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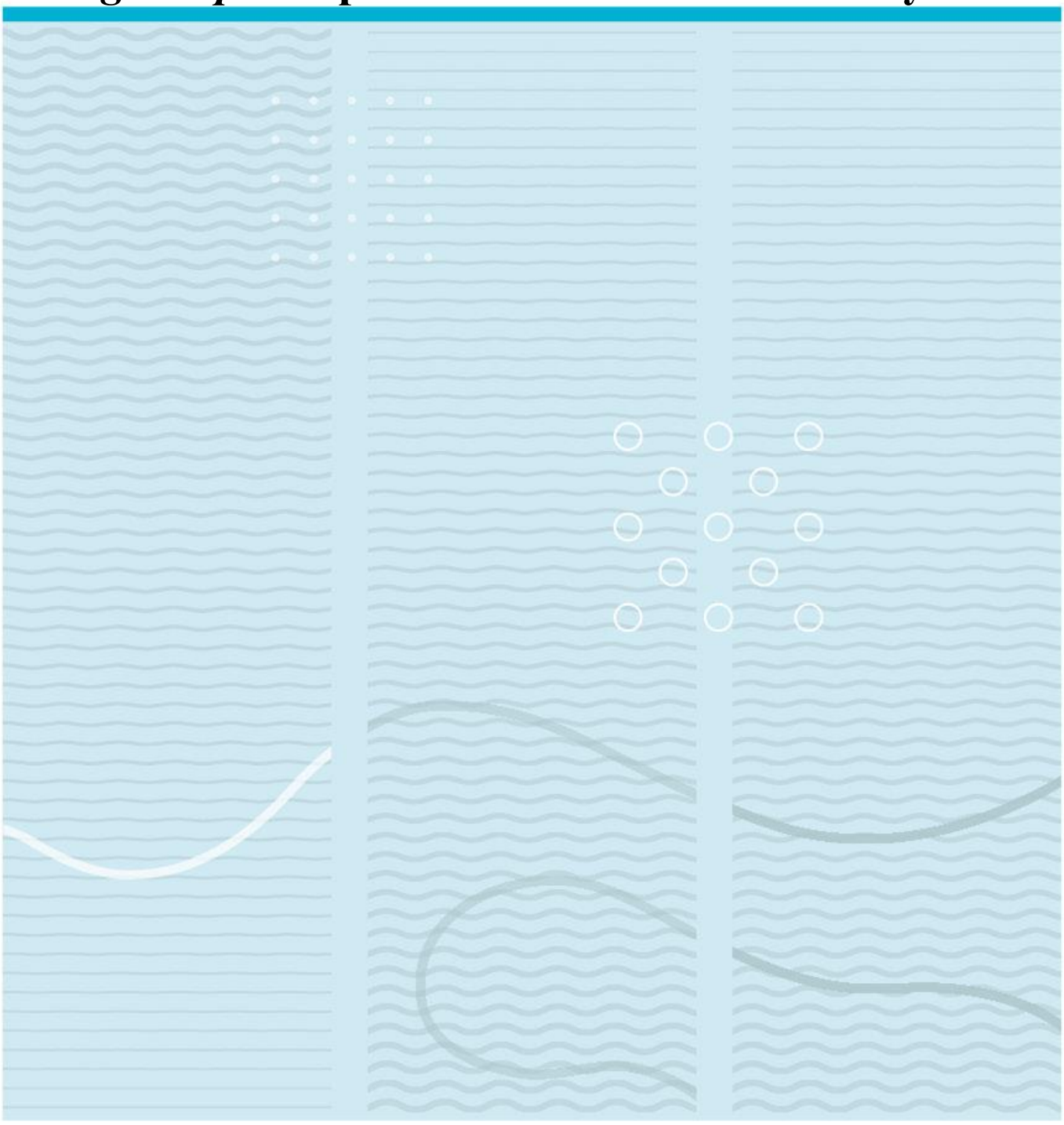
Candidatus Neoehrlichia mikurensis in *Ixodes trianguliceps*. Implications for the infectious cycle.



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This thesis is worth 60 study points

Abstract

Ticks and its pathogen constitute a growing burden for animal and human health in the world. Ticks are blood-feeding parasites, which make them capable of transmitting various pathogens to their hosts that can cause tick-borne infections and tick-borne diseases. These infections and diseases affect wild and domestic animals. The aim of this study was to investigate the presence of the tick-borne pathogen, *Candidatus* Neoehrlichia mikurensis (CNM) in *Ixodes trianguliceps* and its possible role in the infectious cycle. It is an emerging pathogen in Europe. This is the first study of CNM in *Ixodes trianguliceps* in Norway and the first study showing the presence of CNM in *Sorex araneus*. CNM has been described from previous studies in hard tick *Ixodes ricinus* and small mammals as well as in cases of human disease. Ticks were collected from trapped rodents and shrews in 2015 and 2017 along two mountain transects. Spleen samples of 46 small mammals and 116 attached *Ixodes trianguliceps* were investigated by using a real-time PCR to determine the DNA of CNM. Altogether 72% of the spleen samples of the small mammals were positive for the DNA of CNM. Most of the infected small mammals were *Myodes glareolus* (Bank voles) and *Sorex araneus* (common shrew). Altogether 81% of *Ixodes trianguliceps* were positive for DNA of CNM. There was high infection rate of CNM in infected *Ixodes trianguliceps* collected from infected small mammals. However, there were *Ixodes trianguliceps* including larvae, nymphs and adult that were infected although their host animals tested negative for the DNA of *Candidatus* Neoehrlichia mikurensis. Of 15 samples sequenced, 8 samples gave readable sequence, and all of which were confirmed as CNM. There seems to be evidence of transovarial transmission of CNM in *Ixodes trianguliceps* in this study. Although *Ixodes trianguliceps* does not bite humans, it may indirectly be of medical and veterinary importance of its role as a maintenance vector of CNM among small mammals.

Contents

Foreword

This study focused on the presence of *Candidatus* Neoehrlichia mikurensis in *Ixodes trianguliceps* closing the infectious cycle in fulfilment of master's thesis 4317. This was an interesting study knowing that some unexpected discoveries were made during the research like high prevalence of *Candidatus* Neoehrlichia mikurensis in *Ixodes trianguliceps* and *Sorex araneus* (common shrew).

I would like to thank the management of University of South-Eastern Norway and my supervisor, Professor Andrew Jenkins for creating this wonderful opportunity to work on this interesting study. He described and designed the primers that were used, personally trained me on how to use the PCR, made a lot of corrections in the study and made sure I had all necessary supplies for the study. I had a wonderful support and supervision from my supervisor. I would also like to thank my husband, Ambrose Anibueze Ugwu for his love and support. I would like to acknowledge Nicolas De Pelsmaeker who supervised collection and trapping of the small mammals, training me on how to morphologically identify the small mammals and ticks with the help of a rodent specialist, Professor Øyvind Steifetten. I would like to acknowledge Benedikte Nevjen Pedersen, who assisted and trained me on the DNA sequencing protocols and made some corrections in this study. I would like to acknowledge Heidi Storvassli, who worked with me during the morphological identification of the ticks. I would like to acknowledge Jaime Sanchez-Cervera and Laura Mendez that collected the characterized ticks from 2015. And at the same thank the laboratory workers from the department for their assistance.

Bø/ 6th of September 2018

Chinazor Monalisa Ugwu

Nomenclature

List of Abbreviation

Notation	Description
µl	microlitre
Alt	Altitude
CNM	*Candidatus* Neoehrlichia mikurensis
Cq	Quantification cycle
Ct	threshold cycle
dNTP	Deoxyribonucleotide triphosphate
EM	Erythema migrans
F, M, N, L	Female, Male, Nymph and Larvae respectively.
H_0	Null Hypothesis
ID	Identity
Læ	Lærdal
Li	Lifjell
M	Male
ml	millilitre
NA	Not available
Neg	Negative
Neo	*Candidatus* Neoehrlichia mikurensis
Neo 2F	Forward primer for *Candidatus* Neoehrlichia mikurensis
Neo 2R	Reverse primer for *Candidatus* Neoehrlichia mikurensis
nm	Nanometer
No.	Number
PCR	Polymerase chain reaction
Pos	Positive
RNA	Ribonucleic acid
Rpm	revolution per minute
Sp.	Specie
Tm	Melting temperature
UV	Ultraviolet

1 Introduction

Besides mosquitoes, ticks are the most significant disease vectors for human and animal pathogens (de la Fuente, Estrada-Pena, Venzal, Kocan, & Sonenshine, 2008). They may cause direct damage associated with blood feeding and in some cases through the excretion of toxins within their saliva (Cabezas-Cruz & Valdés, 2014). The main importance of ticks is their ability to transmit various pathogens, including viruses, bacteria and protozoa (Jongejan & Uilenberg, 2004). Ticks undergo four stages of metamorphosis (i.e. after being hatched from eggs, they develop from larva to nymph and then become adult) (Bown et al., 2006). Larval ticks hatch with three pairs of legs and after a blood meal from a host, they become nymphs (Pfaff, 2015) and a second blood meal, they become adult (Figure 1-2). While some female ticks feed on large and medium sized mammals to lay eggs. Nymphs and adult have four pair of legs. Ticks are mostly found in warm and humid climates which enhances metamorphosis because low temperatures inhibit the development of larva to adult (Nuttall, 1905). While feeding on a host, each of these stages can transmit and acquire new pathogens (Parola & Raoult, 2001). The transmission of tick-borne pathogens among ticks can be transovarial (which means the pathogen can be transmitted via the eggs from females to their offspring), transstadial (which means the pathogen can be transmitted from larva to nymph and from nymph to adult) and venereal (which means the pathogen can be transmitted during copulation from male to female)(Salman, 2012). Different species of ticks (Figure 1-1) are associated with different pathogens (Dantas-Torres, Chomel, & Otranto, 2012).

1.1 Ixodidae

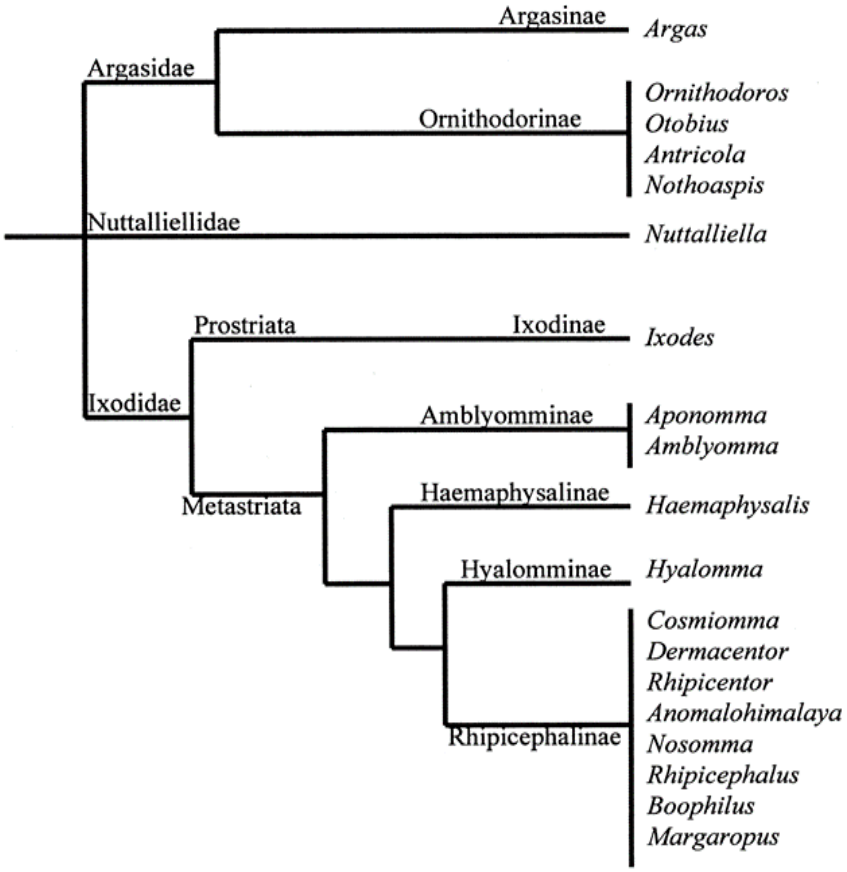


Figure 1-1 Classification of ticks (Parola & Raoult, 2001).

Ixodidae (Figure 1-1) are the family of all hard ticks and their life cycle consist of the egg and three life cycle stages (larva, nymph and adult). Each life cycle stage feed only once but females can detach from their hosts and reattach to the same or a new host and continue feeding (Sonenshine & Roe, 2013). For example, *Ixodes ricinus* females can reattach to a new host and finish their blood meal after 5 days of feeding with a weight of 50mg (Balashov, 1998) while *Hyalomma asiaticum* and *Dermacentor reticulatus* females can reattach to another host and finish their blood meal after 6 or 7 days with a weight of 206mg or 122mg respectively (Sonenshine & Roe, 2013). After a blood meal, detached females can either reattach or lay eggs (Sonenshine & Roe, 2013). Usually, females cannot feed to repletion without mating (Pappas & Oliver Jr, 1971). A few species are parthenogen (which means the females can reproduce without breeding with the males) (Oliver, 1981; Oliver Jr, 1971). For the majority of Ixodid ticks under normal condition, mated females have large blood meals and increase their weight to 100 times

their pre-feeding weight (Sonenshine & Roe, 2013). Fully engorged females drop off their host and lay eggs in suitable microenvironment like leaf litter, crevices or burrows. After depositing the eggs, females live for several days and then die (Sonenshine & Roe, 2013). Depending on the molting of the life cycle stages, Ixodid ticks are classified as three-host life cycle, two-host life cycle or one-host life cycle (Sonenshine & Roe, 2013).

1.1.1 Three-host life cycle

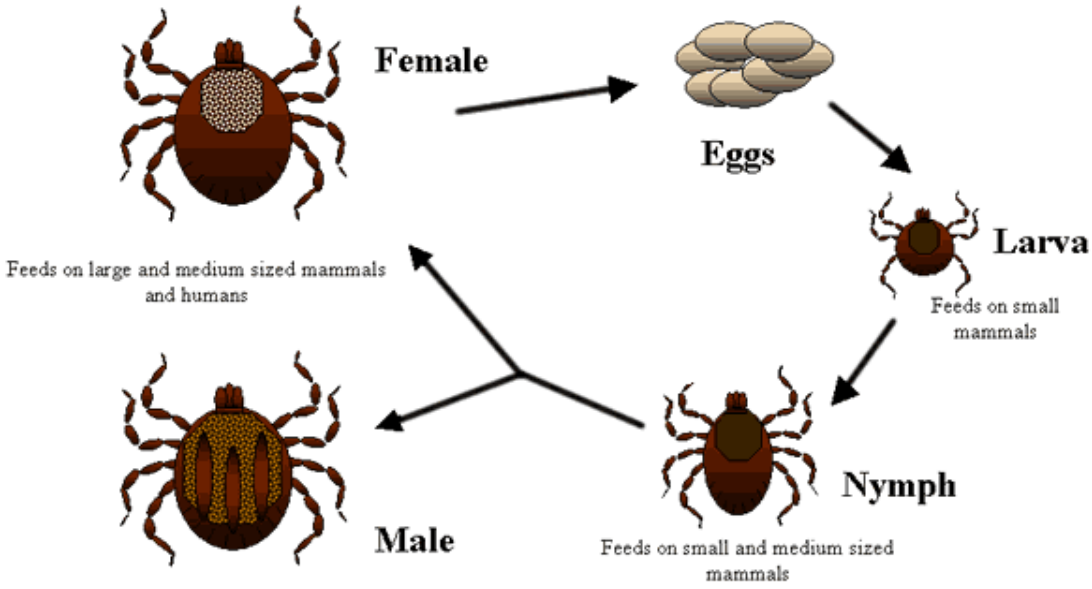


Figure 1-2 Life cycle of Ticks family Ixodidae ((CDC), 2009).

Most of the Ixodid ticks have a three-host life cycle (Sonenshine & Roe, 2013). In three-host cycle (Figure 1-2), the larva drops off from the host after a blood meal and molts to a nymph. The unfed nymph quests for a host and may attach to the same or a new host, drops off after a blood meal and molts to an adult (female or male). The adult finds a host; females then mate, feed, drop off, lay eggs and die (Sonenshine & Roe, 2013). All Ixodid ticks have a single gonotrophic cycle; that is fully engorged mated females lay their eggs continuously for several weeks and then the exhausted females die (Sonenshine & Roe, 2013).

1.1.2 Two- and One-host life cycle.

Some Ixodid ticks from the Metastriata group are known as two or one host life cycle (Sonenshine & Roe, 2013). In two-host life cycle, engorged larva remains on the host and molts to nymph, the nymph feed, engorge, drops off the host and molts to adult in a natural environment (Sonenshine & Roe, 2013). In One-host life cycle, engorged larva and nymph remain on the same host and only engorged female drops off the host (Sonenshine & Roe, 2013).

1.2 Ixodes trianguliceps

Ixodes trianguliceps is also known as vole tick and shrew tick (Figure 1-3). They are nidicolous, of which all their lifecycle stages nest and metamorphosize on a host (Salman, 2012). *Ixodes trianguliceps* belongs to the family of *Ixodidae* (hard) ticks in Great Britain and Atlantic coast countries and they maintain the zoonotic (i.e. diseases that exits normally in animals but can infect humans) of tick-borne diseases (Karbowiak, Biernat, Szewczyk, & Sytykiewicz, 2015). Rodents and shrews have been identified as important host for *Ixodes trianguliceps* (Randolph, 1975). They provide blood meal and maintenance for the different developmental stages of various tick species (Silaghi, Woll, Mahling, Pfister, & Pfeffer, 2012).The shrews host more of the larvae and nymphs of *Ixodes trianguliceps* (Mysterud, Byrkjeland, Qviller, & Viljugrein, 2015). Shrews also play important role as host of *Ixodes ricinus* larvae which varies due to differences in vegetation, humidity and population of the small mammals (Mysterud et al., 2015). Contact with infected ticks and their hosts could be detrimental to human health (Silaghi et al., 2012). Although *Ixodes trianguliceps* does not bite humans (Salman, 2012), it may indirectly be of medical and veterinary importance of its role as a maintenance vector of CNM among small mammals.

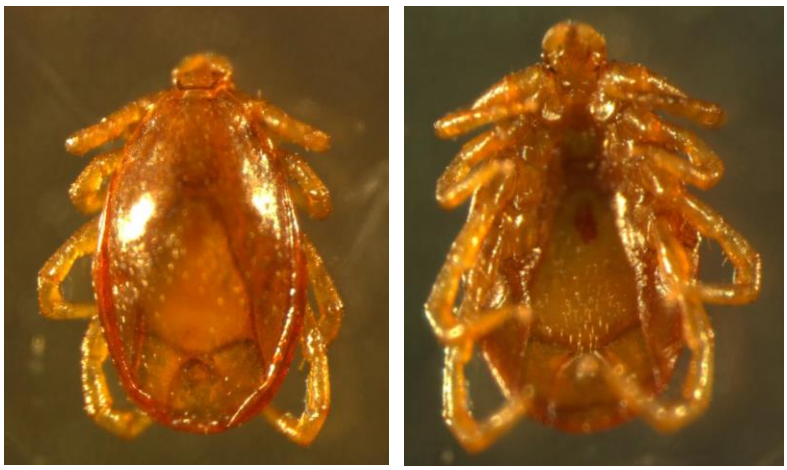


Figure 1-3 Left: Adult male dorsal view of Ixodes trianguliceps, Right: Adult male ventral view of Ixodes trianguliceps (picture by Nicolas De Pelsmaeker)

Ixodes trianguliceps has shown to maintain high level of infections in the reservoirs hosts with regards to *Babesia microti* (Turner et al., 2014) and *Anaplasma phagocytophilum* (Bown et al., 2008; Jahfari et al., 2014). *Ixodes trianguliceps* is known as the specialist tick and play an important role by maintain high infectious levels in the reservoir hosts even if it does not act as vector of disease to human beings and livestock (Mysterud et al., 2015).

1.3 Host-seeking behavior of ticks

Host-seeking behavior of ticks is a system of certain behavioral reactions by ticks that leads to finding a host. Questing behavior of some species ticks can be seasonal and interrupted by unfavorable condition (Sonenshine & Roe, 2013).

1.3.1 Questing Strategies according to their locations

Questing strategies in ticks are divided into two groups according to their locations for questing for their hosts, molting and laying of eggs. They are nidicolous (nest or burrow) and non-nidicolous (pasture type). In most cases, there is no clear border between nidicolous and non- nidicolous.

1.3.1.1 Nidicolous ticks

Nidicolous ticks like *Ixodes trianguliceps* (Salman, 2012), *Ixodes crenulatus* and *Ixodes lividus* meet their hosts in the host's burrow or nest (Glashchinskaya-Babenko, 1956). They rarely feed outside the host's dwelling. This behavior is common among argasid ticks with many species feeding on bats in caves. Nidicolous ticks' day-night drop-off rhythms are fully regulated with the activities of their hosts. All life cycle stages of nidicolous ticks feed on the same of type of host (Sonenshine & Roe, 2013).

1.3.1.2 Non-nidicolous (pasture) ticks

The pasture questing Ixodid ticks seek for their hosts in open places of forest, grassland or brushland habitats. They climb vegetations, spread their first pair of legs which have their sensory organs and the wait for the host (Sonenshine & Roe, 2013). This behavior is common among *Ixodes ricinus*, *Ixodes scapularis* and *Ixodes persulcatus*. Moisture and temperature are major factors that determine these tick species daily rhythm of climbing vegetation and descending back to leaf litter to restore lost water (Sonenshine & Roe, 2013).

1.3.2 Mixed questing strategies

Most species of *Hyalomma* ticks such as *Hyalomma asiaticum* that live in harsh arid environments are distinguished by mixed questing strategies (Balashov, 1998). Their larvae and nymphs are nidicolous ticks by parasitizing on burrowing animals such as gerbils. But the adults are pasture ticks (non-nidicolous ticks) because they feed on large ungulates such as cattle or camels (Sonenshine & Roe, 2013).

1.4 *Candidatus* Neoelchrlichia mikurensis (CNM)

Candidatus Neoelchrlichia mikurensis (CNM*)* is an emerging tick-borne pathogen belonging to the *Rickettsiales* (Kawahara et al., 2004). This pathogen was discovered first as an *Ehrlichia*-like organism, and distinguishable from the analysis of the bacteria from *Ixodes ricinus* ticks in Netherlands, 1999 (Schouls, Van De Pol, Rijpkema, & Schot, 1999) on 16S rRNA sequence, citrate synthase(gltA) and groEL genes (Kawahara et al., 2004). It was discovered in Norway in 2001 (Andrew Jenkins et al., 2001). This organism was discovered later in wild Norwegian rats (Rattus norvegicus)(Szekeres et al., 2015) and

Ixodes ovatus ticks in Japan and described by Kawahara et al in 2004(Kawahara et al., 2004). The description and naming of this *Candidatus* Neolehrlichia mikurensis was an outcome of further findings of the microorganism in rats and *Ixodes ovatus* in Japan and the transmission of the agent in laboratory rats (Kawahara et al., 2004). CNM is a Gram-Negative organism belonging to the family *Anaplasmataceae* (Kawahara et al., 2004) that is being transmitted by the family of ticks called *Ixodidae* (Pfaff, 2015), the hard ticks like *Ixodes ricinus* and *Ixodes trianguliceps*. CNM are mostly found in *Ixodes* in temperate weather and climate (Schouls et al., 1999) of the Northern hemisphere (Blaňarová et al., 2016). However, transovarial transmission in *Ixodes ricinus* has not been reported of this pathogen (Jahfari et al., 2012; A Jenkins & Kristiansen, 2013). Recently, this pathogen was detected in the blood of tick-bitten patients from Agder counties in Southern Norway with *erythema migrans* like skin rash (Quarsten et al., 2017). This pathogen has shown to exist in humans in some countries like Norway, Sweden, China, Switzerland and Czech Republic and as canine pathogen (canine pathogen is a bacteria or a virus that causes high contagious intestinal disease in dogs) in Germany (Grankvist et al., 2014). Majority of the patients were immunocompromised (i.e. weakened immune system by illness or drugs) due to surgical removal of their spleens and symptoms of *neoehrlichiosis* was severe (Szekeres et al., 2015). Symptoms of *neoehrlichiosis* disease in humans are recurring fevers, musculoskeletal pain and deep-vein clotting of the blood (Grankvist et al., 2014). Tetracycline treatment subsides the symptoms (Grankvist et al., 2014; Pfaff, 2015).

