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Doctoral dissertation in medical sciences

Detection and Molecular characterization of *Babesia* spp. and Tick-Borne Encephalitis Virus in ticks from Poland and Ethiopia

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List of Abbreviations

DALY……Disability Adjusted Life Year

NFD ……. Natural Foci Disease

OR…….Odds Ratio

PBS……Phosphate Buffer Saline

PCR……Polymerase Chain Reaction

RBC……Red Blood Cell

RNA……...Ribonucleic Acid

RT-PCR……..Real Time Polymerase Chain Reaction

TBD……Tick-Borne Disease

TBE……Tick-Borne Encephalitis

TBEV…….Tick-Borne-Encephalitis Virus

TBEV_FE……...Tick-Borne Encephalitis Virus Far Eastern

TBEV_Eu……...Tick-Borne Encephalitis Virus European

TBEV_Sib……...Tick-Borne Encephalitis Virus Siberian

UK……...United Kingdom

USA…….United States of America

YLD……Year Lived with Disability

1. Introduction

The majority of the world's population (80%) is at risk of contracting vector-borne diseases, which kill 700,000 people each year. It accounts for 17% of the estimated global burden of all infectious diseases (1–3). After mosquitos, ticks are the second most important vectors of infectious disease causative agents (4). Ticks are blood-feeding parasites that carry a wide range of pathogens, including the agents that cause Lyme disease, tick-borne encephalitis, anaplasmosis, and babesiosis. Ticks are divided into two families: *Argasidae* (soft) and *Ixodidae* (hard). The latter is known to transmit the majority of tick-borne pathogens to humans and animals (5).

Ixodes ricinus (I. ricinus) ticks are considered the most relevant tick species to transmit pathogens of Tick-Borne Disease (TBD)s in Europe (5); however, other ticks, such as *Dermacentor reticulatus (D. reticulatus),* have been linked to the spread of TBDs (6). *Ixodes persulcatus (I. persulcatus)* is common in the subarctic regions of Europe and Russia with a unique association with Tick-Borne Encephalitis Virus (TBEV). The distribution of *I. ricinus* is expanding from central and eastern Europe to northern and western European regions including Sweden and the United Kingdom (UK) (5).

Dermacentor reticulatus is also another suitable vector for pathogens with high resistance to diversified and harsh weather (6). The presence of *D. reticulatus* is reported from north to south of the European continent. It ranges between the Scandinavian and southern Iberian islands. *Dermacentor reticulatus* have epidemic importance because of their involvement in the transmission of tick-borne pathogens like TBEV and ectoparasites like *Babesia* spp. (7). Rodents are important agents in the cycle of TBD causing pathogens transmission by serving as hosts for both ticks and microbes (8). Among others, tick-borne encephalitis (TBE) and babesiosis are the two emerging TBDs seen in the past decade which are being reported in many European countries (9–13). The risk of TBE in humans is

influenced by e.g., socioeconomic changes, climate change, seasonal variations, and individual predispositions. The extended and warmer summer and increased population size of hosts favour the survival of ticks and microbes out of their known habitats (14–16). As there is an extended and warmer summer, the dynamics of the other seasons would also be changed in a way that is comfortable for ticks' life (16).

The duration of the convenient season for tick activity has been expanding since the end of the 1990s (17). In Sweden, the northward movement of ticks has been expanding since the 1980s with evidence of ticks reported in far northern Sweden in 2008 (18). Climate changes affect the survival, reproduction, interaction with their host and environment, and movement of ticks. Cases are reported from new areas, because of occupations related to increased human need and capability of accessing places that were hard to reach in the past (19). Although climate change is mentioned as a major factor for the increased spread of several infectious diseases, including TBE, in humans, socioeconomic changes also play a comparable role. The end of the Soviet era caused a surge in the number of TBE cases in humans among countries that had the influence of the planned economy. This is related to the decreased pesticide use, expansion of subsistence farming and land use, and increased leisure time (20). The distribution in Poland particularly is separated into eastern and western parts of the country with neutral space between the two regions. However, the expansion to the neutral region is significant in recent reports showing that the spread of tick population is crossing the Vistula river from east to west direction (7).

1.1. Ecology of ticks

Tick-borne diseases are known to be one of the Natural Foci Diseases (NFD). For a disease to be called NFD it should have such elements as an agent, susceptible animal host, vector, suitable habitat, and suitable environment. The agents of TBDs can be viruses,

protozoans, and bacteria that are carried by ticks while rodents and other mammals serve as susceptible animal hosts. Plants are considered suitable habitats for both ticks and potential hosts. Moreover, all these elements need a suitable environment for the vector to circulate. The interaction of all these components of NFD facilitates the spread of TBDs. Meanwhile, people get involved with these elements whereby exposing themselves to one of the TBDs etiologic agents (21).

The habitat of ticks and their host is fragmented into different sizes of patches. Small-sized patches can be created because of such activities as agricultural land use, human settlement, and urbanization. The movement of animals between patches is limited so that it can determine the abundance of ticks. The distribution of ticks again impacts the number of human cases of TBDs (22). On the other hand, the afforestation and maintenance of natural reserves connected to residential areas and urban centres promote the interaction of vectors with hosts. Therefore, ticks and hosts get a chance to move between patches (23).

In Europe, the active season of ticks extends between March to November with peak activity in May and June. The period when tick activity is at its peak coincides with the time when the mobility of people is the most (24,25). Increased rainfall and humidity are highly important for ticks to run their biological function. There can be a latency of TBE as has been seen in some parts of the Czech Republic and Germany. This can happen because of problems that could affect one of the elements of NFD. A TBEV latency period would rather be the virus going unreported for that certain period than the virus having disappeared for a while (22).

During winter, ticks hide in the leaf litter or soil and digest the blood ingested during their questing time for their survival. To become active, warmer temperatures and humidity are needed to exit from the soil and ascend into vegetation (19,26). Ticks can be active in dry seasons looking for hosts and food, however, such condition limits their longevity (27).

Weather condition with a temperature of around 8°C and humidity of 70-80% is convenient for ticks' movement and feeding (28). A study conducted in Germany has shown that in the past 50 years there was increased annual rainfall and temperature that contributed to the increased population of ticks (29).

The prevalence of *I. ricinus* depends on altitude in Europe. These ticks are common under the altitude of 700-2,000 meters above sea level. However, altitude is not the only factor that determines the existence of ticks, climatic change and proximity to water bodies also influence the population of ticks (9,30). According to the march 2022 European Centre for Disease Prevention and Control (ECDC) report, *I. ricinus* was found in most European countries including European Russia, and introduced to northern Finland and Iceland (Figure 1).

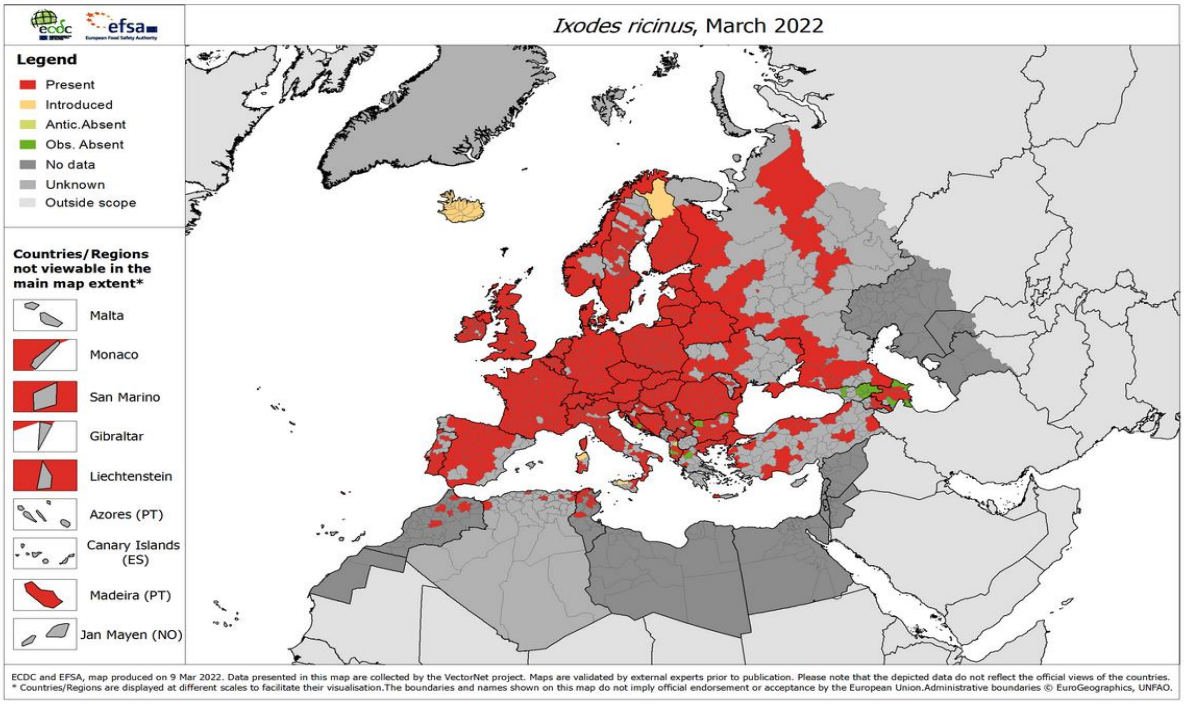


Figure 1. Distribution of *I. ricinus* in Europe as it is reported by ECDC in September 2022

Source: https://register.ecdc.europa.eu/ (accessed on October 25.2022)

Dermacentor reticulatus, the second most reported tick following *I. ricinus* is mainly found in Central and Northern Europe. *Dermacentor reticulatus* can survive for more than

three months under clean water which would be shorter under water bodies with organic contents (31). However, it is also reported in some Mediterranean regions as it has been reported from Portugal and Spain (32). *Dermacentor reticulatus* is reported from Austria, Belarus, Belgium, Croatia, Czech Republic, Germany, Great Britain, Hungary, Latvia, Lithuania, Netherlands, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Switzerland, Turkey, Ukraine (32). Unlike *I. ricinus*, *D. reticulatus* has a lesser affinity to humans yet it remained a vector for TBEV, *Rickettsia* spp., and Omsk haemorrhagic fever virus in addition to the risk it pauses on animals by carrying *Babesia canis (B. canis)* (Figure 2) (31).

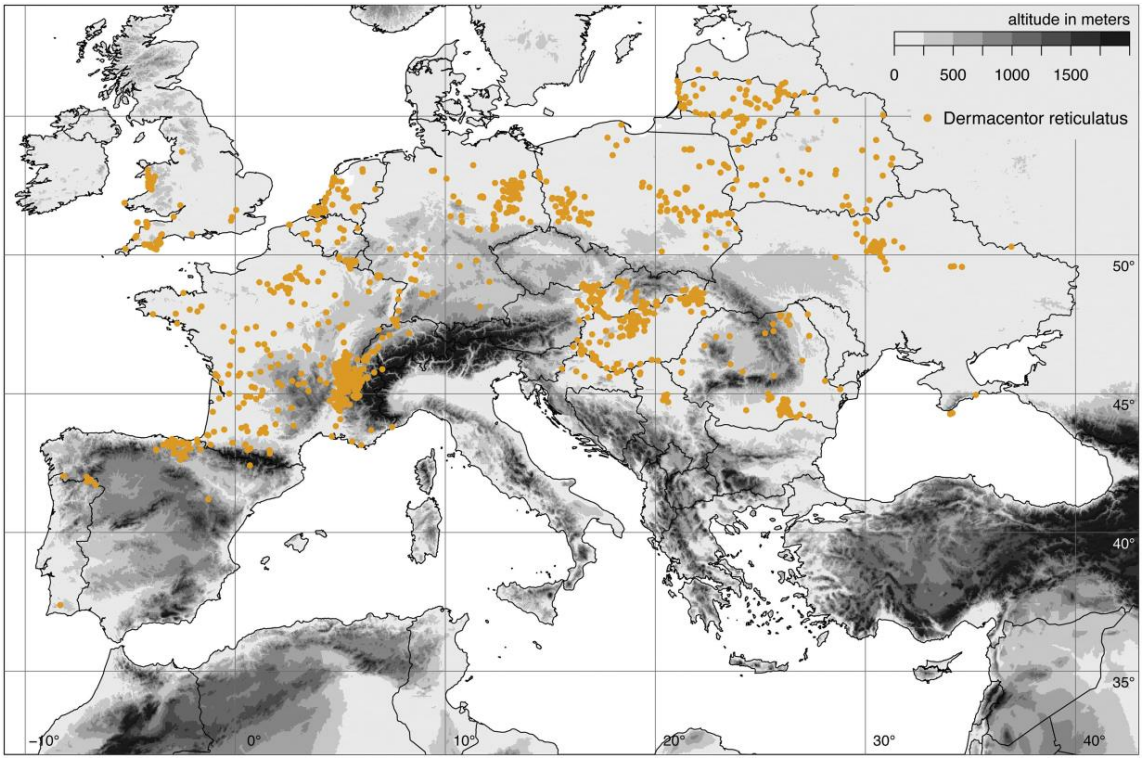


Figure 2. A georeferenced distribution of *Dermacentor reticulatus*

Source: https://doi.org/10.1016/j.ttbdis.2015.10.015 (accessed on October 16.2022)

In Poland, the prevalence of TBEV ranges between 0.99-12% in *D. reticulatus* ticks (33), while 1.6% was reported from *I. ricinus* ticks (34).

1.2. Tick biology

Ticks are arthropods in a class of *Arachnida* from the family of *Ixodidae*, a subfamily of *Ixodidae*, and a genus of *Ixodes* that consists of multiple species including *I. ricinus* and *I. persulcatus*. In the same family, *Rhipicephalinae* is a subfamily that has the genus of *Dermacentor* including species of *D. reticulatus* and *D. marginatus* (35).

Ticks feed mainly on vertebrates once in a developmental stage from days to a few weeks duration. All pass through larvae, nymph, and adult stages of development since once laid as an egg from adult female ticks (22). The size of ticks varies depending on their feeding status and sex and female ticks have a larger size than males. Unfed ticks have a length of 2-20 mm while engorged female ticks have a length that extends between 25-30mm. Larvae ticks have three paired legs while nymphs and adults have eight legs (36).

Ticks have two main parts known as the capitulum and body. The capitulum has palps with numerous chemosensors, a pair of sclerotized tubular chelicerae located medially to the palps that have sharp cutting digits, and hypostomes are used as holdfast and food canal while feeding. The capitulum and body of a tick are attached to a flexible membrane (22,36,37) (Figure 3 – 8).

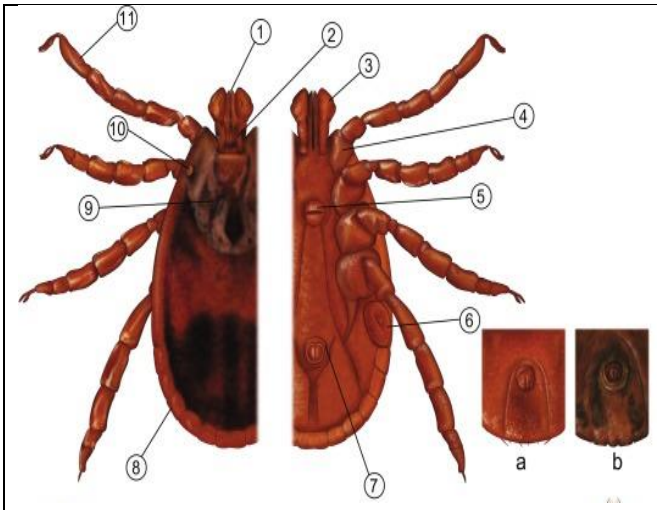


Figure 3. Basic morphology of *Ixodid* ticks

Source: https://doi.org/10.1016/B978-0-12-814112-0.00009-X

(accessed on October 16.2022)

(1) Hypostomum, (2) basis capituli, (3) palps consisting of three segments, (4) coxa I, (5) genital opening, (6) stigmatal plate (spiracles), (7) anus and anal groove, (8) festoons, (9) scutum, ornamented, (10) eye, and (11) tarsus with Haller's organ.

(a) Anus and anal groove of *Ixodes* type: U-shaped anal groove arching anus anteriorly and (b) anal groove arching the anus posteriorly (38)

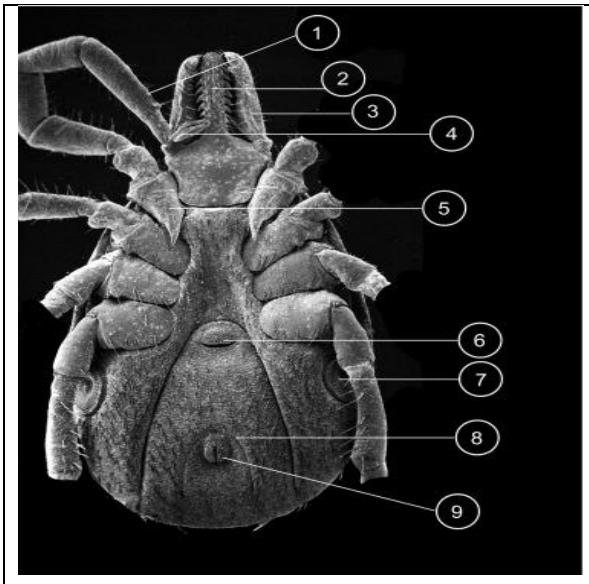


Figure 4. Venteral view of Female *Ixodes ricinus*

Source: https://doi.org/10.1016/B978-0-12-814112-0.00009-X (accessed on October 16.2022)

(1) tarsus with Haller's organ;

(2) hypostome lined with backward-pointing chitin barbs that help anchoring into skin;

(3) palp consisting of three segments;

(4) pulvillus with claws;

(5) well-developed internal spur in coxa I;

(6) genital opening;

(7) stigmatal plate (spiracles);

(8) U-shaped anal groove arching the anus

(9) Anus (38)

The anterior part of the tick's body has legs as extensions and genital pore while the posterior part has spiracles and an anus. The genital aperture doesn't appear in larvae and

nymphs until it gets open in adults (36). In *Ixodid* ticks, female ticks have the scutum covered on the dorsal anterior portion of the body while males have scutum that covers the entire body (22,36,37).

