UNIVERSITA' DEGLI STUDI DI TORINO

DIPARTIMENTO DI SCIENZE VETERINARIE

DOTTORATO DI RICERCA IN SCIENZE VETERINARIE PER LA SALUTE ANIMALE E LA SICUREZZA ALIMENTARE

CICLO XXXII

ONE HEALTH AND EMERGING ZOONOSES: THE PROTOZOA

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ANNI ACCADEMICI 2016-2019

SSD VET-06

La
dedica
di questa tesi
è divisa
in sei modi:
a Luca,
a Mamma, Papà e i Nonni,
a Ezio,
a Stefania,
a Franco e Sandra,
e a te,
se sei
rimasto
con me
fin proprio
alla
fine.

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INTRODUCTION

1. GENERAL INTRODUCTION

Each historical period is remembered for an event, a discovery or an invention that mainly characterized this period. What probably better characterized the last few decades are the major climatic and habitat changes that are occurring on the Earth.
As defined by the Intergovernmental Panel on Climate Change (IPCC), climate change:

> refers to a change in the state of the climate that can be identified […] by changes in the mean and/or the variability of its properties, and that persists for an extended period, typically decades or longer. Climate change may be due to natural internal processes or external forcing such as […] persistent anthropogenic changes in the composition of the atmosphere or in land use" (IPCC, 2014).

One of the most evident driving force of climate change is the global warming, defined as an increase in combined surface air and sea surface temperatures averaged over the globe and over a 30-year period (IPCC, 2019). Given the extreme complexity of the matter, it is difficult to correctly identify all the causes and effects of the climate change, although anthropogenic factors seem to be of pivotal importance among causes. Since the pre-industrial era for example, the emission of greenhouse gases has dramatically increased, driven largely by economic and human population growth. This has led to the increase of the atmospheric concentration of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), which ultimately are responsible for the acidification of the oceans, global warming and ozone layer damaging (IPCC, 2018). While extreme weather events such as extreme precipitation, flooding and long period of drought, together with glaciers melting and the increase of sea level are the most noticeable, climate change is impacting in a less sensational way also on other fields, both at local and global scale. As an example, we can cite the case of the Monarch Butterfly (*Danaus plexippus*), the most familiar North American butterfly. The eastern population of this insect is notable for its annual autumn migration from the Northern regions of the United States and Southern Canada to Mexico, during which they cover thousands of kilometres. More interestingly, circa 4 generations of Monarch Butterflies are necessary to undertake the northward migration, and thus female butterflies lay eggs during the journey. Over the last two decades, the eastern population of these butterflies has declined more than 80% at the overwintering sites (Thogmartin et al., 2017). Collectively, the major threats involved in the decline of Monarch Butterfly

population were the changes in suitable habitat conditions related to climate change, and habitat loss due to anthropogenic factors like deforestation.

Climate change is only the tip of the iceberg of the changes that the Earth is experiencing in these decades, and thus the term “global change” is sometimes more useful to indicate the current situation. In particular, global change refers to the complex of environmental changes that is occurring around the world as a result of the human activities. In particular, drivers for global change not only act on the physical, but also on the social environment on Earth (Sutherst, 2004). Notably, it is possible to distinguish between drivers with global origins and global impact, such as the high level of atmospheric carbon dioxide, and drivers with local origins but global impact, such as land use.

Anthropogenic drivers of global change other than the increase of greenhouse gases and global warming (i.e. acting more specifically on the physical environment) can be grouped in demographic, economic, socio-political and cultural, and scientific and technological drivers (Nelson et al., 2006). Overall, the global population has increased from approximately 2 billion at the beginning of the 20th century to more than 7 billion at the beginning of the 21th century (Roser et al., 2019), and is projected to reach about 10 billion by 2050 (Tilman et al., 2001). Population growth is linked to an increase in food demand and natural resources, thus enhancing crop production, livestock rearing and resource exploitation. Therefore, there is an increasing request of new areas not only for urbanization and agricultural and farming activities, but also for the creation of new infrastructures such as dams, water storage and power plants, that ultimately lead to deforestation and land use change (Sutherst, 2004). Moreover, the more economical mass transport and the liberalization of international trade has increased the number of people and the quantity of goods that travel daily around the world. In fact, if we consider international tourist arrivals only, this number has increased from 25 million in 1950 to more than 1.000 billion arrivals in 2015, and forecasts show that they will be more than 1.8 billion in 2030 (Glaesser et al., 2017). Moreover, several emerging countries such as Thailand and Malaysia are now gaining positions among the top destinations, replacing some developed regions such as Northern Europe (Glaesser et al., 2017).

Another main theme that surely characterize our historical period is the emergence of infectious diseases that represent a threat for the global health. In the first decade after the Second World War there was a consensus among scientists that the fight against infectious diseases, which affected humans for thousands of years, were finally ready to be won. This optimistic vision was supported by scientific discoveries (antibiotics such as penicillin and streptomycin, vaccines against smallpox, pertussis, diphtheria and tetanus, insecticide as DDT for the elimination of malaria and other insect-borne pathogens) and by a general improvement in life conditions, at least in the industrialized countries (Snowden, 2008). In 1962, the immunologist and Nobel Prize winner Sir Frank Burnet quote:

> One can think of the middle of the twentieth century as the end of one of the most important social revolutions in history, the virtual elimination of the infectious disease as a significant factor in social life.

Just a year before, the epidemiologist Aidan Cockburn stated that:

> it seems reasonable to anticipate that within some measurable time, such as 100 years, all the major infections will have disappeared (Cockburn, 1963),

and in 1979, the WHO announced the complete eradication of smallpox from the entire globe. The first and most important fact that undermined this positivistic vision was the outbreak of human immunodeficiency virus (HIV) infection/acquired immunodeficiency syndrome (AIDS) in the 1980s, which has affected almost 75 million people since the start of the epidemic and has led to the death of an estimated number of 32 million people worldwide (UNAIDS, 2019). Following the HIV outbreak, other important events roused the attention of the society in the 1990s and 2000s. In 1991, an epidemic outbreak of cholera occurred in Latin America, after being free of this disease for more than a century, with around 1 million cases and more than 10000 deaths recorded (Pan American Health Organization, 1995). In 1994, an epidemy of plague broke out in India, with around 700 cases and 56 deaths reported, being the most serious outbreak in recent years. In 1997-1998, a large outbreak of Rift Valley fever occurred in East Africa as a result of an increase in rainfall that consequently favoured the hatching of mosquito eggs, vector of the virus. Kenya, Somalia and the United Republic of Tanzania were interested by this epidemy, which resulted in more than 89000 cases in the sole North Eastern province of Kenya and Southern Somalia, with almost 500 reported deaths (World Health Organization, 1998). In 2000s, a severe new disease appeared and frightened the world: Severe Acute Respiratory Syndrome, or SARS. It is caused by a coronavirus and, starting from a province in Southern China, rapidly spread in other countries such as United States and Canada. Globally, 8096 cases and 774 deaths were reported (CDC, 2016). Most recently, an exceptional high number of measles cases has been reported in the past three years in Europe, due to insufficient vaccination coverage, leading to more than 12000 cases from May 2018 to April 2019 (ECDC, 2019).

The aforementioned disease outbreaks throughout the world represent several examples of emerging infectious diseases (EIDs). EIDs are defined as “diseases whose incidence has increased within the past two decades or threatens to increase in the near future” (Lederberg et al., 1992). This increase could be caused by a newly described pathogen (even a pathogen entered for the first time in the human population – e.g. HIV – or a new strain evolved from a pathogen – e.g. chloroquine-resistant malaria), but also by a well-recognized infective agent that, for several reasons, starts to become more common (e.g. Lyme disease) (Jones et al., 2008).

Several factors have been recognized as drivers for the emergence of infectious diseases; among others, the most important are (i) microbial adaptation and change, (ii) susceptibility to infection, (iii) climate, weather and the environment, (iv) economic development and land use, (v) human demographics and behaviour, (vi) technology and industry, (vii) international travel and commerce, (viii) breakdown in public health, (ix) poverty and social inequity, (x) war and conflicts, (xi) urban decay, (xii) lack of political will, (xiii) intentional biological attacks.

Due to their high growth rate, bacteria are particularly prone to develop genetic resistance to antibiotics. This resistance can be acquired not only by random mutations of the genetic materials, but also with the transfer of drug resistance-mediating genes (Barbosa and Levy, 2000). Therefore over time, in response to the use of antibiotics, bacteria have evolved several mechanisms to deal with the actions of these drugs, such as the inactivation of the compound by detoxifying enzymes, reduced cell permeability, expulsion of the drug by specific or non-specific molecular pumps, and modification of the antibiotic targets (Barbosa and Levy, 2000). Along with bacteria, also parasites have showed to develop resistance to drugs, leading to the re-emergence of several diseases. *Plasmodium falciparum* is one of the most intriguing examples. This hematoprotozoan parasite transmitted by anopheline mosquitoes is responsible for almost 200 million new cases of malaria each year and an estimated number of deaths of 435000 (WHO, 2019a). Since 1950s and 1960s, resistance to chloroquine and sulfadoxine-pyrimethamine, widely used as a first line treatment, have quickly developed, while resistance to mefloquine is currently restricted only to some limited areas such as Vietnam, Thailand and Cambodia (White, 2004). Similar to bacteria, resistance onsets in genes encoding or relating to drug targets or influx/efflux pumps that affect intraparasitic concentration of the drug (White, 2004). In Europe, the total consume of antibiotics per year in human medicine has been estimated in 7,000 tonnes (WHO, 2018), although humans are not the greater consumers of drugs worldwide. In fact, the global consumption of antimicrobial in food animal production has been estimated to be almost 63,000 tonnes per year, and is expecting to rise to more than 100,000 tonnes per year due to the increasing demand of animal protein from middle-income countries (Van Boeckel et al., 2015). Thanks to the emergence of drug-resistant strains, we are now assisting to the re-emerging of several bacteria and parasite species, such as methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, multidrug-resistant *Mycobacterium tuberculosis*, artemisinin-based combination therapies (ACT) resistant *P. falciparum* and multidrug-resistant *Leishmania infantum*.

Alongside the emergence of drug-resistant pathogens, there is the increase of human susceptibility to infections. Immune deficiency associated with AIDS, and with chemotherapy for cancer, immune-mediated diseases and organ transplantation has contributed to the enormous global increase in the number of immunocompromised people over the past few decades, with an estimated 1% of the world population being immunosuppressed (Morens et al., 2004). Moreover, the world population is currently grow older, with more than 8% of the total population aged 65 or older, and is expected to

rise further to reach almost 20% over the period between 2025-2050 (He et al., 2016). The rise in number of elder and immunocompromised people has been associated to the risk of re-emergence of several opportunistic infections (Morens et al., 2004). Pathogens such as *Pneumocystis carinii*, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, herpesviruses and various fungi are of particular concern for individuals with increased vulnerability, whether caused by HIV infection or immunosuppressive and cancer therapies used to treat cancer, immune-mediated diseases and for transplantation. Moreover, organ transplantation adds a further risk of infection, due to the possible presence of undetected pathogens in donor tissues (Morens et al., 2004).

Several researches in the past decades have established that temperature and other climatic variables strongly affect the physiology and demography of free-living and parasitic species (Walther et al., 2002). In particular, climate change is expected to have a substantial effect on the burden of infectious diseases, especially those that are transmitted by arthropod vectors and through contaminated water (Shuman, 2010). In the case of vector-borne diseases, the arthropod vector acts as an essential stage for pathogen development, reproduction and transmission from one host to another (Hunter, 2003). Given the fact that most of the vectors spend a considerable part of their life in the environment and only a short period of time on the vertebrate host, therefore they are particularly sensitive to climate changes. Temperature can affect both the distribution of the vector and the effectiveness of pathogen transmission through the vector. In particular, changes in temperature can increase or decrease the survival of the vector, and modify the rate of vector population growth, the feeding behaviour, the susceptibility of vector and the incubation period of the pathogen, and the seasonality of vector activity and pathogen transmission (Gubler et al., 2001). As an example of temperature influence on vector-borne diseases we can cite the northward spread of the Ixodid tick *Ixodes ricinus* that has occurred over the past two decades in Sweden (Lindgren, 1998; Lindgren et al., 2000). In particular, the shift in the northern limit of distribution of the tick species of almost 100 km, together with the higher incidence of Tick-Borne Encephalitis (TBE) between the early 1980s and the mid-1990s, has been linked to the occurrence of warmer summers and winters in this period (Lindgren et al., 2000). A change in temperature is not the only driver affecting the burden of vector-borne infectious diseases. The high concentration of atmospheric CO_2 has been showed to act as a fertilizer on plants, thus enhancing foliage production (Ramirez and Finnerty, 1996). Consequently, the increased density of plant foliage could provide more favourable microclimates for insect vectors, and the extended growth season of plants will effectively increase the duration of these favourable microclimate conditions (Sutherst, 2004). A change in rainfall duration and intensity has been supposed to impact on vector-borne diseases through different ways: increased rainfall could create increased surface water providing breeding sites for several vectors (i.e. mosquitoes), could increase vegetation and allow the expansion of vertebrate hosts population, and flooding deriving from extreme rainfall could force vertebrate hosts into closer contacts with each other (Gubler et al., 2001). In Australia and Papua New Guinea, the heavy rain

and flooding events that are increasingly occurring have been linked to outbreaks of Ross River virus fever, an arbovirosis that cause epidemic polyarthritis, due to the increasing breeding sites available for mosquitoes (Mackenzie et al., 2000). Concerning water-borne infectious diseases, climate change has been supposed to impact on the burden of these diseases through an increase of heavy rainfall, flooding and higher temperature. Several studies in different countries have showed that water-borne disease outbreaks occur more probably soon after heavy rain events (Willocks et al., 1998; Miettinen et al., 2001; Anon., 2000), and the main reasons are that heavy rainfall lead to changes in the direction of flow of water systems and in water channels, the overflow of storm drains that may be combined with the sewage systems, and the additional input of nutrients into water bodies that accompany heavy rainfall (Hunter, 2003). Moreover, increased temperature has been linked to cholera outbreaks. Notably, *Vibrio cholerae* is known to survive in marine waters in a viable form associated with algae and plankton that, in case of temperature increase, bloom, leading to higher incidence of cholera (Hunter, 2003).

Not merely the burden of vector-borne and water-borne infectious diseases can be affected by climate changes, but also diseases that rely on intermediate hosts to complete the life cycle, such as toxoplasmosis. A recent paper has observed a positive correlation between warmer climate in Europe and human toxoplasmosis in Czech Republic in the period between 1951 and 2003, with strong evidence of increasing incidence of this disease (Hubálek, 2005). The author has hypothesized that a warmer year could enhance the population of rodents, some of the most important intermediate hosts of *T. gondii*, thus resulting in an increased infection rate of these animals that ultimately lead to higher infection rate also in the definitive feline host, that could transmit the parasite to humans through oocysts.

As mentioned above, the increasing demands for food and natural resources enhance the use of natural areas for agriculture, livestock rearing and resource exploitation, leading to deforestation and fragmentation of natural habitats. In a recent paper, Gottdenker and colleagues (Gottdenker et al., 2014) have reviewed more than 300 scientific articles investigating the effects of anthropogenic land use changes on infectious disease dynamics, and more than a half of the analysed studies revealed an increased pathogen transmission in response to anthropogenic changes of the habitats. Notably, commonly reported mechanisms by which land use change altered infectious disease transmission included alteration of the vector, host, and pathogen niche; changes in host and vector community composition; changes in behaviour or movement of vectors and/or hosts; altered spatial distribution of hosts and/or vectors; socioeconomic factors and environmental contamination.

Population growth strongly affects the burden of infectious disease, and human population density has been showed to be a primary driver for disease emergence (Jones et al., 2008) In particular, more than 50% of global human population currently resides in urban areas, and this percentage is growing continuously (WHO, 2019b). In developing countries, urbanization is mainly the result of immigration from rural areas, and rapid evolution of urban centres is usually not accompanied by the

creation of adequate safe drinking water supplies and sanitation and garbage disposal, that ultimately results in the formation of breeding sites for arthropod vectors and high risk of water-borne and food-borne pathogen transmission (Macpherson, 2005). In developed countries, the economic mass transportation of goods has broadened the national eating habits, resulting in the consumption of exotic foods originating from many different parts of the world. Nonetheless, millions of people travel each day around the world, thus unwittingly exposing themselves to different pathogens and increasing the risk of acquiring water-, food- and air-borne diseases. Moreover, travellers can import pathogens on the way back and thus starting new outbreaks of infectious diseases, such the case of Chikungunya in France (Delisle et al., 2015). Notably, travel and tourism has been listed as the most important driver for infectious disease events, followed by water and food quality, natural environment, global trade and climate (Semenza et al., 2016). Finally, a change in the human lifestyle, such as the increase of outdoor activities and human encroachment into wild habitats, has been observed to be a driver for vector-borne diseases (Semenza et al., 2016).

As mentioned above, emerging infectious diseases are defined as “diseases whose incidence has increased within the past two decades or threatens to increase in the near future” (Lederberg et al., 1992). Analysing 335 infectious diseases emerged between 1940 and 2004, Jones and colleagues revealed interesting results (Jones et al., 2008). In particular, they observed that almost 60% of EIDs are caused by zoonotic pathogens, i.e. pathogens able to spread between animals and humans. In addition, more than 70% of these zoonotic EIDs are caused by pathogens with a wildlife origin, and the number of these EIDs has increased over time, confirming past suggestions (Weiss and McMichael, Anthony, 2015; Smolinski et al., 2003; Morens et al., 2004; Lederberg et al., 1992; King et al., 2006). In the past, several authors have supported the hypothesis that most emerging pathogens originate in wildlife and then spillover to human hosts as a result of a range of demographic, ecological and socio-economic changes (Morse, 1995; Lederberg et al., 1992; Krause, 1992; Daszak et al., 2000). Causes of novel disease emergence from wildlife are multiple, but anthropogenic interference in wild habitats seems to be the most important. Hunting and outdoor activities, tourism, farming and livestock rearing, pet and livestock trade, wildlife introduction and translocation, and importation of food have showed to influence spillover of wildlife pathogens to humans and livestock (Thompson, 2013). Moreover, pressure of human encroachment into wild habitats have showed to cause increased wildlife population densities in certain areas, thus resulting in a higher risk of emergence of infectious diseases (McCallum and Dobson, 1995; Daszak et al., 2000), as observed for Lyme disease in the United States (Levi et al., 2012) (see Fig. 1 for a summary of drivers for EIDs).

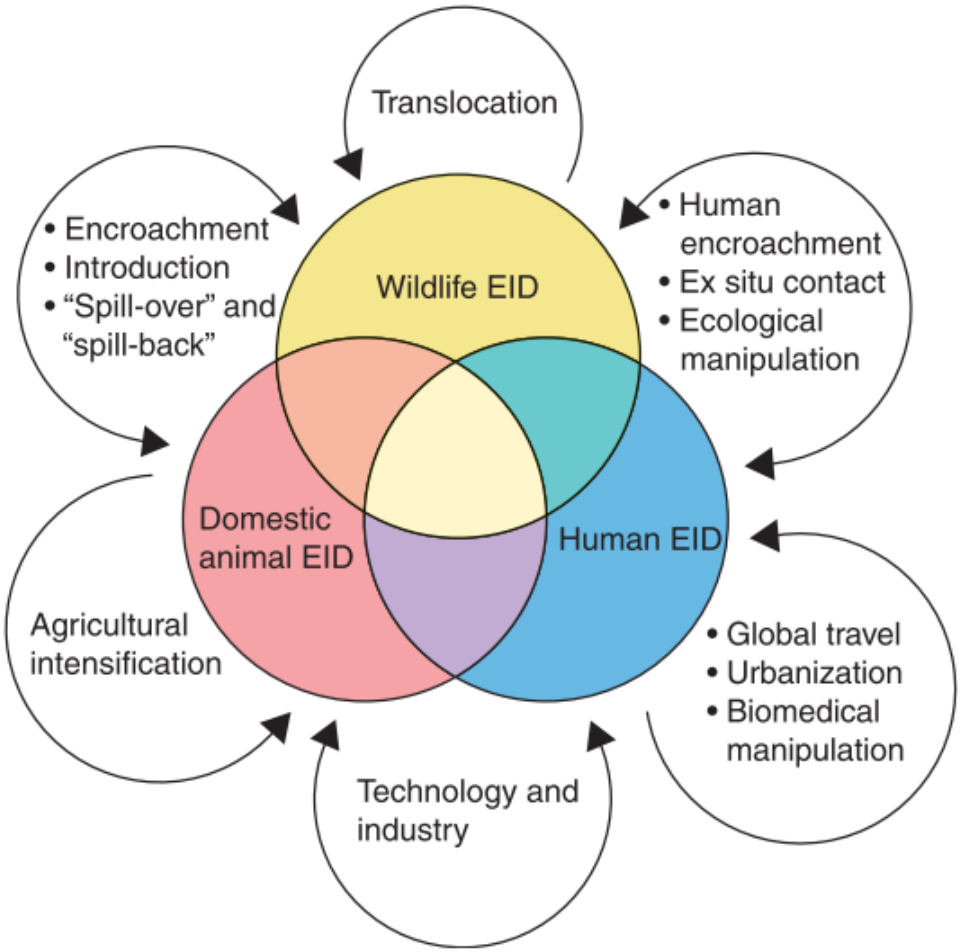

Fig. 1 The host-parasite ecological continuum (adapted from Daszak et al., 2000).

At the same time, several wildlife species have adapted to live in urban and suburban environments, dramatically altering the composition of wildlife communities and thus of the associated pathogens (Bradley and Altizer, 2007). In fact, urban-adapted species usually occur at much higher densities in urban and suburban environments than in less-disturbed habitats, given the abundant food resources accidentally (e.g. household waste) or intentionally (e.g. bird feeders) provided by humans. High population densities can increase contact rates among wildlife species, thus favouring the transmission of parasites. Moreover, in response to resource provisioning, increased birth rates among urban-adapted wild species could enhance parasite transmission by increasing the abundance of susceptible juvenile hosts (Bradley and Altizer, 2007).

Among pathogens responsible for EIDs events, Jones and colleagues have recognized bacteria and rickettsiae as the most represented, followed by viruses, protozoa and helminths that accounted for 25%, 10% and 3% of the EIDs events, respectively (Jones et al., 2008).

Vector-borne pathogens are some of the most likely to emerge. They are responsible for 23% of the emerging human infections (Jones et al., 2008), and their incidence is raising. Some of these

emerging vector-borne pathogens have an exotic origin (e.g. Chikungunya and West Nile virus in Europe and North America), therefore they have been introduced into new regions, while other are endemic species that have greatly increased in incidence (e.g. Lyme disease in North America and TBE in Europe). Factors influencing disease emergence are multiple and acting simultaneously, thus it is extremely difficult to describe them as single entities. Pathogen introduction in new areas is mainly driven by an increase in travel and trade (Kilpatrick and Randolph, 2012), that allow the spread of pathogens through the movement of infected persons, animals or vectors. In contrast, emergence of endemic pathogens has been linked to changes in land use and socioeconomic conditions. A change in land use can affects the incidence of vector-borne pathogens by altering the interaction between pathogens, vectors and hosts (both human and animal). Reforestation in eastern North America in the past few decades, for example, is thought to have allowed recolonization of natural environments by deer, and a consequent expansion of *Ixodes* scapularis, thus underpinning the emergence of Lyme disease occurred in the middle of the 20th century (Barbour and Fish, 1993). Several changes in socioeconomic conditions can drive the increase in incidence of vector-borne pathogens, such as human encroachment and expansion into natural habitats for exploitation, dwelling or recreational activities. Apart from the low-income people that are at risk for vector-borne infections due to the poor living conditions and the exploitation of natural areas for subsistence, it is worthy to note that the risk for tick exposure and *Borrelia* infection has been showed to be particularly high in high income people (Linard et al., 2007). In fact, outdoor recreational activities associated with wealth and residing in peri-urban areas rich of wildlife can increase the exposure to vectors. Other important drivers for vector-borne disease emergence are urbanization, strictly linked to land use change, deforestation and socioeconomic conditions; atmospheric and climate changes, that allow the survival of newly introduced vectors into previously unsuitable areas; development of insecticide and drug-resistance by vectors and pathogens; immunosuppression in hosts (Harrus and Baneth, 2005). Given the fact that most of the vectors spend only a small fraction of their lives on the hosts and the greatest part in the environment, there are particularly sensitive to climatic conditions. The abovementioned climate changes that are occurring in our days are thus very likely to influence the incidence of vector-borne pathogens by altering the distribution of vectors in a certain area. Currently, we are assisting to a northward shift of the distribution limit of *I. ricinus* in Europe (from 61°N up to 66°N), mainly related to a reduction in the number of winter days with temperature below -12 °C that ultimately lead to higher survival rate of overwintering ticks (Lindgren et al., 2000; Gray et al., 2009). Moreover, *I. ricinus* has been observed to move not only in term of latitudinal expansion, but also altitudinal (Omeragic, 2011; Medlock et al., 2013; Danielová et al., 2008; Daniel et al., 2003). Ticks are not the only example of vector expansion as a consequence of climate changes. Sand flies of the genus *Phlebotomus* are the most important vectors of *Leishmania* spp. in the Old World, that is estimated to cause around 70,000 human death each year (Arenas et al., 2017). While considered a tropical disease, *Leishmania* is currently spreading to temperate countries

due to the expansion of sand flies into areas previously free of these vectors (Maroli et al., 2008; Ferroglio et al., 2005) (see Fig. 2 for a summary of drivers associated to vector-borne disease emergence).

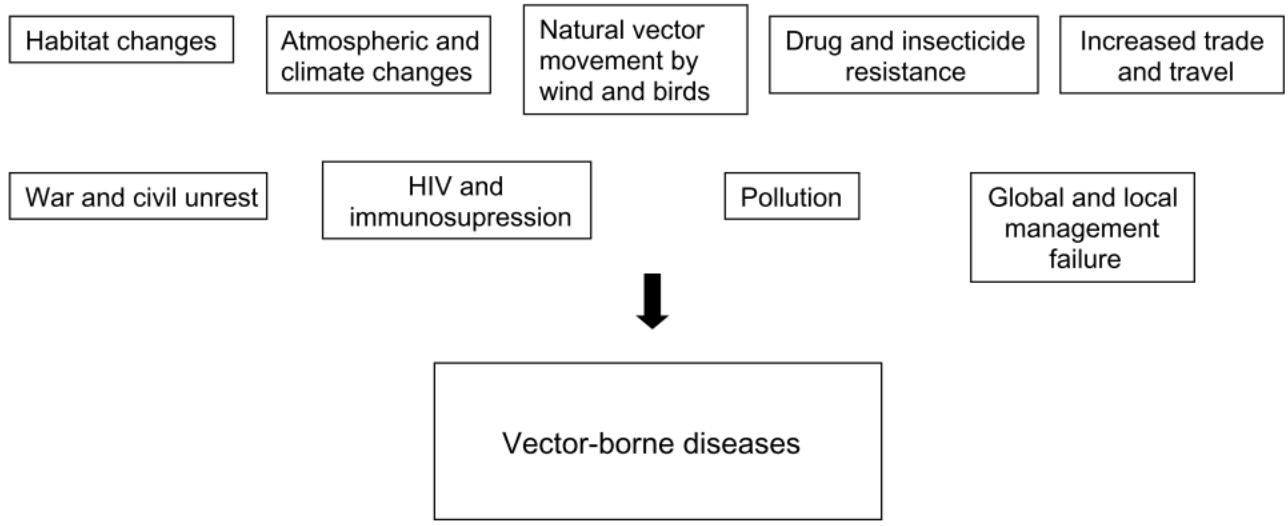

Fig. 2 Major drivers associated to vector-borne disease emergence (adapted from Harrus and Baneth, 2005).

Vector-borne diseases have a considerable impact on human health, especially in tropical and subtropical countries. Malaria, leishmaniasis, African trypanosomiasis and dengue account for thousands of death each year (World Health Organization, 2002). Even if the fatality rate of several vector-borne pathogens occurring in Europe and North America is low, the burden of these diseases is high in terms of prevention, diagnosis, treatments, such as the 2.5 billion dollars over 5 years estimated for Lyme disease prevention in United States (Maes et al., 1998). Not only humans are threatened by emerging vector-borne diseases, but also companion animals, livestock and wildlife species are susceptible to several of these pathogens.

In a recent paper, Semenza and colleagues found out that most of the infectious disease events that have occurred in Europe in the period between 2008 and 2013 have been caused by pathogens transmitted through the consume of contaminated food or water (Semenza et al., 2016). Several factors have been listed as drivers of food-borne parasite emergence, such as (i) changing eating habits with the consumption of exotic, raw or undercooked food, (ii) rapid population growth and shift to urbanized areas, (iii) global market in vegetables, fruit, meat ethnic food and farm animals, (iv) improved transport logistic and conditions, (v) global travelling of humans, (vi) shift from low- to high-protein food consumption and consequent request of meat and fish products worldwide, (vii) higher proportion of immunologically compromised individuals as a consequence of increasing elderly population or patients with immunosuppressive treatments or diseases, (viii) shift in farming

practices, with intensification in developing countries and free-range and organic animal production in developed countries to respond to consumer welfare concerns, (ix) climate change, that favour the presence of parasite intermediate hosts or vectors (Broglia and Kapel, 2011).

In recent years we have assisted to an increasing demand for exotic food and preparations, mostly in developed countries, that is a consequence of enhanced travelling, economic prosperity and mass media influence. In particular, the consume of raw or undercooked foods is mainly associated to the risk of parasitic infections, such as sushi for Anisakis, seafood for *Cryptosporidium* and raw vegetables for *T. gondii* and Giardia (Broglia and Kapel, 2011). However, the most important source of infection is represented by raw or undercooked meat and meat preparations, both from livestock or game meat, that is associated to several parasitic infections such as *T. gondii*, *Taenia solium* and *Trichinella britovi*. Even if intensive farming of livestock is growing in order to answer the increasing demand of food worldwide, public opinion on animal welfare and ecological impacts of food production is moving toward organic farming and a more sustainable agriculture. As a consequence, free-ranging livestock is more exposed to parasitic infections through increased contacts with wildlife and the environment (van der Giessen et al., 2007). Finally, climate changes have showed to impact also on food- and water-borne diseases, mainly by influencing and modifying population dynamics of both definitive and intermediate hosts (Broglia and Kapel, 2011). As an example, we can cite a study analysing the effect of warmer temperatures in Europe on the human seroprevalence of *T. gondii* in Czech Republic, in which the author observed that higher seroprevalence of the parasite correlated with warmer years (Hubálek, 2005). In particular, this finding may be explained by the increased survival of rodents and micromammals, important reservoir for this parasite, during warmer winters.

Given the striking burden of emerging infectious diseases (especially zoonotic diseases) on human and animal health, and the importance of both veterinary and human medicine to face them, in the last decades was coined the concept of One Health. In particular, the One Health approach aim to create inclusive collaborations between physicians, veterinarians, wildlife managers, conservation biologist, environmental scientists and ecologist, in order to face EIDs and to provide health for humans, animals (wildlife, livestock, pets) and the environment in which they all live (Thompson, 2013)

The aim of this doctoral project was to evaluate the epidemiology of three different zoonotic protozoa, *T. gondii*, *Babesia* spp. and *L. infantum*. In particular, the aim was to assess the risk of transmission between animals and humans, and to fill some of the gaps of the existent literature on the prevalence and circulation of these parasites in the studied area. The research project had as common goal and framework the “one-health” concept, thus attempting to investigate the epidemiology of the three parasites by assessing in different ways, each one of the components of the One Health triad (humans, animals and environment).

These pathogens were chosen for their considerable impact on both human and animal health, and due to their emergence in some parts of Europe. In particular, they are perfect examples of how global and local changes can act on the epidemiology of infectious diseases, because each of these parasites is mainly influenced by different drivers.

L. infantum and *Babesia* spp. are vector-borne pathogens with sand flies and ticks as vectors, respectively. Among other drivers, vector-borne pathogens are emerging because of climate and habitat changes, both at global and local scale. As widely discussed in the next sections, sand flies are small insects requiring warm climatic conditions to survive and develop, and in recent decades we have assisted to the northward shift of their distribution in Europe, coupled with the shift of *L. infantum* infection in both humans and dogs. Precisely dogs and humans are the subjects of paper 10, in which the prevalence and circulating strains of *L. infantum* are investigated in these hosts, together with sand fly expansion as a consequence of climate changes. Moreover, the prevalence of *L. infantum* was also assessed on wild carnivores from Western Alps (paper 6), to point out possible sylvatic reservoirs of this parasite. Ticks are arthropod vectors able to transmit protozoa such as *Babesia* and *Theileria*, and bacteria such as Anaplasmataceae, SFG *Rickettsiae* and *B. burgdorferi* s.l. Ticks spend most of their lives in the environment, and thus they are extremely influenced by climate and habitat conditions. In particular, habitat fragmentation and changes in agricultural practices, increase of wildlife hosts (large and small mammals) and human encroachment into wild habitats are some of the most important drivers of tick-borne pathogen emergence. In this PhD project, the presence of *Babesia* spp. and other TBPs was evaluated in wild carnivores (paper 6) and in ticks collected from humans (paper 7) , privately-owned dogs (paper 4 and 5) and wild animals (migratory birds – paper 8). Moreover, a habitat suitability model on the occurrence of *Babesia* is proposed and field validated with dragging results (paper 9).

In contrast to *Babesia* spp. and *L. infantum*, *T. gondii* is a food-borne parasite whose life cycle is based on the presence of definitive and intermediate hosts. Even if moderately influenced by climate and habitat changes, the emergence of *T. gondii* in some regions has been mainly associated to a change in eating habits, lifestyle and food production, such as the consume of raw or undercooked foods, ethnic food, organically-raised livestock products and free-ranging game meat. Circulating genotypes of *T. gondii* in domestic and sylvatic animals from Northwestern Italy are the focus of paper 1, while paper 2 and 3 discuss about the prevalence and viability of *T. gondii* in ewe milk and cheese made from unpasteurized milk, in order to evaluate the risk for humans deriving from the consume of dairy products. Given the high devotion of Umbria region (Central Italy) for ewe farming and traditional cheese production, these studies were carried out on bulk milk collected from flock originating from this region.

2. *TOXOPLASMA GONDII*

2.1. DISCOVERY AND TAXONOMY

Toxoplasma gondii was identified for the first time in 1908 by Splendore in a laboratory rabbit in Brazil, and contemporarily by Nicolle and Manceaux in Tunisia (Dubey, 2009a). The name *Toxoplasma* derives from the Greek terms *toxon* = bow and *plasma* = creature, that recall the half-moon shape of tachyzoites, while gondii indicates the *Ctenodactylus gundi*, a hamster-like rodent in which *Toxoplasma* was firstly identified (Dubey, 2008).

First reports of *T. gondii* infection in humans occurred few years later, when Castellani reported *T. gondii*-like parasites in blood and spleen smears of a young boy from Ceylon who died for a disease characterised by anaemia, fever and splenomegaly (Castellani, 1914). In 1938, the pathologists Wolf, Cowen and Paige were the first to conclusively identified *T. gondii* as a cause of human disease, while Sabin clearly demonstrated that human strains of this parasite were biologically and immunologically identical to those obtained from animals (Weiss and Dubey, 2009). In the next decades, *T. gondii* has been widely studied, and its complete life cycle, transmission routes and genetics has been extensively figured out (Weiss and Dubey, 2009; Dubey, 2009a).

2.2. LIFE CYCLE AND BIOLOGY

T. gondii is a heteroxenous parasite able to infect all warm-blood animals (mammals and birds). Its life cycle is characterised by a sexual phase occurring within the intestine of definitive hosts (represented by feline) alternated by asexual phases within various tissues of intermediate hosts. It belongs to the phylum Apicomplexa, family Sarcocistidae. In contrast to other members of this family which require the presence of both the definitive and the intermediate hosts to close the life cycle, *T. gondii* can be transmitted, apart from definitive to intermediate host (and vice versa) through a prey-predator mechanism, also from intermediate to intermediate and from definitive to definitive hosts, enabling *T. gondii* to be one of the most successful parasite in the world.

This parasite presents three different shape: tachyzoite, bradyzoite and oocyst. The term tachyzoite (from the Greek term *tachos* = speed) is used to indicate the stage that rapidly multiplicate in any cell of the intermediate host and in non-intestinal cells of the definitive host (Dubey and Lindsay, 1998). Bradyzoites are the stage of *T. gondii* that can be found within cysts in various tissues of the intermediate host, and their name derives from the Greek term *brady* = slow, due to their slow replication rate (Dubey and Lindsay, 1998). The latest stage of the parasite, the oocyst, is shed by the definitive feline host at the end of the sexual reproduction of *T. gondii* within the intestinal cells

of the host (Dubey and Lindsay, 1998). All these stages are, with some differences, infective both for the definitive and the intermediate host.

2.2.1. DEVELOPMENT IN THE DEFINITIVE HOST

The definitive host can be infected through the consume of bradyzoites-containing tissue cysts (mainly by carnivorism on infected prey), through contacts with sporulated oocysts and vertically by congenital infection and transplacental transmission of tachyzoites.
Bradyzoites has been proved to be more infective for feline definitive hosts than oocysts. In fact, bioassay studies on experimentally infected cats has showed that, while 100% of cats fed with tissue cysts shed oocysts, less than 30% of cats fed with tachyzoites or oocysts shed oocysts as a consequence of *T. gondii* infection (Dubey and Frenkel, 1976). Moreover, one bradyzoites has proved to be sufficient to initiate the infection and thus to observe oocysts shedding in cats (Dubey, 2001). Bradyzoite-induced cycle in feline hosts has been widely studied. After the ingestion, *T. gondii* starts the merogonic phase of the reproduction, in which five morphologically distinct asexual types are formed within the intestinal cells of the host. Thanks to this process, *T. gondii* rapidly increase in number leading to a massive infection. After this process, merozoites starts to differ in macro- and microgametes that fuse together to produce zygotes, and ultimately the oocysts. The prepatent period in feline, corresponding to the merogonic phase and zygote production, last for 3-10 days in case of bradyzoite-induced infection, while is longer for tachyzoites (more than 18 days) and oocysts (more than 13 days) (Dubey, 1998a).

2.2.2. DEVELOPMENT IN THE INTERMEDIATE HOST

The intermediate host can be infected by *T. gondii* through the ingestion of sporulated oocysts, through the consume of bradyzoites-containing tissue cysts and vertically by congenital infection and transplacental transmission of tachyzoites.
While definitive hosts acquire the infection mainly by bradyzoites, oocysts have been proved to be the most effective infective stage for intermediate hosts. In fact, while cats fed with 10 oocysts did not become infected, experimentally infected mice and pigs fed with 1 oocyst showed symptomatic infections (Dubey, 1998a). Both oocysts and bradyzoites ingested by the intermediate host develop in tachyzoites in the intestine lumen, starting to multiply rapidly and invading extra-intestinal organs within few days post infection. As soon as they reach various extra-intestinal organs, tachyzoites start to convert to the bradyzoite stage, forming tissue cysts containing thousands of bradyzoites.
Compared to oocysts, bradyzoites are less infective to intermediate host. In mice, consistent infections were induced only by feeding 1000 bradyzoites, probably because a proportion of them are destroyed in the lumen of the gut (Dubey, 2007).

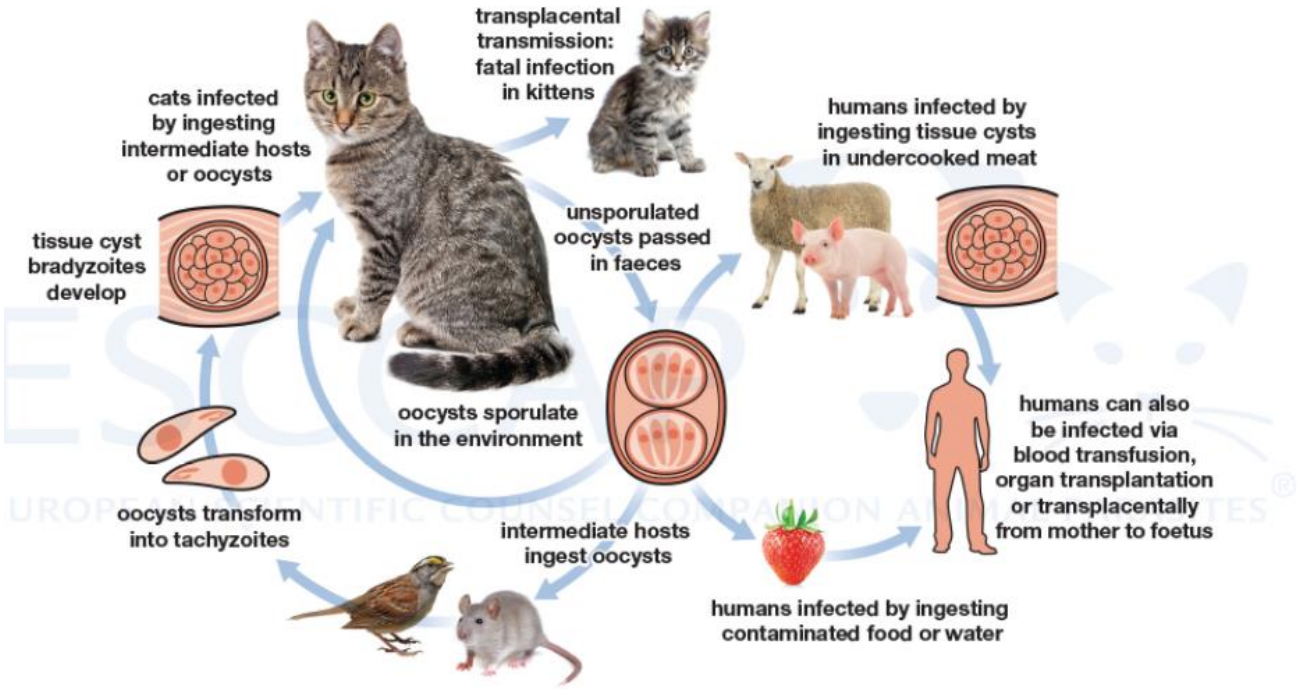

Fig. 3 Life cycle of *T. gondii*. Adapted from ESCAAP.

2.3. IMMUNOLOGY

Cellular immunity is the key component of the host's immune reaction against *T. gondii* (Schluter et al., 1991; Lindberg and Frenkel, 1977). In particular, macrophages, T lymphocytes and "natural killer" cells, together with cytokines, are the major components involved (Filisetti and Candolfi, 2004).

The primary immune response starts with the recognition of *T. gondii*-specific molecules by TLR receptors of macrophages and dendritic cells, and the consequent production of several inflammatory cytokines and chemokines, such as IL-12, IL-6, IL-1, TNF-α and IL-10 (Sasai et al., 2018). All these effectors mediate the activation of NK cells and T cells, producing INF-γ, and the recruitment of neutrophils and inflammatory monocytes to the infected site (Sasai et al., 2018).

In particular, *T. gondii* replication and its destruction is mediated by various effectors: (i) oxidative mechanisms; (ii) non-oxidative mechanisms with the production of nitrogen monoxide (NO) by macrophages activated by IFN-γ; (iii) induction by IFN-γ of indoleamine 2,3-dioxygenase, which degrades the tryptophan required for growth of the parasite (Filisetti and Candolfi, 2004). Apart from the cytotoxic effect of the abovementioned mechanisms, lymphocyte CD8+ are also able to directly kill the parasite. In particular, they are activated by IL-2 secreted by T CD4+ and able to recognize

T. gondii-infected cells by class-I MHC mechanism, and exert their cytotoxic activity by IFN-γ (Filisetti and Candolfi, 2004).

2.4. GENETIC

The sexual reproduction of *T. gondii* that occurs within the intestinal cells of feline definitive host allow the recombination of the genetic material of the parasite. However, first evidences of discrete strains of *T. gondii* came from the analysis of isoenzyme profiles for a large number of isolates of French origin, which showed non-random association of different isoforms for a variety of enzymes and thus the existence of discrete strains (Dardé et al., 1992, 1988). Subsequent genetic analysis confirmed these first observations, leading to the conclusion that the majority of the strains identified in Europe and North America fall into one of three distinct genotypes, called genotype I, II and III (Sibley and Boothroyd, 1992; Sibley, 1995; Ajzenberg et al., 2004). Detailed genetic analysis revealed than within-type variation is extremely rare, probably less than 1% (Su et al., 2006; Fazaeli et al., 2000). Type I strains are extremely virulent in mice and grouped together as monophyletic (Sibley and Boothroyd, 1992), with LD_{100} in mice of 1 tachyzoite (Su et al., 2006). In contrast, type II and III strains are mild virulent in mice ($LD_{100} > 10^3$ tachyzoites) and causing the majority of human and livestock infections (Su et al., 2006; Grigg and Sundar, 2009). The presence of clonal population structure of *T. gondii* is mainly explained by the fact that the sexual cycle of the parasite is not obligatory (although is important in allowing the species to occupy various niches). Indeed, transmission via ingestion of bradyzoites within tissue cysts (mainly by carnivorism and scavenging) between intermediate hosts is possible, and allows the propagation of the parasite in the absence of definitive hosts (Boothroyd and Grigg, 2002). This aspect is clearly demonstrated by the high seroprevalence of *T. gondii* in the arctic foxes (*Vulpes lagopus*) from Svalbard archipelago. In these islands, domestic and wildlife felines are naturally absent, although foxes showed a seroprevalence up to 43%, highlighting the possible propagation of the parasite without the presence of definitive hosts (Prestrud et al., 2007). A second aspect that can contribute to the clonal structure of *T. gondii* is the fact that the parasite is haploid. Thus, a cat infected with only one strain will yield oocysts containing progeny that are genetically identical to the original infecting strain (Boothroyd and Grigg, 2002).

In contrast to the clonal population structure of *T. gondii* typical of Europe and North America, strains identified in both human and animal cases of toxoplasmosis in South America showed high genetic variability and the presence of recombinant alleles (Su et al., 2012; Shwab et al., 2014). However, recombinant “atypical” strains of *T. gondii* are increasingly recognized, especially in wild animals from areas previously reported to be genetically homogeneous (Verin et al., 2013; Mancianti et al., 2013b; D. C. Herrmann et al., 2012; Calero-Bernal et al., 2015; Battisti et al., 2018; Bajnok et al., 2015).

Although different strains of *T. gondii* are known to have different impacts on mice in terms of virulence (Su et al., 2006), no consensus data are present about the impact of several genotypes on human infection. The critical point regarding the study of genotypes in humans is the fact that the majority of human infections are not reported due to the absence of symptoms or the presence of a mild symptomatology. Thus, it is uncertain if the observed frequency of the various strains reflects the distribution of these strains in human infection as a whole, or is biased toward certain strains in the more severe scenarios (Boothroyd and Grigg, 2002). Nevertheless, ocular toxoplasmosis, which is one of the most common sequela of both congenital and acquired infection, has been mainly associated to genotype I and atypical genotypes (Xiao and Yolken, 2015), whereas genotype II seems the most frequently reported in cases of congenital toxoplasmosis (Rico-Torres et al., 2016). However, differences in the frequency of various strains have been reported, such the case of prevalent type II infection in congenital toxoplasmosis in France and of type I in Spain (Rico-Torres et al., 2016). Even if genotype II is the most common genotype infecting immunocompromised persons like AIDS patients (Xiao and Yolken, 2015), genotype I is mainly associated to a more severe symptomatology in these patients (Khan et al., 2005). *T. gondii* infection has also been linked to a higher risk of psychosis and schizophrenia in humans, and again genotype I seems the most commonly associated (Xiao et al., 2009).

