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THE SEARCH FOR TRUE SOURCES OF TBEV AND RELATED PATHOGENS

The danger of professed beliefs on pathogen, host and vector interplays

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Institutions and Supervisors

The master thesis was performed in collaboration with the Norwegian Institute of Public Health and the Norwegian University of Life Sciences, Faculty of Veterinary Medicine and Bioscience, Department of Production Animal Clinical Sciences. The Master Thesis is credited 60 Study Points.

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Summary

Tick-borne disease in general is among the most important vector-borne diseases that are emerging as a threat to humans and is currently identified as a major health problem in many countries. Tick-borne encephalitis and lyme borreliosis are the main diseases transmitted by *Ixodes ricinus* ticks in Europe. Besides TBEV (Tick-borne encephalitis virus) and *Borrelia burgdorferi sensu lato*, *I. ricinus* is known to transmit other pathogenic microorganisms like- louping ill virus, *Anaplasma phagocytophilum*, *Francisella tularensis*, *Coxiella burnetii* and endosymbionts like-*Wolbachia pipientis* and *Midichloria mitochondrii* to humans and animals. Factors like climate change, human behavior and migrating animals are to blame for the spread of tick-borne diseases. The main route of disease transmission is through tick bites, but there is also evidence of infection through alimentary system for serious infectious agents like TBEV.

The knowledge of natural foci and prevalence of these infectious microorganisms is important for risk assessment of human disease. In this study, a total of 3240 nymph and 234 adult ticks were collected from six location sites of three counties of Norway (Hordaland, Vest-Agder and Østfold). The tick samples were analyzed and detected with Real-time PCR, pyrosequencing and direct sequencing to detect the microorganisms. The minimum infection rate or prevalence was calculated from the confirmed observations. Because body fluids of the host animals are valuable epidemiological parameters for TBEV, we also analyzed cow milk and serum from sheep and cows from Hordaland, Vest-Agder and Skedsmokorset. Cow milk was analyzed with PCR and serum with enzyme-linked immunosorbent assay. Two commercial ELISA kits were compared for their sensitivity and specificity.

The study confirms the existence of TBEV endemic foci compared to earlier study. The overall estimated TBEV prevalence in nymphs was 0.12% and in adult 2.13%. The *B. burgdorferi s.l.* prevalence in adult ticks was 6.41%. *A. phagocytophilum* prevalence in nymph was 7.96% and in adult 19.23%. Prevalence of *W. pipientis* in adult ticks was 10.68% and *M. mitochondrii* 83.33%. We also found TBEV prevalence in a new location in Hordaland county. The detection of TBEV in milk and serum from cows has never been reported in Norway before.

Abbreviations

Amplification: Method that makes copy of a specific DNA segment by PCR

Annealing: Process where the primers attach to the template (DNA strand) in real-time PCR when the temperature is lowered to about 50-65 ºC

ATP: Adenosine triphosphate, constitute adenine, sugar-ribose and three phosphate groups. The energy is released when ATP is cleaved to adenosine diphosphate and phosphate

bp: Base pair

cDNA: cDNA is a single stranded DNA which is complementary to and created from RNA template. Complementary DNA

Ct-value: Thershold value. An expression for number of cycle needed to give a PCR product measured by florescence.

Co-localication: Located in the same area

Co-feeding: Ticks feeding on the same host at the same time and transferring infective agents from one to the other

Denaturing: Process where double stranded DNA dissociates into single strands when the temperature is between 95-98 °C

DNA: Deoxyribonucleic acid, a genetic material. Consist of two complementary chains of nucleotides build up by deoxyribose (sugar molecule), phosphate group and the bases adenine (A), thymine (T), cytosine (C) and guanine (G).

dNTP: Deoxyribonuclesidtriphosphate. It consists of dATP, dCTP, dGTP and dTTP. It is building block for DNA strand by binding between the hydroxyl group and phosphate group of next base

ddNTP: Didioxynucleosidtriphosphate. Lacking one hydroxyl group at 3’ end so that is cannot bind to a new phosphate. When ddNTP are bound to the strand, elongation will stop randomly.

DNA-polymerase: It is an enzyme catalyzing synthesis of new DNA strand

Electrophoresis: Process of separation of DNA molecules by electrical charge. Since DNA is negatively charged, it moves from the cathode to the positive anode by electrical charge

EPP: Estimated pooled prevalenc, statistical method for estimating prevalence of pooled sample with condifence interval

Erlichia: Previous name of *A. phagocytophilum*

Foci: It is a small area where infectious agents are distributed within a larger area

Gene: Hereditary part of the chromosome which control the function of all cells. Located in the cell nucleus and code for genetic information by the sequence of nucleic acids

Genome: A cell's total amount of genetic material

Genotype: The genetic basis of the traits.

Host: An organism where the pathogenic microorganim reproduces by the use of the cell nutrients and components from the host

«In house» real-time PCR: Real-time PCR that is develop internally at the institute and not commercially available

ISH: *In Situ* hybridization

***IricES*1**: Previous name of *M. mitochondrii*

LI: Louping ill, neurological disease among sheep and grouse

LIV: Louping ill virus. RNA virus within the family flaviviridae that cause LI.

MGP: Magnetic glass particle. Used in teh extraction of total nucleic acid by MagNa Pure extraction, where nucleic acid bind to the surface of these particles

MIR: Minimum infection rate. Estimation of prevalence without confidence interval

mRNA: Messenger RNA, building block of RNA and that codes for proteins.

MSIS: Meldesystemet for smittsomme sykdommer. National surveillance of infectious diseases in Norway

Meningitis: Inflammation of meninges
Meningoencephalitis: Inflammation of brain and meninges.
Meningoencephalomyelitis: Inflamation of brain, meninges and spinal cord

Nucleic acid: Biochemical substance that are building blocks of DNA and RNA

Oligonucleotide: Short fragments of DNA and RNA (probe and primers)

PCR: Polymerase Chain Reaction. A method to copy specific nucleotide sequencing *in vitro* from a DNA template

Polymerase: DNA-polymerase is a thermo-stable enzyme that catalyzes synthesis of new DNA strand by the use of dNTP from the template.

Pool: Certain amount of arthopods analyzed together in a tube, for example nymphs in a group of ten

Primer: Short synthetic single stranded DNA sequences that bind to the complementary DNA strand (forward primer=at start of the target sequence, reverse primer=end of the target sequence

Probe: Short synthetic single stranded DNA that binds to the PCR product between the forward and reverse primer and emits fluorescent when polymerase dissociates the reporter from the quencher in TaqMan probe. This is detected in real-time PCR when PCR-product is formed.

Quencher: It works as an inhibitor of the reporter in real-time PCR by reducing fluorescence when the quencher and reporter are close. It is attached to 3' end of the probe

Real-time PCR: It is a quantitative measure of PCR product formed in each cycle. Detected by a probe that emits fluorescence when the template is amplified

Reporter: It works as flurorescence signals in real-time PCR by emiting fluorescence as the measures of PCR fragments that are made in real-time when the quencher and reporter are dissociated. It is attached to 5' end of the probe

Revers transkripsjon: A process, where reverse transcriptase enzyme are used to make cDNA from RNA

RNA: Ribonucleic acid. These are located in cell nucleus and cytoplasm. It is genomic material for RNA virus. It is different from DNA by the content of uracil (dUTP) instead of thymine (dTTP), and is often single stranded

RT-PCR: Reverse transcription PCR. RNA is transcribed to cDNA by the enzyme reverse transcriptase.

Subtype: It is genetic variant of viruses like TBEV and LIV.

TBE: Tick-borne encephalitis. In Norwegian:Skogflåttencefalitt. Infection of the central nervous system due to TBEV

TBEV: Tick-borne encephalitis virus. In Norwegian: Skogflåttencefalittvirus. A single stranded RNA virus that belongs to the flaviviridae family causing TBE.

TBEV-Eu: European subtype of TBEV.
TBEV-Fe: Far-eastern subtype of TBEV.
TBEV-Sib: Siberian subtype of TBEV.
TBF: Tick-borne fever.

Transcription: Synthesis of RNA with DNA as a template

Transovarial: In this context when TBEV is transmitted from fertile female ticks to the eggs

Vector: A vector carries the pathogenic microorganism and transfers to a new individual

The aim of the study

Tick-borne diseases have become growing problems in Norway and the whole of Europe during the last decades. The prevalence of pathogens and the abundance of vectors and animals, impact on the risk of human infections with tick-borne diseases. Tick borne encephalitis virus (TBEV) is mainly transmitted by tick bites, but infection also occurs through the alimentary system by consumption of raw (unpasteurized) dairy products from infected animals. The main goal for this master thesis was to investigate the prevalence and the co-infection of tick-borne microorganisms in ticks with the focus on TBEV, detected TBEV in cow milk and to study the prevalence of antibodies against TBEV in sheep and cow sera milk.

The master thesis is a continuation of previous studies at the Norwegian Institute of Public health (FHI) on TBEV in Norway. FHI is the reference laboratory for tick borne encephalitis (TBE) in Norway, and the mapping of TBEV in ticks is important for the national vaccine recommendation. This thesis is a part of two international projects focusing on tick-borne diseases; ScandTick (Interreg IVA project ID.1672226) and Barentsregion project B1412.

The work behind this thesis answers two main research goals and ancillary sub-goals by the application of molecular and serological in ticks and ruminant tissues:

Part 1: **Molecular detection of tick borne viruses and bacteria.**

- Important organisms in the microbiome of ticks: TBEV, Louping-ill virus (LIV), *Borrelia spp*, *Anaplasma phagocytophilum*, *Wolbachia pipientis* and *Midichloria mitochondrii* in ticks from southern- and western Norway.
- Co-infection and co-localization of various tick borne microorganism in ticks.
- Comparison of the prevalence of TBEV in ticks collected in year 2011, 2013 and 2014.
- Establishing nucleic acid extraction methods in milk and investigating the prevalence of TBEV in cow's milk.

Part 2: Serological detection of TBEV

- Establishing detection methods on TBEV IgG antibodies from sheep and cow sera, based on an earlier studies in deer. This part includes the comparison of two different ELISA commercial kits (Immunozym and Enzygnost).
- Investigate the presence of antibodies against TBEV in sera from sheep and cows from sites in southern-, eastern- and western parts of Norway.

TBEV is known to be prevalent in *Ixodes ricinus* from the southern part of Norway. A third aim of this study was to investigate the prevalence of TBEV in new locations.

Introduction

Ticks

Ticks are hematophagous ecto-parasites of mammals, birds and reptiles throughout the world. Most of the tick's pathogens are acquired by blood meals from systemically infected hosts, after which ticks become vectors of disease. All three stages of ticks can acquire the microorganisms when feeding on a systemically infected competent reservoir hosts. Uninfected ticks can also become infected when feeding together with infected ticks by a process called co-feeding, even in the absence of disseminated infection in the host (Randolph, Gern, & Nuttall, 1996). In unfed ticks, *Borrelia* is usually located in the midgut, although different organs may be affected (Barbour & Hayes, 1986). *B. burgdorferi* sensu lato in ticks is trans-stadially transmitted to next stage, but rarely migrates to the ovaries of female ticks and result in trans-ovarial transmission to their larval progeny (Derdakova & Lencakova, 2005; Parola & Raoult, 2001).

After the tick becomes infected, it passes the pathogen between its life stages, known as trans-stadial transmission (Parola & Raoult, 2001). There are two main groups of ticks: the hard ticks (Ixodidae) and the soft ticks (Argasidae). The *Ixodes ricinus* complex is of particular importance in transmitting veterinary and zoonotic disease across the world. This complex comprises 14 tick species and is known to transmit LIV, TBEV, *A. phagocytophilum*, *Babesia* spp. and *B. burgdorferi* spp. *I. ricinus,* which is also one of the most studied species of hard ticks, has low host specificity and can transmit blood parasites, bacteria (*Rickettsiae* and Spirochetes) and viruses (Parola & Raoult, 2001).

I. ricinus

I. ricinus is the most important vector of tick-borne zoonoses in Norway and Europe (Araya-Anchetta, Busch, Scoles, & Wagner, 2015). It is found from Scandinavia down to Northern Africa, and across to Russia and Turkey (Estrada-Pena et al., 1998; Lo et al., 2006). It is suggested that the expansion of tick borne diseases in endemic regions approaches higher altitudes. Climatic changes are most likely, one of the major driving forces for the geographic changes in the distribution of the vectors and diseases (Holzmann et al., 2009). The distribution of *I. ricinus* as far as north-western Europe (Brønnøy, Norway, 1° south of the Arctic) has been known since the 1930s (R, 1983). Ticks quest for blood-meal between spring and autumn. It climbs on vegetation and waits for passing animals or humans. Carbon dioxide given off by the animals attracts ticks.

The habitat of *I ricinus* is situated in the leaf litter and the low strata vegetation of temperate deciduous woodlands and mixed forests. In areas with high rainfall, it also occurs in high densities in coniferous forests and in open areas such as grasslands (J. S. Gray, 1998). *I. ricinus* has particular requirements regarding the humidity of its habitats and a relative humidity (RH) value of >80% is important for the activity and survival of free-living *I. ricinus* (Randolph & Storey, 1999). A wide range of vertebrates such as reptiles, birds, small-, medium- and large sized mammals serve as hosts for *I. ricinus*. It is a three host ticks, and requires three blood meals to complete its molting processes. Small

mammals, birds and reptiles are common hosts for larvae and nymphs; and medium to large sized mammals are parasitized by adult *I. ricinus*. The life cycle lasts for 2-6 years, typically 3 years, since each stage takes about a year to develop to the next instar (J. S. Gray, 1998). *I. ricinus* ticks are active from spring to autumn, generally from February to November and the seasonal activity of the three active stages usually shows a bimodal pattern with high questing activity in the spring (May-June) and another peak in the autumn (September-October). The tick activity in spring is usually greater than in the autumn, except for larvae, which show the opposite situation in some areas (Gern, Burgdorfer, Aeschlimann, & Krampitz, 1993). Ticks are observed to be active when temperatures are above 5-8 ºC and the activity is lower when temperatures are high (Estrada-Pena, Martinez, Sanchez Acedo, Quilez, & Del Cacho, 2004; Lindgren & Gustafson, 2001).

The bacteria transmitted by *I .ricinus* include *Borrelia* species, *A. phagocytophilum*, *Rickettsia* spp., *F. tularensis*, *C. burnetti* and *Bartonella* species (Parola & Raoult, 2001). It transmits viruses like TBEV and LIV as well as protozoan pathogens (*Babesia microti*, *B. divergens*) to humans and animals (Derdakova & Lencakova, 2005). In addition, ticks may carry intracellular bacteria that are apparently harmless to mammals (Cowdry, 1925). The symbionts *Midichloria mitochondrii* is the dominant bacterium in the microbial community of *I. ricinus* (Sassera et al., 2006). However, their biological role in ticks and hosts is still unknown. Competent reservoir hosts are the systemically infected animal species that harbor the pathogen and represent the long-term infection source for the feeding vectors. The difference in tick infestation between woods and pastures is most likely due to the better conditions in host habitats (Walker, Alberdi, Urquhart, & Rose, 2001). For *Borrelia* spp., competent reservoirs are the wood mouse (*Apodemus sylvaticus*), yellow-necked field mouse (*Apodemus sylvaticus*), the black vole (*Clethrionomys glareolus*), meadow vole (*Microtus afrestis*), rats (*Rattus norvegicus* and *Rattus rattus*) and squirrels (*Sciurus vulgaris* and *Sciurus carolinensis*) (Derdakova & Lencakova, 2005).

Tick borne Pathogens

Tick-borne diseases (TBDs) represent a public health problem of growing importance in Norway and across Europe (Andreassen et al., 2012). The emergence and recognition of an increasing number of new TBDs in recent years highlights the significance of their zoonotic aspects (Parola & Raoult, 2001). The occurrence of TBDs depend on geographical locations, tick species and causative agents involved in the natural transmission cycles. In Norway, the incidence of human TBDs is restricted by the presence of *I. ricinus,* being the only significant vector of human tick-borne pathogens (Mehl et al 1987). Animal studies and clinical observations indicate that *I scapularis* ticks require at least 36 hours of attachment in order to transmit *B. burgdorferi* (des Vignes et al., 2001). On the other hand, ticks may transmit *A. phgocytophilum* within 24 hour in mice (des Vignes et al., 2001). The speed of transmission supports that daily inspection of the body for ticks, removal and showering are important as preventative measures of transmission. Risk of exposure to vector-borne pathogens is influenced by the abundance

of the vector and the prevalence of the pathogen within the vector population (Ostfeld, Canham, Oggenfuss, Winchcombe, & Keesing, 2006). It is believed that the risk of human tick-borne disease is also associated with the activity of the reservoir hosts.

Tick-borne encephalitis virus (TBEV)

The family Flaviviridae forms a TBE complex, which includes LIV, Langat virus, Pwassan Virus and Kyasanur Forest disease virus (Kovalev & Mukhacheva, 2014). The existence of tick-borne arboviruses in Norway have been known for years (Traavik, 1979). TBEV is a zoonotic virus that occurs on the Eurasian continent and causes tick borne encephalitis (TBE) in humans (Lindquist & Vapalahti, 2008). The virus is considered the medically most important arthropod transmitted virus (arbovirus) in Europe (Randolph, 2011). In addition to TBEV, the genus *Flavivirus* includes other important pathogens that are endemic throughout the world such as: dengue viruses, yellow fever virus and Japanese encephalitis virus (Dumpis, Crook, & Oksi, 1999). Several synonyms for TBE have been used at different times. The most common are: Früh sommer meningoenzephalitis (FSME or Western Subtype), Central European Encephalitis (CEE), Early Summer Encephalitis, Kumlinge's Disease (Western subtype), and Russian Spring Summer Encephalitis (RSSE) (Kaiser, 2012).

TBEV has three major subtypes: 1) The European subtype (TBEV-Eu), transmitted by *I. ricinus*; 2) Siberian subtype (TBEV-Si) and 3) Far Eastern subtype (TBEV-FE), both subtype transmitted by *I. persulcatus* (Dumpis et al., 1999; Gritsun, Lashkevich, & Gould, 2003; Lindquist & Vapalahti, 2008). The European subtype is widely distributed in Europe and the European part of Russia while far Eastern and Siberian subtypes are spread from Japan and the far east of Russia to the Baltic countries (Lundkvist et al., 2001). In addition to having different geographical distribution and vectors, the subtypes have different clinical manifestations (Lindquist & Vapalahti, 2008). The course and severity of human disease are different between the three subtypes. For the TBEV-FE subtype, a mortality rate of 30% has been reported-while for TBEV-Eu and TBEV-Sib, 1-2% and 6-8% of the infections are fatal, respectively (Gritsun et al., 2003). Transmission of TBEV is seasonal and occurs in the spring and summer, favored by vectors and reservoirs. TBEV is transmitted by 11 tick species, but only two species are main vectors: *I. ricinus* and *I. persulcatus* (Amicizia et al., 2013). TBEV-Eu is mainly maintained in nature by *I. ricinus* and TBEV-Sib and TBEV-FE by *I. presulcatus* (Lindquist & Vapalahti, 2008).

The TBEV in ticks

The virus can chronically infect ticks for the entire life cycle (larva, nymph and adult) and ticks can get infected with the virus by four methods: 1) at any three active stages (larvae, nymph and adult) by blood meal from viraemic hosts 2) infected adult female ticks pass the virus to eggs by trans-ovarial transmission and between mating ticks, 3) the vector remains infected from one life stage to the next, by trans-stadial transmission and 4) during co-feeding (Dumpis et al., 1999). Co-feeding can result in transmission between ticks in proximity even when the host has not yet developed viremia, or has developed immunity to the infection (Dumpis et al., 1999; Kaiser, 2008). TBE cases usually occur in the period between April and November that coincides with the level of tick activity.

Infection with the Eastern subtype of the virus occurs mostly in the spring, while infection with the Western subtype of the virus occurs mostly in the early autumn.

Structure of the virus

The RNA virus is 20-80 nm in diameter and contains a core and an envelope. The core consists of viral RNA and protein C. The envelope is composed of lipids and two glycoproteins, named membrane M and envelope protein E. Protein E is the most immunogenic antigen and induces neutralizing and protective antibodies. The virus is heat-labile and can be inactivated by pasteurization (Dumpis et al., 1999).

Louping ill virus (LIV)

The European TBEV subtype and LIV are two closely related tick-borne flaviviruses (Charrel et al., 2004). LIV causes encephalomyelitis in sheep and red grouse (*Lagopus lagopus scotia*), but is rarely fatal to humans. The virus is considered a major cause of red grouse mortality in endemic areas (Reid, Duncan, Phillips, Moss, & Watson, 1978). Phylogenetic studies indicate that LIV quite recently (first part of 20th century) was introduced to Norway from Scotland (McGuire, Holmes, Gao, Reid, & Gould, 1998). In the 1980s, LIV was isolated from sheep with encephalomyelitis in Southern Norway (Ytrehus, Vainio, Dudman, Gilray, & Willoughby, 2013). The disease is, however, very rare in Norway, in spite of the fact that *I. ricinus* is very common, the sheep population is relatively large in the European setting, sheep are frequently exposed to the ticks, and vaccination against LIV is not performed (Ytrehus et al., 2013). LIV is predominantly distributed on the sheep-rearing hillsides of Scotland, England, Wales and Ireland and has been recognized in the British Isles for at least 200 years (McGuire et al., 1998).There are four subtypes of LIV: British, Irish, Spanish and Turkish. British subtypes occur throughout Scotland, England, Ireland and Norway (McGuire et al., 1998).

Borrelia

The genus *Borrelia* belongs to the family Spirochaetaceae and order Spirochaetales. *I. ricinus* and *I. persculatus*, are the principal vectors of *B. burgdorferi* sensu lato in Europe and Asia, respectively. In the United States, the principal vector is the black-legged or deer tick, *I.scapularis* (Mead, 2015). In the early 1980s a spirochete, *B. burgdorferi*, was isolated and cultured from the midgut of *Ixodes* ticks. It resembles most other spirochetes in that it is a highly specialized, fastidious, motile, two-membrane and spiral-shaped bacterium that lives primarily as an extracellular pathogen. Several of the *B. burgdorferi* sensu lato cause lyme borreliosis that is transmitted by *Ixodes* ticks. Ticks are uninfected when they hatch from eggs; they acquire *B. burgdorferi* by feeding on infected reservoir hosts, principally mice, shrews and other small mammals. Humans are incidental or dead-end hosts that do not sustain large numbers of spirochetes in their tissues (Mead, 2015). Investigators have concluded that roe deer and red deer are incompetent reservoirs of *B. burgdorferi* (O. Rosef, Paulauskas, & Radzijevskaja, 2009). Spirochetes that are sensitive to destruction by the complement system of particular host species are lysed early in the midgut of the feeding tick and are thereby eliminated by the host (Kurtenbach et al., 2006).