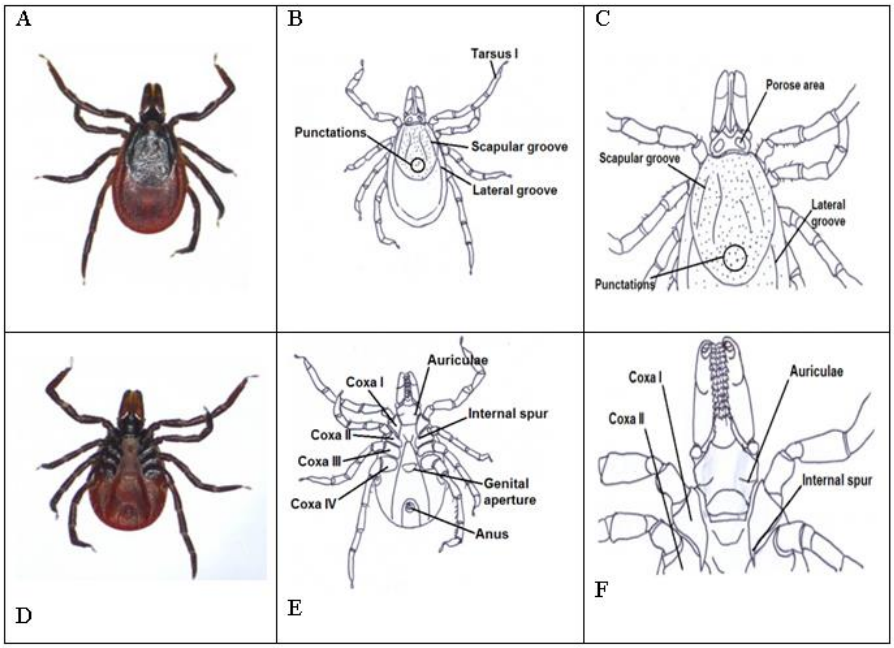


Figure 5.Morphoogy of adult female *I. ricinus* with peculiar features
Dorsal part(A,B,C)-Scapular grooves are present, Cornua absent, Tarsi I is tapered, and Punctations are distinct)
Ventral part (D,E,F)-auriculae are distinct, coxae I: long and distinct internal spurs, coxae II-IV: indistinct external spurs, and genital aperture positioned between coxae IV
Source: http://www.bristoluniversitytickid.uk/page/Ixodes+ricinus/26/#.Y1wMFXZBzIW (accessed on October 28.10. 2022)

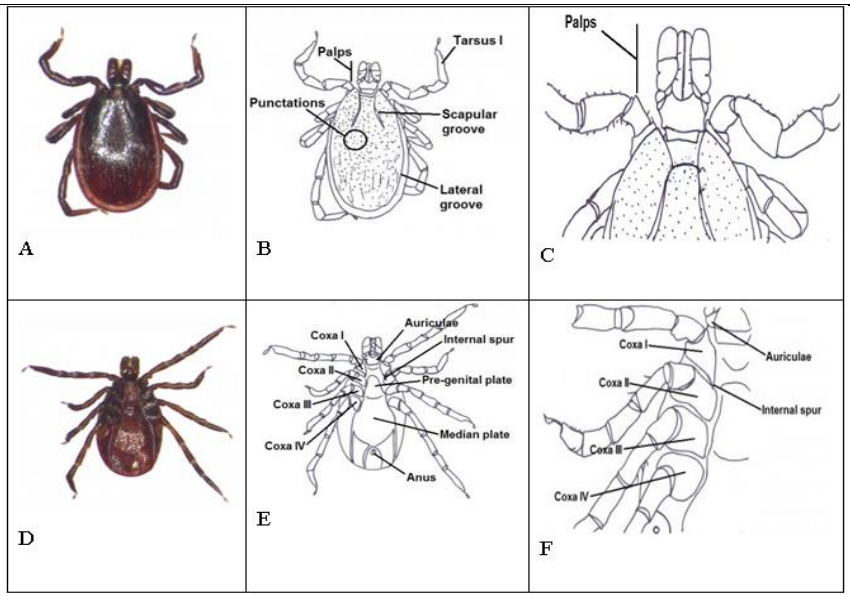


Figure 6. Morphological features of adult male *I. ricinus*

Source: http://www.bristoluniversitytickid.uk/page/Ixodes+ricinus/26/#.Y1wMFXZBzIW (accessed on October 28.10. 2022)

Adult male dorsal part (A,B,C)-palps are small, tarsi I tapered, and punctations are distinct

Adult male ventral part (D,E,F)-auriculae are distinct, coxae I: long and distinct internal spurs, coxae II-IV: indistinct external spurs, pre-genital plate approx. 2x as long as it is broad, and the median plate long and narrow

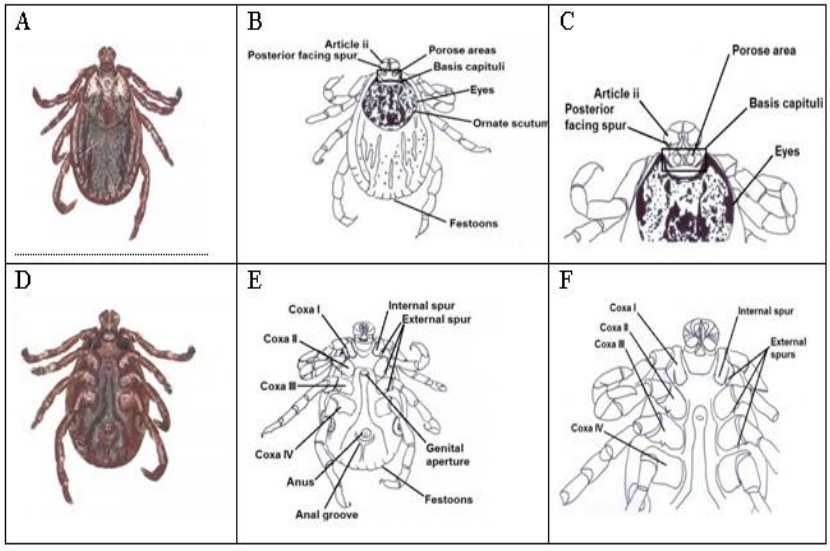


Figure 7. Morphological features of adult female *D. reticulatus*

Source: http://www.bristoluniversitytickid.uk/page/Dermacentor+reticulatus/14/#.Y119wHZBzIU (accessed on October 29.2022)

Adult female dorsal part (A,B,C)-Porose area is broad and oval, article ii of palps has a posterior facing spur, and basis capituli is rectangular in shape and broader than long

Adult female venteral (C,D,E)- Adult female ventral view and adult female close-up of coxae/ventral gnathosoma

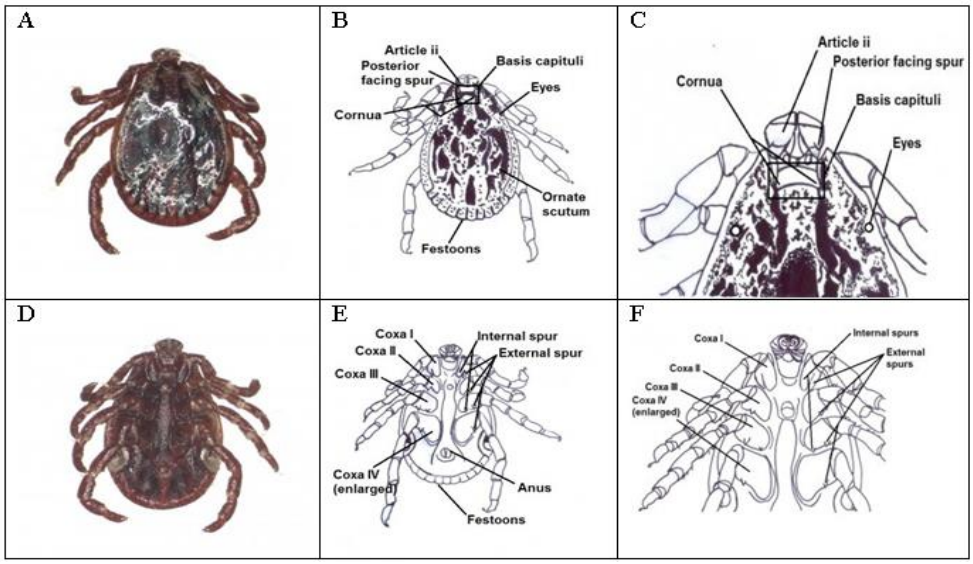


Figure 8. Morphological features of adult male *D. reticulatus*

Source: http://www.bristoluniversitytickid.uk/page/Dermacentor+reticulatus/14/#.Y119wHZBzIU (accessed on October 29.2022)
Adult male dorsal part (A,B,C)-Cornua length is long, article ii of palps have a long posterior facing spur, lateral groove present as punctations only
Adult male ventral part (D,E,F)-Coxae I: external and internal spur present, the gap between each is considered narrow, coxae IV: enlarged, and coxae II-IV: Short external spurs

Hard ticks have endophilous behaviour that they feed on animal burrows for several days to protect from falling. It is a one developmental stage single mandatory feeding that *Ixodid* ticks follow although their host preferences are different (22,39). Each active stage has a preferred host suitable for feeding. Larvae and nymphs choose small mammals and birds, but large mammals are comfortable hosts for adult ticks (40,41). Before feeding on their respective hosts, ticks position on vegetation like a litter of leaves as a questing means to find their host (22). When ticks get their appropriate host, there are particular stages of feeding that starts with puncturing the skin of the host, cementing or attaching with skin, and start suckling blood through peristalsis movement (22,36,37). Eggs get hatched and moult into larves with enough blood meal. Each developmental stage needs enough blood meal to moult and metamorphosis happened (36).

Ticks use odour, vibration, shadowing, and visual appearance stimuli to detect hosts for feeding while a tactile stimulus is used to detect appropriate locations of feeding using their hairlike structures on palps and other body parts. After piercing the skin, the tick secretes saliva, which prevents the blood from clotting, dilates the skin capillaries, digests the host's tissue, causes profuse bleeding, and suppresses the host's inflammatory response. Pheromones are also used to communicate between individual ticks of the same species. Assembly pheromones are used to gather together in the field which helps ticks to find safe microhabitats in the group and find hosts in the meantime. Aggregation and attachment pheromones facilitate the attraction of ticks to specific feeding locations on the host. The other one is the sex pheromone, which attracts male ticks to detach from a nearby feeding site and mate with a female tick ready for mating (6,36).

Engaged female ticks lay thousands of eggs and die as *Ixodide* ticks follow a single gonotrophic cycle (22,37). Mated female ticks ahead of their feeding can take as much as 100 times their size to help lay their eggs. Eventually, the transmission of most pathogens starts in 24 hours mostly, because of their presence in the digestive system. Tick-borne encephalitis virus is transmitted in 30 minutes after tick bite, because it is present in salivary glands (39). Excess water is removed while feeding to create enough space for more blood meals. The lost water is replaced later by water from vegetation and its remaining covered the soil where ticks stay after feeding. Larvae and nymphs may need three to six days for feeding while adult ticks remain for feeding for up to two weeks (22,36).

Digestion of blood meal starts within a few hours of feeding and continues for weeks or more (22,36). It is during digestion that pathogens get the opportunity to cross the gut wall and enter into the hemolymph circulation of ticks. The availability of hosts and combined characteristics of climate matter the feeding pattern of ticks (22).

1.3. Babesiosis

Babesiosis is an emerging tick-borne disease caused by *Babesia* spp. parasites (42,43). Due to their pear-shaped forms inside infected red blood cells, the intraerythrocytic parasites are also known as piroplasms. The transmission of *Babesia* spp. between competent hosts is mainly by *Ixodid* ticks (43). Both *Babesia microti* (*B. microti*) and *Babesia divergens* (*B. divergens)* can be transmitted through blood transfusion (44). The species that cause babesiosis have variations across different geographical locations. In Europe, *B. divergens* is the known cause of babesiosis while *B. microti* is common in the United States (43). However, there were two forester cases infected with *B. microti* reported from eastern Poland (45). The spectrum of babesiosis ranges from silent infection to a malaria-like disease that can end up in death (43). *Plasmodium* and *Babesia* have similar inflammatory and clinical features mainly with the haemolytic and neurological impact of *Plasmodium falciparum* type (46). However, tetrads of merozoites that are arranged in a cross-like pattern are pathognomonic and provide a typical morphology for diagnosis of babesiosis caused by *B. microti* in human erythrocytes (47,48). Babesiosis is commonly seen among immunosuppressed and splenectomised individuals (49–51). The co-occurrence of malaria and babesiosis has increased the burden of illness on persons who contracted both diseases at a time because of the higher parasite density (52–54).

The most common causes of human babesiosis are *B. microti*, *Babesia crassa*-like pathogen, *Babesia duncani* (*B. duncani*), *Babesia divergens* (*B. divergens*), and *Babesia venatorum* (*B.venatorum*). In Europe, *Babesia* pathogens are transmitted by *I. ricinus.* Among others, *B. divergens, B. microti*, and *B. venatorum* are common causes of human babesiosis (47,48,55,56). *Babesia* spp. is divided into small (1.0-2.5 μm) which include *Babesia bovis* (*B. bovis*), *Babesia gibsoni* (*B. gibsoni*), *B. microti*, *Babesia rodhaini*

(*B. rodhaini)*, and others. Large (2.5-5.0 μm) species include *Babesia bigemina* (*B. bigemina*), *Babesia caballi (B. caballi)*, *B. canis*, and others (57).

A study conducted in Poland has shown that the prevalence of *Babesia* spp. was 1.6% in *I. ricinus* ticks (58). The most competent reservoir of *Babesia* spp. in the European context is *Microtus voles*. Their favourite habitats are moist fields, meadows, forest edges, and crop fields, while regular wooded forests are the ideal habitat for ticks, particularly in the United States of America (USA) (58,59). Similarly, in Poland, *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, and *B. microti* were found in ticks where coinfection of either two or three pathogens were also revealed (60).

The distribution of *Babesia* spp. depends on the distribution of its arthropod vector. Only a few places in the world are free of ticks, consequently, *Babesia* spp. is also widely distributed and is an infectious disease of global importance (54,55,61,62). The common clinical findings of malaria and babesiosis usually lead to misdiagnosis. Both diseases have fever, chills, sweat, headache, fatigue, malaise, nausea, vomiting, muscle and joint pain, and haemolysis associated complications (63,62,48). Eventually, babesiosis is not usually diagnosed in malaria endemic areas which have ended cases into complications regardless of any antimalaria provided (55).

The emerging of Southern European ticks (*D. reticulatus* and *Rhipicephalus sanguineus (R. sanguineus)* and the diagnosis of babesiosis in the Netherlands recently are indicative of cross boundary presence of *Babesia* spp. and ticks. Additionally, the extended and warmer summer and increased population size of hosts also favoured the survival of ticks and microbes out of their known habitats (9,14–16,64,65). As there is an extended and warmer summer, the dynamics of the other seasons would also be changed in a way that is comfortable for ticks' life (16). *Dermacentor reticulatus* is a vector of pathogens causing *canine* babesiosis, which is common in the Mediterranean region and this tick species has

been recently found in central and northern parts of Europe due to the increased temperature (66) (Figure 9).

Among other species of *Babesia* spp., *B. bigemina* and *B. bovis*, have been reported in the Sahara and Sub-Saharan regions of Africa with reports commonly involving livestock (49,50,67). The pathogenicity of *Babesia* protozoa varies depending on their strains; however, *B. bovis* is usually more virulent than *B. bigemina* or *B. divergens* (68).

Poland is one of the European countries with the highest number of TBDs reported. The ECDC targets ticks as both medical and veterinary important vectors harbouring multiple microbes, including TBEV and *Babesia* spp. (69). Poland has abundant *I. ricinus* and *D. reticulatus* ticks (24). The eastern and south-eastern part of Poland has the highest prevalence of ticks and TBDs (70,71). Podlaskie is one of the regions with the highest cases within the eastern part of Poland. The country took TBDs as a strategic priority for public health and launched a mandatory national surveillance system of TBDs with involvement since 2000 (72,73). Ticks infected with one or more microbes in Ethiopia are found at an altitude between 900-2586 meters above sea level (74–77).

