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Prevalence of *Babesia* species in *Ixodes ricinus* ticks from Brønnøysund, in Norway

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This thesis is worth 60 study points

Summary

Babesiosis in cattle caused by blood borne parasites in the genera *Babesia*. The disease has shown an increasing trend in Europe over the last decades and are already considered a major threat to livestock in several countries. The only two species of *Babesia* to infect cattle in northern Europe are *Babesia divergens* and *Babesia major* where *B. divergens* being the more common. Knowledge of how these protozoic pathogens is distributed through Europe and how they are transmitted to cattle are of major importance. It is also important to gain knowledge about the tick vector *Ixodes ricinus* and its complex transmission cycle in relation to *Babesia* spp. Climatic changes, and subsequently changes in the distribution and density of different reservoir hosts and the tick vector, might be reasons causing the increase of babesiosis in Europe.

The first aim of this master thesis was to investigate the prevalence of *Babesia* spp. in *I. ricinus* nymph ticks from a farmland of Brønnøysund (Nordland County) which was reported to have cases of babesiosis in cattle. All the ticks were collected by dragging a flannel cloth in a typical tick habitat adjacent to the farm in September 2019. Ten adult ticks were sampled and used for methodological development. 250 nymphs were screened for *Babesia* spp. with an "in house" real-time polymerase chain reaction (PCR). Some *Babesia* positive tick samples were confirmed by pyrosequencing. Due to lack of time, only a subset of seven randomly chosen nymph samples were pyrosequenced. Our results indicated that 51.6% of the ticks were positive in Brønnøysund, which was surprising since such high prevalence of *Babesia* was previously not reported in Norway.

To get more knowledge about infection rate of *Babesia* spp. in Norway, 96 nymph ticks from Hille (Agder County) and 96 nymph ticks from Haugesund (Rogaland County) was collected and analyzed as described above. Hille is an island in the southernmost part of Norway, and the tick sampling location is close to a grazing area for cattle. In the location in Haugesund no cattle were present. Seven random samples from Hille were pyrosequenced. Due to lack of time no samples from Haugesund were confirmed by pyrosequencing. Almost 47% of the ticks at Hille was positive for *Babesia* spp. The most likely explanation for these findings could be due to our modification in methodology. However, a combination of direct and indirect effects on the pathogen by climate, reservoir host and tick population density and

other effects such as migration of birds, rise in cattle movement inland or across neighboring countries can also be reasons for the prevalence of *Babesia* spp. A study with more samples from different sites collected at different seasons need to be conducted to make precise conclusions.

Contents

Preface

This master thesis is a part of the Master program in Environmental Science at The University of South-Eastern Norway (USN), department Bø (Vestfold and Telemark County). All laboratory experiments and data collection were performed at the department of Virology at the Norwegian Institute of Public Health (NIPH) in Oslo. The present study was supported by the EU Interreg, financed by V-A program Scandtick Innovation project (grant number 20200422), and EEA and Norway grants fund for Regional Cooperation, project contract No. 2018-1-0659: TBFVNET: Surveillance and Research on tick-borne Flaviviruses (TBFV). This study is a part of surveillance of tick-borne diseases at the Department of virology and Department of pest control at NIPH.

First, I want to thank all the people of NIPH for giving me opportunity to explore an international master's thesis and letting me work near experts within their fields. Due to the pandemic of SARS-CoV-19 we all had to adapt, and I appreciate the time they took to guide me. Especially thanks to my main supervisor Arnulf Soleng and my internal supervisor Åshild K. Andreassen for great guidance and support through this process. I feel very lucky for having such kind person as my supervisor. I would also like to thank my co-supervisors Rose Vikse, Kristin Edgar and Kristian Alfsnes which have provided me with good feedbacks, laboratory training and help in writing and progress presentations.

My sincere gratitude to Heidi Lindstedt for helping me in map designing of my thesis at NIPH, and to Professor Andrew Jenkins at Department of Nature, Health, and Environment (USN, Campus Bø) for providing me with positive samples required for my research.

Thanks to Alaka Lamsal, Malene Strøm Dieseth and Kristin Edgar with lab training, guidance, support, and advice and thanks to my class fellow Kamilla Häusler for her suggestions in thesis. You have motivated and inspired me to work hard throughout the process. Also, thanks to Kristin Edgar and Arnulf Soleng from the Department of Pest Control for collecting and providing the ticks used in this study.

At last, I want to thank my family and all my friends for the patience and valuable support throughout this Master thesis. This process has brought about a lot of personal growth, and I am very thankful for it.

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Khansaa Islam

Abbreviation and definitions

5'-end: (Pronounced 5 Prime end). Identification of one end of a single strand (DNA/RNA) and indicates the direction of synthesis going from 5'-3'. At the 5' carbon of the sugar-ring (ribose/deoxyribose) a phosphate group is attached.

Annealing: The process of which multiple copies of a specific DNA sequence can be amplified by a PCR reaction.

Arthropod: An invertebrate animal with an exoskeleton, a segmented body, and jointed legs.

Bp: Base pair of Adenine – Thymine and Guanine- Cytosine, held together by hydrogen bonds.

Ct-Value: Threshold cycle value.

DNA (Deoxyribonucleic acid): A genetic material present in almost all living organisms. Two strands consisting of four nucleotides: Adenine (A), Guanine (G), Thymine (T) and Cytosine (C), deoxyribose and phosphate.

DNA-polymerase: A enzyme that synthesize DNA molecules from deoxyribonucleotides.

DNA- Synthesis: The creation of DNA (deoxyribonuclic acid). Occurs when nucleotides (A, T, C, G) are linked together to from DNA molecule.

dNTP´s (Deoxy ribonucleotides): Is the building blocks of DNA strands, consisting of dATP, dCTP, dTTP, dGTP

ELISA: Enzyme-Linked Immunosorbent Assay. Method for detecting and quantifying a specific protein in a complex mixture.

FHI/ NIPH: Folkehelseinstitutettet /The Norwegian Institute of Public Health.

Fluorophore: A fluorescent chemical compound capable of re-emit light upon excitation. Guanidine isothiocyanate: Have in this study been used a as a protection of the nucleoacid during DNA and RNA extraction.

IFAT: Indirect Fluorescent Antibody Technique. Methodology to diagnose infected cells by testing serum samples.

Pathogen: Is defined as an organism that causes disease and illness to its host. A pathogen may be a virus, bacteria, or parasite.

Primers: A short DNA segment that is complimentary to a given DNA sequence and provides a starting point for DNA-polymerase to begin replication.

Quencher: Works as an inhibitor for the reporter during Real-time PCR reaction. The inhibitor keeps the fluorescent dye low when reporter and quencher are physically close to each other.

Real- time PCR: PCR is a process where target area in the DNA is copied millions of times. Real-time PCR shows the results `real-time´ while the machine is running.

Sequencing: A process where the precise order of the nucleotides (A, T, C, G) in a DNA sequence can be revealed.

TaqMan Probe: A short single stranded DNA molecule that binds to the PCR product between the forward and reverse primer. Releases fluorescence during amplification of new PCR strand. TaqMan probes increases the specificity during quantitative PCR.

Transcription: Synthesis from RNA to DNA by RT-PCR reaction.

Vector: An organism that are carrier of a pathogen and can transfer the pathogen to other animals or humans.

Aims of the study

Babesiosis in cattle is caused by blood borne parasites (*Babesia* spp.). The disease has shown an increasing trend in Europe over the last decades. The parasites are transmitted by ticks.

The main objective of this study was to determine the prevalence of *Babesia* spp. in questing *Ixodes ricinus* tick nymphs in Brønnøysund (Nordland County) where babesiosis in cattle was reported.

The results were compared to two other locations (Hille in Agder County and Haugesund in Rogaland County) where no recent babesiosis in cattle was reported. To reach the aims different methods were established including DNA extraction, PCR, and pyrosequencing.

1 Introduction

1.1. Vectors

A biological vector is a living organism that can transmit infectious microorganisms to other organisms and thereby causing disease. Most common vectors are rodents, mites, or insects. Common infectious microorganism are bacteria, viruses, protozoans (single celled parasites), fungal and algae species (Tønjum, 2020). Ticks are considered biological vectors of pathogens and parasites that affect both human and animal health (Estrada-Pena, 2015). Ticks are second worldwide to mosquitoes as vectors of human diseases. They are, however, the most important vectors of disease-causing pathogens in domestic and wild animals. The molecular characterization of tick-pathogen interaction is increasing and providing new ways to control both tick infestations and their competitive pathogens (Fuente et al., 2008).

1.1.1. Ticks - *Ixodes ricinus*

Ticks belongs to the class Arachnida of the phylum Arthropoda. They are ectoparasites which ingest blood from a wide range of hosts like mammals, birds, and reptiles. These blood sucking arthropods are considered first in rank in terms of diversity of pathogens. There are 900 species of ticks. *Ixodes ricinus* (Figure 1), (Castor bean tick) belongs to the family of Ixodidae (hard ticks) that are mostly found in Europe and Asia (Asghar et al., 2014). They can transmit a wide range of microorganisms including viruses (e.g., tick-borne encephalitis virus), bacteria (e.g., Lyme disease spirochaetes and protozoans (e.g., *Babesia* species) that can cause human and animal diseases (Randolph et al., 1999). Ticks have no eyes, but sensory organs at front pair of legs to sense movements, chemicals, environmental changes such as carbon dioxide, humidity, and temperature etc. to recognize the best time to quest and the presence of a host (Asghar et al., 2014).

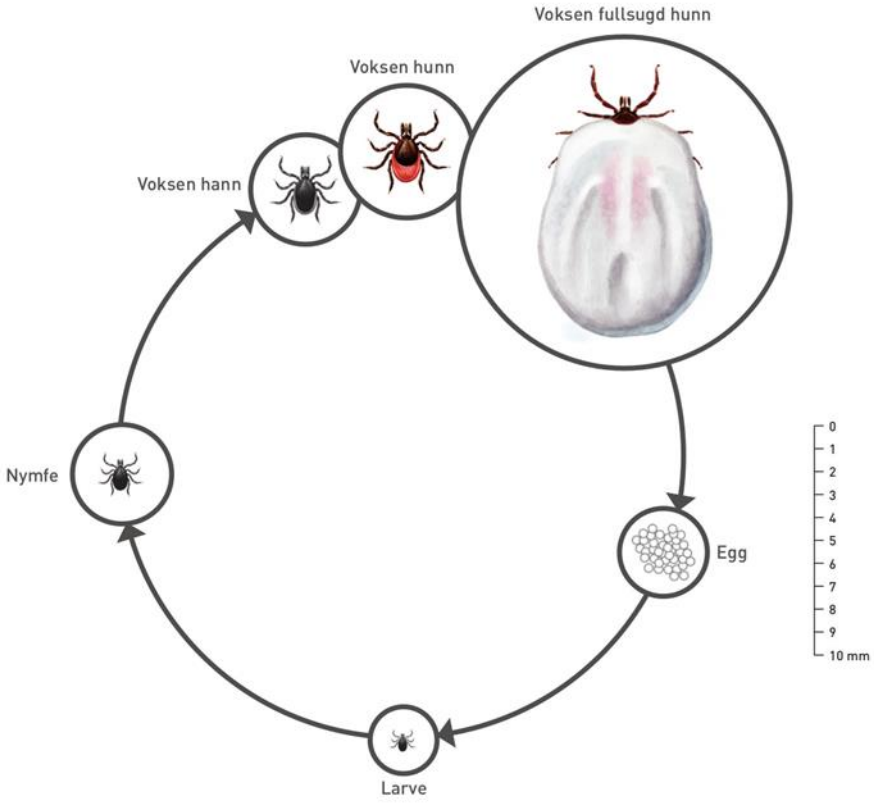

Figure 1. Simple presentation of Ixodes ricinus life cycle. From top right to left showing engorged adult female, unfed adult female with red body, male adult tick fully black, nymph, larvae, and eggs. Illustrated by Hallvard Elven and Preben Ottesen, Folkehelseinstituttet.

1.1.2. Distribution of *Ixodes ricinus*

Ixodes ricinus is distributed all over Europe, from west to east, Asia and from north Sweden to North Africa (Estrada-Pena, 2001). They are widely distributed in north-western Europe. Their distribution range depends upon microclimate, vegetation, and host availability (Milne, 1950). In Norway, viable populations of *I. ricinus* are distributed along the coastline from southeast, approximately 59^{o}N in former Østfold County to approximately 66.2^{o}N in Dønna in Nordland County (Soleng et al, 2018, Hvidsten et al. 2020). They are mostly present in deciduous forests, or grassland including moist vegetation (Milne, 1950). There is a range expansion of ticks in the northern hemisphere at higher latitude and altitude. The distribution range of ticks can be estimated by abiotic factors (i.e., climatic variables) and biotic factors (host availability and vegetation) (Pfäffle et al., 2013; Kjær et al. 2019).

1.1.3. Lifecycle of *Ixodes ricinus*

The average life cycle of *I. ricinus* is 3-6 years depending upon climate and host availability (Asghar et al., 2014). They are oviparous having four life cycle stages: egg, larvae, nymphs, and adults. The three latter stages are called active life stages as they show their parasitic behaviour because they need blood meal to develop from one life stage to next. Each tick life cycle stage feeds only once, but all can transmit *B. divergens* (Zintl et al., 2003). In their life cycle each active stage has a developmental repeating pattern of questing (host seeking), feeding and off-the-host (moulting) in the environment. During questing when the tick finds a host, it will feed for several days. According to a laboratory study, larvae feeds between 2-5 days, nymph feeds between 2-7 days and female adults for 6-11 days (Braks et al., 2016). Larvae and nymphs feed on small animals like reptiles, rodents, and birds, while adults feed on larger mammals like deer, foxes, or wild boars (Asghar et al., 2014). On the average a fertilized female can lay up to 2000-2500 eggs that hatch into six legged larvae (Braks et al., 2016). After the blood meal, a larvae undergo metamorphosis into an eight-legged nymph that undergoes molting into an adult tick after taking another blood meal. Mating usually occurs on the last host when a male adult finds an engorging adult female (Asghar et al., 2014). Adult male ticks can also feed on blood, but they usually quest for hosts to find a female for mating. After mating an adult male tick dies immediately whereas a female dies a few weeks later after laying her eggs (Sonenshine and Roe, 2014).

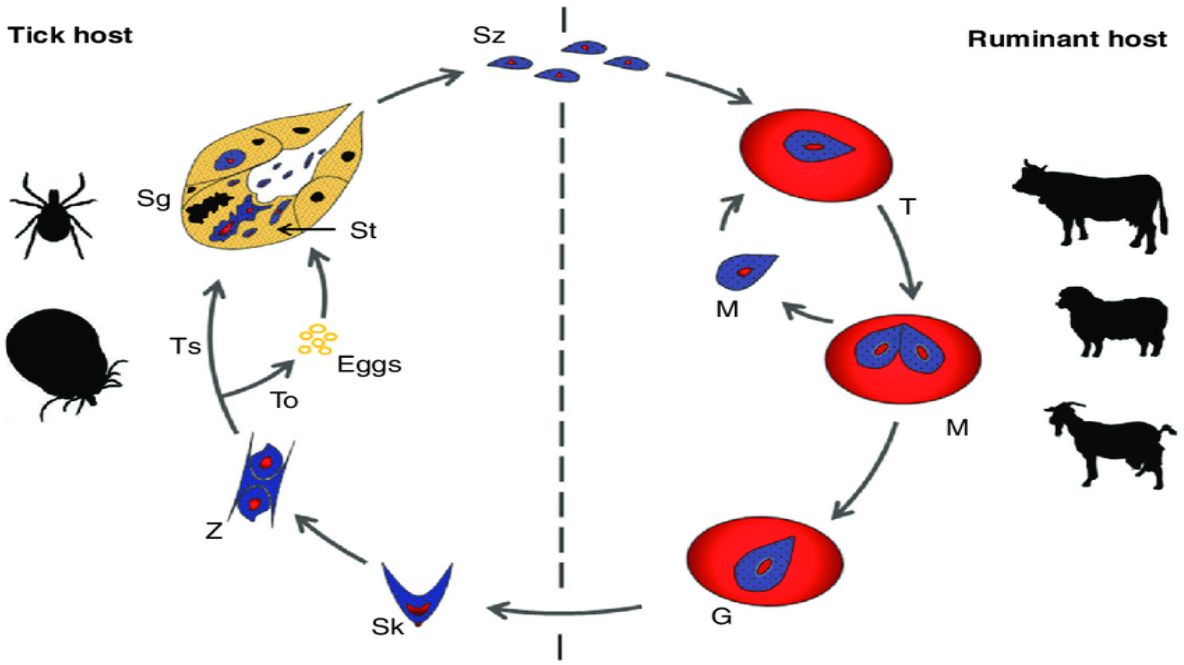

Figure 2. Schematic diagram illustrating life cycle of Babesia spp. in tick and ruminant host. Sporozoites (Sz) are injected into bloodstream of a ruminant host during bloodmeal of an infected tick. After entering erythrocytes, they are called trophozoites (T) which after asexual reproduction divide into two or more cells called merozoites (M). Few merozoite cells stop dividing and convert into gamonts (G). When gamonts enter in the tick by feeding on an infected host they are converted into Strahlenkorper (Sk) in the gut of a tick. Strahlenkorper after fusion form a diploid zygote (Z) (gamogony). Zygotes undergoes meiosis to form haploid kinetes which multiply (sporogony) and replicate in salivary glands (Sg). Kinetes are converted into sporozoites to infect a vertebrate host after tick molting/transstadial transmission stage (Ts). Whereas in infected ruminants, kinetes invade the tick ovaries and eggs and infected sporozoites are formed in salivary glands of next generation larvae stage/transovarial transmission (To) (Schnittger et al., 2012).