Some studies have shown that rodents and small mammals are host reservoirs for *Candidatus* Neoehlrichia mikurensis (Jahfari et al., 2012) and these rodents can transmit this pathogen (Burri, Schumann, Schumann, & Gern, 2014). Wood mouse (*Apodemus sylvaticus*) and Bank voles (*Mayodes glareolus*) proved to play a reservoir role in a Xenodiagnostic study (a diagnostic study whereby the infected tissue is exposed to a clean vector and the examining the vector for the presence of the pathogen) (Burri et al., 2014). This pathogen has not been detected in common shrews (*Sorex araneus)* and yellow necked mouse (*Apodemus flavicollis*) (Jahfari et al., 2012). There is evidence for transplacental transmission of this pathogen in rodents (Obiegala et al., 2014). Detection of a single pathogen relies mostly on PCR (Zweygarth et al., 2014). Previous studies used

quantitative real-time PCR (qPCR) targeting the *groEL* gene for the detection of the pathogen (Andersson, Bartkova, Lindestad, & Råberg, 2013; Jahfari et al., 2012; Vayssier-Taussat et al., 2013). Real-time PCR was chosen for this study because it is rapid, quantitative and operates in a closed system format to prevent contamination (Henningsson et al., 2015). This is first study on *Candidatus* Neoehlrichia mikurensis (CNM) in *Ixodes trianguliceps* and its possible role in the infectious cycle in Norway. The aim of this study was to investigate the presence of the tick-borne pathogen, *Candidatus* Neoehlrichia mikurensis in *Ixodes trianguliceps* and its possible role in the infectious cycle.

1.5 Infectious cycle

Infectious cycle in this study means the tick-host-pathogen cycle. Small mammals are potential reservoir hosts for ticks and their pathogens such as *Candidatus* Neoehrlichia mikurensis (CNM) (Andersson & Råberg, 2011; Andersson, Scherman, & Råberg, 2014; Beninati, Piccolo, Rizzoli, Genchi, & Bandi, 2006; Jahfari et al., 2012; Krücken et al., 2013; Mysterud et al., 2015; Silaghi et al., 2012; Szekeres et al., 2015; Vayssier-Taussat et al., 2012; Víchová et al., 2014). Salivary delivery during tick bite is an important route of pathogen transmission in ticks (Burgdofer, 1992; Burgdorfer & Brinton, 1975; Spielman, Ribeiro, Mather, & Piesman, 1987), although regurgitation may occur during blood meal in some ticks (Connat, 1991). Previous studies in *Borrelia burgdorferi* indicates that infected ticks must feed for certain period before spirochetes would appear in the saliva and become transmittable (Piesman, 1989; Piesman, Maupin, Campos, & Happ, 1991; Spielman et al., 1987). The time delay needed to transmit a pathogen suggests that an infective form can either develop or move to the salivary gland in response to the blood meal (Munderloh & Kurtti, 1995). The transmission of tick-borne pathogens among ticks can be transovarial (which means the pathogen can be transmitted via the eggs from females to their offspring), transstadial (which means the pathogen can be transmitted from larva to nymph and from nymph to adult) and venereal (which means the pathogen can be transmitted during copulation from male to female) (Salman, 2012).

1.6 Polymerase Chain Reaction

Polymerase Chain Reaction is an in vitro significant molecular biology scientific technique used to amplify a single or few copies of DNA template to generate thousands or millions of copies of a DNA sequence (Joshi & Deshpande, 2010). The American biochemist, Kary Mullis developed Polymerase Chain Reaction in 1984 (Joshi & Deshpande, 2010). Kary Mullis received the Japan Prize and Nobel Prize on PCR development in 1993 (Bartlett & Stirling, 2003). This technique has become the most important and significant technique in molecular biology due to its quick, inexpensive and simple process (Joshi & Deshpande, 2010). Even with low DNA concentration, this technique still amplifies the specific DNA fragments (Erlich, 1989). This technique uses specific proteins known as polymerases, which are enzymes that bind individual DNA building blocks to form elongated molecular strands (Joshi & Deshpande, 2010). Polymerases requires a supply of DNA building blocks which are nucleotides consisting of adenine (A), guanine (G), thymine (T) and cytosine (C) (Joshi & Deshpande, 2010).

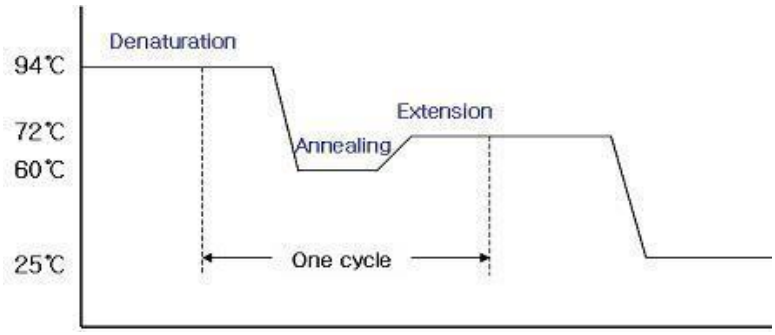


Figure 1-4 Polymerase Chain Reaction(Joshi & Deshpande, 2010)

Short DNA fragment known as the primer is also required to attach to the building blocks and a DNA template for creating a new strand (Joshi & Deshpande, 2010). There are three important steps in PCR technique known as: denaturation, annealing and extension (Joshi & Deshpande, 2010). The first step is denaturation which involves the separation of the double stranded DNA sample into single stranded DNA molecules at high temperatures from 90-97 degree Celsius (Joshi & Deshpande, 2010). The second step involves the annealing of the primers to their complementary DNA template strands using an enzyme that is known as Taq polymerase at lower temperatures from 50-60 degree Celsius to enable duplication of the original DNA (Joshi & Deshpande, 2010). The

third step involves the extension of the annealed primers at their ends by Taq polymerase at temperature approximately 72 degree Celsius for around 2-5 minutes (Joshi & Deshpande, 2010). Each of the new molecules containing one old and one new strand of DNA. Each of these new strands are used to construct two new copies or more (Ochman, Gerber, & Hartl, 1988).

1.6.1 Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction is PCR based molecular biology technique (Joshi & Deshpande, 2010). It is also known as quantitative real time polymerase chain reaction (Joshi & Deshpande, 2010). Real-time PCR allows the analysis of the reaction in progress by using various fluorescent dyes which react with the amplified product and also measured by the instrument in real time (Joshi & Deshpande, 2010). Real-time PCR is known to be highly efficient, rapid, specific and sensitive (Wang et al., 2014). This technique is used in determining the presence of a DNA sequence in a sample and the quantity of its copies available in that sample (VanGuilder, Vrana, & Freeman, 2008). This technique is used in the detection of pathogens and its quantity during analysis (Wang et al., 2014).

1.7 Hypothesis and predictions of the study

The hypothesis and predictions designed for this study were based on known premises and previous studies. These hypotheses were tested through experiments and analysed statistically.

1.7.1 Prediction and hypothesis for *Ixodes trianguliceps*

From previous studies, there is little or no CNM in *Ixodes trianguliceps* (Blaňarová et al., 2016; Obiegala et al., 2014). I therefore predict little or no CNM in *Ixodes trianguliceps.*

Null hypothesis (H_0): There is no CNM in *Ixodes trianguliceps* ……. (H1a)

Null hypothesis (H_0): There is low CNM in *Ixodes trianguliceps* ……(H1b)

Alternative hypothesis (H_1): There is presence of CNM in *Ixodes trianguliceps*……(H1c)

1.7.2 Prediction and hypothesis for the lifecycle stages of *Ixodes trianguliceps.*

Assuming there is presence of CNM in *Ixodes trianguliceps*, I predict no CNM in larvae (not engorged) of *Ixodes trianguliceps* since there no CNM in unfed larvae (not engorged) of *Ixodes ricinus* from previous studies (Blaňarová et al., 2016; Jahfari et al., 2012).

Null hypothesis (H_0): There is no CNM in unfed larvae (not engorged) of *Ixodes trianguliceps*…….. (H2a)

Alternative hypothesis (H_1): There is CNM in unfed larvae (not engorged) of *Ixodes trianguliceps*……..(H2b)

Assuming there is presence of CNM in larvae of *Ixodes trianguliceps*, I predict it would be low since they can only be infected by feeding on infected hosts and no transovarial transmission in larvae of *Ixodes ricinus* from previous studies (Burri et al., 2014; Jahfari et al., 2012; Obiegala et al., 2014; Silaghi et al., 2012). This might make a difference when compared with infection rate of CNM in nymphs of *Ixodes trianguliceps*.

Null hypothesis (H_0)*:* There is no difference between the infection rate of CNM in larvae and nymphs of *Ixodes trianguliceps*. …… (H2c)

Alternative hypothesis (H_1): There is difference between the infection rate of CNM in larvae and nymphs of *Ixodes trianguliceps*…….. (H2d)

I predict no difference in the infection rate of CNM among nymphs and adults of *Ixodes trianguliceps* since they are nidicolous, they must have fed on infected hosts.

Null hypothesis (H_0)*:* There is no difference in the infection rate of CNM between nymphs and adults of *Ixodes trianguliceps* ……..(H2e)

Alternative hypothesis (H_1): There is difference in the infection rate of CNM between nymphs and adults of *Ixodes trianguliceps*……..(H2f)

1.7.3 Prediction and hypothesis for small mammals

Assuming there is presence of CNM in *Ixodes trianguliceps*, I predict there would be no presence of CNM in the host species called *Sorex araneus* due no presence of CNM in *Sorex araneus* from previous studies (Andersson & Råberg, 2011; Jahfari et al., 2012; Kawahara et al., 2004; Li et al., 2013; Silaghi et al., 2012).

Null hypothesis (H_0): There is no prevalence of CNM in *Sorex araneus* …… (H3a)

Assuming there is presence of CNM in *Sorex araneus*, I predict the infection rate would be low which would make a difference when compared with the infection rate of CNM in *Myodes glareolus*.

Null hypothesis (H_0): There is no difference in the infection rate of CNM between *Sorex araneus* or *Myodes glareolus*…… (H3b)

Alternative hypothesis (H_1): There is difference in the infection rate of CNM between *Sorex araneus* or *Myodes glareolus*…… (H3c)

1.7.4 Prediction and hypothesis for location and year (Sites).

Assuming there is presence of CNM in the ticks collected from Lifjell in 2015 and 2017, I predict difference in the infection rate of CNM in ticks collected from Lifjell between 2015 and 2017 since ticks collected in 2015 are more of larvae of *Ixodes ricinus* due no prevalence of CNM in larvae (not engorged) of *Ixodes ricinus* from previous studies (Blaňarová et al., 2016; Jahfari et al., 2012).

Null hypothesis (H_0): There is no difference in the infection rate of CNM in ticks collected from Lifjell between 2015 and 2017 …… (H4a)

Alternative hypothesis (H_1): There is difference in the infection rate of CNM in ticks collected from Lifjell between 2015 and 2017…… (H4b)

Assuming there is presence of CNM in the ticks collected in 2017 from Lærdal and Lifjell, I predict difference in the infection rate of CNM in ticks in 2017 from Lærdal and Lifjell since Lærdal seems to be warmer in summer than Lifjell.

Null hypothesis (H_0): There is no difference in the infection rate of CNM in ticks in 2017 from Lærdal and Lifjell …… (H4c)

Alternative hypothesis (H_1): There is difference in the infection rate of CNM in ticks in 2017 from Lærdal and Lifjell …… (H4d)

1.7.5 Infectious cycle

Assuming *Ixodes trianguliceps* were infected, considering lack of transovarial transmission of CNM in *Ixodes ricinus* from previous studies (Burri et al., 2014; Jahfari et al., 2012; Obiegala et al., 2014; Silaghi et al., 2012), I predict that *Ixodes trianguliceps* were infected through blood meal from infected hosts and possibly linked knowing that they were collected from their hosts.

Null hypothesis (H_0): *Ixodes trianguliceps* were not infected with CNM due to blood meal from infected hosts…… (H5a)

Alternative hypothesis (H_1): *Ixodes trianguliceps* were infected with CNM due to blood meal from infected hosts……(H5b)

Null hypothesis (H_0): There is no difference in the infection rate of CNM between small mammals and *Ixodes trianguliceps* …….. (H5c)

Alternative hypothesis (H_1): There is difference in the infection rate of CNM between small mammals and *Ixodes trianguliceps* …… (H5d)

2 Summary

Various lifecycle stages of ticks were collected from various species of small mammals. DNA extracted from ticks and spleen samples were screened with Real-time PCR. Positive DNA templates were amplified and sequenced and compared to sequences from *Candidatus* Neoehrlichia mikurensis (CNM). Prevalence of *Candidatus* Neoehrlichia mikurensis were calculated using Excel. Confidence Intervals and differences were computed with Pearson's Chi-squared test and Fisher's Exact Test for count data ($n < 30$) using RGui (64-bit) as described in the departmental course, Natural science methods 4301 by Professor Jan Heggenes

2.1 Study area and collection of small mammals (rodents and shrews).

The study areas were, Lifjell in Bø, Telemark county and Lærdal in Sogn og Fjordane county. Small mammals were trapped in both areas along different transects and at 100m, 200m, 300m, 400m, 500m, 600m, 700m, 800m, 900m and 1000m altitudes. Live and lethal traps were used for the capturing of these small mammals (Jones, McShea, Conroy, & Kunz, 1996). All traps were baited with apple slices to allow the live trapped small mammals survive for at least a day (Steen, Mysterud, & Austrheim, 2005). Controlling of the traps was done twice daily during the fieldwork. Live small mammals captured were euthanized by cervical dislocation and transferred to well labelled plastic bag and stored at -20^{o}C and thawed later prior to collection of ticks from them. Each plastic bag had information about the capturing on altitude, trap type, date, session, time and trap number.

2.2 Collection of ticks from small mammals

Each thawed small mammal was placed on a tray and dried with hair dryer. Data from each thawed small mammal plastic bag was transferred to excel sheet. The excel sheet was to be filled with the following data: host identification number, location, altitude, trap type, date captured, time captured, processed date, processed time, host specie, sex of the host, burden, ticks found in the plastic bag of the host, total ticks per host, tick identification number, life stage of the tick, tick specie, other parasites and remarks. All

small mammals were morphologically identified to species level (Mysterud et al., 2015) and confirmation of these species were done by morphological examination of the teeth under microscope. Ticks were picked with tweezer from each small mammal under microscope, preserved in 70% ethanol and stored at -20^{o}C prior to use. Ticks were morphologically identified to species level and characterized by their lifecycle stages; larva, nymph, adult male and adult female (Hillyard, 1996).

The database sheet of ticks collected from small mammals in 2015 was sent by Professor Andrew Jenkins. They were preserved in alcohol, well labelled and stored in the university cold room. Afterwards, they were sorted for DNA extraction.

2.3 Collection of spleen samples from small mammals

The small mammals were thawed overnight prior to use. Each small mammal was placed in dorsal view on a clean filled paper tissue tray and dissected. The spleen was morphologically identified and collected from the right side of the small mammal. All the organs collected were placed separately in different tubes, frozen and thawed prior to DNA extraction. The spleen samples of Small mammals collected in 2015 were not collected and analyzed due to incomplete data set. The available data set had no information on identity. It only had the transect number and altitude which was not enough to determine the tick burden (the total number of ticks per animals). The information was necessary to determine the possible infectious cycle for a tick or certain number of ticks collected from an animal and the prevalence of a certain infectious cycle.

2.4 DNA extraction

DNeasy blood and tissue extraction kit was used for all DNA extractions (QIAGEN, 2013). Ticks and spleen samples of the small mammals were thawed prior to use.

2.4.1 DNA extraction from ticks *(Ixodes ricinus* and *Ixodes trianguliceps*) collected in 2015

Ticks collected in 2015 were extracted using DNeasy Blood & Tissue kit for detection of DNA of Borrelia according to manufacturer's protocol (QIAGEN, 2013) and modified in the following ways. Each tick was removed from excess ethanol, placed in 2ml beadbug tube containing three small alcohol washed steal beads, 180µl PBS lysis buffer was added

and the mixture was disrupted for 3 minutes using BeadBug microtube Homogenizer (Benchmark_Scientific, 2018). The solution was transferred to a 2ml microcentrifuge tube, steel beads were discarded and 180µl Buffer ATL was added to the solution. 40µl of proteinase K was added and the mixture was vortexed and incubated at 56°C for 1 hour using thermomixer (Eppendorf, 2016) at 500rpm to ensure it was completely lysed. After incubation, 400µl of Buffer AL was added, the solution was mixed by pipetting up and down to prevent precipitate from forming and incubated at 70°C for 10 minutes. 460µl of 100% ethanol was added and the solution was mixed thoroughly by pipetting up and down to prevent precipitate from forming. The solution was transferred to DNeasy Mini spin column placed in a 2ml collection tube, centrifuged at 8000rpm for 1 minute and the flow-through and collection tube was discarded (this step was repeated three times because the total volume of the mixture was bigger than the size of the spin-column tube). The DNeasy Mini spin column was placed in a new 2ml collection tube provided, 500µl Buffer AW1 was added, and the mixture was centrifuged at 8000rpm for 1 minute, and the flow-through and collection tube was discarded. The DNeasy Mini spin column was placed in a new 2ml collection tube provided, 500µl Buffer AW2 was added, and the mixture was centrifuged at 13,000rpm for 3 minutes and the flow-through and collection tube was discarded. The DNeasy Mini spin column was placed in a new 2ml collection tube provided, centrifuged at 14,000rpm for 1 minute and the flow-through and collection tube was discarded (This step was carried out to dry the membrane of the DNeasy Mini spin column to prevent ethanol carry over during elution). The DNeasy Mini spin column was placed in a clean 1.5ml microcentrifuge tube, 35µl of Buffer AE was added directly onto the DNeasy membrane, incubated at room temperature for 1 minute and was then centrifuged at 8000rpm for 1 minute for elution. Another 30µl of Buffer AE was added and centrifuged at 8000rpm for 1 minute for elution. This last step was to increase the overall DNA yield. The eluted DNA sample was stored at -20°C prior to use.

2.4.2 DNA extraction from ticks (*Ixodes trianguliceps*) collected in 2017

DNA extraction was done using DNeasy Blood & Tissue kit for detection of DNA of Borrelia according to manufacturer's protocol (QIAGEN, 2013) and modified in the following ways. Tissue lysis was carried out overnight at 56°C using thermomixer (Eppendorf, Hamburg, Germany) at 500rpm to ensure it was completely lysed. Pipetting up and down was

carried out to prevent precipitation from forming. The eluted DNA sample was stored at -20^{o}C prior to use.

2.4.3 DNA extraction from the spleen samples

DNA extraction was carried out using Qiagen spin-column protocol for purification of total DNA from Animal tissue with DNeasy Blood & Tissue kit according to manufacturer's protocol (QIAGEN, 2013). Spleen samples weighed between 0.01 and 0.03g. Tissue lysis was carried out overnight at 56^{o}C using thermomixer (Eppendorf, Hamburg, Germany) at 500rpm to ensure it was completely lysed. Pipetting up and down was carried out to prevent precipitation from forming. The eluted DNA sample was stored at -20^{o}C prior to use.

2.5 Quantity and purity of extracted DNA samples

Concentration and purity of the DNA samples extracted were calculated automatically using a Picodrop and Nanodrop spectrophotometer ng/µl unit (Nanodrop, 2009; Picodrop, 2013). Not all the concentrations and purities of the DNA samples were measured prior to PCR due to a technical fault that developed in the Picodrop. Some of the DNA samples concentrations from *Ixodes trianguliceps* measured between 0.5ng/µl and 50ng/µl while DNA concentrations of the spleen samples measured between 50ng/µl and 100ng/µl.

2.6 Real-time Polymerized Chain Reaction (PCR) and Primer Design

Candidatus Neoehrlichia mikurensis (CNM) was detected using real-time PCR. PCR primers targeting the groEL gene of CNM are described in (Kjelland et al., 2018) and Jenkins et al (unpublished). PrimerExpress v.2.0(Biosystems, 2015) was carried out to select efficient primers, using *Candidatus* Neoehrlichia mikurensis *GroEL* sequence AB084583 as the input sequence and program settings for the design. Primers were selected targeting the region 560-688 in AB084583. The primer sequences were:

Table 2-1 Primers

Primer	Sequence
Forward Primer, Neo2F	GCAAATGGAGATAAAAACATAGGTAGTAAA
Reverse Primer, Neo2R	CATACCGTCAGTTTTTTCAACTTCTAA

Confirmed *Candidatus* Neoehrlichia mikurensis positive tick's DNA templates were used as positive controls and double distilled water without DNA was used as negative control. Real-time PCR was carried out on the Applied Biosystem StepOne(Biosystems, 2015) using Applied Biosystems SYBR-green mastermix. Spleen DNA samples were diluted in 1: 10 ratios to prevent excess DNA that could lead to inhibition of the PCR. Each reaction volume was 25µl containing 12.5µl (2x Applied Biosystems) SYBR Green mastermix, 2µl of 10 µM CNM reverse primer, 2µl of 10 µM CNM forward primer, 3.5µl of double distilled water and 5µl of DNA template. Two positive controls and two negative controls were included in each run of 48 samples. Each DNA sample was duplicated for every PCR run. The PCR program using SYBR-green was 95^{o}C, 10 minutes for complete denaturation of genomic DNA in the sample (95^{o}C, 15seconds; 60^{o}C, 60seconds) x 45 cycles (two-step PCR cycle; denaturation at 95^{o}C, annealing and extension at 60^{o}C). It warmed from 60^{o}C to 70^{o}C, the denaturation analysis was from 70^{o}C to 85^{o}C with 0.1^{o}C increment. Background subtraction, threshold cycle(C_t) setting, melting temperature(Tm), the determination of the quantification cycle (C_q) and PCR efficiency were automatically carried out by the instrument software(StepOne, 2012). Necessary corrections were made on the instrument data. A dissociation or melting temperature (Tm) between 71^{o}C and 76^{o}C and amplification curve that exceeded the background fluorescence were considered positive samples. PCR products were stored at -20^{o}C prior to DNA sequencing.

2.7 DNA sequencing and genetic analysis

DNA sequencing of the PCR amplicons were performed using the following protocols.