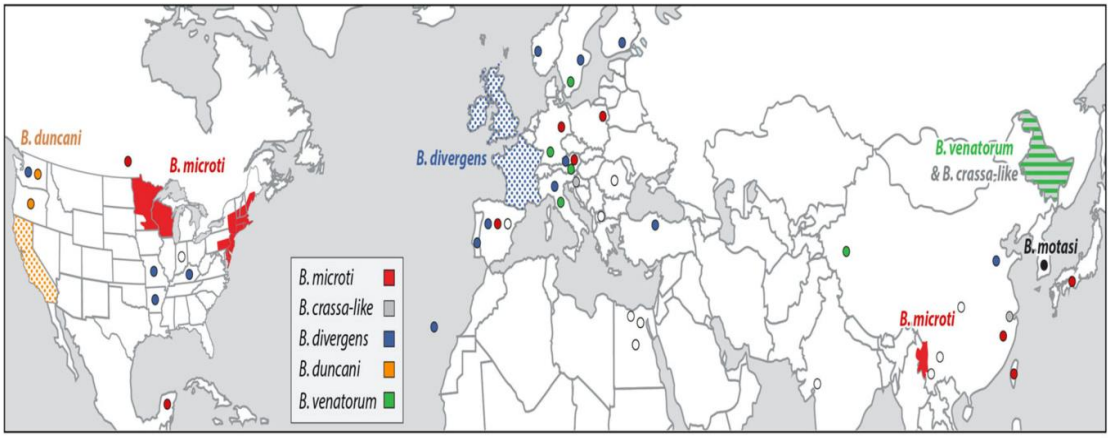


Figure 9. Geographic location of human babesiosis and *Ixodes* tick

Source: doi: https://doi.org/10.3390/pathogens10111447 (accessed on November 05.2022)

1.3.1. Babesiosis in Europe

Over the past 50 years, more than 50 human cases have been reported in Europe. The highest number of babesiosis is mainly reported in the USA followed by Europe in countries like France, England, Ireland, Germany, Spain, Switzerland, Russia, and Poland (47,48,55,56).

Since 1956, the first human case of babesiosis has been reported from the former Yugoslavia (now Croatia) with more than 50 autochthonous cases reported. Twenty two of them were reported until 1998, 10 from France, 6 from the British Isles, 2 from Spain, and 1 from each country of Russia, Sweden, Switzerland, and Croatia. After 1998, 13 cases were reported. Of all non-imported cases, 35 of them had *B. divergens*, 5 *Babesia venaturum (B. venaturum*), and 11 *B. microti*. Thirteen more imported cases had acquired the parasite in the Americas (78).

1.3.2. Babesiosis in Poland

The very first reported case of babesiosis was a 36 years old man who returned from Brazil in 1992 (79). However, there are reports of babesiosis in an immunocompromised patient in 2004 and *B. venatorum/B. divergens* reported from an immunocompetent person in 2010 from south-eastern Poland (80), the first autochthonous *B. microti* infected cases were immunocompetent foresters identified among a group of 58 foresters from eastern Poland in June 2011. Both babesiosis cases were more than 45 years old and had been bitten by *I. ricinus* ticks several times while they were on duty. The parasite in their blood was detected using both conventional and RT-PCR methods (45). A study that involved foresters from central and north-eastern regions of Poland has identified five cases from 114 foresters who had been working in two separate forests (81). Another study from north-eastern Poland has reported six cases with non-specific symptoms of babesiosis caused by *B. microti*. All have reported

their history of tick bit. None of them required anti-babesiosis treatment. These six cases were reported among 548 patients who have been attending an infectious diseases hospital from 2010-2013 (82).

1.3.3. Babesiosis in Africa

Most reports related to TBDs are from the United States of America, Europe, and Asia, however, cases reports have shown that people with a history of travel to African countries like Ethiopia, Uganda, South Africa, Egypt, Gabon, and other countries had one or more tick-borne diseases diagnosed. Two cases of babesiosis were reported in 1990 after visiting Namibia and Zimbabwe who were misdiagnosed with *Plasmodium falciparum* (56). In Tanzania there were indications of babesiosis in one of its districts, however it needs molecular verification since increased parasite load alone can lead to a false positive conclusion (83). A woman had also been misdiagnosed with malaria for babesiosis after multiple trials of treatment failure. The species detected from her was *B. microti* which has not been detected in Equatorial Guinea (84) except in reports from non-human primates (83). Moreover, she had a history of travel to tick prone areas in Spain that posed doubt about where she has gotten the infection (84).

Unlike multiple studies conducted in Ethiopia about *Babesia* spp. in ticks and animals, no published human case is reported so far. A study from the Western part of Ethiopia has shown a 1.5% of prevalence (1.24% for *B. bovis* and 0.248% for *B. bigemina*) (85). Another study conducted in and around Jimma town in the south-western of Ethiopia reported a 2.3% of prevalence rate for bovine babesiosis by *Giemsa* stained blood smears (33.33% for *B. bovis* and 62.96% for *B. bigemina*) (74). Bovine babesiosis is also found in animals with suggestions of presence in humans in the context of Ethiopia (50).

Ethiopia is known for the higher prevalence of both *Plasmodium falciparum* and *Plasmodium vivax* protozoans of malaria. Malaria is endemic to three fourth of Ethiopia's land coverage (75). Among the tick genera prevalent in Africa, the main ticks found in Ethiopia are *Ambylomma* (40 %), *Rhipicephalus (Rh) (Boophilus)* (21 %), *Haemaphysalis* (0.5 %), *Hyalomma* (1.5 %), and *Rhipicephalus* (37 %). Among these, *Amblyomma varigatum* (*A. varigatum*) and *Rh. decoloratus* are the most important and widely distributed (50,74,76). Furthermore, the highest prevalence of babesiosis in domestic animals was recorded during the autumn season (2.99%) while extremely low prevalence in the winter season (0.88%), as indicated in a study conducted in western Ethiopia (85). Similarly, the prevalence of malaria also reached the highest level in autumn (53).

Another report from Bishoftu area, Central Ethiopia, found a prevalence of 0.6%, and similar prevalence values of *B. bigemina* and *B. bovis* (0.3%) were found (12). The result of a microscopic examination of a study from Southern Ethiopia in Teltele District, Borena Zone, indicated the overall prevalence of 16.9% of *Babesia* spp. (9.9% for *B. bovis* and 7% for *B. bigemina*) (87).

1.3.4. Natural history of babesiosis

1.3.4.1.Pathogenesis

The initial targets of *Babesia* spp. sporozoites in a human host are red blood cells (RBCs). In the early stage of *Babesia* spp. in RBCs, its morphology has a ring like structure that is close to the same as that of *Plasmodium* spp.. The replication process follows budding method and the ring like structure forms a "figure eight" shape ahead of division (44). Commonly two and rarely four merozoites are formed during replication (78). The shape created after repeated budding makes "Maltase Cross" which is unique to *Babesia* spp. that helps to microscopically differentiate from *Plasmodium* spp. (44). The replicated merozoites

damage RBCs and exit to look for new cells. Haemolysis occurs at this stage with subsequent clinical manifestations of anaemia, jaundice, and haemoglobinuria. When the affected RBCs are not haemolyzed, the size is reduced so that removed by the spleen (78). Because of the asynchronous multiplication and release of *Babesia* spp., the fever manifested in humans is irregularly intermittent unlike the regularly intermittent fever manifestation in the case of malaria. The multiorgan complication results from proinflammatory cytokine production (78).

1.3.4.2.Clinical features of babesiosis

Babesiosis can present clinically as a subclinical infection or as a fatal severe disease. Babesiosis is severe in the two extreme age groups. Neonates and older age adults manifest the severe form of babesiosis associated with an incompetent immune response to any form of infection. People who underwent splenectomy are at higher risk of complicated babesiosis (44).

Babesia microti has an incubation period of 1-4 weeks after a person is being bitten by *B. microti* infected ticks. After the gradual onset of malaise and fatigue, a fever of up to 40.9°C is manifested (63). Subsequently, the common symptoms are abdominal and back pain, headache, fatigue, haemolysis (78), chills, and sweats (63) along with headache muscle pain, anorexia, non-productive cough, joint pain, and nausea. Moreover, sore throat, vomiting, abdominal pain, photophobia, conjunctival injection, weight loss, depression, emotional lability, and hyperesthesia. Laboratory results may show mild to moderate haemolysis with low haematocrit, haemoglobin, and haptoglobin and elevated reticulocyte count and lactate dehydrogenase level. Thrombocytopenia is also commonly reported (63).

Babesia divergens is common in Europe with sever manifestation compared to *B. microti*. Haemoglobinuria is commonly manifested yet jaundice is also reported due to

haemolysis. Vomiting and diarrhoea are also apparent with subsequent pathological responses of toxins and anoxia resulting from haemolysis. Immunologic responses may cause multiorgan failure (44,88).

1.3.4.3.Prevention and control of babesiosis

Prevention of babesiosis is mainly targeted at ticks and their habitats. Avoidance of ticks and modification of favourable habitats for ticks can reduce the transmission of babesiosis to humans. Using tick repellent is important before entering into tick infested areas (43). Remove ticks immediately if found on the body before attachment. If ticks are feeding on the body, removing ticks is advised to minimize the chance of infection. A study has reported that *B. microti* can be transmitted in 36-48 hours after attachment. Therefore, removing ticks at the earlier possible time is recommended (89). The use of acaricides on animals is also advised despite the possible resistance that ticks could develop (87). There is no vaccine available for humans nor is any trial of vaccine development. Only vaccines for animals, particularly for *B. bovis*, *B. divergens*, and *B. bigemina* are in the making (43). In Europe, destroying the habitats of babesiosis in humans is not recommended as the effect is minor while in the USA use of acaricides and intervening in the habitats of ticks is advised (90). Destroying tick infested area is can't be a successful approach as it is against environmental protection but most importantly ticks have a patchy distribution. Ticks can stay in one patch and bite animals and humans when hosts get access to the area (22,30).

1.4. Tick-borne encephalitis

Tick-Borne Encephalitis is a neuroinfectious diseases caused by tick-borne encephalitis virus (TBEV). European (TBEV_Eu), Siberian (TBEV_Sib), and Far-Eastern (TBEV_FE) are the three common subtypes. *I. ricinus* is the primary vector of TBE_Eu while *I. persulcatus* ticks are known vectors for TBEV_Sib and TBEV_FE (91). The Himalayan

(TBEV-Him) and Baikalian (TBEV-Blk) are among the recently discovered subtype (92,93). It is mainly transmitted through the bite of infected ticks of *Ixodes* spp., *I. ricinus,* and *I. persulcatus,* which are commonly found in Europe and Asia (1,2,94). However, *I. ricinus,* and *I. persulcatus* ticks are considered the primary vectors of TBEV, *D. reticulatus* ticks have also a significant potential of carrying TBE (95). Additionally, TBEV has alimentary transmission rout through consumption of raw milk and milk products (from cows, goats, and sheep) (96), unsafe blood product handling in laboratories, and solid organ transplantation (97) (Figure 10).

Tick-borne encephalitis viruses have a spherical shape that has no projections from their membrane. It has a single stranded positive polarity ribonucleic acid (RNA) molecule of 11kb and a capsid protein (C-12KDa). The lipid membrane also has envelope glycoprotein (E-53K) and glycoprotein membrane (M, 8K). Glycoprotein E contains a viral binding site to interact with host cells (98).

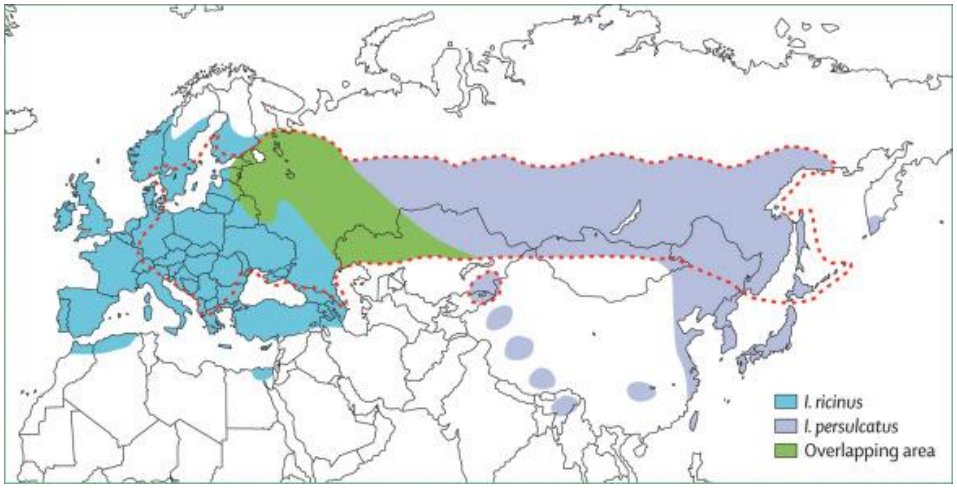


Figure 10. Geographic distribution of *Ixodes* spp. in Europe and Asia

Source: https://doi.org/10.1016/j.idc.2007.12.006 (accessed on October 28.2022)

1.4.1. Tick-borne encephalitis in Europe

The Scandinavian peninsula and central to eastern regions of Europe are referred as TBEV endemic areas. Human TBE instances were found in Europe's Croatia, France, Netherlands, Belgium, and Bulgaria, which were not previously known as TBE endemic regions. Although there have been no reports of TBEV infections in humans in Liechtenstein, Moldova, or South Korea, TBEV was isolated from vectors or animals in these nations (99).

Regardless of the geographical distribution of TBEV subtypes, there is growing evidence showing the cross-boundary presence of cases. A Siberian subtype was reported from South Korea, Mongolia, and Ukraine, and all subtypes were found in Scandinavia (100–103). In recent decades, the European strain of TBEV has spread into non-endemic areas. A phylogenetic-based study conducted in Hungary has revealed that there were strains that have the contents of Finland, Germany, and Russia. Long-distance migratory birds were presumably responsible for the movement of other countries' strains (104). The Siberian subtype was also found in Finland (105), and Estonia and three of the subtypes were found in the Crimean Peninsula (101) (Figure 11).

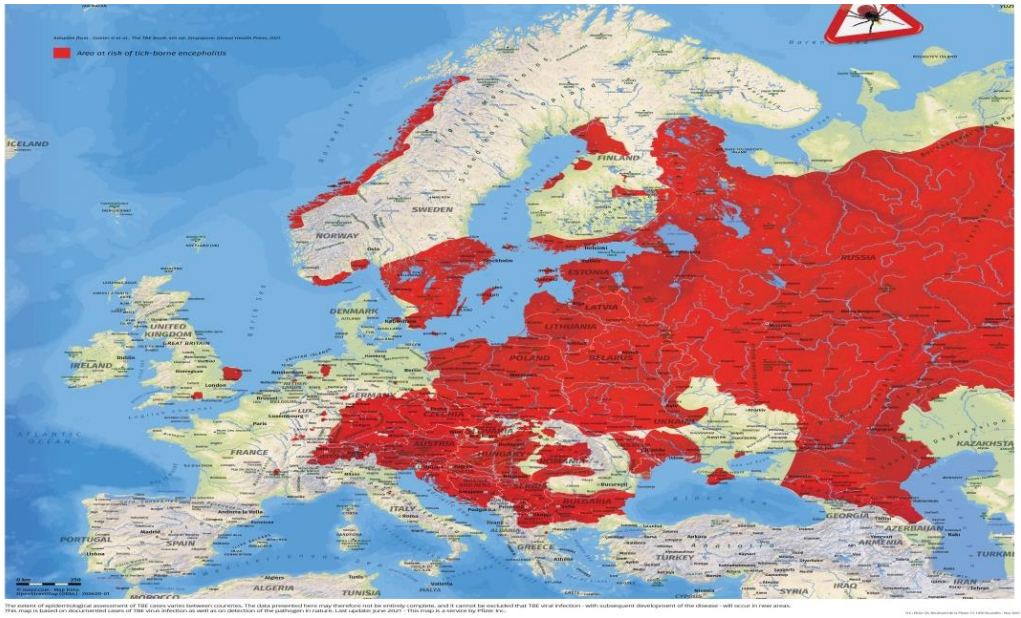
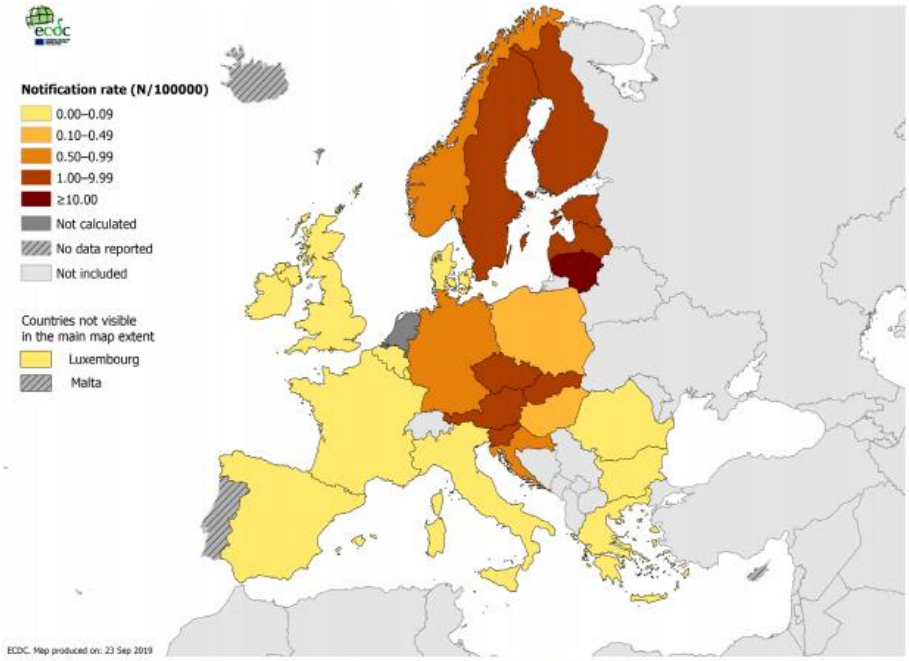


Figure 11. Distribution of TBE in Europe, 2021
Source: map from Wanda (https://www.wanda.be/en/a-z-index/tick-borne-encephalitis-map-of-europe/) (accessed on October 21.2022)

The number of reported TBE occurrences is between 10,000-12,000 per year which is expected to be far lower than the real rate of the disease (9,106). Confirmed cases reported from the European Union/European Economic Area (EU/EEA) countries were 3,092 by the year 2018 with a notification rate of 0.6 per 100,000 population size that has persisted for two more years. In the same area, there is a 60% incidence of TBE in men with the highest rate among those aged 45-64 years (94). The mortality rate related to TBEV-Eu is 0.5-2%, which also causes neurological sequelae in up to 10% of cases contracted of the virus. TBEV-Sib causes a mortality rate of 2-3% and prolonged infection compared to other sub-types. There is also a higher fatality of 40% among TBEV-FE patients with high rates of neurological sequelae outcomes (107,108) (Figure 12).