1.2 *Babesia* species

Babesia spp. are apicomplexan protozoans found in ticks, migratory birds, and mammals, first observed by Victor Babes in 1888 (Figure 2) (Schnittger et al., 2012). It is a blood parasite that infects red blood cells of an organism and are commonly found in mammals (Leonhard et al., 2012). There are different species of *Babesia* residing in different species of ticks and different

reservoir hosts. *Babesia* are divided into two groups based on size: small e.g., *Babesia microti* (1.0-2.5 um) and large e.g. *Babesia bovin* (2.5-5.0 um). *Babesia divergens* appears as small parasites in blood smear (0.4-1.5 um) but it is genetically related to large babesias. These parasites maintain similar appearance in sheep erythrocytes as in cattle erythrocytes. In human these parasites are larger than cattle (Zint et al., 2003). Generally, they are present in pairs lying in red blood cells (Figure 3) (Gonzalez et al., 2019). Under microscope their circular, stout, or pyriform shape can also be seen (Laha et al., 2015). This is called intraerythrocytic parasite as it attacks only red blood cells. It is transmitted by *I. ricinus* (Zintl et al., 2003). It is the main causative agent of bovine babesiosis or red water fever in cattle. In Europe, babesiosis is mainly caused by *B. divergens* and leads to fever, anaemia, jaundice, weakness, change in heart rate and respiratory rate, slow production of milk in adult cows while calves show mild to no effect (Geothert and Telford, 2003). It is less or infrequently infectious to healthy humans (Uguen et al., 1997).

1.2.1 Genomic structure of *Babesia divergens*

The Rouen genome sequence for *Babesia divergens* was reported in 1987. High quality draft of genome of *B. divergens* was isolated from human red blood cell cultures (Gorenflot et al., 1998). The genome was sequenced by three different sequencing platforms. First, Illumina HiSeq 2000 platform was assembled using ALLPATHS-LG to produce total of 34,182,568 reads with 310x coverage (Gnerre et al., 2011). Secondly, Illumina scaffolds or fake Sanger reads were assembled using Newbler v. 2.8 to produce 829,056 reads with 23x coverage. Thirdly, Illumina+454 hybrid was assembled using PBJelly to produce 109,329 PacBio reads (English et al., 2012). At the end ICORN2 platform was used for sequence base error corrections (Otto TD et al., 2010). The GC content was 40% like other *Babesia* genomes (Brayton KA et al., 2007). Eighty percent complete genome was obtained by CEGMA v. 2.5 as 3741 protein coding genes and 10.7Mb high quality draft become the first reference tool to study its whole genomic structure (Parra G et al., 2009).

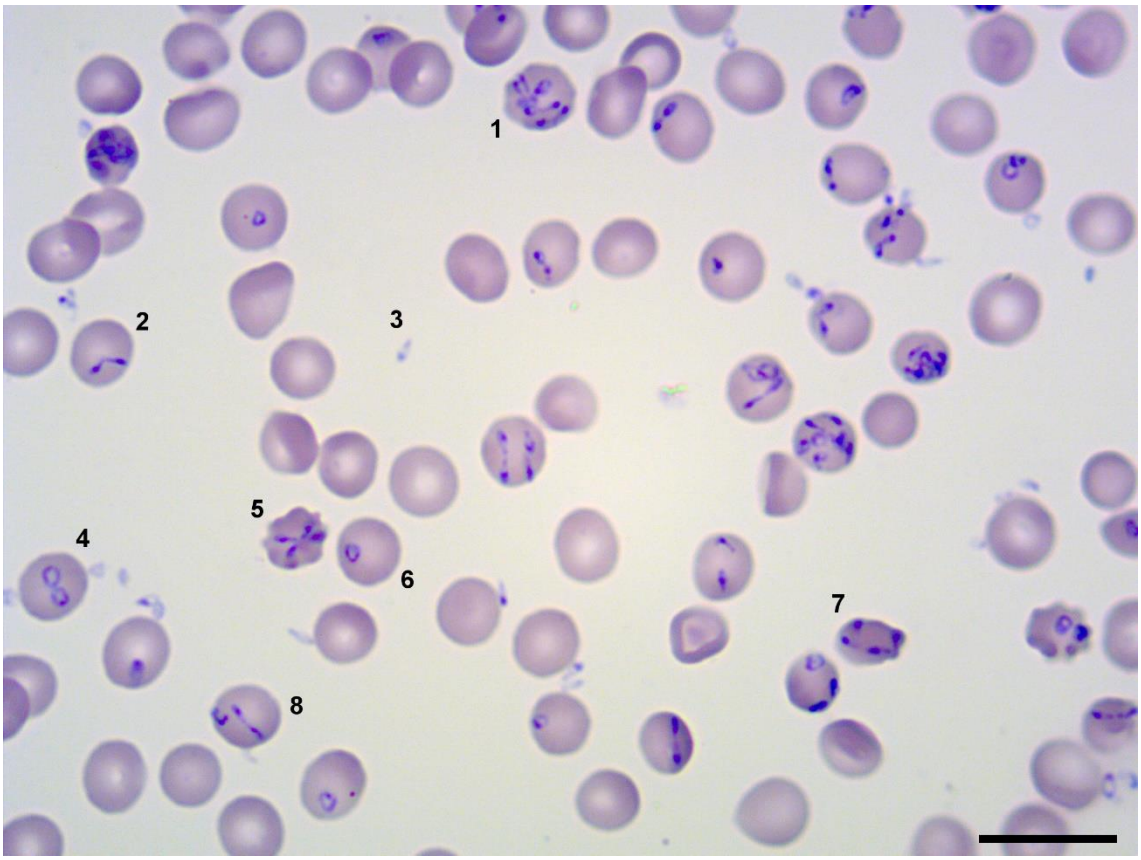

Figure 3. Microscopic representation of invasion and egress process of B. divergens during asexual reproduction by Giemsa staining (1) Multiple parasitic stages formed by more than four parasites within RBCs. (2) Pyriform in pair. (3) independent merozoite (4) double trophozoites (5) Tetrad (6) Single trophozoite (7) Double pyriforms in pairs (8) Transient morphological combination (Gonzalez et al., 2019).

1.2.2 Distribution and prevalence of *Babesia* species

The incidence of bovine babesiosis, caused by *Babesia divergens* has decreased markedly since the 1930 s, but has re-emerged from last decade possibly due to climate change and changes in legislation and pasturing practices. This is a potentially serious disease, with both economical and animal welfare consequences. Therefore, there is a need to survey the distribution of *B. divergens* (Hasle et al., 2010). *Babesia* species is distributed globally between 40^{o}N and 32^{o}S (McCosker, 1981). It is more dependent on geographic availability of competent vectors that commonly includes castor bean tick *(Ixodes ricinus*), black-legged tick *(I. scapularis)* and taiga tick *(I. persulcatus*) (Young et al., 2019). *Babesia divergens* of bovine

origin is reported in human babesiosis from Europe (Gray et al., 2010), *B. microti* which belongs to rodent origin is reported in North America and *B. venatorum* is reported in Asia and other parts of the world (Herwaldt et al., 2012) (Figure 4).

Out of total 111 species of *Babesia*, seven are known to affect cattle (Table 1) (MsCosker, 1981).

Table 1. List of globally distributed Babesia species with their competent vectors. Affecting cattles including Babesia divergens, Babesia bovis and Babesia bigemina.

Species	Vector	Distribution
Babesia divergens	*Ixodes ricinus* *Ixodes persulcatus*	Northern Europe
Babesia bovis	*Boophilus microplus* *Boophilus annulatus* *Boophilus geigyi*	Latin America Southern Europe Africa Asia Australia
Babesia bigemina	*Boophilus decoloratus* *Boophilus microplus* *Boophilus annulatus* *Boophilus geigyi* *Rhipicephalusevertsi*	As for *Babesia bovis*, more widely spread in Africa

Ixodes ricinus and rodents play an important role in epidemiology of zoonotic *Babesia* spp. in south-western Slovakia (Zuzana et al., 2016). These protozoans are mostly found in endemic areas of tick habitat that include forest or temperate regions. It is a common parasite of cattle in tropical and subtropical regions and cause serious zoonosis (Zintl et al., 2003). Infections in both humans and animals mostly occur in spring between April and June and in autumn from August to October due to high active rate of ticks during warm conditions and because people spend more time within tick infested areas during these months (Estrada- Pena et al., 2008). Like in spring, at maximum air temperature between 5-8^{o}C Nymphs and adult ticks become

more active (Donnelley, 1973). In Norway *B. divergens* thrive in *I. ricinus* and cattle is a reservoir. In addition, change in climate and pastures as well as cattle transfer can increase the distribution of bovine babesiosis in Scandinavia, whereas in Norway and Sweden the mostly reported *Babesia* spp. affecting cattle is *B. divergens* (Lindgren et al., 2000; Christensson et al., 1989, Hasle et al., 2010). In Norway babesiosis in cattle is reported in areas where ticks are present i.e., from Østfold in the southeast through south and west coasts of Norway and along Helgeland in the north. According to reports and recent studies, Bovine babesiosis is a limited problem in Norway as being confined to coastal areas in Nordland County but locally it can cause risk to cattle transferred from tick-free inland localities to tick areas (Hasle et al., 2010). In 1933-1940, 1388 cases of babesiosis were reported per year (Tambs-Lyche, 1943). In 1996-2008, 121 cases per year were reported by Norwegian dairy herd recording system. The decrease in babesiosis cases might occur due to destruction of tick habitat, immunity caused by early infection, less movement of cattle from tick-free pasture to pasture with ticks, change in cattle farming causing less exposure to outdoor so less risk of infected ticks (Gray et al., 1996).

In northern European countries, severe cases of *B. divergens* causing infection in humans were reported in Sweden in 1992 and Finland in 2010 (Uhnoo et al., 1992, Haapasalo et al., 2010). The first and only case of *B. divergens* causing disease in humans in Norway was reported in the western part in 2007 (Mørch et al., 2014).

In Norway during 2006-2008 *Babesia* species showed scattered pattern in questing ticks along the *I. ricinus* distribution range from southeast to northwest Norway. All infected ticks with *B. divergens* were found in the west (Utvik) and southern part (Odderøya) of Norway (Øines et al., 2012). In 2001, Skotarczak et al., reported a 0.9 % prevalence rate of *B. divergens* in four different locations of coastal area of southern Norway including Mundheim, Utvik, Tippheia and Fjellværsøy. According to this report only adult female ticks harboured *B. divergens* (Skatorczak et al., 2001).

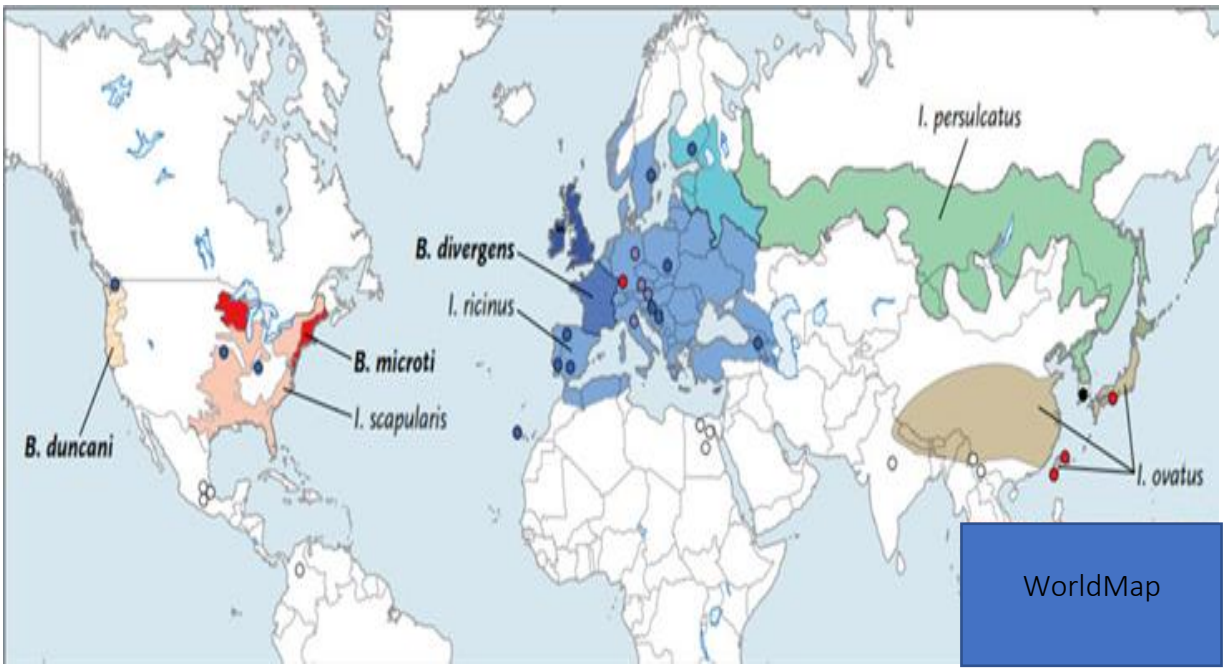

Figure 4. World distribution map of human Babesiosis and the vectors for Babesia strains. The blue area describes the geographical distribution of the vector, I. ricinus, and the most common European strain, B. divergens (New England Journal of Medicine: Vannier and Krause, 2012).

1.2.3 Mode of transmission

Babesia divergens is transmitted in a transstadial mode during each life stage and the natural and dominant mode of transmission is by tick bite from infected *I. ricinus*. However, transovarial modes of transmission is also known, as well as transmission during sexual reproduction (Mehlhorn and Schein, 1984; Mehlhorn et al., 1984; Sonenshine et al., 2014). In the transstadial mode, during the final phase of differentiation multiplicating sporozoites infect the vertebrate host after ticks has molted from larvae to nymphs or from nymphs to adults. On the other hand, in the transovarial mode, infected host cells kinetes invade the tick ovaries and eggs and infected sporozoites are formed in the salivary gland of next generation larvae (Leonhard et al., 2012). After attachment to the host cell, secretory products are released for invagination (Igarashi et al., 1988), the vacuole encloses the sporozoite due to which the vacuole membrane disintegrates. Now the parasite is in direct contact with erythrocyte cytoplasm, so it is called trophozoite or feeding stage where asexual reproduction

occurs by binary fission (Mackenstedt et al., 1990). In trophozoite there is a single haploid nucleus which divides, followed by cell division into two haploid merozoites that invades the new cells in the host. Size of merozoites is variable from 1-3µm depending upon host (Gorenflot et al., 1998). Most of the merozoites continue to multiply by asexual reproduction while a few are non-dividing cells which remain in erythrocytes until they are taken up by ticks during the feeding process (Mackenstedt et al., 1990). One asexual reproduction cycle takes approximately 8 hours in vitro (Figure 5) (Valentin et al., 1991). The large species of *Babesia* like *B. divergens* is transmitted by the transovarial mode. The bloodstream stages of *B. divergens* are divisional so they can be identified in a single blood smear from an infected host (Gorenflot et al., 1998).

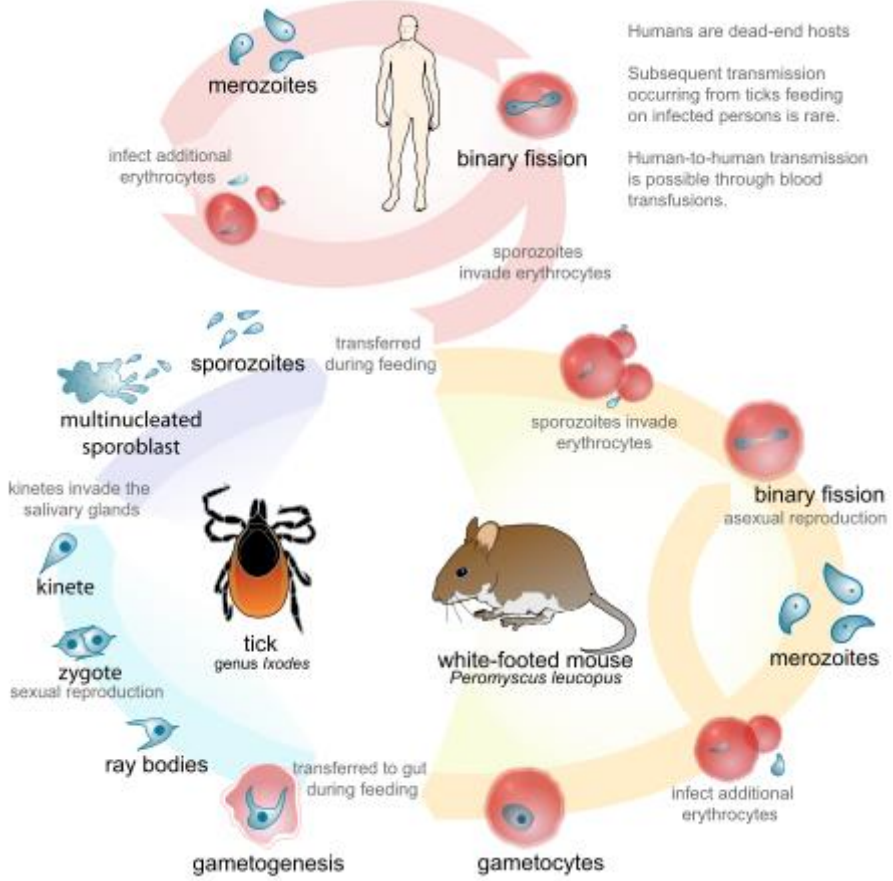

Figure 5. Illustration of the lifecycle of Babesia species showing sexual reproduction by transmission in Ixodes ricinus to the mammalian host showing asexual reproduction and transmission to the dead-end hosts (human) (Jalovecka et al., 2019).

