as has been observed under experimental conditions (Morters et al., 2020). The use of real-time PCR for piroplasms was extremely useful in screening the canine blood samples and in identifying the positives through the melting curve analysis. This diagnostic approach represented an effective and cost-saving way to diagnose piroplasms using molecular technology. However, the use of the same primers for the detection of different protozoa belonging to the same group may represent a limitation of the study, since the species with higher parasitaemia may hide the presence of the other species, resulting in an underestimation of the prevalence of pathogens circulating at a lower level.

Several species of *Babesia* have been reported in domestic cats from different continents. Only some of these species have been associated with clinical disease in cats. Piroplasm species which infect domestic cats are often carried sub-clinically by wild life reservoirs, such as a wild feline species (Penzhorn and Oosthuizen, 2020). To the best of our knowledge, the positive cat reported in the present study is the first report of *Babesia leo* so far in Ethiopia. Previously in Africa, *B. leo* was recorded from South Africa and Mozambique by using 18S rRNA gene (Bosman et al., 2019). Clinical *babesiosis* in domestic cats has mostly been reported from the coastal regions of South Africa where infection is due to *Babesia felis*, a small *Babesia* that causes a disease characterized by anemia and icterus (Penzhorn et al., 2004). It also infects African wild felids including lions, cheetahs and servals (Bosman et al., 2007) and has been reported to cause clinical disease in domestic cats imported into non-endemic countries (Wells, 2012). Other reports of clinical domestic feline *babesiosis* in Africa and other parts of the globe have mostly been sporadic. Reports of the rare occurrence of feline *babesiosis* cases in Europe (France, Germany, Poland, and Spain) and Asia (Israel, India, and Pakistan) are documented (Penzhorn and Oosthuizen, 2020). Several tick spp., including *Ixodes ricinus*, *Ixodes hexagonus*, Dermacentor spp., *R.sanguineus*, and *Haemophysalis* spp., infest cats and are possible vectors of *Babesia*. Other routes of transmission reported in canine babesial infections including vertical and direct transmission have not been consistently described in cats (Solano-Gallego and Baneth, 2011). Piroplasms are probably widely present in African countries, although data are scant and information on their circulation is mostly limited to South Africa (Penzhorn et al., 2018; Penzhorn, 2020; Viljoen et al., 2021).

5.2.7. *Cytauxzoon* spp.

Cytauxzoonosis caused by *C. felis* is most frequently identified in young outdoor cats that have a history of tick attachment (Beugnet and Halos, 2015). In some regions where cytauxzoonosis is endemic, the prevalence in domestic cats may be as high as 30 % (Brown et al., 2010). In the northwest region of Italy, the prevalence of infection in feral cats was 30 % (Carli et al., 2012). However, no associations between breed, gender, age, presence of ticks, clinical status, laboratory findings such as anemia, were found. In another study in North-Eastern Italy

Cytauxzoon spp., DNA was detected in 3.8% of cats (Grillini et al., 2021). So far in Africa, non-fatal cases of *Cytauxzoon* spp., were reported in South African wild felids (Wang et al., 2017). *Cytauxzoonosis* caused by *C. felis* has been found in North America, South America, Europe, and Asia (Wang et al., 2017).

5.2.8. *Bartonella* spp.

Cat scratch disease is the most common zoonotic infection caused by *Bartonella* bacteria. Among the many mammalian species infected with *Bartonella* spp., cats represent a large source for human infection, as they are the main reservoir for *B. henselae, B. clarridgeiae* and *B. koehlerae*. The transmission of *B. henselae* by cat fleas occurs mainly through infected flea faeces (Chomel et al., 2009; Beugnet and Halos, 2015). Previously in Ethiopia *Bartonella henselae* DNA was detected in 6% (2 of 34) of *C. felis* fleas collected from cats (Kumsa et al., 2014a). In our study the prevalence was (2 of 110; 1.8%). The results showed that the blood prevalence was lower than the prevalence found in the collected fleas from cats in the previous study. Since the first isolation of *B. henselae* from a domestic cat in the early 1990s, several studies have been conducted worldwide to determine the importance of cats as reservoirs of this bacterium (Boulouis et al., 2005; Chomel et al., 2006). Prevalence of infection varies considerably among cat populations (stray or pet) with an increasing gradient from cold climates (0 % in Norway) to warm and humid climates (68 % in the Philippines) (Boulouis et al., 2005). Antibody prevalence of *Bartonella henselae* in cats from southern Africa and Zimbabwe was determined by indirect fluorescent antibody assay. Overall, 23% of cats are positive for *B.henselae*, with cats from Zimbabwe (24%) having higher seroprevalences than those from South Africa (21%) (Kelly et al., 1996). In another molecular study from South Africa *Bartonella* species DNA was amplified as *Bartonella henselae* 4.9% followed by *Bartonella clarridgeieae*: 2.9% (Lobettiand Lappin, 2012). Several genotypes have been identified, with two main genotypes designated as Houston-1 (type I) and Marseille (previously BATF) (type II), based on the 16S rRNA gene sequence (Boulouis et al., 2005; Chomel et al., 2004). The respective prevalence of these two genotypes varies considerably among cat populations from different geographical areas. *B. henselae* type Marseille (also known as type II) is the dominant

