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Second Cycle Degree (MSc) in
Biotechnologies for Food Science

Identification of vector-borne diseases in domestic dogs using biomolecular techniques and implications for human health

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Abstract

The number of domestic dog owners has been increased in recent years worldwide and domestic dogs are nowadays the closest animal companion to human beings. Due to these aspects, they can represent a potential reservoir of vector-borne diseases. Canine vector-borne diseases (CVBDs) comprise a group of parasitic and infectious diseases caused by several pathogens (e.g., bacteria, protozoa, and helminths), which are transmitted by arthropod vectors, including ticks, fleas, lice, mosquitoes, and phlebotomine sand flies. Because CVBDs causing pathogens (e.g. *Hepatozoon* spp., *Babesia* spp., *Anaplasma* spp., *Borrelia* spp.) are of major zoonotic concern, they represent an increased public health threat for domestic dogs and their owners. Therefore, the study of CVBDs can help to recognize the disease affecting human health. In this study, 273 blood samples were collected in an area in southern Ethiopia by FTA cards and analyzed by biomolecular techniques (i.e. conventional PCR, real-time PCR and multiple real-time PCR) in order to identify the infection and parasitic/infective agents and emphasized the importance of molecular methods in distinctive CVBD pathogens. *Hepatozoon canis* DNA and *Babesia canis rossi* DNA were detected in 141 (51.6%) and 9 (3.3%) samples, respectively. No *Anaplasma phagocytophilum* DNA and *Borrelia burgdorferi s.l.* DNA were detected in samples by multiple real-time PCR. In this study, *Hepatozoon canis* had the highest detection rate, while Anaplasma and Borrelia appeared to be absent in the study area. The relevance of encountered pathogens should be further investigated.

Keywords: canine vector-borne diseases, biomolecular techniques, pathogens, human health

1. Introduction

1.1 The social relationship between domestic dogs and human

The social relationship between domestic dogs and human is known since a long time. Dogs are currently members of our family and became a part of our daily lives. They're not only pets, but even working, guard and hunting dogs. The number of dogs in the world is increasing. Nowadays, it is estimated that there are around 7 billion dogs on earth and this number will increase to over 10 billion by 2050. Moreover, canine population is growing at a rate of 1% per year, which means that we could be looking at an additional 2 million every day by 2050 (American Veterinary Medical Association, AVMA). Canine diseases are closely related to human health and have drawn new attention to human public health. In fact, several diseases that occur in dogs can also occur in humans, for instance babesiosis, anaplasmosis and Lyme borreliosis, among the canine vector borne diseases (CVBD). Worldwide, vector-borne diseases are one of the greatest threats to public and animal health (Folly et al., 2020). For many diseases considered to be zoonotic, dogs are effective hosts. Therefore, pathogen infection may cause some symptoms or no symptoms in dogs, but dogs infection provides a reservoir of pathogens for disease transmission that eventually affect humans.

1.2. The important vector-borne diseases in domestic dogs

The CVBDs considered in this study are hepatozoonosis, babesiosis, anaplasmosis and Lyme-borreliosis. A detailed description of each pathogen, as well as the main type of carrier, symptoms of infection in domestic dogs and humans, methods of identification and detection of the disease, and prevention of the disease. In addition, among them, several diseases have a zoonotic potential with possible transmission to the human. (Despoina et al. 2020).

1.2.1. Hepatozoonosis

Canine hepatozoonosis is a tick-transmitted disease caused by an intraleukocytic parasite, *Hepatozoon* spp., which is an apicomplexan protozoon belonging to the Order Eucoccidiorida (Grillini et al., 2021). *Hepatozoon canis* was first described in the blood of dogs in India and named *Leukocytozoon canis* initially in the 1900s (Christophers, 1907; Grillini et al., 2021). There are currently over 340 *Hepatozoon* species. They infect both domestic and wild animals like carnivores, reptiles, amphibians, and birds. The two species (*Hepatozoon canis* and *Hepotozoon americanum*) infect domestic and wild dogs inflicting the disease referred to as canine hepatozoonosis (Erol et al., 2021). The main route of transmission of *Hepatozoon* spp. is the ingestion of a tick containing *Hepatozoon* sporozoites but even other routes of transmission are reported (i.e. *Rhipicephalus sanguineus*, *Amblyomma ovale*, placental transmission) (Vannier, 2008; Maia et al., 2014; S. A. Ewingand R. J. Panciera., 2003).

1.2.1.1. *Hepatozoon canis*

Hepatozoon canis is the etiological agent of canine hepatozoonosis commonly infecting dogs in including in Africa, the US and South America and Europe worldwide (Grillini et al., 2021). The main vector is brown dog tick (*Rhipicephalus sanguineus)*. Hepatozoonosis in dogs might gift as a usually milder unwellness caused by *Hepatozoon canis* (i.e. extreme lethargy, cachexia, anemia, inflammatory skeletal lesions and multiple infections). All *Hepatozoon* spp. have a similar life cycle including sexual development and sporogony in a hematophagous invertebrate (i.e.ticks, mites, sand flies, tsetse flies, mosquitoes, fleas, lice), and merogony followed by gamontogony in a vertebrate (i.e. dogs, cats, other mammals, carnivores, reptiles, amphibians and birds). The dogs act as intermediate host and the ticks as definitive host (Baneth et al., 2007). Ticks transfer oocysts, by biting, with multiple sporozoites containing infective spores into the dogs is the basic route of infection (Baneth et al., 2007). As for *Hepatozoon canis* sporozoites, they are released in the intestine of dogs and penetrate the intestinal wall into mononuclear cells. They spread through the blood or lymph to target organs, including bone marrow, spleen, lymph nodes, liver, kidney and lung (Baneth,2011).