1.2.4 Human transmission

Four out of 100 different *Babesia* parasite species can cause diseases in humans i.e., *Babesia divergens*, *Babesia microti*, *Babesia venatorum* and *Babesia duncani* (Spielman et al., 1985). These species are present as pairs in bovine erythrocytes and as tetrads in human red blood cells. *Babesia* is not directly exposed to human for transmission of the disease but exposure to identical or similar parasites such as bitten by an infected tick can transmit *Babesia* in humans (Beattie et al., 2002). It is a causative agent in splenectomised humans (Fitzpatrick et al., 1968). The first human case was documented in Yugoslavia in 1957.Approximately, 30 human cases of babesiosis were reported in Europe in which a majority was splenectomised patients (Folkehelseintituttet, 2016). A severe case of human babesiosis by *B. divergens* was reported in Sweden in 1992 and a fatal case was reported in Finland in 2010 (Uhnoo et al., 1992; Haapasalo et al., 2004). Symptoms occurring in humans are like malarial patients including fever, fatigue, and anaemia etc. The human incubation period from the tick bite to appearance of symptoms can vary from 6 weeks to a few months (Jorge et al., 1981).

1.3 Treatment and vaccination

Appropriate clinical manifestations of infections, inspection of ticks from endemic areas, surveillance of blood transfusion and splenectomy can be used to diagnose *B. divergens* infections. PCR analysis is the most common diagnostic method (Olmeda et al., 1997).
B. divergens cannot be diagnosed by serology as its antibodies are not identified until 7-10 days after the production of haemoglobinuria (Gorenflot et al., 1998). Infected patients require proper treatment like blood exchange transfusion with intravenous clindamycin and oral quinine to prevent kidney failure (Gorenflot et al., 1998). Various antimicrobial agents like Imidocarb and combination of oxomemazine and phenamidine are effective in vitro treatment for cattle (Brasseur et al., 1998).

There are no vaccines reported to protect humans against babesiosis whereas there is developed a vaccine to prevent cattle and other animals against infection with the large strains

of *Babesia* (Barriga, 1994). Use of vaccine with live parasite to immunize cattle from infection of *Babesia* is being used in livestock management (Callow, 1979). Live vaccines can cause problems such as transmission of some other virus or enzootic agents, development of infection in the field, temperature sensitivity during storage and transportation and a limited shelf life of almost seven days (Edelhofer et al., 1998). So, another technique with the development of a recombinant vaccine in which antigens of sporozoites were used (Wright, 1991). In Austria live vaccine was used from 1969-1988 to protect cattle against *B. divergens* by the Federal institute for the Control of Infectious Diseases in Animals. In 1969-1979, a total of 237, 456 bovines were vaccinated, 3795 (1.6%) of them contracted babesiosis due to coinfection and 432 (11.4%) of them died. Due to insufficient protection the vaccine caused coinfections and was therefore not used. In 1981, Hinaidy introduced three dead vaccines with inactivated merozoites (Edelhofer et al., 1998; Hinaidy,1981). All mammalian hosts have been reported to develop immunity after infection. Immunity against babesiosis depends upon both humoral and cellular factors. When the infection occurs first time from a tick bite, sporozoites are free in the bloodstream for a short interval. During this stage, immunoglobin antibodies bind and neutralize sporozoites before they inject their target cells and prevent infection (Zivkovic et al., 1984).

Control of bovine babesiosis can be performed by tick management, immunization, anti-babesia drugs or by a combination of these approaches (Suarez et al., 2011). Sick animals should be treated as soon as possible with an anti-parasitic drug. The success of the treatment depends on early diagnosis and the rapid administration of effective drugs (Vial et al., 2006; Food security and public health, 2011). There is a retention period after anti-parasitic treatment.

1.4 Molecular detection of *Babesia* spp.in ticks

1.4.1 DNA extraction

Important technique involving isolation of DNA from tissue. It is a basic method used in molecular biology and depends upon various factors like type of tissue, integrity of DNA etc. (Silva and Hellberg, 2021). DNA can be isolated from any biological material such as living or conserved tissues, cells, bacterial or viral particles for subsequent downstream processes, analytical or preparative purposes (Wink, 2006). Generally, DNA extraction is divided based on two protocols includes solution based (conventional method) or column based (solid phase nucleic acid extraction) (Figure 6). Conventional methods include phenol chloroform extraction, alkaline extraction, TAB extraction, EtBr-CsCl Gradient centrifugation. Solid phase nucleic acid extraction includes silica matrices, glass particles, diatomaceous earth, magnetic beat-based extraction, and anion exchange materials. Automated nucleic acid extraction of DNA is more commonly used at laboratory scale as it reduces work hours, reduce labor cost and labor hazards (Doyle, 1996). Purification of DNA involves isolation of recombinant DNA like plasmids or bacteriophage and isolation of genomic DNA from eukaryotic or prokaryotic organisms (Doyle, 1996). Purification of DNA consists of four important stages that includes lysis of cells or tissue which interrupts cellular structure to form lysate, denaturation of nucleoprotein complex, inactivation of nuclease such as DNase or RNase and at last desired DNA should be free from contamination including protein, carbohydrate, lipids, salt, or other nucleic acids like DNA free of RNA (Doyle, 1996; Cseke et al., 2004).

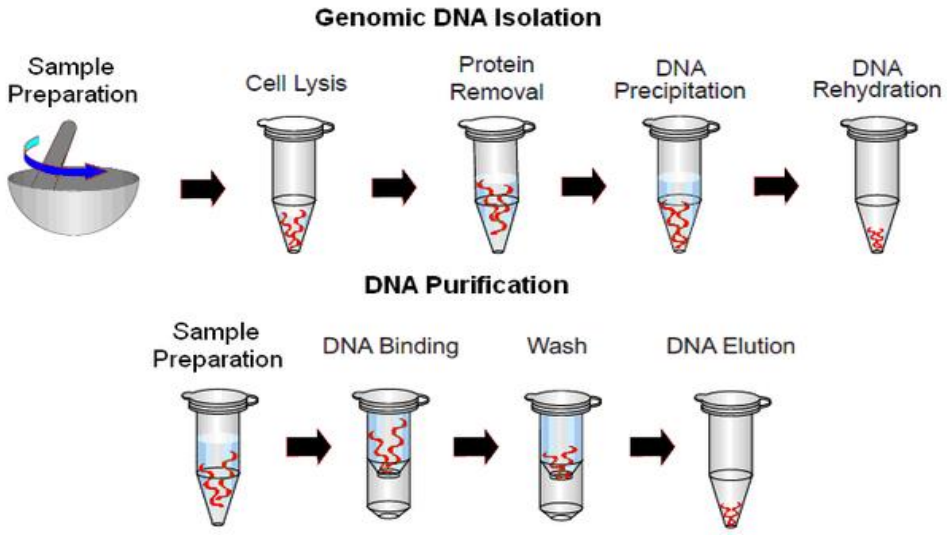

Figure 6. Basic steps involved in genomic DNA and DNA purification illustrating three common steps. Firstly, the membrane needs to be lysed by lysis buffer, secondly DNA needs to be isolated from other cell components in separate washing step. Thirdly, DNA are isolated and eluted in elution buffer (BIO-HELIX, 2016).

1.4.2 Polymerase Chain Reaction (PCR)

It is a scientific technique in molecular biology for detection of wide varieties of templates across range of scientific specialties like bacteriology and virology (Mullis and Faloona, 1987). It amplifies a single or few copies of DNA into millions of copies of a particular DNA sequence (Joshi and Deshpande, 2010). Basic PCR setup require components and reagents to start their reaction (Figure 7). It includes DNA template containing target region to amplify, specific proteins known as polymerases that are enzyme binding DNA building blocks to form long molecular strands, the nucleotides dNTPs consisting of four bases adenine (A), thymine (T), cytosine (C) and guanine (G) which are supply of DNA building blocks (Joshi and Deshpande, 2010). It requires small fragment of DNA called primer which are complementary strands of DNA target to indicate start and the end of the sequence that you want to replicate, buffer solution, bivalent and monovalent cations and water (Joseph and David, 2001). PCR consists of three main steps: denaturation, annealing and extension. In first step DNA is denatured into two strands at high temperature (94-98^{o}C) for 20-30 sec. In second step temperature is

dropped (50-65ºC) for 20-40 sec so both primers can bind to the separated DNA templates where polymerase can create a complementary strand in 5´-3´direction. In third step temperature is increased (65-75^{0}C) to activate polymerase to create a complimentary strand to template DNA in 5´-3´direction (Joseph and David, 2001). Conventional PCR is a basic method that provides qualitative results. It requires gel-electrophoresis or sequencing for detection and visualization of DNA.

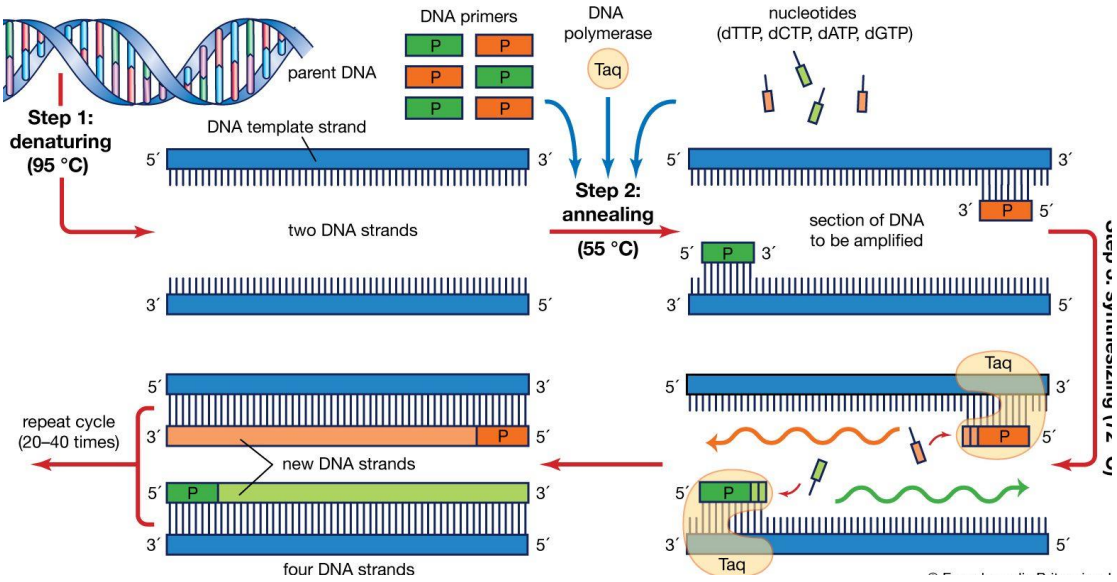

Figure 7. Principles of simple PCR illustrating amplification of specific DNA sequence by denaturation, annealing and extension

(Eric Gregersen, https://www.britanicca.com/science/polmerase-chain-reaction).

1.4.3 Real-time PCR

Real time PCR is developed from conventional PCR and is used in scientific laboratory for virological and bacterial purposes to study different pathogens (Mackay et al., 2002). It is used to detect presence or absence of *Babesia* spp. in ticks while the reaction is running in real time. It is less time consuming than conventional PCR, additional detection and visualization of DNA is not required as it is based on fluorogenic labelled probes or fluorophores and is a sensitive method for detection by fluorogenic emission. In this way it requires less time and

more clear results (Wittwer et al., 1990). Real time PCR consists of two principles method in which first requires a fluorophore like SYBR Green or SYBR Gold that binds to non-specific double stranded DNA (Rychlik et al., 1990). Second principle requires sequence specific probe with a fluorophore and a quencher at its 5´ and 3´ ends, respectively. When Taq polymerase binds to single stranded DNA the probe will breakdown and release fluorophore from the part of the prob with the quencher a fluorescence signal will be emitted which can be detected in the detector within the real time PCR machine (Figure 8) (Wittwer et al., 1990; Rychlik et al., 1990).

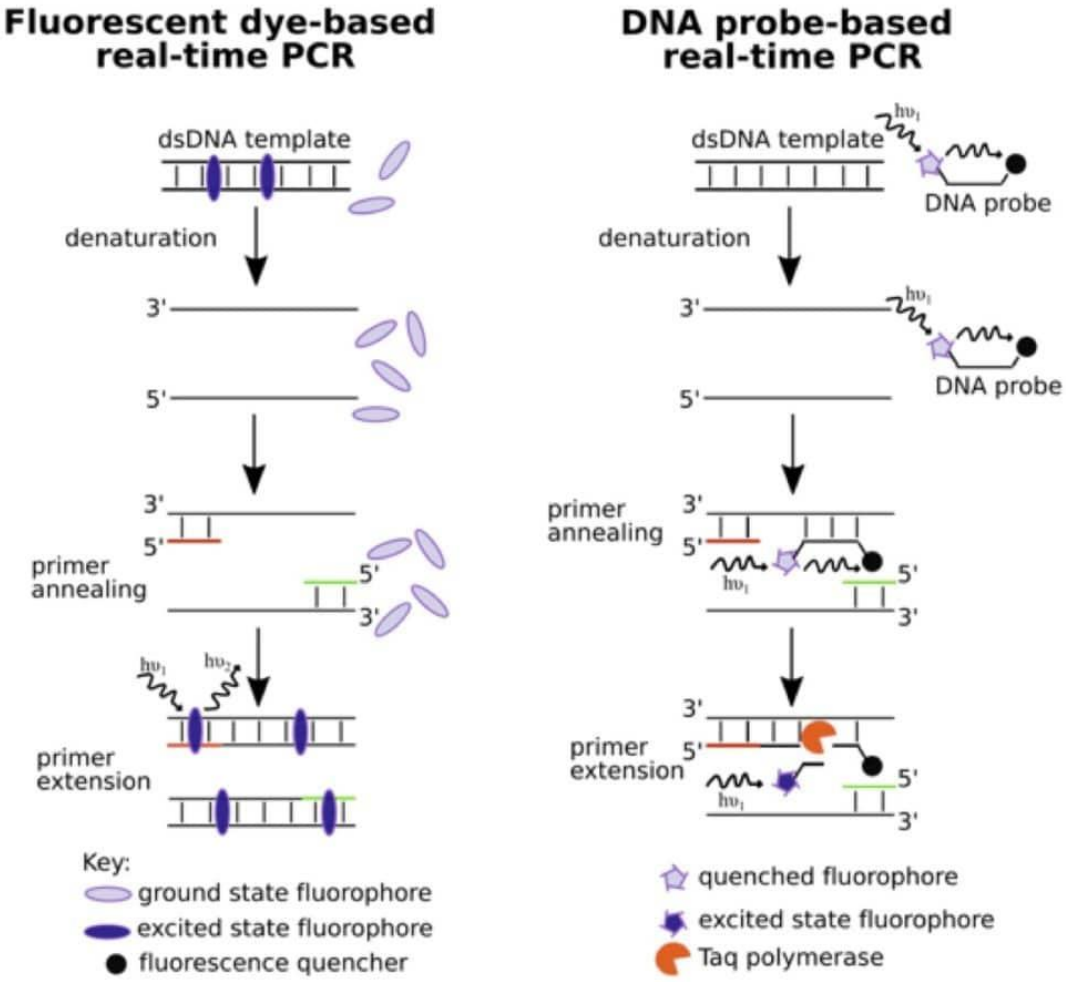

Figure 8. Simple presentation of real-time PCR using both fluorescent dye-based and DNA probe-based principles (Nair et al., 2019).

1.4.4 Pyrosequencing

It is the first alternative method in DNA sequencing after the conventional method of Sanger sequencing for sequencing of small PCR fragments (< 100bp) (Fakruddin et al., 2012). It has revolutionized genetic research in molecular biology (Ahmadian et al., 2006). This method is widely used as single nucleotide polymorphism genotyping and analysis of short nucleotide sequence of DNA, identification of bacteria, genotyping of different pathogens like bacteria or virus. It can detect and sequence small DNA fragments from 10-100bp according to the capacity of machine. This is highly accurate method which can read sequences up to 100 bases (Ahmadian et al., 2006). It is a sequencing technique which depends upon detection of pyrophosphate released during DNA polymerase by enzymes during nucleotides incorporation (Figure 9) (Fakruddin et al., 2012). In pyrosequencing nucleotide removal occur by two methods. Firstly, the solid phase pyrosequencing which involves three-coupled enzymatic process with washing steps and secondly the liquid phase pyrosequencing which involves a cascade of four enzymes with no washing steps (Gharizadeh et al., 2001).

Generally pyrosequencing method have common steps in which sequencing primer is hybridized into single stranded biotin labelled DNA template and mixed with series of four enzymes: DNA polymerase for synthesis of new complimentary DNA strand, adenosine triphosphate (ATP)sulfurylase, luciferase to create light signal and apyrase for degradation of unbound nucleotides and two substrates which are ATP adenosine 5’ phosphosulfate (APS) and luciferin as substrates (Ronaghi, 2001). As Primer will bind to the DNA template, it initiates the starting point of DNA polymerase. The four dNTPs will be added one by one in mixture. Incorporation of dNTP by polymerase will release inorganic pyrophosphate (PPi) molecule that is converted to ATP by ATP-sulfurylase, which subsequently provides energy to luciferase and ultimately oxidize luciferin to generate light signal. Each light signal will be seen as “peaks” on pyrogram and its magnitude corresponds to number of incorporated nucleotides (Fakruddin et al., 2012).