B. burgdorferi sensu lato

The *B. burgdorferi sensu lato* complex is a genetically diverse group of spirochetes and currently comprises at least 18 genospecies, several of which can cause lyme borreliosis or lyme disease (Margos, Vollmer, Ogden, & Fish, 2011). In Europe, at least the following five different genospecies belonging to the *B. burgdorferi* sensu lato complex, have been found; *B.afzelii*, B. *garinii*, *B. burgdorferi sensu stricto* (ss), *B. valaisiana* and *B. lusitaniae*. Different reservoir hosts seem to harbor different genospecies of *B. burgdorferi* sensu lato, which is explained by differential properties of host complement systems that favor certain genospecies (Rauter & Hartung, 2005). Furthermore, there is a relationship between *Borrelia* species and their vectors. *B. burgdorferi sensu lato* has been associated with *I. persculatus* and *B. japonica* with *I. ovatus*, *B. turdi* with *I. turdus* and *B tanukii* with *I. tanuki* (Lee et al., 2000). Infection by multiple *B burgdorferi s.l* genospecies have been observed in ticks in many parts of Europe. However, *B burgdorferi s.s* and *B.lusitaniae* are the two *Borrelia* spp., least involved in co- infections (J. Gray, Kahl, Lane, & Stanek, 2002). The ticks must be attached to the host for at least 24 hours before transmission starts. The most effective transmission of *B. burgdorferi s.*s. occurs after 48 hour of the tick attachment (Ohnishi, Piesman, & de Silva, 2001). In the unfed tick, *Borrelia* is generally located in the midgut where they express outer surface protein A (OspA) but not OspC. On its surface, OspA possesses a receptor for plasminogen of host organisms. After the tick starts to feed on the host, plasminogen changes into plasmin, which facilitates *Borrelia* migration through the midgut wall and hemocoel (Coleman et al., 1997). During the blood meal, the synthesis of OspC is upregulated and the synthesis of OspA is downregulated (Ohnishi et al., 2001). The tick secretes vasoactive mediators and immune-modulators that facilitate the transmission of the pathogens to the host (P. A. Nuttall, Paesen, Lawrie, & Wang, 2000).

Anaplasma phagocytophilum

The Genus *Anaplasma* comprises gram negative, obligate intracellular rickettsiae. It belongs to the family Anaplasmataceae, in the order Rickettsiales. The bacterium *A. phagocytophilum* (formerly *Ehrlichia phagocytophilia*) can cause infection in animals such as; sheep, goats, cattle, horses, dogs, cats, roe deer, reindeer, wild animals (Woldehiwet, 1983) and humans. Infection in animals is prevalent in *I. ricinus* endemic region of Europe (S. Stuen, 2007), but clinical cases have never been discovered outside Europe. Based on phylogenetic analyses, *Ehrlichia* phagocytophilia, *E. equi* and human granulocytic Ehrlicia (HGE) have been reclassified in the genus *Anaplasma* (Bakken & Dumler, 2001). Six variants, based on *16S* rDNA sequencing, have been described (Granquist, Aleksandersen, et al., 2010). Different variants may exist within the same sheep flock and even simultaneously in the same animal. *I. ricinus* has been found to be the main vector of *A. phagocytophilum* in Europe (Strle, 2004). European wild rodents have been suggested to be competent reservoirs of *A. phagocytophilum* (Liz et al., 2000). In addition, roe deer are also reservoir hosts for *Anaplasma spp.* and *I. ricinus* (Walker et al., 2001).

Wolbachia pipientis

W. pipientis is an obligate, intracellular α-proteobacterium and a member of the Rickettsiales family (LePage & Bordenstein, 2013) which was discovered in 1924 in the ovaries of *Culex pipiens* mosquitoes (Hertig & Wolbach, 1924). *Wolbachia* spp. is the predominant bacterial endosymbiont of arthropods and about 40% of all arthropod species harbor the bacterium (Zug & Hammerstein, 2012). The bacteria are found in the reproductive tissues of all major groups of arthropods and transmitted vertically from the female hosts to their offspring, in a pattern analogous to mitochondria inheritance (Frydman, Li, Robson, & Wieschaus, 2006). The most studied biological manipulatory mechanism of *W. pipientis* is the cytoplasmic incompatibility (CI), a type of embryonic lethality that occurs when *Wolbachia*-infected males mate with females that do not harbor the same strain (Iturbe-Ormaetxe & O'Neill, 2007). Other reproductive phenotypes include selective killing of male offsprings, the conversion of genetic males into functional phenotypic females and induction of parthenogenesis (Iturbe-Ormaetxe & O'Neill, 2007; LePage & Bordenstein, 2013). Domestic mosquitoes (*Aedes aegypti*) infected with *Wolbachia* have been shown to inhibit replication of dengue, chikungunya, yellow fever viruses and malaria parasites, hence providing a potential in biological control strategies for human pathogens (van den Hurk et al., 2012). While in bedbug *Cimex lectularius*, *W. pipientis* resides in a bacteriome and appears to be an obligate mutualists (Hosokawa, Koga, Kikuchi, Meng, & Fukatsu, 2010). The phenotypic consequences of *W. pipientis* infection for their hosts are highly diverse and new impacts are regularly discovered (Plantard et al., 2012).

Midichloria mitochondrii

The Order *Rickettsiales*, family *Midichloriaceae* in which *M. mitochondrii* is an intracellular α-proteobacterial symbiotic bacterium that inhabits the germline of its female hosts (Sassera et al., 2006). Residence within host membranes is typical of many intracellular α-proteobacterial genera that are closely related to *M. mitochondrii*, such as *Wolbachia*, *Ehrlichia* and *Anaplasma* (Beninati et al., 2004). Based on phylogenetic evidence from *gyrB* gene sequencing, electron microscopy (EM), in situ hybridization, and PCR sequencing of the *16S* rRNA gene (AJ566640) and gyrB gene (AM159536), Sassera and co-workers named the bacterium *Candidatus* Midichloria mitochondrii (Sassera et al., 2006). This was then in accordance with the guidelines of the International Committee of Systemic Bacteriology, saying that uncultivable bacteria should be classified as *Candidatus* (Murray & Stackebrandt, 1995; Sassera et al., 2006). It is the first and only bacterium identified to reside within animal mitochondria (Ninio et al., 2015; Sassera et al., 2006). The endosymbiotic bacterium is found primarily in the ovaries or malpighian tubules of ticks (Cowdry, 1925; Noda, Munderloh, & Kurtti, 1997). Lewis discovered in 1979, rickettsia-like microorganism in the mitochondria of ovarian cells of female *I. ricinus* that were fed on sheep, infected with *Cytoecetes phagocytophilia* (now *A. phagocytophilum*), the causative agent of tick-borne fever (Lewis, 1979). Initially, the α-proteobacterium was given the temporary designation IricES1 (*I. ricinus* Endosymbiont 1) pending further taxonomic characterization (Beninati et al., 2004).

The *gyrB* gene sequence analysis showed the closeness to sequences from the genera *Anaplasma*, *Ehrlichia*, *Wolbachia*, *Neorickettsia* and *Rickettsia* (Sassera et al., 2006). Figure 1.1 shows the phylogenetic relationship of *M. mitochondrii* to other members of the α-proteobacteria based on *gyrB* sequencing. In EM, a Gram-negative bacterium with bacillus shape, can be seen, that is 0.45 µm in diameter and 1.2 µm in length. In the cytoplasm and intermembrane space of the mitochondria of the ovarian cells, the bacterium varies in number from single to more than 20 (Sassera et al., 2006). The symbiont appears to be ubiquitous in the female *I. ricinus* across its distribution (up to 100% prevalence), while there is a significantly lower prevalence observed in males (44%). Also the bacterial load in males is low compared to females (Lo et al., 2006). The *gyrB* gene is a 145 bp fragment that encodes the protein DNA gyrase subunit. Studies show that the copy number of the *gyrB* gene is high, following engorgement and low following molting from one stage to another (Sassera et al., 2008). The symbiont does not cause sex ratio distortion and is transferred to both males and females, horizontally and vertically (Sassera et al., 2008). *M. mitochondrii*, that resides in the tick's salivary glands have been shown to cause seropositivity in 60% of humans exposed to tick bites (N=80), and close to 1% in healthy blood donors (N=169) (Mariconti et al., 2012). This makes *M. mitochondrii* interesting from a medical perspective.

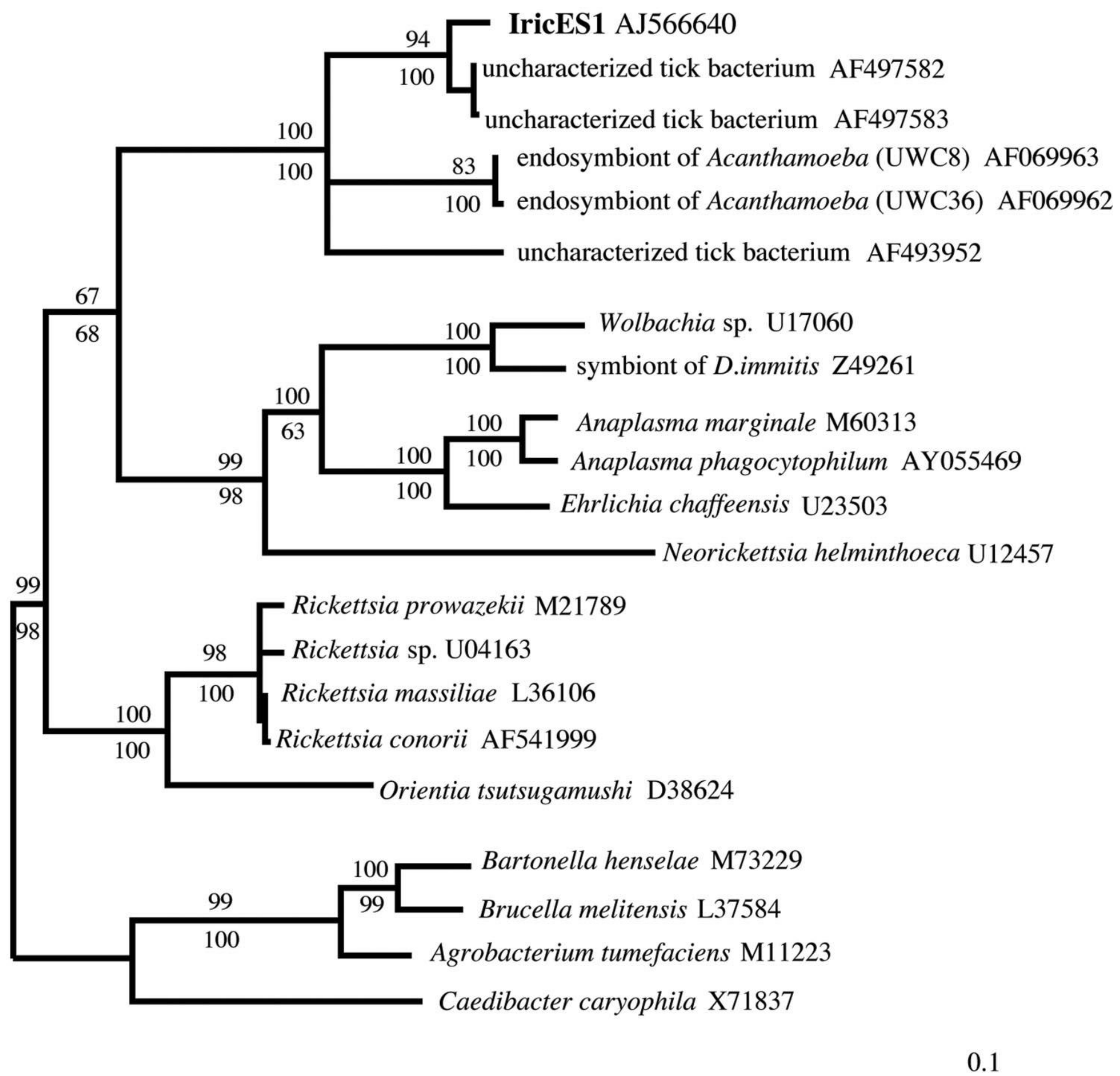

Figure 1. Phylogenetic comparison of the *16S* rRNA of *IricES1 (M. mitochondrii)*, an intracellular bacterium of *I. ricinus*, with the 16S rRNAs of selected members of the α-proteobacteria (Beninati et al., 2004).

Human and animal tick-borne diseases in Norway

Tick-borne encephalitis (TBE)

TBE is a zoonotic disease, affecting the central nervous system in humans. TBEV-associated central nervous system diseases in ruminants are rare (Bago, Bauder, Kolodziejek, Nowotny, & Weissenbock, 2002). The disease was first described by the Austrian physician, Shneider, in 1931 (Amicizia et al., 2013; Dumpis et al., 1999). The first TBE case in Norway occurred in 1997 while in Sweden and Finland first reported in 1954 and 1956, respectively (Skarpaas, Ljostad, & Sundoy, 2004). TBE is a viral tick-borne infectious disease that occurs in endemic areas across large regions of central and

eastern-Europe and Russia (Amicizia et al., 2013). The epidemiology of TBE is closely related to the ecology and biology of Ixodid ticks (Kovalev & Mukhacheva, 2014). The severity of disease depends on the subtype and age of the patient. The reported case fatality rate associated with symptomatic infections is 0.5-2% for the European subtype, 1-2% for the Western subtype and 5-20% for the Eastern subtype (Balogh et al., 2012). There is no cure against TBE and apart from the use of hyperimmunoglobulins in patients above the age of 14, symptomatic therapy is the only means of providing patient support (Lindhe, Meldgaard, Jensen, Houser, & Berendt, 2009). The disease TBE is not regarded as a communicable disease, however the infection can occurs through the alimentary canal by consumption of unpasteurized milk from infected goat, cow and sheep (Cisak et al., 2010).

Route of infection and pathogenesis of TBE

TBEV is transmitted from the saliva of an infected tick, minutes after the tick-bite (Lindquist & Vapalahti, 2008). The TBEV is transmitted to humans, usually by tick bites; occasionally cases occur following consumption of infected unpasteurized milk. Serological surveys suggest that between 70 and 95% of human infections in endemic regions, are sub-clinical (asymptomatic) (Gritsun et al., 2003). In rare condition, the virus may also be transmitted by blood transfusion and during working in laboratory (Suss, 2003). Transmission to a vertebrate hosts during blood feeding, most probably occurs via saliva that contains the virus secreted from infected salivary glands (P. A. Nuttall, Jones, Labuda, & Kaufman, 1994).

Only one-third of patients who are infected with TBEV develop symptomatic disease (Kaiser, 2012). After inoculation through a tick bite, the virus multiples at the Langerhans cell (dendritic skin cells), which are carried to local lymph nodes. The virus replicates in T-lymphocytes, B-lymphocytes and macrophages of the thymus and spleen. High production of viruses is a prerequisite for the virus to cross the blood-brain barrier because the capillary endothelium is not easily infected (Kaiser, 2008). Leukocytes migrate between tick feeding sites bearing infective virions and provide a transport route for the virus between co-feeding ticks, independent of a systemic viraemia (Randolph, 2011). The incubation period ranges from four to 28 days. The incubation period following exposure by the alimentary route is shorter (two days) than by tick bite (Hudopisk et al., 2013). The clinical outcome is biphasic. The symptoms in the first phase are sudden high fever, headache, muscle and joint pain, nausea, vomiting and fatigue. These symptoms cease after 5-10 days and an asymptomatic period of six to10 days follows. In the second febrile phase, high fever and neurological symptoms develop (Balogh et al., 2010). TBE results in meningitis in about 50% of patients, meningoencephalitis in 40%, and meningoencephalomyelitis in 10% (Dumpis et al., 1999; Kaiser, 2008). Meningoencephalomyelitis is the most severe form of the disease. Double infection with TBEV and *B. burgdorferi*, may result in a more severe disease (Kaiser, 2008). Alimentary transmission usually results in milder neurological complications, than transmission by ticks (Gritsun et al., 2003). Pasteurization is confirmed to prevent milk-borne TBEV infection (Dumpis et al., 1999). Prevention is by vaccination of people and dairy animals

in endemic areas (Balogh et al., 2012), although it is not known how long the immunity persists in animals.

Epidemiology of TBE

TBEV is endemic in foci from central Europe and the Scandinavian Peninsula in the west through the Eurasian continent to Far East Asia (Kaiser, 2008). The virus is endemic in 27 European countries. The incidence of clinical cases is reported to be 10,000 to 15,000 per year worldwide (Amicizia et al., 2013). The prevalence of ticks infected with TBEV in endemic areas in Europe usually varies from 0.5% to 5%, but in some regions of Russia, a prevalence of 40% has been reported (Dumpis et al., 1999). One study has reported that the prevalence in Europe varies between 0.1% and 5% in ticks identified by reverse transcriptase (RT-PCR), with an increasing prevalence during the life-cycle of the tick, and up to 10% in engorged ticks removed from individuals (Lindquist & Vapalahti, 2008). The endemic foci of TBEV are increasing. Environmental changes to warmer and more humid conditions support the spread of tick habitats and establishment of new TBEV micro-foci, which pose a threat to development of new and high abundance infection centers (Lindhe et al., 2009). Some studies suggest mild winters and autumns may be responsible for high tick densities (Kaiser, 2008). In addition, with the increase of travelling to endemic regions, the risk for tourists in acquiring TBEV will increase (Dumpis et al., 1999). Competent reservoirs and hosts of the virus are mainly small rodents (voles, mice). Other hosts that support virus circulation are different species of wild and domestic mammals (sheep, cattle, dogs, deer and swine). Humans are incidental and dead-end hosts (Kaiser, 2008). In Norway, *I. ricinus* is most prevalent along the coast from Hvaler in the south-east and along the coast, up to the north-west coast of Norway. However, the tick has been detected in the northern part of Norway as far as Brønnøysund in Nordland County at 66 ºN (Larsen et al., 2014). In Norway, the TBE vaccine is recommended for people with a history of tick bites who engage in outdoor activities in areas with a known occurrence of TBEV. Currently the recommendations include the counties of Agder, Telemark, Vestfold and neighboring areas (Larsen et al., 2014).

TBEV in milk

Raw milk and other dairy products made from unpasteurized milk, may be contaminated with pathogens such as *Mycobacterium bovis*, *Salmonella, Campylobacter*, *Brucella*, *Listeria*, *Shigella*, Shiga toxin-producing *Escherichia coli* (*E.coli* 0157), *Staphylococcus aureus*, *Streptococcus pyogenes*, *Yersinia enterocolitica*, *Coxiella burnettii*, Giardia, norovirus and the tick-borne encephalitis virus (cdc.gov). For many of these, vaccines are not available. The first reported milk-borne TBE epidemic occurred in Roznava, Slovakia, in 1951, where more than 600 people were infected, 271 of whom were hospitalized, after consuming contaminated milk- which was not pasteurized (Balogh et al., 2012). Similar cases in the European part of Russia were identified between 1947 and 1951 and described as' biphasic milk fever'(Gritsun et al., 2003). In the European Union, TBE outbreaks due to the consumption of unpasteurized cow-, sheep- and goats milk have been explained (Balogh et al., 2010; Holzmann et al., 2009; Kerbo, Donchenko, Kutsar, & Vasilenko, 2005; Kohl, Kozuch, Eleckova, Labuda, & Zaludko, 1996; Kriz, Benes, & Daniel, 2009).

A study carried out by Cisak et al found that cow-, sheep- and goat's milk from TBEV endemic areas contained the virus particles in 22.2% (sheep), 20.7% (goats) and 11.1% (cows) respectively. However, the detection of anti TBEV antibody did not correlate with the animals carrying the virus (Cisak et al., 2010). The virus is stable for up to two hours in the gastric juice (pH 1.49-1.80) and even after a meal (pH 2-7). Consumed milk reaches the duodenum within minutes from the stomach (Gritsun et al., 2003). Viable TBEV binds to the microfold cells of the Peyers's patches in the ileum (Balogh et al., 2012). Infected animals (i.e., goats, sheep and cows) can shed the virus into milk for five to 25 days after infection, beginning on the second or third day post-infection in the period when the animals show no clinical signs or fever (Gritsun et al., 2003; Holzmann et al., 2009). It is reported that TBEV has been demonstrated to be virulent for up to eight days after collecting milk from the animal (Suss, 2003). During viremia, TBEV is secreted in the milk of sheep and it is hypothesized that immuno-suppression of the animals due to *A. phagocytophilum* infection, may contribute to shedding of the virus into milk. In the study, it is found that the animals with higher *A. phagocytophilum* titer tend to have lower anti-TBEV titer and vice versa (Zeman et al., 2004).

Borreliosis

Borrelia species are causative agents of lyme borreliosis (LB) and relapsing fever (RF). Lyme borreliosis (LB) represents the most common disease, transmitted by *I. ricinus*. LB is a multi-systemic inflammatory disorder caused by spirochetes of the *B. burgdorferi sensu lato* complex. Annually, 14-140 cases/100,000 inhabitants are reported in Europe (O'Connell, Granstrom, Gray, & Stanek, 1998). Erythema migrans was first described in Europe in 1921 and in the United States in 1970. At that time, it was known that the syndrome was caused by an infectious, nonbacterial, but antibiotic-sensitive agent (Burgdorfer, 1984). Individual ticks can be infected with more than one genospecies of *B. burgdorferi s.l.* and such mixed infections have also been detected in patients (Misonne, Van Impe, & Hoet, 1998). There is currently no vaccine available on the European market against borreliosis (Rizzoli et al., 2011). Most human cases of human borreliosis are transmitted in the summer by the nymphal stages of ticks, except in the Eurasian species *I. persculatus*, where the adult females are mainly responsible (J. S. Gray, 1998). Møre and Romsdal is a high incidence region for Lyme borreliosis in Norway (Eldøen, Vik, Vik, & Midgard, 2001). The mean annual incidence rate in this county, as notified to the Norwegian Surveillance System for Communicable Diseases (MSIS), was 4.4/100,000 in the period 1989-99. Lyme borreliosis exhibits a broad array of clinical manifestations: Inflammation to the skin (erythema migrans) is the characteristic feature; other symptoms are carditis, arthritis and neurological symptoms. The clinical outcome seems to depend on the infecting geno-species. Lyme arthritis has been attributed to infection by *B. burgdorferi s.s.*, neuroborreliosis has been attributed to *B. garinii* and acrodermatitis chronica atrophicans has been attributed to *B. afzelii* (Anthonissen, De Kesel, Hoet, & Bigaignon, 1994).