2.7.1 Step 1

The preparation of the templates was done by rinsing the PCR products with the help of the enzyme EXO STAR(GE Healthcare Illustra ExoProStarTM 1-step, 2013) to remove unincorporated dNTPs and primers efficiently. Each PCR tube had a mixture of 2µl of EXO

STAR and 5µl of PCR product. All were stored cold by using ice cubes below tube stands. The tubes were placed in a PCR machine for the rinsing procedure at 37^{o}C in 15 minutes, 80^{o}C in 15 minutes, 4^{o}C. And the templates were ready for cycle sequencing.

2.7.2 Step 2

Cycle sequencing was performed using BigDye Terminator v1.1 cycle sequencing Kit (Applied Biosystems), DNase free water, previously described and designed primers in this thesis (3.2µM primer: 16µl of 10µM primer + 34µl of DNase free water), sequencing plate, film and rubber. Each reaction volume for each strand was 10µl containing 4.5 µl of DNase free water, 1 µl of BigDye mix(2.5x), 1.5 µl of sequencing buffer(5x), 1 µl of 3.2 µM primer (forward or reverse primer) and 2 µl of the prepared template. Sequencing control was prepared for each strand. Each sequencing control reaction volume for each strand was 10µl containing 1 µl of BigDye, 1.5 of 5xBDbfr, 2 µl of primer M13, 0.75 µl of pGEM-template and 4.75 µl of distilled water. The sequencing plate was covered with plate tape, vortexed for 3 seconds and centrifuged for 10 seconds at 2000rpm. And the sequencing plate was then placed in a PCR machine for 25 cycles at 96^{o}C for 1 minute, (96^{o}C for 10 seconds, 50^{o}C for 5 seconds, 60^{o}C for 4 minutes) and 4^{o}C ∞. After this procedure, the template was ready for step 3 (2.7.3)

2.7.3 Step 3

In this procedure, BigDye XTerminator Purification Kit was used. After cycle sequencing, the plate was centrifuged for 1 minute. The cycle sequencing product was on ice cubes during this process. Each reaction well was added 55 µl of a homogenous solution containing 10 µl of XTerminator solution which captures unincorporated dye terminators and free salts from post cycle sequencing and 45 µl of SAM solution which improves BigDye XTerminator reagent performance and stabilizes the sample after purification. The mixture was vortexed at maximum speed for 10 seconds and was not left on stand for more than 2 minutes, otherwise vortexing would have been repeated to yield a homogenous solution. The mixture was transferred to a new plate. During the transfer, the mixture was kept in motion by pipetting up and down. The plate was covered with plastic film. The mixture was vortexed for 30 minutes at 2000rpm. The vortexing step of the BigDye XTerminator Purification Kit protocol was critical to achieving optimum

performance especially at a minimum of 2000rpm and a maximum orbital diameter of 4mm. Afterwards, the plate was placed in a 3130xl Genetic Analyzer machine and run module and run plate was selected. The generated Sequences were compared with the DNA sequences of *Candidatus* Neoehrlichia mikurensis in Genbank using BLAST after subtraction of the sequencing control (NCBI_Resources, 2016). Sequences were assembled using chromas and identified by BLAST search.

2.8 Statistical Analysis

Confidence Intervals (95%) and differences in the infection rate of *Candidatus* Neoehrlichia mikurensis was computed with Pearson's Chi-squared test and Fisher's Exact Test for count data ($n < 30$) using RGui(64-bit) as described in the departmental course, Natural science methods 4301. P-values < 0.05 were regarded statistically significant. Graphical representations were made using Excel.

3 Results

3.1 Collection of samples.

Detailed results are shown in the annex. A total of 261 ticks were morphologically identified and selected for further analysis. Species of 2 out of 261 ticks could not be determined as they had no heads and they were not used for further analysis. In 2015, a total of 162 ticks were picked from 161 small mammals that were not morphologically identified. The small mammals were collected from Lifjell at altitudes of 100m, 200m, 300m, and 400m. A total of 143 *Ixodes ricinus* were selected which includes 141 larvae, 1 nymph and 1 adult. *Ixodes ricinus* was included to ensure that the procedures were correct and efficient not because of a specified species. A total of *17 Ixodes trianguliceps* from 2015 collection were also selected which includes 14 larvae and 3 nymphs with details shown in Table 2-1.

Table 3-1:Ticks collected in 2015 and 2017

Tick Species	*Year*	*2015*		*2017*		*TOTAL*
	Source	Jaime	Laura	Nicolas	Nicolas	
	Location	Lifjell	Lifjell	Lærdal	Lifjell	
Ixodes ricinus	Larva	123	18	-	-	141
	Nymph	1	0	-	-	1
	Adult	0	1	-	-	1
	unknown	1	0	-	-	1
Ixodes trianguliceps	Larva	7	7	12	27	53
	Nymph	1	2	35	19	57
	Adult	0	0	0	6	6
	unknown	1	0	0	0	1
	Total	**134**	**28**	**47**	**52**	**261**

In 2017, a total of 99 *Ixodes trianguliceps* were picked from 99 small mammals which includes 1 *Apodemus sylvaticus*, 25 *Myodes glareolus* and 20 *Sorex araneus* as shown in Table 3-2. A total of 27 small mammals were collected from Lærdal and 19 small mammals were collected from Lifjell at 100m, 200m, 300m, 400m, 500 and 600m

altitudes. A total of 47 *Ixodes trianguliceps* that includes 12 larvae and 35 nymphs from Lærdal. As shown in Table 3-1, 52 *Ixodes trianguliceps* that includes 27 larvae, 19 nymphs and 6 adults from Lifjell. One small mammal was not available for further analysis due to difficulty in the morphological identification of the specie. It was taken by the rodent specialist for further identification and was not included in this study.

Table 3-2:Small mammals collected in 2017

Location	Altitude	Small mammals			Total
		Apodemus sylvaticus	*Myodes glareolus*	*Sorex araneus*	
Lærdal	100m	-	6	2	8
Lærdal	200m	-	-	4	4
Lærdal	300m	-	4	9	13
Lærdal	500m	-	1	1	2
Lifjell	100m	1	8	2	11
Lifjell	200m	-	2	-	2
Lifjell	300m	-	2	1	3
Lifjell	500m	-	1	-	1
Lifjell	600m	-	1	1	2
Total		1	25	20	46

3.2 PCR result for detection of DNA of *Candidatus* Neoehrlichia mikurensis

As shown in Figure 3-1 amplification curves that exceeded the background fluorescence and with melting temperature between 71°C and 76°C were considered positive samples (Figure 3-2). Positive samples showed the presence of DNA of *Candidatus* Neoehrlichia

mikurensis (CNM) in the DNA of the ticks and spleen samples of the small mammals. Figure 3-3 and Figure 3-4 are amplification plot and melt curve of individual sample.

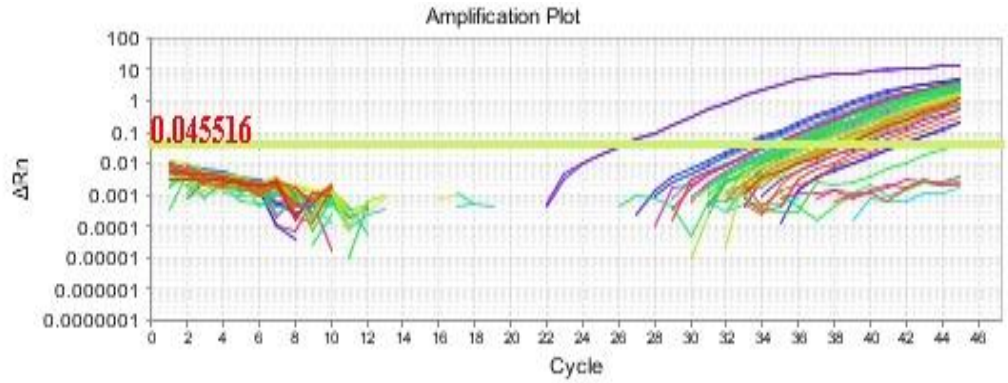


Figure 3-1:Amplification plot of the DNA of CNM in real-time PCR.

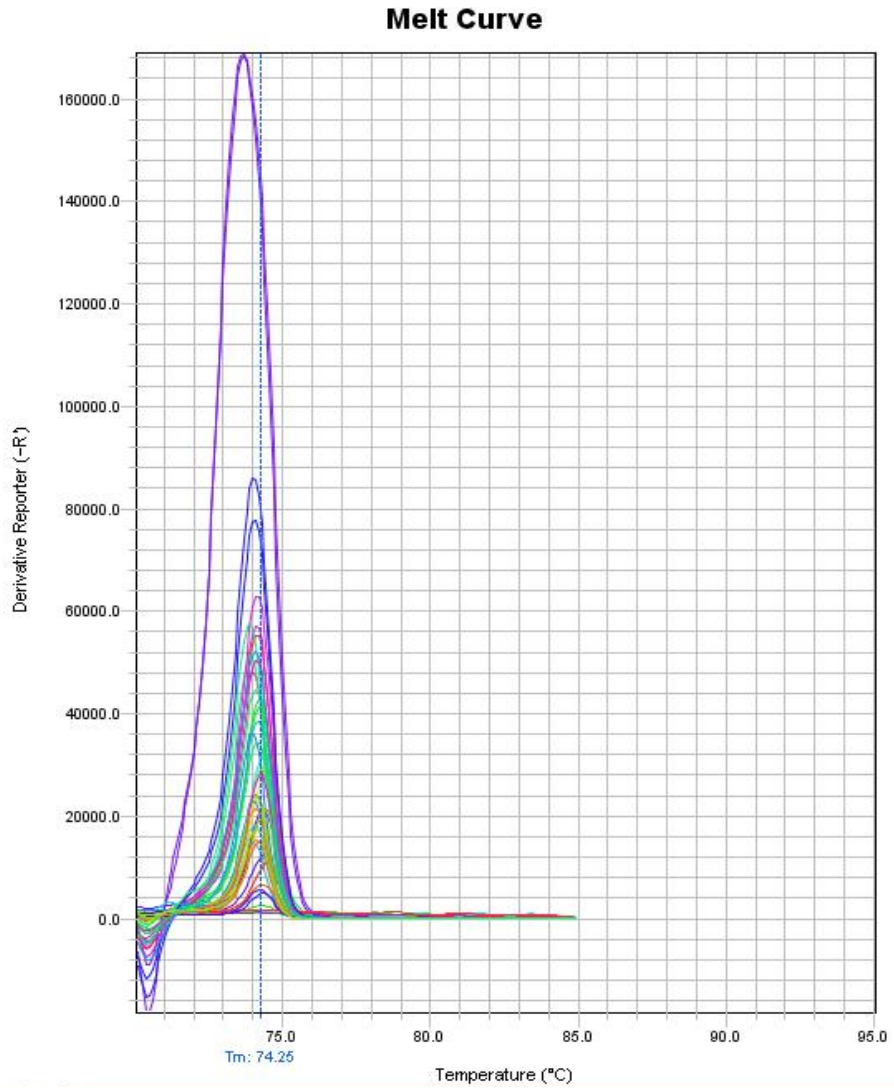


Figure 3-2:Melt curve of the DNA of CNM in real-time PCR

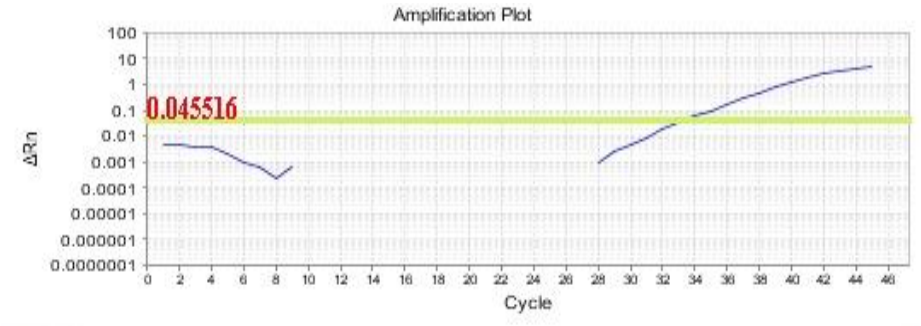


Figure 3-3:Amplification plot of the DNA of CNM in real-time PCR of a sample

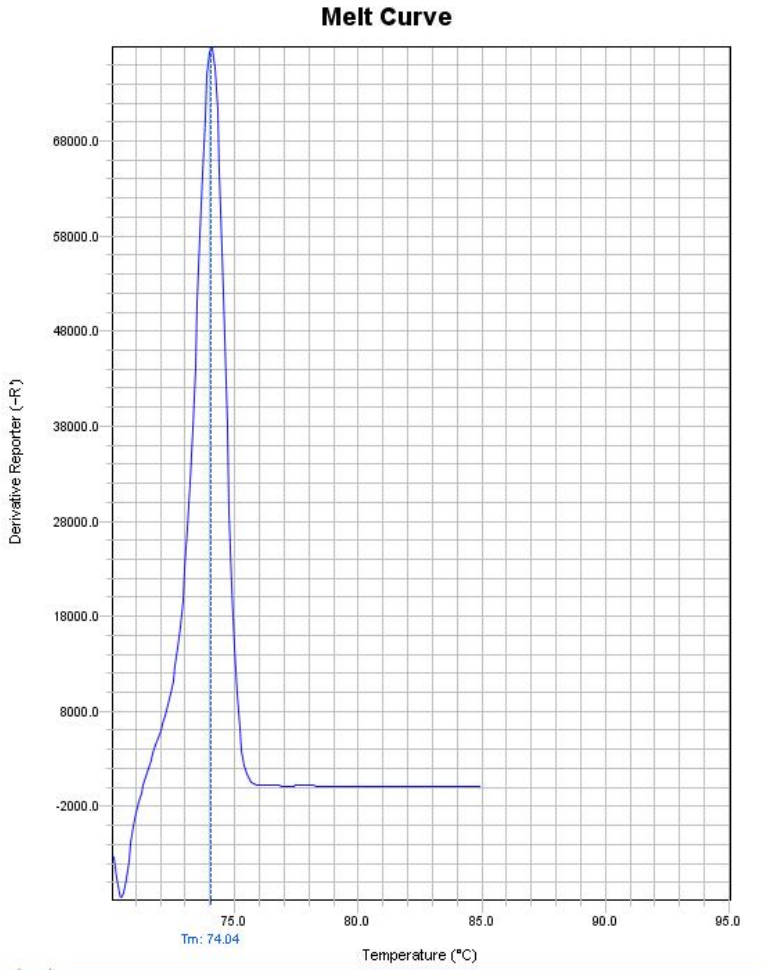


Figure 3-4:Melt curve of the DNA of CNM in real-time PCR of a sample

3.3 Detection of *Candidatus* Neoehlrlichia mikurensis in ticks collected in 2015 and 2017

As shown Table 3-3, all the 160 ticks collected in 2015 were negative which includes 141 larvae, 1 nymph and 1 adult of *Ixodes ricinus* and 14 larvae and 2 nymphs of *Ixodes trianguliceps*.

All ticks analyzed from 2017 were *Ixodes trianguliceps*. Of the *Ixodes trianguliceps* from Lærdal, 12/12 larvae and 32/35 nymphs were positive. Of the *Ixodes trianguliceps* from Lifjell, 26/27 larvae, 18/19 nymphs and 6/6 adults were positive. In total, 94/99 *Ixodes trianguliceps* that were collected from small mammals were positive. Difference in the infection rate of CNM in ticks collected from Lifjell between 2015 and 2017 was statistically significant for Pearson's Chi-squared test at p-value = 0 and Fisher's Exact Test for Count Data at p-value < 2.2e-16 (almost 0) with 95 percent confidence interval: 0.000000000 - 0.002078563. Difference in the infection rate of CNM in ticks collected in 2017 from Lærdal and Lifjell was not statistically significant for Pearson's Chi-squared test at p-value = 0.56490 and Fisher's Exact Test for Count Data at p-value = 0.666 with 95 percent confidence interval: 0.04724933 - 5.39578231. There was 73% prevalence of CNM in *Ixodes trianguliceps* collected small mammals in Lifjell (50/69) and 94% prevalence of CNM in *Ixodes trianguliceps* collected from small mammals in Lærdal (44/47).

Table 3-3: Detection of Candidatus Neoehlrlichia mikurensis *in ticks in 2015 and 2017 divided by species, lifecycle stages and locations.*

Year		2015	2017	
Location		Lifjell (no. pos/no. tested)	Lærdal (no. pos/no. tested)	Lifjell (no. pos/no. tested)
Ixodes ricinus	Larva	0/141	-	-
	Nymph	0/1	-	-
	Adult	0/1	-	-
Ixodes trianguliceps	Larva	0/14	12/12	26/27
	Nymph	0/3	32/35	18/19
	Adult	0/0	0/0	6/6
Total		0/160	44/47	50/52

3.4 Detection of *Candidatus* Neoerlichia mikurensis in spleen samples of the small mammals collected in 2017

As shown in Table 3-4, 33/46 small mammals collected in 2017 showed detectable DNA of *Candidatus* Neoerlichia mikurensis (CNM) in their spleen samples. Of the small mammals collected from Lærdal, 6/11 *Myodes glareolus* and 13/16 *Sorex araneus* were positive. Of the small mammals collected from Lifjell, 9/14 *Myodes glareolus*, 4/4 *Sorex aranneus* and 1/1 *Apodemus sylvaticus* were positive. In 2017, 19/27 (70.4%) small mammals from Lærdal were positive and 14/19 (73.7%) small mammals from Lifjell were positive.

Table 3-4: Detection of CNM in spleen samples of the small mammals in 2017 by locations.

Location	Small mammal species	No. Investigated (no. pos/no. tested)	NA/Void
Lærdal	*Myodes glareolus*	6/11	1 (discarded after the open DNA tube mistakenly dropped on the floor)
	Sorex araenus	13/16	2 (discarded due to error in dilution)
	Apodemus sylvaticus	0/0	-
Lifjell	*Myodes glareolus*	9/14	-
	Sorex araneus	4/4	-
	Apodemus sylvaticus	1/1	-
Total		33/46	3

3.5 The prevalence of *Candidatus* Neoehrlichia mikurensis in the lifecycle stages of Ixodes trianguliceps.

The prevalence of *Candidatus* Neoehrlichia mikurensis (CNM) was divided by their lifecycle stages of *Ixodes trianguliceps*. As shown in *Table 3-5* and *Figure 3-5*, *Ixodes trianguliceps* from both locations, Lærdal and Lifjell collected in 2015 and 2017, 38/53 (72%) larvae were positive, 50/57 (88%) nymphs were positive and 6/6 (100%) adults (2 females and 4 males) were positive. The prevalence of *Candidatus* Neoehrlichia mikurensis was more in nymphs (88%) than larvae (70%). Of the larvae of *Ixodes trianguliceps*, 10/10 partially engorged, 28/29 not engorged and 0/14 no remarks were positive. Difference in the infection rate of CNM between larvae and nymphs of *Ixodes trianguliceps* was statistically significant for Pearson's Chi-squared test at p-value = 0.03582 and not statistically significant for Fisher's Exact Test for Count Data p-value = 0.05505 with 95 percent confidence interval: 0.1117791 and 1.0446303. Difference in the rate of CNM between nymphs and adults of *Ixodes trianguliceps* collected from both locations and years, was not statistically significant for Pearson's Chi-squared test at p-value = 0.36258 and Fisher's Exact Test for Count Data p-value = 1 with 95 percent confidence interval: 0.000000 and 7.554546. There was no significant difference between male and female *Ixodes trianguliceps*. In total 94 out of 116 (81%) *Ixodes trianguliceps* were positive.

Table 3-5:Prevalence of CNM in the lifecycle stages of Ixodes trianguliceps collected in 2015 and 2017

Stage	No. Investigated (no. pos/no. tested)	Prevalence (%)
Larvae	38/53	72
Nymph	50/57	88
Female	2/2	100
Male	4/4	100
Overall	94/116	81

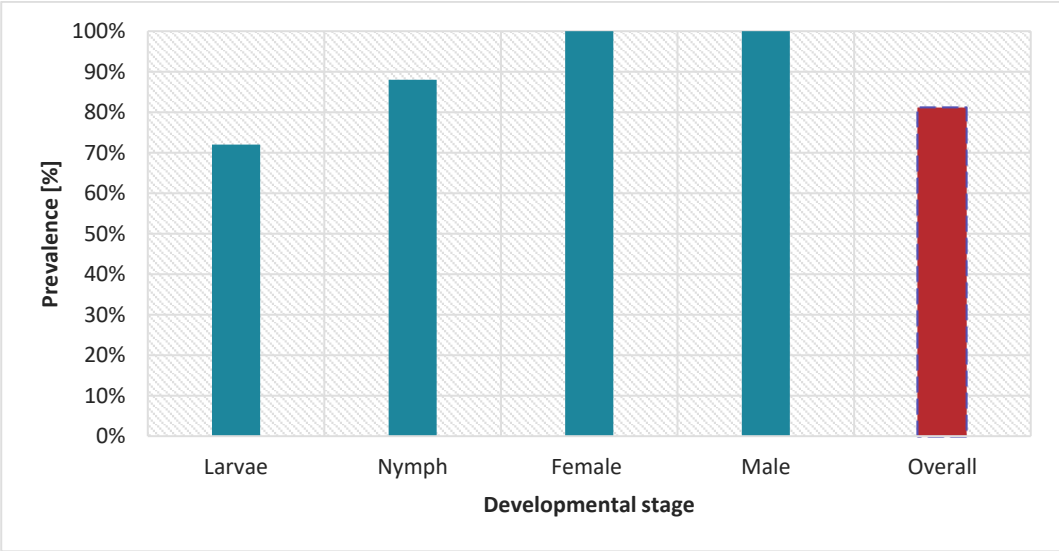


Figure 3-5: Prevalence of CNM relative to lifecycle stages of Ixodes trianguliceps collected in 2015 and 2017

Table 3-6 Status of Larvae of Ixodes trianguliceps collected in 2015 and 2017 with their CNM prevalence

Larvae	No. Investigated (no. pos/no. tested)	Prevalence (%)
Partially engorged	10/10	100
Not engorged	28/29	97
No remarks	0/14	0
Total	**38/53**	**72**

3.6 The prevalence of *Candidatus* Neoehrlichia mikurensis in small mammals collected in 2017

The prevalence of *Candidatus* Neoehrlichia mikurensis in small mammals was also divided by their various species. Small mammals collected in 2017: 17/20 (85%) *Sorex araneus* were positive, 15/25 (60%) *Myodes glareolus* were positive and 1/1 (100%) *Apodemus sylvaticus* were positive as shown in Table 3-7 and Figure 3-6. The prevalence was higher in *Sorex araneus* than *Myodes glareolus*. In total, 33 out 46(72%) small mammals were positive as shown Table 3-6 and Figure 3-6. Difference in the infection rate of CNM between *Sorex araneus* and *Myodes glareolus* was not statistically significant for Pearson's Chi-squared test at p-value = 0.06598 and Fisher's Exact Test for Count Data p-value = 0.0998 with 95 percent confidence interval: 0.7522933 and 24.6591742. *Apodemus sylvaticus* was not compared due to its small sample size (n = 1).

Table 3-7: Prevalence of CNM on spleen samples of various species of small mammals collected in 2017.