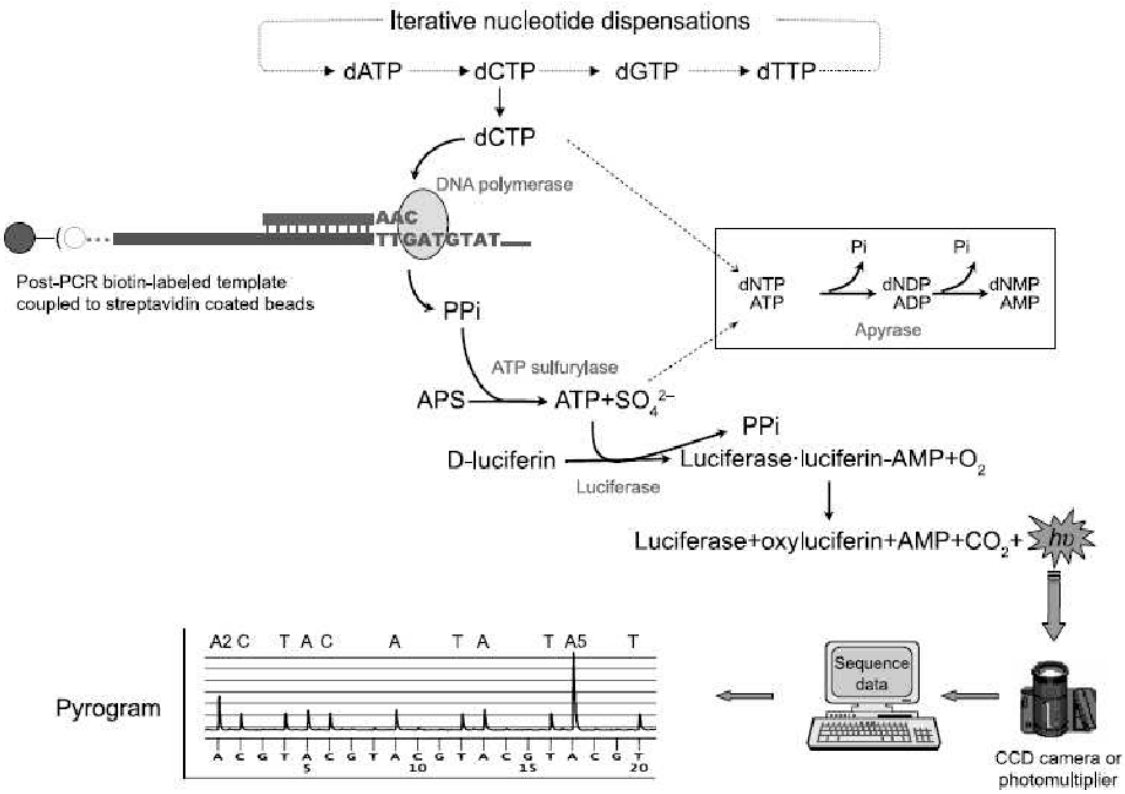

Figure 9. A systematic representation of pyrosequencing presenting synthesis of new strand along the template DNA by polymerase where dNTPs are added separately pyrogram shows incorporated nucleotides as peaks (Fakruddin et al., 2012).

2 Material and methods

2.1 Study area and tick collection

The study consists of *Ixodes ricinus* ticks from three sampling sites. Adult ticks and nymphs were collected in September 2019 from Brønnøysund (Nordland County) in Northern Norway where babesiosis in cattle was reported (Figure 10). The sampling was adjacent to a farmland and was a typical tick habitat with deciduous trees and undergrowth with small bushes, heather, grass, herbs, and ferns. There were several tracks from cervids observed at the cattle pasture and adjacent to the pasture (Table 1). Nymph ticks were also collected in June 2020 from Island Hille (Agder County) in southern Norway where to our knowledge no recent cases of babesiosis have been reported (Figure 10). The area was typically tick habitat dominated with deciduous trees, some conifers, undergrowth with small bushes, heather, grass, herbs, and ferns. There were several tracks and resting places from cervids observed adjacent to a pasture for cattle (Table 2). Nymph ticks were collected in June 2019 from Haugesund (Rogaland County) in Western Norway in an area without cattle (Figure 10). It was at a flat forest area with a mixture of deciduous and coniferous trees. There was undergrowth of small bushes, heather, grass, herbs, and ferns in the area.

Table 2. The three sampling sites of Ixodes ricinus; Brønnøysund, Hille and Haugesund with their latitude and longitude, distance from coastal line and elevation (meters above sea level).

Location	Latitude and longitude	Distance from coastal line	Elevation (meters above sea level)
Brønnøysund	65.3°N 12.1°E	290-420 meters	15-25 meters
Hille	58.0°N 7. 2°E	25-110 meters	15-50 meters
Haugesund	59.2°N 5.3°E	1050-1100 meters	30-35 meters

Ticks were collected by dragging a woollen flannel cloth (size 90 cm x 120 cm) over the dry vegetation. Ticks were collected from the cloth with the help of a tweezer. Nymph ticks were kept in tubes in pools of ten whereas adult ticks were stored individually. All ticks were transported on crushed ice in a cooling bag until reaching the laboratory and subsequently stored at -80^{o} until analysis.

Figure 10. Ticks for this study were collected in 2019-2020 at three different study sites where Brønnøysund is in northern part of Norway, Hille is almost on the southernmost tip of Norway close to Mandal and Haugesund is on west coast of Norway

Map from © Kartverket (Heidi Lindstedt, Norwegian Institute of Public Health).

2.2 Laboratory methods

The overall experimental design to study the ticks to detect *Babesia* spp. is represented by the flowchart (Figure 11).

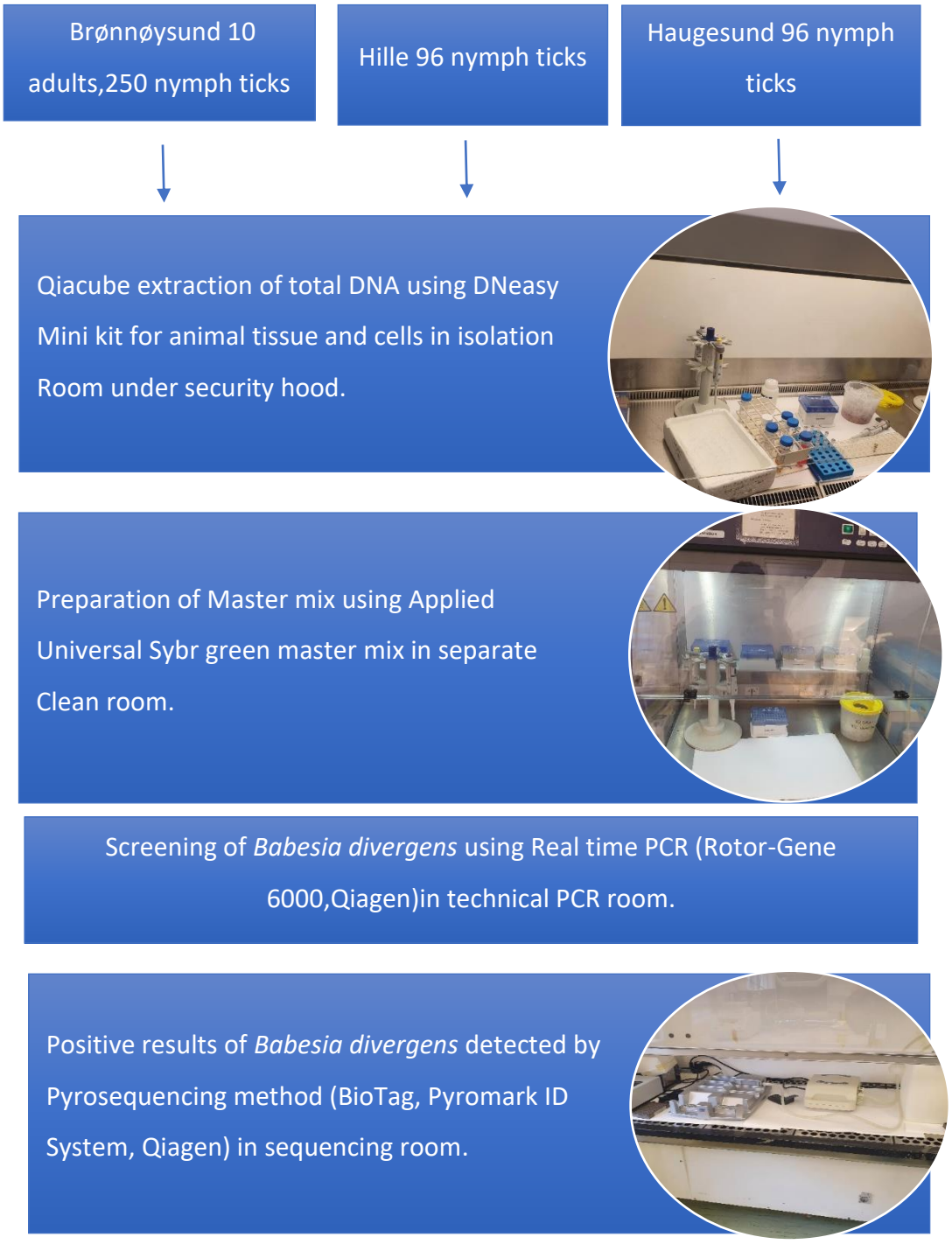

Figure 11. Experimental design explaining methods of screening of ticks to detect Babesia divergens.

2.2.1 DNA extraction from ticks *(Ixodes ricinus)*

Ticks were homogenized in a 2ml MP-Matrix S tube with 6 metal beads (2.8 mm in diameter, MP biomedicals, California) by FastPrep-24 homogenizer (MP-products, California, USA) at the condition of (4.0 M/S, CY: 24x2) for 60 seconds in 200ul PBS solution. Supernatant were transferred to new 2 ml archive tubes and 100 µl Buffer ATL and 20 µl proteinase K was added. The samples were vortexed and incubated on a QBD 2 Grant heating block at 56 °C 1,5 hours and vortexed every 30 minutes. To remove unwanted debris the samples were transferred to new 2ml microtubes and centrifuged at 14000 rpm for 5 minutes. DNA isolation was performed according to QIAamp DNA Mini Kit (QIAGEN Inc., Valenica, CA, USA) protocol for "purification of DNA from tissues" on an automated QIAcube extractor (Qiagen, Valencia, CA, USA) (Figure 12, Appendix 1). The samples were stored at-80^{0}C until Real-time PCR.

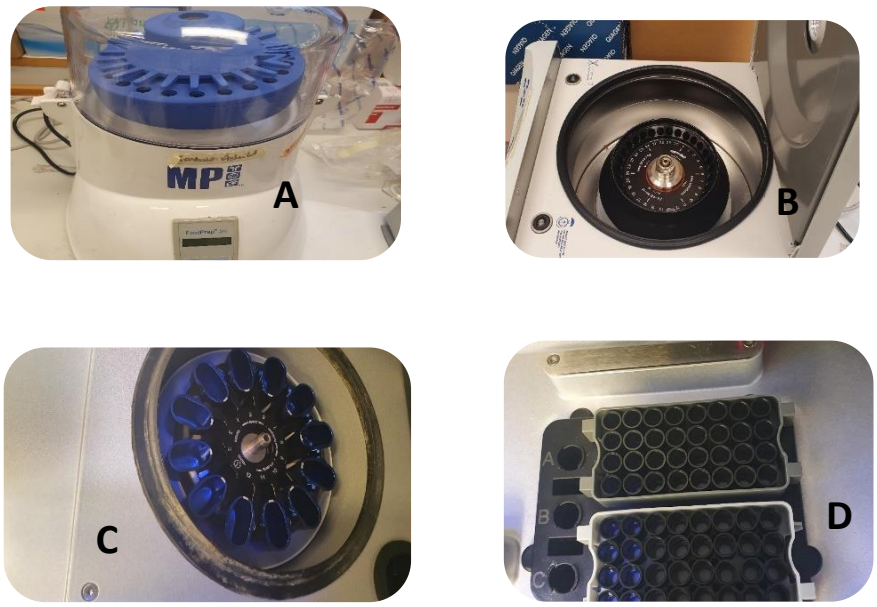

Figure 12. Instruments used for DNA extraction by QIAamp DNA Mini Kit: A) Homogenizer B) Centrifuge C) Rotor adapters in QIAcube D) one row with "ordinary" 1000µl filter tips and one row with wide bore 1000µl filter tips (Photographed by Khansaa Islam).

2.2.2 Screening of *Babesia* from ticks by real-time PCR (QIAGEN GmbH, Hilden, Germany)

The DNA template was screened for *Babesia divergens* by real-time PCR. For detection of *B. divergens* in ticks an "in-house" real-time PCR was designed by Torstein Tengs at the veterinary institute. That method was used to amplify a fragment of 62 bp in the 3' end to *B. divergens (*MG344781.1) (Table 3, Appendix 2).

Table 3. Forward primer, reverse primer and probe used in real-time PCR for detection of B. divergens with nucleotide sequence, genome position and GenBank accession number.

Primer/Probe	Sequence (5`-3`)	Genome position	GenBank accession number
Bdi-F	CAGCTTGACGGTAGGGTATTGG	249-270	MG344781.1
Bdi-R	TCGAACCCTAATTCCCCGTTA	291-311	MG344781.1
Bdi-T	(6-FAM-CGAGGCAGCAACGG-MGB)	276-289	MG344781.1

2.2.3 Establishment of PCR conditions for analyzing *B. divergens*

PCR conditions applied on this study was based on the procedure explained by Øivind Øines et al., about prevalence and diversity of *Babesia* spp. in questing *I. ricinus* ticks from Norway in 2012. Due to high concentration of forward and reverse primers, it was firstly diluted. Bdi-F was diluted from 300uM to 0.3uM and Bdi-R from 900uM to 0.9uM (Table 4, Appendix 3).

Table 4. Dilution of Babesia divergens (positive control) in 2-fold and 10-fold series as 1:2 dilution of 1:64- and 10-fold serial dilution from 10^{-0} to 10^{-6} (1:128, 1:1280, 1:12800, 1:128000, 1:1280000, 1:12800000, 1:128000000)

Positive control	Water	Result
2ul of 1:64	2ul	1:128
2ul of 1:128	18ul	1:1280
2ul of 1:1280	18ul	1:12800
2ul of 1:12800	18ul	1:128000
2ul of 1:128000	18ul	1:1280000
2ul of 1:1280000	18ul	1:12800000
2ul of 1:12800000	18ul	1:128000000

**Positive control dilutions →1:128, 1:1280, 1:128000 and 1:1280 000 Water 4 samples (2 samples of water from clean room+2 water samples from lab to detect contamination)*

2.2.4 In-house real-time PCR

The extracted DNA sample (5ul) was screened for detection of *Babesia* by an "in-house" real time PCR (Øivind Øines et al., 2012) on a Corbett Rotor-Gene 6000 real-time analyser (QIAGEN GmbH, Hilden, Germany) to amplify a fragment of 62bp by Applied universal Sybrgreen Mastermix (2x) (Applied Biosystems, Vilnius, Lithuania) with the following supplements; Bdi-F (0.3-300µM), Bdi-R (0.9-900µM) and water (18 µl).Total volume of 20 ul (15ul Master mix+5ul DNA template) was prepared for PCR analysis (Appendix 2).

All samples were analyzed on a Corbett Rotor-Gene 6000 by the Rotor Gene software (QIAGEN GmbH, Hilden, Germany) under the following conditions: initial denaturation at 50°C for 2 minutes and 95°C for 2 minutes followed by the annealing-elongation step of 45 cycles with (15 second denaturation at 95°C and 1 minute at 60°C). Final extension was at 72°C for 30 seconds (Appendix 2). All real-time PCR analysis were run with nuclease free water as a negative- and a serial diluted positive control of *B. divergens* (of unknown concentration kindly

provided by Andrew Jenkins). Positive control was used to verify that the results were positive and in the expected area of analysis.

Positive results were detected with high S-shaped peaks. Negative results were detected as a straight flat line to check validity of PCR analysis.

2.2.5 Pyrosequencing

The pyrosequencing method was used to verify short segment of 62bp from *Babesia* samples that were confirmed positive by real-time PCR. As 62bp segment was too short to use in direct sequencing according to the principle of Sanger sequencing. The positive samples were verified using Pyro gold SQA reagents (QIAGEN, Hilden, Germany) and SQA analysis on Pyro Mark Q24 system (QIAGEN, Hilden, Germany) according to manufacturer's protocol (Appendix 4). All adult ticks from Brønnøysund that were successfully amplified by PCR were further confirmed by pyrosequencing as part of the methodological development. Only 7 random nymphs from each location (Brønnøysund and Hille) were pyrosequenced because of lack of time.

In the pyrosequencing method a vacuum filter holder is used to bind the mixture of biotinylated PCR product bound to streptavidin. The added streptavidin particles and PCR product attached to the filter are pre-washed in 70% ethanol to get rid of excess primers and salts. Then NaOH is used to denature double stranded DNA bound to streptavidin particles to get rid of the unbound sense-strand (un-biotinylated strand). The filters were then washed in buffer to rinse remnants of PCR products and get rid of salts, primers, ethanol and NaOH. Finally, results are evaluated visually by comparing the sequence of the positive control (*Babesia*) seen on pyrogram with standard positive control samples of PCR product. Sequence similarity of 70% or more is considered positive in this study.

2.2.6 Prevalence calculation

The prevalence was calculated according to the definition given by Margolis et al (1982).

Term: Prevalence (usually expressed as a percentage).[1]

Definition: Number of infected individuals of a species divided by total number of individuals examined.