type in cat populations from the western USA, western Europe (France, Germany, Italy, Netherlands, United Kingdom) and Australia, whereas type Houston-1 is dominant in Asia (Japan and the Philippines) (Boulouis et al., 2005). A few studies in Western Europe and Australia have reported that most human cases of CSD were caused by *B. henselae* type Houston-1 despite the fact that type Marseille was found to be the dominant type in the cat population. This suggests that type Houston-1 strains could be more virulent to humans (Boulouis et al., 2005).

6. CONCLUSIONS AND RECOMMENDATIONS

In conclusion, this study investigated the presence of ectoparasites and vector-borne pathogens in owned dogs and cats living in sympatry in the urban and rural areas of Gamo zone. The dogs and cats in this study share a common environment with humans, which make them the key reservoirs for ectoparasites. This role has the potential to infest humans living in the same surroundings and to transmit zoonotic vector-borne pathogens (VBPs). The data presented in this study are important for building knowledge about the occurrence of ectoparasites infesting dogs and cats in this area of Ethiopia, and consequently to design future surveillance and prevention strategies. More importantly, some epidemiological aspects disclosed by the present investigation, such as the different distribution among dogs and cats and between different environmental settings of arthropod species, are of overall interest. According to our questionnaire survey, most of the pet owners in the area had no knowledge about arthropods and VBDs of dogs and cats, and the majority of them never visited a veterinary clinic.This general absence of knowledge on the importance of dog and cat health status in a public health perspective, in combination with the finding of medium/high burden of ectoparasites acting as vector for important zoonotic VBDs leads to an awareness campaign in the area and to the strengthening of the veterinary care also for companion animals. The more relevant finding of this study is the detection of different species of tick and flea-borne pathogens with zoonotic potential (*A. phagocytophilum*, *B. afzelii*, *B. burgdorferi* s.l, *R. conorii*, *R. monacensis* and *Bartonella* spp.) and in particular the relatively high prevalence of *A. phagocytophilum*. These results demonstrate the circulation of the above-mentioned pathogens in the area, calling for a sensitization of the health authorities and medical personnel. The role of dogs and cats in the epidemiological cycle of these pathogens needs further investigation. Furthermore, the tick species acting as vectors for *A. phagocytophilum* and *B. burgdorferi* s.l., pathogens usually vectored in temperate climates by *Ixodes* sp. (absent in this area), should be investigated. The research enabled the determination of the occurrence of tick and flea-borne pathogens relevant to dog and cat health, revealing the wide diffusion of the relatively harmless *H. canis* and the sporadic presence of other more pathogenic species, such as *B. canis rossi*, *B. leo*, *E. canis* and *A. platys*. Rural dogs seem at higher risk for most of the pathogens, probably due to their shared environment with other wild canids which can act as carriers of pathogens and spreaders of

infected ticks (e.g., black-backed jackal for *Babesia*). However, the large majority of the investigated dogs have a lifestyle at risk, since most of the urban dogs are kept outdoors and left to free roaming during the day. In conclusion, human and veterinary services should pay higher attention to these diseases, whose importance is underestimated in the area, probably due to the difficulties of performing an appropriate diagnosis in both humans and animals in such a context, which is characterized by poor diagnostic facilities.