Human Granulocytic Anaplasmosis and tick-borne fever

The disease caused by *A. phagocytophilum* in domestic ruminants is also called tick-borne fever (TBF) and has been known for at least 200 years (S. Stuen, 2007) in Norway. TBF is of growing concern from the production and animal welfare perspectives in the sheep industry (S. Stuen, Bergstrom, & Palmer, 2002). The bacterium causes high fever, cytoplasmic inclusions in polymorphonuclear cells and severe neutropenia. The disease is seldom fatal, unless being complicated by other infections such as *Pasturella* spp. *E. coli* and *Staphylococcus aureus* (S. Stuen, 2007). Neutrophil granulocytes are the main hosts for *A. phagocytophilum*. They have a short lifespan (6-12 h), but the bacteria have the ability to inhibit apoptosis of the short lived leukocytes, allow intracellular proliferation and significant morula formation before horizontal transmission to other neutrophils can occur (Yoshiie, Kim, Mott, & Rikihisa, 2000). TBF was first recognized in tick infested pastures of Scotland in the 1950s and was described in Norway in 1959 (Woldehiwet, 1983). In sheep, high fever, reduced milk yield, abortions, reduced weight gain and infertility in rams may occur. The bacterium causes a marked immune-suppression in infected animals, and affected flocks may suffer from heavy losses due to mortality, crippling and reduced productivity (S. Stuen, Van De Pol, Bergstrom, & Schouls, 2002). In cattle, the incubation period after experimental inoculation is four to nine days and the fever period may last for one to 13 days. Clinical signs in cattle may include depression, reduced appetite, coughing, nasal discharge, respiratory signs and swelling of the hind limbs (S. Stuen, Oppegaard, Bergstrom, & Moum, 2005). The bacterium has been found to persist in sheep, horse, dogs, red deer and cattle, providing a safe haven for the bacterium between seasons of tick activity. Movement of these infected individuals may contribute to the spread of variants between geographical areas (S. Stuen, 2007). Studies indicate that migrating birds may be important in the disposal of infected *I. ricinus* in Europe (Paulaukas et al, 2009). TBF is a common disease in domestic ruminants along the coast of southern Norway (S. Stuen, I. Van De Pol, et al., 2002). The northernmost case of TBF diagnosed so far has been in the county of Sør-Trøndelag (63º43'N). Except for *B. divergens,* tick-borne infections in mammalians have not earlier been diagnosed in North Norway (S. Stuen et al., 2005). Across Europe, the prevalence of *A. phagocytophilum* in ticks varies from 0.4 to 66.7% (Blanco & Oteo, 2002).

In humans, the disease caused by *A. phagocytophilum* is called Human Granulocytic Anaplasmosis (HGA) and the most common clinical manifestations are flu-like symptoms two to three weeks after tick attachment. Other symptoms are anemia, thrombocytopenia, leucopenia and elevated liver enzymes (Blanco & Oteo, 2002). The consequences of infection vary from asymptomatic infections to severe fatal illness. The seroprevalence in the European population for HGA agents range from 0 to 28%, however clinical symptoms are rarely present (Strle, 2004). *Anaplasma* species may be identified by microscopic detection of morulae, PCR, reverse line blot hybridization and *16s* rDNA sequencing (S. Stuen, I. Van De Pol, et al., 2002).

Prevention of tick-borne disease

Ticks have a barbed, harpoon-like mouthpiece called a hypostome which they insert into their host to suck blood. Many hard ticks (family *Ixodidae*) also secrete cement which further strengthens their attachment (Pitches, 2006). Any attached ticks, should be removed immediately with tweezers if available, by seizing and pulling steadily on the mouthparts, without twisting (Rizzoli et al., 2011). It is important not to squash the body (toxins and microbes could be injected into the host), break of the mouthpiece or leave cement behind (tick proteins could lead to allergic reaction). Few studies have compared the effectiveness of tick removal by chemical and mechanical techniques (Pitches, 2006). It is recommended to remove the ticks by grasping with forceps as close to the skin as possible. Since ticks do not have a high probability of transmitting *Borrelia* until 12-24 hours after beginning to feed, immediate removal of ticks is one of the most effective ways of avoiding *Borrelia* infection. On dissection of ticks, 60% contained spirochetes in the midgut (Burgdorfer, 1984). The site should be monitored for 30 days after the bite for sign of erythema migrans (Rizzoli et al., 2011). In animals, tick repellants are used by dipping or pour on preparations.

Diagnostics and research techniques for studying tick borne diseases

Identification and typing of *Borrelia* species

Several methods have been used to characterize and identify *Borrelia* isolates. These are: protein analyses using monoclonal antibodies, multilocus enzyme electrophoresis, and plasmid profiles analyses, *16S* rDNA sequence analyses, ribotyping and PCR-RFLP analyses of 5S-23S intergenic spacer amplicons (Lee et al., 2000; Postic, Assous, Grimont, & Baranton, 1994). Different single gene loci have been targeted for ecological, epidemiological, phylogeographic and evolutionary studies. These includes intergenic spacer (IGS) regions, *rrs* (16s rRNA) locus, the plasmid located genes encoding the outer surface proteins A and C (*osp*A, *osp*C), decorin-binding protein (*dbp*A), the chromosomally located housekeeping genes recombinase A (*rec*A), *gro*EL, *hbb* or *flagellin* B(*fla*b) (Margos et al., 2011; Postic et al., 1994).

Identification by intergenic space (IGS)

The organization of rRNA genes in *Borrelia* strains is unique among bacteria. There is a single *rrs* (16S) gene and two copies, each of the *rrl* (23S) and *rrf* (5S) genes, which are tandemly duplicated in the order 23S-5S-23S-5S (Postic et al., 1994). The duplication of the 23S and 5S genes is unique to *B. burgdorferi* and was not observed for the closely related species *B. hermsii, B anserine or B. turciatae*. Within each 23S-5S unit, an identical 22-bp spacer separates the 23S and 5S rRNA sequence from each other, and individual copies of the 23S-5S duplication are separated by a 182 bp spacer. The *16S* rRNA gene is separated from 23S-5S gene cluster by more than 2 kb (Schwartz, Gazumyan, & Schwartz, 1992). Loci that have been studied, include the chromosomal

intergenic spacer (IGS) between the single *16S* (rrs) and the first of two *23 S* (rrlA) rRNA genes (Figure 1). The outer forward primer of the intergenic space between *rrs* and *rrlA* was at the 3' end of the *rrs* gene, and the outer reverse primer was in the coding sequence for the *ileT* tRNA gene in the spacer (Bunikis et al., 2004).

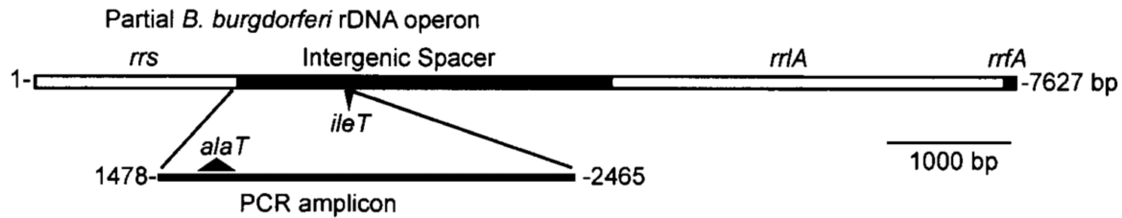

Fig 2. Location map of the partial rRNA operon. The rrs-rrlA intergenic spacer (IGS) separates *rrs* (16S) and *rrl*A.

Real time polymerase chain reaction or quantitative PCR (qPCR)

The quantitative real-time PCR (RT-qPCR) is widely used because of its high sensitivity, good reproducibility and direct detection of viral nucleic acid from the sample and reducing post-reaction analyses. Signals (generally fluorescent) are monitored as they are generated and are tracked after they rise above the background but before the reaction reaches a plateau. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The method utilizes a pair of synthetic oligonucleotides or primers, each hybridizing to one strand of a double-stranded DNA target, with the pair spanning a region that will be exponentially produced in presence of DNA polymerase (Mackay, Arden, & Nitsche, 2002). The important parameter for quantitation is the crossing point of the amplification curve (C_t). The point at which the fluroscence passes from insignificant levels to clearly detectable is called the threshold cycle (C_t) (Mackay et al., 2002). A TaqMan probe is a hydrolysis probe that uses the flurogenic 5' exonuclease activity of Taq-polymerase to measure the amount of target sequences in cDNA samples (Dorak, 2006). Two fluorescent dyes; a reporter (R) (eg. FAM or 6-carboxyfluroescein) and a quencher (Q) (eg. TAMRA or tetramethylrhodamine), are attached to two ends of the probe. The 3' end of the probe is blocked, so it is not extended during the PCR reaction. When both dyes are attached to the probe, reporter dye emission is quenched due to fluorescence energy transfer (FRET) from the reporter dye to the quencher dye. During each extension cycle, the probe is displaced at the 5' end by the DNA polymerase. Taq DNA polymerase then cleaves the reporter dye from the probe via its 5'-3' exonuclease. The exonuclease functions at annealing temperature (50-65 ºC) (www.core-facility.uni-freiburg.de/lc480). TaqMan probes are complementary to specific regions of the target DNA, between the binding sites of the forward and reverse primers for PCR bind (Thieman and Palladino, 2009). SYBR green is a flurogenic minor groove binding dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA (Morrison, Weis, & Wittwer, 1998). Association of a DNA-binding flurophore with primer-dimer or other non-specific amplification products can confuse the results; this can be addressed

with melting curve analysis. Melting temperature (T_m) is the temperature at which 50% of oligoprobe-target duplexes separate (Wetmur, 1991).

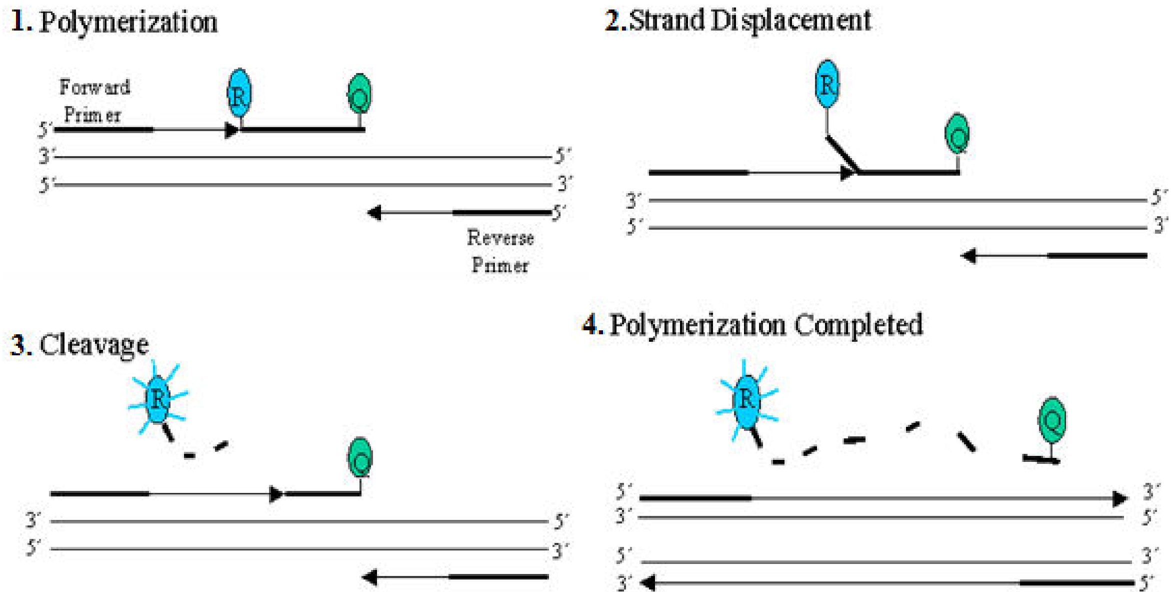

Fig 3. TaqMan (Hydrolysis) probe PCR (www.core-facility.uni-freiburg.de)

Melting Temperature (T_m)

When the temperature is raised, the hydrogen bond between the double-stranded DNA dissociates and a single-stranded structure is formed. This phenomenon is called melting. At melting conditions, the absorbance of nucleic acid under ultraviolet light increases at 260 nm. At the melting temperature, the ratio of the double strands to the single strand becomes equal. T_m is an index of the thermal stability of a nucleic acid, and is dependent on such conditions as the base sequence, base number, nucleic acid concentration, solvent conditions, mismatch etc.

Direct Sequencing

Sequencing experiments are to determine the order of the bases in a nucleic acid containing sample. Fluorescently labeled dyes are attached to bases of the PCR products in the reaction mixture. Dye labels are incorporated using 5'- dye labeled primers or 3'- dye labeled dideoxynucleotide terminators.

Pyrosequencing

Pyrosequencing is a DNA sequencing technique that is based on the detection of released pyrophosphate (PPi) during DNA synthesis. The inorganic PPi is subsequently converted to ATP by ATP sulfurylase, which provides the energy to luciferase to oxidize luciferin which then generates light (Ronaghi, 2001). Pyrosequencing technology on the Biotage/QIAGEN platform is better suited for the analysis of short sequences, sequencing up to 100 nucleotides accurately.

ELISA

Enzyme linked immunosorbent assay is based on either purified virions or recombinant virus-like particles obtained by expression of prM and E proteins as antigen. TBEV-IgM

and IgG antibodies are present in serum and CSF when CNS symptoms manifest in the second phase of the disease. Due to cross reactivity of the antigenic structure in the flaviviruses, it is difficulty in differential diagnoses arising from other flavivirus that circulate a particular area or in individuals that have been vaccinated against TBEV, Japanese encephalitis or yellow fever virus (Lars and Olli 2008).

Materials and Methods

Samples

Collection of ticks

Ticks (both adults and nymphs) were sampled from six locations from South-Eastern and South-Western Norway (Table 1) between June during the year 2011, 2013 and 2014. For convenience, the sampling locations are referred to with universal transverse mercaton coordinates (UTM) in tables 1, 2 and 3. Questing ticks were collected by flagging of a white flannel cloth (1.0m x 0.7m) through low vegetation on animal paths and pastures from three locations: Skånevik (Hordaland), Mandal (Vest-Agder) and Hvaler (Østfold) municipalities. The ticks attached were picked from the cloth with forceps and collected in a cryotube that was kept on crushed ice during transport to the laboratory. In the laboratory, adult male and female ticks were placed in separate tubes. Ten nymph ticks were pooled in each tube. Tubes were labeled with types of tick, site and date of collection, and stored at -80°C awaiting further analysis. *I. ricinus* are routinely collected every year from Vest-Agder as a surveillance program for TBEV and the results are compared each year against variations in temperature, humidity, vegetation, number of deer etc. In this study Hordaland and Kirkøy are new locations.

Figure 4. Map showing four location sites for collection of ticks, milk and serum samples.

Table 1. Collection of ticks from different part of Norway

Collection site, Municipality, County	UTM-Coordinate	Site code	Date of Collection	Nymphs	Adults		
					♂	♀	Total
Tungesvik, Hordaland*	32V 331199 6625361	H2	27.06.2014	310	10	11	**21**
Risøy, Nedenfor veien, Tungesvik, Hordaland*	32V 284322 6644884	H3	28.06.2014	540	17	12	**29**
Risøy, Over veien, Tungesvik, Hordaland*	32V 284322 6644884	H4	28.06.2014	1120	14	14	**28**
Hillevågen, Mandal, Vest-Agder**	32V 403111 6430689	S10	13.06.2014	1170	19	23	**42**
Hillevågen, Mandal, Vest-Agder**	32V 403111 6430689	S10	08.06.2011	460	24	28	**52**
Hillevågen, Mandal, Vest-Agder**	32V 402681 6431194	S11	08.06.2011	260	29	26	**55**
Hillevågen, Mandal, Vest-Agder**	32V 403111 6430689	S10	2013	120			
Kirkøy, Hvaler, Østfold*	32V 616421 6546422	Ø14	2014	30	3	4	**7**
Total	**Nymphs**			**4010**			
	Adults				**113**	**118**	**234**

*- New location, **-Continuation of previous study on TBEV, LIV, *Anaplasma* spp, *Borrelia* spp

Milk and serum samples

Serum (N=54) and milk (N=35) samples from cows and sheep were collected from Hordaland, Vest-Agder and Skedsmokorset for the detection of TBEV. Blood and milk samples were collected by veterinarians. Milk samples were collected and stored in a falcon tubes at -80°C before analysis. Blood samples were drawn in vacuum tubes with no additives, from animals and transported to laboratory. Serum was separated from the whole blood by centrifugation at 3000g for 15 min and stored at -80°C before analysis. Two of cows and two sheeps were vaccinated against TBEV with Ticovac to make positive control. The vaccination procedure is give at appendix 19.

Table 2. Collection of milk from cow

Collection site, Municipality, County	UTM-Coordinate	Number of samples
Hille, 4513 Mandal	32V 403111 6430689	**7**
Hille, 4513 Mandal*	32V 403111 6430689	**2**
Skedsmokorset	32V 612704 6655316	**22**
Skedsmokorset*	32V 612704 6655316	**1**
Total		**32**

*Pooled sample

Table 3. Collection of serum samples from sheep and cow

Collection site, Municipality, County	UTM-Coordinate	Site code	Animal	Number of samples
Skånevik, Hordaland	32V 331199 6625361	H2	Sheep	**20**
Hillevågen, Mandal, Vest-Agder	32V 403111 6430689	S10	Cow (calf)	**10 (9+1)**
Skedsmokorset	32V 612704 6655316		Cow	**24**
Total				**54**

Selection criteria of collection sites

Choice of locations was based on:

- Areas with known high abundance ticks as noticed by local people and tick species confirmed by entomologist to be *I. ricinus.*
- Area having many deer and favorable vegetation. Also known endemic regions of one of six studied organism (Granquist et al., 2014; Skarpaas et al., 2006).
- Cases of tick borne diseases in humans as reported by physicians to the Norwegian Surveillance System for Communicable Disease (MSIS) (Andreassen et al., 2012). Presence of antibodies in blood donors (Larsen et al., 2014) and hunted deer.
- Study of new location

Nucleic acid extraction

Nucleic acid extraction from ticks

Individual adults and pools of ten nymphs were homogenized in FastPrep®-24 5G instrument (MP Biomedical Life Science, CA, USA). Flow chart for analysis is given in Appendix 1. The ticks were placed in a FastPrep® 2 ml tube containing six steel beads (MP Biomedical Life Science, CA, USA) and RLT® lysis buffer (400 μl for nymphs and 350 μl for adults) (RNeasy Mini Kit, QIAGEN Inc., Valencia, CA, USA) was added. β-mercaptoethanol (β-ME) was added to the RLT® lysis buffer in an amount 1:10. β-ME eliminates ribonuclease that is released during cell lysis. Numerous di-sulphide bonds make ribonuclease very stable, so β-ME reduces these bonds and irreversibly denature the protein. Thus RNA is prevented from digestion. The homogenizer; FastPrep®-24 5G instrument was operated at a speed of 4.0 m/sec, with a tube holder CY: 24x2 and at run time 60 seconds for nymphs (additional 45 second for adults). The homogenate was transferred to a new tube and then centrifuged at 14000 rpm for 5 minutes. The obtained liquid part was used for nucleic acid extraction. Nucleic acid extraction was carried out with two different instrument and reagents according to Paulsen *et al*., 2015. Total nucleic acid (TNA) was extracted from adult ticks with MagNa Pure LC 2.0 Instrument and MagNa Pure LC Total Nucleic Acid High Performance Isolation kit (both supplied by Roche Diagnostics GmbH, 82372 Penzberg, Germany) (Appendix 3). In the MagNa Pure LC method the nucleic acid attached to magnetic particles are washed and separated from other elements. The sample volume was 200 μl and elution volume was 60 μl. Total RNA was extracted from nymphs using the RNeasy Mini Kit in a fully automated QIAcube

(both from QIAGEN Inc., Valencia, CA, USA) (Appendix 4). The isolation of RNA is done by binding of RNA to silica-gel-based membrane in a microspin column. Manufacturer's procedure was followed for both extraction methods. The sample volume was 350 μl and the elution volume was 60 μl.

RNA extraction from Milk

Milk samples were thawed at 4 ºC overnight before analysis. Upper white fat deposit layer was removed by suction and vortexed to mix. Aliquot 1000 μl milk to 1.5 μl tube and centrifuged at 6000 g for 10 min. Skimmed milk was drawn up by placing pipette tips below the fat layer and 500 μl was transferred to another tube. RNA was isolated with QIAamp® Viral RNA mini kit from skimmed milk according to Cisak and colleague (Cisak et al., 2010) (Appendix 2, 5). RNA concentration and purity was measured by DeNovix DS-11/DS-11+ Spectrophotometer (Wilmington, Delaware, US) according to the manufacturer's instruction.

Reverse Transcription

Nucleic acid extracted from *I. ricinus* was first reversely transcribed and amplified in real-time PCR for detection of different microorganisms. Those positive in real-time PCR were further confirmed with sequencing and pyrosequencing. RNA and TNA were reversely transcribed to cDNA with the High-Capacity cDNA Reverse Transcription-Kit and 2720 thermal cycler (both supplied by Applied Biosystems, Foster city, CA, USA) according to Andreassen *et al*., 2012. Manufacturer's instructions were followed for component of reverse transcriptase PCR (RT-PCR) mixture and cycle condition (appendix 6). The cDNA was either directly analyzed or stored at -80 °C for further analysis.

Primers and Probes

Probes and primers were custom produced by Life technologies Corporation, Carlsbad, California, USA. Sequences of primers and probes for TBEV, LIV, *B. burgdorferi* sensu lato, *M. mitochondrii*, *W.pipientis* and *A. phagocytophilum* are given in table 4. TBEV primers and probe was designed by Torstein Tengs at the Norwegian Veterinary Institute for the study carried out by Andreassen *et al*., 2012. LIV primer and probes were also designed by Torstein Tengs for the master thesis study of Katrine M. Paulsen, Telemark University College (unpublished). Primers and probe for *B. burgdorferi s.l.* is specific for section of *16S* rRNA gene. The *msp*2 gene encodes major outer membrane proteins 2 in *A. phagocytophilum* and other *Anaplasma* species. The primers were manufactured by TIB Molbiol (Germany) (Granquist, Bardsen, Bergstrom, & Stuen, 2010).

Table 4. List of primers and probes used in real-time PCR and nested PCR for detection of TBEV, LIV, Borrelia, Anaplasma, Wolbachia and Midichloria.