1.2.1.2. *Hepatozoon americanum*

In the Southern USA a separate species, *Hepatozoon americanum*, causes disease in dogs and is transmitted by the tick *Amblyomma maculatum.* In contrast, *Hepatozoon americanum* oocyst containing sporocysts with infective sporozoites from the hemocoel of the infected vector tick by releasing sporozoites from the sporocyst. Dog bile facilitates the release of this sporozoites, which can migrate freely through the intestinal wall or be taken up by host macrophages and then move to target organs through the lymphatic system or blood (Baneth, 2011).

1.2.1.3. Diagnosis and control of hepatozoonosis

Hepatozoonosis in dogs are diagnosed by observing blood smears, histopathology, serology, or PCR. *Hepatozoon canis* and *Hepatozoon americanum* could be distinguished by PCR assay (Kelly, 2022). The three most commonly used are real-time PCR, conventional PCR and nested PCR(Laise et al., 2016). Polymerase chain reaction (PCR) is a powerful method for amplifying specific DNA fragments. DNA polymerases control the synthesis of DNA from deoxynucleotide substrates on single-stranded DNA templates. The DNA polymerase adds nucleotides to the 3' terminal of the custom-designed oligonucleotide when it anneals with longer template DNA. Thus, the DNA polymerase can use the synthesized oligonucleotide as a primer and extend its 3' end to form an extended stretch of double-stranded DNA if it is annealed against a single-stranded template that has a region that is complementary to the oligonucleotide. The principle of real-time PCR is the detection of the fluorescent dye emitted during the PCR assay. Plasmid DNA was quantified using a spectrophotometer (DU 530 life science UV/visible-light spectrophotometer) (Kim et al., 2008). Nested PCR typically involves two consecutive amplification reactions, each using a different primer pair. The product of the first amplification reaction was used as a template for the second PCR initiated by an oligonucleotide located inside the first primer pair. The use of two pairs of oligonucleotides allows for more cycles, thus increasing the sensitivity of PCR. The improved reaction specificity results from the combination of two different sets of primers with the same target template. Nested PCR is an effective method to expand the template fragment, but it needs to understand the target sequence

(Green et la., 2019). If a dog is infected with pathogens and the complete blood cell count may show low erythrocyte and high leukocyte meanwhile there are organisms in the leukocyte under the microscope. Pathogens can be found in muscle tissue or may also cause skeletal lesions, which can be seen on X-rays. It is an effective way that prevention of hepatozoonosis should avoid to ingest infected tick vectors and infected prey.

1.2.2. Babesiosis

Babesiosis canine is a tick-borne protozoan blood parasitosis caused by *Babesia* also known as *Nuttallia* is an apicomplexan parasite (Nowell et al., 2009). There are more than 100 known species of *Babesia*, infections in wild or domestic vertebrates and humans have occurred throughout the world such as in Europe, Africa, Asia, and the United States, where *Babesia microti* is one of the few most common strains that has been shown to cause human infection. The first reported case of human babesiosis in the world was reported in Europe, in 1957.(Boozer and Douglass, 2003; Gorebflot, 1998). The main infections that can cause dogs are *Babesia canis* and *Babesia gibsoni* in Europe (Teodorowski et al., 2022). *Babesi*a species generally can be divided into large and small organisms. So far, only three small *Babesia* species with clinical importance have been described that are *Babesia gibsoni*, *Babesia conradae* and the recently reported *Babesia vulpes* (Teodorowski et al., 2022). As for *Babesia canis* is the larger one which is pyriform and occurs in pairs twice as large as *Babesia gibsoni*. However, *Babesia gibson* occurs in five or six different shapes, usually oval and single, and may show ring-like patterns (Boozer and Douglass, 2003).

1.2.2.1. *Babesia canis*

Babesia canis has three distinct subspecies named *B. canis canis*, *B. canis rossi* and *B. canis vogeli*. They differ considerably in geographical distribution, infectious tick vectors, and clinical characteristics. It first was reported in the United States in 1934 (Eaton, 1934). After being bitten by an infectious tick, the sporozoites are released into the bloodstream and cause dogs to become infected with *Babesia*. For *Babesia canis* must continue last 2-3 days biting to complete transmission. In the host, the organism

attaches to the erythrocyte membrane and is engulfed by endocytosis and then the membrane dissolves, leaving the parasite directly in the cytoplasm (Hauschild and Schein, 1996; Boozer and Douglass, 2003; Oyamada et al., 2005). *Babesia canis* causes a range of clinical symptoms, including fever, anemia, thrombocytopenia, lethargy, anorexia, splenomegaly, hemoglobinuria, bilirubinuria, and jaundice. Severe cases may be accompanied by acute renal failure, acute respiratory distress syndrome (ARDS), cerebral babesiosis and death.

Babesia canis rossi is the most virulent one and that causes South African babesiosis is transmitted by the *Hemaphysalis leachi* may also be transmitted by *Rhipicephalus sanguineus, Rhipicephalus evertsi evertsi,* or *Amblyomma lepidum* (Oyamada et al., 2005). This subspecies causes varying degrees of hemolytic anaemia, splenomegaly, thrombocytopenia and fever and is associated with high mortality (Nowell et al., 2009).