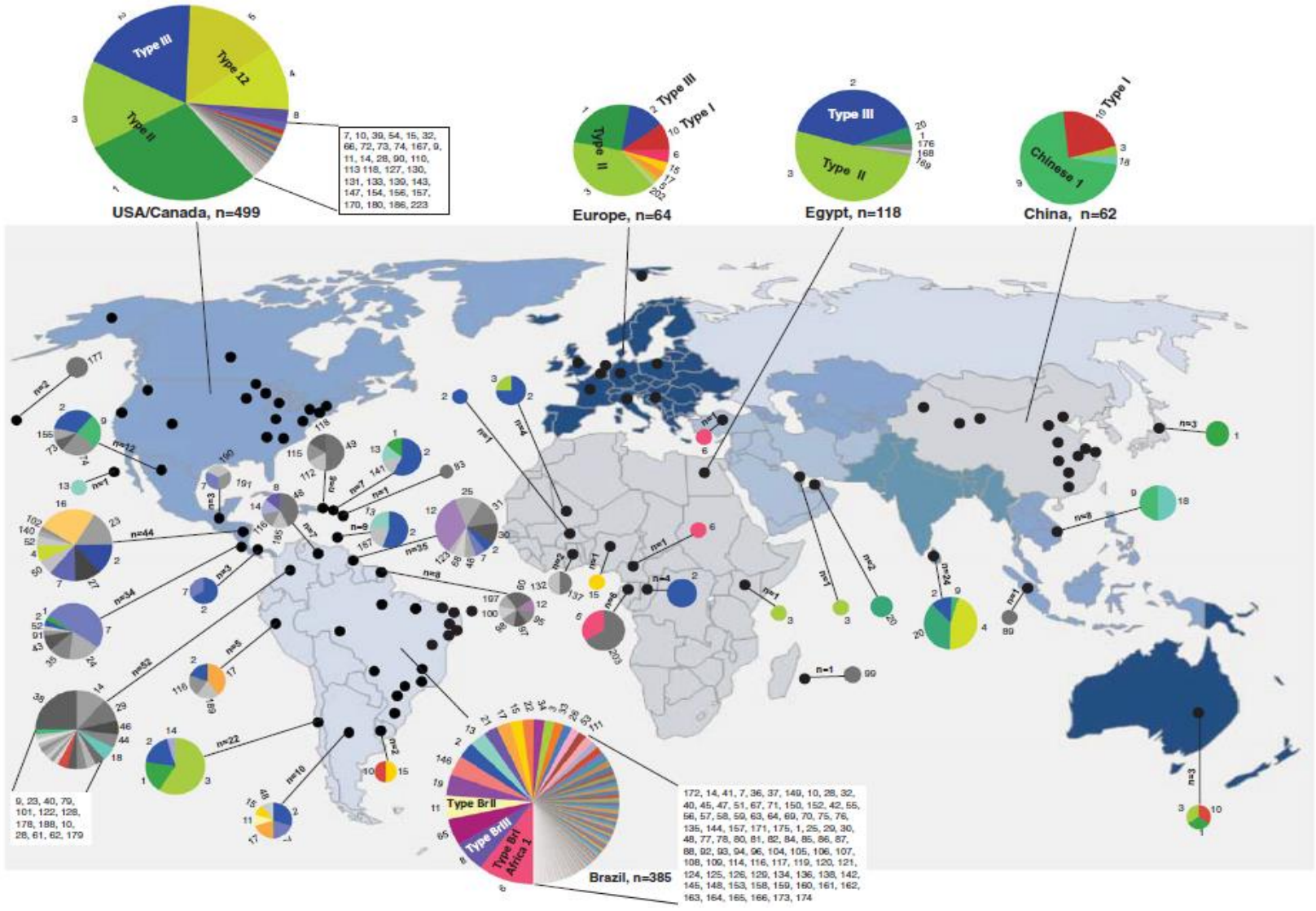

Fig. 4 Geographical distribution of *T. gondii* genotypes across the world and relative frequency (adapted from Shwab et al., 2014).

2.5. TRANSMISSION ROUTES

Intermediate hosts can acquire *T. gondii* infection mainly through (i) ingestion of sporulated oocysts contaminating food and water, (ii) ingestion of bradyzoites by carnivorism on infected preys, (iii) vertical transmission of tachyzoites from mother to foetus.

Oocyst-mediated infection is a common route of infection for intermediate hosts, and the only possible for herbivorous species. After a primary infection, cats or other wild felines can shed more than 100 million oocysts in the environment, thus representing an important source of environmental contamination (Frenkel, 2000; Dubey, 1996). Oocysts are particularly resistant in the environment, remaining infective for up to 18 months in moist soil or sand (Frenkel, 2000). Under laboratory conditions, sporulated oocysts have showed to survive at 4 °C up to 54 months and at -10 °C for 106 days (Dubey, 1998b). However, they are rapidly inactivated by heating to 55-60 °C for 1-2 min (Dubey, 1998b). Moreover, given the low permeability of the oocyst wall, they are also resistant to all the most common disinfectants (Frenkel, 2000). Animals can come in contact with *T. gondii* oocysts in case of feline faecal contamination of feed, pasture or water. The most striking example is the high level of *T. gondii* infection in Southern sea otters (*Enhydra lutis nereis*) in California. The recent population decline of this species has been associated to higher rate of mortality due to protozoal meningoencephalitis caused by *T. gondii* infection (Conrad et al., 2005). In particular, shellfish and bivalves such as *Chlorostoma brunnea, Chlorostoma montereyi* and *Promartynia pulligo* are the main food sources for sea otters, that due to their role as filter feeders, are able to accumulate a large quantity of oocysts (Krusor et al., 2015). The main source of oocyst contamination for Californian sea otters seems to be mainly domestic cats. In fact, even if they showed a lower seroprevalence compared to wild felines such as cougar (*Puma concolor*) and bobcat (*Lynx rufus*), the elevated number and wide distribution of domestic cats make the latter the ultimate responsible for the high rate of infection in sea otters (Vanwormer et al., 2013). Oocyst-mediated infection in humans can be acquired through accidental or intentional contact with feline faeces (e.g. during gardening or cleaning cat litter), although oocysts shed by felines are not immediately infective but require almost 24 hours to sporulate. Globally, from 6% to 17% of human infections have been ascribed to soil contact and oocyst ingestion (Tenter et al., 2000). Oocysts can also contaminate food (e.g. fresh vegetables) and water. Several outbreaks of human toxoplasmosis have been associated to contaminated food or water, such as the outbreak observed in Vancouver in 1995, in which more than 100 people displayed signs of acute toxoplasmosis following the contamination of municipal water system by infected feline faeces (Bowie et al., 1997). Ingestion of contaminated fresh vegetables has been associated to higher risk of congenital toxoplasmosis and postnatally acquired toxoplasmosis in the United States (Torrey and Yolken, 2013).

Even if bradyzoites seems to be less infective for intermediate than definitive hosts, carnivorism on infected prey is an important source of infection for several species, including humans. The number

and localization of tissue cysts within a certain host clearly depend on the intermediate host species. Notably, tissue cysts are more commonly seen in infected pigs, sheep and goats compared to poultry, horses, cattle and buffalo, even if up to 92% of cattle developed antibodies after primary exposure to *T. gondii* (Tenter et al., 2000). In general, the importance of an animal species as a source for human infection is given by (i) the prevalence of infection, (ii) the potential of *T. gondii* to establish as tissue cysts in this species, (iii) the density of tissue cysts in meat, and finally (iv) the regional preferences of humans regarding the sources and the meat preparation (e.g. consumption of raw or undercooked meat) (Schlüter et al., 2014). A recent meta-analysis has investigated the prevalence of *T. gondii* in beef, pigs and sheep meat across the world, reporting an overall prevalence of 2.6%, 12.3% and 14.7%, respectively (Belluco et al., 2016).

Livestock management is extremely important in reducing *T. gondii* infection in meat-producing animals. In particular, intensive farm management with adequate measures of hygiene, confinement and prevention are required, such as (i) keep the animals indoor throughout their life-time, (ii) keep the sheds free of rodents, birds and insects, (iii) feed the animals with sterilized food, (iv) control the access of pet animals (e.g. cats) to sheds and feed stores (Tenter et al., 2000). In recent years, public opinion on animal farming move towards a more “friendly” animal management, with free-ranging livestock (Cornish et al., 2016). One of the most important disadvantage of free-ranging management of livestock is the reduced biosecurity, with higher contacts with wild reservoir of the parasite (van der Giessen et al., 2007). In free-raised animals, *T. gondii* has been recorded with considerable higher prevalence than intensive raised animals (van der Giessen et al., 2007; Kijlstra et al., 2004; Bacci et al., 2015). Moreover, animals with outdoor access may be at greater risk of infection with atypical, virulent strains of *T. gondii* (Dubey and Su, 2009). Also game meat is a source of infection, with prevalence up to 70% in commonly consumed wild animals (Waap et al., 2016; Vikøren et al., 2004; Gauss et al., 2006; Ferroglio et al., 2014; Calero-Bernal et al., 2015; Aubert et al., 2010). Given the fact that both wild animals and the consume of game meat have increased in recent years in same parts of Europe (Côté et al., 2004; Avagnina et al., 2012), the risk of infection from these animals is not negligible.

Tachyzoites are essential in the vertical transmission of *T. gondii* from mother to the foetus. Most of congenital infections are a consequence of primary infection in the pregnant woman, although cases of *T. gondii* transmission that occurred in women that have become infected shortly before the pregnancy and women with immunosuppression and consequent reactivation of the parasite have been observed (Lindsay and Dubey, 2011). During the infection of *T. gondii* in a pregnant woman, tachyzoites invade several tissue including the placenta, by means of which they can reach the foetal circulation and tissues (Tenter et al., 2000). The incidence of congenital infection has shown to vary from 1 to 310 per 10,000 births based on country, ethnicity and exposure to risk factors (Tenter et al., 2000). In particular, the risk of intrauterine infection, development of sequelae and the severity of clinical manifestations strongly depend on the time of maternal infection during pregnancy, the

immunological competence of the mother at the time of the infection, the number and virulence of *T. gondii* strains infecting the pregnant woman, and the age of the foetus at the time of the infection (Tenter et al., 2000). Primary infection occurring in the first trimester of pregnancy shows around 14% of probability to be transmitted to the foetus, while this probability increases to 59% if the primary infection occurs in the last trimester (Chatterton, 1992). On the contrary, clinical manifestations of congenital toxoplasmosis in the foetus are more severe in case of infection occurred in the first trimester of pregnancy, with abortion, neonatal death and Sabin triad (retinochoroiditis, intracranial calcifications and hydrocephalus). In case of congenital infection occurring at the third trimester, most of the infected children are asymptomatic at the birth time (Tenter et al., 2000).

Immunosuppression is responsible for the reactivation of latent *T. gondii* infection and the dissemination of tachyzoites throughout the body. Tissue cysts that resulted from a primary infection of the parasite may accidentally rupture and release bradyzoites. In immunocompetent persons, the dissemination of bradyzoites activate the immunological response that promptly eliminate the circulating parasites and prevent parasitaemia. In immunocompromised patients, such as those with HIV-AIDS, Hodgkin's disease, or treated with immunosuppressive therapies, the release of bradyzoites is not followed by an immune response, thus tachyzoites are free to disseminate throughout the body and cause severe symptomatology, like toxoplasmic encephalitis (Tenter et al., 2000). Disseminated toxoplasmosis can complicate also organ or bone marrow transplantations, either in case of transplant from a *T. gondii*-infected donor to an uninfected recipient or for reactivation of a latent infection in the recipient due to immunosuppressive therapies.

Tachyzoites play a major role in vertical transmission and in the case of reactivation in immunosuppressed persons, but they are usually less important in horizontally transmission of *T. gondii* due to their susceptibility to environmental conditions. Moreover, it is generally though that tachyzoites are sensible to proteolytic enzymes and thus they are rapidly destroyed by the gastric digestion. However, a recent study has showed that tachyzoites are able to survival for a short period of time in acid pepsin solutions, and milk extend their viability in acid conditions (Koethe et al., 2017). Up to now, confirmed cases of acute human toxoplasmosis by ingestion of infected milk have been recorded only for unpasteurized goat's milk (Skinner et al., 1990; Sacks et al., 1982), although the parasite has been observed in the milk of many other species (Veronesi et al., 2018; Mancianti et al., 2013a; Fusco et al., 2007; da Silva et al., 2015) (PAPER 2). However, the finding of *T. gondii* DNA do not necessary imply the presence of viable parasites (Ranucci et al., submitted) (PAPER 3).

2.6. HUMANS

Primary infection in immunocompetent persons is usually asymptomatic and self-limiting (Montoya and Liesenfeld, 2004), although lymph node enlargement, asthenia, headache, fever and weight loss could be observed (Neves et al., 2009). In recent years has become clear that *T. gondii* infection is associated to higher risk of developing schizophrenia, autism, obsessive compulsive disorders and bipolar disorders (Elsheikha et al., 2016). Moreover, *T. gondii* infection has been linked to higher risk of car accident and suicide (Torgerson and Macpherson, 2011).

As for the immunocompetent, congenital infection is asymptomatic in most of the cases (up to 75% of infected newborns). However, if the infection is acquired in the first trimester of pregnancy, abortion, neonatal death and miscarriage can occur (Tenter et al., 2000). Involvement of central nervous system is a hallmark of congenital toxoplasmosis, with retinochoroiditis, intracranial calcifications and hydrocephalus being the most common signs (collectively identified as the Sabin triad) (McAuley, 2014). Other findings that characterized intrauterine transmission are fever, microcephaly, hepatosplenomegaly, jaundice, convulsions, abnormal cerebrospinal fluid, rash (maculopapular, petechial, or both), myocarditis, pneumonitis and respiratory distress, hearing defects, an erythroblastosis-like picture, thrombocytopenia, lymphocytosis, monocytosis, and nephrotic syndrome (McAuley, 2014). Even if parasite transmission occurred, around 80% of the newborns have a subclinical infection and thus they appear healthy at birth (Tenter et al., 2000). However, up to one third of the prenatally infected infants have showed to develop sequelae of the infection (Rothova, 2000; Brezin, 2000), such as chorioretinitis, strabismus, blindness, hydrocephalus or microcephaly, cerebral calcifications, developmental delay, epilepsy, or deafness months or years later (McAuley, 2014).

The involvement of central nervous system is also typical of *T. gondii* infection in immunocompromised patients, in addition to myocarditis and pneumonitis (McAuley, 2014).

3. *BABESIA*

3.1. DISCOVERY AND TAXONOMY

Even if a disease causing blood urination in cattle was described as early as in the Bible, it was at the end of the 19th century that the Romanian microbiologist Victor Babeş discovered the causative agent of what is now recognized as cattle babesiosis (Babeş, 1888). In the same years, the medical doctor Theobald Smith described the agent of Texas fever in USA, naming it *Pyrosoma bigeminum*, and was the first to discover the link between the disease and ticks (Smith and Kilbourne, 1893; Smith, 1889). However, the name *Pyrosoma* was already used for a genus of free-floating colonial tunicates, and therefore the name *Babesia* was proposed by Starcovici in 1893 (Starcovici, 1893). Since then, many different species belonged to genus *Babesia* have been identified, and we currently recognize more than 100 species affecting mammals and birds, including human (Hunfeld et al., 2008). The genus *Babesia*, together with the close-related *Theileria* genus, is classified within the phylum Apicomplexa, suborder Piroplasmidae and family Babesiidae. *Babesia* and *Theileria* can collectively referred as piroplasms, due to the pear-shaped aspect that they assume in the intra-erythrocytic phase of the life cycle.

3.2. LIFE CYCLE AND BIOLOGY

The life cycle of *Babesia* species requires two different hosts: an arthropod (tick), in which *Babesia* undergoes sexual reproduction with zygote formation, and a vertebrate host (mammalian, including humans, or avian hosts) in which *Babesia* multiplies asexually within red blood cells.

3.2.1. DEVELOPMENT IN THE VERTEBRATE HOST

The vertebrate host get infected through the bite of an infected tick, which inject the infective stage of the parasite, the sporozoite, together with the saliva. Once in the blood, sporozoites start to invade the erythrocytes with the help of several proteins secreted by apical secretory organelles of the parasite (Soldati et al., 2001; Dubremetz et al., 1998). Contrary to other Apicomplexa such as *T. gondii* and *P. falciparum*, sporozoites of the genus *Babesia* do not enter the red blood cells with the help of a parasitophorous vacuole but occur freely in the cytoplasm. As soon as inside the cell, sporozoites develop into trophozoites and start to multiplicate asexually giving rise to merozoites, in a process called merogony (Jalovecka et al., 2018). Merozoites are then released from the disrupted erythrocytes and ready to invade other uninfected red blood cells. Asynchronous formation of trophozoites and merozoites is possible, leading to the simultaneous presence of both stages in the

bloodstream of the host (Jalovecka et al., 2016). Merozoites usually occur within the red blood cells as a pear-shaped cell, forming pairs or tetrads. The size of intra-erythrocytic stages of the parasite largely depend on the species and was traditionally used to distinguish between them. Briefly, they can be divided into small Babesiae (trophozoites of 1.0 – 2.5 µm) and large Babesiae (2.5 – 5.0 µm). Example of small Babesiae are *B. gibsoni*, *B. microti* and *B. rodhaini*, while *B. bovis*, *B. caballi* and *B. canis* belonged to large Babesiae. Fig. 5 illustrates the different shapes of *Babesia* merozoites within the vertebrate erythrocytes.

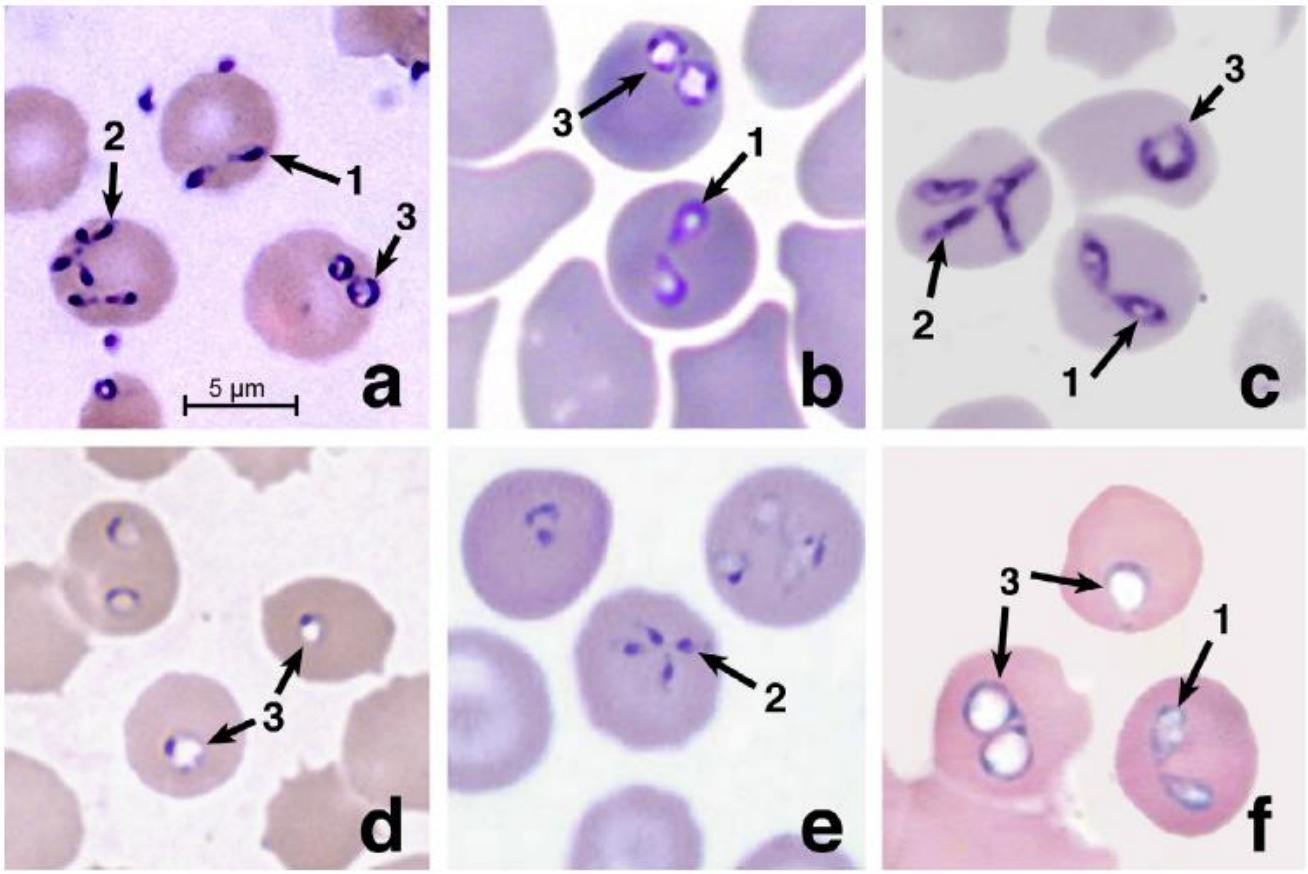

Fig. 5 Merozoites of *Babesia* spp. in human erythrocytes. (a) *B. divergens*, (b) *B. venatorum*, (c) *Babesia* sp. MO1 from Kentucky, (d) *B. microti*, (e) B. duncani, (f) *Babesia* sp. KO1 from Korea. (1) Paired piriforms; (2) Tetrads; (3) Ring forms (figure adapted from Yabsley and Shock, 2013).

3.2.2. DEVELOPMENT IN THE TICK

In the vertebrate host, some merozoites within the erythrocytes develop into gametocytes, the first stage of the sexual reproduction of *Babesia*. During the blood meal, ticks can ingest gametocytes within red blood cells (Jalovecka et al., 2018), which further differentiate into male and female haploid gametes in the tick gut (Strahlenkörper) (Jalovecka et al., 2018). A close contact between two gametes starts the fertilization that lead to the formation of the zygote, a motile stage. During blood

feeding, a peritrophic matrix is formed within the tick midgut dividing it into endo-peritrophic and ecto-peritrophic spaces. The motile zygote is able to cross this matrix with the help of enzymes released by the arrowhead structure of the zygote, entering the ecto-peritrophic space first and the gut epithelial cells subsequently. Once in the epithelium, the zygote hires a rounded shape and starts a meiotic division with the formation of kinetes. These haploid cells are released into the tick haemolymph and disseminate throughout the body. There, kinetes undergo two phases of asexual multiplication, the first one in several tissues such as haemocytes, muscle fibers, Malpighian tubules and peritracheal cells. After the production of secondary kinetes, these invade the salivary gland cells in which the parasite undergoes sporogony, i.e. maturation of sporozoites. This process starts with the formation of a multi-nucleated syncytium from a single invading kinete, called sporoblast. This large cell rapidly evolved into several sporozoites through the multiple fission process, called budding. Time necessary to the formation of sporozoites is variable, but it generally takes at least 48 h (Jalovecka et al., 2018). During tick ecdysis, the sporoblast remains dormant within the salivary gland cells until a new moulting stage is reached and a new host is found by the tick. At this time, sporozoites are formed and ready to be transmitted to a new naïve host. The ability of *Babesia* to lie within the tick during all the moulting phases of its life allows the transstadial transmission of the parasite, increasing the opportunity to survive and be transmitted to a new host. For several *Babesia* species, transovarial transmission is also possible: during the kinete dissemination through the tick body, these can invade ovaries of female ticks which, after mating, transmit the parasite to larvae. Fig. 6 summarizes the life cycle of *Babesia* in the mammalian and ixodid hosts.

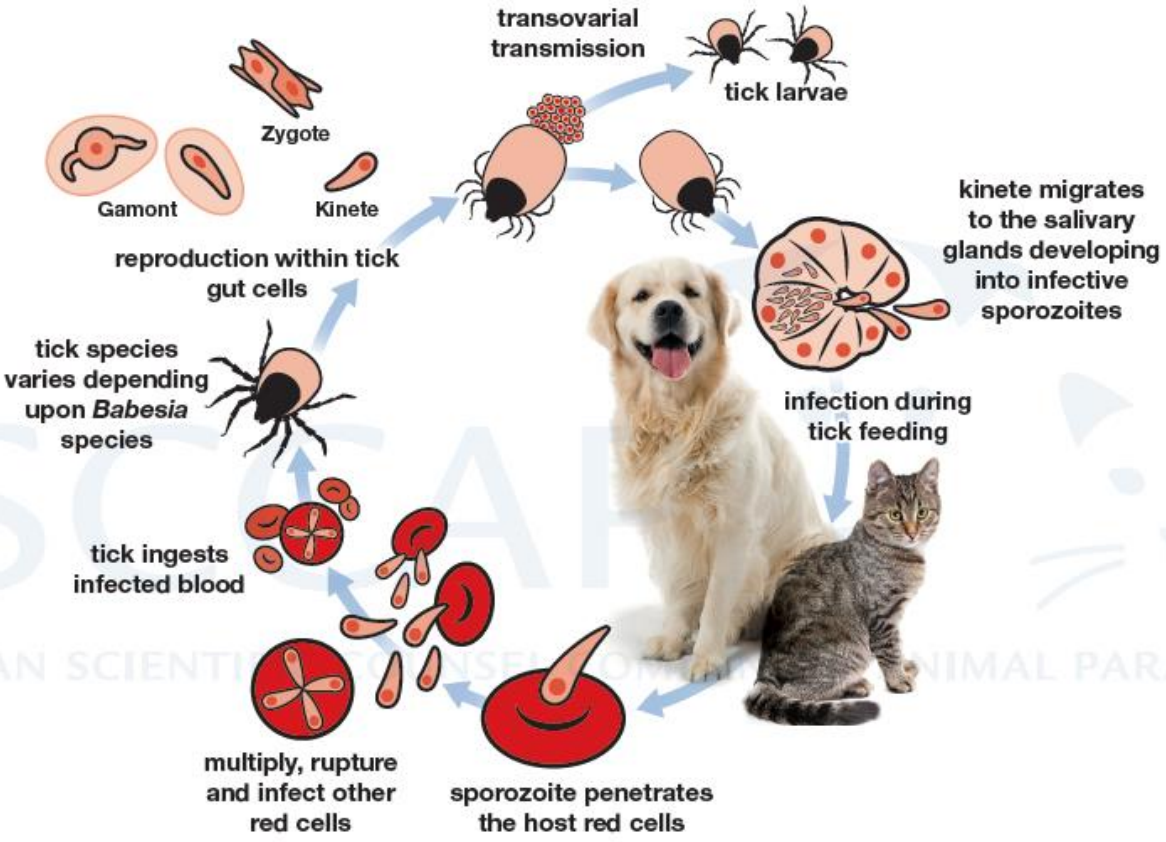

Fig. 6 Summary of *Babesia* spp. life cycle. (adapted from ESCAAP).

3.3. IMMUNOLOGY

Both humoral and cellular factors are involved in the immunity against *Babesia*, although humoral immunity appears to be less important in controlling babesiosis, as demonstrated in B-cell deficient mouse models that remain less susceptible to *Babesia* infection than T-cell receptor deficient that are readily infected (Telford 3rd and Maguire, 2006; Clawson et al., 2002).

The first phase of *Babesia* infection starts with sporozoites invading red blood cells and establishing the intraerythrocytic stage, that coincide with the rise of parasitaemia and acute disease. At this time, innate immune system plays an essential role for controlling the growth rate of the parasites and thus the parasitaemia level (Aguilar-Delfin et al., 2003). In particular, soluble factors such as gamma interferon (IFN-γ) produced by NK cells and tumor necrosis factor alpha (TNF-α), nitric oxide (NO) and reactive oxygen species (ROS) produced by macrophages inhibit the indiscriminate growth of *Babesia*, although it is not clear how these molecules interact with the parasites inside the

erythrocytes. In mouse models, parasitaemia starts to decline approximately 10 days after the infection (Homer et al., 2000). This falling is mainly due to the clearance of the parasites by the hyperreactive spleen. In particular, the T lymphocytes CD4+ IFN-γ producers are responsible for killing the intraerythrocytic parasites and for the resolution of the infection. The importance of cellular immunity for parasite clearance explains why individuals with depressed cellular response are particular prone to develop acute disease and show difficulties in controlling parasitaemia level (Telford 3rd and Maguire, 2006; Kjemtrup and Conrad, 2000; Häselbarth et al., 2007).

3.4. PHYLOGENY

Babesia species have been historically divided into small and large Babesiae based on the morphological aspect and size of trophozoites within red blood cells, and on the vertebrate and tick host specificity. This classification has been partially confirmed by the phylogenetic characterization of the nuclear small subunit ribosomal RNA gene (18S rRNA), which has showed that small and large Babesiae fall into two different phylogenetic clusters. In particular, small Babesiae seems to be more related to *Theileria* genus, with the exception of *B. divergens* that presents small trophozoites but is genetically related to large Babesiae (Homer et al., 2000).

Other authors have classified piroplasms into more than two phylogenetic clusters. Zahler and colleagues have proposed three groups (*B. microti*, Theilerids and Babesids) (Zahler et al., 2000), whereas Kjemtrup et al. and Penzhorn et al. described four groups, one of which grouping *Babesia* isolates from western USA (Penzhorn et al., 2001; Kjemtrup et al., 2000). In 2003, Criado and colleagues proposed five distinct phylogenetic clades: (i) Archeopiroplasmids - *B. microti* group containing *B. rodhaini*, *B. felis*, *B. leo*, *B. microti* and *B. microti*-like isolates; (ii) Prototheilerids - western US *Theileria*-like group, containing *B. conradae*; (iii) Theilerids - *Theileria*-group, containing all *Theileria* species from bovines; (iv) Babesids - a first group of *Babesia* sensu stricto, including *B. canis*, *B. gibsoni*, *B. divergens* and *B. odocoilei*; (v) Ungulibabesids - a second group of *Babesia* sensu stricto, composed of species from ungulates such as *B. caballi*, *B. bigemina*, *B. ovis*, *B. bovis* and other *Babesia* from cattle (Criado-Fornelio et al., 2003a).

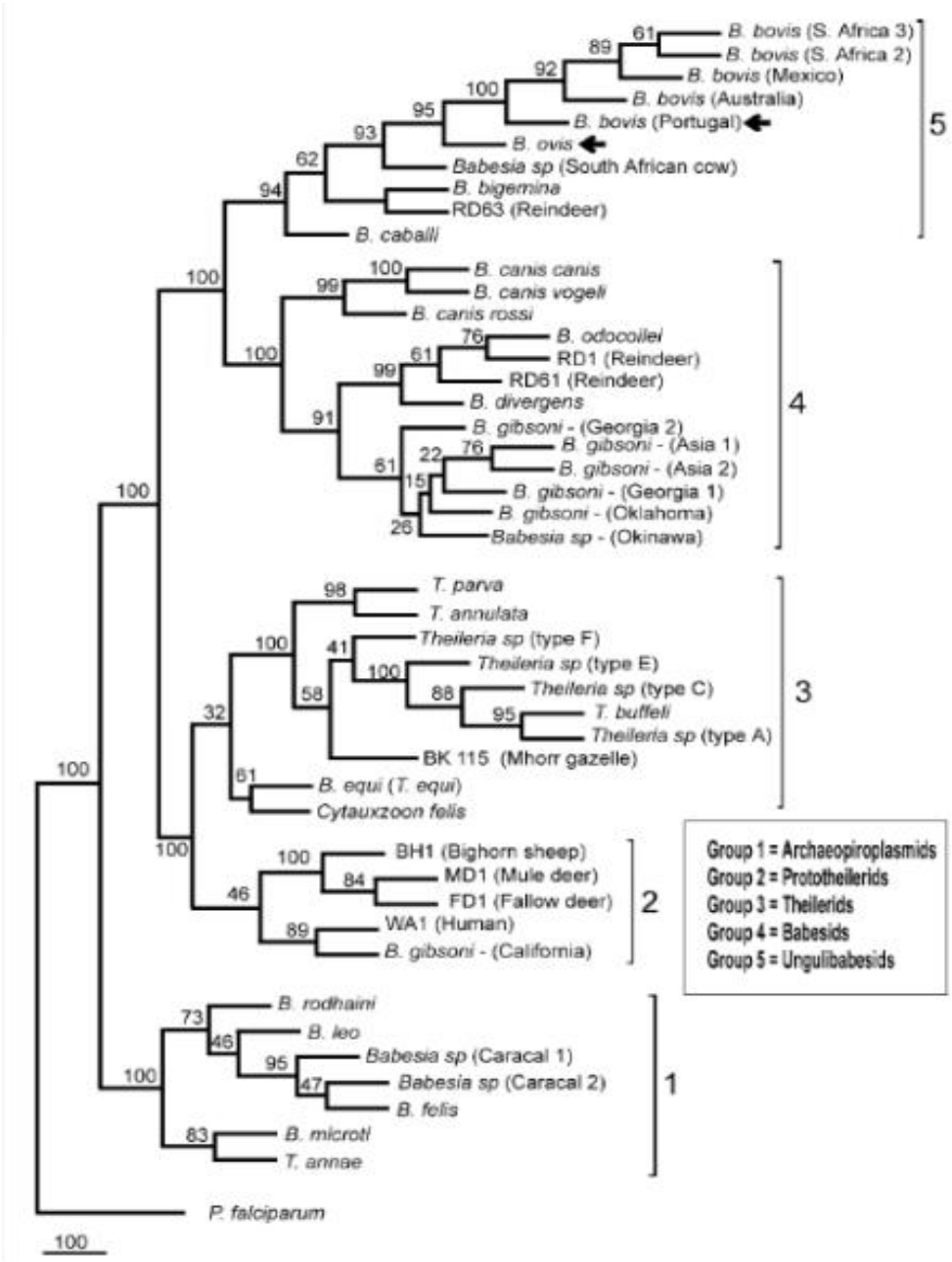

Fig. 7 Phylogenetic tree for a subset of *Babesia* and *Theileria* species showing the five distinct clades proposed by Criado-Fornelio and colleagues (2003). The PARSIMONY method was used (adapted from Criado-Fornelio et al., 2003).

3.5. VECTORS

Transmission of *Babesia* to the vertebrate host occurs through the bite of an infected tick, although human infections by blood transfusion have been observed (Ord and Lobo, 2015; Leiby, 2006). Due to the fact that the most recent common ancestor of Apicomplexa seems to have infected an invertebrate host, it is presumable that piroplasms have evolved from an ancient form infecting an

invertebrate tick-predecessor in a monoxenous life style (Leander, 2008). As a result, adaptive tolerance to *Babesia* has been described in the tick *Rhipicephalus microplus*, suggesting a balance between tick defence mechanisms and tick-pathogen mutual interactions (de la Fuente et al., 2016), with several tick molecules implicated in the acquisition, dissemination and transmission of *Babesia* (Antunes et al., 2017).

While around 700 Ixodid ticks (hard ticks) are known to compose the world's tick fauna (Jongejan and Uilenberg, 2004), five genera have been recognized as *Babesia* vectors: *Ixodes*, *Rhipicephalus*, *Haemaphysalis*, *Hyalomma* and *Dermacentor* (Sonenshine, 2014).

Ixodid ticks are characterized by the presence of a rigid chitinous scutum, which cover the entire dorsal surface of the adult male. In the adult female and in the larval and nymphal stages, the chitinous scutum is extended for only a small area of the body, allowing the extension of the abdomen during the blood feeding. Hard ticks are temporary parasites, and therefore they spend a relatively short period of time on the animal host and long periods in the environment. Before each moult (from larva to nymph and from nymph to adult) and the deposition of eggs by the adult female, ticks are required to take a blood meal on the vertebrate host. Ixodid ticks can be divided in one-, two- or three-host species, depending on the number of vertebrate hosts they attached to during their life for feeding. In one-host species, the entire parasitic development from larva to adult occurs on one host, without free-living periods in the environment. In two-host species, larva and nymph take the blood meals on the same host, then the engorged nymph drops off and moults in the environment, and the resulting adult searches for another suitable vertebrate host (that may or may not be of the same species of the first host). In the three-hosts species, each stage (larva, nymph and adult) takes the blood meal on a different host, and thus the moulting occurs in the environment. The mating usually takes place on the host, and then the engorged female drops off from the animal and lays the eggs in the environment, usually a large batch of several thousands of eggs. Hard ticks are generally long-lived, and certain species have adapted to live most of their lives in the environment, therefore they have evolved several mechanisms to survive. Ticks which inhabit areas with temperate climate, characterized by alternative periods of suitable and unsuitable climatic conditions, have synchronized their life cycle (i.e. seeking for a host and feeding) with the period in which climatic conditions are suitable and the presence of hosts is maximum.

Ixodes is the largest genus in the family Ixodidae, with around 250 species. They have a three-host life cycle, and many species within the genus inhabit nests and burrows (Jongejan and Uilenberg, 2004). *I. ricinus* is the most widespread tick species in Europe, inhabiting wooded and grassy environments. It feeds on a wide range of warm- and cool-blooded vertebrate hosts including small rodents, lizards, passerine birds and larger mammals such as deer, wild boars, livestock and humans (Schwarz et al., 2009; Otranto et al., 2014; Humair et al., 1998, 1993a, 1993b). Immature stages of *I. ricinus* feed on animals of all sizes, from rodents and passerine birds to ungulates, while adults mainly parasitize large animals such as deer and cattle. Large hosts are therefore required for

maintaining tick population, and it has been observed that tick abundance is lower in the absence of these animals (Medlock et al., 2008). *I. ricinus* is considered as an opportunistic tick species, feeding on humans if chances occur for example, and thus is an excellent vector of tick-borne human diseases (Parola and Raoult, 2001).

Rhipicephalus genus comprises around 80 species of ticks, mainly distributed in tropical and subtropical regions. *Rhipicephalus sanguineus*, probably evolved as a parasite of burrowing carnivores in warm climates (Morell, 2003), is now one of the most widespread tick species around the world. This tick species is of great importance for both veterinary and human medicine, given the fact that it is able to transmit a wide range of pathogens to the vertebrate hosts (Gray et al., 2013). *R. sanguineus* is a nidicolous tick with endophilic behaviour (i.e. inhabiting burrows or artificial shelters), although in particular conditions such as in humid habitats it shows exophilic behaviour, ambushing in the vegetation (Parola et al., 2008). Due to its habitat preferences, *R. sanguineus* is mainly found in urban and suburban areas, where it lives in close proximity to the dog, its main host, and humans (Shimada et al., 2003).

Ticks of the genus *Haemaphysalis* are particularly adapted to humid and well-vegetated habitats in Eurasia and tropical Africa (Wall and Shearer, 2001). Birds and small mammals are common hosts for immature stages of this genus, while adults mainly infest large mammals such as livestock (Nosek, 1971). *H. punctata* is widely distributed throughout Europe, Scandinavia, North Africa and central Asia, and is responsible for the transmission of *B. major* and *B. bigemina* in cattle (in England and central Europe, respectively), and of *B. motasi* in sheep (Wall and Shearer, 2001).

The genus *Hyalomma* comprises 27 species mainly present in the Afrotropical region and parts of the Palearctic region (Guglielmone et al., 2014), where they inhabit semi-desert lowlands. The *Hyalomma marginatum* complex is formed by the species *Hyalomma marginatum* sensu strictu, *H. rufipes*, *H. isaaci*, *H. turanicum* and *H. glabrum* (Capek et al., 2014). Apart from being the most important vectors of Crimean-Congo haemorrhagic fever (CCHF) virus, ticks of the *Hyalomma marginatum* complex are able to transmit piroplasms such as *B. caballi*, *B. occultans* and *Theileria annulata* (Walker et al., 2014; de Kok et al., 1993).

Ticks of the genus *Dermacentor* are three-host ticks widely distributed in Europe and North America (Wall and Shearer, 2001). In USA, *D. variabilis* and *D. andersoni* are the most important tick species of this genus, being the vectors of *Rickettsia rickettsia* (agent of the Rocky Mountain Spotted Fever) and *Anaplasma marginale* (Wall and Shearer, 2001). In Europe, *D. marginatum*, also known as the "ornate sheep tick", is widely present in areas with dense bush and tree cover, and infests both domestic and wild mammals. *D. reticulatus* is the second most reported species in Europe after *I. ricinus* and is more common in cooler areas (Rubel et al., 2016). Moreover, it has been showed to survive flooding for a certain period of time, allowing the tick to inhabits alluvial forests and swamps (Rubel et al., 2016). *D. reticulatus* is known to infest dogs, cattle, sheep and horses, to which it can transmit *B. canis*, *B. divergens*, *B. ovis* and *B. caballi*, respectively (Wall and Shearer, 2001).

3.6. RESERVOIRS

Bovine babesiosis is one of the most important disease in cattle worldwide (Uilenberg, 1995), with more than 1 billion of cattle at risk of infection (Bock et al., 2004). The cost of babesiosis in cattle is not only linked to mortality, abortion and loss of milk and meat production, but also to the control measures (acaricide treatments, vaccines and therapies) and impact on international trades, with up to US$20 million spent each year only in Australia (McLeod and Kristjanson, 1999). Clinical signs and symptoms of bovine babesiosis are usually fever, inappetence, depression, increased respiratory rate and weakness, anaemia and jaundice, and haemoglobinuria (Bock et al., 2004). Similar to bovine babesiosis, infection in sheep appear with classical symptoms of fever, anorexia, depression, weakness, dyspnoea, tachycardia, impotence, haemolytic anaemia, thrombocytopenia, icterus, haemoglobinuria, and, in the case of late-stage treatment, death (Sevinc et al., 2018).

Ticks of the genus *Rhipicephalus* are mainly implicated in the transmission of *Babesia* species to livestock. In particular, *B. bovis* and *B. bigemina*, the aetiological agents of bovine babesiosis in tropical and subtropical areas, are transmitted by *R. microplus* and *R. annulatus* (and *R. decoloratus* for *B. bigemina* only) (Gohil et al., 2013), given the fact that these tick species are particularly adapted to warm and humid climates (Estrada-Peña et al., 2006). In contrast, *B. divergens*, the aetiological agent of bovine babesiosis in temperate climates, is transmitted by the tick *I. ricinus* (Chauvin et al., 2009), which is the most widespread tick species in Europe. *B. ovis*, the aetiological agent of babesiosis in sheep, is mainly transmitted by *R. bursa* (Ferrolho et al., 2016).

Dogs are particular susceptible to *Babesia* infection. Symptoms of canine babesiosis vary from subclinical infection to multi-organ failure, depending on several factors such as age, splenectomy, immune competence and concomitant infections or diseases (Solano-Gallego et al., 2016). Common signs are apathy, weakness, anorexia, pale mucous membranes and a poor general condition, fever, enlarged lymph nodes and spleen, anaemia, thrombocytopenia, jaundice and pigmenturia (Solano-Gallego et al., 2016).

Several *Babesia* species are responsible for the infection in dogs. Notably, large Babesiae such as *B. canis*, *B. vogeli* and *B. rossi* and small *Babesia*s such as *B. gibsoni*, *B. conradae* and *B. vulpes* are known to cause canine babesiosis (Solano-Gallego et al., 2016). *B. canis* is mainly transmitted by *D. reticulatus*, which prefers wet and cool climates and thus is widespread in Northern and Central Europe. In contrast, ticks belonging to *R. sanguineus* s.l., also known as the brown dog tick, are able to transmit *B. vogeli* (Cacciò et al., 2002). *R. sanguineus* s.l. is widely present in Mediterranean countries thanks to its preference for warm habitats, although it can be found in Northern countries such as UK due to its endophilic nature, that allows to survive in climatically limiting environments (Hansford et al., 2014). *R. sanguineus* s.l. is also the potential vector for *B. gibsoni* in Europe, while in Asia the transmission is attributable to *Haemaphysalis longicornis* (Iwakami et al., 2014). While

details on the life cycle of *B. vulpes* are largely unknown, *Ixodes hexagonus* has been implicated as a potential tick vector for this species (Miró et al., 2015; Camacho et al., 2003). Ticks of the *Ixodes hexagonus* species show a "pholeophilic" behaviour, thus free-living stages are confined to dens and burrows, and are mainly associated to hedgehog and wild carnivores such as foxes (Solano-Gallego et al., 2016).

Cats are mainly infected by B. felis, although several other *Babesia* species are known to cause infection in feline, such as *B. catii*, *B. canis* subsp. *canis*, *B. canis* subsp. *presentii*, *B. vulpes*, *B. pantherae* and *B. leo* (Taboada and Lobetti, 2006; Shaw et al., 2001; Lopez-Rebollar et al., 1999; Baneth et al., 2004). However, information about tick vectors of these species are lacking.

Aside from domestic animals, *Babesia* infection has been reported also in wildlife. Foxes are known to be infected mainly by *B. vulpes*, with I. *hexagonus* being the most probable vector for this species (Miró et al., 2015; Camacho et al., 2003; Battisti et al., 2020) (PAPER 6). Similarly, *B. vulpes* has been reported also in I. *hexagonus* ticks collected from European badgers (Millán et al., 2016). Apart from *B. vulpes*, other *Babesia* species implicated in wild carnivore infection are *B. canis*, *B. rossi* and *B. vogeli* (most commonly in canids), *B. leo*, *B. felis* and *B. lengau* in wild felids, and two potentially new species (*Babesia* NV1 and *Babesia* UR1) identified in the American mink *Neovison vison* and the Hokkaido brown bear *Ursus arctos yesoensis*, respectively (Alvarado-Rybak et al., 2016).

In Europe, several rodent and shrew species are known reservoir for *B. microti* and *B. microti*-like species, such as yellow-necked mice (*Apodemus flavicollis*), wood mice (*Apodemus sylvaticus*), bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*), common shrews (*Sorex araneus*), and *Mus* spp. (Yabsley and Shock, 2013). *I. ricinus* is the most important vectors of these *Babesia* species (Yabsley and Shock, 2013).

Wild ungulates are natural hosts of several *Babesia* species. In particular, roe deer (*Capreolus capreolus*) is the most important reservoir for *B. capreoli*, a species closely-related to *B. divergens*, and for *B. venatorum*, also known as *Babesia* sp. EU1 (Tampieri et al., 2008; Malandrin et al., 2010; Bonnet et al., 2007; Bastian et al., 2012). Other wild ungulates such as red deer, reindeer and fallow deer have showed to be susceptible to these *Babesia* species (Hoby et al., 2009; Ebani et al., 2016), while fatal *B. capreoli* infections in alpine chamois (*Rupicapra rupicapra*) from Switzerland have been reported (Hoby et al., 2007). As for *B. microti*, *I. ricinus* is the main vector of *B. capreoli* and *B. venatorum* (Yabsley and Shock, 2013).