Primer name	Sequence(5'→3')	Genome position	GenBank accession No.
TBE 320 Forward [1]	GGGAGCGCAAAACTG GAA	1662-1680	U27495.1
TBE 373 Rerverse [1]	TGAGGAGCCCCAAATTC AAC-biotin	1696-1715	U27495.1
TBE 339 probe [1]	(FAM)-AACGCAGAAAGAC-(MGB)	1681-1693	U27495.1
LIV 410 Forward [2]	CCAACAAAATAGTGTACACAGTGAAGGTTG	401-430	D12936.1
LIV 1000 Reverse [2]	CGGGTCAAAGCCATGCAGG	999-1017	D12936.1
LIV 680 probe [2]	(FAM)-AGCATGATGGAAACCCAC-(MGB)	683-702	D12936.1
RF forward primer [3]	GCTGTAAACGATGCACACTTGGT		
RF reverse primer [3]	GGCGGCACACTTAACACGTTAG		
LB probe [3]	6FAM-TTCGGTACTAACTTTTAGTTAA-MGBNFQ		
IGS 1 forward primer [4]	GTATGTTTAGTGAGGGGGGTG	2306-2326	U03396
IGS 1 reverse primer [4]	GGATCATAGCTCAGGTGGTTAG	3334-3313	
IGS 2 forward primer [4]	AGGGGGGTGAACTCGTAACAAG	2318-2339	
IGS 2 reverse primer [4]	GTCTGATAAACCTGAGGTCGGA	3305-3284	
ApMSP2 252F [5]	ACAGTCCAGCGTTTAGCA AGA		
ApMSP2 459R [5]	GCACCACCAATACCATAA CCA		
gyrB-MidiF [6]	CTTGAGAGCAGAACCAC CTA		
gyr-MidiR [6]	CAAGCTCTGCCGCCGAAA TATCTT		
Wolwsp81F [7]	TGGTCCAATAAGTGAAGA AAC		
Wolwsp691R [7]	AAAAATTAAACGCTACTC CA		

1-(Andreassen et al., 2012), 2- Katrine M Paulsen, 3-(Tsao et al., 2004), 4-(Bunikis et al., 2004), 5-(Granquist, Bardsen, et al., 2010), 6-(Sassera et al., 2008), 7-(Braig, Zhou, Dobson, & O'Neill, 1998)

Real-time PCR

TBEV detection

Real time PCR or quantitative PCR (qPCR) method is based on the detection of fluorescent signals when the sample containing DNA or cDNA is amplified. cDNA was analyzed by an in-house real-time PCR that amplifies a 54 bp fragment of the 3' end of the TBEV envelope gene (1662-1715) in a Rotor gene 6000 thermal cycler (QIAGEN Inc., Valencia, CA, USA). This is an established method developed by Andreassen *et al* (2012). TBEV-Eu (Soukupa) isolated from a TBE positive case and cultivated, was run as positive control. Soukupa (supplied by Dr Christian Beuret, Spiez lab, Spiez, Switzerland) contains 50,000 virus particles/ μl. Reversely transcribed virus particles were diluted from 10^{-3} - 10^{-7} and run with each real time PCR as positive control and RNase free water as negative control. The PCR constitution and number of cycle are described in appendix 6. Final concentration of PCR mix consisted of 5 mM $MgCl_2$, 1X AB Buffer, 0.2X dNTP, 0.25 μM TBEV 320 forward primer and TBEV 373 biotin labelled reverse primer, 0.3 µM TBEV probe 339 and 0.19 Units Pt-Taq (Invitrogen Life Technology, Inc., Carlsbad, CA, USA) adjusted with RNase free water (Appendix 7). The extraction and analysis of ticks for TBEV from Tungesvik H4, Hordaland were performed along with Benedikte Nevjen Pedersen and the data is also included in her master thesis.

LIV detection

The primer mix and reaction conditions are given in appendix 8 and primers are given in table 4.

B. burgdorferi s.l. detection

Detection of *B. burgdorferi* s.l. 16S RNA fraction was performed from cDNA instead of DNA with primers and probes (table 4) as described by Tsao *et al*., 2004. Real-time PCR was performed with StepOnePlus™ Real-Time PCR Systems (Applied Biosystems Inc., New Jersy, USA). The mixture consisted of TaqMan Universal PCR master mix (Applied Biosystems Inc.,New Jersey, USA), primers (0.9 μM), probes and template 5μL which was diluted 1:10. PCR components and conditions are described in appendix 9.

A. phagocytophilum detection

The primers amplify 208 basepairs that includes the conserved N-terminal region of *msp*2. LigthCycler® 480 Instrument II (Roche Life Science, Germany) was used for quantitative PCR analysis. SYBR green I assays and melting temperature (T_m calling) of the samples were analyzed with LightCycler® 480 Software, version 1.5. LightCycler® 480 SYBR Green I master (Roche Life Science, Germany), which is a hot start PCR mix, contains FastStart Taq DNA polymerase and DNA double-strand specific SYBR Green I dye for detection. The PCR mix consists of 7.5 units of LightCycler 480® Sybr Green I master and 0.5 μM primers listed in table 3 (appendix 10). In the study total nucleic acid of adult ticks and 1:10 diluted cDNA of nymph ticks were used.

W.pipientis detection

The analysis was performed along with Sveinung Eskeland. *W. pipentis* was detected by SYBR Green real-time PCR for *wsp* (Braig et al., 1998) in a Rotor gene 6000 thermal cycler (QIAGEN Inc., Valencia, CA, USA). The mixture consisted of Perfecta® SYBR® Green FastMix® (Quanta Biosciences Inc., Gaithersburg, USA), primers (0.4 μM) and adjusted with RNase free water for 15 μL total volume. The PCR components and reaction condition are described in appendix 11.

M. mitochondrii detection

The analysis was performed along with Sveinung Eskeland. *M. mitochondrii* was detected by SYBR Green real-time PCR for *gyr*B. The mixture consisted of SYBR® Green Master Mix (Thermo Fisher Scientific Inc., USA), primers (0.4 μM) and adjusted with RNase free water for 15 μL total volume. The PCR components and reaction condition are described in appendix 12.

Nested PCR

The samples that were positive on *Borrelia* spp. *16s* DNA were further amplified with a nested PCR and electrophoresis was run to visualize the amplicons. Intergenic space (IGS) between *rrs*(16S)-*rrl*A(23S) was amplified by nested PCR comprising of 35 cycles in the first reaction (IGS1) and 39 cycles in the second reaction (IGS2). The primer sequences

are listed in table 4. The concentration of each component in 25 μL PCR mix are: 1mM GeneAmp® dNTP (deoxynucleoside triphosphate), 1X PCR Gold buffer, 1 unit AmpliTaq® Gold DNA polymerase, 2.5 mM $MgCl_2$ and 0.8 μM of each primers listed in table 4 (appendix 9).

Gel Electrophoresis

Nested PCR products were run on an agarose gel (1.5%) in 1x TAE buffer at 100V for 30 minutes. The bands were visualized under an UV trans-illuminator, ImageQuant 300 (GE Healthcare Life Sciences, Buckinghamshire, UK). For this, 2.4 grams of agarose gel was mixed with 150 ml of 1x TAE buffer and heated in a microwave for even mix. When the gel returned to approximately 50 ºC, 7.5 μL of SYBR® safe DNA gel stain (Thermo Fisher Scientific, USA) was added, this helps to visualize the bands. The gel was poured into the chamber with comb properly placed in. After the gel had solidified in about 15 minutes, it was immersed in 1x TAE buffer. 2 μL of loading dye was mixed with 10 μL of IGS nested PCR products and this 12 μL of mixture was added into each well. A 100 base pair ladder was diluted 1:10 with water and 6 μL was added in each well in each row. The samples were run on the gel for 30 minutes at 100 V. The gel was viewed under UV trans-illuminator.

Sequencing

Pyrosequencing

Adult, pooled nymph and cow milk samples that were positive with lower C_t values than 40 on real time PCR for TBEV were further confirmed by pyrosequencing. Pyrosequencing was preferred over Sanger sequencing because the amplified PCR products are short fragment of 54 base pairs. Pyrosequencing was performed with a sequence analysis (SQA) protocol in BioTage (PyroMark™ ID) System (Qiagen, Germany). The hybridized primer and biotinylated single-stranded template were incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase, as well as the substrate adenosine 5' phosphposulfate (APS) and luciferin (appendix 13). In the cascade of reactions, inorganic pyrophosphate (iPP) is released which is proportional to the number of incorporation of nucleotides and amount of visible light generated, making it a quantitative analysis.

Sequencing *Borrelia burgdorferi* s.l.

The samples that had fragments between 600-800 bp on electrophoresis were sequenced for intergenic space (IGS) between *rrs* (16S) and *rrrl*A (23S). Nested PCR products were sequenced with IGS2 reverse primer on a 3130 Genetic Analyzer (Applied Biosystems Inc.)

PCR product clean-up

Exo Star contains alkaline phosphatase and exonuclease 1 that removes unincorporated dNTPs and primers prior to downstream analysis. In the PCR tube, 2 μL of EXO STAR (GE Healthcare Life Sciences, Buckinghamshire, UK) was added with 5 μL of IGS2 nested PCR products. The tubes were set at 37 ºC for 15 min, 80 ºC for 15 min and cool down to 4 ºC in the 2720 thermal cycler (Applied Biosystems, Foster city, CA, USA).

Cycle sequencing

Cycle sequencing involves denaturation, annealing and extension in a thermal cycler with only one primer that results in linear amplification of extension products. We used dye terminator based cycle sequencing, where each of the four dideoxynucleotide triphosphate (ddNTP) is tagged with a different fluorescent dye. The chain elongation is terminated when fluorescently labeled ddNTP is incorporated. ddNTP lacks 3'OH group that further cannot make a phosphodiester bond with another nucleotide. Therefore, after completion of cycle sequencing copious amounts of differently lengthened and labelled fragments at 3' side are produced. The reaction mixture contains Taq polymerase, unlabeled dNTPs, fluorescently labeled ddNTPs, buffer, primer and distilled water (Appendix 14). The reaction condition was 96 ℃ for 1 min., 25 cycles of 96 ℃ for 15 second, 50 ℃ for 10 second, 60 ℃ for 4 min and cooled down to 4 ℃. The extension temperature is lowered to 60 ℃ instead of 72 ℃ so that the polymerase has to incorporate ddNTPs. In a 96 well plate 9 μL of above mix were pipetted. The samples which had strong intensity bands were diluted 1:10, 1:20 or 1:40 whereas the rest were processed without dilution. In each wells 1 µl of diluted and undiluted sample were added. Covered with strip caps and put in thermal cycler.

Detection of sequence

The fluorescent- labelled fragments are passed through polyacrylamide gel. The gel is able to separate fragments that differ even with one nucleotide. Negatively charged DNA will migrate towards the positively charged side. As the fragments migrate, each ddNTP would fluorescence a different wavelength when a laser is fired through it. The order of fluorescence gives the order of nucleotide sequence.

Prevalence calculation

Prevalence of TBEV was calculated with two different methods, minimum infection rate (MIR) and estimated pooled prevalence (EPP) (Cowling, Gardner, & Johnson, 1999; Ebert, Brlansky, & Rogers, 2010; Sergeant, 2009). The EPP method estimates prevalence within confidence limits. Based on previous study which shows the prevalence of TBEV in ticks in Norway (Skarpaas et al., 2006), that gives prevalence estimation with a 95% confidence limit and accuracy of ± 0.4 (Andreassen et al., 2012).

Minimum infection rate

$$MIR = \frac{P}{mk} x100$$

Where, P = the number of positive pools

m =the number of pools tested

k = pool size

ELISA test

Qualitative detection of specific IgG antibody against TBEV produced in cow and sheep sera samples from Hille, Skedsmokorset and Skånevik were performed with enzyme immunoassay Enzygnost®Anti-TBE virus (IgG) (Dade Behring, Marburg, Germany) and Immunozyme FSME IgG kit (Progen GmbH, Heidelberg, Germany). The assays were performed manually according to manufacturer's instruction. For Enzygnost, conjugate developed, against bovine IgG (produced in goat) and sheep IgG (produced in rabbit) were used were used, which was supplied by KPL, Inc. Gaithersburg, USA. The test plates were read at 450nm wavelength.

Calculation of titer

The absorbance (A) of anti-TBE reference P/P (positive control) should be within lower and upper margin.

Lower margin ≤ A Rerference P/P ≥ upper margin

Correction factor (Cf) = $\frac{Nominal\ Value}{Refernce\ P/P}$

Cut-off = A reference N/N (Negative control) + 0.200

Absorbance (A) corrected = A x Cf

Interpretation

Anti-TBEV IgG negative	A corrected < cut-off
Anti-TBEV IgG positive	A corrected > cut-off + 0.100
Anti-TBEV IgG equivocal	cut-off ≤ A corrected ≥ cut-off + 0.100

Statistical methods

All statistical analyses were performed in the statistical package STATA (StataCorp LP, TX, USA). Summary statistics were used to calculate the prevalence with Standard Error Mean (SEM) and 95% CI for micro-organisms in ticks and milk samples.

Pairwise comparison of means was used for nymphs to test hypotheses of no significant differences between regions. The cut-off for statistical significance was set to $p<0.05$. The hypotheses testing used a student t-test statistics. The assumptions were that the two samples were independent and representative of the population, that the observations were approximately normally distributed and that the variances in the two samples were approximately equal. Adults were tested for differences between regions by logistic regression, reporting odd ratios. Each tick was given a binary value of 0(negative) or 1(positive). Only results confirmed by pyrosequencing were used in the statistics. A cut-off for statistical significance was set at $p<0.05$.

Results

Real-time PCR and sequencing result from monoinfections in ticks

TBEV in adult and nymph ticks

A Total of 4244 *I. ricinus* (nymphs and adults) were collected by flagging from southern and south-western parts of Norway (Hordaland, Vest-Agder and Østfold Counties). In the study, 3240 nymph ticks i.e. 324 pools, were analyzed out of 4010 ticks collected in the year 2011, 2013 and 2014. The overall prevalence of TBEV in nymph ticks confirmed by pyrosequencing was 0.12%, standard error 0.006 and 95% confidence interval was 0.0003 - 0.024. The estimated pool prevalence (EPP) for different areas ranged from 0 to 0.86% (table 5). The minimum infection rate (MIR) ranged from 0 to 0.83%. The nymph pools from Mandal S10 collected in 2013 have highest prevalence of 0.86%. A total of 12 nymph pools (0.37%) were positive in Real-time PCR. TBEV were not detected with real-time PCR in Hordaland H2, Mandal S10 (2014) and Hvaler Ø14 region. Ticks from Mandal (2011) were negative in pyrosequencing, though three each of them were positive with Real-time PCR.

Table 5. Detection of TBEV in nymph ticks from different regions with real-time PCR and pyrosequencing; their EPP and MIR values

Location (Year of collection)	No. of nymph-pools	No. of positive nymph-pools by RT-PCR	No. of positive nymph pools by pyro-sequencing	EPP*%	MIR*%	SE	95 % CI
Hordaland, Tungesvik, H2 (2014)	31	0	0	0	0	0.02	-0.04, 0.04
Hordaland, Tungesvik, Risøy, Nedenforveien H3 (2014)	54	3	2	0.37	0.37	0.014	0.007, 0.07
Hordaland, Tungesvik, Risøy, Nedenforveien H4 (2014)	78	2	1	0.129	0.128	0.012	-0.012, 0.04
Vest-Agder, Mandal, Hillevågen S10 (2014)	74	0	0	0	0	0.013	-0.025, 0.025
Vest-Agder, Mandal, Hillevågen S10 (2011)	46	3	0	0	0	0.022	-0.042, 0.04
Vest-Agder, Mandal, Hillevågen S11 (2011)	26	3	0	0	0	0.016	-0.032, 0.03
Vest-Agder, Mandal, Hillevågen S10 (2013)	12	1	1	0.86	0.83	0.032	0.02, 0.15
Kirkøy, Hvaler Ø14 (2014)	3	0	0	0	0	0.064	-0.13, 0.13
Overall prevalence estimates	**324**	**12**	**4**	**0.12**	**0.12**	**0.006**	**0.0003-0.024**

***EPP- Estimated Pooled Prevalence. *MIR- Minimum Infection Rate. SE-Standard Error. CI-Confidence Interval**

A total of 234 adult ticks were analyzed by real-time PCR and pyrosequencing. The overall confirmed prevalence was 2.13% (SEM 0.009, 95% CI 0.003-0.04). The prevalence for different areas ranged from 0 to 7.14% (table 6). Hordaland H4 had the highest prevalence rate of 7.14%. No TBEV were detected from Mandal S10 (2014) and Hvaler (Ø14) locations. With real-time PCR, 5.12% of the adult tick samples are positive with TEBV.

Table 6. Prevalence of TBEV in adult ticks from different regions with real-time PCR and pyrosequencing.

Location (Year of collection)	Number of adult ticks	No. of positive adults RT- PCR	No. of positive adults by pyro-sequencing	Prevalence	SEM	95 % CI
Hordaland, Tungesvik, H2 (2014)	21	1	0	0	0.03	-0.06, 0.06
Hordaland, Tungesvik, Risøy, H3 (2014)	29	3	2	6.89 %	0.03	0.016, 0.12
Hordaland, Tungesvik, Risøy, H4 (2014)	28	2	2	7.14 %	0.03	0.02, 0.13
Vest-Agder, Mandal, Hillevågen S10 (2014)	42	0	0	0	0.02	-0.04, 0.04
Vest-Agder, Mandal, Hillevågen S10 (2011)	52	4	1	1.92 %	0.02	0.02, 0.06
Vest-Agder, Mandal, Hillevågen S11 (2011)	55	2	0	0	0.02	-0,38, 0.38
Kirkøy, Hvaler Ø14 (2014)	7	0	0	0	0.05	-0,1, 0.1
Overall prevalence estimates	**234**	**12**	**5**	**2.13 %**	**0.009**	**0.003-0.04**

SEM-Standard error of the mean, CI-Confidence interval

The overall prevalence of TBEV from 3474 ticks analyzed (both nymph and adult) was 0.17%. MIR of three counties was calculated. Nymphs from Hordaland have MIR 0.18% and Mandal, 0.06%. In adults, Hordaland has MIR 5.12% and Mandal, 0.67% (appendix 16).

LIV in adult and nymph ticks

Nymphs and adult sample was tested for detection of LIV but none were found to be positive.

B. burgdorferi s.l. in adult ticks

The adult tick samples were analyzed for the detection of *16S* rDNA, and those being positives were amplified and sequenced at intergenic space (IGS) between *rrs*-*rrl*A regions. All locations were detected with *Borrelia* infection. The percentage of *Borrelia* infection is highest in Hvaler region, 28.57% (table 7), where the number of analyzed ticks was seven. The overall prevalence of adult ticks was 6.41% SEM 0.02 and 95% CI 0.04 - 0.11, which was confirmed to be infected with *Borrelia spp*. With real-time PCR 34.61% of the samples were detected having *Borrelia* 16S rDNA.

Table 7 Prevalence of Borrelia burgdorferi in adult ticks with real-time PCR and sequencing

Location (Year of collection)	Number of adult ticks	No of adult ticks positive by Real-time PCR (%)	No. of adult ticks positive by sequencing	Prevalence of sequenced ticks	SEM	95 % CI
Hordaland, Tungesvik, H2 (2014)	21	7 (33.33)	1	4.76 %	0.06	-0.06-0.16
Hordaland, Tungesvik, Risøy, H3 (2014)	29	12 (41.37)	1	3.45 %	0.05	-0.06-0.13
Hordaland, Tungesvik, Risøy, H4 (2014)	28	3 (10.71)	1	3.57 %	0.05	-0.06-0.13
Vest-Agder, Mandal, Hillevågen S10 (2014)	42	23 (54.76)	6	14.28 %	0.04	-0.03-0.11
Vest-Agder, Mandal, Hillevågen S10 (2011)	52	20 (38.46)	2	3.84 %	0.04	0.06-0.22
Vest-Agder, Mandal, Hillevågen S11 (2011)	55	10 (18.18)	2	3.63 %	0.03	0.00-0.14
Kirkøy, Hvaler Ø14 (2014)	7	6 (85.71)	2	28.57 %	0.10	0.09-0.48
Total	**234**	**81 (34.61)**	**15**	**6.41 %**	**0.02**	**0.04-0.11**

SEM-Standard Error of the Mean, CI-Confidence interval

Except for ticks from Mandal Hille 2014 (p=0.176), the infection rate of *Borrelia* spp was significantly higher in Hvaler (p<0.05). The prevalence of *B. burgdorferi* s.l. in Hordaland was 3.84%, in Mandal 0.67% and in Hvaler, 28.57% (appendix 16).

Determination of *Borrelia* spp

Four genotypes of *Borrelia* spp. were detected in the present study; *B.afzelii*, *B.garini*, *B.burgdorferi sensu stricto* and *B.valaisiana*. Out of 81 positive by real-time PCR, 15 were confirmed positive with sequencing i.e. 6.41% of the total sample. From the confirmed genospecies, *B.garinii* 53.33%, *B.afzelii* 33.33%, *B.burgdorferi* s.s. and *B. valaisiana* 6.66% were detected (table 8).

Table 8. Borrelia genotype in different locations

Location (Year of collection)	No. of adult ticks	Positive in RT-PCR	*B. afzelii*	*B.garinii*	*B. burgdorferi*	*B. valaisiana*	Total
Hordaland, Tungesvik, H2 (2014)	21	7		1			1
Hordaland, Tungesvik, Risøy, H3 (2014)	29	12			1		1
Hordaland, Tungesvik, Risøy, H4 (2014)	28	3	1				1
Vest-Agder, Mandal, Hillevågen S10 (2014)	42	23	1	5			6
Vest-Agder, Mandal, Hillevågen S10 (2011)	52	20		1		1	2
Vest-Agder, Mandal, Hillevågen S11 (2011)	55	10	1	1			2
Kirkøy, Hvaler Ø14 (2014)	7	6	2				2
Total	**234**	**81**	**5 (33.33 %)**	**8 (53.33 %)**	**1 (6.66 %)**	**1 (6.66%)**	**15 (6.41%)**

Gel electrophoresis of nested PCR product

The adult tick cDNA samples, which were positive with *16s* rDNA real-time PCR, were amplified on *rrs-rrl*A region with nested PCR. The PCR products were checked for presence of fragment in gel electrophoresis (figure 3.1). Those which has band at 600-1000 bp were further confirmed with sequencing.

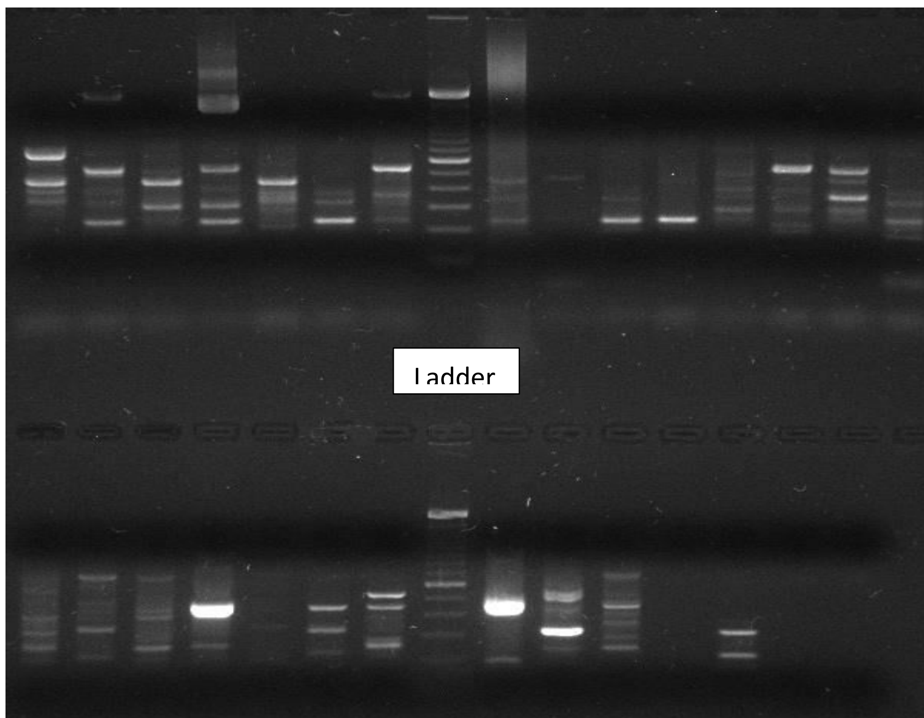

Figure 5. Gel picture of nested PCR amplified at intergenic space between rrs(16S)-rrlA(23S) region.

Anaplasma phgocytophilum in nymph ticks

The nymph tick samples were analyzed for *A. phagocytophilum* by detection of the *msp2* genes with real-time PCR. The estimated pooled prevalence ranged from 2.07% in S10 (2014) to 11.11% in H4 (2014) (table 9). The overall EPP and MIR were 7.96% and 5.64%, respectively with SE 0.27 and 95% CI 0.35-0.46. Nymphs from Hordaland have MIR 5.21%, Mandal 2.91% and Hvaler 3.33% (appendix 16).