Optimally, all ticks found positive by real-time PCR should have been verified by pyrosequencing to find true positives as real-time PCR gives a certain number of false positives. However, due to lack of time only 7 positive nymphs from Brønnøysund and 7 positives from Hille was selected at random and subsequently pyrosequenced. The ratio of true positives versus negatives after pyrosequencing was then used to estimate the probable number of true positives after real-time PCR. This estimated number of true real-time PCR positive nymphs was then divided by the total number of nymphs examined to get the prevalence of *Babesia* spp.

[1] The term "incidence" is frequently misused for this concept, and in Russia and Eastern Europe the term "extensity" is often used.

3.Results

Ticks from Brønnøysund farmland, 250 nymphs were examined by real-time PCR and screened by pyrosequencing to detect the prevalence of *Babesia* spp. These nymph ticks from Brønnøysund were compared with 96 nymph ticks from Hille and 96 nymph ticks from Haugesund. However, 10 adult ticks from Brønnøysund were used as part of the modified method.

3.1 Modification of primer concentration

Modification in forward and reverse primers from the method described by Øyvind Øines et al., (2012) was performed in this present study. Modification was performed by diluting the concentrated primers. As forward and reverse primers, Bdi-F=300µM and Bdi-R=900 µM were highly concentrated it was first diluted to Bdi-F=0.3 µM and Bdi-R=0.9 µM and then again diluted to Bdi-F=0.03 µM and Bdi-R =0.09 µM (Appendix 5).

3.1.1 Analysis of serial dilutions of the positive *Babesia divergens* control

Analysis of the positive control of *B. divergens* was performed by the previously described modified method. Due to high (unknown) concentration of the positive control sample it was necessary to dilute it. Positive control was tested in a 10-fold serial endpoint dilution test from 10^{-0} to 10^{-6} (1:128, 1:1280, 1:12800, 1:128000, 1:1280000, 1:12800000, 1:128000000) and examined by PCR until at least one dilution show negative result. Ct values of the positive diluted controls (1:128,1:1280,1:12800,1:128000) was between 26-34 whereas the last 3 in the series were negative illustrating highly diluted controls (Figure 13, Appendix 6).

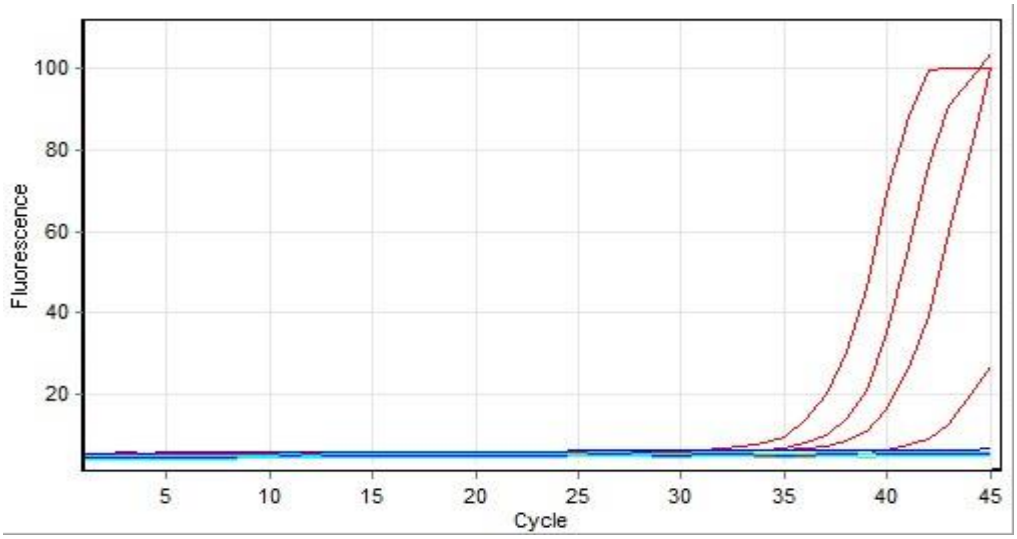

Figure 13. Results from Real-time PCR of a 10-fold serial endpoint dilution of the positive control in which four red curves showing positive control sample result (1:128,1:1280,1:12800,1:128000) from right to left respectively and three bottom dark blue lines showing highly diluted samples (1:1280000 ,1:12800000,1:128000000) with negative result. Light blue negative line represent water to check contaminated result. These results were illustrated using linear scale with threshold value of 0.02.

3.2 Detection of *Babesia* species by real-time PCR

All nymph and adult ticks used in this study were analysed individually. Six adult ticks (out of 10) and 180 nymphs (out of 250) analysed ticks from Brønnøysund were positive for *Babesia* spp. by real time PCR (Figure 14, Table 4). Out of 96 nymphs from Hille 79 were positive (Figure 15, Table 5) and out of 96 nymphs from Haugesund 91 were positive (Figure 16, Table 4). The positive amplified tick samples from Brønnøysund had CT values ranging from 23.8-40.3 (Appendix 7) while the positive amplified tick samples from Hille had CT values ranging from 29.5-38.2 (Appendix 8). The positive amplified tick samples from Haugesund had CT values ranging from 31.1-44.1 (Appendix 9). All the PCR results was analysed by linear scale at threshold level 0.02 on the Rotogene 6000 program.

Table 5. Overview of sampling sites of ticks with their sample name, number of samples analysed individually for Babesia species with their positive results from PCR and range of CT values to analyse Babesia species.

Sampling site	Sample name	Total number of samples analysed individually	PCR positive samples	PCR negative samples	Range of CT value
Brønnøysund	N4b (19)	10 adults 250 nymphs	6 180	4 70	23.84-40.3
Hille	S10 (20)	96 nymphs	79	17	29.5-38.2
Haugesund	H (19)	96 nymphs	91	5	31.2-44.1

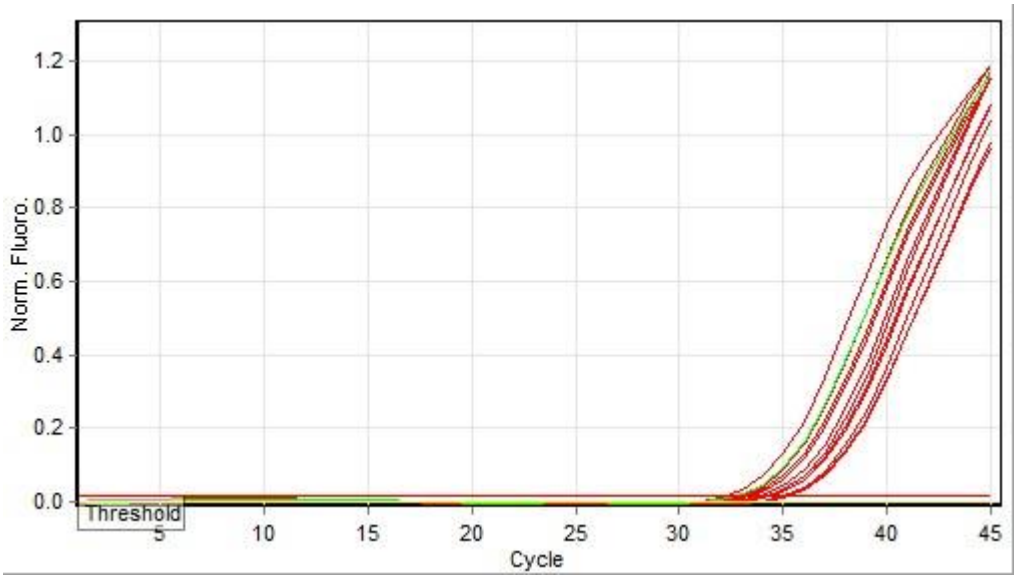

Figure 14. Selected results from Real time PCR of 10 nymph ticks from Brønnøysund with red curves, diluted positive controls with green curves and negative control with two yellow lines. Out of ten nymph ticks, one tick shows negative result as a red straight line at bottom whereas the other nine being positive has CT values ranging from 32.5-35.5. Negative control(water) in yellow straight line at the bottom shows that the result is free from contamination (Appendix 7). These results were illustrated using linear scale with threshold value of 0.02.

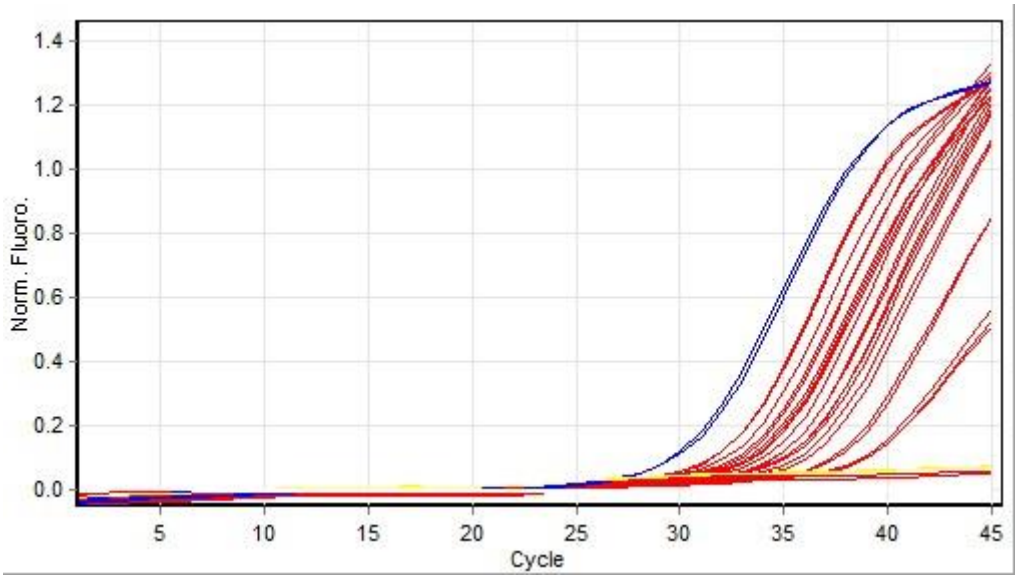

Figure 15. Selected results from Real time PCR of 24 nymph ticks from Hille with red curves, diluted positive controls with blue curves and negative control with yellow lines. Out of 24 nymph ticks, two ticks show negative result in small red curves at bottom whereas the other 22 being positive has CT values ranging from 27.8-37.1. Negative control(water) in yellow straight line at the bottom shows the result is free from contamination (Appendix 8). These results were illustrated using linear scale with threshold value of 0.02.

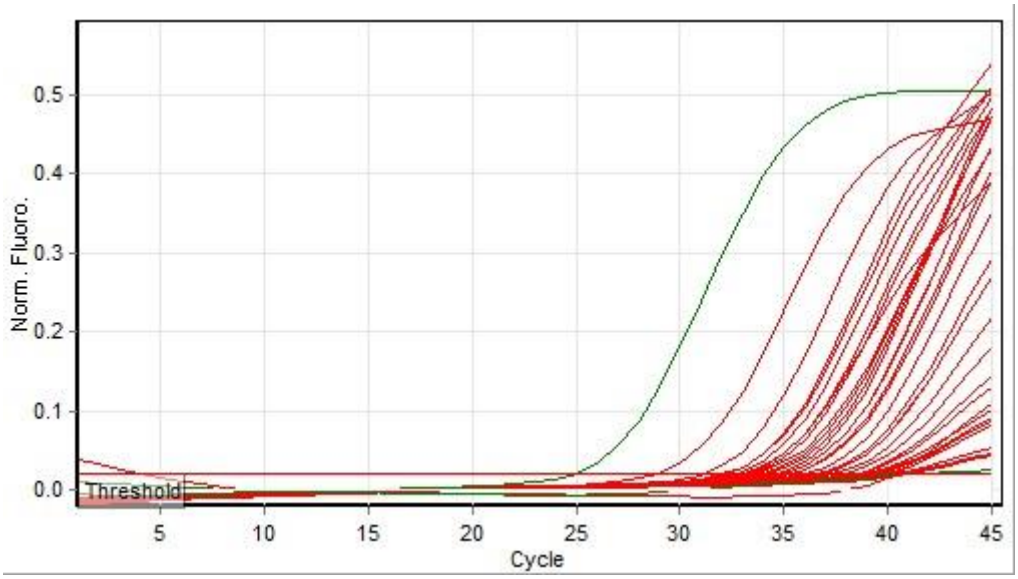

Figure 16. Selected results from Real time PCR of 24 nymph ticks from Haugesund with red curves, diluted positive controls with green curves and negative control with blue lines. Out of 36 nymph ticks, three ticks show negative result in small red curves at bottom whereas the other 33 being positive has CT values ranging from 31.2-44.1. Negative control(water) in blue straight line at the bottom shows the result is free from contamination (Appendix 9). These results were illustrated using linear scale with threshold value of 0.02.

3.3 Screening of *Babesia divergens* by Pyrosequencing

The positive result of tick samples for *Babesia* species was confirmed by pyrosequencing. Due to the high amount of positive tick samples from real-time PCR and lack of time a subset of randomly chosen positive samples were confirmed by screening 17 ticks (10 adults and 7 nymphs) from Brønnøysund and seven nymphs from Hille. In pyrosequencing samples were compared visually with correct control sample sequences (Figure 17, Appendix 13). The pyrosequencing method was run two times as no samples from Haugesund were tested due to time limitation.

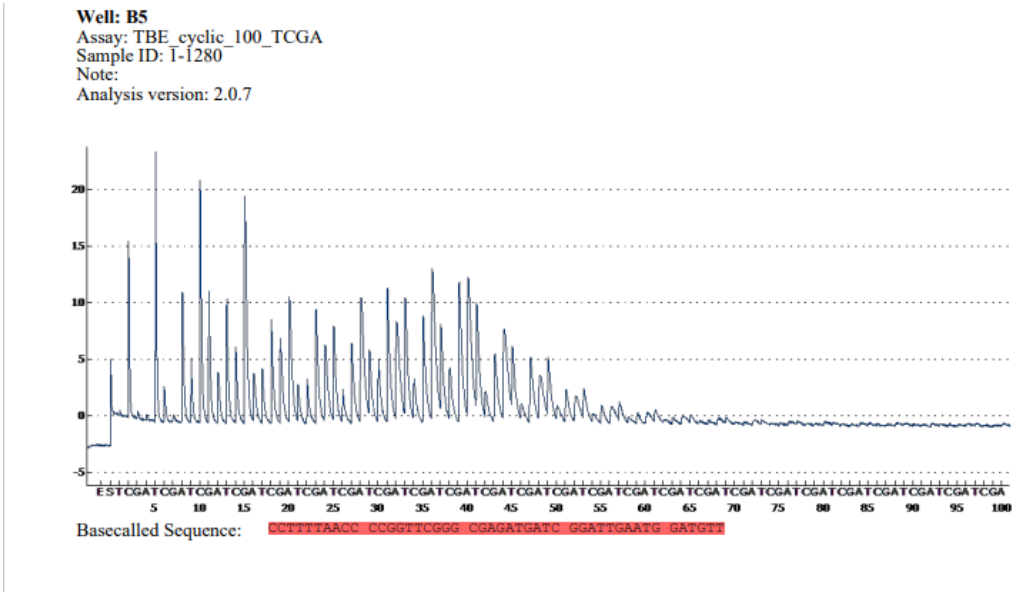

Figure 17. Pyrosequencing graph of control positive sample of Babesia species.

In the first run, ten PCR positive adult ticks from Brønnøysund were analysed by pyrosequencing. Three were confirmed true positive (Figure 18) and one was confirmed weak positive (might be due to high amount of DNA concentration), the other six were showing negative results (Appendix 10).

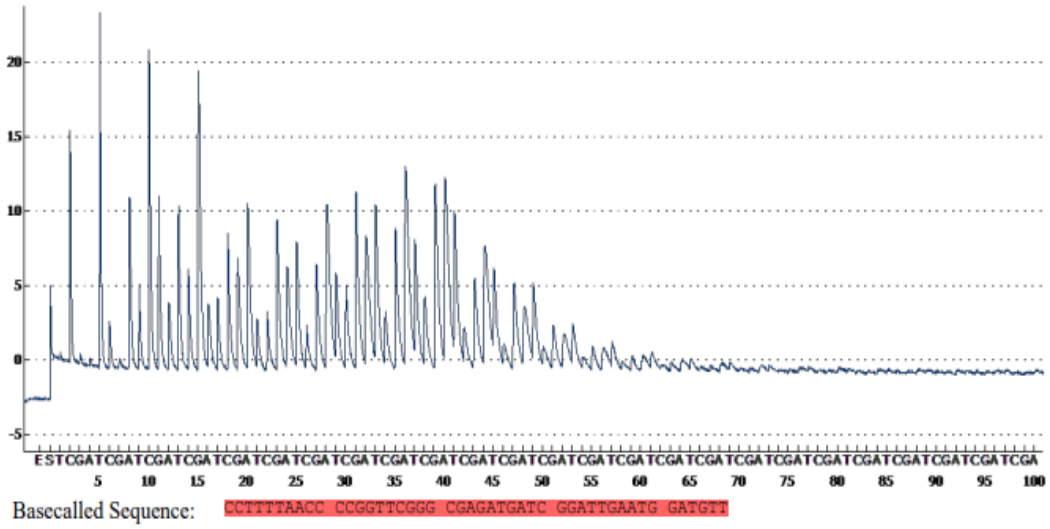

Figure 18. Pyrosequencing graph of adult tick samples from Brønnøysund confirming true positive samples with Babesia species.

In a second run of pyrosequencing, seven nymph samples from Brønnøysund and seven nymph samples from Hille were selected for analysis (Appendix 11). Five nymph ticks from Brønnøysund and four nymph ticks from Hille were confirmed true positive (Figure 19) whereas one nymph tick from Hille showed a weak positive result (might be a problem with the cartridge) (Table 6, Appendix 12).