Source: Country reports from Austria, Belgium, Bulgaria, Croatia, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, the Netherlands, Norway, Poland, Romania, Slovakia, Slovenia, Spain, Sweden and the United Kingdom

Figure 12. Confirmed tick-borne encephalitis case notification rate per 100 000

Source: https://register.ecdc.europa.eu/ (accessed on October 25.2022)

Because of the increased movement of people between forests-suburban areas and urban areas, migration of diseases from the endemic to the non-endemic areas has increased including towns where ticks are not commonly found (9). A study conducted in Moscow revealed that there were six viral isolates of European strains which would have been related to urbanization (109). There are also reports of TBEV cases that came out of the United Kingdom, which otherwise was not known for tick-borne encephalitis (110). Migrating birds also play a role in the dispersal of TBEV infected ticks (111). Migratory birds that moved through Scandinavia had 1-5 ticks, which therefore could facilitate the spread of TBEV (111). For the above reasons, TBE is considered an international public health problem in the field of travel medicine since the migration of subtypes would have been related to the globalized movement of people and other hosts (14,112,113).

The transmission of TBEV occurs by co-feeding, trans-stadial (horizontal), vertical transmission, and sexual transmission. Ticks got infected while co-feeding on hosts without having viremia (39,114). After ingesting blood meal, pathogens remain with the blood ingestion. The TBEV is internalized into gut cells through endosomes while trying to digest the ingested meal. Enveloped TBEV makes fusion to cross the membrane and arrive at the cytoplasm (39).

1.4.2. Tick-borne encephalitis in Poland

According to the Polish National Public Health Institute, the total number of TBE cases reported from the 16 voivodships of Poland from 1999 to 2019 was 4792 (115). From 1999 to 2019, the national average incidence of TBE was 0.6 per 100,000 people, with an average annual number of cases of 228. Among all regions, Podlaskie had the highest average incidence of TBE, with 9 cases per 100,000 inhabitants and an average of 107 cases per year. In Poland, most of the cases were reported from the three eastern and north-eastern regions. Podlaskie had the most with a 2263 total number of cases which encompasses 47.23% of the total cases reported in the past 21 years. The second most affected region that borders Podlaskie in the north is Warminisko-Mazurskie with a case number of 1246 from 1999-2019. Podlaskie and Warminisko-Mazurskie continued as the most affected regions above the national average rate of TBE per 100000 population size from 1999 to 2019 (115).

Although most of the cases were reported from eastern and north-eastern regions, Dolnoslaskie came to be the fourth affecter region with 188 cases situated in the western part of Poland. Mazowieckie had a lesser number of cases in the early 2000s, however, the number of cases made the third most affected region except in 2005 when Opolskie dominated it. Since 2010 the curve of Dolnoslaskie had continuously increased except in

2015 when the number slightly declined. Since 2015, Dolnoslaskie and Lubelskie have shown a persistently increasing record of cases (115) (Table 1).

Table 1. Number of TBE cases and TBE incidence in Poland and Podlaskie Voivodeship in the years 1999-2019

Year	TBE cases in Poland	TBE cases in Podlaskie Voivodeship	TBE incidence in Poland	TBE incidence in Podlaskie Voivodeship
1999	101	42	0.26	3.43
2000	170	62	0.44	5.07
2001	210	104	0.54	8.52
2002	126	70	0.33	5.79
2003	339	160	0.89	13.27
2004	262	113	0.69	9.39
2005	177	94	0.46	7.83
2006	317	155	0.83	12.94
2007	233	98	0.61	8.2
2008	202	97	0.53	8.14
2009	351	139	0.92	11.67
2010	294	137	0.77	11.52
2011	221	89	0.57	7.4
2012	190	105	0.49	8.75
2013	227	111	0.59	9.28
2014	195	109	0.51	9.13
2015	149	77	0.39	6.47
2016	283	160	0.74	13.47
2017	283	160	0.74	13.5
2018	197	73	0.51	6.17
2019	265	108	0.69	9.16

However, Mazowieckie had the third most frequent number of cases Opolskie and Swietokrzyskie had a greater average rate of 0.60 and 0.39 cases in 100,000 from 1999-2019 populations at risk respectively. The 21 years national average rate was 0.6 while Podlaskie and Warminisko-Mazurskie had a far greater average rate of 9 and 4.13 cases per 100,000 populations, respectively. In recent years, the number of cases and rate of TBE in Dolnoslaskie and Lubelskie is rising although the curve of other regions is variable with

time. This shows that TBE is spreading from eastern and north-eastern Poland to south-eastern and south-western Poland. This needs further investigation on whether the number of reported cases emerging in these regions is associated with domestic expansion or migration from neighbouring countries (115).

1.4.3. Natural history of tick-borne encephalitis

1.4.3.1.Pathogenesis

Once TBEV enters the human body it takes on average 7-14 days of incubation period which can also be extended between 2-28 days (116–118). In the cases of milk-borne acquired TBEV, the incubation period is 3-4 days (118). TBE induced by TBEV_Eu has a bi-phasic course while the other sub-types manifest central nervous system manifestations (118,119). In the biphasic course, during the early days of the infection, non-specific flue like manifestations occur including febrile body temperature, fatigue, headache, and muscle ache. The incubation of TBEV starts at the site of entry, particularly at the Langerhans cells (dendritic skin cells) that take the virus to the nearby lymph nodes. Subsequently, T-cells, B-cells, and macrophages are the subsequent sites of incubation. The virus leaves the lymph nodes and joins the bloodstream and reaches the brain (116). The virus could cross the blood-brain barrier through one or more such ways as peripheral nerves, highly susceptible olfactory neurons, transcytosis through vascular endothelial cells of brain capillaries, and diffusion of the virus between capillary endothelial cells. The primary targets of TBEV infection in the central nervous system are neurons (118).

TBE can be presented as meningitis, meningoencephalitis, and meningoencephalomyelitis. It affects all age groups with the highest burden on the older age group with long-term complications of sequelae (116,117). The brain and meninges of both cerebral and spinal are affected by the virus. Defused infiltration on the meninges and

oedema in the brain with microscopic lesions are apparent in most parts of the central nervous system (117).

1.4.3.2.Clinical features of tick-borne encephalitis

After the incubation period, two-thirds of adults experience a prodromal period, which is more severe in meningoencephalitis and meningitis than in encephalomyelitis. The prodromal symptoms include headache, fatigue, muscle pain, and fever up to 39℃ lasting for 2–7 days. In the second phase of TBE, fever with a 1-2℃ rise from the prodromal period presents as meningitis in 50%, meningoencephalitis in 40%, and meningoencephalmyelitis in 10% of patients (107,116). TBE also affects cranial nerves that cause cranial neuritis mostly unilateral with the involvement of facial, ocular, vestibular, and pharyngeal nerves (107). A chronic progressive form of TBE is far from happening in the case of TBE_FE, however, there are indications of long-term outcomes in TBE_FE. The TBE_FE has such clinical manifestations as Kozhevnikov's epilepsy, neuritis of the shoulder plexus, progressive muscle atrophy, lateral sclerosis, and Parkinson-like disease. Physical deterioration is frequently accompanied by mental deterioration (120).

1.4.3.3.Tick-borne encephalitis control and prevention

TBE vaccination is advised for those over the age of 1 year in areas with a high incidence (5 cases/100,000/year) however those who are at risk in places with a lower frequency are also recommended to take it. Those visiting endemic regions who would involve in engaging in significant outdoor activity also should get vaccinated (121). The four vaccines available are based on formalin inactivated viruses. Two of them FSME-IMMUN®/TicoVac® have been produced by Baxter (Vienna, Austria) which has its market

authorization from Pfizer. Encepur® has been manufactured by Novartis (Marburg, Germany) now owned by (Bavarian Nordic) and is the second vaccine produced based on a European strain. The other two vaccines which have been manufactured based on the Far Eastern subtype are TBE-Moscow (Chumakov Institute, Moscow) and EnceVir (Microgen, Tomsk). The latter two are not authorized in Europe. Additionally, China has also vaccines based on the Fare-Eastern subtype manufactured by the Changchun Institute of Biological Products (121). The vaccine failure rate is below 5%, most importantly those who have started the vaccine after the age of 50 years could have more failure rate (122).

2. Aim

Babesiosis is an emerging disease of importance to both humans and livestock. In humans, it is more important in USA and Europe than in Africa. This study is interested in investigating the proximity and variations between *Babesia* spp. of Poland and Ethiopia. The sequencing part of this study will be served as input for further studies on whether *Babesia* spp. in the two areas is risky for human health. Meanwhile in Ethiopia, it will open a new idea of investigating diseases with similar manifestations like malaria.

Controlling TBE is becoming more difficult as the number of cases grows due to the emergence of new variants and transboundary migration of subtypes (9,111). Some preceding studies have recommended further investigations on the contents of the virus to step up into considerations of evidence based TBE interventions (123–125). Moreover, the migration of subtypes out of their previous habitat required molecular evidence along with the depth of the problem so that the right intervention is provided as per the strain of the virus (9,111,113).

Therefore, the aims of the study were:

1. To detect and characterize *Babesia* spp. and TBEV using PCR assays in ticks collected from Poland and Ethiopia.
2. To investigate the prevalence and strain variation between *Babesia* spp. of Poland and Ethiopia.
3. To analyse the potential predictors (temperature, humidity, developmental stage, and species) of tick-borne pathogens detection in ticks collected from Poland and Ethiopia.

3. Material and methods

3.1. Tick collection

The ticks used in this study were collected from Knyszyn forest landscape park in Poland's north-east region, as well as multiple sites in Ethiopia. Knyszyn Forest Landscape Park is located in the Podlaskie region at the coordinate point of 53°14′00″N 23°23′00″E (126) (Figure 13). The region is known to have reported the highest number of TBD (127). The ecology of the park has the entire essentials for ticks' shelter. There are rodents and other vertebrates that boost the movement of ticks in the park along with varied fauna. The park is located around the touristic town of Supraśl and 19 km away from the capital of Podlaskie Białystok (126). In Supraśl, a town where the park surrounds it, the highest temperature is recorded from May 16-September 8 with an average daily temperature of 18.33ºC. July is the hottest month with a low of 1.67℃ and a high 24℃ while the coldest month in the area is January with -5.26℃ to -0.56℃ (128). The preliminary observation within the park has demonstrated that there were ticks at different developmental stages. Therefore, this site is chosen as a site for tick collection. Ticks were also collected from multiple sites in Ethiopia, to achieve one of the study's objectives, which is to compare *Babesia* spp. in Ethiopia and Poland.

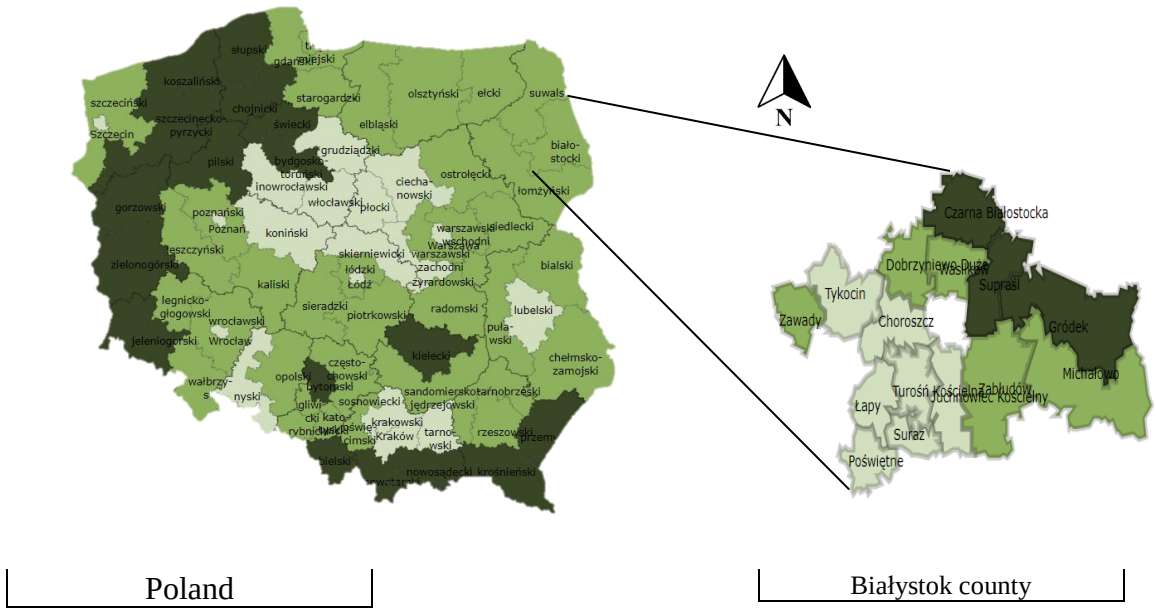


Figure 13. Map of Poland with Białystok county where ticks were collected

Source: http://swaid.stat.gov.pl/EN/SitePages/StronaGlownaDBW.aspx (accessed on November 13.2022

Questing *I. ricinus* and *D. reticulatus* ticks were collected using white flagging from Knyszyn forest near Białystok in north-eastern Poland. The park is located 19 km from Białystok which surrounds a small town called Supraśl. It is a natural reserve for plants and animals. It has pine trees, meadows, rodents, lynxes, European bison, European beaver, red deer, fox, moose, mouse, voles, wolves, birds, and other animals. People are visiting the park for a picnic, beery picking, and forestry. There are also residents in some parts of it. Supraśl is also a known tourist destination in the region. Both *I. ricinus* and *D. reticulatus* were collected between September-November 2020 and May 2021. Temperature, humidity, and precipitation during the time of collection were registered.

The Supraśl river and its tributaries run through the park, with a small beach and pound near an open market. There is a bike ride route from Białystok to Supraśl. There are swampy areas within the park. Ferns, mosses, liverworts, and lichens are abundantly available.

Non-rainy weather was convenient to collect ticks. Each tick was stored in 2ml Eppendorf tube. Morphological identification of ticks' species, developmental stage, sex,

and presence of any material on the body was identified ahead of further processing. Before extraction of nucleic acid, ticks were kept at 4^{o}C. Ticks that developed fungus were excluded from further investigation. Both dead and alive ticks were used to extract RNA and DNA.

For *Babesia* spp., the difference between ticks of Poland and Ethiopia was conducted however, there was not enough evidence to conclude there was a statistically significant difference between the two countries *Babesia* spp. detection rate. Ticks from Ethiopia were collected from Dembidollo, Gambella, Bishoftu, and Boset districts. These areas are know to have domestic and wild animals. Previous reports have shown that ticks and tick-borne diseases of animals are common in these areas (129) (Figure 14).

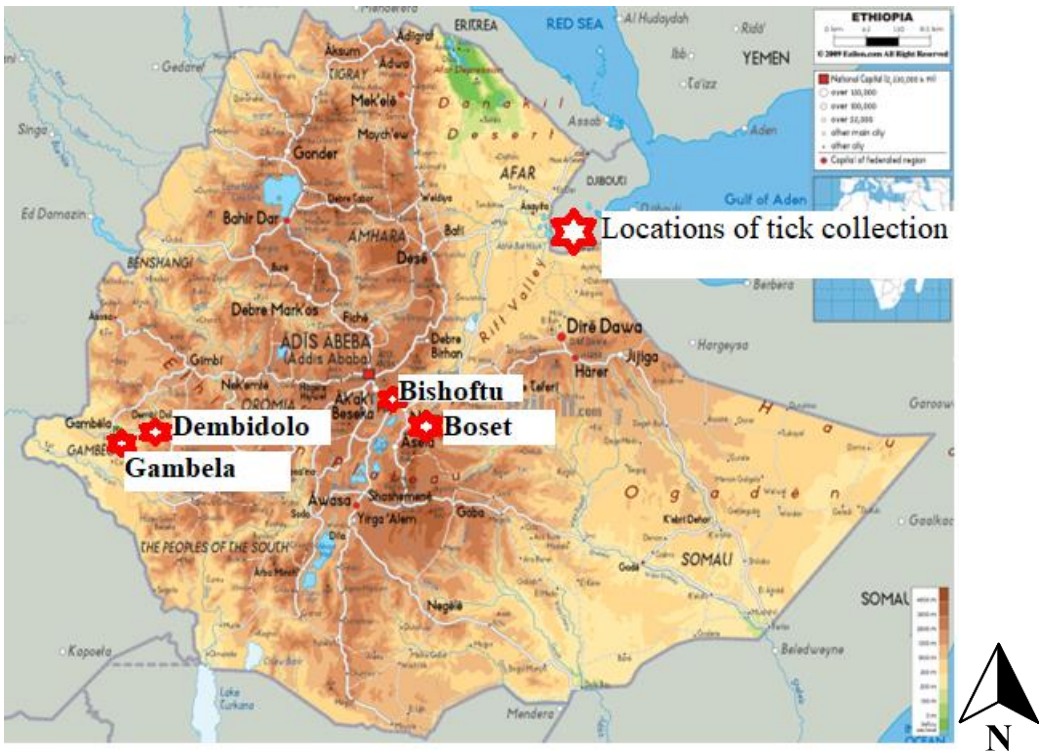


Figure 14. Locations of tick collection in central and western Ethiopia

The developmental stage of ticks was classified depending on developmental stage (larve, nymph and adult), sex (male and female) and temperature as categories of 10-14.9°C and 15-17°C. Classification of temperature was based on the effect of temperature on the

life of ticks. A temperature of <5°C is convenient for adult ticks to start questing while 10°C is for nymphs and larvae. Ticks are stable at 15-25°C. On the other hand, the density of ticks is exponential below 15°C then gets linear between 15-17°C (130).