Small mammals	No. Investigated (no. pos/no. tested)	Prevalence (%)
Sorex araneus	17/20	85
Myodes glareolus	15/25	60
Apodemus sylvaticus	1/1	100
Total	33/46	72

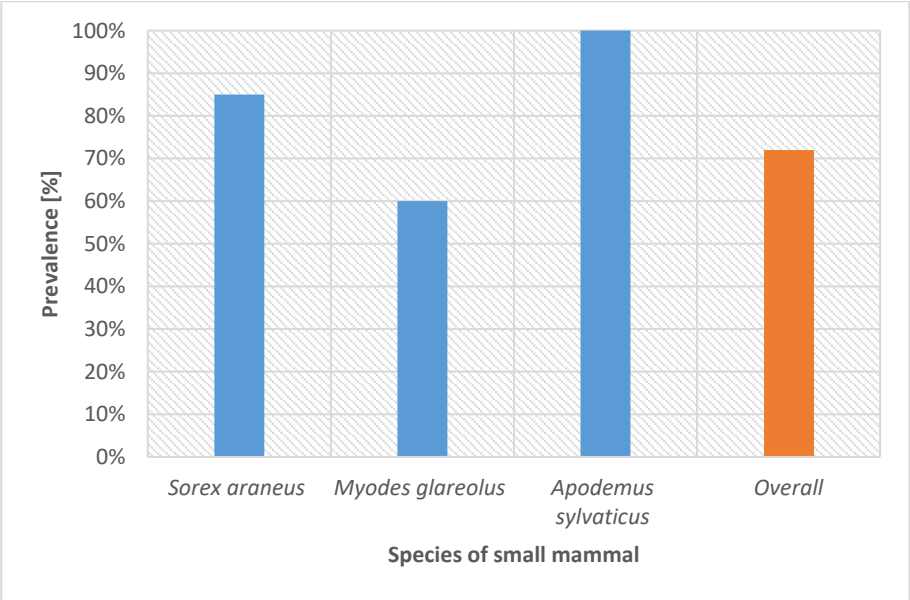


Figure 3-6: Prevalence of CNM on spleen samples of various species of small mammals collected in 2017.

3.7 Possible sources of infection

Possible sources of infection of *Ixodes trianguliceps* were determined by their host animals. As shown in Table 3-8 and Figure 3-7, the *Ixodes trianguliceps* and small mammals collected in 2017, 23 infected larvae were collected from infected small mammals, 14 infected larvae were collected from small mammals that did not have DNA of *Candidatus* Neoehrlichia mikurensis and 1 uninfected larva (not engorged) was collected from an infected small mammal. A total of 34 infected nymphs were collected from infected small mammals, 14 nymphs were collected from small mammals that did not have DNA of *Candidatus* Neoehrlichia mikurensis, 2 uninfected nymphs were collected from infected small mammals and 1 uninfected nymph was collected from small mammal that did not have DNA of *Candidatus* Neoehrlichia mikurensis. A total of 5 infected adults (2 female and 3 male) were collected from infected small mammals and 1 infected male adult was collected from small mammal that did not have DNA of *Candidatus* Neoehrlichia mikurensis. Difference in the infection rate of CNM between small mammals and *Ixodes trianguliceps* tested was not statistically significant for Pearson's Chi-squared test at p-value = 0.1949 and Fisher's Exact Test for Count Data p-value = 0.2088 with 95 percent confidence interval: 0.2528773 and 1.4424391.

Table 3-8: Infection status of ticks and their host animals.

Spleen sample	Larva		Nymph		Adult	
	Pos	Neg	Pos	Neg	Pos	Neg
Pos Spleen	23	1	34	2	5	0
Neg Spleen	14	0	14	1	1	0
NA/void	1	0	2	1	0	0

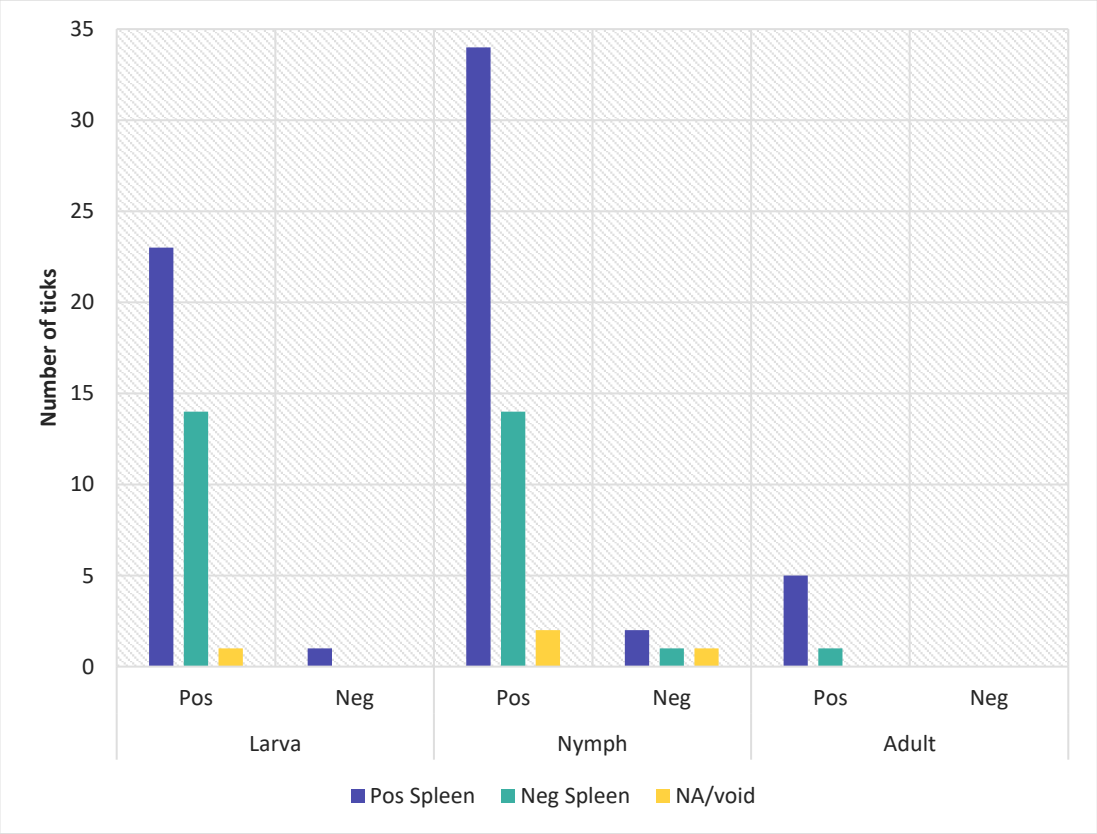


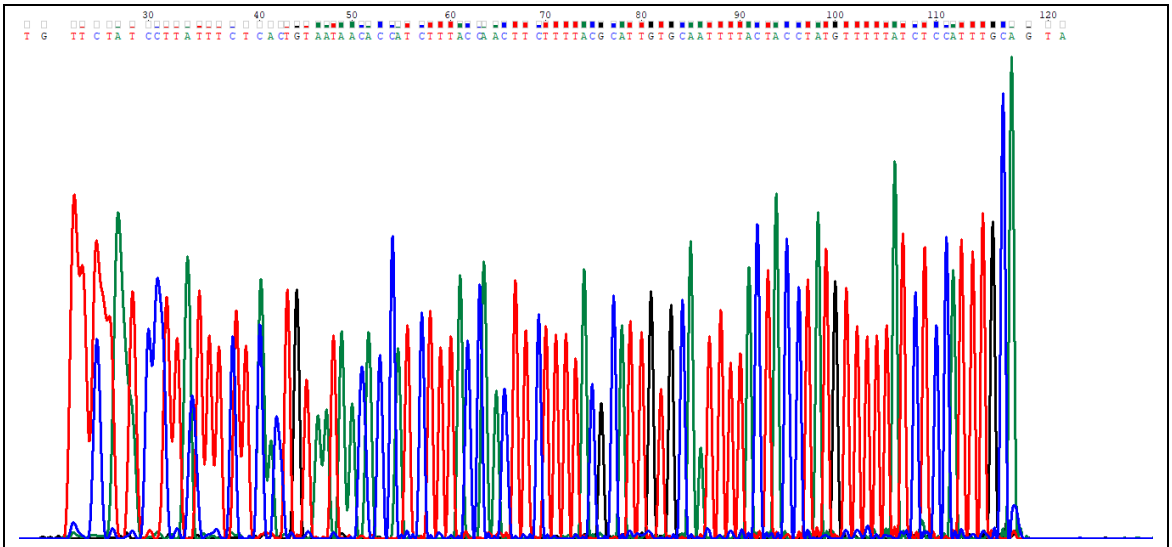
Figure 3-7: A bar chart showing infection status of ticks and their host animals

3.8 DNA Sequencing

The real-time PCR products of the DNA of *Candidatus* Neoehrlichia mikurensis from ticks and spleen samples of the small mammals were sequenced and compared with each other and with *Candidatus* Neoehrlichia mikurensis sequences available in Genbank. Sequences from *Sorex araneus*, *Myodes glareolus* and *Apodemus sylvaticus* spleen samples were 79% to 98% similar to CNM sequences in Genbank. Sequences from larvae of *Ixodes trianguliceps* were 83% to 98% similar to sequences in Genbank. Of 15 samples sequenced, 8 samples gave readable sequence, and all of which were confirmed as CNM

Table 3-9 The readable sequences confirmed as CNM

	species	No confirmed /no tested
Tick	*Ixodes trianguliceps*	1/3
Small mammals	*Sorex araneus*	2/3
	Myodes glareolus	4/8
	Apodemus sylvaticus	1/1
Overall		8/15

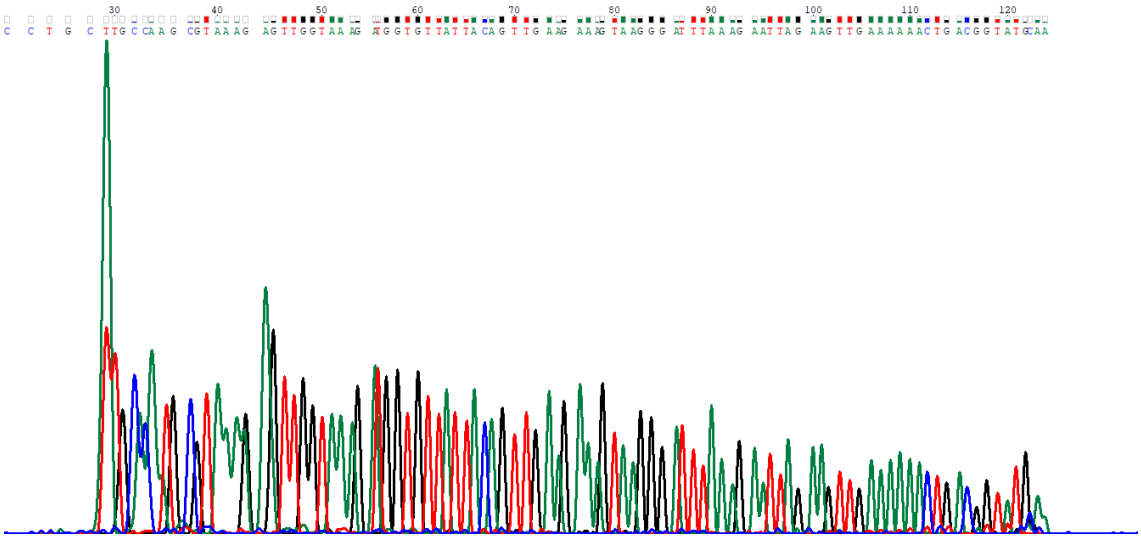


>E6-283_R

AGGCTGACAGCATACTGACCCTGTTCTATCCTTATTTCTCACTGTAATAACACCATCTTT

ACCAACTTCTTTTACGCATTGTGCAATTTTACTACCTATGTTTTTATCTCCATTTGCAGT

A



>E6-283_F

TAGATCGTGCTGCAGTCCTCCTCCCTGCTTGCCAAGCGTAAAGAGTTGGTAAAGATGGTG

TTATTACAGTTGAAGAAAGTAAGGGATTTAAAGAATTAGAAGTTGAAAAAACTGACGGTA

TGCAA

Figure 3-8: Chromatograms showing the Sequencing of the DNA of CNM of a sample

4 Discussion

In this study, real-time PCR was used to target *groEL* gene of *Candidatus* Neoehrlichia mikuensis (CNM). The purpose of this study was to test the presence of CNM in *Ixodes trianguliceps* and its possible role in the infectious cycle.

I predicted little or no CNM in *Ixodes trianguliceps* (H1a and H1b (see section 1.7)). In this study, there was presence and high prevalence of CNM in *Ixodes trianguliceps*. I predicted no CNM in unfed larvae (not engorged) of *Ixodes trianguliceps* assuming there was presence of CNM in *Ixodes trianguliceps* (H2a). In this study, there was presence of CNM in unfed larvae of *Ixodes trianguliceps*. I predicted difference in the infectious rate of CNM between larvae and nymphs of *Ixodes trianguliceps* assuming there is low prevalence of CNM in larvae of *Ixodes trianguliceps* (H2c). In this study, the difference in the infection rate of CNM between larvae and nymphs of *Ixodes trianguliceps* was statistically significant for Pearson's Chi-squared test and was not statistically significant for Fisher's Exact test. I predicted no difference in the infection rate of CNM between nymphs and adults of *Ixodes trianguliceps* assuming they were infected by feeding on infected hosts (H2e). In this study, the difference in the rate of CNM between nymphs and adults of Ixodes trianguliceps collected from both locations and years, was not statistically significant for Pearson's Chi-squared test and Fisher's Exact Test for Count Data. I predicted no presence of CNM in the spleen samples of *Sorex araneus* due to lack of evidence of CNM in *Sorex araneus* from previous studies (H3a) (Andersson & Råberg, 2011; Jahfari et al., 2012; Kawahara et al., 2004; Li et al., 2013; Silaghi et al., 2012). This study showed presence of CNM in *Sorex araneus*. I predicted difference in the infection rate of CNM between *Sorex araneus* and *Myodes glareolus* assuming there was presence of CNM in *Sorex araneus* (H3c). In this study, the difference in the infection rate of CNM between *Sorex araneus* and *Myodes glareolus* was not statistically significant. I predicted difference in the infection rate of CNM in ticks collected from Lifjell between 2015 and 2017 since ticks collected in 2015 are more of larvae of Ixodes ricinus (H4b). In this study, the difference in the infection rate of CNM in ticks collected from Lifjell between 2015 and 2017 was statistically significant for Pearson's Chi-squared test and Fisher's Exact test for Count Data. I predicted difference in the infection rate of CNM in ticks collected in 2017 from Lærdal and Lifjell (H4d). In this study, the difference in the infection rate of CNM in ticks collected in 2017 from Lærdal and Lifjell was not statistically significant for

Pearson's Chi-squared test and Fisher's Exact test for Count Data. I predicted lack of transovarial transmission of CNM in *Ixodes trianguliceps* due to lack of transovarial transmission of CNM in *Ixodes ricinus* (H5b) (Burri et al., 2014; Jahfari et al., 2012; Obiegala et al., 2014; Silaghi et al., 2012). This study showed possibility of transovarial transmission of CNM in *Ixodes trianguliceps.*

4.1 CNM in ticks

In this study, the presence of *Candidatus* Neoehrlichia mikuensis in *Ixodes trianguliceps*, was investigated. The null hypothesis that was tested for CNM in *Ixodes trianguliceps* stated that there was no presence of CNM in *Ixodes trianguliceps*. The alternative hypothesis stated there was presence of CNM in *Ixodes trianguliceps*. In this study, there was presence and high prevalence of *Candidatus* Neoehrlichia mikuensis in the *Ixodes trianguliceps* collected from small mammals in 2017, higher (81%) than in studies from Slovakia (2.7%) (Blaňarová et al., 2016) and Germany (2.5%) (Obiegala et al., 2014). This result leads to rejection of the null hypothesis. This result is also contrary to my prediction that stated little or no prevalence of CNM in *Ixodes trianguliceps*. The result did not only show presence of CNM but also showed high prevalence (81%) of CNM in *Ixodes trianguliceps* in this study. Considering that all lifecycle stages of *Ixodes trianguliceps* were found on small mammals, the high prevalence of CNM in *Ixodes trianguliceps* could be caused by their feeding strategy. *Ixodes trianguliceps* are nidicolous ticks, in which all their lifecycle stages feed and metamorphosize within the confines of a single host and its nest (Salman, 2012). They may re-use a host after metamorphosis. This could increase their chance of transmitting CNM from tick to rodent and from ticks to ticks through co-feeding (transmission of infection by ingestion of the infected salivary secretions of a neighboring tick without the host itself becoming infected) (Voordouw, 2015). This could lead to the acceptance of the alternative hypothesis that stated there was presence of CNM in *Ixodes trianguliceps*.

All ticks collected in 2015 were negative and most of the ticks were larvae of *Ixodes ricinus.* 141 out of 160 ticks collected in 2015 were larvae of Ixodes ricinus. Previous studies have shown no prevalence in the larvae of *Ixodes ricinus* (Blaňarová et al., 2016; Jahfari et al., 2012). Their hosts were not investigated since *Ixodes ricinus* were not the

point of interest in this study. Considering that previous studies have shown lack of transovarial transmission of CNM in *Ixodes ricinus* (Burri et al., 2014; Jahfari et al., 2012; Obiegala et al., 2014; Silaghi et al., 2012), it could be that these larvae of *Ixodes ricinus* are probably not engorged or have not fed on blood meal from their host or their hosts were not infected. This result was expected from *Ixodes ricinus* considering that CNM is not found in larvae of *Ixodes ricinus* and transovarial transmission of CNM is rare or absent (Burri et al., 2014; Jahfari et al., 2012; Obiegala et al., 2014; Silaghi et al., 2012). Larvae of *Ixodes ricinus* cannot infect the rodents because they do not have this pathogen until their first blood meal from an infected host, and they feed only once before metamorphosing to nymphs and adults(Burri et al., 2014; Silaghi, Beck, Oteo, Pfeffer, & Sprong, 2016).

The null hypothesis that was tested for unfed larvae stated no CNM in unfed larvae (not engorged) of *Ixodes trianguliceps* collected from small mammals in 2017. The alternative hypothesis stated prevalence of CNM in unfed larvae (not engorged) of *Ixodes trianguliceps* collected from small mammals in 2017. In this study, DNAs of CNM were found in 28/29 (96.5%) of unfed (not engorged) larvae of *Ixodes trianguliceps* collected from small mammals in 2017. This result leads to the rejection of the null hypothesis. This result suggests transovarial transmission of CNM in larvae of *Ixodes trianguliceps.* This is unlike unfed larvae of *Ixodes ricinus* that lack transovarial transmission of CNM (Burri et al., 2014; Jahfari et al., 2012; Obiegala et al., 2014; Silaghi et al., 2012). These larvae of *Ixodes trianguliceps* were collected from small mammals and visibly examined using microscope to find out if they were engorged or not. There could also be a possibility of being engorged but not visibly engorged. The result may lead to the acceptance of the alternative hypothesis that stated prevalence of CNM in unfed larvae of *Ixodes trianguliceps.*

The null hypothesis that was tested for the infection rate between larvae and nymphs of *Ixodes trianguliceps* stated no difference in the infection rate of CNM between larvae and nymphs of *Ixodes trianguliceps*. The alternative hypothesis stated difference in the infection rate of CNM between larvae and nymphs of *Ixodes trianguliceps*. In this study, the difference in the infection rate of CNM between larvae and nymphs of *Ixodes*

trianguliceps was statistically significant for Pearson's Chi-squared test ($P<0.05$). But the difference in the infection rate of CNM between larvae and nymphs of *Ixodes trianguliceps* was not statistically significant for Fisher's Exact Test. Because Fisher's Exact test for Count Data is mainly used for sample sizes, the null hypothesis that stated no difference in the infection rate of CNM between larvae and nymphs of *Ixodes trianguliceps* may be accepted.

The null hypothesis that was tested for the infection rate of CNM between nymphs and adults of *Ixodes trianguliceps* stated no difference in the infection rate of CNM between nymphs and adults of *Ixodes trianguliceps*. The alternative hypothesis stated difference in the infection rate of CNM between nymphs and adult of *Ixodes trianguliceps*. In this study, the difference in the infection rate of CNM between nymphs and adults of *Ixodes trianguliceps* was not statistically significant for Pearson's Chi-squared test and Fisher's Exact Test. The result may lead to the acceptance of the null hypothesis that stated no difference in the infection rate of CNM between nymphs and adults of *Ixodes trianguliceps*. This result is contrary to other tick-borne pathogens such as TBE- virus, *Borrelia* and *Anaplasma phagocytophilum* in adult and nymphs questing ticks, for which the infection rate was higher in adult than in nymphs (Pettersson, Golovljova, Vene, & Jaenson, 2014; Rauter & Hartung, 2005; Z. Svitálková et al., 2015).

The null hypothesis that was tested for the infection rate of CNM in ticks collected from Lifjell in 2015 and 2017 stated no difference in the infection rate of CNM in ticks collected from Lifjell in 2015 and 2017. The alternative hypothesis stated difference in the infection rate of CNM in ticks collected from Lifjell in 2015 and 2017. In this study, the difference in the infection rate of CNM in ticks collected from Lifjell in 2015 and 2017 statistically significant for Pearson's Chi-squared test and Fisher's Exact Test. The difference might be explained by sample degradation in ticks collected in 2015 or seasonal variation between 2015 and 2017. Aside having more of larvae of *Ixodes ricinus* (141 larvae of *Ixodes ricinus* out of 160 ticks) in ticks collected in 2015, of which the presence of CNM was not expected because they lack transovarial transmission (Burri et al., 2014; Jahfari et al., 2012; Obiegala et al., 2014; Silaghi et al., 2012) , a different DNA extraction method was used for the ticks collected in 2015. Some of the concentrations of the DNA samples

extracted from ticks collected in 2015 were as low as 0.5ng/µl. The concentration of all the DNA samples were not measured due to a technical problem that developed in the spectrophotometer. Considering the time given to this study, detection of DNA of the tick species using PCR was not done. All these could be reason for no detection of CNM in ticks collected in 2015 but has not been concluded. If the distribution of ticks has increased so much since 2015, it could also lead to increase in their tick-borne pathogens. This may lead to the acceptance of the alternative hypothesis that stated difference in the infection rate of CNM in ticks collected from Lifjell in 2015 and 2017

The null hypothesis that was tested for the infection rate of CNM in ticks collected from in 2017 from Lærdal and Lifjell stated no difference in the infection rate of CNM in ticks collected from in 2017 from Lærdal and Lifjell. The alternative hypothesis stated difference in the infection rate of CNM in ticks collected in 2017 from Lærdal and Lifjell. In this study, the difference in the infection rate of CNM in ticks collected in 2017 from Lærdal and Lifjell was not statistically significant for Pearson's Chi-squared test and Fisher's Exact Test. This result may lead to the acceptance of the null hypothesis that stated no difference in the infection rate of CNM in ticks collected from in 2017 from Lærdal and Lifjell. There seems to be more breeding population of this pathogen in *Ixodes trianguliceps* in Lærdal (94%) than in Lifjell (73%) not withstanding that both locations showed high prevalences.

4.2 CNM in small mammals collected in 2017

Silaghi et al demonstrated that spleen is the one of the best organ materials for the detection of the DNA of *Candidatus* Neoehrlichia mikuensis (Silaghi et al., 2012). In this study, there was 72% (33 out of 46) prevalence of *Candidatus* Neoehrlichia mikurensis in the spleen samples of the small mammals which was similar to study in Germany(Silaghi et al., 2012). The prevalence of *Candidatus* Neoehrlichia mikuensis in *Myodes glareolus* (60%) were higher than studies from France (1.8%) (Vayssier-Taussat et al., 2012), Germany (29%-55%) (Krücken et al., 2013; Obiegala et al., 2014; Silaghi et al., 2012), Netherlands (11%) (Jahfari et al., 2012), Slovakia (11%) (Víchová et al., 2014), Sweden (9.1%-19%) (Andersson & Råberg, 2011; Andersson, Scherman, et al., 2014) and

Switzerland (8%) (Burri et al., 2014). Small mammals play important role for the development of lifecycle stages of Ixodid ticks especially ticks that feed on them (Fehr et al., 2010; Vayssier-Taussat et al., 2012). There is a possibility that nidicolous ticks like *Ixodes trianguliceps* (Salman, 2012) could play an important role in high prevalence of CNM in *Myodes glareolus* in this study.