3.7. HUMANS

Several *Babesia* species are known to be zoonotic, thus able to infect humans. In Europe, most of the cases of human babesiosis are caused by *B. divergens*, with 50 confirmed infection so far (Hildebrandt et al., 2013). Most of the cases have been recorded in British island and France (Krause, 2019), although confirmed infections have been reported also from other European

countries such as Croatia, Finland, Norway, Poland, Portugal, Spain and Sweden (Krause, 2019). *B. divergens* infection in humans is usually severe, with haemoglobinuria being the presenting symptoms. Other common symptoms are jaundice due to haemolysis, vomiting and diarrhoea. Moreover, toxins and anoxia, resulting from the haemolysis and the host immunological response, may cause respiratory, cardiac, renal, or hepatic failure (Hunfeld et al., 2008).

B. venatorum was first recognized as human pathogen in 1998, and up to now few confirmed cases of human infection have been reported from Austria, Germany, Italy and Sweden (Hunfeld et al., 2008; Herwaldt et al., 2003; Gray et al., 2010). Clinical manifestations of *B. venatorum* infection ranged from mild to moderately severe, and reported cases were observed in immunocompromised patients (i.e. Hodgkin's disease, large B cell lymphoma, immunosuppressive treatments) (Hildebrandt et al., 2013). The few cases recorded may suggest the low circulation or low infectivity of this species to humans; however, a large epidemiological study carried out in China revealed that approximately half of the human cases of *B. venatorum* infection were asymptomatic, thus underlying the possible underestimation of the prevalence of this parasite (Ord and Lobo, 2015).

B. microti infection is rare outside Americas, with few confirmed cases in Europe. The first autochthonous case of *B. microti* infection in humans was observed in an immunocompromised woman from Jena which presented fever, heavy chest pain and acute myocardial infarction (Hildebrandt et al., 2007). Other two asymptomatic cases of infection have been reported in forestry workers from Poland (Hildebrandt et al., 2013).

4. *L. INFANTUM*

4.1. DISCOVERY AND TAXONOMY

The existence of *Leishmania*-like parasites in prehistorical times have been documented in fossil ambers containing the extinct sand flies *Palaeomyia burmitis* and *Lutzomyia adiketis* (Steverding, 2017). First reports of cutaneous lesions reminiscent of Oriental sore in humans are dated back to the 7th century BC, while the first convincing reports of visceral leishmaniasis (VL) appeared in the 19th century (Steverding, 2017). During the 20th century, some of the most important scientists working on *Leishmania* were Sir William Boog Leishman, a Scottish pathologist that served with the British Army in India, and the Irish doctor Charles Donovan, that are credited together for the discovery of *Leishmania donovani*, a causative agent of visceral leishmaniasis (Steverding, 2017). The related VL-causing species *L. infantum* was first identified by Charles Jules Henry Nicolle while working on children suffering for splenic anaemia in Tunisia (Nicolle, 1908).
L. infantum belongs to the phylum Euglenozoa, class Kinetoplasta, Order Trypanosomatida. Together with Trypanosoma, *Leishmania* is the only member of the family Trypanosomatidae.

4.2. LIFE CYCLE AND BIOLOGY

The life cycle of *L. infantum* requires two different hosts: a phlebotomine sand fly of the Psychodidae family (Order Diptera), and a vertebrate host represented by mammals of several species, including humans. The transmission of this parasite occurs during the blood feeding of an infected phlebotomine sand fly on a mammal, that acquires the infection and thus is able to transmit the parasite to uninfected sand flies during subsequent blood meals.
Sand flies acquire the parasite from an infected host during the blood meal, that is carried out by the females for egg production. During the blood taking, sand flies ingest the amastigote form of the parasite, characterized by a non-flagellated round-shape, and reach the intestine of the insect. Here amastigotes start to develop into elongated, motile promastigotes with an external flagellum and rapidly dividing by binary fission (Killick-Kendrick, 2002). In the intestinal lumen of the insect, blood meal and promastigotes are surrounded by the peritrophic membrane by the cells of the midgut. Thus, promastigotes escape from the membrane and invade the abdominal gut where they attach to the wall of the gut by inserting flagella between microvilli. Here they intensively multiplicate, and then they move to the thoracic midgut where they attach to the stomodaeal valve of the insect (Killick-Kendrick, 2002). At this site, promastigotes start to mutate to metacyclic promastigotes, the infective stage of the parasite for vertebrate host. At the time of blood meal, metacyclic promastigotes are regurgitated on the skin of the host during the insect bite, probably due to the damage of the

stomodaeal valve by the promastigotes (Killick-Kendrick, 2002). In the skin of the mammal, they are phagocytized by macrophages, and within these cells they change back to the amastigote rounded form and divide rapidly, causing the rupture of the macrophages and the invasion of other mammalian cells (Killick-Kendrick, 2002). Parasites and infected macrophages can be localized only in skin and mucosae of the host (in case of the cutaneous form of leishmaniasis) or move to other tissue and organs, causing visceral leishmaniasis (Mcgwire and Satoskar, 2014).

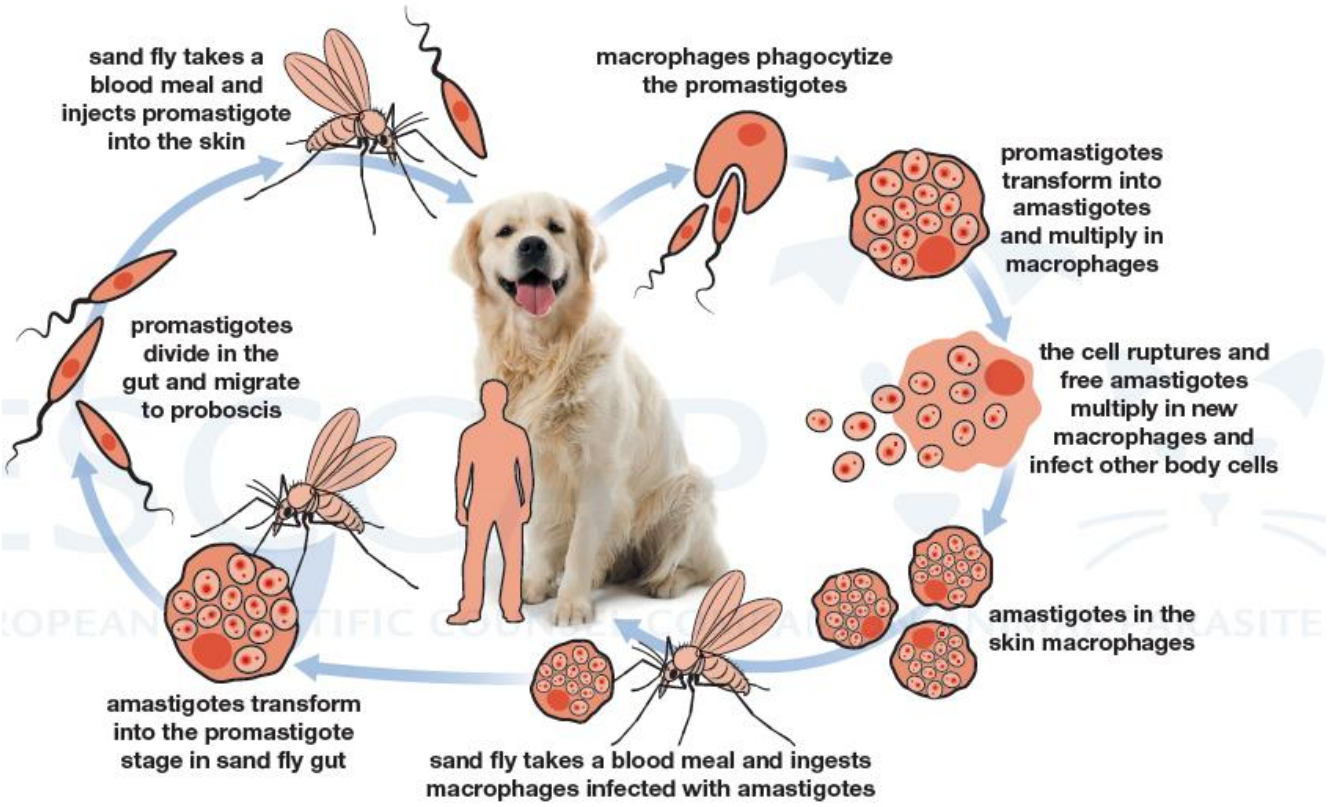

Fig. 8 Life cycle of *Leishmania* spp. in the vertebrate and invertebrate hosts (adapted from ESCAAP)

4.3. IMMUNOLOGY

Mammal host protection against *L. infantum* infection is based on Th1-type immunity that triggers antimicrobial activity of macrophages. In particular, antigen-presenting cells are responsible for production of IL-12 while T cells of IFN-γ, that ultimately lead to the activation of macrophages and the development of acquired resistance (Nylén and Sacks, 2007). Another important cytokine involved in the resistance against *L. infantum* is TNF-α produced by T-cells, showing a role in granuloma formation and maintenance. The development of functional granuloma around the

parasite has showed to be essential for parasite resistance, within which *L. infantum* is controlled by NO production (Murray et al., 2000). However, high level of TNF-α can lead to the production of IL-10 as a homeostatic response to excessive level of inflammation. The immunosuppressive cytokine IL-10 has a key role in the development of clinical signs of visceral leishmaniasis and active disease. As abovementioned, IL-10 production is triggered by an excessive inflammatory status of tissues, protecting them collateral damages. However, in case of visceral leishmaniasis, high level of IL-10 has been linked to limited parasite control, enhanced parasite replication and disease progression (Nylén and Sacks, 2007). Several factors are responsible for the different outcome of *L. infantum* infection. Apart from the genetic background of the host, some environmental factors that contribute to the production of high level of IL-10 and thus disease progression are malnutrition, drugs, and concurrent infections such as HIV or helminthiases (Nylén and Sacks, 2007).

4.4. PHYLOGENY

First attempts to evaluate the genetic variability of *L. infantum* were made by using isoenzyme analysis (Kreutzer and Christensen, 1980). In particular, *L. infantum* has been subdivided into zymodemes MON with starch gel Multilocus Enzyme Electrophoresis technique (MLEE), which is based on the following 15 enzymatic systems: DIA (NADH diaphorase), FH (fumarate hydratase), G6PD (glucose-6-phosphatedehydrogenase), GLUD (glutamate dehydrogenase), GOT1 and GOT2 (glutamate oxaloacetate transaminase), GPI (glucose phosphate isomerase), IDH (isocitratedehydrogenase), MDH (malate dehydrogenase), ME (malic enzyme), MPI (mannose-phosphateisomerase), NHI and NHD (nucleoside hydrolase), 6PGD (6-phosphogluconate dehydrogenase) and PGM (phosphoglucomutase). A zymodeme is composed by isolates showing the same isoenzyme profile (Hide et al., 2001). Unfortunately, this technique requires parasite isolation, that is not routinely carried out for diagnostic purpose, and due to the low variability of aminoacidic sequence of enzymes, this tool is not applicable for epidemiological studies (Botilde et al., 2006). In the Mediterranean area, the most widespread zymodeme is MON-1 (Jimenez et al., 1995). By using the RAPD (Random Amplified Polymorphic DNA) technique, it has been observed the presence of heterogenicity within the MON-1 zymodeme, with some isolates phylogenetically more similar to strains classified as non-MON-1 than to MON-1 isolates (Hide et al., 2001). In recent years, molecular methods other than RAPD technique have been used for strain typing, such as (i) PCR amplification of antigen-encoding genes (gp63 and cpb) followed by analysis of restriction fragment length polymorphism (Quispe Tintaya et al., 2004), (ii) PCR-RFLP of kDNA minicircles (Ferroglio et al., 2006b), (iii) Multilocus microsatellite typing (Ochsenreither et al., 2006). Among these methods, PCR-RFLP of kDNA and microsatellite typing have shown to have the best resolution (Botilde et al., 2006).

L. infantum typing is a helpful tool in epidemiological studies, i.e. to distinguish between autochthonous and imported cases of leishmaniasis in recently established endemic areas, but also in clinical studies, such as to establish the correlation between clinical outcome, preferential host (immunocompromised or immunocompetent humans, dogs, etc.) and strains (Bulle et al., 2002). Several foci of both canine and human leishmaniasis have been investigated for strain typing (Ferroglio et al., 2018; Chicharro et al., 2013) (PAPER 10).

4.5. VECTOR

L. infantum is transmitted to mammals by the bite of an infected sand fly (Order: Diptera, Family: Psychodidae, Subfamily: Phlebotominae). The name sand fly relies on the yellowish colour of these insects, although their colour can range from white to almost black based on the species (Killick-Kendrick, 1999). They have a small size (circa 3 mm) and a hairy body. When at rest, they typically hold their wings at an angle above the abdomen, and they are silent during blood feeding and flying (Killick-Kendrick, 1999). Sand flies are mainly active at crepuscular and nocturnal hours, while they spend the day in cool and humid resting places such as houses, latrines, cellars and stables, caves, fissures in walls, rocks or soil, dense vegetation, tree holes and buttresses, burrows of rodents and other mammals, bird's nests and termitaries (Killick-Kendrick, 1999).
Within the subfamily Psychodidae there are 6 different genera, although only two have a medical importance: *Phlebotomus* in the Old World (Europe, Africa and Asia) and *Lutzomyia* (Americas). In Europe, the most important and proved vectors of *L. infantum* are *P. ariasi*, *P. balcanicus*, *P. kandelakii*, *P. langeroni*, *P. neglectus*, *P. perfiliewi*, *P. perniciosus* and *P. tobbi* (Alten et al., 2016). In Italy, *P. perniciosus* and *P. perfiliewi* are the most widespread and important vectors of *L. infantum*, followed by *P. ariasi* and *P. neglectus* (Bongiorno et al., 2003). In particular, *P. perniciosus* is able to colonize rural, peri-urban and urban areas, and known to be an opportunistic feeder, thus taking the blood meal on several animal species such as dogs, horses, birds and humans (Bongiorno et al., 2003).

4.6. RESERVOIRS

Domestic dogs is the main reservoir of *L. infantum* in the Mediterranean region, with 2.5 million dogs estimated to be infected in southwestern Europe alone (Moreno and Alvar, 2002). Canine leishmaniasis is a complex disease that shows considerable differences in clinical presentation and infection prevalence, with up to 80% of dogs population infected in certain endemic areas (Solano-Gallego et al., 2001). In Mediterranean areas, dog prevalence ranges from 5% to 30% depending on the regions (Solano-Gallego et al., 2009). Even if the prevalence of parasite infection in dogs can

be relevant, the rate of clinical disease is usually low (Baneth et al., 2008). The development of clinical signs mainly depends on the immunological status of the dog and its ability to mount an effective immune response against the parasite. The disease can manifest mainly by two ways: (i) the presentation of severe clinical signs shortly after the infection, probably due to the inability of the dog to develop an effective immune response, and (ii) the appearance of clinical signs after changing the immunological status of a previously infected dog, i.e. by administrating immunosuppressive therapies, mainly due to the reactivation of a latent infection (Baneth et al., 2008). Most of the dogs with clinical signs present poor body condition, generalized muscular atrophy, lymph adenomegaly and excessive skin scaling (Koutinas et al., 1999). Moreover, renal involvement is frequent, with glomerulonephritis, interstitial nephritis and glomerular deposition of parasite antigen (Costa et al., 2003). Even though dogs with clinical presentations have showed to be highly infectious to sand flies, also asymptomatic dogs are able to transmit the parasite during sand flies blood feeding (Baneth et al., 2008). In recent years, both sand flies and *L. infantum* have showed a geographical expansion, mainly due to climate and habitat changes. One of the most striking examples is the northward spread of this parasite and its vectors in Italy. In northern Italy, a traditional *Leishmania*-free area, first cases of autochthonous canine leishmaniasis has been increasingly reported in 1990s, together with the first findings of efficient vectors of this parasite (Maroli et al., 2008; Ferroglio et al., 2005). At present, the prevalence of *L. infantum* in both dogs and humans in this area has increased in respect to the past (Ferroglio et al., 2018) (PAPER 10).

Even though domestic dogs are the main reservoir of *L. infantum*, other wild animals have been proposed as alternative reservoirs. Control measures of canine leishmaniasis and prevention of human leishmaniasis are mainly based on the treatment of dogs with permethrin, which shows a contemporary antifeeding and knock-down activity on sand flies (Ferroglio et al., 2008). However, several reports have highlighted the lack of efficacy of these control measures, and the presence of wild reservoirs could be one of the causes of this lacking (Millán et al., 2014). In addition, several *L. infantum* vectors such as *P. perniciosus* show high adaptability to both rural and urban habitats, leading to a possible overlap of domestic and sylvatic cycle of this parasite (Millán et al., 2014). Most of the studies attempted to find a sylvatic reservoir of *L. infantum* focused on carnivores, lagomorphs or rodents. Among carnivores, red fox is one of the most studies animals, probably due to its phylogenetic relationship with domestic dog and the fact that is the most abundant and widespread carnivore in Europe (Fuller et al., 2010). As for domestic dogs and humans, seroprevalence in foxes is much lower than molecular prevalence, suggesting a sort of resistance to the infection (Millán et al., 2014). Apart from foxes, other wild carnivores have showed moderate to high prevalence of *L. infantum*, such as wolves and badgers (Oleaga et al., 2018; Del Río et al., 2014) (Battisti et al., submitted – PAPER 4), although their ability as reservoir host of the parasite is not proved.

Concerning lagomorphs, the Iberian hare (*Lepus granatensis*) have showed to be the reservoir of an outbreak of human leishmaniasis in Spain, where surprising 446 cases of infection in

immunocompetent persons have been recorded between 2009 and 2012. In particular, the ability of asymptomatic hares to transmit the parasite to sand flies have been demonstrated by xenodiagnoses (Molina et al., 2012). Apart from Iberian hare, the capacity of infected animals to transmit *L. infantum* to sand flies has been proved only in the black rat (*Rattus rattus*) (Pozio et al., 1985). Moreover, Zanet and colleagues (Zanet et al., 2014a) have recently shown that on the island of Montecristo (Italy), where no dogs or other carnivores are present, up to 15.5 % of black rats were infected by *L. infantum*. Thus, given their high abundancy, their infectivity to the vector and their ability to maintain the parasite in habitats that are free of domestic dogs, black rats can be considered as potential reservoir of *L. infantum* (Millán et al., 2014).

4.7. HUMANS

Human leishmaniasis is considered as a neglected tropical disease, with 1.3 million new cases detected each year (1 million cases of cutaneous leishmaniasis, 0.3 million cases of visceral leishmaniasis) (Alvar et al., 2012). In Europe, the annual incidence of visceral leishmaniasis caused by *L. infantum* is of approximately 1100-1900 cases (Gradoni et al., 2017). Human leishmaniasis is considered as an emerging or re-emerging disease in Europe, mainly due to (i) natural spread of the parasite and its vectors from endemic Mediterranean regions to temperate non-endemic countries for climate changes, and (ii) the increase of immunosuppressed people showing *L. infantum* as a coinfection (Ready, 2010). As for the dog, parasite infection in humans is not always associated to clinical manifestation of symptoms. In fact, it has been estimated that only 1 infected patient out of 20-30 will develop a clear leishmaniasis symptomatology (Miró and López-Vélez, 2018). The appearance of symptoms can be subacute and slowly progressing, consisting of general malaise, fever, weight loss and abdominal pain; or acute and rapidly progressing, especially in children (Miró and López-Vélez, 2018). Signs and symptoms of visceral leishmaniasis are anaemia, hepatosplenomegaly, enlarged lymph nodes, while advanced disease is associated with cachexia, oedema and ascites. Moreover, spontaneous bleeding, chronic diarrhoea and malabsorption can occur.

EXPERIMENTAL PART

The following papers are the results of the research activities carried out during the PhD under the framework of the project:

Battisti E., Zanet S., Trisciuoglio A., Bruno S., Ferroglio E. (2018). Circulating genotypes of *T. gondii* in Northwestern Italy. *Vet Parasitol*. 253, 43-47 doi: 10.1016/j.vetpar.2018.02.023 **PAPER 1**

Veronesi F., Chiesa F., Zanet S., **Battisti E**., Sebastiani V., Branciari R., Valiani A., Ranucci D. (2018). Screening of *T. gondii* positive sheep flocks in Perugia province (Umbria region, central Italy) using bulk milk analyses. *Large Animal Review*. 24(5):185-187 https://cms.sivarnet.it/it/lar **PAPER 2**

Ranucci D., **Battisti E**., Veronesi F., Diaferia M., Morganti G., Branciari R., Ferroglio E., Chiesa F. (2020). Absence of viable *T. gondii* in raw-milk ewe cheese derived from naturally infected animals. *Microorganisms.* 8(1):143 doi.org/10.3390/microorganisms8010143 **PAPER 3**

Maurelli MP., Pepe P., Colombo L., Armstrong R., **Battisti E**., Morgoglione ME., Counturis D., Rinaldi L., Cringoli G., Ferroglio E., Zanet S. (2018) A national survey of Ixodidae ticks on privately owned dogs in Italy. *Parasites & Vectors*. 11:420 doi: 10.1186/s13071-018-2994-2 **PAPER 4**

Zanet S., **Battisti E**., Pepe P., Ciuca L., Colombo L., Trisciuoglio A., Ferroglio E., Cringoli G., Rinaldi L., Maurelli MP. Tick-borne pathogens in Ixodidae ticks collected from privately-owned dogs in Italy: a country-wide molecular survey. Accepted for publication in *BMC Veterinary Research* **PAPER 5**

Battisti E., Zanet S., Khalili S., Trisciuoglio A., Hertel B., Ferroglio E. (2020). Molecular survey on vector-borne pathogens in alpine wild carnivorans. *Frontiers in Veterinary Science*. 7:1 doi: 10.1186/s13071-018-2994-2 **PAPER 6**

Battisti E., Zanet S., Boraso F., Minniti D., Giacometti M., Ferroglio E. (2019) Survey on tick-borne pathogens in ticks removed from humans in Northwestern Italy. *Veterinary Parasitology: Regional Studies and Reports.* 18. 100352 doi.org/10.1016/j.vprsr.2019.100352 **PAPER 7**

Battisti E., Urach K., Cardinale M., Felsberger G., Ferroglio E., Fusani L., Hodžić A., Hufnagl P., Duscher GG. Zoonotic pathogens in ticks from migratory birds, Italy. Submitted to *Emerging Infectious Diseases* **PAPER 8**

Zanet S., **Battisti E**., Ferroglio E., Tizzani P. Ecological niche modeling of *Babesia* sp. infection in wildlife experimentally evaluated in questing *I. ricinus.* Accepted for publication with minor revision in *Geospatial Health.* **PAPER 9**

Ferroglio E., **Battisti E**., Zanet S., Bolla C., Concialdi E., Trisciuoglio A., Khalili S., Biglino A. (2018) Epidemiological evaluation of *L. infantum* zoonotic transmission risk in the recently established endemic area of Northwestern Italy. *Zoonoses Public Health*. 65(6):675-682 doi: 10.1111/zph.12477 **PAPER 10**

5. *TOXOPLASMA GONDII*

5.1. Circulating genotypes of *T. gondii* in Northwestern Italy – PAPER 1

5.1.1. Materials and methods

The genotyping study of *T. gondii* was performed on 65 animals originating from the Northwestern part of Italy, comprising Piedmont and Lombardy regions (Fig. 9). Samples derived from swine *Sus scrofa domesticus* (n=15), cattle *Bos taurus* (n=11), wild boar *Sus scrofa* (n=18), red fox *Vulpes vulpes* (n=18) and roe deer *Capreolus capreolus* (n=3), for an overall number of 65 animals. Wild animals were culled or accidentally found dead between 2009 and 2012, while domestic animals were sampled at the time of the slaughter in the same period. These animals previously tested positive for *T. gondii* by using conventional PCR targeting the 200-300 fold repetitive 529 bp DNA fragment (Ferroglio et al., 2014). For each animal, a portion of the skeletal muscle tissue of approximately 25 mg was used for DNA extraction by using the commercial kit GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA).

For this study, *T. gondii* genotyping was performed by using the PCR-RFLP method. This technique is based on the ability of restriction endonucleases to recognize single nucleotide polymorphisms (SNPs), to digest PCR products and consequently display distinct DNA banding patterns on agarose gel by electrophoresis (Sibley and Boothroyd, 1992; Sibley, 1995).

PCR protocols and target genes used in this study were adapted from a paper by Su and colleagues (Su et al., 2010). In this paper, authors have developed a multiplex multilocus nested PCR-RFLP method consisting in the analysis of 10 genetic markers (i.e. SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and APICO). This technique has been elaborated in order to improve the sensibility compared to conventional PCR-RFLP, which is carried out targeting single-copy genes, and is based on the concomitant use of a multiplex and a nested approach. In particular, all markers are pre-amplified by multiplex PCR using external primers in a single reaction, and the pre-amplified PCR products are used as template to amplify each individual marker separately by nested PCR (Fig. 10). Once the PCR amplicons is obtained, this is digested by specific restriction endonucleases and visualized by electrophoresis. In case of SNPs, different band patterns will be observed, each of them peculiar to one different genotype of the parasite.

In the present study, 6 DNA fragments were used for genotyping: GRA6, alt. SAG2, BTUB, C22-8, 5' SAG2 and SAG1 (Tab.1). Briefly, PCR reaction for the first step contained 12.5 µl of PCR Master Mix (Promega, Madison, WI), 4 pmol of the external primers targeting two different DNA fragments (e.g. GRA6 and alt. SAG2) and 4 µl of extracted DNA, in a total volume of 25 µl. The PCR reaction was carried out by using the MJ Mini Thermal Cycler (Bio-Rad, Hercules, CA) with the following

thermal conditions: 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, and a final elongation step of 72 °C for 10 min. PCR product of the first step was used as a template for the second step. Briefly, the reaction contained 12.5 µl of PCR Master Mix (Promega, Madison, WI), 8 pmol of internal primers targeting only one DNA fragment (e.g. GRA6) and 3 µl of amplification product, for an overall volume of 25 µl. During each PCR, a positive and a negative control were processed in parallel in order to minimize the risk of contamination. PCR amplicons were visualized on a 2% agarose gel with a UV transilluminator (GelDoc 1000, Bio-Rad, Hercules, CA). For RFLP typing, 3 µl of PCR product was incubated with 0.2 µl of restriction endonuclease (20000 units/ml - New England BioLabs) (see Tab. 1 for the list of enzymes used for each marker) and 2 µl of 1 X NEB buffer, for a total volume of 20 µl. Results were visualized on a 2.5% agarose gel. Together with RFLP typing using restriction endonucleases, an innovative in-silico RFLP typing was carried out. Briefly, positive amplicons were purified by using the Nucleospin Extract II Kit (Macherey-Nagel, Düren, Germany) and sent for sequencing (Macrogen Europe, The Netherlands). RFLP analysis was performed by using the free online software NEBCutter (New England BioLabs) (Vincze et al., 2003).

Statistical analysis was carried out with the R software (R Development Core Team, 2018).

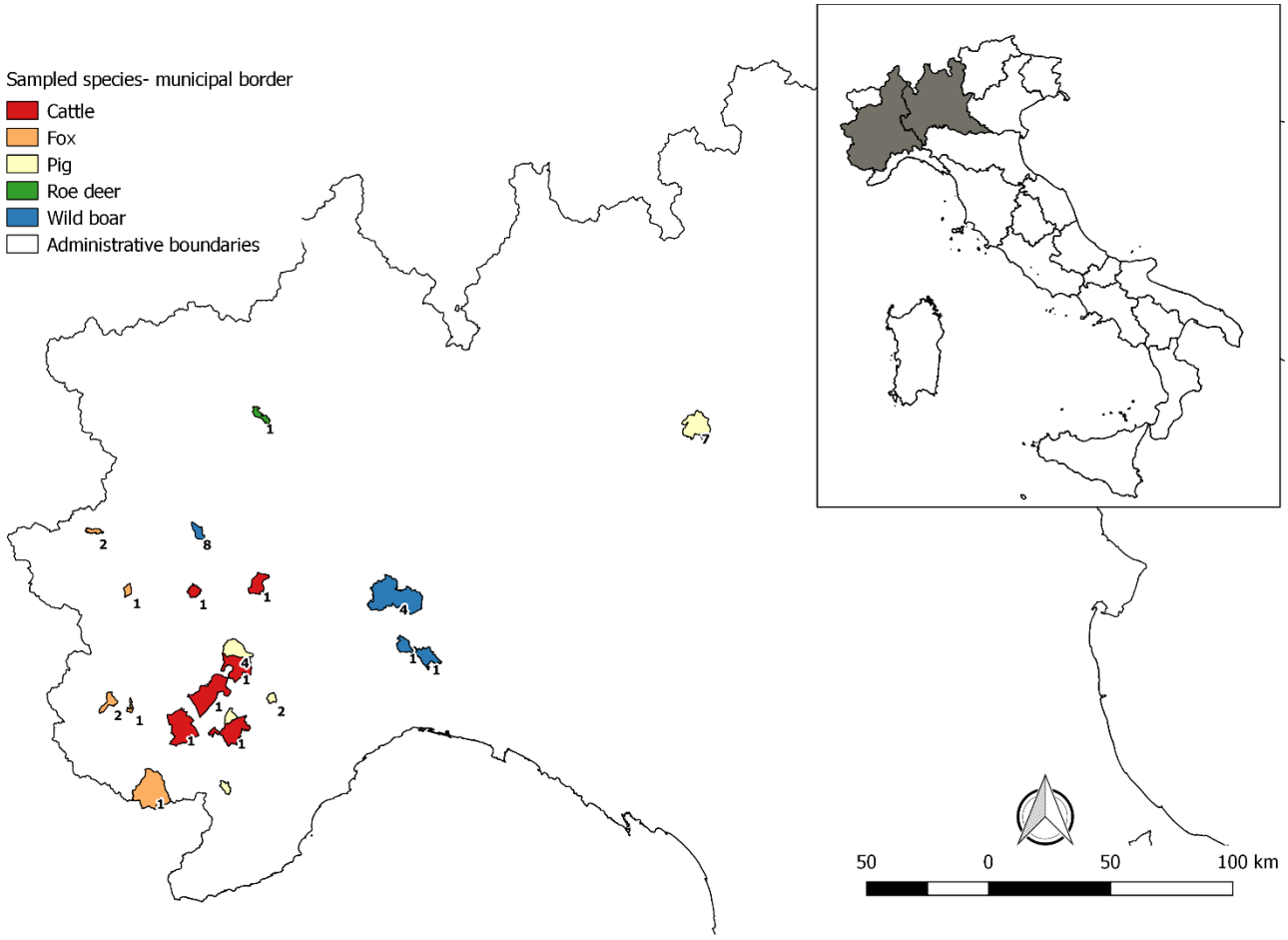

Fig. 9 Sampling sites for wildlife and livestock analysed in this study. Coloured areas displayed the municipal borders in which samples were collected; for each area, the number of collected samples is shown.

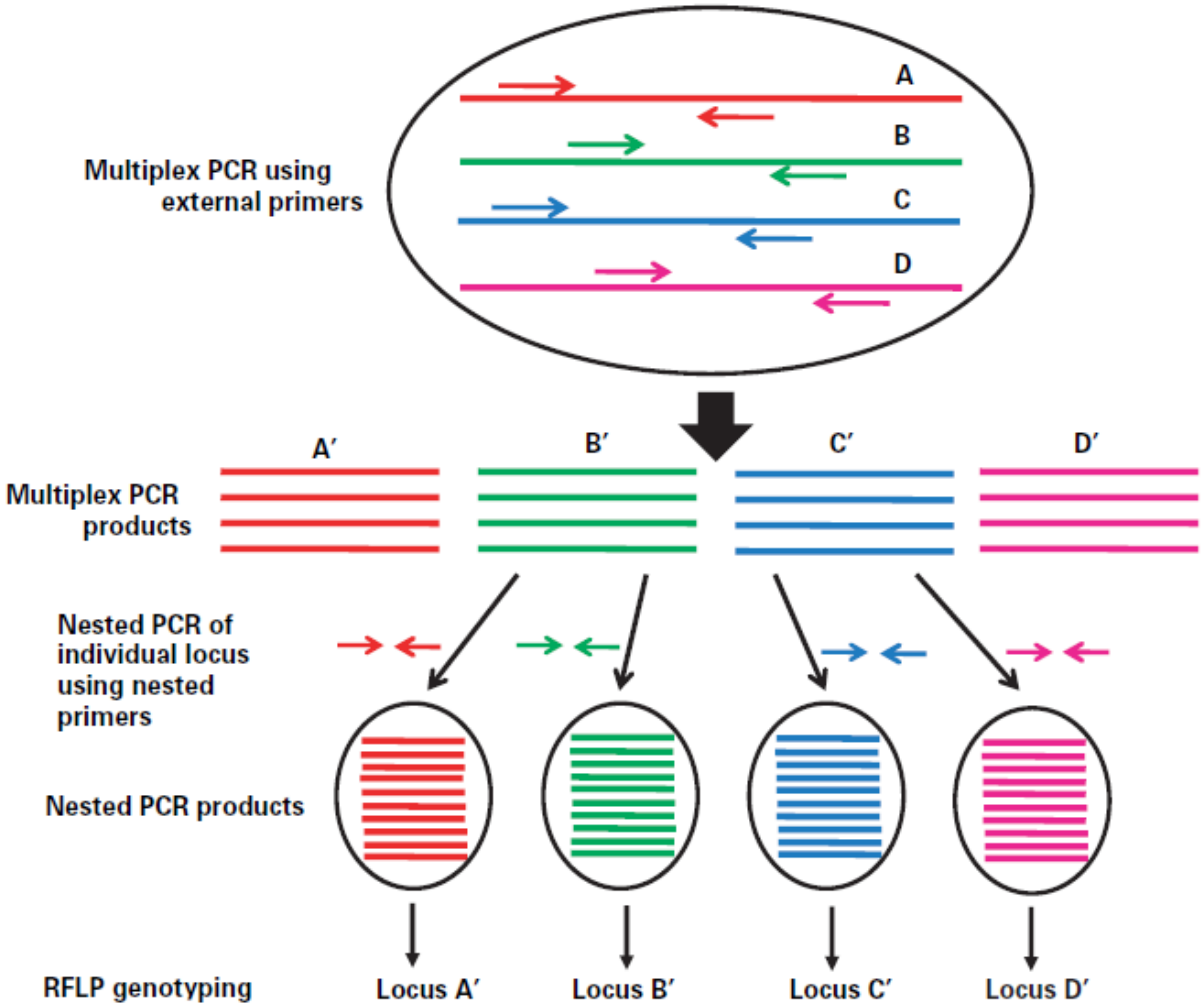

Fig. 10 Summary of the multiplex multilocus nested PCR-RFLP technique (adapted from Su et al. 2010).

Markers	Multiplex PCR primers (external primers)*	Nested PCR primers (internal primers)	Nested PCR (bp)	Restriction enzymes, NEB buffers, incubation temperature and time	Reference
SAG1	F: GTTCTAACCACGCACCCTGAG R: AAGAGTGGGAGGCTCTGTGA	F: CAATGTGCACCTGTAGGAAGC R: GTGGTTCTCCGTCGGTGTGAG	390	Sau96I + HaeII (double digest). NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Grigg *et al.* 2001)
5′-SAG2	Not needed. The DNA fragment for 5′-SAG2 is covered by the external primers of alt. SAG2.	F: GAAATGTTTCAGGTTGCTGC R: GCAAGAGCGAACTTGAACAC	242	MboI, NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Howe *et al.* 1997; Su *et al.* 2006)
3′-SAG2	F: TCTGTTCTCCGAAGTGACTCC R: TCAAAGCGTGCATTATCGC	F: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	222	HhaI, NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Howe *et al.* 1997)
alt. SAG2	F: GGAACGCGAACAATGAGTTT R: GCACTGTTGTCCAGGGTTTT	F: ACCCATCTGCGAAGAAAACG R: ATTTCGACCAGCGGGAGCAC	546	HinfI + TaqI, NEB3, BSA, 37 °C 30 min, 65 °C 30 min. 2·5% gel.	(Khan *et al.* 2005*b*; Su *et al.* 2006)
SAG3	F: CAACTCTCACCATTCCACCC R: GCGCGTTGTTAGACAAGACA	F: TCTTGTCGGGTGTTCACTCA R: CACAAGGAGACCGAGAAGGA	225	NciI, NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Grigg *et al.* 2001)
BTUB	F: TCCAAAATGAGAGAAATCGT R: AAATTGAAATGACGGAAGAA	F: GAGGTCATCTCGGACGAACA R: TTGTAGGAACACCCGGACGC	411	BsiEI + TaqI (double digest), NEB4, BSA, 60 °C 1 h. 2·5% gel.	(Khan *et al.* 2005*b*; Su *et al.* 2006)
GRA6	F: ATTTGTGTTTCCGAGCAGGT R: GCACCTTCGCTTGTGGTT	F: TTTCCGAGCAGGTGACCT R: TCGCCGAAGAGTTGACATAG	344	MseI, NEB2, BSA, 37 °C 1 h. 2·5% gel.	(Khan *et al.* 2005*b*; Su *et al.* 2006)
C22-8	F: TGATGCATCCATGCGTTTAT R: CCTCCACTTCTTCGGTCTCA	F: TCTCTCTACGTGGACGCC R:AGGTGCTTGGATATTCGC	521	BsmAI + MboII (double digest), NEB2, BSA, 37 °C 30 min, 55 °C 30min. 2·5% gel.	(Khan *et al.* 2005*b*; Su *et al.* 2006)
C29-2	F: ACCCACTGAGCGAAAAGAAA R: AGGGTCTCTTGCGCATACAT	F: AGTTCTGCAGAGTGTCGC R:TGTCTAGGAAAGAGGCGC	446	HpyCH4IV + RsaI (double digest), NEB1, BSA, 37 °C 1 h. 2·5% gel.	(Khan *et al.* 2005*b*; Su *et al.* 2006)
L358	F: TCTCTCGACTTCGCCTCTTC R: GCAATTTCCTCGAAGACAGG	F: AGGAGGCGTAGCGCAAGT R: CCCTCTGGCTGCAGTGCT	418	HaeIII + NlaIII (double digest), NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Khan *et al.* 2005*b*; Su *et al.* 2006)
PK1	F: GAAAGCTGTCCACCCTGAAA R: AGAAAGCTCCGTGCAGTGAT	F: CGCAAAGGGAGACAATCAGT R: TCATCGCTGAATCTCATTGC	903	AvaI + RsaI (double digest), NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Khan *et al.* 2005*b*; Su *et al.* 2006)
Apico	F: TGGTTTTAACCCTAGATTGTGG R: AAACGGAATTAATGAGATTTGAA	F: GCAAATTCTTGAATTCTCAGTT R: GGGATTCGAACCCTTGATA	640	AflII + DdeI (double digest), NEB2, BSA, 37 °C 1 h. 3% gel.	(Su *et al.* 2006)

* F, forward primer; R, reverse primer.

Tab. 1 Summary of primers and restriction enzymes used in this study (adapted from Su et al., 2010).

5.1.2. Results

Overall, PCR amplification and sequencing of the inquired markers (GRA6, alt. SAG2, BTUB, C22-8, 5' SAG2 and SAG1) was obtained for 43 samples (6 bovines, 15 pigs, 7 foxes, 1 roe deer and 14 wild boars). Nucleotide sequences obtained were deposited in GenBank™ (accession number from MG587956 to MG588017).

For one sample (wild boar 817/11) it was possible to amplify all the markers, while most of the samples were genotyped at less than 6 alleles due to amplification fail. GRA6 marker was statistically more amplified than the others (χ^2 = 10.5; p = 0.001).

Thirty-one samples showed the presence of type I allele at least at one locus [31/45], with an overall prevalence of 72.09% (CI95% 57.31% - 83.25%). Two samples showed the presence of type II allele [2/43] (p = 4.65%; CI95% 57.31 - 83.25%), while type III allele was present in three samples [3/43] (p = 6.98%; CI95% 2.40% - 18.61%). Seven samples [7/43] (p = 16.28%; CI95% 8.12% - 29.97%) showed mixed type alleles. Results are summarized in Tab. 2.

Sample ID		PCR-RFLP markers					
		alt.SAG2	GRA6	SAG1	BTUB	5'SAG2	C22-8
Roe deer	12/11	I	I	na	na	na	na
Fox	116/11	I	I	na	na	na	na
Fox	324/11	na	I	na	na	na	na
Fox	335/11	na	III	na	na	na	II
Fox	339/11	I	I	na	na	na	na
Wild boar	697/11	III	na	na	na	na	na
Wild boar	698/11	I	III	na	na	na	na
Wild boar	699/11	I	III	na	na	na	na
Wild boar	700/11	I	I	na	na	na	na
Wild boar	723/11	na	I	na	na	na	na
Wild boar	730/11	I	I	na	na	na	na
Wild boar	766/11	III	na	na	na	na	na
Wild boar	780/11	I	I	na	na	na	na
Wild boar	783/11	na	I	na	na	na	na
Fox	806/11	I	I	na	na	na	na
Wild boar	807/11	na	I	na	na	na	na
Wild boar	817/11	II	II	II/III	II	II	II
Wild boar	846/11	III	I	na	na	na	na
Wild boar	847/11	I	III	na	na	na	na
Fox	869/11	na	I	na	na	na	na
Fox	876/11	II	I	II/III	na	III	II
Wild boar	890/11	I	na	na	na	na	na
Pig	A4	na	I	na	na	na	na
Pig	A7	na	I	na	na	na	na
Pig	B8	na	I	na	na	na	na
Pig	C5	na	I	na	na	na	na
Pig	P51	na	I	na	na	na	na
Pig	P54	na	I	na	na	na	na
Pig	P55	I	I	na	na	na	na
Pig	P60	I	I	na	na	na	na
Pig	Q64	III	II	na	na	na	na
Pig	Q67	I	I	na	na	na	na
Pig	Q68	na	I	na	na	na	na
Pig	Q69	na	I	na	na	na	na
Pig	Q70	na	I	na	na	na	na
Pig	Q72	I	I	na	na	na	na
Pig	Q73	I	I	na	na	na	na
Cattle	6	I	I	na	na	na	na
Cattle	41	I	I	na	na	na	na
Cattle	60	I	I	na	na	na	na
Cattle	63	III	na	na	na	III	na
Cattle	64	na	II	na	na	na	na
Cattle	91	na	I	na	na	na	na

Tab. 2 Results of the genotyping study (adapted from Battisti et al., 2018, paper n°1). The first two columns showed the animal species and the ID number. The other columns indicated the allele type obtained from the PCR-RFLP analysis (na = marker region could not be amplified).

Analysing results for species, higher prevalence of type I allele was recorded in domestic pigs than in foxes and wild boars ($X^2 = 5.6$; $p = 0.018$). Furthermore, mixed type alleles were more frequently

recorded in wildlife species (foxes, wild boars and roe deer) than in livestock ($X^2 = 6.6$; $p = 0.010$) (Fig. 11).

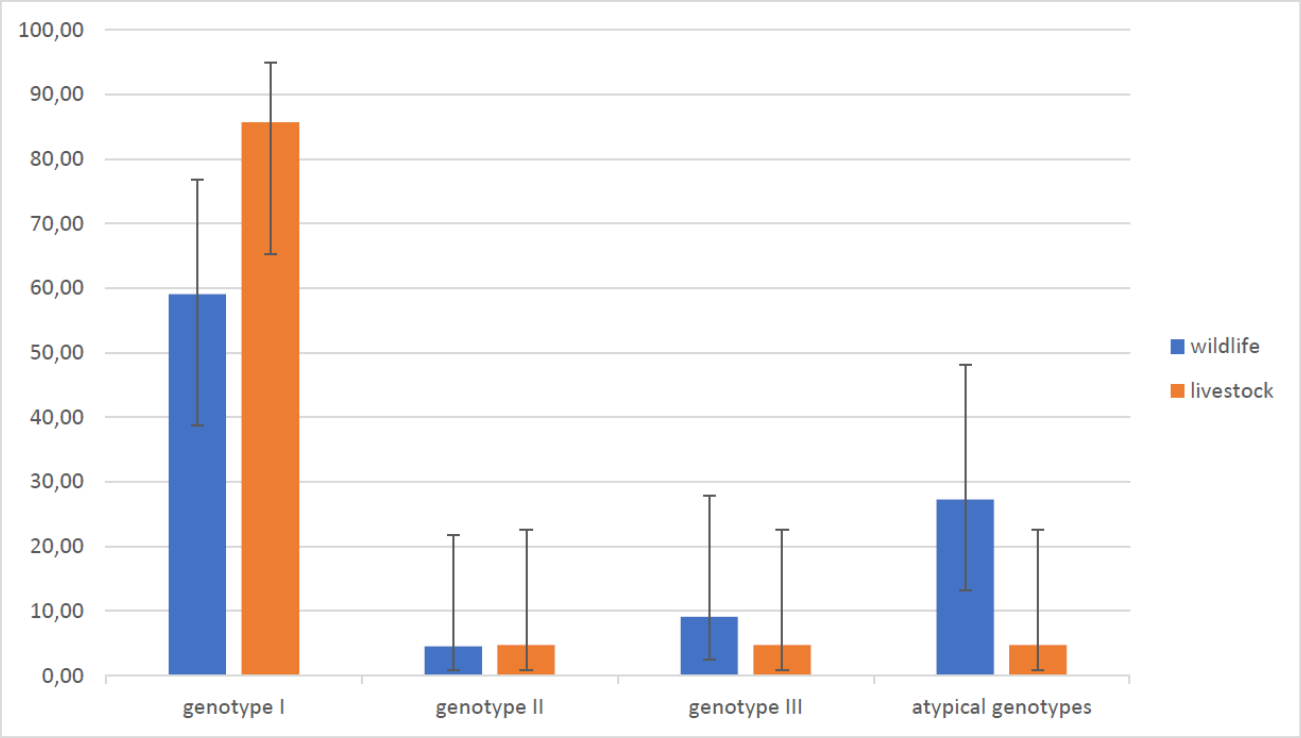

Fig. 11 Prevalence of *T. gondii* genotypes within domestic and sylvatic animals (adapted from Battisti et al., 2018; paper n°1). Significative differences ($p < 0.05$) were observed in the prevalence of atypical genotypes between wildlife and domestic population. Whiskers represent 95% confidence intervals.