3.2. DNA isolation

Mortar and pestle were used to crush ticks using 1.65mL of Phosphate Buffer Saline (PBS). After centrifugation (8000 rpm by 5 min) about 1 ml of supernatant was removed and rest of homogenate was used for nucleic acid extraction. Half a portion of the homogenate was used for DNA isolation while the remaining was used for RNA isolation. DNA extraction was conducted using extraction kit (EurX DNA isolation kit, Gdansk, Poland) according to the manufacturer's instruction. An amount of 100µL was collected from each tick sample and put in a storage of temperature -20℃ until further use of the samples.

3.3. RNA isolation

From the half homogenate of each tick, RNA was extracted. Qiagen RNeasy Kits (Qiagen, Germany) was used as per the instruction of the manufacturer. Finally, 60µL of RNA extract was collected and stored at -20℃ temperature. To prevent damage during thawing and refreezing 14.6µL of RNA isolate was stored in separate tubes from each sample at a -80℃ temperature. This portion was used in cases of positive in Real-time PCR TBEV samples for sequencing.

3.4. Polymerase chain reaction - amplification

All Polymerase Chain Reaction (PCR) reactions for *Babesia* spp. were run using SensQuest Labcycler (SensoQuest, Göttingen, Germany). *Babesia* spp. was detected using a fragment of the *18S rDNA* gene that located at the conserved region of V4. The reaction

protocols are designed based on previous methods used by other researchers. The primers (F2 (5` GAC ACA GGG AGG TAG TGA CAA G 3`) and R2 (5`- biotin CTA AGA ATT TCA CCT CTG ACA GT 3`)) for *Babesia* protozoa manufactured by Sigma Aldrich (Germany) were utilised to perform end-point PCR by using *Taq PCR Core Kit* (Qiagen, Germany). The total volume used in the reaction was 15 µl that included 3 µl of DNA for *Babesia* spp., 4 µl buffer x 10 with 15 mM $MgCl_2$ (Qiagen, Germany), 1 µl 25mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl 20 µM of each primer 0.125 µl (5U/µl) Taq DNA polymerase (Qiagen, Germany) and 9,375 µl of ultra-pure water (Sigma Aldrich, Germany). The Amplification program have used 94ºC for 3 minutes for initial denaturing, 40 cycles (94ºC for 40 seconds to denature, 58ºC for 60 seconds to anneal, and 72ºC for 60s for extension) and 72ºC for 10 minutes to make the final extension (82,131–133).

3.5. Electrophoresis

Specificity of the received PCR products was confirmed by electrophoresis in 2% agarose gel (Sigma- Aldrich, Germany, 3gm 10% TBE with 5µL Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany), in 90V by 55 minutes for *Babesia spp.* The results obtained were visualized by Gel Logic System 100 camera (Kodak, Imaging System, Inc., United States of America). As a positive control were used extracts from mause whole blood infected in laboratory with *Babesia* parasite, which indicates high level of parasitemia. Size of positive amplicons was from 410 – 600 bp. To each course of electrophoresis was used Molecular Weight Marker (BLIRT-DNA Gdańsk, Poland).

3.5.1. Sequencing analysis for *Babesia* spp.

Samples positive for *Babesia* spp. were sequenced by Macrogen (Amsterdam, The Netherlands). 5 µl of obtained amplification products were mixed with specific primers

previously used for end-point PCR. Prepared samples were sent to Macrogen, where they were sequenced from both sides (forward and reverse).

The obtained sequences were compared with sequences collected in the NCBI database (http://www.ncbi.nlm.nih.gov) and check for homology and cover index.

3.6. Tick-borne encephalitis virus detection

The detection of RNA of TBEV was conducted using amplification of RNA products was conducted using Amplisense® TBE-FRT PCR kit. Such components of mixes as FEP/FRT TBE-10μL, FPE/FRT (mix2)-5μL, polymerase (TaqF)-0.5μL, TM-Revertase (mmlv)-0.25μL, RT-G-mix-2-0.25μL, and internal control of 5μL per each sample were used. The amplification program was as it is shown in the table below (Table 2).

Table 2. Amplification program used to detect TBEV using qualitative RT-PCR

Cycle	Temperature in ℃	Time
Hold	50	30 minutes
Hold 2	95	15 minutes
Cycling	95	10 seconds
	65	45 seconds
	72	15 seconds
Cycling 2	95	10 seconds
	60	45 seconds
	72	15 seconds

3.7. Sequencing analysis for tick-borne encephalitis virus

Heminested Reverse Transcription-PCR for the detection of Flaviviruses targeted to a conserved region of the NS5 Gene was used (134).

Positive PCR samples were sequenced. All sequences obtained from ticks were identical to each other as shown here:

ATAGAAGAGTCCTCCATTCAGAGTTGACAACTTCTTGAGGTGCCAGCCCA
GATAGTTCAAGCTTATTCCCTCAACTCCAGCTCCACTGGACTCTCTAGAGGCCC
AATGGTCTTCATTCAAGAATCCAAGAGCCTCGAACTCCAGAAAGCGACTCCCC
AGCCACATGT

3.8. Statistical analysis

Statistical analysis was conducted using STATA software version 14. The proportion of positive samples against temperature, humidity, sex of ticks, the season of tick collection, developmental stage of ticks, species of ticks, and life status (dead or alive) at the time of extraction.

Binary and ordinal logistic regression between each factor mentioned above against the proportion of TBEV was conducted to evaluate the influence of predictor variables. Both univariable and multivariable logistic regression were employed with a p-value of statistical significance <0.05.

4. Results

4.1. Characteristics of ticks

A total of 995 (727 from Poland and 268 from Ethiopia) ticks were collected and examined to detect whether ticks were infected with TBEV and/or *Babesia* spp.. A total of 703 RNA samples were extracted from ticks collected in Poland. Thirty-nine (5.55%) of ticks were positive for TBEV out 703 ticks. Eighty-five (9.51%) of ticks out of 894 ticks were positive for *Babesia* spp. Among the 626 ticks from Poland 61 (9.74%) and out of the 268 ticks from Ethiopia, 24 (8.96%) of them were positive for *Babesia* spp. A total of 601 ticks have been tested for both *Babesia* spp. and TBEV. Seven (1.17%) out of the 601 ticks had co-infection of *Babesia* spp. and TBEV while 86 (14.31%) of ticks had monoinfection. Slightly more than half (54.36%) of the ticks collected from Poland were nymphs while the remaining were adult ticks. Two hundred ninety-two (41.24%) of the ticks were collected between the temperature of 10-14.9°C while 416 (58.76%) had 15-17°C of ambient temperature. Only 25 (2.51%) were alive during crushing. *D. reticulatus* accounts for 446 (61.35%) while the remaining were *I. ricinus*- 281 (38.65%). Among ticks collected from Ethiopia *A. gamla* and *A. veriegatun* account for 60 (22.39%) each species followed by 47 (17.54%) *R. pulchellus* and *A. cohaerens* 39 (14.55%) (Table 3).

Table 3. Characteristics of ticks examined to detect *Babesia* spp. and TBEV

Characteristics	n	%
Country (n=995)		
Ethiopia	268	26.93
Poland	727	73.07
Babesia spp.(n=894)		
Positive	85	9.51
TBEV (n=703)		
Positive	39	5.55
Co-infection(n=601)		
Co-infected	7	1.17
Monoinfection	86	14.31
Sex (n=734)		
Nymph	399	54.36
Male	113	15.4
Female	222	30.25
Species (n=995)		
D. reticulatus	446	44.82
I. ricinus	281	28.24
Amblyomma vergienatum	36	3.618
Amblyomma cohaerens	39	3.92
Amblyomma gamla	60	6.03
Amblyomma variegatum	60	6.03
Recipephalus trurcusus	8	0.804
Rhipicephalus decoloratus	18	1.809
Rhipicephalus pulchellus	47	4.724
Temperature in ℃ (n=708)		
10-14.9	292	41.24
15-17	416	58.76
Life status (n=995)		
Alive	25	2.51
Dead	970	97.49
Species ticks from Poland (n=727)		
D. reticulatus	446	61.35
I. ricinus	281	38.65
Ticks Species from Ethiopia (n=268)		
Amblyomma vergienatum	36	13.43
Amblyomma cohaerens	39	14.55
Amblyomma gamla	60	22.39
Amblyomma variegatum	60	22.39
Recipephalus trurcusus	8	2.99
Rhipicephalus decoloratus	18	6.72
Rhipicephalus pulchellus	47	17.54

4.2. *Babesia* spp. and tick-borne encephalitis in *D. reticulatus* ticks

Babesia spp. was detected in 40 (9.83%) of the 407 *D. reticulatus* ticks. Of all 432 *D. reticulatus* ticks, 38 (8.81%) were positive for TBEV. From *D. reticulatus* ticks 6 (1.75%) had co-infection of TBEV and *Babesia* spp., 142 (31.35%) were female, and 66 (14.57%) were male. Two hundred thirty-three (52.95%) of the *D. reticulatus* ticks were collected under the ambient temperature of 15-17^{o}C. The majority (429 (94.7%) of *D. reticulatus* ticks were dead during extraction (Table 4).

Table 4. Characteristics of *D. reticulatus* ticks examined to detect TBEV and *Babesia* spp.

Characteristics	n	%
Babesia spp. (n=407)		
Positive	40	9.83
TBEV (n=432)		
Positive	38	8.81
Co-infection(n=399)		
Co-infected	7	1.75
Monoinfection	64	16.04
Sex (n=453)		
Nymph	245	54.08
Female	142	31.35
Male	66	14.57
Temperature (n=440)		
10-14.9	207	47.05
15-17	233	52.95
Life status (n=453)		
Alive	24	5.3
Dead	429	94.7

4.3. *Babesia* spp. and tick-borne encephalitis virus in *I. ricinus* ticks

In *I. ricinus* ticks *Babesia* spp. was detected in 21 (9.59%) ticks while TBEV was detected in 1 (0.37%). There was no *Babesia* spp. and TBEV coinfection in *I. ricinus*. Slightly

more than half (54.08%) of *I. ricinus* ticks were nymphs, while 80 (28.47%) were adult females. The ambient temperature during tick collection was registered at 15-17℃ for 181 (68.05%) *I. ricinus* ticks (Table 5).

Table 5. Characteristics of *I. ricinus* ticks examined to detect TBEV and *Babesia* spp.

Characteristics	n	%
TBEV (n=271)		
Positive	1	0.37
Babesia spp. (n=219)		
Positive	21	9.59
Co-infection(n=216)		
coinfection		
Monoinfection	22	10.19
Sex (n=281)		
Nymph	154	54.8
Female	80	28.47
Male	47	16.73
Temperature (n=266)		
10-14.9	85	31.95
15-17	181	68.05
Life status(n=281)		
Alive	1	0.36
Dead	280	99.64

4.4. *Babesia* spp. sequencing

Through the sequencing analysis of *Babesia* spp. 70.59% (60/85) of the samples were *B. microti* with a mean homology of 87.56% that ranges between 82.29%-100%. Apart from *B. microti*, *Theileria velifera* 8.24% (7/85), *B. capreoli* 4.71% (4/85), *B. venatorum* and *B. canis* each 3.53% (3/85), and *Theileria mutans* 2.35% (2/85) were also detected via sequencing (Table 6).

Table 6. Number and percentage of *Babesia* spp. and *Theileria* spp. subtypes in ticks collected from Poland and Ethiopia (n=85)

Babesia sub-types	n	%
B. canis	3	3.53
B. capreoli	4	4.71
B. divergens	1	1.18
B. microti	60	70.59
B. odocalei	1	1.18
B. venatorum	3	3.53
Babesia bigemina	1	1.18
Babesia spp. Badger type B	1	1.18
Theileria equi	1	1.18
Theileria mutans	2	2.35
Theileria species	1	1.18
Theileria velifera	7	8.24
Total	85	100

Of all the *Babesia* spp. detected 49.18% (30/61), 16.39% (10/61), and 34.43% (21/61) were detected from an adult female, male, and nymph ticks collected from Poland respectively. Close to half (24 of the 45) of *B. microti* were detected from adult female ticks while 4 and 17 were detected from adult male and nymph ticks respectively (Table **7**).

Table 7. *Babesia* spp. sub-types per sex and developmental stage in ticks collected from Poland (n=61)

Genospecies	Female	Male	Nymph	Total	%
B. canis	3	0	0	3	4.92
B. capreoli	2	2	0	4	6.56
B. divergens	0	1	0	1	1.64
B. microti	24	4	17	45	73.77
B. odocalei	0	0	1	1	1.64
B. venatorum	0	1	2	3	4.92
Babesia spp. Badger	0	1	0	1	1.64
Theileria equi	1	0	0	1	1.64
Theileria mutans	0	1	0	1	1.64
Theileria velifera	0	0	1	1	1.64
Total	30	10	21	61	100

Among the *Babesia* spp. detected from ticks in Poland, 73.77% (45/61) of them were *B. microti* with a mean homology of 97.69% that ranges between 82.29-100%. *B. microti* was detected from 29 and 16 *D. reticulatus* and *I. ricinus*, respectively (Table 8).

Table 8. *Babesia* spp. and *Theileria* spp. subtypes per type of tick species in ticks collected from Poland

	Species of ticks		
Genospecies	*D. reticulatus* (n=407)	*I. ricinus* (n=219)	Total (n=626)
B. canis	3 (0.49)	0 (0)	3 (0.48)
B. capreoli	2 (0.74)	2 (0.91)	4 (0.64)
B. divergens	0 (0)	1 (0.46)	1 (0.16)
B. microti	29 (7.13)	16 (6.85)	45 (7.03)
B. odocalei	1 (0.25)	0 (0)	1 (0.16)
B. venatorum	2 (0.49)	1 (0.46)	3 (0.48)
Babesia spp. Badger	1 (0.25)	0 (0)	1 (0.16)
Theileria equi	0 (0)	1 (0.46)	1 (0.16)
Theileria mutans	1 (0.25)	0 (0)	1 (0.16)
Theileria velifera	1 (0.25	0 (0)	1 (0.16)
Total	40 (9.83)	21 (9.59)	61 (9.74)

With regard to the distribution of *Babesia* spp. strains in *D. reticulatus* and *I. ricinus*, there were seven identified strain. The overall *Babesia* spp. among ticks collected from north-eastern Poland was 9.29% with highest incidence of *B. microti* that accounts 7.19% prevalence in 626 tick sample (Table 9).

Table 9. *Babesia* spp. strain subtypes per type of tick species in ticks collected from Poland

	Species of ticks					
	D. reticulatus (n=407)		*I. ricinus* (n=219)			Homology
Genospecies	n	%	n	%	Mean	Min/max
B. canis	3	0.74	0	0.00	99.53	99.18/99.72
B. capreoli	2	0.49	2	0.91	97.52	93.85/99.72
B. divergens	0	0.00	1	0.46	87.90	
B. microti	29	7.13	16	7.31	98.61	89.36/100
B. odocalei	1	0.25	0	0.00	82.29	
B. venatorum	2	0.49	1	0.46	99.15	98.87/99.42
Babesia spp. Badger	1	0.25	0	0.00	88.0	
Total	38	9.34	20	9.13		

The combined prevalence of *Babesia* spp. and *Theileria* spp. in ticks from Ethiopia was 8.96% (24/268). The prevalence of *B. microti* accounts for 5.61% (15/268) and 11 had infected *A. gamla* and 4 infected *A. vergienatum*. *Theileria* spp. were also detected in 2.24% (6/268) ticks collected from Ethiopia. Six of the eight *Theileria* spp. were *Theileria velifera* detected from 4 *A. cohaerens* and 2 from *R. decoloratus* (Table 10).

Table 10. Number of *Babesia* spp. and *Theileria* spp. detected in ticks collected from Ethiopia (n=268).