Based on the above concluding reamrks the following recommendations are forwarded:

- ✓ Detailed study on arthropods and their associated pathogens of dogs and cats need to be further investigated in dffernet parts of Ethiopia.
- ✓ Much work needs to be carried out on public awareness creation on arthropods and the associated zoonotic VBDs from dogs and cats in Ethiopia.
- ✓ A prevention and control program needs to be implemented on arthropods and zoonotic pathogens from dogs and cats in Ethiopia.

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Appendices

Appendix 1. Dog and cat information collection sheet

S/n	Owners name	Code	Kebele	District	Agroecology	Species	Age	Sex	Life-style	Agro-ecology	Pet origion	Last time pet visit to vet. clinc	Reson for visit	Blood on FTA card	Tick	Flea	Lice

Sheet _____

Appendix 2. Owner's information collection sheet

S/n	Name	Age	Gender	Occupation	Level of education	Kebele	District	Owned spp. Of pet	Porpoise of keeping pet	Knowledge about VBDs	Person infected by VBD in the household	Knowledge about transmission

Sheet _____

Appendix 3. Sample pretreatment and DNA extraction

Samples collected by FTA cards (Whatmann® FTA™, Merck KGaA, Darmstadt, DE) from Ethiopia were analyzed in this study. From each FTA card, a diameter of approximately 1 cm dried blood spot was cut into different Eppendorf tubes and consecutively numbered with the same number reported on FTA cards. The samples were processed using NucleoSpin™ Tissue extraction kit (Macherey-Nagel, Düren, DE). The first step was a pre-lyse phase in which 180 µl of Buffer T1 was added in each tube and incubated at 95°C for 10 minutes. Then, 25 µl of Proteinase K was added to digest proteins and samples were incubated for 90 minutes at 56°C. The second step consisted in a further lysis phase in which 200 µl of Buffer B3 was added in each tube and incubating at 56°C for 10 minutes. At the end of incubation, the 1 cm piece of FTA card paper was removed from each tube. The third step concerned the adjusting of DNA binding conditions; for this aim 210 µl of ethanol (97% concentration) was added in each tube and the mixture was left to swirl for 30 minutes. This is followed by the fourth step of DNA binding, where the sample is transferred from the Eppendorf tube to the NucleoSpin™ Tissue column with a silica membrane, included in the kit, placed into a Collection tube. All samples were centrifuged for 1 minute at 13,000 x g. The waste liquid in the collection tube is discarded into a waste liquid cylinder for centralized collection. The fifth step involved the silica membrane washing in a two-step process that allowed removing proteins and contaminant. First, 500 µl of Buffer BW was added and the tube centrifuged for 1 minute at 13000 x g. Then, 600 µl of Buffer B5 was added into the tube and centrifuged again at the same condition. Next, silica membrane was dried thanks to a further centrifugation, without adding any reagent, for 1 min at 11000 x g, to remove the residual ethanol. Finally, NucleoSpin™ Tissue column was placed in a new Eppendorf tube and the DNA was eluted adding 100 µl of Buffer BE and letting it at room temperature for 1 minute before a centrifugation (1 min at 11000 x g). After this phase, purified DNA was obtained and stored in the freezer at -20°C.

Appendix 4. Real-Time PCR assay for the detection of Hepatozoon spp., and Babesia spp.