Babesia canis canis causes babesiosis in Europe and Asia is transmitted by the *Dermacentor reticularis.* This subspecies causes fewer clinical symptoms and has a lower mortality rate, but when death does occur it is attributed to liver and kidney damage.

Babesia canis vogeli causes relatively mild disease in the United States, tropical and subtropical areas is transmitted by the brown dog tick (*Rhipicephalus sanguineus*)(Eaton, 1934; Boozer and Douglass et al., 2003). It is comparatively weaker than other two subspecies (*Babesia canis rossi* and *Babesia canis canis*) (Oyamada et al., 2005; Boozer and Douglass, 2003).

1.2.2.2. *Babesia gibsoni*

Unlike *Babesia canis* described in the appeal, *Babesia gibsoni* was first demonstrated in 1904 and has since been reported in Asia, North and East Africa, Brazil but rarely in Europe (Patton, 1910; Zahler et al., 2000). *Babesia gibsoni* is transmitted by ticks of the *Rhipicephalus sanguineus* and *Haemphysalis longicornis* (Teodorowski et al., 2022), and causes a range of clinical symptoms, such as hemolytic anemia, thrombocytopenia, lethargy, and splenomegaly. In the United States, the disease primarily affects American pit bull dogs. However, other breeds of dogs are rarely infected, and if they are, there may be a link to pit bull fighting (Boozer, 2005). Other routes of transmission could be blood transfusion, placental transmission or open wounds from fight (Teodorowski et al., 2022; Boozer, 2005).

1.2.2.3. Diagnosis and control of babesiosis

The diagnosis of *Babesia canis* and *Babesia gibsoni* is usually made by identifying the organism in blood smears with microscope, fluorescent antibody (FA) staining of the organism, enzyme-linked immunosorbent assay tests (ELISA) and PCR test. It can detect all four species of *Babesia*. Serological and antibody testing show limitations, as a positive test result depends on antibody production in the infected dog, which can take up to ten days. Once dogs own antibodies of babesiosis that may remain in their bodies for many years (Teodorowski et al., 2022; Solano-Gallego, 2016; Boozer, 2005). Molecular analysis can also be used, such as real-time PCR, conventional PCR and nested PCR. The sensitivity of conventional PCR is not as significant as that of real-time PCR and nested PCR. However, these three methods are commonly used to identify babesiosis(Kim et al., 2008).

The most effective way to prevent babesiosis is to combat the vector by using topical insecticides, cleaning the dog's premises or removing ticks from the dog to reduce the chance of the dog being exposed to ticks. In European, there is a vaccine to against *Babesia canis* is available to protect dogs (Solano Gallego et al., 2016).

1.2.3. Anaplasmosis

Anaplasmosis is caused by several bacterial species of the genus *Anaplasma*. *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophilum*) is a Gram-negative and uncommon in response to neutrophils bacterium that is the most important representative and has been detected in blood samples from a wide range of wild and domestic animals (i.e. goat, cattle, horse and dog) (Farhan et al., 2021; Dumler et al., 2001). It was first described as a veterinary pathogen, and after identifying the organism in the cytoplasm of leukocytes from sheep in Scotland. Also known as tick-borne fever in European domestic ruminants (i.e. goat, cattle, horse). The first reported dogs with *Anaplasma phagocytophilum* infection were recorded in California in 1982 (Carrade et al., 2009). In 1990, human granulocytic anaplasmosis (HGA) was first identified in a Wisconsin patient who died with a severe febrile illness 2 weeks after a tick bite (Dumler et al., 2005). Transmission of *Anaplasma phagocytophilum* from ticks to mammals is completed approximately one or two days of sustained biting or adsorption.

It spreads through the blood or lymph system in mammals with an incubation period of 1 to 2 weeks. After adhesion through the abundant P-selectin on the bacterial surface and they enter neutrophils by endocytosis (Dumler et al., 2009). *Anaplasma phagocytophilum* cause a range of symptoms including lethargy, loss of appetite and acute fever. Less common symptoms include lameness, cough, polydipsia, intermittent vomiting and bleeding. In humans, the symptoms are fever, headache, myalgia, malaise, absence of skin rash, leukopenia, thrombocytopenia, and mild injury to the liver (Dumler et al., 2005).

1.2.3.1. Diagnosis and control of anaplasmosis

Anaplasmosis can be diagnosed using immunofluorescent antibody techniques, using immunofluorescent antibody techniques in combination with serology, and DNA detection by PCR using primers specific for *Anaplasma phagocytophilum* (Dumler et al., 2001,2005,2009; Carrade et al., 2009). Duplex real-time PCR, multiple real-time PCR, conventional PCR, nested PCR can be used to detect pathogens. Multiplex real-time PCR is a variation of the conventional PCR. The basic principle of multiplex PCR is the same as that of the conventional PCR, with the exception that more than one pair of primers are used in same reaction. Different primers can bind to specific templates and amplify new DNA fragments. This method not only retains the advantages of real-time PCR high sensitivity, but is also faster and more economical. Multiple DNA fragments can be amplified one reaction.

When visiting woodlands or tick habitats, use a tick repellent spray and wear long sleeves and pants to avoid contracting anaplasmosis. In unfortunate cases where the tick is already attached to the skin, the tick should be immediately and correctly removed from the skin and the bite treated.