	Species				
Genospecies	*A. vergienatum*	*A. cohaerens*	*A. gamla*	*R. decoloratus*	Total
B. microti	4	0	11	0	15 (5.61)
B. bigemina	0	0	0	1	1 (0.37)
T. mutans	0	1	0	0	1 (0.37)
Theileria spp.	0	0	0	1	1 (0.37)
T. velifera	0	4	0	2	6 (2.24)
Total	3 (1.12)	5 (1.87)	11 (4.10)	4 (1.49)	24 (8.96)

4.5. Evolutionary relationships of taxa

The evolutionary history of the various *Theileria* and *Babesia* genospecies was inferred by using the Neighbor-Joining method (135). Tamura-Nei method was used to compute the evolutionary distances (136) considering base substitution in each site as a unit while MEGA X was used to run the evolutionary analyses (137). Interactive tree of life -iTOL v61 was also used for subsequent phylogenetic tree visualization (138) (Figure 15, Figure 16). This analysis involved 74 nucleotide sequences for *Babesia* spp. isolated from *D. reticulatus, I. ricinus, A. vergienatum,* and *R. decoloratus* (364 bp) 11 nucleotide sequences for *Theileria* isolated from *A. cohaerens R. decoloratus D. reticulatus,* and *I. ricinus* (373 bp) (Table 11).

Table 11. Subtypes of *Babesia* spp. and *Theileria* spp. identified using sequencing

Lab Nr	Genospecies	Homology (%)	Query Cover (%)	Gen Bank Accession Number	Bp	Tick Species	Stage/Sex	Country
2	*Theileria equi*	100.00	6	MT613662.1	434	*Ixodes ricinus*	Female	Poland
8	*B. microti*	98.94	99	MH351724.1	379	*Dermacentor reticulatus*	Female	Poland
12	*B. microti*	100.00	80.00	OL549366.1	484	*Dermacentor reticulatus*	Female	Poland
29	*B. microti*	98.21	91.00	MG199177.1	406	*Dermacentor reticulatus*	Female	Poland
49	*B. microti*	91.82	90.00	OL549366.1	419	*Ixodes ricinus*	Female	Poland
54	*B. microti*	99.49	85.00	JX627356.1	455	*Dermacentor reticulatus*	Female	Poland
56	*Theileria velifera*	96.07	62	MK792966.1	555	*Amblyomma cohaerens*	Adult	Ethiopia
58	*Theileria mutans*	85.56	78	KX882754.1	449	*Amblyomma cohaerens*	Adult	Ethiopia
63	*Theileria velifera*	99.47	85	MK792966.1	510	*Amblyomma cohaerens*	Adult	Ethiopia
64	*Theileria velifera*	96	97	LC431549.1	392	*Amblyomma cohaerens*	Adult	Ethiopia
65	*Theileria velifera*	96.40	88	MK792966.1	538	*Amblyomma cohaerens*	Adult	Ethiopia
66	*B. microti*	99.22	97.00	KX591647.1	517	*Amblyomma variegatum*	Adult	Ethiopia
114	*Theileria velifera*	87.83	65	LC431551.1	488	*Rhipicephalus decoloratus*	Adult	Ethiopia
118	*Babesia bigemina*	99.13	73	MH050387.1	542	*Rhipicephalus decoloratus*	Adult	Ethiopia
122	*Theileria velifera*	92.33	85	LC431551.1	459	*Rhipicephalus decoloratus*	Adult	Ethiopia
127	*Theileria species*	96.25	75	MT239527.1	568	*Rhipicephalus decoloratus*	Adult	Ethiopia
220	*B. microti*	98.69	74	MH553358.1	514	*Amblyomma gamla*	Adult	Ethiopia
223	*B. microti*	99.08	27	KP055639.1	588	*Amblyomma gamla*	Adult	Ethiopia
224	*B. microti*	100.00	99.00	KU550682.1	474	*Amblyomma gamla*	Adult	Ethiopia
227	*B. microti*	99.54	98.00	MH628094.1	457	*Amblyomma gamla*	Adult	Ethiopia
228	*B. microti*	99.46	78	MH553358.1	530	*Amblyomma gamla*	Adult	Ethiopia
229	*B. microti*	99.47	81.00	KT869379.1	463	*Amblyomma gamla*	Adult	Ethiopia
230	*B. microti*	99.25	64	MH628094.1	479	*Amblyomma gamla*	Adult	Ethiopia
230	*B. microti*	93.61	52.00	MW554606.1	500	*Amblyomma gamla*	Adult	Ethiopia
242	*B. microti*	98.94	66.00	OM0066130.1	457	*Amblyomma gamla*	Adult	Ethiopia

Lab Nr	Genospecies	Homology (%)	Query Cover (%)	Gen Bank Accession Number	Bp	Tick Species	Stage/Sex	Country
245	*B. microti*	98.68	74.00	MH628094.1	511	*Amblyomma vergienatum*	Adult	Ethiopia
250	*B. microti*	99.70	72	KU955526.1	517	*Amblyomma vergienatum*	Adult	Ethiopia
255	*B. microti*	100.00	99.00	MH351713.1	515	*Amblyomma vergienatum*	Adult	Ethiopia
323	*B. microti*	98.96	96.00	KX161765.1	400	*Dermacentor reticulatus*	Female	Poland
325	*B. microti*	98.78	47	KY649347.1	518	*Dermacentor reticulatus*	Female	Poland
328	*B. capreoli*	97.87	93	MN296295.1	543	*Dermacentor reticulatus*	Male	Poland
330	*B. microti*	99.96	97.00	MW791419.1	496	*Dermacentor reticulatus*	Female	Poland
332	*Theileria mutans*	84.68	53	MK792976.1	401	*Dermacentor reticulatus*	Male	Poland
333	*B. microti*	98.74	82	MN355504.1	501	*Dermacentor reticulatus*	Female	Poland
335	*B. microti*	98.72	97	MH35173.1	401	*Dermacentor reticulatus*	Female	Poland
338	*B. microti*	89.36	90.00	MW791419.1	530	*Dermacentor reticulatus*	Female	Poland
339	*B. microti*	96.34	94.00	MW791419.1	404	*Dermacentor reticulatus*	Female	Poland
371	*B. microti*	97.86	69	MN355504.1	403	*Dermacentor reticulatus*	Male	Poland
468	*Theileria velifera*	92.88	73	AF097993.1	454	*Dermacentor reticulatus*	Nymph	Poland
510	*B. microti*	99.49	94	MN355504.1	457	*Dermacentor reticulatus*	Nymph	Poland
633	*B. odocalei*	82.29	71	MT830846.1	538	*Dermacentor reticulatus*	Nymph	Poland
635	*B. microti*	99.73	99.00	MH628094.1	457	*Ixodes ricinus*	Nymph	Poland
646	*B. venatorum*	98.87	81	MH351698.1	530	*Dermacentor reticulatus*	Nymph	Poland
652	*B. capreoli*	93.85	42	MT151374.1	454	*Ixodes ricinus*	Male	Poland
669	*B. microti*	98.68	92	KT869379.1	409	*Ixodes ricinus*	Nymph	Poland
675	*B. venatorum*	99.16	44	JX051870.1	549	*Ixodes ricinus*	Male	Poland
688	*B. microti*	98.36	67	OM0066130.1	534	*Ixodes ricinus*	Female	Poland
737	*B. microti*	93.06	69	MN355504.1	520	*Ixodes ricinus*	Nymph	Poland
783	*B. canis*	99.72	61	MN173223.1	556	*Dermacentor reticulatus*	Female	Poland
789	*B. canis*	99.18	89	MN173223.1	407	*Dermacentor reticulatus*	Female	Poland
795	*B. canis*	99.7	81	MK591947.1	512	*Dermacentor reticulatus*	Female	Poland

Lab Nr	Genospecies	Homology (%)	Query Cover (%)	Gen Bank Accession Number	Bp	Tick Species	Stage/Sex	Country
797	*B. capreoli*	99.71	94	MN296295.1	540	*Dermacentor reticulatus*	Female	Poland
799	*B. microti*	100.00	80	KX161765.1	423	*Dermacentor reticulatus*	Nymph	Poland
803	*B. microti*	100.00	94	KC470047.1	408	*Dermacentor reticulatus*	Nymph	Poland
804	*B. microti*	98.91	77	AF373332.1	480	*Ixodes ricinus*	Male	Poland
807	*B. microti*	99.46	91	AF494286.1	573	*Ixodes ricinus*	Female	Poland
808	*Babesia spp. Badger type B*	88.00	24.00	MK733579.1	402	*Dermacentor reticulatus*	Male	Poland
813	*B. microti*	99.17	65	MH628094.1	551	*Dermacentor reticulatus*	Female	Poland
816	*B. microti*	99.62	56	MN355504.1	481	*Dermacentor reticulatus*	Female	Poland
818	*B. microti*	97.89	69	EF523092.1	405	*Ixodes ricinus*	Female	Poland
824	*B. microti*	98.68	79	KU550681.1	476	*Dermacentor reticulatus*	Female	Poland
840	*B. capreoli*	98.66	98	MN296295.1	528	*Ixodes ricinus*	Female	Poland
849	*B.microti*	99.22	94	MN355504.1	410	*Ixodes ricinus*	Nymph	Poland
856	*B. microti*	99.23	77	MH628094.1	410	*Dermacentor reticulatus*	Male	Poland
883	*B. microti*	99.21	77.00	KC007119.1	491	*Ixodes ricinus*	Female	Poland
885	*B. microti*	98.68	94	MW791419.1	399	*Dermacentor reticulatus*	Male	Poland
887	*B. microti*	98.63	75	MH523092.1	483	*Dermacentor reticulatus*	Female	Poland
895	*B. microti*	99.48	56	AB085191.1	516	*Ixodes ricinus*	Female	Poland
901	*B. microti*	99.25	64	MN355504.1	484	*Ixodes ricinus*	Female	Poland
906	*B. microti*	100.00	89	KU955532.1	519	*Dermacentor reticulatus*	Female	Poland
915	*B. divergens*	87.90	40	MT550684.1	398	*Ixodes ricinus*	Male	Poland
917	*B. microti*	98.42	87.00	MH523092.1	468	*Ixodes ricinus*	Nymph	Poland
922	*B. microti*	100.00	56.00	MF440670.1	452	*Ixodes ricinus*	Female	Poland
926	*B. microti*	99.21	72	MN355504.1	538	*Dermacentor reticulatus*	Nymph	Poland
928	*B. microti*	99.73	83	KY319204.1	479	*Dermacentor reticulatus*	Nymph	Poland
929	*B. microti*	98.70	80	MH553358.1	476	*Dermacentor reticulatus*	Nymph	Poland

Lab Nr	Genospecies	Homology (%)	Query Cover (%)	Gen Bank Accession Number	Bp	Tick Species	Stage/Sex	Country
930	*B. microti*	99.73	76	MH553358.1	483	*Ixodes ricinus*	Nymph	Poland
931	*B. microti*	99.16	76	KU955532.1	470	*Ixodes ricinus*	Nymph	Poland
932	*B. microti*	100.00	74	KP055639.1	439	*Dermacentor reticulatus*	Nymph	Poland
933	*B. microti*	99.22	90	MH553358.1	426	*Ixodes ricinus*	Nymph	Poland
934	*B. microti*	100.00	96.00	MW554593.1	492	*Dermacentor reticulatus*	Nymph	Poland
935	*B. venatorum*	99.42	81	MG344777.1	424	*Dermacentor reticulatus*	Nymph	Poland
936	*B. microti*	99.47	91	KY319204.1	417	*Dermacentor reticulatus*	Nymph	Poland
124e	*B. microti*	99.21	94	KX161765.1	412	*Amblyomma gamla*	Adult	Ethiopia
226e	*B. microti*	99.08	58	KY319204.1	479	*Amblyomma gamla*	Adult	Ethiopia

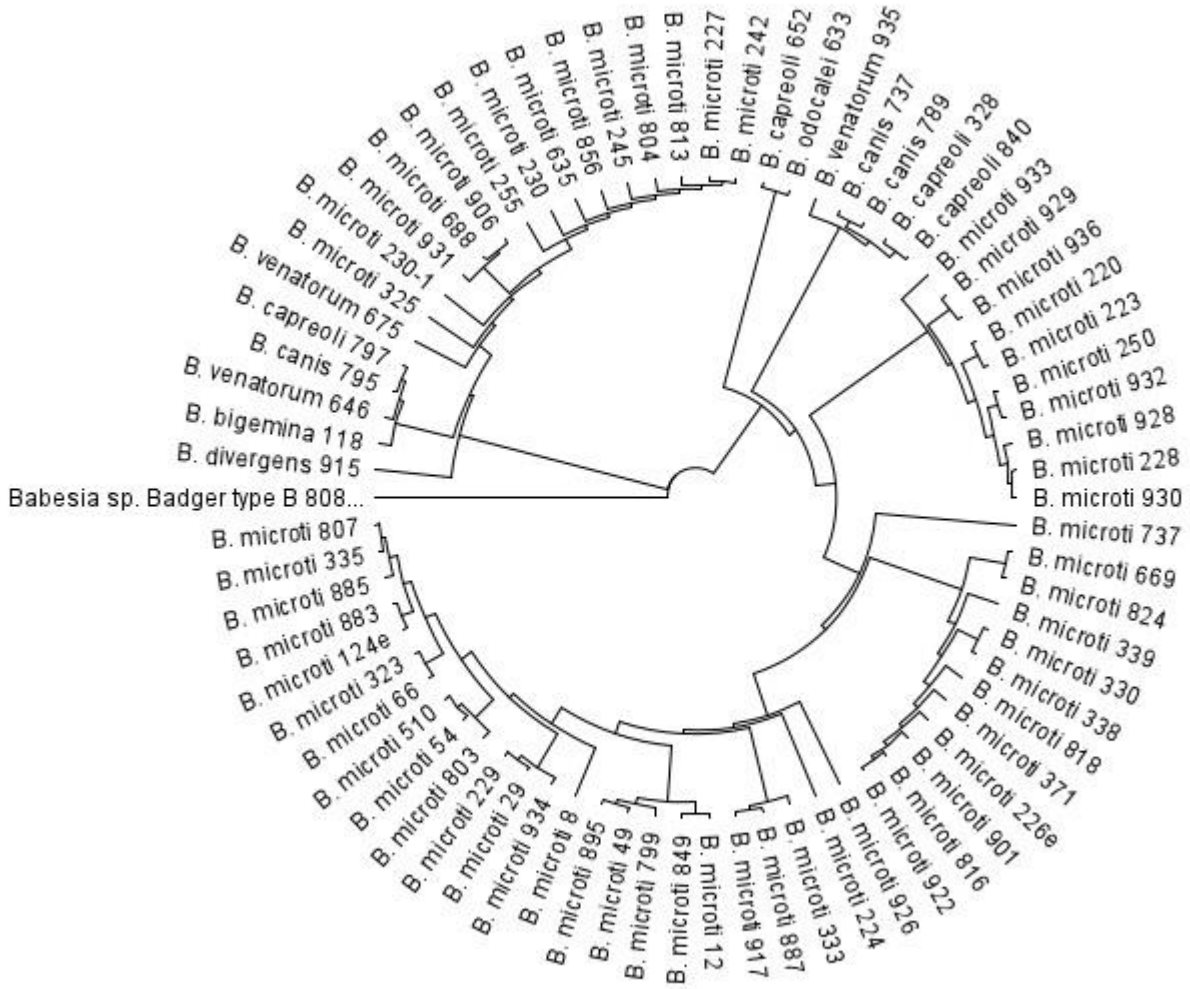


Figure 15. Phylogenetic analysis of the *Babesia* spp. sequences

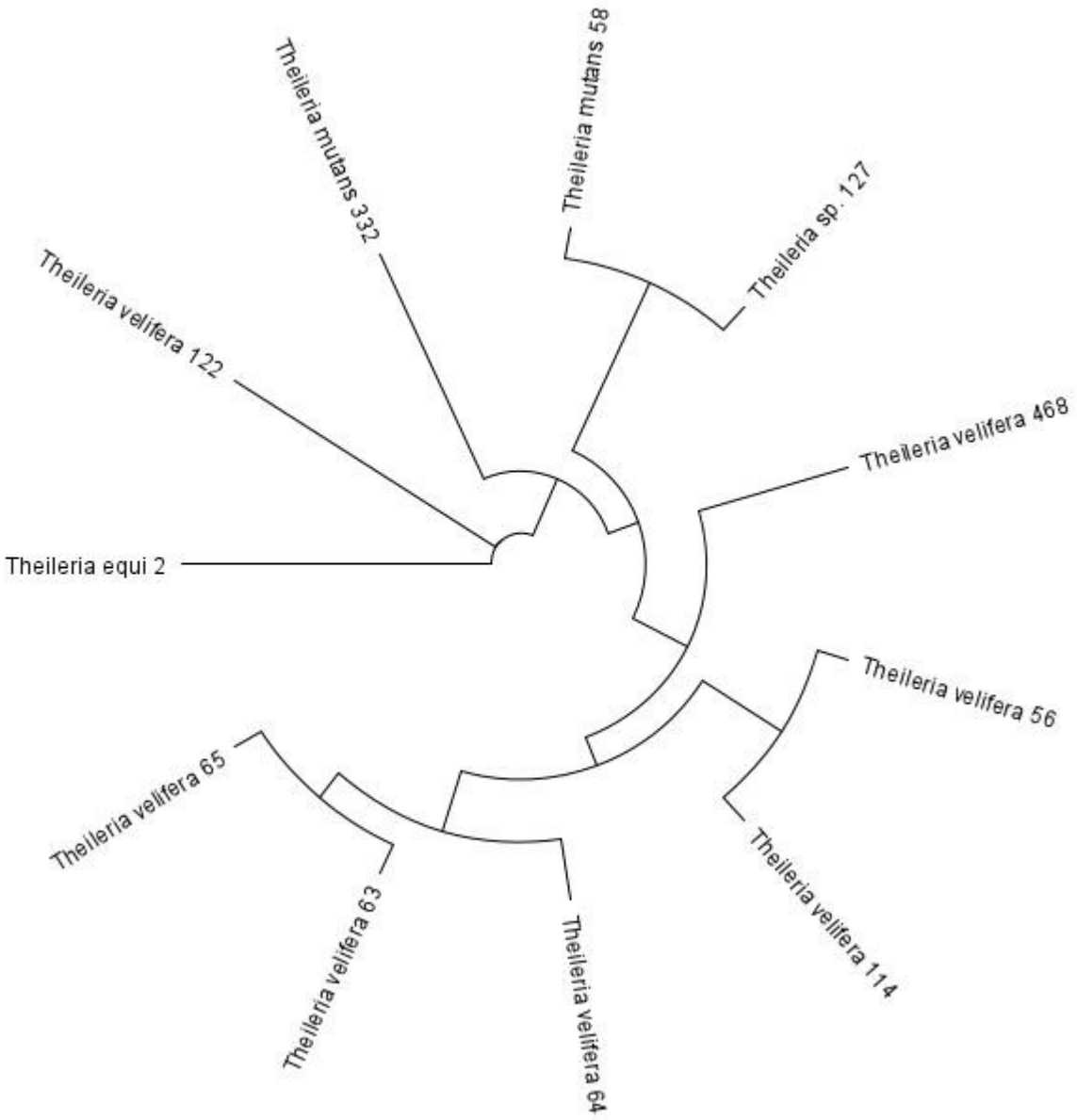


Figure 16. Phylogenetic analysis of the *Theileria* spp. sequences

4.6. Tick-borne encephalitis virus sequencing result

The sequencing result for TBEV shows that all TBEV strains were European subtype with a homology of 165/167 (98.8%) similarity with tick-borne encephalitis virus Hypr polyprotein gene, complete cds Sequence ID: U39292.1 (European subtype).