5.1.3. Discussion

The present study aimed to investigate, through the use of the PCR-RFLP technique, the circulating genotypes of *T. gondii* in wildlife and livestock samples collected from the same area. In Italy, previous studies have focused on a singular animal species, even domestic or sylvatic (Verin et al., 2013; Mancianti et al., 2014, 2013a; Formenti et al., 2016; Dubey et al., 2008; Bacci et al., 2015).
The PCR-RFLP method is one of the most commonly used for *T. gondii* genotyping, both in human and animal samples (Thomasson et al., 2011; Richomme et al., 2009; Prestrud et al., 2008; Mancianti et al., 2014, 2013a; Kik et al., 2015; Khan et al., 2005; Herrmann et al., 2013; D. C. Herrmann et al., 2012; Alessia Libera Gazzonis et al., 2018; Formenti et al., 2016; Ferreira et al., 2008; Dubey et al., 2008; Calero-Bernal et al., 2015; Burrells et al., 2013; Bajnok et al., 2015; Bacci et al., 2015; Aubert et al., 2010, 2008). This technique is based on the ability of restriction endonucleases to recognize single nucleotide polymorphisms (SNPs), to digest PCR products and

consequently display distinct DNA banding patterns on agarose gel by electrophoresis (Sibley and Boothroyd, 1992; Sibley, 1995). Among the genotyping methods (e.g. microsatellites, MLST technique), PCR-RFLP is simple and cost effective, and thus is considered one of the most useful for epidemiological studies (Su et al., 2010). However, this technique showed several problems during the present study, the most important of which was the low sensitivity of the PCR protocol. In particular, it is clear that high quantity and quality of DNA extracted from a sample is pivotal for a successful outcome of the PCR amplification and thus of the genotyping. In literature, PCR-RFLP results are controversial and mainly dependent on the type of sample used for genotyping. Authors using the mouse bioassay (Dubey et al., 2008) as a starting point for genotyping have showed to obtain better results in terms of number of amplified markers than authors that have carried out PCR-RFLP without a prior bioassay step (D. C. Herrmann et al., 2012; Calero-Bernal et al., 2015; Berger-Schoch et al., 2011)(Daland C. Herrmann et al., 2012; Calero-Bernal et al., 2015). Mouse bioassay is based on the oral administration or, more commonly, on the subcutaneous inoculation of homogenized tissue from the investigated animal to the mouse, and the subsequent analysis of tissues or serum of the infected mice. Infected mice used for the bioassay usually show a certain level of immunosuppression, and thus *T. gondii* is free to multiply and spread to all the mouse tissues that are used for genotyping. Even if mouse bioassay allows to obtain a large amount of high-quality DNA, necessary for the amplification of all the genotyping markers, it is extremely expensive and time consuming. Furthermore, it requires the recovery of viable *T. gondii* from investigated animals, that is nevertheless not always possible in case of epidemiological studies on wildlife, for which sampling activities may occur days after the death of the animals.

Although amplification for most of the markers failed, type I allele seemed to be the most prevalent in the analysed animals, followed by atypical genotypes (mixed type alleles), while type II and III showed a significative lower prevalence. As described in the introduction section, the genetic population of *T. gondii* in Europe is mainly based on three clonal genotypes (Ajzenberg et al., 2004), which genetically differ each other by less than 1% (Su et al., 2006). Previous studies in Europe showed considerable differences among the countries. In France, studies on both wildlife and livestock have showed the presence of the genotype II only (Richomme et al., 2009; Dumètre et al., 2006; Aubert et al., 2010, 2008). A similar situation has been observed in rodents from Czech Republic (Machačová et al., 2016) and The Netherlands (Kik et al., 2015), and in lagomorphs from Finland (Jokelainen et al., 2011). In other countries, the situation is somewhat different. In Spain, Calero-Bernal and colleagues reported the presence of genotype II from all the analysed wildlife species, but also of genotype III in red deer, Spanish ibex and foxes, of genotype I in wild boars and of atypical genotypes in wild boars, red deer and foxes (Calero-Bernal et al., 2015). In Germany, the presence of genotype II only has been reported in European beavers and wildcat (Herrmann et al., 2013), while genotype II, III and atypical have been observed in foxes (D. C. Herrmann et al., 2012). In Switzerland, type II alleles has been found to be predominant in sheep, while mixed type or

atypical infections have been recorded in bovines (Berger-Schoch et al., 2011). Mixed type alleles have been also reported in arctic foxes from Norway (Prestrud et al., 2008), and genotype II and III in foxes from Belgium (De Craeye et al., 2011). In Italy, previous studies carried out on wildlife have showed the presence of the three clonal and atypical genotypes. Genotype II has been reported from alpine chamois and wild boars (Alessia Libera Gazzonis et al., 2018; Formenti et al., 2016), while atypical genotypes have been the only genotypes observed in wild birds of the genus *Anas* (Mancianti et al., 2013b). Regarding carnivores, foxes from Central Italy have been showed to be infected with genotype I, III and atypical genotypes, while no clonal genotype II has been recorded (Verin et al., 2013). Concerning domestic animals, type II alleles are the only genotype observed in chickens (Dubey et al., 2008). Genotype II and III are the most commonly reported also in goats, donkeys and pigs, with low prevalence of genotype I and atypical genotypes (Mancianti et al., 2014, 2013a; Bacci et al., 2015). Although it is difficult to compare results among the studies due to differences in exploited techniques and other peculiarities, it seems clear that the robust clonal structure of *T. gondii* previously thought to exist in Europe it is not such accurate. In fact, several studies reported the presence of atypical genotypes, defined as mixed type alleles (Verin et al., 2013; Prestrud et al., 2008; Mancianti et al., 2013b; Daland C. Herrmann et al., 2012; Calero-Bernal et al., 2015; Berger-Schoch et al., 2011; Bacci et al., 2015). Furthermore, while genotype II seems to be the most or even the only genotype recorded in many studies (Richomme et al., 2009; Herrmann et al., 2013; Alessia Libera Gazzonis et al., 2018; Formenti et al., 2016; Dumètre et al., 2006; Calero-Bernal et al., 2015; Berger-Schoch et al., 2011; Aubert et al., 2010, 2008), there are some exception. In particular, Verin and colleagues have observed, similar to the present study, high prevalence of atypical genotypes and low prevalence of genotype II in wildlife (Verin et al., 2013).

Statistical analysis on our results revealed the higher prevalence of type I allele in domestic pigs than in foxes and wild boars ($X^2 = 5.6$; $p = 0.018$). In contrast, mixed type alleles were more frequently recorded in wildlife species (foxes, wild boars and roe deer) than in livestock ($X^2 = 6.6$; $p = 0.010$). These results are in accordance with previous findings (Grigg and Sundar, 2009). In this paper, authors have described two different pathways of *T. gondii* life cycle: a domestic (comprising humans and livestock) and a sylvatic cycle that concerns wildlife. Within domestic cycle, clonal lineages of *T. gondii* have been identified as the most frequently reported in Europe and North America, revealing little variation across different isolates. However, cats (one of the most important definitive host for *T. gondii*) are highly prevalent and widely distributed in the domestic environment, and therefore the sexual recombination of *T. gondii* in the definitive host should be extremely frequent. In order to explain the lack of genetic variability of the parasite in the presence of high number of felines, parasite propagation based on either oral transmission through the ingestion of bradyzoites cysts or by inbreeding (self-fertilization of *T. gondii* within the definitive host) was proposed (Sibley and Boothroyd, 1992; Dardé, 1996). In contrast, wildlife samples have showed higher prevalence of atypical genotypes of *T. gondii*, that could be linked to the higher rate of genetic variability of the host

compared to domestic animals. In the present study, we supposed that pigs are more likely infected with clonal lineages of *T. gondii* due to the intensive breeding systems in which they are raised. In these systems, animals could accidentally come in contact with the parasite through the ingestion of infected prey such as rodents or sporulated oocysts shed by felids. In both these cases, the infection cycle is more probably limited to the breeding area, without contacts with wildlife. In contrast, foxes and wild boars showed higher prevalence of mixed type infections that could be linked to the scavenging habits of these species. In fact, they feed on several preys infected with different genotypes of *T. gondii*, leading to a superinfection with different strains. For instance, they could come in contact with migrating birds which act as biological carriers and reservoir hosts of the parasite (Yan et al., 2016), due to the fact that *T. gondii* infection has been linked to reduced motility of birds and hence can increase the susceptibility to predation (Hubalek, 2004). Moreover, organically-raised pigs, which are free to raise outdoor, have showed higher risk of acquiring *T. gondii* infection compared to pigs raised in confinement (van der Giessen et al., 2007; Dubey, 2009b), and higher risk of infection with atypical lineages (Bacci et al., 2015).

5.1.4. Conclusions

Despite the burden of human toxoplasmosis, that could be even greater to that of salmonellosis, campylobacteriosis and other food-borne pathogens (Scharff, 2012), *T. gondii* is still considered a neglected parasite (Jones et al., 2014). Even though it is frequently underreported as a food safety concern, *T. gondii* is considered a re-emerging pathogen due to several reasons, such as changing eating habits (Dorny et al., 2009). In Northwestern Italy, human toxoplasmosis has been reported as the second most frequent zoonosis, with 805 cases (with all the plethora of typical symptoms including retinochoroiditis, meningoencephalitis and myocarditis) between 1999 and 2012 (Ferroglio et al., 2016).

In spite of the small number of genotyped loci, results showed the high prevalence of genotype I and type-I alleles, together with recombinant non-clonal genotypes in both wildlife and livestock. The wide spectrum of symptoms caused by *T. gondii* depends also on the genotype of the parasite. In mice, genotype I has showed a LD_{100} of 1 tachyzoite, underlying a high virulence of these strains, while genotypes II and III have been considerable less virulent, showing a LD_{100} of more than 1000 tachyzoites (Sibley and Boothroyd, 1992). In humans, genotype I and some atypical strains have been associated to a more severe symptomatology in immunocompromised individuals (Khan et al., 2005) and newborns (Xiao and Yolken, 2015). Together with the increased number of susceptible people due to cancer rise, ageing, malnutrition and HIV infection (WHO, 2014; Dorny et al., 2009), these results should put the focus on *T. gondii* and its consequences for human health, in order to act accordingly. In particular, it is necessary to increase prevention through raising awareness on

the problem, and to progress in the genotyping methods, because up to now the PCR-RFLP technique without bioassay presents several problems, such as low sensitivity.

Battisti E., Zanet S., Trisciuoglio A., Bruno S., Ferroglio E. (2018). Circulating genotypes of *T. gondii* in Northwestern Italy. *Vet Parasitol*. 253, 43-47 doi: 10.1016/j.vetpar.2018.02.023

5.2. Screening of *T. gondii* positive sheep flocks in Perugia province (Umbria region, Central Italy) using bulk milk analysis – PAPER 2

5.2.1. Materials and methods

For the evaluation of *T. gondii* prevalence and viability in dairy products, only sheep flocks devoted to milk production and possessing more than 300 units were included in the study. Therefore, 36 herds complying with these criteria were chosen and sampled in the period between June and September 2016. Sampling activities were performed three times for each flock, with three weeks interval between each sampling, for an overall number of 108 collected specimens of 100 ml bulk milk. Samples were aseptically removed from the daily production and immediately refrigerated. Two aliquots were obtained from each specimen: one (50 ml) was analysed for the presence of anti-*T. gondii* IgG antibodies at the Dept. of Veterinary Medicine of the University of Perugia (Italy) by the laboratory staff, and the other one (50 ml) was sent at the Dept. of Veterinary Sciences of the University of Torino (Italy) for molecular analysis. The detection of anti-*T. gondii* IgG antibodies was performed by using an immunofluorescence antibody test (IFAT) with sheep anti-IgG antibodies (Sigma®) conjugated to fluorescein isothiocyanate, a cut-off value of 1/64, tachyzoites of the RH strain as antigens (MegaScreen® FLUOTOXOPLASMA g., Diagnostik Megacor) and positive and negative control sera in all the reactions. For the molecular analysis of milk samples, an isothermal amplification with LAMP technology was used, following the protocol by Trisciuoglio and colleagues (Trisciuoglio et al., 2015). Briefly, the LAMP (Loop-mediated isothermal amplification) technology is based on amplification of DNA at constant temperature of 60-65 °C, using 2 primer pairs designed to amplify 6 specific regions of the target gene (Notomi et al., 2000). In the present study, primer pairs targeted the SAG2 gene. Each LAMP reaction contained 8 U of *Bst* Polymerase (New England BioLabs), and 2.5 µl of buffer. Amplification was carried out at 65 °C for 60 min, followed by 20 min at 80 °C to inactivate the polymerase. LAMP products were visualized on a 1.5% agarose gel with a UV transilluminator (GelDoc 1000, Bio-Rad, Hercules, CA). Positive and negative controls were processed in each assay in order to minimize the risk of contamination. Positive samples were amplified by using a conventional PCR, targeting the GRA6 gene, to confirm the result.

Briefly, PCR reaction for the first step contained 12.5 µl of PCR Master Mix (Promega, Madison, WI), 4 pmol of the external primers targeting the GRA6 fragment and 4 µl of extracted DNA, in a total volume of 25 µl. The PCR reaction was carried out by using the MJ Mini Thermal Cycler (Bio-Rad, Hercules, CA) with the following thermal conditions: 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, and a final elongation step of 72 °C for 10 min. PCR product of the first step was used as a template for the second step. Briefly, the reaction contained 12.5 µl of PCR Master Mix (Promega, Madison, WI), 8 pmol of internal primers and 3 µl of amplification product, for

an overall volume of 25 µl. Agreement between IFAT and LAMP on bulk milk was calculated by K statistic (K) and McNemar's Chi squared-test.

5.2.2. Results

Out of the 36 analysed flock, 8 were positive for the presence of *T. gondii* by using both methods (IFAT and LAMP), with an overall prevalence of 22.2% (CI95% 11.72% - 38.08%), while 7 tested negative (Tab. 3). For remaining flocks, positivity was observed by using either LAMP or IFAT method, for an overall prevalence at herd level of 80.6% (CI95% 64.97% - 90.25%).
In 30% of the flocks tested positive by using IFAT, the presence of anti-*T. gondii* antibodies was confirmed in all the three samples collected from each herd. Concerning LAMP results, one herd that tested positive was confirmed in two out of the three samples tested, while remaining flocks showed positivity only in one of the replicates (Tab. 4). Agreement analysis showed a k-value of 0.125.

Method	% of positive flocks	CI 95%
IFAT	55.56	47.39 - 63.72
LAMP	44.44	36.27 - 52.61
Total	80.56	74.05 - 87.06

Tab. 3 Percentage of positive flocks to *T. gondii* by applying the two different methods (adapted from Veronesi et al., 2018; paper n°2).

Methods	Number of positive flocks	Flocks with 3 positive samples	Flocks with 2 positive samples	Flock with 1 positive sample
IFAT +	20	6 (30% of the positives)	6 (30% of the positives)	8 (40% of the positives)
LAMP +	16	0	1 (6.25% of the positives)	15 (93.75% of the positives)

Tab. 4 Number of flocks found positive to *T. gondii* during replicate sampling (adapted from Veronesi et al., 2018; paper n°2).

5.2.3. Discussion

Results of *T. gondii* prevalence in sheep herds confirmed the wide distribution of this parasite and were in line with previous studies carried out throughout Italy. In particular, data on the seroprevalence obtained by using IFAT, MAT or ELISA have showed that 77.8%, 87%, 87.5% and 97% of the herds had at least one *T. gondii* positive animal in Campania, Sicily, Lombardy and

Tuscany region respectively (Vesco et al., 2007; Gazzonis et al., 2015; Fusco et al., 2007; Cenci-Goga et al., 2013). The high prevalence of *T. gondii* infection in sheep could be due to the rearing system adopted for these animals, which is partially or totally free-ranging. As discussed previously for *T. gondii* genotypes in pigs, free-ranging rearing system has been associated to higher risk of acquiring an infection (van der Giessen et al., 2007; Dubey, 2009b) due to contacts with domestic and feral cats, as well as with rodents and wildlife (Gazzonis et al., 2015).

It is worthy to note the lack of concordance between IFAT and LAMP techniques, with lower prevalence of infection recorded with molecular analysis. These methods are based on two different assumption: while IFAT measure the presence of anti-*T. gondii* antibodies and thus give and information about the immunological response of the animal against the parasite, LAMP technique is a direct method that evaluate the presence of *T. gondii* DNA. For pathogens like *T. gondii*, that can be transmitted to humans through the ingestion of products derived from animals such as meat or milk, it is essential to use alternative matrixes to sera for pathogen screening. Given the fact that sheep milk can be a source of infection to humans, this matrix should be used for the evaluation of *T. gondii* prevalence, as strongly recommended also for goats (A. L. Gazzonis et al., 2018). However, the low agreement among the techniques obtained in the present study could suggest that bulk milk is less effective than sera for the detection of *T. gondii*, probably due to the non-homogeneous nature of this matrix. Moreover, it has been showed that a limited number of positive animals are present within a flock even in highly infected areas (Cenci-Goga et al., 2013), thus a dilution effect of the parasite DNA in bulk milk could be observed. One more possible explanation of this discrepancy could be the non-continuous shedding of *T. gondii* tachyzoites in the milk of positive animals, as previously observed in mice (Pettersen, 1984) and cats (Powell et al., 2001). In fact, the presence of parasite tachyzoites in milk of infected animals depends on several factors such as the immunological status of the animal and the infection phase (Bezerra et al., 2015), and thus not all the serologically positive animals eliminate the parasite through milk. Therefore, the concordance between positive sera and bulk milk samples should be carefully considered for a proper interpretation of epidemiological studies results, and the adoption of bulk milk for *T. gondii* screening in sheep flocks needs attention if only one method is adopted along with a sampling protocol without replication.

5.2.4. Conclusions

The present study highlights the considerable prevalence of *T. gondii* in ewe flocks from Umbria, a region highly devoted to sheep farming and traditional cheese production. The need of sampling replicates and the low concordance of the two methods employed underline the fact that bulk milk is not completely suitable for *T. gondii* screening, despite its higher availability compared to blood.

Further studies are needed to investigate the effective risk for human health deriving from milk and other dairy products obtained from sheep milk.

Veronesi F., Chiesa F., Zanet S., **Battisti E**., Sebastiani V., Branciari R., Valiani A., Ranucci D. (2018). Screening of *T. gondii* positive sheep flocks in Perugia province (Umbria region, central Italy) using bulk milk analyses. *Large Animal Review*. 24(5):185-187 https://cms.sivarnet.it/it/lar

5.3. Absence of viable *T. gondii* in raw-milk ewe cheese derived from naturally infected animals – PAPER 3

5.3.1. Material and methods

Eight herds, found positive both to LAMP and IFAT in the previous study, were chosen to evaluate *T. gondii* viability in raw milk and cheese. Briefly, twenty litres of bulk milk were collected at each farm and immediately transported refrigerated at the Dept. of Veterinary Medicine of the University of Perugia (Italy). Here the milk was stored at 4-6 °C and processed within 48 h. Samples of bulk milk were divided in 2 batches of 10 litres each and separately processed for cheese preparation by the laboratory staff of the Department. Milk was heated to 37 °C and added with started culture of *Lactobacillus lactis* subsp. *helveticus* and *Streptococcus termophylus* (Laboratorio Prodor, Bobbio, PC, Italy). After 30 min of rest, calf rennet was added (Laboratorio Prodor, Bobbio, PC, Italy), in order to obtain coagulation of the milk. The curd was then cut in pieces of approximately 3 cm in size each, transferred to cylindrical cheese moulds and pressed to drain the whey. Cheese was then stored at 30 °C for 1 h, to facilitate the proliferation of lactic acid bacteria, and stored at 7 °C overnight. Marine salt was finally added on the surface of each cheese and stored in a ripening chamber at 10 °C and 90% of relative humidity. At 5 (T_5) and 15 (T_{15}) days after production, 5 gr of each cheese were subjected to analytical determination. Briefly, pH was measured by inserting a pHmeter probe (Crison Instruments, Barcelona, Spain) in the inner part of each cheese sample; water activity (a_w) was measured by using a hygrometer (Series 3 TB, Decagon Devices Inc., Pullman, WA, USA); finally, the salt content was determined by using the Volhard method 935.43 (AOAC, 2000). All analyses were performed in triplicate.

At the same time, specimens of bulk milk, T_5 and T_{15} cheese from each herd were sent refrigerated to the Dept. Veterinary Sciences of the University of Torino for molecular analysis on *T. gondii* by using LAMP and RT-PCR. For nucleic acid extraction, 50 ml of bulk milk were centrifuged at 1000 g for 15 min a 4 °C, to obtain fat separation (Mura et al., 2013). Pellet was resuspended in 200 µl of PBS (pH 7.2) and 1 ml of RNAlater (Qiagen, Milan, Italy), and washed by repeated steps of pelleting and resuspending with a solution of 200 µl of TE buffer [1 mM EDTA, 10 mM Tris-HCl (pH 7.6)] and 300 µl of EDTA (pH 8). The final pellet was resuspended with 600 µl of RLT buffer (Qiagen, Milan, Italy). DNA extraction was performed by using the GenElute Mammalian Genome DNA Miniprep (Sigma-Aldrich, St. Louis, MO, USA), while RNA was extracted by using the RNAeasy Mini Kit (Qiagen, Milan, Italy). For nucleic acid extraction from cheese, 5 gr of material was added with 20 ml of RNAlater (Qiagen, Milan, Italy) and homogenized with a stomacher machine. One ml of suspension was then mixed with 9 ml of trisodium citrate (2 % w/w), and 1 ml of this latest suspension was recovered and centrifuged at 6000 g for 5 min at 4 °C. Pellet was washed with a solution of 1 ml of TE buffer [1 mM EDTA, 10 mM Tris-HCl (pH 7.6)] and 1 ml of EDTA (pH 8), and resuspended

in 1 ml of TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) and 80 µl of proteinase K (20 mg/ml). With the addition of 200 µl of chloroform (Sigma-Aldrich, St. Louis, MO, USA), three different phases appeared in the solution: a colourless upper aqueous phase containing RNA, an interphase containing DNA, and a red organic lower phase containing proteins. DNA isolation was performed following the TRI reagent protocol (Sigma-Aldrich, St. Louis, MO, USA), while RNA was recovered from the aqueous phase and isolated by using the RNAeasy Mini Kit (Qiagen, Milan, Italy). In order to check the quality of extracted DNA and RNA, nucleic acids were visualized by using a 2% agarose gel electrophoresis followed by image analysis with Bio-Rad ChemiDoc XRS + Molecular Imager (Bio-Rad Laboratories Inc., U.S.A.), and by OD 260/280 nm ratio by using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). In order to confirm samples positivity, DNA extracted from both milk and cheese was used as template for LAMP amplification, as previously described (Veronesi et al., 2018). For *T. gondii* viability assay, RNA was first subjected to reverse-transcriptase PCR (RT-PCR) by using the QuantiTect reverse transcription kit (Qiagen, Milan, Italy), following manufacturer's instructions. Obtained cDNA was used as template for a conventional PCR targeting the SAG1 gene of the parasite (Mahittikorn et al., 2010). Briefly, the PCR mixture contained 2.5 µl of 10X PCR buffer, 2.5 UI of HotStarTaq Polymerase (Qiagen, Milan, Italy), 0.5 µl of dNTPs mix (10 mM of each dNTP, Sigma-Aldrich, St. Louis, MO, USA), 8 pmol of each primer and 4 µl of cDNA, for a total volume of 25 µl. Thermal cycler conditions were 95 °C for 15 min, followed by 40 cycles of 95 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. PCR products were visualized on a 2% agarose gel with UV transilluminator (GelDoc 1000, Bio-Rad, Hercules, CA). The validation of the SAG1 RT-PCR protocol adopted was assessed according to previous reports (Mahittikorn et al., 2010). Briefly, T. gondii-free milk and cheese homogenates were added with serial 10-fold dilutions of RH strain tachyzoites obtained from Vero cell cultures, ranging from 10^4 to 10^{-3}. These milk and cheese specimens were then processed similarly to the samples to evaluate the presence of PCR inhibitors and to assess the sensitivity of the protocol. In order to evaluate the specificity, RNA extraction from RH tachyzoites inactivated by heating (100 °C for 20 min) was used as template for SAG1 RT-PCR amplification.

5.3.2. Results

The eight flocks that tested positive for the presence of *T. gondii* by using LAMP and IFAT were subsequently analysed for parasite viability in milk and cheese. On these eight flocks, LAMP investigation on a new batch of bulk milk revealed the presence of *T. gondii* DNA in two herds. Results of RT-PCR on bulk milk samples from these two positive flocks showed the presence of viable *T. gondii*. Only cheese obtained from these two herds were further analysed for the viability of the parasite, with results of chemical and physical analysis, LAMP and RT-PCR summarized in Tab. 5. Totally, 32 heads of cheese were obtained from the two positive herds, all positive for the

presence of *T. gondii* DNA by using LAMP method. However, no viability of the parasite was recorded using the RT-PCR. The sensitivity of SAG1 RT-PCR was found to be of 10 tachyzoites per reaction, and negative results were obtained from RH tachyzoites inactivated by heating.

Parameters	Cheese (5 days of ripening)		Cheese (15 days of ripening)	
	LAMP[a]	RT/PCR[b]	LAMP[a]	RT-PCR[b]
***T. gondii* positive**	32/32	0/32	32/32	0/32
NaCl %	2.48 ± 0.22		3.12 ± 0.14	
pH	5.56 ± 0.31		4.93 ± 0.04	
Aw	0.972 ± 0.010		0.962 ± 0.008	

Tab. 5 Summary of LAMP, RT-PCR, chemical and physical analysis results obtained from ripened cheese.

5.3.3. Discussion

The aim of this study was to evaluate, through the use of RT-PCR technique, the viability of *T. gondii* in ewe's milk, in order to assess the risk for human health deriving from the consume of unpasteurized milk and cheese.

Regarding cheese obtained from positive herds, physical and chemical analysis showed the reduction of pH during the ripening due to lactic acid bacteria proliferation, even in the presence of lower ripening temperature than other cheese making processes from ewe milk (Branciari et al., 2012). The a_w value was affected by the whey draining and moisture reduction (due to NaCl addition) during ripening. Moreover, salt content was in line with that previously reported from other authors in raw milk cheese with similar or longer ripening process (Fuka et al., 2013; Branciari et al., 2015).

To date, human infection with *T. gondii* through the consume of raw milk was confirmed only for goats (Chiari Cde and Neves, 1984); however, phylogenetic proximity between goat and sheep suggest that also the latter could be a source of infection. In this study, the viability of *T. gondii* is confirmed through the use of RT-PCR targeting SAG1 gene mRNA, which is only produced by metabolically active parasites (Rousseau et al., 2019, 2018). Even if the persistence of mRNA in dead organisms has been reported for other pathogens (Xiao et al., 2012; Habtewold et al., 2015), other proposed methods for the evaluation of *T. gondii* viability, such as Propidium Monoazide (PMA)-PCR, have showed to underestimate the number of viable parasites (Rousseau et al., 2019).

The absence of *T. gondii* viability was observed as early as after 5 days of ripening, before major physical and chemicals changes took place. The cheese making process occurred without heating, thus the effect on the viability of the parasite could be due to pH conditions (mean value 5.5) and salt content (mean value 2.48%) that probably represent an unsuitable environment for tachyzoites. Limited data about the survival of *T. gondii* tachyzoites in cheese making procedures are present in literature (Dubey et al., 2014). In meat, addition of salt to dry fermented meat (>1.3% in the final product) and a pH in the range between 4.6 and 5.2 have showed to inactivate *T. gondii* bradyzoites within 4 hours (Hill et al., 2018; Fredericks et al., 2019). Even if the pH value measured in our samples was higher, it is possible that the ripening procedure, characterized by reducing pH due to acid lactic bacteria proliferation and increasing NaCl values, may strongly affect the viability of *T. gondii* in cheese.

5.3.4. Conclusions

The presence of viable *T. gondii* in raw milk confirmed the shed of the parasite by naturally infected sheep and the risk for humans deriving from the consume of unpasteurized raw milk. Based on the results of the present study, it can be concluded that the cheese making procedures are able to inactivate the parasite, likely due to a change in pH and NaCl values, even in 5 day-ripened cheese made with unpasteurized milk. Nevertheless, this study did not provide quantitative data related to the amount of *T. gondii* in the sample milk, thus further studies are needed to define the fate of the parasite in case of high loads of tachyzoites in the milk.

Ranucci D., **Battisti E**., Veronesi F., Diaferia M., Morganti G., Branciari R., Ferroglio E., Chiesa F. (2020). Absence of viable *T. gondii* in raw-milk ewe cheese derived from naturally infected animals. *Microorganisms.* 8(1):143 doi.org/10.3390/microorganisms8010143

6. *BABESIA* SPP.

6.1. A national survey of Ixodidae ticks on privately owned dogs in Italy – PAPER 4

6.1.1. Materials and methods

The present survey on ticks collected from owned dogs throughout Italy was conducted in the period between February 2016 and September 2017. Dogs enrolled in the study were homogeneously distributed across the Italian peninsula using a criterion of proportional allocation, i.e. the number of enrolled dogs was proportional to the overall number of dogs registered within each region (Italian Ministry of Health, 2013). This project was based on the voluntary participation of veterinary practitioners; each veterinary practice was provided with the study protocol, 60 questionnaires, 60 tubes filled with 70% ethanol for tick storage and a tick removal hook. Over the 20-month study period, each veterinarian was instructed to examine at least five dogs per month, randomly chosen without prior knowledge of their tick infestation status. During the visit, dogs were visually and manually examined for 15-20 min to detect any possible tick present. In particular, 11 body regions were analysed: head, muzzle, neck, armpits, back, abdomen, arts, tail, anus, vagina and interdigital spaces. Collected ticks were carefully removed, stored in ethanol tubes at room temperature and sent to the Dept. of Veterinary Sciences of the University of Turin and to the Dept. of Veterinary Medicine of the University of Naples. The questionnaire requested information on the location of the sampled dog (post code of the owner or, if not given, practice postcode), breed, sex, age, hair length and recent ectoparasiticidal treatments (the last treatment and the drug used). Additional information was: the type of housing (indoor/outdoor/kennel), the environment in which the dog is usually moved (urban/rural/sylvatic), and the attachment site of the ticks if present. An individual identification number (ID) was assigned to each animal, questionnaire and vial.

Collected ticks were identified to the species or complex level, life stage (e.g. larva, nymph or adult) and gender (male or female) by using a stereomicroscope and morphological keys (Walker, 2003; Estrada-Peña et al., 2004a; Dantas-Torres et al., 2013).

Positive dogs for each tick genera were geo-referenced using a geographical information system (GIS, ArcGIS version 10.3 ESRI), referring to the owner postcode or, if no postcode was reported, to the veterinary practice postcode. GIS was also used to analyse spatial information on body locations of ticks on examined dogs. Differences in tick infestation were analysed in association with dog sex, age, hair length (long or short), habitat (indoor/outdoor/kennel) and environment (urban/rural/sylvatic) by using the Chi-square test, with presence or absence of ticks as dependent variable. Regarding dog gender, four categories were used: male, female, neutered male and neutered female. Dogs were classified in five groups for age analysis: puppy (less than 1 year of

age), young-adult (1-3 years), adult (4-6 years), old (7-10 years) and very old (more than 10 years of age). The efficacy of acaricide treatment was evaluated for those dogs for which complete information on the product used, date of sampling and date of last acaricide treatment was available. Chi-square tests and logistic regression were performed by using SPSS Statistics 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA).

For ticks difficult to identify morphologically, a molecular approach was used. Briefly, partial mitochondrial 12S rRNA and 16S rRNA gene sequences were generated and analysed by using primers and PCR conditions previously described (Dantas-Torres et al., 2013; D'Oliveira et al., 1997). PCR products were visualised in 2% agarose gel by using an UV transilluminator (GelDoc 1000, Bio-Rad, Hercules, CA). Amplicons were purified using a commercial kit (Nucleospin Extract II Kit, Macherey-Nagel, Düren, Germany) and sent for sequencing (Macrogen Europe, The Netherland). Sequences were analysed using the Chromas version 2.1.1 software and compared with the partial mitochondrial 12S and 16S rRNA gene sequences
in GenBank.

6.1.2. Results

One-hundred and fifty-three veterinary practices from 64 provinces of Italy were enrolled for the present study, covering 17 regions out of 20 (corresponding to 85% of the Italian peninsula) (Fig. 12).

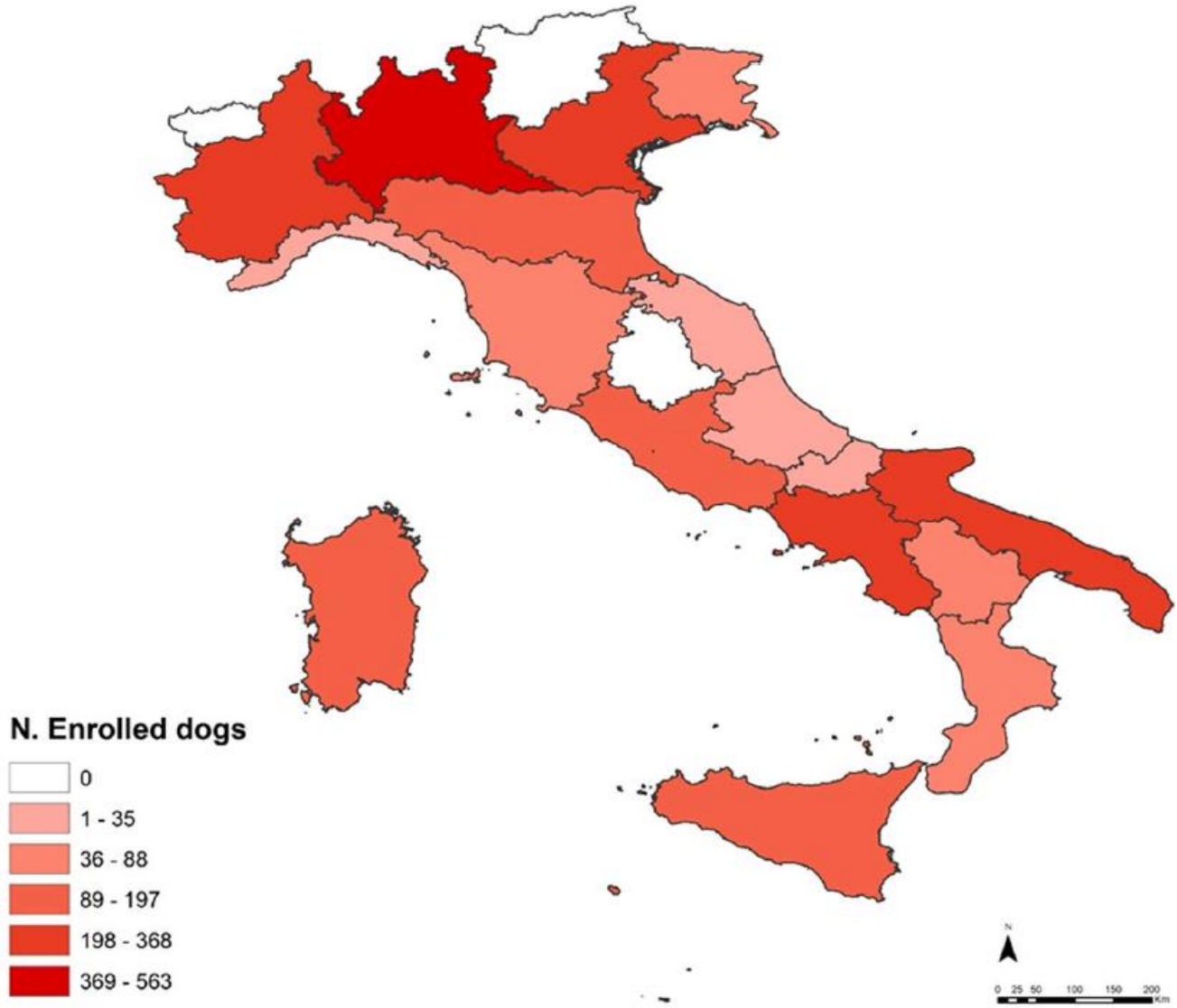

Fig.12 Number of dogs enrolled for each Italian region (adapted from Maurelli et al., 2018; paper n°4).

During the study period, 3026 dogs were analysed for tick presence and had a complete questionnaire: 1520 from Northern, 283 from Central and 1223 from Southern Italy. Regarding gender, dogs were divided as follow: 835 intact (27.6%; CI95% 26.0% - 29.2%) and 562 neutered females (18.6%; CI95%17.2% - 20.0%), 1430 intact (47.3%; CI95% 45.5% - 49.1%) and 199 neutered males (6.6%; CI95% 5.7% - 7.5%). The age of animals ranged from 1 month to 17 years (median age of 4 years). The dogs belonged to over 100 different breeds, of which cross-bred were the most prevalent (39.3%; CI95% 37.5% - 41.0%). Most had short hair (56.6%; CI95% 54.9% - 58.4%) and were housed rather than kennelled (57.2%; CI95% 55.5% - 59.0%).

Overall, 1383 dogs (45.7%; CI95% 43.9 – 47.5%) carried at least one tick, although tick specimens were submitted for only 1217 dogs. Ticks were mainly localized on the head (37.4%), the neck (28.8%), the muzzle (15.5%) and the back (15.3%) (Fig. 13). Statistical analysis revealed no

significative differences between the overall tick preference for location of attachment on dogs or between the different tick genera regarding their location of preference on the dog's body. However, a higher prevalence of *Rhipicephalus* spp. was found in interdigital spaces (10.8%) compared to *Ixodes* spp. (0.2%) (Fig. 13).

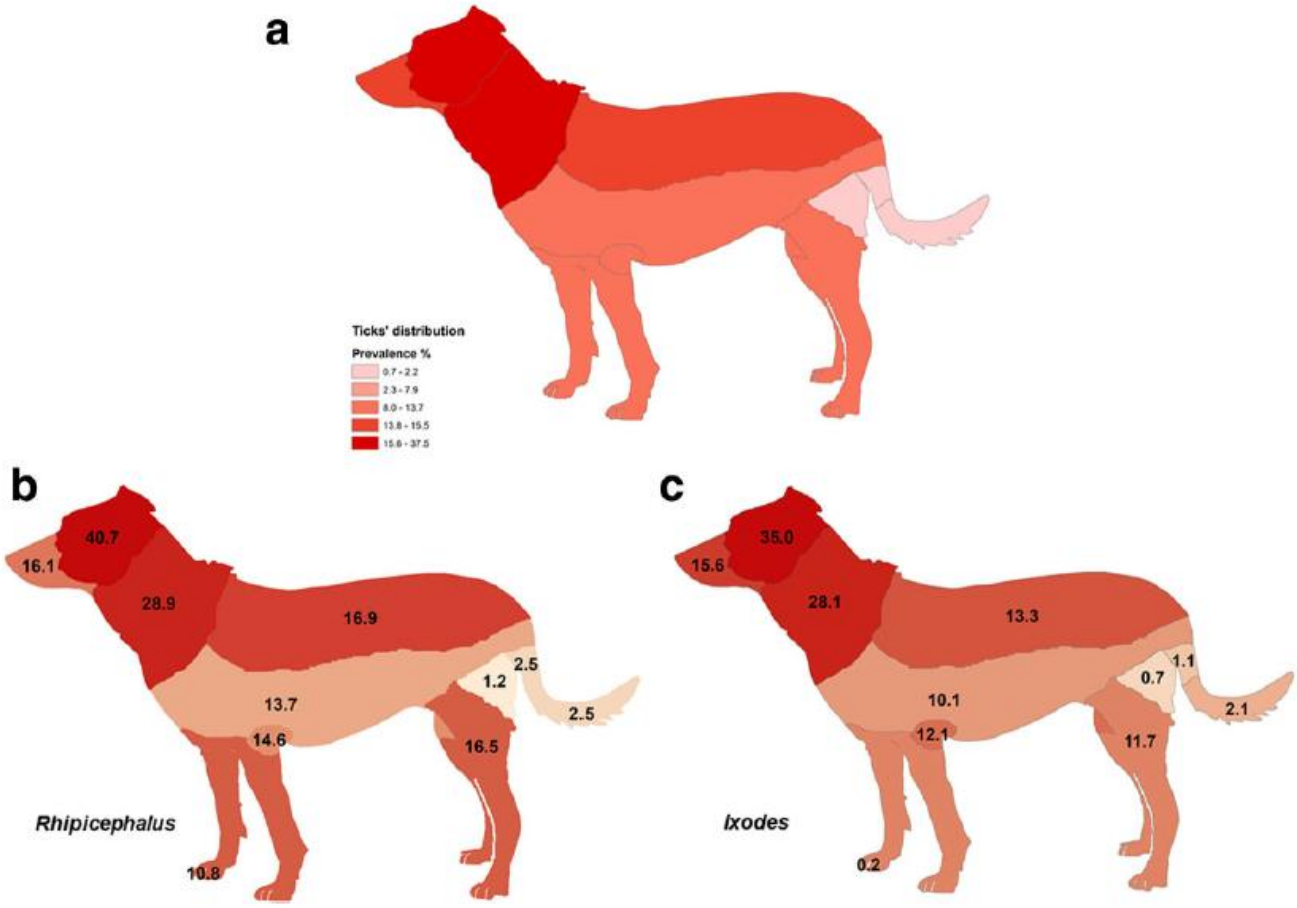

Fig. 13 Distribution of ticks on the body of enrolled dogs: **a.** overall ticks collected; **b.** *Rhipicephalus* spp.; **c.** *Ixodes* spp. (adapted from Maurelli et al., 2018; paper n°4).

Totally, 2439 ticks were collected from the analysed dogs. The mean number of ticks per dogs was 1, with a range between 1 and 44. Regarding the stage, the majority of the ticks were adults (2056, corresponding to 84.3%), followed by nymphs (325, corresponding to 13.3%) and larvae (58, 2.4%). Ticks belonged to four different genera and 14 species (Tab. 6). The most prevalent genus was *Rhipicephalus*, with 27.5% of infested dogs in Northern Italy and 36.1% of the infested dogs in Central-Southern Italy. *Ixodes* spp. was the second most frequent genus, with 25.6% of dogs in the North and 10.8% in Central-Southern Italy. Less common genera were *Dermacentor* spp., with 0.6% of dogs infested, and *Haemaphysalis* spp. (0.2% of dogs). *R. sanguineus* group was the most prevalent among the tick species/complex (63.6%), followed by *I. ricinus* (30.6%), I. *hexagonus* (5.6%), *R. bursa* (0.6%), *D. marginatus* (0.4%), *D. reticulatus* (0.3%), *H. punctata* (0.3%), *Ixodes*

arboricola (0.2%), *I. canisuga* (0.2%), *I. gibbosus* (0.2%) and *I. festai* (0.08%) (see Tab. 6 and 7 for a summary of collected ticks classified by life stages and dog infestation by tick species).

Tick group/species	Larvae	Nymphs	Adults		Total no. of specimens
			Females	Males	
Rhipicephalus sanguineus group[a]	21	295	881	625	1822
Ixodes ricinus	5	18	399	46	468
Ixodes hexagonus	0	7	73	3	83
Dermacentor marginatus	0	0	4	1	5
Rhipicephalus bursa	0	1	7	3	11
Dermacentor reticulatus	0	0	2	5	7
Haemaphysalis punctata	0	2	2	0	4
Ixodes arboricola	32	0	0	0	32
Ixodes canisuga	0	2	2	0	4
Ixodes gibbosus	0	0	2	0	2
Ixodes festai	0	0	1	0	1
Total	58	325	1373	683	2439

Tab. 6 Tick species collected from privately owned dogs in Italy classified by life-stage and sex (larvae, nymphs, females and males) (adapted from Maurelli et al., 2018; paper n°4).

Tick species	Number of dogs	Prevalence (%)[a]	95% CI
Rhipicephalus sanguineus group	769	63.6	60.40–65.89
Ixodes ricinus	372	30.6	28.00–33.26
Ixodes hexagonus	68	5.6	4.39–7.07
Rhipicephalus. bursa	7	0.58	0.25–1.24
Dermacentor marginatus	5	0.41	0.15–1.02
Dermaceontor reticulatus	3	0.25	0.06–0.78
Haemaphysalis punctata	3	0.25	0.06–0.78
Ixodes arboricola	2	0.16	0.03–0.66
Ixodes canisuga	2	0.16	0.03–0.66
Ixodes gibbosus	2	0.16	0.03–0.66
Ixodes festai	1	0.08	0.00–0.53

Tab. 7 Prevalence of tick infestation on dogs surveyed in Italy classified by tick species (adapted from Maurelli et al., 2018; paper n°4).

The distribution across Italy of the 14 recorded species showed a different pattern, explained in Tab. 8. Briefly, species such as *R. sanguineus* group, *I. ricinus* and I. *hexagonus* were detected in all the analysed regions throughout the peninsula, while others were typical of only one area. For instance, both the *Dermacentor* species, *I. arboricola*, *I. canisuga* and *I. festai* were collected solely in the Northern Italy, while *H. punctata* and *I. gibbosus* only from the South. Mixed infestations with more than one tick species were recorded on 24 dogs (Tab. 9).

Tick species	Geographical origin	Season
Dermacentor marginatus	North (Lombardy, Piedmont)	Spring, Summer
Dermacentor reticulatus	North (Lombardy, Piedmont)	Spring, Summer
Haemaphysalis punctata	South (Calabria, Campania)	Summer, Winter
Ixodes arboricola	North (Veneto)	Summer
Ixodes canisuga	North (Lombardy)	Summer
Ixodes festai	North (Piedmont)	Summer
Ixodes gibbosus	South (Calabria, Campania)	Spring
Ixodes hexagonus	North (Emilia Romagna, Friuli Venezia Giulia, Lombardy, Piedmont, Veneto), Center (Lazio, Molise, Tuscany), South (Calabria, Campania)	Spring, Summer, Autumn, Winter
Ixodes ricinus	North (Emilia Romagna, Friuli Venezia Giulia, Lombardy, Piedmont, Veneto), Center (Lazio, Molise, Tuscany), South (Basilicata, Calabria, Campania, Apulia, Sardinia)	Spring, Summer, Autumn, Winter
Rhipicephalus sp. I	South (Basilicata, Apulia)	Spring, Summer
Rhipicephalus bursa	Center (Lazio), South (Calabria, Campania, Sicily)	Summer, Winter
Rhipicephalus pusillus	Center (Lazio), South (Basilicata, Calabria, Campania, Apulia)	Spring, Summer, Autumn, Winter
Rhipicephalus sanguineus (sensu lato)	North, Center, South (all the regions investigated)	Spring, Summer, Autumn, Winter
Rhipicephalus. turanicus	Center (Lazio), South (Basilicata, Sardinia)	Spring, Summer, Autumn, Winter

Tab.8 Collected ticks classified by geographical origin and season of detection (adapted from Maurelli et al., 2018; paper n°4).

Tick species	Number of dogs
Rhipicephalus sanguineus group + *Ixodes ricinus*	10
Rhipicephalus sanguineus group + *Ixodes hexagonus*	5
Ixodes ricinus + *Ixodes hexagonus*	5
Rhipicephalus sanguineus group + *Rhipicephalus bursa*	2
Rhipicephalus sanguineus group + *Ixodes canisuga*	1
Rhipicephalus sanguineus group + *Dermacentor reticulatus*	1
Total	24

Tab. 9 Mixed type infestation observed in this study (adapted from Maurelli et al., 2018; paper n°4).

Out of the 86 specimens analysed with molecular biology for species identification, 80 useful sequences were obtained and compared with those deposited in GenBank®. In particular, 37 partial 12S rRNA and 43 partial 16S rRNA were obtained. The length of the 12S rRNA and 16S rRNA gene sequences alignments were of 370 and 330 bp, respectively. This sequence analysis showed 99–100% identity to GenBank sequences of *R. sanguineus* (sensu lato) (accession numbers: KU255852, KU255849, KU255848, KU556694, KX553960), *R. turanicus* (accession number: KC243822) and *Rhipicephalus* sp. I (accession number: KC243794). Additionally, *D. marginatus* (accession number: JX051098) and *D. reticulatus* (accession number: JF928493) were 99–100% identity to GenBank sequences. Finally, five partial 16S rRNA gene sequences were identified as *I. canisuga* (accession number: KY962075) and I. festai (accession number: KU170522).