1.2.4. Lyme disease

Lyme disease, also known as Lyme borreliosis (LB), is a zoonosis caused by spirochetes that is transmitted by *Ixodes scapularis* and *I. ricinus,* respectively in USA and in Europe. It is caused by 4 main species of spirochetes called *Borrelia burgdorferi* and *Borrelia mayonii* in the United States, *Borrelia afzelii* and *Borrelia garinii* in

Europe and Asia (Allen et al., 2016). In North America, the most *Borrelia burgdorferi* positive dogs show no clinical signs. The two main clinical manifestations of *Borrelia burgdorferi* infection in dogs are Lyme arthritis and Lyme nephritis. As for Europe, it has not been shown to cause clinical symptoms in dogs (Krupka & Reinhard., 2010). Lyme disease is transmitted to humans by the bites of infected ticks of the genus *Ixodes*. The disease does not appear to spread from person to person, however through other infected animals with the pathogen. *Borrelia burgdorferi* can spread throughout the body during the course of the disease, and has been found in the skin, heart, joints, peripheral nervous system, and central nervous system (Allen et al., 2016,2004). During the initial tick bite, an erythema migrans (EM) rash may appear within a mounth, with an expanding red area that is sometimes transparent in the center, forming a 'bull eye'. There is no itching or pain, but it feels warm to the touch. Erythema migrans is one of the hallmarks of Lyme disease, but not all people with Lyme disease develop a rash. They may also be accompanied by fever, chills, fatigue, body aches, headache, neck stiffness, and swollen lymph nodes (Pachner and Steiner, 2007; Allen et al., 2016, 2004). If left untreated for a long time, it can cause pain and swelling that can move from one joint to the other. Sometimes weeks, months, or even years after infection, inflammation of the membranes around the brain (meningitis), temporary paralysis of one side of the face commonly defined Bell's palsy, numbness or weakness of the limbs, and muscle movement disorders may develop (Eugene D., 2014; Pachner &Steiner, 2007; Allen et al., 2004). *Borrelia burgdorferi* is quite different from typical Gram-negative bacteria since outer membrane lacks lipopolysaccharide, which an important environmental signal during transmission of *B. burgdorferi* between tick vectors and mammalian hosts. However, the lipoproteins on the outer membrane surface of *B. burgdorferi* can vary according to the environment. It uses differential gene expression to survive in different environments of tick vectors and mammalian hosts. A tick bite penetrating the host skin can transport *B. burgdorferi* deep into the dermis near blood vessels. Tick salivary proteins can cause blood clotting, fibrinolysis, and immune responses that help pathogen establish infection (Allen et al., 2016).

1.2.4.1. Diagnosis and control of Lyme disease

The diagnosis of Lyme disease is complex and difficult, because early diagnosis can only be based on erythema migrans (EM) lesions of Lyme disease and non-specific clinical signs and symptoms, but can be achieved through serological testing including sensitive enzyme immunoassays (EIA) or immunofluorescence assays. Sometimes the results are ambiguous since these tests may lack in specificity, and Western blotting might be used to further confirm these obtained results (Bobe et al., 2021). The simplest and fastest test is PCR(i.e. Duplex real-time PCR, multiple real-time PCR, conventional PCR and nested PCR).

The best way to prevent Lyme disease is to avoid deer tick habitats, especially overgrown and wooded areas and choose for long-sleeved clothing and insect repellant if necessary. If you are bitten by a tick, you need to remove it. Lyme disease in dogs can be prevented by vaccination (Allen et al., 2016).

1.3. The impact of vector-borne diseases on human health

Vector-borne parasitic and infectious diseases are one of the major global public health concerns and they can infect humans in both developed and developing countries (Zahid and Mondal., 2020). The transmission of these diseases does not occur directly from human to human, but an arthropod vector is necessary. Major vector-borne diseases in humans include malaria (i.e. P*lasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale and Plasmodium knowlesi*) (Kariuki et al, 2020), ehrlichiosis (i.e.*Ehrlichia chaffeensis*)(Marcos, 2018), haemoplasmosis (i.e.*Candidatu*s mycoplasma haemohominis) (Hattoti et al., 2020), schistosomiasis (i.e. *Schistosoma mansoni*, *Schistosoma japonicum*) (Kamdem et al., 2018), hepatozoonosis (i.e. *Hepatozoon canis* and *Hepatozoon americanum*), babesiosis (i.e. *Babesia canine* and *Babesia gibsoni*), anaplasmosis(i.e. *Anaplasma phagocytophilum*), Lyme disease(i.e. *Borrelia burgdorferi* and *Borrelia mayonii*). They account for quite 17% of all infectious diseases and cause more than 700,000 deaths every year (World Health Organization, WHO, 2020). Moreover, their impact causes huge economic losses and limits the development in both rural and urban areas. The burden of those diseases is greatest in tropical and climatic zone regions, and that they disproportionately have an effect on the poorest populations.

2. Objective of the study

As the populations of dogs gradually increased, the social roles that dogs have in many countries (e.g. guide dogs for people with disabilities) and their growing relationship with humans raise new concerns for human public health. In recent years, 'one health' concept has been raised by infectious disease experts worldwide.

The present work is inserted in the framework of a collaboration between the University of Padova and the Addis Ababa University (AAU) in Ethiopia, aimed at the support of the PhD program held at the College of Veterinary Medicine and Agriculture of the AAU. Specifically, this thesis focuses on the some vector-borne diseases (hepatozoonosis, babesiosis, anaplasmosis and Lyme borreliosis) in domestic dogs and the consequent risk for humans (Michael., 2011), as part of a PhD research project on canine and feline vector-borne pathogens in selected Districts of Ethiopia. In this study, molecular biotechnology was used in order to identify the etiological agents of selected CVBDs and to understand the potential risk for human health, using dog blood samples previously collected.