Table 9. Detection of Anaplasma phagocytophilum in nymph ticks by real-time time PCR

Location (Year of collection)	Number of nymph pools	No.of nymph pool positive by Real-time PCR	EPP*	MIR*	SE	95 % CI
Hordaland, Tungesvik, H2 (2014)	31	6	2.12 %	1.9 %	0.08	0.032-0.36
Hordaland, Tungesvik, Risøy, Nedenforveien H3 (2014)	54	25	6.02 %	4.63 %	0.06	0.34-0.59
Hordaland, Tungesvik, Risøy, Nedenforveien H4 (2014)	78	54	11.11 %	6.92 %	0.05	0.59-0.79
Vest-Agder, Mandal, Hillevågen S10 (2014)	74	14	2.07 %	1.89 %	0.05	0.08-0.29
Vest-Agder, Mandal, Hillevågen S10 (2011)	46	12	6 %	4.61 %	0.09	0.28-0.64
Vest-Agder, Mandal, Hillevågen S11 (2011)	26	17	4.5 %	3.7 %	0.07	0.24-0.50
Vest-Agder, Mandal, Hillevågen S10 (2013)	12	3	2.83 %	2.5 %	0.13	-0.01-0.51
Kirkøy, Hvaler Ø14 (2014)	3	1	3.97 %	3.33 %	0.26	-0.19-0.85
Total nymphs	**234**	**132**	**7.96 %**	**5.64 %**	**0.027**	**0.35-0.46**

EPP*-Estimated pooled prevalence from real-time PCR, MIR*-Minimum infection rate from real-time PCR, SE-Standard Error, CI-Confidence interval

Anaplasma phgocytophilum in adult ticks

The overall prevalence of *Anaplasma* in adult ticks was 19.23% with SE 0.14 and 95% CI -0.28-0.28. The prevalence rate ranged from 2.38% in S10 (2014) to 39.28% in H4 (2014) (table 10). No *msp2* was detected from Hvaler region. The prevalence for *Anaplasma* was higher in H4 region for both adult and nymphs. MIR of adults from Hordaland was 3.84% and Mandal 16.77% (appendix 16)

Table 10. The prevalence of Anaplasma phagocytophilum in adult ticks with real-time PCR and sequencing.

Location (Year of collection)	Number of adult ticks	No of adult ticks positive by RT-PCR	No of adult ticks positive by sequencing	Prevalence (from confirmed value)	SE	95 % CI
Hordaland, Tungesvik, H2 (2014)	21	6	5	23.80 %	0.08	0.08-0.40
Hordaland, Tungesvik, Risøy, H3 (2014)	29	4	4	13.79 %	0.07	-0.00-0.28
Hordaland, Tungesvik, Risøy, H4 (2014)	28	11	11	39.28 %	0.07	0.25-0.53
Vest-Agder, Mandal, Hillevågen S10 (2014)	42	5	1	2.38 %	0.06	-0.09-0.14
Vest-Agder, Mandal, Hillevågen S10 (2011)	52	15	6	11.53 %	0.05	0.01-0.22
Vest-Agder, Mandal, Hillevågen S11 (2011)	55	19	18	32.72 %	0.05	0.23-0.43
Kirkøy, Hvaler Ø14 (2014)	7	0	0	0	0.14	-0.28-0.28
Total	**234**	**60**	**45**	**19.23**	**0.14**	**-0.28-0.28**

SE-Standard Error, CI-Confidence interval

W. pipientis in adult ticks

Adult tick samples were analyzed for detection of *wsp* genes for *Wolbachia spp* by real-time PCR. The sample that had a C_t value higher than 20, melting point 80 ± 3 and a melting curve that fell within the positive control curve were considered as positive. The prevalence ranged from 5.77 to 25% (table 11). The overall *W. pipientis* prevalence was 10.68%, SEM 0.02 and 95% CI 0.06-0.15. In Hvaler, *W. pipientis* was not detected. Adult ticks from Hordaland have MIR 16.66% and Mandal have 8.05% (appendix 16).

Table 11. The prevalence of Wolbachia from adult ticks with real-time PCR.

Location (Year of collection)	No. of adult ticks	No. of adult ticks positive for *Wolbachia* by Real-time PCR	*Wolbachia* Prevalence	Standard Error	95 % CI
Hordaland, Tungesvik, H2 (2014)	21	4	19.04 %	0.067	0.06-0.32
Hordaland, Risøy, H3 (2014)	29	2	6.9 %	0.057	-0.04-0.18
Hordaland, Risøy, H4 (2014)	28	7	25 %	0.06	0.14-0.36
Vest-Agder, Mandal, Hillevågen S10 (2014)	42	5	11.9 %	0.05	0.03-0.21
Vest-Agder, Mandal, Hillevågen S10 (2011)	52	3	5.77 %	0.04	-0.03-0.14
Vest-Agder, Mandal, Hillevågen S11 (2011)	55	4	7.27 %	0.04	-0.01-0.15
Kirkøy, Hvaler Ø14 (2014)	7	0	0	0.12	-0.23-0.23
Total percentage	**234**	**25**	**10.68 %**	**0.02**	**0.06-0.15**

M. mitochondrii in adult ticks

For the detection of *M. mitochondrii, gyr-B* gene was used. The sample that had a C_t-value higher than 12, melting point of 77 ± 1 and melting curve overlap with the positive control curve, in real-time PCR was considered positive. The total prevalence of *M. mitochondrii* in this study was 83.3% SEM 0.024 and 95% CI 0.79-0.88. The prevalence of *Midichloria* was highest in H4 (2014) and S10 (2014), 92.85%. In Hvaler region 57.14% of ticks were positive (table 12). Hordaland has MIR 83.33%, Mandal 84.56% and Hvaler 57.14% (appendix 16).

Table 12. Prevalence of Midichlori from adult ticks with real-time PCR.

Location (Year of collection)	No. of adult ticks	No. of adult ticks positive for Midichlori by PCR	*Midichloria* Prevalence	Standard Error	95 % CI
Hordaland, Tungesvik, H2 (2014)	21	18	85.71 %	0.08	0.70-1.01
Hordaland, Risøy, H3 (2014)	29	21	72.41 %	0.07	0.59-0.86
Hordaland, Risøy, H4 (2014)	28	26	92.85 %	0.07	0.80-1.07
Vest-Agder, Mandal, Hillevågen S10 (2014)	42	39	92.85 %	0.06	0.82-1.04
Vest-Agder, Mandal, Hillevågen S10 (2011)	52	39	75 %	0.05	0.65-0.85
Vest-Agder, Mandal, Hillevågen S11 (2011)	55	48	87.27 %	0.05	0.78-0.97
Kirkøy, Hvaler Ø14 (2014)	7	4	57.14 %	0.14	0.30-0.84
Total percentage	**234**	**195**	**83.33 %**	**0.024**	**-0.23-0.23**

Co-infections in ticks

From 234 adult ticks, 205 ticks were infected either with single microorganism or multiple organisms (table 13). Twenty-nine ticks (12.39%) were not infected with any

microorganism. Single and up to three microorganisms were detected in each of the positive ticks.

Only one tick was infected with *B. burgdorferi s.l., W. pipientis* and *M. mitochondrii*. Six ticks were infected with combination of *A. phagocytophilum W. pipientis* and *M. mitochondrii*.

Table 13. Co-infections of different organism

Organisms	Location	H2 (2014)	H3 (2014)	H4 (2014)	S10 (2014)	S10 (2011)	S11 (2011)	Ø14 (2014)	Total
Total Adults		21	29	28	42	52	55	7	**234**
TBEv + *Midichloria*			2	1		1			**4**
TBEv +*Anaplasma*				1					**1**
Borrelia + Midichloria		1	1	1	5	1	3		**12**
Anaplasma +Midichloria		4	1	6	1	5	14		**31**
Wolbachia +Midichloria		2	1	4	4	3	2		**16**
Anaplasma +Borrelia +Midichloria						1	1		**2**
Borrelia +Wolbachia +Midichloria					1				**1**
Anaplasma +Wolbachia +Midichloria		1		3			2		**6**
Anaplasma +Wolbachia			1						**1**
Borrelia						1		2	**3**
Midichloria		10	16	11	28	28	26	4	**123**
Anaplasma			2	1			1		**4**
Wolbachia		1							**1**
No organism		**2**	**5**		**3**	**12**	**6**	**1**	**29**

Real-time PCR result for TBEV from milk

Thirty two milk samples were collected and analyzed for TBEV, from different regions of Norway were analyzed for TBEV with real-time PCR and then confirmed by pyrosequencing. Of the total 29 individual cow milk, 25% were found to contain viral RNA (table 14). From Hille, 42.85% of the individual milk sample was infected with TBEV and both pooled milk sample contained TBEV. From Skedsmokorset, 18.18% of individual cow milk sample was infected.

Table 14. Prevalence of TBEV RNA in milk samples from cows.

Collection site, Municipality, County	Number of samples	Positive in real-time PCR	Prevalence
Vest-Agder, Mandal, Hillevågen	7	3	42.85 %
Vest-Agder, Mandal, Hillevågen*	2	2	100 %
Skedsmokorset	22	4	18.18 %
Skedsmokorset*	1	0	0
Total	32	8	25 %

*Pooled sample

ELISA results for cow and sheep sera

Twenty sheep and 34 cow serum samples were analyzed for immunoglobulin G produced against TBEV. Two kits were compared for sensitivity and specificity (table 15). With Enzygnost kit, it shows 12.96% prevalence and with Immunozym kit, 3.55%. In most, that borderline with Immunozyme was found to be positive with Enzygnost.

Table 15. Serum positivity of sheep and cow sera

Collection site, Municipality, County	Animal	Number of serum sample	Positive on Immunozyme (Prevalence)	Positive on Enzygnost (Prevalence)
Skanevik, Hordaland	Sheep	20	1 (1 borderline) (5%)	3 (1 borderline) (15%)
Hillevågen, Mandal, Vest-Agder	Cow (+calf)	10 (9+1)	(1 borderline)	0
Skedsmokorset	Cow	24	(2 borderline)	2 (1 borderline) (8.33 %)
Skanevik	Cow	2	2 (100%)	2(100%)
Total		**56**	**3 (3.55%)**	**7 (12.96 %)**

Discussion

Presence of microorganisms in tick

Tick-borne encephalitis virus in *Ixodes ricinus*.

The estimated overall prevalence or MIR of TBEV in nymph and adult *I. ricinus* from all three counties in this study were, 0.12% and 2.13%, respectively. This finding is in agreement with the average prevalence in foci of endemic areas in Europe that ranges from 0.1-5% (Suss, 2003). For Northern Europe, MIR for adult and nymph has been found to be 0.28% (Pettersson, Golovljova, Vene, & Jaenson, 2014). Earlier TBEV prevalence has been reported to be 0.53% in Norway (Andreassen et al., 2012). In the present study there are great variations between the different locations. Lower MIR compared to previous data was due to variation between and within the different sites (Gibory, 2013). The MIR ranged from 0-7.14% in adults and 0-0.83% in nymphs for different locations. The highest MIR for adult ticks was 7.14% in H4, Hordaland (new location) and for nymphs was 0.83% in S10 (2013) Mandal. A previous study on ticks from north-western coast of Norway found that adults have 3.08% prevalence from Møre og Romsdal and 0.41% in nymph from Hitra and Frøya (Paulsen et al., 2015). Furthermore, it has also been described that in Europe, TBEV prevalence varies between 0.1% and 5% in ticks identified by reverse transcriptase (RT-PCR), with an increasing prevalence during the life-cycle of the tick, and is up to 10% in engorged ticks removed from individuals (Lindquist & Vapalahti, 2008).

In the present study MIR of TBEV in nymphs from Mandal S10 (2013) was 0.83%. Previously, Katrine M Paulsen, Telemark University College, in 2014 detected 1.08% from the same location in her thesis (personal communication). Those ticks were collected in 2013. Another study from Mandal region had a prevalence of 0.70 to 1.22% (Andreassen et al., 2012). These are the same areas where human TBE cases have been reported every year (MSIS). In addition, the first human case in Norway was detected from Vest-Agder county (Skarpaas et al., 2006). This means that, Mandal in Vest-Agder is an endemic area for TBEV.

No TBEV was detected from ticks collected in 2014 from Mandal. Nymph ticks collected in 2011 from same location, no TBEV was detected when analyzed in 2013 by Gibory for his master thesis (Gibory, 2013). In the present study no TBEV from nymphs collected in 2011 was detected either. However, adult ticks from S10 (2011) showed 1.92% prevalence; Katrine Mørk Paulsen found a pooled prevalence of 1.14% from the same location in a previous study (personal communication). Unlike Lyme *Borrelia* spp. in endemic regions, TBE risk areas are distributed in a patchwork pattern, sometimes the situation remains stable, sometimes changes occur due to altered climatic conditions or other factors (Suss, 2011). The numbers of ticks were small in this study (46 pools) the sample number may not be sufficient to represent the true prevalence. According to Epitool, it is necessary to analyze 740 nymphs i.e. 74 pools when the estimated prevalence is 0.3% (Cowling et al., 1999; Ebert et al., 2010).

The prevalence of TBEV is higher in adult ticks compared to nymph ticks because of the probability for an extra blood meal in their life cycle (Pettersson et al., 2014). The TBEV is rarely transmitted via trans-ovarial routes; however ticks carrying the infection at the larval stage will remain infected until adult stage. In addition, the titration will be high in adults if they feed infected animals at every life stage. Pettersson and colleague found a prevalence of 4.48% in adult ticks and 0.51% in nymphs from Herrahamra, south-east Sweden (Pettersson et al., 2014). This is in accordance with our findings, the adult ticks from Mandal and Hordaland (0.67% and 5.12%, respectively), had higher prevalence when compared to nymphs (0.063% and 0.18%, respectively).

Viral existence and the maintenance of TBEV microfoci not only require a microhabitat favorable for *Ixodes* ticks, but suitable hosts and host population dynamics are also important (Lindhe et al., 2009). Other factors such as seasonal variation, vector-host interactions are also important factors for influencing TBEV prevalence. Drier climates and habitats without dense matt of plant on the soil surface will be inhibitory to *I. ricinus* (Walker et al., 2001). Each year from 1970 to 1980, *I. ricinus* and TBEV has been reported to reach higher altitudes in the mountains (Holzmann et al., 2009; Suss, 2011). Moreover, the spread of ticks and TBEV has been detected towards north in Europe (Holzmann et al., 2009; Lindgren & Gustafson, 2001; Skarpaas et al., 2006). In Sweden, it has been shown a relation of increase in TBE incidence with periodic change towards milder winters and early arrival of spring (Lindgren & Gustafson, 2001). Also it has been documented that the larvae develops into nymph if the temperature are favorable 8- 10 ºC.

TBEV circulates in a triangle of interaction between virus, vector tick and tick host and is able to persist in a given habitat over long periods of time (Patricia A Nuttall, 1999). It has been observed that the tick-borne virus survival is greatly dependent on persistent infections in tick populations (P. A. Nuttall et al., 1994). Hosts preferences are also important for ticks getting infected. Small mammal like rodent act as main transmission hosts to ticks because they are able to transmit the virus without themselves being viremic which may result in efficient co-feeding (Suss, 2011).

Host and reservoir animal population especially, small and medium sized mammals, such as roe deer, have increased as a result of favorable conditions created by the milder winters and longer vegetation periods (Lindgren & Gustafson, 2001).

Jääskeläinen and colleague in 2006, detected *I. persculatus* and the Siberian subtype from Kokkola region in western Finland, 300 km south of the Arctic Circle, which was the first finding of the TBEV-Sib in northern Europe (Jaaskelainen et al., 2006). Before that, it was believed that boundaries exist between Europe and Russia with *I. persculatus* occurring on the Russian side. The northern-most detection of TBEV-Eu from *I. ricinus* so far, is from Møre og Romsdal which is approximately 6,426 kilometers (3,993 miles) from the Arctic circle (Paulsen et al., 2015). The present study shows that TBEV varies within and between locations. But all the ticks in the present study were *I. ricinus* and all TBEV was of the TBE-Eu strain.

Detection of TBEV in milk and anti-TBEV antibody in serum

Several studies on TBEV from Mandal, Hille show that it is an endemic region. If the virus persists in ticks, the ruminants could be bitten and infected while grazing. Serological examination of sentinel animals helps to identify the natural foci and support the evidence that the virus persist in the ecosystem. Detection of the virus and serology may give the correct epidemiological indications. But, serological test interpretation is often influenced by other factors such as cross-reaction with other flaviviruses, past infections and vaccination (Schwaiger & Cassinotti, 2003). In Norway, cow milk is consumed most often than milk from other animals; we examined cow milk from Mandal, Hille and Skedsmokorset. It was found that 42.85% of the cow milk sample was detected with TBEV from Mandal. The finding is in accordance with the study in TBEV endemic area in Poland, where 11.1% of cow milk was detected with TBEV (Cisak et al., 2010). This is first study on cow milk that have been performed in Norway. There are many studies on animal sera for anti-TBEV antibody. When compared with previous studies, Traavik in 1973, investigated 81 cow sera from Farsund to Ørskog in Møre og Romsdal and found 14 animals seropositive for TBE viruses (Traavik, 1973). He also analysed 341 human patients sera from Hordaland, Sogn, Fjordane and Møre og Romsdal counties. With hemagglutination inhibition test (HI) and gel diffusion test it was found 19.6% of the humans were positive with TBEV antibody (Traavik, 1979). Ytrehus and colleague found antibody against TBEV in 22 of 54 cervids from Farsund, Southern Norway (Ytrehus et al., 2013).

Since both the bulk milk samples were confirm positive with pyrosequencing, we can assume that more than one cow in the pool must have been shedding virus in the milk, otherwise lesser chance of detection due to dilution effect or single cow is shedding copious amount of virus.

We compared the findings of Mandal with another study area, Skedsmokorset, where no human cases have been reported before and the site was hypothesized to be negative TBEV region. In our study, cow milk from Skedsmokorset was 9.09% prevalent with the viral RNA. In fact, one of the cow milk with TBEV was also found to have anti-TBEV IgG with Enzygnost kit and borderline with Immunozym kit. Another cow milk sample that was positive with TBEV RNA detection, was borderline with Enzygnost kit. The study is first of its kind to show that TBEV actively persist in the location.

A study from Østfold County showed that the human population had a seroprevalence of 0.65% and that the estimated pool prevalence in ticks was 0.14%, despite any reported TBE cases from the same region (Larsen et al., 2014). We were not able to detect any TBEV from Hvaler in Østfold, this may be due to the low number of tick collected, 7 adults and 30 nymphs. But it does not exclude TBEV being not present in the area. It could be that the population might be more aware of tick bites or the infection might have been unnoticed. In the future, a study with more representative numbers should be done and serological examination on host will be helpful in knowing the infection rate of TBEV.

In endemic regions 70 to 95% of human infections are either subclinical or totally asymptomatic (Gritsun et al., 2003). In a study on dogs, antibodies to TBEV were detected in 16.4% in Aust-Agder county of Southern Norway and it is the same area where numerous human cases have been reported (Csango, Blakstad, Kirtz, Pedersen, & Czettel, 2004). In Denmark, antibodies to TBEV were detected 31% from dogs from the same location as seropositive cases of forest workers were detected (Lindhe et al., 2009). Similarly, in Austria horses were 26.1% seropositive (Rushton et al., 2013). The study of antibodies against TBEV in animals indicates the presence of the agent in that location even though no human cases have been reported earlier. Importantly, the animals can be considered as sentinel hosts for monitoring the spread of TBEV (Rushton et al., 2013). We assume that the risk of human infection is directly related to infected ticks and animals. Transmission between ticks and rodents may occur in a relatively small area, called the micro-focus (Dobler, Hufert, Pfeffer, & Essbauer, 2011). Humans are infected by entering the micro-focus and large animals help to disperse in larger areas.

The fact that TBEV were found in milk sample is alarming news for the public health interest. Until 1955, it was not known that infected milk would transmit the virus through oral route; however it was known that goat shed the virus in milk (Van Tongeren, 1955). Further, it has been shown that infected goats do not show any clinical signs or fever; importantly they are able to shed virions in milk for 8-19 days (Balogh et al., 2012). Same must be for cow and other animals. In Europe several, single cases and outbreaks due to consumption of TBEV infected sheep, goat and cow milk and other dairy products have been described (Aendekerk, Schrivers, & Koehler, 1996; Balogh et al., 2010; Caini et al., 2012; Holzmann et al., 2009; Hudopisk et al., 2013; Kerbo et al., 2005; Kohl et al., 1996; Kriz et al., 2009). Though, no outbreak of TBEV due to dairy consumption in Norway has been in the past.

TBEV is secreted in milk during the viremic phase of the infection, and it is hypothesized that immunosuppression of the animals due to *A. phgocytophilum* infection could help TBEV to get into the milk (Zeman et al., 2004). As shown in this study, *A. phagocytophilum* were detected in ticks from the same areas as TBEV. If there is a correlation between TBEV in milk and infection with *A. phagocytophilum* needs to be further studied.

Detection of the virus in ticks would support the evidence that TBEV is prevalent in Skedsmokorset. Further examination of antibodies against TBEV in humans should be done to find out if human has subclinical infection. This will support our detection of TBEV in cow milk from Skedsmokorset. Detection of TBEV and anti-TBEV antibody in animals is good indication for infected ticks in the area. This is also because many ticks bite the animal. When the prevalence of TBEV in ticks was compared between counties both in nymphs and adults, Hordaland had higher prevalence compared to Mandal. In addition to this, sheep sera have 15% prevalence and cow sera was 100%, while no anti-TBEV IgG was detected in Mandal. This has raised the question have the epidemic-foci shifted to further north-west.

In serological study, anti-TBEV antibody cross-reacts with anti-LIV antibody, because of their close phylogenetic relationship (Klaus, Ziegler, Kalthoff, Hoffmann, & Beer, 2014). In our study we could not exclude the detection of anti-LIV antibody. It is also known that clinical signs of LIV disease in sheep are severe, while TBE infection in ruminants is subclinical (Gao et al., 1993). False positive results increase with older animals (Klaus et al., 2011). In our study, the sheep and cows were of reproductive age.

The milk should be pasteurized before consuming to prevent milk-borne infections due to TBEV since heat inactivates the virus. Pasteurization has been confirmed to prevent milk-borne TBEV infection (Dumpis et al., 1999). Even boiling the milk for 3 minutes would kill the virus, but if the viral content is very high even heating 65 ºC for 30 minute, still the milk is infectious (Balogh et al., 2012). People consume raw milk because of the conception that heat will destroy the nutrients, taste and medicinal value. Local farmers and people should be informed about the risk of drinking raw milk in the endemic region.

Earlier it has been shown that ELISA-test negative by Immunozym kit was positive by neutralization test. The sensitivity has been shown 57%, but with 100% specificity (Klaus et al., 2011). In our study, two serum samples that were borderline with Immunozym were positive with Enzygnost. All-in-all Immunozym is all species ELISA kit, so the main purpose is to screen different animal species and so it may not be sensitive enough for cow and sheep sera. Enzygnost kit is used for diagnostic purpose and is sensitive to avoid false negative. The lower specificity can be compensated by re-testing in the highly sensitive neutralization test. Those positive sera samples should be further tested to avoid false positive.