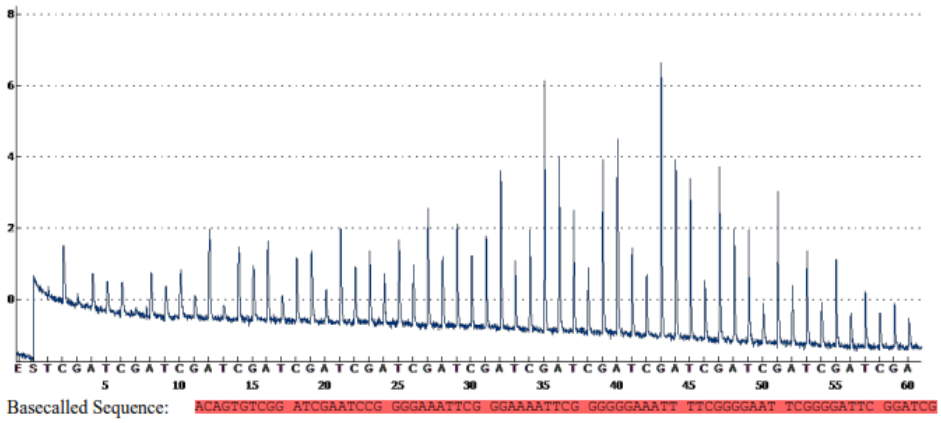

Figure 19. Pyrosequencing graph of nymph tick samples from Hille confirming true positive samples with Babesia species.

Table 6. Illustration of number of Ixodes ricinus nymphs examined, positive by real-time PCR and pyrosequencing, and estimation of prevalence at the two sampling locations (Brønnøysund and Hille).

Location	Total number examined	Number of positive by RT-PCR	Number pyro-sequenced	Number of true positives by pyrosequencing	Estimated number of true positives after RT-PCR*	Prevalence**
Brønnøysund	250	180	7	5 (71.4 %)	129	51.6 %
Hille	96	79	7	4 (57.1%)	45	46.9 %

*(Number of positive by RT-PCR) x (% true positive by pyrosequencing) / (100)

**(Estimated number of true positives after RT-PCR) / (total number of nymphs examined) x (100)

3.3.1 BLAST run for detection of *Babesia* species in positive results

One of the confirmed positive samples was analysed further to detect species of *Babesia* by BLAST and compared to similar sequences (BLAST: Basic Local Alignment Search Tool (nih.gov). In this search there was several *Babesia* species detected with similar sequence as the positive sample. By this we anticipated that the positives were confirmed by pyrosequencing and that most probably all positive samples were *Babesia divergens*. *Babesia* species might be confirmed by Sanger sequencing or next generation sequencing but due to limited time, this was not performed (Appendix 14).

3.4 Prevalence of confirmed positive samples

The prevalence for *Babesia* species in Brønnøysund samples in this study was 51.6%, while for samples from Hille it was 46.9% (Table 6).

4. Discussion

The overall aim of this master thesis was to get a better understanding of the prevalence of the protozoan *Babesia* spp. in questing *I. ricinus* ticks in Brønnøysund (Nordland County) where babesiosis in cattle was reported. Babesiosis causes major economic problems for farmers in milk production. The ticks from Brønnøysund were compared with ticks collected in the south (Hille, Agder County) and the southwestern part (Haugesund, Rogaland County) of Norway.

4.1 Prevalence of *Babesia* species in *Ixodes ricinus*:

Babesiosis is endemic in the northeastern and upper midwestern regions of the United States. The disease is affecting humans sporadically in other parts of the United States, Europe, Asia, Africa, and South America (Hildebrandt et al., 2021). It is a growing health concern in Scandinavia and throughout Europe though it is not endemic, and it is attracting increased attention as a worldwide emerging zoonosis (Vannier et al., 2008; Hildebrandt et al., 2021). The health authorities in Europe depend on surveillance and information of the distribution of ticks and the prevalence of *Babesia* spp. in each country to give updated information of the risk for human and animal infections.

Records of tick-borne diseases available from the Norwegian Cattle Health Recording system (NCHRS) reported that infection rate of livestock diseases decline towards the North where climate is colder, and diseases occur most frequently along the coast where the ticks thrive. According to their analysis from 2009-2016 the infection rate of reported diseases (babesiosis in cattle and anaplasmosis in sheep) is lower in eastern Norway due to low vector abundance and lower pathogen prevalence as compared to the western part of Norway. The eastern part of Norway (Akershus and Østfold County) was reported from the year 2014-2016 where roe deer and moose were commonly found whereas domestic sheep was abundant in some study area. The western part of Norway (Sogn and Fjordane County) was reported from the year 2009-2016 where red deer and moose was commonly found. These were forested areas from south to middle boreal zone with oceanic climate where temperature generally

declines from the coast to the inland and from the lowland to higher altitudes (Østerås et al., 2007; Mysterud et al., 2017; Mysterud et al., 2018).

The multiplex real time PCR method was used for detection of babesiosis in cattle giving a prevalence of 34.3% in United States (Courtney et al., 2004; Mysterud et al., 2017). *Babesia* can develop in a range of different vertebrate hosts and are specifically adapted to use ticks as vectors (Chauvin et al., 2009). Generally, there are few molecular studies to detect *Babesia* species in nature due to its scattered distribution, so preventive measures to control babesiosis in cattle is of importance.

The first aim in the current study was to detect the prevalence of *Babesia* species in questing ticks collected close to a cattle field where babesiosis had been reported. Babesiosis is a disease that is spreading mostly in cattle at higher rate in Norway during the grazing as ticks are mostly active in that season. There was limited updated information of cows getting fever, diarrhea and decreased appetite and milk production in the farmland near Brønnøysund. This made it important to study *Babesia* species in *I. ricinus* ticks. *Babesia* was first discovered in Romania (Ristic et al., 2012), but several studies have indicated that *Babesia* spp. is prevalent at multiple sites in Scandinavia causing babesiosis (Andersson et al., 2017).

In Norway, *I. ricinus* is a vector for *Babesia divergens* which is causing babesiosis in domestic cattle (Radzijevskaja et al., 2008). Radzijevskaja et al, (2008) collected 224 ticks from four different locations in the coastal area of southern Norway (Hvasser and Mølen in Vestfold County) in which only two ticks were found positive of *B. divergens*. The prevalence of *B. divergens* at these two sites varied between 0-3%. A study from Øines et al, (2012) collected 639 female, 568 male and 701 *I. ricinus* nymphs from 22 locations from southeast to northwest Norway during the summer seasons of 2006-2008. Out of 1908 questing *I. ricinus* ticks, 17 (0.9%) was detected positive for *Babesia* spp. by real-time PCR. *Ixodes ricinus* harboring *Babesia* spp. was found in 9 out of 22 localities. Furthermore, molecular analysis of DNA from these positive ticks detected presence of *Babesia venatorum*, *Babesia divergens*, *Babesia capreoli* and currently undescribed species of *Babesia* in Norwegian ticks. Out of these *B. venatorum* was dominant with 71% of the positive ticks (Øines et al., 2012). Surprisingly, in the present study the prevalence was found to be much higher in Brønnøysund and Hille with 51.6% and 46.9% positive ticks, respectively.

4.2 Screening of *Babesia* species by real-time PCR and pyrosequencing

In the present study all the three study sites showed positive results for *Babesia* species using real-time PCR, and this was confirmed by pyrosequencing a random selection of ticks from two of the locations. However, the prevalence found in the present study is much higher and does not correspond to the previous findings of *Babesia* spp. in ticks from Norway. There are many possible causes that can explain this high prevalence.

The methodology used for real-time PCR was according to Øines et al, (2012) but with modifications. The primer was highly positive so the methodology was modified by diluting primers up to the lowest level until it was detected positive by real-time PCR as highly concentrated primers can cause false signals due to primer looping and not show proper sequence during pyrosequencing (Gharizadeh et al., 2006). This might be the reason for the low prevalence reported by Øines et al, (2012) and the high prevalence in the present study. Other explanations might be differences in methodology, differences in the sensitivity of the detection methods including PCR, pyrosequencing etc. done in previous studies (Øines et al., 2012; Radzijevskaja et al., 2008). Due to high positive samples screened by PCR, only a few were selected randomly for pyrosequencing. This small sample size might bias the present results as for optimal results all positive samples should be pyrosequenced. Moreover, sequencing all these positive samples was time consuming and expensive and due to less time, it was not possible to distinguish species by Sanger sequencing or NGS. It might be argued that the high prevalence in the present study is caused by contamination, but because there were both negative samples from the study areas and negative controls included in all analysis this explanation is not likely.

Babesia divergens is the least prevalent pathogen among other *Babesia* species. The common method to diagnose *B. divergens* is by serological testing of blood samples from cows by an antibody test. Hasle et al, (2010) collected blood samples from 306 healthy cows in 2004 and 2005 from 24 farmlands along the southern Norwegian coast. Positive samples were found in 17 farms by indirect fluorescence antibody test (IFAT). By this same method, 27% of cattle from farms in forested areas were seropositive for *B. divergens.* In another study *Babesia* from roe deer, cattle and human blood was isolated and serological testing was

performed by IFAT methodology which indicated that the parasites isolated from roe deer are *B. capreoli* but there are no intraspecific variations in the 18S rDNA as *B. divergens* and *B. capreoli* are 99.83% identical in their morphology and serological cross reactions. (Malandrin et al., 2010). This might indicate that the method of Radzijevskaja et al, (2008) does not detect correct prevalence in ticks as there are positive cattle but no *Babesia* are found in the ticks.

Andersson et al, (2017) tested 71 blood samples of cattle that were genetically analyzed for detection of *Babesia*. Out of these 71 cattle,39 was diagnosed with babesiosis and 32 did not show any infection signs. Real-time PCR indicated 38 of 71 (53%) positive results for *B. divergens* and 17 of 71 (24%) positive results for *Anaplasma* species. Moreover 18% of infected cattle with a *Babesia* spp. had a simultaneous co-infection with *Anaplasma* spp. (Andersson et al., 2017). One more study in southern Sweden was reported by Karlsson et al, (2016) in which 519 *I. ricinus* ticks (10 larvae, 402 nymphs and 107 adults) were collected from four different localities in 2013 and 2014. The results indicated 2.8% prevalence of *Babesia* spp. in adult ticks and 4.4% positive nymphs whereas no larvae were detected positive for *Babesia* spp. (Karlsson et al., 2016). One more study in Central Europe was done in Slovenia where ticks were collected and 2.2% infection rate was detected with *B. divergens*-like parasites (Duh et al., 2001). This indicate that the prevalence of *Babesia* species can differ due to their scattered distribution, climatic effects and differences in host diversity and density.

4.3 Climatic effect

Climatic changes due to global warming can cause a prolonged period of tick activity and cause changes in distribution of risk areas (Zintl et al., 2003). In addition, global warming has caused 2-3 weeks shortening of winter season and an earlier warmer spring in the coastal areas of Norway (Karlsen et al., 2009). A strong effect of climate change operating via tick vector in Scotland is also one example to explain increase in infectious rate of disease by increased temperature causing prolong duration of the tick questing season (Gilbert et al., 2016). Similarly, temperature change and tick activity are correlated for the determination of infection rate of *Babesia* spp. Babesiosis transmitted by tick activity has a bimodal seasonal distribution with a spring peak between April and June and an autumn peak from August-October (Donnelly et al., 1970). In the spring, overwintering nymphs and adult ticks are active whereas larvae are mostly active between spring and autumn. Temperature has little or no

effect on tick activity once the threshold temperature is exceeded (Gray J. S. ,1980). This indicates that temperature variation also can be an important factor for high prevalence. In the present study, the samples were collected from each location at a specific time, so it might be a chance of having different a prevalence rate at other time of the year with a different temperature.

The prevalence rate in our study is different from other studies in Norway and Europe. In a previous report from Norway in 2008 the prevalence of questing *I. ricinus* was 0.9% (Øines et al., 2012) and this is comparable to the prevalence of 1.3% - 4.1% detected in other European countries including Estonia, Belgium, Southern Germany, and Poland (Katargina et al., 2011; Lempereur et al., 2011; Overzier et al., 2013; Silaghi et al., 2012, Welc-Faleciak et al., 2012). A report was conducted that globally represent quite high pooled prevalence of babesiosis in six continents including South America (64%), Australia (61%), North America (52%), Africa (27%), Europe (22%) and Asia (19%) (Jacob et al., 2020).

4.4 Effect of host population density

Cattle are the main reservoir of *B. divergens* in Europe and field observations also suggest that cattle are mostly parasitized by adult ticks (Chauvin et al., 1999). But calves up to 1 year of age, although completely susceptible to infection are resistant to disease (Joyner et al., 1979). Moreover, roe deer and red deer have also been studied as babesiosis and lyme disease are somehow associated with high deer population density (Cézanne et al., 2017; Mysterud et al., 2016). This association might be because deer are important hosts for adult ticks (Mysterud et al., 2017). A study in Ireland explained the potential presence of competent reservoir host such as deers for *B. divergens* in Irish woodlands. The study was done to check infectious rate of *B. divergens* in ticks by collecting 1369 *I. ricinus* nymph ticks from three different sites consisting of farmland, woodland, and limestone pavement sites in the Burren region in western Ireland by describing the fragmented nature of the Irish landscape, deer that stay close to cattle farmland and cattle stay near woodland and deer (Mckiernan et al., 2022).

The overall prevalence of *B. divergens* were 0.3% in ticks from farmland and 1.3% from woodland while none of the ticks from limestone pavement site tested positive (Mckiernan et al., 2022). As nymphs tend to quest close to where the larval stage may have dropped off the

previous host could indicate the importance of presence of competent reservoir host like deer for *B. divergens* (Malandrin et al., 2010). Considering that the parasites like *B. capreoli* which are reported 99% identical to *B. divergens* in their 18S rRNA gene described from Irish red deer and roe deer (Mckiernan et al., 2022; Malandrin et al., 2010). Somehow, in the present study it might be a chance of cross reaction with other parasites that closely resemble the fragments of 18S rRNA gene of *Babesia* species giving a high prevalence. The increase in number of cervids along the coast of Norway might also give a higher prevalence than in earlier studies. Due to lack of time, we could not distinguish the species of *Babesia* so it might be a chance that these isolates are indeed *B. divergens* or a different species that closely resemble to it in the rRNA locus like *B. capreoli* (Mckiernan et al., 2022).

Deer, as roe deer, plays a potential role as a reservoir host for *B. divergens*. Infection rate also depends upon nature of the landscape (Remesar et al., 2019). Analysis of roe deer and cattle from the three studied location where ticks were collected can help to explain the high prevalence in ticks. An analysis in Spain detected the prevalence of *Babesia* in spleen samples taken from 174 roe deer, and 89.7% were positive. This shows that cervids can act as reservoirs for several *Babesia* species (Remesar et al., 2019). In the present study, several tracks from cervids were observed at the collection sites in Brønnøysund and Hille that consists of high densities of ticks, indicating that high positive results might be due to high number of competent reservoirs hosts such as roe deer in the sampling location (Remesar et al., 2019).

According to the varying landscape and nature, co-existence of roe deer and cattle, increasing number of ticks at a sampling location might be some of the explanations of the large variation in prevalence rate of *Babesia* species. However, the extent of spatial and temporal variation in its distribution and its reservoir hosts are not well explained (Yabsley et al., 2013).

It might be high chances of ticks getting infected by *Babesia* species due to persistence and long lifespan of *Babesia* species in ticks. Earlier research suggested that the infection was mostly transmitted by larvae (Joyner et al., 1963) but Grey in 1980, suggested that adult ticks play an important role for parasite transmission after comparing seasonality of Redwater fever and tick activity (Grey, 1980). A study from Piesman et al., (1986) reported that infected larva of *Ixodes dammini* did not survive into the adult stage but infected nymph of *I. dammini* survived into the adult stage however salivary glands of *I. dammini* was more infected in

nymphal stage (Piesman et al., 1986). It might explain that survival of *Babesia* species depends upon the lifespan of tick activity. *Babesia microti* were kept alive for one year in salivary glands of nymphal *I. dammini* in laboratory experiment demonstrating its long lifespan in ticks (Piesman et al., 1987).

4.5 Other effects causing high prevalence

Ticks from migrating birds can be one explanation of introduction of *Babesia* species in areas where there are persistent populations of *I. ricinus*. For instance, Hasle et al., (2011) examined four bird observatories along the southern Norwegian coast during spring migrations of 2003, 2004 and 2005. Overall, 713 birds carried a total of 517 larvae and 1440 nymphs in which 512 examined ticks showed a prevalence of *Babesia* of 1.0% (Hasle et al., 2011). Thus, migrating birds can introduce *Babesia*-infected ticks to Norway. Similarly, a study was done by Wilhelmsson et al., (2021) in south-eastern Sweden. In total 4601 migratory birds were captured at Ottenby Bird Observatory, in March to November 2009 and examined for ticks. Out of 1102 ticks, 1051 ticks were *I. ricinus*, 24 were *I. frontalis*, 12 were *Haemaphysalis punctata,* 4 were *Hyalomma marginatum* and 11 could not be identified by molecular analysis due to unreadable sequences. *Babesia venatorum* was most common with a prevalence of (58%) followed by *Babesia microti* (38%) and *Babesia capreoli* (4.0%) (Wilhelmsson et al.,2021). Since high prevalence of *Babesia* species is found in Sweden (Andersson et al., 2017), the present study can explain that migratory birds certainly disseminate ticks and tick-borne microorganisms to different areas.