3. Materials and methods

3.1. Sample pretreatment and DNA extraction

Two hundred seventy-three samples collected by FTA card (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 to remove 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.

3.2. Piroplasms detection (*Hepatozoon* spp. and *Babesia* spp.)

3.2.1 Real-Time PCR assay

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, DE). 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 (Fig. 1). 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 delle Venezie.

Samples were tested in duplicate.

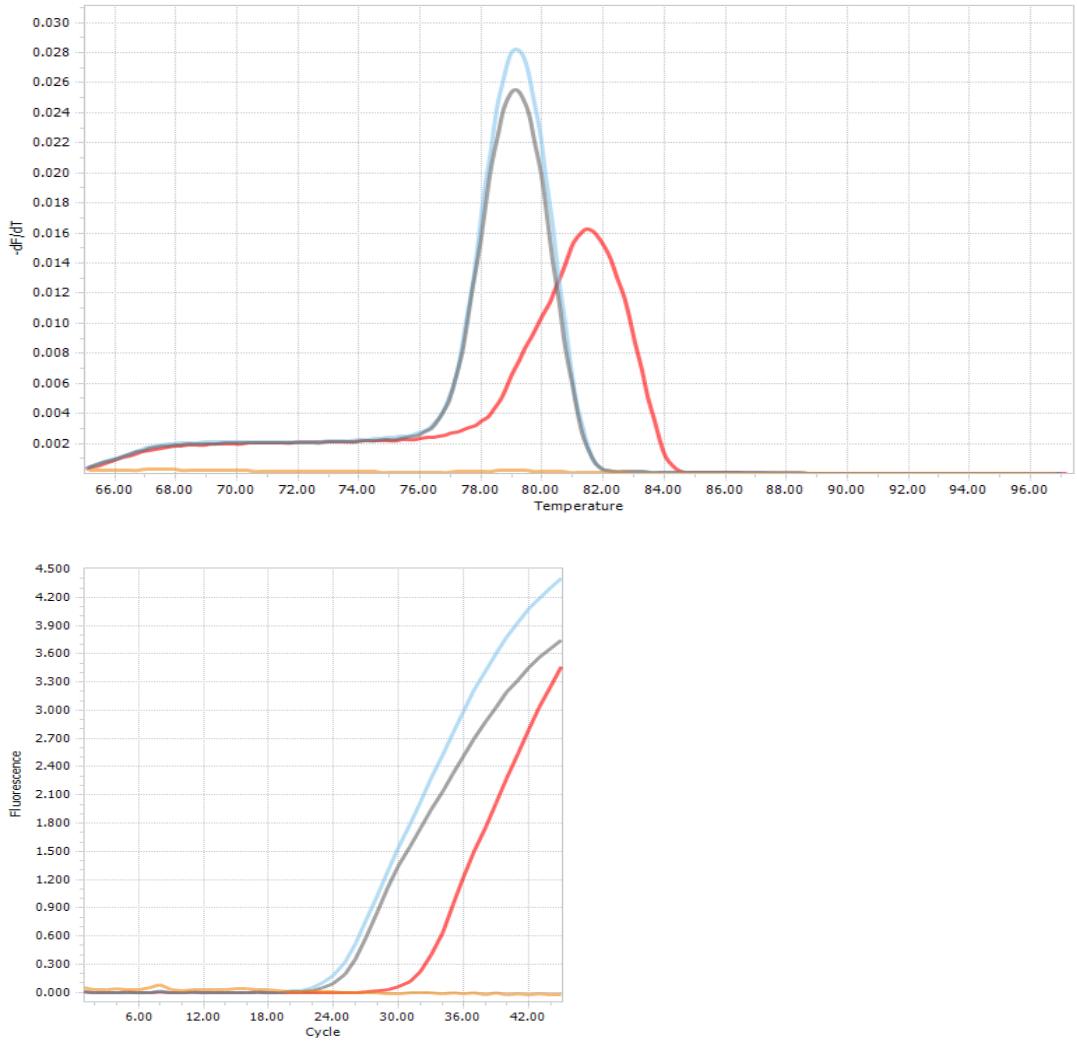

Figure. 1 - Specific temperature of melting (Tm) and cycle threshold (Ct) for *Hepatozoon canis* (light blue and purple lines), *Babesia canis canis* (red line) and negative control (orange line)

3.2.2 Conventional PCR assay

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, $MgCl_2$), 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, then a software for the gel reading was used to take photos and read the results.

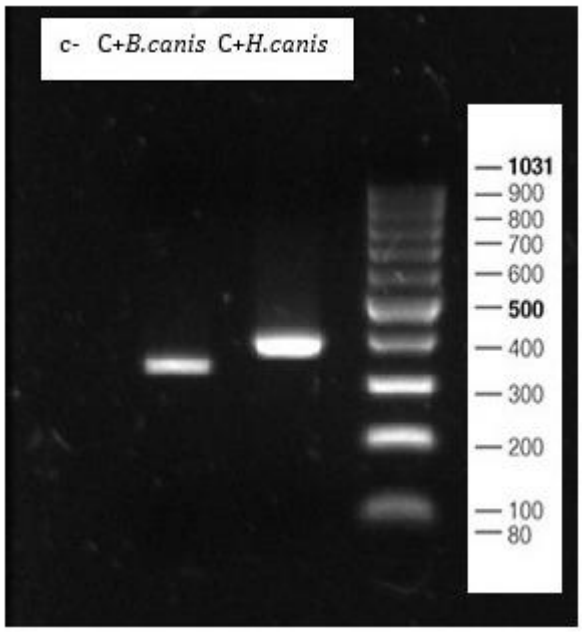

Figure. 2 – Electrophoretic gel depicting negative control, *Hepatozoon canis* and *Babesia canis canis*

3.3. Detection of *Anaplasma* and *Borrelia*

3.3.1 Multiple Real-Time PCR assay

This assay was used to detect multiple DNA targets simultaneously in a single tube. The thermocycler Roche LightCycler® 96 was used in this analysis too. The reaction mix included forward and reverse primers, DNase/RNase- free water and QuantiNova Probe PCR Kit (QIAGEN Group, Hilden, DE).