Neutralization test is very specific and efficient methods for detection of anti-TBEV antibodies in the serum. The scope of this study did not include the test. However, it should be confirmed to know the true epidemic of the infection. In those sera which were positive with ELISA and real-time PCR, we could know if the cow was still infective if we had also detected IgM antibody.

LIV in *I. ricinus*

Clinical and histological cases of Louping-ill-like disease were reported in sheep from Vest-Agder and Etne, Hordaland back in 1980s (Ytrehus et al., 2013). However, in the present study no LIV were detected by real-time PCR.

Prevalence of *Borrelia spp* in *I. ricinus*

The overall prevalence of *Borrelia* spp in questing ticks from seven locations was 6.41%. Previous studies have reported a prevalence of *B. burgdorferi sensu lato* infection in ticks from southern Norway of 0-38% (Jenkins et al., 2001; Kjelland, Stuen, Skarpaas, & Slettan, 2010; Paulauskas, Ambrasiene, Radzijevskaja, Rosef, & Turcinaviciene, 2008). In the current study, the prevalence of different locations ranged from 3.45 to 28.97%. The prevalence of *B burgdorferi* s.l. in nymphs and adults collected in 2007 from Mandal was found to be 25.2% (Kjelland et al., 2010) by real-time PCR. In this study, ticks collected in 2014 had a prevalence of a 54.76% and the total number of tick from same locations

from different years was 35.57%. Some decades ago, it was reported to be 4% (Mehl, Sandven, & Braathen, 1987). This may indicate either that the numbers of ticks are increasing or that the bacteria are spreading due to other factors related to the reservoir host. It could also be due to more reliable detection methods in later years.

Roe deer and red deer are incompetent reservoir host for *B. burgdorferi* (O. Rosef et al., 2009). It has been shown inverse relationship between red deer density and prevalence of *Borrelia* spp. (Mysterud, Easterday, Qviller, Viljugrein, & Ytrehus, 2013). The higher the number of deer, the higher the number of ticks will be, but the serum incompetence will reduce infection to ticks and the risk of borreliosis transmission. In this study we have collected ticks from areas with a high abundance of wild ruminants. According to various studies on *B. burgdorferu sensu lato* in *I. ricinus* ticks in Europe from 1984 to 2003, the overall mean prevalence of *Borrelia* was 13.7% (from total 112,579 ticks). The infection rate increases from western to eastern Europe in adult and latitude had no effect in the prevalence of tick infection (Rauter & Hartung, 2005). Infection rate for *B. burgdorferi s.l* was 15.5% in Denmark (Vennestrom, Egholm, & Jensen, 2008) and 24.1% in Sweden (Rauter & Hartung, 2005). Furthermore, host associations substantially shape *Borrelia* populations by impacting their dispersal patterns and geographical distributions (Margos et al., 2011). Not only specific manifestations of *Borrelia* spp, distinct genospecies are also considered to be preferentially associated with different reservoir host. In addition, distribution of distinct genospecies varies with the different geographic area and over a time (Derdakova & Lencakova, 2005).

Compared to nymphs, adults had a considerably higher infection rate (Rauter & Hartung, 2005). The deer serves as a principal source of all stages of *I. ricinus*, however, it is not a major reservoir of *B. burgdorferi* (Jaenson & Talleklint, 1992; Telford, Mather, Moore, Wilson, & Spielman, 1988). So when adult ticks feed on deer, which are immune to *B burgdorferi*, the deer is important in the ecology of disease by transporting ticks and maintaining the tick populations. In the paper by Hubalek and Halouzka, the average infection prevalence of questing *I. ricinus* in Europe was 1.9% (0-11%) for larvae, 10.8% for nymphs (2-43%) and 17.4% for adults (3-58%) (Hubalek & Halouzka, 1998). The infection prevalence varied geographically and according to the used method of detection (Hubalek & Halouzka, 1998). The geographic distribution of *B.burgdorferi s.l* genospeies in Europe is variable. Based on review article in 26 European countries 501 isolates were classified as *B. garinii* (39.7%), *B.afzelii* (37.1%), *B.burgdorferi* s.s. (15.9%), *B. valaisiana* (6.7%) and *B. luisitanae* (0.6%) (Hubalek & Halouzka, 1998). In our study *B. garinii* (53.33%), *B. afzelii* (33.33%), *B burgdorferi s.s* (6.66%) and *B valaisiana* (6.66%) were detected. *B.garinii* is the most frequent genospecies detected among *I ricinus* ticks in Europe (Rauter & Hartung, 2005). But also, *B.garinii* and *B.afzelii*, occur alternately as a dominant genospecies in most of the studied European countries, however, distribution of *Borrelia genospecies* can vary even over relatively small areas as well as over the time period (Hanincova et al., 2003). In earlier studies from Mandal *B garinii* (50%) was the dominating species, followed by *B.afzelii* (28%), *B burgdorferi* s.s (13%) and *B valaisiana* (9%) (Kjelland et al., 2010).

Prevalence of *A. phagocytophilum* in *I. ricinus*

The disease caused by *A. phagocytophilum*, TBF is a common disease in domestic ruminants along the coast of southern Norway (S. Stuen, I. Van De Pol, et al., 2002) only few human cases though (S. Stuen & Bergstrom, 2008). It is no surprise that the bacteria are persistent in *I. ricinus* in the grazing grasslands. In our study *A. phagocytophilum* prevalence in nymphs and adults were, 7.96% and 19.23%, respectively. The prevalence of the bacterium in questing ticks is usually higher in adult ticks than in nymphs and ranges from zero to > 30% (Strle, 2004). The prevalence of *A. phagocytophilum* varies from area to area and between development stages of tick. The prevalence in nymphs has been described to vary from 0.25-25% (Walker et al., 2001). From west coast of Norway 8.8% (Mysterud et al., 2013) has been described and Henningsson and colleague have found 3% *A. phagocytophilum* prevalence in ticks collected from northern Norway (Nordland, Troms and Finnmark) (Henningsson et al., 2015). It has been reported 2.55% from Romania (Dumitrache et al., 2015), 7.9% in Italy (Aureli et al., 2015) and 20.5% has been described in Spain (Ruiz-Fons, Fernandez-de-Mera, Acevedo, Gortazar, & de la Fuente, 2012). The prevalence of *Erlichia* was 11.5% from Southern Norway (Jenkins et al., 2001). In Holland, Schouls et al found members of the *E. phgocytophila* genogroup in more than 60% of *Erlichia*-positive ticks. This is in accordance with the resulted presented in this thesis which identified a substantial variation in the prevalence between sampling sites. The prevalence of *A. phagocytophilum* was highest in new the location south-western Norway, Hordaland (H4) than in southern Norway, Vest-Agder (S10). The prevalence of *A. phagocytophilum* was significantly higher in nymphs from Hordaland, H4 compared to other locations (P<0.05). The prevalence of *A. phagocytophilum* in adults is higher than previous finding. The area must have high activity of deer. High prevalence of *Anaplasma*, 19.4% has been reported from Hitra, north-western Norway, which has a very high density of roe deer and red deer (Olav Rosef, Radzijevskaja, Paulauskas, & Haslekås, 2009). Roe deer from southern Norway were found to be 96% seropositive, and also in moose and red deer antibodies were found (Snorre Stuen, Åkerstedt, Bergström, & Handeland, 2002). The prevalence of *A. phagocytophilum* in adults was higher compared to nymphs within the same location. Similar to other micro-organism, the chance of ticks to acquire infectious agent gets higher with higher number of blood meals. Larvae do not get infected with *A. phagocytophilum* trans-ovarially (Macleod, 1936; Walker et al., 2001) thus the risk of infection is only with later stage tick. Lower prevalence were found from Brønnøysund, Northern Norway 0.8% in nymphs and 4.6% in adult ticks (Soleng & Kjelland, 2013). In earlier study from Southern Norway, *A. phagocytophilum* prevalence ranged from 0 – 23% (O. Rosef et al., 2009). The prevalence of *A. phagocytophilum* detected in the present study was within the range as reported in previous mentioned Norwegian studies.

Prevalence of *Wolbachia pipientis* in *I. ricinus*

This is the first study in Norway detecting *W. pipientis*. The overall estimated prevalence of *W. pipientis* in this study from adult ticks was 10.68%. In the previous study *W. pipientis* was not detected in ticks from *A. phagocytophilum*-epidemic areas from west coast of Norway (Granquist et al., 2014). However, it has been reported 0.9% from

Southern Germany (Hartelt et al., 2004), 1.0% in adult and 27.3% in nymphs from France (Reis, Cote, Paul, & Bonnet, 2011) and 0.66 % from Thailand (Foongladda, Inthawong, Kositanont, & Gaywee, 2011). In a study it has been found 99.2 % prevalence in *I. ricinus* which has been parasitoid by a wasp *Ixodiphagus hookeri* (Plantard et al., 2012). Also, 87 % was reported from Netherland (Tijsse-Klasen, Braks, Scholte, & Sprong, 2011). The finding of *W.pipientis* in *I.ricinus* should be further investigated.

Prevalence of *M. mitochondrii* in *I. ricinus*

In our study 195 adult ticks (121 females and 113 males) were detected with *M. mitochondrii* by real-time PCR. In addition the difference between males and females prevalence was small 89.25% female ticks were infected and 76.99% male ticks were infected. It suggests that the presence of the bacterium causes no harm to the reproduction alterations and skewed sex ratios. Further, almost equal numbers of ticks are infected with *M. mitochondrii* this rule out cytoplasmic incompatibility. N. Lo *et al* 2006 found *M. mitochondrii* in 100% of all female *I. ricinus* (n=128), while in males 44% detected (n=108) based on screening with *16S* rRNA (Lo et al., 2006). Harmless endosymbiotic bacteria are primarily found in the ovaries or malpighian tubules of ticks. This tissue specificity reduces the chances of the bacteria being transferred to the tick's vertebrate host during a blood meal, thereby reducing the probability of horizontal transfer to other blood-sucking arthropods. They are more likely to spread via transmission to the eggs (Lo et al., 2006). ISH and PCR screening of various tick tissues by using *IricES1*-specific oligonucleotides revealed that the bacterium is restricted to the ovarian tissues (Beninati et al., 2004). In an earlier study using DGGE method, *M. mitochondrii* was present in more than 25% of the samples (N=120) (Tveten, Riborg, & Vadseth, 2013).

This study is in accordance with others, suggesting that Males harbor fewer bacteria than females. If an infected egg develops into a female, the bacterium continues to survive in primordial ovarian tissues. In contrast, if the egg develops into a male, most or all of the bacteria are lost. Maintenance in the lab for several generations appears to lead to a loss of *IricES1* in majority of female hosts. So, the symbionts are not obligatory. *M. mitochondrii* is found in female ovaries of ticks, suggesting that it is an important component of the tick microbial community and possibly transmission of pathogens. The very high female-specific prevalence of *M. mitochondrii* in *I. ricinus* obtained from all parts of Europe examined, argues against parasitism and commensalism (Beninati et al., 2004). Absence of negative effect and high prevalence of *M.mitochondrii*, it is possible that the bacterium is involved in an obligate mutualism with *I. ricinus*. However, our study did not show 100% infection in all ticks and suggests that commensal or transient mutualism occurs. Since the bacterium is present inside mitochondria, which is powerhouse of cell, it role in metabolism should be identified. A study shows no effect on the load of *M. mitochondrii* in adult ticks injected with antibiotics and in larvae feed on antibiotic treated animals (Ninio et al., 2015).

Presence of all organisms in single ticks

A number of factors influence the prevalence, such as tick behavior and survival, and survival of the pathogen within the ticks over the season (Herrmann, Voordouw, & Gern,

2013). It has been observed that having moist summers, but a relatively harsh winter, increases the prevalence of pathogens (Mysterud et al., 2013). Except for eight ticks that were infected with single organism almost in all ticks were associated with *Midichloria*. The duration of the life cycle of ticks can vary from one habitat to another and also regionally, and can be affected by microclimatic factors and host density (Gray 1991). Ticks acquire the bacteria primarily through a single blood meal per life stage, higher prevalence of *Borrelia* in adult ticks versus nymphs is expected (J. S. Gray, 1998). Same applies for other organisms, if the tick acquires virus or bacteria at every life stage the concentration would be higher than when it acquire only during larvae stage due to dilution effect. These results are also reflected in this study. With each microorganism we detected, two very close sites H3 and H4 from Hordaland were different in the prevalence. These two places are separated by a man-made road; ticks might not have crossed it unlike mosquito. The road becomes a barrier for easy passage of rodents and deer. Not all real-time PCR positive samples were positive in pyrosequencing or sequencing. It might be due to lower nucleic acid for the microorganism in the tick. Intensity of the ticks receiving the microorganism also depends on how much concentration in the reservoir host. In the host, multiple numbers of infected ticks attached will transmit copious amount of infectious microorganism otherwise it will be diluted. The amount of pathogen DNA in the tick will thus vary on an individual basis. The study of tick-borne infection prevalence should be known in a region, as well as accurate identification, reservoir prevalence and human exposure to understand the epidemiology of the disease and plan preventive strategies. Since ticks can carry more than one pathogen at a time, there is possibility to get infected with multiple microorganisms. A synergistic effect between co-infecting microorganisms can be favorable for pathogenic microorganisms and alter their pathogenesis (Swanson, Neitzel, Reed, & Belongia, 2006). *I. ricinus* acquires microorganisms both during feeding on one or more hosts. The presence or absence of any microorganism in ticks does not seem to affect the presence of any other specific pathogen (Tveten et al., 2013). The increasing number of vectors in new and existing areas, and a spread of vector borne diseases, is likely to be consequences of the predicted climate changes, dismantling farms and forestry, and increasing populations of wild ruminants (Lindgren & Gustafson, 2001; Randolph, 2004).

Comparison of animal infection with tick infection

In this study, the prevalence of various organisms was different in different locations. To prevent contamination of TBEV in the food chain, preventive measures such as vaccination of animals and human populations and pasteurization of milk should be done. The serological investigation of zoonotic infections may provide valuable epidemiological information. However, serological test interpretation is often influenced by other factors such as cross-reaction with other flaviviruses, past infections or vaccination (Schwaiger & Cassinotti, 2003). Though there are uncertainties related to cross-reaction in both by PCR and serological test. The detection of infectious agents in ticks, animal sera and milk from same location may indicate that the virus is circulating and may represent as a risk for the human population living in those areas, even though there are not reported cases. However, this need to be further investigated.

Conclusion

All three components: pathogens, vectors and reservoirs are important in the ecology of zoonotic diseases. Assumptions are commonly made that the vector is the only danger in transmission of pathogens. However, the present study shows that new thinking is needed, especially in the field of food safety and contact with domestic ruminants. The risk factors to human infection are not only suitable tick habitats, high deer density and outdoor activities, but also consumption of products from animals carrying the virus. Certain occupations and hobbies increase the risk of infection. In this study Ticks, domestic ruminants and their products are shown to carry the tick borne encephalitis virus. A thorough risk assessment is therefore needed to evaluate the consequences of consuming raw milk and dairy products from ruminants in areas where ticks are known to carry the virus. Further studies are needed to identify epidemiological parameters and causal relationships in order to control zoonotic infections in the ticks, animal and human interface.

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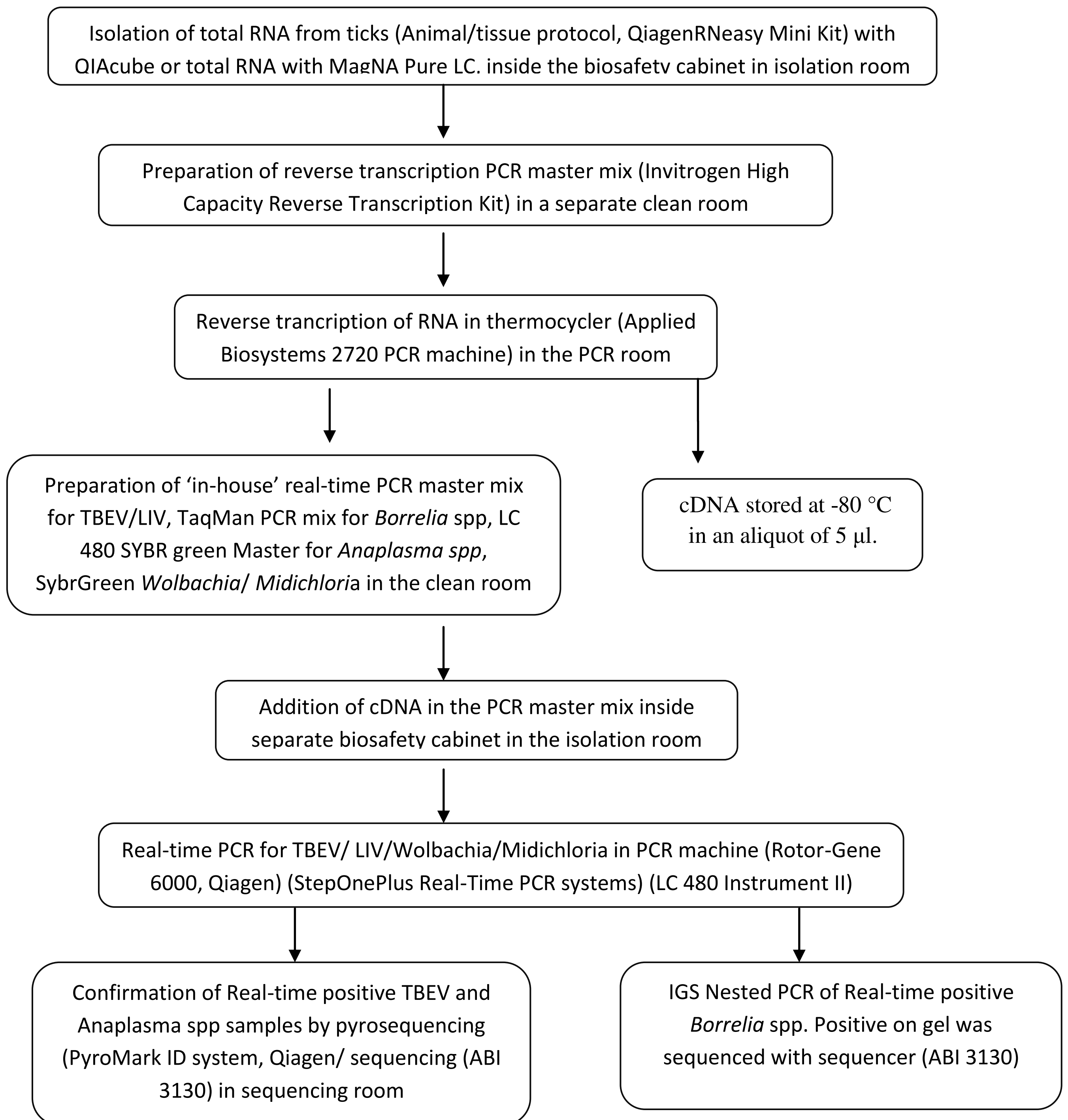
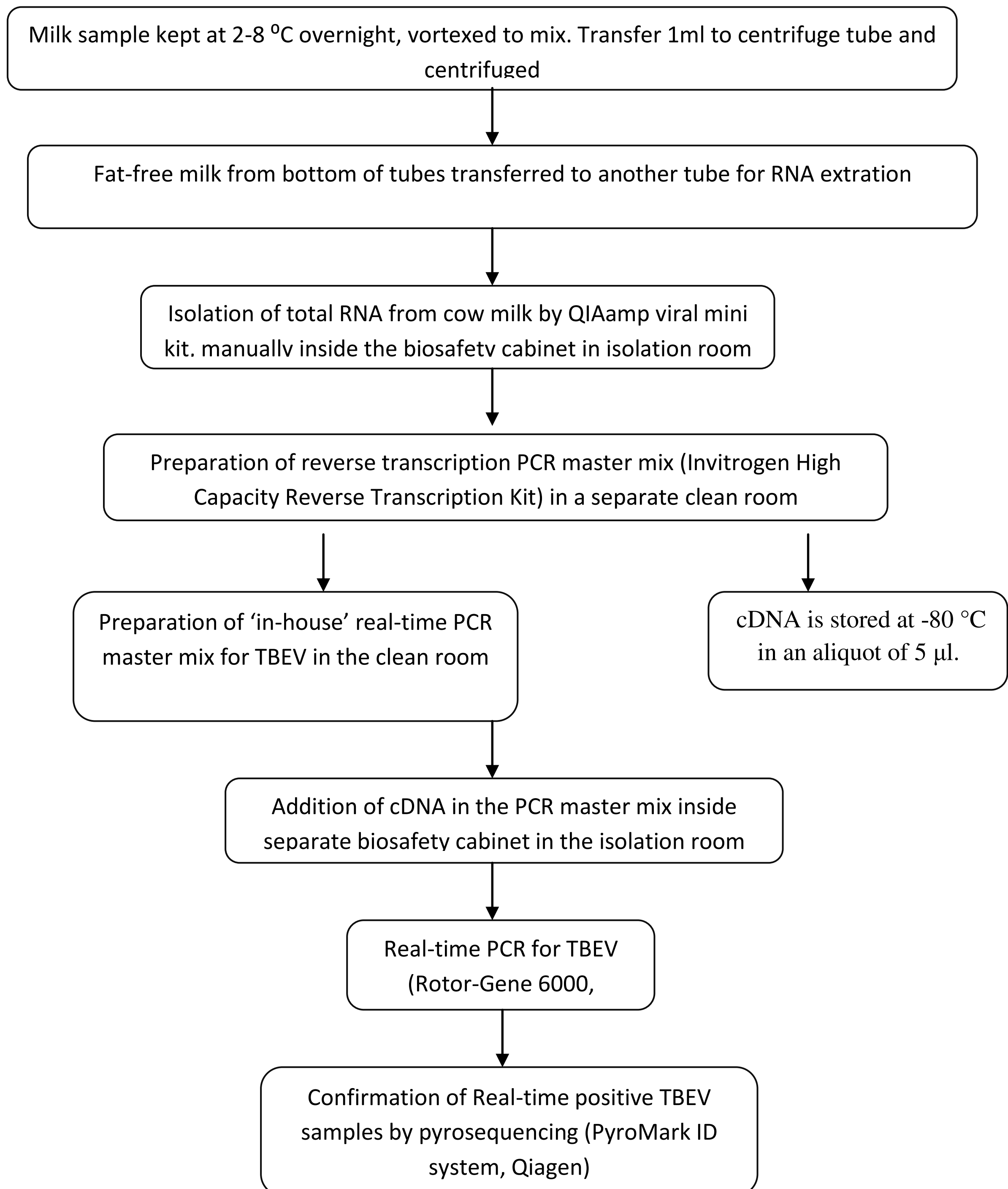
Appendix 1: Flowchart for Analysis of *Ixodes ricinus*



Appendix 2: Flowchart for Analysis of cow milk



Appendix 3: MagNA Pure LC protocol

MagNA Pure LC 2.0 instrument with software v 1.1.24 was installed. The instrument is based on magnetic bead technology for isolation of nucleic acid. MagNA Pure LC Total Nucleic Acit Isolation Kit-High Performance version 08 was used.