4.7. Factors associated with the detection of tick-borne encephalitis Virus

Univariate analysis of variables associated with TBEV detection in ticks showed that *I. ricinus* ticks were 96.2% less likely to have been infected with TBEV compared to *D. reticulatus* species of ticks ($p \leq 0.001$). Ticks collected during an ambient temperature of 15-17℃ were 96.9% less likely to have TBEV compared to those collected under a temperature of less than 15℃ ($p \leq 0.001$). Adult ticks (OR: 25.37) were more likely to have been detected with TBEV than nymphs ($p \leq 0.001$). On the other hand, females (OR: 25.32) and males (OR: 25.47) were more likely to have been infected with TBEV as compared to nymphs ($p<0.001$).

A multivariable logistic regression has also shown a similar direction of the association. Regarding species of ticks, *I. ricinus* was 93.7% less likely to have TBEV compared to *D. reticulatus* species of ticks as it is adjusted for ambient temperature and developmental stage of ticks ($p= 0.007$). Ticks collected during an ambient temperature of 15-17℃ were 95.8% less likely to have been infected with TBEV compared to those collected under a temperature of less than 15℃ adjusted for species and developmental stage of ticks ($p \leq 0.001$). As for the developmental stage of ticks adjusted for species and temperature, adult ticks (OR=23.66) were more likely to have been infected with TBEV ($p \leq 0.001$) (Table 12).

Table 12. Factors associated with TBEV detection in ticks using univariable and multivariable logistic regression

	TBEV detected				TBEV detected (n=687)			
Characteristics	OR	[95% CI]		p	AOR	p	[95% CI]	
Species (n=703)								
D. reticulatus	1							
I. ricinus	0.038	0.052	0.281	0.001	0.063	0.007	0.008	0.475
Temperature in °C (n=687)								
10-14.9	1							
15-17	0.031	0.007	0.13	<0.001	0.042	<0.001	0.011	0.181
Development (n=703)								
Nymph	1							
Adult	25.37	6.064	106.14	<0.001	23.66	<0.001	5.542	101.029
Sex (n=703)								
Nymph	1							
Female	25.32	5.92	108.29	<0.001				
Male	25.47	5.65	114.73	<0.001				

5. Discussion

Tick-borne pathogens with an increased detection rate both in vectors and hosts signal alarm to the coming danger posed to human and animal health. The prevalence of *Babesia* spp. in ticks has shown a staggering pattern. In this study, the overall prevalence of *Babesia* spp. in both tick species was 9.51% with 9.59% in *I. ricinus* and *D. reticulatus* 9.83%. In a study conducted in northern Poland the overall prevalence of *Babesia* spp. was 10.6% with 7.7% in *I. ricinus* and 18.9% in *D. reticulatus* ticks (139). *Babesia* spp. has been also been detected in 2.0% *I. ricinus* and 7.0% of *D. reticulatus* ticks collected from a forest in north-eastern Poland which is close to the study area where this study has been conducted (131).

The prevalence of the *Babesia* strain varies across parts of the world where *B. divergens* is common in Europe while *B. microti* is common in the United States (43). This study and other studies that used samples from ticks and humans have reported *B. microti* as the most frequently detected strain of *Babesia*. In this study, four strains of *Babesia* spp. were detected in ticks collected from north-eastern Poland. The overall prevalence of *Babesia* spp. in *I. ricinus* ticks was 9.13% with *B. microti* at 7.31%, *B. capreoli* 0.91%, *B. divergenes* at 0.46%, and *B. venaturum* at 0.46%. A study conducted in the eastern part of Poland has reported three identified and one unidentified strain of *Babesia* spp. with an overall prevalence of 4.6% where *B. microti* was detected in 2.8% of *I. ricinus* ticks. *Babesia microti* has the highest detection rate in both studies. Grochowska A. et al. have reported 15 out of the 17 detected *Babesia* spp. in *I. ricinus* ticks were *B. microti* (131). Subsequent studies conducted on people at risk of acquiring *Babesia* spp. have shown that *B. microti* was the commonest cause of babesiosis. The first two autochthonous cases had *B. microti* (45) and five foresters among 114 of their colleagues

had *B. microti* antibody (81). The other six patients who attended health facilities had also *B. microti* (82). All these studies signal that *B. microti* dominates other *Babesia* strains in the north-eastern and eastern regions of Poland.

Similarly, in this study, the frequently detected strain of *Babesia* in *D. reticulatus* ticks was *B. microti*. The prevalence of *B. microti* in *D. reticulatus* ticks was 7.37%, however Grochowska A. et al. have detected 15 *B. canis* of the 16 *Babesia* spp. and only one out of the 16 was *B. microti* detected in *D. reticulatus* ticks (131). In this study 3 out of the 38 *Babesia* spp. were *B. canis* in *D. reticulatus* ticks. Another study conducted in the eastern part of Poland has shown that the prevalence of *B. microti* in adult *D. reticulatus* was 4.5% (140). It is difficult to reach at a defined conclusion about the prevalence difference between this study and other studies. Therefore, further studies that can cover a wider area may help to understand about the prevalence of *Babesia* strains in different species of ticks.

In the coming years babesiosis can be more prevalent mainly in the north-eastern and eastern parts of Poland. Two studies conducted in the north-eastern part of Poland has reported 11 human cases of babesiosis (81,82). Studies in the regions also show an increasing trend of tick-borne pathogens (80,131,141). Risk factors that promote TBDs in the area is high. Some of the indicators are forest coverage, availability of favourable hosts, interaction of hosts with ticks and human, agricultural land use, and movement of people (142). The forest coverage in Podlaskie fore example is 30.5-39.8% (143). The weather condition of the area from May to November is the peak season of ticks' activity. Natural reserve parks, forests, connected to the towns and villages are abundantly available for ticks to reproduce and interact with their hosts (131,144). The overall prevalence of tick-borne

pathogens is higher in areas with monthly average temperature of more than 20℃ (9) which coincides with Suprasl's July temperature which is 22℃ (128).

In Ethiopia *Babesia* spp. were detected in ticks and both domestic and wild animals. *Babesia* spp. and *Thelieria* spp. were detected in 8.96% of ticks with *B. microti* at highest rate of detection reported in 5.61% ticks. A study has reported 15.53% *B. bigemina* and 6.17% of cattles in southern part of Ethiopia while this study has detected only one tick infected with *B. bigemina* (145). This study has identified one *T. mutans* and four *T. velifera* strains from *A. cohaerens* ticks and one unidentified strain of *Theileria* spp. from *R. decoloratus* ticks. Another study conducted in the northern part of Ethiopia which claims the first to report *T. mutans* strains for the first time in the country reported that *T. orientalis* complex, *T. mutans, T. velifera*, and *T. annulata* from blood samples of domestic ruminants (146). Another study conducted in the southern Ethiopia has also detected two *B. bovis* in 9.9%, *B. bigemina* in 7% of cattle involved in the study (87). A study reported from the western part of Ethiopia had also detected *T. orientalis*, *T. velifera*, and *T. ovis* strains from *R. decoloratus R. evertsi evertsi* ticks (147). This shows that the both *Babesia* and *Theileria* are detected in all the corners of the country. The impact of these pathogens on the health animals is immense therefore causes economic damage to many Ethiopians who are dependent on animal husbandry.

Another interesting TBD worthy to investigate is TBE. The European Centre for Diseases Prevention and Control has reported an increasing number of TBEV cases in the past decade. Along with an increased detection rate and widening geographic coverage. The overall prevalence of TBEV in this study was 5.55 %, while the disaggregated prevalence in *I. ricinus* and *D. reticulatus* was 0.37 % and 8.81 %, respectively. A study conducted in the neighbouring Lublin region has shown a prevalence of TBEV at 1.6 % in *I. ricinus* and

10.8 % in *D. reticulatus* ticks. The study conducted in the Lublin region pooled a group of ticks together to detect the prevalence considering there could be a single infection in a group. Additionally, this study is conducted in a region where the highest prevalence of TBEV in both ticks and humans is observed. The Podlaskie region contributes the highest incidence of TBEV cases reported amongst the other regions of Poland. On the other hand, a multi-site study was conducted in Lublin that reported a prevalence of TBEV up to 14.03% in *D. reticulatus* which is relatively closer to the prevalence in this study (148). In the neighbouring Belarus, the prevalence of TBEV in *D. reticulatus* and *I. ricinus* was 16.47% (149).

Despite *I. ricinus* being considered to be the primary vector of TBEV, this study and other studies mentioned above have revealed the prevalence is higher in *D. reticulatus*. A study aimed to evaluate the detection capacity of vectors of TBEV revealed that female *D. reticulatus* were competent vectors, but has a lower capability of transmitting the virus while co-feeding compared to *I. ricinus* ticks. The time to have a detectable virus transmitted to co-feeding nymphs was also longer (150). Therefore, in this study only we don't have enough evidence to override the status of *I. ricinus* as the primary vector to transmit TBEV although the detection rate is higher in *D. reticulatus* ticks.

In *I. ricinus* ticks, females (2.7%), males (4.35%), and nymphs (3.97%) were positive for TBEV. In the north-eastern part of Poland including the region where this study was conducted, females (1.59%), males (1.14%), and nymphs (1.0%) with an aggregate proportion of TBEV reported 1.29%. A relatively lower number of positive cases were also reported from Pomorskie and Warmińsko-Mazurskie. The relative difference needs further investigation to stop the transmission into humans and animals (151). The year difference might have an impact on the number of TBEV infected ticks. In the north-eastern region,

the number of cases reported to the ECDC accounts for 90% of human cases (152). Recent studies including this study have shown an increasing prevalence of TBEV in ticks, which has similarly increased the pattern of TBE human cases reported to the Polish national public health institute, particularly between 2011-2017. This is happening despite the increasing pattern of the number of people who got vaccinated (153).

A multisite study that has examined the prevalence of TBEV in *D. reticulatus* ticks collected from 2007-2010 both from the urban and natural environment has reported an infection rate of 0.99% to 12.5% with an overall mean rate of 2.12%. One of the sites was in the Podlaskie region where TBEV was detected in 1.58% of ticks. There was also no significant difference between male and female ticks of TBEV detection, which was also attested in our study (154).

The prevalence of TBEV in adult ticks was significantly higher than that of nymphs both in this study and one from Sweden. In this study, adult ticks had an odd of 25.37 more than nymphs ($p<0.001$). Similarly in Sweden, the mean minimum infection rate in nymphs was 0.1% which was lower than the adult rate of 0.55% ($p=0.003$) (155). On the other hand, the detection rate in adults could have a relation with the feeding frequency. Ticks at younger stages could get infected either through transovarial transmission or during few frequencies of feeding compared to adults. Adult ticks have at least one more feeding time and longer duration of feeding which could facilitate the risk of getting an infection compared to nymphs (40,41).

Microclimate factors and the ever-changing climate influence the life of ticks and their interaction with their respective hosts eventually favour the transmission of tick-borne pathogens within and between the vector and host population. Ticks' survival was highly dependent on the combined variation of microclimate and habitat. Ticks collected at the

ambient temperature of 10-14.9 ℃ had a higher chance of acquiring TBEV compared to 15-17℃. This resembles a field and laboratory combined study which has found ticks survived only up to 4 hours at -10℃ while none of the adult ticks laid eggs at 0℃ and over. The higher temperature has also affected ticks' survival as it is stipulated ticks held at 32℃ had declined mean survival time to 12 days from 230 days at 4℃ (156). This has also gone with the finding of a study that examined the effect of climate on pathogens detection rate in *I. ricinus* ticks. The mean monthly temperature in January affects the detection rate of tick-borne pathogens. Mean temperature >0℃ had contributed to a higher detection rate of pathogens in *I. ricinus* (9). A model-based study that projected the transmission of TBEV has reported an increased density of ticks that could promote co-feeding. The basic reproduction number of potential transmission is expected to increase by 31% from 2021-2050 and 50% from 2071-2100 as predicted based on the data from 1961-1990 in Hungary (157).

Climate change over the past decade has shown a significant change in the pattern of seasons. Snow covers become thinner than before and thaw easily that causes an acute rise in spring temperature. Ticks start to wake in the early days of the spring season. On the other hand, the lowest temperature fluctuation during winter favoured the activity of ticks in the subsequent spring season (158). A model that aimed to predict the effect of seasonal variation and tick population suggested that after variable lower temperatures, ticks' abundance to feed starts in the early weeks of spring (159). Neither temperature rise nor fluctuation is the only driver of tick population abundance, other special factors like vegetation cover and altitudinal level are also important. Vegetation cover promotes ticks' activity while the activity of ticks becomes low in high altitudes (160). Summer temperature has also an impact on the activity of ticks. When the temperature rises too high in summer, ticks become inactive and elongate their activity in autumn (161).

The density of ticks plays an important role in the spread of *Babesia* spp. A study conducted in north-eastern Poland has shown up to 6.3 *D. reticulatus* ticks per 100 m^2 space. The density was higher in the urban area (162). Another study from eastern Poland also reported 96.8 *D. reticulatus* ticks in a 100 m^2 area. Higher density was registered in spring and autumn (163). The density of ticks is a pretext for uninfected ticks to either co-feeding or get access to infected hosts meanwhile the detection rate of pathogens in ticks will be higher (114). Apart from the density of ticks, the prevalence of tick-borne pathogens is endemic in these areas (5). Ticks infected with more than one pathogen have multiplicated effects on the health of people who get infected by those pathogens. Therefore, the exposure to developing multiple infections at a time is possibly higher. Infected ticks are fitter than their uninfected counterparts by inducing heat shock proteins and glycoproteins. The induction of those proteins again facilitates the fitness of ticks to remain warmer and fit to look for hosts to feed on. The fact that, ticks infected with TBEV and *Borrelia* spp. prefer higher parts of grass litter and small trees is related to the boosted fitness following the existence of these pathogens (164).

Tick-borne diseases will have more public health importance in the future than now. Climate change will play a significant role in it. Climate change causes both latitudinal and altitudinal expansion of ticks' survival. The spread to northern Europe like Sweden (165), Denmark (166), and Norway (80) suggests latitudinal expansion while expansion to high altitude was observed in Serbia (167) and the Czech Republic (168). A prediction model showed that ongoing climate change promotes the reproduction of ticks. The ecology of ticks is also expanding due to climatic changes. The climate in central and eastern Europe gets more convenient than ever before. A prediction model to examine the effect of changing climate on the distribution of *D. reticulatus*, *D. marginatus,* and *I. ricinus* revealed that more areas will be colonized by these ticks in the future. In this model, each month's minimum

temperature all around the year has positively predicted the survival of *I. ricinus* (169). More infected ticks have been reported from the northern parts of Europe over the past years. The number of people with indications of babesiosis is also increasing. Since the first case was reported in Sweden in 1989 the second case was diagnosed in 2015. In the following years more cases of *Babesia* spp. infection were reported. A seroprevalence of 2.5% in healthy and 16.3% in cases with *Borrelia burgdorferi* was detected (165). Another study from Sweden again reported a seroprevalence of 4.4% *B. microti*. Similarly, the antibody of *B. microti* was reported from southern Norway (80). In line with the changing climate that ticks can inhabit, migratory birds facilitate the cross-boundary movement of ticks and tick-borne pathogens. Ticks found on the body of migratory birds were found positive for TBEV. This indicates that infected ticks can move long distances attached to birds. The source of infection for those ticks is most likely before attaching to birds since birds are less likely to be competent hosts for TBEV (111) and *Babesia* spp. (170). Because of climate change, suitable habitat is waiting for ticks to harbour in new areas. Therefore, the reproduction of ticks and the spread of tick-borne pathogens will be imminent in the future.