Oligonucleotide probe and primer sequences were designed to be specific for the *msp2* gene of *Anaplasma phagocytophilum* and for the *23S* rRNA gene of *Borrelia burgdorferi sensu lato* (*s.l.*) as described by Countney et al (2004). *Anaplasma phagocytophilum* primer sequences ApMSP2f (5'-ATGGAAGGTAGTGTTGGTTATGGTATT-3') and ApMSP2r (5′-TTGGTCTTGAAGCGCTCGTA-3') were used in conjunction with TaqMan probe ApMSP2p-HEX (5′-TGGTGCCAGGGTTGAGCTTGAGATTG-3'). Primer sequences specific for *B. burgdorferi s.l* were Bb23Sf (5′ - CGAGTCTTAAAAGGGCGATTTAGT) and Bb23Sr (5′-GCTTCAGCCTGGCCATAAATAG) to generate a 75 bp fragment with a TaqMan probe identified as Bb23Sp-FAM (5′-AGATGTGGTAGACCCGAAGCCGAGTG).

The reaction mixture was transferred in a real-time PCR plate: 5 µl of mix and 2 µl of target DNA were put in each well. In each run at least a positive control (i.e. DNA of positive field sample confirmed by sequencing) and a negative control (no DNA added) were analyzed.

The plate was covered and centrifuged to help the mixing between samples DNA and reaction mixture and the convergence of the liquid at the bottom. Then it has been placed into the thermocycler using the following amplification cycle: Preincubation at 95°C for 2 min, followed by 50 cycles of 3 step amplification for 95°C for 5 sec, 60°C for 5 sec and 72°C for 30 sec.

Samples were tested in duplicate.

3.4. 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).

4. Results

The results of real-time PCR analysis of the target pathogens (i.e. *Hepatozoon canis, Babesia canis rossi, Anaplasma phagocytophilum, Borrelia burgdorferi s.l.*) are shown in Table 1. Out of 273, 150 (54.9%) samples showed positive results. The most common pathogen in this study was *H. canis* (141/273; 51.6%), followed by *B. canis rossi* (9/273; 3.3%). On the contrary, *A. phagocytophilum* and *B. burgdorferi s.l.* were not detected in the samples.

Table 1. Frequencies (%) of vector-borne pathogens in dogs.

Pathogen	Positive	Frequency (%)
Hepatozoon canis (79/79.5°C)	141	51.6%
Babesia canis rossi (81.5°C)	9	3.3%
Anaplasma phagocytophilum	0	0.0%
Borrelia burgdorferi s.l.	0	0.0%
Negative	123	45.1%
Total	273	100.0%

Among the 150 positive samples, 88 were randomly selected for cPCR analysis and part of these (40/88) were also Sanger sequenced to obtain a further confirmation of the implemented methodological process. Indeed, the results obtained were consistent with what previously determined using melting temperatures (Table 2). Finally, 59/150 samples were evaluated only by real-time PCR, considering them positive according to their melting temperature.

Figure 3 shows that 141 positive samples of *Hepatozoon canis* were detected by real-time PCR, 88 samples were randomly selected for cPCR analysis, and 40 samples were randomly selected for sequencing. As for *Babesia canis rossi,* 9 positive samples of *Babesia canis rossi* were detected by cPCR and real-time PCR. Only 6 samples were randomly selected for sequencing.

Table 2. Results of each molecular analysis in the procedure for the identification of piroplasms.

real-time PCR		**cPCR**		**Sequencing**		**(%)**	**Interpretation**
analysis result	**N**	**analysis result**	**N**	**analysis result**	**N**		
POS to *Hepatozoon canis* (79/79.5°C)	141	POS	88	sequenced	40	12%	positive to *H. canis* based on real-time PCR, cPCR and sequencing
				not sequenced	48	18%	positive to *H. canis* based on real-time PCR and cPCR
		not analysed	59	not sequenced		22%	positive to *H. canis* based on real-time PCR
POS to *Babesia canis rossi* (81.5°C)	9	POS	9	POS	6	2%	positive to *B. canis rossi* based on real-time PCR, cPCR and sequencing
				not sequenced	3	1%	positive to *B. canis rossi* based on real-time PCR and cPCR
Negative	123	not analysed		not sequenced		45%	negative
Total number	273					100%	