For isolation of total nucleic acid (DNA and RNA) following protocol was followed:

- Start the instrument and software
- Select the appropriate protocol: Total NA Hp 200
- Specify number of samples, sample volume (200 µl) and elution volume (60 µl), this will give volume of reagent required
- Fill the reagents and tips in the appropriate container
- Run the machine.

Appendix 4: QIAcube protocol

In the QIAcube, protocol for purification of total RNA, including small RNAs, from animal tissue and cells (aqueous phase) was chosen. Ethanol (70%) provided ideal conditions for binding and, washing and elution occurred in the RNeasy mini column. From approximately 350 ml of homogenate 30-100 µl of purified RNA was extracted.

For isolation of total RNA following protocol was followed:

- Turn of the QIAcube machine
- Fill 1000 µl pipette tips in the appropriate rack
- Place the spin column and elution tube on the rotor adaptor
- Buffer RW1, Buffer RPE, 70% Ethanol and 10 mM Tris pH 8.0, on the reagent bottle
- Place the sample tube in the sample rack
- Select RNA RNeasy Mini Animal tissues and cells Two elution steps (2x30 µl) start
- Extraction takes approximately 30 minute. RNA is further reverse transcribed or stored at -80 °C

Appendix 5: QIAamp® Viral RNA mini kit

Cow milk sample was kept at 2-8 ºC overnight before viral RNA extraction. About 1 ml milk sample was added to a micro-centrifuge tube and centrifuged at 6000 g for 10 minute. Pipette 400-500 µl of fat-free milk from below the fat layer. The milk was processed manually with a QIAamp viral mini kit as described in the manufacturer protocol.

Procedure

1. Buffer AVL 560 µl and 5.6 µl of carrier RNA was pipetted into 1.5 ml microcentrifuge tube.
2. Milk sample (140 µl) was added to the buffer AVL-carrier RNA. Mixed in a vortex for 15 second
3. Incubate at room temperature for 10 min
4. 560 µl of ethanol (96-100%) was added to the mix and vortex for 15 second. Briefly centrifuged to remove liquid attach to the lid
5. 630 µl of above mix was added to the QIAamp Mini column with 2ml collection tube. Centrifuged at 6000x g for 1 min. This step was repeated.
6. On the QIAamp Mini column, 500 µl of Buffer AW1 was added. The cap closed and centrifuged at 6000x g for 1 min. The column was placed on a clean 2ml collection tube
7. On the QIAamp Mini column, 500 µl of Buffer AW2 was added and centrifuged at 20,000x g for 3 min.
8. The column was placed on a clean 1.5 ml micro-centrifuge. Buffer AVE (60 µl) was added to the column. After incubation for 1 min, the column was centrifuged to get the total RNA.

Appendix 6: Reverse transcription PCR

Composition of reverse transcription PCR mixture

Component	Amount (µl)/ test
10X RT Buffer	2.0
25X dNTPMix (100 mM)	0.8
10X RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Rnase Inhibitor	1.0
Nuclease-free water	8.2
Total mix	15 µl

Total volume, 20 µl = 15 µl (Mix) + 5 µl RNA/TNA (approx. 500ng)

PCR condition

Temperature	Time
25 °C	10 min
37 °C	120 min
85 °C	5 min
4 °C	∞

Appendix 7: Real-time PCR mix for TBEV

Components	Amount (µl)/ test
50 mM $MgCl_2$	2.5
10 X AB buffer	2.5
25 mM dNTP	0.2
25 µM 320 F	0.25
25 µM 373 R+Biotin	0.25
25 µM 339 Probe	0.3
Pt-Taq enzyme *	0.19 (Units)
RNase-free water	15.81
Total mixture	22

*Platinum®Taq DNA polymerase enzyme from Invitrogen™

Total volume, 25 µl = 22 µl Mix + 3 µl cDNA

10 X AB buffer components

Component	Volume
1 M Tris pH 8.8	75 ml
$(NH4)_2$ $S0_4$	20 ml
Tween 20	0.2 ml
RNase free water	4.8 ml
Total	100 ml

PCR condition

Temperature	Time	No of cycle
95 ºC	2 min	1
95 ºC	15 second	
60 ºC	45 second	48
72 ºC	30 second	

Appendix 8: Real-time PCR mix for LIV

Component	Amount (µl)/ test
5 mM Qiagen onestep-RT-PCR buffer (5X)	5
MgCl2 5mM	1.55
dNTP 25 mM	0.4
LIV 410F forward primer (25 µM)	0.4
LIV 1000R reverse primer (25 µM)	0.4
LIV 680 MGB probe (25 µM)	0.3
Pt-Taq enzyme**	0.19
RNase-free water	13.76
Total volume	22

Total volume, 25 µl = 22 µl Mix + 3 µl cDNA

PCR condition

Temperature	Time	No. of cycle
95 ºC	2 min	1
95 ºC	15 sec	
54 ºC	30 sec	45
72 ºC	30 sec	

Appendix 9: Real-time PCR mix for *Borrelia* spp.

Compositions of PCR mix for identification of *Borrelia burdorferi* s.l. spp. in Stepone Plus real time PCR system

Component	Amount (µl)/ test
TaqMan Universal PCR Master Mix	12.5
RF forward primer (100 µM)	0.225
RF reverse primer (100 µM)	0.225
LB probe	0.05
Nuclease-free water	7
Total volume	20

Total volume, 25 µl = 20 µl Mix + 5 µl cDNA

PCR condition

Temperature	Time	No. of cycle
50 ºC	2 min	1
95 ºC	10 min	1
94 ºC	30 sec	
53 ºC	30 sec	55
72 ºC	30 sec	
4 ºC	∞	

Eight samples from H4 V14 and H2V14, which is 1:10 dilution and undiluted templates in different volumes: 2 µl, 4 µl and 6 µl were used to establish the method. Based on this results undiluted 4 µl of sample template were used in first nested PCR reaction. For the second nested PCR, 3 µl of PCR products from first nested PCR were used.

Components of Nested PCR for Intergenic space (IGS) between rrs-rrlA are given below

First IGS nested PCR

Components	**Amount (µl per reaction)**
IGS 1 forward primer (20 µM)	1
IGS 1 reverse primer (20 µM)	1
10mM dNTP mix	2.5
10X PCR buffer	2.5
25mM MgCl2	2.5
Taq Pol (Gold)	0.2
Nuclease free water	11.3
Total volume	**21**

Total volume, 25 µl = 21 µl Mix + 4 µl template cDNA

PCR condition

Temperature	Time	No. of cycle
95 ºC	5 min	1
94 ºC	30 sec	
52 ºC	30 sec	35
74 ºC	3 min	
74 ºC	7 min	1
4 ºC	∞	

Second IGS nested PCR

Components	**Amount (µl per reaction)**
IGS 2 forward primer (20 µM)	1
IGS 2 reverse primer (20 µM)	1
10mM dNTP mix	2.5
10X PCR buffer	2.5
25mM MgCl2	2.5
Taq Pol (Gold)	0.2
Nuclease free water	12.3
Total volume	**22**

Total volume, 25 µl = 22 µl Mix + 3 µl amplicon from first nested PCR

PCR condition

Temperature	Time	No. of cycle
95 ºC	5 min	1
94 ºC	30 sec	
57 ºC	30 sec	39
74 ºC	3 min	
74 ºC	7 min	1
4 ºC	∞	

Appendix 10: Real-time PCR mix for *A. phagocytophilum*

Roche LightCycler 480 (LC 480) in use for PCR analysis is 96 well plate based machine. It has Xenon lamp as a light source and is possible to choose number of excitation/ emission wavelength. With SYBR Green I master we used 483/35 nm excitation and 533/ 20 emission wavelength.

LightCycler® 480 SYBR Green I master consists of DNA double strand specific dye and FastStartTaq DNA polymerase. In this 'hotstart' reaction the modified enzyme is inactive at room temperature. The FastStart Taq polymerase is activated when the blocking amino acid residues are removed at high temperature (pre-incubation at 95 ºC for 5 minutes).

During the cycling process, fluorescence emission is measured every cycle at the appropriate wavelength within each well.

Components	**Amount (µl per reaction)**
LightCycler® 480 SYBR Green I master	7.5
ApMSP2 252 forward primer (10 µM)	0.75
ApMSP2 459 reverse primer (10 µM)	0.75
RNase-free water	4
Total	13

Total volume, 15 µl = 13 µl Mix + 2 µl template cDNA

PCR condition

Temperature	Time	Ramp rate	No. of cycle
95 ºC	5 sec	4.4	1
Amplification			
95 ºC	10 sec	4.4	
56 ºC	10 sec	2.2	50
72 ºC	10 sec	4.4	
Melting curve			
95 ºC	5 sec	4.4	1
65 ºC	60 sec	2.2	
97 ºC	Cont.	0.11	
4 ºC	∞		

Appendix 11: Real-time PCR mix for *W.pipientis*

Components	Amount (µl per reaction)
Perfecta® SYBR® Green FastMix®	7.5
Wolwsp81F forward primer (10 µM)	0.6
Wolwsp691R reverse primer (10 µM)	0.6
RNase-free water	3.3
Total volume	12

Total volume, 15 µl = 12 µl Mix + 3 µl template cDNA

PCR condition

Temperature	Time	No. of cycle
Hold		
95 ºC	5 min	1
Amplification		
95 ºC	30 sec	
56 ºC	30 sec	45
72 ºC	30 sec	
Melting curve		
95 ºC	5 sec	1
65 ºC	60 sec	
97 ºC	Cont.	
4 ºC	∞	

Melt Curve 65 ºC to 99 ºC with increasing increments of 0.2 ºC per cycle.

Appendix 12: Real-time PCR mix for *M. mitochondrii*

Components	Amount (µl per reaction)
Perfecta® SYBR® Green FastMix®	7.5
*gyr*B-MidiF forward primer (10 µM)	0.6
*gyr*B-MidiR reverse primer (10 µM)	0.6
RNase-free water	5.1
Total volume	13.8

Total volume, 15 µl = 13.8 µl Mix + 1.2 µl template cDNA

PCR condition

Temperature	Time	No. of cycle
Hold		
95 ºC	5 min	1
Amplification		
95 ºC	30 sec	
56 ºC	30 sec	45
72 ºC	30 sec	
Melting curve		
95 ºC	5 sec	1
65 ºC	60 sec	
97 ºC	Cont.	
4 ºC	∞	

Melt Curve 65 ºC to 99 ºC with increasing increments of 0.2 ºC per cycle.

Appendix 13: Pyrosequencing

In the pyrosequencing reaction, biotinylated single-stranded DNA templates are bound to Streptavidin Sepharose™ beads (GE Healthcare, Little Chalfont, UK) in a solution containing enzymes and substrates. When deoxyribonucleotide triphosphate (dNTP) is incorporated in to the DNA by DNA polymerase, inorganic pyrophosphate (iPP) is released. The iPP is converted into ATP in presence of ATP sulfurylase and adenosine 5'-phosphosulfate (APS). ATP converts luciferin to oxyluciferin in presence luciferase and in this light signal is produced. Apyrase in the mix degrades any unincorporated nucleotides.

<u>Pyrosequencing SQA Protocol Using the PyroMark™ ID (Biotage)</u>

<u>Equipments</u>

PyroMark™ ID Instrument, Software installed PC, PyroMark™ ID Vacuum Prep workstation, Vacuum tool, Orbital Plate Shaker, Heat block, 96 well plate.

<u>Reagents</u>

Binding buffer mix	Primer mix
• Binding buffer- 17µl • Streptavidin Sepharose™ beads (properly mixed)- 3 µl • Nuclease-free water	• Annealing Buffer- 43 µl • 25 µM TBE 320 F primer- 0.8 µl • Nuclease-free water- 1.2 µl

Procedure

Preparation of master mix and primer mix

1. Before making the mix the reagents are brought from 4 ºC to room temperature. Streptavidin Sepharose™ beads should be mix properly. 60 µl of the binding buffer mix is added to each well of 8 stripped tubes without cap. Inside the biosafety cabinet 20 µ of PCR product is added.
2. With covers on mix the tubes in orbital plate shaker for 10 minutes at 1400 rpm.
3. Prepare primer mix and add 45 µl in test well of 96 well plate.

Washing steps

1. Set plastic reagent tray in the designated positions on the Vacuum Prep Workstation and fill with 70% ethanol, denaturation solution (NaOH), wash buffer and distilled deionized water.
2. The heating block is set at 80 ºC.
3. Turn on the vacuum switch for both the Vacuum prep workstation and the vacuum pump, and allow flow of water for few second. This is to check the filter of the vacuum tool if it is blocked. Let the tool sit on water until ready to use.
4. After shaking of sample and streptavidin is complete, the cover is gently removed inside the biosafety cabinet. It is placed in respective position on the Vacuum Prep Workstation.

5. Turn on the vacuum pump. Flush the sample and reagents through the vacuum tool in following order.
    - Put the vacuum tool on the sample tubes for 1 minute or when entire volume has been collected. The tip of the vacuum tool contains filter probes that captures the immobilized amplicons-streptavidin.
    - Transfer the vacuum tool on 70% ethanol tray for 5 second. This step allows any unbound amplicon or unincorporated reagents from the real time PCR reactions to be washed off.
    - Transfer the Prep tool on NaOH tray for 5 second. This step allows for the denaturation of the double-stranded DNA amplicon containing a biotin-tagged, single-stranded, DNA template. The complementary strand synthesized from the non-biotin primer will be washed away
    - Transfer the Prep tool on wash buffer tray for 5 second.
6. Lift the Prep tool at 90º and turn off the vacuum to allow all liquid to drain out. Place it on the plate containing primer mix for 5 minutes. Shake the vacuum tool side to side for 10 second to release bead bound to the single-stranded template.
7. Put the plate on a pre heated block at 80 ºC for 2 minute to allow denaturation of template and primer mix.
8. Allow to cool on a bench for 5 minute to anneal. By the time it cools, turn the PC on and enter sample name.

Enzyme mix

It consists of all enzyyme that is needed for pyrosequencing reaction. DNA polymersase incorporate nucleotide, ATP sulpharase that transmit pyrophosphate to ATP, Luciferase gives light signal and apyrase degrades unbounded nucleotides and ATP. Single stranded binding protein (SSB) is added to the mix to destroy secondary structure in the template.

Substrate mix

The substrate mix consists of adenosine 5' phosphosulphate (APS) that is need for production of ATP and luciferin.

Pyrosequencing PyroMark ID and Software

1. Turn the PC and pyrosequencing machine on.
2. Log into PyroMark ID.
3. Select SQA-run and duplicate the last run.
4. Write run name as TBE SQA year_day_month
5. In the 'New Run Setup' highlight all the wells that are to be used and drag the dispensation order to the wells. Write sample identification name.
6. Click on the 'Tools' tab to reveal a drop-down menu. Select 'Pre Run Information' to see the volumes of the Enzyme (E), substrate (S) and nucleotides (A, C, G and T) that need to be added to the cartridge. Fill them in correct order without bubble.

Figure: PyroMark ID cartridge setup

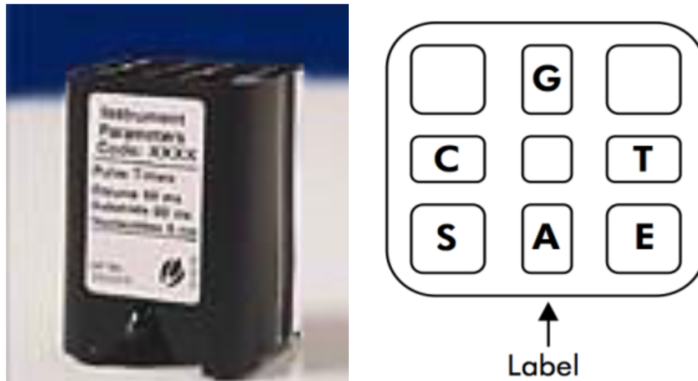


Figure. PyroMark Q96 block and template. The template shows where the enzyme, substrate and dNTPs should be added.

7. Load the cartridge containing enzyme, substrate and nucleotides onto the PyroMark ID instrument, with the cartridge label facing front towards the user.
8. Load the plastic plate containing template and primer into the PyroMark ID instrument.
9. Select ‘run’ to start. Substrate peak appears which ensure that the enzyme and substrate are working well.

Cleaning the Instrument

1. Clean the Prep tool with distilled deionized water on the tray. Turn on the vacuum and flush the tool for 10-20 second. Discard the remaining reagents and wash the trays.
2. After pyrosequencing run is complete, remove the plate and discard. Remove cartridge from the instrument and wash 3 times with water. Check all dispensation pins are clean by applying pressure to the top of each of the channels so that water should stream straight down out of the pin.

After run is complete, select ‘SQA Full Reprot’ under report tab. This will generate a document containing pryrograms and the results of the analysis.

Images of Pyrosequencing pyrogram

Positive control - Sokoupa 10^{-3} dilution

Sample positive - H4V(14)-78

Negative control - Water

Appendix 14: Direct sequencing

Genetic Analyser ABI 3130

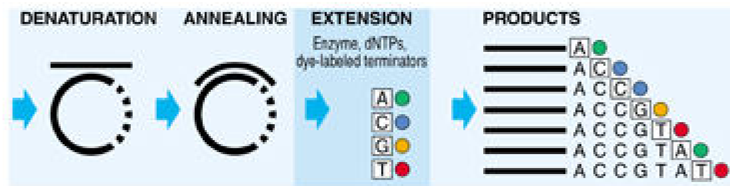


Fig. Dye labeled terminator reactions (www.appliedbiosystems.com)

Reaction mixture for cycle sequencing

Components	Volume (µl per reaction)
DyeBig ver1.1 (2.5x)	5.5
ABI buffer (5x)	1
Rerverse IGS2 primer (3.2 µM)	1
Nuclease free water	5.5
Total	9

Total volume, 10 µl = 9 µl cycle sequencing mix + 1 µl Exo Star cleaned nested PCR product

Appendix 16: Comparison of various microorganisms from different counties

Comparison of TBEV in nymph tick

Collection site, Municipality, County	No of Nymph pool	No of positive nymph pools by RT-PCR	No of positive nymph pools by pyrosequencing	MIR
Hordaland, Tungesvik	163	5	3	0.18 %
Mandal, Vest-Agder	158	7	1	0.06%
Hvaler	3	0	0	0
Total	324	12	3	0.0009%

Comparison of TBEV in adult tick

Collection site, Municipality, County	No of adult ticks	No of positive adult by RT-PCR	No of positive adult by pyrosequencing	MIR
Hordaland, Tungesvik	78	6	4	5.12 %
Mandal, Vest-Agder	149	6	1	0.67 %
Hvaler	7	0	0	0
Total	234	12	5	2.13

Comparison of *Borrelia* spp in adult tick

Collection site, Municipality, County	No of adult ticks	No of positive adult by RT-PCR	No of positive adult by direct sequencing	MIR
Hordaland, Tungesvik	78	22 (28.2%)	3	3.84 %
Mandal, Vest-Agder	149	53 (35.57 %)	1	0.67 %
Hvaler	7	6 (85.71%)	3	28.57 %
Total	234	81 (34.61 %)	5	2.13

Comparison of *Anaplasma* spp in nymphs

Collection site, Municipality, County	No of Nymph pool	No of positive nymph pools by RT-PCR	MIR
Hordaland, Tungesvik	163	85	5.21 %
Mandal, Vest-Agder	158	46	2.91 %
Hvaler	3	1	3.33
Total	324	132	4.07%

Comparison of *Anaplasma* spp in adult tick

Collection site, Municipality, County	No of adult ticks	No of positive adult by RT-PCR	No of positive adult by direct sequencing	MIR
Hordaland, Tungesvik	78	21 (26.9 %)	20	3.84 %
Mandal, Vest-Agder	149	39 (35.57 %)	25	16.77 %
Hvaler	7	0	0	0
Total	234	60 (25.64 %)	45	19.82 %

Comparision of *W.pipientis* in adult tick

Collection site, Municipality, County	No of adult ticks	No of positive adult by RT-PCR	MIR
Hordaland, Tungesvik	78	13	16.66 %
Mandal, Vest-Agder	149	12	8.05 %
Hvaler	7	0	0
Total	234	25	10.68 %

Comparision of *M.mitochondrii* in adult tick

Collection site, Municipality, County	No of adult ticks	No of positive adult by RT-PCR	MIR
Hordaland, Tungesvik	78	65	83.33 %
Mandal, Vest-Agder	149	126	84.56 %
Hvaler	7	4	57.14
Total	234	195	83.33 %

Appendix 17: Detection of TBEV in cow milk

Table: Cow milk from Mandal, Vest-Agder

Samples ID from Mandal, Vest-Agder	Cow milk sample with positive Ct value by RT-PCR	Cow milk sample positive by pyrosequencing
222		
223		
237	32.86	
164	35.29	Positive
212		
232	34.41	Positive
202		
Tank 1	33.58	Positive
Tank 2	35.45	Positive

Table: Cow milk from Skedsmokorset

Samples ID	Cow milk sample with positive Ct value by RT-PCR	Cow milk sample positive by pyrosequencing
545		
546		
579		
591		
603	31.07	Positive
605		
613		
614	37.55	Positive
819		
626		
630		
641		
645	37.37	
655		
658		
659		
660		
662		
663		
667		
669	36.47	
656		
Tank 1		

Appendix 18: Comparison of Immunozym and Enzygnost ELISA kit

Table: ELISA result on sheep serumsamples from Skänevik, Hordaland

Sheep Samples	Immunozym	Enzygnost
40012		
40013		
40015		
40016		
40017		
40020		
40033		Positive
40034		
40035		
40039		Borderline
40041		
40042		
40046		
40050		
40052		
40056		
40057		
40063		Positive
40070	Borderline	Positive
40093		
40021 Negative control		
40031 Negative control		
3002 Unvaccinated		
3003 Unvaccinated		
3002 Vaccinated	Positive	Positive
3003 Vaccinated	Borderline	Positive

Table: ELISA result on cow serum from Skedsmokorst compared with milk result

Cow serum from Hille	Immunozym	Enzygnost	RT-PCR of cow milk	Cow milk Positive by pyrosequencing
542				
545				
546				
579				
591				
603		Borderline	31.07	Positive
605				
613				
614	Borderline	Positive	37.55	Positve
626				
630				
641		Positive		
645			37.37	
646				
655				
658				
659				
660				
662				
663				
667				
669			36.47	
819	Borderline			
Cow serum from Mandal, Hille				
189				
196				
203				
211				
212	Borderline			
222				
223				
232			34.41	
237			32.86	
Cow serum from Skanevik				
0245	Positive	Positive		
0248	Positive	Positive		
Positive controls				
749 unvaccinated				
751 unvaccinated				
749 vaccinated	Borderline	Positive		
751 vaccinated	Positive	Positive		

Appendix 19: Application and approval of vaccination trail of sheep and cow

Søker og medarbeidere

Institusjon	011 NMBU, Seksjon for eksperimentell biomedisin, (inngår i 170)
Adresse	Postboks 8146 Dep. 0033 Oslo
Telefon	22964574
E-post	erik.ropstad@nvh.no
Personell med særskilt kontrollansvar	Erik Ropstad
Forsøksansvarlig	Erik Georg Granquist - Forsker (Ansvarlig søker/Prosjektansvarlig) (Kurs i forsøksdyrlære)
Medarbeider 1	Åshild Andreassen - Forsker (Ansvarlig søker/Prosjektansvarlig)
Søknadsdato	11.09.2015

Generelle opplysninger

Id	8135
Søkerens ref.nr.	TICOVAC 2015
Forsøkets arbeidstittel	Tick borne encephalitis in the food chain - dairy cows and sheep.
Dyreart	Pattedyr - drøvtyggere (Ruminantia) - Storfe-kveg (Bos taurus) Pattedyr - drøvtyggere (Ruminantia) - Sau (Ovis aries)
Institusjon	011 NMBU, Seksjon for eksperimentell biomedisin, (inngår i 170)
Type søknad	Pilotforsøk - Feltforsøk (jr. § 10. 1. ledd)
Type søknad som må sendes Mattilsynet for avgjørelse	Feltforsøk (jr. § 10. 1. ledd)
Akutt forsøk	Nei
Tidligere erfaring med tilsvarende forsøk	Ja
Forskningen er finansiert av	På oppdrag fra offentlig etat
Planlagt start	01.10.2015
Planlagt slutt	15.11.2015

Beskrivelse

The project will use serological assays (ELISA), both commercial and new assays, to diagnose cattle and sheep which are positive for TBE and Louping-ill virus in Norway. The pilot study will have impact on how we perform risk assessments of products from sheep and cattle for human handling and consumption. To obtain positive control samples for serological assays we need to vaccinate two lambs/young sheep and two calves (approx. six months) with a human vaccine for TBE virus. We intend to vaccinate these animals on a farm belonging to Georg Molvig on Skedsmo, Norway (Producer Number 0231012238). The vaccine: TicoVac (Pfizer Limited, Storbritannia) Content: 1 dosage (0,5 ml) contains: Tick borne encephalitis virus (TBE-virus) (strain Neudørfl) 2,4 µg. Cultured in Chicken Embryofibroblast Cells (CEF-celler), adsorbed to aluminium hydrokside, hydrated (0,35 mg Al^{3+}), human albumine, Sodium chloride, di-sodium phosphate di-hydrate, Potassium di-hydrogen phosphatet, sucrose, water for injectionfluids. Potassium and sodium levels are <1 mmol /dosage, in practice free of sodium and potassium. The vaccine is based on a complete inactivated virion. The adverse effects are reported for humans and include: mild lethargy, mild fever, nausea, local inflammation in the skin at the injection site. We have contacted the Norwegian Medicines agency to determine the withdrawal period of the vaccine. There is no possibility of transmission of infective agents as the vaccine is inactivated. The procedure will include three vaccines on days 0, 7 and 21. Then collect blood samples by vacutainers and subdermal needles on day 0, 28 and 45.