Statistical analysis showed that tick presence was positively associated with long hair ($\chi^2 = 5.07$; *df* = 1; p = 0.024), outdoor as a type of housing ($\chi^2 = 175.3$; *df* = 1; p < 0.0001) and rural/sylvatic as a frequented environment ($\chi^2 = 287.1$; *df* = 1; p < 0.0001).

Most of the specimens were received in the period between May 2016 and July 2017, with fewer submissions at the beginning and at the end of the project, probably due to reduced compliance of the practitioners. More dogs showed the presence of ticks in the period between April and August, while fewer dogs were infested from October to February. However, *Rhipicephalus* and *Ixodes* were collected during all the year. In particular, *R. sanguineus* group showed a peak of infestation during spring and summer, while *I. ricinus* and I. *hexagonus* had a lower variation throughout the year (Fig. 14).

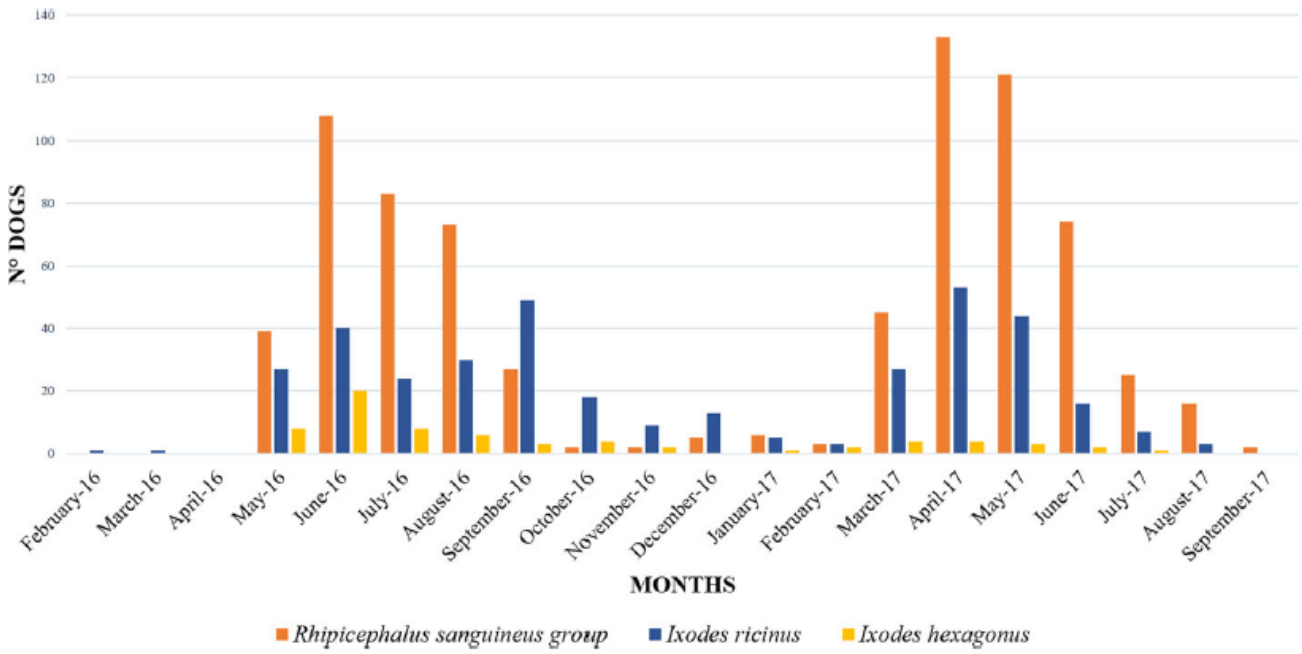

Fig. 14 Monthly seasonal dynamics of the three most prevalent ticks (*R. sanguineus* group, *I. ricinus* and I. *hexagonus*) found in this study (adapted from Maurelli et al., 2018; paper n°4).

Acaricide treatments were reported for 2180 dogs (72.0%), although complete information on the treatment were present for only 2016 dogs. Remaining dogs were not treated with any ectoparasiticide product (687 dogs) or no information reporting the drug used were present (159 dogs). Out of the treated dogs, the majority were treated with one product (1930/2016; 95.7%). The most frequent formulation type for acaricide treatment was the topical spot-on formulation (1278/1930; 66.2%), followed by oral formulations (348/1930; 18.0%) and collars (283/1930; 14.7%). Other formulations type, such as injectable products, spray, shampoo and powder, were lesser used, with <1% of dogs treated. Eighty-six dogs were treated with a combination of two products, with spot-on and collars being the most common association (38 dogs reported).

The efficacy of the acaricide treatment was evaluated by comparing tick infestation in treated and untreated dogs. In particular, only dogs for which complete information on the product used and the date of the last treatment were present were considered in the treated group. Totally, there were 1320 dogs with complete ectoparasiticide information, and 687 untreated dogs. In general, acaricide treatment was significantly protective against tick infestation ($\chi 2 = 196.89$, df = 1, $p < 0.0001$). However, statistical analysis showed significative differences in the tick control efficacy among the formulations. In fact, oral formulations provided the highest protection, with 90.1% (CI95% 84.91% - 93.65%) of the treated dogs showing no tick infestation compared to 69.18% (CI95% 61.28% - 76.10%) of dogs free of ticks treated with antiparasitic collars and 53.37% (CI95% 48.94% - 57.75%) of dogs free of ticks after spot-on acaricide treatment. In particular, Chi square test revealed a χ^2 of 22.87 for oral vs collar treatments ($p < 0.0001$) and of 77.08 for oral vs spot-on treatments ($p < 0.0001$). Moreover, spot-on treatments were considerably more effective than collars ($\chi^2 = 11.46$; $p < 0.001$). An overall number of 32 different commercial products were reported on questionnaires, consisting in 13 different active compounds (or active compounds combinations). Only 5 active compounds were used on more than 50 dogs (Tab. 10). No significative differences were recorded between these compounds by generalized linear model (GLM) ($p > 0.05$); however, the majority of treated dogs that did not report tick infestation were treated with fluralaner (89.5%).

Regarding owner compliance to treatments, it was observed that owner respect to recommended retreatment intervals was greater for collars (95.4% of valid treatments) than for oral (70.8% of valid treatments) and spot-on formulations (52.6% of valid treatments). It is also noteworthy the lack of knowledge on acaricide products of some owners and practitioners, because 31 dogs were reported to be treated with products without acaricidal effect.

Compound	Dogs without ticks	Dogs with ticks	Total no. of dogs	Infestation prevalence (%)
Deltamethrin	39	25	64	39.06
Fipronil	117	133	250	53.20
Flumethrin - Imidacloprid	61	18	79	22.78
Fluralaner	137	16	153	10.46
Permethrin - Imidacloprid	69	55	124	44.35

Tab. 10 Ectoparasiticide compounds used on at least 50 dogs in the present study, with infestation prevalence (adapted from Maurelli et al., 2018; paper n°4).

6.1.3. Discussion

The aim of the present survey was to evaluate what kind of tick species infest privately and healthy owned dogs throughout Italy. In fact, previous studies performed on dogs were focused on limited areas of the Italian peninsula or only on dogs with clinical signs of TBDs (Trotta et al., 2012; Lorusso et al., 2010). In contrast, dogs enrolled for the present study originated from 17 out of 20 Italian

regions, for an overall number of 78 provinces and 1455 municipalities analysed. This survey was based on the voluntary enrolment of veterinary practitioners, as previously carried out in other countries including Belgium (Claerebout et al., 2013), Spain (Estrada-Peña et al., 2017) and UK (Abdullah et al., 2016).

The geographical distribution of the four genera of ticks found across Italy in the present survey is consistent with maps of tick distribution in Europe (ESCCAP, 2012; Beugnet et al., 2017), and represent the different climatic and environmental features of the Italian peninsula. *Rhipicephalus* is the most widespread tick genus in the Mediterranean area, while *Ixodes* spp. and *Dermacentor* spp. show higher adaptability to cold temperatures, thus they are widespread in areas with continental climates. The genus *Haemaphysalis* was only detected on dogs living outdoor in rural and sylvatic environments in Southern Italy. This finding is consistent with the tick behaviour, because *H. punctata* is the primary species infesting domestic ruminants in this area (Rinaldi et al., 2004; Cringoli et al., 2002). Collectively, 14 tick species were recorded from enrolled dogs, confirming the high biodiversity of Ixodid ticks in Italy (Manilla, 1998). *R. sanguineus* group was the most prevalent (63.6%), especially in Central and Southern regions of Italy (36.1% of prevalence). In particular, the distribution of *R. sanguineus* group is resemble the regions in which the climate is more suitable for the maintenance and development of the tick species. Among the *R. sanguineus* group, *R. sanguineus* s.l. was the most prevalent tick species, with remarkable concern for public health because it is the vector for various TBDs, such as ehrlichiosis, anaplasmosis and babesiosis. Other species belonging to the genus were identified only in Southern Italy, but with much lower prevalence (less than 1%), including *R. bursa*, *R. pusillus*, *R. turanicus* and *Rhipicephalus* sp. I. In particular, the record of *R. bursa* and *R. turanicus* on dogs from Basilicata, Campania, Latium, Sicily and Sardinia from this study is consistent with previous reports of these species in livestock from the same regions (Torina et al., 2006; Toma et al., 2017; Rinaldi et al., 2004; Dantas-Torres et al., 2013; Cringoli et al., 2002; Chisu et al., 2014).

After *R. sanguineus* s.l., *I. ricinus* was the second most common tick species in Italy (29.2%). In particular, higher prevalence was recorded in Northern Italy (20.9%), although was also present in Central and Southern regions with a prevalence of 9.5%. *I. ricinus* is well known as vector of Lyme borreliosis, thus the high prevalence of this species highlights the potential risk for this disease across Italy.

Other *Ixodes* species recorded were I. *hexagonus* (4%), *I. arboricola*, *I. canisuga* and *I. festai* (less than 1% each) in Northern Italy, and I. *hexagonus* (1.2%) and *I. gibbosus* (0.2%) in Southern regions. The general spatial distribution of ticks in Italy observed in the present study is consistent with findings of large-scale studies conducted in Mediterranean countries such as Spain (Estrada-Peña et al., 2017), Cyprus (Tsatsaris et al., 2016) and Greece (Latrofa et al., 2017). In these studies, *R. sanguineus* group has been the most prevalent tick species, while surveys conducted in temperate

European countries including the Netherland (Nijhof et al., 2008), Belgium (Claerebout et al., 2013) and UK (Abdullah et al., 2016) reported higher prevalence of *I. ricinus* and I. *hexagonus*.

Even if the prevalence of dogs infested by ticks is high (45.7%), this finding should be carefully interpreted due to the nature of the study. In fact, there is the possibility of an over-reporting bias by veterinary practitioners that reported only the tick-infested dogs, as already hypothesized in a large-scale UK survey (Abdullah et al., 2016).

Mixed infestation was quite rare in the present study, as previously reported (Latrofa et al., 2017; Estrada-Peña et al., 2017), with 24 dogs reported to carry more than one tick species. Regarding tick developmental stages, the majority of the specimens were adults, and this could be due to the difficulties in detecting smaller tick-life stages like larvae and nymphs during clinical examination (Latrofa et al., 2017; Abdullah et al., 2016).

Head, neck, thorax and abdomen areas were reported to be the most frequently infested by ticks, probably because these are the most exposed sites for tick attachment, as reported previously (Claerebout et al., 2013; Brianti et al., 2013). Another possible explanation is the difficulties for dogs in grooming ticks from these sites, as well as the skin thickness and local odours could explain this multifocal distribution (Lorusso et al., 2010; Brianti et al., 2013). In particular, *R. sanguineus* was frequently found in interdigital spaces, confirming this attachment site as a favourite for this species (Silveira et al., 2009; Dantas-Torres, 2010). This result highlights the importance of systemic acaricidal treatments compared to topical ones; in fact, products applied topically and externally acting are less effective in reaching acaricidal concentrations on distant locations such as foot.

Regarding risk factors for tick infestation, hair length and dog lifestyle (indoor vs outdoor; urban vs rural) were significant predictors. In fact, long hair was positively associated to tick infestation, probably because of the greater difficulties for the owner to see and collect the ticks, but also because ticks grab more easily on long hair compared to short hair. This finding is consistent with one previous study (Silveira et al., 2009), but in contrast with another (Jennett et al., 2013). In the latter, short-haired dogs were significantly more infested than long haired ones. Finally, dogs living in rural/sylvatic environments or with outdoor lifestyle were more often infested by ticks than pets living in urban areas or with indoor lifestyle, as already observed (Latrofa et al., 2017). However, both the categories (urban vs rural dogs, indoor vs outdoor) showed the presence of *R. sanguineus* s.l. as the main tick species. Moreover, no effect of age or sex was reported to act of the probability of tick infestation. In literature, it seems clear that tick presence strongly correlated with tick exposure rather than any specific dog characteristic (Jennett et al., 2013; Abdullah et al., 2016). Results of the present survey agree with these previous findings, and confirms the inconsistency of this relationship seen among prior tick risk surveys (Abdullah et al., 2016).

Seasonal tick species distribution in Italy showed considerable variation between genera. *R. sanguineus* group was predominant mainly during the spring and summer, with an activity peak between April and August. In contrast, *I. ricinus* and I. *hexagonus* were mainly active during all

seasons, as observed in other European countries (Széll et al., 2006; Pfister and Armstrong, 2016; Földvári and Farkas, 2005; Estrada-Peña et al., 2017; Georg G. Duscher et al., 2013; Dantas-Torres and Otranto, 2013). The finding that several tick species across Italy are active throughout the year highlights the importance of years round effective acaricidal treatments. Moreover, it is also evident the lack of knowledge on acaricide products of some owner and practitioners who treated dogs with compounds without any proved effect on ticks. This may be due to the fact that most of the ectoparasiticidal products are over-the-counter medications that facilitate the availability of these products by the owners but at the same time lead to an improper use of these drugs. It is noteworthy to say that the European Scientific Counsel for Companion Animal Parasites (ESCCAP) provides several advices on tick prevalence, treatments and tick-borne pathogen risk, thus providing a reliable resource for tick control information and guidelines for owners and veterinary practitioners.

6.1.4. Conclusions

The present study is the first nationwide survey on ticks and associated pathogens on companion animals in Italy. Its main aim was to provide a comprehensive spatial understanding of tick distribution and species abundance, showing that different tick species parasitize dogs in this country. Risk factors for tick infestation vary by dog phenotype and lifestyle, while seasonal species distribution showed considerable variation between tick genera.
Further investigations are needed in order to clarify the environmental and host factors that influence tick species infestations on companion animals, in order to develop and plan effective control measures.

Maurelli MP., Pepe P., Colombo L., Armstrong R., **Battisti E**., Morgoglione ME., Counturis D., Rinaldi L., Cringoli G., Ferroglio E., Zanet S. (2018) A national survey of Ixodidae ticks on privately owned dogs in Italy. *Parasites & Vectors*. 11:420 doi: 10.1186/s13071-018-2994-2

6.2. Tick-borne pathogens in Ixodidae ticks collected from privately-owned dogs in Italy: a country-wide molecular survey – PAPER 5

6.2.1. Materials and methods

The aim of the present study was to investigate the distribution of tick-borne pathogens in ticks collected from privately-owned dogs across Italy. In particular, we focused on the protozoa *Babesia* and *Theileria*, and on the bacteria of the family Anaplasmataceae and *Borrelia burgdorferi* s.l., chosen for their impact on human and animal health.

Results of the morphological and molecular identification of the ticks analysed in the present study are reported in paper 4. For TBPs detection, we included in this study only tick species that are commonly reported to feed on dogs (Tab. 11).

A total of 2681 Ixodidae ticks grouped into 1578 homogeneous pools were then analysed (Table 12), originating from 1454 privately-owned dogs from 78 Italian NUTS3 provinces (hereinafter NUTS3, Nomenclature of Territorial Units for Statistics, level 3), (mean = 18.64 dogs/province, standard deviation = 24.75) and 1389 municipalities (LAU2, Local Administrative Units, level 2).

For pathogen detection, ticks collected from the same dog were divided into pools homogeneous for species or complex, developmental stage, gender and engorgement status, while ticks for identification were processed individually. Specimens were finely minced with a sterile scalpel and homogenized in TRI-Reagent® (Sigma-Aldrich, Italy). Total DNA was extracted according to the manufacturer's instructions with additional overnight incubation in Proteinase K (0.8 mg) and 500 µl of TRI-Reagent.

To detect piroplasms, a semi-nested PCR targeting the V4 hypervariable region of the 18S rRNA using primers RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3'), RLB-R2 (5'-CTAAGAATTTCACCTCTGACAGT-3') and RLB-FINT (5'-GACAAGAAATAACAATACRGGGC-3') was performed (Zanet et al., 2014b). Briefly, the reaction mixture for the first step contained 1X of PCR Master Mix (Promega Corporation, WI, USA), 20 pmol of each primer and 5µl of DNA, in an overall volume of 25 µl. The thermal cycler conditions used were an initial denaturation step at 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 50°C for 45 s and 72°C for 90 s, and a final elongation step at 72°C for 10 min. Amplicons from the first step (1 µl) were used as templates for the second step with internal primer RLB-FINT instead of RLB-F2. Protocol and thermal cycler conditions were identical to the first step except for the annealing temperature at 55°C and for the cycling number of 40.

For *Ehrlichia* and *Anaplasma* detection, the 16S rRNA was targeted using primers PER1 (5'-TTTATCGCTATTAGATGAGCCTATG-3') and PER2 (5'-CTCTACACTAGGAATTCCGCTAT-3') (Goodman et al., 1996). The PCR reaction mixture contained 1X of PCR buffer, 25 pmol of each primer, 0.5 mM of $MgCl_2$, 2.5 U of HotStarTaq DNA Polymerase (Qiagen, Milan, Italy), 0.2 mM of

dNTPs mix (Sigma-Aldrich, St. Louis, MO, USA) and 1 µl of DNA, in a total volume of 25 µl. The thermal cycler conditions were an initial denaturation step at 95°C for 15 min, followed by 40 cycles at 94°C for 1 min, 52.4°C for 45 s, 72°C for 1 min and a final elongation step at 72°C for 10 min.

B. burgdorferi s.l. was detected using the primers FlaF (5'-AGAGCAACTTACAGACGAAATTAAT-3') and FlaR (5'- CAAGTCTATTTTGGAAAGCACCTAA-3'), targeting a conserved region of the *fla* gene (Kurtenbach et al., 1998). The reaction mixture contained 1x PCR buffer, 0.4 µM of each primer, 1.5 mM of $MgCl_2$, 2.5 U of HotStarTaq DNA Polymerase (Qiagen, Milan, Italy), 0.2 mM of dNTP mix (Sigma-Aldrich, St. Louis, MO, USA) and 3 µl of DNA, in a total volume of 50 µl. The thermal amplification conditions were an initial denaturation step at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 54°C for 45 s, 72°C for 45 s and a final elongation step at 72°C for 3 min.

Positive and negative controls were included in each PCR reaction and all necessary measures were taken to minimize the risk of contamination. The PCR results were expressed as a minimum infection rate (MIR) or the minimum percentage of ticks in a pool with detectable DNA for each specific pathogen. This calculation was based on the assumption that a PCR-positive pool contains only one positive tick (Kramer et al., 1999). PCR-positive amplicons were purified using a commercial kit (Nucleospin Extract II Kit, Macherey-Nagel, Düren, Germany) and sequenced on both strands (Macrogen Europe, Spain) for species identification. The resulting nucleotide sequences were analyzed using MEGA X software (Kumar et al., 2018) and compared to those available in GenBank.

Distributions of tick samples and pathogens were geo-referenced using a Geographical Information System (QGIS, version 3.2, QGis Development Team, 2018), entering the owner's postcode or, if missing, the location of the veterinary practice that enrolled the dog.

Chi-square tests, logistic regressions and confidence intervals at 95% were calculated using R 3.4.4 (R Development Core Team, 2018). Differences were considered significant at $p<0.05$.

Genera	**Species**	**N. of Ticks (n. of pools)**	**Adults**			**Nymphs**	**Larvae**
			Males	Females	Engorged females		
Dermacentor	*D. marginatus*	5 (2)	1 (1)	4 (1)	0	0	0
	D. reticulatus	7 (6)	4(3)		3 (3)	0	0
Haemaphysalis	*H. punctata*	4 (3)	0	2 (2)	0	2(1)	0
Ixodes	*I. canisuga*	2 (1)	0	2(1)	0	0	0
	I. hexagonus	112 (96)	4(4)	48(41)	45(39)	14(11)	1(1)
	I. ricinus	611 (516)	64 (34)	319 (285)	195 (172)	26 (22)	7 (3)

Rhipicephalus	*R. bursa*	10 (5)	3 (1)	6 (3)	0	1 (1)	0
	R. sanguineus group	1930 (949)	628 (236)	761(484)	189 (122)	330 (94)	22(13)
Total		2681 (1578)	704 (279)	1142 (817)	432 (336)	373 (129)	30 (17)

Tab. 11 Genera, species and number of ticks (plus number of homogeneous pools) per species, life stage and engorgement status included in the molecular study.

6.2.2. Results

Regarding *Babesia*, DNA of piroplasms was detected in 435 pools (MIR = 27.57%; CI95% 25.42% - 29.82%) from 395 dogs.

A significantly higher prevalence was found in *I. ricinus* ($\chi^2 = 5.5$, $p < 0.05$) and in ticks belonging to *R. sanguineus* group ($\chi 2 = 4.1$, $p<0.05$) compared to other tick species as well as in adult ticks ($\chi^2 = 9.99$, $p < 0.05$) and engorged females ($\chi^2 = 15.82$, $p < 0.05$). Dogs living in urban environments were at a lower risk of being parasitized by piroplasms-infected ticks ($\chi^2 = 109.04$, $p < 0.05$; odds ratio (OR) = 0.31; CI95% 0.25% - 0.39%) compared to dogs living in rural and forest habitats, while housing (indoor, garden, kennel) did not influence the risk of being parasitized by an infected tick ($p > 0.05$). Geographical distribution at the NUTS3 level of piroplasms-infected ticks is reported in Figure 15. Positive ticks were detected in 53 provinces (53/78 = 67.95%, 95% CI = 56.96-77.25%) (Figure 15a) with significant differences among the provinces ($p < 0.05$). Considering NUTS3 provinces where at least 20 dogs were sampled, piroplasms were detected with MIR values ranging from 0% (CI95% 0.00% - 17.59%) to 61.90% (CI95% 40.88% - 79.25%) (Figure 15b).

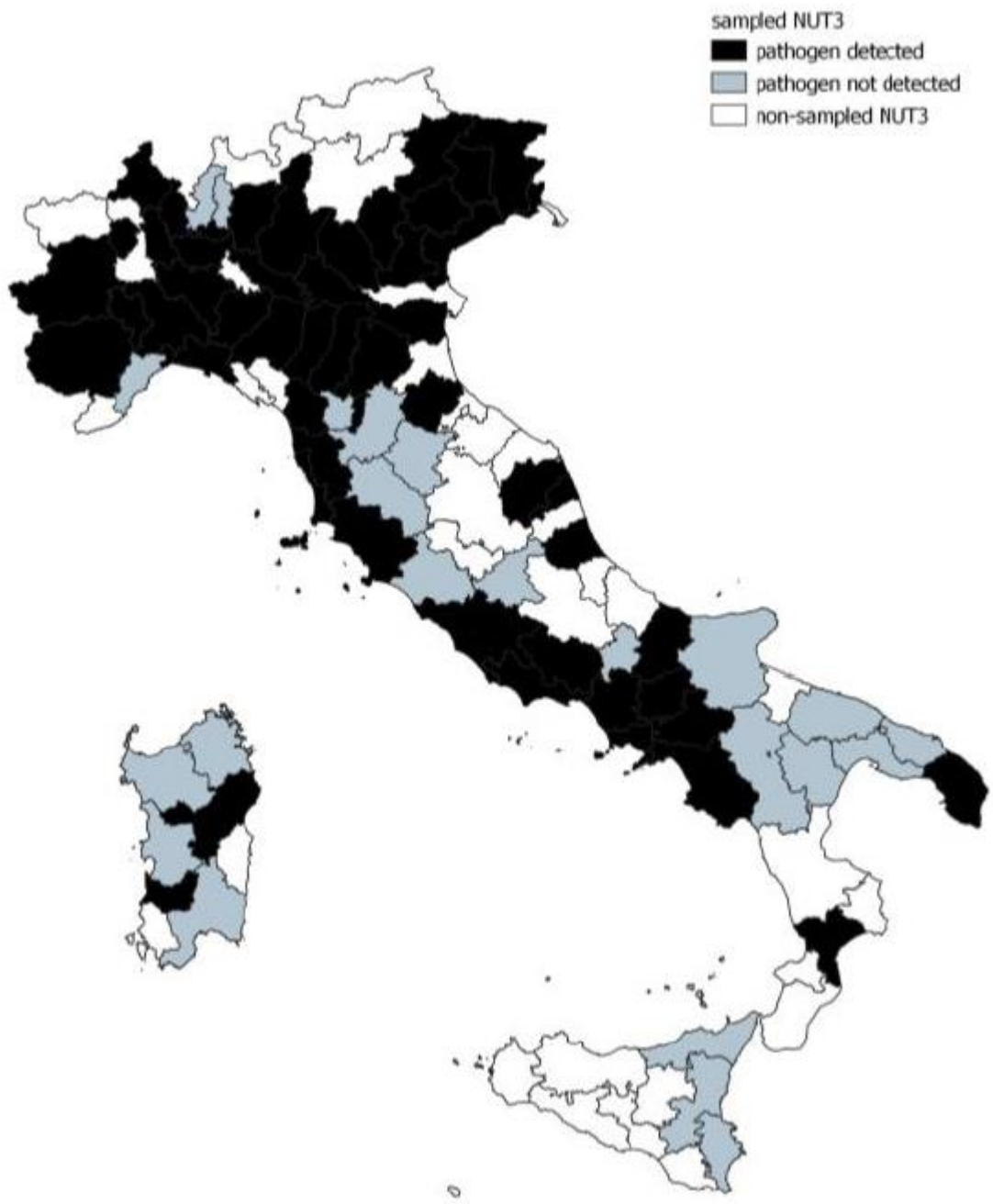

Figure 15a. Geographical distribution, at the NUTS3 level, of ticks infected with *Babesia*/*Theileria* piroplasms.

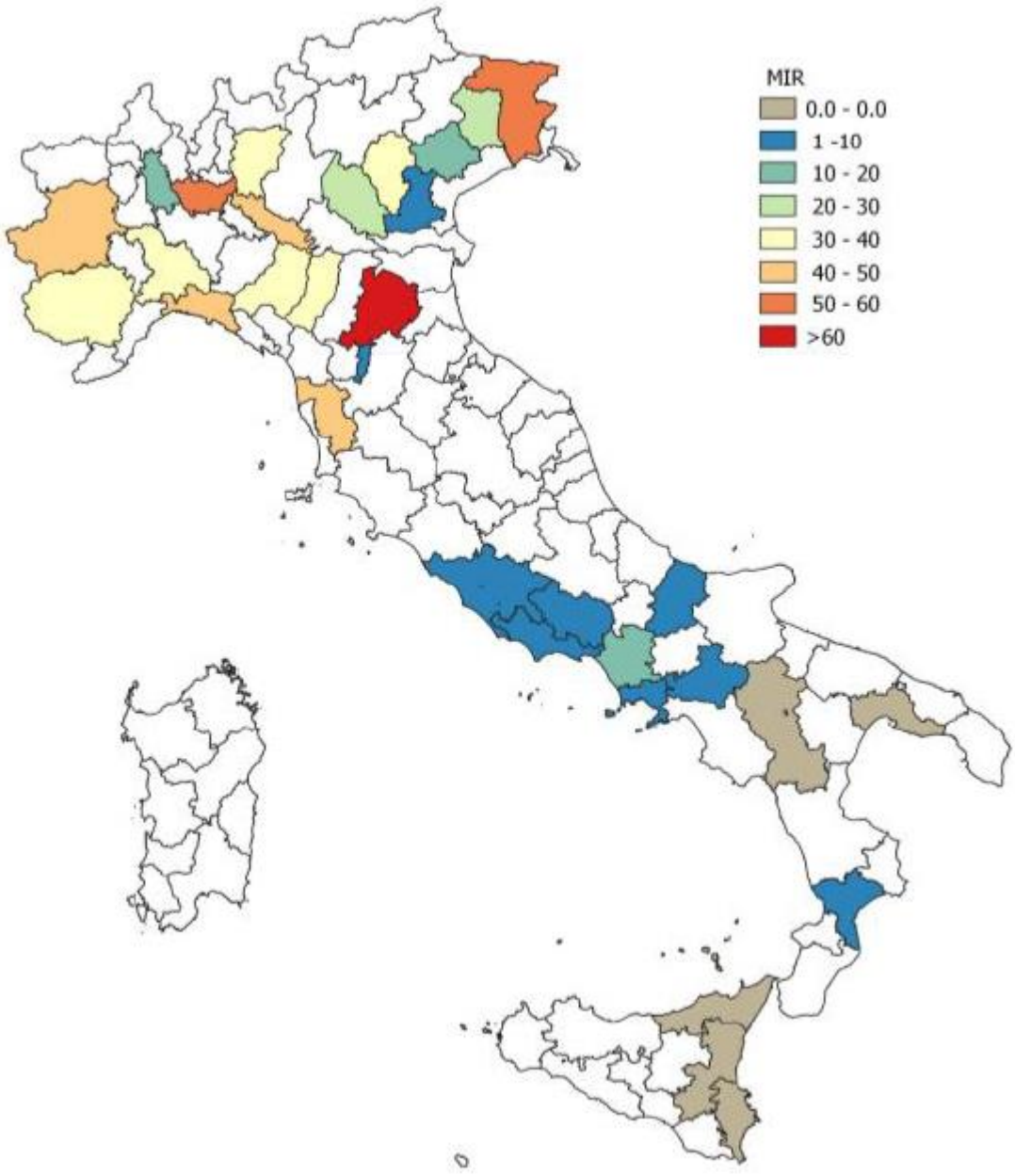

Fig. 15b Minimum Infection Rate (MIR%) in NUTS3 provinces, where at least 20 dogs were sampled, for *Babesia*/*Theileria*.

Regular antiparasitic treatment significantly reduced the risk of being parasitized by positive ticks (χ^2 = 144.97, p < 0.05; OR = 0.24; CI95% 0.19% - 0.31%), even if dogs treated with collars (χ^2 = 53.60, p < 0.05; OR = 6.99; CI95% 3.89% - 12.55%) and spot-on products (χ^2 = 119.29, p < 0.05; OR = 7.75; CI95% 5.18% - 11.59%) were more likely to be parasitized than those treated with oral formulations. Sequencing results showed the presence of at least 9 species of the genus *Babesia* and 5 species belonging to the genus *Theileria,* as reported in Table 12.

Tab. 12 Pathogen species and number of homogeneous tick pools positive for each species, Minimum Infection Rate (MIR), and MIR confidence intervals (CI) at 95% are reported below.

Species	Positive Pools	*D. marginatus*	*I. canisuga*	*I. hexagonus*	*I. ricinus*	*R. sanguineus* group	*H. punctata*	Query Coverage	Max Identity	GenBank Accession Number
B. canis	6; (0.38%; 0.17 - 0.83%)					6 (0.63%; 0.29 - 1.37%)		100%	100%	MK571831
B. capreoli	56; (3.55%; 2.74 - 4.58%)			9 (9.37%; 5.01 - 16.86%)	35 (6.78%; 4.92 - 9.29%)	12 (1.26%; 0.72 - 2.20%)		100%	99-100%	KX839234
B. capreoli/divergens	9; (0.57%; 0.3 - 1.08%)				8 (1.55%; 0.79 - 3.03%)	1 (0.11%; 0.02 - 0.59%)		60-90%	87-100%	KX839234
B. microti	38; (2.41%; 1.76 - 3.29%)	1 (50%; 9.45 -90.55%)		3 (3.13%; 1.07 - 8.79%)	12 (2.33%; 1.34 - 4.02%)	22 (2.32%; 1.54 - 3.49%)		94-100%	99-100%	MG182158 FJ608739
B. microti-Munich type	4; (0.25%; 0.1 - 0.65%)					4 (0.42%; 0.16 - 1.08%)		100%	100%	AB071177
Babesia spp.	69; (4.37%; 3.47 - 5.50%)		1 (100%; 20.66% - 100%)	3 (3.13%; 1.07 - 8.79%)	14 (2.71%; 1.62 - 4.50%)	51 (5.37%; 4.11 - 7.00%)		100%	100%	KJ486571/ KT182986/KY290979/ KJ486571
B. venatorum	119; (7.54%; 6.34 - 8.95%)			4 (41.67%; 1.63 - 10.23%)	54 (10.47%; 8.11 - 13.40%)	61 (6.43%; 5.04 - 8.17%)		100%	100%	KX857480 / MF510178
B. vogeli	10; (0.63%; 0.34 - 1.16%)					10 (1.05%; 0.57 - 1.93%)		100%	100%	KY290979
B. vulpes n. sp.	12; (0.76%; 0.44 - 1.32%)			3 (3.13%; 1.07 - 8.79%)	7 (1.36%; 0.66 - 2.77%)	2 (0.21%; 0.06 - 0.77%)		100%	98%	KT223483 / FJ608737
T. buffeli/ sergenti/orientalis	51; (3.23%; 2.47-4.22%)			2 (2.08%; 0.57 - 7.28%)	13 (2.52%; 1.48 - 4.26%)	36 (3.79%; 2.75- 5.21%)		95-100%	98-100%	MH327771
T. cervi	9; (0.57%; 0.3 - 1.08%)				7 (1.36%; 0.66 - 2.77%)	2 (0.21%; 0.06 - 0.77%)		100%	97%	MG041373

T. equi	6; (0.38%; 0.17 - 0.83%)		4 (0.78%; 0.30 - 1.98%)	2 (0.21%; 0.06 - 0.77%)		100%	100%	KJ787768
T. ovis	6; (0.38%; 0.17 - 0.83%)		1 (0.19%; 0.03 - 1.09%)	5 (0.53%; 0.23 - 1.23%)		100%	100%	KT851432
Theileria spp.	3; (0.19%; 0.06 - 0.56%)		1 (0.19%; 0.03 - 1.09%)	2 (0.21%; 0.06 - 0.77%)		100%	97%	KF270741
A. ovis	3; (0.19%; 0.06 - 0.56%)			3 (0.32%; 0.11 - 0.93%)		100%	100%	MG869525
A. phagocytophilum	80; (5.07%; 4.09 - 6.27%)	4 (41.67%; 1.63 - 10.23%)	59 (11.43%; 8.97 - 14.47%)	17 (1.79%; 1.12 - 2.85%)		98%	100%	KY924885 / MG637125 / MH122891 / MK271308
A. platys	13; (0.82%; 0.48 - 1.4%)	1 (1.04%; 0.18 - 5.67%)	6 (1.16%; 0.53 - 2.51%)	6 (0.63%; 0.29 - 1.37%)		100%	100%	MH762081
Anaplasma spp.	36; (2.28%; 1.65 - 3.14%)		24 (4.65%; 3.15 - 6.83%)	12 (1.26%; 0.72 - 2.20%)		100%	100%	KY924885
E. canis	21; (1.33%; 0.87 - 2.03%)	2 (2.08%; 0.57 - 7.28%)	16 (3.10%; 1.92 - 4.98%)	2 (0.21%; 0.06 - 0.77%)	1 (33.33%; 6.15 - 79.23%)	99%	100%	KY594915
Ehrlichia spp.	12; (0.76%; 0.43 - 1.32%)	2 (2.08%; 0.57 - 7.28%)	8 (1.55%; 0.79 - 3.03%)	2 (0.21%; 0.06 - 0.77%)		96-98%	96-100%	MF142766 / LC120821 / AY098730
B. afzelii	4; (0.25%; 0.1 - 0.65%)	1 (1.04%; 0.18 - 5.67%)	3 (0.58%; 0.20 - 1.70%)			100%	100%	KY213885
B. burgdorferi s.l.	6; (0.38%; 0.17 - 0.83%)		1 (0.19%; 0.03 - 1.09%)	5 (0.53%; 0.23 - 1.23%)		100%	100%	KX646201

For 37 PCR-positive samples, sequencing was not possible due to low-quality DNA. The zoonotic *B. venatorum* was the most prevalent species (MIR = 7.52%; CI95% 6.32% - 8.92%), followed by unspecified *Babesia* spp. (MIR = 4.42%; CI95% 3.51% - 5.55%) and *B. capreoli* (MIR = 3.54%; CI95% 2.73% - 4.57%). Other zoonotic isolates belonged to the *B. microti* group, which were reported with MIR = 2.40% (CI95% 1.75% - 3.28%). For 4 tick-pools, it was possible to specifically determine the presence of *B. microti* "Munich-type" (MIR = 0.25%; CI95% 0.1% - 0.65%). *Babesia* species with the domestic dog as their primary reservoir host were reported with a lower prevalence (*B. canis* MIR = 0.38%, CI95% 0.17% - 0.82%; *B. vogeli* MIR = 0.63%, CI95% 0.34% - 1.16%). The geographical distribution of zoonotic and dog-related piroplasms is reported in Figure 16.

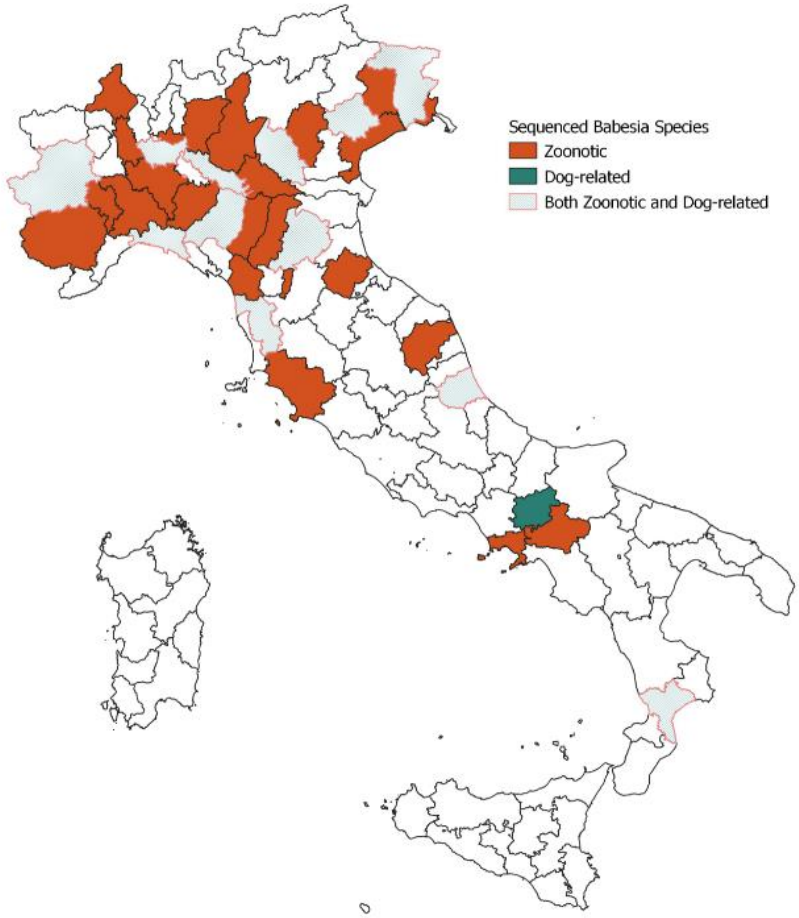

Fig. 16 Zoonotic and dog-related *Babesia* spp. geographical distribution at NUTS3 level.

Regarding *Anaplasma* and *Ehrlichia* spp., genomic DNA of any of these two Gram-negative bacteria was detected in 165 tick pools (MIR = 10.46%; CI95% 9.26% - 11.79%) from 160 dogs.
A significantly higher prevalence was found in *I. ricinus* (χ^2 = 93.53%, $p < 0.05$; OR = 5.33; CI95% 3.70% - 7.67%), while ticks of the genus *Rhipicephalus* were significantly less infected (χ^2 = 94.43, $p < 0.05$; OR = 0.19; CI95% 0.13% - 0.27%). Among different developmental stages, engorged *I.*

ricinus females were significantly more infected than the others ($\chi^2 = 15.16$, $p < 0.05$; OR = 2.39; CI95% 1.48% - 3.53%). Higher infection prevalence, although not statistically significant, was found in tick pools of dogs from forest environments compared to dogs living in only urban or rural environments ($\chi^2 = 4.63$, $p > 0.05$). Housing and use of antiparasitic treatment had no effect on the risk of being parasitized by infected ticks ($p > 0.05$). Geographical distribution at NUTS3 level of *Anaplasma/Ehrlichia*-infected ticks is reported in Figure 17.

Anaplasma/Ehrlichia DNA was detected in 46 of the 78 provinces sampled (P = 58.97, CI95% 47.89% - 69.22%) (Figure 17a) with significant differences between the NUTS3 provinces ($p < 0.05$). Considering NUTS3 where at least 20 dogs were sampled, *Anaplasma/Ehrlichia* DNA was detected with MIR values ranging from 0% (CI95% 0.00% - 15.46%) to 22.73% (CI95% 10.12% - 43.44%) (Figure 17b).

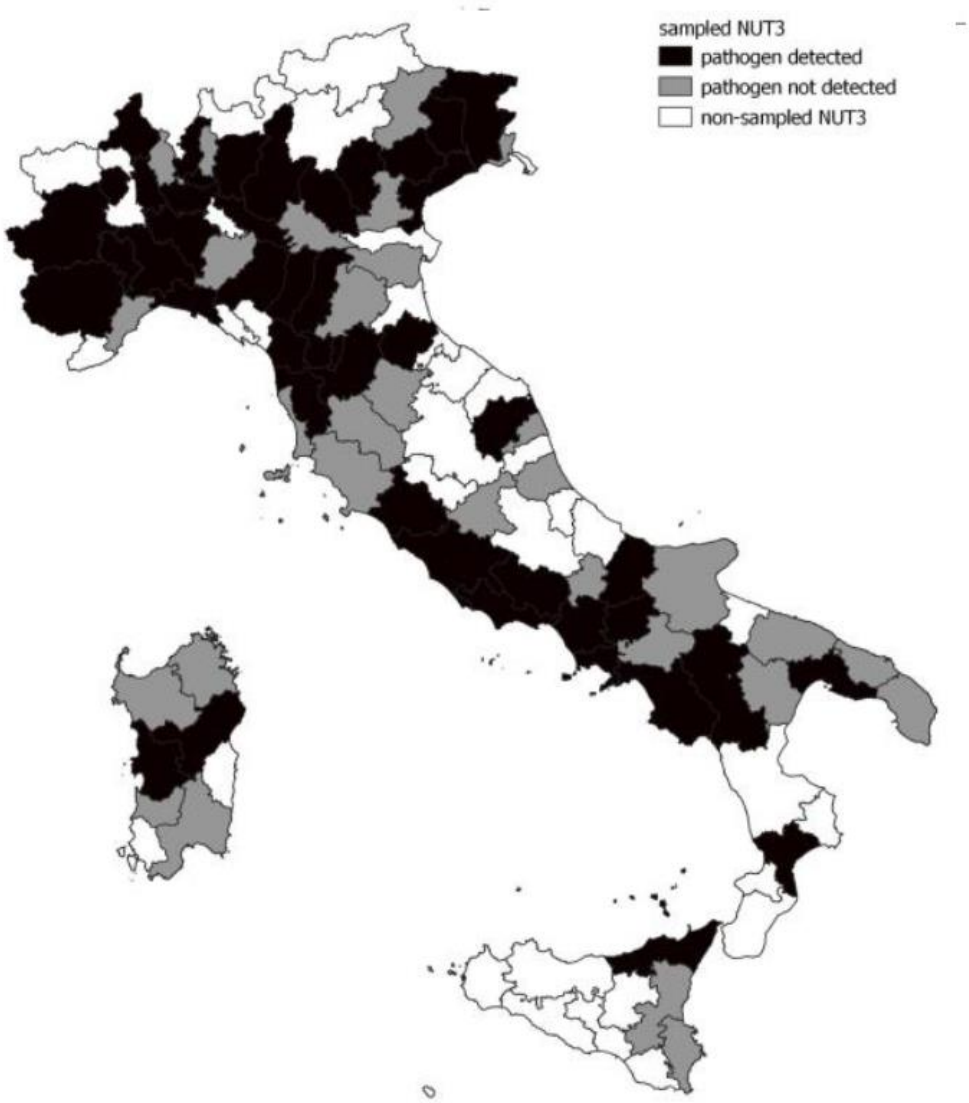

Figure 17a. Geographical distribution, at the NUTS3 level, of ticks infected with *Anaplasma*/*Ehrlichia* spp.

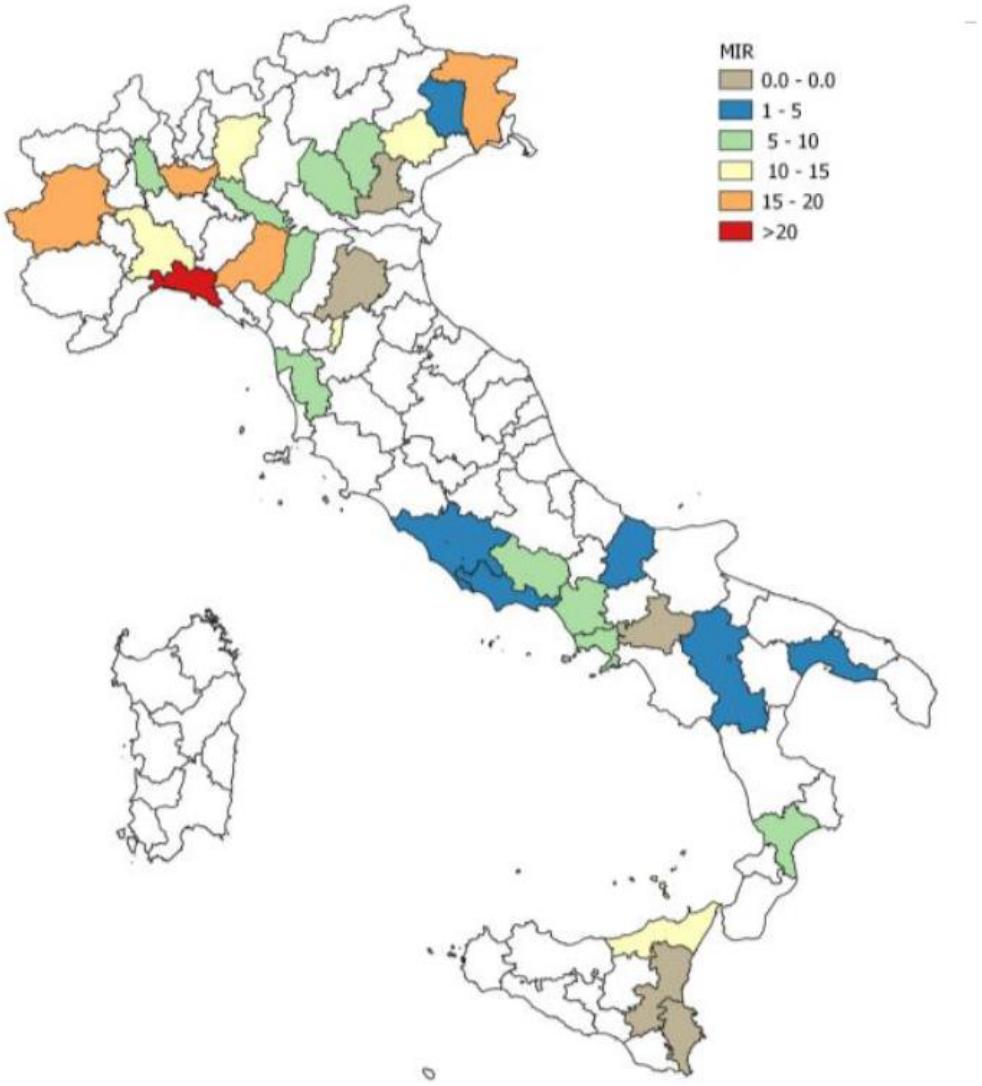

Figure 17b. Minimum Infection Rate (MIR%) in NUTS3 provinces, where at least 20 dogs were sampled, *Anaplasma*/*Ehrlichia*.