The null hypothesis that was tested for CNM in *Sorex araneus* stated no prevalence of CNM in *Sorex araneus*. The alternative hypothesis stated prevalence of CNM in *Sorex araneus* which was what was found. This study is the first study that showed the presence and prevalence (85%) of *Candidatus* Neoehrlichia mikuensis in *Sorex araneus*. Even though previous studies showed lack of *Candidatus* Neoehrlichia mikuensis infection for shrews (Andersson & Råberg, 2011; Jahfari et al., 2012; Kawahara et al., 2004; Li et al., 2013; Silaghi et al., 2012). The result leads to the rejection of the null hypothesis. The alternative hypothesis that stated prevalence of CNM in *Sorex araneus* may be accepted. There is a possibility that nidicolous ticks like *Ixodes trianguliceps* (Salman, 2012) could play an important role in high prevalence of CNM in *Sorex araneus* in this study.

The null hypothesis that was tested for the infection rate of CNM between *Sorex araneus* and *Myodes glareolus* stated no difference in the infection rate of CNM between *Sorex araneus* and *Myodes glareolus*. The alternative hypothesis stated difference in the infection rate of CNM between *Sorex araneus* and *Myodes glareolus*. In this study, the difference in the infection rate of CNM between *Sorex araneus* and *Myodes glareolus* was not statistically significant. This led to acceptance of the null hypothesis that stated no difference in their infection rates. This means that both species of small mammals are important reservoir hosts for the tick-borne pathogen, *Candidatus* Neoehrlichia mikuensis.

Only one *Apodemus sylvaticus* was collected from Lifjell in 2017 was available for this study. CNM in *Apodemus sylvaticus* has also been detected in studies from China (Li et al., 2013), Germany (Obiegala et al., 2014), Netherlands (Jahfari et al., 2012) and Sweden (Andersson & Råberg, 2011). The sample size (n=1) of the *Apodemus sylvaticus* was too low to draw a conclusion on its prevalence.

4.3 Infectious Cycle of CNM in Ixodes trianguliceps

Infectious cycle in this study means the tick-host-pathogen cycle. Ticks feed on blood meal from their hosts (Andrew Jenkins et al., 2001; Parola & Raoult, 2001; Pfaff, 2015; Silaghi et al., 2012). Small mammals are known to be the potential reservoir hosts for ticks and their pathogens such as *Candidatus* Neoehrlichia mikurensis (Andersson & Råberg, 2011; Andersson, Scherman, et al., 2014; Beninati et al., 2006; Jahfari et al., 2012; Krücken et al., 2013; Mysterud et al., 2015; Silaghi et al., 2012; Szekeres et al., 2015; Vayssier-Taussat et al., 2012; Víchová et al., 2014).

The null hypothesis that was tested for infection rate of CNM between small mammals and *Ixodes trianguliceps* stated no difference in the infection rate of CNM between small mammals and *Ixodes trianguliceps*. The alternative hypothesis stated difference in the infection rate of CNM between small mammals and *Ixodes trianguliceps*. In this study, the prevalence of CNM in ticks (84%; 94/116) was higher than the prevalence in the small mammals being their host (72%; 33/46) though the difference in the infection rate of CNM between small mammals and *Ixodes trianguliceps* was not statistically significant. This could explain their infection cycle but to a limit. To think that larvae of *Ixodes trianguliceps* are only infected by feeding on infected hosts and then CNM becomes transstadial in nymphs and adults, would have been easier but that is not the case in this study. There were infected *Ixodes trianguliceps* larvae on uninfected small mammals. These infected larvae of *Ixodes trianguliceps* on uninfected small mammals suggest transovarial transmission of CNM in *Ixodes trianguliceps*. Previous studies that suggested that transovarial transmission of CNM does not occur in *Ixodes ricinus*(Burri et al., 2014; Jahfari et al., 2012; Obiegala et al., 2014; Silaghi et al., 2012) could not support this result in this study. Infected unfed nymphs and adults were also found on uninfected small mammals which indicates transstadial transmission since they could not have acquired their infection from their hosts instead, they must have been infected prior to attachment.

4.4 DNA sequencing

Amplified DNA of *Candidatus* Neoehrlichia mikurensis from *Ixodes trianguliceps* were sequenced and compared with CNM sequences in the GenBank database using BLAST showed 79%-98% similarities (Jahfari et al., 2012; Silaghi et al., 2012). Of 15 samples sequenced, 8 samples gave readable sequence, and all of which were confirmed as CNM. These samples include DNA of CNM detected in *Sorex araneus*, *Myodes glareolus*, *Apodemus sylvaticus*, and larvae of *Ixodes trianguliceps*. This is the first detection of DNA of *Candidatus* Neoehrlichia mikurensis in *Sorex araneus* and 2/3 sequenced DNA of CNM in *Sorex araneus* showed 79%-98% similarities.

5 Conclusion

This study strongly indicates the presence of *Candidatus* Neoehrlichia mikurensis (CNM) in *Ixodes trianguliceps* and *Sorex araneus* (common shrew). This is based on their high detection rate and prevalence. In this study, there seems to be evidence for transovarial transstadial transmission of CNM in *Ixodes trianguliceps.* Further investigation on *Candidatus* Neoehrlichia mikurensis in *Sorex araneus* and *Ixodes trianguliceps* is recommended to enable a conclusion on the recent outcome from this study. Not engorged infected larvae of *Ixodes trianguliceps* were collected from infected hosts. Uninfected *Ixodes trianguliceps* were also collected from infected host animals. This study has shown high prevalence of *Candidatus* Neoehrlichia mikurensis, which means there could be a possibility of CNM outbreak in Norway and some issues raised in this study are yet to be known. Although *Ixodes trianguliceps* does not bite humans (Salman, 2012), it may indirectly be of medical and veterinary importance of its role as a maintenance vector of CNM among small mammals.

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Annex 1: Detection of *Candidatus* Neoehrlichia mikurensis in various tick species and estimated rates of its prevalence worldwide

Country	Source	Tick species	year	Pos/Total	Prev %	References
Austria	Questing	*Ixodes ricinus*	na	19/86	22	(Derdáková et al., 2014)
		Ixodes ricinus	2002 - 2003	22/518	4.2	(Glatz et al., 2014)
Baltic region	Birds	*Ixodes ricinus*	2009	1/135	0.7	(A Movila, Alekseev, Dubinina, & Toderas, 2013)
	Birds	*Ixodes frontalis*	2009	1/4	25	(A Movila et al., 2013)
Belgium	Questing	*Ixodes ricinus*	na	6/373	1.6	(Jahfari et al., 2012)
	Vegetation	*Ixodes ricinus*	2012 - 2014	na	0.4	(Heylen, Fonville, van Leeuwen, & Sprong, 2016)
	Hedgehogs	*Ixodes ricinus*	2014 - 2015	na	2.7	(Jahfari et al., 2017)
	Hedgehogs	*Ixodes hexagonus*	2014 - 2015	na	0.09	(Jahfari et al., 2017)
China	Questing	*Ixodes persulcatus*	2010	6/316	1.9	(Li et al., 2012)
	Questing	*Haemaphysalis concinna*		2/187	0.8	(Li et al., 2012)
Czech Republic	Questing	*Ixodes ricinus*	na	3/138	2.2	(Derdáková et al., 2014)
	Questing	*Ixodes ricinus*	na	2/20	10	(Richter & Matuschka, 2011)
	Vegetation	*Ixodes ricinus*	2010 - 2014	na	0.4-10	(Derdáková et al., 2014; Richter & Matuschka, 2011; Venclíková et al., 2016; Venclikova, Rudolf, Mendel,

						Betasova, & Hubalek, 2014)
	Sheep	*Ixodes ricinus*	2013 - 2014	na	30.7	(Venclíková et al., 2016)
Denmark	Vegetation	*Ixodes ricinus*	2011	3/2625	0.1	(Fertner, Mølbak, Pihl, Fomsgaard, & Bødker, 2012)
	Vegetation	*Ixodes ricinus*	2008 - 2012	2350/(in pools)	0.2-0.9	(Michelet et al., 2014)
Estonia	Vegetation	*Ixodes ricinus*	2006 - 2013	na	1.3	(Ivanova et al., 2017)
France	Questing	*Ixodes ricinus*	na	1/60	1.7	(Richter & Matuschka, 2011)
Germany	Wild boars	*Ixodes ricinus*	2010 - 2013	1/16	6.2	(Silaghi, Pfister, & Overzier, 2014)
	Dogs	*Ixodes ricinus*	2010 - 2011	32/773	4.1	(Krücken et al., 2013)
	Dogs	*Ixodes hexagonus*	na	10/151	6.6	(Krücken et al., 2013)
	Questing	*Dermacentor reticulatus*	2010 - 2011	1/1237	0.08	(Krücken et al., 2013)
	Questing	*Ixodes ricinus*		13/192	6.8	(Richter, Kohn, & Matuschka, 2013)
	Questing	*Dermacentor reticulatus*		0/283	0	(Richter et al., 2013)
	Questing	*Ixodes ricinus*	na	44/542	8.1	(Richter & Matuschka, 2011)
	Humans	*Ixodes ricinus*	na	9/111	8.1	(Richter & Matuschka, 2011)
	Rodents	*Ixodes ricinus*	2012 - 2013	32/918	3.5	(Obiegala et al., 2014)
	Rodents	*Ixodes trianguliceps*	2012 - 2013	0/7	0	(Obiegala et al., 2014)

	Rodents	*Dermacentor reticulatus*	2012 - 2013	1/40	2.5	(Obiegala et al., 2014)
	Questing	*Ixodes ricinus*	2009 - 2013	51/2315	2.2	(Obiegala et al., 2014)
	Vegetation	*Ixodes ricinus*	2008 - 2013		2.2-24.2	(Obiegala et al., 2014; Richter & Matuschka, 2011; Silaghi et al., 2012)
	Rodents	*Ixodes ricinus*	2010 - 2013		3.8-6.4	(Silaghi et al., 2012)
	Rodents	*Dermacentor reticulatus*	2010 - 2011		7.7	(Silaghi et al., 2012)
	Rodents	*Ixodes triangulicep*s	2012 - 2013		2.5	(Obiegala et al., 2014)
	Rodents	*Ixodes spp.*	2010 - 2011		100	(Silaghi et al., 2012)
	Rodents	*Unidentified larva*	2010 - 2011		100	(Silaghi et al., 2012)
Hungary	Questing	*Ixodes ricinus*	2012	3/34	8.8	(Szekeres et al., 2015)
	Questing	*Dermacentor reticulatus*		0/64	0	(Szekeres et al., 2015)
	Questing	*Haemaphysalis concinna*		0/62	0	(Szekeres et al., 2015)
Italy	Humans	*Ixodes ricinus*		10/357	2.8	(Brouqui, Sanogo, Caruso, Merola, & Raoult, 2003)
	Humans	*Ixodes ricinus*		2/64	3.1	(Otranto et al., 2014)
	Questing	*Ixodes ricinus*	2006 - 2008	20/193	10	(Capelli et al., 2012)
	Rodents	*Ixodes ricinus*	2011 - 2013		5.3	(Baráková et al., 2018)

	Humans	*Ixodes ricinus*	1995 - 2011		0.5	(Otranto et al., 2014)
Japan	Vegetation	*Ixodes ovatus*	2000 - 2001	?/164	?	(Kawahara et al., 2004)
Moldova	Questing	*Ixodes ricinus*	1960	1/126	0.79	(Alexandru Movila, Toderas, Uspenskaia, & Conovalov, 2013)
The Netherlands	Roe deer	*Ixodes ricinus*		8/121	6.6	(Schouls et al., 1999)
	Questing	*Ixodes ricinus*		21/180	12	(Van Overbeek et al., 2008)
	Humans	*Ixodes ricinus*		31/289	11	(Tijsse-Klasen et al., 2011)
	Questing	*Ixodes ricinus*	2000 - 2009 & 2006 - 2010	300/5343	5.6	(Coipan et al., 2013)
	Questing	*Ixodes ricinus*		160/2002	8.0	(Jahfari et al., 2012)
	Red deer	*Ixodes ricinus*		26/409	6.4	(Jahfari et al., 2012)
	Wild boars	*Ixodes ricinus*		4/84	8.3	(Jahfari et al., 2012)
	Sheep	*Ixodes ricinus*		33/264	12	(Jahfari et al., 2012)
	Mouflon	*Ixodes ricinus*		10/233	4.3	(Jahfari et al., 2012)
	Vegetation	*Ixodes ricinus*	2000 - 2012		2.4-11.7	(Coipan et al., 2013; Michelet et al., 2014; Van Overbeek et al., 2008)
	Humans	*Ixodes ricinus*	2007 - 2008		5.4	(Jahfari et al., 2016)
Nigeria	Dogs	*Rhipicephalus sanguineus,*	2011	4/76	5.3	(Kamani et al., 2013)

		Haemaphysalis lechi				
Norway	Questing	*Ixodes riinus*	1998 - 1999	8/341	2.3	(Andrew Jenkins et al., 2001)
Poland	Questing	*Ixodes ricinus*		3/1325	0.23	(Welc-Falęciak et al., 2014)
	Questing	*Ixodes ricinus*		0/40	0	(Richter & Matuschka, 2011)
	Vegetation	*Ixodes ricinus*	2011		0.3	(Welc-Falęciak et al., 2014)
	Dogs	*Ixodes ricinus*	2013 - 2014		8.1	(Król, Obiegala, Pfeffer, Lonc, & Kiewra, 2016)
	Dogs	*Ixodes hexagonus*	2013 - 2014		0.7	(Król et al., 2016)
Portugal	Questing	*Ixodes ricinus*		0/101	0	(Richter & Matuschka, 2011)
Romania	Human	*Ixodes ricinus*	2013	1/1	100	(Andersson, Zaghdoudi-Allan, Tamba, Stefanache, & Chitimia, 2014)
	Vegetation	*Ixodes ricinus*	2013 - 2014		5.3-14.6	(Kalmár et al., 2016; Raileanu et al., 2017)
Russia	Questing	*Ixodes persulcatus*	2002	2/53	3.8	(Shpynov, FOURNIER, Rudakov, Tarasevich, & Raoult, 2006)
	Questing	*Ixodes ricinus*		21/295	7.1	(Alekseev, Dubinina, Van De Pol, & Schouls, 2001)
	Birds	*Ixodes frontalis/Ixodes ricinus*		2/139	1.4	(A Movila et al., 2013)
	Questing	*Ixodes persulcatus*		8/3552	0.22	(Rar et al., 2010)
	Questing	*Ixodes persulcatus*	2003 - 2008	5/2590	0.19	(Rar et al., 2010)

Serbia	Vegetation	*Ixodes ricinus*	NA		4.2	(Potkonjak et al., 2016)
Slovakia	Questing	*Ixodes ricinus*		47/1311	3.6	(Derdáková et al., 2014)
	Questing	*Ixodes ricinus*	2008 - 2010	16/670	2.4	(Pangrácová et al., 2013)
	Questing	*Ixodes ricinus*	2006	2/68	2.9	(Spitalská, Boldis, Kostanová, Kocianová, & Stefanidesová, 2008)
	Vegetation	*Ixodes ricinus*	2006 - 2013		1.1-11.6	(Blaňarová et al., 2016; Derdáková et al., 2014; Pangrácová et al., 2013; Spitalská et al., 2008; Z. H. Svitálková et al., 2016)
	Rodents	*Ixodes ricinus*	2011 - 2014		0.3-1.3	(Blaňarová et al., 2016; Z. H. Svitálková et al., 2016)
	Rodents	*Ixodes trianguliceps*	2011 - 2013		2.7	(Blaňarová et al., 2016)
Spain	Cows	*Ixodes ricinus*	2013	2/200	1.0	(Palomar, García-Álvarez, Santibáñez, Portillo, & Oteo, 2014)
Sweden	Questing	*Ixodes ricinus*	2010 - 2011	57/949	6	(Andersson et al., 2013)
	Birds	*Ixodes ricinus*	2009		2.1	(Sandelin et al., 2015)
Switzerland	Birds	*Ixodes ricinus*	2007 - 2010	7/215	3.3	(Lommano, Dvořák, Vallotton, Jenni, & Gern, 2014)
	Questing	*Ixodes ricinus*	2009	1916(5-10)	3.5-8.0	(Maurer et al., 2013)

	Rodents		2011 - 2012	15/575	2.6	(Burri et al., 2014)
	Questing	*Ixodes ricinus*	2009 - 2010	52/818	6.4	(Lommano, Bertaiola, Dupasquier, & Gern, 2012)
Uk	Questing	*Ixodes ricinus*		0/954	0	(Hansford, Fonville, Jahfari, Sprong, & Medlock, 2015)
	Questing	*Dermacentor reticulatus*		0/61	0	(Tijsse-Klasen et al., 2013)
	Questing	*Haemaphysalis punctata*		0/100	0	(Tijsse-Klasen et al., 2013)
	Various	*Ixodes ricinus*		0/338	0	(Jahfari et al., 2012)
	Various	*Dermacentor reticulatus*		0/63	0	(Jahfari et al., 2012)

Annex 2: Detection of *Candidatus* Neoehrlichia mikurensis in various small mammal species and its estimated rates of prevalence worldwide

Country	Rodent species	Common name	Pos/total	Prev (%)	References
China	*Rattus norvegicus*	Brown rat	3-4/15	20-27	(Pan, Liu, Ma, Tong, & Sun, 2003)
	Apodemus agarius	Striped field mouse	14/117	12	(Li et al., 2013)
	Apodemus sylvaticus	Wood mouse	5/40	12	(Li et al., 2013)
	Apodemus draco	South China field mouse	1/7	14	(Li et al., 2013)
	Apodemus peninsuale	Korean field mouse	5/57	8.8	(Li et al., 2013)
	Eothenomys custos	Southwest China vole	2/8	25	(Li et al., 2013)

	Myodes rufocanus	Grey red-backed vole	4/83	4.8	(Li et al., 2013)
	Niviventer confucianus	Chinese white-bellied rat	1/52	1.9	(Li et al., 2013)
	R.norvegicus	Brown rat	1/87	1.1	(Li et al., 2013)
	Tamias sibiricus	Siberian chipmunk	1/7	14	(Li et al., 2013)
	Clethrionomys rufocanus	Grey red-backed vole	5/109	4.6	(Li et al., 2012)
	R.norvegicus	Brown rat	2/35	5.7	(Li et al., 2012)
	Tamias sibiricus	Siberian chipmunk	1/3	33	(Li et al., 2012)
France	*Myodes glareolus*	Bank Vole	5/276	1.8	(Vayssier-Taussat et al., 2012)
Germany	*Myodes glareolus*	Bank Vole	16/56	29	(Krücken et al., 2013)
	Microtus arvalis	Common vole	4/11	36	(Krücken et al., 2013)
	Microtus agrestis	Field vole	2/2	100	(Krücken et al., 2013)
	Apodemus flavicollis	Yellow-necked mouse	10/82	12	(Krücken et al., 2013)
	Apodemus agarius	Striped field mouse	4/78	5.2	(Krücken et al., 2013)
	Apodemus flavicollis	Yellow-necked mouse	24/37	65	(Silaghi et al., 2012)
	Apodemus agarius	Striped field mouse	1/3	33	(Silaghi et al., 2012)
	Myodes glareolus	Bank Vole	23/42	55	(Silaghi et al., 2012)
	Myodes glareolus	Bank Vole	125/396	32	(Obiegala et al., 2014)
	Apodemus sylvaticus	Wood mouse	1/36	2.8	(Obiegala et al., 2014)
	Apodemus flavicollis	Yellow-necked mouse	50/178	28	(Obiegala et al., 2014)
	Microtus arvalis	Common vole	4/7	57	(Obiegala et al., 2014)
Hungary	*Apodemus flavicollis*	Yellow-necked mouse	3/67	4.5	(Szekeres et al., 2015)
	Apodemus agarius	Striped field mouse	3/92	3.3	(Szekeres et al., 2015)

Italy	*Clethriononyms glareolus*	Bank Vole	1/34	2.9	(Beninati et al., 2006)
Japan	*Apodemus speciosus*	large Japanese field mouse	5/55	9.1	(Tabara et al., 2007)
	Apodemus argenteus	Small Japanese field mouse	2/7	28	(Tabara et al., 2007)
	R.norvegicus	Brown rat	7/15	47	(Kawahara et al., 2004)
The Netherlands	*Apodemus sylvaticus*	Wood mouse	5/23	22	(Jahfari et al., 2012)
	Microtus arvalis	Common vole	2/8	25	(Jahfari et al., 2012)
	Myodes glareolus	Bank Vole	4/35	11	(Jahfari et al., 2012)
Russia	*M.rufocanus*	Grey red-backed vole	1/606	0.17	(Rar et al., 2010)
	Apodemus peninsuale	Korean field mouse	3/236	1.3	(Rar et al., 2010)
	Microtus spp	Vole	1/38	2.6	(Rar et al., 2010)
Slovakia	*Apodemus spp. & Myodes glareolus*	Mice and voles	31/286	11	(Víchová et al., 2014)
	Apodemus spp. & C.glareolus	Mice and voles	0/30	0	(Spitalská et al., 2008)
Sweden	*M.glareolus*	Bank Vole	50/261	19	(Andersson, Scherman, et al., 2014)
	M.glareolus	Bank Vole	64/705	9.1	(Andersson & Råberg, 2011)
	Microtus agrestis	Field vole	2/24	8.3	(Andersson & Råberg, 2011)
	Apodemus sylvaticus	Wood mouse	1/10	10	(Andersson & Råberg, 2011)
	Apodemus flavicollis	Yellow-necked mouse	1/25	4	(Andersson & Råberg, 2011)
Switzerland	*Apodemus spp. & Myodes glareolus*	Mice and voles	8/100	8	(Burri et al., 2014)

Annex 3: European patients with *Candidatus* Neoehrlichia mikurensis infection

Country	Year	Age	Gender	Medical condition	References
Czech Republic	2008	55	F	Mantle cell lymphoma, asplenic	(Pekova et al., 2011)
	2009	58	M	Liver tranplantation, sclerosing cholangitis, splenectomy	(Pekova et al., 2011)
Germany	2007	69	M	Chronic inflammatory demyelinating polyneuropathy	(von Loewenich et al., 2010)
	2008	57	M	Previously healthy	(von Loewenich et al., 2010)
Netherlands	2007-2008	63	F	Tick bite, arthralgia	(Jahfari et al., 2016; Welinder-Olsson, Kjellin, Vaht, Jacobsson, & Wennerås, 2010)
		79	M	Tick bite	
		40	M	EM	
		60	F	EM	
		61	F	EM, headache, myalgia, pain in limbs	
		48	M	EM, tingling in limbs	
		71	M	Tick bite	
Norway	2014-2015	55(mean)	1.6F: 1M (ratio)	EM (7), fatique(1)	(Quarsten et al., 2017)

Poland	2012	44.1(mean)	4M & 1F	Asymptomatic, previously healthy (foresters with high risk of tick bites	(Welc-Falęciak et al., 2014)
Sweden	2009	77	M	B cell chronic lymphocytic leukaemia, asplenic	(Welinder-Olsson et al., 2010)
	2011	75	M	B cell chronic lymphocytic leukaemia, splenectomy	(Grankvist et al., 2014)
	2011	67	F	Follicular lymphoma, systemic lupus erythematosus, (inborn) asplenic	(Welinder-Olsson et al., 2010)
	2013	67	F	T cell large granular lymphoma, psoriasis arthropathy, splenectomy	(Welinder-Olsson et al., 2010)
	2013	54	M	Psoriasis, immunosuppressive therapy	(Welinder-Olsson et al., 2010)
	2013	59	M	Diffuse large cell B lymphoma, rheumatoid arthritis splenectomy	(Welinder-Olsson et al., 2010)
	2014	71	F	Rheumatoid arthritis, recurrent fever, immunosuppressive therapy	(Andréasson et al., 2015)
	2015	78	M	Rheumatoid arthritis	(Grankvist, Moore, et al., 2015)
	2015	55	M	Granulomatosis with polyangiitis	(Grankvist, Moore, et al., 2015)
	2015	57	F	EM, tick bite, Anaplasma phagocytophilum-	(Grankvist, Sandelin, et al., 2015)

				specific IgG antibodies	
	2015	68	F	EM, tick bite, Borrelia-specific IgM and IgG antibodies	
	2015	57	M	Pre-B cell acute lymphocytic leukaemia	(Grankvist, Moore, et al., 2015)
	N.A	65	F	Autoimmune haemolytic anaemia	(Wennerås et al., 2017)
Switzerland	2009	61	M	Coronary artery bypass grafting, septicaemia	(Fehr et al., 2010)
	2011	68	M	Chronic lymphocytic leukaemia, asplenic	(Maurer et al., 2013)
	2012	58	M	Follicular lymphoma	(Maurer et al., 2013)

F, female; M, male; N.A, not available.