Following climate change happening and the expansion of TBDs territory, the burden of disease from tick-borne pathogens are soaring in the years to come. According to a study based on ECDC surveillance reports, the number of TBE has shown a linear growth of about 74.3% from 2012 to 2020 and is predicted to increase to 141% in 2025 while the Disability Adjusted Life Year (DALY) was 71.3% and Year Lost with Disability (YLD) was 71.75% from 2021 to 2020 (171). The burden of diseases from TBE and babesiosis mainly considers, the symptomatic period of the diseases. There is not enough evidence on the long-term complications and associated burden of TBEV and babesiosis or other TBDs. Nonetheless, some biomarkers are suggestive of complications of TBE. Some are also serving as neurodegenerative biomarkers. A study investigating the level of biomarkers in

neurodegenerative disease also showed elevated levels after TBEV infection. Among the biomarkers, studies signalled an elevated level of NSE, YK-40, and tau both during illness and after recovery (172–174). If elevated biomarker levels after recovery were due to neurodegeneration as a complication of TBE, the disease burden would be much greater than the YLD and DALY above. Therefore, follow-up studies to examine the long-term complications of TBE is important to understand the full course of TBE in human.

The tick-borne disease will be a public health concern in the coming years for some important reasons. The first and foremost reason is climate change which has an effect on all infectious diseases. By the end of the 21st century, global temperature is predicted to rise by 1-3.5℃ which will positively impact the livelihood of vector-borne diseases. The tick-borne disease will also benefit from this factor to expand and affect more people and animals (171).

Prevention of tick-borne pathogens is difficult because of the complex pathways that the vector passes through. Ticks like *I. ricinus* are widely distributed but *D. reticulatus* ticks have a patchy distribution. The movement of host animals and humans helps different species of ticks to interact while co-feeding. Yet it is possible to access ticks, eliminating the vector and pathogens is difficult since ticks can hide and pass harsh conditions (9,30). Mandatory vaccination is the most advised intervention to control TBEV. The vaccine uptake in most countries with high incidence rates is low despite the public health authorities' advice to take it (175). Except for Austria and Switzerland, other European countries didn’t embrace national vaccination packages for TBE. Most countries recommend giving vaccines to either a specific group of people or places where the incidence rate is higher. Some countries like the Czech Republic, Germany, Sweden, and Slovenia recommend vaccination to people who have access to endemic areas (175). Poland

has TBE endemic regions in the east and north-east of the country, the vaccination rate remains low with only 1.1% of its population vaccinated (153), which is far less than the percentage of people protected from TBEV in Switzerland. A study from Switzerland has reported that 41.7% of its population has taken at least one dose of the anti-TBEV vaccine (176). The number of people who got anti-TBEV vaccines is increasing, and the incidence rate of TBE can't show any sign of reduction (153). A study from north-eastern Poland has shown that only a third of survey participants showed their willingness to be vaccinated or vaccinate their children (177).

This study is limited to short time and places. The study used ticks randomly collected from specific places. There was no clustering of study areas used to collect ticks. The species type and developmental stage of ticks were also taken randomly without considering the density of ticks and their capacity of transmitting tick-borne pathogens. Ticks were also collected within a short time. The seasonal variation, microclimates, and involvement of humans and other hosts were not also considered. The density of the tick population in the study area along with variations with time and season is not also observed. A study with wider area coverage clustered based on ticks' density, and prevalence of tick-borne pathogens in animals and humans would be more informative that can help to design either public health or veterinary interventions to deter the spread of tick-borne pathogens. With these limitations, we cannot generalize the findings of this study to the bigger tick population of the study area, so further studies are needed.

Summing up, the detected strains of *Babesia* spp., *Theileria*, and TBEV have both public and veterinary health importance. Further studies with wider temporal and special coverage are mandatory to understand the full course of ticks and pathogens. The interaction of hosts and ticks is mainly important to deter the transmission of tick-borne pathogen

transmission. In Poland particularly, the high detection rate of both pathogens in tick populations requires public health intervention that can halt their spread.

6. Conclusions

1. *Babesia* spp. and *Theileria* spp. are prevalent in *Ixodidae* ticks both in Poland and Ethiopia. *B. microti* is the most frequently detected strain of *Babesia* spp. in ticks from both countries. All the TBEV detected from *D. reticulatus* and *I. ricinus* were European subtypes. The detection rate of both pathogens was higher in *D. reticulatus* ticks than *I. ricinus*.

2. In ticks from both countries; *B. microti*, *T. mutans*, and *T. velifera* were detected. *Babesia* spp. detected only in Poland were *B. canis*, *B. capreoli*, *B. divergenes*, *B. odocalei*, and *Babesia* spp. *Badger* while *B. bigemina* was detected from Ethiopian tick. *Theileria* detected from ticks of the two countries were *T. mutans* and *T. velifera* while *T. equi* was detected only from Polish ticks.

3. Ambient temperature during tick collection, developmental stage of ticks, and species of ticks had a significant role in the detection rate of TBEV. Ambient temperature of 15-17℃, and nymph stage of ticks were against the detection rate of TBEV, while *D. reticulatus* has a positive impact on the detection of TBEV compared to *I. ricinus* ticks.

7. Abstract

Introduction: Babesiosis and tick-borne encephalitis are emerging vector-borne diseases associated with favourable habitats of ticks and their hosts. Ticks are blood-feeding parasites that carry a wide range of pathogens, including the agents that cause Lyme disease, tick-borne encephalitis, anaplasmosis, and babesiosis. *Ixodidae* (hard) ticks are known to transmit the majority of tick-borne pathogens to humans and animals. *Ixodes ricinus* ticks are considered the most relevant tick species to transmit pathogens of Tick-Borne Diseases (TBDs) in Europe; however, other ticks, such as *Dermacentor reticulatus,* are also linked to the spread of TBDs. Babesiosis is an intraerythrocytic infection caused by *Babesia* spp. parasites. Tick-borne encephalitis is an infectious disease of the central nervous system caused by Tick-borne encephalitis virus (TBEV). Both babesiosis and tick-borne encephalitis are mainly transmitted through tick bite. In Poland, close to half of the TBE cases were reported from the Podlaskie region over the past 20 years. *Babesia* spp. in Africa is mainly limited between animals and ticks however there are few cases with substantial evidence of babesiosis. Ethiopia is known for its high prevalence of malaria which shares most of its features with babesiosis. This study intended to investigate the strains of *Babesia* spp. in Ethiopia and eventually will contribute to the existing understanding of the two diseases.

Aims: This study aimed to detect and molecularly characterize *Babesia* spp. and tick-borne encephalitis virus in ticks from Poland and Ethiopia. The detailed aims were:

1. To detect and characterize *Babesia* spp. and TBEV using PCR assays in ticks collected from Poland and Ethiopia.
2. To investigate the prevalence and strain variation between *Babesia* spp. of Poland and Ethiopia.
3. To analyse the potential predictors (temperature, humidity, developmental stage, and species) of tick-borne pathogens detection in ticks collected from Poland and Ethiopia.

Material and Method: The ticks used in this study were collected from Knyszyn forest landscape park in Poland's north-east region, as well as multiple sites in Ethiopia. The ecology of tick collection sites had the entire essentials for ticks' shelter. DNA was extracted from ticks collected in both Poland and Ethiopia while RNA was extracted from ticks collected in Poland only. Ticks were crashed and nucleic acid from each tick was collected independently. After detecting *Babesia* spp. and TBEV using conventional PCR and qualitative RT-PCR methods respectively, positive samples were sequenced. The

association between the developmental stage of ticks, ambient temperature during tick collection, species, and sex of ticks and TBEV detection rate was conducted.

Results: A total of 995 (727 from Poland and 268 from Ethiopia) ticks were collected and examined to detect whether ticks were infected with TBEV and/or *Babesia* spp.. Eighty-five (9.51%) of ticks out of 894 ticks were positive for *Babesia* spp. Among the 626 ticks from Poland 61 (9.74%) and out of the 268 ticks from Ethiopia, 24 (8.96%) of them were positive for *Babesia* spp. Seven (1.17%) out of the 601 ticks had co-infection of *Babesia* spp. and TBEV while 86 (14.31%) of ticks had monoinfection. The prevalence of *Babesia* spp. in ticks has shown a staggering pattern. In this study, the overall prevalence of *Babesia* spp. in both tick species was 9.51% with 9.59% in *I. ricinus* and *D. reticulatus* 9.83%. In a study conducted in northern Poland the overall prevalence of *Babesia* spp. was 10.6% with 7.7% in *I. ricinus* and 18.9% in *D. reticulatus* tick. *Babesia* spp. has been also been detected in 2.0% *I. ricinus* and 7.0% of *D. reticulatus* ticks collected from a forest in north-eastern Poland which is close to the study area where this study has been conducted. Sequencing analysis of *Babesia* spp. showed *B. microti* in 70.59% (60/85) of the samples with a mean homology of 87.56% that ranges between 82.29-100%. Apart from *B. microti*, *Theileria velifera* 8.24% (7/85), *B. capreoli* 4.71% (4/85), *B. venatorum* and *B. canis* each 3.53% (3/85), and *Theileria mutans* 2.35% (2/85) were also detected via sequencing. A multivariable logistic regression has shown, *I. ricinus* was 93.7% less likely to have TBEV compared to *D. reticulatus* species of ticks (p= 0.007). Ticks collected during an ambient temperature of 15-17℃ were 95.8% less likely to have been infected with TBEV compared to those collected under a temperature of less than 15℃ ($p \leq 0.001$). As for the developmental stage of ticks adjusted for species and temperature, adult ticks (OR=23.66) were more likely to have been infected with TBEV ($p \leq 0.001$). The prevalence of the *Babesia* strain varies across parts of the world where *B. divergens* is common in Europe while *B. microti* is common in the United States. This study and other studies that used samples from ticks and humans have reported *B. microti* as the most frequently detected strain of *Babesia* spp. in the eastern and north-eastern parts of Poland. All TBEV were the European subtype.

Conclusions: *Babesia microti* was the commonest strain detected in ticks collected from both Poland and Ethiopia. *Theileria* spp. were detected from both countries' ticks. All TBEVs detected in ticks from Poland were European subtypes. The temperature during tick collection, species of ticks and their developmental stages determine the detection rate of

TBEV. The detection rate of *Babesia* spp. and TBEV in ticks is increasing in recent years which needs further investigation on the interaction of ticks and hosts. Public health interventions including promoting vaccine campaigns are relevant to prevent the growing incidence of TBDs.

Streszczenie

Wprowadzenie: Babeszjoza i kleszczowe zapalenie mózgu (KZM) to choroby przenoszone przez kleszcze, powiązane z korzystnymi warunkami siedliskowymi i obecnością żywicieli. Kleszcze to żywiące się krwią pajęczaki, które przenoszą szeroką gamę patogenów, w tym czynniki wywołujące boreliozę, KZM, anaplazmozę i babeszjozę. Wiadomo, że kleszcze *Ixodidae* (twarde) przenoszą większość patogenów przenoszonych przez kleszcze na ludzi i zwierzęta. *Ixodes ricinus* jest gatunkiem najczęściej przenoszącym patogeny wywołujące choroby przenoszone przez kleszcze (TBD) w Europie; jednak inne kleszcze, takie jak *Dermacentor reticulatus,* są również powiązane z rozprzestrzenianiem się TBD. Babeszjoza to wewnątrzerytrocytarna infekcja wywołana przez pasożyty *Babesia* spp. Kleszczowe zapalenie mózgu jest chorobą zakaźną ośrodkowego układu nerwowego, wywoływaną przez wirus kleszczowego zapalenia mózgu (TBEV). Zarówno babeszjoza, jak i KZM są przenoszone głównie przez pokłucie przez kleszcza. W Polsce, w ciągu ostatnich 20 lat, blisko połowa przypadków KZM została zgłoszona w województwie podlaskim. *Babesia* spp. w Afryce ogranicza się głównie do zwierząt i kleszczy, potwierdzono niewiele przypadków z objawową babeszjozą. Etiopia znana jest z wysokiej częstości występowania malarii, która ma większość cech wspólnych z babeszjozą.

Celem tego badania było zbadanie występowania *Babesia* spp. w Etiopii i lepsze zrozumienie patogenezy i epidemiologii tych dwóch chorób.

Cele: Niniejsze badanie miało na celu wykrycie i molekularną charakterystykę *Babesia* spp. i TBEV u kleszczy z Polski i Etiopii. Szczegółowymi celami były:

4. Wykrycie i charakterystyka *Babesia* spp. i TBEV przy użyciu testów PCR u kleszczy zebranych w Polsce i Etiopii.
5. Zbadanie częstości występowania i zmienności *Babesia* spp. w Polsce i Etiopii.
6. Analiza potencjalnych predyktorów (temperatura, wilgotność, stadium rozwojowe i gatunek) wykrywania patogenów przenoszonych przez kleszcze u kleszczy zebranych z Polski i Etiopii.

Materiał i metoda: Kleszcze wykorzystane w badaniach zostały zebrane z Knyszyńskiego Parku Krajobrazowego w północno-wschodniej Polsce oraz z wielu

stanowisk w Etiopii. Ekologia miejsc zbierania kleszczy zawierała wszystkie niezbędne elementy bytowania kleszczy. DNA zostało wyekstrahowane z kleszczy zebranych zarówno w Polsce, jak i w Etiopii, podczas gdy RNA zostało wyekstrahowane z kleszczy zebranych tylko w Polsce. Kleszcze rozbito i z każdego kleszcza ekstrahowano kwas nukleinowy. Po wykryciu *Babesia* spp. i TBEV przy użyciu konwencjonalnych metod PCR i jakościowych metod RT-PCR, zsekwencjonowano pozytywne próbki. Przeprowadzono analizę zależności między etapem rozwojowym kleszczy, temperaturą otoczenia podczas pobierania kleszczy, gatunkiem i płcią kleszczy, a wskaźnikiem wykrywalności TBEV.

Wyniki: W sumie zebrano 995 (727 z Polski i 268 z Etiopii) kleszczy i przeprowadzono w nich badania molekularne w celu wykrycia zakażenia TBEV i/lub *Babesia* spp. U 85 (9,51%) kleszczy z 894 wykryto DNA *Babesia* spp. Spośród 626 kleszczy z Polski 61 (9,74%) i spośród 268 kleszczy z Etiopii 24 (8,96%) było dodatnich w kierunku *Babesia* spp. U 7 (1,17%) z 601 kleszczy stwierdzono koinfekcję *Babesia* spp. i TBEV, podczas gdy u 86 (14,31%) kleszczy obserwowano monoinfekcję. Częstość występowania *Babesia* spp. u obu gatunków kleszczy wynosiła 9,51%, 9,59% u *I. ricinus* i 9,83% u *D. reticulatus*. W badaniu przeprowadzonym w północnej Polsce ogólny odsetek występowania *Babesia* spp. wynosił 10,6%, 7,7% u kleszczy *I. ricinus* i 18,9% u kleszczy *D. reticulatus*. *Babesia* spp. wykryto również u 2% kleszczy *I. ricinus* i 7% kleszczy *D. reticulatus* zebranych z lasu w północno-wschodniej Polsce, blisko obszaru badań. Analiza sekwencjonowania *Babesia* spp. wykazał *B. microti* w 70,59% (60/85) próbek ze średnią homologią 87,56%, która waha się między 82,29-100%. Oprócz *B. microti,* poprzez sekwencjonowanie zostały zidentyfikowane *Theileria velifera* 8,24% (7/85), *B. capreoli* 4,71% (4/85), *B. venatorum* i *B. canis* po 3,53% (3/85) oraz *Theileria mutans* 2,35% (2/85). Wieloczynnikowa regresja logistyczna wykazała o 93,7% mniejsze prawdopodobieństwo stwierdzenia wirusa KZM w *I. ricinus* w porównaniu z gatunkami kleszczy *D. reticulatus* ($p = 0,007$). W kleszczach zebranych w temperaturze otoczenia 15-17 ℃ było o 95,8% mniejsze prawdopodobieństwo stwierdzenia wirusa KZM w porównaniu z kleszczami zebranymi w temperaturze poniżej 15 ℃ ($p \leq 0,001$). Jeśli chodzi o etap rozwojowy kleszczy dostosowany do gatunku i temperatury, dorosłe kleszcze (OR=23,66) były bardziej narażone na zakażenie TBEV ($p < 0,001$). Częstość występowania *Babesia* spp. jest różna w różnych częściach świata, gdzie *B. divergens* jest powszechna w Europie, podczas gdy *B. microti* jest powszechna w Stanach Zjednoczonych. To badanie i inne badania, w których analizowano próbki kleszczy i ludzi, wykazały, że *B. microti* jest najczęściej wykrywanym

gatunkiem *Babesia* spp. we wschodniej i północno-wschodniej części Polski. Wszystkie TBEV były podtypem europejskim.

Wnioski: *Babesia microti* była najczęściej wykrywanym gatunkiem u kleszczy pobranych zarówno z Polski, jak i z Etiopii. *Theileria* spp. wykryto u kleszczy z obu krajów. Wszystkie TBEV wykryte u kleszczy z Polski były podtypami europejskimi. Temperatura podczas zbierania kleszczy, gatunki kleszczy i ich stadia rozwojowe decydują o wykrywalności TBEV. Wskaźnik wykrywalności *Babesia* spp. i TBEV u kleszczy wzrasta w ostatnich latach, co wymaga dalszych badań nad interakcjami kleszczy i żywicieli. Interwencje w zakresie zdrowia publicznego, w tym kampanie promujące szczepienia, są istotne dla zapobiegania rosnącej częstości występowania TBD.

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10. List of tables

11. List of Figures