The thermocycler Roche LightCycler® 96 was used in the analysis. Before the running of Real-time quantitative polymerase chain reactions (real-time PCR), it's necessary to prepare the reaction mix including forward and reverse primers, DNase/RNase- free water and QuantiNova SYBR Green PCR Kit (QIAGEN Group, Hilden, Germany). The primers were already described by Tabar et al. (2008) based on 373 bp fragment of Piroplasmid 18S-rRNA gene (Primer pair 5'-CCAGCAGCCGCGGTAATTC-3' and 5'-CTTTCGCAGTAGTTYGTCTTTAACAAATCT-3'). The reaction mixture was transferred in the real-time PCR plate, and 17 µl of mix and 3 µl of DNA, together with positive (i.e. DNA of sequenced field sample) and negative (no DNA added) controls were put in each well. Finally, the plate was covered and centrifuged for 30 seconds to help the mixing between samples DNA and reaction mixture and the convergence of the liquid at the bottom. The plates were placed into the thermocycler and analyzed under the following amplification cycle: preincubation at 95°C for 2 min, followed by 45 cycles of amplification steps at 95 °C for 5 sec and 60 °C for 10 sec, concluding with the melting steps at 95°C for 10 sec, 65 °C for 1 min and finally it increases up to 97 °C. The rate of temperature increase was 1°C/sec, as well as the fluorescence was continuously acquired. Target pathogens (i.e.*Hepatozoon canis*, *Babesia canis*) were identified by the melting temperature. The specific melting temperatures (i.e. 79/79.5°C for *Hepatozoon canis*, 81.5° for *Babesia canis*) at real-time PCR analysis were identified based on preliminary analysis conducted on positive controls and confirmed by sequencing. The positive control for *Hepatozoon canis* was obtained through preliminary analysis of 20 samples of the present study. The samples with best performances in the shape of the melting temperature peak and cycle threshold (Ct) (26-28 Ct) were selected for conventional PCR (cPCR) and sequencing to confirm the specific identification. The positive control for *Babesia canis canis* was a field sample provided by the Istituto Zooprofilattico Sperimentale delleVenezie. Samples were tested in duplicate.

Appendix 5. Conventional PCR assay for the detection of Hepatozoon spp., and Babesia spp.

Some of the samples that resulted positive at real-time PCR analysis (i.e. 79/79.5°C for *Hepatozoon canis*, 81.5° for *Babesia canis*) were submitted to conventional PCR (cPCR) analysis targeting the 18S-rRNA gene, using the same primers as before (Tabar et al. 2008), to confirm the specificity of melting curve temperature. The reaction mix including DNase/RNase-free water, Deoxyribonucleotide triphosphate (dNTP), forward and reverse primers, reagents (i.e. Buffer, MgCl2), and Invitrogen Taq DNA polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA), together with positive (i.e. DNA of sequenced field sample) and negative (no DNA added) controls was loaded in tubes. The tubes were centrifuged for a few seconds to help the mixing between sample DNA and reaction mixture. The samples were amplified according to Tabar et al. (2008) protocol with slight modifications: initial step at 94°C for 2 minutes, followed by a denaturation step at 94°C for 30 seconds, an annealing step from 64°C to 60°C with 0.5°C decrements per cycle (touchdown), a final extension step at 72°C for 30 seconds, and a final extension cycle at 72°C for 2 minutes. At the same time, 2% agarose gel was heated in the microwave until it dissolved, then Invitrogen SYBR® Safe DNA gel stain (Thermo Fisher Scientific Inc., Waltham, MA, USA) was added to visualize the DNA in gel. When the PCR run was concluded, 9 µl of each PCR product and 1.8 µl of Blue Gel Loading Buffer (Jena Bioscience GmBH, Jena, DE) for tracking dye during electrophoresis were loaded into gel wells, together with 4 µl of 100 bp DNA ladder (MassRuler™ Low Range DNA Ladder, Thermo Fisher Scientific Inc., Waltham, MA, USA). During the electrophoresis step, gel was run in Tris/Borate/EDTA (TBE) buffer (i.e. a buffer solution containing Tris base, boric acid and Ethylenediaminetetraacetic acid) at 100V for 30 minutes. The gel was removed from electrophoresis machine and placed under UV light, and then software for the gel reading was used to take photos and read the results.

Appendix 6. Purifying and Sequencing

PCR product was purified using 1 µl of Exo and 1 µl of SAP (ExoSAP-IT™ PCR Product Cleanup, Thermo Fisher Scientific Inc., Waltham, MA, USA) for each 5 µl of amplified sample. A unique amplification cycle was run to activate the cleanup reagents as follow: 35°C for 5 minutes and 80°C for 10 minutes. Then, 5 µl of purified sample was added to 5 µl of forward primer (5µm concentration) and other 5 µl to 5 µl of reverse primer (5µm concentration). Purified products were sequenced following Sanger technology (Macrogen Spain, Madrid, ES) and the obtained nucleotide sequences were compared to those deposited in GenBank® using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast) (accessed date: 2 August 2021).