Annex 4: Statistical analysis of differences

Difference in the infection rate of *Candidatus* Neoehrlichia mikurensis between *Sorex araneus* and *Myodes glareolus* collected in 2017

H_0 – The null hypothesis states that there is no difference in the infection rate of *Candidatus* Neoehrlichia mikurensis between *Sorex araneus* and *Myodes glareolus* collected in 2017

Small mammals	Positive	Negative
Sorex araneus	17	3
Myodes glareolus	15	10

Pearson's Chi-squared test

X-squared	3.3804
Degree of freedom	1
p-value	0.06598

Fisher's Exact Test for Count Data

p-value	0.0998
alternative hypothesis	true odds ratio is not equal to 1
95 percent confidence interval	0.7522933 - 24.6591742
sample estimates: odds ratio	3.669329

Difference in the infection rate of *Candidatus* Neoehrlichia mikurensis in larvae and nymphs of *Ixodes trianguliceps*

H_0 – The null hypothesis states that there is no difference in the infection rate of *Candidatus* Neoehrlichia mikurensis in larvae and nymphs of *Ixodes trianguliceps*

Stage	Positive	Negative
Larvae	38	15
Nymphs	50	7

Pearson's Chi-squared test

Since the P-value=0.03582 is less than 0.05, it is statistically significant which means the null hypothesis would be rejected.

X-squared	4.4058
Degree of freedom	1
P-value	0.03582

Fisher's Exact Test for Count Data

p-value	0.05505
alternative hypothesis	true odds ratio is not equal to 1
95 percent confidence interval	0.1117791 - 1.0446303
sample estimates: odds ratio	0.3580426

Difference in the infection rate of *Candidatus* Neoehrlichia mikurensis between nymphs and adults of *Ixodes trianguliceps* collected in 2015 and 2017

H_0 – The null hypothesis states that there is no difference in the infection rate of *Candidatus* Neoehrlichia mikurensis between nymphs and adults of *Ixodes trianguliceps*

Lifecycle stage	Positive	Negative
Nymphs	50	7
Adults	6	0

Pearson's Chi-squared test

X-squared	0.82895
Degree of freedom	1
P-value	0.36258

Fisher's Exact Test for Count Data

P-value	1
alternative hypothesis	true odds ratio is not equal to 1
95 percent confidence interval	0.000000 -7.554546
Odd ratio	0

Difference in the infection rate of *Candidatus* Neoehrlichia mikurensis in *Ixodes trianguliceps* and their host animals

H_0 – The null hypothesis states that there is no difference in the infection rate of *Candidatus* Neoehrlichia mikurensis in *Ixodes trianguliceps* and their host animals

	Positive	Negative
small mammals	33	13
Ixodes trianguliceps	94	22

Pearson's Chi-squared test

X-squared	1.6803
Degree of freedom	1
p-value	0.1949

Fisher's Exact Test for Count Data

p-value	0.2088
alternative hypothesis	true odds ratio is not equal to 1
95 percent confidence interval	0.2528773 1.4424391
sample estimates: odds ratio	0.5961758

Difference in the infection rate of *Candidatus* Neoehrlichia mikurensis in ticks collected from Lifjell between 2015 and 2017

H_0 – The null hypothesis states that there is no difference in the infection rate of *Candidatus* Neoehrlichia mikurensis in ticks collected from Lifjell between 2015 and 2017

Site/year	Positive	Negative
Lifjell 2015	0	160
Lifjell 2017	50	2

Pearson's Chi-squared test

X-squared	201.3
Degree of freedom	1
P-value	0

Fisher's Exact Test for Count Data

P-value	< 2.2e-16
alternative hypothesis	true odds ratio is not equal to 1
95 percent confidence interval	0.000000000 - 0.002078563
Odd ratio	0

Difference in the infection rate of *Candidatus* Neoehrlichia mikurensis in ticks collected in 2017 from Lærdal and Lifjell

H_0 – The null hypothesis states that there is no difference in the infection rate of *Candidatus* Neoehrlichia mikurensis in ticks collected in 2017 from Lærdal and Lifjell

Site	Positive	Negative
Lærdal	44	3
Lifjell	50	2

Pearson's Chi-squared test

X-squared	0.33130
Degree of freedom	1
P-value	0.56490

Fisher's Exact Test for Count Data

P-value	0.666
alternative hypothesis	true odds ratio is not equal to 1
95 percent confidence interval	0.04724933 - 5.39578231
Odd ratio	0.589826

Annex 5: Task Description and Work Plan

To be updated.

Execution Plan	2017					2018							
	Aug	Sept	Oct	Nov	Dec	Jan	Feb	March	April	May	Aug	Sept	Oct
Initialization													
Training													
Literature review													
Collection of samples													
Acquisition of materials													
Methods													
Identification of samples													
DNA extrection from ticks													
DNA extraction from spleen samples													
Detection procedures													
PCR													
DNA sequencing													
Finialization													
Result analysis													
Discussion and conclusion													
Submission of 1st draft													
Correction													
Final submission													
Presentation													

Annex 6: Result summary of *Ixodes trianguliceps* and their hosts collected in 2017

Host ID	Life stage	Tick ID	Tick status	Host Status	site	Alt [m]	Host Species	Tick Remark
155	2	109	Positive	positive	Læ	300	Sorex sp.	partially engorged
155	2	111	Positive	positive	Læ	300	Sorex sp.	partially engorged
167	2	96	Positive	negative	Læ	300	Sorex araneus	partially engorged
172	1	105	Positive	positive	Læ	300	Sorex araneus	physically not engorged
172	1	106	Positive	positive	Læ	300	Sorex araneus	Not engorged
172	2	107	Positive	positive	Læ	300	Sorex araneus	Engorged
172	1	108	Positive	positive	Læ	300	Sorex araneus	partially engorged
180	2	112	Positive	positive	Læ	300	Sorex araneus	partially engorged
180	2	121	Positive	positive	Læ	300	Sorex araneus	partially engorged
190	2	90	Positive	NA	Læ	300	Sorex sp.	Not engorged
199	2	98	Positive	positive	Læ	200	Sorex araneus	Not engorged
199	1	99	Positive	positive	Læ	200	Sorex araneus	Not engorged
199	2	100	Positive	positive	Læ	200	Sorex araneus	Not engorged
226	2	87	Positive	positive	Læ	100	Myodes glareolus	
234	2	104	Negative	negative	Læ	100	Myodes glareolus	
236	2	81	Positive	positive	Læ	300	Myodes glareolus	Not engorged
273	2	113	Positive	negative	Læ	300	Myodes glareolus	partially engorged
283	2	103	Positive	negative	Læ	200	Sorex araneus	partially engorged
283	2	110	Positive	negative	Læ	200	Sorex araneus	partially engorged
283	2	116	Positive	negative	Læ	200	Sorex araneus	Not engorged
283	1	117	Positive	negative	Læ	200	Sorex araneus	Not engorged
283	2	118	Positive	negative	Læ	200	Sorex araneus	partially engorged
286	2	82	Positive		Læ	100	Myodes glareolus	
295	2	91	Positive	positive	Læ	200	Sorex araneus	partially engorged
304	2	68	Positive	positive	Læ	100	Sorex araneus	
304	2	69	Positive	positive	Læ	100	Sorex araneus	
304	2	77	Positive	positive	Læ	100	Sorex araneus	
304	1	101	Positive	positive	Læ	100	Sorex araneus	partially engorged
304	1	102	Positive	positive	Læ	100	Sorex araneus	partially engorged
304	1	114	Positive	positive	Læ	100	Sorex araneus	partially engorged
304	1	115	Positive	positive	Læ	100	Sorex araneus	partially engorged
304	1	119	Positive	positive	Læ	100	Sorex araneus	partially engorged
304	1	120	Positive	positive	Læ	100	Sorex araneus	partially engorged
307	2	97	Positive	negative	Læ	300	Myodes glareolus	Engorged
317	2	79	Positive	positive	Læ	300	Sorex araneus	partially engorged
344	2	86	Positive	positive	Læ	300	Sorex araneus	partially engorged
359	2	95	Positive	positive	Læ	100	Myodes glareolus	partially engorged
364	2	78	Positive	positive	Læ	300	Sorex araneus	partially engorged
365	2	89	Positive	positive	Læ	300	Sorex araneus	Not engorged
366	2	88	Positive	positive	Læ	200	Sorex araneus	Not engorged
403	2	38	Positive	negative	Li	100	Myodes glareolus	
505	3	34	Positive	negative	Li	300	Myodes glareolus	Adult male
553	2	25	Positive	positive	Li	100	Myodes glareolus	partially engorged
565	3	50	Positive	positive	Li	200	Myodes glareolus	Adult male
682	3	29	Positive	positive	Li	100	Myodes glareolus	Adult male
682	2	30	Positive	positive	Li	100	Myodes glareolus	
888	2	37	Positive	positive	Li	100	Apodemus sylvaticus	
923	1	67	Positive	positive	Li	100	Sorex araneus	partially engorged
923	1	83	Positive	positive	Li	100	Sorex araneus	Not engorged
923	2	27	Positive	positive	Li	100	Sorex araneus	

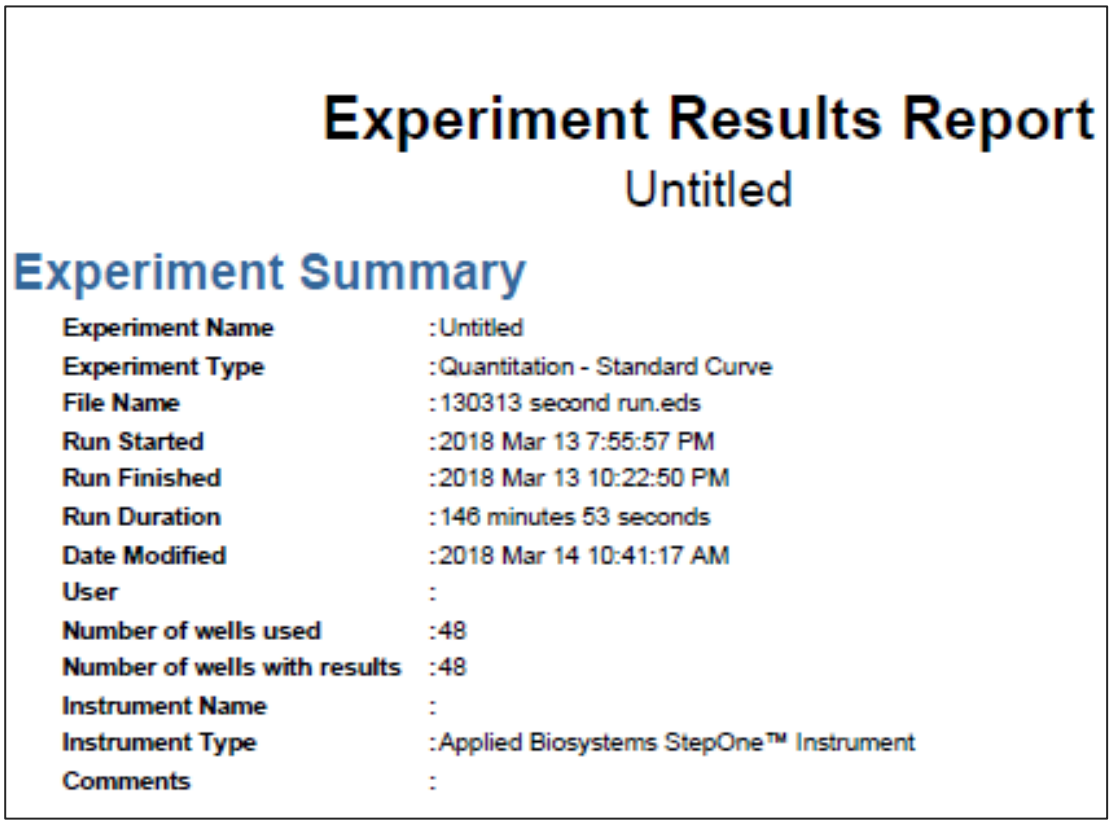
923	2	28	Positive	positive	Li	100	Sorex araneus	
927	2	39	Positive	positive	Li	100	Myodes glareolus	partially engorged
927	2	40	Positive	positive	Li	100	Myodes glareolus	partially engorged
931	2	26	Positive	positive	Li	100	Sorex araneus	partially engorged
932	1	80	Positive	positive	Li	100	Myodes glareolus	Not engorged
932	2	35	Positive	positive	Li	100	Myodes glareolus	
932	3	36	Positive	positive	Li	100	Myodes glareolus	female adult
1015	2	41	Positive	negative	Li	100	Myodes glareolus	
1015	2	42	Positive	negative	Li	100	Myodes glareolus	
1538	2	1	Positive	positive	Li	600	Sorex araneus	
1619	2	13	Positive	negative	Læ	500	Myodes glareolus	
1662	2	6	Negative	positive	Læ	300	Myodes glareolus	
1690	1	54	Positive	positive	Li	300	Myodes glareolus	Not engorged
1690	1	63	Positive	positive	Li	300	Myodes glareolus	Not engorged
1690	1	70	Positive	positive	Li	300	Myodes glareolus	Not engorged
1690	1	71	Positive	positive	Li	300	Myodes glareolus	Not engorged
1690	3	10	Positive	positive	Li	300	Myodes glareolus	Adult female
1698	1	56	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	57	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	59	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	60	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	61	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	62	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	65	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	72	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	75	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	76	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	84	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	85	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	1	92	Positive	negative	Li	200	Myodes glareolus	Not engorged
1698	2	5	Positive	negative	Li	200	Myodes glareolus	
1698	2	12	Positive	negative	Li	200	Myodes glareolus	
1765	1	55	Positive	positive	Li	500	Myodes glareolus	Not engorged
1765	1	73	Negative	positive	Li	500	Myodes glareolus	Not engorged
1765	1	74	Positive	positive	Li	500	Myodes glareolus	Not engorged
1765	2	2	Positive	positive	Li	500	Myodes glareolus	
1868	1	58	Positive		Læ	500	Sorex araneus	Not engorged
1868	2	15	Negative		Læ	500	Sorex araneus	
1966	2	33	Positive	positive	Læ	100	Sorex araneus	
2113	2	4	Positive	negative	Li	100	Myodes glareolus	
2238	1	64	Positive	positive	Li	300	Sorex araneus	partially engorged
2238	1	66	Positive	positive	Li	300	Sorex araneus	Not engorged
2238	2	24	Positive	positive	Li	300	Sorex araneus	partially engorged
2256	1	93	Positive	positive	Li	600	Myodes glareolus	partially engorged
2256	1	94	Positive	positive	Li	600	Myodes glareolus	Not engorged
2256	2	3	Negative	positive	Li	600	Myodes glareolus	
2401	3	7	Positive	positive	Li	100	Myodes glareolus	Adult male, partially engorged
2433	2	51	Positive	positive	Læ	100	Myodes glareolus	
2435	2	49	Positive	positive	Læ	100	Myodes glareolus	

ID: Identity; Life stage: Life cycle stage (1= larva; 2= nymph; 3= Adult); Læ: Lærdal; Li: Lifjell; Alt: Altitude.

Range of ticks per animal

Host ID	Larva	Nymph	Adult	Tick burden
155	0	2	0	2
167	0	1	0	1
172	3	1	0	4
180	0	2	0	2
190	0	1	0	1
199	1	2	0	3
226	0	1	0	1
234	0	1	0	1
236	0	1	0	1
273	0	1	0	1
283	1	4	0	5
286	0	1	0	1
295	0	1	0	1
304	6	3	0	9
307	0	1	0	1
317	0	1	0	1
344	0	1	0	1
359	0	1	0	1
364	0	1	0	1
365	0	1	0	1
366	0	1	0	1
403	0	1	0	1
505	0	0	1	1
553	0	1	0	1
563	0	0	1	1
682	0	1	1	2
888	0	1	0	1
923	2	2	0	4
927	0	2	0	2
931	0	1	0	1
932	1	1	1	3
1015	0	2	0	2
1538	0	1	0	1
1619	0	1	0	1
1662	0	1	0	1
1690	4	0	1	5
1698	13	2	0	15
1765	3	1	0	4
1868	1	1	0	2
1966	0	1	0	1
2113	0	1	0	1
2238	2	1	0	3
2256	2	1	0	3
2401	0	0	1	1
2433	0	1	0	1
2435	0	1	0	1
Overall	39	54	6	99

Annex 7: Real-time PCR Experiment summary



Experiment Results Report

Untitled

Experiment Summary

Experiment Name	:Untitled
Experiment Type	:Quantitation - Standard Curve
File Name	:130313 second run.eds
Run Started	:2018 Mar 13 7:55:57 PM
Run Finished	:2018 Mar 13 10:22:50 PM
Run Duration	:146 minutes 53 seconds
Date Modified	:2018 Mar 14 10:41:17 AM
User	:
Number of wells used	:48
Number of wells with results	:48
Instrument Name	:
Instrument Type	:Applied Biosystems StepOne™ Instrument
Comments	:

Results Summary

Sample	Target	Quantity (Mean)	Quantity (Std Dev)	CT (Mean)	CT (Std Dev)
LXF09 -1	SYBR				
LXF09 -2	SYBR				
NEG	SYBR				
Sample 1	SYBR			38.76	
Sample 10	SYBR				
Sample 11	SYBR			33.23	
Sample 12	SYBR			35.72	
Sample 13	SYBR			34.72	
Sample 14	SYBR			37.20	
Sample 15	SYBR			44.99	
Sample 16	SYBR			38.05	
Sample 17	SYBR			42.06	
Sample 18	SYBR			36.55	
Sample 19	SYBR				
Sample 2	SYBR			37.98	
Sample 20	SYBR			37.28	
Sample 21	SYBR				
Sample 22	SYBR			38.42	
Sample 23	SYBR			41.79	
Sample 24	SYBR			34.78	
Sample 25	SYBR			41.18	
Sample 26	SYBR			38.60	
Sample 27	SYBR			36.12	
Sample 28	SYBR			34.94	
Sample 29	SYBR			39.76	
Sample 3	SYBR			36.41	
Sample 30	SYBR			37.18	
Sample 31	SYBR			39.46	
Sample 32	SYBR			37.77	
Sample 33	SYBR			35.33	

Experiment:Untitled | Experiment Results Report | Applied Biosystems StepOne™ Instrument

Sample	Target	Quantity (Mean)	Quantity (Std Dev)	CT (Mean)	CT (Std Dev)
Sample 34	SYBR			33.91	
Sample 35	SYBR			38.23	
Sample 36	SYBR			34.68	
Sample 37	SYBR			38.64	
Sample 38	SYBR			38.97	
Sample 39	SYBR			35.97	
Sample 4	SYBR			36.85	
Sample 40	SYBR			38.27	
Sample 41	SYBR			37.83	
Sample 42	SYBR			38.35	
Sample 43	SYBR			35.80	
Sample 44	SYBR			37.18	
Sample 5	SYBR			33.62	
Sample 6	SYBR			35.39	
Sample 7	SYBR			40.43	
Sample 8	SYBR			38.62	
Sample 9	SYBR			36.20	