Due to extended agricultural system within the European Union, there is an increase of cattle movement into several countries of Europe where disease is prevalent. This can lead to an increase in vector tick populations and introduce new tick species in Norway from neighboring countries that can cause babesial infections in cattle, dogs, and other animals (Zintl et al., 2003). For example, a case of canine babesiosis was reported in Norway in a dog with recent travel history to Central Europe (Øines et al., 2010). Similarly, the first case of babesiosis caused by *Babesia canis canis* was reported in a Norwegian dog that showed no travel history from the last 10 months. There was no report of transmission of *Babesia canis* by *I. ricinus* in Scandinavia (Uilenberg et al., 1989). *Dermacentor reticulatus* is a vector of

Babesia canis canis, but this species is not common in Norway and no viable populations exists. However, a larvae of *D. reticulatus* was found once on migrating bird on an island off the southern Norwegian coast (Hasle, 2013). Therefore, transmission of babesiosis in dogs in Norway might be explained by accidental import of domestic tick-infested animals (Øines et al., 2010). According to State Agricultural Administration of Norway, over the last 5 years the number of cattle grazing has increased up to 8.7% and from January 1st, 2014, it is obligatory to pasture cattle for a minimum of eight weeks. In this way producing a good habitat to the ticks can cause transmission of tick-borne diseases to the livestock (Mysterud et al., 2017). Cattle from tick free areas are known to be susceptible to *Babesia* spp. The farmer at Hille had previously observed that imported cattle from a tick free area got infected and sick when arriving at Hille. We have no information about importation of cattle to the farm in Brønnøysund. It would be interested to get blood samples from cattle at these two farms and compare the prevalence of *Babesia* with the results from Andersson et al., (2017).

4.6 Human babesiosis in Norway

There is only one human case of babesiosis in Norway that was reported in 2007. The patient was an old male who worked with cattle and frequently were exposed to tick bites in an endemic area of bovine babesiosis in western Norway. This babesiosis case were diagnosed as *Babesia divergens* by IFAT (Mysterud et al., 2017; Mysterud et al., 2018; Mørch et al., 2015).

Earlier, analysis of *Babesia* prevalence in ticks suggest that the risk of human babesiosis is low. Pawełczyk et al, (2021) reported in four-year research from 2016-2019 the prevalence of *Babesia* species and co-infection in *I. ricinus* removed from humans throughout Poland. Molecular screening, Sanger sequencing and PCR-RFLP analysis was done for this analysis. Over all 1.3% of *I. ricinus* ticks were infected by *Babesia* species. The prevalence of *Babesia* in ticks was 0.9% in 2017 and 2.4% in 2016 and was higher in adults compared to nymphs (Pawelczyk et al., 2021). The overall risk of human babesiosis after tick bite in Europe is 4% (Hofhuis et al., 2017). However, the results from the present study, with significantly higher prevalence detected in questing nymphs, might challenge this risk assessment if the results are correct.

5. Conclusions

Babesia spp. are protozoan parasites transmitted by ticks causing important disease in cattle, subsequently leading to economic loss for farmers. Furthermore, babesiosis is attracting increasing attention as a worldwide emerging zoonosis.

The main purpose in this study was to investigate the prevalence of *Babesia* species in *I. ricinus* tick nymphs from a farmland in Brønnøysund in northern Norway. These were compared with the prevalence in *I. ricinus* nymphs from two other sampling sites: Haugesund in southwestern Norway and Hille in the south. The prevalence for Brønnøysund and Hille was 51.6% and 46.9% respectively whereas the prevalence of Haugesund was not confirmed by pyrosequencing due to lack of time, however 91 nymph ticks out of 96 showed positive result from RT-PCR. The prevalence in Brønnøysund and Hille is much higher than reported in earlier studies from Norway and Sweden.

The most likely explanation for these findings could be due to our modification in methodology. However, a combination of direct and indirect effects on the pathogen by climate, reservoir host and tick population density and/or availability, migration of birds, and rise in cattle movement both inland and across neighboring countries can also be reasons explaining the high prevalence of *Babesia* spp. A study with more samples from different sites and different seasons should be conducted to make precise conclusions.

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6. Further studies

The present study is a small initiative to find the prevalence of *Babesia* in questing ticks in Norway. It is very important to continue surveillance of ticks in endemic and non-endemic areas to predict future scenarios, especially regarding effects caused by climate change. Such knowledge will be significant regarding vaccine development for tick-borne diseases, as well as recommendations towards the public. Our data indicate that *Babesia* may be more widespread in ticks in Norway than earlier suggested. Additional studies are warranted to elucidate both the distribution and prevalence of *Babesia* as well as determining which species of *Babesia* that are involved.

In more detail, further studies should aim to:

- Analyze the presence of parasite and their vector by recognizing their favorable habitat like nymph and adult ticks will be found in well-maintained permanent pasture according to their environment. Similarly, area infected by *Babesia* species can be recognized if pastures are unimproved under grazed, hedges, and headlands (Zintl et al., 2003; Joyner et al., 1963). In this way by analyzing the infected area, control mechanisms can be performed to lower *Babesia* infections in the tick population. Control mechanism such as use of combinations of antimalarials to cure Babesiosis, boost the immune system of living organisms in that area etc.
- Investigate the role of deers, cattle and other hosts in endemic areas of ticks. Especially if we determine the immune status of host and virulence of the infecting strain, it would be easy to diagnose the infectious rate of pathogen like *Babesia* spp. whether it is mild, severe, or fatal will help to treat the infection (Purnell et al., 1976).

- Use of IFAT methodology, which is serological technique to detect viral proteins in cells via blood samples. For example, to detect babesiosis in cattle or other infectious livestock diseases as it is an easily automated method for large number of samples and interpretation of these results is less subjective. For example, *Babesia* bloodstream stages do not divide simultaneously (Zintl et al., 2003). All stages can be observed in a single blood smear from a parasitized host and would be less time consuming. But molecular detection techniques are more reliable as they can directly determine

presence of parasites. In IFAT methodology, antibodies may remain in the body for long periods even after no symptoms of disease, due to which no information can be utilize that when infection occurred, as this method is used only for serum samples (Joyner et al., 1972). So, a less time consuming and more reliable methodology should be developed to diagnose the pathogen and disease even at early stages. For example, a "Universal" PCR method is developed for the identification of nine most common pathogenic bovine, equine and rodent piroplasms including *B. divergens* (Cacciò et al., 2000).

- Modification of methodology done in the present study could be used to examine study sites from earlier research to compare the results. Moreover, Sanger sequencing or NGS should be developed to analyze the present samples to bring more accurate knowledge on the *Babesia* species involved.

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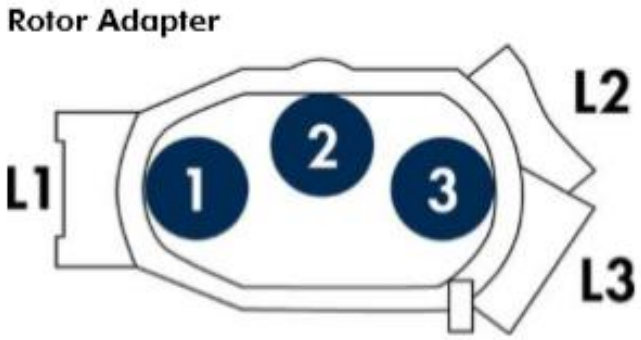
7 Appendixes

7.1 Laboratory protocols

Appendix 1: QIAcube protocol DNA

Extraction of total DNA using QIAcube are the protocol for DNA purification from tissues and cells performed by QIAamp DNA mini and blood mini kit (QIAGEN Inc, CA, USA).
Turn on the QIAcube. Place one empty prelabeled elution tube and one spin column in rotor adapter according to that picture:



Position	Labware	Lid position
1	QIAamp spin column	L1
2	–	–
3	1.5 ml collection tube†	L3

The rotor adapters (with QIAamp spin column and 1,5 ml collection tubes) were placed in the centrifuge and the samples were placed in the shaker, in 2 ml microtubes. Buffers were added in reagent bottles according to the position mentioned in DNA mini kit.

Rack labeling strip	QIAamp DNA

Position	Reagent
1	–
2	Buffer AL
3	100% ethanol
4	Buffer AW1
5	Buffer AW2
6	Buffer AE

For the DNA Mini kit one row with “ordinary” 1000µl filter tips and one row with wide bore 1000µl filter tips were used as shown in the figure 13(D).

Appendix 2: Protocol for Babesia real-time PCR master mix

Real time PCR setup of master mix for detection of *Babesia* with “in-house” Real time PCR machine and Rotor Gene software method

Components	1x sample µL	Final concentration
Perfecta Sybrgreen Fastmix (2x)	10	
Bdi-F (30 µM)	0,2	0,3 µM
Bdi-R (90 µM)	0.2	0,9 µM
RNase-free H_2O	4,6	
Total mix	15 µL	

* PowerUp Sybrgreen Mastermix (Applied Biosystems, Vilnus, Lithuania)

15 µL Mix + 5 µL DNA = 20 µL Total volume

Appendix.3 Protocol for dilution of concentrated forward and reverse primers; Bdi-F (0.3-300µM), Bdi-R (0.9-900µM), respectively.

PCR condition:

Temperature	Time	Cycles
50℃	2min	Hold 1
95℃	2min	Hold 2
95℃	15sec	45 cycles
60℃	1min	
72℃	30sec	

Appendix 3: Protocol for dilution of concentrated primers

To dissolve primers:

Formula:

X nMol primer/0,250nmol= Volume of solution

Bdi-F 300uM = 0,300 nmol

Concentration nMol/0,300nMol = X water

Bdi-R 900 µm = 0,900nMol

Concentration nMol/0,900nmol = X water

Appendix 4: Protocol Pyrosequencing

Instrument: PyromarkTM Q24 (Biotage), QIAGEN Germany.

Kit: Pyrogold SQA reagents, QIAGEN Germany.

Program: SQA analysis program calibrated for TBEV- Norwegian strand

1. Before going to start pyrosequencing:

- Make a worksheet of sequenced samples with names, PCR tube number and date of PCR analysis before going to start pyrosequencing.
- Turn on heating block 80 ° C 15 minutes before starting pyrosequencing.
- Check once diluted forward primer to 25 microns and include RNase-free water and pyromarks kit.

- Make sure you have all enzymes, substrates and buffers in room temperature on bench.
- Take out washing buffer and place it on the bench with a vacuum machine.

2. Prepare worksheet and Pyromark machine on computer

- First turn on the computer and Pyro mark machine. Select username / Password: CAS1200 / polymer1.
- Start program Pyro Mark Q24 and Pyro Mark Q24 machine and click the new run.
- Give a name to plate_ID
- Right click on track A1 in sheet view, then click load assay and select the desired assay (TBE-cyclic 60-TGAA-pyrosetup). Get assay of file or document.
- Choose Tools-Prerun Information, and the computer calculates the amount of enzyme (E), the substrate (S) and nucleotides (ATCG). Notes amounts of the worksheet
- Save run the file on the USBand transfer to Pyro Mark Q24. Give it a name according to date and samples. Do not press start run.

3. Label PCR strips and prepare Master mix and primer mix

Preparation of Master mix: (Shake well the beads)

Component	1xul
RNase free water	40
Binding buffer	18
Streptavidin	2

Aliquot 60ul in each tube and mix it well before use.

Preparation of Primer mix

Component	1x ul
Annealing buffer	24.7
Sequencing primer(30uM)	0.3

Aliquot 25ul in each tube.

- Make master mix and primer mix and aliquot master mix in strips.
- Put primer mix in the refrigerator and aliquot 60 µl master mix in strips

- Put arm cap and work in Kojarbenk: Add 20 µl of PCR product wells in strip and place the lid after a strip is completed. Transport the strips using another rack to the workstation, place the rack fastened with tape on shaker and secure it with rubber band) and shake it for 10 minutes (room temperature).
- Aliquot the primer mix in 25 µl each of Pyro Mark Q24 sequencing plate.
- After shaking, open the strips in cabinet (just use hands to take the strips into the cabinet as we have three stripes for a full run.
- Place plate with primer mix in stand with NO.1 top left.

4. Washing of PCR products

- Keep plastic vessels in vacuum workstation pyrosequencing and fill wash buffer, NaOH), sterile water ,70% ethanol.
- Turn on the vacuum machine, check that it works by putting the washing device in the tub with sterile water, and let it run for about 20 seconds.
- Put scrubber in sample tubes for 1 min. Look after the sample is drawn up and the tubes are properly empty. Shake it after some time.
- Put scrubber in 70% ethanol for 5 seconds when the liquid enters the tube.
- Transfer the scrubber to denaturing buffer, NaOH and wash for approximately 5 seconds.
- Transfer the scrubber to WASH wait until you see the fluid enters the hose and wash in approximately 10 sec.
- Lift the scrubber vertically. Set the switch on the washing device in the off position and wait 5 seconds until there is no vacuum longer, turn off the vacuum pump.
- Keep the washing device in the wells of pyrosequencing plate to elute the sample from the filter.
- After 5 minutes discard the strips in yellow bucket in bench.
- Keep pyrosequencing plate on pre-heated block.

Cleaning: Keep scrubber in 1st vessel with water for 10 seconds by shaking it without vacuum. Soak up about 70 ml of water under vacuum. Lift it vertically and hold for 5 seconds to remove remaining liquid. Turn off the washing device and tighten end of the vacuum pump.

5. Start the program:

- Add enzyme (E), substrate (S) and nucleotides (ATCG) which the computer has calculated.

- Put the block in the machine with the label towards you by taking off the bottom and secure it with locking pin.
- Select the correct program and push start run.

6. After pyromark run is complete

- Running file is automatically saved on the USB. Move USB to the PC in room 008.
- Get the run from USB.
- Click "analyzes all the wells".
- Press report SQA full report, and SQA pyrogram report(landscape). Save it as PDF on F, and print it.
- Throw pyrosequencing plate and clean block properly with sterile water.

Appendix 5: Modified protocol of primer dilution

According to dissolving primers formula in Appendix 3:
X nM primer/0,250nmole= Volume of solution
250uM=250pmole/ul=0.250nmole/ul
As Bdi-F 300uM=0.300 nmole
We have 49.3 nmol
49.3nmole/0.300nmole=164.3ul Water
As Bdi-R 900uM=0.900nmol
We have 59.1 nmole
59.1nmole/0.900nmole=65.7 ul Water

Appendix 6: Protocol for dilution of primers in 10-fold series (10^{-0} to 10^{-6})

PCR tests for diluted primers by 10-fold serial dilution from 10^{-0} to 10^{-6} (1:128, 1:1280, 1:12800, 1:128000, 1:1280000, 1:12800000, 1:128000000) with their Ct value.

Diluted primers	Ct value	Result by PCR
1:128	24.94	+
1:1280	26.82	+
1:12800	30.48	+
1:128000	32.22	+
1:1280000	-	-
1:12800000	-	-
1:128000000	-	-

Appendix 7: RT-PCR report of Brønnøysund samples

PCR test of 10 nymph tick samples from Brønnøysund with 2 positive control dilutions showing full quantitation concise report with graph.

www.qiagen.com

1. Quantitation Report

Experiment Information

Run Name	10 nymph tick samples Brønnøysund with 2 dilutions of positive control (128000,1280000)29.10.20
Run Start	10/29/2020 11:11:55 AM
Run Finish	10/29/2020 1:14:50 PM
Operator	Khansaa
Notes	Babesia spp(10 nymph tick samples and 2 dilutions 128000,1280000)
Run On Software Version	Rotor-Gene Q Software 2.3.1.49
Run Signature	The Run Signature is valid.
Gain Green	10.

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (copies/ul)	Calc Conc (copies/ul)
5		N4b (19)T1	Unknown	35.28			
6		N4b (19)T2	Unknown	33.39			
7		N4b (19)T3	Unknown	32.44			
8		N4b (19)T4	Unknown	34.07			
9		N4b (19)T5	Unknown	34.64			
10		N4b (19)T6	Unknown	35.50			
11		N4b (19)T7	Unknown	34.63			
12		N4b (19)T8	Unknown	34.38			
13		N4b (19)T9	Unknown	33.65			
14		N4b (19)T10	Unknown		NEG (NTC)		
17		128000	Unknown	32.94			
18		1280000	Unknown		NEG (NTC)		
21		N.6	Unknown		NEG (NTC)		
22		N.6	Unknown		NEG (NTC)		

Appendix 8: RT-PCR report of Hille samples

PCR test of 24 nymph tick samples from Hille with 4 positive control dilutions showing full quantitation concise report with graph.

www.qiagen.com

2. Quantitation Report

Experiment Information

Run Name	Babesia S10(20) Hille25-48 and 1:1280-1:128000013.04.21
Run Start	4/13/2021 11:22:35 AM
Run Finish	4/13/2021 1:25:04 PM
Operator	Khansaa
Notes	Babesia S10(20) Hille25-48 and dilutions 13.04.21
Run On Software Version	Rotor-Gene Q Software 2.3.1.49
Run Signature	The Run Signature is valid.
Gain Green	9.33
Machine Serial No.	120803

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (copies/ul)	Calc Conc (copies/ul)
1		S (10)20(25)	Unknown	31.67			

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (copies/ul)	Calc Conc (copies/ul)
2		S (10)20(26)	Unknown	31.42			
3		S (10)20(27)	Unknown	30.40			
4		S (10)20(28)	Unknown	33.44			
5		S (10)20(29)	Unknown		NEG (Multi Ct)		
6		S (10)20(30)	Unknown	27.68			
7		S (10)20(31)	Unknown	29.50			
8		S (10)20(32)	Unknown		NEG (Multi Ct)		
9		S (10)20(33)	Unknown	31.00			
10		S (10)20(34)	Unknown	36.78			
11		S (10)20(35)	Unknown	32.61			
12		S (10)20(36)	Unknown	36.27			
13		S (10)20(37)	Unknown	35.09			

14		S (10)20(38)	Unknown	33.80			
15		S (10)20(39)	Unknown	29.54			
16		S (10)20(48)	Unknown	32.84			
17		S (10)20(41)	Unknown	33.50			
18		S (10)20(42)	Unknown	32.49			
19		S (10)20(43)	Unknown	34.32			
20		S (10)20(44)	Unknown	37.06			
21		S (10)20(45)	Unknown	31.71			
22		S (10)20(46)	Unknown	31.71			
23		S (10)20(47)	Unknown	30.84			
24		S (10)20(40)	Unknown	35.58			
25		1:1280	Unknown	27.82			
26		1:12800	Unknown	27.93			

-

27		1:128000	Unknown				
28		1:1280000	Unknown				
32		neg.clean	Unknown				
33		neg.clean	Unknown				

Appendix 9: RT-PCR report of Haugesund samples

PCR test of 36 nymph tick samples from Haugesund with two positive control dilutions showing full quantitation concise report with graph.