Appendix 7. Sample transportation invoice

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ESONERO DI RESPONSABILITÀ. IN NESSUN CASO FEDEX SARÀ RESPONSABILE PER EVENTUALI DANNI, SIANO ESSI DIRETTI, INDIRETTI, ACCIDENTALI, PARTICOLARI O EMERGENTI, PER UN VALORE SUPERIORE A QUELLO DICHIARATO AI FINI DELLA RESPONSABILITÀ DEL VETTORE (INCLUSA, MA SENZA LIMITAZIONE ALCUNA, LA PERDITA DI ENTRATE O GUADAGNI) OPPURE PER IL REALE VALORE

DELLA SPEDIZIONE, SE INFERIORE, INDIPENDENTEMENTE DAL FATTO CHE FEDEX FOSSE O NO A CONOSCENZA DELL'EVENTUALITÀ CHE TALI DANNI POTESSERO VERIFICARSI. FedEx non è responsabile per

atti o omissioni del mittente, inclusi ma senza limitazione alcuna: un'errata dichiarazione di carico, imballaggi, misure di sicurezza, contrassegni o indirizzo insufficienti o inadeguati; oppure per atti o omissioni del destinatario o di chiunque altro che abbia un interesse nella spedizione; oppure per violazioni dei termini del presente accordo da parte di uno dei contraenti. FedEx non è responsabile per eventuali danni, ritardi, carenze, errata o mancata consegna, errata o mancata comunicazione di informazioni in relazione a spedizioni di contanti, valuta o altri articoli proibiti, oppure in presenza di situazioni al di fuori del nostro controllo, come cause di forza maggiore, pericoli dell'aria, condizioni meteorologiche, ritardi di natura meccanica, atti di nemici pubblici, guerra, sciopero, guerra civile, oppure per atti od omissioni di autorità pubbliche (tra cui funzionari doganali e sanitari) con autorità reale o apparente. NESSUNA GARANZIA. Non diamo alcuna garanzia, né esplicita né implicita. RECLAMI PER PERDITE, DANNI O RITARDI. TUTTI I RECLAMI DEVONO ESSERE FATTI PER SCRITTO, ENTRO I TERMINI

PRESCRITTI. PER I DETTAGLI, VI INVITIAMO A CONSULTARE LE TARIFFE, LA GUIDA AI SERVIZI FEDEX IN VIGORE OPPURE LE CONDIZIONI STANDARD DI TRASPORTO. La Convenzione di Varsavia prevede

particolari procedure scritte per le richieste di risarcimento per danni, ritardi o la mancata consegna della spedizione. Inoltre, l'interpretazione e l'azione delle disposizioni di reclamo della Convenzione di Varsavia possono variare da un paese all'altro. Per conoscere i termini entro cui inoltrare la richiesta relativa alla vostra spedizione, si prega di far riferimento alla Convenzione. Il diritto alla richiesta di risarcimento di danni nei nostri confronti si

intenderà scaduto se entro due anni non è stata intentata causa, secondo quanto stabilito dalla Convenzione. FedEx non ha alcun obbligo di agire nei confronti di una richiesta fino al completo pagamento di tutte le spese di

spedizione. L'ammontare della richiesta di risarcimento non è deducibile dalle spese di trasporto. Se il destinatario accetta la spedizione senza annotare alcun danno sulla bolletta di consegna, FedEx presume che la spedizione è stata consegnata in buone condizioni. Affinché un reclamo per danni possa essere preso in considerazione, il contenuto, il cartone di spedizione originale e l'imballaggio devono essere tenuti a nostra disposizione per il controllo. LEGISLAZIONE COMPETENTE. Nella misura in cui una disposizione contenuta o a cui si fa riferimento nella presente Lettera di Vettura di trasporto via aerea è contraria a trattati internazionali in vigore, leggi, regolamenti governativi, ordini o requisiti, essa resta valida in quanto facente parte del nostro accordo, purché non sia dirimente. La nullità o la non applicabilità di qualsiasi disposizione non ha alcun effetto sulle altre parti della presente Lettera di Vettura di trasporto via aerea. Salvo altrimenti indicato, FEDERAL EXPRESS CORPORATION, 2005 Corporate Avenue, Memphis, TN 38132, USA, è il primo vettore di questa spedizione. Indirizzo e-mail

indicato in www.fedex.com.

Dopo aver stampato la Lettera di Vettura:

COPIA LETTERA DI VETTURA FEDEX – INSERIRE DIETRO ALLA COPIA DESTINATARIO

Piegate la pagina stampata lungo la linea orizzontale.