Considering sequencing results, the zoonotic *A. phagocytophilum* was identified in 80 pools (MIR = 5.07%, CI95% 4.09% - 6.27%) from 35 provinces, while *A. platys* and *E. canis,* which cause cyclic canine thrombocytopenia and canine monocytic ehrlichiosis, were detected in 13 (MIR = 0.82%; CI95% 0.48% - 1.4%) and 21 (MIR = 1.33%; CI95% 0.87% - 2.03%) pools respectively. *A. ovis* was detected in 3 tick-pools from the sole Catania province (Sicily, Southern Italy) (MIR = 0.19%, CI95% 0.06% - 0.56%). Uncultured *Anaplasma* spp. was amplified from 36 pools (MIR = 2.28%, CI95% 1.65% - 3.14%) and uncultured *Ehrlichia* spp. from 12 pools (MIR = 0.76%, CI95% 0.43% - 1.32%),

including 1 isolate from northeastern Italy of *Candidatus E. walkerii* [GenBank: AY098730], previously identified in *I. ricinus* ticks attached to asymptomatic human patients from the same part of Italy (Brouqui et al., 2003). Table 2 reports the overall sequencing results for *Anaplasma/Ehrlichia* related to tick species. Figure 18 shows the geographical distribution of zoonotic and canine-related Anaplasmataceae (*A. platys* and *E. canis*).

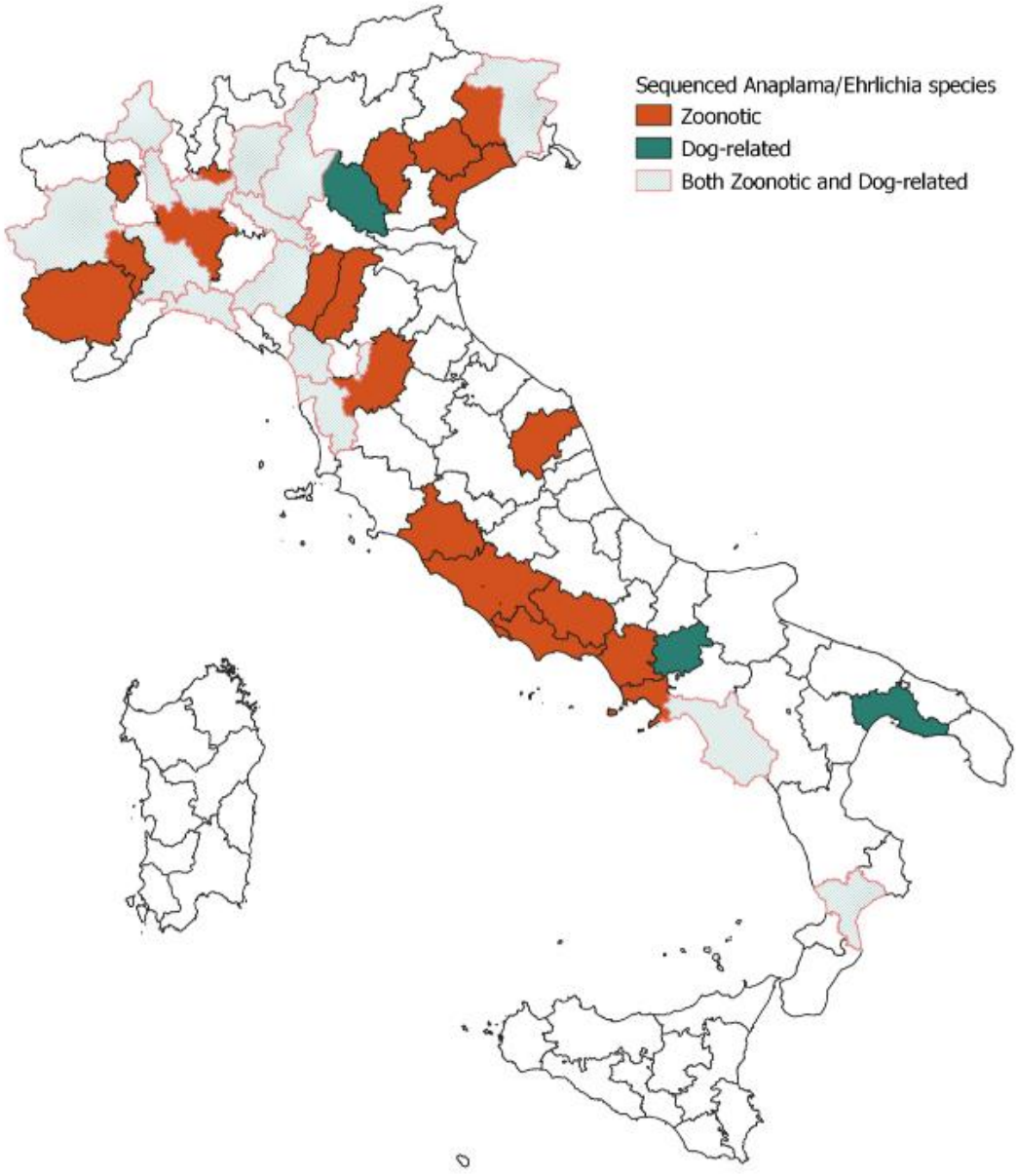

Fig. 18 Zoonotic and dog-related *Anaplasma* and *Ehrlichia* spp. geographical distribution at NUTS3 level.

Regarding *Borrelia spp.*, *B. burgdorferi* s.l. DNA was detected in 10 tick pools (MIR = 0.63%, CI95% 0.34% - 1.16%) from 10 different dogs. Infected ticks were all adults (n=8 non engorged adults and n=2 engorged females). Infected pools belonged to the genera *Ixodes* (*I. ricinus* n=4, *I. hexagonous* n=1) and *Rhipicephalus* (*R. sanguineus* s.l. n=4, *R. turanicus* n=1), with no statistically significant differences among genera or species due to the small number of positive samples. All dogs with *B. burgdorferi* s.l. positive ticks were housed indoors with access to a garden. Seven dogs regularly attended rural and forest environments, while 3 lived exclusively in an urban setting. Antiparasitic treatment was reported for 6 dogs, but active in only 2 dogs. Sequencing identified n=6 *B. burgdorferi* s.l. and n=4 *B. afzelii* (Table 13). Geographical distribution at NUTS3 level of *B. burgdorferi s.l.* is reported in Figure 18. *B. burgdorferi s.l.* was detected in 11.54% of the sampled NUTS3 provinces (CI95% 6.19% - 20.50%).

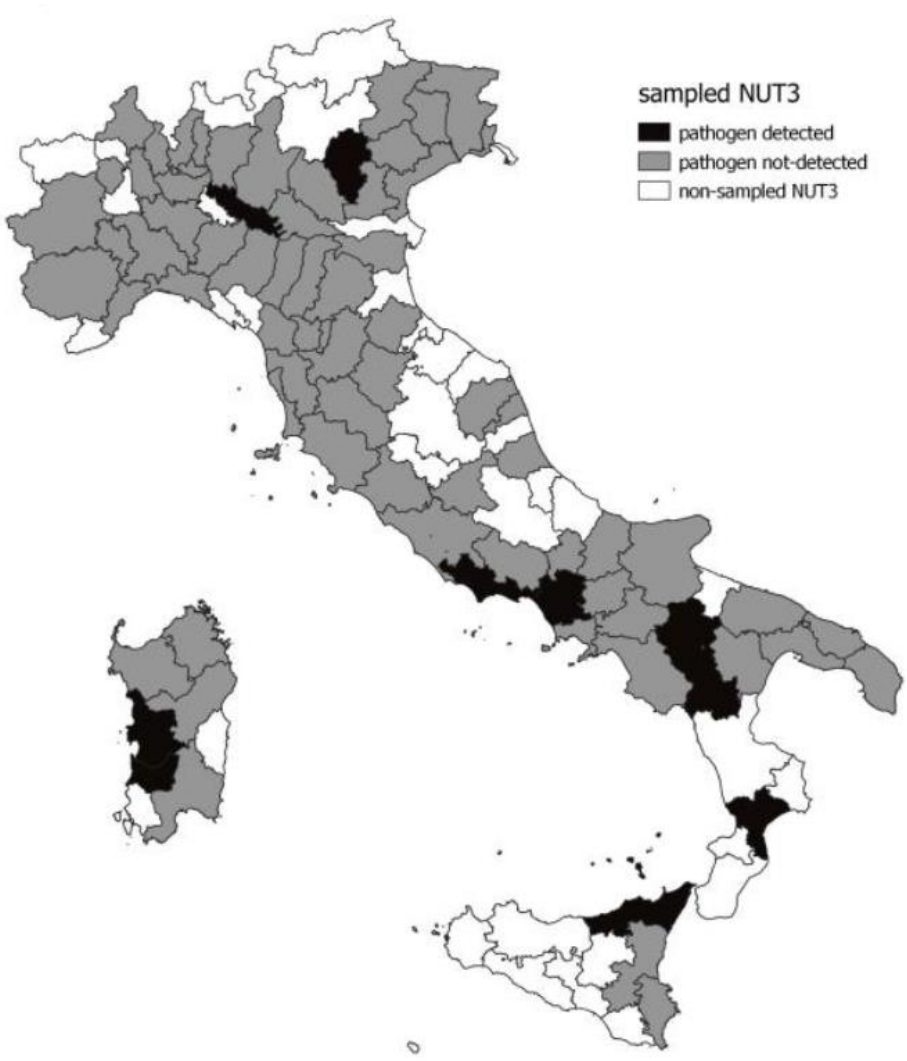

Fig. 18a Geographical distribution, at the NUTS3 level, of ticks infected with *B. burgdorferi* s.l.

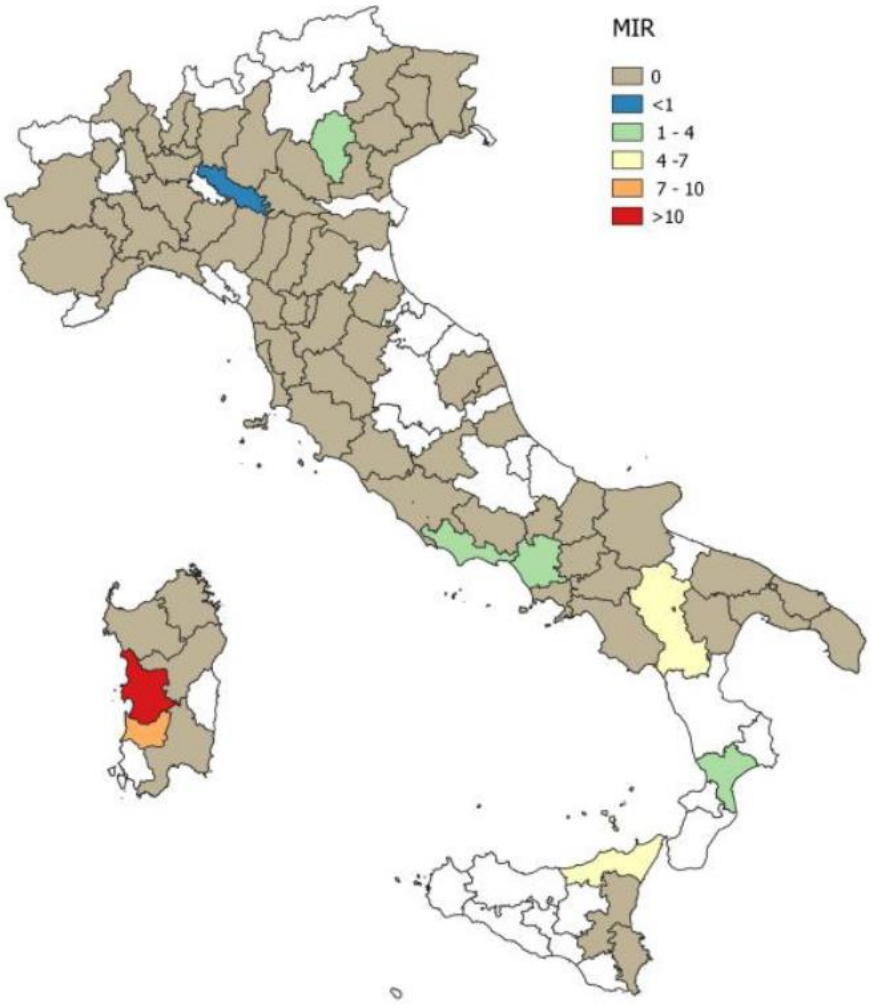

Fig. 18b Minimum Infection Rate (MIR%) in NUTS3 provinces, where at least 20 dogs were sampled, for *B. burgdorferi* s.l.

6.2.3. Discussion

As already explained in the introduction, ticks and tick-borne diseases have showed a general emergence over the last decades (Colwell et al., 2011). Moreover, when pets such as domestic dogs are involved, they are perceived by public opinion as a significant threat to both animal and human health.

Among the analysed tick pools, 27.57% were positive for *Babesia*/*Theileria* DNA, with higher prevalence in *I. ricinus* if compared to the other tick species. The importance of *I. ricinus* in relation to the epidemiology of piroplasms is confirmed by the large variety of species found to infect this tick. In particular, high prevalence of piroplasms for which wild animals are the definitive reservoir hosts were recorded in *I. ricinus*, such as *B. venatorum*. Given its widespread distribution, feeding habits and anthropophagic behaviour, *I. ricinus* can transmit a wide variety of pathogens, linking together sylvatic, rural and peri-urban environments (Estrada-Peña and Jongejan, 1999). It is

noteworthy to say that other zoonotic *Babesia* species, such as *B. microti* and *B. microti* "Munich-type", were detected not only in *I. ricinus* but also in *R. sanguineus*s.l., I. *hexagonus* and *D. marginatus*. Although this is the first report of *B. microti* and *B. microti* "Munich-type" in these tick species, it is not possible to state that *R. sanguineus* s.l., I. *hexagonus* and *D. marginatus* are vectors for these piroplasms. However, no other ticks were found to parasite their respective hosts, thus excluding an infection through co-feeding. Further studies are required to assess the role of these ticks as competent vectors for *B. microti* and *B. microti* "Munich-type". *B. vulpes* was detected with higher prevalence in I. *hexagonus* ticks, although its presence was recorded also in *I. ricinus* and R. sanguineous s.l., as already reported (Solano-Gallego et al., 2016; Lledó et al., 2014). Even if the finding of *B. vulpes* in feeding I. *hexagonus* has no direct implication on its vectorial capacity, this finding in the present study supports the hypothesis of the hedgehog tick I. *hexagonus* as a likely vector of *B. vulpes* (Checa et al., 2018). Despite the high prevalence of infection with *B. vulpes* in red fox, there are very few cases of symptomatic infection described in naturally infected foxes (Clancey et al., 2010). On the contrary, severe symptomatology has been reported in dogs infected with this piroplasm, with symptoms including pale mucous membranes, anorexia, apathy and fever, severe macrocytic/hypochromic regenerative anaemia and thrombocytopenia (Miró et al., 2015; Guitián et al., 2003; Camacho et al., 2005, 2004; Baneth et al., 2019). Given the possible risk for dogs health, particular attention should be paid to this emergent canine pathogen, which is considered to be endemic in most European countries (Nayyar Ghauri et al., 2019). The low percentage of positive tick pools found on dogs which attend exclusively urban environments reflects the lower burden of canine piroplasms (*B. canis* and *B. vogeli*), which were detected only in the competent vector *R. sanguineus* s.l. (Dantas-Torres, 2008). In particular, *B. canis* showed a prevalence of 0.38% in the sequenced tick pools, while *B. vogeli* of 0.63%. Regular antiparasitic treatments in dogs are important not only for preventing tick-infestation and canine TBPs, but also and especially in the context of public health. Geographical analysis of our results confirms the widespread nationwide presence of piroplasms, with 67.95% of the sampled provinces positive for *Babesia* or *Theileria*. Higher prevalence of infection was reported in northern Italy, particularly in near mountainous or hilly areas. In Mediterranean coastal areas, especially those overlooking the Tyrrhenian Sea, piroplasms were consistently detected with MIR levels ranging from 4% to 12%.

DNA of any of the *Ehrlichia* or *Anaplasma* species was reported in 46 of the NUTS3 provinces (58.97% of the territory included in the study), with an overall prevalence of 10.46% of tick pools. Higher prevalence of infection was recorded in Northern Italy, except for the province of Messina (Sicily region), an area traditionally endemic for *Anaplasma* spp. (Torina et al., 2008). In this province, 3 pools of *R. sanguineus* were infected with *A. ovis*.

Engorged females of *I. ricinus* were the most infected class of ticks, followed by I. *hexagonus*. However, *R. sanguineus* was found to be infected with the highest variety of Anaplasmataceae species, despite the low prevalence of infection. Regarding species, *A. phagocytophilum* was the

most widespread species detected, with the highest MIR in I. *hexagonus* (MIR = 41.67%), followed by *I. ricinus* (MIR = 11.43%) and *R. sanguineus* s.l. (MIR = 1.79%). Although *I. ricinus* is the primary vector of *A. phagocytophilum* in Europe, the high rate of infection of I. *hexagonus* confirms the important role of the hedgehog tick and its animal reservoir in the epidemiology of *A. phagocytophilum* in Europe (Silaghi et al., 2012). In Italy, previous reports of *A. phagocytophilum* in ticks collected from domestic dogs and wild carnivores showed a prevalence ranging from 0% to 16.6% (Vascellari et al., 2016; Solano-Gallego et al., 2006; Morganti et al., 2017; Geurden et al., 2018; Ebani, 2019; Ebani et al., 2011, 2008; Da Rold et al., 2018; Baráková et al., 2018; Aureli et al., 2015).

DNA of *A. platys* and *E. canis* were homogeneously reported in tick pools from both Northern and Southern Italy, in contrast with previous records of higher seroprevalence in dogs from Southern regions (Solano-Gallego et al., 2006; Ramos et al., 2014) and Sardinia (Cocco et al., 2003). Despite the report of *E. canis* in *R. sanguineus* s.l., that is the proved vector of this pathogen in the Mediterranean area (Stich et al., 2008), it is interesting to note the higher MIR detected in *I. ricinus* and I. *hexagonus*, as well as in *Haemaphysalis punctata*. H. punctate is a known vector and reservoir for a variety of tick-borne pathogens, such as *Babesia bigemina*, *Coxiella burnetii* and *Rickettsia massiliae* (Tijsse-Klasen et al., 2013), but up to now no reports of *E. canis* DNA in this tick species have been published. However, due to the very limited number of ticks belonging to this species included in this study (n = 4), no inference can be made on the possible role of this tick species in the epidemiology of *E. canis*.

B. burgdorferi s.l. DNA was detected with low prevalence across the country, both in *I. ricinus* and in *R. sanguineus* s.l. Regarding Italian provinces, 8 out of 78 examined showed the presence of infected tick pools. In particular, only isolated tick pools were positive for this pathogen, except for the province of Oristano (Sardinia region), in which 2 tick pools from 2 different dogs were found infected with *B. burgdorferi* s.l. In Sardinia, a previous survey reported a seroprevalence of 6.1% in teenagers, although it has showed no association between seropositivity and pet ownership (Castiglia et al., 2004). Across Italy, anti-B. burgdorferi antibodies are present in humans with a prevalence ranging considerably between geographical areas (from 0% to 23.2%) (Santino et al., 2006). Results of the present study confirm the highly localized distribution of *B. burgdorferi* spirochete; moreover, the low number of ticks submitted from the Northeastern part of the country (traditionally endemic for *B. burgdorferi* s.l.) (Santino et al., 2006) did not allow a detailed assessment of the epidemiological situation of ticks infesting dogs in this area. Finally, it is noteworthy that tick positivity to B. burgdorferi s.l. was observed not only in dogs living in rural and sylvatic environments, but also in ticks from dogs exposed to urban environment, highlighting a possible risk for public health.

6.2.4. Conclusions

The present study is the first nationwide survey on tick-borne pathogens on companion animals in Italy. Results highlight the high variability of piroplasms, Anaplasmataceae and Spirochetae in these ticks. Moreover, our data confirm that the emergence of TBPs which have mainly wild reservoir hosts (i.e. roe deer for *B. venatorum*, wild rodents for *A. phagocytophilum* and *B. burgdorferi* s.s.) (Malandrin et al., 2010; Gern et al., 1998), are not limited or confined to sylvatic and rural environments but are increasingly reported in anthropic biological communities (human, pets and ectoparasites of owned/pet dogs). The reported high prevalence of TBPs in ticks from privately-owned dogs reflects the importance of an in-depth understanding of ticks and TBPs by veterinary practitioners and authorities, which must duly inform pet owners and assist them in accessing preventive care through ectoparasitic treatments.

Zanet S., **Battisti E**., Pepe P., Ciuca L., Colombo L., Trisciuoglio A., Ferroglio E., Cringoli G., Rinaldi L., Maurelli MP. Tick-borne pathogens in Ixodidae ticks collected from privately-owned dogs in Italy: a country-wide molecular survey. Accepted for publication in *BMC Veterinary Research*

6.3. Molecular survey on vector-borne pathogens in alpine wild carnivorans – PAPER 6

6.3.1. Materials and methods

For this study, 235 wild carnivorans (157 foxes, 45 badgers and 33 wolves) were collected in the period between 2009 and 2017. All the animals were road-killed, with the exception of red foxes that were culled during the official hunting seasons as part of the culling program for fox population control, and carcasses were brought to the Department of Veterinary Science, University of Turin, for necropsy. For each animal, information such as age (estimated by dental conditions and body size measurements), sex and area of origin (mountain/flat/hill region) were recorded. Spleen was collected from each animal and individually stored at−20◦Cuntil further analysis. Fig. 19 shows the spatial distribution of sampled animals.

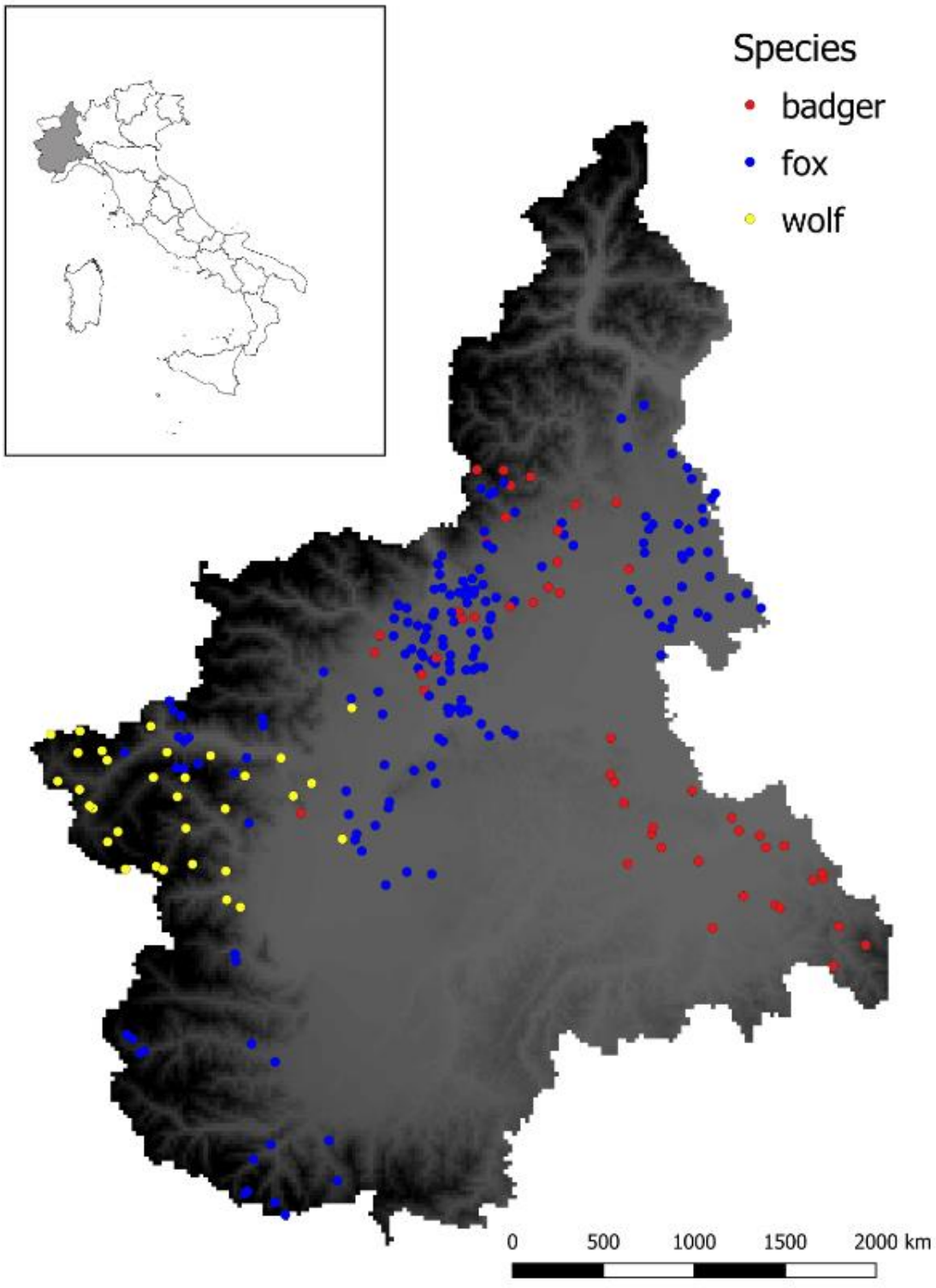

Fig. 19 Spatial distribution of foxes, wolves and badgers analysed in this study.

For pathogen detection, DNA was extracted from approximately 10 mg of spleen by using the commercial kit GenElute Mammalian Genome DNA Miniprep (Sigma-Aldrich, St.Louis, MO, USA). Extracted DNA was then used as template for PCR analysis.

To detect piroplasms, a semi-nested PCR targeting the V4 hypervariable region of the 18S rRNA of both *Babesia* and *Theileria* genera, using primers RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3'), RLB-R2 (5'-CTAAGAATTTCACCTCTGACAGT-3') and RLB-FINT (5'-GACAAGAAATAACAATACRGGGC-3'), was performed (Zanet et al., 2014b). Briefly, the reaction mixture for the first step contained 1X of PCR Master Mix (Promega Corporation, WI, USA), 20 pmol of each primer and 5µl of DNA, in an overall volume of 25 µl. The thermal cycler conditions used were an initial denaturation step at 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 50°C for 45 s and 72°C for 90 s, and a final elongation step at 72°C for 10 min. Amplicons from the first step (1 µl) were used as templates for the second step with internal primer RLB-FINT instead of RLB-F2. Protocol and thermal cycler conditions were identical to the first step except for the annealing temperature at 55°C and for the cycling number of 40.

For *Ehrlichia* and *Anaplasma* detection, the 16S rRNA was targeted using primers PER1 (5'-TTTATCGCTATTAGATGAGCCTATG-3') and PER2 (5'-CTCTACACTAGGAATTCCGCTAT-3') (Goodman et al., 1996). The PCR reaction mixture contained 1X of PCR buffer, 25 pmol of each primer, 0.5 mM of $MgCl_2$, 2.5 U of HotStarTaq DNA Polymerase (Qiagen, Milan, Italy), 0.2 mM of dNTPs mix (Sigma-Aldrich, St. Louis, MO, USA) and 1 µl of DNA, in a total volume of 25 µl. The thermal cycler conditions were an initial denaturation step at 95°C for 15 min, followed by 40 cycles at 94°C for 1 min, 52.4°C for 45 s, 72°C for 1 min and a final elongation step at 72°C for 10 min.

For *Hepatozoon* detection, the 18S rRNA was targeted by using primers HepF (5'-ATACATGAGCAAAATCTCAAC-3') and HepR (5'-CTTATTATTCCATGCTGCAG-3'). The reaction contained 12.5 µl of Taq PCR Master Mix (Qiagen, Milan, Italy), 50 pmol of each primer and 2 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 53 °C for 30 sec, 72 °C for 1 min, and a final elongation step at 72 °C for 10 min.

For each PCR, positive and negative controls were processed together with samples and all the precautions were taken to minimize the risk of contamination.

Selected positive amplicons were purified using a commercial kit (Nucleospin Extract II Kit, Macherey-Nagel, Düren, Germany) and sent for sequencing (Macrogen Europe, The Netherland).

Logistic regression was performed by using R software (3.5.1) (R Development Core Team, 2018), to investigate possible risk factors for TBD infection (age, sex, area of origin and year of sampling).

6.3.2. Results

DNA of at least one target pathogen was detected in 93.6% [220/235] of the analyzed animals (Tab. 13). In particular, 94.9% of the foxes [149/157], 84.8% [28/33] of the wolves and 95.6% [43/45] of the badgers tested positive for any of the analyzed vector-borne pathogen (VBP).

The prevalence of *Babesia* spp. was significantly higher ($p<0.05$) in foxes (89.7%, CI95% 83.63-93.63%) [130/145] and badgers (91.1%, CI95% 79.27-96.49%) [41/45] than in wolves (39.4%, CI95% 24.68-56.32%) [13/33], while *Hepatozoon* spp. showed higher prevalence ($p<0.05$) in wolves (75.76%, CI95% 58.98-87.17%) [25/33] than foxes (5.1%, CI95% 2.62-9.79%) [8/156]. None of the badgers tested positive for *Hepatozoon* spp., although they showed higher prevalence ($p<0.05$) of *Anaplasma*/*Ehrlichia* infection (62.22%, CI95% 47.63-74.89%) [28/45] (see Tab.13).

Species	**Prevalence (Confidence Interval 95%)**		
	***Babesia* spp.**	**Anaplasmataceae**	***Hepatozoon* spp.**
Red fox (*Vulpes vulpes*)	89.7% (83.63-93.63%)	10.97% (6.96-16.86%)	5.1% (2.62-9.79%)
Wolf (*Canis lupus*)	39.4% (24.68-56.32%)	11.43% (4.54-25.95%)	75.76% (58.98-87.17%)
European badger (*Meles meles*)	91.1% (79.27-96.49%)	62.22% (47.63-74.89%)	0% (0.00-7.87%)

Tab.13 Summary of TBPs prevalence in the analysed species.

Results of the sequencing are listed in Table 14. Among the positive samples, 65 amplicons were chosen for sequencing due to the high quality of the PCR products.

In foxes, *B. vulpes* was the most prevalent species (10.34%, CI95% 6.37-16.37%), with sequences showing 99-100% of similarity with a sequence described by Duscher and colleagues (Duscher et al., 2014) in hunted foxes from Austria [GenBank: KM115977]. One fox tested positive for *Babesia* sp. DO23163 (0.69%, CI95% 0.12-3.80%), with sequence showing 100% similarity to a sequence obtained from a racoon dog in Osaka, Japan [GenBank: AB935167]. Moreover, 8 foxes tested positive for *H. canis*, and sequences showed 100% similarity with *H. canis* described in hunting dogs from the Czech Republic (Mitková et al., 2016) [GenBank: KU893127]. Three wolves were found to be infected by *B. capreoli*, whose sequence were 100% similar to *B. capreoli* [GenBank: KX839234] identified in horses from Northwestern Italy (Zanet et al., 2017), and 21 by *H. canis*. Sequences of *H. canis* obtained in wolves showed 97-100% similarity to those described in hunting dogs from the Czech Republic (Mitková et al., 2016) [GenBank: KU893127], in Eurasian golden jackals from

Austria (Mitková et al., 2017) [KX712123], and in foxes and ticks from Italy (Gabrielli et al., 2010) [GenBank: GU371448]. Regarding badgers, *Babesia* sp. isolate badger type A was the most prevalent recorded piroplasm (15.56%, CI95% 7.75-28.78%), followed by *Babesia* sp. DO23163 (4.44%, CI95% 1.23-14.83%), *Babesia* sp. isolate badger type B (2.22%, CI95% 0.39-11.57%) and *B. capreoli* (2.22%, CI95% 0.39-11.57%). *Babesia* sp. isolate badger type A sequences showed 100% similarity to a sequence described by Bartley and colleagues (Bartley et al., 2017) in badgers from Scotland [GenBank: KX528553], while the positive samples for *Babesia* sp. DO23163 showed 100% similarity with the sequence we found in one fox in this study. *Babesia* sp. isolate badger type B, found in one badger, was 100% similar to a sequence described by Barandika and colleagues (Barandika et al., 2016) in badgers from Northern Spain [GenBank: KT223485], while *B. capreoli* was 100% similar to *B. capreoli* identified in 3 wolves from this study and horses from Northwestern Italy (Zanet et al., 2017). Finally, *A. phagocytophilum* was detected in 3 badgers out of 8 samples sequenced (6.67%, CI95% 2.29-17.86%), while 5 isolates showed 99% similarity with a novel *Ehrlichia* sp. found in a badger in Northern Spain (García-Pérez et al., 2016) [GenBank: KR262717]. None of the Anaplasmataceae positive samples obtained from foxes and wolves were sequenced due to the poor quality of the amplicons.

Logistic regression showed higher risk of infection for animals collected in flat or hilly areas (below 600 m a.s.l.) than in mountain areas (above 600 m a.s.l.) (AUC=0.79). In particular, higher risk of Anaplasmataceae infection in foxes ($p<0.05$; OR=7.16) and of Babesia sp. in badgers ($p<0.01$; OR=22.50) was recorded.

Pathogen species	Host species	Sequenced amplicons	Prevalence	Confidence Interval (95%)	Percent Identity	GenBank Accession Number
B. vulpes	Fox	15	10.34%	6.37-16.37	99-100%	KM115977
B. sp DO23163	Fox	1	0.69%	0.12-3.80	100%	AB935167
B. capreoli	Wolf	3	9.09%	3.14-23.57	100%	KX839234
	Badger	1	2.22%	0.39-11.57	100%	KX839234
B. badger type A	Badger	7	15.56%	7.75-28.78	100%	KX528553
B. badger type B	Badger	1	2.22%	0.39-11.57	100%	KT223485
A. phagocytophilum	Badger	3	6.67%	2.29-17.86	100%	KC800985
Ehrlichia sp.	Badger	5	11.11%	4.84-23.50	99%	KR262717
H. canis	Fox	8	5.13%	2.62-9.79	100%	KU893127
	Wolf	21	63.64%	46.62-77.81	100%	KU893127

Tab. 14 Prevalence, confidence intervals and identity of each sequenced pathogen divided by host species.

6.3.3. Discussion

With 23% of emerging infectious diseases actually transmitted by arthropods, vector-borne pathogens have a considerable impact on human health (Jones et al., 2008). Several factors have been implicated in this emergence; among others, the increase of human encroachment into wild habitats, climate change and the consequent territorial expansion of vector arthropods are the most important (Gortázar et al., 2007; Cardoso et al., 2013).The recent increase of some wildlife populations (Gortázar et al., 2007) is expected to influence the epidemiology of vector-borne diseases, as several sylvatic species are known or suspected reservoirs of VBPs.

Few data are available on VBP presence and prevalence in carnivorans from the alpine region (Zanet et al., 2014b). In our study, the prevalence of *Babesia* in foxes is one of the highest reported so far in Europe. Similar prevalence has been found in Portugal, in which Cardoso and colleagues have reported a prevalence ranging from 78 to 100%, depending on the type of sample (blood or bone marrow) and the analyzed area (northern or southern part of the country) (Cardoso et al., 2013). Lower prevalence has been detected in Spain (Checa et al., 2018), Germany (Najm et al., 2014a), Hungary (Farkas et al., 2015) and Slovakia (Koneval et al., 2017) (72.2%, 46.4%, 20% and 9.7% respectively). In Italy, previous findings have showed variable results depending on the geographical area, ranging from less than 1% to 54% (Zanet et al., 2014b; Santoro et al., 2019; Ebani et al., 2017; Da Rold et al., 2018). Most of the foxes in the present study were found to be infected with *B. vulpes*, as already reported in Spain (Criado-Fornelio et al., 2003b), Italy (Tampieri et al., 2008), Croatia (Dezdek et al., 2010), Germany (Najm et al., 2014a), Portugal (Cardoso et al., 2013) and Austria (Duscher et al., 2014). Despite the severe symptomatology reported in dogs infected with *B. vulpes* (Guitián et al., 2003; Camacho et al., 2005, 2004), only one case of symptomatic infection in a fox has been reported so far (Clancey et al., 2010). This finding, together with the high rate of infection reported, may indicate a role of foxes in the sylvatic cycle of this parasite, although more evidences are needed. To date, no proven tick vectors for *B. vulpes* have been observed. The hedgehog tick *I. hexagonus* has been proposed as the main vector of this parasite based solely on the association between the occurrence of this tick and the infection in dogs (Camacho et al., 2003). Moreover, the detection of *B. vulpes* DNA in unfed *D. reticulatus* ticks in Austria (Hodžić et al., 2017b) may suggest a possible role of this tick species as well. In the study area, both *I. hexagonus* and *D. reticulatus* have been described infesting privately owned dogs (Maurelli et al., 2018).

To the best of our knowledge, only two previous studies have investigated the occurrence of *Babesia* spp. in free-ranging wolves in Europe, reporting a prevalence of 20% in Croatia (Beck et al., 2017) and 7% in Italy (Santoro et al., 2019). Similar to the present study, Beck and colleagues (Beck et al., 2017) reported the presence of wolves infected by piroplasms having wild ungulates as natural hosts. In particular, we detected *B. capreoli* DNA in 3 wolf samples, with sequences showing 100% similarity to those reported from sympatric roe deer, red deer, horses and ticks collected from owned dogs (Zanet et al., 2017, 2014b). Additionally, the same parasite was also found in 1 badger from

this study, suggesting a broader host specificity for *B. capreoli* than previously observed. However, due to the limited number of sequenced amplicons, we are not able to speculate any further, and more studies are needed in order to better understand the role of wolves and badgers in the epidemiology of this Babesia species. Moreover, most of the badgers and one positive fox were infected with mustelid-related *Babesia* species, such as *Babesia* sp. DO23163, *Babesia* sp. badger type A and type B (Hornok et al., 2018; Bartley et al., 2017; BARANDIKA et al., 2016) that belong to the *B. microti* group (Hornok et al., 2018). In Italy, badger-associated *Babesia* infection has been observed also in a wolf from Southern Italy (Santoro et al., 2019), highlighting the circulation of these species within wild carnivorans of the order Caniformia.

H. canis was the only *Hepatozoon* species detected, with 5% of prevalence in foxes and more than 75% in wolves. Previous reports of *H. canis* in foxes from Italy (Gabrielli et al., 2010), Croatia (Dezek et al., 2010), Bosnia and Herzegovina (Hodžić et al., 2015) and Spain (Gimenez et al., 2009; Criado-fornelio et al., 2006) showed a prevalence ranging from 13% up to 90%, while to the best of our knowledge this is the first epidemiological study investigating the occurrence of this parasite in free-ranging wolves. In contrast to *Babesia*, infection with *H. canis* is acquired by the mammal host through the ingestion of an infected tick rather than tick bite. The main vector of this parasite is *R. sanguineus* s.l., the kennel tick, which is widely distributed in Southern Europe and strongly associated with dog presence (Dantas-Torres, 2010). However, the occurrence of *H. canis* has been reported in wildlife from areas in which *R. sanguineus* s.l. is not endemic such as Austria (Georg Gerhard Duscher et al., 2013), Slovakia (Majláthová et al., 2007) and Germany (Najm et al., 2014b), suggesting the role of other tick species as vectors of *H. canis*. To date, only *R. turanicus* has been considered an additional definitive host for this parasite (Giannelli et al., 2017), while *Dermacentor* spp., *Haemaphysalis concinna* and *I. ricinus* have proved to harbor parasite DNA (Hornok et al., 2013b; Gabrielli et al., 2010). In Northern Italy, both the proved and the suspected vectors of *H. canis* have been reported in dogs (Maurelli et al., 2018) and humans (PAPER 7). Predation has been proved to be an alternative route for *H. americanum* infection, a closely related species endemic in the United States (Johnson et al., 2008), thus suggesting a similar transmission way for *H. canis*. In particular, the consume of infected carrions and prey such as rodents carrying tissue cysts of the parasite may be a possible infection route for both wild and domestic carnivorans (e.g. shepherd and hunting dogs) (Hornok et al., 2013b). Finally, transplacental transmission of *H. canis* has been proved in dogs (Murata et al., 1993) and foxes (Hodžic et al., 2018).

Several studies have investigated the prevalence of Anaplasmataceae in wild carnivorans, showing considerable differences among the species. In foxes, infection with *A. phagocytophilum* has been reported from Italy (Ebani et al., 2011), Germany (Härtwig et al., 2014), Poland (Karbowiak et al., 2009), The Netherland (Jahfari et al., 2014), Romania (Dumitrache et al., 2015), Switzerland (Hofmann-Lehmann et al., 2016), Czech Republic (Hodžić et al., 2017a), Austria (Hodžic et al., 2018) and Hungary (Tolnai et al., 2015), while none of the foxes analyzed in Spain were positive for this

bacterium (García-Pérez et al., 2016). In contrast to the red fox, badgers and wolves have been less investigated in Europe. No badgers showed positivity for *A. phagocytophilum* in The Netherland (Jahfari et al., 2014), Czech Republic (Hodžić et al., 2017a), and Spain (Millán et al., 2016), where negative results have been obtained also for the wolf (García-Pérez et al., 2016). Notably, only two positive badgers out of 114 were found during a study on the occurrence of VBPs in mustelids from Belgium and The Netherland (Hofmeester et al., 2018). The prevalence of *A. phagocytophilum* obtained in the present study is in line with that reported previously, showing low occurrence of this bacterium in badger and maybe suggesting the poor role of this mustelid species in the epidemiology of Anaplasmataceae. Conversely, additional studies are needed to further investigate the presence of this bacteria in the wolf, due to limited existing information.

Statistical analysis on risk factors showed a higher risk of Anaplasmataceae infection in foxes and of *Babesia* spp. in badgers collected in flat or hilly areas (below 600 m a.s.l.) than in mountain areas (above 600 m a.s.l.). This could be accounted to higher abundance of vectors (Ixodid ticks) in hilly areas than in the mountains due to more suitable environmental characteristics and to a higher presence of other sylvatic hosts for ticks as wild ungulates.

6.3.4. Conclusions

With the molecular analysis of 235 specimens collected from 2009 to 2017, this study provides valuable information about the situation of vector-borne pathogens in wild carnivorans from Northwestern Italy, showing high level of infection in all target species. Moreover, we reported for the first time the presence of *B. capreoli* in wolves and badgers, two unexpected hosts for this parasite, and of *H. canis* in wolves.

This survey highlights the presence of several VBP in the study area, many of which capable to infect domestic animals and humans. The high occurrence of VBPs in sylvatic carnivorans could pose a risk for both animal and human health, especially in an area with growing urbanization and increasing wildlife population as in many parts of Europe, that lead to more close contacts between humans, wildlife, livestock and pets.

Battisti E., Zanet S., Khalili S., Trisciuoglio A., Hertel B., Ferroglio E. (2020). Molecular survey on vector-borne pathogens in alpine wild carnivorans. *Frontiers in Veterinary Science*. 7:1 doi: 10.1186/s13071-018-2994-2

6.4. Survey on tick-borne pathogens in ticks removed from humans in Northwestern Italy – PAPER 7

6.4.1. Materials and methods

From January to December 2018, 6 healthcare facilities located in the western part of the Piedmont region, Turin province, were enrolled for the study. Following the patient's informed consent, ticks were collected from patients reaching the healthcare facilities for a tick bite and stored in ethanol 70% until further processing. Moreover, some information about the age, gender, jobs and frequented environments were collected using a questionnaire. Up to 3 months after the tick collection, patients were interviewed by phone about the presence of signs and symptoms related to the tick bite.

Ticks were identified to the species or complex level by using the standard morphological keys (Estrada-Peña et al., 2004b) and DNA was extracted by using the TRIreagent (Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's instructions.

For pathogen detection, ticks from the same patient were homogeneously pooled based on the species, sex and developmental stage.

The presence of *Babesia spp.*, *Anaplasma/Ehrlichia spp.*, SFG *Rickettsiae* and *B. burgdorferi s.l.* was investigated with protocol described elsewhere (Zanet et al., 2014b; Roux et al., 1996; Liebisch et al., 1998; Goodman et al., 1996).

To detect piroplasms, a semi-nested PCR targeting the V4 hypervariable region of the 18S rRNA using primers RLB-F2 (5'-GACACAGGGAGGTAGTGACAAG-3'), RLB-R2 (5'-CTAAGAATTTCACCTCTGACAGT-3') and RLB-FINT (5'-GACAAGAAATAACAATACRGGGC-3') was performed (Zanet et al., 2014b). Briefly, the reaction mixture for the first step contained 1X of PCR Master Mix (Promega Corporation, WI, USA), 20 pmol of each primer and 5µl of DNA, in an overall volume of 25 µl. The thermal cycler conditions used were an initial denaturation step at 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 50°C for 45 s and 72°C for 90 s, and a final elongation step at 72°C for 10 min. Amplicons from the first step (1 µl) were used as templates for the second step with internal primer RLB-FINT instead of RLB-F2. Protocol and thermal cycler conditions were identical to the first step except for the annealing temperature at 55°C and for the cycling number of 40.