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3. Quantitation Report

Experiment Information

Run Name	Haugesund samples 61-96(9.11.21)
Run Start	11/9/2021 1:30:47 PM
Run Finish	11/9/2021 3:30:57 PM
Operator	Khansaa
Notes	Haugesund last 36 samples Babesia and 4 positive control dilutions of Babesia
Run On Software Version	Rotor-Gene Q Software 2.3.1.49
Run Signature	The Run Signature is valid.
Gain Green	5.33
Machine Serial No.	120803

-

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (000)	Calc Conc (000)
1		H (19)61	Unknown	32.96			
2		H (19)62	Unknown	40.64			
3		H (19)63	Unknown	37.78			
4		H (19)64	Unknown	38.55			
5		H (19)65	Unknown	40.08			
6		H (19)66	Unknown		NEG (NTC)		
7		H (19)67	Unknown	35.94			
8		H (19)68	Unknown	32.63			
9		H (19)69	Unknown	38.49			
10		H (19)70	Unknown	40.19			
11		H (19)71	Unknown	39.41			
12		H (19)72	Unknown	28.78			
13		H (19)73	Unknown	32.04			
14		H (19)74	Unknown	38.65			

15		H (19)75	Unknown	34.72			
16		H (19)76	Unknown	39.09			
17		H (19)77	Unknown	33.91			
18		H (19)78	Unknown	36.64			
19		H (19)79	Unknown	37.10			
20		H (19)80	Unknown	40.90			
21		H (19)81	Unknown	33.43			
22		H (19)82	Unknown	35.22			
23		H (19)83	Unknown	36.39			
24		H (19)84	Unknown	35.24			
25		H (19)85	Unknown		NEG (Multi Ct)		
26		H (19)86	Unknown	38.37			
27		H (19)87	Unknown	36.70			
28		H (19)88	Unknown	34.81			
29		H (19)89	Unknown		NEG (NTC)		

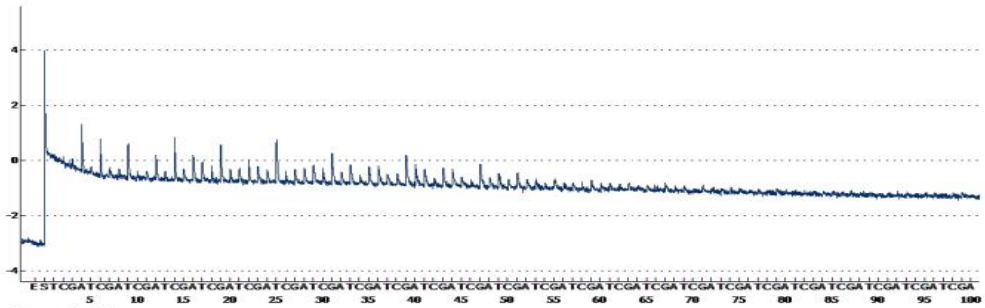
-

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (000)	Calc Conc (000)
30		H(19)90	Unknown	34.15			
31		H(19)91	Unknown	33.22			
32		H(19)92	Unknown	39.89			
33		H(19)93	Unknown	33.14			
34		H(19)94	Unknown	31.17			
35		H(19)95	Unknown	32.19			
36		H(19)96	Unknown	34.71			
37		n.c	Unknown		NEG (NTC)		
38		n.c	Unknown		NEG (NTC)		
39		1:128	Unknown	40.90			
40		1:1280	Unknown	24.86			

Appendix 10: Pyrosequencing graph of Brønnøysund sample

Pyrosequencing graph of Brønnøysund adult tick sample N4b (19)T12 confirming negative result might be due to high concentration of DNA, problem in cartridge or absence of Babesia species in ticks.

Well: B3
Assay: TBE_cyclic_100_TCGA
Sample ID: T-12
Note:
Analysis version: 2.0.7



Basecalled Sequence:

Quality	Number of bases
Passed	0
Check	0
Failed	0

Quality at end of Quality Window (20 bases): InsufficentDataForAnalysis

Warnings:
Not analyzable due to lack of data

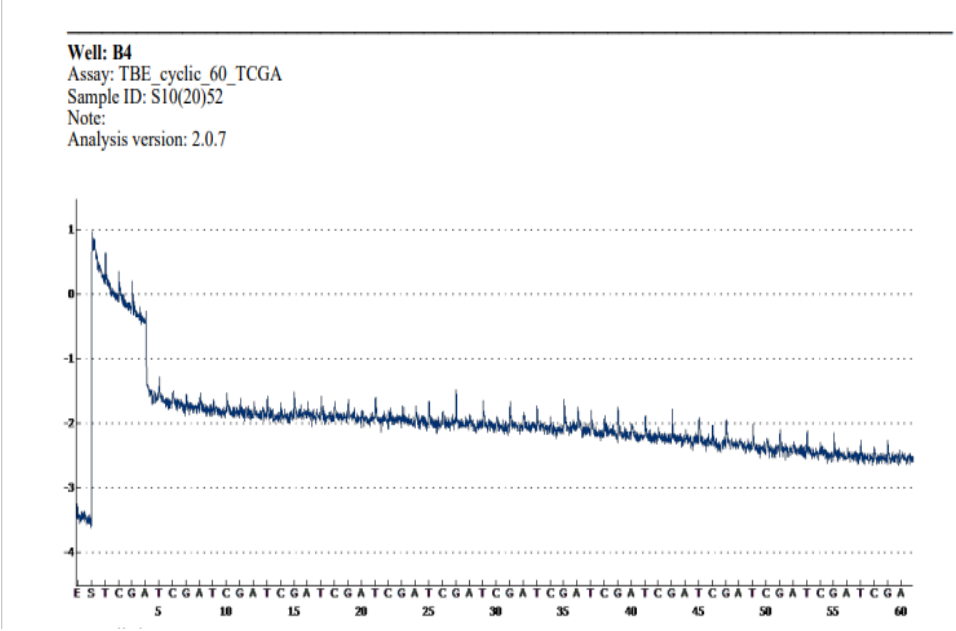
Appendix 11: List of Brønnøysund and Hille samples with their Ct values used for pyrosequencing

List of seven nymph tick samples from Brønnøysund and seven nymph tick samples from Hille with Ct value selected for second run of pyrosequencing.

Sampling Site	Sample Name	Ct value
Brønnøysund	N4b (19)28	36.31
	N4b (19)90	35.51
	N4b (19)77	36.31
	N4b (19)158	40.30
	N4b (19)178	33.63
	N4b (19)105	31.41
	N4b (19)123	31.58
Hille	S10(20)24	37.30
	S10(20)44	37.06
	S10(20)48	32.84
	S10(20)51	30.33
	S10(20)52	38.16
	S10(20)62	39.73
	S10(20)66	38.24

Appendix 12: Pyrosequencing graph of Hille sample

Pyrosequencing graph of Hille nymph tick sample S10(20)52 confirming negative result might be due to high concentration of DNA, problem in cartridge or absence of Babesia species in ticks.



Appendix 13: Sequence of positive controls of Babesia species analysed by pyrosequencing.

Babesia 1:1280

CCTTTTAACC CCGGTTCGGG CGAGATGATC GGATTGAATG GATGTT

Babesia 1:12800

CTTACCGTGG CAGTAACGGT TAACGGGGGA ATTAGGGGTT

Babesia 1:128000

CTTACCGTGG CAGTAACGGT TAACGGGGGA ATTAGGT

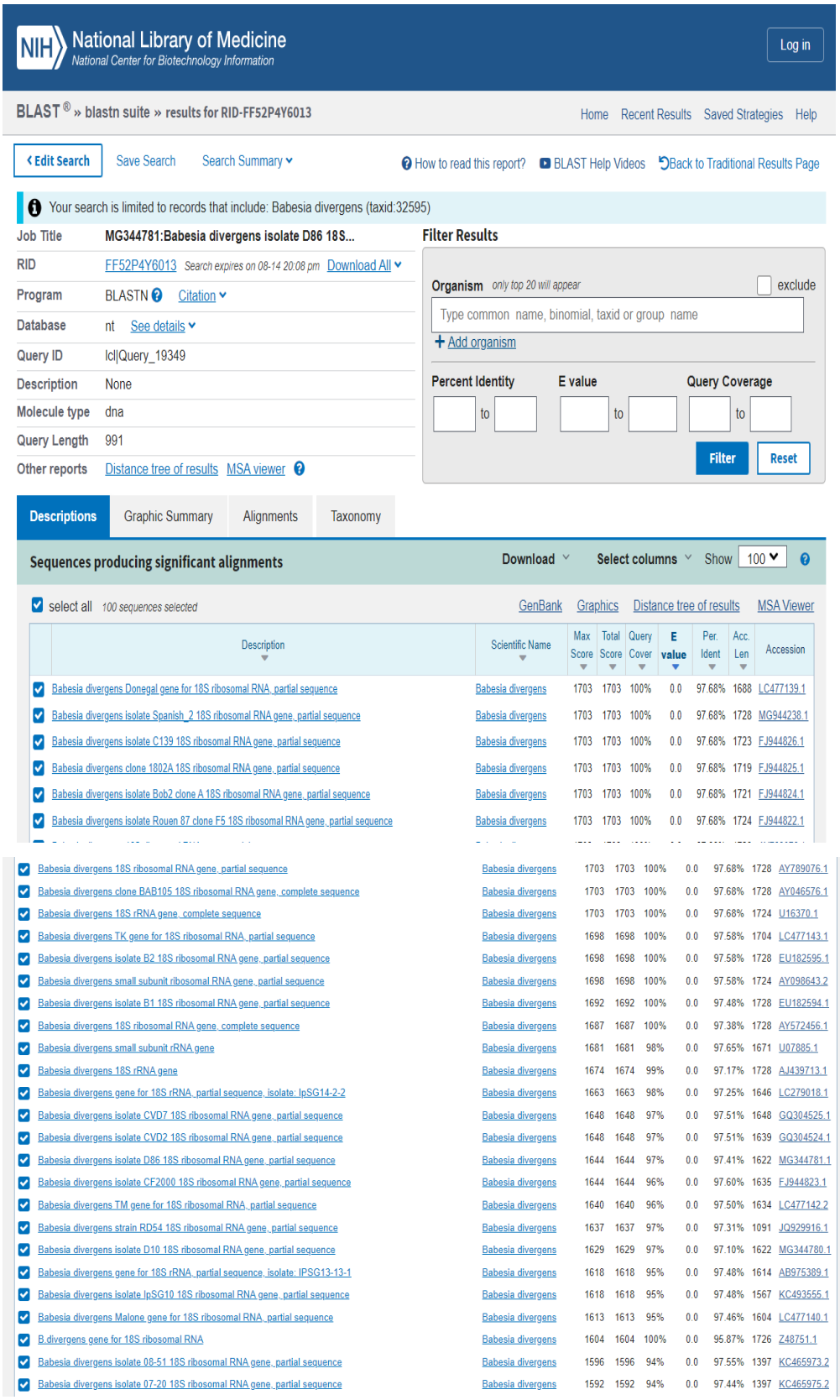
Babesia 1:1280000

GGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTTTGTT TT

Babesia 1:12800000

GGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTTTTTTTT

Appendix 14: Blast run on positive sequence confirmed by pyrosequencing to detect similar sequence of Babesia species



Babesia sp. EU1 isolate Arnhem 18S ribosomal RNA gene, partial sequence

GenBank: GQ888709.1

GenBank Graphics

>GQ888709.1 Babesia sp. EU1 isolate Arnhem 18S ribosomal RNA gene, partial sequence

TGGTTGATCCTGCCAGTAGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTACAAACTTTT
TACGGTGAAACTGCGAATGGCTCATTACAACAGTTATAGTTTCTTTGGTATTCGTTTTCCATGGATAACC
GTGCTAATTGTAGGGCTAATACAAGTTCGAGGCCTTTTGGCGGCGTTTATTAGTTCTATAACCACCCTTT
TGGTTTTCGGTGATTCATAATAAACTCGCGAATCGCAATTTATTGCGATGGACCATTCAAGTTTCTGACC
CATCAGCTTGACGGTAGGGTATTGGCCTACCGAGGCAGCAACGGGTAACGGGGAATTAGGGTTCGATTCC
GGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGA
CACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCAATTGTCTTGTAATTGGAATGATGGTGACCTAA
ACCCTCACCAGAGTAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGC
GTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCGTTATCGAGTTATTGACTCTTGT
CTTTAATCGATTTCGCTTTTGGGATTTATCCCTTTTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGACT
TTTGTCTTGAATACTTCAGCATGGAATAATAGAGTAGGACTTTGGTTCTATTTTGTTGGTTTTTGAACCT
TAGTAATGGTTAATAGGAACGGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGT
TAAAGACGAACTACTGCGAAAGCATTTGCCAAGGACGTTTCCATTAATCAAGAACGAAAGTTAGGGGATC
GAAGACGATCAGATACCGTCGTAGTCCTAACCATAAACTATGCCGACTAGGGATTGGAGGTCGTCATTTT
TCCGACTCCTTCAGCACCTTGAGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTG
AAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGG
GAAACTCACCAGGTCCAGACAATGTTAGGATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTG
GTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCT
GCTAACTAGTACCCGTAAAAAGGTTCGTCCGTTACGGTTTGCTTCTTAGAGGGACTTTGCGGCTCTAAGC
CGCAAGGAAGTTTAAGGCAATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCTGCACGCGCGCTACAC
TGATGCATTCATCGAGTTTAATCCTGTCCCGAAAGGGCTGGGTAATCTTTAGTATGCATCGTGACGGGGA
TTGATTTTTGCAATTCTAAATCATGAACGAGGAATGCCTAGTATGCGCAAGTCATCAGCTTGTGCAGATT
ACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATCGAGTGATCCGGTGAATTATTCGGACC
GTGGCTTTTCCGATTCGTCGGTTTTGCCTAGGGAAGTCTCGTGAACCTTATCACTTAAAGGAAGGAGAAG
TCGTAACAAGGTTTCCGTAGGTGAA

Babesia capreoli isolate CVD5 18S ribosomal RNA gene, partial sequence

GenBank: GQ304526.1

GenBank Graphics

>GQ304526.1 Babesia capreoli isolate CVD5 18S ribosomal RNA gene, partial sequence

GCCATGCATGTCTAAGTACAAACTTTTTACGGTGAAACTGCGAATGGCTCATTACAACAGTTATAGTTTC

TTTGGTATTCGTTTTCCATGGATAACCGTGCTAATTGTAGGGCTAATACAAGTTCGAGGCCTTTTGGCGG
CGTTTATTAGTTCTAAAACCATCCCTTTTGGTTTTCGGTGATTCATAATAAACTTGCGAATCGCAATTTT
TTGCGATGGACCATTCAAGTTTCTGACCCATCAGCTTGACGGTAGGGTATTGGCCTACCGAGGCAGCAAC
GGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGC
AGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGCAATTG
TCTTGTAATTGGAATGATGGTGACCTAAACCCTCACCAGAGTAACAATTGGAGGGCAAGTCTGGTGCCAG
CAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATT
TTTGCGTGGTGTAAATATTGACTGATGTCGAGATTGCACTTCGCTTTTGGGATTTTTCCCTTTTTACTTT
GAGAAAATTAGAGTGTTTCAAGCAGACTTTTGTCTTGAATACTTCAGCATGGAATAATAGAGTAGGACTT
TGGTTCTATTTTGTTGGTTTGTGAACCTTAGTAATGGTTAATAGGAACGGTTGGGGGCATTCGTATTTAA
CTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCAAGGACGTTTTC
ATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCCTAACCATAAACTATG
CCGACTAGGGATTGGAGGTCGTCATTTTTCCGACTCCTTCAGCACCTTGAGAGAAATCAAAGTCTTTGGG
TTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAG
CCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACAATGTTAGGATTGACAGATTGA
TAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTA
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GATGTCCTGGGCTGCACGCGCGCTACACTGATGCATTCATCGAGTTTTATCCCTTCCCGAAAGGGCTGGG
TAATCTTTAGTATGCATCGTGACGGGGATTGATTTTTGCAATTCTAAATCATGAACGAGGAATGCCTAGT
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TCGAGTGATCCGGTGAATTATTCGGACCGTGGCCTTTCCGATTCGTCGGTTTGGCCTAGGGAAGTCTTGT
GAACCTTATCACTTAAAGGAAGGAGAAGTCGAGCAA