Inserite la Lettera di Vettura nell'apposita busta autoadesiva e incollatela sulla spedizione.

ORIGIN ID:ADDA 911715485
Prof. Bersissa Kumsa
Addis Ababa University
P.O.Box 34, Bishoftu (Debre Zeit),
Department of Parasitology and Path
Bishoftu,
ETHIOPIA, ET

SHIP DATE: 13MAY21
TOTWGT: 2.25 KG
CAD: 100016244/INET4340

DIMS: 33x22x18 CM
BILL SENDER
EIN/VAT:

TO **Prof. A. Frangipane - Rudi Cassini**
Universita di Padova (MAPS)
Viale dell'Universita, 16
Department of Animal Medicine
LEGNARO, 35020
ITALY, IT

0498272969

FedEx Express

(IT)

J21132103120uv

AWB

FB QPAT8

PKG:CUSTOMER

TRK# 7737 1159 9890 Form 0430

1 of 2

PM
INTL PRIORITY

REF:
DESC1:SAMPLE FOR UNIVERSITY RESEARCH
DESC2:
DESC3:
DESC4:

COUNTRY MFG: EE
CARRIAGE VALUE: 0.00 EUR
CUSTOMS VALUE: 5.00 EUR

SIGN: Prof. Bersissa Kumsa
T/C: S 317407068
D/T: R

FEDEX AWB COPY - PLEASE PLACE IN POUCH

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COPIA DESTINATARIO – POSIZIONARE DAVANTI NELLA BUSTA AUTOADESIVA

Piegate la pagina stampata lungo la linea orizzontale.

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Avvertenza: Per la spedizione, utilizzate esclusivamente la Lettera di Vettura stampata originale. Utilizzare per la spedizione una fotocopia dellaLettera di Vettura è illegale e potrebbe dar luogo a oneri supplementari di fatturazione, insieme alla cancellazione del vostro Codice Cliente FedEx.

TERMINI LEGALI E CONDIZIONI DELLE DEFINIZIONI DI SPEDIZIONE FEDEX. In questa Lettera di Vettura, "noi", "nostro", e "FedEx" si riferiscono a Federal Express Corporation, le sue filiali e consociate e i rispettivi dipendenti, agenti e appaltatori indipendenti. Con “voi” e “vostro” si fa riferimento al mittente, suoi dipendenti, committenti e agenti. Qualora la spedizione abbia origine al di fuori degli Stati Uniti, il contratto di vettura si intende stipulato con la consociata, la filiale o l'appaltatore indipendente FedEx che per primo ha accettato la vostra spedizione. Il termine "collo" indica qualsiasi contenitore o busta accettati da noi per la consegna, inclusi quelli da voi presentati a FedEx utilizzando i nostri sistemi automatici, misurazioni, manifesti o Lettere di Vettura. Il termine "spedizione" indica tutti i colli che sono presentati a FedEx e accettati da FedEx su un'unica Lettera di Vettura.

NOTA SUL TRASPORTO AEREO. A qualsiasi spedizione internazionale per via aerea si applicano la Convenzione di Varsavia e i relativi emendamenti. Dunque la Convenzione di Varsavia, con i relativi emendamenti, disciplina e in molti casi limita la responsabilità di FedEx per perdite, ritardi o danni subiti dalla vostra spedizione. La Convenzione di Varsavia, come emendata, limita la responsabilità di FedEx. Per esempio negli Stati Uniti la responsabilità è limitata a 9,07 dollari statunitensi per libbra (20 dollari per chilo), a meno che non sia stato

dichiarato un valore superiore ai fini della responsabilità del vettore, come spiegato qui di seguito, e non abbiate pagato tutte le spese supplementari applicabili. L'interpretazione e l'applicazione dei limiti di responsabilità della Convenzione di Varsavia possono variare a seconda dei paesi. Non vi sono specifiche località d'arresto ufficiali, e FedEx si riserva il diritto di far transitare le spedizioni in qualsiasi modo gli sembri più appropriato. NOTA AL TRASPORTO SU STRADA. Le spedizioni effettuate unicamente su strada da o verso un paese che ha firmato la