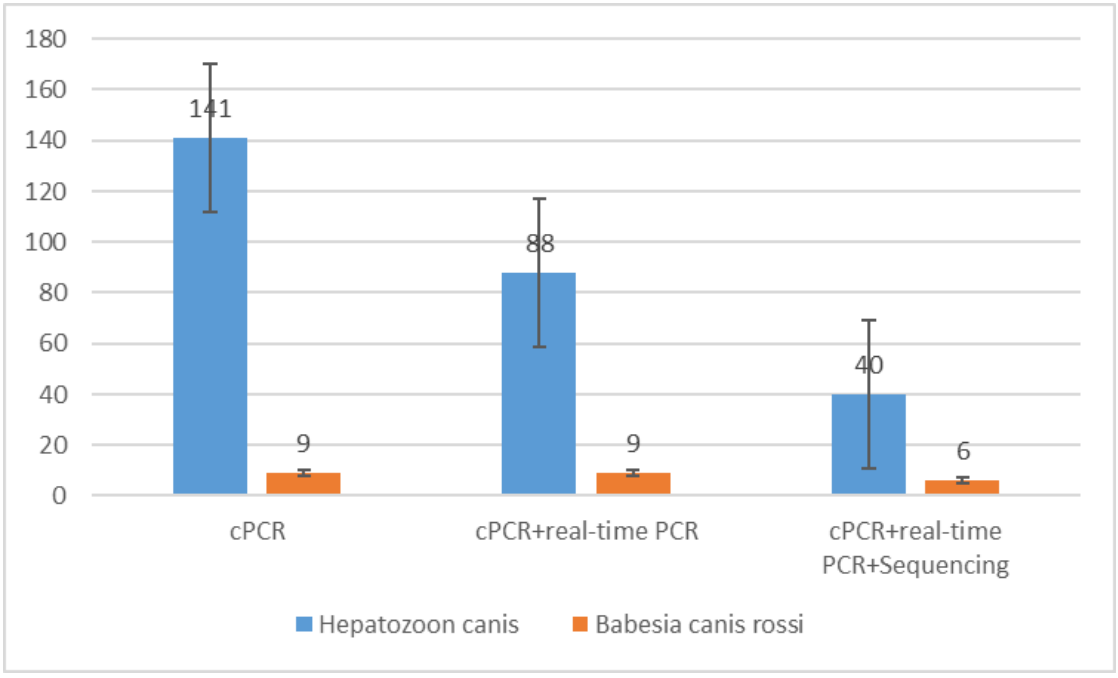

Figure 3 – The number of each molecular analysis in the procedure for the identification of piroplasms.

The BLAST analysis retrieved 96.9-100% homology with sequences deposited as *H. canis* isolated from black-backed jackal (*Canis mesomelas*) in South Africa (Viljoen et al., 2021), grey wolf (*Canis lupus)*, in Germany (Hodzic et al., 2020), and dog in India (Lakshmanan et al., 2018).

Regarding *B. canis rossi*, the same analysis retrieved 96.8–98.9% identity from black-backed jackal (*Canis mesomelas*) in South Africa (Penzhorn et al., 2017).

5. Discussion and conclusions

Among 273 dog samples, 141 (51.6%) resulted positive for *Hepatozoon canis* and 9 (3.3%), for *Babesia canis rossi*. On the contrary, none of them showed positivity to *Borrelia burgdorferi s.l.* and *Anaplasma phagocytophilum*.

Hepatozoon canis infections have been reported mainly from tropical, subtropical, and temperate climate regions where vector tick species are abundant. In Europe, infections occur mainly around the Mediterranean basin. It has been reported from Turkey (Karagenc et al., 2006), Bulgaria (Tsachev et al., 2008), Italy (Gavazza et al., 2003), France (Rioux et al., 1964), Spain (Tabar et al., 2009). In Africa from Sudan (Oyamada et al., 2005), Nigeria (Sasaki et al., 2008) and South Africa (McCully et al., 1975). In this study, 141/273 (51.6%) *H. canis* positive samples were obtained from dogs' samples, indicating that the pathogen is commonly found in the Ethiopian territory. In a study conducted in Sudan (Oyamada et al., 2005) 33/78 dogs (42.3%) were found positive for *Hepatozoon canis* and in another study, 81/400 (20.3%) samples were positive for *Hepatozoon canis* in Nigeria (Sasaki et al., 2008). These studies confirm the tendency of this parasite to circulate in African dog populations with medium or high prevalence value, as in our area. Compared with the traditional blood smear examination of blood, PCR detection of hepatozoonosis is more sensitive. For example, the prevalence of hepatozoonosis in 349 dogs reported by Karagenc et al. (2006) in Turkey was 10.6% by blood smear and 25.8% by blood PCR.

According to the experimental results, all *Babesia* spp. positive samples (9 of 273) were *Babesia canis rossi* that is the most virulent one and has only been reported from sub-Saharan Africa (Penzhorn et al., 2017; 2018). In sub-Saharan Africa, babesiosis in domestic dogs is mainly caused by *Babesia canis rossi*. The reservoir in African country is represented by black-backed jackals (*Canis mesomelas*) and they can transmit the pathogen to domestic dogs. The study by Penzhorn et al (2017) demonstrated that infections are common in free-ranging jackals, indeed, nearly a third of jackals (29.7%) were infected by *Babesia canis rossi*. Black-backed jackals are found mainly in Northeast Africa (Somalia and Eastern Ethiopia southward to Tanzania) and South-western Africa (from South-western Angola and Zimbabwe to the Western Cape Province, South Africa) (Penzhorn et al., 2017; 2018). It is therefore reasonable to suspect that black-backed jackals are responsible for *B. canis rossi* infection also in Ethiopian dogs.