User: | 3 | Printed:2018 Sep 08 11:05:56 AM

Experiment:Untitled

Experiment Results Report

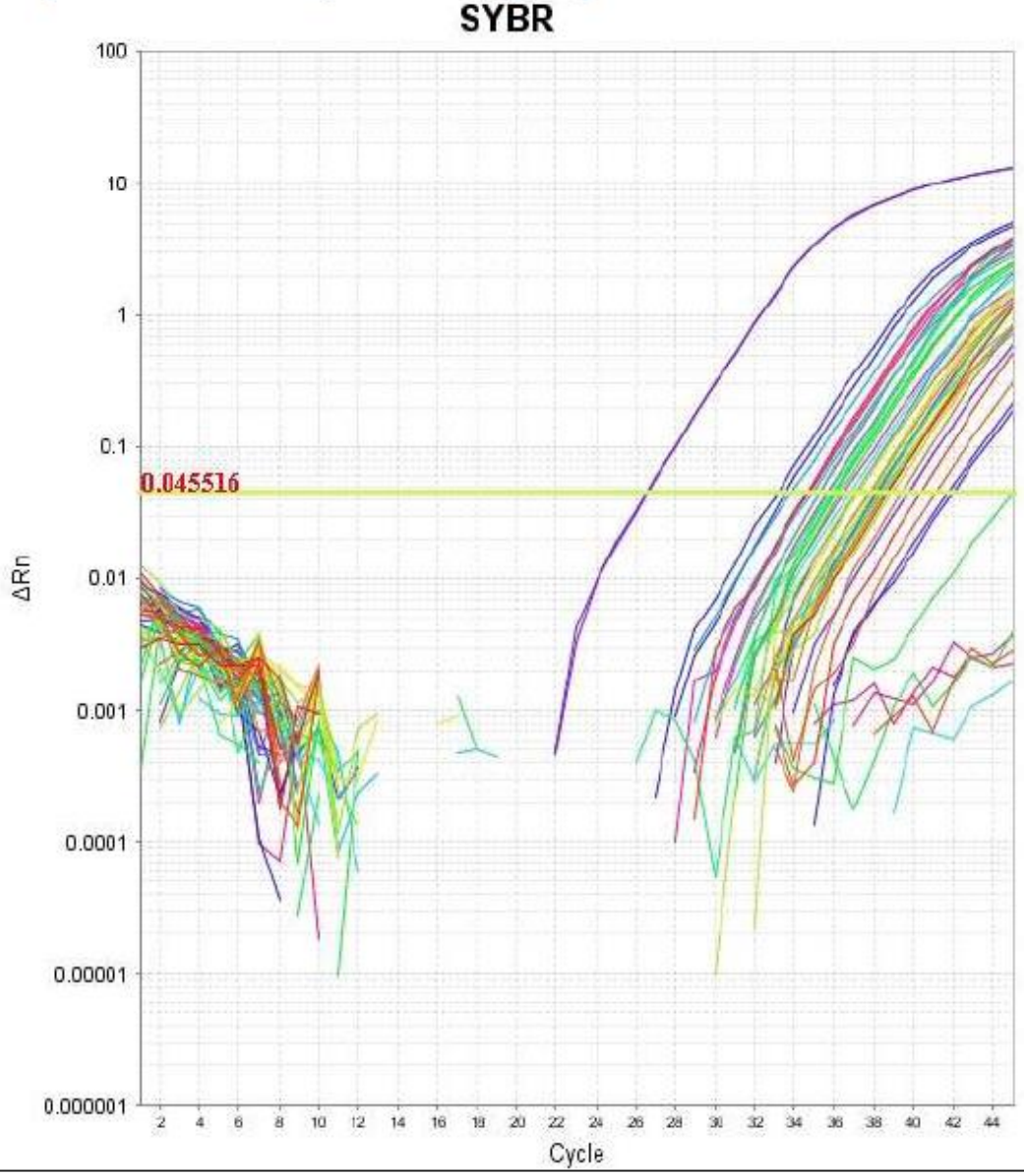
Applied Biosystems StepOne™ Instrument

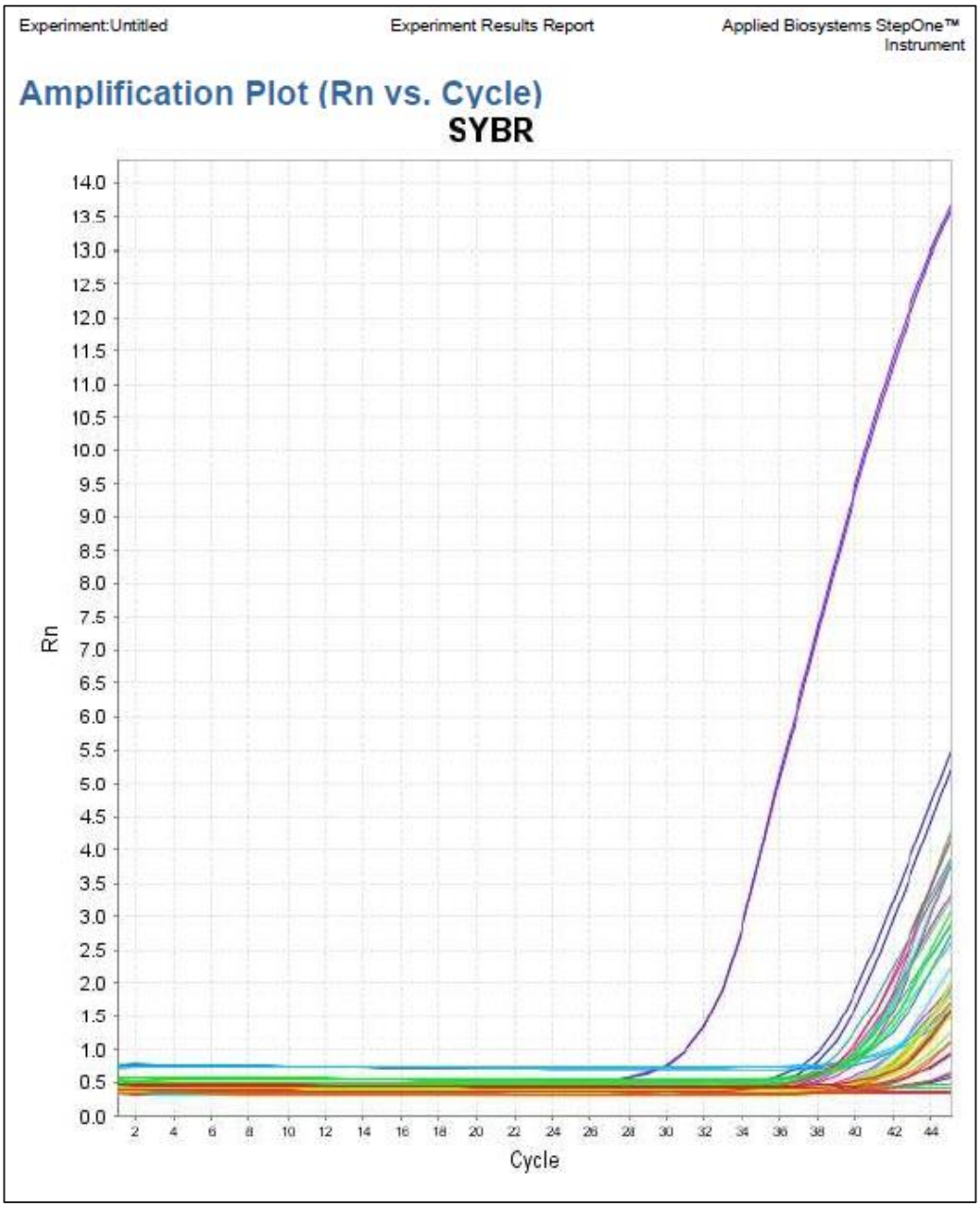
Plate Layout

	1	2	3	4	5	6	7	8
A	Sample 1 SYBR ∞ SYBR-None Ct: 38.76 Tm1: 74.39	Sample 7 SYBR ∞ SYBR-None Ct: 40.43 Tm1: 74.49	Sample 13 SYBR ∞ SYBR-None Ct: 34.72 Tm1: 74.14	Sample 19 SYBR ∞ SYBR-None Ct: Undetermined Tm1: 73.72	Sample 25 SYBR ∞ SYBR-None Ct: 41.18 Tm1: 74.34	Sample 31 SYBR ∞ SYBR-None Ct: 39.48 Tm1: 74.24	Sample 37 SYBR ∞ SYBR-None Ct: 38.84 Tm1: 74.09	Sample 41 SYBR ∞ SYBR-None Ct: 37.83 Tm1: 74.09
B	Sample 2 SYBR ∞ SYBR-None Ct: 37.98 Tm1: 74.39	Sample 8 SYBR ∞ SYBR-None Ct: 38.02 Tm1: 74.29	Sample 14 SYBR ∞ SYBR-None Ct: 37.2 Tm1: 74.09	Sample 20 SYBR ∞ SYBR-None Ct: 37.28 Tm1: 74.09	Sample 26 SYBR ∞ SYBR-None Ct: 38.6 Tm1: 74.39	Sample 32 SYBR ∞ SYBR-None Ct: 37.77 Tm1: 74.29	Sample 38 SYBR ∞ SYBR-None Ct: 38.97 Tm1: 74.14	Sample 42 SYBR ∞ SYBR-None Ct: 38.35 Tm1: 74.09
C	Sample 3 SYBR ∞ SYBR-None Ct: 38.41 Tm1: 74.24	Sample 9 SYBR ∞ SYBR-None Ct: 36.2 Tm1: 74.19	Sample 15 SYBR ∞ SYBR-None Ct: 44.09 Tm1: 74.29	Sample 21 SYBR ∞ SYBR-None Ct: Undetermined Tm1: 73.22	Sample 27 SYBR ∞ SYBR-None Ct: 36.12 Tm1: 74.14	Sample 33 SYBR ∞ SYBR-None Ct: 35.33 Tm1: 74.09	Sample 39 SYBR ∞ SYBR-None Ct: 35.97 Tm1: 73.95	Sample 43 SYBR ∞ SYBR-None Ct: 35.8 Tm1: 73.9
D	Sample 4 SYBR ∞ SYBR-None Ct: 36.85 Tm1: 74.34	Sample 10 SYBR ∞ SYBR-None Ct: Undetermined Tm1: 72.6	Sample 16 SYBR ∞ SYBR-None Ct: 38.05 Tm1: 74.04	Sample 22 SYBR ∞ SYBR-None Ct: 38.42 Tm1: 73.99	Sample 28 SYBR ∞ SYBR-None Ct: 34.84 Tm1: 74.19	Sample 34 SYBR ∞ SYBR-None Ct: 33.91 Tm1: 74.09	Sample 40 SYBR ∞ SYBR-None Ct: 38.27 Tm1: 74.04	Sample 44 SYBR ∞ SYBR-None Ct: 37.18 Tm1: 73.99
E	Sample 5 SYBR ∞ SYBR-None Ct: 33.62 Tm1: 74.04	Sample 11 SYBR ∞ SYBR-None Ct: 33.23 Tm1: 74.04	Sample 17 SYBR ∞ SYBR-None Ct: 42.06 Tm1: 74.34	Sample 23 SYBR ∞ SYBR-None Ct: 41.79 Tm1: 74.24	Sample 29 SYBR ∞ SYBR-None Ct: 39.76 Tm1: 74.39	Sample 35 SYBR ∞ SYBR-None Ct: 38.23 Tm1: 74.34	LN99-1 SYBR 1.5E4 SYBR-None Ct: 28.64 Tm1: 73.7	LN99-2 SYBR 1.5E3 SYBR-None Ct: 29.62 Tm1: 73.65
F	Sample 6 SYBR ∞ SYBR-None Ct: 35.39 Tm1: 74.14	Sample 12 SYBR ∞ SYBR-None Ct: 35.72 Tm1: 74.14	Sample 18 SYBR ∞ SYBR-None Ct: 36.55 Tm1: 74.24	Sample 24 SYBR ∞ SYBR-None Ct: 34.78 Tm1: 74.14	Sample 30 SYBR ∞ SYBR-None Ct: 37.18 Tm1: 74.09	Sample 36 SYBR ∞ SYBR-None Ct: 34.68 Tm1: 73.99	NEG SYBR SYBR-None Ct: Undetermined Tm1: 84.24	NEG SYBR SYBR-None Ct: Undetermined Tm1: 78.42

Experiment:Untitled | Experiment Results Report | Applied Biosystems StepOne™ Instrument

Amplification Plot (ΔRn vs. Cycle)




Experiment:Untitled
Experiment Results Report
Applied Biosystems StepOne™ Instrument
Amplification Plot (Rn vs. Cycle)
SYBR
Rn
Cycle
14.0
13.5
13.0
12.5
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
4
6
8
10
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16
18
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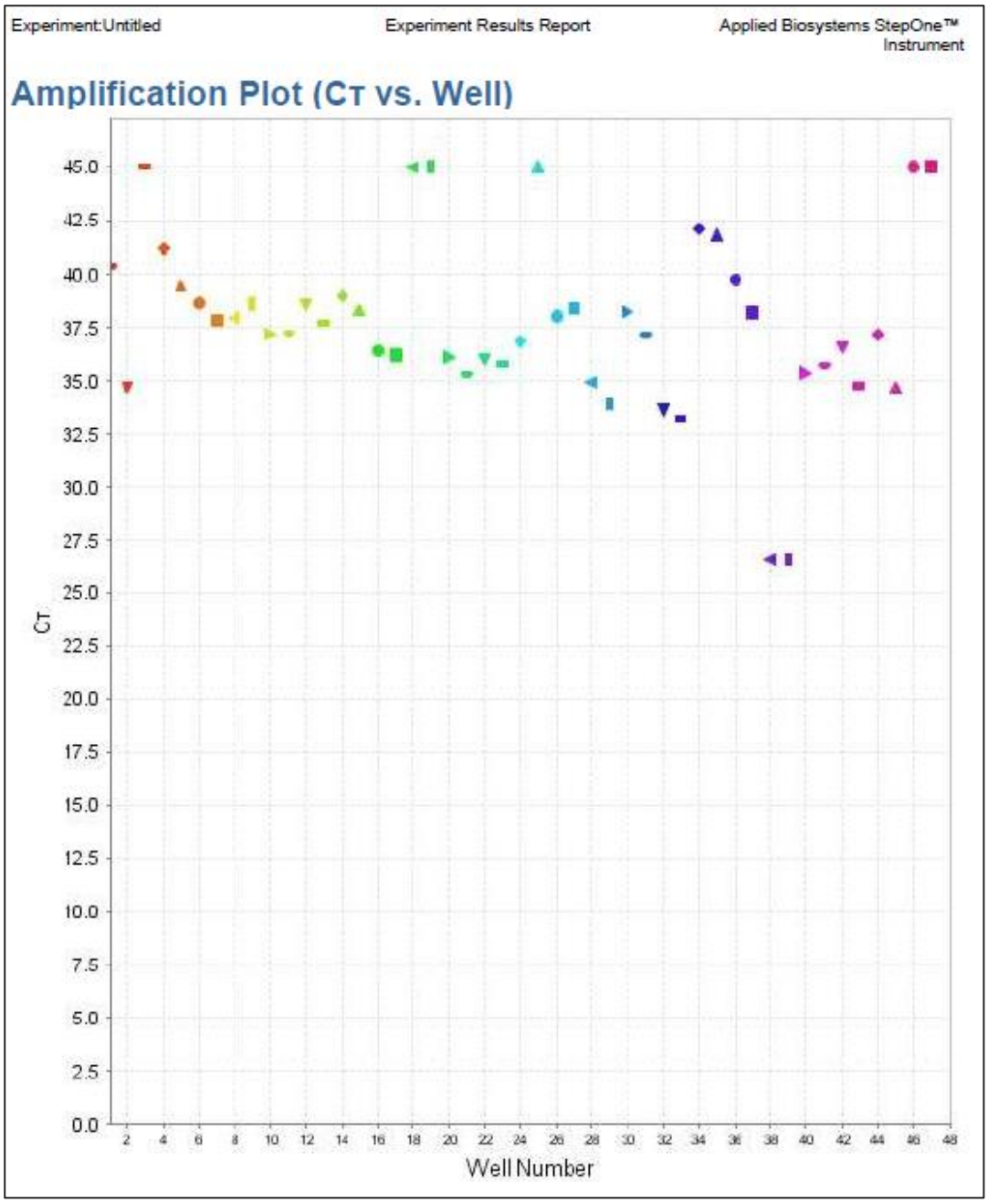
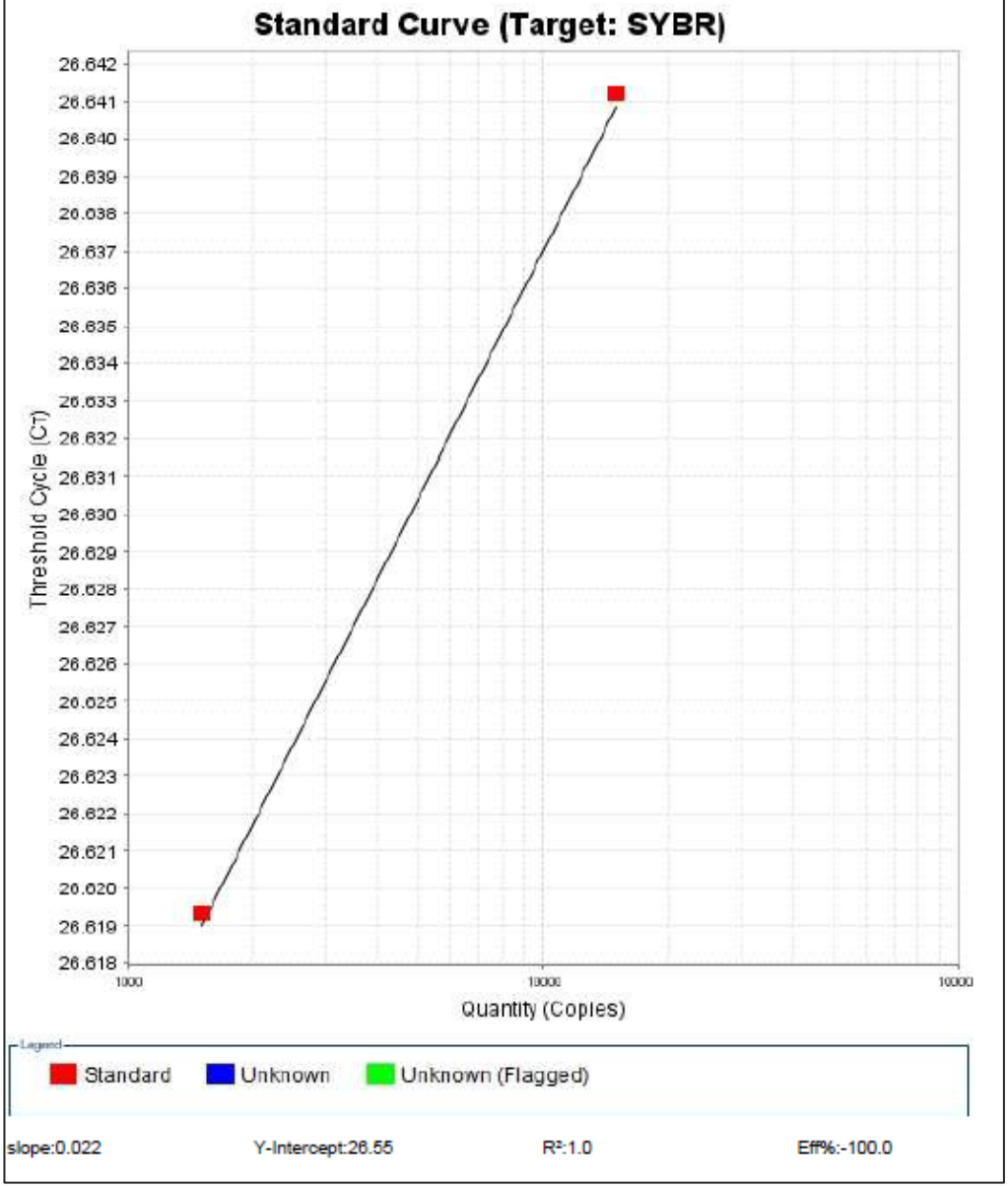
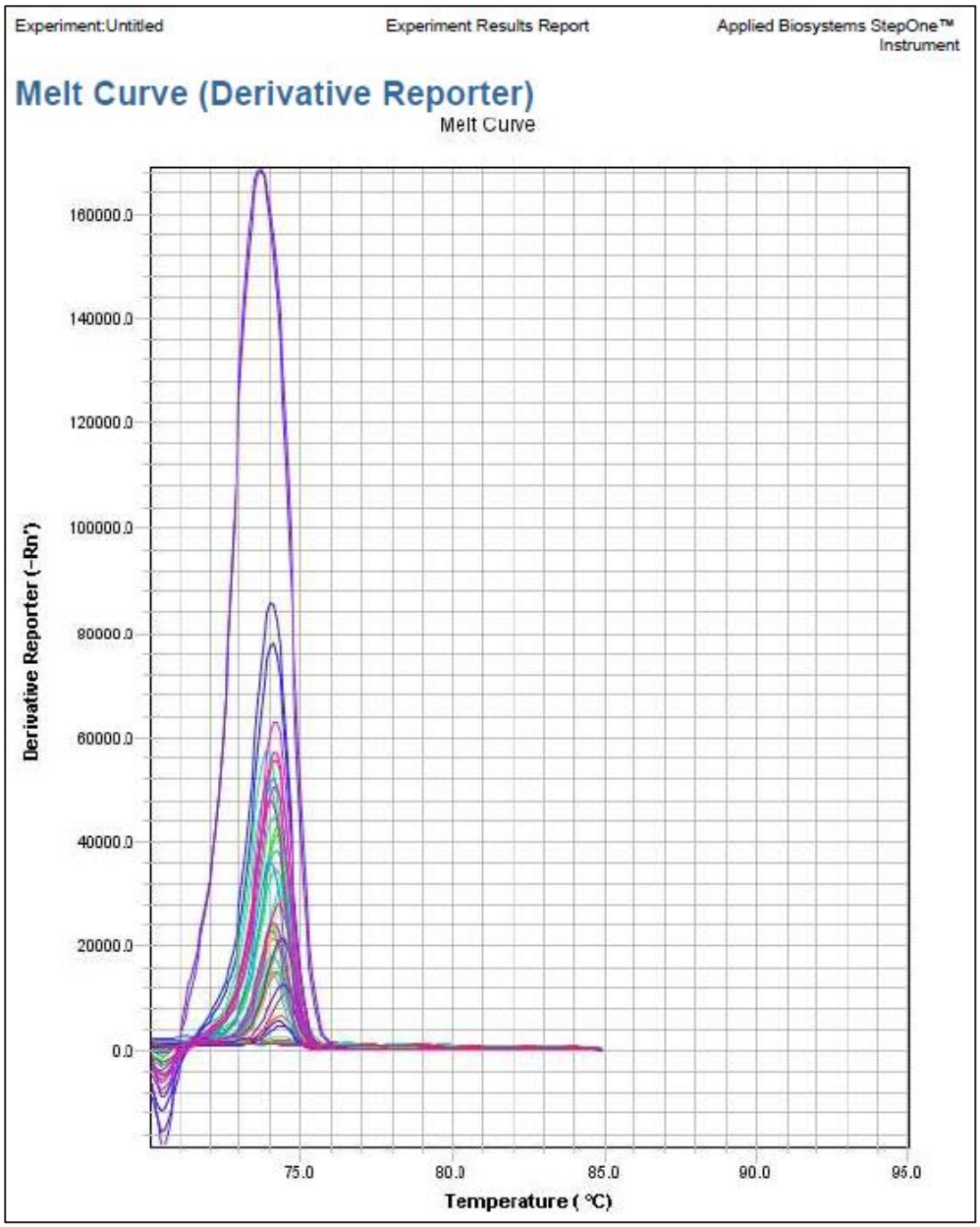
22
24
26
28
30
32
34
36
38
40
42
44


Experiment:Untitled
Experiment Results Report
Applied Biosystems StepOne™ Instrument
Amplification Plot (CT vs. Well)
CT
Well Number
45.0
42.5
40.0
37.5
35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48