Convenzione di Varsavia o il Contratto per il trasporto internazionale di merci su strada (il "CMR") sono soggette ai termini e alle condizioni del CMR, a prescindere da qualsiasi altra disposizione contraria della presente Lettera di Vettura. Per quanto riguarda le spedizioni effettuate unicamente su strada, in caso di disaccordo tra le disposizioni della CMR e la presente Lettera di Vettura, prevalgono i termini della CMR. LIMITAZIONE DI

RESPONSABILITÀ. Se non diversamente previsto dalla Convenzione di Varsavia, il CMR o altri trattati internazionali, leggi, regolamenti governativi, ordini o requisiti, la massima responsabilità di FedEx per danno, perdita, ritardo, carenze, errata o mancata consegna, errata o mancata comunicazione di informazioni in rapporto alla vostra spedizione è limitata dal presente Accordo, secondo i termini e condizioni stipulati nel contratto di trasporto. Invitiamo a consultare il contratto di trasporto stabilito nella Guida ai servizi FedEx in vigore, o il suo equivalente, per determinare le limitazioni contrattuali. FedEx declina ogni responsabilità sul carico o assicurazione a copertura globale, ma è possibile pagare un supplemento per ogni 100 USD supplementari di valore dichiarato ai fini della responsabilità del vettore (o l'equivalente nella valuta locale del paese d'origine). Qualora sia stato dichiarato un valore superiore ai fini della responsabilità del vettore e sia stato pagato il supplemento, la massima responsabilità di FedEx sarà pari all'importo che risulta inferiore tra il valore dichiarato e i reali danni subiti.

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indicato in www.fedex.com.

This invoice must be completed in English
(Questa fattura deve essere compilata in inglese)

PRO FORMA INVOICE
(Fattura proforma)

EXPORTER (ESPORTATORE):
Tax ID# (Codice fiscale):
Contact Name (Nome del Contatto): Prof. Bersissa Kumsa
Telephone No. (N. telefono): 911715485
E-Mail (e-mail): mbe522@mbe.it
Company Name/Address (Nome/indirizzo società):
Addis Ababa University
P.O.Box 34, Bishoftu (Debre Zeit), Department
of Parasitology and Path

Bishoftu
Country/Territory (Paese/territorio): Ethiopia
Parties to Transaction(Parti della transazione):
☐ **Related** (in relazione) ☐ **Non-Related** (Non in relazione)
Payment Terms (Termini di pagamento):
Purpose of Shipment (Scopo della spedizione): Sample

Ship Date (Data spedizione): 13 May, 2021 13 Mag, 2021
Air Waybill No. / Tracking No. / Bill of Lading (N. Lettera di Vettura aerea / N. monitoraggio / Polizza di carico): 773711599890
Invoice No. (N. fattura): **Purchase Order No.** (N. riferimento interno):

CONSIGNEE (DESTINATARIO):
Tax ID# (Codice fiscale):
Contact Name (Nome del Contatto): Prof. A. Frangipane - Rudi Cassini
Telephone No. (N. telefono): 0498272969
E-Mail (e-mail):
Company Name/Address (Nome/indirizzo società):
Universita di Padova (MAPS)
Viale dell'Universita, 16
Department of Animal Medicine

LEGNARO 35020
Country/Territory (Paese/territorio): Italy

SOLD TO (if different from Consignee) (VENDUTO A (se diverso dal Destinatario)):
☑ **Same as CONSIGNEE** (Stesso del Destinatario):

Tax ID# (Codice fiscale):
Company Name/Address (Nome/indirizzo società):

Country/Territory (Paese/territorio):

If there is a designated broker for this shipment, please provide contact information (Se è stato designato un broker per questa spedizione, fornitene i dati di contatto)

Name of Broker (Nome del Broker) **Tel No.** (Tel.) **Contact Name** (Nome del Contatto)

Duties and Taxes Payable by(Oneri doganali per il brokeraggio pagabili da) ☐ **Exporter**(Esportatore) ☑ **Consignee**(Destinatario) ☐ **Other**(Altro) **If Other, please specify**(Se Altro, specificare)

No. of Packages (Numero dicolli)	No. of Units(Numero di unità)	Unit of Measure (Unità di misura)	Description of Goods (Descrizione delle merci)	Harmonized Tariff Number (Numero Harmonized Tariff)	Country/Territory of Origin (Paese/territorio di origine)	Unit Value (Valore unitario)	Total Value (Totale valore)
	2.00	PCS	Sample - SAMPLE FOR UNIVERSITY RESEA RCH		EE	2.500000	5.00