The results of real-time PCR mainly showed two melting peaks (i.e., 79/79.5 C°and 81.5 C°). Therefore, we suspected that the 79/79.5°C sample was *Hepatozoon canis*, and the 81.5°C sample was *Babesia canis.* Contrarily to our results, Buddhachat et al. (2020) reported different temperature for *Babesia canis* and *Hepatozoon canis* (82.41°C and 78.56°C°, respectively). As our experimental samples came from Ethiopia, Africa, the report on *Babesia* spp. infection in dogs lacks detailed data support. *Babesia canis* has three subspecies, *Babesia canis vogeli* is mainly distributed in Europe, and *Babesia canis rossi* is widespread in Africa. In this study, nearly half of the cPCR positive samples were randomly selected for sequencing. This is an effective and more cost-effective approach to study and diagnose vector-borne disease using molecular technology due to the fact that, once we have confirmation for a relevant part of the samples, we can say with a good level of certainty that the melting peak temperature corresponds to that specific pathogen.

The negativity to *Anaplasma* may be caused by the type of matrix or to epidemiological reason. As already reported, *A. phagocytophilum* is mainly present in white blood cells, especially neutrophils. The normal leukocyte (WBC) value (including all white blood cell types) is 5,500-16,900 per microliter. Compared to the number of red blood cells between 55.5 million-8.5 million cells per microliter of blood. Neutrophils are usually the most common white blood cell. They make up about 60 to 70 percent of WBC in the blood of healthy dogs (Kidd, 2003). Therefore, the negativity for A. *phagocytophilum* could be due to the small amount of blood analyzed which did not allow to detect the infected granulocytes. Secondly, *A. phagocytophilum* is mainly reported in temperate climate and concerning the African continent, in South Africa. However, there are few reports from Ethiopia, where a prevalence of 2.73% of *A. phagocytophilum* was found in cattle (Teshale et al., 2018). Also other studies on *A. phagocytophilum* were concentrated domestic ruminants in Ethiopia (Teshale et al., 2015; 2016). As for domestic dogs have not been reported.

B*orrelia burgdorferi s.l.* has never been reported in Ethiopia. Nevertheless, new *Borrelia* species (i.e. *Borrelia theileri* and *Borrelia lonestari*) have been reported in Ixodid ticks from Ethiopia by Kumsa et al. In this study, the researchers speculated that *Borrelia* DNA could be found in ticks removed from several species of animals (e.g. cattle, sheep, dogs, and cats). However, *Borrelia* DNA was not detected in ticks taken from dogs (0/23) or cats (0/6) (Kumsa et al, 2015). Therefore, it is reasonable to suspect that *Borrelia burgdorferi s.l.* is only frequently transmitted in the United States, Europe,

and Asia, and it's not so relevant in sub-saharan Africa (Kumsa et al., 2015), confirming the results of this study. In the study of Bouattour et al. (2004) *Borrelia burgdorferi s.l.* has been found in North Africa (Tunisia) but was very rare. Forest workers in Tunisia are considered at high risk for *Borrelia burgdorferi s.l.*, but only 4% of them are infected.

Lyme disease and anaplasmosis are two very common vector-borne zoonoses in the temperate climate, and their etiological agents (*B. burgdorferi and A. phagocytophilum*) share vectors, hosts and geographical distributions. Multiplex real-time PCR assay is the most useful and economical tool to detect these pathogens in their endemic areas. Although these pathogens do not seem to be present in our study area, the assay used in our research was a cost-effective approach, since it could detect simultaneously both pathogens (Countney et al., 2004). Due to we use the oligonucleotide probe and primer sequences were designed based on msp2 genes (*Anaplasma phagocytophilum*) and 23S rRNA (*Borrelia burgdorferi s.l.*). The reason is that the genome sequence of *B. burgdorferi* has been determined and the 23S rRNA gene targeted by the detection target has two highly conserved copies. On the basis of the outer surface protein C gene's (ospC) sequence variability, rRNA genes, intergenic spacers, and fla genes, the Lyme disease bacteria were characterized as *B. burgdorferi sensu lato* (Wormesr et al., 2008; Countney et al., 2004). Therefore, based on the genotypic heterogeneity of *Borrelia spp.*, the high conservation of 23S rRNA gene has become the main target of detection. The 23S rRNA primers and probes will amplify only DNA fragments of *B. burgdorferi* in multiplex real-time PCR. The msp2 genes encode a 44-kDa immunodominant outer membrane proteins that are considered unique to the intragranulocytic agent of *Anaplasma phagocytophilum* (Lin et al., 2004; Countney et al., 2004; Park et al., 2003). The multiplex real-time PCR assay is therefore specific and the primers do not amplify the DNA of any other bacterial species tested.

In conclusion, in this work four target disease (i.e. hepatozoonosis, babesiosis, anaplasmosis and Lyme borreliosis) were detected and analyzed using three PCR technologies (real-time PCR, multiple real-time PCR, and conventional PCR), and our hypothesis (melting peaks at 79/79.5 C° is *Hepatozoon canis* and at 81.5 C° is *Babesia canis rossi*) was confirmed. *Babesia canis rossi* has been found to infect domestic dogs in Ethiopia, and *Hepatozoon canis* is the predominant piroplasm. Sound information on *Anaplasma phagocytophilum* and *Borrelia burgdorferi s.l.* from domestic dogs in Africa (Ethiopia) is still lacking, but our findings suggest a limited importance for these

pathogens in the area. Further studies on domestic dogs and on human population are needed to unveil the zoonotic relevance of these pathogens and their diffusion. In this specific context, pathogen lifecycles, pathogenicity, mode of transmission and the role of local African wildlife reservoirs and of different arthropod vectors remain open research questions to be addressed.

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