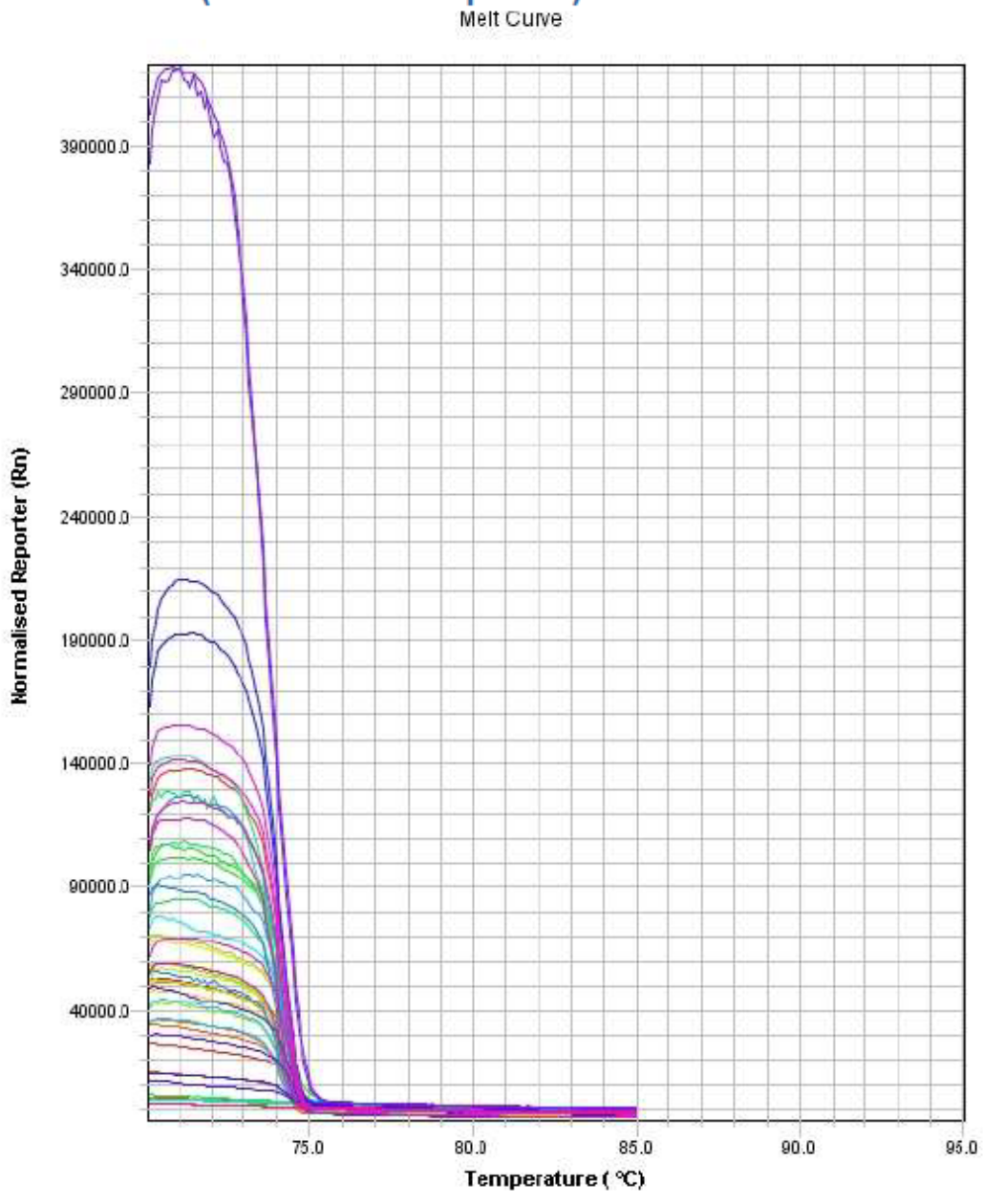
tandard Curves



slope:0.022 Y-Intercept:26.55 R²:1.0 Eff%:-100.0


Experiment:Untitled
Experiment Results Report
Applied Biosystems StepOne™ Instrument
Melt Curve (Derivative Reporter)
Melt Curve
160000.0
140000.0
120000.0
100000.0
80000.0
60000.0
40000.0
20000.0
0.0
Derivative Reporter (-Rn')
75.0
80.0
85.0
90.0
95.0
Temperature (°C)

Melt Curve (Normalized Reporter)



Results Table

Well	Sample	Target	Task	Quantity	CT	Tm1	Tm2	Tm3
A1	Sample 1	SYBR	UNKNOWN	∞	38.7589	74.3926		
A2	Sample 7	SYBR	UNKNOWN	∞	40.4299	74.4921		
A3	Sample 13	SYBR	UNKNOWN	∞	34.7225	74.1439		
A4	Sample 19	SYBR	UNKNOWN		Undetermined	78.7194	81.0569	71.7567
A5	Sample 25	SYBR	UNKNOWN	∞	41.1824	74.3429		
A6	Sample 31	SYBR	UNKNOWN	∞	39.4563	74.2434		
A7	Sample 37	SYBR	UNKNOWN	∞	38.6428	74.0942		
A8	Sample 41	SYBR	UNKNOWN	∞	37.8341	74.0942		
B1	Sample 2	SYBR	UNKNOWN	∞	37.9781	74.3926		
B2	Sample 8	SYBR	UNKNOWN	∞	38.6162	74.2931		
B3	Sample 14	SYBR	UNKNOWN	∞	37.204	74.0942		
B4	Sample 20	SYBR	UNKNOWN	∞	37.2828	74.0942		
B5	Sample 26	SYBR	UNKNOWN	∞	38.5971	74.3926		
B6	Sample 32	SYBR	UNKNOWN	∞	37.7656	74.2931		
B7	Sample 38	SYBR	UNKNOWN	∞	38.9688	74.1439		
B8	Sample 42	SYBR	UNKNOWN	∞	38.3547	74.0942		
C1	Sample 3	SYBR	UNKNOWN	∞	36.4136	74.2434		
C2	Sample 9	SYBR	UNKNOWN	∞	36.1954	74.1937		
C3	Sample 15	SYBR	UNKNOWN	∞	44.9877	74.2931		
C4	Sample 21	SYBR	UNKNOWN		Undetermined	78.2221	83.1458	72.8011
C5	Sample 27	SYBR	UNKNOWN	∞	36.1171	74.1439		
C6	Sample 33	SYBR	UNKNOWN	∞	35.3261	74.0942		
C7	Sample 39	SYBR	UNKNOWN	∞	35.9709	73.945		
C8	Sample 43	SYBR	UNKNOWN	∞	35.7999	73.8953		
D1	Sample 4	SYBR	UNKNOWN	∞	36.8541	74.3429		
D2	Sample 10	SYBR	UNKNOWN		Undetermined	72.6022	71.2594	74.7407
D3	Sample 16	SYBR	UNKNOWN	∞	38.0503	74.0445		
D4	Sample 22	SYBR	UNKNOWN	∞	38.4174	73.9947		
D5	Sample 28	SYBR	UNKNOWN	∞	34.9429	74.1937		
D6	Sample 34	SYBR	UNKNOWN	∞	33.9145	74.0942		

Experiment:Untitled | Experiment Results Report | Applied Biosystems StepOne™ Instrument

Well	Sample	Target	Task	Quantity	Cr	Tm1	Tm2	Tm3
D7	Sample 40	SYBR	UNKNOWN	∞	38.2692	74.0445		
D8	Sample 44	SYBR	UNKNOWN	∞	37.1831	73.9947		
E1	Sample 5	SYBR	UNKNOWN	∞	33.6248	74.0445		
E2	Sample 11	SYBR	UNKNOWN	∞	33.2274	74.0445		
E3	Sample 17	SYBR	UNKNOWN	∞	42.0617	74.3429		
E4	Sample 23	SYBR	UNKNOWN	∞	41.7884	74.2434		
E5	Sample 29	SYBR	UNKNOWN	∞	39.7596	74.3926		
E6	Sample 35	SYBR	UNKNOWN	∞	38.2312	74.3429		
E7	LXF09 -1	SYBR	STANDARD	15000	26.6412	73.6963		
E8	LXF09 -2	SYBR	STANDARD	1500	26.6193	73.6466		
F1	Sample 6	SYBR	UNKNOWN	∞	35.3879	74.1439		
F2	Sample 12	SYBR	UNKNOWN	∞	35.723	74.1439		
F3	Sample 18	SYBR	UNKNOWN	∞	36.5489	74.2434		
F4	Sample 24	SYBR	UNKNOWN	∞	34.7809	74.1439		
F5	Sample 30	SYBR	UNKNOWN	∞	37.1783	74.0942		
F6	Sample 36	SYBR	UNKNOWN	∞	34.6767	73.9947		
F7	NEG	SYBR	NTC		Undetermined	84.2399	82.7479	72.2043
F8	NEG	SYBR	NTC		Undetermined	78.421	71.508	81.5543

Experiment:Untitled | Experiment Results Report | Applied Biosystems StepOne™ Instrument

QC Summary

Total Wells	48	Processed Wells	48	Targets Used	1
Well Setup	48	Flagged Wells	5	Samples Used	47

Flag	Name	Frequency	Locations
AMPNC	Amplification in negative control	0	
BADROX	Bad passive reference signal	0	
BLFAIL	Baseline algorithm failed	0	
CTFAIL	C_T algorithm failed	0	
EXPFAIL	Exponential algorithm failed	3	A4, C4, D2
HIGHSD	High standard deviation in replicate group	0	
MTP	Multiple Tm peaks	5	A4, C4, D2, F7, F8
NOAMP	No amplification	3	A4, C4, D2
NOISE	Noise higher than others in plate	0	
NOSIGNAL	No signal in well	0	
OFFSCALE	Fluorescence is offscale	0	
OUTLIERRG	Outlier in replicate group	0	
SPIKE	Noise spikes	0	
THOLDFAIL	Thresholding algorithm failed	0	

Annex 8: Qiagen protocol for purification of total DNA from ticks

QIAGEN Supplementary Protocol:

Purification of total DNA from ticks using the DNeasy® Blood & Tissue Kit for detection of *Borrelia* DNA

This protocol provides recommendations for DNA purification from ticks, for use in real-time PCR detection of *Borrelia* spp., using, for example, the QIAGEN® *artus*® Borrelia LC PCR Kit (cat. no. 4551063 or 4551065, not available in the USA).

Introduction

Due to generally low numbers of *Borrelia* in ticks, both complete digestion of tick tissue (except the exoskeleton) and removal of inhibitors are crucial to guarantee the highest possible sensitivity in downstream PCR.

In general, for DNA preparation single, whole ticks are used, except for ticks that are engorged with blood. Preparation of the whole tick is only useful if the body does not exceed 5 mm. Larger ticks most probably have recently sucked blood and therefore may inhibit downstream PCR analysis due to the release of inhibitors from digested blood.

Therefore, use only the head section of large and often dark colored ticks (females). Cut off the abdomen containing most of the digested blood with a sharp scalpel. Frequently, relatively small ticks (males) can be found fixed to the ventral body of a much bigger female. In this case only the females (head) have to be analyzed, because the males did not have contact with the host.

IMPORTANT: Please read the *DNeasy Blood & Tissue Handbook*, paying careful attention to the "Safety Information" and "Important Notes" sections, before beginning this procedure.

DNeasy Blood & Tissue Kits: For Research Use Only. Not for use in diagnostics procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease. The *artus* Borrelia LC PCR Kit is not available in the USA. Purification of total DNA from ticks using the DNeasy Blood & Tissue Kit and detection of *Borrelia* DNA using the *artus* Borrelia LC PCR Kit is a research application.

Equipment and reagents required

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- DNeasy Blood & Tissue Kit (cat. no. 69504 or 69506)
- Pipets and pipet tips
- Scalpel
- Vortexer
- Microcentrifuge tubes (1.5 ml or 2 ml)

- Microcentrifuge with rotor for 1.5 ml and 2 ml tubes
- Thermomixer, shaking water bath, or rocking platform for heating at 56°C and 70°C
- Ethanol (96–100%)*
- Carrier RNA solution, 10 mg/ml (e.g., poly A RNA Homopolymer, Amersham Biosciences, cat. no. 27-4110-01)†
- Optional: Internal control DNA (e.g., as provided in the QIAGEN *artus* Borrelia LC PCR Kit, cat. no. 4551063 or 4551065, not available in the USA)

Important points before starting

- If using the DNeasy Blood & Tissue Kit for the first time, read "Important Notes" in the *DNeasy Blood & Tissue Handbook*.
- All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5–10 s.

Things to do before starting

- Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 4.
- If using frozen samples, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.

Procedure

1. **Place the whole tick or the head in a 1.5 ml microcentrifuge tube with 180 µl Buffer ATL. Vortex thoroughly.**
2. **Draw the moistened body of the tick with the tip of a pipette to the upper rim of the opened microcentrifuge tube and cut it with a scalpel once longitudinally and once diagonally (depending on the sample size, further cuts may be necessary).**

   Cutting the tick is necessary so that the tick tissue is thoroughly lysed and the *Borrelia* contained in it can be quantified.
3. **Close the microcentrifuge tube and centrifuge briefly to collect all tissue pieces at the bottom of the tube.**

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

† This is not a complete list of suppliers and does not include many important vendors of biological supplies.

4. **Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed (only the exoskeleton remains). Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking shaker.**

   Lysis time varies depending on the size of the tissue pieces. Lysis is usually complete in 30–60 min. If it is more convenient, samples can be lysed overnight; this will not affect them adversely.

   Heat the thermomixer, shaking water bath, or rocking shaker to 70°C after this step if it will be used for the incubation in step 5.

5. **Vortex for 15 s. Add 200 µl Buffer AL (without added ethanol) to the sample, and mix thoroughly by vortexing. Incubate at 70°C for 10 min.**

   Ensure that ethanol has not been added to Buffer AL (see "Buffer AL" in the *DNeasy Blood & Tissue Handbook*). Buffer AL can be purchased separately.

   It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL. This precipitate does not interfere with the DNeasy procedure.

6. **Add 1 µl of carrier RNA (10 mg/ml), and mix thoroughly by vortexing. Then add 230 µl ethanol (96–100%), and mix again thoroughly by vortexing.**

   Optional: Add internal control DNA with the carrier RNA. Adjust the amount of internal control DNA according to the final elution volume. For very small ticks (<5 mm), a final elution volume of 60 µl is recommended. For larger ticks (or part of one), use elution volumes up to 200 µl. When using the *artus* Borrelia LC PCR Kit, add 6 µl or up to 20 µl, respectively, of the Internal Control provided in the kit.

   It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. This precipitate does not interfere with the DNeasy procedure.

7. **Pipet the mixture from step 6 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.***
8. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.***

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See *DNeasy Blood & Tissue Handbook* for safety information.

9. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.**

   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

   Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

10. **Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 35 µl Buffer AE (for small ticks, <5 mm) or 105 µl Buffer AE (for large ticks) directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x g (8000 rpm) to elute.**

11. **Pipet another 30 µl Buffer AE (for small ticks, <5 mm) or 100 µl Buffer AE (for large ticks) directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x g (8000 rpm) to elute.**

    This step leads to increased overall DNA yield.

    Eluting with 35 µl + 30 µl Buffer AE results in an eluate volume of approximately 60 µl. Eluting with 105 µl + 100 µl Buffer AE results in an eluate volume of approximately 200 µl.

    The microcentrifuge tube from step 10 should be reused for this elution step.

    Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

Troubleshooting

For general troubleshooting, please consult the Troubleshooting Guide in the *DNeasy Blood & Tissue Handbook*.

Annex 9: Qiagen protocol for purification of DNA from animal tissue

Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol)

This protocol is designed for purification of total DNA from animal tissues, including rodent tails.

Animal Tissues (Spin-Column Protocol)

Important points before starting

- If using the DNeasy Blood & Tissue Kit for the first time, read "Important Notes" (page 15).
- For fixed tissues, refer to the pretreatment protocols "Pretreatment for Paraffin-Embedded Tissue", page 41, and "Pretreatment for Formalin-Fixed Tissue", page 43.
- All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5–10 s.
- Optional: RNase A may be used to digest RNA during the procedure. RNase A is not provided in the DNeasy Blood & Tissue Kit (see "Copurification of RNA", page 19).

Things to do before starting

- Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 2.
- If using frozen tissue, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.

Procedure

1. **Cut up to 25 mg tissue (up to 10 mg spleen) into small pieces, and place in a 1.5 ml microcentrifuge tube. For rodent tails, place one (rat) or two (mouse) 0.4–0.6 cm lengths of tail into a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Earmark the animal appropriately.**

   Ensure that the correct amount of starting material is used (see "Starting amounts of samples", page 15). For tissues such as spleen with a very high number of cells for a given mass of tissue, no more than 10 mg starting material should be used.

We strongly recommend to cut the tissue into small pieces to enable more efficient lysis. If desired, lysis time can be reduced by grinding the sample in liquid nitrogen* before addition of Buffer ATL and proteinase K. Alternatively, tissue samples can be effectively disrupted before proteinase K digestion using a rotor–stator homogenizer, such as the QIAGEN TissueRuptor, or a bead mill, such as the QIAGEN TissueLyser (see page 56 for ordering information). A supplementary protocol for simultaneous disruption of up to 48 tissue samples using the TissueLyser can be obtained by contacting QIAGEN Technical Services (see back cover).

For rodent tails, a maximum of 1.2 cm (mouse) or 0.6 cm (rat) tail should be used. When purifying DNA from the tail of an adult mouse or rat, it is recommended to use only 0.4–0.6 cm.

2. **Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform.**

   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h or, for rodent tails, 6–8 h. If it is more convenient, samples can be lysed overnight; this will not affect them adversely.

   After incubation the lysate may appear viscous, but should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, see the "Troubleshooting Guide", page 47, for recommendations.

   Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature before continuing with step 3.

   Transcriptionally active tissues such as liver and kidney contain high levels of RNA, which will copurify with genomic DNA. For tissues that contain low levels of RNA, such as rodent tails, or if residual RNA is not a concern, RNase A digestion is not necessary.

3. **Vortex for 15 s. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.**

   It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

4. **Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard flow-through and collection tube.***

5. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x *g* (8000 rpm). Discard flow-through and collection tube.***

6. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.**

   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

   Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

7. **Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x *g* (8000 rpm) to elute.**

   Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

8. **Recommended: For maximum DNA yield, repeat elution once as described in step 7.**

   This step leads to increased overall DNA yield.

   A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

   Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 8 for safety information.

Annex 10: *Candidatus* Neoehrlichia mikurensis PCR protocol

Candidatus Neoehrlichia mikurensis PCR Protocol

Lab: PCR1 Preparation of mastermix

Prepare mastermix according to the following formula:

Reagents	1 sample	10 samples	20 samples	50 samples	100 samples
Primer NeoR,10µM	2 µl	20 µl	40 µl	100 µl	200 µl
Primer NeoF, 10µM	2 µl	20 µl	40 µl	100 µl	200 µl
Water	3.5 µl	35 µl	70 µl	175 µl	350 µl
2x Applied Biosystems SYBR Green mastermix	12.5 µl	125 µl	250 µl	625 µl	1250 µl

Mix reagents in 1.5 ml polypropylene microcentrifuge tube

Add mastermix last. Mix by pipetting up and down a few times at the final pipetting stage.

Take a small portion of TE buffer for use as a negative control.

Do not wear a standard labcoat in PCR1. Do not bring in any equipment or materials from another laboratories

Lab: PCR 2. Portioning of mastermix and PCR setup

1. Assemble and arrange your samples in a suitable rack. Fill out a PCR form indicating the positions of the samples in the PCR plate.
2. Ad 20 µl of mastermix to the wells of a 48- or 96-well PCR plate. An empty pipette tip box makes a convenient stand.
3. Add 5 µl of sample to the appropriate well(s). **Change the pipette tip between each pipetting**.
4. Include at least one positive and one negative control
5. Cover the plate with adhesive plastic film. Smooth the film into place using the plastic tool provided.

Avoid touching the optical surfaces of the plate with your fingers or other surfaces. Many common substances, such as glove dust and fabric fibers fluoresce. This can interfere with your results. Wear gloves and one of the blue lab coats provided. Take the labcoat off before you leave the lab.

Lab PCR3: Running PCR.

1. Open the door of the real time PCR instrument and place the plate in the plate holder with the beveled corner matching the corresponding mark on the plate. Close the door.
2. Click 'StepOne software'. Log in as GUEST
3. Open the appropriate template file and make any necessary changes. If you need to make large-scale changes, do this before you start the PCR setup to avoid having your PCR setup standing around unnecessarily.

1. Save your updated file in your folder in the Experiments folder. Give the file a distinctive and understandable name that you are likely to recognize in six months' time. It is a good idea to start the file name with the date in the form yymmdd. File names can have spaces, hyphens (-), underscore (_) and brackets () but most other symbols will confuse the PC.
2. Click on the green RUN button. Wait a few minutes to ensure that the instrument initializes correctly.
3. When your PCR is finished, discard the used plate in the plastic or ordinary waste container, unless you wish to keep the material for post-PCR analysis. In this case, mark the edge of the plate with your initials and other identifying information before starting the PCR. Unmarked plates will be discarded.

The PCR program is:

95°C, 10min. (To achieve complete denaturation of genomic DNA in the sample)

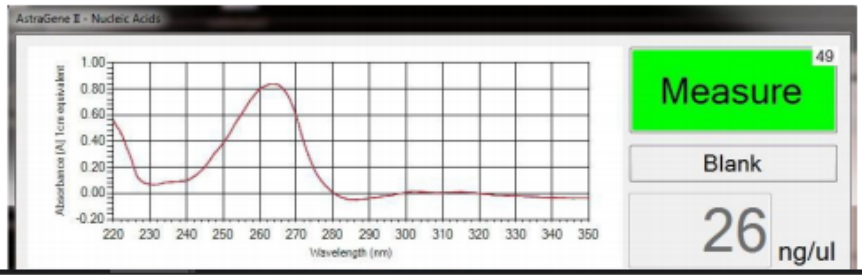
{95°C, 15s; 60°C, 60s} x 45 cycles (two-step PCR cycle; denaturation at 95°C, annealing and extension at 60°C).

95°C, 15s; 60°C, 15s (the machine puts this in automatically; it's not necessary); warm from 60°C to 70°C. Denaturation analysis from 70°C to 85°C, measurement every 0.1°C.

Annex 11: Picodrop protocol for DNA measurement

Nucleic Acid Measurement

- General application information and background is described in the Appendix.
- Select the relevant nucleic acid mode (dsDNA, ssDNA, RNA) from the dialogue box.
- Pipette 2.5µl of 'Blank' solution into a tip and place the tip and pipette adapter
- into the pipette holder (see Correct pipette use and sample handling).
- Click the button marked 'Blank'. The software will run a reference scan on the spectrophotometer and set the baseline to zero. For several seconds the LED on
- the front of the unit will turn orange; the buzzing noise is due to the xenon lamp.
- If for any reason (including air bubbles or settling turbidity in the sample) a blank cannot be completed successfully a message requesting that you're- blank' will be displayed. If this occurs simply try blanking again, and if the procedure continues to fail, try following the lens cleaning protocol detailed in the Trouble Shooting Section.
- Once the instrument has been successfully 'Blanked' then the red 'Measure' button
- will change to green signifying that you can commence measuring your samples.
- Insert the pipette with sample and press the 'Measure' button.
- After a few seconds the concentration (in ng/µl) will appear in the bottom right hand window, the spectrum will be plotted and details displayed in the table below the graph
- It is not necessary to 'save' the results as all results are done so automatically.



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Abstract:

Ticks and its pathogen constitute a growing burden for animal and human health in the world. Ticks are blood-feeding parasites, which make them capable of transmitting various pathogens to their hosts that can cause tick-borne infections and tick-borne diseases. These infections and diseases affect wild and domestic animals. The aim of this study was to investigate the presence of the tick-borne pathogen, *Candidatus* Neoehrlichia mikurensis (CNM) in *Ixodes trianguliceps* and its possible role in the infectious cycle. It is an emerging pathogen in Europe. This is the first study of CNM in *Ixodes trianguliceps* in Norway and the first study showing the presence of CNM in *Sorex araneus*. CNM has been described from previous studies in hard tick *Ixodes ricinus* and small mammals as well as in cases of human disease. Ticks were collected from trapped rodents and shrews in 2015 and 2017 along two mountain transects. Spleen samples of 46 small mammals and 116 attached *Ixodes trianguliceps* were investigated by using a real-time PCR to determine the DNA of CNM. Altogether 72% of the spleen samples of the small mammals were positive for the DNA of CNM. Most of the infected small mammals were *Myodes glareolus* (Bank voles) and *Sorex araneus* (common shrew). Altogether 81% of *Ixodes trianguliceps* were positive for DNA of CNM. There was high infection rate of CNM in infected *Ixodes trianguliceps* collected from infected small mammals. However, there were *Ixodes trianguliceps* including larvae, nymphs and adult that were infected although their host animals tested negative for the DNA of *Candidatus* Neoehrlichia mikurensis. Of 15 samples sequenced, 8 samples gave readable sequence, and all of which were confirmed as CNM. There seems to be evidence of transovarial transmission of CNM in *Ixodes trianguliceps* in this study. Although *Ixodes trianguliceps* does not bite humans, it may indirectly be of medical and veterinary importance of its role as a maintenance vector of CNM among small mammals.