Total No. of Packages (Totale Numero di colli): 2
Total Weight (Indicate LBS or KGS) (Totale Peso (indicate libbre o kg)): 2.25kg

Special Instructions (Istruzioni speciali):

Declaration Statement(s) (Dichiarazione(i)):

I declare that all the information contained in this invoice to be true and correct (Dichiaro che tutte le informazioni contenute in questa fattura sono vere ed esatte)

Originator or Name of Company Representative if the invoice is being completed on behalf of a company or individual (Mittente o Nome del rappresentante dell'azienda se la fattura deve essere compilata in nome di un'azienda o di un singolo):

Signature / Title / Date (Firma / Titolo / Data)

Terms of Sale (Terms of Sale):	EXW
Subtotal (Totale parziale):	5.00
Insurance (Assicurazione):	0.00
Freight (Nolo):	0.00
Packing (Imballaggio):	0.00
Handling (Trasporto speciale):	0.00
Other (Altro):	0.00
Invoice Total (Fattura totale):	5.00
Currency Code (Codice valuta):	EUR

13 May, 2021 13 Mag, 2021

This invoice must be completed in English (Questa fattura deve essere compilata in inglese)

PRO FORMA INVOICE

EXPORTER (ESPORTATORE): **Tax ID#** (Codice fiscale): **Contact Name** (Nome del Contatto): Prof. Bersissa Kumsa **Telephone No.** (N. telefono): 911715485 **E-Mail** (e-mail): mbe522@mbe.it **Company Name/Address** (Nome/indirizzo società): Addis Ababa University P.O.Box 34, Bishoftu (Debre Zeit), Department of Parasitology and Path Bishoftu **Country/Territory** (Paese/territorio): Ethiopia **Parties to Transaction**(Parti della transazione): ☐ **Related** (in relazione) ☐ **Non-Related** (Non in relazione) **Payment Terms** (Termini di pagamento): **Purpose of Shipment** (Scopo della spedizione): Sample	**Ship Date** (Data spedizione): 13 May, 2021 13 Mag, 2021 **Air Waybill No. / Tracking No. / Bill of Lading** (N. Lettera di Vettura aerea / N. monitoraggio / Polizza di carico): 773711599890 **Invoice No.** (N. fattura): **Purchase Order No.** (N. riferimento interno):
CONSIGNEE (DESTINATARIO): **Tax ID#** (Codice fiscale): **Contact Name** (Nome del Contatto): Prof. A. Frangipane - Rudi Cassini **Telephone No.** (N. telefono): 0498272969 **E-Mail** (e-mail): **Company Name/Address** (Nome/indirizzo società): Universita di Padova (MAPS) Viale dell'Universita, 16 Department of Animal Medicine LEGNARO 35020 **Country/Territory** (Paese/territorio): Italy	**SOLD TO (if different from Consignee)** (VENDUTO A (se diverso dal Destinatario)): ☑ **Same as CONSIGNEE** (Stesso del Destinatario): **Tax ID#** (Codice fiscale): **Company Name/Address** (Nome/indirizzo società): **Country/Territory** (Paese/territorio):

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Total No. of Packages (Totale Numero di colli): 2	**Total Weight (Indicate LBS or KGS)** (Totale Peso (indicate libbre o kg)): 2.25kg	**Terms of Sale (Terms of Sale):**	EXW
Special Instructions (Istruzioni speciali):		**Subtotal** (Totale parziale):	5.00
		Insurance (Assicurazione):	0.00
		Freight (Nolo):	0.00
		Packing (Imballaggio):	0.00
Declaration Statement(s) (Dichiarazione(i)):		**Handling** (Trasporto speciale):	0.00
		Other (Altro):	0.00
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Originator or Name of Company Representative if the invoice is being completed on behalf of a company or individual (Mittente o Nome del rappresentante dell'azienda se la fattura deve essere compilata in nome di un'azienda o di un singolo):		**Currency Code** (Codice valuta):	EUR
Signature / Title / Date (Firma / Titolo / Data)		13 May, 2021 13 Mag, 2021	

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