For *Ehrlichia* and *Anaplasma* detection, the 16S rRNA was targeted using primers PER1 (5'-TTTATCGCTATTAGATGAGCCTATG-3') and PER2 (5'-CTCTACACTAGGAATTCCGCTAT-3') (Goodman et al., 1996). The PCR reaction mixture contained 1X of PCR buffer, 25 pmol of each primer, 0.5 mM of $MgCl_2$, 2.5 U of HotStarTaq DNA Polymerase (Qiagen, Milan, Italy), 0.2 mM of dNTPs mix (Sigma-Aldrich, St. Louis, MO, USA) and 1 µl of DNA, in a total volume of 25 µl. The

thermal cycler conditions were an initial denaturation step at 95°C for 15 min, followed by 40 cycles at 94°C for 1 min, 52.4°C for 45 s, 72°C for 1 min and a final elongation step at 72°C for 10 min.
For *B. burgdorferi* s.l. detection, the 16S rRNA was targeted by using primers BOR-ALLG-F (5'-ACGCTGGCAGTGCGTCTTAA-3') and BOR-ALLG-R (5'- CTGATATCAACAGATTCCACCC-3') (Liebisch et al., 1998). Briefly, for each reaction there was 1X of 5X Green Reaction Buffer, 10 pmol of each primer, 1.25 U of GoTaq HotStar Polymerase (Promega Corporation, WI, USA), 0.2 mM of dNTPs mix (Promega Corporation, WI, USA) and 5 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were 94 °C for 2 min, followed by 40 cycles at 94 °C for 1.5 min, 63 °C for 2 min and 72 °C for 2 min, and a final elongation step at 72 °C for 10 min.
For SFG *Rickettsiae* detection, the OmpA (outer membrane protein A) gene was targeted by using primers Rr190-70 (5'-ATGGCGAATATTTCTCCAAAA-3') and Rr190-701 (5'-GTTCCGTTAATGGCAGCATCT-3') (Roux et al., 1996). The PCR reaction contained 1X of 5X Green Reaction Buffer, 10 pmol of each primer, 1.25 U of GoTaq HotStar Polymerase (Promega Corporation, WI, USA), 0.2 mM of dNTPs mix (Promega Corporation, WI, USA) and 5 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were an initial denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 sec, 47 °C for 30 sec and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min.
Positive amplicons were sequenced (Macrogen Europe, Spain; LGC Genomics, Germany) and obtained sequences were compared with those deposited in GenBank®. Logistic regression was performed by using R software (3.5.1) (R Development Core Team, 2018), to investigate possible risk factors for TBD infection.

6.4.2. Results

An overall number of 92 patients (55 men and 37 women) were enrolled in this study, with a median age of 34 (range 2-81). The majority (62%, 57/92) of the patients reported the tick bite during outdoor activities (hiking, mushroom picking), followed by leisure activities as gardening (28,3%, 26/92) and job activities (forestry workers) (3,3%, 3/92). Remaining patients did not specify anything in the questionnaire. All the tick bites occurred in the geographical area of the study, except one patient that reach the healthcare facilities after a travel in Sardinia region (Italy).
One hundred and twenty-eight ticks were collected, most of them belonging to the genus *I. ricinus* (97.7%, 125/128), while three specimens were identified as *R. sanguineus* s.l. (2.3%, 3/128). Nymphs were most frequently collected (78.9%, 101/128), followed by adults (13.3%, 17/128) and larvae (7.8%, 10/128). The three *R. sanguineus* s.l. specimens were a nymph and two adults. Among adults, 5 females were fully engorged at the time of removal. Ticks were collected from April to October, with a peak of frequency in June, from 4 out of the 6 healthcare facilities enrolled in the study. No ticks were collected during cooler months (from November to March).

Overall, 93 pools consisting of all the collected ticks were processed for pathogen identification. Among these, 30 pools (2 larva pools, 26 nymph pools and 2 adult pools all consisting of *I. ricinus*) tested positive for at least one pathogen, with a Minimum Infection Rate (MIR) of 32,26%% (CI95% 23.62-42.30%). In particular, *Babesia* spp. showed a MIR of 31.18% (29/93; CI95% 22.67-41.19%), while 1 out of 93 pools tested positive for SFG *Rickettsiae* (MIR = 1.08%; CI95% 0.19-5.84%). No samples tested positive for *A. phagocytophilum* and *Borrelia* spp.

Nine selected positive amplicons for *Babesia* spp. and the positive sample for SFG *Rickettsiae* sequenced, and results revealed the presence of *B. venatorum* in 8 out of 9 positive amplicons (MIR = 88.9%; CI95% 56.50-98.01%), with sequences showing 100% similarity to those reported from ticks from the Czech Republic [GenBank: KX857480]. The remaining amplicon was positive for *Theileria buffeli/orientalis* complex (MIR = 11.1%; CI95% 1.99-43.50%), showing 100% similarity to those reported from Asia [e.g. KX965722], while the SFG *Rickettsiae* positive amplicon was identified as *R. monacensis*.

Regarding symptoms, 2 patients (2.15%) reported a local rash in the weeks after the tick bite that heal spontaneously without treatment. Ticks collected from these patients tested negative for all the analysed pathogens. No other patients reported any signs or symptoms that could have indicated a tick-borne infection.

Statistical analysis did not show association between tick positivity for any of the pathogens and possible risk factors as age, gender, frequented environments and activities at the time of tick bite.

6.4.3. Discussion

As already discussed before, the incidence of ticks and tick-borne diseases is rising (Colwell et al., 2011). However, few data on ticks and TBDs in humans are available in Europe (Wilhelmsson et al., 2013, 2010; Liebisch et al., 1998; Gargili et al., 2012) In Italy, previous studies have been carried out in the Southern and Eastern parts of the nation (Otranto et al., 2014; Beltrame et al., 2018).

The greater prevalence of *I. ricinus* in comparison to *R. sanguineus s.l.* found in the present study can be explained by the activities carried out by patients at the time of the tick bite and the frequented environments. In fact, *I. ricinus* is the most widespread tick species in Northern Italy that prefer natural areas characterized by deciduous and coniferous woodland, forests, urban and peri-urban park. For this reason, human encroachment into natural habitats for hiking, gardening or hunting may expose people to contacts with this tick species. In contrast, *R. sanguineus* s.l. is an endophilic tick species preferring animal burrows and nests, and mainly associated to dog presence and warmer climates.

Regarding pathogens, almost a third of all the analysed pools were infected. It is noteworthy that *B. venatorum*, the most prevalent reported species, and *R. monacensis* are zoonotic species able to cause from moderate to severe infections in humans. In particular, *B. venatorum* was recorded as

the etiological agent of babesiosis in asplenic and immunocompromised patients in Europe (Yabsley and Shock, 2013), and has the roe deer as reservoir host. In Northwestern Italy, previous findings of this species were reported in wild ungulates (Zanet et al., 2014b), ticks collected from owned dogs (PAPER 5) and questing (PAPER 9), highlighting the circulation of this parasite at the sylvatic-domestic interface. Moreover, human cases of babesiosis are increasingly reported in Europe, likely due to both an increase in the actual incidence as well as increased awareness of the disease (Homer et al., 2000). In contrast, *T. buffeli/orientalis* complex consist of several isolates with uncertain phylogeny and no zoonotic potential, even though is responsible for benign bovine babesiosis (Gubbels et al., 2000). *R. monacensis* is a recently described SFG *Rickettsia* causing a Mediterranean spotted fever-like disease (Madeddu et al., 2012) and its presence has been observed in ticks collected from several urban parks across Europe (Szekeres et al., 2016; Simser et al., 2002). With nearly 85,000 cases of Lyme borreliosis per year in Europe (ECDC, 2011), *B. burgdorferi* s.l. is an important threat for human health. Although no ticks tested positive in our study, up to 7% of ticks collected from humans were infected with this pathogen in Northern Italy (Beltrame et al., 2018). Together, these data highlight the importance of passive surveillance to assess the epidemiology of TBDs that pose a threat to human health.

6.4.4. Conclusions

The present study highlights the risk for human health deriving from a tick bite. In particular, a considerable prevalence of wildlife-related *Babesia*, such as *B. venatorum*, was found. All together, these data highlight the importance of passive surveillance to assess the epidemiology of TBDs that pose a threat to human health and should get the attention of physicians that are called to identify first symptoms of TBPs infection.

Battisti E., Zanet S., Boraso F., Minniti D., Giacometti M., Ferroglio E. (2019) Survey on tick-borne pathogens in ticks removed from humans in Northwestern Italy. *Veterinary Parasitology: Regional Studies and Reports.* 18. 100352 doi.org/10.1016/j.vprsr.2019.100352

6.5. Zoonotic pathogens in ticks from migratory birds, Italy – PAPER 8

6.5.1. Materials and methods

Fieldwork activities were conducted during the spring 2016 and 2017 at the Ponza Ringing Station on the island of Ponza (Central Tyrrhenian sea, Italy, 40°55' N, 12°58' E). Collected ticks were identified to the species or complex level by using standard morphological keys (Estrada-Peña et al., 2004b) and PCR amplification and sequencing of the ITS region where possible (Lv et al., 2014). Two commercial kits were used for RNA (High Pure Viral Nucleic Isolation Kit, Roche Diagnostics GmbH, Mannheim, Germany) and DNA (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany) extraction. RealStar®CCHFV RT-PCR Kit 1.0 (Altona Diagnostics, Hamburg, Germany) was used for CCHF virus detection, while the presence of *Babesia* spp., *Anaplasma* and *Ehrlichia* spp., SFG *Rickettsiae* and *Borrelia* spp. DNA was investigated by using conventional PCR, with protocols described elsewhere (Roux et al., 1996; Liebisch et al., 1998; Hodžić et al., 2015; Brown et al., 2001).

For *Babesia* spp. detection, the 18S rRNA gene was targeted by primers BTH-1 F (5'-CCTGAGAAACGGCTACCACATCT-3') and BTH-1 R (5'-TTGCGACCATACTCCCCCCA-3') (Hodžić et al., 2015). Briefly, the PCR reaction mixture contained 1X of 5X Green Reaction Buffer, 10 pmol of each primer, 1.25 U of GoTaq HotStar Polymerase (Promega Corporation, WI, USA), 0.2 mM of dNTPs mix (Promega Corporation, WI, USA) and 1 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were 94 °C for 2 min, followed by 40 cycles at 94 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min.

In order to detect *Anaplasma* and *Ehrlichia* spp., the 16S rRNA of the bacteria was used as target for primers EHR16SD (5'-GGTACCYACAGAAGAAGTCC-3') and EHR16SR (5'-TAGCACTCATCGTTTACAGC-3') (Brown et al., 2001). The PCR reaction contained 1X of 5X Green Reaction Buffer, 10 pmol of each primer, 1.25 U of GoTaq HotStar Polymerase (Promega Corporation, WI, USA), 0.2 mM of dNTPs mix (Promega Corporation, WI, USA) and 1 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were 94 °C for 2 min, followed by 40 cycles at 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min.

For *B. burgdorferi* s.l. detection, the 16S rRNA was targeted by using primers BOR-ALLG-F (5'-ACGCTGGCAGTGCGTCTTAA-3') and BOR-ALLG-R (5'- CTGATATCAACAGATTCCACCC-3') (Liebisch et al., 1998). Briefly, for each reaction there was 1X of 5X Green Reaction Buffer, 10 pmol of each primer, 1.25 U of GoTaq HotStar Polymerase (Promega Corporation, WI, USA), 0.2 mM of dNTPs mix (Promega Corporation, WI, USA) and 5 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were 94 °C for 2 min, followed by 40 cycles at 94 °C for 1.5 min, 63 °C for 2 min and 72 °C for 2 min, and a final elongation step at 72 °C for 10 min.

For SFG *Rickettsiae* detection, the OmpA (outer membrane protein A) gene was targeted by using primers Rr190-70 (5'-ATGGCGAATATTTCTCCAAAA-3') and Rr190-701 (5'-GTTCCGTTAATGGCAGCATCT-3') (Roux et al., 1996). The PCR reaction contained 1X of 5X Green Reaction Buffer, 10 pmol of each primer, 1.25 U of GoTaq HotStar Polymerase (Promega Corporation, WI, USA), 0.2 mM of dNTPs mix (Promega Corporation, WI, USA) and 5 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were an initial denaturation step at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 sec, 47 °C for 30 sec and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. A subset of OmpA-positive ticks were also screened with primers RpCS.877p (5'-GGGGGCCTGCTCACGGCGG-3') and RpCS.1258n (5'-ATTGCAAAAAGTACAGTGAACA-3') targeting a fragment of the gltA (citrate synthase) gene (Hodzic et al., 2017). Briefly, each reaction tube contained 1X of 5X Green Reaction Buffer, 10 pmol of each primer, 1.25 U of GoTaq HotStar Polymerase (Promega Corporation, WI, USA), 0.2 mM of dNTPs mix (Promega Corporation, WI, USA) and 5 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were an initial denaturation step at 95 °C for 2 min, followed by 40 cycles at 95 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min.
Selected positive amplicons were sent for sequencing (LGC Genomics, Germany) for tick identification and pathogen detection, and sequences were compared with those deposited in GenBank®.

6.5.2. Results

Overall, seven-hundred and forty-four migratory birds, belonging to 20 different species (Tab. 15), were captured during regular ringing procedures and checked for the presence of ticks. Fourteen species were long-distance migratory birds, known to winter in sub-Saharan Africa, while 6 were partial migrants, such as the Blackbird (*Turdus merula*), the Dunnock (*Prunella modularis*), the Eurasian Blackcap (*Sylvia atricapilla*), the European Robin (*Erithacus rubecula*), the Song Thrush (*Turdus philomenos*) and the Subalpine Warbler (*Sylvia cantillas*).
Totally, 231 engorged ticks were collected. With the help of PCR amplification and sequencing of the ITS region, 94 ticks were identified at the species level: *Hyalomma marginatum* complex (5 larvae, 82 nymphs), *Ixodes frontalis* (3 nymphs), *I. ventalloi* (3 nymphs) and *Amblyomma marmoreum* (1 nymph). For the remaining ticks, amplification of the ITS region failed, and therefore only the genus was identified, namely *Hyalomma* spp. (1 larva, 118 nymphs) or *Ixodes* spp. (3 larvae, 14 nymphs and 1 adult).
Among the analysed ticks, 50 tested positive for SFG *Rickettsiae* DNA, with an overall prevalence of 21.7% (50/231; CI95% 16.8% - 27.4%). Sequencing results showed the presence of *Rickettsia aeschlimannii* in 47 out of 50 ticks (P=94.0%; CI95% 83.8% - 97.9%) (Table 15). In particular, 46 sequences were 100% identical to each other and to *Rickettsia aeschlimanni* from different countries

[e.g. GenBank® HQ335157.1] while 1 sequence was 100% identical to *Rickettsia aeschlimanni* strain RH from Senegal [GenBank® HM050286] and differed at 1 nucleotide compared to the others (T instead of C at the 425 base pair). Two sequences showed 100% identity to each other and to *Rickettsia africae* [e.g. GenBank® HQ335132] (2/50; P=4.0%; CI95% 1.1% - 13.5%), while 1 sequence was 100% identical to *Rickettsia raoultii* [e.g. GenBank® MF166732] (1/50; P=2.0%; CI95% 0.4% - 10.5%). The gltA sequencing of selected OmpA-positive ticks confirmed the species for all the specimens.

No ticks tested positive for any of the other investigated pathogens.

Bird species	**No. of birds investigated**	**Tick species found**	**No. of pathogen-positive ticks / No. of tested ticks**	***Rickettsia***		
				R. aeschlimanni	***R. africae***	***R. raoultii***
2016						
Barn Swallow (*Hirundo rustica*)	18	NA*	NA			
Blackbird (*Turdus merula*)	1	*Ixodes ventalloi*	0/1			
Black Redstart (*Phoenicuros ochruros*)	29	*Ixodes ventalloi*	0/2			
		Hyalomma sp.	1/3	1	0	0
Eurasian Blackcap (*Sylvia atricapilla*)	1	NA	NA			
European Robin (*Erithacus rubecula*)	22	*Ixodes frontalis*	0/1			
Garden Warbler (*Sylvia borin*)	83	NA	NA			
Icterine Warbler (*Hippolais icterina*)	19	*Hyalomma* sp.	1/2	0	1	0
Northern Weathear (*Oenanthe oenanthe*)	1	*Hyalomma* sp.	1/1	1	0	0
Pied Flycatcher (*Ficedula hypoleuca*)	21	*Hyalomma* sp.	1/5	1	0	0
Redstart (*Phoenicuros phoenicuros*)	14	*Hyalomma* sp.	7/9	7	0	0
Spotted Flycatcher (*Muscicapa striata*)	25	NA	NA			
Subalpine Warbler (*Sylvia cantillas*)	1	*Ixodes frontalis*	0/1			
Tree Pipit (*Anthus trivialis*)	1	*Hyalomma* sp.	1/2	1	0	0
Whinchat (*Saxicola rubetra*)	38	*Ixodes frontalis*	0/1			
		Hyalomma sp.	4/13	4	0	0
Whitethroat (*Sylvia communis*)	92	*Ixodes* sp.	0/1			
		Hyalomma sp.	10/24	9	0	1

Willow Warbler (*Phylloscopus trochilus*)	1	NA	NA			
Wood Warbler (*Phylloscopus sibilatrix*)	1	*Hyalomma* sp.	0/3			
2017						
Barn Swallow (*Hirundo rustica*)	20	*Hyalomma* sp.	0/1			
Black Redstart (*Phoenicuros ochruros*)	3	*Hyalomma rufipes*	1/1	0	1	0
Collared Flycatcher (*Ficedula albicollis*)	1	*Hyalomma rufipes*	1/1	1	0	0
Dunnock (*Prunella modularis*)	1	*Hyalomma* sp.	0/1			
Eurasian Blackcap (*Sylvia atricapilla*)	48	NA	NA			
European Robin (*Erithacus rubecula*)	39	*Hyalomma rufipes*	2/10	2	0	0
Garden Warbler (*Sylvia borin*)	30	NA	NA			
Icterine Warbler (*Hippolais icterina*)	41	NA	NA			
Northern Weathear (*Oenanthe oenanthe*)	1	*Hyalomma rufipes*	1/1	1		
Pied Flycatcher (*Ficedula hypoleuca*)	30	*Hyalomma* sp.	0/1			
Redstart (*Phoenicuros phoenicuros*)	25	*Hyalomma rufipes*	8/12	8	0	0
Song Thrush (*Turdus philomenos*)	4	*Hyalomma rufipes* *Hyalomma* sp.	1/1 0/3	1	0	0
Spotted Flycatcher (*Muscicapa striata*)	24	*Hyalomma rufipes*	1/2	1	0	0
Subalpine Warbler (*Sylvia cantillas*)	1	*Hyalomma* sp.	0/1			
Tree Pipit (*Anthus trivialis*)	2	*Amblyomma marmoreum* *Hyalomma* sp.	1/1 0/1	1	0	0
Whinchat (*Saxicola rubetra*)	43	*Hyalomma rufipes*	5/7	5	0	0
Whitethroat (*Sylvia communis*)	57	*Hyalomma rufipes*	2/5	2	0	0
Willow Warbler (*Phylloscopus trochilus*)	1	*Hyalomma* sp.	0/1			
Wood Warbler (*Phylloscopus sibilatrix*)	5	*Hyalomma rufipes* *Hyalomma* sp.	1/1 0/4	1	0	0

Tab 15 Summary of investigated bird species, collected ticks and *Rickettsia* PCR positivity divided by year of sampling.

6.5.3. Discussion

Ticks of the *Hyalomma marginatum* complex (i.e. *Hyalomma marginatum marginatum*, *H. rufipes*, *H. turanicum* and *H. isaaci*) are some of the most widespread ticks in Africa (Horak et al., 2007), and species such as *H. marginatum marginatum* belongs to the endemic ixodid fauna of the Mediterranean and Eastern European countries (Estrada-Peña et al., 2004a). However, several sporadic reports have been recorded from Northern European countries such as Germany (Kampen et al., 2007), Hungary (Hornok et al., 2013a), Russia (Movila et al., 2013), Finland (EFSA, 2010) and The Netherland (ICTTD, 2008).

Moreover, ticks of the *H. marginatum* complex are the most important vectors of Crimean Congo Haemorrhagic Fever virus (CCHFv) that causes a life-threatening disease in humans and occurs mainly in Africa, Asia and the Middle East (Ergonul and Whitehouse, 2007). *Hyalomma* ticks act both as vectors and reservoirs of this virus and birds, as main hosts for the immature stages of these ticks, can participate in the virus maintenance and its spread into new areas through migration (Palomar et al., 2016).

R.aeschlimannii is the microbial agent of a rickettsiosis that clinically resemble the Mediterranean spotted fever disease, which is caused by *R.conorii* and widespread in Mediterranean regions. It is transmitted by ticks of the *H. marginatum* complex, and recently recognized as human pathogen (Raoult et al., 2002). *R.africae* is the agent of the African tick-bite fever; it is transmitted by ticks of the genus *Amblyomma*, as *A.hebraeum* and *A.variegatum*, and is the most common cause of systemic febrile illness among travellers in this continent after Malaria (Freedman et al., 2006). *R. aeschlimannii* and *R. africae* are zoonotic bacterial species that occur mainly in African continent; however, their presence have been reported in ticks from other geographical areas, such as Oceania, Caribbean islands and Europe (Palomar et al., 2016; Kelly, 2006; Eldin et al., 2011). In Europe, autochthonous cases of human rickettsiosis caused by *R. aeschlimannii* have been recently described in Greece (Germanakis et al., 2013) and Italy (Tosoni et al., 2016). *R. raoultii* is mainly recognized as the agent of the Tick-borne lymphadenopathy (TIBOLA), also known as *Dermacentor*-borne necrosis and lymphadenopathy (DEBONEL). It is transmitted by *Dermacentor marginatum* and *D.reticulatus* in Europe and Asia.

Unexpectedly, both *R. africae* and *R. raoultii* were detected in tick species that are not the proved vectors for these pathogens (*H. rufipes* and *Hyalomma* spp., respectively). Given the fact that birds were not tested for *Rickettsia* and that ticks were engorged, we cannot exclude the possibility that ticks acquired these microorganisms by feeding on positive birds, and thus it is impossible to speculate any further about the vector role of these tick species. Nevertheless, our results are in line with those observed in Italy in a previous study (Toma et al., 2014) and confirm the circulation of these Rickettsia species in non-endemic areas and the important role of migratory birds in the passive transportation of infected ticks, thus emphasizing the risk for human health.

Although no ticks tested positive for CCHF virus in the present study, there are papers reporting the presence of this virus in *H. marginatum* complex ticks attached to birds migrating from Africa to Europe (Mancuso et al., 2019), and dispersion through migratory birds seems to be one of the possible explanation for the establishment of the enzootic focus of CCHF in Spain (Negredo et al., 2017). Moreover, the current climate changes could lead to the establishment of autochthonous population of *Hyalomma* ticks in areas previously free of these vectors. Finally, the RNA of another relevant human pathogen, the recently discovered Alkhurma haemorrhagic virus (Hoffman et al., 2018), has been detected in ticks of the *H. marginatum* complex, but further studies are needed in order to clarify the role of these ticks in the epidemiology of this flavivirus.

Battisti E., Urach K., Cardinale M., Felsberger G., Ferroglio E., Fusani L., Hodžić A., Hufnagl P., Duscher GG. Zoonotic pathogens in ticks from migratory birds, Italy. Submitted to *Emerging Infectious Diseases*

6.6. Ecological niche modelling of *Babesia* spp. infection in wildlife experimentally evaluated in questing *I. ricinus* – PAPER 9

6.6.1. Materials and methods

The habitat suitability model (HSM) for *Babesia* sp. was estimated by using Maxent 3.3.3 (Phillips and Dudik, 2008; Phillips et al., 2006). Like the other modelling methods, Maxent is able to predict environmental suitability for a target species as a function of the given environmental variables. It is based on the idea of the ecological niche, defined as a subdivision of the habitat containing the environmental conditions that enable individuals of a species to survive and reproduce (Grinnell, 1917). Maxent is a general-purpose method for making predictions or inferences from incomplete information. In particular, it is based on a presence-only modelling approach, and it estimates a target probability distribution (*Babesia* sp. in this study) by finding the probability distribution of maximum entropy (i.e. the most spread out, or closest to uniform) within a set of constraints that represent the available information about the environmental requirements of the target distribution (Phillips et al., 2006).

For this analysis, default settings were used for the regularization multiplier, maximum number of iterations, convergence threshold and maximum number of background points. Background is limited to the Piedmont regional territory with the exclusion of all urban areas as unsuitable for *Babesia* hosts and vectors (Hirzel et al., 2006; Elith et al., 2011). Models were generated randomly by assigning 75% of occurrences as training data and the remaining 25% as test data. For each model, five cross-validate replicates were run. The selection of predictors was carried out automatically following the default rules dependent on the number of presence records (Phillips et al., 2006; Elith et al., 2011). The output was given in a logistic format conditioned on the environmental variables in each grid cell with suitability values ranging from 0 (unsuitable environmental condition) to 1 (optimal environmental condition) (Hu and Jiang, 2011).

The threshold value above which there is a substantial probability of presence of *Babesia* sp. was set for each model to the value that maximizes training sensitivity and specificity. Values of habitat suitability above threshold were divided in four quartiles for graphical visualization and further on-field validation. Model evaluation and best-model selection was carried out using the area under receiver-operator curve (AUC) (Merow et al., 2013) and lower model complexity (Elith and Leathwick, 2009). The best performing model ($\Delta AUC \leq 2$) and lower number of covariates was chosen. Covariates to be retained were selected by backward step-wise model selection excluding at each step the feature with the lowest permutation importance (Merow et al., 2013).

An overall number of 25 presence points (PP) were used to train the Maxent model (Fig. 20). The occurrence of *Babesia* sp. in sylvatic animals from the Western Alps has been fully described by Zanet and colleagues (Zanet et al., 2014b). Only those PP for which exact coordinates were

available have been used to train/test the model. For these PP, GPS coordinates are known (UTM, datum ED50; Fig. 19) for the location where the infected animal was shot or found dead. Roe deer (n=10), Red deer (n=8) and Alpine chamois (n=7) are the three species of ungulates included in the model, that were found infected with *Babesia* sp.

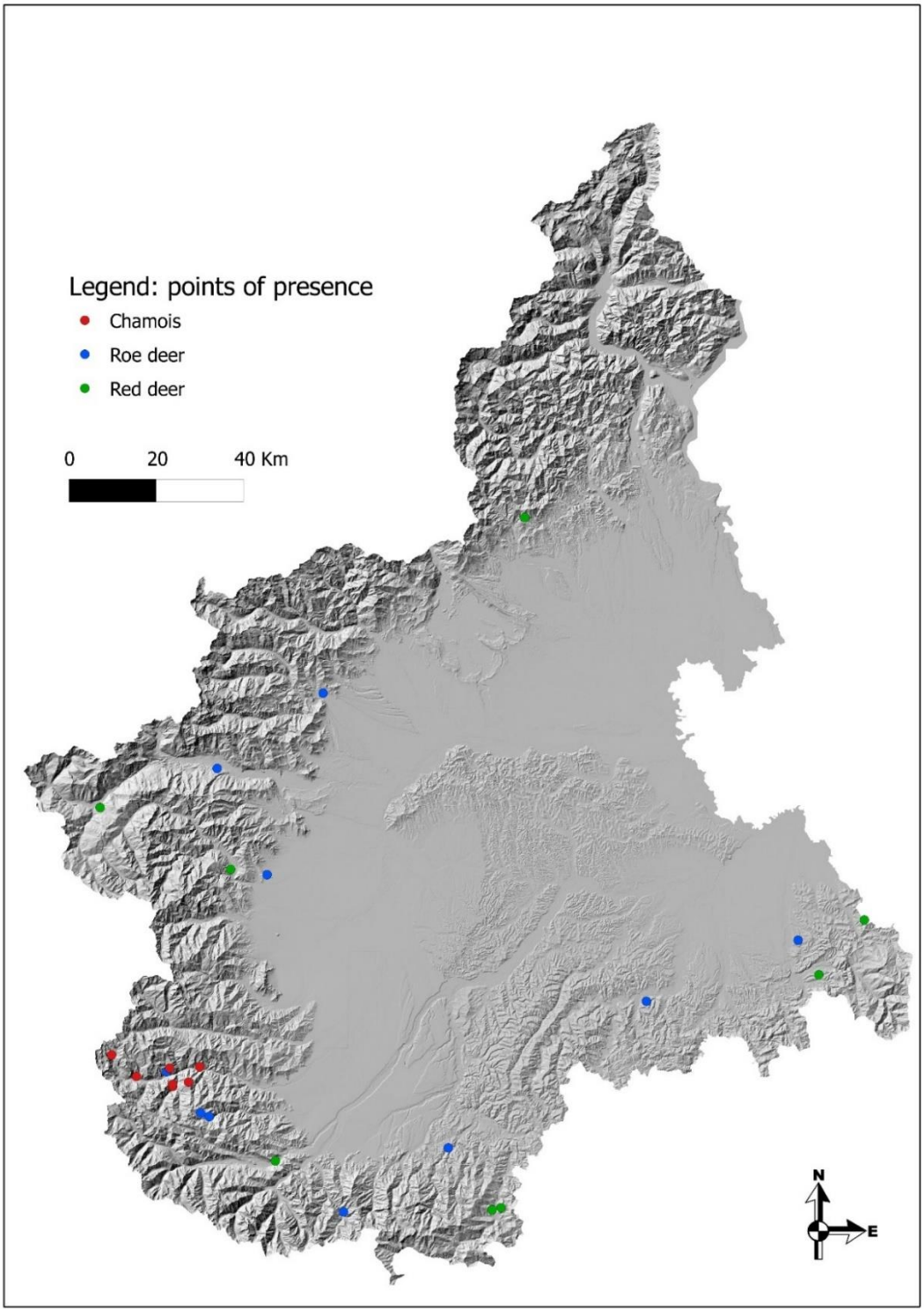

Fig. 20 Presence points of the *Babesia*-infected ungulates included in the model, divided by species.

In the present study, three models have been developed to estimate the probability of presence of *Babesia* sp. in Piedmont. Model 1 considered cervids as target hosts, and thus used only PP data of *Babesia* sp. isolated from either red deer or roe deer. Where present, the habitat of red deer overlaps over roe deer's presence area, and tick-borne diseases are probably shared among both species (Tampieri et al., 2008; Duh et al., 2005; Bastian et al., 2012). Model 2 was trained with alpine chamois PP data only, due to the fact that *Babesia* sp. infection cases in this species has been reported in a restricted area of the Maritime Alps (Val Maira - Southwestern Piedmont), at higher altitude and in a different environment than that of cervids. Last, Model 3 used all the PP data from both cervids and alpine chamois in order to summarize the piroplasm circulation in the three most frequently infected wild ungulates species in Western Alps.

An overall number of 21 potential predictors were selected based on the ecological requirements of *Babesia* definitive hosts and vectors. Appropriate biotic and abiotic priors were chosen considering specific literature on the species of ungulates mostly involved in piroplasmids epidemiology in Europe and Italy (Zintl et al., 2011; Tampieri et al., 2008; Malandrin et al., 2010; Kauffmann et al., 2017; Hoby et al., 2009, 2007; Ebani et al., 2016; Duh et al., 2005; Cézanne et al., 2017; Bastian et al., 2012) as well as the environmental factors that influenced the potential presence of *I. ricinus* (Rizzoli et al., 2004; Remesar et al., 2019; Overzier et al., 2013). The spatial resolution of all priors as well as of the final model was 1000 m.

Regarding host species priors, presence and mean density of hunted ungulates/km^2 (Roe deer, Red deer and Chamois) were inferred from the Official Hunting dataset (Osservatorio Faunistico Regionale) and normalized for hunting effort.

For vector priors, four abiotic candidate priors were included in the model: i) Altitude (ALT), ii) annual solar exposure (total KWatthour/m^2 received during a year period; SOLAR), iii) surface aspect (downslope direction of the maximum rate of change in value from each cell to its neighbors; ASPECT), iv) slope (steepness of land surface measured in degrees from horizontal; SLOPE) and Brightness Temperature (a measurement of the radiance of the microwave radiation traveling upward from the top of the atmosphere to the satellite, expressed in units of the temperature of an equivalent black body; BT). Among biotic priors, the performance of Normalized Difference Vegetation Index (NDVI) and Land cover was tested. NDVI was considered separately for summer months (vegetational peak season from May to September, n= 34 Landsat images) and winter (from November to March, n= 29 Landsat images) (http://glovis.usgs.gov). For each season we evaluated the fit of PP data on the minimum, maximum and mean NDVI. NDVI was assumed as indicator of tick-adequate vegetation coverage and soil relative humidity (Estrada-Pena, 2001). The same Landsat image collection was used for calculating summer and winter BT (minimum, maximum and mean BT) as indicator of relative ground temperature (Hönig et al., 2011; Eisen et al., 2010). Landcover raster map (http://www.ruparpiemonte.it/geocatalogorp/main/?sezione=catalogo)

updated to the year 2010, was also evaluated as model covariate. To exclude autocorrelation among covariates, independence was assessed using a correlation matrix.

Maxent graphical outputs were analyzed and visualized using QuantumGIS 3.4 (QGIS Developmental Team, 2018). Statistical analysis was performed using R 3.4.4 (R Development Core Team, 2018).

To further evaluate the validity of the model, ticks were collected by using the dragging method from 8 randomly selected locations uniformly distributed across the study area and processed for *Babesia* sp. detection. In particular, locations were classified by Model 1 as moderately suitable (Q2 - 4 locations) and highly suitable (Q4 - 4 locations) for *Babesia* sp. In each one of these areas, a monthly sampling by dragging was performed from May to October, in order to evaluate the presence of questing *I. ricinus*, for an overall number of forty transects (5 transects for each location, each transect covering an area of 100 m^2). Before each sampling session the exact coordinates of the sampled transect, temperature and humidity were recorded. Collected ticks were preserved in 70% ethanol, and identified to species level, life-stage and gender under a stereomicroscope using appropriate morphological keys (Walker, 2003; Estrada-Peña et al., 2004b, 2004a). Identified ticks were divided into pools comprised of specimens collected from the same transect and homogeneous for species, developmental stage, and sex. Ticks pools were mechanically homogenized using Qiagen TissueLyser LT (Qiagen, Milan Italy). Total genomic DNA was extracted by using TRI-Reagent® (Sigma-Aldrich, Italy), according to the manufacturer's instructions with additional overnight incubation in Proteinase K (0.8 mg) and 500 µl of TRI-Reagent. To detect piroplasms, a specific fragment of *Babesia* sp. DNA was amplified by PCR targeting the V4 hypervariable region of the 18S rDNA (Zanet et al., 2017). Briefly, the reaction mixture for the first step contained 1X of PCR Master Mix (Promega Corporation, WI, USA), 20 pmol of each primer and 5µl of DNA, in an overall volume of 25 µl. The thermal cycler conditions used were an initial denaturation step at 95°C for 5 min, followed by 25 cycles at 95°C for 30 s, 50°C for 45 s and 72°C for 90 s, and a final elongation step at 72°C for 10 min. Amplicons from the first step (1 µl) were used as templates for the second step with internal primer RLB-FINT instead of RLB-F2. Protocol and thermal cycler conditions were identical to the first step except for the annealing temperature at 55°C and for the cycling number of 40. All PCR-positive amplicons were purified using a commercial kit (Nucleospin Extract II Kit, Macherey-Nagel, Düren, Germany) and sequenced on both strands (Macrogen Europe, Spain) for species identification. The resulting nucleotide sequences were analyzed using MEGA X software (Kumar et al., 2018) and compared to those available in GenBank (www.ncbi.nlm.nih.gov/genbank). The PCR results were expressed as a minimum infection rate (MIR) or the minimum percentage of ticks in a pool with detectable DNA of *Babesia* sp. (Kramer et al., 1999).

Tick abundance in Q2 and Q4 locations was statistically compared using Student's T test for paired data. Statistical analysis was performed using R 3.4.4 (R Development Core Team, 2018).

6.6.2. Results

Correlation matrix of covariates dependence allowed to exclude from contemporary use in Maxent analysis SLOPE (cor = 0.71 with ALT) and maximum summer TB (cor = 0.70 with ALT).
Regarding *Babesia* sp. distribution in cervids (Model 1), the best performing model (AUC = 0.937) was generated using as covariates the following parameters: roe deer and red deer abundance, mean summer NDVI, SLOPE, SOLAR, and LAND USE. Red deer abundance, together with Mean Summer NDVI, SLOPE and SOLAR have a direct correlation with *Babesia* sp. occurrence probability, while *Babesia* sp. Suitability reached a peak in correspondence to roe deer abundance of 4 individuals/km^2. The most suitable land use categories for *Babesia* sp. occurrence were broad-leaved forests (category 9), ecotonal shrubs areas (category 10), high altitude pastures and meadows (category 14) and grassland (category 7). The suitable area for *Babesia* sp. infection in wild Cervids was estimated of 956 km2 (3.98% of the regional territory; threshold value for Maximum Training Sensitivity plus Specificity 0.443). Logistic probability of *Babesia* sp. infection in cervids is spatially represented in Fig. 21.

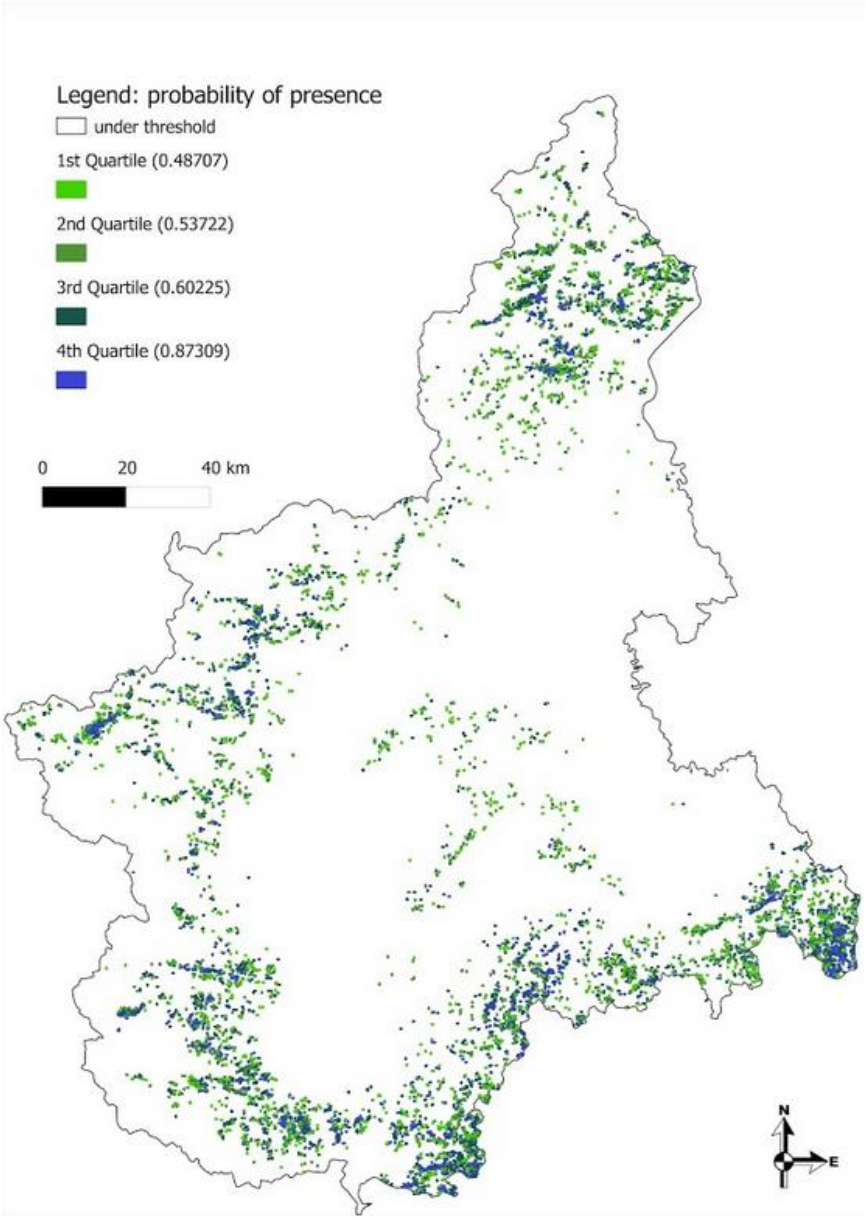

Fig. 21 Spatial representation of logistic probability of *Babesia* sp. infection in cervids.

Concerning Model 2 (*Babesia* sp. occurrence in alpine chamois), the best performing model (with AUC = 0.993) was generated using as covariates the following parameters: chamois density, ALT, SOLAR LAND COVER, and mean summer NDVI. All continuous variables showed a direct correlation with *Babesia* sp. probability of occurrence, while the land use categories most suitable to infection are high altitude pastures and meadows (category 14), grassland (category 7), and ecotonal shrubs areas (category 10). The estimated suitable area for *Babesia* sp. infection in Chamois is of 81 km^2 (0.3% of the regional territory; threshold value for Maximum Training Sensitivity plus Specificity 0.465).

Model 3 estimated the occurrence of *Babesia* sp. in cervids and alpine chamois together, because several studies have reported that natural infection in the latter is frequently acquired from sympatric roe deer (Zanet et al., 2014b; Tampieri et al., 2008; Hoby et al., 2009, 2007) (Fig. 22).

The overall suitable area for *Babesia* sp. in this simulation is of 3723.3 $km^{2,}$ which correspond approximately to 15.5% of the background regional territory (Fig. 22). The AUC of the model is 0.91 and was generated using the following covariates: mean summer NDVI, SOLAR, and the reclassified version of LandCover. The probability of presence for *Babesia* sp., peaked when NDVI reaches 0.65, value that corresponds to broad-leaved forests areas, that are notoriously the most suitable environments for *Ixodidae* ticks (Estrada-Peña and Venzal, 2006; Estrada-Peña, 2001; Daniel et al., 1998). LandCover confirmed the high suitability of broad-leaved forests (class 9, in the LandCover predictor), and solar exposure showed a direct correlation with increasing *Babesia* habitat suitability.

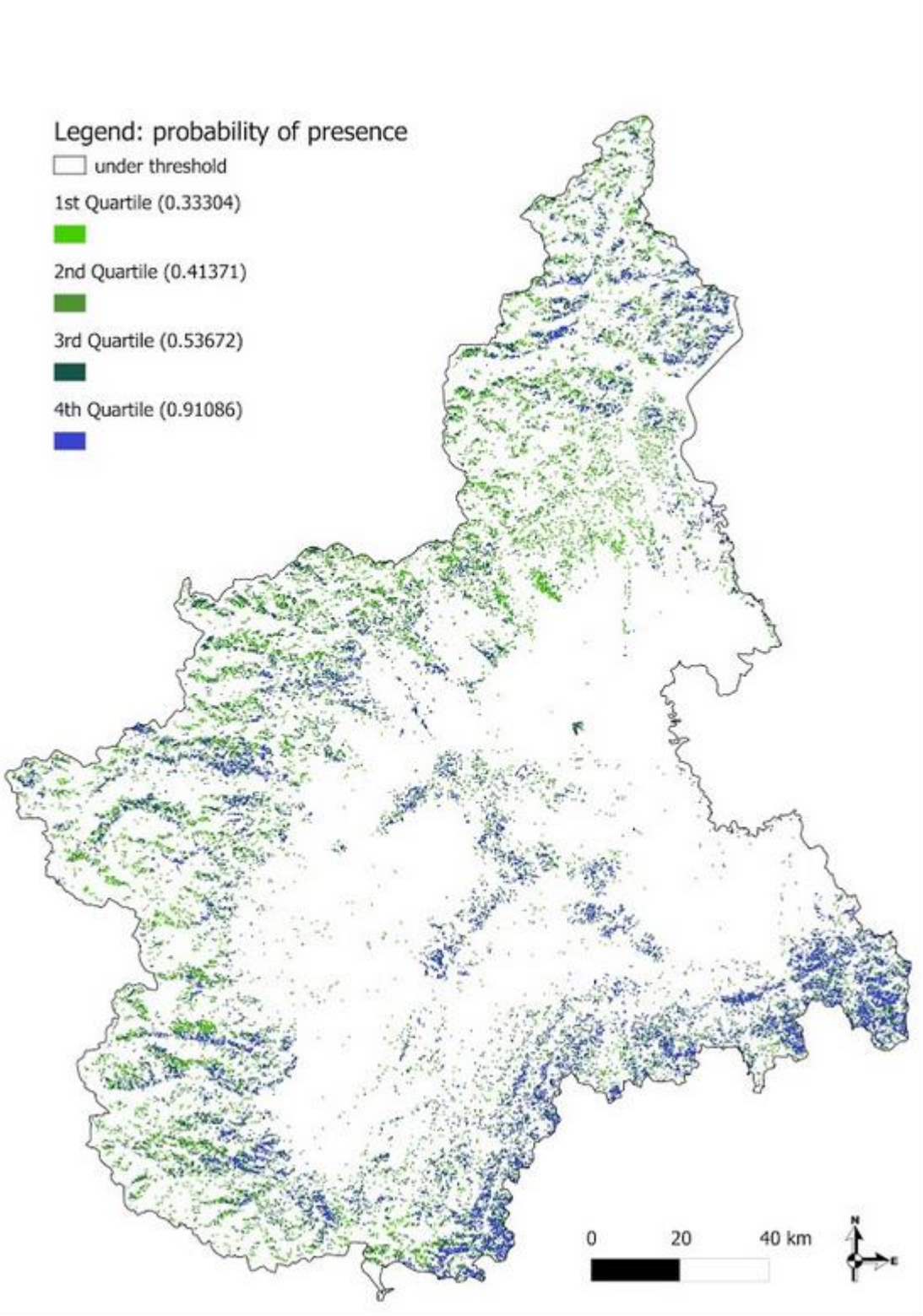

Fig. 22 Spatial representation of logistic probability of *Babesia* sp. infection in wild ungulates (cervids and alpine chamois).

Over the 6 months of sampling an overall number of 1555 ticks were collected and morphologically identified as *I. ricinus* (n=1553) and *I. hexagonus* (n=2). The number of ticks collected from each location is reported in Tab. 16.

ID	Coordinates	Valley	Quartile	Larvae [pools]	MIR *Babesia* (CI95%) [positive pools]	Nymphs [pools]	MIR *Babesia* (CI95%) [positive pools]	Adults [pools]	MIR *Babesia* (CI95%) [positive pools]
Q2				224 [38]	26.32% (14.97-42.01) [10]	87 [37]	27.03% (15.4-42.98) [10]	1 [1]	100% (20.65-100) [1]
a	45°01’03.9”N 6°48’44.8”E	Susa	Q2	0 [0]	-	2 [2]	0.00% (0.00-65.76) [0]	0 [0]	-
c	44°28’50.0”N 7°20’47.5”E	Maira	Q2	22 [8]	0.00% (0.00-32.44) [0]	27 [11]	18.18% (0.51-47.7) [2]	0 [0]	-
e	45°04’56.2”N 7°20’35.0”E	Sangone	Q2	34 [9]	44.44% (18.87-73.33) [4]	43 [13]	46.15% (23.21-70.86) [6]	1 [1]	100% (20.65-100) [1]
g	44°31’45.0”N 8°25’26.7”E	Bormida	Q2	168 [21]	28.57% (13.81-49.96) [6]	15 [11]	18.18% (5.14-47.7) [2]	0 [0]	-
Q4				1078 [46]	45.65% (32.15-59.82) [21]	163 [37]	40.54% (26.35-56.51) [15]	2 [2]	100% (34.24-100) [2]
b	45°06’13.3”N 6°55’04.3”E	Susa	Q4	284 [19]	31.58% (15.36-53.99) [6]	54 [13]	15.38% (4.33-42.23) [2]	1 [1]	100% (20.65-100) [1]
d	44°29’17.8”N 7°02’22.4”E	Maira	Q4	0 [0]	-	0 [0]	-	0 [0]	-
f	45°01’46.7”N 7°17’12.7”E	Sangone	Q4	238 [15]	73.33% (40.05-89.10) [11]	70 [16]	68.75% (44.4-85.84) [11]	1 [1]	100% (20.65-100) [1]

h	44°31’02.4”N 8°26’53.3”E	Bormida	Q4	556 [12]	33.33% (13.81-60.94) [4]	39 [8]	25% (7.15-59.07) [2]	0 [0]	-
total				**1302 [84]**	**36.90% (27.37-47.58) [31]**	**250 [74]**	**33.78% (24.05-45.12) [25]**	**3[3]**	**100% (43.85-100) [3]**

Tab. 16 Ticks collected by dragging in the present study, divided by locations and developmental stages. The MIR of *Babesia* sp. for each stage is indicated.