Offentlighet

Inneholder søknaden opplysninger som ønskes unntatt fra offentlighet?	Nei
Hvis ja, angi relevante lover og paragrafer (f. eks. Offentlighetsloven, § 13, 1. avsnitt og Forvaltningsloven, § 13, 1. avsnitt, 2. punkt).	- - -

Hvis ja, beskriv hvilke opplysninger som ønskes unntatt fra offentlighet.

- - -

Bakgrunn og hensikt

Gi en kort presentasjon av bakgrunn og hensikt med forsøket (maksimalt 500 ord), i en allment tilgjengelig språkform. Angi eventuell hypotese som skal testes. Angi særskilt hvis spesielle lovbestemmelser/krav fra offentlige myndigheter krever at forsøket skal utføres.

Skogflåttencefalitt (TBEvirus) er klassifiser i smittekategori 3: I Forskrift om tiltaksverdier og grenseverdier for fysiske og kjemiske faktorer i arbeidsmiljøet samt smitterisikogrupper for biologiske faktorer (forskrift om tiltaks- og grenseverdier) står det i Vedlegg 2 under Liste over klassifiserte biologiske faktorer at: Smitterisikogruppe 3 er en biologisk faktor som kan forårsake alvorlig infeksjonssykdom hos mennesker og utgjøre en alvorlig fare for arbeidstakerne, det kan være risiko for spredning til samfunnet, men det finnes vanligvis effektive forebyggende tiltak eller behandling. Det registreres årlig mellom seks og 14 nye tilfeller av skogflåttencefalitt i Norge og det er grunn til å tro at denne sykdommen er underdiagnostisert. Vi har indikasjoner fra tidligere utførte studier (Ewa Cisak et. al. 2010) at TBE finnes i melk fra sau (22.2%), geit (20.7%) og ku (11.1%). Vår vurdering er at husdyr representerer et smittepotensiale for TBE, via melk til mennesker fra dyr der nisjeproduksjon av upasteuriserte meieriprodukter selges fra gård eller butikk. Det foreligger ingen kunnskap om utbredelse eller risiko for TBE smitte hos landpattedyr i Norge. Vi har startet en pilotstudie for å finne gode diagnostiske (serologi og PCR) verktøy for påvisning av TBE hos lakterende landpattedyr og differensiering av TBE og Louping-ill virus infeksjon hos ku og sau. Dette vil være viktige verktøy for å kartlegge epidemiologien av disse to beslektede virusene samt å bidra til en god risikovurdering av melk fra gårder som selger eller konsumerer upasteurisert melk. Hensikten med søknaden er å be om tillatelse til å vaksinere to sau og to kalver slik at vi kan oppnå serumpositivitet som kontroll i vårt studium. Vi skal vaksinere med vaksinen TICOVAC som er en human vaksine, og har bedt legemiddelverket se på hvordan vaksinerte dyr skal håndteres med tanke på tilbakeholdelsestid og eventuell isolering og destruksjon. Det er meget få bivirkninger forbundet med vaksinering av mennesker og det forventes minimal lidelse påført vaksinerte dyr. Likevel vil det antakelig være noe mer enn et nålestikk. I tillegg skal det tas serumprøver (dag 0, 28 og 45) av dyrene etter tre intramuskulære vaksinasjoner på 0, 7 og 21 dager med vanlig blodprøvetakingsutstyr (vacutainer) fra vena jugularis. Denne delen av studien er svært viktig da den verifiserer positive prøver i vårt materiale og kan være med på å sikre serologitestens kvalitative egenskaper.

Beregning av antall dyr

Gi en begrunnelse for antall forsøksdyr. Ved usikkerhet om populasjonsstørrelse skal det gjennomføres pilotforsøk, jf. forskriftens § 13. Søk hjelp hos statistiker dersom du er i tvil.

To dyr av hver art skal benyttes fordi individuell respons på vaksiner vil avhenge av forskjellige faktorer som ernæringsstatus, generell immunstatus og responsivness for antigenet som appliseres. Det er derfor vensentlig å benytte minst to dyr av hver art for å få frem den individuelle forskjellen. I og med at dette er en pilotstudie, ønsker vi ikke å benytte mer enn disse fire individene for å unngå unødig lidelse.

Gi en oversikt over samtlige forsøksgrupper og gruppestørrelser. Legg gjerne ved en tabell som vedlegg til søknaden.

to værlam og to oksekalver benyttes. De oppstalles etter gjeldende forskrift om hold av storfe og forskrift om velferd for småfe.

Hvilken metode er brukt for beregning av antall dyr.	Ikke aktuelt

Hvis "Power analyse"/"Ressursligning": Hvilke input er lagt inn?
Hvis "Annen metode": Gi en detaljert beskrivelse av den metoden som er benyttet.
Hvis "Ikke aktuelt": Beskriv hvorfor statistiske metoder ikke kan benyttes.

Dette er en pilotstudie og vi vil benytte det minste antallet dyr som er mulig i en slik sammenheng (uttak av positivt serum som kontroll).

Alternativer/3R

Erstatning ("replacement"): Hvorfor kan man ikke oppnå forsøkets hensikt uten å benytte levende dyr? Hvilke alternativer er vurdert og hvorfor er de forkastet?

Det ble vurdert å hente inn serum fra utlandet, men på grunn av ukjent smittestatus på dyr i utlandet vil vi ikke ha interfererende serumkomponenter i vårt kontrollserum. Da dette er et flåttoverførbart agens var det ønskelig å benytte kontrolldyr fra områder med lite flått. Skedsmo (Oslo omegn) er et slikt område. I tillegg tilhører den nevnte besetning, ambulatorisk klinikks (NMBU) praksisområdet og sykdomsstatus i besetningen er meget godt kjent for oss fra før.

Hvilke databaser ble det søkt i og hvilke søkeord ble benyttet for å finne alternativer?

Det er ikke søkt etter alternativer i databaser. Vår kunnskap om skogflåttencefalitt på husdyr er meget begrenset og gjenspeiler det omfang av studier som er gjort nasjonalt og internasjonalt på TBE og Louping-Ill. Derfor er det viktig å innhente kontrollsera fra de dyrearter som inngår i pilotstudien.

Reduksjon ("reduction"): Når bruk av dyr er uunngåelig: Hvilke tiltak, steg og forholdsregler har du brukt for å minimalisere antall dyr og fremdeles oppnå valide vitenskapelige resultater?

Vi har ikke et ønske om å bruke fler dyr enn det som er beskrevet i denne søknaden. Vi mener antall dyr bør være tilstrekkelig for å oppnå formålet om serumpositivitet hos disse og at dette serum kan inngå i vårt vitenskapelige arbeide som går ut på å utvikle en diagnostisk test for serumdiagnostikk på norske husdyr.

Raffinering ("refinement"): Når bruk av dyr er uunngåelig: Hvilke forbedringer av stell og prosedyrer er gjort for å minimalisere smerte, lidelse, ubehag og varig skade og for å øke dyrevelferden i forhold til tidlige lignende forsøk? (Stikkord: anestesi, analgesi, endepunkter, miljøberikelse, operasjonsteknikk, prøvetakningsteknikk osv).

Dyrene skal oppstalles i sitt vante miljø og håndteres av dyreeier. Vi vil ha kontakt med dyrene gjennom vaksinasjonsprosedyrer og ved blodprøvetaking. Dyrene vil også følges opp av ambulatorisk klinikks personell samt ved behov for dyrehelsehjelp relatert til vaksinasjonen eller uforutsette hendelser som måtte intreffe under forsøksperioden. Personen som skal utføre vaksinasjon og prøvetaking er meget godt trenet i dette.

Metodebeskrivelse

Forberedelsen av dyrene før inngrep:
For feltforsøk: Beskriv evt. sporing, innfanging, fikseringsmetode, transport osv.
For labforsøk: Beskriv evt. innkjøp, transport, karantene/akklimering, oppstalling, miljøberikelse, fóringsregime, merking, veiing osv.
Storfe: Dyrene er merket og identitetsnummeret vil benyttes for identifikasjon i forsøket. Dyrene fikseres med grime under prosedyren, klippes på injeksjonsstedet og over vena jugularis. Områdene for injeksjon og prøvetaking desinfiseres med 70% enthanol.
Sau: Dyrene er merket og identitetsnummeret vil benyttes for identifikasjon i forsøket. Dyrene fikseres ved at en person holder dyrets hode under prosedyren. Dyrene klippes på injeksjonsstedet og over vena jugularis. Områdene for injeksjon og prøvetaking desinfiseres med 70% enthanol.

Hvilke inngrep (kirurgi, administrasjon av teststoff, merking av viltlevende dyr, fysiske behandlinger m.m.) skal gjøres på dyret under selve forsøket? Legg evt. ved tegninger, protokoller, tidslinjer (aktivitetskart) eller lignende som vedlegg til søknaden.
Det skal injiseres vaksine i m. supraspinatus på høyre side ved de tre nevnte anledninger (dag 0, 7 og 21). Det skal tas blodprøve av den venstre v. jugularis ved tre anledninger (dag 0, 28 og 45).

Hvilke registreringer skal gjøres og hvilke prøver skal tas i løpet av forsøket?
Rektaltemperatur skal måles daglig i løpet av første uke etter første vaksinasjon, deretter annenhver dag i løpet av den resterende forsøksperioden. Dyrene skal observeres for apetitt, hydreringsgrad og vannopptak. Det skal observeres om dyrene har smerte under forsøksperioden og gårdbrukeren skal rapportere dette til forsøksansvarlig. Dersom det oppstår smerte eller ubehag skal forsøksansvarlig kalles ut som besøksveterinær og tilby symptomatisk behandling. Det skal føres journal for disse besøkene. Det skal tas serumprøver på dag 0, 28 og 45.

Angi oppfølging og overvåkning av dyrene under hele forsøket (før, under og etter aktuelle inngrep). Legg gjerne ved relevant scoringsskjema:
Dyrene skal oppstalles hos dyreeier og vil få daglig stell som normalt. Dyrene skal overvåkes for tegn på smerte, stress og urolig atferd. Dyrene skal overvåkes for mat -og vanninntak og det skal registreres rektaltemperatur daglig de første syv dagene av forsøksperioden, deretter annenhver dag. Ved tegn på feber eller andre sykdomstegn skal veterinær (forsøksansvarlig) tilkalles. Den lokale vaksineresponsen måles med skyvelær for å bedømme hevelse på injeksjonsstedet.

Angi avlivingsmetode og hvorfor denne metoden er valgt. Ved bruk av preparater oppgi generisk navn, preparatnavn og dosering:
Ikke relevant

Angi kriterier for humane endepunkter (dvs. kriterier for å avbryte forsøket for det enkelte dyr/grupper av dyr fordi belastningen for dyret er større enn det som er nødvendig for å oppnå formålet med forsøket).
Det antas at dyrene ikke vil vise alvorlig smerte eller ubehag. Dersom dette inntreffer vil forsøket stanses umiddelbart.

Hvilke tiltak vil bli aktuelt å iverksette hvis dyrene når humant endepunkt (f. eks. behandling av symptomer, redusere eksponering, avliving)?
Hvis dette inntreffer vil veterinær (forsøksansvarlig) vurdere hvorvidt det er rom for symptomatisk behandlig for å kontrollere evt. smerte eller ubehag. Alternativt vil dyrene avlives av dyrevernmessige hensyn med boltpistol og avblødning etter standard prosedyre.

Forsøksdyr (art, medikamentbruk og smertevurdering)

Dyreart	Pattedyr - drøvtyggere (Ruminantia) - Sau (Ovis aries)
Linje/Stamme	NKS
Kjønn	Hann
Antall	2
Vekt ved oppstart	65-75
Vekt ved avslutning	65-75
Alder	6-10 mnd
Antall dyr ved gjenbruk (jf. § 15)	2
Erfaring med denne dyreart	Ja
Beskriv fordeling av antall dyr i forhold til kjønn, vekt og alder	Vi må ha en homogen gruppe, dvs to nokså like dyr
Varighet av hele forsøket for det enkelte dyr (d, t, min).	48

Dyr med en avvikende fenotype (se prinsipputtalelse).

Har dyrene arvelig sykdom/lidelse som kan påvirke deres veldferd (eksempler: diabetes, autoimmun sykdom, økt forekomst av tumor, lidelser i bevegelsesapparatet, tanndefekter m.m.)?
- - -

Slike lidelser er ikke kjent/beskrevet i litteraturen ☑

Hvilke tiltak/behandling skal iverksettes for å sikre velferden for dyr med arvelig/medfødt sykdom/lidelse nevnt over, og når regner du med at det blir nødvendig?
- - -

Slike tiltak vil ikke bli nødvendig ☑

Sedasjon, analgesi og anestesi

Periode	Type	Preparat	Induksjonsdose (mg/kg)	Vedlikeholdsdose (mg/kg)	Administrasjonsmåte

Annen medikamentering (alle andre medikamenter/testsubstanser som anvendes)
- - -

Neuromuskulære blokkere vil bli benyttet ☐

Begrunnelse for bruk av neuromuskulær blokker:
- - -

Smerte og ubehag

Forsøket innebærer smerte, men analgesi må utelates ☑

Begrunnelse for at analgesi unnlates
Smerten antas å være raskt forbigående og vil være lokal på injeksjonsstedet (vaksinasjon) og ved blodprøvetaking. Dette er normale prosedyrer som omhandler både sau og storfe i ordinær veterinærpraksis.

Forsøket anses å innebære betydelig/ vedvarende smerte eller ubehag. ☐

Begrunnelse for vurderinger
- - -

Styrke av smerte/ubehag	Lite
Varighet av smerte/ubehag	1 dager

Begrunnelse for valg av dyremodell

Gi en begrunnelse for valg av dyremodell, jf. forskriftens § 8 - dyreart, linje, kjønn, alder, spesielle egenskaper, genmodifikasjoner
Vi har innhentet prøvermateriale for serologisk testing fra sau og storfe og må derfor ha positive kontrolldyr av tilsvarende art.

Forsøksdyr (art, medikamentbruk og smertevurdering)

Dyreart	Pattedyr - drøvtyggere (Ruminantia) - Storfe-kveg (Bos taurus)
Linje/Stamme	Jersey
Kjønn	Hann
Antall	2
Vekt ved oppstart	180
Vekt ved avslutning	180
Alder	6 mnd
Antall dyr ved gjenbruk (jf. § 15)	2
Erfaring med denne dyreart	Ja
Beskriv fordeling av antall dyr i forhold til kjønn, vekt og alder	Det benyttes hanndyr for å unngå videreføring av seropositivitet til neste generasjon via råmelk.
Varighet av hele forsøket for det enkelte dyr (d, t, min).	48 dager

Dyr med en avvikende fenotype (se prinsipputtalelse).

Har dyrene arvelig sykdom/lidelse som kan påvirke deres veldferd (eksempler: diabetes, autoimmun sykdom, økt forekomst av tumor, lidelser i bevegelsesapparatet, tanndefekter m.m.)?
\- - -

Slike lidelser er ikke kjent/beskrevet i litteraturen ☑

Hvilke tiltak/behandling skal iverksettes for å sikre velferden for dyr med arvelig/medfødt sykdom/lidelse nevnt over, og når regner du med at det blir nødvendig?
\- - -

Slike tiltak vil ikke bli nødvendig ☑

Sedasjon, analgesi og anestesi

Periode	Type	Preparat	Induksjonsdose (mg/kg)	Vedlikeholdsdose (mg/kg)	Administrasjonsmåte

Annen medikamentering (alle andre medikamenter/testsubstanser som anvendes)
\- - -

Neuromuskulære blokkere vil bli benyttet ☐

Begrunnelse for bruk av neuromuskulær blokker:
\- - -

Smerte og ubehag

Forsøket innebærer smerte, men analgesi må utelates ☑

Begrunnelse for at analgesi unnlates
Det vil være minimal smerte forbundet med forsøket og det vil derfor ikke være tilstrekkelig indikasjon på bruk av analgesi. Skulle det derimot vise seg at enkeltdyr viser tegn på smerte eller andre kliniske ytringer, vil symptomatisk behandling iverksettes.

Forsøket anses å innebære betydelig/ vedvarende smerte eller ubehag. ☐

Begrunnelse for vurderinger
\- - -

Styrke av smerte/ubehag	Lite
Varighet av smerte/ubehag	1 dager

Begrunnelse for valg av dyremodell

Gi en begrunnelse for valg av dyremodell, jf. forskriftens § 8 - dyreart, linje, kjønn, alder, spesielle egenskaper, genmodifikasjoner
Det er innhentet serologisk prøvemateriale fra storfe of sau. Det er derfor nødvendig med seropositive kontroller for å verifisere den diagnostiske testen.

Vedlegg

Mattilsynet

MATTILSYNET

Deres ref:	Vår ref:	Dato:
TICOVAC 2015		22.09.2015

erikgeorg.granquist@nvh.no

VEDTAK OM BRUK AV FORSØKSDYR - FOTS I D 8135

Behandlet av Mattilsynet, 22.09.2015.
Dokumenter i saken:
1. Søknad fra FOTS, id 8135, fra Erik Georg Granquist, datert 11.09.2015
2. Etterspurt vedlegg, fra Erik Georg Granquist, datert 21.09.2015.

Mattilsynets behandling:
Vedtak:
Mattilsynet, region Sør og Vest, avdeling nasjonale oppgaver, gir tillatelse til Georg Granquist, i perioden 01.10.2015 til 15.11.2015, ved seksjon for eksperimentell biomedisin, NMBU til å gjøre forsøk med dyr, iht søknad, jfr. Forskrift 18.juni 2015 om bruk av dyr i forsøk § 6. Mattilsynet gir med dette også unntak fra påbudet om at forsøk skal finne sted i godkjente lokaler, jf. forskriftens § 12.

Begrunnelse:
Søknaden gjelder 2 voksne sauer og 2 kalver som vaksineres mot skogflått encephalitt for å produsere serum som kan benyttes som positiv kontroll i en oppfølgende studie. Formålet med hele prosjektet er å undersøke om besetninger av storfe og sau er smittet i områder hvor viruset opptrer hos mennesker. Videre om dyr er smittet med levende virus eller om de har antistoffer etter en tidligere gjennomgått infeksjon, og i tillegg så skal melk fra storfe undersøkes for viruspartikler og antistoff. Dersom man finner positive prøver så vil dette bety at man må vurdere risiko for smitte fra dyr til mennesker og fra animalske produkter til menneske i matkjeden. Forsøket skal utføres utenfor godkjente lokaler fordi dyrene skal stå i sitt vante miljø og håndteres av dyreeier under hele forsøket, og belastningsgraden på dyrene blir dermed mindre.

Mattilsynet mener at formålet med forsøket og bruken av dyr er tilfredsstillende beskrevet i søknad, slik at kravene i forsøksdyrforskriften § 10 (formål med forsøket), § 11 (metoder, teststrategier og endepunkter) og § 9 (erstatning, reduksjon og forbedring) er oppfylt. På bakgrunn av disse vurderingene mener Mattilsynet at dyr ikke utsettes for unødige belastninger, jf forsøksdyrforskriften § 1.
Dersom det skal gjøres endringer ift det godkjente forsøket som forventes å svekke dyrevelferden må dette omsøkes som endring av forsøket. For endringer som forventes å ikke svekke dyrevelferden skal det sendes endringsmelding. Alle avvik forventes loggført og håndtert på en dyrevelferdsmessing forsvarlig måte, jf. forsøksdyrforskriften § 28.
Det forutsettes at alle som deltar i forsøket har fått tilstrekkelig utdanning og opplæring, og at deres kompetanse vedlikeholdes, jvf forskriftens § 24.

Mattilsynet klassifiserer forsøket som lett belastende, jf forsøksdyrforskriften, vedlegg B.

Vedtak kan påklages til Mattilsynet, jfr. lov 10 feb 1967 om behandlingsmåten i forvaltningssaker(forvaltningsloven) § 28. Klagefristen er 3 uker fra mottak av dette brev, jfr.forvaltningsloven § 29. Klagen stiles til Mattilsynet, Hovedkontoret, men sendes via avdeling for nasjonale oppgaver.

Med hilsen for Mattilsynet

sign
Marianne Waldum Furnes
for Gunnar M. Gunnarsson

www.mattilsynet.no

Mattilsynet
Avdeling for nasjonale oppgaver

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MATTILSYNET

Deres ref:	Vår ref:	Dato:
TICOVAC 2015		22.09.2015

Kopi: personell med særskilt kontrollansvar
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