The most abundant life stage of Ixodid ticks collected in all sampling locations was larvae (n = 1302), followed by nymphs (n=250) and adults (n=3). For PCR analysis, ticks were grouped in 161 homogeneous pools depending on the site of collection, species and developmental stage (3 pools consisting of adult ticks, 74 of nymphs and 84 of larvae). The DNA of *Babesia* sp. was detected in 59 pools, with an overall MIR of 36.65% (CI95% 29.60% - 44.32%). Adults showed considerably higher infection ($p < 0.05$) than the other stages, with a MIR of 100% (CI95% 43.85% - 100%), although only 3 specimens were recorded. The MIR of *Babesia* sp. in larvae was 36.90% (CI95% 27.37% - 47.58%) [31/84], while 25 out of 74 nymph pools tested positive (MIR= 33.78%; CI95% 24.05% - 45.12%). From locations moderately suitable for *Babesia* sp. (Q2) we consistently recovered a lower number of ticks ($p < 0.05$) compared to highly suitable areas (Q4) with the exception of one Q4 location (location d, Table 16) where no ticks were recovered. Results from location d were possibly impaired by the use of part of the sampling area as pasture for cattle. MIR was significantly higher in Q4 areas than in Q2 ($\chi^2 = 5.05$; $p < 0.05$) with Odds Ratio of 2.12 (CI95% 1.1% - 4.1%). The zoonotic B. venatorum was the most prevalent species with a MIR of 23.60% (CI95% 17.71 – 30.73). B. capreoli was reported with an overall MIR of 3.11% (CI95% 1.33 – 7.06), followed by B. microti and B. vulpes which were both detected with a MIR of 2.48% (CI95% 0.97 – 6.21%). Protozoa of the genus Theileria were detected in 8 pooled samples with a MIR of 4.97% (CI95% 2.54 – 9.50%). All species were detected with higher prevalence in Q4 areas compared to Q2 (Table 17).

Species	Total Positive Pools (MIR, CI95%)	Q2 Positive Pools (MIR, CI95%)	Q4 Positive Pools (MIR, CI95%)	Query Coverage	Percent Identity	GenBank Accession Number
B. venatorum	38 (23.60%; 17.71 – 30.73)	14 (18.42%; 11.30 – 28.58)	24 (28.24%; 19.77 – 38.58)	100%	99.5 %	KX857480
B. microti	4 (2.48%; 0.97 – 6.21)	1 (1.32%; 0.23 – 7.08)	3 (3.53%; 1.21 – 9.87)	100%	100%	FJ608739
B. vulpes	4 (2.48%; 0.97 – 6.21)	1 (1.32%; 0.23 – 7.08)	3 (3.53%; 1.21 – 9.87)	100%	100%	KI175166
B. capreoli	5 (3.11%; 1.33 – 7.06)	2 (2.63%; 0.72 – 9.10)	3 (3.53%; 1.21 – 9.87)	100%	100%	KU145465
Theileria spp.	8 (4.97%; 2.54 – 9.50)	3 (3.95%; 1.35 – 10.97)	5 (5.88%; 2.54 – 13.04)	100%	100%	MH327771, AJ616717, KX965721

Tab. 17 Pathogen species and number of homogeneous tick pools positive for each species, Minimum Infection Rate (MIR), and MIR confidence intervals (CI) at 95% are reporte

6.3.3. Discussion

Predictive modelling of species geographical distribution based on environmental conditions of the locations in which the species is known to occur constitutes an important technique in analytical biology, with applications in conservation and reserve planning, ecology, evolution, epidemiology, invasive-species management and other fields (Phillips et al., 2006). Niche-based models obtained from presence-only data, such as those estimated with Maxent, are particularly helpful when absence data are not available or arguable.

The models presented in this study are intended to be a tool to better understand the geographical occurrence and the epidemiology of *Babesia* sp. infecting wild ungulates in Northern Italy. Babesiosis plays a key role as pathogen in livestock (Schnittger et al., 2003; Criado-Fornelio et al., 2003b; Bock et al., 2008) and is currently emerging as a human disease (Vannier and Krause, 2015, 2012; Kjemtrup and Conrad, 2000; Hildebrandt et al., 2013). However, several aspects of *Babesia* sp. infections are still unknown, especially for what concerns the epidemiology of these parasites in wildlife hosts and wildlife-related ticks and environments (Yabsley and Shock, 2013).

The models described in this study covered a variety of different environments: from low-altitude broadleaved forests and agricultural areas to high altitude Alpine areas. Thanks to the precise resolution (1000 m), models described and predicted suitable areas for highly selective tick vectors which can persist only in particular areas where a set of environmental characteristics have to occur together with the presence of suitable animal hosts. Biotic and abiotic priors were selected and modeled on the requirements of adult *I. ricinus*, which is most strictly associated to rural environments and wild ungulates (Rizzoli et al., 2004). All three models fit very well (high values of AUC) to the variance of our data, and the priors included in the models are highly responsive in depicting suitable presence areas. Among environmental features, the most informative were NDVI (the mean value computed during summer months), slope steepness, solar exposure, altitude and land cover. In particular, even though each model was best described by different priors, all agreed that areas well exposed to sun light and covered with a rich vegetation (shrubs areas, and broad-leaved forests) are the most suitable for *Babesia* sp. occurrence, corresponding to the most suitable areas for the tick vector *I. ricinus* (Rizzoli et al., 2004; Remesar et al., 2019; Overzier et al., 2013). Instead, the suitability for *Babesia* sp. occurrence of the pasture and alpine meadows resulting from this study was probably due to the presence of the alpine chamois in the model, which typically inhabits high altitude open areas. Taking into account Model 3, that estimates the occurrence of *Babesia* sp. infection by using presence data of both cervids and alpine chamois, the overall extension of *Babesia* sp. suitable area corresponded to 15% of the territory considered in the study (i.e. the whole Piedmont region). Human infection occurs mostly in rural or natural environments due to the changes in social and economic behaviours that are currently arising (increase of outdoor activities and re-naturalization of rural areas and territorial expansion of wildlife) (Kjemtrup and

Conrad, 2000). Moreover, the population of wild ungulates and especially of roe deer in Italy have been increasing at growing rates since the last decades (Carnevali et al., 2009). Together with ungulates, ticks have also increased (De Meneghi, 2006) and their geographical range has expanded to include areas at higher latitude and/or altitudes, possibly due to the influence of climate changes on tick habitats (Tälleklint and Jaenson, 1998; Materna et al., 2008; Lindgren et al., 2000; Jore et al., 2014; Estrada-Peña and Venzal, 2006; Daniel et al., 2003). A study by Stainforth and colleagues (Stainforth et al., 2013), documented for the study area a decrease between 5% and 10% of number of winter nights that fall below zero. Higher winter temperatures favor overwintering and extend the activity season of the vector ticks (Materna et al., 2008; De Meneghi, 2006), thus increasing the risk of tick-borne diseases.

Field sampling of Ixodid ticks in the study area was used to validate the *Babesia* sp. occurrence model with empirical data. Results highlighted a significant positive association ($p < 0.05$) between *Babesia* sp. MIR in collected ticks and highly suitable areas for parasite occurrence (Q4 areas), thus underlying the ability of the model to predict habitat suitability for *Babesia* sp. The higher prevalence of infection in Q4 than in Q2 areas was confirmed for each of the Piroplasmid species identified by sequencing. Most of the species of *Babesia* identified in sampled ticks had been previously reported in the study area. The Roe deer used as PP, were infected with *B. venatorum*, *B. capreoli* and *B. bigemina* (Zanet et al., 2014b). Both *B. venatorum* and *B. capreoli* have Roe deer as main reservoir hosts (Michel et al., 2014; Malandrin et al., 2010) while *B. bigemina* is together with *B. divergens* mainly associated to bovine babesiosis (Zintl et al., 2003; Hilpertshauser et al., 2007). *B. capreoli* was the most prevalent species found in Red deer used as PP, followed by *Theileria* spp. (Zanet et al., 2014b) which was also detected in the ticks used for empirical validation. *B. vulpes* had been previously reported from the same study area to infect Red foxes and horses (Zanet et al., 2017, 2014b). *B. vulpes* is also recognized as a pathogenic species in dogs with symptoms of clinical infection ranging from pale mucous membranes, anorexia, apathy and fever with severe macrocytic/hypochromic regenerative anaemia and thrombocytopenia (Guitián et al., 2003; Baneth et al., 2019). Two zoonotic species of Piroplasms were detected in ticks, namely *B. venatorum* and *B. microti*. Both species have *I. ricinus* as main vector (Gray et al., 2002; Bonnet et al., 2007). *B. venatorum* was detected in 6 of the 8 sampling locations, both in Q2 and Q4 areas. It was not detected only from sites B (Q2) and F (Q4) where *Babesia* spp. was generally detected with lower MIR values or no ticks where collected. *B. microti* was instead reported only from locations C (Q2) and G (Q4) where *Babesia* spp. was also reported with the highest MIR in larva and nymph pools.

6.3.4. Conclusions

Habitat suitability models that describe the spatial distribution of pathogens and their vectors are pivotal for an accurate understanding of the epidemiology of TBDs, and represent a helpful tool in public health to bolster the efforts on disease prevention and management (Brownstein et al., 2003). Future applications for the model include the possibility of making predictions on how climate changes will affect *Babesia* sp. occurrence in temperate regions such as Northern Italy. Also, the relationships occurring between free-ranging animals and domestic livestock is an issue that needs to be examined in depth to better understand the role of wildlife in the epidemiology of tick-borne diseases and zoonoses.

Zanet S., **Battisti E**., Ferroglio E., Tizzani P. Ecological niche modeling of *Babesia* sp. infection in wildlife experimentally evaluated in questing *I. ricinus.* Accepted for publication with minor revisions in *Geospatial Health.*

7. *L. INFANTUM*

7.3. Epidemiological evaluation of *L. infantum* zoonotic transmission risk in the recently established endemic area of Northwestern Italy – PAPER 10

7.3.3. Materials and methods

For the evaluation of *L. infantum* circulation in humans and domestic animals, five different study areas were identified within the Piedmont region (namely area A, B, C, D and E) (Tab. 18), distinct for topographical, altitudinal, land cover and demographical characteristics. In these areas, an overall number of 815 human serum samples and 803 blood samples from dogs were collected in the period 2007-2009, from December to March of each year to maintain a suitable time distance from sand fly active season.

Name	Topography	Mean elevation (m asl)	Land cover	No. dogs	No. humans
Susa Valley (A)	Mountain	922	Low urbanized natural areas	418	130
Asti (B)	Hill	200	Agricultural	208	243
Alessandria (C)	Plain	140	Agricultural	44	193
Chivasso and Ivrea (D)	Hill	252	Highly urbanized	133	83
Pinerolo (E)	Hill	400	Low urbanized	0	166

Tab. 18 Study sites: details of topography, elevation and land cover in the five study sites are reported together with the number (No.) of dog and human samples from each area. The mean elevation of each study area is reported as metres above sea level (m asl) (adapted from Ferroglio et al., 2017; paper n°10).

Human samples were collected from anonymous blood donors, together with some information such as sex, age, living place, hobby, job and contact with dogs, by using a questionnaire. Dogs were randomly sampled by local veterinarians in all the study areas except one (area E), and for each dog, information on individual and environmental factors that might influence their exposure to *L. infantum* (breed, age, sex, municipality of origin, coat type, housing, sleeping sight, frequentation of endemic sites) were collected. In the area B, a subset of 112 human subjects were sampled together

with their dogs (n=121), in order to compare the infectious status. All the samples were stored at -20 °C until further use. In the same areas, an entomological survey on phlebotomine sand flies has been performed in July 2007, to confirm the local presence of *L. infantum* vectors. Sand flies were collected by using the sticky traps, consisting in 20 x 20 cm paper sheets embedded with castor oil and placed in suitable sites for phlebotomine sampling (Killick-Kendrick, 1999). Briefly, from 10 to 20 sticky traps were placed for one night in 105 sampling points distributed among the areas (A-E), for an overall sticky trap area of 0.4 m^2 to 0.8 m^2. Insect were then stored in 70% alcohol until further use. *L. infantum* diagnosis was made by using both a serological technique – Western blotting (WB) – and a molecular technique – PCR targeting a specific fragment of parasite kDNA. WB was performed according to the protocol described by Ferroglio and colleagues (Ferroglio et al., 2007). For PCR analysis, genomic DNA was extracted from 200 µl of dog's blood by using the commercial kit GenElute Mammalian Genomic MiniKit (Sigma-Aldrich) following manufacturer's instructions; for human samples, DNA was extracted from white blood cells pelleted from 2 ml of serum by using the same kit. Amplification of the specific 145 bp fragment of kDNA was performed according to Zanet and colleagues (Zanet et al., 2014a), by using the endpoint PCR. Briefly, the reaction mixture contained 2.5 µl of 10X PCR buffer, 5 UI of HotStarTaq Polymerase (Qiagen, Milan, Italy), 0.5 µl of dNTPs mix (10 mM of each dNTP, Sigma-Aldrich, St. Louis, MO, USA), 22.5 pmol of each primer and 2.5 µl of DNA, for an overall volume of 25 µl. Thermal cycler conditions were 95 °C for 15 min, followed by 45 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min, and a final step of 72 °C for 10 min. PCR products were visualized on a 2% agarose gel with UV transilluminator (GelDoc 1000, Bio-Rad, Hercules, CA). All positive amplicons were purified by using the QIAQuick PCR purification kit (Qiagen, Milan, Italy) and sent for sequencing (Macrogen, the Netherlands) to confirm *L. infantum* identification. Given the fact that in an endemic focus of leishmaniasis the number of PCR positive individuals exceed the number of seropositive individuals (Baneth et al., 2008), blood samples from dogs were first tested all with PCR, and only PCR-positive samples were screened with WB, in order to obtain information on the prevalence of *L. infantum* in the study area. On the contrary humans, for which only the serum was available instead of whole blood, were first all screened with WB, and only WB-positive individuals were tested with PCR for confirmation.

PCR positive samples of both dogs and humans were digested with restriction enzymes in order to evaluate polymorphisms in the RFLP patterns of *L. infantum*. The enzymes M1sI (MscI) and BseLI (Bs1I) (Fermentas, Milan, Italy) were used, as previously described by Ferroglio and colleagues (Ferroglio et al., 2006b). Briefly, 0.3 µl of each enzyme was incubated overnight in 25 µl of PCR products, and results were visualized on 2.5% agarose gel. At the same time, PCR positive samples were sequenced (Macrogen, the Netherlands), and sequences used for in silico RFLP (Vincze et al., 2003), as reported also for *T. gondii* (PAPER 1).

To identify possible associations between *L. infantum* infection status and anamnestic variables, we used Pearson's Chi square implemented in the statistical environment R version 3.0.2 (R

Development Core Team, 2018). Cohen's kappa coefficient (k) was used to assess test agreement between PCR and WB on autochthonous dogs, and between RFLP and in silico RFLP (Landis and Koch, 1977). A Geographic Information System (GIS) environment (QGIS 2.8.0; QGIS Development Team, 2015) was used to calculate the Minimum Convex Polygon (MCP) area (for patterns shared between
n ≥ 3 individuals) or the linear distance (for patterns shared between *n* = 2 individuals) of each RFLP pattern.

7.3.4. Results

Concerning sand fly distribution, *P. perniciosus* was identified in all the five study areas (A-E) with an overall frequency of positive trapping sites of 42.86% (CI95% 15.82% - 74.95%; 45/105 positive trapping stations), and an average density of 15.2 individuals/m^2. The mean density, absolute number of specimens and capture frequency of *P. perniciosus* are listed in Tab. 19, together with those of Sergentomya minuta. No significative differences were recorded among the capture frequency or the density values of *P. perniciosus* in the different areas.

Study area	*P. perniciosus* M	*P. perniciosus* F	Capture frequency *P. perniciosus*	*S. minuta* M	*S. minuta* F	Capture frequency *S. minuta*
A	8 (88)	9 (99)	28.57% (6/21)	14 (154)	3 (33)	100% (21/21)
B	10 (90)	8 (72)	57.14% (12/21)	6 (55)	8 (72)	100% (21/21)
C	5 (55)	9 (90)	47.62% (10/21)	4 (43)	11 (121)	100% (21/21)
D	7 (77)	8 (88)	52.38% (11/21)	10 (110)	9 (99)	100% (21/21)
E	9 (81)	3 (27)	28.57% (6/21)	12 (108)	13 (117)	100% (21/21)

Tab. 19 The mean density (number of specimens/m^2), absolute number of specimens (in parenthesis) of *P. perniciosus* (males M; females F) and *Sergentomya minuta* (males M; females F) captured in each study area. The capture frequency (percentage of positive trapping stations) is also reported for each area for both *P. perniciosus* and *S. minuta* (adapted from Ferroglio et al., 2017; paper n°10).

PCR results on dog samples showed an overall prevalence of *L. infantum* of 40.35% (CI95% 37.01% - 43.78%), with 324 positive dogs out of 803 tested. The prevalence of *L. infantum* recorded on autochthonous dogs was 48.52% (CI95% 42.62% - 54.46%), with 131 positive dogs out of 270 tested. The seroprevalence on autochthonous dogs was 42.22% (CI95% 36.48% - 48.18%) with 114/270 subjects testing positive. The *k* coefficient of agreement between PCR and WB on autochthonous dogs showed substantial to almost perfect agreement (depending on the study area, see Tab. 20). From area C, no sera from autochthonous dogs were available. Statistically significative differences were recorded among the prevalence of *L. infantum* on autochthonous dogs

in the different study areas. In particular, sites B and D, representing agricultural and urbanized hilly areas, showed higher prevalence and seroprevalence of the parasite compared to site A, a low urbanized and natural mountain area (Tab. 20). No other significative differences among the study areas were recorded.

Area	PCR prevalence all (95% CI)	No. PCR positive/ tested	PCR prevalence autochthonous (95% CI)	No. PCR positive autochthonous/tested	WB seroprevalence autochthonous (95% CI)	No. WB positive/ autochthonous tested	k (p)
A	40.67% (36.07–45.44)	170/418	27.27% (16.35–41.85)	12/44	20.45% (11.15–34.50)	9/44	.81 (<.001)
B	38.94% (32.57–45.71)	81/208	47.30% (39.42–55.31)	70/148	42.57% (34.89–50.62)	63/148	.84 (<.001)
C	9.09% (3.59–21.16)	4/44	nd	nd	nd	nd	Nd
D	51.88 (43.46–60.20)	69/133	60.26% (49.16–70.39)	47/78	53.85% (42.86–64.47)	42/78	.76 (<.001)

Tab. 20 Summary of the PCR prevalence and 95% confidence intervals for dogs from study areas A, B, C and D, together with PCR and WB seroprevalence results and relative 95% confidence intervals for autochthonous dogs only. PCR and WB results on autochthonous dogs from study area C are not reported (not determined, nd) as no dogs were exclusively autochthonous. For each study area, the number (No.) of dogs tested by PCR and WB is reported together with the number of subjects positive to each diagnostic assay. Cohen's kappa coefficient of agreement (*k*) between PCR and WB on autochthonous dogs (and the relative *p*-value) is reported for each area (adapted from Ferroglio et al., 2017; paper n°10).

WB results on human samples showed an overall seroprevalence of 16.81% (CI95% 14.40% - 19.53%), with 137 positive individuals out of 815 tested. Of these, 100 individuals were also positive by PCR (P = 72.99%; CI95% 65.01% - 79.73%). Individuals living in sites B and C, mainly agricultural areas, showed significative lower seroprevalence compared to the other sites, i.e. $\chi^2 = -0.59163$ ($p = 0.0124$) and $\chi^2 = -0.5043$ ($p = 0.0396$) respectively. No significative association was found between *L. infantum* in humans and period stay in traditionally endemic areas nor with owning a dog, not even if the dog is infected.

The RFLP analysis showed an overall number of 116 patterns of *L. infantum* in 424 dog and human PCR-positive samples. Among all, 17 patterns were detected in two or more individuals, for an overall number of 66 dogs and/or humans infected with these patterns. Figure 23 illustrate the geographical distribution of the most frequently occurring patterns. In particular, pattern 7, 11 and 14 were the three most common.

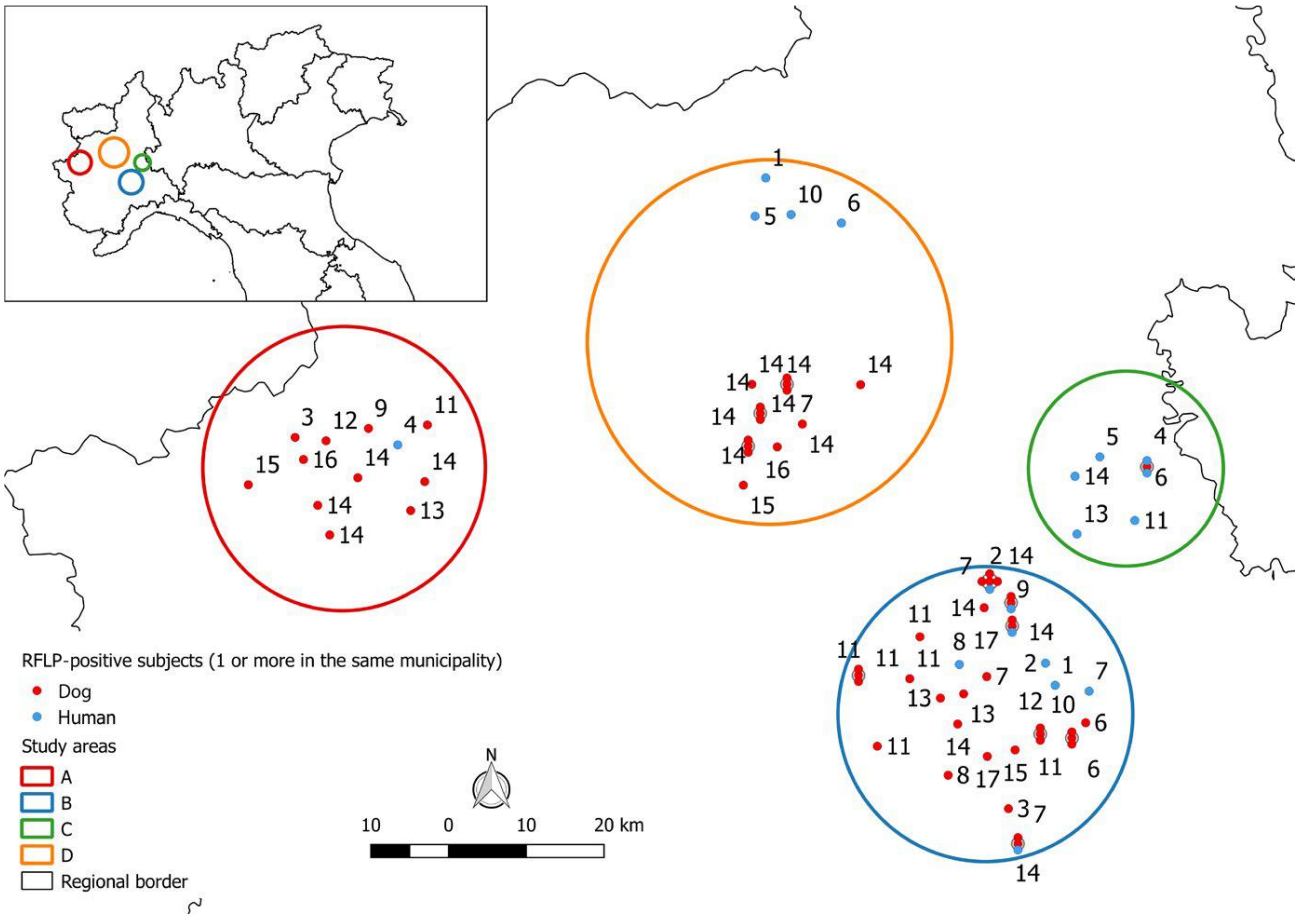

Fig. 23 Geographical distribution of RFLP patterns identified in dogs and humans in study areas A, B, C and D. The pattern identification number is reported close to each identifier on the map (adapted from Ferroglio et al., 2017; paper n°10).

Pattern 7 was recorded in four municipalities of area B (reported from two persons and two dogs) and from a municipality of area D. The MCP area of pattern 7 is 435 km^2. Pattern 11 was reported from areas A (in one dog), B (in six dogs) and C (in 1 human), with an overall MCP of 1,691 km^2. Pattern 14 was recorded in areas A (in five dogs), B (in six dogs and 1 human), C (one human) and D (seven dogs). The MCP for pattern 14 is 3,154 km^2 , the biggest reported from the present study. Notably, pattern 14 from area B was recorded in one human and one dog residing in the same municipality.

Pattern 2, 8 and 17 showed a localized distribution, being reported only from specific areas and shared among two subjects each. Moreover, they were recorded exclusively in municipalities of area B.

Similarly, patter 16 was found only in two dogs from two municipalities of area D, with MCP of 4.8 km. The other 10 patterns shared among two individuals were reported from dogs and/or humans residing in different study areas.

A perfect agreement (k = 1) was reported between RFLP and in silico RFLP.

7.3.5. Discussion

As stated previously, the epidemiology of leishmaniasis has changed rapidly in the recent years. Particularly in Europe and the Mediterranean Basin, *L. infantum* has showed to spread to higher altitudes and latitudes (Guernaoui et al., 2006; Ballart et al., 2012), becoming endemic in areas with continental climate previously free from this parasite, in which active foci of both canine and human leishmaniasis has been recorded (Ferroglio et al., 2005; Biglino et al., 2010).

Results of the entomological survey on Phlebotomine sand flies confirmed the presence of *L. infantum* vectors in all the five study areas, as reported in a previous study (Ferroglio et al., 2005). In particular, while the density values of *P. perniciosus* were comparable with those obtained in the same areas in the years 2000 and 2001, the capture frequencies increased from 21.8% in 2000 - 2001 (Ferroglio et al., 2005) to 42.86% of positive trapping stations in the present survey.

The entomological survey was conceived as a single sampling during seasonal abundance peak to maximize capture sensitivity. As a consequence, no inference can be made on eventual differences in seasonal dynamics nor abundance among the study areas.

A previous study carried out in the period 2000-2001 on the epidemiology of *L. infantum* in the same areas reported a seroprevalence tested by indirect fluorescent antibody test (IFAT) in dogs ranging from 3.9% to 5.8% (Ferroglio et al., 2005). In the same area, another study showed that the seroprevalence by WB in asymptomatic healthy humans reached 7.41% (Biglino et al., 2010). In the present survey, referring to the period 2007-2009, data showed a marked increase in the prevalence of the parasite both in the dog and human population, with 16.81% of seroprevalence in humans and 40% of dogs tested positive by PCR. In particular, the highest prevalence of *L. infantum* was reported from autochthonous dogs, in which 48% of the individuals were positive. Interestingly, dogs living in areas B and D were significatively more infected than animals from the other sites and, even if not significative, also the capture frequencies of *P. perniciosus* were higher in these two study areas. These results could suggest that to a more frequent presence of competent vectors within an area correspond to an increase in host-vector contacts and thus the risk of *L. infantum* infection (Ferroglio et al., 2006b, 2006a, 2005). However, this hypothesis seems to not be true for humans. WB results showed an overall seroprevalence of more than 16% in human samples tested, and 73% of the WB-positive individuals showed also the presence of DNA of the parasite. However, humans residing in sites B and C, the most agricultural areas, showed significative lesser prevalence of *L. infantum* than the other study areas. In particular, it is worthy to note that in area B there is a contemporary high prevalence of infection in dogs and a low prevalence in humans. Moreover, also owning a dog positive to *L. infantum* was found not to be a risk factor for human infection, as shown by the subset of dogs and owners tested in parallel. Further researches are needed to assess why there is no direct relation between the infection rate in dogs and humans, and to evaluate risk factors for human infection.

The fact that the infection status of dogs seems not to be a critical point for human infections in the immediate surrounding, at least in the first years after the disease becoming endemic, is further corroborated by the RFLP patterns analysis. Results showed the presence of a high number of circulating strains that confirms the multiple-introduction origin of *L. infantum* in the analysed area, as previously illustrated in classical endemic areas (Millán et al., 2011). In particular, patterns 7, 11 and 14, widely found in a high number of subjects from all the investigated areas, could be strains that have been circulating in Piedmont for a long time. On the contrary, patterns 2, 8, 16 and 17 are strains localized to limited areas and thus suggesting that have been somehow recently introduced into the territory or have been weakly spread among a small number of subjects. Thanks to the frequent travels and journeys to which the population is subject and to the expanding presence of the vectors in terms of capture frequency, these are now circulating in much of the Piedmont territory, probably in a stable manner considering the size of their presence area. The complete agreement between RFLP and in silico RFLP confirms the possibility of using either technique with the same degree of accuracy in pattern determination.

7.3.6. Conclusions

Due to several factors such as climate and habitat changes, arthropod vectors such as sand flies have showed to expand their territorial distribution (Medlock et al., 2014). The consequent establishment of vector-borne diseases into naïve areas is of great concern for both public and veterinary health authorities (Githeko et al., 2000), especially if taking into account diseases with high social impact such as zoonotic visceral leishmaniasis. In order to understand the transmission mechanisms and patterns of disease establishment of a vector-borne disease, it is essential to monitor vector and disease expansion in areas of recent colonization (Bern et al., 2008).

Ferroglio E., **Battisti E**., Zanet S., Bolla C., Concialdi E., Trisciuoglio A., Khalili S., Biglino A. (2018) Epidemiological evaluation of *L. infantum* zoonotic transmission risk in the recently established endemic area of Northwestern Italy. *Zoonoses Public Health*. 65(6):675-682 doi: 10.1111/zph.12477

7.4. Molecular survey on vector-borne pathogens in alpine wild carnivorans – PAPER 4

7.4.3. Materials and methods

For this study, 235 wild carnivorans (157 foxes, 45 badgers and 33 wolves) were collected in the period between 2009 and 2017. All the animals were road-killed, with the exception of red foxes that were culled during the official hunting seasons as part of the culling program for fox population control, and carcasses were brought to the Department of Veterinary Science, University of Turin, for necropsy. For each animal, information such as age (estimated by dental conditions and body size measurements), sex and area of origin (mountain/flat/hill region) were recorded. Spleen was collected from each animal and individually stored at−20◦Cuntil further analysis. Fig. 24 shows the spatial distribution of sampled animals.

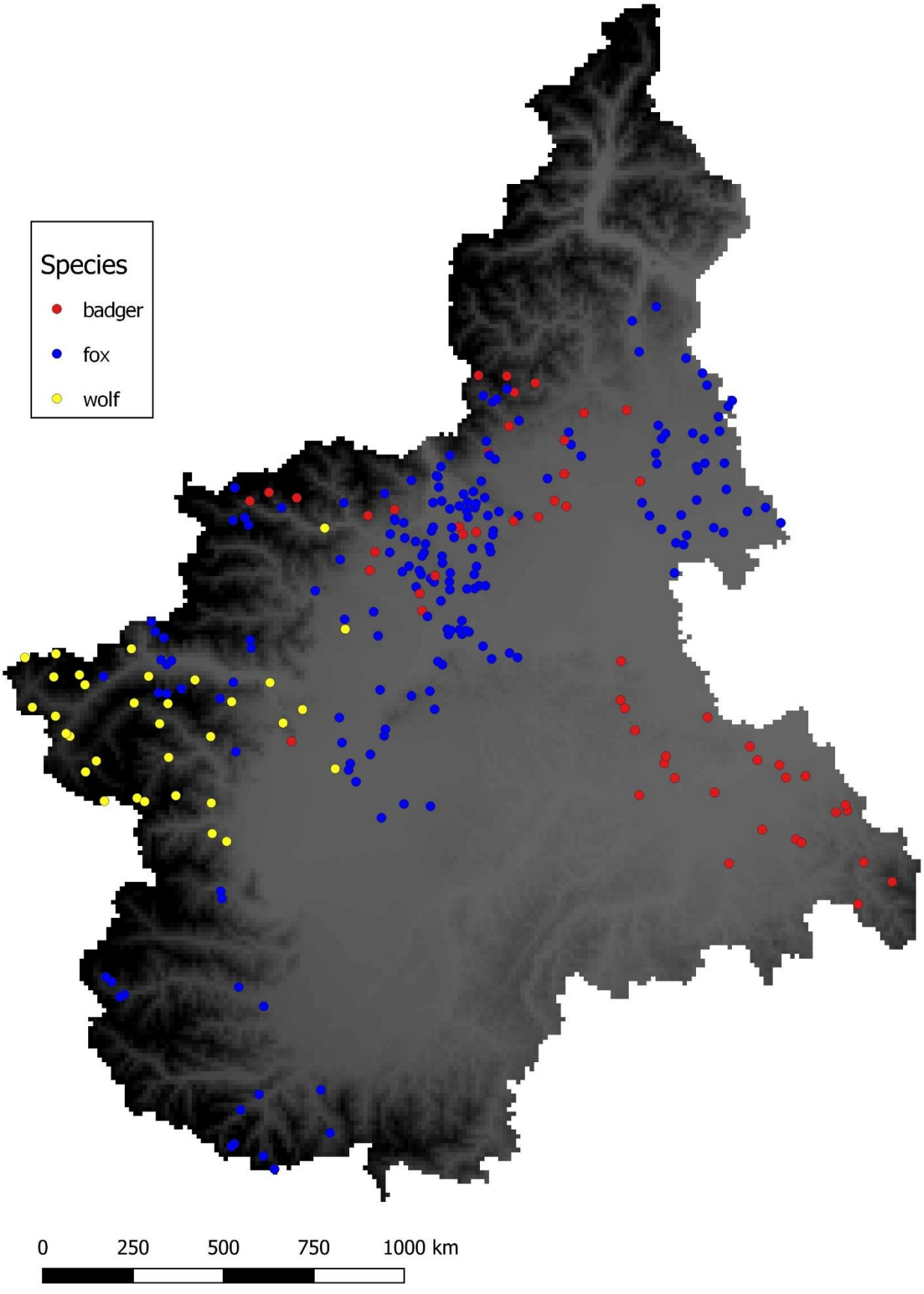
Species
badger
fox
wolf
0
250
500
750
1000 km

Fig. 24 Spatial distribution of foxes, wolves and badgers analysed in this study.

For *L. infantum* detection, a fragment of the highly reiterated minicircles of kDNA was used as target for primers RV1 (5′ - CTTTTCTGGTCCCGCGGGTAGG - 3′) and RV2 (5′ - CCACCTGGCCTATTTTACACCA - 3′) (Ferroglio et al., 2006b). Briefly, the reaction mixture contained 1X of PCR buffer, 22.5 pmol of each primer, 2.5 U of HotStarTaq DNA Polymerase (Qiagen, Milan, Italy), 0.2 mM of dNTPs mix (Sigma-Aldrich, St. Louis, MO, USA) and 2.5 µl of DNA, in a total volume of 25 µl. Thermal cycler conditions were 95 °C for 15 min, 45 cycles of 94 °C for 1 min, 62 °C for 1.5 min and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min.
For each PCR, positive and negative controls were processed together with samples and all the precautions were taken to minimize the risk of contamination. Logistic regression was performed by using R software (3.5.1) (R Development Core Team, 2018), to investigate possible risk factors for TBD infection (age, sex, area of origin and year of sampling).

7.4.4. Results

The overall prevalence of *L. infantum* in the analysed wildlife samples was 21.28% (CI95% 16.53% - 26.95%) [50/235]. In particular, significative higher ($p < 0.05$) prevalence of parasite DNA was reported from badgers (P = 53.33%; CI95% 39.08% - 67.06%) [24/45] compared to foxes (P = 12.10%; CI95% 7.89% - 18.13%) [19/157] and wolves (P = 21.21%; CI95% 10.68% - 37.75%) [7/33]. Among the positive samples, 50 amplicons were chosen for sequencing due to the high quality of the PCR products. Results of the sequencing are listed in Tab. 21.

Pathogen species	Host species	Sequenced amplicons	Prevalence	Confidence Interval (95%)	Percent Identity	GenBank Accession Number
L. infantum	Fox	19	12.26%	7.99-18.35	100%	HF937257
	Wolf	7	25.71%	14.16-42.07	100%	HF937257
	Badger	24	53.33%	39.08-67.06	100%	HF937257

Tab. 21 Prevalence, confidence intervals and identity of each sequenced pathogen divided by host species.

7.4.5. Discussion

As discussed in the introduction of this thesis, current climate changes are estimated to impact on infectious diseases (Shuman, 2010). In particular, the territorial expansion of arthropods due to climate changes (Medlock et al., 2013), the increase of human encroachment into wild habitats

(Semenza et al., 2016) and the rise of wildlife population (Gortázar et al., 2007) are expected to impact on vector-borne pathogens, which already account for 23% of emerging infectious diseases (Jones et al., 2008).

More than half of the badgers in our survey tested positive for *L. infantum*, while the prevalence in the other two species is significantly lower (25.71% in wolves and 12.26% in foxes). The occurrence of *L. infantum* has been largely assessed in wild carnivorans, especially in canids, due to their phylogenetic closeness to dogs that are the main reservoir of this parasite (Millán et al., 2014). The prevalence of *L. infantum* found in foxes in this study is lower than reported previously in Central and Southern Italy (Verin et al., 2010; Dipineto et al., 2007), confirming the recently established endemic area of transmission in Northern Italy where autochthonous dogs showed more than 40% of seroprevalence (Ferroglio et al., 2018). The prevalence in foxes from Spain ranges from 14% (Sobrino et al., 2008) to 75% (Criado-Fornelio et al., 2000), while in Portugal the prevalence is much lower (Abranches et al., 1983, 1982). Although several studies have been performed in order to detect *L. infantum* in wolves in Europe (Sobrino et al., 2008; Oleaga et al., 2018; Beck et al., 2008), to our knowledge this is the first epidemiological study on this parasite in the Italian wolf population. Our results are in line with those obtained from Spain (Oleaga et al., 2018), suggesting a similar epidemiological situation. Compared with foxes and wolves, the presence of *L. infantum* in badgers has been generally less evaluated. However, the moderate to high prevalence observed in this and other studies (Oleaga et al., 2018; Del Río et al., 2014) could suggest a role of this species in the epidemiology of *L. infantum*, at least in its sylvatic life cycle. Nevertheless, further studies are needed to assess the capacity of badgers to infect sandflies, a fundamental ability for a competent reservoir (Millán et al., 2014).

7.4.6. Conclusions

With the molecular analysis of 235 specimens collected from 2009 to 2017, this study provides valuable information about the situation of vector-borne pathogens in wild carnivorans from Northwestern Italy, showing high level of infection in all target species. Moreover, we reported for the first time the presence of *B. capreoli* in wolves and badgers, two unexpected hosts for this parasite, and of *H. canis* in wolves.

This survey highlights the presence of several VBP in the study area, many of which capable to infect domestic animals and humans. The high occurrence of VBPs in sylvatic carnivorans could pose a risk for both animal and human health, especially in an area with growing urbanization and increasing wildlife population as in many parts of Europe, that lead to more close contacts between humans, wildlife, livestock and pets.

Battisti E., Zanet S., Khalili S., Trisciuoglio A., Hertel B., Ferroglio E. (2020). Molecular survey on vector-borne pathogens in alpine wild carnivorans. *Frontiers in Veterinary Science*. 7:1 doi: 10.1186/s13071-018-2994-2

8. FINAL CONSIDERATIONS

The aim of this PhD project was to assess the epidemiology of three different emerging protozoa, namely *T. gondii*, *Babesia* spp. and *L. infantum*, with a One Health approach. The One Health concept was borne to face emerging infectious disease events with a multidisciplinary approach, bringing together experts of human and veterinary medicine, wildlife managers, conservation biologist, environmental scientists and ecologist. In fact, One Health recognizes that the health of humans, animals (sylvatic and domestic) and the environment in which they live is deeply connected, and thus zoonotic infectious diseases are a problem to deal with together.

The parasites investigated in the project were chosen due to their considerable burden on human and/or veterinary health, and since some information regarding the epidemiology of these protozoa in the study area were lacking. Circulating genotypes of *T. gondii* were investigated in domestic and sylvatic animals from Northwestern Italy, highlighting the presence of type I and non-clonal atypical alleles in these samples. In particular, while livestock showed higher prevalence of type I allele, wildlife were considerably more infected with non-clonal strains of the parasite, suggesting a difference between the domestic and the sylvatic cycle of *T. gondii*. The prevalence of type II and III alleles was unexpectedly low (4.65% and 6.98%, respectively), in contrast with what observed in other European countries such as France (Richomme et al., 2009; Aubert et al., 2010). Genotype II has long been considered the most prevalent genotype circulating in Europe, both in animals and humans (Howe et al., 1997). However, recent findings have showed a more variable situation, with considerable prevalence of genotype I and atypical genotypes in wild animals from Spain, Germany and Italy (Verin et al., 2013; D. C. Herrmann et al., 2012; Calero-Bernal et al., 2015). While the role of meat as a source of infection with *T. gondii* is well understood, less data is present regarding the transmission of this parasite through dairy products. To date, only raw goat's milk has been implicated as a cause of human infection with *T. gondii* (Sacks et al., 1982), although tachyzoites have been observed in the milk of several other species (Mancianti et al., 2014; Bezerra et al., 2015). For this reason, we investigated the presence and viability of *T. gondii* in ewe milk and cheese made from unpasteurized milk, in order to evaluate the risk for human health deriving from the consume of dairy products from naturally infected sheep. Results showed that, even if *T. gondii* DNA was present in around 50% of the tested flocks and viable in raw milk, no viable tachyzoites were observed in 5- and 15-days ripened cheese, suggesting that the ripening procedures may inactive the parasite. Further researches on *T. gondii* should focus on the improvement of the genotyping technique, because the current PCR-RFLP method lacks in sensitivity and thus shows poor results in the genotyping of wildlife samples. Moreover, it would be interesting to evaluate the genotypes circulating in humans and in the definitive host, in order to better understand the epidemiology in the study area and the differences between domestic and sylvatic cycle of the parasite. Concerning dairy

products, further investigations are needed to evaluate the viability of *T. gondii* in cheese compared to the amount of tachyzoites in the milk used for cheese production.

In this project, the prevalence of *Babesia* spp. and other relevant tick-borne pathogens was investigated in sylvatic carnivores, revealing high prevalence for all the analysed pathogens. In particular, foxes and badgers were considerably more infected with *Babesia* spp. than wolves, with a prevalence of 89.7%, 91.1% and 39.4%, respectively. The high prevalence of tick-borne pathogens in wild carnivores may suggest a role for these animal species in the epidemiology of the parasites, although it is clear that high level of infection is not a proof of being a reservoir host. Nevertheless, this study points out the presence of relevant pathogens for human and animal health, such as *A. phagocytophilum* and *B. vulpes*, in sylvatic animals, suggesting a potential risk for humans and their pets attending wild habitats. The presence of *Babesia* species linked to sylvatic habitats, such as *B. venatorum*, *B. capreoli* and *B. microti*, was also observed in ticks collected from humans and privately-owned dogs, confirming the circulation of wildlife-related species in domestic environments likewise. Previous data on the positivity of *Babesia* spp. in wild ungulates from Northwestern Italy were used for modelling the occurrence of this parasite in wildlife, showing that around 15% of the analysed area is suitable for *Babesia* occurrence. The model was further validated with field sampling of ticks by dragging method, and results confirmed the ability of the model to correctly identify the most suitable areas for the parasite. Thanks to the passive transportation using animal hosts, ticks are able to travel for even thousands of kilometres, crossing sea and deserts. Migratory birds are well-known carriers of infected ticks, allowing the spread of these vectors into new ecological niches, and consequently introducing new pathogens into non-endemic areas. For this reason, we investigated which kind of pathogens could be found in ticks collected from migratory birds travelling northward from sub-Saharan Africa to Europe. Results showed the absence of *Babesia* spp., Anaplasmataceae, *B. burgdorferi* s.l. and CCHFv, but the presence of SFG *Rickettsiae*. In particular, we detected *R. aeschlimanni* and *R. africae*, that are typical species of the African continent and known to cause infection in humans. This study confirmed the role of migratory birds as carriers of infected ticks and thus the possible introduction of exotic pathogens in Europe, posing a risk for human health and a challenge for physicians.

The presence and circulating strains of *L. infantum* were investigated in 5 different areas in Piedmont region, and results showed considerable prevalence in both human and dog samples analysed (16.81% and 40.35%, respectively). Moreover, similar RFLP patterns were observed in humans and dogs from the same municipalities, although statistical analysis revealed that the infection status of dogs in the immediate surrounding is not a critical point for human infection, because owning a dog positive for *Leishmania* was not a risk factor for humans. High prevalence of *L. infantum* was also observed in wild carnivores, especially in badger, but further studies are needed to evaluate the role of this species in the wild cycle of the parasite, and to assess the presence of sylvatic reservoirs of *L. infantum*.

As discussed in the introduction part, several infectious diseases are emerging or re-emerging because of changes occurring at the interface between the pathogen, the hosts and the environment in which they live, that lead to closer contacts and higher risk of transmission. There are multiple drivers influencing the emergence of pathogens that act simultaneously, both at global and local level.

Results of this PhD project clearly indicate a northward shift in the geographical distribution of *L. infantum* towards previously non endemic areas such as Northern Italy. This shift has been triggered by the concurrent expansion of *Phlebotomus* sand flies, that is ultimately due to climatic changes such as milder winter temperatures. Favourable climatic conditions not only influence the survival and viability of sand flies, but also of ixodid ticks. However, it is clear that the occurrence and distribution of ticks in a certain area is influenced by habitat and environmental changes likewise, such as the fragmentation of habitats, the resurgence of natural and sylvatic areas and the increase of wildlife. Moreover, the emergence of tick-borne pathogens affecting human health can be also attributable to cultural and behavioural changes, that are leading to human encroachment into natural habitats and closer contacts with pathogens, vectors and hosts (both sylvatic and domestic). Cultural changes are also the most important drivers of food-borne pathogens emergence. In particular, the growing demand of exotic foods, game meat and organically-raised livestock products can increase the risk of transmission and negatively influence the human health.

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